THE ACTION OF PEROXYNITRITE ON PROTEINS

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ABSTRACT

Oxidative damage to proteins can occur under physiological conditions through the action of reactive oxygen species, including those containing nitrogen such as peroxynitrite. Peroxynitrite has been shown in vitro to target tyrosine and tryptophan residues in proteins through free radical addition to produce 3-nitrotyrosine and nitrotryptophan. In this work, we show that mass spectral patterns associated with 3-nitrotyrosine and nitrotryptophan containing peptides allow identification of peptides containing these modifications. Quadrupole time-of-flight mass spectrometry (Q-TOF MS) was used to characterise several peptides containing 3-nitrotyrosine and one containing nitrotryptophan, derived from albumin and transferrin treated with either peroxynitrite or 3-morpholino-sydnonimine (SIN-1). A unique series of modified peptides were also found for the reacted proteins that did not relate to the nitration of tyrosine residues. Sequencing of these peptides found that nitration is not the only modification that tyrosine can undergo. Tyrosine was found to also undergo dinitration, oxidation and dimerisation. It was also found that other amino acids underwent modification when exposed to peroxynitrite or SIN-1. Histidine showed modifications of +126 Da and +252 Da, lysine +28 Da, cysteine +25 Da and threonine –18 Da. Several proteins containing 3-nitrotyrosine were isolated from the cerebral spinal fluid of acute lymphoblastic patients using western blotting with the antibody antinitrotyrosine. One of these proteins was identified as albumin using matrix-assisted laser desorption time-of-flight mass spectrometry (MALDI-TOF MS). These observations suggest that Q-TOF MS/MS can provide a selective method for the analysis and characterisation of amino acids modified by peroxynitrite and that a combination of western blotting and MALDI-TOF MS can be utilised to isolate and identify proteins containing 3-nitrotyrosine. However, it does highlight that caution is required when attributing observed changes to protein activity, after exposure to peroxynitrite, to only the nitration of tyrosine residues.
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>ALL</td>
<td>acute lymphoblastic leukaemia</td>
</tr>
<tr>
<td>ARDS</td>
<td>acute respiratory disease</td>
</tr>
<tr>
<td>BH₄</td>
<td>tetrahydrobiopterin</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CaM</td>
<td>Calmodulin</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CSF</td>
<td>cerebral spinal fluid</td>
</tr>
<tr>
<td>ECD</td>
<td>electrochemical detection</td>
</tr>
<tr>
<td>ESI</td>
<td>electrospray ionisation</td>
</tr>
<tr>
<td>HPLC</td>
<td>high-performance liquid chromatography</td>
</tr>
<tr>
<td>MALDI-TOF</td>
<td>Matrix assisted laser desorption ionisation time-of-flight</td>
</tr>
<tr>
<td>MS</td>
<td>mass spectrometry</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>NO⁺</td>
<td>nitrosyl cation</td>
</tr>
<tr>
<td>NO⁻</td>
<td>nitroxy1 anion</td>
</tr>
<tr>
<td>NO₂⁻</td>
<td>nitrite</td>
</tr>
<tr>
<td>NO₃⁻</td>
<td>nitrate</td>
</tr>
<tr>
<td>NO₂</td>
<td>nitrogen dioxide radical</td>
</tr>
<tr>
<td>NOS</td>
<td>nitric oxide synthase</td>
</tr>
<tr>
<td>NO₂⁻-Tyr</td>
<td>3-nitrotyrosine</td>
</tr>
<tr>
<td>O₂⁻</td>
<td>superoxide</td>
</tr>
<tr>
<td>ONOO⁺</td>
<td>peroxynitrite</td>
</tr>
<tr>
<td>ONOOH</td>
<td>peroxynitrous acid</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>Q-TOF</td>
<td>quadrupole time-of-flight</td>
</tr>
<tr>
<td>SIN-1</td>
<td>3-morpholino-sydnonimine</td>
</tr>
<tr>
<td>SOD</td>
<td>superoxide dismutase</td>
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Peptide masses (unless otherwise stated) are $[\text{M+H}]^+$
ACKNOWLEDGEMENTS

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1.1 Review of Acute Lymphoblastic Leukaemia

Current medical treatments for childhood acute lymphoblastic leukaemia (ALL) have improved the outlook such that 70-80% can expect a five-year survival rate or cure (Peckham 1991). However, investigations monitoring the short and long-term outcomes for these patients have found evidence of neuropsychological sequelae, academic performance deficits and some behavioural difficulties. These effects may not become apparent for some years after the cessation of treatment (Copeland et al. 1996; Kato et al. 1993).

The treatment of ALL involves five phases of treatment, induction, early intensification, presymptomatic CNS treatment (PCNS), late intensification and continuing, the total length of treatment takes approximately 100 weeks. Patients are randomised at the PCNS phase of treatment into separate groups depending on the risk of developing CNS leukaemia. Patients with a high risk of developing CNS leukaemia (presenting with a white blood cell count $> 50 \times 10^9$ /L) are randomised into groups that receive cranial radiotherapy or intrathecal methotrexate plus high-dose intravenous methotrexate with folinic acid rescue. Low risk patients are randomised for PCNS treatment into groups receiving intrathecal methotrexate (ITMTX) alone, or intrathecal methotrexate plus high-dose intravenous methotrexate (HDMTX) with folinic acid rescue. There are therefore two groups receiving different doses of methotrexate given by different routes, with other treatments remaining the same. All patients receive intrathecal methotrexate during the induction, early intensification, PCNS, and late-intensification (plus late-consolidation if given) phases of treatment. Patients who receive cranial radiotherapy (24 Gy over 3 weeks) do not have continuing intrathecal methotrexate, while all others receive intrathecal methotrexate every 12 weeks for 2 years during continuing treatment, and both before and after the late-intensification phase of treatment. The dose of intrathecal methotrexate is usually between 7.5 and 12.5 mg depending on age and groups without cranial radiotherapy receive a total of 16 treatments. In addition,
the HDMTX group receives three infusions of high-dose methotrexate (6 or 8 g/m² according to age) with folinic acid rescue 36 hours later during the PCNS phase of treatment. In all patients remission is induced using a combination of prednisolone, vincristine, asparaginase, daunorubicin, and intrathecal methotrexate. The early and late intensification blocks consist of prednisolone, vincristine, daunorubicin, etoposide, cytarabine, thioguanine, and intrathecal methotrexate. The late consolidation block (when given) consists of dexamethasone, vincristine, asparaginase, cyclophosphamide, cytarabine, thioguanine, and intrathecal methotrexate. Continuing treatment is given in 12-week cycles and consists of prednisolone, vincristine, mercaptopurine, oral methotrexate, and (excepting the children who receive PCNS with radiotherapy) intrathecal methotrexate.

Goff et al (1980) reported on 37 long-term ALL survivors, 13 of 30 children less than 8 years of age at diagnosis showed lower scores on intellectual and academic achievements tests, non-verbal problem solving tasks, and auditory language tasks. Only 1 in 7 children 8 years or older at diagnosis was impaired in neuropsychological testing. Radiotherapy is now not recommended for use on patients under two at the onset of treatment, as this young age group appears to be most vulnerable to the deleterious effects of treatment. Sex differences have also been reported with females being more at risk than males. Sex exerts its effect most strongly on verbal skills, with girls showing selective impairment on verbal IQ (Christie et al. 1995). It has become clear that presymptomatic central nervous system treatment with combined cranial radiotherapy and methotrexate can cause these late intellectual deficits (Jereb et al. 1994; Stehbens et al. 1991).

Evidence has shown that cranial irradiation is a major cause of the deficits reported especially in children under the age of two at diagnosis (Eiser 1980 and Moss et al 1981). Histological and radiographic studies have revealed two forms of delayed neuropathy after cranial irradiation; leukoencephalopathy and mineralising microangiopathy. These are not
usually apparent until several months after cranial irradiation and are characterised by damage to areas surrounding the basal ganglia, including myelin degeneration, ventricular dilatation, and cerebral calcifications (Paakko et al. 1992; Price and Jamieson 1975; Tsuruda et al. 1987). Symptoms associated with these problems include seizures, ataxia and slurred speech and occur most often after high doses of cranial irradiation (Ochs 1989).

Although the neurological effects of chemotherapeutic agents, particularly methotrexate, are less well documented, a review (Valk and van-der-Knaap 1992) has suggested that damage has been observed in the white matter after chemotherapy alone, with the mechanism being similar to that described after cranial irradiation and including vascular and glial injury (Ball-WS et al. 1992). In general, findings have indicated some association between the type and severity of brain abnormality as seen by computer topography, and patterns of neuropsychological dysfunction (Brouwers et al. 1985; Brouwers and Poplack 1990). In these studies CNS abnormalities are most commonly described in the region of the basal ganglia, particularly the caudate, which has extensive neural connections to the frontal lobes.

Evidence for deficits occurring after combined cranial radiotherapy and methotrexate have been well documented, however the effect of chemotherapy alone is more controversial. The majority of recent studies show no difference in outcome between groups receiving presymptomatic CNS treatment with a combination of radiotherapy and chemotherapy or chemotherapy alone (Ochs et al. 1991; Williams et al. 1991), but others have shown that chemotherapy alone causes clinically insignificant (Bakke et al. 1993; Copeland et al. 1996) or no effect (Anderson et al. 1994; Butler et al. 1994).

Methotrexate is associated with a number of gastrointestinal, hematopoietic and neurological toxicities (Quinn and Kamen 1996). Methotrexate neurotoxicity can be classified into three syndromes
according to time of onset: acute syndrome, subacute syndrome and a delayed leukoencephalopathy (Gay et al. 1989; Jaffe et al. 1985; Walker et al. 1986). The delayed leukoencephalopathy occurs weeks to months following therapy, it may be progressive and it is characterised by disturbances of higher cognitive functions. Methotrexate neurotoxicity may arise from three main mechanisms: the interference of methotrexate with the metabolism of adenosine, biopterins, or homocysteine.

There has been found to be a time elapse between the disease and the onset of cognitive deficits, with the decrease in IQ and learning first being noted at three years from the cessation of treatment with a further decrease at five years post treatment (Cousens et al. 1988; Brown et al. 1992; Mulhern et al. 1992), this suggests that the capacity for new learning is affected rather than the ability to retain already learned skills. New learning requires the ability to form new synapses. Hebb's rule (1949) states that a synapse that repeatedly becomes active at the same time when the postsynaptic neurone fires will cause changes in the structure or chemistry of that synapse that will strengthen it (synaptic plasticity). It has been revealed recently that nitric oxide plays an important role in synaptic plasticity and is necessary for new learning in experimental studies (Holscher 1997). I therefore propose to study CNS nitric oxide metabolism in acute lymphoblastic leukaemia.

1.2 Biochemistry

Nitric oxide is a free radical gas essential for normal function of CNS (Dawson et al. 1992). However in excess it is toxic (Lipton et al. 1998). In this section I am going to discuss the synthesis of NO, the molecular isoforms of NOS, the substrates and products of NO synthesis and the cofactors required.

Central to the pathway in figure 1.1 is tetrahydrobiopterin (BH₄), which is an essential co-factor for nitric oxide synthases (NOS). NOS catalyses
the formation of NO from arginine and molecular oxygen. BH$_4$ is also an essential co-factor for several mono-oxygenases that shown in figure 1 is phenylalanine mono-oxygenase. BH$_4$ is derived from two sources: de novo synthesis from guanosine triphosphate along a methotrexate insensitive pathway and salvage of oxidised biopterin intermediates. The neurotoxicity of methotrexate may be mediated in part by its interference with the recycling of BH$_4$. The salvage of BH$_4$ from oxidised biopterin species occurs along at least three pathways:

(1) quinonoid dihydrobiopterin (qBH$_2$) is reduced to BH$_4$ by dihydropteridine reductase (DHPR) in an NADPH- (or NADH-) dependent reaction (Kaufman S 1964);
(2) 7,8-dihydrobiopterin (7,8-BH$_2$), which is a nonenzymatic conversion product of qBH$_2$, is reduced to BH$_4$ by DHPR, also requiring NADPH (Kaufman S 1967); and
(3) qBH$_2$ may be reduced to BH$_4$ by the enzyme 5,10-methylenetetrahydrofolate reductase (MTHFR) in the presence of 5-methyltetrahydrofolate (5CH$_3$FH$_4$) in a reaction that regenerates 5,10-methylenetetrahydrofolate (5,10CH$_2$FH$_4$) (Matthews and Kaufman 1980).

Methotrexate has been demonstrated to inhibit the enzyme DHPR (Craine et al 1972; Liang et al 1981), that is believed to be the main source of BH$_4$ salvage. Dihydrofolate reductase activity can readily be inhibited by methotrexate, and this may also block a minor pathway for BH$_4$ regeneration from 7,8-BH$_2$. Finally the most intriguing, the depletion of reduced folate intermediates resulting from methotrexate administration may result in decreased conversion of qBH$_2$ to BH$_4$ by the enzyme MTHFR because of a lack of 5-methyltetrahydrofolate (Matthews and Kaufman 1980).
Figure 1.1. An overview of the biochemical pathway studied in this project.
(BH$_4$ - tetrahydrobiopterin, qBH$_2$ - quinoid dihydrobiopterin, Phe - phenylalanine, Tyr - tyrosine, Arg - arginine, Cit - citrulline, ONOO$^-$ - peroxynitrite, NO-Tyr - 3-nitrotyrosine)
1.2.1 Pterin metabolism in ALL

It has been noted (Leeming et al. 1976) that administration of methotrexate causes an increase in serum total biopterin concentration (total biopterin is the sum of biopterin, BH$_4$ and BH$_2$), which is maintained for up to two weeks. Further work has revealed that this increase in total biopterin is due to the accumulation of BH$_2$ as methotrexate inhibits its recycling back to BH$_4$ (Heales and Hyland 1989).

In addition, clearance of phenylalanine in methotrexate treated patients is slower than normal (Goodfriend and Kaufman 1961) clearly indicating the impact of methotrexate on phenylalanine metabolism and suggesting a decrease in BH$_4$ availability. A rise in phenylalanine might also affect arginine availability to the brain by competition with its transport across membranes (Neame 1961). Such substrate and cofactor limitation might cause nitric oxide synthase to switch to the production of superoxide and peroxynitrite anions (Mayer and Werner 1995).

1.2.2 Evidence for nitric oxide’s role in acute lymphoblastic leukaemia

A pilot study performed by Surtees et al (1998) found that the stable breakdown products of nitric oxide, nitrate and nitrite were increased in eleven leukaemia patients on presentation of symptoms. As treatment progressed the levels of nitrite and nitrate were found to increase during the induction and consolidation periods of treatment then the levels slowly declined so by the end of treatment the levels were not significantly different from controls. Nitrate and nitrite are stable breakdown products of nitric oxide and the peroxynitrite anion (Hevel and Marletta 1994). In fasted humans the breakdown of nitric oxide and peroxynitrite anion is the major source of nitrate and nitrite in biological fluids (Rhodes et al. 1995). In rodents, brain nitrate and nitrite concentrations were found to correlate
accurately with brain nitric oxide synthase activity (Salter et al. 1996). Cisternal CSF concentrations were found to parallel those of brain and there was no effect of plasma concentrations upon brain concentrations (Salter et al. 1996).

The findings reported by Surtees et al (1998) imply that brain nitric oxide synthase activity is increased in children with acute lymphoblastic leukaemia and rises further during induction and consolidation therapy, but normalises by the end of treatment. It has been shown that serum TNFα concentrations are raised in children with acute lymphoblastic leukaemia, returning to normal with remission of the disease (Saarinen et al. 1990), and a substantial proportion of malignant cells from acute lymphoblastic leukaemia express TNFα and IL-1β messenger RNA (Kurzrock et al. 1993). Leukaemic cells have also been found to elaborate both TNFα and IL-1β (Kurzrock et al. 1993) and during destruction of the tumour mass in the early stages of treatment of ALL, it is likely that these are released. These cytokines might cause the induction of iNOS resulting in large quantities of nitric oxide being produced for sustained periods (Nathan and Xie 1994).

The time elapse between the disease ALL and the onset of cognitive deficits makes it appear that synaptic plasticity is affected. Nitric oxide's association with synaptic plasticity is thought to be through its interaction with N-methyl-D-aspartate (NMDA) receptors. The NMDA receptor is a class of glutamate receptor that is critical for development, learning and memory in the CNS (Hollmann and Heinemann 1994; McBain and Mayer 1994). However excessive activation of this receptor has been shown to contribute to the pathological processes of stroke, epilepsy and several neurodegenerative disorders (Choi 1988; Meldrum and Garthwaite 1990; Lipton and Rosenberg 1994).
1.3 Biochemistry of the NOS enzyme isoforms

Since the discovery of the first biological effects of nitric oxide, the pursuit of evidence of the ubiquitous involvement of this simple free radical in cell signalling and regulation has grown at a phenomenal rate. NO is a relatively long-lived free radical species, with high diffusibility and surprisingly selective reactivity, both of which are useful assets in its role as a signalling molecule. NO generated by constitutive isoforms of NOS plays a critical role in normal physiology by regulating vasomotor tone (Palmer et al. 1987), host defence against infectious agents, tumours and is also an essential neurotransmitter (Mayer et al. 1989). NO is produced by a family of nicotinamide adenine dinucleotide phosphate (NADPH)-dependent NOS enzymes which catalyse the conversion of L-arginine and O$_2$ to L-citrulline and NO.

The NOS enzyme functions as a dimer consisting of two identical monomers, which can be functionally (and structurally) divided into two major domains: a C-terminal reductase domain, and an N-terminal oxygenase domain (Leber et al. 1999) (figure 1.2). The C-terminal contains binding sites for one molecule each of NADPH, FAD and FMN, whereas the N-terminal binds haem and BH$_4$, as well as the substrate L-arginine. Between these two regions lies the calmodulin (CaM) binding domain, which plays a key role in both the structure and function of the enzyme. Three distinct isoforms of NOS have been cloned (Stuehr 1997): neuronal NOS (nNOS, type I), inducible NOS (iNOS, type II), and endothelial NOS (eNOS, type III). nNOS and eNOS are also referred to as constitutive NOS, while the expression of iNOS requires induction by microbial endotoxins or cytokines. With tightly bound calmodulin, iNOS is fully active at basal Ca$^{2+}$ levels, whereas constitutive nNOS and eNOS activity depends on the elevation of intracellular Ca$^{2+}$. Although derived from distinct genes and chromosomes, the three NOS isoforms share similarities in their structure and catalytic mechanisms. They also share regions of high homology namely the oxygenase and reductase domains,
but each isoform exhibits distinctive features, which reflect their specific in vivo functions.

Figure 1.2 Scheme of the domain structure of the NOS dimer, showing cofactor and substrate binding sites.

1.3.1 The NOS-catalysed reaction

Biosynthesis of NO involves a two-step oxidation of L-arginine to L-citrulline, with concomitant production of NO (figure 1.3). The reaction consumes 1.5 mol of NADPH, and 2 mol of oxygen per mol of L-citrulline formed. The reaction involves an initial hydroxylation of L-arginine, leading to the formation of $N^0$-hydroxy-L-arginine that can also act as a substrate for NOS (Griffith and Stuehr 1995). This is followed by oxidation of the intermediate, using a single electron from NADPH (Abu-Soud et al. 1997), to form L-citrulline and NO. Although this scheme represents the reaction assumed to be catalysed by NOS the enzyme is also capable of catalysing the production of additional products, notably superoxide anion (O$_2^-$), depending on the conditions (Heinzel et al. 1992; Mayer et al. 1991).
1.3.2 The NO synthase dimer

The two dimers (reductase and oxygenase) are capable of functioning independently under certain circumstances. However NO synthase activity is only carried out by the homodimer. The cofactor haem plays an essential role in the formation of the dimer. When absent, NOS exists as two monomers. These monomers however are unable to bind BH$_4$ or a substrate analogue and do not catalyse L-citrulline/NO production (Baek et al. 1993; Klatt et al. 1996; List et al. 1997). Haem is the sole cofactor for which there is an absolute requirement for the formation of active nNOS dimers (Klatt et al. 1996), it is also a key factor in eNOS dimerisation (Baek et al. 1993). The actual characteristics differ, but the haem plays a similar role in iNOS dimerisation (List et al. 1997).

Evidence indicates that BH$_4$ plays a critical role in increasing the rate of nitric oxide production, it enhances L-arginine binding and couples NADPH to L-arginine oxidation. It has been previously shown that nNOS and eNOS can form dimers in the absence of the cofactor BH$_4$ (Venema et al. 1997), iNOS dimerisation has been reported to require the presence of the pteridine (Baek et al. 1993), although dimers have been formed in
E. Coli in the absence of BH$_4$. Furthermore BH$_4$ stabilises the nNOS and eNOS dimers once formed, and also the iNOS dimer, though not to the same extent (Klatt et al. 1995; List et al. 1997; Mayer et al. 1997; Venema et al. 1997).

### 1.3.3 Calcium dependence

Dependence on Ca$^{2+}$ is a key distinguishing feature between the constitutive and inducible isoforms of NOS. eNOS and nNOS are both activated by an elevation in intracellular Ca$^{2+}$, followed by the subsequent binding of Ca$^{2+}$/CaM. In contrast, iNOS contains irreversibly bound CaM, and is hence largely independent of Ca$^{2+}$. Only the Ca$^{2+}$-bound formation conformation of CaM can activate the enzyme. This dependence on Ca$^{2+}$ may be ultimately due to the presence of an autoinhibitory sequence which is present in the FMN - binding region of the Ca$^{2+}$ - dependent isoforms, but is not found in iNOS (Salerno et al. 1997). The CaM-binding region in eNOS is directly involved in membrane association, specifically to anionic phospholipids such as phosphoserine, and this association prevents the binding of CaM to eNOS and hence catalytic activity (Venema et al. 1995). CaM binds to both the isolated reductase domain of nNOS as well as the full length enzyme, and stimulates the rate of electron transfer within the reductase domain (Abu-Soud et al. 1994). CaM is furthermore essential for the transdomain transfer of electrons to the haem (Abu-Soud and Stuehr 1993).

### 1.3.4 nNOS

nNOS is the largest of the three isoforms due to the addition of a 300 amino acid stretch at the N-terminus. This region contains a PDZ domain (named after three of the proteins in which it was first described (Kennedy 1995), also called a discs-large homologus region (DHR) or GLGF amino acid repeat), which is approximately 90-residues long. It is a protein-
recognition module responsible for the association of nNOS with other proteins containing this motif, including dystrophin at the sarcolemmal membrane (Brenman et al. 1995) and PSD-95, a channel-associated protein in the brain.

nNOS is capable of producing not only NO, but also $O_2^-$ and peroxynitrite. This unusual property is a consequence of the dimeric nature of the enzyme, in which the two subunits are able to function independently (Gorren et al. 1997). In fact, the purified nNOS dimer normally consists of one BH$_4$-containing subunit and one BH$_4$-free subunit, due to the large difference in binding affinity between the first and second BH$_4$-binding sites (Gorren et al. 1996). This negative cooperativity of BH$_4$ binding means that only one subunit will have BH$_4$ bound over a wide range of BH$_4$ concentrations (up to 1μM). This has important implications for the outcome of the catalytic reactions, since only at very high BH$_4$ concentrations will nNOS function purely as a NO synthase.

In the presence of low concentrations or the absence of L-arginine, the heme center of the oxygenase-domain of nNOS catalyses the uncoupled reduction of oxygen, leading to the production of $O_2^-$ and $H_2O_2$ (Heinzel et al. 1992; Mayer et al. 1991). Saturating L-arginine concentrations and the presence of sub-saturating levels of BH$_4$ lead to the simultaneous production of NO and $O_2^-$, by the BH$_4$-containing and the BH$_4$-free subunits respectively. These two products can react together to form peroxynitrite (Beckman and Koppenol 1996). The physiological outcome depends on levels of GSH and SOD (Mayer et al. 1998), as these compounds scavenge NO. Recent work using genetically engineered nNOS constructs has shown that the reductase domain of the nNOS enzyme generates superoxide by a calcium/calmodulin dependent mechanism (Miller et al. 1997). This gives evidence that both domains of the nNOS enzyme are capable of producing superoxide, though the physiological reason for this has not been elucidated yet.
1.3.5 eNOS

eNOS is active, submaximally, at the concentration of Ca\(^{2+}\) found in resting endothelial cells (around 100nM) (Presta et al. 1997). The enzyme is generally fully activated by an increase in intracellular Ca\(^{2+}\), or from release from intracellular stores. The activation of eNOS can be induced by hormones such as catecholamines and vasopressin, autacoids such as bradykinin and histamine, and platelet derived mediators such as serotonin and ADP, via receptor-mediated activation of G-proteins.

\(\text{O}_2^-\) generation from eNOS is dependent on the presence of Ca\(^{2+}\)/calmodulin, suggesting that \(\text{O}_2^-\) synthesis requires electron transfer from reductase domain to the oxygenase domain. The onset of \(\text{O}_2^-\) generation from eNOS appears to be triggered by a different mechanism compared with the other two NOS isoforms. nNOS and iNOS generate \(\text{O}_2^-\) under conditions of L-arginine depletion, therefore \(\text{O}_2^-\) synthesis is triggered by low levels of L-arginine. \(\text{O}_2^-\) generation from eNOS is not similarly affected by L-arginine, in the absence of BH4, \(\text{O}_2^-\) production from eNOS is essentially unchanged even in the presence of high levels of L-arginine (1mM). Conversely, BH4 blocks this \(\text{O}_2^-\) formation in a dose-dependent manner. Thus, eNOS-mediated \(\text{O}_2^-\) generation is triggered and controlled by decreased availability of BH4 rather than L-arginine.

1.3.6 iNOS

iNOS in cells of macrophage/monocyte lineage generate functionally important amounts of \(\text{O}_2^-\) and NO, leading to \(\text{ONOO}^-\) formation. \(\text{O}_2^-\) synthesis from iNOS mainly occurs at the flavin-binding sites of its reductase domain. This is different from the process of \(\text{O}_2^-\) generation from nNOS, which is thought to occur primarily at the oxygenase domain. iNOS and nNOS exhibit very different L-arginine-dependent inhibition of
$\text{O}_2^-$ generation. Inhibition of $\text{O}_2^-$ generation from iNOS is seen only at high L-arginine concentrations. Whereas 100$\mu$M L-arginine can totally block $\text{O}_2^-$ generation from nNOS, iNOS mediated $\text{O}_2^-$ formation is essentially unaltered by 100$\mu$M L-arginine.

1.3.7 Peroxynitrite inhibition of the NOS enzymes

Studies looking at the feedback mechanism of nNOS have shown that nNOS is also inhibited by peroxynitrite (Huhmer et al. 1996). One mechanism of this feedback has been elucidated and involves the regulatory protein CaM. This protein contains nine methionine residues, all of which are susceptible to oxidation by peroxynitrite. The oxidation of Met-36 was found to significantly reduce CaM's ability to stimulate nNOS. Calmodulin is therefore a target for chemical oxidation by peroxynitrite, though these experiments were performed on a model system and how this would relate to physiological processes would depend on the flux of peroxynitrite formation and the presence of antioxidants. However calmodulin oxidation may be physiologically relevant process as calmodulin isolated from the brains of aged rats have been shown to contain high levels of methionine sulfoxide.

Robinson et al (2001) recently showed that peroxynitrite also has an inhibitory action on iNOS NO production, this was shown to be a dose-dependent inhibition. Both peroxynitrite and the peroxynitrite donor SIN-1 inhibited NO accumulation. The mechanism of iNOS inhibition by peroxynitrite has been previously shown not to involve the CaM protein (Huhmer et al. 1997). CaM is tightly bound to the iNOS enzyme and this binding seems to offer protection to the methionine residues in the CaM protein, which seems to be due to the competitive reaction of ONOO$^-$/ONO$OOH$ with iNOS instead of CaM. Even when the iNOS/CaM complex was reacted with high concentrations of peroxynitrite, none of the methionine residues were found to be oxidised.
Given the number of other potential amino acid targets for peroxynitrite, including cysteine, methionine, tryptophan, tyrosine and the number of protein sequences that can effect iNOS function, it is not surprising that peroxynitrite could interact with iNOS and alter its function. This would create a negative feedback loop at inflammatory sites where the generation of superoxide occurs and may be one mechanism of attenuating iNOS activity in vivo. The other NOS enzymes eNOS and nNOS both have inhibitory feedback mechanisms involving NO, but in the case of iNOS, additional NO was found to have no effect on enzyme activity. This is probably due to the fact that iNOS produces >1000 fold higher levels of NO than eNOS or nNOS, therefore it is probable that the NO inhibitory sites are saturated and increasing the concentration of NO has little effect on the enzymes activity.

The combined evidence of increased levels of nitrite and nitrate in the initial stages of acute lymphoblastic leukaemia and that methotrexate can reduce the availability of BH₄ and L-arginine for the NOS enzyme, suggests that peroxynitrite might be produced in significant quantities in acute lymphoblastic leukaemia. The production of peroxynitrite during the development of the disease may have numerous effects as the reactivity of peroxynitrite and other NO free radicals are very complex.

1.4 Peroxynitrite

Peroxynitrite is a reactive and short-lived species that promotes oxidative molecular and tissue damage (Beckman et al. 1990;Ischiropoulos et al. 1992;Radi et al. 1991). In addition to the generation of a pro-oxidant species, the formation of peroxynitrite results in decreased bioavailability of NO, therefore diminishing both its physiological functions (Ignarro 1990;Moncada and Higgs 1993) and its strong antioxidant action over free radical and metal-mediated processes (Radi 1996;Rubbo et al. 1994).
1.4.1 Peroxynitrite formation reactions

The biological formation of peroxynitrite anion (ONOO\(^-\)) is mainly due to the fast reaction between NO\(^-\) and O\(_2\)^\(^-\). This radical-radical combination reaction undergoes with a second order rate constant that has been independently determined as 4.3, 6.7 and 19 x 10\(^9\) M\(^-1\)s\(^-1\) (Goldstein and Czapski 1995; Huie and Padmaja 1993; Kissner et al. 1997), and therefore one can safely assume a value of \(= 10^{10} \text{M}^{-1}\text{s}^{-1}\), which indicates a diffusion controlled reaction.

Since both precursor radical species, NO\(^-\) and O\(_2\)^\(^-\) are transient in nature, the biological formation of peroxynitrite requires the simultaneous generation of both radicals which, in addition, must approach and react within the same compartment. However, while NO\(^-\) has a biological half-life in the range of seconds and readily diffuses across membranes (Denicola et al. 1996b; Lancaster, Jr. 1994), O\(_2\)^\(^-\) lasts less than milliseconds and permeates membranes only via anion channels (Fridovich 1995). Thus, due to both the greater half-life and facile diffusion of NO\(^-\) compared to O\(_2\)^\(^-\), peroxynitrite formation will predominantly occur nearer to the O\(_2\)^\(^-\) formation site.

The peroxynitrite anion exists in protonation equilibrium with peroxynitrous acid (ONO\(_2\)H, pKa = 6.8) (Radi et al. 1991). Thus under biological conditions both ONOO\(^-\) and ONOO\(_2\)H will be present, the ratio depending on local pH (i.e. At pH 7.4 80% of peroxynitrite will be in the anionic form). This is relevant because both species have different reactivities and diffusional properties.
1.4.2 Peroxynitrite diffusion

Due to target molecule reactions, the biological half-life of peroxynitrite is estimated to be less than 100 ms (Lymar et al. 1996;Radi 1998). This half-life is long enough for peroxynitrite to potentially travel some distances (e.g., 5-20 nm) across extra- and/or intracellular compartments. However, in addition to the estimated diffusion in aqueous environments, the biological effects and detection of peroxynitrite will be influenced by its ability to permeate cell membranes. In this regard, both ONOO− and ONOOH can cross biological membranes, via anion channels and passive diffusion, respectively (Denicola et al. 1998;Marla et al. 1997).

The diffusion distances of peroxynitrite will critically influence the distribution of oxidative modifications within a tissue and the reaction yields with reporter molecules.
1.4.3 Reactivity of Peroxynitrite

Figure 1.4 Peroxynitrite reaction pathways. Numbers I to V indicate possible fates of peroxynitrite: direct reactions include the one-electron oxidation of transition metal centres (Fe, Mn, Cu) (I); the two-electron oxidation with a target substrate (RH) (II) and the formation of nitroso-peroxocarboxylate (III), that rapidly decomposes to secondary radicals in 35% yield. Peroxynitrous acid undergoes homolysis at 0.9 s\(^{-1}\) to yield free radicals in 30% yields (IV) or rearrange to nitrate (V).
Peroxynitrite is a potent one-electron and two-electron oxidant and can therefore oxidise protein and non-protein sulfhydryls. Peroxynitrite is also a nitrating agent; this is supported by the observation that both 3-nitrotyrosine and 8-nitroguanine are found in cells that have been exposed to peroxynitrite.

Peroxynitrite promotes its biological effects via different types of reactions (fig 1.4), which could be classified in three main groups: (1) direct redox reactions (I and II), (2) reaction with carbon dioxide (III) and (3) homolytic cleavage of ONOOH (IV). In the direct reactions (e.g. Interactions with metal centres, thiol oxidation), peroxynitrite can promote one- or two-electron reactions.

1.4.4 Peroxynitrite Modulators

The most important modulator of peroxynitrite, both in vitro and in vivo, is carbon dioxide. The rate constant for the reaction of peroxynitrite anion with carbon dioxide is $3 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$ (Denicola et al. 1996a); this indicates that CO$_2$ greatly accelerates the decomposition of peroxynitrite. This is one of the fastest reactions known for peroxynitrite. Given the high concentrations of CO$_2$ (up to 1 mM) in biological fluids, it is believed that the reaction of peroxynitrite with CO$_2$ will be the predominant pathway for decay of peroxynitrite in vivo. The initial intermediate for this reaction is postulated to be the nitroso-peroxycarbonate ion, $O=N$–OOCOO$^-$, which may rearrange to the nitrocarbonate ion, $O_2N$–OCO$_2^-$. This compound then undergoes homolysis to a carbonate radical (CO$_2^*$) and nitrogen dioxide (NO$_2^*$) in 35% yields (Bonini et al. 1999; Lymar and Hurst 1998). The carbonate radical is a relatively strong one-electron oxidant and also a nitrating agent; therefore the radical products arising from ONOOOCOO$_2^-$ decomposition promote secondary oxidation events. The remaining 65% undergo homolysis to form nitrate and carbon dioxide, nitrate is an
unreactive molecule which can be used as a measurement of peroxynitrite activity.

The other main reaction mechanism is the homolysis of ONOOH to OH and NO₂⁻, with a first order rate constant of 0.9 s⁻¹ at pH 7.4 and 37°C in; 30% yields, while the rest of ONOOH isomerises directly to nitrate (NO₃⁻) (Augusto et al. 1994; Beckman et al. 1990; Merenyi and Lind 1998). In the absence of targets, the proton- or carbon dioxide-catalysed decomposition of peroxynitrite mainly yields nitrate, due to recombination of the radical intermediates arising from homolysis. However, in the presence of targets, most peroxynitrite yields nitrite (NO₂⁻).

An important aspect to unravelling the formation of peroxynitrite in biology is to recognise the differential reactivities (and effects) of NO⁻ and peroxynitrite over cell and tissues. In particular, NO⁻ is neither a strong oxidant nor a nitrating agent; it mostly participates in reversible interactions with iron centres, radical-radical combination reactions (e.g., with lipid radicals to terminate lipid oxidation chain reactions, with O₂⁻ to form peroxynitrite) and nitrosylation reaction via intermediate formation of dinitrogen dioxide (N₂O₃). On the other hand, peroxynitrite is a strong oxidant and nitrating agent and a poor nitrosylating agent. Thus, several oxidation and nitration reactions measured in probes or bimolecules secondary to NO⁻-formation reflect the presence of peroxynitrite.

1.4.5 Nitroxyl anion and the nitrosyl cation

Other possible nitrating agents that have to be taken into consideration are NO⁻ (nitroxyl anion) and NO⁺ (nitrosyl cation). The species nitroxyl anion and the nitroxyl cation are related to nitric oxide by reduction and oxidation of NO⁻ respectively, and each has distinctive chemistry unique to itself (Bonner and Stedman 1996). The nitroxyl anion can be formed in the cellular environment by several routes and it can exert direct and varied effects on biological molecules and systems. It has been
proposed that the nitroxyl anion may be formed directly from nitric oxide synthase and its chemistry is related to that of peroxynitrite as the nitroxyl anion reacts with dioxygen to give peroxynitrite (Arnelle and Stamler 1995; Butler et al 1995). The nitroxyl anion formation is catalysed by superoxide dismutase, it is also formed by cytochrome c and haemoglobin (Gow and Stamler 1998).

The nitroxyl ion is a short-lived species in solution, decomposing via dimerisation and dehydration to give nitrous oxide. The nitroxyl ion, however, will react with a variety of targets. The reaction with thiols is a good example of such reactions, nitroxyl's reaction with cysteine leads to the nitrosylation of this residue (Sharpe and Cooper 1998). This reactive anion does not however lead to the nitration of residues except through the formation of peroxynitrite.

The reaction to form peroxynitrite is an important factor to consider, the reaction of nitric oxide and superoxide is favoured by a factor of 300 when compared to the reaction of the nitrosyl anion with dioxygen (Beckman and Koppenol 1996). However, intracellular concentrations of superoxide are low, due to the efficient catalysis by superoxide dismutase. Basal release of superoxide from cultured endothelial cells is too low to be measured (Rosen and Freeman 1984). Concentrations of dioxygen could be up to a thousand times greater than those of superoxide, supporting the suggestion that the reaction between dioxygen and nitroxyl ion is a significant peroxynitrite source. However the rate of reaction between nitroxyl ion and dioxygen is difficult to estimate as there are competing reactions, for example with haem and iron-sulphur centres and dimerisation. Another factor involves the spin state of the nitroxyl ion, which means it can exist in the singlet or triplet states. Only the triplet state reacts with dioxygen, thus the magnitude of the pathway for peroxynitrite formation involving nitroxyl ion is difficult to place on a quantitative basis. Nevertheless, Sharpe and Cooper (1998) have produced evidence to show that the nitroxyl anion formed by reduction of NO• by ferrocytochrome c reacts with dioxygen to form
peroxynitrite. These results show that peroxynitrite can be formed in the absence of superoxide by the reaction between dioxygen and nitroxy ion.

The nitrosyl cation, $\text{NO}^+$, is the oxidised form of nitric oxide, is related to nitrous acid and is the key species in the process of nitrosation, in which the NO$^+$ group is transferred (usually from a carrier compound) to a nucleophilic center, often to a sulphur or nitrogen lone pair of electrons (Williams 1988; Ridd 1979). The nitrosyl cation is rapidly hydrolysed in aqueous solution to give nitrous acid. At pH values of biological relevance, nitrosation is most likely to occur through the action of NO$^+$ carriers such as S-nitrosothiols rather than through the action of the NO$^+$ ion itself as this ion is only found in solution at very high acidity. Nitrosyl complexes in which the nitrosyl group is formally NO$^+$, for example sodium nitroprusside may be very effective nitrosating agents at pH 7, particularly towards sulphur centres. Such NO$^+$ nitrosyl complexes may be formed intracellularly by reaction of NO with Fe(III) centres.

1.4.6 Peroxynitrite’s mediated reactions

Peroxynitrite has been presumed to be a mediator of cellular and tissue injury in various pathological situations. Its biological effects are due to its reactivity towards a large range of molecules including amino acids such as cysteine, methionine, tyrosine and tryptophan, nucleic bases and antioxidants (e.g. phenolics, selenium- and metal-containing compounds, ascorbate and urate).

1.4.6.1 Reaction with DNA

The treatment of DNA with peroxynitrite leads to both oxidisation and nitration and may potentially cause strand breakage due to attack on the sugar-phosphate backbone. Strand breakages have been observed in naked DNA treated with peroxynitrite at levels as low as 2-5μM (Salgo et
The most susceptible base in DNA is guanine, with 30% of the guanine reacting with peroxynitrite (Burney et al. 1999). The other bases do also react, but to a significantly smaller degree. The main products of the reaction of guanine with peroxynitrite are 8-nitroguanine (Yermilov et al. 1995) and 8-oxoguanine (Kennedy et al. 1997), though 8-oxoguanine will react further with peroxynitrite. The modification to 8-oxoguanine has been shown to cause G:C→ A:T transitions, no mutagenicity data is available on 8-nitroguanine.

1.4.6.2 Peroxynitrite reaction with Lipids and mitochondria

Peroxynitrite acts as an oxidative reagent in respect to lipids and is capable of modifying unsaturated phospholipids, glycolipids and cholesterol in cell membranes. This leads to lipid peroxidation which is a degenerative process that perturbs structure and function of the target system, often with cytopathological consequences (Girotti 1985; Girotti 1990; Halliwell and Gutteridge 1990; Lin and Girotti 1993). Lipid peroxidation has been linked to a variety of disorders, including atherogenesis, ischemia-reperfusion injury, and UV-induced carcinogenesis (Halliwell and Gutteridge 1990). It may also play a role in the cytotoxic effects of oxidant-based chemotherapeutic and phototherapeutic drugs (Girotti 1990).

Addition of peroxynitrite to mitochondria causes extensive protein modification and cross-linking, and lipid peroxidation. In turn this causes irreversible inhibition of respiration at a number of sites: complex I, complex II and complex IV (Barker et al. 1996; Bolanos et al. 1995; Brookes et al. 1998). This inhibition requires high levels of peroxynitrite (100-500 μM), though Radi et al. (1994) showed that addition of 1.3 mM peroxynitrite over a period of 10 minutes (resulting in a low micromolar steady-state level of peroxynitrite) caused the same level of inhibition as the bolus addition. The mechanism of this inhibition is very complex and involves peroxynitrite’s ability to oxidise lipids, DNA,
proteins and GSH, there is also evidence of damage being induced through the nitrosylation of proteins. Peroxynitrite also causes opening of the permeability transition pore (PTP) (Packer and Murphy 1995; Schweizer and Richter 1996), and opening of this pore causes loss of cytochrome C (Liu et al. 1996; Skulachev 1998) which may contribute to peroxynitrite-induced inhibition of respiration. Pore opening is also likely to uncouple the mitochondria by increasing the proton permeability. Because of these opening the PTP, this causes irreversible mitochondrial death. Peroxynitrite also caused rapid oxidation of mitochondrial NAD(P)H (Scarlett et al. 1996; Schweizer and Richter 1996) by unknown mechanisms (possibly direct oxidation), followed by hydrolysis of NAD by a cyclosporin-inhibitable mechanism (Schweizer and Richter 1996). The NAD(P)H oxidation might be related to the fact that peroxynitrite also rapidly oxidises mitochondrial glutathione (Scarlett et al. 1996).

1.4.7 Peroxynitrite's reaction with amino acids

As mentioned above peroxynitrite is capable of modifying DNA, lipids and mitochondrial-metabolism, these actions of peroxynitrite are important in the pathological processes of oxidative stress and have now been implicated in a number of diseases. The action of peroxynitrite on proteins and individual amino acids, however, is one of the least understood actions of peroxynitrite. Initially this interaction was thought to be purely detrimental and an extension of oxidative stress induced damage, but recent work has shown that it may play an important role in normal physiology. It is therefore important that the actions of peroxynitrite on amino acids and proteins are elucidated.

Peroxynitrite has previously been shown to initiate three types of reactions with amino acids: oxidation, nitration and nitrosylation. It is a potent oxidant when reacting with cysteine and methionine, however when reacting with tryptophan and tyrosine it has been shown to act as a
nitrating agent. The most reactive amino acids with peroxynitrite are cysteine, methionine and tryptophan.

### 1.4.7.1 Reaction with Methionine

Cysteine and methionine both contain sulphur groups, which are susceptible to attack by peroxynitrite. Peroxynitrite reacts with methionine in both a one-step oxidation, which leads to ethylene and a two-step reaction that gives methionine sulfoxide (MetSO) (Pryor et al. 1994). Methionine is readily oxidised to methionine sulfoxide by numerous oxidative species. This oxidation of surface methionine is thought to act as a protective mechanism to prevent the oxidation of other amino acids within a protein. The oxidation of methionine residues within the protein α-2-Macroglobulin has been studied in detail. This protein often acts at sites of inflammation where reactive oxygen and nitrogen species are at relatively high concentrations. This protein when initially studied was found to be resistant to oxidation by these species (Reddy et al. 1989), on closer examination of the protein it was found that the methionine residues were oxidised, but that this had no effect on function of the protein (Reddy et al. 1994). When continuously exposed to oxidative agents a single tryptophan residue was found to oxidise and with this there was a concomitant loss of antiproteinase activity. All the oxidised methionine residues were surface exposed (Keck 1996) and were clustered in an array around the entrance to the active site bay of glutamine synthetase. In other words the methionine residues are acting as guards, protecting the active site and possibly the susceptible tryptophan residue (Levine et al. 1999). Methionine sulfoxide can be reduced back to methionine by the enzyme methionine sulfoxide reductase (Brot and Weissbach 1983), providing a catalytic amplification of the antioxidant potential of each methionine residue. The potential of methionine sulfoxide reductase to contribute to a cells antioxidant defence is clear. In yeast the gene for methionine sulfoxide reductase was disrupted and the yeast cells found to be normal until challenged with
an oxidant challenge where a significantly higher fraction of the mutants died when stressed (Moskovitz et al. 1997). The antioxidant effects of methionine residues are starting to be elucidated and seem to play an important function in cell viability.

1.4.7.2 Reaction with cysteine

The sulphur group on cysteine residues can undergo both oxidation and nitrosylation reactions. The oxidation reaction is very similar to that mentioned above for methionine and may play a similar function (Quijano et al. 1997). Nitrosylation is the addition of NO to the sulphur group of the thiols homocysteine, cysteine, glutathione and N-acetylcysteine. The nitrosylation reaction seems to be a mechanism for extending the biological half-life of NO. NO has a very short half-life and two of its functions are vasodilation of blood vessels and inhibition of ADP-induced platelet aggregation. Previous work has shown that both low molecular weight S-nitrosothiols and S-nitrosoproteins have increased half-life’s (S-nitrosoproteins being the longest (Stamler et al. 1992)) and that both these compounds are capable of vasodilation of blood vessels and inhibition of ADP-induced platelet aggregation (Ignarro et al. 1981; Mellion et al. 1983). S-nitrosothiol group in proteins may serve as intermediates in the cellular metabolism or bioactivity of NO and their formation may represent an important cellular regulatory mechanism.
1.4.7.3 Reaction with tryptophan

Peroxynitrite's reaction with tryptophan yields nitro-tryptophan as the predominant product, in a transition metal-independent mechanism (Alvarez et al. 1996).

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\text{Figure 1.5 Reaction of tryptophan with peroxynitrous acid, leading to the formation of nitro-tryptophan.}
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The effects of this reaction have not been elucidated yet but nitrotryptophan formation can inhibit the formation of tryptophan-derived neurotransmitters (i.e. Serotonin). Nitration of critical tryptophan residues in proteins may interfere with function of the protein, both by affecting critical catalytic motifs or via deformation of the three-dimensional protein structure.

A single tryptophan residue in human Cu,Zn-superoxide dismutase has been found to nitrate when exposed to peroxynitrite (Yamakura 2001) in a potassium phosphate buffer containing 25mM sodium bicarbonate. Peroxynitrite-modified enzymes showed a 30% decrease in the original enzyme activity. The absence of carbon dioxide reduced the nitration of the tryptophan residue by 20%, carbon dioxide seems to stimulate the nitration of the tryptophan residue, probably due to the formation of the nitrocarbonate ion.

None of the reactions initiated by peroxynitrite mentioned above are useful as a marker for peroxynitrite activity. The oxidation of methionine or cysteine residues is not specific to peroxynitrite, as there are many
oxidising reagents capable of modifying these two residues. The nitrosylation of cysteine etc. is also non-specific as other nitrating agents exist. The products formed by these two types of reaction are also transient in nature and therefore are not a good product to measure peroxynitrite activity. The nitration of tryptophan does seem at present to be specific to the actions of peroxynitrite, but this amino acid is only present at low concentrations in most proteins (1.1% mol), reducing its usefulness as a marker for peroxynitrite activity. The next reaction of peroxynitrite is the nitration of tyrosine residues, tyrosine residues have a higher abundance in proteins and the reaction is more specific to peroxynitrite, therefore the main product of the reaction 3-nitro-tyrosine is often used as a marker of peroxynitrite activity.

1.5 Tyrosine Nitration

Tyrosine nitration by peroxynitrite is closer to constituting a specific footprint then the other reactions described above as NO⁻ is incapable of directly promoting nitration reactions. However it has become apparent that other mechanisms of biological nitration may exist in addition to peroxynitrite, including (i) the H₂O₂-NO₂⁻-hemeperoxidase (Eiserich et al. 1998) (ii) the reactions of NO₂⁻ (formed from the oxidation of NO) with tyrosine residues (Squadrito and Pryor 1998) and (iii) the oxidation of unstable nitrosotyrosine (Gunther et al. 1997). Importantly, these alternative mechanisms of nitration appear to be more restricted than the nitration mediated by peroxynitrite. Indeed, the peroxidase mechanism requires the presence of a specific enzyme (e.g., myeloperoxidase or eosinophil peroxidase) in the site of formation of oxygen radicals and NO⁻. This may be limited to those tissue regions or compartments and processes involving an important participation of activated inflammatory cells.

Tyrosine nitration is a common modification made by protein chemists using tetranitromethane to probe the function of tyrosine residues in vitro
Nitrogen dioxide, which is a common component in cigarette smoke, will nitrate tyrosine by a two-step mechanism (Prutz et al. 1985). A single nitrogen dioxide molecule is required to combine with tyrosine to form 3-nitrotyrosine. This mechanism occurs readily when gaseous nitrogen dioxide is bubbled over protein solution, but slows rapidly when nitrogen dioxide becomes dilute because the mechanism depends upon the square of nitrogen dioxide concentration. Furthermore, nitrogen dioxide is a promiscuous oxidant like hydroxyl radical and will attack a wide range of substrates with a similar rate constants. As a consequence, inclusion of a small amount of ascorbate or glutathione will effectively stop tyrosine nitration in vivo. Nitric oxide itself does not produce substantial amounts of nitrogen dioxide in vivo because the rate of reaction with oxygen is extremely slow at physiologically relevant concentrations of nitric oxide. Consequently, the formation of nitrogen dioxide is not likely to be a major source of tyrosine nitration in vivo.

Finally the rapid reaction of tyrosyl radical with NO transiently yields nitrosotyrosine, in a reaction that appears to be reversible. In the case of free tyrosine, most nitrosotyrosine dissociates back to tyrosyl radical and NO to ultimately yield dityrosine (Goldstein et al. 2000). However in some proteins containing adjacent redox centres (e.g., prostaglandin synthase) (Gunther et al. 1997), protein-bound nitrosotyrosine may be further oxidised to 3-nitrotyrosine by a site-specific one-electron oxidation to iminoxyl radical, followed by a second one-electron oxidation nitrotyrosine. This mechanism would be limited to a small number of proteins.

Peroxynitrite promotes both the oxidation and nitration of tyrosine residues. The addition of peroxynitrite to free tyrosine leads to the formation of 3-nitrotyrosine and dityrosine (van der Vliet et al. 1995). 3-hydroxy-tyrosine is also formed in small quantities, following the addition reaction of OH with tyrosine (Ramezanian et al. 1996). The oxidation reactions can also be accomplished by other oxidant systems such as OH directly and oxo-iron complexes. HPLC analysis of the products
formed from the reaction of peroxynitrite with tyrosine have noted the presence of other products that have not been identified. These by-products probably include dinitrated tyrosine and other combinations of oxidation and nitration reactions.

The nitration process involves free radical mechanisms in which one-electron oxidants derived from peroxynitrite attack the aromatic ring leading to the formation of tyrosyl radicals, which then rapidly combine with NO· to yield 3-nitrotyrosine.
Figure 1.6 Tyrosine oxidation pathways by peroxynitrite. Compounds shown are (i) tyrosine, (ii) tyrosyl radical, (iii) tyrosine-hydroxyl radical adduct, (iv) dityrosine, (v) 3-nitro-tyrosine and (vi) 3-hydroxytyrosine. All of these structures can be yielded during the reaction of peroxynitrite-derived oxidants with tyrosine. Both CO$_3^-$ and NO$_2$ can perform a one-electron abstraction of tyrosine to yield tyrosyl radical, while OH predominantly leads to the formation of a radical adduct that can either decay by water elimination to tyrosyl radical, or be oxidised to 3-hydroxy-tyrosine.
Peroxynitrite does not react with tyrosine at any appreciable rate in the absence of carbon dioxide (Alvarez et al. 1999). The formation of the intermediate nitrocarbonate ion is an important factor in the nitration of tyrosine residues.

1.5.1 Effect of carbonate on tyrosine nitration

Peroxynitrite can be formed and mediate reactions in the extracellular environment (White et al. 1994). Since reticuloendothelial cell-derived superoxide is primarily produced by the plasma membrane-bound NADPH oxidase (Gabig and Babior 1979) and intracellular derived nitric oxide readily diffuses to the extracellular space (Denicola et al. 1996b; Lancaster, Jr. 1994). In endothelial cells, peroxynitrite can be formed in both intra- and extracellular compartments because these cells are rich sources of both NO and $O_2^{-}$ generation, with the extracellular release or generation of $O_2^{-}$ being readily detectable, especially in instances of metabolic stress (White et al. 1994). The participation of peroxynitrite in vascular dysfunction and oxidative stress in the intravascular space and interstitial compartments has been demonstrated in atherosclerotic human coronary arteries and vessels of hypercholesterolemic animal models (White et al. 1994).

One of the most abundant constituents of the extracellular milieu is the inorganic anion bicarbonate, present at a concentration of 25mM. The HCO$_3^{-}$/CO$_2$ system is one of the major blood buffers. The yield of tyrosine nitration by ONOO$^{-}$ at pH 7.4 has been shown to increase twofold in the presence of 10mM bicarbonate (Berlett et al. 1998). The rate of aromatic nitration increases in the presence of bicarbonate to a similar extent as the decomposition of ONOO$^{-}$. This indicates that the rate-limiting step is the reaction of ONOO$^{-}$ with CO$_2$. It has also been shown that the nitration of tyrosine residues within the protein albumin is increased in the presence of 10mM bicarbonate (Gow et al. 1996). The
presence of CO$_2$ also inhibits the oxidation of methionine residues supporting the theory that the nitrating and oxidating effects of peroxynitrite are instigated through the formation of two different reactive products of peroxynitrite decomposition.

### 1.5.2 Nitration of free tyrosine

3-nitrotyrosine has been measured in numerous samples including plasma, CSF and various tissues. Tohgi et al (1999) measured the concentration of tyrosine and 3-nitrotyrosine in control subjects and Alzheimer patients (AD). It was found that control subjects had a 3-nitrotyrosine concentration ranging from 0.2 to 2.3 nM, and tyrosine concentration from 4.8 to 7.9 μM, the 3-nitrotyrosine/tyrosine ratio was $0.5 - 3.7 \times 10^{-4}$. In control subjects both the 3-nitrotyrosine and 3-nitrotyrosine/tyrosine ratio were found to increase with age, the tyrosine concentration was found to remain unaltered. In patients with AD, tyrosine concentration did not change significantly with age. However, both 3-nitrotyrosine and 3-nitrotyrosine/tyrosine ratio were significantly raised compared to age-matched controls. These results show that 3-nitrotyrosine in CSF moderately but significantly increased in AD patients. As the free tyrosine concentration didn't decrease the increase in 3-nitrotyrosine did not appear to be due to nitration of free tyrosine. Rather the increase in 3-nitrotyrosine was due to an increase in proteins or an increase in degradation of 3-nitrotyrosine containing proteins.

Studies measuring the nitrite and nitrate levels in CSF of Alzheimer patients have found conflicting results ranging from a decrease to an increase in the levels. The levels of nitrite and nitrate, however, are stage dependent with increases in levels being observed in the early phase of AD and this then decreasing back to control levels as disease progresses. The increase in 3-nitrotyrosine may reflect this early increase in nitrite and nitrate, during which tyrosine residues within
proteins may become nitrated, and then the subsequent degradation of these nitrated proteins will increase the levels of free 3-nitrotyrosine.

1.5.3 Tyrosine nitration in proteins

Numerous studies of nitration of tyrosine residues have now been performed, the initial studies have used an in vitro method and have revealed some of the mechanisms of tyrosine nitration.

Souza et al (1999) indicated that neither the number of tyrosine residues nor the abundance of the protein determines the protein target that is modified by nitrating agents in vitro. In their 1999 study they determined the effects of nitrating agents on three model proteins, lysozyme, ribonuclease A (RNAse A), and phospholipase A2. These proteins were selected because they have similar molecular weight but differing percentages of tyrosine residues. They found that not all the tyrosine residues were available for nitration, tyrosine residues not exposed to the solvent phase were not nitrated. In contrast to the other two aromatic residues, phenylalanine and tryptophan, and the nonpolar side chain amino acids, tyrosine residues do not pack into the interior of proteins. The fraction of tyrosine residues buried at least by 95% in proteins is 0.15 which is similar to charged amino acids (glutamate, 0.18) compared to 0.5 for phenylalanine, 0.27 for tryptophan, and 0.54 for valine. Most tyrosine residues exposed to the surface were a target for nitration. However, few exposed tyrosine residues were not highly susceptible to nitration. Analysis of the residues that were susceptible to nitration found that there was an absence of cysteine and methionine residues, both of these residues being alternative targets for peroxynitrite modification. The secondary structure also appears to play an important role of nitration susceptibility, the nitrated tyrosine residues were shown to be found mainly in loop structures. Peptides with a glutamate at position -1 relative to the tyrosine residue have been found to show the highest efficiency for nitration. The precise role of the negative charge in
directing nitration to a neighbouring tyrosine is not clear. Suggestions made have been that the carboxyl group of glutamate may react with peroxynitrite forming an acetyl nitrate intermediate that is the proximal agent responsible for nitrating tyrosine residue(s) in bovine Cu,Zn superoxide dismutase (Ischiropoulos et al. 1992). Crow et al (1997) suggested that the carboxyl group of acidic residues facilitates the nitration of nearby tyrosine residues by hydrogen bonding with one of the two equivalent hydrogen atoms at the ortho position of the tyrosine. An alternative suggestion made by Souza et al (1999) is that the electrostatic repulsion of negatively charged nitrating agents by the carboxyl group directs the nitrating agent toward the aromatic ring of the neighbouring tyrosine. This will effectively increase the local concentration of the nitrating agent over that of the bulk solvent in the vicinity of the tyrosine residue leading to selective nitration. Conversely the presence of positively charged residues in the vicinity of tyrosine residues is expected to reduce the local concentration of nitrating agent. The interaction of negatively charged residues with a positive charge results in protonation and rapid decomposition of the nitrating agent before its encounter with the aromatic ring of tyrosine.

Protein Kinase C (PKC) an important intracellular signalling molecule whose activity is essential for a number of aspects of neuronal function including proliferation, differentiation, immune response, transcriptional regulation, synaptic plasticity and learning and memory has also been found to be susceptible to nitration (Knapp et al. 2001). The regulation of this protein by peroxynitrite is complex, low levels of peroxynitrite (1 μM) activates PKC in hippocampal homogenates but not in purified preparations, suggesting that peroxynitrite interacts with a component in the homogenate. Products of lipid oxidation have been shown to increase PKC activity and this may be the mechanism peroxynitrite is acting via at low concentrations. At higher peroxynitrite concentrations PKC activity was reduced in both hippocampal homogenate and purified rat brain PKC. This reduction in activity by 250 μM peroxynitrite was correlated to the nitration of tyrosine residues on PKC, though low
concentrations of peroxynitrite (100 μM) were also found to nitrate tyrosine residues. This suggests that as the concentration of peroxynitrite increases a less accessible, critical tyrosine residues becomes nitrated or that there are a critical number of tyrosine residues that must become nitrated before a detrimental effect is observed. There is evidence that PKC activity is decreased in a number of neurodegenerative diseases such as AD, amyotrophic lateral sclerosis, Huntingdon disease and Parkinson disease, which makes it reasonable to hypothesise that loss of PKC function may contribute to the etiology of these disorders.

Other studies looking at tyrosine nitration in vitro have studied the susceptibility of a wide range of proteins, and these studies allow detailed study of the process of nitration. With the use of techniques such as mass spectrometry the nitrated tyrosine can be identified and its position and role in the protein monitored, but these studies don't reflect the in vivo situation. For example the protein albumin has been shown to be nitrated in vitro, 3 tyrosine residues are susceptible to nitration, but when mixed with other more susceptible proteins its nitration rate is very low, it is therefore important to monitor the nitration of proteins in vivo. It is also important to be able to relate the nitration of a tyrosine residue with a change in function.

1.5.4 Protein nitration in animal models of disease

The recent development of an antibody to 3-nitrotyrosine has now allowed nitrated proteins to be monitored in vivo. Protein 3-nitrotyrosine has been detected in a number of human and animal models of disease (Amirmansour et al. 1999). Studies have identified specific proteins modified by nitration in animal and cellular models of disease. The SERCA2a isoform of sarcoplasm reticulum Ca-ATPase was found to be nitrated and inactivated in aged rat skeletal muscle (Viner et al. 1996). Stimulation of rat mesangial cells with interleukin-1β results in the
selective nitration and inactivation of prostacyclin synthase (Zou et al. 1997).

Tyrosine hydroxylase, the rate limiting step in catecholamine synthesis, is modified by nitration in the striatum of mice in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) model of Parkinson’s disease (Ara et al. 1998). The nitration of a single tyrosine residue in tyrosine hydroxylase results in loss of enzymatic activity and a concomitant decrease in the dopamine levels in the brain of these mice. Nitration of tyrosine residues in vitro results in inactivation of a vast number of mammalian proteins whose activity is dependent on the tyrosine residues. Nitration of the tyrosine residue results in a marked shift of the local pKₐ from 10.07 of the OH group of tyrosine to 7.5 of 3-nitrotyrosine that is expected to change the hydrophobicity, hydrogen bonding, and electrostatic interactions within the protein. The pKₐ provides the biological explanation for the inhibition in the rate of phosphorylation by tyrosine kinases as well as for the inactivation of protein function.

These results indicate that not all proteins are modified by nitration in vivo. These observations also indicate that selective nitration of proteins, quite often nitration of a single tyrosine residue, is sufficient to induce changes in function of critical proteins. The alterations in function may contribute to the pathogenic mechanism of disease.

1.5.5 Protein nitration in human disease

Recent studies have also demonstrated widespread nitration of tyrosine residues in the brain of both Alzheimer and Parkinson patients. When transition metal ions or certain metalloproteins such as superoxide dismutase (SOD) are present, the rate of tyrosine nitration is high. This nitration has been suggested to be pathologically important in certain neurodegenerative diseases. Aoyama et al (2000) identified that manganese superoxide dismutase (Mn-SOD) isolated from CSF was
nitrated in neurological patients with Parkinson’s, Alzheimer’s and amyotrophic lateral sclerosis. Mn-SOD is a primary antioxidant enzyme that functions to remove superoxide radicals in mitochondria. Nitration of the Tyr-34 residue of Mn-SOD leads to inactivation of the enzyme. This residue has a specific position in the three-dimensional structure of human Mn-SOD. Structural studies on human Mn-SOD along with other studies on other bacterial Fe-SODs, Mn-SODs and cambialistic SOD, show that Tyr-34 is conserved in all these SODs and that the phenolic oxygen atom of Tyr-34 is located 5.4 Å from manganese at the vertex of the substrate funnels. These funnels are proposed to lead the substrate O$_2^-$, from the bulk solvent toward the metal ions by an electrostatic guidance mechanism. Peroxynitrite may be introduced to manganese through a similar mechanism as O$_2^-$. The peroxynitrite may react with manganese to form an active nitrating species, probably nitronium ion (NO$^{2+}$), this cation then reacts with the adjacent tyrosine residue directly. A pathologically significance of the nitration of the tyrosine residue in Mn-SOD has been suggested. Although physiological concentrations (<1 μM) of NO reversibly inhibit cytochrome oxidase in an oxygen concentration-dependent manner (Brown and Cooper 1994) and therefore NO has been proposed as a regulator of mitochondrial respiration under these conditions (Poderoso et al. 1996; Takehara et al. 1996), a higher concentration of NO (5-200 μM) is proposed to cause mitochondrial dysfunction (Cassina and Radi 1996). In the latter case, NO will compete with Mn-SOD for mitochondrially produced O$_2^-$, production of which may be enhanced by the inhibition of cytochrome oxidase with NO, to form peroxynitrite. Nitration and inactivation of Mn-SOD by peroxynitrite could be a turning point in this mitochondrial dysfunction, because inactivation of Mn-SOD leads to accumulation of O$_2^-$ and as a consequence more accumulation of peroxynitrite. Both accumulated O$_2^-$ and peroxynitrite may cause oxidative damage of mitochondrial components. Nitration and inactivation of Mn-SOD has also been observed in a tissue homogenate of transplanted allogripha during chronic rejection (MacMillan et al. 1996).
The presence of tyrosine nitration has also been reported in acute respiratory distress syndrome (ARDS) (Gole et al. 2000). In this study, nitrated proteins were isolated using immunoprecipitation from the plasma of 12 ARDS patients. These proteins were then visualised using Western blotting and 5 main protein bands were observed, these bands corresponded to the proteins ceruloplasmin, transferrin, α1-antichymotrypsin, α1-protease inhibitor, and β-chain fibrinogen and appear to fall into three major categories: proteins that regulate metal binding, proteolysis, and coagulation. The activities of these proteins are critical for maintaining normal homeostasis and protecting the lung from oxidative injury. Some of these proteins may be acting in a similar way to the methionine residues in α-2-macroglobulin, mentioned previously, and may be protecting the more susceptible proteins at low concentrations of peroxynitrite. Exposure of plasma proteins to nitrating agents in vitro resulted in the inhibition of function (α1-protease inhibitor), a gain of function (fibrinogen), or no effect (α1-antichymotrypsin, transferrin). The exposure of fibrinogen to nitrating agents results in the acceleration of the interaction with thrombin and clot formation. Both ceruloplasmin and α1-protease inhibitor are sensitive to oxidation, and, therefore, it is likely that oxidation and nitration may contribute to the loss of function. Nitration of α1-protease inhibitor has been shown to inactivate the inhibitory function of this protein toward elastase but not toward trypsin and chymotrypsin inhibitory activity. However, exposure of α1-protease inhibitor to peroxynitrite in the absence of CO₂ was also shown to inactivate the elastase activity by oxidation of methionine residue(s). In contrast to the loss of elastase activity, the function of α1-antichymotrypsin is not inhibited by exposure of nitrating agents and the α1-antichymotrypsin activity in the plasma of patients with ARDS did not vary from that of control.

Fibrinogen, transferrin, plasminogen and ceruloplasmin have also been identified as being nitrated in smokers and lung cancer patients (Pignatelli et al. 2001), fibrinogen was also found to be oxidised in these subjects.
The level of nitrated proteins were found to be significantly higher in lung cancer patients than smokers.

1.5.6 Tyrosine nitration’s effect on protein degradation

Nitrated proteins have been detected in post-mortem brain lesions associated with major neurodegenerative disorders, and in other major pathologies of lung, heart, liver, and kidney. There is an apparent need for removal and repair of these nitrated proteins, since this posttranslational modification induces both short- and long-range changes in protein structure and conformation that alter protein function and may interfere with tyrosine phosphorylation signalling pathways. Work performed by Souza et al (2000) shows how the nitration of tyrosine residues accelerates the rate of protein degradation by the 20S proteasome. Cu,Zn superoxide dismutase was used as a model protein and it was found that only one tyrosine residue was nitrated, Tyr-108 (Smith et al. 1992). The addition of a hydrophilic NO$_2$ group induced a small twist in the tyrosine ring and resulted in a slight outward displacement of the neighbouring glutamate 107 without affecting the catalytic activity of the protein (Smith et al. 1992). Therefore, in the absence of any other modification of the protein the nitration represented a single event that augmented the degradation of the protein by the proteasome. The single or double nitration of the tyrosine hydroxylase protein (Ara et al. 1998) also induces proteolytic degradation of the protein by proteasome. The degradation of the nitrated tyrosine hydroxylase is highly selective, as there is no further loss of the native tyrosine hydroxylase after the nitrated molecules are removed (Souza et al. 2000).

Chymotrypsin activity may be critical for the removal of nitrated proteins in plasma, as circulating chymotrypsin activity appears to rise in response to injury, and relatively high plasma levels of nitrated proteins have been detected in a variety of human diseases. Chymotrypsin is a serine
endoprotease that hydrolyses peptide bonds in the carboxyl group of the aromatic residues tryptophan, phenylalanine, and tyrosine. Nitration of tyrosine residues appears to significantly reduce the susceptibility to chymotrypsin, thereby providing a potential mechanism to explain the detection of high levels of nitrated proteins. Plasma chymotrypsin activity is also regulated by α₁-antichymotrypsin, which is also elevated in response to injury, but its activity is not affected by nitration. In contrast to nitration, another posttranslational modification of tyrosine residues in proteins, tyrosine sulfation, renders proteins resistant to chymotryptic cleavage.

Nitration of tyrosine residues has also been shown to inhibit the rate of tyrosine phosphorylation (Gow et al. 1996). The ability of protein tyrosine kinases to phosphorylate a synthetic peptide was found to be inhibited by peroxynitrite mediated nitration of tyrosine. Exposure of endothelial cells to peroxynitrite was found to decrease the intensity of tyrosine phosphorylated proteins and increased the intensity of 3-nitrotyrosine containing proteins. Previous work has shown that the phosphorylation of tyrosine is reliant on the pKₐ of the phenolic hydroxy group (Haddad et al. 1993) and the nitration of the tyrosine residue shifts the pKₐ to 7.5 (Gow et al. 1996), which may provide the mechanism for the significantly reduced rate of phosphorylation.

Overall, nitration of protein tyrosine residues appears to be a biological process with potentially significant implications, and the elimination of nitrated proteins may represent another level of cellular defence from oxidative stress.

1.5.7 Protein Denitrase

Protein nitration has been used as an indication of peroxynitrite formation, though protein nitration can occur through peroxynitrite-independent
mechanisms. The nitration has been shown to alter protein's conformation and structure, catalytic activity, and/or susceptibility to protease digestion. It has also been shown that tyrosine nitration can diminish a protein's effectiveness as a substrate for tyrosine kinases. Thus, the present view is that protein nitration is a purely irreversible detrimental effect. Recent work by Kamisaki et al. (1998), however, has provided evidence that protein nitration may be a post translational modification. Post-translational modifications of proteins can participate in many regulatory processes. The phosphorylation, sulfation, adenylation, acylation, and other protein modifications can have dramatic effects on a protein's structure, intracellular compartmentation, catalytic activity, or rate of degradation and turnover. Presumably, protein nitration is but one of many possible post-translational modifications a protein may undergo. Often these modifications are reversible processes and, as noted above, they can have functional significance.

Using Western immunoblots Kamisaki et al. (1998) found several extracts from several rat tissues that could decrease nitrated BSA on incubation. Homogenates of rat kidney, rat liver, and human erythrocytes could decrease nitrated BSA levels when samples were incubated in the absence, but not the presence, of various protease inhibitors. The activity was found predominantly in 100,000 x g supernatant fractions of spleen homogenates, was time- and protein-concentration dependent, and was liable to heating or trypsin treatment, further supporting the view that the activity is a protein and probably an enzyme.

It has been shown that peptides and proteins that are nitrated on the tyrosine residues are poor substrates for tyrosine kinases. Presumably the nitration of tyrosine on the 3-position and phosphorylation of the hydroxyl group on the 4-position interfere with each other. Thus, tyrosine nitration may compromise cell signalling via tyrosine kinase pathways. Therefore an activity that removes the nitrate as a "3-nitrotyrosine denitrase" could have profound and important effects in cell signalling pathways. The denitration of proteins could permit the restoration of their
function, allow them to become substrates for tyrosine kinases, and have important effects on cell signalling processes.

Greenacre et al (1999) have studied the formation and loss of nitrated proteins in peroxynitrite treated rat skin in vivo. They found that an injection of peroxynitrite into the rat skin caused a dose-dependent increase in nitrated proteins and albumin was identified as the major nitrated protein. The clearance of these nitrated proteins was found to be biphasic, and it was found that there was an initial rapid clearance of nitrated proteins ($t_{1/2} = 2h$) and then a slower loss ($t_{1/2} = 22h$). The rapid clearance probably reflects the time taken for nitrated albumin to be cleared from the extravascular skin site, since control albumin was initially cleared at the same rate. The slower rate of clearance may include proteins other than albumin, or it may be that nitrated albumin sticks or cannot leave the skin as readily as non-nitrated albumin. Alternatively, it may be that this slow clearance is due to the cleavage of the nitrate group from the tyrosine residue rather than the removal or degradation of the protein.

1.5.8 One physiological function of tyrosine nitration

Initially tyrosine nitration was considered to have only detrimental effects. However, Herrero et al (1999) have produced evidence of at least one physiologically significant process of tyrosine nitration. Sperm capacitation can be defined as a maturational process that occurs in vivo in the female genital tract (Chang 1951; Austin 1952), and it can accomplished in defined media in vitro. The main purpose of capacitation is to ensure that spermatozoa will reach the oocyte at the appropriate time and in the appropriate state to fertilise the oocyte. Under physiological conditions human spermatozoa generate small amounts of $O_2^-$ and NO during the process of capacitation. During this stage of sperm maturation it appears likely that low levels of peroxynitrite are produced. Herrero et al (2001) have shown that tyrosine nitration does
occur when sperm are incubated for 8hr under conditions conducive to
capacitation. This tyrosine nitration has been shown to modulate sperm
function, decreasing sperm linearity and increasing sperm velocity and
amplitude of lateral head displacement, without modifying the percentage
of motile cells. When nitrated proteins were isolated from donor sperm
and visualised using western blotting Herrero et al (2001) observed the
spontaneous nitration of proteins, the pattern of nitration was consistent
across a number of donor samples, the only differences observed were in
the relative intensity of some of the bands. Evidence strongly suggests
that nitration was due to peroxynitrite and because this was not observed
when the sperm was incubated with either NO\ alone or nitrite.

Other groups, however, have shown a detrimental effect of sperm
incubation with peroxynitrite, though in these experiments a higher
concentration of peroxynitrite was used. The difference in results may
again highlight that low levels of tyrosine nitration have a functional
aspect to the physiology of the cell and it is only when the exposure to
peroxynitrite is increased that a detrimental affect is observed.

The activity of peroxynitrite has been shown to increase in disease, and
may play role in the progression of disease states, however the effects of
peroxynitrite don’t seem to be purely detrimental and may play an
important role in normal cell physiology. The scope of this project is to
detect the presence of nitrated tyrosine in both the free state and within
proteins in the acute lymphoblastic leukaemia patients. The presence of
nitration events will be assessed as being detrimental to the patient, but it
will be important to consider if any of the effects found could be related to
any normal functions of peroxynitrite.
1.6 Aims of Present study

The aims of the present study were to investigate cerebrospinal fluid concentrations of 3-nitrotyrosine and to detect the presence of protein's containing 3-nitrotyrosine in patients with acute lymphoblastic leukaemia. In order to do this, a HPLC with electrochemical detection method had to be validated to measure the concentration of 3-nitrotyrosine in cerebrospinal fluid. To measure the presence of 3-nitrotyrosine containing proteins, methods using MALDI-TOF MS and Q-TOF MS/MS had to be devised and the sensitivity of these methods assessed using the control proteins transferrin and albumin.
1.7 References


Sawa, T., Akaike, T., and Maeda, H. (2000). Tyrosine nitration by peroxynitrite formed from nitric oxide and superoxide generated by xanthine oxidase. J. Biol. Chem. 275,


2.1 Introduction

As previously described the concentrations of nitrite and nitrate in ALL patients have been measured and were found to be increased at the onset and early stages of the treatment of ALL (Surtees et al. 1998). The measurement of nitrite and nitrate gives an indirect measurement of the level of peroxynitrite activity, but does not measure it directly.

The detection of peroxynitrite in biological systems has been a challenge because of (i) the rapid breakdown of peroxynitrite that precludes its direct isolation and detection, (ii) necessity to find detector molecules that can efficiently outcompete the multiple reactions that peroxynitrite can undergo, (iii) non-existence of footprints totally specific of peroxynitrite reactions, and (iv) the difficulty to discriminate between the biological effects of peroxynitrite versus that of its precursors, NO\(^\cdot\) and O\(_2\)\(^{2-}\), and other NO-derived oxidants. The detection of peroxynitrite relies on either (i) modification of exogenously added probes, or (ii) footprinting reactions on endogenous molecules. However, these are not straightforward procedures; at present there are no totally specific modifications of either probe or biomolecules that can directly and unambiguously assure the formation of peroxynitrite. The use of probes to measure the activity of peroxynitrite was not a suitable procedure for the detection of peroxynitrite activity in patients with acute lymphoblastic leukaemia therefore it was decided to study footprinting reactions on endogenous molecules.

Peroxynitrite formation and reactions can be evidenced through the detection of oxidative modifications that peroxynitrite promotes in target biomolecules. Oxidative modifications can be performed by different oxidants and/or may be readily reversed by appropriate repair systems, therefore ideal modifications to measure would be those that are (i) more specific for peroxynitrite versus other oxidant systems, and (ii) relatively permanent and stable. These modifications involve oxidation reactions in
proteins, DNA or lipids, and most notably nitration of phenolic compounds.

Peroxynitrite promotes nitration and oxidation of phenolic compounds such as tyrosine or ρ-hydroxyphenylacetic acid (p-HPA). Nitration leads to the formation of 3-nitrotyrosine or 3-nitro-pHPA (Ischiropoulos 1998; Ischiropoulos et al. 1992), while oxidation results in dimerisation (van der Vliet et al. 1995) and hydroxylation reactions (Ramezanian et al. 1996), which can also be accomplished by other oxidant systems such as 'OH and oxo-iron complexes. Thus, nitration of phenolic probes is thought to be a surrogate measure of peroxynitrite and has been utilised as a more specific modification induced by peroxynitrite and potentially other 'NO-derived oxidants. However 'NO reacting with tyrosine will cause the transient formation of nitrosotyrosine (Gunther et al. 1997). Nitrosotyrosine and other nitrosophenols are apparently not stable (Ischiropoulos et al. 1996; Eiserich et al. 1995). However, an electron paramagnetic resonance signal of prostaglandin H synthase-2 closely resembling tyrosyl imminoxyl radical, the one-electron-oxidised nitrosotyrosine, was present, suggesting that nitrosotyrosine was sufficiently stable to be oxidised (Gunther et al. 1997). An additional one electron oxidation of tyrosyl imminoxyl radical leads to the formation of 3-nitrotyrosine. However, in the case of free tyrosine, most nitrosotyrosine dissociates back to tyrosyl radical and 'NO, to ultimately predominate ly yield 3,3-dityrosine (Goldstein et al. 2000). The 'NO₂ mechanism appears to be of minor relevance because under normal or low tissue oxygen tensions, the oxidation of 'NO to 'NO₂ is a rather slow reaction, and also, because the initial reaction of 'NO₂-mediated thiol oxidation will be kinetically favoured. Therefore the measurement of free 3-nitrotyrosine is a reasonable marker of peroxynitrite activity.

Immunohistochemical-based methods rely on the use of anti-nitrotyrosine antibodies (Ye et al. 1996) and have been used extensively to detect 3-nitrotyrosine in various tissue types and cell cultures. The advantage of this method is that it can identify the particular cell types in which tyrosine
is nitrated. However, immunological detection methods, including immunohistochemistry and ELISA can only be semi-quantitative, because the antibodies may exhibit some non-specific binding, and the antibody affinity may vary with the nature of the proteins. In addition, 3-nitrotyrosine at some sites may not be completely accessible to the antibody.

3-nitrotyrosine is a stable product and can be directly assessed spectrophotometrically, since it has a characteristic absorbance in the 350-450 nm region that is strongly pH-dependent (Crow and Ischiropoulos 1996). While at low pH (<5.5) 3-nitrotyrosine has a peak absorbance at 360 nm, at alkaline pH maximum absorption is at 430 nm. Since dissociation of the aromatic hydroxyl group in 3-nitrotyrosine is responsible for the shift in absorbance to 430 nm (pKa = 7.5), samples can be quantitated at 430 nm by the difference between absorbance values at pH <5.5 and pH≈10. The limit of sensitivity using this method is ≈ 1 μM and it is potentially subject to interference from other compounds that may absorb in the same region, therefore it is only useful for relatively concentrated and pure samples.

More sensitive and specific detection of 3-nitrotyrosine requires high performance liquid chromatography (HPLC) or gas chromatography (GC) separation and different alternative methods for detection, including UV/VIS, electrochemical (EC), fluorescent, and mass spectrometry (MS), depending on required detection limits and equipment availability and expertise (table 2.1). This methodology also allows the concomitant detection of the parent aromatic compound i.e. tyrosine and other oxidised forms including dimeric and hydroxylated products.

Several HPLC methods have been developed to measure the concentration of 3-nitrotyrosine, the vast majority rely on reverse phase HPLC (Eliasson et al. 1999; Crow and Ischiropoulos 1996; Shigenaga et al. 1997; Hensley et al. 1998; Hensley et al. 1997; Crow J 1999; Herce-
Pagliai et al 1998) (table 2.1). UV-VIS detection can be accomplished by the use of acidified samples and simultaneous detection at 280 and 360 nm. The $A_{280}/A_{360}$ ratio for 3-nitrotyrosine is 2.3, while tyrosine and other oxidation products such as 3-hydrotyrosine and dityrosine do not absorb at 360 nm.
<table>
<thead>
<tr>
<th>Method</th>
<th>Analyte</th>
<th>Sensitivity (pmol)</th>
<th>Comments</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPLC/UV-VIS</td>
<td>3-nitrotyrosine</td>
<td>10</td>
<td>Widespread availability and simple. No derivatisation required. Low sensitivity</td>
<td>Crow and Ischiropoulos (1996)</td>
</tr>
<tr>
<td></td>
<td>3-3'-dityrosine tyrosine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HPLC/EC</td>
<td>N-acetyl-3-aminotyrosine</td>
<td>0.02</td>
<td>Improved signal to noise ratio by detection of 3-aminotyrosine. Alternative method for the detection of tyrosine needed.</td>
<td>Shigenaga et al. (1997)</td>
</tr>
<tr>
<td>HPLC/EC</td>
<td>3-nitrotyrosine</td>
<td>10</td>
<td>Allows detection of multiple analytes in a single run.</td>
<td>Hensley et al. (1998)</td>
</tr>
<tr>
<td>Array system</td>
<td>3-aminotyrosine</td>
<td></td>
<td>Increase direct specificity by EC signature.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3,3'-dityrosine</td>
<td>1.0</td>
<td>Detection system not readily available</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3-hydroxytyrosine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>tyrosine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HPLC/Fluo</td>
<td>3-aminotyrosine</td>
<td></td>
<td>More sensitive than UV-VIS. Minimally explored and validated</td>
<td>Van der vliet et al. (1996)</td>
</tr>
<tr>
<td></td>
<td>3-3'-dityrosine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GC/MS^o</td>
<td>3-nitrotyrosine</td>
<td>0.05</td>
<td>Highly sensitive and specific. Requires preparative steps and specialised equipment</td>
<td>Crowley et al. (1998)</td>
</tr>
<tr>
<td>(NICI)</td>
<td>3-aminotyrosine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3,3'-dityrosine</td>
<td>3-hydroxytyrosine</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>tyrosine</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*a* Sensitivity values for 3-nitrotyrosine and 3-aminotyrosine are independently indicated.

*b* Chromatographed and measured as n-propyl heptafluorobutyl-derivatives

*c* The limit of detection of the n-propyl heptafluorobutyl-derivatives

HPLC = high performance liquid chromatography; EC = electrochemical; GC = gas chromatography; MS = mass spectrometry; NICI = negative ion chemical ionisation.

Table 2.1 Selected methods for the detection of 3-nitrotyrosine.
Electrochemical detection however can achieve over 100-fold higher sensitivity than UV-VIS and various EC methods have been developed. An intrinsic problem of EC-detection of 3-nitrotyrosine relies on the high voltage required for a response (= 800 mV), which results in instability of baseline values and decreased specificity, since other compounds that may coelute with 3-nitrotyrosine and even components of the mobile phase may provide an electrochemical signal. Various approaches have been used to overcome this problem, including the use of array detector systems and/or reduction of 3-nitrotyrosine to 3-aminotyrosine, a treatment that substantially reduces the potential for oxidation (= 70-100 mV). 3-Nitrotyrosine is resistant to reduction by common reductants such as ascorbate and dithioeitol, but can be readily reduced by addition of sodium dithionite under alkaline conditions. 3-Aminotyrosine is colourless and fluoresces at pH = 3.0-3.5 with $\lambda_{\text{exc}} = 277$ nm and $\lambda_{\text{em}}$ of 308 and 350 nm, which can serve for fluorescence detection purposes. Since tyrosine also fluoresces at 308 nm, the $\lambda_{\text{em}}$ at 350 nm is the one of choice, when assessing 3-aminotyrosine by fluorescent detection methods. 3-Aminotyrosine typically has a poor retention in reverse-phase HPLC columns, thus either derivatisation (e.g., acetylation) (Shigeno et al. 1997; Shigenaga M 1999) or use of ion pairing mobile phases (Hensley et al. 1998) have been designed to shift 3-aminotyrosine retention to a convenient elution time. Commercial standards of 3-aminotyrosine are available; however, the reduction of 3-nitrotyrosine to 3-aminotyrosine is in many cases not complete and 3-aminotyrosine can progressively be air-oxidised back to 3-nitrotyrosine.

### 2.1.1 Nitrotyrosine Detection – HPLC with electrochemical detection

Even with the problems associated with this method, HPLC with electrochemical detection (ECD), is still the technique of choice for sensitive, selective, and facile instrumental determination of 3-nitrotyrosine. Electrochemical detectors have long been used to measure catecholamines, xenobiotics, and oxidised nucleotides (Hensley et al.
but only recently has ECD been brought to bear on protein and lipid analysis. Phenolic compounds are ideal candidates for ECD as they typically oxidise at convenient potentials (200-800 mv) and are well-retained on reverse phase columns.

Tyrosine and 3-nitrotyrosine were the first compounds to be measured and an assay was developed to try and measure their concentration in CSF of both ALL patients and controls. It was necessary to measure both compounds, as the measurement 3-nitrotyrosine alone does not give any indication of whether it is bound or free tyrosine being nitrated. HPLC with electrochemical detection was chosen as the methodology of choice as this allows the most selective and sensitive measurement of 3-nitrotyrosine with the equipment available. We decided not to use methods involving the reduction of 3-nitrotyrosine to 3-aminotyrosine due to the number of extra steps involved which increases the chance of introducing experimental errors.

2.2 Materials

3-nitrotyrosine, tyrosine, dibutylamine, tetrahydrofuran, sodium dithionite were obtained from Sigma Chemical Co. (Poole, Dorset, UK). Apex 5μM ODS columns were obtained from Analytichem International (Harbor City, USA). High-performance liquid chromatography solvents were obtained from BDH (Poole, Dorset, UK).

2.3 Electrochemical Detection

The electrochemical detector used was a coulochem ESA 5010 dual cell detector (Bedford, Massachussets, USA). In this detector there are two porous graphite electrodes arranged in series in a flow-through electrolysis cell, both of which can operate coulometrically. The principle of electrochemical detection is that a fixed potential is applied across a
compound dissolved in a conducting medium, oxidation or reduction of that compound results in a measurable electric current. The current produced is proportional to the amount of compound present. High Performance liquid chromatography with flow through electrochemical detector cell will produce a current signal proportional to the concentration of the analyte as it is eluted from the column when the applied potential is constant and the electrolytic current is measured. When only partial oxidation or reduction of the analyte occurs the electrode response is termed as amperometric. However, should all of the analyte undergo oxidation or reduction at the electrode the response is defined as coulometric. Not all compounds are electrochemically active and those that are oxidise or reduce at different potentials. The current producing reaction takes place at the electrode surface and is sensitive to changes in solution conditions such as pH, solute concentration and organic modifiers.

The presence of two electrodes in series increases the selectivity of detection. Selectivity is controlled by the magnitude of the applied potential, at higher potentials more compounds are likely to undergo oxidation or reduction. To increase the selectivity at a given potential a dual cell electrochemical detector can be used in the screen or redox modes. In the screen mode the upstream detector is set at a potential just lower than that required to detect the analyte of interest. The upstream detector will therefore cause electrolysis of all interfering compounds whose electrolytic potentials are less than the analyte of interest. The downstream detector is used to detect the analyte of interest at its appropriate potential. If the electrolysis reaction of the analyte of interest is chemically reversible the redox mode can be used. 3-Nitrotyrosine and tyrosine can both be electrochemically oxidised.
2.3.1 High Performance Liquid Chromatography

Static phase was a (25 x 0.4cm I.D) Apex 5µM ODS column, set at room temperature. Initial mobile phase was 90mM Sodium Acetate, 35mM Citric acid, 130µM Sodium dodecyl sulphate (SDS), 460µM Ethylenediaminetetraacetic acid (EDTA), pH 4.35 and flow rate set at 0.7ml/min.

Using this method 3-nitrotyrosine had a retention time of 37 min and because of the long retention time the peaks observed were broad and ill-defined. It was also found that at this long retention time the sensitivity of detection was significantly reduced. To decrease the elution time of nitrotyrosine methanol was added to the mobile phase (figure 2.1). This however decreased the elution time of tyrosine and it was found to elute close to the solvent front so a variety of methanol concentrations were tried to optimise the system (table 2.2).
Figure 2.1 Comparison between the chromatograms obtained when the mobile phase contained no methanol top trace RT= 31.3 min and when the mobile phase contained methanol 11.3 min. Though both injections were exactly the same (500μM NO$_2$-Tyr) the bottom trace shows a sharper more defined trace.
Table 2.2. The different concentrations of methanol used to modify the retention time of the nitrotyrosine peak and the respective retention times.

A methanol concentration of 7.5% was chosen as the optimum, as this produced a well-defined peak with good definition between peaks, and was used through the continuing work.

### 2.3.2 Current-voltage relationships

The current-voltage relationships of tyrosine and 3-nitrotyrosine were measured. Tyrosine was measured at the first electrode whereas nitrotyrosine was measured at the second electrode with the first set at 0.5V. 50μL of 10μM of both tyrosine and nitrotyrosine were injected into the column and the electrolytic current produced by each measured as the percentage of the full-scale deflection. The potential applied across the electrodes was varied from 0.1 to 0.95 volts between injections. The results are shown in figure 2.2.
Figure 2.2 Current-voltage relationship for tyrosine and nitrotyrosine.

This shows that tyrosine and 3-nitrotyrosine can both be electrochemically oxidised with 3-nitrotyrosine requiring higher applied potentials for oxidation than tyrosine. Due to the high applied potential required to oxidise 3-nitrotyrosine it was measured at the second electrode with the first set at 0.5V to measure tyrosine and act as a screen for 3-nitrotyrosine.

To check that the method was sensitive enough to measure tyrosine and nitrotyrosine concentrations in cerebral spinal fluid the electrolytic current generated by varying concentrations of tyrosine and nitrotyrosine were measured. Standards as low as 2.5pmol 3-nitrotyrosine and 0.5pmol tyrosine were readily detectable and the response was linear (figures 2.3, 2.4). Validation experiments were performed using standards and the coefficient of variation was found to be < 10 % within day and 7.75 % between days.
Figure 2.3 Linearity of electrochemical response to 3-nitrotyrosine. FSD is full-scale deflection.

Figure 2.4 Linearity of electrochemical response to tyrosine.
2.3.3 Recovery of added tyrosine and 3-nitrotyrosine

Adding known amounts of tyrosine and 3-nitrotyrosine to CSF assessed the recovery from the assay. 50 μl of such spiked CSF was injected onto the high performance liquid chromatography system; eluant detection used electrochemical detection with the analytical electrodes set at + 0.4 and +0.9 volts. The recovery was calculated after calibration with a standard.

Figure 2.5 The recovery of tyrosine from spiked CSF.

Figure 2.6 The recovery of 3-nitrotyrosine from spiked CSF.
2.3.4 Analysis of ALL patient samples

Using the method developed for standards patient CSF was injected down the column spiked with 3-nitrotyrosine. The 3-nitrotyrosine peak was found to be eluting close to a large unknown peak (figure 2.7).

![Unknown Peak](image)

Figure 2.7. Trace showing the large unknown peak coeluting with NO₂-Tyr.

To evaluate if this unknown peak was affecting the measurement of the genuine 3-nitrotyrosine peak, values obtained from spiked CSF were compared with standards (table 2.3).
Table 2.3 The percentage difference between results obtained from the spiked CSF and the standard.

<table>
<thead>
<tr>
<th>Conc. of spike/standard</th>
<th>% difference between spike/standard</th>
</tr>
</thead>
<tbody>
<tr>
<td>100µM</td>
<td>3.16</td>
</tr>
<tr>
<td>50µM</td>
<td>4.16</td>
</tr>
<tr>
<td>10µM</td>
<td>39.13</td>
</tr>
<tr>
<td>1µM</td>
<td>132.14</td>
</tr>
<tr>
<td>500nM</td>
<td>47.87</td>
</tr>
</tbody>
</table>

The spiked samples were always measured as having higher 3-nitrotyrosine concentrations than the standards, but these increases were not consistent over a range of concentrations and could not be related to a fixed amount of native 3-nitrotyrosine being present.

As this caused unreliable measurement of the 3-nitrotyrosine peak the retention time of 3-nitrotyrosine was altered to try and separate it from the large unknown peak. The methanol percentage and pH of the mobile phase were found to have an effect on the separation of these two peaks, but not to the point of giving two clearly defined peaks. Other modifiers added to the mobile phase to try and improve the peak separation were dibutylamine (500µL/L) and tetrahydrofuran (1%). Dibutylamine is an ion pair reagent. Ion pair chromatography relies upon the addition of ionic compounds to the mobile phase to promote the formation of ion pairs with charged analytes. These reagents are comprised of an alkyl chain with an ionisable terminus. When used with an hydrophobic HPLC phase in the reverse phase mode, ion pair reagents can be used to selectively increase the retention of charged analytes. Tetrahydrofuran is added to mobile phase because it competes strongly for the active sites within the column and hence leaves less time for the sample molecules to be absorbed into the column giving rapid elution. Tetrahydrofuran also accelerates straight-chained compounds with respect to cyclic...
compounds. The addition of these two chemicals allowed the concentration of methanol to be reduced and did produce better chromatography, but still did not allow reliable data to be gathered.

Deproteinisation of the cerebral spinal fluid was tried using perchloric acid (1:4 CSF:acid) or methanol (1:4 CSF:methanol) to elute out the proteins from CSF. This improved the general chromatography, but again did not remove the large unknown peak (figures 2.8, 2.9).
Figure 2.8 Untreated CSF gave a complex trace and the NO$_2$-Tyr peak was coeluting with a large unknown peak.

Figure 2.9 Deproteinisation of the CSF before analysis by HPLC-ECD improved the overall chromatography, it did not however, separate the unknown and 3-nitrotyrosine peaks.
Since the original development of this method a report (Kaur et al., 1998) has shown a similar problem with a large coeluting peak in brain tissue from patients with Parkinson's disease, Huntingdon's chorea, multiple system atrophy, and Alzheimer's disease, but not in control tissue. This peak was observed using both UV and electrochemical detection methods. The absorbance spectrum, fragmentation pattern on mass spectrometry, and electrochemical profile of this peak did not match that of authentic 3-nitrotyrosine. The peak was lost on reduction with sodium dithionite, a criterion often used to identify 3-nitrotyrosine, as was our unknown peak (figure 2.10).

Figure 2.10. Trace showing the reduction of NO₂-Tyr and the unknown peak.

To determine the identity of the unknown peak Kaur et al (1998) compared the HPLC-EC spectrum of numerous compounds including, L-DOPA, dopamine, p-, m-, and o-tyrosines, 3-chlorotyrosine, 3-nitrotyrosine, 3-aminotyrosine, serotonin, tyramine, etc. analysed before and after reaction with peroxynitrite with that of the unknown peak. They also used the fragmentation data obtained from the unknown peak to search databases for a matching compound. None of these studies elucidated the identity of this unknown peak although they did eliminate the most likely candidates. This peak remains unidentified at present.
As mentioned previously another option to try and measure the concentration of 3-nitrotyrosine in CSF was to convert the 3-nitrotyrosine to 3-aminotyrosine by reduction with dithionite and then measuring this compound using either HPLC/ECD or HPLC/Fluo. There are, however, similar problems associated with these methods as found using the method described here. Using a method like this would also require the inclusion of an internal standard, usually $^{13}$C$_6$-3-nitrotyrosine. Yi et al (2000) found that during sample work up 3-nitrotyrosine can be generated in situ from tyrosine by the action of an unidentified nitrating species. It is important to note that this spontaneous generation of 3-nitrotyrosine was only observed in biological samples and not in standards. It was also noted that the simple addition of an internal standard increased the spontaneous production of 3-nitrotyrosine. The main culprit for this artefactual formation of 3-nitrotyrosine was thought to be nitrate and the methods used to purify the sample seems to increase its ability to act as a nitrating agent. As nitrate cannot be easily removed from biological samples this is a major hindrance in the measurement of free 3-nitrotyrosine.

2.4 Discussion

Using HPLC with electrochemical detection we have been unable to develop a method for the reliable measurement of 3-nitrotyrosine in the CSF of patients with ALL. The method developed produced reliable results with linearity across a range of concentrations; it was also sensitive enough for the detection of the expected physiological levels of tyrosine and 3-nitrotyrosine. Unfortunately due to the presence of the large unknown peak the results obtained from patient samples were unreliable and could not be used to draw any conclusions from this work.

Though other groups have previously reported the measurement of free 3-nitrotyrosine using an HPLC methodology, these results have to be assessed carefully as it is possible that a number of groups have actually
been measuring this large unidentified peaks and therefore the results may be false. It is also necessary to assess whether artefactual production of 3-nitrotyrosine has been taken into account. Methods that measure the formation of spontaneous 3-nitrotyrosine during sample workup and therefore allow a more accurate measurement of the true levels of 3-nitrotyrosine are currently being developed. Whether these methods will allow accurate measurement of 3-nitrotyrosine in a range of tissue samples, and if these methods can then be developed to have any diagnostic value remains to be seen. For the purpose of this study we decided to use another method for the detection of nitration events in the CSF of ALL patients namely protein nitration detected through the use of mass spectrometry.
2.5 References


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3.1 Introduction

Due to the problems in measuring the presence of free 3-nitrotyrosine in CSF it was decided to look for the presence of 3-nitrotyrosine residues within proteins. The nitration of tyrosine residues within proteins depends on numerous factors. It is apparent from the limited number of experiments simulating in vivo conditions that tyrosine nitration is a selective process. Not all proteins or all tyrosine residues of a protein are targets for nitration. Neither the abundance of a protein nor the abundance of tyrosine residues in a protein can predict a target for nitration.

Previous studies have shown that surface exposure of the tyrosine residues is an important factor in determining which tyrosine residues will be nitrated (Souza et al. 1999). For example egg-white lysozyme has three tyrosine residues at sites 20, 23 and 53. Tyrosine residues Y$_{20}$ and Y$_{23}$ are located in a loop and are exposed to solvent, whereas Y$_{53}$ is located in a $\beta$ sheet and is not readily exposed as arginine R$_{68}$ appears to block the access of the aromatic ring to the solvent phase (Souza et al. 1999). Tyrosine Y$_{23}$ is more readily nitrated than tyrosine Y$_{20}$ and Y$_{53}$ remains unnitrated. The lower yield of nitration of residue Y$_{20}$ may be due to the electrostatic interference from nearby arginine which appears to block solvent access to at least one of the two equivalent carbons (position 3 or 5) of the aromatic ring. Therefore, in addition to surface exposure, electrostatic forces in close vicinity to the tyrosine residue may influence the site of nitration.

Due to the limited knowledge of tyrosine nitration it was decided to nitrate proteins in vitro to try to understand possible outcomes before trying to detect nitration sites in vivo. Albumin and transferrin were used as models for probing peroxynitrite reactivity toward proteins. Albumin was chosen because nitration of this protein has been studied previously and this allowed a basis for comparison. It is also found in relatively high concentrations in both plasma and CSF making it accessible for study from samples. The nitration of the protein transferrin has not been
studied, but this protein has been well defined both in the literature and previously in our laboratory. A high percentage of transferrin in CSF is from production within the brain and this would give an indication of peroxynitrite activity within the brain.

3.1.1 Albumin

Albumin is the most abundant protein in plasma (Carter and Ho 1994). Its physiological roles include the maintenance of colloid osmotic pressure (Carter and Ho 1994) and the transport of a wide variety of different ligands (Goodman, 1958; Daughaday, 1959; Yates and Urguhart, 1962; Jacobsen, 1969; Klopfenstein, 1969; Burke et al. 1971; Unger, 1972; Westphal and Harding, 1973; Beaven et al. 1974; Jacobsen, 1977; Richardson et al., 1977; Spector and Fletcher, 1978; Brodersen, 1979; Adams and Berman, 1980; Savu et al. 1981; Roda et al. 1982). In addition, albumin may have important roles as an extracellular antioxidant: by ligating free metals and scavenging reactive species (Cha et al. 1996). It may also serve as a transport molecule for nitric oxide (Stamler et al. 1992). This well characterised 66-kDa protein has 18 tyrosine, 6 methionine, 1 tryptophan, 17 sulphur bridges, and only 1 free cysteine in a total of 585 amino acids, and no prosthetic groups (Peters, T 1985).

Alaveraz et al (1999) studied the effect of peroxynitrite on human serum albumin (HSA) and found that the sulphur containing residues accounted for 65% of the reactivity of HSA toward peroxynitrite. The critical role of the free cysteine residue was evident in the fact that tyrosine nitration increased in sulfhydryl-blocked HSA. This increase may in part be due to simple kinetic competition of the sulfhydryl for reaction with peroxynitrite, though this didn’t explain the total increase in nitration, suggesting that additional mechanisms may be operative; such as the sulfhydryl acting as a radical sink (Davies et al 1993).
Sarver et al (2001) used a prototype MALDI orthogonal quadrupole-TOF (Q-TOF) to study modifications when BSA was treated with tetranitromethane, a nitrating agent. The linear matrix-assisted laser desorption time-of-flight mass spectrometry (MALDI-MS) spectrum of nitrated BSA showed a slight increase in the mass (≈ 420 Da) relative to unreacted BSA. After proteolysis of untreated and nitrated BSA with trypsin, both mass/charge spectra showed numerous peptides, which could be readily assigned to the sequence of BSA. However, in the tryptic digest of nitrated BSA sample, several changes were evident. Two new peptides were observed that relate to the substitution of a hydrogen with NO$_2$, a mass increment of + 45 Da. The total protein mass increase of ≈ 420 Da probably relates to other modifications, which weren't analysed in this study, e.g., oxidation of methionine residues.

3.1.2 Transferrin

No published work has been completed on the nitration of transferrin. Transferrins are a family of iron-binding proteins that provide transport of iron from plasma to cells and buffer the iron level in plasma (Harris and Aisen 1989; Chasteen and Woodworth 1990). This ≈ 80 kDa protein has 26 tyrosine, 9 methionine, 8 tryptophan, 19 sulphur bridges, and no free cysteine in a total of 679 amino acids, it also has two N-linked glycosylation sites at Asn-413 and Asn-611 and one O-linked glycosylation site at Ser-32.

Iron is held in transferrin in two deep clefts located in the N- and C- lobes by co-ordinating to four amino acid ligands, Asp$_{(63)}$, Tyr$_{(95)}$, Tyr$_{(188)}$ and His$_{(249)}$. Iron is bound octahedrally by co-ordinating to these four ligands and 2 oxygen atoms from a synergistic anion, carbonate. Single mutations of these four residues have shown to significantly alter the binding properties to iron. When Tyr$_{(188)}$ is mutated to phenylalanine the protein is unable to bind iron under normal conditions (He et al. 1998). The mutations of the other three ligand binding residues leads to changes
in the binding properties, but does not abolish iron binding; and leads to the appearance of novel iron-binding sites of varying structure and stability (He et al. 2000). Other metal ions having a similar positive charge and ionic radius can occupy the binding site of the N-lobe.

There are five methionine residues in the N-lobe of transferrin; most are located on the surface of the protein molecule. These methionine residues are widely distributed, but they have no specific function nor do they contact, directly or indirectly, functional residues. Mutations in four of these methionine residues have been shown to have no significant effect on the binding properties of transferrin, however when Met(109) was mutated to leucine the iron-release rate was approximately half of that of the wild type (He et al. 1999).

The tyrosine residues involved in the binding of iron may be susceptible to modification by peroxynitrite, which may alter the function of the protein. In the N-lobe of transferrin, iron binds octahedrally to four ligands: Tyr 95, Tyr 188, Asp 63, and His 249. When the two tyrosine residues were mutated to phenylalanine the iron binding ability of transferrin was greatly reduced (He et al. 1997). The nitration of these tyrosine residues may have a similar effect and may be another physiological method of controlling the activity of transferrin.

3.2 Materials

SIN-1, antinitrotyrosine antibody, protein G-sepharose beads were obtained from Calbiochem-Novabiochem Corporation (San Diego, CA). Trypsin was obtained from Promega Ltd. (Southampton, U.K.). Replecote VS was obtained from BDH (Poole, Dorset, U.K.). All other reagents and solvents were research grade and obtained from Sigma Chemical Co. Ltd. (Poole, Dorset, UK). The sources of other materials are indicated in the text.
3.3 Protein exposure to peroxynitrite

Proteins were either exposed to a bolus addition of peroxynitrite or to 3-morpholino-sydnonimine (SIN-1), which produces peroxynitrite in situ. In vitro exposure methods to peroxynitrite vary greatly; initially the use of bolus addition of peroxynitrite was one of the most popular methods as the concentration of peroxynitrite could be measured accurately and easily (Beckman, J 1996; van der Vliet et al. 1995; Gow et al. 1996; Ischiropoulos et al. 1992). Although this method is useful for the detection of susceptible amino acids and to assess the type of modification that occurs, but it may not represent the in vivo situation accurately. The exposure of proteins to bolus peroxynitrite reveals the presence of susceptible residues, but during this exposure method the residues are exposed to a quick, relatively high concentration of peroxynitrite, whereas in vivo, proteins will be exposed longer to lower levels of peroxynitrite therefore, other exposure methods have been developed. SIN-1 has been shown to release both nitric oxide and $O_2^-$ (Hogg et al. 1992) leading to the formation of ONOO$^-$ at physiological pH. SIN-1 can therefore be regarded as an ONOO$^-$ donor rather than purely a NO$^-$ donor. It was important to look at these two different mechanisms of peroxynitrite production because of the controversy about the relevance of exposing proteins to bolus addition of peroxynitrite. Pfeiffer and Mayor (1998) recently reported that the chemical reactivity of ONOO$^-$ generated from NO$^-$ and $O_2^-$ in situ appeared to be different from that of chemically synthesised ONOO$^-$. They found that NO/$O_2^-$ did not nitrate tyrosine residues. Hence, they questioned the significance of formation of ONOO$^-$ as a source of tyrosine nitration in vivo. In their experiments, NO$^-$ and $O_2^-$ were generated by using an NO donor (spermine NONOate; $t_{1/2}$ 230 min) and xanthine oxidase with hypoxanthine as a substrate. Subsequent work by Sawa et al (2000) has argued that NO/$O_2^-$ fluxes have the ability to produce significant amounts of 3-nitrotyrosine and that the levels of 3-nitrotyrosine produced were similar to that of authentic ONOO$^-$ over a range of tyrosine concentrations. Other studies have also claimed that low level steady flux of NO/$O_2^-$ are capable of producing
similar results to that of bolus addition of ONOO⁻. These opposing results appear to be consequences of differing experimental design, Pfeiffer and Mayor (1998) did not consider that the reaction mixture would produced significant concentrations of uric acid. Uric acid is produced due to the oxidation of hypoxanthine by xanthine oxidase, used in their experimental design. Uric acid is a potent ONOO⁻ scavenger (Becker, B 1993) and this would reduce the concentration of ONOO⁻ available for tyrosine nitrination. The type of exposure system used is relevant when comparing the results back to the *in vivo* situation as the NOS enzymes produce peroxynitrite due to the simultaneous formation and reaction of O₂⁻ and NO. This will produce long lasting, low concentrations of peroxynitrite, represented in vitro better by reaction with SIN-1 or NO/O₂⁻ fluxes instead of by reaction with bolus peroxynitrite.

Proteins, transferrin and albumin were exposed to both bolus addition of peroxynitrite and SIN-1 to try and identify possible tyrosine residues that will undergo nitrination and to compare any different nitrination patterns caused by these two exposure methods. These results will then be compared to transferrin and albumin isolated from CSF samples taken from acute lymphoblastic leukaemia patients.

### 3.3.1 Peroxynitrite Synthesis

Peroxynitrite is a very unstable product and was therefore not available commercially until very recently. It was therefore necessary to synthesise peroxynitrite. Numerous synthetic methods have been reported for the preparation of peroxynitrite. These include (i) the reaction of ozone with azide ions in the presence of a low concentration of alkali (Pryor et al. 1995; Uppu et al. 1996), (ii) the autooxidation of hydroxylamine in a moderately alkaline solution containing 0.1-0.5 N NaOH (Hughes and Nicklin 1971; Koppenol et al. 1996), (iii) the reaction of nitrite with acidified hydrogen peroxide followed by a base quench (Hughes and Nicklin 1968), (iv) the reaction of H₂O₂ with 2-ethoxyethyl nitrite in a basic...
medium (Leis et al. 1993), (v) the reaction of NO with solid $K^\cdot O_2^-$ (Koppenol et al. 1996), (vi) the reaction of NO with tetramethylammonium superoxide in liquid ammonia (Bohle et al. 1994). Some of these methods produce peroxynitrite solutions that contain excess alkali as well as other impurities such as azide, 2-ethoxyethanol, $H_2O_2$, hydroxylamine, nitrite, nitrate, and/or tetramethylammonium hydroxide (Uppu et al. 1996). A new method of peroxynitrite formation has been reported by Uppu and Pryor (1996), this method gives peroxynitrite solutions containing less nitrite, when expressed as a percentage of peroxynitrite formed, than those prepared by ozonation of azide (Pryor et al. 1995; Uppu et al. 1996), the reaction of nitrite with acidified $H_2O_2$ (Hughes and Nicklin 1968; Beckman et al. 1994), or the autooxidation of hydroxylamine (Hughes and Nicklin 1971).

The synthesis of ONOO$^-$ by the method developed by Uppu and Pryor (1996) uses a two-phase system and involves a displacement reaction by the hydroperoxide anion (in the aqueous phase) on isoamyl nitrite (in the organic phase) (Eqs. 1-3). The product peroxynitrite remains in the aqueous phase, whereas isoamyl alcohol forms a new organic phase along with the unreacted isoamyl nitrite. The aqueous phase contains some 0.15 M isoamyl alcohol and the unreacted hydrogen peroxide, but no isoamyl nitrite. Removal of isoamyl alcohol or traces of isoamyl nitrite is accomplished by washing the aqueous phase with dichloromethane, chloroform, or hexane. A near total removal of hydrogen peroxide is then achieved by reacting the solutions with manganese dioxide, which can then be removed by filtration.
Eqs. 1-3. This shows the reaction steps in the formation of peroxynitrite. Hydroperoxide anion reacts with oxygen atom to form hydroperoxynitrite and isoamyloxide. The hydroperoxynitrite then spontaneously ionises to form peroxynitrite and a hydrogen ion. The isoamyloxide and hydrogen ions react together to form isoamyl alcohol.

Deionised water (resistance ≥ 18 MΩ) was used for rinsing of glassware and preparation of buffers and reagents. Just before use Isoamyl nitrite and hexane were both washed with 3 x 2 volume of water using a separating funnel. The reaction to produce peroxynitrite required 200µM diethylenetriaminepentaacetic acid (DETAPAC), 1M sodium hydroxide, 22ml hydrogen peroxide, 58ml water, 26.6ml isoamyl nitrite was added last and the mixture was vigorously stirred for 2hrs and maintained on ice throughout the reaction time. Using the separating funnel the non-aqueous phase was removed and discarded. The aqueous phase was then washed with hexane three times to remove any contaminating isoamyl nitrite and isoamyl alcohol. Activated manganese oxide was then added to the aqueous phase to remove any remaining hydrogen peroxide. The resulting solution was then filtered to remove the manganese oxide and the concentration of peroxynitrite measured using a spectrophotometer at wavelength 302nM (Eₐ=1670) (Hughes and Nicklin 1968).
The formation of peroxynitrite was monitored by withdrawing aliquots (~1.5ml) every 15 or 30 mins, separating the aqueous phase after brief centrifugation, diluting the aqueous phase 500- or 1000-fold with 0.1 N NaOH, and then assaying for peroxynitrite at 302 nm ($E_a=1670$) (figure 3.1).

Figure 3.1 Time course of the formation of peroxynitrite. Aliquots were taken from the reaction mixture at intervals of 15 or 30 min.

### 3.3.2 Proteins exposure to peroxynitrite

The concentration of peroxynitrite was spectrophotometrically measured at 302 nm ($E_a=1670$) prior to each experiment, this was to take account of decomposition of the peroxynitrite. The peroxynitrite was added to the samples as a small drop along the wall of the tube just above the reaction mixture and rapidly mixed by vortexing.

The reaction of peroxynitrite with the proteins was carried out as described previously (Ischiropoulos and Al-Mehdi 1995). Each protein was diluted in a potassium phosphate buffer (100mM) containing 100µM DETAPAC, 25mM sodium bicarbonate pH7.4. They were then exposed to differing concentrations of peroxynitrite for 5 mins. The final pH after the completion of the reaction was measured and kept at 7.4. To control
for the potential effect of nitrite, nitrate and H$_2$O$_2$, which may be present in the reaction mixture, peroxynitrite was allowed to decompose for 5 min in the phosphate buffer before the addition of the protein. To insure that peroxynitrite was decomposed, we measured the absorbance of peroxynitrite in the phosphate buffer. The absorbance at 302 nm of the decomposed peroxynitrite was the same as the buffer alone.

After reaction any unreacted products were removed from the reaction mixture using 30 KDa filters (millipore, Watford, U.K.). The reaction mixture was added to the filter and centrifuged for 10 mins at 10,000 x g. The remaining protein was then washed 3 times by the addition of phosphate buffer to the filter and centrifugation after each addition for 10 mins at 10,000 x g. This removes the unreacted products as anything with a molecular weight less than 30 KDa passes through the filter and is discarded.

### 3.3.3 Carboaminomethylation and tryptic digest of proteins

To measure the concentration of protein remaining after the above protocol a BCA protein assay (Sigma) was performed. The proteins were derivatised and digested using the method developed by Mills et al (2000). 100µgs of reacted protein was then dried and reconstituted in 100µl TRIS-HCl pH 7.0 (10mM), 0.1% SDS and 0.15% dithiothreitol (reducing agent). During proteolysis it is generally desirable to cleave the disulphide bridges prior to digestion in order to make the protein more accessible to the protease. Therefore, the proteins were reduced by heating at 90°C for 60 secs. To prevent the free cysteine residues polymerising during sample work up they were then reacted with 5µls of iodoacetamide (0.5M) and the solution was incubated at 37°C for 30 minutes (in darkness). This reaction carboxymethylates the cysteine residues causing a mass increase of + 57 Da which is taken into account during the analysis of results. Excess reaction reagents were removed using 30KDa filters and 10µls of protein was added to 190µls of
ammonium bicarbonate pH 8.5 (50mM). Then trypsin 20:1 (protein:protease) was added and incubated at 37°C overnight, the incubation was stopped by heating at 90°C for 30 seconds and the peptides were cleaned using C18 columns. The C18 columns are used as a desalting process and contain 5mgs of C18 stationary phase (Jones Chromatography, Bargoed, U.K.). Trifluoroacetic acid (TFA) is used throughout the desalting procedure as it is an ideal mobile phase modifier for RP-HPLC of peptides. Its properties as a strong, ion pairing acid allow it to complex with large biomolecules, increasing their retentivity and minimising their interaction with the HPLC column packing. The standard RP-HPLC gradient for peptides is a water to acetonitrile gradient with 0.1% TFA. The C18 columns were treated with 200 µls of silanising solution (2% Repelcote vs hexane), and primed by the stepwise addition of 1ml of 90% acetonitrile (0.1% trifluoroacetic acid [TFA]), 1ml of 10% acetonitrile (0.1% TFA), 1ml of H2O (0.1% TFA). The sample is applied to the column, allowed to pass through the column under gravity and washed with 800µl of H2O (0.1% TFA). The desalted peptides were then eluted with 3 x 75µls of 50% acetonitrile (0.1% TFA). At no time in the procedure was the C18 stationary phase bed allowed to dry out.

3.3.4 Proteins exposure to SIN-1

Proteins were also exposed to two concentrations of 3-Morpholinosydnonimine (SIN-1). Using molecular oxygen, this chemical liberates both the superoxide anion and nitric oxide that spontaneously combine to form peroxynitrite. The proteins were again diluted into the potassium phosphate buffer (100mM) containing 100µM DTPAC, 25mM sodium bicarbonate pH7.4. SIN-1 was added at two concentrations (5mM and 10mM) and allowed to react for 1-2hrs at 37°C with vortexing every 10 minutes. The proteins were cleaned and digested using the same protocol described above.
3.3.5 Isolation of nitrated peptides

Once the proteins have been nitrated through exposure to bolus peroxynitrite or SIN-1; the peptides obtained through digestion with trypsin will contain a mixture of modified and unmodified peptides. Modified peptides may not be detected on the spectrum due to the presence of other peptides at a similar or the same mass. It is also possible that the modified peptides peaks may be suppressed due to their lower concentration in the peptide mixture. It was therefore decided to isolate the peptides containing a nitrated tyrosine residue using the anti-nitrotyrosine antibody. The isolation of nitrated peptides was based on the method developed by Gole et al. (2000).

The proteins were exposed to peroxynitrite using the same method described above. After exposure the mixture of control and nitrated peptides were cleaned using a C18 column. The elutant was then dried and resuspended into 100µl of potassium phosphate buffer (50mM). 15µl of protein-G-sepharose beads was added and the mixture was incubated at 4°C for 1hr. After incubation the solution was centrifuged at 10,000rpm for 2min. The supernatant was collected, 2µg of anti-nitrotyrosine antibodies was added and incubated at 4°C overnight. 30µl of protein-G-sepharose beads was added and incubated for 1½ hr. The beads were then washed three times with phosphate buffer, after the last wash most of the supernatant was removed and 30µl of 0.1% TFA in water was added. The acidic nature of this solution causes the dissociation of the bound peptides from the antibody. The peptides were cleaned again using a C18 column. The peptides were then analysed using MALDI-TOF MS.
3.3.6 Gel Electrophoresis

Previous work performed by Ischiropoulos and Al-Mehdi (1995) studying the effect of peroxynitrite on Albumin showed that when reacted protein was analysed using SDS-PAGE analysis modifications to the protein had occurred. They found that no significant protein aggregation had occurred, due to the formation of dityrosine. They did notice that a percentage of the protein had increased in mass. A percentage of the protein had also decreased in mass, shown by an increase in the band intensity of protein fragments with molecular weight lower than 66,000 Da. The aggregation and fragmentation of the protein would go unnoticed using the MALDI-TOF technique alone, as once a tryptic digest has taken place it would mask these effects.

To visualise the effects of transferrin's and albumin's exposure to bolus addition of peroxynitrite, exposed proteins and controls were run on a 1-D 12% SDS gel. Transferrin and albumin were exposed to increasing concentrations of peroxynitrite, using the same method described previously with the addition of protease inhibitor cocktail, Sigma Chemical Co. Ltd. (Poole, Dorset, UK). to the reaction mixture. Again control protein was added to the buffer 5 minutes after the addition of peroxynitrite. All proteins were placed in 30 KDa filters and unreacted products were removed by washing with fresh buffer. A BCA protein assay was performed and 4µg of protein was loaded onto a 12% acrylamide gel.

3.3.6.1 Gel electrophoresis theory

Polyacrylamide gel is polymerised from a solution of acrylamide monomers into crosslinking chains, forming a semisolid matrix of suspensions in water (Lodish et al. 2000). Separation of proteins in this matrix can be enhanced by varying the ratio of the bis-acrylamide cross-linker (N,N'-methylene-bis-acrylamide) to the acrylamide monomer. The
gel formulation below yields 30% total acrylamide composed of 29.2% monomer to 0.8% cross-linker, or a ratio of 36.5 : 1. Higher ratios will allow most proteins to migrate faster and also will promote easier transfer for immunoblots. Lower ratios yield firmer gels and slower rates of migration. Not all proteins will be affected equally, and changing the ratio of monomer to cross-linker can be used to gain better separation between some proteins. This matrix is buffered to weakly basic; so most proteins will be anionic and migrate to the anode of the gel when an electric current is applied. The rate at which the proteins move in the gel is affected by the electric current, the pore size of the gel, and the size of the protein (i.e. smaller proteins move faster in the gel) (Horton et al. 2002).

Sodium dodecyl sulphate (SDS) is an anionic detergent with a long hydrophobic tail that binds to the hydrophobic side chains of amino acids at a constant ratio of 1.4g of SDS to 1g of polypeptide, proportional to the molecular weight of the protein (Hames, 1998). Larger proteins bind to more SDS molecules. This ratio ensures that all SDS-protein complexes have a similar mass:charge ratio, eliminating the intrinsic charge of the protein as a factor affecting migration (Rybicki and Purves, 2001). SDS denatures the protein and dissociates multimeric proteins into their subunits, causing extended conformations in the polypeptides and thus eliminating the effects of the shape of the protein in migration (Lodish et al. 2000). This leaves protein size (mass) as the only factor affecting the separation of proteins in electrophoresis.

SDS is added to the gel and protein before and during electrophoresis, and is often accompanied by heating the protein at 100°C for a few minutes to denature the protein (Lodish et al. 2000). Reducing agents are used to reduce disulfide bonds (e.g. dithiothreitol (DTT)). The proteins are stained after electrophoresis and bands appear on the gel, showing the migration distances of different sized proteins. Migration distances are characteristic to specific proteins, and can be used to assess the purity of protein. If the protein of interest is unidentified, it can be compared to the
migration distances of a known protein for an estimation of molecular weight.

### 3.3.6.2 Gel electrophoresis material and methods

The resolving gel consisted of 40mls of acrylamide (30% sol), 490μls of thiosulphate, 25mls of TRIS-HCl (1.34M) pH 8.8, 0.120g of N,N’-methylene-bis-acrylamide (BIS) and 1ml of 10% SDS, this was then made up to 100mls with H₂O. To this the catalysts for acrylamide gel polymerisation were added, 100mgs ammonium persulphate (APS)/1ml and 50μls of N,N,N’N’-tetramethylethylenediamine (TEMED), and the gel poured immediately after this addition. A thin layer of saturated butanol was poured over the gel to remove any bubbles. The resolving gel should finish approx. 4cm from the top of the gel frame. The gel was then left to set for approx. 45 mins. The butanol was poured off the set gel. The stacking gel consisted of 6.67mls acrylamide, 53mgs of BIS, 12.5mls TRIS-HCl (0.5M) pH6.8 and 0.5mls of 10% SDS, this was then made up to 50mls with H₂O. To this 250μls of 10% APS and 25μls of TEMED was added and the gel then immediately poured on top of the main gel. The spacing comb was immediately placed in the stacking gel and left for at least 30mins to set. The proteins to be run were then made up to the required concentration in 25μls of H₂O. To this 25μls of loading buffer was added. The loading buffer consisted of 2.5ml TRIS-HCl (0.5M) pH 6.8, 4ml of 10% SDS, 2ml glycerol, 2mg bromophenol blue. This was made up to 10ml and then aliquoted into 1ml eppendorfs, which were then kept frozen at −70°C until required. Before use 31mg of DTT was added to 1ml of loading buffer.

To denature the protein before running it in the gel the protein/buffer mixture was heated at 90°C for 1 min. The comb was then removed from the set stacking gel and the proteins loaded into the channels, loading buffer was also put into any empty channels. The apparatus was then loaded into the electrophoresis tank and 6Ls of tank buffer added, making
sure that there was tank buffer in the top section covering the platinum wires. The tank buffer consists of 89.1g glycine, 22.65g TRIS and 6g SDS made up to 6L with millipore water. The apparatus was then attached to the cooling system and the electrophoresis power supply was set at 30V and 30mA per gel. The gel was allowed to run at these settings until the blue line from the loading gel has moved to the interface between the stacking gel and the resolving gel, at this point the voltage was then increased to 250V. The gels were then left to run for approx. 4-5hrs. Once the blue marker reached the base of the gel, the power was turned off and the gel was removed from the apparatus and placed in fixative (50% methanol : H2O, 1% glacial acetic acid) from 2hr to overnight with shaking. The gels were then ready to be stained.

3.3.6.3 Silver staining of gels

The silver staining of the gels was based on the method described by Shevchenko *et al* (1996). The gels were placed on a shaking platform throughout the staining process and were initially washed three times in deionised H2O with 10 minutes for each wash. The gels were then sensitised by incubation with sodium thiosulphate (0.2g/L) for 2 min, this was washed off with two 5 min washes with H2O followed by another 5 min H2O (0°C) wash. The gel was then treated with silver nitrate (1g/L) at 0°C for 30 mins, this was washed off with two 2 min washes of room temperature H2O. The gels were then exposed to the developing solution (sodium carbonate 1 ml/L, formaldehyde 1 ml/L) until the protein spots showed the required strength of colour. The staining process was stopped by the addition of 5% acetic acid for 10 mins and then the gels were placed back in fixative. Excised spots for protein digestion should be washed with distilled water and stored in Eppendorf tubes.
3.3.6.4 In-gel Digestions

The gel spots were cut out using a scalpel; those not required immediately were frozen at -70°C. The gel slices were then carboaminomethylated and digested. The gel slices were initially washed with 200µls of Ammonium bicarbonate buffer (100mM), this buffer was then discarded and 500µls of acetonitrile was added. The gel slices were then placed on a rotator for 15mins to dehydrate the slice. The acetonitrile was then removed and the gel slice dried in the speed vacuum (25mins, heat setting 4). The gel slice had to be dried sufficiently to allow the reabsorbance of another liquid into the gel slice. The gel slices were then rehydrated in 200µls of Ammonium bicarbonate buffer (100mM) containing DTE (10mM) and heated at 56°C for 1hr. After the incubation the supernatant was removed and all the cysteines were then derivatised by the addition of 200µls of iodoacetamide (55mM) in Ambic buffer (100mM), this was incubated at 37°C for 45 mins. The supernatant was then removed and the gel slices dried again by the addition of 500µls of acetonitrile for 15 mins and then 45 mins drying in the speed vacuum (heat setting 4). To perform the in-gel digestion the gel slices were rehydrated in 30µls trypsin (20µg trypsin in 1.2mls Ambic buffer [50mM]) and left overnight at 37°C. At the end of the incubation 500µls of Urea was added to the gel slice/trypsin solution and rotated for 1hr. The peptides in the urea were then cleaned up using C18 columns. These peptides were analysed using the MALDI-TOF MS.
3.4 Matrix-assisted laser desorption time-of-flight mass spectrometry

3.4.1 Introduction

Various forms of mass spectrometry that have the capability to analyse protein structure have been introduced over the past 10-15 years. These techniques have served a central role in the advancement of biotechnology during this time period. As an approach to protein identification, database searching of peptide mass fingerprints is not as informative as homology searching of amino acid sequence data. However, it is much faster than automated Edman sequencing, and much more specific than fingerprints based on PAGE migration patterns or HPLC retention times. Evaluation exercises in a number of laboratories have shown that a typical protein can be uniquely identified from a database such as SwissProt using just 4 or 5 peptide mass values, and mass measurement errors of 1 or 2 Da have little effect.

One possible reason for the surge of interest in peptide mass fingerprinting is that it has become much easier to acquire the experimental data. In 1989, the majority of peptide and protein work was still performed on relatively complex instruments using fast atom bombardment ionisation. Such instrumentation struggled to achieve the sensitivity levels required to present a realistic alternative to automated Edman sequencing. However, more recently mass spectrometry has seen the introduction of new ionisation techniques that are comparable with the sensitivity of Edman chemistry. In addition, these techniques are more readily incorporated into instrumentation suitable for a general laboratory environment. For peptide and protein work, two types of mass spectrometry now dominate: matrix-assisted laser desorption (MALDI) coupled to a time-of-flight mass analyser and electrospray ionisation (ESI) coupled to a quadrupole mass analyser.
The development of modern laser technology has provided a means of directing a large amount of energy into a sample, leading to the desorption of intact molecules rather than the thermal decomposition seen in other types of mass spectrometry. MALDI-TOF MS uses pulses of laser light to desorb the analyte from a solid phase directly to an ionised gaseous state. Any molecule naturally possesses rotational, vibrational and electronic energy. When exposed to a laser pulse (or beam) a molecule's internal energy is vastly increased in a very short period of time. This leads to melting (with increased rotational, vibrational and electronic energy), vaporisation (desorption; with increased kinetic or translational energy), some ionisation (electronic excitation energy causing the ejection of an electron) and possibly some decomposition (increase in total energy sufficient to cause bond breaking). If enough energy is deposited into the sample in a very short space of time, the energy cannot be dissipated into the surroundings and the sample is desorbed from the target area because of a large gain in kinetic energy. Laser desorption ionisation is the process of beaming laser light, continuously or in pulses, onto a small area of a sample specimen in order to desorb ions; these ions are accelerated in an electric field (receiving constant kinetic energy) and ‘fly’ down to the detector. The lighter ions can fly faster than the heavier ions and reach the detector first. By measuring this time of flight of an ion, applying the law that the time of flight of an ion is proportional to the square root of its mass (equation 4), and combining this data with internal calibrations for the instrument it is possible to measure the molecular weight of a peptide.

Equation 4. \( \sqrt{m} = At + B \)

\( m \) = mass of the ion, \( A \) and \( B \) are instrument constants, \( t \) = time of flight of the ion
3.4.2 Other considerations on laser desorption ionisation

Consider a laser emitting radiation of energy, $E$. For a substance to absorb that energy, it must have an absorption spectrum (ultraviolet, visible or infrared) that matches that energy. If the substance cannot absorb the energy the laser radiation will be reflected and none of the energy absorbed. When the energy can be absorbed the energy must then be dissipated, causing the effects mentioned above. For MALDI the sample is mixed or dissolved in a matrix material that has an absorption spectrum that matches closely to the energy of the laser radiation. On irradiating the matrix alone, it will rapidly increase in energy and will desorb and ionise quickly. When mixed with the sample some of the energy absorbed by the matrix is passed onto the sample causing it to desorb and ionise. Commonly the photoactive compounds such as gentistic acid, 4-HCCA (alpha-cyano-4-hydroxycinnamic acid), or dithranol are used to provide the matrix. As the sample does not fragment during desorption, MALDI is often referred to as being “soft”.

3.4.3 Time-Of-Flight mass spectrometry

When analyte molecules and peptide fragments are accelerated in an electrostatic field to a common kinetic energy, lighter ions travel faster and heavier ions – with the same momentum – travel more slowly. The ionised particles enter one end of the time-of-flight tube, and the number of ions reaching a detector at the other end is recorded in a time-dependent manner. One problem inherent with time-of flight analysis is due to the fact that not all ions of any one given m/z value reach the same velocity after acceleration nor are they all formed at exactly the same point on the ion source. Therefore, even for any one m/z value, the ions reach the detector over an interval of time instead of all at once (figure 3.2). Clearly, where separation of flight times is very short, as with TOF instruments, the spread for individual ion m/z values means there will be overlap in arrival times between ions of closely similar m/z values.
This effect decreases available (theoretical) resolution. The resolution can be improved by including a reflectron (figure 3.3).

Figure 3.2 This figure shows a schematic of the ionisation process seen in MALDI, resulting in not all ions emerging from the source at the same velocity. This discrepancy results in the ions E1 and E2 (same mass but slightly different velocity) reaching the detector over a time period of T1. This time difference results in peak broadening and reduces the isotopic resolution.

Figure 3.3 With the reflectron on, the ion E1 with the higher velocity than the ion E2 penetrates deeper into the retarding field of the reflectron and takes longer to emerge. This allows the ion E2 to ‘catch up’ with the faster ion (E2) and both emerge from the reflectron at the same time. Thus the time discrepancy T1 is compensated for and with the resulting improvement in peak resolution.
To improve the focusing of an ion band the TOF geometry can be changed by adding a reflectron to the end of the flight tube (figure 3.3). A reflectron or “ion mirror” consists of a series of electrostatic and magnetic fields that collect and redirect the ions in a controlled manner. The reflectron allows ions with greater kinetic energies to penetrate deeper into the reflectron than ions with smaller kinetic energies. The ions that penetrate deeper will take longer to return to the detector. If a packet of ions of a given mass-to-charge ratio contains ions with varying kinetic energies, then the reflectron will decrease the spread in the ion flight times and reduce the effects of initial kinetic energy differences, therefore improving the resolution of the time-of-flight mass spectrometer. Reflectron based TOF tubes give sharper signals by reducing the effects of initial kinetic energy differences, as reflectrons effectively increase the TOF free-flight path, they increase resolution and therefore improve mass accuracy.

3.4.4 MALDI-TOF Methods

The peptide samples were prepared for MALDI-TOF MS analysis using the method described by Mills et al (2000). Mass spectrometry was carried out on a matrix assisted laser desorption ionisation, time of flight instrument, fitted with a reflectron and a 337nm u.v. laser (TOF Spec E, Micromass, Manchester, U.K.). Analysis settings were performed at a source voltage of 20,000V, extraction 19,950V, focus 16500V and reflectron voltage of 25,000V.

1.5µl of the digestion solution was taken and placed in an eppendorf, to this 1.5µl of matrix was then added. The matrix consisted of 1:1 [vol:vol] solution of acetonitrile / ethanol [50:50] : fucose [50mM], containing 10mgs of α-cyano-4-hydroxy-cinnamic acid. The matrix and the digest solution were thoroughly mixed by taking up / expulsion in a pipette tip. 1µl was added to the MALDI-TOF target to form a droplet. The target
was then allowed to air dry at room temperature for at least 15 mins before being placed into the mass spectrometer.

### 3.4.5 Analysis of MALDI-TOF data

The MALDI-TOF MS was used to obtain a spectrum giving the m/z of the peptides produced from the digest. The masses obtained from the MALDI-TOF MS analysis were analysed using the on-line database Protein prospector (http://prospector.ucsf.edu, accessed March 2002), which in turn uses SWISS-PROT (http://us.expasy.org/sprot/, accessed March 2002). SWISS-PROT is a reference database of proteolytic peptide mass values, created by applying the appropriate enzyme cleavage rules to the collection of sequence data. The cleavage rules are chosen to simulate as closely as possible the real life behaviour of the enzyme. In calculating the molecular weights of the proteolytic peptides, it is essential that the mass values for individual amino acid residues correspond to those found in the samples. The most common ambiguity is the state of the cysteine residues. In our experiments the cysteine residues were reduced and then carboaminomethylated, this modification has to be taken account of when searching the database. Post-translational modifications present a more difficult problem. A significant proportion of database entries are derived from conceptual translations of nucleic acid sequences, and so contain no information on post-translational modifications. Some of these post-translational modifications can be taken account of; for example, where a sequence is marked as being N-terminal acetylated. Others are not accounted for in the databases, but fortunately, in most cases, the mass spectrum of a digest contains such extensive redundancy that failure to obtain positive matches for peptides which incorporate post-translational modifications does not jeopardise the overall chances of success.
3.4.6 Selecting experimental masses for a search

If it is assumed that proteins can be treated as being composed of truly random sequences of the 20 standard amino acids, then it is not difficult to calculate the peptide size distribution to be expected from any given enzyme. In the case of trypsin, 10% of the peptides are longer than 20 residues. Because of the relative scarcity of these larger peptides, it is the higher experimental mass values that provide the greatest discrimination power in a search. When selecting experimental mass for use in a search, it is advisable to choose those that are large enough to offer good discrimination, yet not so large as to be likely to be extended partials.

3.4.7 Search Schemes

The calculated peptides masses for each entry in the sequence database are compared with the set of experimental masses. Each calculated value that falls within a given mass tolerance of an experimental value counts as a match. The score is simply the number of matched peptides per entry. A molecular weight range for the intact protein can be used as a pre-filter. The database program protein prospector was used in the analysis of our proteins, this program is separated into separate programs for differing types of searching; to identify a protein from its resulting peptides after a trypsin digest we used MS-FIT. This uses the database SWISS-PROT to compare the experimental masses to theoretical masses of known proteins. The MS-FIT program does a simple comparative search as mentioned above, but it also gives a molecular weight search (MOWSE) score.
The MOWSE score reported by MS-FIT is based on the scoring system described by Pappin et al (1993). As MS-FIT offers several options not available in the initial version of MOWSE, several modifications have had to be made.

After the species and molecular weight pre-searches the remaining proteins undergo theoretical digestion. The resulting peptides are then placed in bins based on their molecular weight and the intact molecular weight of undigested protein they originated from. There are eleven intact molecular weight bins. Under 100000 Da there are 10 bins of width 10000 Da. The other bin contains all the proteins over 100000 Da. There are thirty peptide molecular weight bins of width 100 amu between 0-3000 Da. Peptides above 3000 Da are not binned. Peptides with no missed cleavages contribute 1.0 to the bin total whereas peptides containing missed cleavages contribute pfactor (a user supplied parameter).

Bin frequency values are then calculated by dividing the bin totals by the sum of the bin totals for each 10000 Da protein interval. The bin frequency values are then normalised to the largest bin frequency value to yield frequency values between 0 and 1.

Masses in the theoretical digestion which match masses in the data set are divided into scoring matches and non-scoring matches. Scoring matches include unmodified peptides and acrylamide modified Cys and N-terminal Gln to pyroGlu and oxidation of Met in the presence of the unmodified peptide. Non-scoring matches include pyroGlu and oxidation of Met in the absence of the unmodified peptide, acetylated N-termini, phosphorylation of serine, threonine and tyrosine and single amino acid substitutions. Unmatched masses are ignored. The score for each matching mass is assigned as the appropriate normalised distribution frequency value. In the case of multiple matching masses the scores are multiplied together. The final product score is inverted and normalised to an average protein molecular weight of 50 kDa.
Using the above methods, we went on to examine protein nitration in two sample proteins, transferrin and albumin. These proteins were exposed to both SIN-1 and bolus peroxynitrite and the modifications monitored using MALDI-TOF MS.
3.4 References


CHAPTER 4. MALDI-TOF MS ANALYSIS OF PROTEIN NITRATION

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4.1 Introduction

MALDI-TOF MS was used to analyse the peptide masses gained from the tryptic digest of the proteins transferrin and albumin. Control and samples exposed to SIN-1 and bolus peroxynitrite were compared, the exposed samples were monitored for the occurrence of any peptides containing tyrosine, showing a modification of + 45 Da, that would indicate the nitration of the tyrosine residue.

4.2 Tryptic digest of control transferrin and albumin

When control transferrin and albumin were analysed using MALDI-TOF MS and the protein prospector database, only a percentage of the protein can be observed: transferrin 47% and albumin 32%. Those sections of the proteins that cannot be observed using this method are those peptides produced using trypsin digest which are unsuitable for ionisation. This may be because the peptides are too heavy, because of the length of the sequence or the presence of glycosylation sites that will also increase the mass of the peptide.

It is possible to increase the power of the MALDI-TOF MS analysis by using different enzymes to digest the protein and by using PNGase F to cleave off the glycans (Mills et al 2000); using both these methods it is possible to increase the total coverage of the protein. However, in respect to this study the coverage obtained using digestion by trypsin is sufficient for the initial studies as a percentage of the peptides observed contain tyrosine and methionine residues as shown in table 4.1 and 4.2, figures 4.1 and 4.2 give examples of the ionic spectrum obtained from the tryptic digest of transferrin and albumin respectively.
Figure 4.1  Spectrum obtained from MALDI-MS analysis of tryptic digest of transferrin. Y-axis shows the relative ion abundance, this is an arbitrary unit based on a percentage of the largest peak, the x-axis shows the mass-charge ratio for each individual ion. Arrows indicate the main peptides from transferrin and their position in the protein molecule. Inset shows a closer view of one of the peptides, showing the isotopic resolution obtained using MALDI-TOF MS. Here the different proportions of $^{13}$C and $^{14}$C in a peptide causes increases of m/z ratio by single mass units.
Figure 4.2 Spectrum obtained from MALDI-TOF MS analysis of tryptic digest of albumin. Y-axis shows the relative ion abundance, this is an arbitrary unit based on a percentage of the largest peak, x-axis the mass-charge ratio for each individual ion. Arrows indicate the peptides from albumin and their position in the protein molecule.
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Table 4.1 Peptide masses observed from the tryptic digest of control transferrin. Sites of possible: tyrosine nitrations are marked in red, tryptophan in green and methionine oxidation in blue. * Standard 3 letter and 1 letter abbreviations of amino acids are given in appendix 1.
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<td>168</td>
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<td>RHPFYAPELLEYANK</td>
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</tbody>
</table>

Table 4.2 Peptide masses observed from the tryptic digest of control albumin. Sites of possible: tyrosine nitration are marked in red, tryptophan nitration in green and methionine oxidations in blue. * See appendix 1.

4.3 Tryptic digest of transferrin and albumin exposed to peroxynitrite

The exposure of both transferrin and albumin to peroxynitrite caused an increase in the total coverage of the protein, it was also observed that a significant proportion of higher mass peptides could be observed (figures 4.3 to 4.8) (Table 4.3). The exposure of transferrin and albumin to SIN-1 didn’t significantly change the trace obtained from the MALDI-TOF MS.
Figure 4.3 MALDI-TOF MS ion spectra for tryptic digest of transferrin. Lower graph is a typical trace for control transferrin and the upper trace is transferrin treated with peroxynitrite. The traces show that when transferrin has been treated with peroxynitrite there are a larger number of peptides observed, especially the higher mass peptides.
Figure 4.4 MALDI-TOF MS ion spectra for tryptic digest of albumin. Lower graph is a typical trace for control albumin and the upper trace is albumin treated with peroxynitrite. The traces show that when albumin has been treated with peroxynitrite there are a larger number of peptides observed, especially the higher mass peptides.
Figure 4.5 An amino acid map of transferrin, the red lines represent the peptides observed after carboamidomethylation and trypsin digest and analysed using MALDI-TOF MS. The observed peptides cover 47% of the protein.

Figure 4.6 An amino acid map of transferrin, the red lines represent the peptides observed after nitration with peroxynitrite, carboamidomethylation, trypsin digest and analysis by MALDI-TOF MS. The observed peptides cover 64% of the protein.
Figure 4.7 An amino acid map of albumin, the red lines represent the peptides observed after carboaminomethylation and trypsin digest and analysed using MALDI-TOF MS. The observed peptides cover 32% of the protein.

Figure 4.8 An amino acid map of albumin, the red lines represent the peptides observed after nitration with peroxynitrite, carboaminomethylation and trypsin digest and analysis by MALDI-TOF MS. The observed peptides cover 42% of the protein.
<table>
<thead>
<tr>
<th></th>
<th>Peptide Coverage</th>
</tr>
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<tbody>
<tr>
<td>Transferrin (Control)</td>
<td>47%</td>
</tr>
<tr>
<td>Transferrin (Peroxynitrite)</td>
<td>64%</td>
</tr>
<tr>
<td>Albumin (Control)</td>
<td>32%</td>
</tr>
<tr>
<td>Albumin (Peroxynitrite)</td>
<td>42%</td>
</tr>
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</table>

Table 4.3. The percentage of the protein covered by the observed peptides.

Even though the results from the MALDI-TOF MS showed that exposure to peroxynitrite was changing the peptide masses obtained for both albumin and transferrin, when the masses were compared to the theoretical mass obtained from the nitration of tyrosine and tryptophan residues no matches were found (Tables 4.4 and 4.5). The peptides containing methionine residues were also monitored as peroxynitrite has been shown previously to oxidise this residue, this modification can not, however, be used as a measure of peroxynitrite activity as the sample work up and the MALDI-TOF MS process itself can also cause the oxidation of methionine residues. The percentage of methionines oxidised was not found to differ significantly between controls and peroxynitrite exposed proteins.
<table>
<thead>
<tr>
<th>Theoretical mass of peptide</th>
<th>Amino acid sequence</th>
<th>Possible modification masses</th>
</tr>
</thead>
<tbody>
<tr>
<td>878.4624</td>
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<td>YLGEE YVK</td>
<td>1045.499 1090.499</td>
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<td>1328.57 1373.57 1418.57</td>
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<td>1354.6313</td>
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Table 4.4 The theoretical masses of transferrin tryptic peptides modified by exposure to peroxynitrite. Tyrosine residues are highlighted in red and tryptophan residues in green.
### Table 4.5: Theoretical Masses of Albumin Tryptic Peptides Modified by Exposure to Peroxynitrite

<table>
<thead>
<tr>
<th>Theoretical mass of peptide</th>
<th>Amino acid sequence</th>
<th>Possible modification masses</th>
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</table>

Table 4.5: The theoretical masses of albumin tryptic peptides modified by exposure to peroxynitrite. Tyrosine residues are highlighted in red and tryptophan residues in green.
As the proteins in these experiments were exposed to peroxynitrite and then digested and analysed using MALDI-TOF MS, a mixture of control and nitrated peptides would have been present. If a high percentage of control peptides are present this would suppress the nitrated peptides and the nitrated masses may remain undiscovered. To try and further understand the effect of peroxynitrite on the proteins transferrin and albumin the proteins were run on a 12% SDS PAGE gel to try and isolate nitrated proteins as these would have an increased mass and would run higher on the gel.

4.4 Visualisation of peroxynitrite modified proteins using electrophoresis and silver staining

Transferrin and albumin were exposed to increasing concentrations of peroxynitrite (0.1μM-5μM), using the methods described in chapter 3.3.2. When the proteins were visualised using silver staining it was found that as the concentration of peroxynitrite increased, the bandwidth of both proteins increased (figures 4.9 and 4.10)

![Figure 4.9 Exposure of transferrin to increasing amounts of ONOO, 4μg of protein was loaded into each lane.](image)

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Figure 4.10 Exposure of albumin to increasing amounts of ONOO, 4µg of protein was loaded into each lane.

As the concentration of peroxynitrite increased, it was noted that a percentage of the protein increased in mass, this increase in mass was more distinct in the case of albumin than transferrin. The increase was expected as the nitration modifications would increase the overall mass of the protein, previous work measuring the overall mass of albumin has shown that exposure to tetranitromethane caused an increase of 420 Da (Sarver et al 2001). This slight increase in mass would not be resolved using gel electrophoresis, however, the exposure to bolus peroxynitrite may cause a greater number of modifications and therefore cause the increase in mass observed. It was also noted that transferrin showed a greater increase in mass than albumin which is probably due to transferrin having a greater number of possible nitration sites than albumin.

A decrease in mass was also observed when transferrin was exposed to peroxynitrite. This is unlikely to be due to enzymatic degradation of the protein during work up because protease inhibitors were added to the reaction mixture. The reduction in mass may be due to peroxynitrite causing direct degradation of the protein or the cleavage of the glycans.
The decrease in mass seems to be fairly large and suggests that exposure of transferrin to peroxynitrite has a detrimental effect on protein structure.

The bands were then sectioned and excised from the gel. The bands were sectioned into above (2 sections), the same and below the control band. These gel slices were then washed and digested as described in chapter 3.3.9.

4.5 Analysis of proteins excised from the 1-D gels

The different sections obtained from the gel showed differences in their MALDI-TOF MS spectra. The gel band cut out on a level with the control transferrin showed very little difference to that of the control transferrin. 19 peptides were found which matched to that of the protein transferrin giving a peptide coverage of 33%. The band 0.5-1cm above the control was found to have 12 matching peptides giving a coverage of 20%. There was also an increase in the number of unknown peptides that could not be assigned to keratin or trypsin, none of these unknown peptides however matched with the theoretical increase in mass when a tyrosine residue undergoes nitration. On closer inspection it was found that even though none of the unknown peptide masses correlated with nitrated tyrosine, some of the tyrosine residues had undergone a modification as the mass correlating to their control value had disappeared from the trace (table 4.6) (figure 4.11).
<table>
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<th>Control Mass</th>
<th>Sequence</th>
</tr>
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<td>1952.938</td>
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</table>

Table 4.6 Peptides that could not be observed in peroxynitrite treated transferrin that could be observed in control transferrin.
Figure 4.11 MALDI-TOF MS ion spectra after in-gel tryptic digestion of transferrin. Upper trace shows the peptides in the control sample, the peptide at 1283.64 relates to the peptide EDYYDYGAFR, this peptide can be seen to be missing in the trace below, which is the trace obtained from the gel slice taken above the control height.
Analysis of the bands produced by peroxynitrite treatment of albumin revealed similar results. Digestion of the control band produced 13 peptides matching albumin covering 26% of the protein. When the band above the control level was analysed only 9 peptides were found that matched the protein this gave a coverage of 18%. The peptide masses that weren’t observed in the band above control level again contained at least one tyrosine residue (table 4.7)(figure 4.12).

<table>
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</table>

Table 4.7 The masses not observed in nitrated albumin that were present in control albumin

In the case of both transferrin and albumin it is worth noting that in-gel digestion of proteins produces a lower coverage of the protein, less peptides are observed when compared to the in-solution digestions performed earlier.

All the peptides relating to the missing masses contain at least one tyrosine residue. This showed that the reaction of albumin and transferrin with peroxynitrite did modify some of the tyrosine residues. However even though the peptides containing tyrosine have been modified no masses were observed at the expected increments of + 45 Da.

The results up to this point have suggested that modification of tyrosine residues is occurring, however, the modifications have remained undetected. This may still be due to modified peaks being hidden by control peaks, it could also be due to the presence of other modifications.
Figure 4.12 MALDI-TOF MS ion spectra after in-gel tryptic digestion of albumin. Lower trace shows the peptides in the control sample, the peptide at 2045.028 relates to the peptide RHPYFYAPEL LYANK, this peptide can be seen to be missing in the trace above, which is the trace obtained from the gel slice taken above the control height.
other than just tyrosine nitration. To try and visualise the modifications a method was used to isolate the peptides with at least one nitrated tyrosine residue.

4.6 Separation of peptides containing nitrated tyrosine residues using the antinitrotyrosine antibody.

Peptides from both transferrin and albumin, containing nitrated tyrosine residues, were isolated using the method described in chapter 3.3.5. Using the MALDI-TOF MS transferrin and albumin control peptides were compared to nitrated peptides. The spectrum obtained using the MALDI-TOF MS are shown in figures (4.13 and 4.14).
Figure 4.13 MALDI-TOF MS analysis of tryptic digest of transferrin. The y-axis all show the relative ion current and the x-axis the mass/charge ratio. The upper trace shows the peptides observed in control transferrin, the middle trace the peptides obtained when transferrin was reacted with peroxynitrite and the lower trace shows the lack of any discernible peptide peaks when peptides containing at least one nitrated tyrosine residue were isolated using the antibody antinitrotyrosine.
Figure 4.14 MALDI-TOF MS analysis of tryptic digest of albumin. The y-axis all show the relative ion current and the x-axis the mass/charge ratio. The upper trace shows the peptides observed in control transferrin, the middle trace the peptides obtained when transferrin was reacted with peroxynitrite and the lower trace shows the lack of any discernable peptide peaks when peptides containing at least one nitrated tyrosine residue were isolated using the antibody antinitrotyrosine.
In the case of both transferrin and albumin no peptides were observed using the method described above. It is possible that no actual tyrosine nitrations are occurring and that the differences in traces are due purely to other effects of peroxynitrite. It may also be that because both transferrin and albumin contain numerous methionine residues these residues are being oxidised in preference to the nitration of tyrosine residues. However, after analysis of the number of peptides with oxidised methionine residues there was no difference between control and peroxynitrite treated proteins.

4.7 Discussion

The results obtained using the MALDI-TOF MS have not revealed any sites of either tyrosine or tryptophan nitration. The loss of peptides containing tyrosine residues does suggest that modifications are occurring. It is possible that the tyrosine nitration modification affects peptide ionisation and therefore prevents peptide analysis using MALDI-TOF MS. Previous work using a prototype MALDI orthogonal quadrupole-TOF (Q-TOF) MS to analyse the nitration of peptides have shown the presence of nitrated peptides. Sarver et al (2001) found that when BSA was nitrated two peptides were significantly reduced in relative ion abundance, m/z 927.4 and 1479.4. They also found that two new ions appeared in the same mass region that would normally correspond to the substitution of a hydrogen with NO\(_2\) (+ 45 Da) at m/z 972.5 and 1524.6, as would be expected if 3-nitrotyrosine was present in these peptides; neither of these modified masses were observed in the work presented here. These two new nitro-tyrosine-containing peptides also appeared to have associated ions that were 16 and 32 Da lower in mass. These associated peptides suggest that 3-nitrotyrosine is undergoing a series of photodecomposition reactions involving the loss of one or two oxygens. The loss of a single oxygen from the aromatic nitro group to form the nitroso analogue is relatively straightforward and can be rationalised as involving a two electron reduction process accompanied
by a transfer of two protons and loss of water. Such a pathway is a common photodecomposition product of many nitroaromatic compounds in the presence of proton donors (Ho and Chow 1996), which could be the MALDI matrix and/or residual solvent. The loss of two oxygens from the nitrotyrosine side chain is more problematic, and probably involves multiple steps. It is conceivable that the second loss of oxygen could involve participation of the neighbouring hydroxyl group, accompanied by a reduction step from the matrix. Sarver et al (2001) also examined the effects of time (i.e., number of laser shots), laser power, and peptide concentration on the formation of the photodecomposition fragments. It was found that time and laser power had no discernible effect on the spectra. However a marked difference was observed with peptide concentration. At very high amounts photodecomposition products were considerably lower in abundance than the molecular ion. At successive 10-fold dilution to 2.5 pmol, the abundance of the photodecomposition products increased.

Since the development of this method a study performed by Petersson et al (2001) have also found that the nitration of BSA by tetranitromethane does not reveal the presence of nitrated peptides when analysed by MALDI-TOF MS. They theorised that this may be due to suppression of signal due to the presence of non-nitrated peptides or that the photodecomposition of the NO₂ group reduces the concentration of the nitrated ions to a point below the level of detection of the MALDI-TOF MS. This may explain why no nitrated peptides were noted in this study with the use of the antinitrotyrosine antibody as the concentration of nitrated peptides may be below detection levels. They also found that when the nitration of BSA was analysed using Q-TOF MS/MS the NO₂ group did not undergo photodecomposition this would significantly increase the concentration of the nitrated peptides and allows analysis of these peptides using methods other than MALDI-TOF MS.
Due to the inability to isolated nitrated peptides from a mixture of peptides it was then decided to study the effect of both peroxynitrite and the peroxynitrite generator SIN-1 on individual peptides.
4.8 References


CHAPTER 5. MALDI-TOF MS ANALYSIS OF PEPTIDE NITRATION

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5.1 Introduction

The exposure of proteins to peroxynitrite did not result in the identification of any peptides containing a tyrosine nitration. It is possible that the treatment of a whole protein with peroxynitrite did not result in the nitration of tyrosine because there are numerous other targets for peroxynitrite in a protein. It is also possible that the presence of non-nitrated peptides suppresses signals from nitrated peptides. It was therefore decided to look at isolated peptides to try and discern any reactions occurring. The presence of only one peptide also makes the spectrum obtained using the MALDI-TOF MS simpler and allows analysis of any unexpected peaks.

5.2 Methods

A range of peptides were chosen to study, ranging from peptides containing one, two tyrosine residues and peptides that do not contain methionine residues. The peptides chosen are shown in table 5.1 below.

<table>
<thead>
<tr>
<th>Name</th>
<th>m/z</th>
<th>Peptide sequence *</th>
</tr>
</thead>
<tbody>
<tr>
<td>Angiotensin</td>
<td>1296.5</td>
<td>Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu</td>
</tr>
<tr>
<td>ACTH clip (18-39)</td>
<td>2465.7</td>
<td>Arg-Pro-Val-Lys-Val-Tyr-Pro-Asn-Gly-Ala-Glu-Asp-Glu-Ser-Ala-Glu-Ala-Phe-Pro-Leu-Glu-Phe</td>
</tr>
<tr>
<td>N-acetyl-renin</td>
<td>1801.1</td>
<td>Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-Leu-Tyr-Ser</td>
</tr>
<tr>
<td>Substance P</td>
<td>1347.6</td>
<td>Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met</td>
</tr>
<tr>
<td>ACTH 4-10</td>
<td>962.1</td>
<td>Met-Glu-His-Phe-Arg-Trp-Gly</td>
</tr>
</tbody>
</table>

Table 5.1 The theoretical mass and the sequence of the peptides used to study the affects of peroxynitrite. * See appendix 1.

The peptides were exposed to peroxynitrite using a similar method to that used to expose proteins. The peptide was diluted in a phosphate buffer
(100mM) and then exposed to either SIN-1 (5mM) for 1½ hrs or a bolus addition of peroxynitrite (0.5µM) for 5 mins. After exposure the peptides were cleaned using a C18 column and were then prepared for MALDI-TOF MS analysis.

5.3 Results

When the fragment 18-39 of adrenocorticotropic hormone was exposed to SIN-1 a modification of +28 Da was observed. The sequence of this peptide is ‘Arg-Pro-Val-Lys-Val-Tyr-Pro-Asn-Gly-Ala-Glu-Asp-Glu-Ser-Ala-Glu-Ala-Phe-Pro-Leu-Glu-Phe’. It has only one tyrosine residue and no nitration of this tyrosine residue was observed. The bolus addition of peroxynitrite had no discernible effect on the spectrum (figure 5.1).

Figure 5.1 The control trace for the peptide RPKVYPNGAEDESAEAFP is shown at the top with the SIN-1 treated peptide at the bottom, a mass increment of 28 Da can be seen clearly.
Substance P contains one methionine residue and it was noted that a small percentage of control substance P contains oxidised methionine. This may be due to the method used to prepare the peptide as the processes of using the C18 column, the matrix and ionisation method used in the MALDI-TOF MS technique can cause oxidation of methionine residues. It was, however, noted that after exposure to SIN-1 the percentage of methionine oxidised increased significantly and only a small percentage remained unmodified. This supports the previous work showing that methionine residues are particularly susceptible to oxidation.

When Substance P (1364.92) was exposed to SIN-1 (5mM) a peptide was observed at m/z 1392.87 which again is an increment of + 28 Da (figure 5.2). Other smaller peptides were also observed at m/z 1376.92 and 1408.92. These two peptides may relate to peptide at m/z 1392.87 i.e., the loss and gain of 16 Da, which could be rationalised as being the loss and gain of an oxygen. This would suggest that the increment of + 28 is a form of nitrosylation reaction on one of the amino acids within the peptide, as the –NO group has previously been shown to undergo photodecomposition reactions to –N and to further oxidise to –NO$_2$ (Sarver et al. 2001; Gunther et al. 1997). The addition of –NO to an amino acid was theorised to cause a mass increase of + 29 Da as this would be the addition of an NO group with the loss of one hydrogen, however it is possible that depending on the position of bonding another hydrogen is lost. Which amino acid is involved in this reaction cannot be elucidated using the MALDI-TOF MS data gained in these experiments due to the lack of sequencing data. None of the amino acids contained within the sequence of both ACTH$_{18-39}$ and substance P have been previously shown to be nitrosylated when reacted with peroxynitrite. This may be a previously unreported effect of peroxynitrite or it may be due to the by-products formed by the reaction SIN-1 as this product of + 28 was not observed when these peptides were treated with bolus addition of peroxynitrite.
Figure 5.2 MALDI-TOF MS spectra of Substance P, lower trace shows the control peptide, 1349.09 is the unmodified peptide and 1365.02 is the peptide containing an oxidised methionine residue. The upper trace shows the spectrum when substance P is reacted with SIN-1, the spectrum shows both the unmodified and methionine oxidised, 1392.87 is the peptide with the + 28 Da modification with the loss and gain of 16 Da at m/z 1376.92 and 1408.92.
The spectrum of angiotensin 1 exposed to 5mM of SIN-1 shows that the main modification is again the addition of 28 Da (m/z 1324.5) (figure 5.3). A peptide associated with this increment of +28 can be observed at m/z 1340.24, which is again an addition of +16. However, additional modifications of +16 were also observed at m/z 1312.45 and 1356.2. The peptide at m/z 1312.45 relates to the oxidation of the control peptide and the peptide at m/z 1356.2 relates to the oxidation of the already modified peptide i.e., total increment of +60. This suggests that as well as the hypothesised nitrosylation reaction an amino acid is also undergoing an oxidation reaction.

Though these experiments were performed in the presence of bicarbonate (10mM) which is thought to inhibit oxidation reactions, these results show that oxidation seems to be the main reaction of peroxynitrite. It is possible that because of the reduced number of possible sites of reaction when compared to whole proteins reactions are occurring that would not occur in vivo.

It was also noted that peptides reduced in mass could also be observed after exposure to SIN-1. These peptides were observed at m/z 1046.69 and 1074.55, again there is a difference between these two peptides of 28 Da, which suggests that they are related to the control mass and they were not observed in control spectrum. These reduced masses relate to the theoretical mass of angiotensin 1 with the two end residues, Leu and His cleaved off. This cleavage seems to be an effect of the exposure to SIN-1 and may be a previously unreported effect of low levels of peroxynitrite.
Figure 5.3 MALDI-TOF MS spectrum of angiotensin 1 (m/z 1296.5) exposed to 5mM SIN-1, the main modification is the addition of 28 Da (m/z 1324.5), additional modifications of +16 Da can be observed at m/z 1312.45 and 1356.2. A peptide can also be observed at m/z 1046.69, this peptide relates to the theoretical mass of angiotensin 1 with the end two amino acids cleaved off, and again the associated + 28 Da modification at m/z 1074.55.
The exposure of ACTH (4-10) to both SIN-1 and bolus addition of peroxynitrite produced complex spectra (figures 5.4 and 5.5). The sequence of ACTH (4-10) is Met-Glu-His-Phe-Arg-Trp-Gly; the main target for peroxynitrite in this peptide is the methionine residue. As shown previously the percentage of methionine oxidised increased (m/z 978.02) when exposed to bolus addition of peroxynitrite, this, however, wasn't the only modification observed.

When ACTH (4-10) was exposed to SIN-1 successive increments were observed, the peptide at m/z 1036.75 can be explained as being the addition of 28 Da as observed previously, this peptide showed related peptides at both −16 and +16 Da. There was, however, also an initial addition of +30 Da which was not observed in any other peptide sequence, this peptide also had a related peptide at −16 Da. This may still be a type of nitrosylation reaction, but may cause a different mass increase because the reaction occurs with a different amino acid leading to different bonding properties and may occur without the loss of hydrogen. This peptide also revealed an additional oxidation at m/z 1078.97 similar to that observed with the peptide angiotensin.
Figure 5.4 MALDI-TOF MS spectra of ACTH 4-10 (m/z 961.99), lower trace is the control peptide and shows that a percentage of the peptide contains an oxidised methionine residue. The upper trace shows that when the peptide is reacted with SIN-1 successive modifications can be observed.
Figure 5.5 MALDI-TOF MS spectrum showing the peptides observed when ACTH 4-10 (m/z 962.14) was reacted with peroxynitrite, the trace shows successive oxidation reactions.
When ACTH (4-10) was reacted with bolus peroxynitrite at least 5 successive oxidations were observed after the initial oxidation of methionine. Methionine residues can undergo two oxidation reactions as shown in figure 5.6; this would explain the increments of +16 and +32. The other oxidation reactions remain unexplained at present. These spectra again show the differences obtained through the reaction with bolus peroxynitrite and SIN-1, this may be due to SIN-1 producing by-products which may then go on to react with the peptide.

\[
\begin{align*}
\text{Methionine} & \quad \text{Sulfoxide} & \quad \text{Sulfone} \\
\text{CH}_3 & \quad \text{CH}_3 & \quad \text{CH}_3 \\
\uparrow & \quad \uparrow & \quad \uparrow \\
\text{S} & \quad \text{S}=\text{O} & \quad \text{O}=\text{S}=\text{O} \\
\text{CH}_2 & \quad \text{CH}_2 & \quad \text{CH}_2 \\
\uparrow & \quad \uparrow & \quad \uparrow \\
\text{CH}_2 & \quad \text{CH}_2 & \quad \text{CH}_2 \\
\uparrow & \quad \uparrow & \quad \uparrow \\
-\text{NH-CH-CO-} & \quad -\text{NH-CH-CO-} & \quad -\text{NH-CH-CO-} \\
\end{align*}
\]

Figure 5.6 Reaction mechanism for the single and double oxidation of methionine
The exposure of N-acetyl-renin substrate tetradecapeptide, mass 1801.1, to SIN-1 and bolus peroxynitrite, gave complex spectra, three main types of events occurring. The first spectrum shows oxidation and nitration events, second spectrum shows dimerisation events and the third spectrum shows cleavage events (figures 5.7 to 5.9).

One important point is that there is no evidence of a mass increase of 28, this may be due to other targets of peroxynitrite being favoured. The peptide's reaction with SIN-1 caused the appearance of peptides at m/z 1817.32 and 1846.34. The peptide at m/z 1817.32 is presumably due to an oxidation reaction The peak at m/z 1846.34 corresponds to the substitution of a hydrogen with NO₂ as would be expected if nitro-tyrosine was present in this peptide. The new nitro-tyrosine-containing peptide ion appears to have an associated ion that is 16 Da lower in mass, i.e., m/z 1830.29. This suggests that 3-nitrotyrosine is undergoing photodecomposition involving the loss of an oxygen atom, this could be accompanied by further reactions that weren’t visible in the spectrum. The loss of a single oxygen from the aromatic nitro group to form the nitroso analog [Tyr(NO)] is relatively straightforward and can be rationalised as involving a two electron reduction process accompanied by the transfer of two protons and loss of water (figure 5.10). Such a pathway is a common photodecomposition product of many nitroaromatic compounds in the presence of proton donors, which in this case would be the MALDI matrix and/or residual solvent.
Figure 5.10 Photodecomposition reaction leading to the formation of nitrosotyrosine.

The second spectrum (figure 5.8) shows that nitration isn’t the only reaction occurring when this peptide is reacted with SIN-1 or bolus addition of peroxynitrite. The peptide observed at m/z 3856.06 (SIN-1) and 3855.63 (bolus peroxynitrite) corresponds to the dimerisation of the peptide plus an addition of $\approx 254$. If two of the possible four tyrosine residues contained in the dimerised peptide are nitrated and there is an addition of another free tyrosine residue this would account for this mass increase. Spectrum three (figure 5.9) shows that cleavage events are occurring when this peptide is exposed to SIN-1 and it is therefore possible that free tyrosine residues are being generated through this process. As there are two tyrosine residues within this peptide, it is possible that one tyrosine residue is responsible for the dimerisation of the peptide and that the other tyrosine residue dimerises with a lone tyrosine residue. The other peptides observed in spectrum two are possible due to the dimerisation of the peptide with cleaved peptides present in the mixture. The results obtained from this one peptide show that the effects of peroxynitrite whether bolus addition or low levels (SIN-1) cannot be isolated into oxidation or nitration reactions alone as it seems capable, in vitro at least, of causing other more complex reactions.
Figure 5.7 MALDI-TOF MS spectra of the exposure of N-acetyl-renin (m/z 1801.45) to bolus peroxynitrite and SIN-1. The lower trace shows the control spectrum and the middle spectra shows the peptides observed when reacted with bolus peroxynitrite. The upper trace gives evidence of oxidation and nitration events occurring when N-acetyl-renin is reacted with SIN-1.
Figure 5.8 MALDI-TOF MS spectra of N-acetyl-renin (m/z 1801.45) when reacted with bolus peroxynitrite (lower trace) and SIN-1 (upper trace). Both traces show evidence of dimerisation events with peptides observed at m/z 3855.63 and 3856.06 corresponding to dimerisation plus nitration of tyrosine residues.
Figure 5.9 MALDI-TOF MS spectrum of the exposure of N-acetyl-renin (m/z 1801.45) to SIN-1. The arrows indicate the theoretical peptide fragments.
As previously mentioned the third spectrum obtained from the reaction of N-acetyl-Renin with SIN-1 shows that cleavage events are occurring. The spectrum obtained from the reaction of N-acetyl-Renin with SIN-1 is similar to the results that would be obtained if MALDI-TOF MS was used to produce fragmentation data. The mass spectrometer generates a series of fragmentation ions by shattering the parent ion into many fragments of varying size, with the masses of these fragments recorded. Each of these fragments differs from the next by one amino acid (in the simplest of cases) and each amino acid in the sequence is identified from the mass difference between successive peaks. However, none of the peptides observed in this spectrum are seen in control N-acetyl-Renin, which underwent exactly the same sample work up except for the addition of SIN-1, suggesting that the fragmentation is due to an effect of the reaction with SIN-1 and not due to the MALDI-TOF MS itself. As can be seen in the figure, the reaction with SIN-1 seems to cleave between amino acids and I have postulated possible structures for some of the observed peaks, these show that the peptide is losing successive amino acid from the C-terminus. Some of the peaks remain unidentified at present. This is probably because of a combination of modifications occurring, i.e., one or both of the tyrosines may be nitrated, dinitrated or oxidised, it is also possible that other free amino acids bind to the peptide making identification of the peak difficult. Fragmentation of proteins by reaction with bolus peroxynitrite has been shown previously by Ischiropoulos and Al-Mehdi (1995), who, studying albumin, found that when reacted with peroxynitrite and visualised using electrophoresis, there was an increase in band intensity of protein fragments with molecular weight lower than 66,000 Da (the molecular weight of albumin).
5.4 Discussion

<table>
<thead>
<tr>
<th>Name</th>
<th>Peptide sequence</th>
<th>Modifications with SIN-1</th>
<th>Other</th>
</tr>
</thead>
<tbody>
<tr>
<td>Angiotensin</td>
<td>DRVYIHFPHL</td>
<td>+ 28</td>
<td>Fragmentation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2 x + 16</td>
<td></td>
</tr>
<tr>
<td>ACTH clip (18-39)</td>
<td>RPVKVYPNGAESAEAFPLEF</td>
<td>+ 28</td>
<td></td>
</tr>
<tr>
<td>N-acetyl-renin</td>
<td>DRVYIHFPFLLVYS</td>
<td>+ 45</td>
<td>Dimerisation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+ 16</td>
<td></td>
</tr>
<tr>
<td>Substance P</td>
<td>RPKPQQFFGLM</td>
<td>+ 28</td>
<td>Fragmentation</td>
</tr>
<tr>
<td>ACTH 4-10</td>
<td>MEHFRWG</td>
<td>+ 28</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>+ 30</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>+ 16</td>
<td></td>
</tr>
</tbody>
</table>

Table 5.2 A summary of the results obtained in these experiments.

A summary of the results obtained from these experiments is given in table 5.2. When comparing the reactions of SIN-1 with the various peptides used in these experiments it was observed that the addition of + 28 seems to be a universal reaction with the exception of N-acetyl-renin. This peptide may not undergo this reaction because there are numerous other preferred reactions. A further result is that the peptides angiotensin and N-acetyl-renin revealed different reactions, and these two peptides only differ by 4 amino acids, N-acetyl-Renin having Leu-Val-Tyr-Ser as extra amino acids. It is possible that the addition of a second tyrosine residue increases the ability of the peroxynitrite to nitrate one of the residues. It has been shown previously that the amino acids surrounding the tyrosine residue do affect its ability to be nitrated (Souza et al. 1999) and this may be having an influence here.

The data obtained by reacting peptides with bolus peroxynitrite and SIN-1 shows that peroxynitrite does not simply nitrate tyrosine or oxidise methionine residues; there are a number of other reactions occurring. As previously shown the oxidation of methionine residues seems to be the main target for peroxynitrite, though it is capable of oxidising other residues.
Previous work by Alvarez et al. (1999) found that the most reactive amino acids were Cys, Met and Trp. The other amino acids were 1-3 orders of magnitude less reactive, but most amino acids were found to react with peroxynitrite to a degree. Histidine, leucine and phenylalanine, however, were found to undergo no discernible reaction with peroxynitrite.

When comparing the results obtained from the different peptides used in the experiments, it was found that three of the peptides showed additional increments of + 16 Da unrelated to the addition of 28. By comparing these peptides that did not show an addition of 16 Da it was noted that the amino acid residue histidine was present in the peptides showing an increment of + 16 and was not present in the other peptides. Histidine has been previously reported to undergo metal-catalysed oxidation reactions to form 2-oxo-histidine, characterised by a gain of 16 atomic mass units in molecular weights (Uchida and Kawakishi 1994).

A possible scheme of formation of 2-oxo-histidine has been suggested by Zhao et al (1997) and is shown in Figure 5.11.
The results obtained from the peptide work gives strong evidence that SIN-1 can oxidise histidine residues, whether the reaction is the same as the one postulated above is unknown at present. It is possible that there is a quantity of copper in the reaction mixture used, but the mechanism may utilise a different pathway. Bolus addition of peroxynitrite was also found to oxidise the peptide N-acetyl-Renin suggesting that this oxidation is not due to histidine reacting with by-products of the SIN-1 decomposition.
Out of all the peptides investigated only N-acetyl-renin revealed the presence of a nitrated tyrosine residue, this is an unusual observation as previous work has shown that when proteins or isolated peptides are reacted with either bolus peroxynitrite or SIN-1 tyrosine nitration is a common occurrence (Jiao et al. 2001; Souza et al. 1999; Alvarez et al. 1999 and Greis et al. 1996). The difference in my results and the published papers may be due to differing exposure techniques and levels of exposure.

The tyrosine nitration that was observed in the case of N-acetyl-renin was only a small percentage of the overall reactivity and in relation to the work performed with whole protein shows that a nitrated peptide could easily be missed due to the presence of unmodified peptides and the occurrence of other reactions. It also shows that when the antibody was used to isolate the nitrated peptides the concentrations of nitrated peptide would be very low and may be lost during the required clean up steps.
5.5 References


CHAPTER 6. Q-TOF MS MATERIALS AND METHODS

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6.1 Introduction

The results obtained from the MALDI-TOF MS on both whole proteins and peptide fragments, have revealed that the nitration of tyrosine or oxidation of methionine residues are not the only peroxynitrite initiated modifications. The main problem with MALDI-TOF MS data is that modifications observed cannot be assigned to specific amino acids, therefore it was decided to sequence the same proteins and two of the peptides using a quadrupole time-of-flight mass spectrometer (Q-TOF MS)

6.2 Quadrupole time-of-flight mass spectrometry (Q-TOF MS)

The quadrupole-time of flight mass spectrometer (Q-TOF MS) is technically a hybrid mass spectrometer. It is the combination of two mass spectrometry technologies to create the most powerful commercially available mass spectrometer for proteomic analyses. The Q-TOF is the amalgamation of quadrupole / collision cell technology coupled to an orthogonally situated time of flight analyser (figure 6.1).
Figure 6.1. A solution containing the analyte of interest is sprayed from the end of a capillary by application of a high electric potential. The resulting charged droplets are stripped of solvent and ions formed from analyte molecules are directed electrically into the mass spectrometer (Z-spray). The ion beam passes into a quadrupole analyser, which may be operated in a narrow band mode so as to transmit ions of defined m/z values or in its wide bandpass mode in which all ions are transmitted irrespective of m/z value. There is a further focusing hexapole, after which the ion beam is focused and accelerated by an electric lens before being passed into the TOF analyser in front of a deflector electrode. A high electric potential is applied to this electrode in pulses so that, at each pulse, a section of the ion beam is deflected and accelerated into the TOF analyser. After reflection by the reflectron, the ions are detected at a microchannel plate multipoint collector. The reflectron is used mainly to increase the time intervals at which successive m/z values are detected at the collector. The quadrupole is operated in the narrow bandpass mode for MS/MS and in its wide bandpass mode for obtaining a full spectrum by the TOF analyser.
6.2.1 Electrospray

A solution of an analyte in a solvent can be sprayed (nebulised) from an electrically-charged narrow tube to give small electrically-charged droplets, which desorb solvent molecules to leave ions of the analyte. This atmospheric pressure ionisation is known in various forms, the Q-TOF contains a Z-spray, that is an adaptation of electrospray.

Electrospray is one method effectively used for differential solvent removal. A sample for mass spectrometry is often dissolved in an organic or an aqueous solvent, the sample to be examined is in solution and cannot be put straight into a mass spectrometer without first removing most of the solvent and without, of course, removing the dissolved sample. The solution is passed along a short length of stainless steel capillary tube, to the end of which is applied a high positive or negative electric potential, typically 3-5 kV. When the solution reaches the end of the tube, the powerful electric field causes it to be almost instantaneously vaporised (nebulised) into a jet or spray of very small droplets of solution in solvent vapour. Before entering the mass spectrometer proper this mist of droplets flows along an evacuated tube which is continuously pumped down to a modest vacuum and the walls of which are heated slightly to prevent condensation. As the droplets move through this region, solvent evaporates rapidly from their surfaces and the droplets get smaller and smaller. In addition to producing the spray, this method of rapid vaporisation leaves no time for equilibrium to be attained and a substantial proportion of the droplets have an excess of positive or negative charge which resides on their surfaces. Thus as the droplets get smaller the electrical surface charge density increases until such a time that the natural repulsion between like charges cause ions as well as neutral molecules to be released from the surface (figure 6.2). The charges are statistically distributed amongst the analyte's available charge sites, leading to the possible formation of multiply charged ions. The end of the capillary tube is aimed somewhat like a gun at a small hole (target) at the opposite end of this vacuum region.
Figure 6.2 After being formed as a spray, many of the droplets contain some excess positive (or negative) charge. Solvent (S) evaporates from the droplets to form smaller ones, until eventually, ions (MH⁺, SH⁺) from the sample M and solvent begin to evaporate to leave even smaller drops and clusters (SnH⁺; n=1, 2, 3 etc.). Later, collisions between ions and molecules leave MH⁺ ions which proceed on into the mass analyser.

6.2.2 Z-spray combined inlet/ion source

As an adaptation of electrospray, Z-spray is cleaner and more efficient for the generation and separation of analyte ions from solvent and buffer agents. In conventional electrospray sources droplets issue from the end of a narrow inlet tube as a cone-shaped spray, in which small molecular mass solvent molecules tend to diffuse away towards the edges of the cone and high molecular mass analyte ion continue along the axis of the cone until they enter the mass spectrometer analyser through a small orifice (the skimmer). Thus the narrow solution inlet, the cone axis and the position of the orifice lie all in one straight line-of-sight (figure 6.3a). Approximately, ions produced in such a situation travel along a linear
trajectory from formation to entering the analyser. Ions going through the skimmer are accompanied by small quantities of neutral materials and some of these as well as ions strike the edges of the skimmer and are deposited there. Gradually, the skimmer hole becomes blocked. The Z-spray inlet/ionisation source has a geometry such that the trajectory of the ions can be likened to a somewhat flattened Z-shape (figure 6.3b) and hence the name. However a final skimmer is set off to one side of the spray instead of being in-line. Any neutral species continue on to the vacuum pumps so that build-up of deposits and blockages are greatly reduced.
Figure 6.3 (a) The trajectory of analyte ions from a conventional electrospray inlet/ion source is essentially a straight line between the inlet tube carrying the solution of interest and a skimmer orifice placed a short distance away. The neutral solvent molecules are shown diffusing away from the main ion beam over a roughly cone-shaped space.

(b) The trajectory of analyte ions from a Z-spray inlet/ion source follows a sort of flattened Z-shape in going from the inlet tube to the final skimmer. Again neutral solvent molecules are shown diffusing away from the main ion beam over a roughly cone shaped space. However, unlike the situation in (a), the ion beam first passes through an initial skimmer orifice placed such that it lies at right angles to the direction of the spray. Electrical potentials in the source cause the ion beam to bend towards the skimmer orifice and, having passed through it, to bend once again so as to pass the extraction cone.
6.2.3 Hexapoles

The ion beam then passes into a hexapole. A hexapole is made up of six rods (poles) evenly spaced around a central axis (figure 6.4). The hexapole cannot act as a mass filter by applying a DC field and is used only in its all (radiofrequency) RF mode, in which its allows all ions in an ion beam to pass through whatever their m/z values. In doing this, the ion beam is constrained so that it leaves the hexapole as a narrow beam. This is important because the ion beam from the inlet system tends to spread due to mutual ion repulsion and collision with residual air and solvent molecules. By injecting this divergent beam into a hexapole unit, it can be ‘refocused’.

Figure 6.4. A diagrammatic representation of a hexapole unit. The left-hand of the figure shows the end view of the unit, the ends of the six poles being represented as open circles and the ion beam by the black circle. The right-hand of the figure shows an angled side view of the unit, showing the six parallel poles (or rods), with the ion beam passing through the middle of them.
6.2.4 A quadrupole in wide bandpass mode

All ions from the ion source are injected into the quadrupole via a hexapole unit. In the ion source, the ions produced have approximate thermal energies corresponding to a room temperature ground state. On passing through the skimmer, the ions are accelerated to supersonic speeds by the gas expansion that occurs. The first hexapole has a high enough gas pressure to slow down the ions to thermal energies once more. Next, the quadrupole allows all of the ions to pass through in its wide bandpass mode. The ion beam is then collimated by two further hexapoles before passing through an electric lens, which defines the beam and accelerates the ions to a kinetic energy of about 40 volts. The ions reach the pusher electrode and a full mass spectrum is obtained in the TOF analyser. Thus, in this configuration, ions are simply transmitted from an ion source through the quadrupole and into the TOF analyser and the mass/charge ratio is measured.

As the analyser measures the m/z ratio only when the peptides contain a single charge will the mass of the peptide be observed on the spectrum, when peptides hold a double charge the mass observed will be halved, peptides with three charges will have a mass a third of the expected value etc. The mass difference between the isotopic peaks can be used to indicate how many charges the peptide is holding, as the mass difference between the different isotopes of carbon is 1 Da. When the peptide is doubly charged the mass difference between isotopes becomes ½ Da and when the peptide holds three charges the difference between isotopes becomes ⅓ of a Da.

Calculating the mass of a protein from the mass spectrum is performed in two stages. The first is the determination of the charge state of the ions and the second involves combining this data with the mass to charge ratio to give the molecular mass of the peptide. As mentioned above the charge state of the ions can be determined simply by studying the
spectrum obtained. The molecular mass of the peptide can be calculated using the following equation:

Molecular mass = [ (m/z) x charge state of the ion] – the charge state of the ion.

Figure 6.5 Graphs showing singly, doubly and triply charged peptides. (A) singly charged peptide (mass difference 1 Da), (B) doubly charged peptide (mass difference ½ Da), (C) triply charged peptide (mass difference ½ Da).
6.2.5 A quadrupole in narrow bandpass mode and a hexapole collision cell

Ions from an ion source are injected into the quadrupole via the hexapole unit as before. Now however, ion selection is made. By adjusting DC and RF voltages on the poles, ions of a particular m/z value are chosen (figure 6.6). Ions of m/z values not selected, do not pass through but strike the poles (rods) and are lost. By continuously adjusting the voltages on the poles, ions of successive m/z values can be allowed to pass through the assembly in turn so as to give a mass spectrum of all of the ions injected into the front end (e.g., from an ion source). This configuration of electric fields is often termed the narrow bandpass mode and effectively acts as an electronic gate (sometimes erroneously called a mass filter). By opening or closing the gate, ions of selected m/z values can be allowed to traverse from one end of the quadrupole assembly to the other. The middle hexapole (figure 6.1) can become a gas collision cell by increasing the background gas pressure within this assembly (this is the reason the assembly is shown encased in a container having holes just large enough to transmit the ion beam but small enough that it is possible to maintain fairly easily a small gas pressure against the effect of the vacuum pumps). The selected ion's emerging from the quadrupole, collide with gas molecules in this hexapole, and gain sufficient internal energy to fragment. This is usually done by colliding the ions with a neutral gas in a process called collision-induced dissociation (CID). Collision-induced dissociation works because if an ion collides with a neutral atom or molecule e.g. argon, some of the ions energy can be converted into internal energy. This is called collision activation. If there is enough excess internal energy to break chemical bonds, the ion will decompose. Thus, selected precursor ions are caused to dissociate to give product (fragment) ions. The product ions and any unchanged precursor ions travel on to the TOF analyser, in which a mass spectrum is obtained.
Figure 6.6  A schematic drawing of ions in a beam entering a quadrupole assembly. The shaded circles represent ions of one particular m/z value that are to be selected. The open circles represent all ions of other m/z values that will not be selected. Diagram (a) represents the wide bandpass mode and all ions (shaded and unshaded) are transmitted. Diagram (b) represents the narrow bandpass mode, the shaded ions have been selected and pass through the quadrupole assembly but other ions follow trajectories that lead to them striking the quadrupole rods and being lost; for the sake of clarity, only two such incidents are shown.

6.2.6 Fragmentation

The mass spectrometer does not sequence from any particular terminus, it generates a series of fragmentation ions by shattering the parent ion into many fragments of varying sizes. Each of these fragments differs from the next by one amino acid (in the simplest of cases) and each amino acid in sequence is identified from the mass difference between successive peaks. In peptides the fragmentation generally occurs at the peptide backbone, however cleavage at peripheral bonds may occur. In
order to understand peptide fragmentation a nomenclature system was developed by Roepstorff and Fohlman (1984) (figure 6.7)

\[ R_1 \quad A_1 \quad B_1 \quad C_1 \quad A_2 \quad B_2 \quad C_2 \quad A_3 \quad B_3 \quad C_3 \]

Figure 6.7 Example of the possible fragmentation sites in a generic peptide backbone, \((R_{1-4})\) represents the different structural groups found on amino acids. Fragment ions of the type \(a_n, b_n,\) and \(c_n\) are generated when the charge is retained on the N terminus of the peptide. The \(x_n, y_n\) and \(z_n\) ions are formed if the charge is retained at the C terminus. Fragment ions corresponding to side chains are also seen in some spectra and are denoted \(d_n\) (n-terminus), \(v_n\) (C-terminus) and \(w_n\) (C-terminus). The \(d_n\) ions, corresponding to side chain ions, are very informative as they provide a means to differentiate between the isomeric amino acids leucine and isoleucine. If leucine were the amino acid at the terminus, an isopropyl ion would be eliminated. On the other hand, isoleucine would eliminate a methyl or ethyl ion, these different ions would be observed in the spectrum.

Figure 6.8 shows an example of a peptide from control transferrin, carboaminomethylated and digested with trypsin. The peaks on the spectrum relate to various fragmentation events. For simplicity, only the \(y\)-ions are labelled in figure 6.8. To be able to decipher the data obtained using the Q-TOF the masses of the fragments are entered into the MS-FIT section of the online database proteinprospector. This database searches through its database for matching peptides, it uses the mass of the parent ion and a range of possible masses for the protein (usually set as 1000-100,000) as pre-filters, it can also use species as a pre-filter.
In the case of the example peptide only two matches are given by the database both relating to the same peptide, but from two different species, rabbit and human (figure 6.9). As with the MALDI-TOF MS data it is important to search all species, instead of just the one of interest, in case of contamination. The information obtained from the database identifies the fragments observed in the spectrum (figure 6.9), where there is no data underneath the mass, these are masses which are not related to fragments. The unidentified masses may be due to unexpected fragmentation events, or to contaminating peaks. Figure 6.10 shows other data that can be obtained using this database, this gives the full list of the possible fragments of this peptide.
Figure 6.8 Q-TOF MS/MS spectrum of the control transferrin peptide EGYGYTGAFR (AA's 512-522). The brackets between peaks represent the mass relating to amino acids. As amino acids are removed from the N-terminus the mass decreases, for example the mass 1154.62 represents the peptide minus a glutamate residue. The extrapeaks include losses from the C-terminus of the peptide and losses of simple neutrals for example NH$_2$ and H$_2$O.
Figure 6.9 Example of the results obtained from the online database proteinprospector. The matching peptide is highlighted in red, in blue are the MS-digest index numbers and the SWISS-PROT accession numbers. The masses entered into the database are in bold and underneath each of these numbers are the matching fragments. Where there is no matching fragment in the database, the box underneath the submitted value is blank.
Figure 6.10  A full list of the possible fragmentation masses obtained from the peptide EGYGYTGAFR (AA’s 512-522).
6.3 Sequencing of a control peptide using Q-TOF MS/MS

6.3.1 Method

Q-TOF MS/MS was first used to examine the modification of 28 found when angiotensin was reacted with SIN-1. The peptide was reacted with 5µM SIN-1 for 2hr, cleaned using C18 columns and then analysed using the Q-TOF MS. All experiments were performed on a Micromass Q-TOF mass spectrometer (Micromass, Altrincham, U.K.), equipped with a HPLC column to separate the samples before being introduced to the Z-spray source. The capillary voltage used was 2800 V, and the cone voltage was 40 V.

6.3.2 Sequencing data obtained from the SIN-1 reacted peptide angiotensin

When the spectrum for angiotensin was examined fragmentation data was acquired for a peptide with two charged states, these had mass/charge ratios of 442.227 Da and 662.835 Da. The first charge state at m/z 442.227 Da was observed to be triply charged (mass difference between isotopes of carbon = 28 Da) and related to a peptide of 1324.681 Da and the second charge state at m/z 662.83 Da was observed to be doubly charged (mass difference between isotopes of carbon = 14 Da) and related to a peptide of 1324.67 Da. The control mass of this peptide was 1296.5 Da, therefore when the peptide has been exposed to 5 mM of SIN-1 for 2 hr a modification of +28 Da occurs. This result using Q-TOF MS was the same as that found using MALDI-TOF MS. The full MS/MS spectrum obtained from the peptide 442.227 Da, mass 1324.681 Da is shown in figure 6.11, the data obtained from this spectrum could not be entered directly into MS-FIT in proteinprospector as the modification is unknown and therefore cannot be accounted for in the database. The peaks obtained in the spectrum were
therefore compared to the control values to isolate differences between control and modified. Using the program MS-Product within proteinprospector, it is possible to enter a sequence of amino acids and obtain the theoretical fragmentation data (figure 6.12).
Figure 6.11 Q-TOF MS/MS spectrum of the peptide angiotensin 1, exposed to SIN-1. The brackets between peaks represent the masses relating to amino acids. This peptide can be sequenced fully up to the valine residue.
Figure 6.12 MS-Product theoretical fragmentation data for the peptide DRVYIHPFHHL (angiotensin).

When the experimental data obtained from Q-TOF MS/MS sequencing of the peptide 1324.67 Da was compared to the theoretical fragmentation data of the angiotensin peptide it was found that the y-ions matched the theoretical data up to y₆, this is shown in figure 6.11. This means that the
VYIHPFHL section of the peptide remains unmodified by the exposure to SIN-1 and that the observed modification of + 28 Da involves either the aspartate or arginine residue. Unfortunately the y-ion data does not reveal any more information as no discernible peak is observed after the y₀ ion. It was therefore necessary to try assign peaks to the other types of fragmentation. Figure 6.13 shows the data obtained at the lower end of the spectra for m/z 442.227 and 662.835 Da.
Figure 6.13 The two spectra show part of the fragmentation data obtained from the ions 442.227 (bottom), 662.835 (top). The relevant y ions can be observed, but the b ions are not present.
Table 6.1 shows a comparison of experimental and theoretical masses due to the fragmentation of the peptide angiotensin. The modification was again found to involve either the aspartate or arginine residue as the $b_2$ ion was found to contain a modification of + 28 Da. This $b_2$ ion relates to the mass of the first two ions sequenced from the N-terminus of the peptide i.e. Asp-Arg. Further examination of the experimental data revealed the presence of the $c_1$ ion (133.01 Da), this ion relates to the first amino acid sequenced from the N-terminus plus NH$_3$, this gives evidence that the modification occurs after the aspartate residue, i.e. the modification is associated with the arginine residue. Further support for this is the presence of a fragment at 274.17 Da, this fragment has a molecular mass 28 Da greater than that of the fragment Arg-Val, again suggesting that the modification is occurring on the arginine residue.

<table>
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<tr>
<th>Experimental Mass Observed</th>
<th>Matching Theoretical Mass</th>
<th>Fragment</th>
</tr>
</thead>
<tbody>
<tr>
<td>86.105</td>
<td>86.10</td>
<td>Ile</td>
</tr>
<tr>
<td>110.074</td>
<td>110.07</td>
<td>His</td>
</tr>
<tr>
<td>133.0</td>
<td>133.01</td>
<td>$c_1$</td>
</tr>
<tr>
<td>138.075</td>
<td>138.07</td>
<td>His</td>
</tr>
<tr>
<td>217.145</td>
<td>217.13</td>
<td>Pro-His - 28</td>
</tr>
<tr>
<td>223.167</td>
<td>223.16</td>
<td>Ile-His - 28</td>
</tr>
<tr>
<td>251.167</td>
<td>251.15</td>
<td>Leu-His</td>
</tr>
<tr>
<td>269.176</td>
<td>269.16</td>
<td>$y_2$</td>
</tr>
<tr>
<td>274.17</td>
<td>256.18</td>
<td>RV + 28</td>
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<tr>
<td>285.142</td>
<td>285.14</td>
<td>Phe-His</td>
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<tr>
<td>300.137</td>
<td>272.14</td>
<td>$b_2$ + 28</td>
</tr>
</tbody>
</table>

Table 6.1 The theoretical fragmentation data compared to the experimental data obtained from the sequencing of angiotensin exposed to SIN-1 using Q-TOF. The left-hand column shows the experimental data, the middle column the theoretical data and the right hand column the fragment the mass relates to. Highlighted in red are the two masses which reveal the modification of arginine by +28 Da.
6.4 Discussion

The evidence obtained from the sequencing of this peptide confirms the findings of the modification of +28 Da observed with MALDI-TOF MS analysis of peptide data. The data obtained from using Q-TOF MS/MS suggests that it is the arginine residues that are capable of undergoing a modification of +28 Da when exposed to 5μM SIN-1 for 2 hr.

Previous work by Natake and Ueda (1986) revealed that when the protein albumin was reacted with 25 mM nitrite (NO$_2^-$) for 24 hr at pH 3, there was a 25% loss of the arginine residues. This supports the theory that SIN-1 can modify this amino acid. At present the exact nature of this modification remains unclear.
6.5 References


CHAPTER 7. Q-TOF MS/MS ANALYSIS OF PROTEIN NITRATION

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7.1 Introduction

MALDI-TOF MS analysis of the proteins transferrin and albumin when incubated with both SIN-1 and peroxynitrite did not reveal evidence for the presence of tyrosine nitration. The data obtained from their reaction with peroxynitrite however, did show that a number of peptides containing tyrosine residues observed in control samples were missing from the reacted samples. Though no masses were observed using MALDI-TOF MS with increments of 45 Da, this does suggest that a modification is occurring, the same proteins were therefore sequenced using Q-TOF MS/MS to try and assign what, if any modifications were occurring.

7.2 Methods

Transferrin and albumin were exposed to both peroxynitrite and SIN-1 as described in sections 3.3.2 and 3.3.4. Control proteins were prepared as before, with the protein being added to the reaction mixture 5 mins after the addition of the bolus peroxynitrite. The proteins were then cleaned using 30 kDa filters, carboamidomethylated and digested with trypsin. All experiments were performed on a Micromass Q-TOF mass spectrometer (Micromass, Altrincham, U.K.), equipped with a HPLC column to separate the samples before being introduced to the Z-spray source. The capillary voltage used was 2800 V, and the cone voltage was 40 V.

7.2.1 Analysis of peptides observed from control transferrin and albumin.

Control transferrin and albumin were analysed first using Q-TOF MS. An example of a spectrum obtained from control transferrin is shown in figure 7.1. This spectrum shows that tryptic digestion of transferrin produces peptides that are singly, doubly and triply charged.
Figure 7.1 Q-TOF MS/MS spectrum of control transferrin. Insets show examples of singly (A), doubly (B) and triply (C) charged peptides.
The peptide coverage of transferrin found using Q-TOF MS (47%) was similar to that when using the MALDI-TOF MS. The coverage map, however, was found to be different. Table 7.1 shows a list of the peptides sequenced using Q-TOF MS/MS. Figures 7.2 and 7.3 shows the coverage maps obtained using MALDI-TOF MS alone and MALDI-TOF MS and Q-TOF MS combined. It was found that when these two methods were used in conjunction the total coverage of the protein transferrin was 71% (482/679 aa's), greatly increasing the power of these methods.

<table>
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<tr>
<th>mass/charge</th>
<th>Peptide Mass [M+H]+</th>
<th>Residue no.</th>
<th>Amino acid sequence*</th>
<th>Modifications</th>
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<tr>
<td>421.76</td>
<td>842.42</td>
<td>1-7</td>
<td>VPDKTVR</td>
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<td>512-522</td>
<td>EGGYGYTGAFR</td>
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<td>434-445</td>
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<td>313-324</td>
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<td>789.41</td>
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<td>815.4</td>
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<td>EDPQTFYYAVAVVK</td>
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</tbody>
</table>

Table 7.1 The peptides identified from the carboaminomethylatation and tryptic digest of control transferrin. The first column shows the mass/charge ratio observed on the spectrum, the second shows the mass of the peptide, the third shows where this peptide occurs in the transferrin sequence, the fourth the amino acid sequence this relates to and the fifth shows any modifications that have occurred to the amino acids in the sequence, in the case shown this is the oxidation of the methionine residue. * Standard one and three letter abbreviations of amino acids are given in appendix 1.
Figure 7.2 Peptides observed using MALDI-TOF MS, after carboaminomethylation and trypsin digest of transferrin.

Figure 7.3 Peptides observed when combining MALDI-TOF MS and Q-TOF MS/MS analysis of transferrin after carboaminomethylation and trypsin digest. The red lines represent peptides observed using MALDI-TOF MS and blue lines represent peptides observed using Q-TOF MS/MS.
Q-TOF MS/MS analysis of albumin was found to give a protein coverage of 27% table 7.2 shows the peptides sequenced using Q-TOF MS/MS of albumin. When MALDI-TOF and Q-TOF analysis were combined the total protein coverage increased to 34%. Figures 7.4 and 7.5 show the coverage maps for MALDI-TOF MS alone and MALDI-TOF MS and Q-TOF MS/MS combined.

<table>
<thead>
<tr>
<th>mass/charge ratio</th>
<th>peptide mass [M+H]*</th>
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Table 7.2  The peptides identified from the carboaminomethylation and tryptic digest of control albumin. * Standard one and three letter abbreviations of amino acids are given in appendix 1.
Figure 7.4 Peptides observed using MALDI-TOF MS, after carboaminomethylation and trypsin digest of albumin.

Figure 7.5 Peptides observed when combining MALDI-TOF MS and Q-TOF MS/MS analysis of albumin after carboaminomethylation and trypsin digest. The red lines represent peptides observed using MALDI-TOF MS and blue lines represent peptides observed using Q-TOF MS/MS.
7.2.2 Analysis of proteins exposed to peroxynitrite and SIN-1

All control peptides could be analysed using the database proteinprospector mentioned above, however modified peptides presented a problem because the automated database searches could not be used. This is due to the fact that the peptide mass has been altered by the modification and that at least one of the amino acid’s mass has been altered and the expected modification isn’t recognised by the database proteinprospector. The analysis of modified peptides had to be performed by hand.

First, a tag of at least three consecutive amino acids had to be identified. This was done purely by examining the trace and measuring the gap between peaks, to find those that related to an amino acid. This tag can then be used to identify possible peptide sequences using the program PAWS. PAWS is a program containing the full sequence of transferrin and albumin. It is possible to perform an automated search through the amino acid sequence to find a sequence of amino acids. It is also possible to perform a theoretical digest of the sequence, giving the mass of relevant peptides. The tag of three amino acids can be used to identify a number of possible peptide matches. These possible matches can then be compared back to the raw data to assign which peptide the raw data relates to. The difference between the theoretical and experimental mass also gives an indication of the modification occurring, for example if the mass difference is + 45 Da, this would suggest a nitration event occurring on either a tyrosine or tryptophan residue.
7.2.3 Sequencing of peptides observed when albumin was incubated with SIN-1

7.2.3.1 Modification of tyrosine

When Albumin was reacted with SIN-1 it was found that the peptide LGEYGFQNALIVR was modified by + 45 Da. Examination of the fragmentation data obtained from this peptide showed that the modification was associated with the first tyrosine residue found in this peptide (figure 7.6). This modification is associated with the substitution of a hydrogen with an –NO₂ group. This gives evidence that tyrosine nitration is occurring when albumin is reacted with SIN-1.

Further analysis of the peptides obtained using Q-TOF MS/MS also showed that this peptide also undergoes a modification of + 16 Da. This modification was again associated with the first tyrosine residue and is presumably the oxidation of the tyrosine residue. Figure 7.7 shows the most likely structure of the modified tyrosine residue, this is the addition of an oxygen to one of the hydrogen molecules, at which position the oxygen is added is unknown at present.

The peptide DAFLGFLYEYSR was also noted to undergo tyrosine nitration, the nitration was associated with the second tyrosine residue in the peptide (figure 7.8).
Figure 7.6 Q-TOF MS/MS spectrum of the albumin peptide LGEYGFQNALIVR (AA's 421-433) after exposure to SIN-1. The brackets between peaks represent the masses relating to amino acids. The tyrosine residue can be observed to have a mass increment of 45 Da, this is presumed to be due to the nitration of the tyrosine residue.
Figure 7.7  Q-TOF MS/MS spectrum of the albumin peptide LGEYGFQNALIVR (AA's 421-433) after exposure to SIN-1. The brackets between peaks represent the masses relating to amino acids. The tyrosine residue can be observed to have a mass increment of 16 Da, this is presumed to be due to the oxidation of the tyrosine residue.
Figure 7.8 Q-TOF MS/MS spectrum of the albumin peptide DAFLGSFLYEYSR (AA's 347-359) after exposure to SIN-1. The brackets between peaks represent the masses relating to amino acids. The tyrosine residue can be observed to have a mass increment of 45 Da, this is presumed to be due to the nitration of the tyrosine residue.
7.2.3.2 Modification of lysine

An unexpected modification was found when the doubly charged peptide at m/z 585.85 was analysed. This relates to the peptide 1170.7, and analysis of the fragmentation data obtained from this peptide revealed that the lysine residue has been modified by + 28 Da (figure 7.09). This modification also remains unexplained at present, but the lysine modification may be similar to that found with arginine. Both arginine and lysine have a free –NH$_2$ group and it is possible that this is the group that is being modified by SIN-1.
Figure 7.9 Q-TOF MS/MS spectrum of the albumin peptide KQTALVELLK (AA's 548-557), after exposure to SIN-1. The brackets between peaks represent the masses relating to amino acids. The lysine residue can be observed to have a mass increment of 28 Da, this modification is unexplained at present.
7.2.3.3 Modification of cysteine

The amino acid cysteine was also found to be modified, four different cysteine residues were found to be modified by + 25 Da. These residues were found at Cys 288, 471, 500, and 510 (figures 7.10-7.13); all of these cysteine residues are part of a disulphide bond. The modification found does not relate to the expected increase of + 57 Da found when cysteines are carboxymethylated by iodoacetamide, or the increase of + 29 Da found when cysteine residues are nitrosylated.
Figure 7.10 Q-TOF MS/MS spectrum of the albumin peptide YICDNQDTISSK (AA's 286-297), after exposure to SIN-1. The brackets between peaks represent the masses relating to amino acids. The cysteine residue can be observed to have a mass increment of 25 Da, this modification is unexplained at present.
Figure 7.11 Q-TOF MS/MS spectrum of the albumin peptide CTEDYLSLILNR (AA’s 471-481), after exposure to SIN-1. The brackets between peaks represent the masses relating to amino acids. The cysteine residue can be observed to have a mass increment of 25 Da, this modification is unexplained at present.
Figure 7.12 Q-TOF MS/MS spectrum of the albumin peptide CTESLVNR (AA's 500-507), after exposure to SIN-1. The brackets between peaks represent the masses relating to amino acids. The cysteine residue can be observed to have a mass increment of 25 Da, this modification is unexplained at present. The peptide has also a cysteine residue cleaved off.
Figure 7.13 Q-TOF MS/MS spectrum of the albumin peptide CFSALTPDETYVPK (AA's 510-523), after exposure to SIN-1. The brackets between peaks represent the masses relating to amino acids. This peptide has had the two end amino acids cleaved off RP, and the cysteine residue has a mass increment of 25 Da.
7.2.3.4 Cleavage events occurring due to exposure to SIN-1

The peptides obtained from albumin reacted with SIN-1 also showed evidence of fragmentation events occurring. The peptides 946.46, 1491.78 and 1595.88 Da all related to peptides with at least one of the amino acids cleaved off. The peptide 946.46 has an amino acid sequence of CTESLVNR and relates to the control peptide CCTESLVNR with the terminal C cleaved off (figure 7.12). The peptide 1491.78 has a sequence of FYAPELLYYANK and relates to the control peptide HPFYAPELLYYANK with HPY cleaved off (figure 7.14). The peptide 1595.88 has a sequence of CFSALTPDETYVPK and relates to the control peptide RPCFSALTPDETYVPK with RP cleaved off (figure 7.13). These cleavage reactions must be related to the presence of SIN-1 in the reaction mixture as it is not observed in control and the two proteins undergo the same process except for the addition of SIN-1.
Figure 7.14 Q-TOF MS/MS spectrum of the albumin peptide FYAPELLYYANK (AA's 173-183), after exposure to SIN-1. The brackets between peaks represent the masses relating to amino acids. This peptide has had the amino acids HPY cleaved off.
A full list of modifications observed when albumin was reacted with SIN-1 are shown in table 7.3.

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Table 7.3 The peptides observed using Q-TOF MS/MS when albumin was modified by incubation with SIN-1. * Standard one and three letter abbreviations of amino acids are given in appendix 1.
7.2.4 Sequencing of peptides observed when transferrin was incubated with SIN-1

7.2.4.1 Modification of tyrosine

When transferrin was incubated with SIN-1, the only modifications found was the nitration of the tyrosine residue in the peptide MYLGYEYVTAIR (1523.74 Da) (figure 7.15) and the oxidation of two methionine residues. The oxidation of the methionine residues may be due to the SIN-1 as this residue was not found to be oxidised in the control protein. A list of modifications are shown in table 7.4

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Table 7.4 The peptides observed using the Q-TOF when transferrin was incubated with SIN-1. * Standard one and three letter abbreviations of amino acids are given in appendix 1.
Figure 7.15 Q-TOF MS/MS spectrum of the transferrin peptide MYLGYEYVTAIR (AA's 457-470), after exposure to SIN-1. The brackets between peaks represent the masses relating to amino acids. The tyrosine residue can be observed to have a mass increment of 45 Da, this is presumed to be due to the nitration of the tyrosine residue.
7.2.5 Sequencing of peptides observed when albumin was incubated with peroxynitrite

When the peptides produced from the reaction of bolus addition of peroxynitrite with albumin were analysed the first observation made was that a greater number of modifications had occurred.

7.2.5.1 Tyrosine modifications

Tyrosine residues at Tyr 161, 364, 424 and 519 were found to be nitrated (figures 7.16-7.19). The tyrosine residue at Tyr 424 was found to also undergo dinitration, this presumably is the substitution of –H with –NO₂ at positions 3 and 5 of the ring structure (figure 7.20). Two tyrosine residues, Tyr 163 and 424 were also found to undergo a modification of + 252 Da. The modification of 252 to the tyrosine residues can be explained by the formation of dityrosine with two nitration sites on the tyrosine residues. It has been shown previously that free tyrosine is capable of dimerisation during the reaction with peroxynitrite to form dityrosine. Examples of this modification are given in figures 7.16 and 7.21.
Figure 7.16 Q-TOF MS/MS spectrum of the albumin peptide YLYEIAR (AA's 161-167), after exposure to peroxynitrite. The brackets between peaks represent the masses relating to amino acids. The first tyrosine residue in the peptide can be observed to have a mass increment of 45 Da, whereas the second tyrosine residue has a mass increment of 252 Da, these modifications are presumed to be due to nitration and dimerisation plus two nitrations of the respective tyrosine residues.
Figure 7.17 Q-TOF MS/MS spectrum of the albumin peptide RHPEYAVSVLLR (AA’s 360-371) exposed to peroxynitrite. The brackets between peaks represent the masses relating to amino acids. The tyrosine residue can be observed to have a mass increment of 45 Da, this is presumed to be due to the nitration of the tyrosine residue. The histidine residue can be observed to have a mass increment of +126 Da, this modification is unexplained at present.
Figure 7.18 Q-TOF MS/MS spectrum of the albumin peptide LGEYGFQNALIVR (AA's 421-433), after exposure to peroxynitrite. The brackets between peaks represent the masses relating to amino acids. The tyrosine residue can be observed to have a mass increment of 45 Da, this is presumed to be due to the nitration of the tyrosine residue.
Figure 7.19 Q-TOF MS/MS spectrum of the albumin peptide RPCFSALTPDETYVPK (AA's 508-523), after exposure to peroxynitrite. The brackets between peaks represent the masses relating to amino acids. The tyrosine residue can be observed to have a mass increment of 45 Da, this is presumed to be due to the nitration of the tyrosine residue.
Figure 7.20 Q-TOF MS/MS spectrum of the albumin peptide LGEYGFQNALIVR (AA's 421-433) after exposure to peroxynitrite. The brackets between peaks represent the masses relating to amino acids. The tyrosine residue can be observed to have a mass increment of 90 Da, this is presumed to be due to the double nitration of the tyrosine residue.
Figure 7.21 Q-TOF MS/MS spectrum of the albumin peptide LGEYGFQNALIVR (AA’s 421-433) after exposure to peroxynitrite. The brackets between peaks represent the masses relating to amino acids. The tyrosine residue can be observed to have a mass increment of 252 Da, this is presumed to be due to dimerisation of the tyrosine residue plus two nitations.
7.2.5.2 Modification of histidine

Four histidine residues (His 42, 361, 402 and 487) were also found to be modified, the modification was + 126 Da (figures 7.22, 7.17, 7.23 and 7.24 respectively), this is not the same modification observed when albumin was reacted with SIN-1 which was a modification of + 54 Da. The modification of + 126 Da is unexplained at present, but may be a form of dimerisation reaction similar to that observed with tyrosine residues. Though there was no evidence of fragmentation occurring when albumin was reacted with bolus peroxynitrite it is possible that this is occurring, leading to free amino acids being available for binding. The evidence obtained from the peptide data (MALDI-TOF MS) and the reaction with SIN-1 shows that histidine residues are sensitive to reaction with peroxynitrite and this may lead to the amino acid being available for binding to other amino acids.
Figure 7.22 Q-TOF MS/MS spectrum of the albumin peptide FKDLGEEHK (AA's 35-44), after exposure to peroxynitrite. The brackets between peaks represent the masses relating to amino acids. The histidine residue can be observed to have a mass increment of 126 Da, this modification is unexplained at present.
Figure 7.23 Q-TOF MS/MS spectrum of the albumin peptide HLVDEPQNLK (AA's 402-412), after exposure to peroxynitrite. The brackets between peaks represent the masses relating to amino acids. The histidine residue can be observed to have a mass increment of 126 Da, this modification is unexplained at present.
Figure 7.24 Q-TOF MS/MS spectrum of the albumin peptide LCVLHEK (AA's 483-489), after exposure to peroxynitrite. The brackets between peaks represent the masses of amino acids. The histidine residue can be observed to have a mass increment of 126 Da, this modification is unexplained at present.
7.2.5.3 Modification of threonine

One threonine residue was also found to react with bolus addition of peroxynitrite, but this was found to be a reduction in mass - 18 Da (figure 7.25). This reaction seems unusual as all the previously described reactions of peroxynitrite, my work and published work, show that the reactions of peroxynitrite are addition reactions i.e. oxidation, nitrosylation and nitration. The explanation for this modification is unknown at present. One possible explanation is that this modification involves the loss of a water molecule.
Figure 7.25 Q-TOF MS/MS spectrum of the albumin peptide KVPQVSTPTLVEVSA (AA's 437-451), after exposure to peroxynitrite. The brackets between peaks represent the masses relating to amino acids. The tryptophan residue can be observed to have a mass decrement of 18 Da, this modification is unexplained at present.
A full list of modifications observed when albumin was incubated with bolus peroxynitrite are shown in table 7.5.

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<td>1524.76</td>
<td>421-433</td>
<td>LGEYGFQNALIVR</td>
<td>Y +45</td>
</tr>
<tr>
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<td>1569.76</td>
<td>421-433</td>
<td>LGEYGFQNALIVR</td>
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</tr>
<tr>
<td>820.482</td>
<td>1639.964</td>
<td>437-451</td>
<td>KVPQVSTPTLVEVSR</td>
<td></td>
</tr>
<tr>
<td>831.439</td>
<td>2492.317</td>
<td>45-65</td>
<td>GLVIAFSQLQQCPFD</td>
<td></td>
</tr>
<tr>
<td>866.3</td>
<td>1731.6</td>
<td>421-433</td>
<td>LGEYGFQNALIVR</td>
<td>Y +252</td>
</tr>
<tr>
<td>958.95</td>
<td>1916.9</td>
<td>508-523</td>
<td>RPCFSALTPDETYVPK</td>
<td>Y +45</td>
</tr>
</tbody>
</table>

Table 7.5 The peptides observed using Q-TOF MS/MS when albumin was incubated with peroxynitrite. * Standard one and three letter abbreviations of amino acids are given in appendix 1.
7.2.6 Sequencing of peptides observed when transferrin was incubated with peroxynitrite

7.2.6.1 Modification of tyrosine

When transferrin was incubated with bolus peroxynitrite four tyrosine residues were found to be nitrated, Tyr 95, 96, 397 and 468 (figures 7.26-7.28). Two tyrosine residues were found to be dimerised and dinitrated i.e. a modification of + 252 Da, Tyr 650 and 655 (figure 7.29). Another tyrosine modification was also noted, when the doubly charged peptide m/z 704.15 was analysed it was found to relate to the peptide 1409.3 Da. This peptide related to the sequence of EGYGYTGAFR with a theoretical mass of 1283.58 giving a modification of + 126 Da (figure 7.30). This modification was again associated with a tyrosine residue (the second in the sequence). This modification is the same as that observed with respect to histidine residues and suggests that it is related to the addition of a free amino acid, further study is required to confirm this theory.
Figure 7.26 Q-TOF MS/MS spectrum of the transferrin peptide EDPQTFYYAVAVK (AA's 89-102), after exposure to peroxynitrite. The brackets between peaks represent the masses relating to amino acids. Both tyrosine residues can be observed to have mass increments of 45 Da, this is presumed to be due to the nitrination of both the tyrosine residues.
Figure 7.27  Q-TOF MS/MS spectrum of the transferrin peptide IMNGEADAMSLDGGFVYIAGK (AA's 381-401), after exposure to peroxynitrite. The brackets between peaks represent the masses relating to amino acids. The tyrosine residue can be observed to have a mass increment of 45 Da, this is presumed to be due to the nitration of the tyrosine residue. Both the methionine residues were found to oxidised.
Figure 7.28 Q-TOF MS/MS spectrum of the transferrin peptide TAGWNIPMGGLYNK (AA's 457-470), after exposure to peroxynitrite. The brackets between the peaks represent the masses relating to amino acids. The tyrosine residue can be observed to have a mass increment of 45 Da, this is presumed to be due to the nitration of the tyrosine residue. In this peptide the methionine residue was found to be oxidised.
Figure 7.29 Q-TOF MS/MS spectrum of the transferrin peptide YLGEEYVK (AA's 650-657), after exposure to peroxynitrite. The brackets between peaks represent the masses relating to amino acids. The two tyrosine residues can both be observed to have mass increments of +252 Da, this is thought to be due to dimerisation of the tyrosine residue plus two nitration.
Figure 7.30 Q-TOF MS/MS spectrum of the transferrin peptide EGYGYTGAFR (AA's 512-522), after exposure to peroxynitrite. The brackets between peaks represent the masses relating to amino acids. The tyrosine residue can be observed to have a mass increment of +126, this modification is unexplained at present.
7.2.6.2 Modification of histidine

Three histidine residues were found to have the modification of +126 kDa, His 207, 535 and 585 (figures 7.31-7.33). However, another histidine modification was also noted when the doubly charged peptide at m/z 709.7, mass 1418.4 Da was analysed. This peptide related to the sequence HQTVPQNTGGK, theoretical mass 1166.6, giving a modification of +252 Da (figure 7.34). This modification supports the theory that the action of peroxynitrite induces histidine to bind with a free amino acid, because the value of +252 Da could be due to the binding of a free dinitrated tyrosine residue.
Figure 7.31 Q-TOF MS/MS spectrum of the transferrin peptide HSTIFENLANK (AA's 207-217), after exposure to peroxynitrite. The brackets between peaks represent the masses relating to amino acids. The histidine residue can be observed to have a mass increment of 126 Da, this modification is unexplained at present.
Figure 7.32 Q-TOF MS/MS spectrum of the transferrin peptide HQTVPQNTGGK (AA’s 535-545), after exposure to peroxynitrite. The brackets between peaks represent the masses relating to amino acids. The histidine residue can be observed to have a mass increment of 126 Da, this modification is unexplained at present.
Figure 7.33 Q-TOF MS/MS spectrum of the transferrin peptide APNHAVVTR (AA’s 582-590) after exposure to peroxynitrite. The brackets between peaks represent the masses relating to amino acids. The histidine residue can be observed to have a mass increment of 126 Da, this modification is unexplained at present.
Figure 7.34 Q-TOF MS/MS spectrum of the transferrin peptide HQTVPQNTGGK (AA's 535-545), after exposure to peroxynitrite. The brackets between peaks represent the masses relating to amino acids. The histidine residue can be observed to have a mass increment of 252 Da, this modification is unexplained at present.
When two doubly charged peptides were analysed m/z 633.24 and 647.3, relating to the peptides 1265.48 and 1294.6 they were found to relate to the same sequence of SASDLTWDNLK. Fragmentation data of these two peptides showed that in both cases the tryptophan residue was the residue being modified, in the first peptide the tryptophan residue was oxidised and in the second peptide the tryptophan residue was nitrated (figures 7.35 and 7.36). As can be seen in figure 7.35 the actual site of tryptophan oxidation is unknown, the diagram shows various options.
Figure 7.35 Q-TOF MS/MS spectrum of the transferrin peptide SASDLTWDLNK (AA's 435-445), after exposure to peroxynitrite. The brackets between peaks represent the masses relating to amino acids. The tryptophan residue can be observed to have a mass increment of 16 Da, this is presumed to be due to the oxidation of the tryptophan residue.
Figure 7.36  Q-TOF MS/MS spectrum of the transferrin peptide SASDLTWDNLK (AA's 435-445), after exposure to peroxynitrite. The brackets between peaks represent the masses relating to amino acids. The tryptophan residue can be observed to have a mass increment of 45 Da, this is presumed to be due to the nitration of the tryptophan residue.
A full list of modifications occurring when transferrin was incubated with bolus peroxynitrite is given in table 7.6.

<table>
<thead>
<tr>
<th>mass/charge</th>
<th>Peptide Mass [M+H]*</th>
<th>Residue no.</th>
<th>Amino acid sequence*</th>
<th>Modification</th>
</tr>
</thead>
<tbody>
<tr>
<td>482.77</td>
<td>964.54</td>
<td>582-590</td>
<td>APNHAVVTR</td>
<td></td>
</tr>
<tr>
<td>489.05</td>
<td>978.1</td>
<td>197-206</td>
<td>DGAGDVAFVK</td>
<td></td>
</tr>
<tr>
<td>497.94</td>
<td>1493.82</td>
<td>279-291</td>
<td>SKEFQLFSSPHGK</td>
<td></td>
</tr>
<tr>
<td>545.73</td>
<td>1090.46</td>
<td>582-590</td>
<td>APNHAVVTR</td>
<td>H +126</td>
</tr>
<tr>
<td>583.8</td>
<td>1166.6</td>
<td>535-545</td>
<td>HQTVPQNTGGK</td>
<td></td>
</tr>
<tr>
<td>598.29</td>
<td>1195.58</td>
<td>104-113</td>
<td>DSGFQMNQLR</td>
<td></td>
</tr>
<tr>
<td>606.28</td>
<td>1212.56</td>
<td>104-113</td>
<td>DSGFQMNQLR</td>
<td>M +16</td>
</tr>
<tr>
<td>625.3</td>
<td>1249.6</td>
<td>435-445</td>
<td>SASDLTWDLNK</td>
<td></td>
</tr>
<tr>
<td>633.24</td>
<td>1265.48</td>
<td>435-445</td>
<td>SASDLTWDLNK</td>
<td>W +16</td>
</tr>
<tr>
<td>637.33</td>
<td>1273.66</td>
<td>207-217</td>
<td>HSTIFENLANK</td>
<td></td>
</tr>
<tr>
<td>646.75</td>
<td>1292.5</td>
<td>535-545</td>
<td>HQTVPQNTGGK</td>
<td>H +126</td>
</tr>
<tr>
<td>647.3</td>
<td>1294.6</td>
<td>435-445</td>
<td>SASDLTWDLNK</td>
<td>W +45</td>
</tr>
<tr>
<td>689.36</td>
<td>1377.72</td>
<td>434-445</td>
<td>KSASDLTWDLNK</td>
<td></td>
</tr>
<tr>
<td>700.29</td>
<td>1399.58</td>
<td>207-217</td>
<td>HSTIFENLANK</td>
<td>H +126</td>
</tr>
<tr>
<td>705.15</td>
<td>1409.3</td>
<td>512-522</td>
<td>EGYGYTGAFR</td>
<td>(2nd) Y +126</td>
</tr>
<tr>
<td>709.7</td>
<td>1418.4</td>
<td>535-545</td>
<td>HQTVPQNTGGK</td>
<td>H +252</td>
</tr>
<tr>
<td>752.07</td>
<td>1504.14</td>
<td>650-657</td>
<td>YLGEEMYVK</td>
<td>Both Y +252</td>
</tr>
<tr>
<td>819.91</td>
<td>1638.82</td>
<td>457-470</td>
<td>TAGWNIPMGLLYNK</td>
<td>Y +45 M +16</td>
</tr>
<tr>
<td>837.92</td>
<td>1674.84</td>
<td>89-102</td>
<td>EDPQTFYYAYAVVK</td>
<td>(2nd) Y +45</td>
</tr>
<tr>
<td>860.4</td>
<td>1719.8</td>
<td>89-102</td>
<td>EDPQTFYYAYAVVK</td>
<td>Both Y +45</td>
</tr>
<tr>
<td>923.33</td>
<td>1845.66</td>
<td>457-470</td>
<td>TAGWNIPMGLLYNK</td>
<td>M +16 Y +252</td>
</tr>
<tr>
<td>1118.54</td>
<td>2237.08</td>
<td>381-401</td>
<td>IMNGEADAMSLDGGF VYIAGK</td>
<td>Both M +16 Y +45</td>
</tr>
</tbody>
</table>

Table 7.6 The peptides observed by Q-TOF when transferrin was reacted with peroxynitrite. * Standard one and three letter abbreviations of amino acids are given in appendix 1.
7.3 Summary of results

Table 7.7 shows all the modifications found with the two proteins being incubated to either SIN-1 or bolus addition of peroxynitrite.

<table>
<thead>
<tr>
<th>Albumin (SIN-1)</th>
<th>Albumin (ONOO')</th>
<th>Transferrin (SIN-1)</th>
<th>Transferrin (ONOO')</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y₁₆₁ (+ 45)</td>
<td>Y₁₆₃ (+ 252)</td>
<td>Y₃₁₉ (+ 45)</td>
<td>Y₉₅ (+ 45)</td>
</tr>
<tr>
<td>Y₁₆₃ (+ 252)</td>
<td>Y₃₆₄ (+ 45)</td>
<td>Y₉₆ (+ 45)</td>
<td>Y₃₉₇ (+ 45)</td>
</tr>
<tr>
<td>Y₃₅₇ (+ 45)</td>
<td>Y₄₂₄ (+ 45)</td>
<td>Y₄₆₈ (+ 45)</td>
<td></td>
</tr>
<tr>
<td>K₂₈₈ (+ 25)</td>
<td>Y₄₂₄ (+ 90)</td>
<td>Y₅₁₅ (+ 126)</td>
<td></td>
</tr>
<tr>
<td>C₄₇₁ (+ 25)</td>
<td>Y₄₂₄ (+ 252)</td>
<td>Y₄₆₈ (+ 252)</td>
<td></td>
</tr>
<tr>
<td>C₅₁₀ (+ 25)</td>
<td>H₄₂ (+ 126)</td>
<td>Y₅₅₅ (+ 252)</td>
<td></td>
</tr>
<tr>
<td>H₃₆₁ (+ 126)</td>
<td>H₂₀₇ (+ 126)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H₄₀₂ (+ 126)</td>
<td>H₅₃₅ (+ 126)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H₄₈₇ (+ 126)</td>
<td>H₅₈₅ (+ 126)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T₄₄₃ (- 18)</td>
<td>H₅₃₅ (+ 252)</td>
<td>W₄₄₁ (+ 252)</td>
<td>W₄₄₁ (+ 16)</td>
</tr>
</tbody>
</table>

Table 7.7 The residues modified by treatment with either peroxynitrite or SIN-1. (Y - tyrosine, H - histidine, M - methionine, W - tryptophan, C - Cysteine and T - Threonine). Number in brackets is the modification in Da.

It can be seen that peroxynitrite caused a greater number of modifications than SIN-1. Treatment of transferrin with SIN-1 was found to cause only the nitration of the tyrosine residue Y-319 (+ 45 kDa). This tyrosine residue is found in the N-lobe of the transferrin molecule and is exposed to the surface to the protein (figure 7.37).
The exposure of albumin to SIN-1 was found to cause a greater number of modifications than transferrin. SIN-1 was found to modify tyrosine by both 16 and 45 mass units. The increase of 16 was oxidation of the tyrosine residue, which shows that SIN-1 has the ability to both nitrate and oxidate the tyrosine residue. Whether the oxidation site is the same as the nitration is unknown at this time. Other modifications found on the albumin protein were the modification of cysteine (+25) and Lysine (+28) residues. Cysteine has been shown previously to react with peroxynitrite through a nitration reaction, giving a mass increase of 29 Da giving Cys-NO (Stamler et al. 1992). The lysine modification remains unexplained at present, but the lysine modification may be similar to that previously found with arginine. Both arginine and lysine have a free –NH$_2$
group and it is possible that this is the group that is being modified by SIN-1.

Peroxynitrite treatment of transferrin caused the nitration of tyrosine (+ 45 Da) and tryptophan residues (+ 45 Da). Tryptophan was also found to undergo oxidation (+ 16 Da). Other modifications observed were an addition of 126 Da to histidine and tyrosine residues and a modification of 252 Da to tyrosine residues. The modification of 252 Da to the tyrosine residues can be explained by the formation of dityrosine with two nitration sites on the tyrosine residues. It has been shown previously that free tyrosine is capable of dimerisation during the reaction with peroxynitrite to form dityrosine. The tyrosine residues undergoing a modification of 252 Da are shown in figure 7.38. These three tyrosine residues are exposed to the surface and therefore it is possible for dimerisation to occur.
Figure 7.38 Atomic model of the polypeptide backbone of sections of the N-lobe of transferrin. These images show the three tyrosine residues found to have a mass increase of 252 in transferrin. In red are the peptide fragment measured on the Q-TOF (a) 650-657 (b) 457-470 and in blue are the tyrosine residues. As can be seen from these pictures these tyrosine residues are exposed to the surface.
7.4 Discussion

The modifications found within the transferrin protein may have important implications on the function of this protein. Transferrin reversibly binds iron in blood plasma and transports iron to cells requiring iron (Harris and Aisen 1989; Chasteen and Woodworth 1990 and Griffiths, E. 1987). The full-length transferrin molecule has two similar halves, the N-lobe and C-lobe, linked by a short bridging peptide. Each lobe carries as iron-binding site, surrounded by two dissimilar domains (NI and NII domains for N-lobe and CI and CII domains for C-lobe) which make up the binding cleft. When iron is released, the two domains within each lobe rotate around two hinges to form an “open” conformation (Anderson et al. 1990). Crystallographic studies of four homologus proteins, human lactoferrin (hLF) (Anderson et al. 1987), human serum transferrin (hTF) (Zuccola, H. J. 1993), rabbit serum transferrin (rTF) (Bailey et al. 1988), and chicken ovotransferrin (oTF) (Kurokawa et al. 1995), have shown that the iron co-ordination in the binding sites is the same, with the ferric ion bound octahedrally to four protein ligands (two tyrosines, one aspartate, and one histidine) and two oxygens from the synergistic anion, carbonate. In the sequence of the N-lobe in human serum transferrin the ligands are Tyr 95, Tyr 188, Asp 63, and His 249, with Asp 63 on the NI domain, Tyr 188 on the NII domain, and Tyr 95 and His 249 on the hinge strands.

Recombinant DNA techniques have been used to explore the specific role a binding ligand plays in the metal-binding properties of transferrin. Mutants of the histidine ligand and the aspartate ligand in the N-lobe of both lactoferrin (Faber et al. 1996 and Nicholson et al. 1997) and human serum transferrin (Woodworth et al. 1991; Grady et al. 1995; Zak et al. 1995 and He et al. 1997) have been prepared and characterised. Not surprisingly, structural and metal-binding studies have demonstrated that mutations of these ligands substantially alter the co-ordination and metal-binding properties of the proteins (Faber et al. 1996 and Nicholson et al. 1997). These mutants generally do not lose their iron-binding ability completely even when the ligand residue is changed to a noncoordinating
residue such as alanine or phenylalanine (Nicholson et al. 1997 and He et al. 1997). When the two tyrosine residues were mutated to phenylalanine they showed different iron-binding properties. The mutation of Tyr 95 to phenylalanine still allows iron-binding to occur although binding is weak, whereas the mutation of Tyr 188 to phenylalanine completely destroys the proteins ability to bind iron (He et al. 1997).

Using the Q-TOF MS/MS data we found that the Tyr 95 ligand was modified, but that the other three ligands remained unaffected. The modification of Tyr 95 would be expected to have a detrimental effect on the action of the protein, and further studies are required to investigate this. The transferrin molecule when reacted when bolus peroxynitrite showed numerous modifications and it is possible that a combination of these modifications would reduce the proteins ability to bind iron. The nitration of transferrin needs to be studied in detail in vivo to discover if any of these effects have a physiological function.

The protein albumin binds numerous different ligands and each appears to have a different binding sites, at present not many of the amino acids involved in binding have been elucidated, but the general effect of both SIN-1 and bolus peroxynitrite can be expected to disrupt at least some of these binding properties of albumin. Further studies would be required to assess the effects these modifications have on the action of albumin.

Modifications of both methionine and tryptophan have been reported previously (Alvarez et al., 1996; Pryor et al., 1994), however, a significant amount of work has only concentrated on nitration of tyrosine. These results show that a bolus addition of peroxynitrite to a protein causes more modifications than nitration of tyrosine residues alone. Previous methods using a bolus addition of peroxynitrite to measure the effect of nitration on the activity of a protein can not attribute the recorded changes to protein activity to only the nitration of tyrosine residues due to the possibility of other undetected modifications, as found in this study.
These results cast doubt on results in published work claiming that nitration of tyrosine causes the increase in a protein's degradation and changes protein function because, unless the protein is sequenced, it is impossible to rule out the occurrence of other modifications. The increased degradation rate of a protein has also been attributed to the presence of nitrated tyrosine residues, but this work shows that peroxynitrite may directly cause protein degradation to occur. Proteins in vivo may be exposed to high concentrations of peroxynitrite for example at sites of inflammation, the concentration of peroxynitrite in these situations may result in the modifications observed in vitro.
7.5 References


Zuccola, H. J. (1993). The crystal structure of monoferric human serum transferrin. Thesis, Georgia Institute of technology, Atlanta, GA.
8.1 Introduction

MALDI-TOF MS analysis of peptides resulting from the carboaminomethylation and tryptic digest of albumin and transferrin did not reveal the presence of any tyrosine nitration, though it did reveal the absence in the peroxynitrite treated proteins of peptides containing tyrosine residues. To further understand the results obtained from the MALDI-TOF MS analysis of the proteins the two sets of data were compared.

8.2 Transferrin

When transferrin was analysed by MALDI-TOF MS it was found that five peptides observed in the control samples could not be observed in the peroxynitrite treated samples. These peptides are listed in table 8.1.

<table>
<thead>
<tr>
<th>Control Mass [M+H]⁺</th>
<th>Sequence *</th>
</tr>
</thead>
<tbody>
<tr>
<td>1000.49</td>
<td>YLGEEYVK</td>
</tr>
<tr>
<td>1283.569</td>
<td>EGYGYTGAFR</td>
</tr>
<tr>
<td>1577.815</td>
<td>TAGWNIPMGLLYNK</td>
</tr>
<tr>
<td>1586.774</td>
<td>KPVEEYANCHLAR</td>
</tr>
<tr>
<td>1952.938</td>
<td>NLNEKDYELLCLGTR</td>
</tr>
</tbody>
</table>

Table 8.1 Peptides that could not be observed in peroxynitrite treated transferrin that could be observed in control transferrin. Possible sites of tyrosine nitration are highlighted in red. * Standard one and three letter abbreviations of amino acids are given in appendix 1.

As previously mentioned all these peptides listed in table 8.1 contain at least one tyrosine residue. Three of these peptides, 1000.49, 1283.569 and 1577.815, can be observed and sequenced in the control transferrin when analysed by Q-TOF MS. The other two however were not observed in the control sample. Q-TOF MS/MS analysis of peroxynitrite
treated transferrin revealed that three of these peptides had been modified by exposure to peroxynitrite, modifications are listed in table 8.2. The other two peptides, 1586.774 Da and 1952.938 Da, were not observed in the control sample and no modification of these peptides were observed in the peroxynitrite sample, therefore no modification can be assigned to these peptides.

<table>
<thead>
<tr>
<th>Control Mass [M+H]*</th>
<th>Sequence *</th>
<th>Q-TOF MS modified mass</th>
<th>Modification</th>
</tr>
</thead>
<tbody>
<tr>
<td>1000.49</td>
<td>YLGEEYVK</td>
<td>1504.14</td>
<td>Both Y + 252</td>
</tr>
<tr>
<td>1283.569</td>
<td>EGYYGYTGAFR</td>
<td>1409.3</td>
<td>2nd Y + 126</td>
</tr>
<tr>
<td>1577.815</td>
<td>TAGWNIPMLLYNK</td>
<td>1845.66</td>
<td>Y + 252</td>
</tr>
<tr>
<td></td>
<td></td>
<td>or 1638.82</td>
<td>M +16</td>
</tr>
<tr>
<td></td>
<td></td>
<td>or</td>
<td>Y + 45</td>
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<td></td>
<td>or</td>
<td>M + 16</td>
</tr>
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<td>KPVEEYANCHLAR</td>
<td>No match</td>
<td></td>
</tr>
<tr>
<td>1952.938</td>
<td>NLNEKDYELLCLGTR</td>
<td>No match</td>
<td></td>
</tr>
</tbody>
</table>

Table 8.2 Comparison between peptides not observed by MALDI-TOF MS analysis of transferrin exposed to peroxynitrite and Q-TOF MS/MS analysis of the same peptides. * Standard one and three letter abbreviations of amino acids are given in appendix 1.

As previously mentioned three of the peptides show the presence of a modification of the tyrosine residue, it was also observed that no unmodified version of these peptides remained in the sample. This suggests that there was sufficient quantity of peroxynitrite to modify all of the protein present in the reaction mixture, or that any remaining unmodified version of this peptide was in too small a quantity to be observed using this method. These results explain why these peptides were not observed in the MALDI-TOF MS analysis of transferrin exposed to peroxynitrite.
8.3 Albumin

When albumin was analysed by MALDI-TOF MS it was found that four peptides observed in the control samples could not be observed in the peroxynitrite treated samples. These peptides are listed in table 8.3.

<table>
<thead>
<tr>
<th>Control Mass [M+H]+</th>
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<td>1439.812</td>
<td>RHPEYAVSVLLR</td>
</tr>
<tr>
<td>1724.835</td>
<td>MPCTEYLSILNR</td>
</tr>
<tr>
<td>1747.705</td>
<td>YNGVFQECQDK</td>
</tr>
<tr>
<td>2045.028</td>
<td>RHPYFYAPELLYYANK</td>
</tr>
</tbody>
</table>

Table 8.3 Peptides that could not be observed in peroxynitrite treated albumin that could be observed in control albumin. Possible sites of tyrosine nitration are highlighted in red. * Standard one and three letter abbreviations of amino acids are given in appendix 1.

Of these peptides three were also observed in control albumin when analysed by Q-TOF MS/MS: 1439.812 Da, 1724.835 Da and 2045.028 Da. The peptide 1747.705 was not observed in the control sample when analysed by Q-TOF MS/MS. Only one of the peptides revealed a modification when albumin was exposed to peroxynitrite, this was 1439.812, that showed two types of modification, with the tyrosine being modified by +45 and the histidine by +126. The other two peptides observed in the control sample did not reveal any modification, but these two peptides were not present in the peroxynitrite treated albumin, suggesting that a modification has occurred, but unfortunately there is no sequencing data at present to determine which modification has occurred.
8.4 Discussion

The modifications noted using the Q-TOF MS/MS data were compared with the MALDI-TOF MS data. Though some of the modifications present are now known, there was still no evidence that these peptide masses were present in the spectra from the MALDI-TOF MS analysis. This shows that MALDI-TOF MS experiments are not suitable for analysing modifications arising from reaction with peroxynitrite.

This suggests that MALDI-TOF MS, as currently set up is insufficient to analyse all modifications in proteins caused by peroxynitrite. Whether this is because MALDI-TOF is not optimally tuned to ionise the modified peptides or whether the TOF MS is of insufficient sensitivity to detect the ions will be addressed in chapter 10.
CHAPTER 9. THE ISOLATION OF NITRATED PROTEINS FROM PLASMA AND CSF USING WESTERN BLOTTING

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9.1 Introduction

Previous research has revealed that CSF concentrations of nitrate and nitrite are increased at presentation of symptoms and during the induction and consolidation periods of treatment of acute lymphoblastic leukaemia (Surtees et al. 1998). Nitrite and nitrate are the stable breakdown products of nitric oxide and the peroxynitrite anion (Hevel and Marletta 1994; Rhodes et al. 1995). There is also evidence that the levels of TNFα are increased during the early stages of acute lymphoblastic leukaemia (Saarinen et al. 1990), this cytokine can induce the enzyme iNOS to increase its production of nitric oxide (Nathan and Xie 1994). This evidence strongly suggests that significant levels of peroxynitrite are produced during the early stages of acute lymphoblastic leukaemia. The work performed to date has revealed some of the effects peroxynitrite can have on proteins in vitro, but doesn’t reveal which proteins are sensitive to nitration in vivo. It was decided, therefore, to isolated nitrated proteins from the CSF of patients with acute lymphoblastic leukaemia and identify them by using MALDI-TOF MS and online database searching.

Increased levels of protein-bound 3-nitro-tyrosine have been reported in bronchoalveolar lavage fluids from patients with acute respiratory distress syndrome (Petruzzelli et al. 1997, Lamb et al. 1999). Moreover, a recent study identified ceruloplasmin, transferrin, α1-antichymotrypsin, α1-protease inhibitor, and the β-chain fibrinogen as the major nitrated plasma proteins in patients with acute respiratory distress syndrome. Pignatelli et al. (2001) studied the presence of nitrated proteins in the plasma of lung cancer patients and controls and identified the proteins fibrinogen, transferrin, plasminogen, and ceruloplasmin as nitrated proteins.
9.2 Materials and Methods

All chemical reagents used were research grade and obtained from Sigma-Aldrich Company (Poole, Dorset, UK) unless stated otherwise.

Because of the relatively large amount of protein required for this method and the scarcity of sample from acute lymphoblastic leukaemia patients this method was initially set up using plasma proteins. Plasma samples were incubated with either 5mM or 10mM SIN-1 for 2hr as a control and compared to unexposed plasma, the transferrin nitrated in earlier experiments was used as a positive control. Once the technique was perfected it was used to analyse control and ALL patient CSF samples.

An aliquot of 125µg (plasma or CSF) protein was used to perform immunoprecipitation experiments based on the method reported by Gole et al. (2000). The sample volume was adjusted to 500µl with immunoprecipitation buffer [20 mM Tris-base (pH 7.4), 140 mM NaCl, 10% glycerol, 1% Triton X-100, 4 mM EGTA, supplemented with 10µg/ml aprotonin and 1 mM phenylmethylsulfonyl fluoride in methanol] and precleaned by incubation with 15µl of Protein G Plus-Agarose beads (Calbiochem) at 4°C with shaking for 1hr. This product, which is ready to use, is 30% (v/v) agarose by volume and contains 20mg IgG binding capacity/ml packed beads, it does not however, have sites for binding BSA unlike other protein G products. Any non-specific binding between the Protein G Plus-Agarose beads and plasma (CSF) proteins will be removed at this point, significantly reducing the occurrence of non-specific binding. After centrifugation at 10,000 x g for 2 min, the supernatant was transferred to a new tube and incubated with 2 µl (1 µg/µl) of mouse anti-nitrotyrosine (Calbiochem) overnight at 4°C, followed by incubation with 30µl of Protein G Plus-Agarose beads at 4°C for 1hr. After centrifugation at 10,000 x g for 2 min, the pellet was washed with 0.5 ml of immunoprecipitation buffer. This procedure was repeated four times. After the last centrifugation, the pellet was resuspended in 30µl of gel loading buffer and heated for 5 min at 90°C. The loading buffer
consisted of 2.5ml TRIS-HCl (0.5M) pH 6.8, 4ml of 10% SDS, 2ml glycerol, 2mg bromophenol blue. This was made up to 10ml and then aliquoted into 1ml eppendorfs, which were then kept frozen at -70°C until required. Before use 31mg of DTT was added to 1ml of loading buffer. The immunoprecipitated proteins were separated on a 10% SDS-PAGE and then gels were either Coomassie blue stained or electroblotted to a nitrocellulose paper for enhanced chemiluminescence detection.

9.2.1 Coomassie Staining

The gel was placed in staining solution (0.1% Coomassie blue, 50% methanol, 5% acetic acid and 45% milliQ water) for 2hr on a rocking table. The gel was then washed in destaining solution (50% methanol, 5% acetic acid and 45% milliQ water) until the background became clear, renewing the destaining solution approximately every 30 minutes.

9.2.2 Detection of proteins using enhanced chemiluminescence

For transferral to nitrocellulose, the gel was first placed in the western blotting solution (25mM Tris, 190mM Glycine, 0.1% (wt/vol) SDS and 16% methanol) for 10 min. The gel was then loaded into the western blotting apparatus and the voltage was set at 100V/1amp. The gel was left to transfer for 1hr. On removal, the membrane was placed in a blocking solution of Tris buffered saline (TBS), (150mM NaCl, 2.68mM KCl and 24.8mM Tris Base) with 10% milk powder, at pH 7.4 overnight. This was followed by three 10 min washes in TBS, pH 7.4 - 0.1% Tween (TBS-T). The blot was then incubated for 2hr with monoclonal anti-nitrotyrosine antibody diluted to 1:2,000 in the TBS-T with 1% dry milk powder. After being washed with four 10 min washes of TBS-T, the blot was incubated for 1 hr with goat anti-mouse IgG diluted 1:5,000 in antibody buffer. Blots were then washed extensively in TBS-T followed by two ten minute washes in TBS and developed with the enhanced
chemiluminescence western blotting system (figure 9.1) (Amersham Pharmacia Biotech).

Figure 9.1 Schematic of western blotting method. The protein of interest is bound to the nitrocellulose paper (Hybond ECL in this figure), the primary and secondary (bound to horse radish peroxidase (HRP) antibodies) are then bound to the protein of interest. Combined HRP and peroxide catalyse the oxidation of luminol generating thousands of acridinium ester intermediates per minute. These intermediates react with peroxide under slight alkaline conditions to produce a sustained, high intensity chemiluminescence with maximum emission at a wavelength of 430nm. This light emission is recorded using hyperfilm ECL, which a form of photographic paper.

The gels that were Coomassie stained were analysed visually and the relevant bands were excised and prepared for peptide analysis on the
MALDI-TOF MS (as described in chapter 3.3.10). Though MALDI-TOF MS has been shown in previous chapters to be insufficient for the detection of nitrated peptides within a protein, it is unlikely that more than one or two tyrosine residues will be modified, therefore this will allow enough unmodified peptides to be present to allow accurate database searching.

9.3 Identification of nitrated plasma proteins by immunoprecipitation and Western blot analysis

An example of the western blots obtained from the immunoprecipitation of proteins from plasma by anti-nitrotyrosine antibody is shown in figure 9.2. Both control and SIN-1 treated plasma samples revealed two bands at approximately 55,000 kDa. These bands were also visible on the Coomassie blue stained gels (figure 9.3) and the bands were excised and analysed using MALDI-TOF MS. The bands were confirmed to be the heavy-chain polypeptides in the Y structure of either the mouse anti-nitrotyrosine or the goat anti-mouse antibodies or a mixture of both. It is unlikely to be the human version of the same protein as the band is observed in the negative control. This showed that during the process of immunoprecipitation some of the antibody was released from the G-protein beads and loaded onto the gel. It was therefore necessary to be aware of the presence of these bands during the analysis of both the nitrated plasma samples and the CSF of ALL patients.

The visualisation of bands using chemiluminescence (figure 9.2) showed the same bands as those seen by Coomassie blue staining (figure 9.3). The plasma samples nitrated by SIN-1 showed approx. 7 bands that could not be observed in the control samples. These bands were also observed in silver stained gels. These bands were excised and proteins were digested using trypsin and analysed using MALDI-TOF MS.
Figure 9.2 Shows the results of western blotting of plasma, visualised by chemiluminescence. The control plasma lane shows the presence of no other proteins apart from the anti nitrotyrosine antibody, the lanes containing plasma incubated with 5mM or 10 mM SIN-1 reveal the presence of other proteins containing nitrotyrosine residues in addition to the antibody proteins, and the ‘+’ve control is transferrin reacted with SIN-1 used in the MALDI-TOF MS and Q-TOF MS/MS experiments. In this lane only the nitrated transferrin can be seen, the antibody bands were too faint to be observed. The boxes indicate which proteins were identified using MALDI-TOF MS.
Figure 9.3 Coomassie blue staining of the duplicate gel, shown above in the chemiluminescence results (figure 9.2). Again the three lines containing plasma show the presence of the antibody. The control plasma and the plasma incubated with 5mM SIN-1 do not show the presence of any other proteins. The lack of proteins containing nitrotyrosine residues observed with plasma reacted with 5mM SIN-1 is probably due to the reduced sensitivity of Coomassie blue staining when compared to chemiluminescence. The boxes indicate the proteins identified using MALDI-TOF MS.

The peptide data obtained from band 1 (highlighted on the gel) was found to correlate to the protein complement C3 precursor, MW 187165.7 Da. Figure 9.4 shows the peptides obtained from the trypsin digest of band 1, the annotated peaks are the identified peptides that relate to either the standards used to calibrate the spectrum, tryptic autodigest and those peptides relating to the protein complement C3.
Figure 9.4 MALDI-TOF MS spectrum obtained from the tryptic digest of band 1. Arrows indicate the identified peptide peaks.
When the peptide peaks observed in the MALDI-TOF MS spectrum were entered into the online database MS-FIT, a match was observed with the protein complement C3 precursor (figure 9.5). This was a good match with a high percentage of experimental masses being within 0.2 Da of the theoretical masses (figure 9.6). There were only three unmatched peptides, giving a high degree of confidence that this is a true match. A search was then performed on the unmatched peptides and two of the unmatched peptides, molecular weight 1707.87 and 2211.51 Da were found to relate to the autodigestion of trypsin. An important note is that the search shown above is of all species, to check for any contaminants occurring during protein work up. When the search was completed just looking at human proteins the next best fit after complement C3 precursor was phosphatidylinositol 3-kinase catalytic subunit, gamma isoform, that showed a MOWSE score of 121 (described in chapter 3.4.7) but had only 5/16 matched peptides.
### Detailed Results

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Click link below to search for another component.
3 unmatched masses
Click individual mass to do a non-specific cleavage search.
1520.6400 1707.8700 2211.5100

Figure 9.6 Detailed results from the peptide search in the database protein prospector.

The spectrum obtained from the tryptic digest of band 2 is shown in figure 9.7, the data from this band was then entered into MS-FIT and was found to match the protein albumin (figures 9.8; 9.9).
Figure 9.7 MALDI-TOF MS spectrum obtained from the tryptic digest of band 2. Arrows indicate the identified peptide peaks.
### Parameters Used in Search

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### Result Summary

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Figure 9.8 Results showing the top 5 matches when the peptide masses obtained from band 2 were entered into the online database proteinprospector.

### Detailed Results

1. 7/12 matches (58%). 69367.4 Da, pI = 5.92. Acc. # P02768. HUMAN. Serum albumin precursor.

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Click link below to search for another component.

5 unmatched masses:

Click individual mass to do a non-specific cleavage search.

1320.3800 1524.8200 1753.3800 1808.2100 2182.5000

The matched peptides cover 17% (106/609AA's) of the protein.

Coverage Map for This Hit (MS-Digest index #): 13727

Percent TIC = 58.3 Mean Error = 0.1656 Da Data Tolerance = 0.3618 Da Mean Missed Cleavages = 0.6

Figure 9.9 Detailed results from the peptide search in the database proteinprospector.
The results obtained from MS-FIT showed that 7/12 of the entered masses matched the theoretical digest of human albumin, the detailed analysis of this data showed that most of the experimental masses matched the theoretical masses within 0.2 Da, and the MOWSE score was found to be 54.9. This allows band 2 to be confidently identified as being human albumin. This shows that when plasma is reacted with SIN-1 the proteins complement C3 precursor and albumin are nitrated. From the evidence obtained using chemiluminescence other proteins are also nitrated, but these proteins were not identified using MALDI-TOF MS.

This work with plasma shows that this is a method that can be used to identify proteins that have been nitrated. Unfortunately not all the proteins observed in the gel can be identified, this is mainly because there was not enough protein to obtain a clear spectrum. It is also possible that more than one protein is contained within a band, this hinders the identification using the MALDI-TOF MS. A way to combat this would be to separate the proteins on a 2-D gel instead of the 1-D gel used. This would separate the proteins in two dimensions by both mass and pl value. A 2-D gel would give more information before digest and help in the identification of the protein, it would also be useful to use other enzymes to digest the protein as this would again give more information.

9.3.1 Analysis of ALL patient samples

Five control and five ALL patient CSF samples, taken at the start of treatment, were then analysed using these methods. Only two bands were observed in the CSF of ALL patients and none in the control samples. These two bands were excised and analysed using the MALDI-TOF MS. The top band unfortunately did not yield enough peptides to be able to identify the protein, but it is known from the gel to be of molecular mass approx. 90,000 Da. The lower band produced a
very clear spectrum (figure 9.10), and this was analysed and found to be albumin (figure 9.11).
Figure 9.10 MALDI-TOF MS spectrum obtained from the tryptic digest of band 3. Arrows indicate the identified peptide peaks.
### Parameters Used in Search

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### Result Summary

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<td>Serum albumin precursor</td>
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</table>

Figure 9.11 Results showing the top 5 matches when the peptide masses obtained from the CSF band were entered into the online database proteinprospector.
As can be seen from the results obtained for this protein 9/13 of the peptides observed can be accurately matched to albumin (figure 9.12). As seen with the results obtained for the plasma protein a high percentage of the experimental masses matched within 0.2 Da of the theoretical masses. This protein was not observed in the control samples.

Figure 9.12 Detailed results from the peptide search in the database protein prospector.
The results obtained from the western blotting experiments show that albumin is nitrated both when plasma is reacted with SIN-1 and at the onset of treatment for ALL. Further work could be carried out to monitor the levels of nitrated proteins during the progression of treatment. Because of the limited data obtained from the MALDI-TOF MS (see chapter 8) identifying which tyrosine residues are nitrated in albumin, albumin containing nitrotyrosine residues was isolated from patient CSF samples and analysed using the Q-TOF MS/MS. Two samples were further analysed by sequencing with Q-TOF MS/MS.

9.4 Rivanol precipitation of albumin and transferrin from control and patient CSF

Albumin and transferrin were isolated from CSF using Rivanol precipitation based on the method described by Charlwood et al (1998). CSF (0.5 ml) was added to 0.2 ml deionised water and the solution was vortex mixed. All other proteins except for transferrin, albumin and immunoglobulins were precipitated by the addition of 0.3 ml of Rivanol (3% w/v, 6,9-diamino-2-ethoxyacridine lactate, Sigma, Poole, UK). The suspension was centrifuged at 10,000 x g for 10 min in an MSE benchtop centrifuge. The yellow supernatant was collected and 0.43 ml of NaCl (25% w/v) was added. The mixture was vortex mixed and centrifuged at 10,000 x g for 10 min. The clear supernatant was collected and 0.8 ml of saturated ammonium sulphate was added per ml of supernatant. The solution was vortex mixed and allowed to stand for 10 min. It was then centrifuged for 10 min at 10,000 x g. The supernatant was then cleaned using 30KDa exclusion filters (Millipore, Watford, UK). The supernatant was first filtered using a benchtop centrifuged, then washed with water, the filter was then inverted and the cleaned sample collected. The protein concentration was determined using standard methods (Smith et al. 1985).
Transferrin and albumin isolated using this method were then separated on 12% acrylamide gels, by the method described in chapter 3.3.8. The gel was then stained using silver stain (chapter 3.3.9). The gel showed two bands, transferrin and albumin, no other contaminants were observed. These two bands were excised and digested by trypsin. The samples were then ready for analysis using the Q-TOF. It was decided, because of the presence of both the proteins in the gel, to analysis both of these proteins by Q-TOF MS/MS though the evidence from the western blotting results showed only the presence of nitrated albumin.

9.4.1 Analysis of control and patient albumin

The analysis of control albumin, table 9.1, revealed 17 peptides that matched albumin, this gives a protein coverage of 27%.

<table>
<thead>
<tr>
<th>m/z</th>
<th>Peptide mass [M+H]</th>
<th>Residue nos.</th>
<th>Amino acid sequence</th>
<th>Modifications</th>
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<tbody>
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<td>789.48</td>
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<td>LVTDLTK</td>
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</tr>
<tr>
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</tr>
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<td>161-167</td>
<td>YLYEIAR</td>
<td></td>
</tr>
<tr>
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<td>960.54</td>
<td>425-433</td>
<td>FQNALLVR</td>
<td></td>
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</tbody>
</table>

Table 9.1 Peptides observed and sequenced when albumin from control CSF was analysed by Q-TOF MS/MS. The first column shows the mass to charge ratio, the second the mass of the peptide, the third reveals the position the peptide has within the protein, the fourth gives the sequence of the peptide and the fifth any modification observed.
ALL patient albumin give a higher coverage of the protein (table 9.2), 24 peptides were identified that matched with albumin, which gave a protein coverage of 39%.

<table>
<thead>
<tr>
<th>m/z</th>
<th>Peptide mass [M+H]+</th>
<th>Residue nos.</th>
<th>Amino acid sequence</th>
<th>Modifications</th>
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<tbody>
<tr>
<td>395.24</td>
<td>789.48</td>
<td>257-263</td>
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<td>438.24</td>
<td>875.48</td>
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Table 9.2 Peptides observed and sequenced when albumin from patient CSF was analysed by Q-TOF MS/MS.

Unfortunately no tyrosine nitrations were observed, this may be due to the relevant peptide not being analysed during this experiment. This may be due to the relevant peptide not being conducive with the Q-TOF MS process and not being available for analysis, further detailed work is required to try and assert which peptide is nitrated and then to sequence this peptide. This may be possible using HPLC as nitrated peptides may have a different retention time to the non-nitrated version and this could be used to isolate the relevant peptide for sequencing. One observation made in these experiments concerns the peptide 568-579 that contains a
methionine residue. In the control sample this peptide shows both the unmodified and the modified form, with the methionine residue being oxidised. In the patient sample, however, only the oxidised form of the peptide is present (figure 9.13). All other peptides observed in control samples were also observed in patient samples, suggesting that the unmodified form of this peptide is absent in patient samples rather than it just not being analysed using Q-TOF MS/MS in patient samples. All samples underwent the same sample work up, therefore the loss of the unoxidised methionine residue may show that there is an increase in oxidative damage during ALL.
Figure 9.13  Q-TOF MS/MS spectrum of control and patient albumin. The upper trace shows the spectrum obtained from control albumin containing both the unoxidised and oxidised form of the peptide AVMDDFAAFVEK and the lower trace shows patient albumin containing only the oxidised form of the same peptide.
9.4.2 Analysis of control and patient transferrin

When transferrin from control CSF was analysed, 12 peptides were sequenced relating to transferrin giving a protein coverage of 17% (table 9.3).

<table>
<thead>
<tr>
<th>m/z</th>
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<th>Residue nos.</th>
<th>Amino acid sequence</th>
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</tbody>
</table>

Table 9.3 Peptides observed and sequenced when transferrin from control CSF was analysed by Q-TOF MS/MS. The first column shows the mass to charge ratio, the second the mass of the peptide, the third reveals the position the peptide has within the protein, the fourth gives the sequence of the peptide and the fifth any modification observed.
Transferrin isolated from patient CSF was found to give 14 peptides relating to transferrin this gave a protein coverage of 22% (table 9.4).

<table>
<thead>
<tr>
<th>m/z</th>
<th>Peptide mass [M+H]^*</th>
<th>Residue nos.</th>
<th>Amino acid sequence</th>
<th>Modification</th>
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Table 9.4 Peptides observed and sequenced when transferrin from patient CSF was analysed by Q-TOF MS/MS.

A similar observation was made when patient transferrin was compared to control CSF as to that found in albumin. The peptide MYLGYEYVTAIR, in the control samples was found in two forms, one with the methionine unmodified (1478.74) and one with the methionine oxidised (1494.24). When the patient samples were analysed it was found that only the peptide form with the methionine oxidised was observed (1494.6) (figure 9.14). Again all peptides observed in control were observed in patient transferrin.
Figure 9.14 Q-TOF MS/MS spectra of control and patient transferrin. The upper trace shows the spectrum obtained from control transferrin containing both the unoxidised and oxidised form of the peptide MYLGYEYVT AIR and the lower trace shows patient transferrin containing only the oxidised form of the same peptide.
9.5 Discussion

Protein oxidation and nitration have previously been utilised as indirect indices of oxidative stress in human and animal models of disease (Amirmansour et al. 1999; Viner et al. 1996; Aoyama et al. 2000). However, in the majority of studies, the specific protein(s) modified by oxidation and nitration in human disease are not known. Consistent with previous observations (Gole et al. 2000; Ara et al. 1998; Gow et al. 1997), only a few proteins are modified by nitration in the CSF of ALL patients. Two proteins were observed to be nitrated in the CSF of ALL patients, one remains unidentified at present, whereas the other was identified as albumin using MALDI-TOF MS. Albumin has been shown previously to have a low reactivity with peroxynitrite when compared to other plasma proteins (Shacter et al. 1994), this raises the possibility that other proteins within the CSF of ALL patients are nitrated, but are in too low a concentration to be detected using Western blot analysis. Though both MALDI-TOF MS and Q-TOF MS/MS methods were used to analyse the nitrated albumin, unfortunately the peptide containing the nitration site was not revealed. The isolation of the nitration site might be accomplished by using different enzymes to cleave the protein e.g. chymotrypsin and V8(DE), these would give different peptide patterns and use of a combination of these three enzymes would increase the protein coverage and would hopefully isolate the nitration site.

Q-TOF MS/MS analysis of the proteins albumin and transferrin found evidence of increased levels of methionine oxidation in proteins from ALL patients. This methionine oxidation could be due to peroxynitrite activity and when combined with the results obtained from the western blots and the previous work performed by Surtees et al. (1998), this is a strong possibility. Other mechanisms causing methionine oxidation are also a consideration and further work is required to be able to isolate the mechanism causing the oxidation of methionine residues. Further work is
also required to compare the various stages of the disease and levels of albumin nitration as this would clarify whether concentrations of nitrite and nitrate in CSF are related to the levels of protein nitration in CSF.
9.6 References


CHAPTER 10. CONCLUSIONS

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10.1 Conclusions

In this work, the detection and characterisation of peroxynitrite modification at the peptide and protein level has been investigated using MALDI-TOF MS and Q-TOF MS/MS.

The use of HPLC with electrochemical detection was found to be an insufficient method for the detection of 3-nitrotyrosine. This was due to the presence of a large co-eluting peak. Various methods were used to try and separate the two peaks, but a level of separation to allow accurate measurement of the 3-nitrotyrosine peak was not achieved. Another contemporary study by (Kaur et al. 1998) also found the presence of this peak and was not able to characterise it, even though they compared it to various known compounds. These results cast doubt on the results published using this method to measure 3-nitrotyrosine concentrations in various samples, as it is possible that previous studies have actually been measuring the unknown peak and not 3-nitrotyrosine. Further work is required in this area to try and identify the unknown peak and to assess the validity of previously published results.

When solutions of albumin and transferrin were exposed to SIN-1 and bolus peroxynitrite no modifications relating to the nitration of tyrosine residues were noted using the MALDI-TOF MS technique. However it was found that a number of peptides (5 for transferrin and 4 for albumin) containing tyrosine residues observed in control samples could not be observed in exposed proteins. This led to the conclusion that modifications did occur, but that the modifications were either not the expected increase of + 45 Da or that the modification of tyrosine residues was not revealed by analysis using MALDI-TOF MS. Contemporary work performed by Petersson et al (2001) also found that MALDI-TOF MS was an insufficient technique for the analysis of nitrated peptides within a complex mixture of nitrated and non-nitrated peptides. This may be due purely to the suppression of nitrated peptides signal due to the presence of non-nitrated peptides. Another reason for this may be the
photodecomposition of NO$_2$ side-chain, which is a phenomenon noted with MALDI (see chapter 4.7), but not with the electrospray ionisation technique used with Q-TOF.

The analysis by MALDI-TOF MS of individual peptides exposed to SIN-1 found that the addition of +28 Da was the most common reaction and that only one peptide (N-acetyl-Renin) underwent a nitration reaction. The nitrated N-acetyl-Renin peptide was found to be only a small percentage of the total peptide signal, this signal could easily be lost when contained within a complex peptide mixture suggesting that MALDI is not a sensitive enough method for the detection of nitrated peptides within a complex mixture. When Q-TOF MS/MS was used to analyse one of the peptides showing an increment of +28 Da it was found that this modification related to the arginine residue. Arginine has been shown previously to react with NO$_2^-$ (Natake and Ueda 1986), but the exact nature of this modification remains unexplained at present.

Using the same two sample proteins, I found that Q-TOF MS/MS was capable of identifying 3-nitrotyrosine modifications in a complex peptide mixture. The nitration of the tyrosine residues, however, was not the only modification that occurred. As well as the nitration of tyrosine, other modifications were the oxidation, dinitration and dimerisation of the tyrosine residues, and modifications of histidine, cysteine, lysine, threonine and tryptophan residues. The nitration and oxidation of tryptophan has been reported previously and cysteine is known to react with peroxynitrite, although the modification found in this study was not the same as reported previously (Ferranti et al. 1997). The modifications of the other amino acids, have not been reported previously. This is probably because previous work has concentrated solely on the nitration of tyrosine and most studies have not had access to sequencing data (Souza et al. 1999; Sarver et al. 2001).

The nitration of tyrosine residues has been reported to be useful as a method of assessing the activity of peroxynitrite in vivo. At low steady
state concentrations of peroxynitrite, represented in this study by the use of SIN-1, this is a reasonable assumption. However, the use of MALDI-TOF MS to assess the presence of 3-nitrotyrosine in samples would give a lot of false negatives, because of the insensitivity of this method for modified tyrosine residues and also because of the expected low concentration of 3-nitrotyrosine in vivo. MALDI-TOF MS as used here is not a suitable method for the measurement of 3-nitrotyrosine in a complex mixture and cannot be a proxy method to assess activity of peroxynitrite. It also does not take into account the other possible amino acid modifications shown now to occur.

Q-TOF MS/MS analysis of samples is the preferred method because this is significantly more sensitive to the presence of 3-nitrotyrosine and allows for the analysis other possible modifications. The unique modifications, especially that of histidine, which is a common modification, discovered in this study may be a better markers of peroxynitrite activity because other mechanisms of tyrosine nitration are present in vivo. These other modifications require further study and may lead to a more specific biological marker for peroxynitrite activity.

The detection of nitrated proteins in CSF samples from ALL patients was possible using a combination of immunoprecipitation and MALDI-TOF MS. It was found that patient samples of CSF contained nitrated proteins which were not present in controls, this does suggest that there is an increase in peroxynitrite production at the start of treatment. This may be due to alteration in the iNOS enzyme function, resulting in the production of peroxynitrite instead of nitric oxide. The production of peroxynitrite may affect learning directly or it may act through alterations in protein function. Extensive research is required to assess if and how the increase in peroxynitrite production may produce a long-term biological affect.
10.2 Future work

A number of the modifications observed using Q-TOF MS/MS analysis remain unexplained at present. To try and discover the nature of these modifications it will be necessary to combine HPLC to isolate and purify the peptides containing a modification with a mass spectrometry method to sequence the relevant peptide. Q-TOF MS/MS could again be utilised by first optimising the instrument for the expected mass of this peptide to increase the mass resolution, this should allow a more detailed analysis of the fragmentation data obtained from the relevant peptide. It may also be possible, by increasing the cone voltage, to fragment the amino acid side-chains, giving data specific to the modifications.

Further work should also be performed using the antinitrotyrosine antibody in combination with Q-TOF MS/MS analysis. The antibody could be used to isolate nitrated proteins and Q-TOF MS/MS to identify the protein and sequence peptides containing nitrated tyrosine residues. This could lead to further understanding of the process of nitration in disease states; it would also be necessary to be aware of other possible modifications due to the presence of peroxynitrite.
10.3 References


## Appendix 1

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