Mechanism of Nucleotide Exchange by Guanine Nucleotide Exchange Factors on the Ras Superfamily of Small G-Proteins

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Supervisor Dr J.F. Eccleston
for my mum

I know you wouldn’t have understood this
but you would have been very proud!

Thank you
Abstract

The Ras superfamily of small G-proteins cycle between an ‘active’ GTP bound form and an ‘inactive’ GDP bound form. This cycle is controlled by GTPase activating proteins (GAPs) and guanine nucleotide exchange factors (GEFs). Sos, a GEF for Ras proteins, is a ubiquitously expressed 150 kDa protein that is recruited to the cell membrane by Grb2 on phosphorylation of a receptor, where it promotes GDP release from Ras, enabling formation of the active Ras.GTP complex.

Study of the interaction of the catalytic domain of mSosl (CmSosl) with N-Ras in vitro was hampered by low expression levels of CmSosl in E.coli. The gene for CmSosl, coding for residues 577-1076, was reconstructed following codon optimisation and expressed in E.coli. Yields for a 4 Litre culture were 120 mg of pure CmSosl protein, an ~100 fold enhancement in expression levels. This enabled the interaction of CmSosl with N-Ras to be studied, using the fluorescent analogue of GDP, 2'-deoxy-3'-O-N-methlyanthraniloylGDP (mdGDP) for transient kinetic and equilibrium measurements.

The mechanism of nucleotide exchange has been proposed to be a substituted mechanism, exchange factors stabilising a nucleotide free binary complex of GEF.G-protein allowing rebinding of nucleotide via a transient ternary complex. The formation of a weak ternary N-Ras.mdGDP.CmSosl complex has been studied using fluorescence anisotropy measurements. Addition of excess GDP to N-Ras.mdGDP.CmSosl enabled the release of mdGDP from the ternary complex to be analysed. These processes have been followed over millisecond time scales using stopped-flow apparatus. The nucleotide free N-Ras.CmSosl complex has been purified and characterised. The reverse reaction, analysing the association of nucleotide to the N-Ras.CmSosl complex, has been studied. Computer simulation of this data has led to rate and equilibrium constants for the steps of the reaction being proposed. The interaction of different nucleotides and nucleotide bound states has also been observed.

The interaction of the proposed catalytic domain of αPIX (a Dbl homology exchange factor) with Rac1 has been studied. Low exchange activity has been observed, leading to the proposal that other factors are required for the observed in vivo activity of PIX on Rho family proteins.
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<th>Description</th>
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<td>BSA</td>
<td>Bovine serum albumin</td>
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<td>CmSos1</td>
<td>Catalytic domain of the mSos1 protein (577-1076)</td>
</tr>
<tr>
<td>DH</td>
<td>Dbl homology domain</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiotreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediamine tetraacetic acid</td>
</tr>
<tr>
<td>GAP</td>
<td>GTPase-activating protein</td>
</tr>
<tr>
<td>GEF</td>
<td>Guanine nucleotide exchange factor</td>
</tr>
<tr>
<td>GMPPNP</td>
<td>Guanylyl imidodiphosphate</td>
</tr>
<tr>
<td>GST</td>
<td>Glutathione S Transferase</td>
</tr>
<tr>
<td>GTPase</td>
<td>Guanosine triphosphatase</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl β-D-thiogalatopyranoside</td>
</tr>
<tr>
<td>$k_{obs}$</td>
<td>Observed rate constant</td>
</tr>
<tr>
<td>$K_d$</td>
<td>Equilibrium dissociation constant</td>
</tr>
<tr>
<td>kD</td>
<td>kilodaltons</td>
</tr>
<tr>
<td>Mant</td>
<td>2'(3')-O-N-methylantraniloyl</td>
</tr>
<tr>
<td>mantGDP</td>
<td>2'(3')-O-N-methylantraniloyl-GDP</td>
</tr>
<tr>
<td>mdGDP</td>
<td>2'deoxy-3'-O-N-methylantraniloyl-GDP</td>
</tr>
<tr>
<td>PH</td>
<td>Pleckstrin homology domain</td>
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<tr>
<td>PMSF</td>
<td>Phenylmethylsulphonyl fluoride</td>
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<tr>
<td>TEMED</td>
<td>NNN’N’-tetramethylene diamine</td>
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Chapter 1

Introduction

Ras genes were first identified as the transforming principle of the Harvey (Harvey, 1964) and Kirsten (Kirsten & Mayer, 1967) strains of rat sarcoma viruses (rat sarcoma, ras), two acute transforming retroviruses generated by transduction of the rat H-ras-1 and K-ras-2 genes respectively (Defeo et al., 1981, Ellis et al., 1981). Then, together with a third ras gene N-Ras, that was identified from a neuroblastoma cell line (Hall et al., 1983), they were found to be transforming genes present in ~30% of all human tumours. Therefore, extensive research has been carried out on these proteins, not only to gain understanding of the pathogenesis of human cancer, but also because it was shown that ras genes were highly conserved among eukaryotes and coded for proteins that bind guanine nucleotides. They were found to have GTPase activity and were associated with the plasma membrane, suggesting a role in the transduction of signals across the plasma membrane (Barbacid, 1987, for a review of early work).

Ras proteins were the first of a large superfamily to be discovered, the Ras superfamily of small G-proteins. They have been classed as a subgroup of the GTP binding protein family, which includes the other subgroups: heterotrimeric G-proteins and translation factors. Common to all Ras family small G-proteins is the property that they form relatively stable complexes with their substrate, GTP, and product, GDP, and are able to inter-convert between the ‘active’ GTP bound state and the ‘inactive’ GDP bound state to regulate many events within the cell. They have a slow
intrinsic rate of GTP hydrolysis, accelerated by GTPase activating proteins (GAPs), and a slow intrinsic rate of GDP release, promoted by guanine nucleotide exchange factors or GEFs (Boguski & McCormick, 1993). This cycle is shown in figure 1a.

As an introduction to my work I will briefly summarise what is known of the cellular functions of the Ras superfamily proteins before discussing the detailed molecular mechanism of the action of these proteins in order to lead onto the aims of the project.

There is a subset of families within the Ras super-family related by their sequence homology: the Ras, Rho, Rab, Arf and Ran families (figure 1b). The structure of Ras has been well defined (de Vos et al., 1988, Milburn et al., 1990) as well as others of the super-family; Rac1 (Hirshberg et al., 1997), RhoA (Wei et al., 1997), Arf-1 (Greasley et al., 1995). These will be discussed in detail in section 1.3. Not surprisingly from their sequence homology they are structurally similar, yet they perform specific functions within the cell, interacting with different effector molecules. For example, the Ras family are mainly implicated in cell growth and differentiation whereas Rho family proteins are mainly implicated in the cytoskeleton, Rab family in vesicle transport and Ran in nuclear transport. An overview of what is known of these cellular functions is followed by a discussion of the biochemical properties that enable these functions to be performed.

1.1 Cellular functions of the Ras superfamily proteins

1.1.1 Ras subfamily proteins.

The three Ras proteins (H, K and N) are ubiquitously expressed and no clear differences in function have been documented, although the abundance of mRNA
A  **The GTPase cycle**

G-proteins cycle between the ‘inactive’ GDP bound and ‘active’ GTP bound forms. Exchange factors promote the formation of the GTP bound form, GTPase activating proteins catalyse the formation of the GDP bound form. In the GTP bound form G-proteins can interact with downstream effector molecules. Guanine nucleotide dissociation inhibitors (GDIs) hold some G-protein family members in the ‘inactive’ GDP bound form.

B  **The G-protein family**

The G-protein family consists of heterotrimeric G-proteins, translation factors and the Ras superfamily. The Ras superfamily has been divided into five subfamilies (Ras, Rho, Rab, Ran and ARF). Some of the members of these families have been highlighted.
Figure 1.1

A  Exchange Factors
Dissociation Inhibitors
'inactive'
Protein.GDP
Protein.GTP
'active'
GTPase Activating Proteins
Downstream Effectors

B  Translation Factors
-H,N,K Ras
Rap (1a,1b,2a,2b)
R-Ras, TC21
Raf (A & B)
Rheb

Heterotrimeric G-proteins
-Transducin

Ras family
-Rho (A,B,C)
Rac (1,2)
Cdc42, G25K
Rho D,E & G
TC 10

Rho family
Ras family
R-huper-family
Rab family
Ran family
Arf family
does vary between tissues (Leon et al., 1987), so the following observations apply to all three isoforms. Early investigations led to the conclusion that Ras proteins mediated signal transduction downstream of cell surface tyrosine kinase receptors and upstream of MEK and ERK that in turn activated transcription factors. More recently, Ras has been shown to be part of a multi-component signalling network which is activated by a variety of upstream receptors and which activate a variety of downstream signals and pathways between which there is much cross-talk (figure 1.2).

A diverse collection of extracellular ligands can stimulate Ras by activating their receptors, such as neurotransmitters, hormones and growth factors causing a transient increase in Ras.GTP levels. Upon stimulation, the level of Ras.GTP in the total Ras protein pool increases rapidly from ~1% to 70-80%. This enables interaction with downstream 'effector' molecules that bind specifically to the GTP bound form of G-proteins. The most characterised effector of Ras proteins is the serine-threonine kinase Raf1. Direct binding of Raf1 to Ras.GTP (Vojtek et al., 1993) activates a kinase cascade in the cytoplasm before translocation of MAPK (ERK) into the nucleus, where the activity of transcription factors (Elk) is initiated (Egan & Weinburg, 1993). Other documented effectors include phosphatidylinositol-3 kinase (PI-3K, Rodriguez-Viciana et al., 1994), which is thought to mediate protein kinase B (PKB/AKT) activation, one function of which is in protection against apoptotic signals (del Peso et al., 1997). PI-3K also activates Rac, a Rho like GTPase, a good example of cross-talk between signal transduction pathways (Hawkins et al., 1995). Other targets of Ras are a family of GEFs for Ral, a member of the Ras subfamily, such as RalGDS (Feig et al., 1996). Also RIN1 (Han & Colicelli, 1995) and AF6 (Kuriyama et al., 1996) are
Figure 1.2

The Ras signal transduction cascade

Association of ligands with several classes of cell surface receptors results in GEF mediated conversion of Ras into the active GTP bound form. Active Ras can then interact with a number of putative effector molecules (yellow) to elicit downstream signalling as described in the text.

Abbreviations

SR         G protein-coupled seven membrane spanning receptor
Gi         heterotrimeric G protein
R-PTK       receptor protein tyrosine kinase
PI3K        phosphatidylinositol 3 kinase
MAPK        mitogen activated protein kinase. (also known as ERK)
MEK         MAPK Kinase
MEKK        MEK Kinase
JNK         Jun nuclear kinase (also known as SAPK)
JNKK        JNK kinase (or SEK 1)
PLD         Phospholipase D
SRF         Serum response factor
PIP3        Phosphatidylinositol trisphosphate

Chapter 1 Introduction

Figure 1.2

Growth Factors
Hormones
Cytokines
Neurotransmitters

SR
G
RAS
GEFS

Raf
p120GAP
AF6
Ral GDS
P13K

Abl
JNK
MEK
p190
MEKK
JNK
MAPK

Rac
PLD
RBP1
SOS
VAV
AKT

AFT2
Jnk
Elk
NF-kB
SRF

CDC42
RAC

Gene Expression -
Cell growth and differentiation
Apoptosis

Actin Cytoskeleton
Cell Survival

20
putative effector molecules. To date Raf1 is the only Ras target protein for which genetic studies confirm its fundamental role in Ras signalling in a normal cellular context. However, interaction with these putative target proteins is likely to be crucial for mediating the role of oncogenic Ras in malignant transformation (Vojtek & Der, 1998). Much less is understood of the function of the other members of the Ras subfamily; Ral, TC21, R-Ras, Rap and Rheb (Bos, 1997).

An additional role for Ras has been found in continuously proliferating cells (Taylor & Shalloway, 1996). Ras activation peaks in mid-G1 of the cell cycle (Mulcahy et al., 1985) and only inhibition of Ras during the G1 phase affects the cell cycle. Ras is also thought to aid in the control of the G1-S transition, differences in Ras activation could determine the decision between proliferation and differentiation (Peeper et al., 1997).

1.1.2: Rho family

Rho A was the first of this family cloned. It was found to be able to induce transformation in some cell types but not in others and was certainly not as potent at transforming cells as Ras and has not been isolated from human cancer or a retrovirus as an oncogene (Ridley 1996 and Symons 1996 for reviews). The first clue to a biological function came from the observation that C3 transferase, a bacterial (Clostridium botulinum) exoenzyme, ADP-ribosylates and inactivates Rho proteins (Sekina et al., 1989), which causes animal cells to lose their actin stress fibres and round up (Paterson et al., 1990). Since then Rho family (Rho, Rac and Cdc42) proteins have been shown to mediate growth factor signalling pathways that regulate actin assembly in cells, much of the information coming from microinjection experiments in human fibroblast cells (Nobes & Hall, 1994). Rac has also been
Chapter 1  

Introduction

implicated in a pivotal role in NADPH oxidase activation in macrophages (Abo et al., 1991). Rho, Rac and Cdc42 are predominantly bound to GDP-dissociation inhibitors (GDIs) in the cytoplasm (section 1.1.6).

1.1.3: Ran, Rab and Arf families

Ran (Ras related nuclear protein) appears to play a crucial role in many nuclear transport pathways. It is thought that a high concentration of Ran.GTP in the nucleus (majority of Ran.GEF in the nucleus) and Ran.GDP in the cytoplasm (majority of RanGAP in the cytoplasm) helps determine directionality of nuclear transport (Moore, 1998).

ADP-ribosylation factors (ARFs) were first identified as cofactors for the ADP-ribosylation of the α-subunit of Gi by cholera toxin, but they are now known to be critical components of membrane trafficking pathways in all eukaryotic cells and activators of specific phospholipase Ds (PLDs) (Moss & Vaughan, 1998). It cycles from the cytosol in its GDP bound form to a coatamer-associated protein complex on the vesicle membrane in its GTP-bound state.

Members of the Rab subfamily regulate ‘membrane trafficking’ or the transport of vesicles between intracellular compartments. It is a large family (more than 30) whose role appears to be in the formation of v-SNARE·t-SNARE complexes, recruiting specific docking factors from the cytosol (Schimmoller et al., 1998, for a recent review).
1.1.4: Activation by Guanine nucleotide exchange factors, GEFs

In general, guanine nucleotide exchange factors are large multi-domain proteins. Each sub family of the Ras super-family of small G-proteins has an associated group of exchange factors. For example, proteins that have a domain with sequence homology with CDC25 (the first exchange factor discovered for Ras super-family proteins by Camonis et al., 1986)) appear to catalyse exchange of GDP for GTP, both in vivo and in vitro, on Ras sub-family proteins. Those with a domain with sequence homology to Dbl (DH domain) are proposed to be specific for the Rho sub-family (Cerione & Zheng, 1996). Dbl, a transforming protein, was initially isolated from a diffuse B-cell lymphoma (Eva & Aaronson, 1985). Interestingly, a number of Dbl homology proteins were isolated as oncogenes from tumours, suggesting a role for some Rho family proteins in some types of cancer. These are the most characterised groups, but the other sub-families are thought also to have a specific domain in common.

My investigation has involved two exchange factors, one CDC25 homology activator of Ras, mSosl (mouse son of sevenless gene product 1) and one Dbl homology protein and proposed activator of Rac1 in vivo, αPIX (PAK interacting exchange factor). The Sos protein is the most studied exchange factor for Ras, at a genetic and cell biological level. Two isoforms (1 & 2) have been found in mouse (Bowtell et al., 1992) and human cell lines (Chardin et al., 1993), since the Sos gene was first discovered to function upstream of Ras in the Drosophila eye (Simon et al., 1991). Sos is a ubiquitously expressed 150kD protein, the primary sequence of which is shown in figure 3a and compared to other CDC25 homology exchange factors. As well as a CDC25 homology domain, it can be seen that Sos proteins also have a DH, a PH and an SH3 domain. The SH3 domain has been shown to bind the SH2 domains
Figure 1.3

The domain structure of the CDC25 homology and Dbl homology exchange factors

A  CDC25 homology proteins, activators of Ras family G-proteins

The primary sequence of the mSos1 protein is shown, and compared with other known members of the CDC25 homology GEF’s; RasGRF (CDC25\textsuperscript{Mm}, a brain specific Ras GEF), CDC25 (Ras specific yeast GEF), C3G (specific for Rap proteins) and RasGRP (a recently cloned Ras GEF, Ebinu et al. 1998)

B  Dbl homology proteins, activators of Rho family G-proteins

The primary sequence of the two PIX isoforms is shown, and compared with other known members of the Dbl homology GEF’s; Dbl (Rho and CDC42Hs GEF), CDC24 (CDC42 GEF), Tiam-1 (GEF for Rac1 and CDC42Hs) and Vav (Rac1 GEF)

Key

- CDC25 homology domain
- Dbl homology domain
- Pleckstrin homology (PH) domain
- Src homology 3 (SH3) domain
- IQ, calmodulin binding domain
- DAG (diacylglycerol) binding
- Calponin related
- Ca\textsuperscript{2+} binding EF hand
- Myosin-like sequence
- Vimentin homology
- SH2 domain
### Figure 1.3

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</tr>
<tr>
<td></td>
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</tr>
<tr>
<td></td>
<td>C3G</td>
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<tr>
<td></td>
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<td></td>
<td>Tiam-1</td>
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</tr>
<tr>
<td></td>
<td>Vav</td>
<td>95</td>
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of the adaptor protein Grb2 (Rozakis-Adcock et al., 1993). In unstimulated cells, Sos appears to be found in a complex with Grb2 in the cytosol (Schlessinger, 1993). Membrane localisation of Sos or CDC25 by fusion of a membrane targeting signal to the C-terminus has been shown to be sufficient for activation of Ras (Aronheim et al., 1994 and Overbeck et al., 1995). It has been proposed, therefore, that the function of Grb2 is to recruit Sos to the membrane on phosphorylation of a receptor (via interaction of the SH2 domain of Grb2), promoting nucleotide exchange and hence activating Ras (figure 1.4). It has also been suggested that other adaptor molecules are involved in this process (such as Shc, Schlessinger, 1993) and tyrosine phosphatases such as PTP2 have also been implicated (Li et al., 1994). The function of the N-terminal domains of Sos remains unclear. The DH domain of Sos has recently been shown to stimulate nucleotide exchange on Rac in vivo (Nimnual et al., 1998), suggesting a possible coupling of the Ras and Rac signalling pathways. PH domains have been implicated in the recruiting of signalling molecules to the membrane (Lemmon et al., 1997). As for the CDC25 homology catalytic domain, what is known of the mechanism of nucleotide exchange by this domain on Ras family proteins is discussed in section 1.4.

PIX (PAK interacting exchange factor) family proteins were identified by co-purifying with PAK (p21 interacting kinase), binding tightly via an SH3 domain (Manser et al., 1998). They are proposed to recruit, or be recruited with, PAK to peripheral focal complexes along with the adaptor protein Nck, where PIX can activate Rac1. PIX has been shown to promote nucleotide exchange on Cdc42 and Rac1 in vitro, but only Rac1 in vivo. Two isoforms of PIX have been discovered (α and β), their primary structures are compared to other DH domain exchange factors in
Figure 1.4

*Adapter mediated translocation of Sos to the membrane*

In the unstimulated cell (A), the Grb2.Sos complex is in the cytoplasm and Ras is in the inactive GDP bound form. On stimulation of a receptor (B), autophosphorylation of tyrosine residues causes translocation of the Grb2.Sos complex to the plasma membrane, enabling stimulation of active Ras. GTP formation. (Taken from Quilliam *et al*, 1995)
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Figure 1.4

A

Receptor protein tyrosine kinase

Grb2

Inactive Ras

B

Growth Factors

Sos

Ras

GDP

GTP

Signal

Ligand-stimulated Ras activation
figure 3b. All DH homology proteins have a DH domain in tandem with a PH domain, the significance of this is not fully understood. Although many (>15) DH homology proteins have now been isolated and characterised, little information is known of the molecular mechanism of activation of Rho family proteins.

1.1.6: Inactivation by GTPase activating proteins, GAPs

The first GAP protein for Ras superfamily proteins was discovered by Trahey & McCormick, 1987. This protein exists in two forms; a 120 kDa protein and an alternatively spliced 100 kDa protein (p120-GAP and p100-GAP). A C-terminal domain of ~300 amino acids was found to contain GAP activity towards Ras proteins (Skinner et al., 1991). Neurofibromin (NF1) is the human protein defective in von Recklinghausen neurofibromatosis was found to have a domain homologous to p120GAP and shown to increase the hydrolysis rate of GTP by Ras proteins (Martin et al., 1990). Both these proteins also share homology with IRA 1 and 2 that have GAP activity towards Saccharomyces cerevisiae RAS proteins (Tanaka et al., 1989).

Each subfamily of the Ras superfamily has an associated GAP family. RhoGAP was the first GAP described for the Rho subfamily (Garrett et al., 1991). It is a 50 kDa protein, the GAP domain being contained within the C-terminal 27 kDa of the protein (Lancaster et al., 1994). More than twelve GAPs for Rho proteins have been discovered (Lamarche & Hall, 1994). As GEFs, GAPs contain a diverse range of other domains associated with signal transduction such as SH3, SH2 and PH domains providing potential for regulation of function.
1.1.7: Guanine nucleotide dissociation inhibitors, GDIs

As yet GDIs have only been described for Rab and Rho family proteins. They are thought to act in the cytosol and regulate both the GTP / GDP cycles and membrane association and dissociation of Rho and Rab proteins. The dissociation of GDIs from these proteins is a prerequisite of membrane association and activation by GEFs (Olofsson, 1999).

So what are the biochemical and biophysical properties of small G-proteins that enable the performance of such diverse functions and interaction with their regulatory and effector molecules?

1.2: Biochemistry of small G-proteins

The proteins of the superfamily are all globular proteins of a molecular weight of ~ 21 kDa (Ras proteins are often referred to as p21ras). There is 80% sequence identity between Ras family proteins, 30 % sequence identity between the Ras and Rho family proteins and approximately 10 % identity between Ras and Arf and Ran family proteins. This identity is confined mainly to the nucleotide-binding region within the N-terminal 165 amino acid catalytic domain. This domain can be purified independently and has been shown to contain all the residues required to bind and hydrolyse guanine nucleotides. Many of the Ras super-family proteins have now been purified enabling in vitro studies to be carried out and the equilibrium binding constants and rate constants of the steps in these processes to be described. As mentioned earlier they all have a high affinity for GTP and GDP and slow intrinsic ability to hydrolyse GTP to GDP + P\(_i\). The basic mechanism is shown in scheme 1.1.


**Scheme 1.1**

\[
\begin{align*}
\text{Ras} + \text{GTP} & \overset{k_{+1}}{\underset{k_{-1}}{\leftrightarrow}} \text{Ras.GTP} & \overset{k_{+2}}{\underset{k_{-2}}{\leftrightarrow}} \text{Ras.GDP.Pi} & \overset{k_{+3}}{\underset{k_{-3}}{\leftrightarrow}} \text{Ras.GDP} & \overset{k_{+4}}{\underset{k_{-4}}{\leftrightarrow}} \text{Ras} + \text{GDP}
\end{align*}
\]

For Ras (H- and N-) the dissociation equilibrium constants for GTP and GDP (\(K_{d1}\) and \(K_{d4}\)) have been shown to be approximately \(10^{-11}\) M with rate constants of nucleotide dissociation of \(10^{-4} - 10^{-5}\) s\(^{-1}\) for GDP and GTP (Neal et al., 1988). The association rate constant for N-Ras to GTP was shown to be 12 fold higher than that for GDP (3 fold higher for H-Ras, John et al., 1990). Rate constants for the hydrolysis step (\(k_{+2}\)) are also in the range of \(10^{-4}\) s\(^{-1}\) (Neal et al., 1988). It is important to note that rate constants and affinities of nucleotide binding and hydrolysis do vary considerably between and within families of small G-proteins.

However, scheme 1.1 is probably an over-simplification of the actual mechanism, conformational changes and different nucleotide bound states having been suggested by several groups. For example, a two step binding mechanism, scheme 1.2, for both GDP and GTP has been proposed involving a rapid weak binding step followed by an isomerisation reaction (John et al., 1990)

**Scheme 1.2**

\[
\begin{align*}
\text{H-Ras} + \text{GXP} & \overset{k_{+1}}{\underset{k_{-1}}{\leftrightarrow}} \text{H-Ras.GXP} & \overset{k_{+2}}{\underset{k_{-2}}{\leftrightarrow}} \text{H-Ras*.GXP}
\end{align*}
\]

Early biochemical work shed light of the detail of the intrinsic GTP hydrolysis properties of Ras proteins. By labelling the thiophosphate group of the GTP analogue
**Chapter 1  Introduction**

GTP\(_7\)S with oxygen isotopes to make it chiral, Feuerstein *et al.* (1989) showed that the configuration of the phosphorus atom was inverted on transfer of the group to water. This suggested the mechanism was a single step in-line transfer of the phosphate from GTP to water, not involving a phosphoenzyme or other phosphorylated intermediate in a similar manner to that of EF-Tu (Eccleston & Webb, 1982).

Small G-proteins bind nucleotides as a complex with Mg\(^{2+}\). The affinity of guanine nucleotides is increased and dissociation rates decreased in the presence of divalent metal ions, for example the affinity between Ras and GDP is increased >500 fold in the presence of Mg\(^{2+}\) (Hall & Self, 1986). Physiological concentrations of Mg\(^{2+}\) (mM) have been shown to inhibit the release of nucleotide from Ras proteins *in vitro*. The GTPase rate is also affected (John *et al.*, 1993). Indeed, most phosphotransferases require at least one divalent cation complexed directly to phosphoryl group oxygens for catalytic activity.

The single point mutations leading to naturally occurring Ras oncogenes were localised to codons 12, 13, and 61 (Barbacid, 1987). *In vitro* mutagenesis techniques have enabled the effects of these mutations to be studied biochemically and also revealed many other mutations that give rise to a transforming phenotype. Some of these will be discussed in relation to the three dimensional structure of Ras proteins in section 1.3.
1.2.1: Membrane localisation

As is apparent from the signal transduction pathways described, plasma membrane localisation of most Ras superfamily proteins is crucial for their function. I will describe this for Ras proteins, although similar principles apply to others of the superfamily. Two signals are required for Ras proteins to be transported to the plasma membrane. The first is farnesylation of the cysteine of the C-terminal CAAX motif followed by proteolytic removal of the AAX residues and carboxyl methylation of the C terminus. This 15-carbon farnesyl prenoid modification provides hydrophobicity for membrane association but a second component is required for stable membrane binding and the observed selective localisation of Ras to the membrane. This has been shown to be S-acylation (palmitoylation) of cysteine residues in N- and H-Ras proteins and a polybasic stretch of amino acids in K-Ras4B, both of these signals being found in the hypervariable domain between the catalytic domain and the CAAX motif (Magee & Marshall, 1999 for a recent review). Rho family proteins are modified with a geranyl-geranyl group. Ran, however, does not appear to gain any post-translational modifications having an acidic -DEDDDL at the C-terminus which is conserved across the family (Richards et al., 1995) This supports its role in the cytoplasm and nucleus as discussed earlier. The effect of these post-translational modifications must be considered in any in vitro functional studies of Ras superfamily proteins.

1.3: The three dimensional structure of small G-proteins

Recently, X-Ray diffraction studies have greatly advanced the understanding of the mechanism of action of small G-proteins. The core nucleotide binding domain of a G-protein was first described at low resolution 20 years ago for the bacterial elongation
factor Tu (Morkawa, 1978). Since then a many structures have been reported. These are discussed below using Ras as the example because this protein has been extensively studied and major differences between families are noted.

Most crystallographic studies on Ras have used a truncated form of H-Ras (N-terminal 166 or 171 amino acids). Truncated forms have been shown to have very similar GTPase activity to the full-length proteins (John et al., 1989) and the structure of full length (189 residue) H-Ras showed the C-terminal residues to be poorly ordered (Milburn et al., 1990). This core catalytic domain common to all G-proteins has a core structure of 5 α-helices and a six-stranded β-sheet in which five strands are parallel and one is anti-parallel, shown clearly in the topological structure of Ras (figure 1.5a).

1.3.1: Nucleotide binding pocket

The GDP binding pocket is defined by four loop sequences, three of which are common to all G-proteins (the fourth varies between families). These have been highlighted in the sequence alignments shown in figure 1.5b. Motif 1 (GxxxxGK(S/T)), residues 10-17, is also found in proteins that bind other purine nucleoside triphosphates (ATP synthetases, myosin and some kinases) and is referred to as the phosphate binding loop (P-loop) or glycine rich loop. Motif 2 (DxxG), residues 57-60, confers the conformational change between GDP and GTP forms and motif 3 (NKxD), residues 116-119, determines the specificity for guanosine. The fourth motif (SAK), residues 145-147, is not absolutely conserved, varying between families.
**Figure 1.5**

**Three dimensional structure of Ras Proteins I**

**A** Topological structure of Ras proteins

Both GDP and GTP analogue complexes of Ras proteins have the same topological structure as represented here. β-strands are represented by arrows and α-helices by cylinders. Residue numbers at the beginning and end of each secondary structural element are listed below:

- **β-strands**
  - β1 (1-9), β2 (38-46), β3 (50-58), β4 (77-84), β5 (110-117), β6 (140-144)

- **α-helices**
  - α1 (15-26), α2 (67-75), α3 (87-104), α4 (126-137), α5 (151-171)

- **Loops**
  - L1 (10-14), L2 (27-37), L3 (47-49), L4 (59-66), L5 (76), L6 (85-86), L7 (105-109), L8 (118-125), L9 (138-139), L10 (145-150).

(Taken from Milburn et al., 1990).

**B** Table to show the nucleotide binding motifs found in all G-proteins. Selected G-proteins are shown from the Ras superfamily (H-Ras, Rap-1A, Ran and ARF-1A), elongation factors (EF-Tu) and heterotrimeric (G_iω) G-protein families. Numbers preceding the sequences correspond to the first residue number of the motif.

*Abreviations*: H (Human), B (Bovine) and Ec (*Escherichia coli*).

(Adapted from Sprang, 1997)
Figure 1.5

A

B

<table>
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<td>VGNKCD</td>
<td>YIEITSAK</td>
</tr>
<tr>
<td>Rap1A (H)</td>
<td>GSGGVGKS</td>
<td>ILDTAQTE</td>
<td>VGNKCD</td>
<td>FLGSSAK</td>
</tr>
<tr>
<td>Ran (H)</td>
<td>GDGTGKS</td>
<td>NWDTAQOE</td>
<td>CGNKVD</td>
<td>YIEITSAK</td>
</tr>
<tr>
<td>ARF1A (H)</td>
<td>GLGAAKGT</td>
<td>VWVDVGQD</td>
<td>FANKCD</td>
<td>IQATCAT</td>
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<tr>
<td>EF-Tu (Ec)</td>
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<td>HVDSGPHQA</td>
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<td>Gua (B)</td>
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<td>FLNKD</td>
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</table>
Chapter 1

Introduction

The γ- and β- phosphates of the nucleotide are bound by the P-loop, four backbone amides are hydrogen bonded to the phosphate oxygens. Important contributions to γ-phosphate binding are made by the main chain NH of Thr-35 and Gly-60. The α-phosphate is only hydrogen bonded weakly to Ala-18, possibly explaining the weak affinity of Ras for GMP. The major interaction with the base is by Asp-119 with the exocyclic amino group and N1 of the guanine base. Ala-146 interacts with the exocyclic oxygen of the base and important contributions are made by aromatic interaction with Phe-28 and hydrophobic interaction with Lys-117. A schematic diagram to illustrate the residues involved in binding of the GTP analogue, GMPPNP, to Ras is shown in figure 1.6a.

1.3.2: Mg²⁺ binding site

The co-ordination of Mg²⁺ as observed in the Ras.GMPPNP crystal structure (Pai et al., 1990) is shown in figure 1.6b. The Mg²⁺ ion is co-ordinated to 6 oxygens in a perfect octahedron, complexed to two phosphate oxygens of GMPPNP, side chain oxygens of Ser-17 and Thr-35 and two oxygens from water molecules. In the GDP bound form Ser-17, four water molecules and the β phosphate are the ligands. The fact that Thr-35 is only complexed to Mg²⁺ in the GTP bound form has also been shown by site directed mutagenesis studies (John et al., 1993). John et al. also showed Mg²⁺ to be in rapid equilibrium with the solvent and to dissociate more quickly and independently of GDP. The dissociation constant between Mg²⁺ and H-Ras.GDP was determined to be 2.8 μM.
Three dimensional structure of Ras Proteins 2

A  Nucleotide binding site of the H-Ras protein

Schematic drawing showing the interactions between GMPPNP and H-Ras or water molecules. All dashed lines correspond to hydrogen bonding interactions (distance between donor and acceptor atom $<3.4 \, \text{Å}$).

(Taken from Pai et al., 1990)

B  Magnesium binding site of the H-Ras protein

Schematic drawing of the $\text{Mg}^{2+}$ binding site showing the ligands of the first coordination sphere of the metal ion and some of the interactions of these ligands (GMPPNP bound H-Ras structure).

(Taken from Pai et al., 1990)
1.3.3: Switch regions

A crystal structure of the GDP bound form has been determined for truncated H-Ras (Tong et al., 1991) as well as the structure when bound to the GTP analogues GMPPNP (Pai et al., 1990) and GMPPCP (Milburn et al., 1990). The only major differences between the two structures are localised in two regions whose conformation ‘switches’ on replacement of GTP for GDP. These are highlighted in figure 1.7 and have been named ‘switch 1’ and ‘switch 2’. Switch 1, which spans residues 30 – 38 (corresponding to loop 2), has been implicated in effector and GAP binding (see section 1.3.5) and is therefore also known as the effector loop. Switch 2, consisting of residues 60 – 76 (loop 4 and α-helix 2) has been shown to be highly mobile, having weak electron density and also appears to be affected by crystal packing. It was also seen to be flexible in the NMR structure of Ras.GDP (Kraulis et al., 1994). Mutational studies have implicated also this region in exchange factor binding (Quilliam et al., 1995 and Mosteller et al., 1994).

1.3.4: Effector interactions

The binding of GTP to a G-protein stabilises the switch regions to enable association with an effector molecule. One of the effectors of Ras is Raf-1 as mentioned earlier. Raf-1 contains an 80-residue Ras binding domain (RBD) that is sufficient for GTP-dependent binding to Ras and a three dimensional structure of this domain with Rap1A has been determined (Nasser et al., 1995). As predicted from mutagenesis, the effector loop forms the site recognised by the RBD. The switch 2 domain does not participate directly in the interaction of the RBD, but indirectly as its conformational change is coupled to that of the effector loop. The switch 2 domain is therefore available for interaction with GAPs.
Three dimensional structure of Ras Proteins 3

The conformational change in the ‘switch’ regions of the H-Ras protein is highlighted in this schematic diagram comparing the GDP bound (bottom) and GMPPCP bound (top) H-Ras crystal structures. The switch regions have been shaded. The conformational changes in these regions can be clearly seen as well as the lack of change throughout the rest of the molecule. The nucleotide is depicted by the ball and stick model and the Mg$^{2+}$ ion by a single sphere in both diagrams.

(taken from Sprang 1997)
Figure 1.7

A

B

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Introduction
1.3.5: Mechanism of hydrolysis and GAP action

As described earlier, the mechanism of hydrolysis proceeds via a single step in-line transfer of the phosphate from GTP to water. A water molecule (175) in the high resolution structure of H-Ras.GMPPNP (Pai et al., 1990) is a candidate to perform this nucleophilic attack. Thr-35 and Gln-61 could hydrogen bond to the water molecule making it more nucleophilic and Lys-16 and the Mg\(^{2+}\) interacting with the \(\gamma\)-phosphate would make the \(\gamma\)-phosphate more electrophilic. There is some opposition to this mechanism, Prive et al. (1992) having proposed that Gln-61 is involved as a general base in the reaction.

This intrinsic hydrolysis can be stimulated by up to five orders of magnitude by GAPs (Eccleston et al., 1993). Two possible models exist for GAP action; either GAPs lower the energy barrier for efficient hydrolysis by Ras without directly participating in the chemical cleavage step or GAPs supply residues to enhance the hydrolysis rate. The catalytic domains of both RasGAP (Scheffzek et al., 1996) and RhoGAP (Barrett et al., 1997) are entirely \(\alpha\)-helical and both have an exposed arginine residue, shown to be required for efficient catalytic activity. This 'arginine finger' has been shown to be essential for efficient catalysis biochemically for RasGAP (Scheffzek et al., 1997), NFl (Sermon et al., 1998) and RhoGAP (Graham et al., 1999). A second absolutely conserved arginine plays a vital role in stabilising the catalytically competent conformation of the loop presenting the catalytic arginine residue to the Ras active site (Ahmadian et al., 1997). This theory has been supported by the publications of the crystal structures of RasGAP in complex with Rap1A (Scheffzek et al., 1997) and RhoGAP in complex with RhoA (Rittinger et al., 1997).
GAPs have been well studied and extensive information is available on the mechanism of catalysis of GTP hydrolysis of small G-proteins (Gamblin & Smerdon 1998 and Noel 1997, for recent reviews). I will concentrate on the less understood activators of Ras superfamily proteins, the guanine nucleotide exchange factors.

1.4: The mechanism of GEF action

As described earlier, there is a considerable amount of published data concerning the cellular and molecular biology of exchange factors and recently the mechanism of action of GAPs on small G-proteins has been studied extensively. Despite this there has been little progress made towards understanding the molecular mechanism of GEF action on small G-proteins. Mechanisms for GEF-catalysed exchange have been proposed in other similar G-protein systems. The interaction between bacterial elongation factor Tu (EF-Tu) with its exchange factor elongation factor Ts (EF-Ts) has been well characterised and a substituted mechanism is proposed (figure 1.8, scheme 1). In this mechanism EF-Ts catalyses the release of GDP by stabilising EF-Tu in a nucleotide free state before GTP binds (Chau et al., 1981, Eccleston 1984 and Hwang & Miller 1985). The recently solved crystal structure of the complex EF-Tu.EF-Ts suggests EF-Ts acts by disrupting the Mg\(^{2+}\) binding site on EF-Tu (Kawashima et al., 1996). In contrast, evidence exists for both a substituted mechanism (Rowlands et al., 1988) and a sequential mechanism (Dholakia & Wahba 1989) (figure 1.8, scheme 2) for the nucleotide exchange on the G-protein eIF2 (eukaryotic elongation initiation factor 2) by the GEF eIF2B. This sequential mechanism involves the possible binding of GTP to eIF2B and formation of a transient quaternary complex (eIF2.eIFB.GDP.GTP).
Figure 1.8

Substituted and sequential nucleotide exchange mechanisms

Scheme 1
The substituted nucleotide exchange mechanism as proposed for the EF-Tu and EF-Ts system. EF-Ts stabilises EF-Tu in a nucleotide free state, a binary complex (♦) being formed as GDP is substituted for GTP.

Scheme 2
The sequential mechanism of nucleotide exchange, involving the formation of a quaternary complex (♦) before the release of the exchange factor and GDP.
Figure 1.8

**Scheme 1**

EF-Tu.GDP

EF-Tu.GDP.EF-Ts

EF-Tu.GTP.EF-Ts

EF-Tu.GTP

**Scheme 2**

eIF2.GDP

eIF2B eIF2B.eIF2.GDP

GTP

eIF2B.GTP eIF2B.GTP + GDP

eIF2.GDP eIF2.GTP
Chapter 1  Introduction

As for GEF interaction with small G-proteins, the mechanism of nucleotide exchange by RCC1 on Ran (Ras related nuclear G-protein) has been studied by equilibrium and transient kinetic measurements (Klebe et al., 1995), and has been proposed to follow the same scheme as that of EF-Tu. Other groups have reported that GEFs stabilise small G-proteins in a nucleotide free state (Lai et al., 1993, Chuang et al., 1994, Hart & Powers, 1995, Jacquet et al., 1995), having been able to show binding of exchange factors to the nucleotide free form of Ras, but not nucleotide bound forms. These findings also suggest a substituted mechanism. Klebe et al., (1995) in their work on the interaction Ran with RCC1 proposed that; ‘the exchange reaction does not favour the formation of the Ran.GTP complex but rather accelerates the formation of the equilibrium dictated by the relative affinities of Ran for GTP/GDP and the respective concentrations of the nucleotide in the cell’. The ratio of GTP:GDP in the cell has been suggested to be in the range of 3 to 30:1 (Kleinecke et al., 1979 and Pan & Wessling-Resnick, 1998). Evidence suggests that GEFs accelerate the dissociation rate of nucleotide as opposed to stimulating the association rate to Ras proteins. Jacquet et al. (1995), working on the interaction of H-Ras with the catalytic domain of cdc25Mm (RasGRF), suggested this to be the case. They purified the nucleotide free H-Ras.cdc25Mm and observed only a small increase in the association rate of nucleotide with this complex as compared with nucleotide free H-Ras protein, but a >1400 fold enhancement of the dissociation rate of H-Ras.GDP by cdc25Mm. However, Haney & Broach (1994) and Mosteller et al., (1994) both proposed that the nucleotide exchange on the yeast protein Ras2 depends on the nature of the free nucleotide, GTP being more efficient than GDP. Haney & Broach (1994) concluded that the rate-limiting step is the nucleotide association step.
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During this work great advances have been made in our knowledge of the mechanism of nucleotide exchange with the publication of the high-resolution crystal structures of several exchange factors. RCC1 (regulator of chromosome condensation gene product) an exchange factor for Ran (Renault et al., 1998) and ARNO (ARF nucleotide-binding-site opener, Cherfils et al., 1998) were the first to be solved. Both have strikingly different folds to each other and to EF-Ts (Kawashima et al., 1996) and the NMR solution structure for Mss4 (exchange factor for Rab, Yu et al., 1995). Failed attempts to thread CDC25 homology domains onto these folds (Pai, 1998) for structure prediction suggested a different structure again. Structures have also been published for the systems on which this work is based; for the DH domain of the Sos protein (Soisson et al., 1998) and more significantly the first structure of a complex between a Ras superfamily member and its exchange factor. Boriack-Sjodin et al., (1998) solved the structure of H-Ras with the catalytic domain of Human Sosl protein. These structures will be discussed in more detail in relation to results obtained during this work in the concluding chapter of this thesis.

Despite this apparent structural variance between exchange factors is there a general nucleotide exchange mechanism? Indeed, would exchange factors for different family members be expected to have the same mechanism? They perform significantly different roles, for example Sos is thought to function to amplify growth factor signals whereas Pix is found at more stable focal complexes with Rac. Different cellular roles would require different adaptation. The only way to answer this question is to study the mechanism of a member of each family in detail, to uncover the mechanism of action of each GEF individually.
Chapter 1  Introduction

The aim of the project is to deduce the reaction mechanism for exchange factor action on small G-proteins, specifically the mechanism of action of Sos on Ras proteins and PIX on Rho proteins. By reaction mechanism, elucidation of each step in the process is implied, characterising intermediates and measuring rate and equilibrium constants of these steps.

1.5:  Techniques used in this project

There are two kinetic approaches to the study of enzyme kinetics; steady state and transient (pre-steady state) kinetics. Steady-state kinetics aims to infer the reaction mechanism (the combination of the elementary steps) from analysis of the overall reaction in which substrates are converted into products in the presence of a catalytic amount of enzyme, without examining the enzyme molecule itself. The requirement of only small amounts of enzyme make this technique widely applicable but information about intermediates and measurement of rate constants of their formation and decay have a tendency to be indirect and ambiguous. The detection of intermediates and direct measurement of rate constants of formation and decay requires methods to study enzymes at reagent concentrations on a time scale approaching steady state.

1.5.1: Transient pre-steady state kinetics

As stated above, in order to measure the rate constants of the individual steps in a reaction pathway or detect intermediates, it is necessary to measure the rate of approach to steady state, i.e. pre-steady state. The enzyme is used at concentrations greater than that of the substrate and consequently the single turnover of substrate occurs as opposed to the multiple cycling for steady state calculations. Therefore,
generally, several exponential processes can be observed as opposed to the linear plots of product formation associated with Michaelis-Menton kinetics. Two advances have revolutionised this technique for studying enzyme kinetics. Firstly, techniques of large scale recombinant protein expression and purification enabling the production of milligram quantities of enzyme and secondly, the development of laboratory apparatus for following these fast reactions in the approach to steady state. These techniques involve either observing relaxation back to equilibrium after rapid changes in conditions, such as temperature and pressure, or the rapid mixing of solutions such as continuous, quenched and stopped-flow techniques.

1.5.2: The development of rapid mixing techniques

The continuous-flow method was first introduced by Hartridge & Roughton in 1923 for the study of the interaction of solutions of haemoglobin and oxygen. Two syringes, one filled with enzyme and one containing the substrate, push the solutions into a mixing chamber and into a flow tube. Measurements made along the length of the tube allow the time course of the reaction to be followed. The development from continuous-flow to stopped-flow techniques by Chance in 1940 and later by Gibson, in 1954, enabled rapid reactions to be observed using lower quantities of enzyme. Here the two syringes containing enzyme and substrate are mechanically stopped after expelling 50-200 μl from each. When the flow is stopped the reaction can be measured over time after mixing. Detection of events occurring after 1-5 milliseconds is possible. Detailed analysis of these techniques and their application to the study of enzyme mechanisms can be found in numerous publications (Gutfreund, 1995, Hiromi, 1979, Fersht, 1995). The stopped-flow apparatus has been used extensively in
this work. Such rapid mixing methods are only of use if an optical probe exists in the system, either intrinsic or extrinsic, to report any changes that occur.

1.5.3: Fluorescent analogues

The major analytical tool used in this study was fluorescence, still the most sensitive probe for studying protein-protein and protein-ligand interactions. G-protein interaction could be followed by intrinsic protein fluorescence, with fluorescently labelled protein, extrinsic fluorescent probes or with fluorescently labelled nucleotides (intrinsic fluorescence of common nucleotides is too low to be of use). If the nucleotide can be isolated spectroscopically, then a fluorophore that can be localised to a defined position exists in the system. Therefore, extensive work has been performed to improve fluorescently modified nucleotide probes. Whereas any modification of the purine ring of the guanine nucleotide results in loss of binding to G-proteins (Eccleston et al., 1989), modification of the ribose moiety, in most cases, results in satisfactory analogues of the parent nucleotide. This can be rationalised on analysis of the structures of G-proteins, such as EF-Tu and H-Ras, where the 2',3'-hydroxyl groups, to which the probe binds, project out of the nucleotide binding domain. A review of such analogues can be found in Eccleston & Jameson, (1997). Some of the best characterised are the 2'(3')-O-anthraniloyl (ant) and 2'(3')-O-methylanthraniloyl (mant) derivatives, first synthesised by Hiratsuka, (1983). The reaction of isatoic or methylisatoic anhydride with nucleotide produces ribose modified analogues with the fluorophore attached by an acyl linkage (figure 1.9a). Such monoacyl derivatives of cis 1,2-diol systems undergo isomerisation by acyl migration. Cremo et al. (1990) demonstrated that an equilibrium does indeed exist between the isomers of the mant derivatives (70% 3' isomer, 30% 2' isomer) (figure
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Figure 1.9

Fluorescent analogues of guanine nucleotides

A  The synthesis of mant nucleotides by reaction of methylisatoic anhydride.
(taken from Eccleston and Jameson, 1997)

B  Isomerisation between the 2’- and 3’-oxygen atoms of nucleotide acyl derivatives.
(taken from Eccleston and Jameson, 1997)

C  Fluorescence enhancement on binding mantGDP and mantGTP to N-Ras protein.
(taken from Neal et al., 1990).
Figure 1.9

A

\[ \begin{align*}
&\text{O}_3\text{P}\text{O} \quad \text{O}_3\text{P}\text{O} \quad \text{O}_3\text{P}\text{O} \\
\text{HO} & \quad \text{CH}_2 \\
\end{align*} \quad \text{B} + \quad \begin{array}{c}
\text{O} \\
\text{CH}_3 \\
\end{array}
\]

B

\[ \begin{align*}
&\text{O}_3\text{P}\text{O} \quad \text{O}_3\text{P}\text{O} \quad \text{O}_3\text{P}\text{O} \\
\text{HO} & \quad \text{CH}_2 \\
\end{align*} \quad \text{B} \quad \text{C} = \text{O} \\
\text{NHCH}_3 \\
\]

C

![Fluorescence graph](image)
1.9b). This is important as different isomers could have different binding properties and be hydrolysed at different rates.

There have been several reports of kinetic studies on the interaction of mant nucleotides with the Ras family proteins, with N-Ras (Neal et al., 1990) and H-Ras (John et al., 1990, Rensland et al., 1991). They have also been used to study interaction with GAPs (Moore et al., 1993). A two to three fold enhancement in the fluorescence intensity of the probe is observed on binding to the G-protein, and then a decrease in the fluorescence on hydrolysis of GTP to GDP (figure 1.9c). It has a similar affinity for Ras as GDP (Neal et al., 1990) and does not appear to perturb the GEF-catalysed nucleotide exchange on Ras from preliminary kinetic studies performed on cdc25 by Lenzen et al., (1995). These observations have been rationalised by Scheidig et al., (1995) who solved the structure of Ras bound to mantGMPPNP. They showed that the mant group was on the surface of the protein and suggested that it interacted with Tyr-32 relieving a quenching effect of the guanine base on the mant moiety that exists in solution.

1.6: Project aims

The aim of this project is to study the mechanism of release of nucleotide from Ras super-family proteins by nucleotide exchange factors by in vitro kinetic experiments using the fluorescent nucleotide analogues. These studies involve large amounts of pure protein. The Ras superfamily proteins (N-Ras and Rac1) and PIX exchange factor express well in E.coli and 30-200mg of protein can be purified from 4-40 litre cultures. However, the catalytic domain of mSos1 (CmSos1, GST or His-tagged) did
not express well enough in *E.coli* to provide concentrated samples of the purified protein.

*E.coli* shows a distinct bias in its utilisation of codons, low-usage codons being clearly avoided in genes encoding abundant proteins (Zhang *et al.*, 1991) and shown to affect translation rates in *E.coli* (Sorensen *et al.*, 1989). This bias varies between species leading to suggestions that mammalian genes may have an inappropriate distribution of codons for high level expression in *E.coli*. The Cmsos1 gene (the region encoding the CDC25 homology catalytic domain) contains many codons that are very rarely found in *E.coli*, especially those encoding arginine where 36 out of 38 are coded for by 'bad' *E.coli* codons. It was decided to redesign the gene fragment, optimising the codon usage for expression in *E.coli*, and reconstruct the gene by the method of Stemmer *et al.*, (1994). This process, leading to a 60 - 120 fold increase in CmSos1 protein production, is described before a detailed kinetic and equilibrium analysis of the interaction of the catalytic domain of mSos1 and N-Ras. A study of the interaction of αPIX and Rac1 was also attempted.
Chapter 2

Materials & Methods

Materials

Unless otherwise stated, standard reagents were from Sigma and fine chemicals were from Boehringer Mannheim.

2.1: Protein Purification

This study required the large-scale purification of three Ras super-family proteins (N-Ras H-Ras and Rac1) and the catalytic domains of two GEFs mSos1 (CmSos1) and αPIX. The materials and methods used for their purification are described.

2.1.1: Expression and purification of N-Ras.GDP

The purification of full length human N-Ras.GDP has been described previously (Moore, 1992). The procedure is described here, modifications to this protocol will be found in section 3.1.2. The E.coli (GC42) expression system (a gift from Prof. A. Hall) contains the gene for full length N-Ras under the control of the trp promoter (expression induced by 3β-indole acrylic acid, IAA) and ampicillin resistance.

A forty litre culture was grown as described previously (Hall & Self, 1986) by M. Goggin, (N.I.M.R). Typically 100-200g of cells were obtained, which were broken in a French Press in 500ml buffer (20mM Tris.HCl pH 7.5, 20 mM NaCl, 1 mM MgCl₂, 5 mM DTT, 1 mM PMSF and 20 μM GDP). Cell debris was removed by centrifugation at 20000 rpm (Sorvall RC-5B) for 30 minutes and the supernatant loaded onto a Q-sepharose ion-exchange column (3 cm x 30 cm) equilibrated with the
above buffer at 2.0 ml.min\(^{-1}\). Proteins that did not bind to the column were discarded and bound protein eluted with a 3 litre gradient from 20–250 mM NaCl at 2.0 ml.min\(^{-1}\). The eluant was monitored by absorbance at 280 nm and N-Ras.GDP fractions were located by filter binding assays and SDS-PAGE (see sections 2.5.1 and 2.2.3 for methods). The protein pool was then concentrated by using a 65% ammonium sulphate precipitation. This also acts as a significant purification step with a considerable percentage of the undesired protein remaining in solution (Moore, 1992). The next stage in the protocol was a gel filtration step, which was modified in order to speed up the procedure. This is described in chapter 3, section 3.1.2.

2.1.2: Expression and purification of the GST-fusion proteins: Rac1, H-Ras, CmSosl and αPIX

The full-length mSosl (mouse) clone was a gift from D.Bowtell (University of Melbourne, Australia). The catalytic domain, CmSosl (residues 577–1076), was cloned into a pGEX-2T vector by K. Nurmohamed and Dr. M. Strom (N.I.M.R.). The αPIX expression construct of the DH and PH domains (PIX-DP, residues 236–547) was a gift from Dr. E. Manser (Glaxo-IMCB, Singapore). It was given as a GST fusion system, the gene having been cloned into the pGEX-4T-1 vector. A pGEX-2T expression system for full length Rac1 was obtained from A. Newcombe (N.I.M.R.) although the protein product was always N-terminally truncated by 7 or 8 amino acids due to proteolysis during preparation. A pGEX-2T expression system was obtained for full-length H-Ras from A. Parmeggiani (Ecole Polytechnique, France).
A general protocol followed for each of these proteins is shown here. Deviations from this protocol, problems encountered, typical yields and characterisation of the final product are covered individually for each protein.

For the preparation of these GST-fusion proteins, a single transformed *E.coli* colony (BL21 DE3 strain, Stratagene) was picked into 100-250 ml LB-amp (Lauria Broth, NIMR media supplies, containing 100 μg.ml⁻¹ ampicillin) and left shaking at 225 rpm (New Brunswick Scientific, model G25) overnight at 37°C. This starter culture was diluted 50-500 times in LB-amp (1-10 ml in 500 ml in 2 litre flasks) and left shaking at 225 rpm at 37°C until an optical density at 600 nm of approximately 0.8 was reached. Expression of fusion protein was induced with 1.0 mM isopropyl β-D-thiogalactoside (IPTG). Three hours after induction the cells were harvested by centrifugation at 4000rpm (Beckman J6-HC rotor) for 15 minutes and stored at -80°C, re-suspended in 50 ml of buffer. The composition of buffer varied for the three proteins and was:

- **CmSos1** - 50 mM Tris.HCl pH 7.5, 1 mM dithiotreitol (DTT), 50 mM NaCl
- **PIX-DP** - 50 mM Tris.HCl pH 7.5, 5 mM DTT, 50 mM NaCl
- **Rac1** - 20 mM Tris.HCl pH 7.5, 1 mM MgCl₂
- **H-Ras** - 20 mM Tris.HCl pH 7.5, 1 mM DTT, 1 mM MgCl₂.

The cells were lysed by sonication for 4 x 2 minutes (1 minute continuous, 1 minute pulsed) on ice (1 mM PMSF added) using an output setting of 6 and 50% duty cycle with a Vibra Cell sonicator (Sonics & Materials Inc.). The cell debris and insoluble material was removed by centrifugation at 40000 rpm (Beckman 45Ti rotor) for 1
hour. The supernatant was loaded onto a 10-20 ml Glutathione Sepharose column (Pharmacia) at 0.5 ml.min\(^{-1}\) (column equilibrated with buffer). After washing with buffer, a 5 ml p-aminobenzamidine agarose (Sigma) column was attached in series and the columns were washed with buffer (plus 2 mM CaCl\(_2\)). Thrombin (250 units of human plasma, high activity protein from Calbiochem) was added to 50 ml buffer (plus 2 mM CaCl\(_2\)) and cleaved protein was eluted at 0.1 ml.min\(^{-1}\) overnight. Cleaved protein was pooled based on the absorbance trace at 280 nm and by analysis of fractions using SDS PAGE. Concentration to 0.5–2.0 ml was achieved by centrifugation at 3000 rpm (Beckman J6-HC) using 10 kD molecular weight cut off concentrators (‘Centriprep’ by Amicon or ‘Vivaspin’ from Vivascience). The concentrated sample was dialysed back into buffer using ‘Slide-a-lyser’ cassettes (Pierce) overnight before aliquoting out, freezing on dry ice and storing at -80°C.

The purification yields and characterisation of the CmSos1 protein is described in chapter 3 and for PIX-DP in chapter 9. H-Ras.GDP and Rac1.GDP were both purified from existing protocols so are described below.

The purification procedure for H-Ras.GDP was adapted from Parrini et al., (1995). A four-litre culture produced 5 mg of protein of thrombin cleaved H-Ras.GDP. From SDS-PAGE analysis, it appeared some proteolysis may have occurred, a double band clearly visible (data not shown). Mass spectrometry suggested three protein forms existed in the final sample (figure 2.1). The molecular weights did not match the predicted molecular weights for C-terminal proteolysis. Therefore, the DNA was sequenced and it was shown that in addition to the glycine and serine expected from the pGEX-2T vector at the N-terminus, the DNA coded for a phenylalanine and
Figure 2.1

Mass spectrometry of the H-Ras protein

Mass spectroscopy was performed by Dr. S. Howell (as described in section 2.2.4). It can be seen that three species of H-Ras were observed. The nature of these were calculated from the molecular weights and sequencing results as shown in table 2.1 below.

Table 2.1

Proteolysis of the H-Ras protein

The calculated molecular weights of the H-Ras species resolved by mass spectroscopy are shown and compared to theoretical molecular masses for proteolytic products. It can be seen that these are very similar suggesting that these are the H-Ras species present in the preparation.
Table 2.1

<table>
<thead>
<tr>
<th>Species</th>
<th>Mass-Spectrometry MW (kDa)</th>
<th>H-Ras Protein</th>
<th>Theoretical MW (kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>21676.55</td>
<td>GSPHMTE...GCMSCKCVLS (full length)</td>
<td>21676.52</td>
</tr>
<tr>
<td>B</td>
<td>21273.99</td>
<td>GSPHMTE...GCMSCK (4 residues cleaved)</td>
<td>21274.01</td>
</tr>
<tr>
<td>A</td>
<td>20954.76</td>
<td>GSPHMTE...GCM (7 residues cleaved)</td>
<td>20955.62</td>
</tr>
</tbody>
</table>
histidine before the N-terminal methionine of H-Ras. This explained the mass spectrometry data (see table 2.1), showing that as well as full-length H-Ras, species existed lacking the last four and seven C-terminal residues. This protein was used for analysis of the interaction with CmSos1 despite this proteolysis having occurred.

The Rac1 purification was taken from Newcombe (1999). A four litre culture of cells produced 25mg of >95 % pure Rac1 protein. No further purification steps were required. Mass spectrometry gave a molecular weight of 20549 Da., which is consistent with the truncation of the last 8 C-terminal residues, as expected for this purification procedure. This protocol has since been improved to avoid the proteolysis observed. Full-length Rac1 used in this work was purified and characterised by C.Davis (N.I.M.R.).

2.2: Protein concentration, purity determination and characterisation

Protein concentrations were calculated using two methods; the Bio-Rad assay and absorbance at 280nm. Purity was determined by SDS-PAGE.

2.2.1: Bio-Rad Assay

The assay (Bio-Rad laboratories) is based on the Bradford dye-binding procedure (Bradford, 1976). Protein samples were added to a 1 in 5 dilution of Bio-Rad reagent (0.2ml in 1ml of water) and left at room temperature for 10 minutes before the absorbance at 595 nm was measured. Bovine serum albumin (BSA) was used to create a standard curve from which the desired protein concentration could be estimated.


2.2.2: Calculated Extinction Coefficients

Molar extinction coefficients were calculated based on theoretical absorbance contributions at 280 nm of tryptophan (5540 M\textsuperscript{-1}cm\textsuperscript{-1}) and tyrosine (1480 M\textsuperscript{-1}cm\textsuperscript{-1}) residues and GDP (7950 M\textsuperscript{-1}cm\textsuperscript{-1}) (Mach et al., 1992). Calculated extinction coefficients for Ras.GDP, Rac1.GDP, CmSos1 and PIX-DP were 19790, 29828, 51380 and 25260 M\textsuperscript{-1}cm\textsuperscript{-1} respectively.

2.2.3: SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

A Hoefer SE600 series gel system was used with gels of 180 x 80 x 0.75 mm. The stacking gel was 0.13% bis-acrylamide, 5% acrylamide, 130 mM Tris.HCl pH 6.8 and 0.1% w/v SDS. The resolving gel consisted of 0.087% bis-acrylamide, 15% acrylamide, 375 mM Tris.HCl pH 8.8 and 0.1% w/v SDS. TEMED (0.02% v/v) and ammonium persulphate (0.1% w/v) were added to each to induce polymerisation. Protein samples were heated to 95°C for 5 minutes in 50 mM Tris.HCl pH 6.8, 100 mM DTT, 2% w/v SDS, 0.1% w/v bromophenol blue and 5% v/v glycerol. 2-20 μl (3-5 μg protein) was loaded onto the gel. The electrophoresis was performed at 30 mA in 25 mM Tris, 250 mM glycine and 0.1% w/v SDS for 1-2 hours. Gels were stained with 0.5% w/v Coomassie blue R250 in 45% v/v methanol, 45% water and 10% acetic acid for 15 minutes and protein bands visualised after several washes in a de-staining solution (stain solution minus Coomassie blue).

2.2.4: Mass Spectrometry

Mass spectrometry of proteins was carried out by S.Howell (N.I.M.R.) using electrospray ionisation mass spectrometry. Reverse phase chromatography (Poros RII,
Perceptive Biosystems) was performed on a 130A HPLC (Perkin Elmer) that was coupled to a platform electrospray mass spectrometer (Micromass).

2.3: Synthesis and purification of guanine nucleotide analogues

The fluorescent analogue of GDP, \(2'(3')-O-(N\text{-methylantranioloyl})\)-GDP (mantGDP), was synthesised as previously described by the method of Hiratsuka, 1983, and modified by Neal et al., 1990. GDP (0.113 mmole) was dissolved in 4 ml of water at \(37^\circ\)C and the pH adjusted to 9.6 with 1 M NaOH. Methylisatoic anhydride (0.226 mmole) was added to the stirred solution and the pH maintained at 9.6 using 1 M NaOH. On completion of the reaction (the pH remains constant) the pH was lowered to 7.6 with 1 M HCl. MantGDP was separated from the unreacted species by ion exchange. The reaction mix was diluted to 50 ml in 10 mM triethylammonium bicarbonate (TEAB, pH 7.6), loaded onto a DEAE-bicarbonate column and eluted with a gradient of 10 mM to 600 mM TEAB (3 litres, overnight at 2 ml.min\(^{-1}\)). MantGDP eluted as the third peak (absorbance at 254 nm), recognised by its characteristic absorption profile. Fractions were pooled and concentrated by rotary evaporation. Three washes with 50 ml methanol removed residual TEAB. The nucleotide analogue was taken up in 2 x 1 ml water and stored at -20 \(^\circ\)C. The final concentrated 1 ml stock was 86 mM and total yield approximately 32 %. Purity was checked by HPLC to be >95% and identical to previously synthesised, fully characterised material. The same method was used to synthesise 2'-deoxy3'-O-(N-Methylanthraniloyl)-GDP (mdGDP), except that the concentration of reactants (2'deoxy-GDP and methylisatoic anhydride was halved. The mantGTP analogue, mantGMPPNP, was synthesised in the same way by C.Davis, N.I.M.R.
2.3.1: Preparation of nucleotide analogue small G-protein complexes

A 25 fold excess of mant-nucleotide was incubated with 1-2 mg of Ras protein, in fast exchange buffer (100 mM ammonium sulphate, 40 mM EDTA and 20 mM Tris.HCl, pH 7.5) to a total volume of 0.2-1.0 ml, for 10 minutes at room temperature. MgCl₂ (80 mM final) was added to stop the reaction and the complexes were separated from free nucleotide by gel filtration using a PD-10 column (Amersham Pharmacia) in 20 mM Tris.HCl pH 7.5, 2 mM MgCl₂ and 1 mM DTT at 4 °C. The elution was visualised under UV light, the complex being the first to elute. Concentration was determined by absorbance at 350 nm (the extinction coefficient of the mant group being 5700 M⁻¹ cm⁻¹).

2.3.2: HPLC separation and analysis of nucleotides

Mant guanine nucleotides (mdGDP and mdGTP) were separated on a Waters Nova-Pak® C₁₈ reversed phase column (3.9 x 150 mm). Isocratic elution with 100 mM KPO₄ plus 12.5 % acetonitrile at 1 ml.min⁻¹ gave enabled clear separation of the diphosphate and triphosphate nucleotides (retention times of ~ 9.0 minutes for mdGTP and ~ 13.0 minutes for mdGTP). Eluant was monitored with a Hitachi F-1050 spectrophotometer, exciting at 360 nm and recording emission at 440 nm. An HP 3390 integrator was used to calculate the area under the elution peaks.

2.4: Fluorescence measurements (Anisotropy and Intensity)

All slow time course fluorescence measurements were performed on an SLM 8000S spectrofluorimeter. Anisotropy was recorded in the T-format, with excitation at 366 nm polarised vertical to the laboratory axis, monitoring emitted light parallel and
perpendicular to the exciting plane simultaneously through Schott KV399 (399nm) cut off filters. Anisotropy was calculated as:

\[
\text{Anisotropy} = \frac{I_{\text{parallel}} - I_{\text{perpendicular}}}{I_{\text{parallel}} + (2 \times I_{\text{perpendicular}})} \quad \text{(Equation 2.1)}
\]

Intensity measurements can also be recorded the T-format as for anisotropy but are calculated using the equation:

\[
\text{Intensity} = I_{\text{parallel}} + (2 \times I_{\text{perpendicular}}) \quad \text{(Equation 2.2)}
\]

Intensity measurements were also recorded in the L-format, without exciting with polarised light, exciting at 366 nm and monitoring emission through a monochromator at 440 nm. All reactions were performed at 30°C with the stated buffer in 4 mm by 4 mm quartz cuvettes. Data were analysed using the software packages ‘Grafit’ (Erithacus software) and Microsoft Excel.

2.4.1: Stopped-Flow fluorescence measurements

Stopped-flow experiments were performed using a Hi-Tech Scientific SF-61MX spectrofluorimeter. As for the slow time course fluorescence experiments, data was recorded in the L-format or T-format. All concentrations quoted in the text are after mixing of the two solutions. Data were collected using the High-Tech Scientific software package ‘RK2’ and analysed using Excel and Grafit programmes.
Chapter 2  
**Materials and Methods**

2.5: N-Ras.\[^3\text{H}\]GDP complex formation

N-Ras.GDP (~ 1 mg) was incubated with 100 pmoles of \[^3\text{H}\]GDP in fast exchange buffer (100mM ammonium sulphate, 40 mM EDTA and 20 mM Tris.HCl, pH 7.5) for 10 minutes at room temperature. MgCl\(_2\) (80 mM) was added to stop the reaction and the complex was separated from free \[^3\text{H}\]GDP by gel filtration using a PD-10 column (Amersham Pharmacia) in 20 mM Tris.HCl pH 7.5, 2 mM MgCl\(_2\) and 1 mM DTT at 4 °C. N-Ras.\[^3\text{H}\]GDP was pooled after scintillation counting (see section 2.5.1 below) and measurement of absorbance from 220 to 320 nm.

2.5.1: Filter Binding Assays

Filter binding assays were performed to follow the dissociation of \[^3\text{H}\]GDP from N-Ras.\[^3\text{H}\]GDP and for the qualitative identification of \[^3\text{H}\]GDP binding to Ras protein during purification. Details of the reactions are described in the text. The reactions were quenched with 5 ml of ice cold wash buffer (50 mM Tris.HCl, 150 mM NaCl and 10 mM MgCl\(_2\)). This solution was filtered through 0.45 μm nitrocellulose membranes (Millipore) and washed with 3 x 5 ml wash buffer to remove unbound \[^3\text{H}\]GDP. The filters were dried and added to 5 ml of scintillant (0.5 % butyl-PBD in toluene). Counts were recorded using a Beckman LS7000 scintillation counter. All reactions were performed in duplicate and counts averaged over 10 minutes.

2.5.2: GTP\(_\gamma\)S incorporation assays

Nucleotide exchange was also measured by the incorporation of \[^{35}\text{S}]\text{GTP}\(_\gamma\)S Rac1 protein. This method was taken from Manser et al. (1998). Rac1.GDP (0.2 μM) was incubated with and without PIX-DP (0.067 μM) in 300 μl of 25 mM Tris HCl, 4.5 mM HEPES, 0.5 mM DTT, 5 mM EDTA, 10 mM MgCl\(_2\) and 20 μM GTP\(_\gamma\)S (0.74
MBq, Amersham) at 30°C. Duplicate 20 μl aliquots were taken at intervals up to 6 hours and one sample overnight. The reaction was quenched by the addition of 2 ml of ice cold 25 mM Tris-HCl (pH 8), 100 mM NaCl, 30 mM MgCl₂. These solutions were filtered through a nitrocellulose membrane and washed with quench buffer (3 x 2 ml). The membranes were dried and 5 ml of scintillation fluid (toluene based, Jencons) added. Counts per minute (cpm) were averaged over ten minutes (Beckman LS 6000SC). The specific activity (cpm.pmole⁻¹) of the [³⁵S]GTPγS (dilutions spotted onto filters, dried and counted as above) was used to calculate the pmoles of GTPγS incorporated. The maximum GTPγS incorporation was taken as 100 % of Rac1 bound with GTPγS.

2.6: Molecular biology

The techniques and materials used for the optimisation of the codon usage of the CmSos1 gene are described. Firstly, for the design and synthesis of the optimised gene and then for the general molecular biology techniques used for cloning into expression vectors.

2.6.1: Design of the optimised mSos1 gene

The programme ‘CODOP’, written by Dr. E. Carpenter, was used to assign codons to the CmSos1 gene to give an ‘E.coli codon distribution’ according to their R.S.C.U. (relative synonymous codon usage) value. This is explained in section 4.1. The programme reads in a 5’ to 3’ DNA sequence (CmSos1 sequence), adds the reverse strand and translates the DNA sequence into an amino acid sequence. It reads in a
specified codon usage table (in this case of course an *E. coli* codon usage table (gcg). The programme allows codons with an RSCU value of less than 0.6 (any value can be specified) to be omitted as it is believed that clusters of these low usage codons particularly are a reason for low expression of heterologous genes in *E. coli*. (Kane 1995) and some of these codons never appear in highly expressing genes (Sharp et al., 1988). Therefore, codons with an R.S.C.U of less than 0.6 are given a weighting of zero and proportion of the remaining codons is recalculated to give the same ratio as found in *E. coli*. The optimised sequence is then generated randomly using the calculated codon proportions and then output sequence converted into oligonucleotides of 42 (specified) base pairs in length (reversing the second strand ensuring the oligonucleotides are 5’ to 3’).

The programme also incorporates procedures to; check annealing temperature of created oligonucleotides, find the probability of non-specific oligonucleotide binding and calculate the chance of secondary structure formation in the transcribed mRNA sequence. The gcg programme ‘map’ was used to check for undesired restriction sites in the optimised sequence. The sequence was manually altered to disrupt long strings of A or T (not good for PCR fidelity) and to ensure an even spread of a particular codon. Enzyme restriction sites for cloning into expression vectors were added to both ends; BamH1, NdeI and EcoR1 at the 5’ end and Hind III, PstI and EcoR1 after two termination codons at the 3’ end for cloning into the vectors pGEX2T, pET28a, pMal and pCal.
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The oligonucleotides were ordered from GibcoBRL and reconstituted in water to a concentration of 100 µM. 5 µl of each oligonucleotide was mixed together (final concentration of total nucleotide 100 µM).

2.6.2: PCR synthesis and amplification of the optimised gene

A schematic diagram to describe the method of gene construction is shown in figure 2.2. The optimised gene can be assembled by the design of overlapping oligonucleotides for the entire top and bottom strands of the DNA. DNA polymerase builds increasingly longer DNA during a gene synthesis PCR step. A second PCR step amplifies the desired gene DNA fragment from a mix of synthesised DNA. Pfu polymerase (Stratagene) was used for its improved fidelity over Taq polymerase due to the additional 3' to 5' exonuclease proof reading activity it possesses. Deoxynucleotide triphosphates (dNTP's) were from Pharmacia. PCR was performed with an Omni Gene Hybrid PCR cycler (a layer of mineral oil was added to each reaction, to prevent sample evaporation).

The gene synthesis reaction mixture consisted of: 1 µM (1 µl of 100 µM) oligonucleotide mix, 1 µl of 100 mM dNTP mix (25 mM of each dNTP), 10 µl of 10X Pfu buffer, 2 µl Pfu (20 units) and 86 µl water. PCR cycling was as follows: 50°C for 2 minutes, 74°C for 15 seconds, followed by 40 cycles of 94°C for 15 seconds, 50°C for 30 seconds and 74°C for 25 seconds (increased by 6 seconds per cycle).
Figure 2.2

**Gene synthesis and amplification protocol**

A schematic diagram to show the synthesis of the optimised CmSos1 gene. PCR cycling 74 oligonucleotides of 42 base pairs in length in the ‘gene synthesis’ reaction produced a mix of DNA species. The amplification reaction, using 5’ oligonucleotide primers for the plus and minus strands, enabled synthesis of the desired construct of CmSos1. Restriction sites, incorporated into the construct during amplification, were used for cloning into the expression vector of choice (pGEX 2T).
Figure 2.2

74 42-mers

 Gene Synthesis

 Gene Amplification

Clone into expression vector
e.g. pGEX 2T

BamH1 EcoRl

BamH1 EcoRl

pGEX2T
For the amplification reaction 20 µl of the gene synthesis reaction was added to; 1µl of 100 µM dNTP mix, 1 µl each of 5' end oligonucleotides (100 µM) from the plus and minus strands as primers, 10 µl of 10X Pfu buffer, 2 µl Pfu and 65 µl water. PCR cycles (40) of 94°C for 45 seconds then 74°C for 5 minutes were performed to recover the desired fragment. Amplification of the correct sized fragment was observed on a 1% agarose gel. DNA from successful PCR reactions was purified using the ‘Wizard PCR preps’ purification kit (Promega).

2.6.3: Agarose gel electrophoresis of DNA.
DNA was analysed on 1% w/v agarose gels in TAE buffer (40 mM Tris-acetate, 1 mM EDTA) and 0.5 µg.ml⁻¹ ethidium bromide. Gel loading buffer (6x buffer of 0.25% bromophenol blue, 0.25% xylene cyanol FF and 30% glycerol) was added to all samples and gels were run at 400 mA for 45-60 minutes with a Hoefer submarine electrophoresis unit (Pharmacia). DNA bands were visualised under UV light.

2.6.4: Cloning into plasmid expression vectors
The fragments of DNA were first blunt end ligated into the shuttle vector pUC 18 (Pharmacia) then cut and ligated into the pGEX2T (Pharmacia) expression vector as described in the following sections.

Blunt - end ligations
T4 polynucleotide kinase (PNK, New England Biolabs) was used to phosphorylate the 5' hydroxyl termini of the insert DNA fragments. The DNA fragments (20 µl) were incubated with 3 µl (10 mM) ATP, 3 µl 10X PNK buffer and 2 µl PNK (20 units) at 37°C for 2 hours. The DNA was phenol/chloroform extracted, ethanol precipitated
(Sambrook et al., 1989) and re-suspended in 10-15 µl of water. 1 µl (50 ng) of pUC18 (ordered pre-cut with Smal and dephosphorylated, Pharmacia) was incubated at 16°C overnight with 2 µl (or 250 ng) purified, phosphorylated DNA insert and 1 µl 10X T4 ligase buffer, 1 µl T4 ligase (400 units, New England Biolabs) and 5 µl water.

**Transformation into E.coli.**

Transformation of the constructs into *E. coli* XL1Blue super-competent cells (Stratagene) was by heat shock. Aliquots of cells (20 µl) were thawed on ice and 1-5 µl of ligation mix was added, keeping the cells on ice for a further 15 minutes before heat-shocking at 42°C for 40 seconds. The cells were put back on ice for 2 minutes prior to adding 300 µl of LB and shaking (225rpm) at 37°C for 30 minutes. The cells were spread on to LB-amp plates and incubated overnight at 37°C.

**DNA preparation**

Single colonies were picked into 5ml LB-amp and incubated overnight with shaking (250rpm) at 37°C. Each overnight culture was mini-prepped using the 'Wizard miniprep' system from Promega. Small-scale restriction digests were performed to detect positives; 2 µl pUC18 construct mini-prep, 1 µl each of BamH1 and EcoR1 (Boeringer Mannheim), 1 µl 10X buffer B (Boeringer Mannheim) and 5 µl water were incubated at 37°C for 2 hours. The sample was analysed on a 1% agarose gel.

**Sticky-end ligation**

Fragments were cut out of pUC18 with BamH1 and EcoR1, insert DNA was purified from a 1% agarose gel and ligated into pGEX-2T (a 1:1 ratio of insert to vector was used, otherwise ligation was as for into pUC18). Colonies were mini-prepped and
positives detected as for pUC18. The same methods were employed for ligation into
the pET28a vector, but 50 \( \mu \text{g.mL}^{-1} \) kanamycin replaced ampicillin and pUC18 and
pET28a were cut with NdeI and EcoRI before ligation.

2.6.5: **Small scale protein expression and solubility testing**

Single colonies were picked from plates into 3 ml LB-amp in 15ml sterile tubes and
left shaking at 250 rpm overnight at 37°C. LB-amp (25ml) was inoculated with 0.5ml
of the starter culture and grown at 37°C and shaking at 250 rpm to an OD\textsubscript{600nm} of 0.8.
Expression was induced with 1 mM IPTG, and left for 3 hours. Pre- and post-
induction samples were taken for analysis by SDS-PAGE (section 2): 1 ml of cells
were centrifuged at 13000 rpm (Biofuge 13, Heraeus) for 5 minutes and the pellet re-
suspended in 65 \( \mu \text{l} \) of 6x gel loading buffer (section 2.4) and heated at 95°C for 5
minutes. After 3 hours, 15 ml of cells were centrifuged at 3000 rpm for 20 minutes.
The pellet was re-suspended in 0.5 ml of buffer (50 mM Tris-HCl pH 7.5, 150 mM
NaCl, 1 mM DTT). Cells were broken by sonication (4x30 seconds using a microtip,
setting 1). Insoluble material was centrifuged down at 13000 rpm (Biofuge 13,
Heraeus) for 30 minutes. Supernatent (50 \( \mu \text{l} \) ) was added to 50 \( \mu \text{l} \) of 2X gel loading
buffer and a scraping of pellet added to 100 \( \mu \text{l} \) of 2X gel loading buffer. Samples were
vortexed and heated at 95 °C for 5 minutes. 15 \( \mu \text{l} \) of pre-induction, 5 \( \mu \text{l} \) of post-
induction samples, 20 \( \mu \text{l} \) of supernatant (soluble) and 5 \( \mu \text{l} \) of pellet (insoluble) sample
were analysed on a 15% SDS gel.

2.6.6: **DNA Sequencing**

DNA sequencing was performed on an ABI Prism 377 automated sequencer (Perkin
Elmer) using the Prism\textsuperscript{TM} Dye Terminator Cycle Sequencing Ready Reaction Kit (as
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protocol. Oligonucleotides used in the gene synthesis process and 5’ and 3’ pGEX-2T sequencing primers (Pharmacia) were used in order to sequence both top and bottom strands of the DNA. Some sequencing was performed by Cambridge Biosciences.
Chapter 3

**The interaction of CmSos1 with N-Ras.mantGDP**

The aim of the project was to elucidate the mechanism of nucleotide exchange on Ras superfamily proteins by guanine nucleotide exchange factors (GEFs). As described in the Introduction, the weight of evidence suggested the interaction of N-Ras.GDP with CmSos1 would follow a substituted mechanism, as shown in scheme 3.1 below.

**Scheme 3.1**

\[
\begin{align*}
N\text{-Ras.GDP} + \text{CmSos1} & \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} N\text{-Ras.GDP.CmSos1} \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} N\text{-Ras.CmSos1} + \text{GDP} \\
N\text{-Ras.CmSos1} + \text{GTP} & \underset{k_{-3}}{\overset{k_{+3}}{\rightleftharpoons}} N\text{-Ras.GTP.CmSos1} \underset{k_{-4}}{\overset{k_{+4}}{\rightleftharpoons}} N\text{-Ras.GTP} + \text{CmSos1}
\end{align*}
\]

The aim was to prove whether this was indeed the case and to measure the rate constants for each step. Also, it was intended to observe any additional isomerisations or conformational changes that might occur and to characterise the intermediates in the process. This was to be attempted using the fluorescent analogue of GDP, mantGDP. This chapter describes the purification of the catalytic domain of the GEF mSos1 (CmSos1, from the original low expression clone) and full length N-Ras proteins, the characterisation of the intrinsic properties of the N-Ras.mantGDP complex and preliminary studies of the interaction of N-Ras.mantGDP with the CmSos1.
3.1: **Protein purification**

This study has involved the large-scale purification of the catalytic domain of the guanine nucleotide exchange factor, mouse Sos1 (CmSos1) and the Ras subfamily member N-Ras. Both these proteins were purified using *E.coli* expression systems. The expression and purification of the putative catalytic domain of mSos1 (residues 577-1076) will be described as well as alterations to a protocol for purification of full length human N-Ras.GDP.

### 3.1.1: Purification of the catalytic domain of the mouse Sos1 protein (CmSos1)

The protocol for the expression and purification was designed by K.Nurmohomed and M.Strom (N.I.M.R.). The details of the purification procedure are described in section 2.1.2. Cleavage with thrombin of GST-CmSos1 bound to the glutathione sepharose column resulted in the production of 2-4 mg of CmSos1 protein from a 6-litre culture of *E.coli*. The elution profile is shown in figure 3.1. The reason for the low yields can be seen to be due to low expression levels from the SDS-PAGE gel of the pre- and post-induction samples (shown in figure 3.1). It can be seen that there is also contamination of a 70 kD protein. The purity of the CmSos1 protein was preparation dependent and ranged from 50% to 75%. This contaminant could be removed by ion exchange using a 1ml MonoQ column (Amersham Pharmacia) and applying a salt gradient from 50 to 500 mM NaCl using an FPLC system (Amersham Pharmacia). This reduced the yield of CmSos1 protein to 0.5 to 1.0 mg from a 6-litre culture (data not shown). The preliminary kinetic data was performed using 75% pure CmSos1 protein as high concentrations of CmSos1 were required.
Figure 3.1

**SDS-PAGE analysis of the CmSos1 purification**

A SDS-PAGE analysis of the elution of CmSos1 from a glutathione sepharose column upon cleavage of the fusion protein GST-CmSos1. A 10 μl aliquot of each fraction of the thrombin wash was prepared in sample buffer (as section 2.2.2) and loaded onto a 15% gel.

SDS-PAGE of the whole cell extracts of *E.coli* transformed with the CmSos1 clone, pre- and post- induction with IPTG are also shown. 1 ml of cells (taken just before addition of IPTG, pre-induction, and just before harvesting, post-induction) were spun at 13000 rpm for 10 minutes and the supernatant discarded. The cell pellet was re-suspended in 60 μl of sample buffer and prepared as section 2.2.2. Pre-induction (14 μl) and post-induction (4 μl) samples were loaded onto a 15% gel.
Figure 3.1

A
3.1.2: Modification of a protocol for the purification of full length human N-Ras.GDP

As stated in section 2.1.1 the first step of N-Ras.GDP purification was over a Q-sepharose ion exchange column. The N-Ras.GDP protein peak was identified by SDS-PAGE and filter binding assays for $[^3H]$GDP and subsequently concentrated using a 65% ammonium sulphate precipitation as described. The final purification step, gel filtration, was modified as follows. Instead of a G-75 column (two columns 2.5 cm by 90 cm in series) run at 0.3-0.4 ml.min$^{-1}$ over 2 days, a Superdex-75 column (HiLoad 26/60, Amersham Pharmacia) was used. The concentrated protein sample was applied to the column, equilibrated in buffer A, at 1.0 ml.min$^{-1}$ and fractions collected every 3 minutes. Similar separation to that with the G-75 columns was achieved but in less than 3 hours as opposed to 2 days (figure 3.2). The pooled protein was concentrated using Vivaspin concentrators (10 kD cut off, Vivascience). This procedure yielded approximately 60-100 mg of active N-Ras.GDP protein (measured using a mantGDP binding assay, section 2.3.2) per preparation. Mass spectrometry revealed a molecular weight of 21229 kDa, as expected, some preparations showing some proteolysis of a methionine residue revealing a second species of 21096 kDa.

The intrinsic GTP hydrolysis and nucleotide release properties of N-Ras have been studied extensively in this laboratory (Neal et al., 1988 & 1990) and others have performed detailed studies on the H-Ras proteins (Feurstein et al., 1987, and Sweet et al., 1984). Feurstein et al. (1987), first purified the highly unstable nucleotide free Ras apoprotein for kinetic study. Neal et al. (1988) were able to adapt this for the study of nucleotide association to the N-Ras apoprotein. The consensus is that nucleotides (GTP and GDP) bind to Ras proteins with dissociation constants in the picomolar
Modification of the N-Ras.GDP purification procedure

A Absorbance trace at 280 nm of the elution from the S-75 gel filtration column during the purification of N-Ras.GDP. The fractions marked were pooled after analysis of the fractions by SDS-PAGE (shown in figure 3.2 b) and filter binding assays.

B SDS-PAGE analysis of the elution profile from the S-75 gel filtration column during the N-Ras.GDP purification. A 10 µl aliquot was taken from a range of fractions and analysed on a 20 % gel. The position of N-Ras on the gel was known by running an N-Ras sample from a previous purification and by comparison with [³H]GDP binding data from filter binding assays (data not shown).
Figure 3.2

**A**

Absorbance (280nm)

**B**

Fraction

N-Ras
range. John et al. (1990) developed a protocol for the purification of larger quantities of nucleotide free H-Ras protein and have proposed a two step binding mechanism for the association of GDP and GTP. This involves a fast weak initial binding step followed by a slow, rate limiting isomerisation process.

Due to the difficulty of obtaining stable apoprotein, characterisation of the N-Ras protein was performed using a nucleotide bound form. A 1:1 complex of either N-Ras.mantGDP or N-Ras.[³H]GDP can be relatively easily formed as described in sections 2.3.2 and 2.5. MantGDP fluorescence has been shown to increase 2.5–3.0 fold on binding to Ras proteins as described in the Introduction, so release of mant nucleotides can be followed by a decrease in fluorescence intensity. [³H]GDP release can be studied using filter-binding assays as described in section 2.5.1. With these two probes a dissociation rate constant can be measured by a simple displacement reaction using unlabelled nucleotide as shown in the scheme below. A concentration of unlabelled nucleotide is used in large excess such that once the labelled nucleotide dissociates it cannot re-associate, therefore the observed rate constant depends solely on $k_{-1}$.

**Scheme 3.2**
3.2: Intrinsic release of mantGDP from N-Ras.mantGDP

A 1:1 complex of N-Ras.mantGDP was made as described in section 2.3.2. 0.5 μM N-Ras.mantGDP was incubated at 30°C in 20 mM Tris.HCl pH 7.5, 2 mM MgCl₂ and 1 mM DTT (buffer B) and fluorescence intensity and anisotropy followed in the T-format in the SLM spectrofluorimeter. Intensity and anisotropy (0.188) remained constant over a period of up to 12 hours. After this time, both intensity and anisotropy began to decay suggesting release of nucleotide as the protein denatured (data not shown). This suggested the complex was stable over a period of 12 hours at 30°C. Readings were taken at regular intervals between which the shutters were kept closed to avoid photobleaching of the fluorophore. Also, in order to counter fluctuations in the lamp intensity over such long time scales, the intensity measurement was always corrected to a solution with an intensity that would not alter over that time (usually mantGDP).

The intrinsic rate of release of mantGDP could be followed using a displacement reaction with excess free unlabelled GDP as described above. N-Ras.mantGDP (0.5 μM) was incubated as above but 100 μM GDP was added and intensity and anisotropy followed over time. Intensity and anisotropy were observed to decrease as expected (figure 3.3a). The intensity data could be fitted to a single exponential decay with an observed rate constant of $7.2 \times 10^{-5}$ s⁻¹. The anisotropy data appears to deviate from that expected for a single exponential process. However this can be explained. The observed anisotropy is made up of the fractional intensity of light emitted from the bound and free species as well as their individual intensities, i.e. at a given time:

$$A_{obs} = f_m A_m + f_r A_r$$  \hspace{1cm} (Equation 3.1)
Figure 3.3

Displacement of mantGDP and[^3H]GDP from N-Ras in the absence of CmSos1

A Displacement reaction with unlabelled GDP to show release of mantGDP from N-Ras.mantGDP. Ras.mantGDP (0.5 μM) was incubated in buffer B at 30°C and the decrease in intensity and anisotropy on addition of 100 μM GDP was observed. Measurements were recorded in the T-format in the SLM spectrofluorimeter to enable the conversion of the data into fluorescence intensity (○) and anisotropy (●) as described in the text. Intensity measurements are fitted to a single exponential function and anisotropy data to an anisotropy decay (with a D value of 0.417) giving observed rate constants of $7.2 \times 10^{-5}$ s$^{-1}$ and $6.0 \pm 0.5 \times 10^{-5}$ s$^{-1}$ respectively.

B Comparison of the displacement reactions of mantGDP from 0.5 μM N-Ras.mantGDP (open circles) and[^3H]GDP from 0.5 μM N-Ras.[^3H]GDP (closed circles). The fluorescence intensity and scintillation data has been converted into percentage of nucleotide released and plotted against time. Both reactions were performed at 30°C in buffer B and in the presence of 100 μM GDP. Both are fitted to a single exponential function with rate constants of $7.2 \times 10^{-5}$ s$^{-1}$ (mantGDP) and $5.5 \times 10^{-5}$ s$^{-1}$ ([^3H]GDP).
Figure 3.3

A

B

Chapter 3  Interaction of CmSos1 with N-Ras.mantGDP
Chapter 3  
Interaction of CmSos1 with N-Ras.mantGDP

Where  
\( A = \text{Anisotropy}, \ f = \text{Fractional Intensity} \)

\( m = \text{mantGDP}, \ r = \text{N-Ras.mantGDP} \)

The observed anisotropy at any given time can be described as:

\[
A_{\text{obs}} = \frac{(A_m - A_r)}{(1-D) + D e^{kt}} + A_r \quad (\text{Equation 3.2})
\]

Where;  
\( D = \text{factor by which} \ m \ \text{is more fluorescent than} \ r. \)

The anisotropy data shown in figure 3.3a has been fitted to equation 3.2 (Jameson & Sawyer, 1995). The anisotropy decreased from a value of 0.188 for N-Ras.mantGDP to 0.027 for free mant GDP and an observed rate constant of \(6.0 \pm 0.5 \times 10^{-5} \text{ s}^{-1}\) was calculated. There is greater error associated with this fit to the anisotropy decay and therefore intensity measurements were used to calculate the rate constant of mantGDP release from N-Ras.mantGDP.

The experiment was repeated monitoring intensity in the L-format. An observed rate constant of \(7.8 \times 10^{-5} \text{ s}^{-1}\) was obtained for the intensity decrease which is comparable to that obtained from measurements in the T-format. The intensity decrease due to dissociation of mantGDP from N-Ras.mantGDP was found to be 2.4 fold. Observed rate constants for the release of mantGDP from N-Ras.mantGDP were obtained also at 0.1 and 5 \(\mu\text{M} \) N-Ras.mantGDP and were shown not to vary significantly giving an average observed rate constant of \(7.6 \pm 0.5 \times 10^{-5} \text{ s}^{-1}\).
In order to compare the dissociation kinetics of mantGDP with GDP, the above experiment was repeated but observing release of $[^3H]GDP$ from N-Ras.$[^3H]GDP$ using filter binding assays. An N-Ras.$[^3H]GDP$ complex was made as described in section 2.5.1. N-Ras.$[^3H]GDP$ (0.5 µM) was incubated in buffer B at 30°C and after addition of 100 µM GDP, aliquots were removed and filtered onto nitrocellulose membranes for scintillation counting. A control in the absence of excess GDP was also performed. Figure 3.3b shows a comparison of the $[^3H]GDP$ and mantGDP release from N-Ras.$[^3H]GDP$ and N-Ras.mantGDP respectively. It can be seen that there is less than a two-fold difference in observed rate constant. This has also been documented elsewhere, but it has been shown here that under these conditions mantGDP behaves in a similar fashion to GDP.

3.3: The interaction of CmSos1 with N-Ras.mantGDP

If CmSos1 catalyses the release of nucleotide from Ras proteins as described in the literature then CmSos1 should accelerate the release of mantGDP from N-Ras.mantGDP. Therefore, a decrease in fluorescence intensity and anisotropy would be expected on addition of CmSos1 to N-Ras.mantGDP at a faster rate than that observed in the absence of the exchange factor as the nucleotide was released.

N-Ras.mantGDP (0.5 µM) was incubated at 30°C in buffer B (figure 3.4a). On addition of 10 µM CmSos1, no change in the fluorescent intensity of the fluorophore was observed (data not shown) but an increase in the fluorescence anisotropy value from 0.188 to 0.205 was recorded. On addition of excess unlabelled nucleotide, a decrease in intensity and anisotropy was observed at a faster rate than that for the intrinsic release of nucleotide by N-Ras. It can be seen that the catalysed reaction is
Chapter 3 Interaction of CmSos1 with N-Ras.mantGDP

Figure 3.4

The interaction of CmSos1 with N-Ras.mantGDP

A In red is shown the fluorescence anisotropy of a solution 0.5 μM N-Ras.mantGDP, in buffer B at 30°C, recorded with time. On addition of CmSos1 to final concentration of 10 μM an increase in anisotropy is observed from 0.188 to 0.208. GDP was then added to a concentration of 100 μM and the anisotropy is observed to decrease towards that of free mantGDP, shown in green (0.5 μM mantGDP). Measurements were recorded in the T-format in the SLM spectrofluorimeter.

B A figure to show the dependence of the fluorescence anisotropy of N-Ras.mantGDP on the concentration of CmSos1. CmSos1 was titrated into a solution of 0.5 μM N-Ras.mantGDP, in buffer B at 30°C, and the fluorescence anisotropy recorded after the solution reached equilibrium. The data points represent two separate titrations. The solid line is the best fit to equation 4.4 and gives a value of the $K_d$ for the interaction of N-Ras.mantGDP and CmSos1 of 13.5 ± 4.4 μM and a limiting anisotropy value of 0.233 ± 0.006 units.
Figure 3.4

A

B
95% complete after 8 minutes whereas the intrinsic mantGDP release rate from N-Ras.mantGDP has a half-life of 152 minutes.

3.3.1: The interaction of N-Ras.mantGDP with CmSos1 in the absence of excess unlabelled GDP.

The increased anisotropy of N-Ras.mantGDP on addition of CmSos1 (before addition of GDP) implies that the fluorophore has an increased rotational correlation time. This suggests an increase in size that could be due to the association of CmSos1 to N-Ras.mantGDP to form a ternary N-Ras.mantGDP.CmSos1 complex. In published data so far (for other exchange factors), it has been suggested that a transient ternary complex is formed (Ras.GDP.GEF) before the release of nucleotide and the formation of the stable binary complex, Ras.GEF (as described in the Introduction). However, as yet no group has been able to measure directly the dissociation constant for this ternary complex, it has only been indirectly calculated for other Ras superfamily systems (Klebe et al., 1995).

A titration of CmSos1 into N-Ras.mantGDP was performed in the absence of any free unlabelled nucleotide and followed by anisotropy in an attempt to observe an equilibrium binding process. N-Ras.mantGDP (0.5 μM) was incubated in buffer B at 30°C and the fluorescence anisotropy value recorded. CmSos1 (0.1–30 μM) was titrated into this solution (0.5 μM N-Ras.mantGDP was added to the CmSos1 to counter dilution effects) and the anisotropy value recorded after each addition. As can be seen from figure 3.4b there was an anisotropy increase with increasing CmSos1 concentration which began to saturate at high concentrations of CmSos1 enabling an
apparent $K_d$ for the binding of CmSosl to N-Ras to be estimated. An equilibrium dissociation constant for the reaction can be derived from the following equations.

$$K_d = \frac{[RmG][SOS]}{[RmG.SOS]} \quad \text{(Equation 3.3)}$$

$RmG$ represents N-Ras.mantGDP and SOS represents CmSosl. The unknown N-Ras.mantGDP.CmSosl concentration ([RmG.SOS]) can be expressed as;

$$[RmG.SOS] = \frac{([RmG]_t + [SOS]_t + K_d) \pm \sqrt{([RmG]_t + [SOS]_t + K_d)^2 - 4([RmG]_t[SOS]_t)}}{2}$$

Therefore, the anisotropy at any total concentration of CmSosl is given by;

$$A_r = \frac{A_{RmG}([RmG]_t - [RmG.SOS]) + A_{RmG.SOS}Q[RmG.SOS]}{[RmG]_t - [RmG.SOS] + Q[RmG.SOS]} \quad \text{(Equation 3.4)}$$

Where $RmG_t$ and $SOS_t$ represent the total amount of N-Ras.mantGDP and CmSosl present, $A_r$ is the total observed anisotropy and $Q$ is the fluorescence intensity of the N-Ras.mantGDP.CmSosl complex relative to the N-Ras.mantGDP complex (Jameson & Sawyer, 1995 and Graham, 1999). No evidence for a change in fluorescence was observed so $Q$ was equal to 1.

The experiment was performed twice and all data points are shown in figure 3.4b. The solid line is a fit to equation 3.4 and gives an apparent $K_d$ of 13.5 ± 4.4 μM. This
implies a weak association of CmSosl to the GDP bound form of N-Ras, without the subsequent release of mantGDP.

**3.3.2: The interaction of N-Ras.mantGDP with CmSosl in the presence of excess unlabelled nucleotide.**

On addition of excess GDP (100 μM) to 0.5 μM N-Ras.mant GDP and 10 μM CmSosl, the anisotropy value decreases exponentially towards that of free mantGDP (figure 3.3). This enables the study of the kinetics of the release of mantGDP from N-Ras.mantGDP catalysed by CmSosl.

CmSosl (2, 10 and 30 μM) was added to 0.5μM N-Ras.mantGDP in buffer B at 30°C and fluorescence anisotropy and intensity monitored in the T-format in the SLM spectrofluorimeter. Excess (100 μM) GDP, was then added and a decrease in anisotropy and intensity was observed. Shown in figure 3.5a are the intensity decreases observed after addition of GDP for each CmSosl concentration and these are compared with the displacement of mantGDP from N-Ras by GDP in the absence of CmSosl. The intensity decreases were fitted to a single exponential function and the observed rate constants were plotted against CmSosl concentration (figure 3.5b). At a CmSosl concentration of 30 μM, an observed rate constant of $3.8 \pm 0.1 \times 10^2 \text{s}^{-1}$ was observed, a 420 fold stimulation of the rate of mantGDP release over the intrinsic rate, and this observed rate constant was linear with CmSosl concentration (figure 3.5b). A fit to a straight line gave a gradient of $1.1 \times 10^{-3} \text{μM} \text{s}^{-1}$ and an intercept of $5.7 \times 10^{-3} \text{s}^{-1}$. 

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The dissociation of mantGDP from N-Ras catalysed by CmSos1 in the presence of excess GDP

A To a solution of 0.5 μM N-Ras.mantGDP and 0 μM (black), 2 μM (green), 10 μM (red) and 30 μM (blue) CmSos1 was added GDP to a final concentration of 100 μM and the decrease in fluorescence intensity monitored with time (GDP added at time 0). The experiments were performed in buffer B at 30°C and measurements recorded in the T-format in the SLM spectrofluorimeter.

B Data as shown in figure 4.2a were fitted to a single exponential function. The observed rate constants from these fits are plotted against CmSos1 concentration. It can be seen that there is a linear relationship up to 30 μM CmSos1. The solid line is a best fit to a straight line with a slope of $1.1 \times 10^3 \text{μM}^{-1}\text{s}^{-1}$ and intercept of $5.7 \times 10^3 \text{s}^{-1}$. 
Figure 3.5

A

B

$|\text{CmSos1}|$ (µM)

$k_{\text{obs}}$ (s$^{-1}$)

Time (sec)

Relative Intensity

0 200 400 600 800

0.01 0.02 0.03 0.04

0 10 20 30
As can be seen from figure 3.5a, the first 10–20 seconds of the reaction is effectively lost (dashed line for each trace) due to manual mixing time limitations. Any transient intermediates in the reaction are likely to occur within this time scale (such as the formation of the N-Ras.mantGDP.CmSos1 ternary complex). If these were to be observed the reaction needed to be carried out in a stopped-flow apparatus.

3.3.3: The association of mantGDP to CmSos1

As shown above (figure 3.3), on addition of 10 μM CmSos1 to 0.5 μM N-Ras.mantGDP, fluorescence anisotropy increased from 0.188 to 0.208. On addition of excess (100 μM) GDP, the anisotropy decreased from 0.208 to 0.068 over 500 seconds. The anisotropy value would be expected to reach that of free mantGDP (0.027) once all the mantGDP had been released from N-Ras, as was observed with the release of mantGDP from N-Ras.mantGDP by displacement with GDP in the absence of CmSos1 (figure 3.3a). This is obviously not the case in the presence of 10 μM CmSos1 as stated above. The endpoint anisotropy not decreasing to that of free mantGDP could be due to two possibilities:

1 complete release of mantGDP from N-Ras.mantGDP not occurring, some mantGDP remaining bound to N-Ras or bound in the ternary complex N-Ras.mantGDP.CmSos1; or
2 a non-specific interaction between mantGDP and the CmSos1 protein (or contaminant in the protein preparations)

To establish whether this increase in the endpoint anisotropy was due to an interaction between CmSos1 and mantGDP, a titration of CmSos1 into mantGDP was performed.
MantGDP (0.5 μM) was incubated in buffer B at 30°C into which 0.25-20 μM CmSos1 was titrated and the anisotropy value measured at each concentration point (measurements recorded in the T-format in the SLM spectrofluorimeter). The time course of this titration is shown in figure 3.6a. Anisotropy increased from 0.029 (free mantGDP) to 0.086 after addition of 20 μM CmSos1. Overlaid on figure 3.6a is the data from figure 3.4a (red). This demonstrates the endpoint anisotropy after the addition of 10 μM CmSos1 and 100 μM GDP to 0.5 μM N-Ras.mantGDP corresponds to the anisotropy after addition of 10 μM CmSos1 to 0.5 μM mantGDP. Therefore, the endpoint anisotropy value, after displacement of mantGDP from N-Ras.mantGDP by excess GDP in the presence of CmSos1, is due to an interaction of mantGDP with CmSos1. This is likely to be via a hydrophobic interaction with the mant moiety rather than the nucleotide because the same interaction occurs between mantGDP and CmSos1 in the presence of a 200 fold excess of GDP over mantGDP as in the absence of GDP. This interaction also caused an increase in fluorescence intensity, a 10% increase in intensity of the fluorophore observed on addition of 20 μM CmSos1 to 0.5 μM mantGDP (data not shown).

Figure 3.6b shows a plot of the average anisotropy value at each CmSos1 concentration of the titration into 0.5 μM mantGDP as described above. The solid line is a fit to an anisotropy binding curve as described in section 3.2.1. In order to fit this data the ratio of the intensities of the two species (mantGDP and mantGDP.CmSos1) is required. As the fractional intensity of mantGDP was not known, but was greater than that of free mantGDP (10% increase observed on addition of 20 μM CmSos1 but not a saturating value), fits to the data were performed using a Q value of 1.1, 1.2, 1.3,
A CmSos1 (0.25-20 μM) was titrated into 0.5 μM mantGDP in buffer B at 30°C and followed in the T-format with the SLM spectrofluorimeter (blue). It can be seen that the anisotropy increases with each addition of CmSos1. Also shown is data from figure 3.4a (red), showing the anisotropy observed on addition of 10 μM CmSos1 to 0.5 μM N-Ras.mantGDP followed by the addition of 100 μM GDP. This enables comparison of the endpoint anisotropy of this reaction to the anisotropy after the addition of 10 μM CmSos1 to 0.5 μM mantGDP.

B The anisotropy after addition of CmSos1 (0-20 μM) to 0.5 μM mantGDP was plotted for each CmSos1 concentration (average anisotropy value calculated from the data recorded with time shown in the above figure (blue)). The solid line is a fit to an anisotropy binding equation as described in section 3.2. This fit is with a Q value of 1.3 (ratio of the fractional intensities of mantGDP.CmSos1 and mantGDP, described in the text) giving a K_d of 21.7 ± 6 μM and a maximum anisotropy of 0.13 ± 0.015. The average of fits using Q values of 1.1, 1.2, 1.3, 1.4 and 1.5 gives a similar value.
Figure 3.6

A

![Graph A showing anisotropy over time with CmSos concentration and GDP interaction](image)

B

![Graph B showing anisotropy vs. CmSos concentration](image)
1.4 and 1.5. A fit of the data with Q as a variable gave a similar fit with Q value of 1.29. The dissociation constants obtained from these fits were averaged to give a $K_d$ of $21.7 \pm 6 \mu M$ and an anisotropy of $0.13 \pm 0.015$ was obtained for the mantGDP.CmSosl species.

The interaction between the small hydrophobic mant group and hydrophobic patches on the CmSosl protein or a contaminant in the CmSosl preparation needs to be accounted for in future fluorescence anisotropy and intensity calculations.

3.4: Conclusions

A protein of the proposed catalytic domain of mSosl has been purified and shown to be active in promoting the rate of release of GDP from N-Ras. Due to the availability of small amounts of CmSosl protein, only preliminary experiments have been performed. The weak ternary complex, CmSosl.N-Ras.mantGDP, has been observed on titration of CmSosl into N-Ras.mantGDP ($K_d$ of 13.5 $\mu M$) with apparently no release of nucleotide from N-Ras. Due to the weak nature of this reaction and the low expression levels of the CmSosl protein, one titration needed all the CmSosl protein from one purification procedure from a six-litre preparation of E.coli.

The catalysis of the displacement of mantGDP from N-Ras.mantGDP by CmSosl (in the presence of excess unlabelled GDP) has been studied. A saturating value for this process could not be achieved due to the low expression levels of the CmSosl protein. The mant moiety has been shown to associate to the CmSosl protein or a contaminant in the CmSosl preparation with a $K_d$ of $21.7 \pm 6 \mu M$.
It has been possible to study the formation of the N-Ras.mantGDP.CmSosl ternary complex and the subsequent release of nucleotide. However, in order for the reaction to be studied in greater detail (to achieve accurate rate and equilibrium constants) and to study the reaction over a faster time scale, the expression levels of the CmSosl protein had to be improved.
Chapter 4

High level expression and characterisation of CmSosl protein

As described previously the catalytic domain of mSosl (CmSosl) expressed poorly as a GST fusion protein in *E.coli* enabling purification of less than 1 mg of pure protein from 30g of cells (6 litre culture). Different growth conditions (varying time of induction, IPTG concentration, time before harvesting cells) did not affect the CmSosl expression level. Cloning of CmSosl into the His-tag vector, pRSET-C, was also attempted (by K.Nurmohammed, N.I.M.R.) without greatly increasing expression levels.

As alluded to in the Introduction, *E.coli* show a distinct bias in their utilisation of synonymous codons (that code for the same amino acid). Low-usage codons are clearly avoided in genes encoding abundant proteins (Zhang *et al.*, 1991) and are shown to affect translation rates in *E.coli*. (Sorensen *et al.*, 1989). This bias varies between species, leading to suggestions that mammalian genes may have an inappropriate distribution of codons for high level expression in *E.coli*. This is thought to be due to selection against codons specifying tRNA molecules of low abundance in the cell. The Cmsos1 gene (the region encoding the catalytic domain) contains many codons that are very rarely found in *E.coli*, especially those encoding arginine where 36 out of 38 are coded for by 'bad' *E.coli* codons. It has been shown that insertion of rare codons into homologous genes can increase translation times and lower expression levels (Sorensen *et al.*, 1989, Robinson *et al.*, 1984). More importantly codon optimisation (silent codon changes, replacing codons that are rare
in *E. coli* with those that occur more frequently) has been shown to increase expression levels of proteins (Hale & Thompson, 1998, Rangwala *et al.*, 1992, Williams *et al.*, 1988).

It was decided that the low expression level was likely to be due to the unfavourable codon usage of the CmSosl gene and that this needed to be corrected before increased production of CmSosl protein could be achieved. As there were so many rare *E. coli* codons distributed throughout the CmSosl gene, the whole CmSosl region of the mSosl gene was 'optimised' in collaboration with Dr. E. Carpenter (Protein Structure, N.I.M.R.). To construct the optimised gene, a two step PCR method of 'gene synthesis' was adapted from Stemmer *et al.* (1994) and Hale & Thompson (1998) as described in section 2.6.2. As stated in that section, the optimised gene is assembled after design of overlapping oligonucleotides (42 base pairs in length) for the entire top and bottom strands of the DNA. DNA polymerase builds increasingly longer DNA during a gene synthesis PCR step, before a second PCR step amplifies the desired gene DNA fragment from the mix of elongated DNA. The process of gene synthesis, amplification, cloning into an expression vector and purification of high levels of active CmSosl protein is described in this chapter.

### 4.1: Codon optimisation of the catalytic domain of the mSosl gene

The optimised codon usage of the CmSosl gene designed as described in section 2.6.1 is shown in figure 4.1. Those codons common to CmSosl but very rare in *E. coli*, so not included in the optimised gene, have been highlighted (R.S.C.U value of less than 0.6). It can be seen that the other codons of the optimised CmSosl
Figure 4.1

Codon optimisation of the CmSos1 gene

A figure to show the optimised codon distribution in the CmSos1 gene created using the programme ‘CODOP’ as described in the text. The distribution of codons within the genes of *E.coli.* (E.coli Distbn) is given as the RSCU value (the observed number of codons divided by the number expected if all codons for that amino acid were used equally). To show how the R.S.C.U. value is calculated, the amino acid alanine has been taken as an example in the table below.

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<tr>
<th>Amino acid</th>
<th>Codons</th>
<th>Proportion in <em>E.Coli.</em> (a)</th>
<th>Sum of proportions</th>
<th>Sum / no. of codons (b)</th>
<th><em>E.coli</em> Distribution (RSCU value) (a / b)</th>
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The greater the RSCU value, the greater the usage of that codon in *E.coli.* in relation to the other codons for that amino acid. Those highlighted have an RSCU value of less than 0.6 and are common in the CmSos1 gene. They, therefore, could be a cause of poor expression.
### Chapter 4  
**High level expression and characterisation of CmSos1 protein**

#### Figure 4.1

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Codon</th>
<th>E. Coli Distbn.</th>
<th>No. in CmSos1</th>
<th>Optimised Gene</th>
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gene have been given an 'E.coli distribution'. The 74 oligonucleotides (appendix 1) had annealing temperatures between 58°C and 68°C and none showed any sign of mismatch at these temperatures. There were no strings of only adenosine or thymine greater than 6 bases in length and no long runs of the same codon were included (the position of some codons being manually changed). A single Kpn1 site was present a third of the way through the gene (base 502), so no additional restriction sites needed to be added for removal of mistakes in the synthesis of the gene.

Oligonucleotides were designed to create smaller fragments of the putative catalytic domain by PCR amplification (QE, QK, AE, AK, RE, and RK) (figure 4.2). This was performed both in order to find a minimal protein domain required for activity and to increase the chances of crystal formation for X-ray crystallographic purposes. The reasons for choosing these fragments were as follows. Fragment QE is the same length as CmSosl, which has been shown to soluble and active, catalysing the release of mantGDP from N-Ras.mantGDP in the previous section. The smallest fragment, RK, contains the most conserved regions of cdc25 homology exchange factors. The C-terminus of RasGRF (cdc25^Mm), a GEF closely related to Sos, ends in alignment with the residues HPK of mSosl, suggesting that any residues after this would not be required for activity. The fragment AK is equivalent to the minimal domain required for in vivo activity and in vitro activity for RasGRF (Jacquet et al., 1994, Coccetti et al., 1995).

4.2: PCR synthesis and amplification of the optimised CmSosl gene fragments

Having designed the oligonucleotides, the gene synthesis and amplification of the optimised CmSosl gene was performed as described in section 2.6.2. The optimised
Chapter 4  High level expression and characterisation of CmSos1 protein

Figure 4.2

Fragments of the CmSos1 protein

A schematic representation (not to scale) of the mSos1 protein to show the fragments it was decided to attempt to express and purify. At the top is the full length mSos1 protein, the domains are highlighted:

- Dbl homology (DH)
- Cdc25 homology
- Pleckstrin homology (PH)
- SH3

Within the cdc25 homology domain two other regions have been highlighted:

- 3 regions of high conservation among cdc25 homology proteins,
- Conserved region found only in RasGEFs.

Fragments

1. QE (residues 577-1076)
2. AE (residues 613-1076)
3. RE (residues 883-1076)
4. QK (residues 577-1044)
5. AK (residues 613-1044)
6. RK (residues 883-1044)
Chapter 4  High level expression and characterisation of CmSos1 protein

Figure 4.2

![Diagram showing sequence of amino acids and phosphorylation sites.]

1. $\rightarrow$ Q [Sequence representation with phosphorylation sites indicated.]
2. $\rightarrow$ A [Sequence representation with phosphorylation sites indicated.]
3. $\rightarrow$ R [Sequence representation with phosphorylation sites indicated.]
4. $\rightarrow$ Q [Sequence representation with phosphorylation sites indicated.]
5. $\rightarrow$ A [Sequence representation with phosphorylation sites indicated.]
6. $\rightarrow$ R [Sequence representation with phosphorylation sites indicated.]
DNA fragments were obtained consistently using the protocol described. The results of each PCR step are described in this section.

4.2.1: Step 1 - Gene synthesis

A successful gene synthesis reaction was shown by DNA streaking occurring on a 1% agarose gel either side of desired fragment size as a mix of DNA lengths were created. Streaking of DNA only above or below the expected size of the DNA fragment desired, suggested a failed synthesis reaction. Initial failures were due to not allowing Pfu time to synthesise long enough fragments (500 phosphodiester bonds per minute). Once this was corrected, the conditions required for successful synthesis were achieved (optimum conditions are described in the methods section). MgCl₂, KCl and other additives were not required. Figure 4.3a shows the analysis of synthesis reactions, using conditions as described in section 2.6.2, varying the concentration of the oligonucleotide mix from 1-4 μM. It can be seen that at each oligonucleotide concentration, DNA streaking occurred either side of the expected size (the total length of the optimised CmSosl gene fragment is 1557 base pairs) suggesting successful gene synthesis. However, amplification of DNA fragments from these gene synthesis mixes was not always possible. The amplification reactions are discussed below.

4.2.2: Step 2 - Gene Amplification

Figure 4.3a shows the amplification of the QE fragment (1557 base pairs) from the gene synthesis reactions at 1-4 μM oligonucleotide concentrations as described above. The PCR amplification procedure was performed exactly as section 2.6.2. It can be seen that amplification of the QE fragment was only achieved from the gene synthesis
Figure 4.3

Agarose gel analysis of the gene synthesis and amplification reactions

A  Agarose gel electrophoresis of four different gene synthesis reactions and attempted amplification of the QE gene fragment from each synthesis mix. Synthesis reactions were performed as in section 2.6.2, except that the concentration of oligonucleotide was varied from 1-4 μM. Amplification reactions were performed using oligonucleotides 1 and 80 (appendix 2) to recover fragment QE as described in section 2.6.2. 10μl of the synthesis mix (S) and amplification mix (A) were run on a 1% agarose gel. It can be seen that a 1 μM oligonucleotide concentration gave good recovery of the desired QE DNA fragment (1557 base pairs) after the amplification reaction. Higher oligonucleotide concentrations were unsuccessful.

B Amplification of all the optimised CmSos1 DNA fragments visualised by agarose gel electrophoresis. Gene synthesis and amplification was performed as described (section 2.6.2). 10μl of the synthesis reaction (S) and amplification mixes (A1-6) were run on a 1% agarose gel. Amplification reactions were as follows;

<table>
<thead>
<tr>
<th>Amplification Reaction</th>
<th>Fragment recovered</th>
<th>Oligonucleotides used (appendix 2)</th>
<th>Length of DNA fragment (base pairs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>QE</td>
<td>1 &amp; 80</td>
<td>1557</td>
</tr>
<tr>
<td>A2</td>
<td>QK</td>
<td>1 &amp; 81</td>
<td>1446</td>
</tr>
<tr>
<td>A3</td>
<td>AE</td>
<td>76 &amp; 80</td>
<td>1329</td>
</tr>
<tr>
<td>A4</td>
<td>AK</td>
<td>76 &amp; 81</td>
<td>1218</td>
</tr>
<tr>
<td>A5</td>
<td>RE</td>
<td>77 &amp; 80</td>
<td>927</td>
</tr>
<tr>
<td>A6</td>
<td>RK</td>
<td>77 &amp; 81</td>
<td>816</td>
</tr>
</tbody>
</table>
Figure 4.3

(A) Schematic representation of the experimental setup for the study of CmSos1 protein expression and characterisation. The figure shows the effect of different concentrations of 1μM, 2μM, 3μM, and 4μM on the expression levels, with markers (MkS) at various points (S and A). The QE fragment is indicated, and the bands at 1636 and 1018 are highlighted.

(B) Further analysis with different concentrations A1, A2, A3, A4, A5, and A6. The bands at 2036, 1636, 1018, and 517 are noted in this section.
mix created using a final oligonucleotide concentration of 1 μM. PCR amplification of the different sized CmSosl fragments (QE, QK, AE, AK, RE and RK) from the gene synthesis mix was very reproducible using the conditions described in section 2.6.2 (shown in figure 4.3b). The amplified fragments were purified for ligation into expression vectors.

4.3: Cloning of the optimised CmSosl DNA fragments into the pGEX-2T expression vector.

The amplified DNA fragments were cloned into vectors for protein expression in *E. coli*. It was planned to cut the fragments with the appropriate restriction enzymes (EcoR1 and BamH1 for ligation into pGEX-2T) and ligate directly into the cut vector. However, this was not successful, presumably due to the restriction enzymes not cutting the amplified fragments, despite adding the required number of additional bases to each end of the DNA (Boeringer Mannheim manual). Therefore the purified fragments were blunt-end ligated into the shuttle vector pUC18. Figure 4.4 shows the cloning of DNA fragment QE into pGEX-2T via the shuttle vector pUC18. Mini-prepped pUC18 constructs were numbered (for example QE 1-12) as incorrect fragments could have been cloned and different inserts of the correct size could contain mutations from PCR infidelity. Each construct was cut with EcoR1 and BamH1 and the correctly sized inserts gel purified before ligation into a pGEX-2T vector. Positive pGEX-2T constructs were identified by small-scale restriction tests with BamH1 and EcoR1 to identify those containing the correct sized inserts.
Figure 4.4

The cloning of gene fragment QE into the pGEX-2T vector

A  Agarose gel electrophoresis of small scale restriction digests of pUC18 constructs (QE 1-12) with BamHI and EcoRI. Positive clones, containing the QE insert (seen by a band just below the 1636 base pair marker), have been highlighted (QE 1, 3, 4, 8 and 11). These inserts were cut out of pUC18 and ligated into pGEX2T.

B  Agarose gel electrophoresis of small scale restriction digests of pGEX2T constructs with BamHI and EcoRI, to show that the clones did contain the QE insert and were not just re-linearised vector. Two colonies were miniprepped from each ligation plate (no transformed colonies were obtained from the ligation of insert QE 11 into pGEX2T). As can be seen, each miniprepped plasmid contained the QE fragment. Expression and solubility tests were performed on these clones.
Figure 4.4

A

<table>
<thead>
<tr>
<th>Markers</th>
<th>QE 1</th>
<th>QE 2</th>
<th>QE 3</th>
<th>QE 4</th>
<th>QE 5</th>
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<th>QE 8</th>
<th>QE 9</th>
<th>QE 10</th>
<th>QE 11</th>
<th>QE 12</th>
</tr>
</thead>
</table>

3054 →
2036 →
1636 →
1018 →
pUC18
QE

B

<table>
<thead>
<tr>
<th>Markers</th>
<th>QE 1</th>
<th>QE 3</th>
<th>QE 4</th>
<th>QE 8</th>
</tr>
</thead>
</table>

5000 →
1636 →
pGEX2T
QE
Chapter 4  
High level expression and characterisation of CmSos1 protein

Constructs containing the correct sized insert were obtained for QE, AE, RE and RK fragments. However, these did not necessarily have the right sequence, due to possible errors in oligonucleotide synthesis or because of Pfu polymerase infidelity. Constructs were tested for protein expression and activity before sequencing, to reduce the amount of sequencing required.

4.4: Expression of protein, solubility and activity testing

Small-scale expression tests were performed on each positive construct obtained as described in section 2.6.5. It was found that 50 to 75% of the clones obtained, and tested for protein expression upon induction with ITPG in this way, expressed protein of the expected size by SDS-PAGE analysis of whole cell extracts (figure 4.5). This expression was significantly higher than from the original CmSos1 clone, including the fragment of the same size (QE) (figure 4.5c). Small-scale solubility tests showed, however, that all the protein fragments expressed were highly insoluble (figure 4.5c).

In an attempt to reduce the problem of expression of insoluble protein, the temperature of cell growth on induction with IPTG was decreased from 37°C to 28°C. This produced some soluble material on induction of protein expression using AE clones 4, 9 and 11, although approximately 90-95% of the protein was still found in the insoluble fraction. Therefore, a large-scale purification of protein from clone AE 11 was performed. A four-litre preparation of AE 11 expressing BL21 cells and purification using a glutathione sepharose matrix (as described for CmSos1 in section 2.1.2) yielded 20mg of thrombin cleaved soluble protein. This protein was tested for exchange activity on N-Ras.mantGDP. On addition of 5 μM AE 11 to 0.5 μM N-Ras.mantGDP in the presence of excess GDP (as described in section 3.3.2), no
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Figure 4.5

Protein expression and solubility testing

A  Testing AE protein expression (GST-fusion) from the pGEX-2T AE clones shown to contain the correct sized insert. Whole cell extracts before induction with IPTG (-) and three hours after induction (+) were analysed by SDS-PAGE on a 15% gel. It can be seen that for three of the six clones, protein of approximately 70 kDa was expressed (AE 4, AE 9 and AE 11). The pGEX-2T positive expression control (2T control) produces GST protein on induction by IPTG.

B  A SDS-PAGE gel as figure 4.5a, but showing expression of RE and RH protein. Three of the four RE clones (RE 1, RE 7 and RE 9), expressed protein of approximately 50 kDa. RK 3, the only RK clone obtained containing an insert of the correct size, caused overproduction of protein of approximately 45 kDa in size.

C  Small-scale QE protein solubility tests were performed from two pGEX-2T QE clones found to express protein of approximately 80 kDa (QE 4 and QE 8). Whole cell extracts (before (-) and after (+) addition of IPTG) and samples from the supernatant (soluble) and pellet (insoluble) after cell lysis were run on a 15% gel as described in section 2.6.5. The comparison of protein solubility after growth temperatures of 37 and 28°C is shown. It can be seen that the protein is found in the pellet. It appears as if some soluble QE 4 protein is present in the supernatant after growth at 28°C, but this was not a reproducible result.
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Figure 4.5

A

2T
control
AE 1
AE 3
AE 4
AE 9
AE 10
AE 11

M&rs
- + - + - + - + - +

AE protein

B

RE 1
RE 5
RE 7
RE 9
RK 3

+ - + - + - +

RE RK

C

QE 4, 37°C
QE 4, 28°C
QE 8, 37°C
QE 8, 28°C

M&rs
- + S P
- + S P
- + S P
- + S P

QE
catalysis of the release of mantGDP was observed by AE 11. DNA sequencing revealed that the sequence was correct and analysis by circular dichroism (S. Martin, N.I.M.R.) suggested similar folding to the active CmSosl fragment, being approximately 40% helical (data not shown). A double band was observed by SDS-PAGE suggesting the protein is susceptible to proteolysis (data not shown).

No soluble protein could be purified using the clone RK3, the smallest fragment containing the most conserved residues of CDC25 like exchange factors. Despite very high expression levels, the protein was found to be highly insoluble. DNA sequencing (Cambridge Biosciences) confirmed the correct DNA sequence.

Soluble protein was also recovered from the clone QE 4 (1.5mg from a 4 litre culture, as described for AE 11 above). As for AE 11 no catalysis of mantGDP release from N-Ras.mantGDP was observed (data not shown). However, DNA sequencing showed a base deletion at position 1349 (a string of only four adenine bases as opposed to five) in the gene, showing that the last 52 amino acids were incorrect. To resolve this, the engineered Kpn1 site at position 502 of the synthesised gene was used (section 4.1). The pGEX-2T constructs AE 11 and QE 4 were cut with EcoR1 and Kpn1. The DNA fragment coding for the correct 3' terminus of AE 11 was ligated to the correct 5' sequence of the QE 4 construct (in the pGEX-2T vector) to leave a pGEX-2T-QE construct of correct sequence, named QEc (figure 4.6a). This construct was sequenced and the mutation was found to have been corrected (figure 4.6b and 4.6c).
Deletion correction and sequencing of the QE clone

A Representation of the correction of the single base deletion found in clone QE 4 (see sequencing figure 4.6b below). QE 4 and AE 11 (insert regions of the pGEX-2T constructs are coloured) were cut with EcoRI and Kpn1. The fragment of correct sequence from AE 11 was ligated into the QE 4-pGEX-2T fragment creating the construct QEc. This was shown to contain an insert of the correct sequence (4.6b and c).

B Sequencing of the QE 4 insert was performed on an ABI prism automated sequencer. Eight reactions were analysed using oligonucleotides templates to cover the top and bottom strand. The oligonucleotides used were the pGEX 2T 5' and 3' primers (Pharmacia) and 9,18,27,48,57 and 66 (Appendix 1). The elution profile shown is from oligonucleotide 27 to highlight the only deviation from the expected sequence, an adenine deletion at base position 1349.

C Identical primers were used to sequence the QEc clone. This sequencing was performed by Cambridge Biosciences. Again the elution profile shown is from the reaction using oligonucleotide 27 as the primer. It can clearly be seen that the deletion has been corrected. The rest of the insert was also of the correct sequence.
Chapter 4  High level expression and characterisation of CmSos1 protein

Figure 4.6

A

<table>
<thead>
<tr>
<th></th>
<th>Kpn1</th>
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<td></td>
<td>X</td>
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</tr>
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<tr>
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<td></td>
</tr>
</tbody>
</table>

B

```
1349
GATGGAAAAAGAGTT
```

C

```
1349
GATGGAAAAAGAGTT
```
4.5: Purification of active CmSos1 protein from the QEc clone

E. coli BL 21 cells were transformed with the QEcl clone and a four-litre culture was grown to an OD$_{600nm}$ of 0.8 at 37°C before induction with 1mM IPTG. The temperature was lowered to 28°C and cells were harvested after 3 hours. The purification was performed as previously described for CmSos1 (section 2.1.2) except that buffer A also contained 150 mM NaCl. Approximately 50% of the GST-fusion protein was found in the soluble fraction. Cleavage of the fusion protein from the glutathione sepharose column with thrombin yielded 120 mg of protein after concentration and dialysis. SDS PAGE analysis of stages of the purification is shown in figure 4.7. It can be seen that the CmSos1 protein was >95% pure. The protein was dialysed into 20 mM Tris.HCl pH 7.5, 50 mM NaCl, 2 mM MgCl$_2$, and 1 mM DTT. After dialysis for approximately 1 hour the solution in the dialysis cassette had become very cloudy due to the protein precipitating. On dialysis back into the above buffer plus 100 mM NaCl, the solution turned clear as the protein went back into solution. Therefore, the solubility of the CmSos1 protein is salt dependant at high concentrations (> 3 mg.ml$^{-1}$).

A mantGDP release assay was performed to test for nucleotide exchange activity on N-Ras. QEcl protein (2 μM) was added to 0.5 μM N-Ras.mantGDP, incubated buffer A at 30°C, in the presence of excess (100 μM) GDP as in section 3.3.1. On addition of QEcl to N-Ras.mantGDP (and excess GDP) the fluorescence intensity decreased exponentially as mantGDP was released. Fitting of this data to a single exponential gave an observed rate constant of 2 x 10$^3$s$^{-1}$ (data not shown). This compares with the observed rate constants calculated with the original CmSos1 protein (section 3.3.1). Therefore, the disruption of the last 60 amino acids of QE 4 protein appears to have
Figure 4.7

*SDS-PAGE analysis of the purification of CmSos1*

SDS-PAGE analysis of the elution of CmSos1 from the glutathione sepharose column upon cleavage of the fusion protein GST-CmSos1 with thrombin. Pre- and post-induction samples were prepared as described previously. 2 μl of each fraction of the thrombin wash were prepared in sample buffer (as section 2.2.3) and analysed on a 15% gel.
Figure 4.7

![Diagram showing Thrombin wash fractions with markers (Mkrs), Pre Ind, Post Ind, Pellet, and Sup. The fractions are labeled 2, 4, 6, 8, 10, 12, 14, and 16. The diagram also highlights GST-QEc and QEc proteins.](image)
been the cause of the observed insolubility and lack of exchange activity. Correction of the mutation in the QE 4 construct led to the purification of 120 mg of soluble, active protein.

Analysis of the purified protein by gel filtration revealed a small peak eluted in the void volume of the column. This was shown to be predominantly CmSosl protein by SDS-PAGE, suggesting a small amount of aggregated protein was present after purification. Therefore, an additional gel filtration step was added to the purification protocol. Protein pooled from the glutathione sepharose column was concentrated to approximately 30 mg.ml\(^{-1}\) and stored at \(-80^\circ\text{C}\) in 1-1.2ml aliquots. Each aliquot was passed down an S-75 Gel filtration column (HiLoad 26/60, Amersham Pharmacia) equilibrated with 20 mM Tris.HCl pH7.5, 100 mM NaCl, 2 mM MgCl\(_2\) and 1 mM DTT (buffer A) at 1 ml.min\(^{-1}\) collecting fractions every 3 minutes. The elution profile is shown in figure 4.8. The CmSosl peak was pooled and concentrated to approximately 30 mg.ml\(^{-1}\) as before and stored at \(-80^\circ\text{C}\) in 50 µl aliquots.

4.5.1: Characterisation of the CmSosl protein

From SDS PAGE analysis (shown in figure 4.7), it can be seen that the CmSosl protein is >95 % pure. Mass spectrometry gave a molecular weight of 58898 kD (theoretical calculated to be 58894.4) (figure 4.9) confirming no proteolysis had occurred and together with the fact that the DNA sequence was correct it could be said that the purified CmSosl was the correct amino acid sequence.
Figure 4.8

_Preparative gel filtration of CmSos1 protein_

Elution profile, recording absorbance at 280 nm, of the CmSos1 protein from the S-75 gel filtration column as described in the text. The flow rate was set at 1 ml.min\(^{-1}\) and fractions were collected every 3 minutes. SDS-PAGE analysis showed that both of these peaks were >95% CmSos1 (data not shown). Analytical gel filtration had shown the first peak eluted in the void of the column suggesting aggregated protein. The second peak was pooled and concentrated.
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High level expression and characterisation of CmSos1 protein

Figure 4.8
Figure 4.9

*Mass spectrometry of the CmSos1 protein*

Mass spectrometry was performed by Dr S. Howell as described in section 2.2.4. A molecular weight of 58898 kD was obtained.
Figure 4.9

CP SOS 22Jul99
97584 188 (3.47% Sn (Mn, 2x3.00); Sb (0.10.00); Cm (111:272)
A58 1016.5
A57 1034.3
A54 1019.6
A51 1123.1
A50 1179.0
A49 1229.9
A48 1254.1
A47 1281.3
A46 1310.0
A45 1370.9
A44 1339.9
A43 1370.9
A42 1403.5
A41 1437.7
A40 1473.8
A39 1511.5
A38 1548.0
A37 1582.6
A36 1617.1
A35 1651.6
A34 1686.1
A33 1720.7
A32 1755.2
A31 1790.8
A30 1825.3
A29 1860.8
A28 1896.4
A27 1931.9
A26 1967.5
A25 2003.0
A24 2038.5
A23 2074.1
A22 2110.6
A21 2146.1
A20 2181.6
A19 2217.1
A18 2252.7
A17 2288.2
A16 2323.7
A15 2359.3
A14 2394.8
A13 2430.4
A12 2465.9
A11 2501.5
A10 2537.0
A9 2572.6
A8 2608.1
A7 2643.7
A6 2679.3
A5 2714.8
A4 2750.4
A3 2786.0
A2 2821.6
A1 2857.2
A0 2892.8
  100 95 90 85 80 75 70 65 60 55 50 45 40 35 30 25 20 15 10 5 0
m/z
700 750 800 850 900 950 1000 1050 1100 1150 1200 1250 1300 1350 1400 1450 1500 1550
Scan E3+
A: 8.96e5
58859.06±5.29
4.5.2: Nucleotide exchange activity of the CmSos1 protein from the high expression clone.

The availability of large quantities of CmSos1 protein enabled the use of fast mixing apparatus to study the interaction with N-Ras in greater detail. These experiments will be discussed in chapters 5, 6 and 7. However, a preliminary experiment performed in the stopped-flow apparatus is described here. N-Ras.mantGDP (0.5 μM) was rapidly mixed with 30 μM CmSos1 in buffer A (20 mM Tris.HCl pH7.5, 100 mM NaCl, 2 mM MgCl₂ and 1 mM DTT) in the presence of 100 μM unlabelled GDP (displacement reaction as section 4.2.2) at 30°C. As expected, a decrease in intensity was observed down towards that of free mantGDP. However, this decrease could be fitted better to a double exponential decay than to a single exponential function (data not shown). This could be due to the mant group reporting two events on the interaction of CmSos1 with N-Ras.mantGDP. Another possibility for the appearance of two processes is the fact that mantGDP exists as an equilibrium mixture of the 2'-0 and 3'-0 isomers in solution as discussed in the Introduction.

It was feasible that the isomers either bind with different affinities to N-Ras or affect the interaction with CmSos1 differently, altering the rate of nucleotide release from N-Ras. It is possible to synthesise the 3’-(N-methylantraniloyl)-2’-deoxy-GDP (mdGDP) isomer as 2’-deoxy-GDP (and 2’-deoxy-GTP) is commercially available. The addition of the methylantraniloyl group to the 3’ OH group of the deoxy-nucleotide was performed as described in section 2.3. Therefore, a way to test this theory was to synthesise mdGDP, form a 1:1 complex with N-Ras as described in section 2.3.1 and compare the interaction of CmSos1 with N-Ras.mantGDP and N-Ras.mdGDP.
4.6: Characterisation of the N-Ras.mdGDP complex and the interaction with CmSos1.

The intrinsic release rate of mdGDP from N-Ras.mdGDP was investigated using the displacement reaction with excess unlabelled GDP as described for the release of mantGDP from N-Ras.mantGDP in section 3.2. N-Ras.mdGDP (0.5 μM) was incubated in buffer A at 30°C and the decrease in fluorescence intensity and monitored after the addition of 100 μM GDP. The intensity decrease could be fitted to a single exponential decay with an observed rate constant of $4.3 \times 10^{-5}s^{-1}$. This compares with rate constants of $7.6 \times 10^{-5}s^{-1}$ and $6.0 \times 10^{-5}s^{-1}$ for N-Ras.mantGDP and N-Ras.[3H]GDP respectively.

On addition of 30 μM CmSos1 to 0.5μM N-Ras.mdGDP in the presence of 100 μM unlabelled GDP, a decrease in fluorescence intensity was observed that could be fitted well to a single exponential function. This is compared with the interaction with N-Ras.mantGDP in figure 4.10a. The residuals to single exponential fits are compared in figure 4.10b. This shows that the two isomers behave slightly differently for the interaction of CmSos1 with N-Ras.mantGDP. These observed rate constants were within a factor of two of each other ($4.8 \times 10^{-2}s^{-1}$ for N-Ras.mantGDP and $2.7 \times 10^{-2}s^{-1}$ for N-Ras.mdGDP) but for all future experiments the 2'-deoxy-mant nucleotides (mdGDP and mdGTP) were used.

4.6.1: The interaction of CmSos1 with N-Ras.[3H]GDP

It was also important to show that the fluorescent nucleotide analogue did not greatly effect the interaction of CmSos1 with N-Ras. Therefore, the interaction of CmSos1 with N-Ras.[3H]GDP was studied. The experiment was designed as for the study of
Chapter 4  High level expression and characterisation of CmSos1 protein

Figure 4.10

Comparison of the interaction of CmSos1 with N-Ras.mantGDP and N-Ras.mdGDP

A 0.5µM N-Ras.mantGDP (blue), 0.5µM N-Ras.mdGDP (red) or buffer A (black) were incubated with 30 µM CmSos1 in buffer A at 30°C before being rapidly mixed with 100 µM GDP in the stopped-flow apparatus. Fluorescence intensity was measured in the L-format. All traces are an average of 3 pushes.

B The data from the mixing of N-Ras.mantGDP and N-Ras.mdGDP with CmSos1 shown in figure 4.10a (above) were fitted to single exponential decays. The residuals of both fits are shown here. It can be seen that the interaction of N-Ras.mdGDP (red) with CmSos1 shows little deviation from a fit to a single exponential whereas the intensity decrease from the interaction of N-Ras.mantGDP (blue) with CmSos1 shows significant deviation.
Figure 4.10

A

![Graph A: Relative Intensity vs. Time (sec)]

B

![Graph B: Residuals vs. Time (sec)]
the intrinsic dissociation rate of $[^3\text{H}]\text{GDP}$ from N-Ras.$[^3\text{H}]\text{GDP}$ described in section 3.2, except that 5 μM CmSosl was added to 0.5 μM N-Ras.$[^3\text{H}]\text{GDP}$ in the presence of 100 μM GDP (buffer A 30°C). The experiment was performed in duplicate and the results shown in figure 4.11. A fit to a single exponential function gave an observed rate constant of $5.8 \pm 0.1 \times 10^{-3} \text{ s}^{-1}$. This compares well with the observed rate constant obtained from the decrease in intensity on addition of 5μM CmSosl to 0.5 μM N-Ras.mdGDP under the same conditions of $5.1 \times 10^{-3} \text{ s}^{-1}$ (Chapter 5, section 5.2).

4.6.2: The interaction of CmSosl with mdGDP

Having shown mantGDP non-specifically bound to CmSosl (or to contamination in the protein preparation, section 3.3.3), it was necessary to investigate whether this was also the case with the new CmSosl preparation and mdGDP. CmSosl (5 to 35 μM) was titrated into 0.5 μM mdGDP in buffer A at 30°C. Fluorescence measurements were recorded in the T-format in the SLM spectrofluorimeter and anisotropy values calculated at each CmSosl concentration. The result is shown in figure 4.12. It can be seen that the anisotropy value increases linearly from 0.027 to 0.046 in the presence of 0 to 35 μM CmSosl. A similar result is observed on titration of CmSosl into mantGDP (figure 4.12). The difference from the result obtained in section 3.3.3 using the CmSosl protein obtained from the low expression clone (highlighted in figure 4.12) is most likely to be due to the increased NaCl concentration in buffer A (100 mM NaCl) as compared to buffer B (none).
Figure 4.11

The interaction of CmSos1 with N-Ras.\textsuperscript{[^3]H}GDP

N-Ras.\textsuperscript{[^3]H}GDP (0.5 μM) was incubated with 5 μM CmSos1 in buffer A at 30°C. 100 μM GDP was added and aliquots removed to be analysed using filter binding assays as described in section 2.5.1. The counts per minute (cpm) recorded at time zero (before GDP was added) was taken as 100% GDP bound. The solid line is the best fit to a single exponential function giving a rate constant of $5.8 \times 10^{-3}$ s\(^{-1}\).
Figure 4.11
Figure 4.12

The interaction of CmSos1 with mdGDP

CmSos1 (0.5 μM to 35 μM) was added to 0.5 μM mdGDP (○) or mantGDP (●) in buffer A at 30°C and fluorescence anisotropy followed in the T-format on the SLM spectrofluorimeter. It can be seen that there is an interaction between CmSos1 and mdGDP and mantGDP but it is significantly lower than previously observed with the CmSos1 from the low expression clone in buffer B (□, data as shown figure 3.6b)
Figure 4.12

![Graph showing CmSos1 concentration (µM) vs. Anisotropy.](image)

CmSos1 concentration (µM)

Anisotropy
4.7: Conclusions

The yield of active CmSos1 protein has been increased from only 0.25-0.5 mg.litre\(^{-1}\) of cells to 30 mg.litre\(^{-1}\). This exceptional increase resulted from the reconstruction of the CmSos1 gene omitting codons that are never found in highly expressed native *E.coli* proteins and giving other codons an "*E.coli* distribution". The process, synthesis and amplification of the optimised gene, has been shown to be both accurate and reproducible.

Attempts to create smaller protein fragments have proved unsuccessful as, despite good expression being achieved for AE, RE and RH fragments, the protein was highly insoluble. AE 11 and RH 3 clones were shown to be the correct DNA sequence, but the protein produced was insoluble. The small amounts of soluble AE protein purified, although apparently folded correctly (by circular dichroism), was found not to be active in promoting the release of mantGDP from N-Ras.

The CmSos1 protein has been characterised by SDS-PAGE, mass spectrometry, and gel filtration. The solubility of the domain has been found to be salt dependent, a concentration of 100 mM NaCl being required to keep the protein in solution. The interaction of CmSos1 with N-Ras.mantGDP was shown to be different with each isomer and therefore a complex of N-Ras with the 2'deoxy-3'methlyanthraniloyl-GDP isomer (mdGDP) was characterised. All subsequent experiments described will be performed with N-Ras.mdGDP in the presence of 100 mM NaCl. The fluorescent analogue mdGDP has been shown to interact with CmSos1. This has been shown to be a weak interaction in high salt buffer that could need to be considered in future experiments. The catalysis of the release of \(^{3}\text{H}\)GDP from N-Ras.\(^{3}\text{H}\)GDP by
CmSos1 has been shown to be comparable to the catalysis of the release of mdGDP from N-Ras mdGDP, suggesting that the fluorescent analogue does not interfere with CmSos1 action.

These achievements enabled a detailed kinetic and equilibrium analysis of the interaction between the catalytic domain of mSos1 and N-Ras. These observations are described in the following chapters.
The design and synthesis of a high expression CmSos1 clone, leading to the purification of 60-120 fold higher levels of protein to greater than 95% purity made possible a detailed examination of the interaction of CmSos1 with N-Ras. The preliminary kinetic data obtained using the CmSos1 protein from the low expression clone suggested that:

1. the formation of a ternary N-Ras.mantGDP.CmSos1 complex could be followed by fluorescence anisotropy on addition of CmSos1 to N-Ras.mantGDP in the absence of excess unlabelled nucleotide; and

2. the release of mantGDP from the ternary complex could be observed by anisotropy and intensity on addition of excess unlabelled nucleotide.

In chapter 3 it was shown that on addition of CmSos1 to N-Ras.mantGDP an increase in anisotropy was observed. This implied the formation of the ternary N-Ras.mdGDP.CmSos1 complex, with an apparent $K_d$ of 13.5 $\mu$M. However, due to the time limitations of manual mixing and low yields of CmSos1, it was not possible to measure the association rate constant of this process. Therefore, using the 2'deoxy-mantGDP analogue (mdGDP) bound to N-Ras (see section 4.6.1) the kinetics of the formation of this complex was studied using the stopped-flow apparatus.
5.1 Stopped-flow analysis of the interaction between N-Ras.mdGDP and CmSos1

The stopped-flow apparatus was configured in the T-format to enable the determination of both fluorescence intensity and anisotropy changes. A 1:1 complex of N-Ras.mdGDP was prepared in buffer A (20 mM Tris-HCl pH 7.5, 100 mM NaCl, 2 mM MgCl₂ and 1 mM DTT). Buffer A is the same as buffer B that was used for experiments between CmSos1 and N-Ras.mantGDP in chapter 3 but with 100 mM NaCl added. This was necessary due to the insolubility of the CmSos1 in buffer of a lower ionic strength as described in section 4.5. This could weaken the association of CmSos1 with N-Ras.mdGDP, but does have the advantage that the ionic strength of buffer A is closer to physiological conditions (~150 mM) than buffer B.

A solution of 0.5 μM N-Ras.mdGDP was rapidly mixed with buffer A in order to observe the extent of any photobleaching event. The slit widths, controlling the amount of light reaching the sample from the lamp, were reduced until photobleaching of the fluorescence of the mant group was found to be negligible. This also enabled the fluorescence anisotropy and intensity of the N-Ras.mdGDP molecule to be calculated. An anisotropy value of 0.167 for N-Ras.mdGDP was observed. This does vary from that recorded in the SLM spectrofluorimeter (0.188) and is due to slight differences in the alignments of the polarisers on the two instruments.

As an introduction to the experiment, on rapidly mixing N-Ras.mdGDP with CmSos1 there was an immediate increase in anisotropy (figure 5.1a). Recording the data over 50 msec showed that this increase has occurred in the dead time of the instrument.


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Figure 5.1

Anisotropy and intensity changes on rapid mixing of N-Ras.mdGDP and CmSos1

A  N-Ras.mdGDP (0.5 μM) was rapidly mixed with buffer A (black) and 100 μM CmSos1 (blue) and the change in anisotropy over time measured using the Stopped-flow apparatus in the T-format at 30 °C.

B  The data collected in figure 5.1a (above) was converted into fluorescence intensity and plotted against time. The solid line is a fit to a single exponential (plus an offset). The observed rate constant for this fit is $1.29 \pm 0.03 \text{ s}^{-1}$ with an amplitude of $0.023 \pm 0.002$ intensity units (a 2.3 % decrease in intensity). The intensity measurements are shown relative to an intensity of 1 at the beginning of the reaction.
Figure 5.1

A

![Graph A showing anisotropy over time.](image)

B

![Graph B showing relative intensity over time.](image)
The dead time of the apparatus was measured using the reaction of N-acetyl tryptophanamide with N-bromosuccinimide (described by Peterman, 1979) and found to be 6 ms (J.F. Eccleston, N.I.M.R., data not shown). When this anisotropy data was converted to fluorescence intensity a small exponential decrease in intensity could be seen on this time scale. This is shown in figure 5.1b fitted to a single exponential function with an observed rate constant of $1.29 \pm 0.03 \text{s}^{-1}$ and an amplitude of 2%. The effect of CmSos1 concentration on these processes was studied. Increasing concentrations of CmSos1 (5-150 μM) were added to 0.5 μM N-Ras.mdGDP and the fast (anisotropy) and slow (intensity) phases were analysed at each concentration as described in the following sections.

5.1.1: The fast phase – an anisotropy increase.

N-Ras.mdGDP (0.5 μM) was rapidly mixed with CmSos1 (5-150 μM) in buffer A at 30°C. Measurements were recorded in the T-format and converted into fluorescence anisotropy and intensity as described previously. Figure 5.2a shows the anisotropy data recorded for each concentration of CmSos1 mixed with 0.5 μM N-Ras.mdGDP (if any change in fluorescence intensity occurred at this time scale it was very small and could not be distinguished from fluctuations in the lamp intensity). As stated above, the initial reactions were too fast to be measured. It can be seen that anisotropy increases with increasing CmSos1 concentration.

The effect of CmSos1 concentration on the anisotropy immediately after mixing with N-Ras.mdGDP was measured in an attempt to define the $K_d$ of the interaction (figure 5.2b). However, there can be seen to be a linear dependence of anisotropy on CmSos1 concentration (up to 150 mM CmSos1) rather than a hyperbolic dependence.
Chapter 5

The interaction of CmSos1 with N-Ras.mdGDP and N-Ras.mdGTP

Figure 5.2

The rapid anisotropy increase on mixing N-Ras.mdGDP with CmSos1.

A Data from the stopped flow apparatus on mixing 0.5 μM N-Ras.mdGDP with;
Buffer A (—), 10 μM CmSos1 (—), 20 μM CmSos1 ( ), 50 μM CmSos1 ( ),
100 μM CmSos1 ( ) and 150 μM CmSos1 ( ). Data was recorded in the T-
format, and data converted into anisotropy as described in section 2.4. All reactions
were performed in buffer A at 30°C and each trace is an average of 3 or 4 data sets.

B From the data shown in A, the anisotropy of the fluorophore immediately after
mixing of N-Ras.mdGDP and CmSos1 was calculated and plotted against CmSos1
concentration. It can be seen that the dependence of the anisotropy increase on
CmSos1 concentration is linear up to 150 μM.
Figure 5.2

A

B
suggesting that the binding is very weak. The relevance of this observation is discussed in section 5.1.3.

5.1.2: The slow phase – an intensity decrease

As alluded to earlier, on mixing 0.5 μM N-Ras>GDP with CmSos1 (5-150 μM), a slow phase (after an initial fast phase of anisotropy increase) is reported by a decrease in fluorescence intensity. The decrease in intensity observed was very small, however not too small to measure. Shown in figure 5.3a is data recorded in the T-format, converted into intensity data as described in section 2.4, and normalised to a starting intensity of 1. This alteration was made because there were small fluctuations in the starting intensity value due to changes in lamp intensity over the course of the experiment. These intensity decreases (figure 5.3a) were each fitted to a single exponential function. The rate constant and amplitude of this exponential decrease was found to increase with increasing CmSos1 concentration.

As the observed decrease was very small, the experiment was repeated in the L-format recording only intensity measurements. The slow phase was again observed and the observed rate constants and amplitude of the process recorded at each CmSos1 concentration. Figures 5.3b and 5.3c show the dependence on CmSos1 concentration of these processes averaged over three experiments in the L-format (error bars are calculated from the standard deviation over the three experiments). There is a hyperbolic dependence of k<sub>obs</sub> on CmSos1 concentration with a half maximal value at 25 ± 7.9 μM and a limiting rate constant of 1.03 ± 0.06 s<sup>-1</sup>. The amplitude of the signal also displays hyperbolic characteristics with a half-maximal value at 46.9 ± 20.5 μM CmSos1, a limiting value suggesting a maximum of 4.8 ±
Chapter 5  The interaction of CmSos1 with N-Ras.mdGDP and N-Ras.mdGTP

Figure 5.3

The intensity decrease on mixing N-Ras.mdGDP with CmSos1

A  Stopped-flow fluorescence intensity data recorded in the T-Format, on mixing 0.5 μM N-Ras.mdGDP against; buffer A ( ), 10 μM CmSos1 ( ), 50 μM CmSos1( ), 100 μM CmSos1 ( ) and 150 μM CmSos1 ( ) in buffer A at 30°C. The data is shown as relative intensity, normalised to a starting intensity of 1 for each data set.

B  Rate constants obtained from the fitting of the data shown in figure A, above, to a single exponential function are plotted against CmSos1 concentration. The data points are the average of 3 experiments (two recorded in the L-format, 1 in the T-format) and the standard deviation of those points is shown. The solid line is a fit to a hyperbolic function, giving a half maximal value of 25 ± 7.9 μM and a limiting value of 1.03 ± 0.06 s⁻¹ and an intercept of 0.42 ± 0.04 s⁻¹.

C  The amplitudes of the process obtained from fits to single exponential functions as described in figure B, above, are plotted against CmSos1 concentration. The solid line is a fit to a hyperbola with half maximal value at 46.9 ± 20.5 μM CmSos1, a limiting value of 0.048 ± 0.009 units (a 4.8% decrease in intensity) and an intercept of 0.002 ± 0.001 units (0.35%).
Figure 5.3

A

B

C

1

0.98

0.96

0.94

0.92

0.90

0.88

0.86

0.84

0.82

0.80

0.78

0.76

0.74

0.72

0.70

0.68

0.66

0.64

0.62

0.60

0.58

0.56

0.54

0.52

0.50

0.48

0.46

0.44

0.42

0.40

0.38

0.36

0.34

0.32

0.30

0.28

0.26

0.24

0.22

0.20

0.18

0.16

0.14

0.12

0.10

0.08

0.06

0.04

0.02

0.00

0.00

0.04

0.08

0.12

0.16

0.20

0.24

0.28

0.32

0.36

0.40

0.44

0.48

0.52

0.56

0.60

0.64

0.68

0.72

0.76

0.80

0.84

0.88

0.92

0.96

1.00

Time (sec)

[CMC] (μM)

k_{obs} (s^{-1})

Amplitude

[CMC] (μM)
0.9% intensity decrease (a value of 0.048 relative to a starting intensity of 1. There are clearly large errors associated with the amplitude data. The significance of these observations is discussed both qualitatively and quantitatively in the following section.

5.1.3: Significance of the anisotropy and intensity changes

It is obviously essential to assign a reason for these observed changes in anisotropy and intensity. The initial rapid increase in anisotropy is most likely due to the association of CmSos1 to N-Ras.mdGDP. However, it was necessary to prove that this was not just an effect of adding high concentrations of protein, as the observed increase is small. Therefore, negative and positive controls were undertaken.

The negative control was to observe the effect of CmSos1 on proteins to which it should not associate and observe any anisotropy changes. It was decided to observe the interaction of CmSos1 with Rac1.mdGDP (a characterisation of this complex can be found in chapter 8). The Cdc25 homology domain of mSos1 has previously been shown not to be an exchange factor for Rac1 and in this work no evidence has been found for exchange activity on Rac1 by CmSos1 (data not shown). The experiment was repeated exactly as for the interaction with N-Ras.mdGDP. Rac1.mdGDP (0.5 μM) was mixed with increasing concentrations of CmSos1 (5-80 μM) in buffer A at 30°C using the stopped flow apparatus. No change in anisotropy (figure 5.4a) or intensity (data not shown) was observed.

A positive control was performed rapidly mixing CmSos1 (5-80 μM) with 0.5 μM H-Ras.mdGDP as CmSos1 has been reported to act in a similar manner on each of N,K
Figure 5.4  Comparison of the interaction of CmSos1 with N-Ras.mdGDP, H-Ras.mdGDP and Rac1.mdGDP

A  A comparison of the anisotropy increase on mixing of increasing concentrations of CmSos1 with 0.5 μM N-Ras.mdGDP (○), H-Ras.mdGDP (▲) and Rac1.mdGDP (●). All experiments were performed in the Stopped-flow apparatus at 30°C in buffer A. Measurements were made in the T-format. The solid lines are linear fits to the data (the dashed line is a linear fit to the data on mixing H-Ras.mdGDP with CmSos1).

B  The observed rate constants for the slow phase of intensity decrease on mixing of increasing concentrations of CmSos1 with 0.5 μM N-Ras.mdGDP (○) and H-Ras.mdGDP (●) in the stopped flow apparatus (buffer A at 30°C). Measurements were recorded in the T-format and converted into intensity data. The data were fitted to a single exponential function to obtain the observed rate constants used. Both data sets are fitted to a hyperbolic function. The H-Ras.mdGDP data has a limiting value of 0.65 s⁻¹, a half-maximal value of 19.7 μM and an intercept of 0.16 s⁻¹. The fit for the N-Ras.mdGDP data is as described in figure 5.3b (limiting value 1.03 s⁻¹, half maximal value of 25.6 μM and an intercept of 0.42 s⁻¹).

C  The amplitudes of the fits to a single exponential function of the data as described in figure 5.4b, above, are plotted against CmSos1 concentration for the mixing with N-Ras.mdGDP (○) and H-Ras.mdGDP (●) as described above. The N-Ras.mdGDP data is fitted to a hyperbola as described in figure 5.3c, the H-Ras.mdGDP data to a hyperbola with half maximal value of 105 ± 45 μM, maximal value of 0.18 ± 0.05 units (18% decrease) and intercept of 0.009 ± 0.004 units.
Figure 5.4

A

Relative Anisotropy

\[ \text{[CmSos1]} \ (\mu \text{M}) \]

B

\[ k_{\text{obs}} (s^{-1}) \]

\[ \text{[CmSos1]} \ (\mu \text{M}) \]

C

Amplitude

\[ \text{[CmSos1]} \ (\mu \text{M}) \]
and H-Ras proteins. Again a fast anisotropy increase was observed within the dead
time of the instrument followed by a slow decrease in intensity and anisotropy (these
processes are described in comparison with those of N-Ras.mdGDP below). This
suggests that the anisotropy increase is reporting a real interaction and that the effect
is specific for Ras subfamily proteins.

Figure 5.4a shows a comparison of the amplitude of the fast phase of anisotropy
increase on mixing of increasing concentrations of CmSos1 with N-Ras.mdGDP, H-
Ras.mdGDP and Rac1.mdGDP. For H-Ras and N-Ras there was a linear increase in
anisotropy with CmSos1 concentration. However, with Rac1 there was no increase in
anisotropy. This suggests that the increase in anisotropy is due to an interaction
between CmSos1 and N-Ras.mdGDP. A comparison of the intensity decrease
(amplitude and observed rate constant) when fitted to a single exponential function for
N-Ras.mdGDP and H-Ras.mdGDP is shown in figures 5.4b and c. Qualitatively, the
rate constants and amplitudes of the process show the same dependence on CmSos1
concentration, but there are quantitative differences. These differences have not been
investigated in this work due to low yields of the H-Ras protein and time constraints,
but they may shed light on specificity of CmSos1 for different members of the Ras
family proteins for which there is little data in the literature.

The slower phase described by the intensity decrease could be due to one of two
possibilities:

(1) an isomerisation in the ternary complex occurring after the initial binding of
CmSos1, or
(2) release of nucleotide from the ternary complex on association of CmSos1, the quantity released being governed by the relative dissociation constants of CmSos1 and mdGDP for N-Ras.

Release of nucleotide from the ternary complex would be expected to cause a decrease in intensity, but nucleotide release needs to be shown to occur by a different method to prove this hypothesis. In order to investigate whether there was indeed any release of nucleotide on the addition of CmSos1 to N-Ras.GDP, an assay was required to detect directly any free nucleotide produced. One method of achieving this was by equilibrium ultrafiltration. The aim was to add CmSos1 to N-Ras.[\textsuperscript{3}H]GDP and allow the reaction to reach equilibrium ( > 25 seconds from the Stopped-flow data). A small portion of this solution is then spun through a 10 kD centricon concentrator membrane (Amicon). In theory, only free [\textsuperscript{3}H]GDP can pass through the membrane, which can be detected by scintillation counting. The volume of the filtrate that was collected after centrifugation needed to be less than 10 % of the total volume in order that the changes in volume had a minimal effect on the equilibrium.

*Equilibrium ultrafiltration analysis*

There were three major problems with this approach that needed to be accounted for in control experiments;

1. Would [\textsuperscript{3}H]GDP partition across the membrane well
2. Would high concentrations of CmSos1 effect the partitioning of [\textsuperscript{3}H]GDP across the membrane in the absence of N-Ras.[\textsuperscript{3}H]GDP,
Would N-Ras,$[^3]H$GDP stay in the retentate and not pass through or denature on the membrane.

Therefore, three controls were designed to test this. Firstly 1ml of $[^3]H$GDP (50 nM, 0.5 μM and 5 μM in buffer A) was incubated at 30°C for five minutes before being spun in a Centricon 10 concentrator (Amicon) at 2000 g for 1.6 minutes to force 70 μl across the membrane. The radioactivity in 50 μl of this filtrate and 50 μl of the retentate was measured, allowing the partitioning of the $[^3]H$GDP to be calculated. The experiment was performed in duplicate for each concentration. The partitioning was found to be not to be equal between the retentate and filtrate. For 50 nM, filtrate concentrations of $[^3]H$GDP were 79 % of the retentate concentrations, for 0.5 μM it was 80 % and for 5μM, 85 %. Therefore, a similar percentage of the $[^3]H$GDP is retained in the concentrator over a 100 fold range of $[^3]H$GDP concentrations. This needed to be corrected for in final calculations.

Secondly, CmSos1 (10 and 50 μM) was added to 5 μM $[^3]H$GDP and treated in exactly the same way as above. It was found that with 10 μM CmSos1, the concentration of $[^3]H$GDP in the filtrate was 79 % of the retentate concentrations, and with 50 μM CmSos1 this figure decreased to 76 %. High concentrations of CmSos1 also affect the partitioning of GDP across the membrane. During the preparation of CmSos1, there is some loss of protein during concentration with concentrators such as the Centricon 10, so this effect of CmSos1 on the partitioning of $[^3]H$GDP is probably due to a partial blocking of the membrane by CmSos1. To account for the results of both control 1 and 2 it was decided to use the average of the above results. Therefore, all the experimental data was corrected, assuming that the radioactivity in the filtrate
was only 80% of the true value due to the behaviour of GDP and CmSos1 across the membrane.

The third control was to spin N-Ras.[^3H]GDP through the membrane to ensure no[^3H]GDP was detectable in the filtrate. Therefore, 0.5 and 5 µM N-Ras.[^3H]GDP was incubated at 30°C for five minutes before being centrifuged as above in duplicate. For 0.5 µM N-Ras.[^3H]GDP 16 % of the[^3H]GDP was in the filtrate, for 5 µM only 2.4%.

It appeared that the same amount of[^3H]GDP was being released, which at higher concentrations of N-Ras.[^3H]GDP, led to a lower overall percentage. Therefore, 5 µM N-Ras.[^3H]GDP was used for all the experiments with CmSos1 and the 2.4 % release of[^3H]GDP was taken account of in the final analysis. A summary of these controls is found in table 5.1a. A background measurement, from addition of 50 µl of buffer A to scintillant, was also made.

Increasing concentrations of CmSos1 (0.1-50 µM) were added to 5 µM N-Ras.[^3H]GDP in a final volume of 1ml in buffer A and incubated at 30°C for 5 minutes. This mixture was centrifuged at 2000g for 1.6 minutes and then 50 µl of the filtrate added to scintillant and the radioactivity measured. The measurement at each CmSos1 concentration was performed in duplicate. The raw data was then corrected using the control experimental data. For example, the correction of the 50 µM CmSos1 data is shown in table 5.2. The behaviour of GDP, CmSos1 and N-Ras on the membrane makes a true quantitative analysis of the results difficult, but what can be concluded is that on addition of CmSos1 to N-Ras.[^3H]GDP there is a small (~6.5 %) release of nucleotide. Fitting of the data to a hyperbola gives an apparent $K_d$ of 1.8 µM (figure 5.5). Error bars have not been put on the data points as they are hard to
Equilibrium ultrafiltration analysis

Table 5.1 A

Table showing the data for the three controls for the equilibrium ultrafiltration experiments. The counts per minute have had the background count of 22 cpm subtracted.

Table 5.1 B

Table showing the conversion of the raw data to the final percentage of nucleotide release for the addition of 50 μM CmSos1 to 5 μM N-Ras.[³H]GDP followed by equilibrium ultrafiltration. From the raw data (radioactivity recorded in the filtrate and retentate) was subtracted the background count before calculating the radioactivity recorded in the filtrate as a percentage of the radioactivity in the retentate. The effect of controls 1 and 2 was that filtrate concentrations of [³H]GDP were an average of 80% of the value expected from the radioactivity in the retentate. Therefore the percentage of radioactivity recorded in the filtrate was increased accordingly before 2.4% was subtracted due to control 3 (loss of [³H]GDP from 5 μM N-Ras.[³H]GDP in the absence of CmSos1) to give the percentage of nucleotide release. Data at each CmSos1 concentration was treated in the same manner.
### Table 5.1A

<table>
<thead>
<tr>
<th>Control</th>
<th>Counts per minute (cpm)</th>
<th>[^3^H\text{GDP in filtrate (% of retentate)}]</th>
<th>Average (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>filtrate</td>
<td>retentate</td>
<td></td>
</tr>
<tr>
<td>1 GDP &amp; Partitioning</td>
<td>50 nM</td>
<td>8302</td>
<td>10579</td>
</tr>
<tr>
<td></td>
<td>8366</td>
<td>10525</td>
<td>79.5</td>
</tr>
<tr>
<td></td>
<td>0.5 μM</td>
<td>6117</td>
<td>7586</td>
</tr>
<tr>
<td></td>
<td>5929</td>
<td>7427</td>
<td>79.8</td>
</tr>
<tr>
<td></td>
<td>5 μM</td>
<td>6584</td>
<td>7542</td>
</tr>
<tr>
<td></td>
<td>6365</td>
<td>7630</td>
<td>83.4</td>
</tr>
<tr>
<td>2 CmSos1 effect on 5 μM GDP partitioning</td>
<td>10 μM</td>
<td>7714</td>
<td>10125</td>
</tr>
<tr>
<td></td>
<td>8020</td>
<td>9620</td>
<td>83.4</td>
</tr>
<tr>
<td></td>
<td>50 μM</td>
<td>8254</td>
<td>11104</td>
</tr>
<tr>
<td></td>
<td>8372</td>
<td>10871</td>
<td>77.0</td>
</tr>
<tr>
<td>3 N-Ras.[^3^H\text{GDP}]</td>
<td>0.5 μM</td>
<td>320</td>
<td>1930</td>
</tr>
<tr>
<td></td>
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</table>

### Table 5.1B

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<th>Raw data (cpm)</th>
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<th>Average</th>
<th>Control 1+2 (%)</th>
<th>Control 3 (%)</th>
<th>Release of nucleotide (%)</th>
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</thead>
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<td>Filtrate</td>
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<td>1206</td>
<td></td>
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</tbody>
</table>
Figure 5.5

Equilibrium ultrafiltration analysis of the interaction of N-Ras.\(^3\)H]GDP with CmSos1

The percentage release of \(^3\)H]GDP from N-Ras.\(^3\)H]GDP, calculated as described in table 5.1b for each CmSos1 concentration, is plotted against CmSos1 concentration. The solid line is a fit to a hyperbola with a half-maximal value of 0.84 \(\mu\)M and a limiting-value of 6.5 %. 
Figure 5.5
quantify, but it can be said that they are large. However, this does show that \(^3\text{H}\)GDP is released from N-Ras.[\(^3\text{H}\)]GDP in the presence of CmSos1 over the same concentration range as used in the stopped-flow experiments with mdGDP.

Therefore, with the knowledge that there is some release of nucleotide on addition of CmSos1 to N-Ras.GDP, the most likely explanation for the observed slow phase of intensity decrease in the Stopped-flow apparatus is release of mdGDP from the ternary N-Ras.mdGDP.CmSos1 complex. It appears that the reaction shown in scheme 5.1 is being observed.

**Scheme 5.1**

\[
\begin{align*}
K_1 & \quad \text{N-Ras.mdGDP + CmSos1} \\
K_2 & \quad \text{N-Ras.mdGDP.CmSos1} \\
& \quad \text{N-Ras.CmSos1 + mdGDP}
\end{align*}
\]

The results obtained on the interaction of N-Ras.mdGDP with CmSos1 can be explained as CmSos1 initially binds to N-Ras-mdGDP in less than 6 ms and at high concentrations a small fraction of the mdGDP subsequently dissociates from this ternary complex. A more quantitative treatment with the aid of computer simulations is given later (chapter 7).

5.2: **Interaction of CmSos1 with N-Ras.mdGDP in the presence of excess unlabelled GDP**

More information about the release of nucleotide can be obtained from the effect of CmSos1 concentration on the displacement of mdGDP from N-Ras.mdGDP using an excess of unlabelled nucleotide. In section 4.2 it was shown that the addition of 5 \(\mu\text{M}\)
CmSosl to 0.5 μM N-Ras.mdGDP in the presence of 100 μM GDP (analysed on the Stopped-flow apparatus) gave an exponential decrease in intensity with an observed rate constant of $5.1 \times 10^3 \text{s}^{-1}$ (a 670 fold enhancement of the intrinsic rate constant).

The dependence of this process on CmSosl concentration was investigated by mixing increasing concentrations of CmSosl (5-100 μM), containing 100 μM GDP, with 0.5 μM N-Ras.mdGDP under the same conditions (buffer A at 30°C), in an attempt to observe a limiting value for the process. The results are shown in figure 5.6a. It can be seen that there is a large decrease in intensity at all concentrations of CmSosl. All data sets could be fitted very well to a single exponential function, suggesting that only one rate-limiting step is being observed. A plot of the observed rate constants against CmSosl concentration is shown in figure 5.6b. A linear relationship was observed with a slope of $8 \times 10^4 \mu\text{M}^{-1}\text{s}^{-1}$. Therefore at a concentration of 100 μM CmSosl, a 200 fold excess over N-Ras.mdGDP, the observed rate constant for the decreasing intensity is still increasing linearly and 1900 fold enhancement over the intrinsic N-Ras nucleotide exchange activity is observed (figure 5.6b).

5.2.1: Interaction of CmSosl with N-Ras.mdGDP in the presence of excess unlabelled GTP.

As stated in the Introduction, Haney & Broach (1994) and Mosteller et al. (1994) both proposed that the nucleotide exchange on the yeast protein Ras2 is dependent on the nature of the free nucleotide. Haney & Broach (1994) noted that in the presence of free GTP $k_{\text{cat}}$ for the CDC25 mediated nucleotide exchange on Ras2.[$^3\text{H}]\text{GDP}$ was two fold higher than in the presence of free GDP. They concluded that the rate-limiting step is the nucleotide association step. Therefore, it was of interest to study
Figure 5.6

*The interaction of N-Ras.mdGDP with CmSos1 in the presence of excess GDP.*

**A**  Stopped-flow data following the decreases in fluorescence intensity on mixing 0.5 μM N-Ras.mdGDP with 100 μM GDP and; Buffer A (----), 5 μM (-----), 10 μM (-----), 20 μM (-----) and 50 μM (-----) CmSos1. Measurements were recorded in the L-format in buffer A at 30°C. All the data sets could be fitted well to a single exponential function. Each data set was recorded until an endpoint was reached although the data is shown here plotted over the first 200 seconds.

**B**  The observed rate constants from single exponential fits to data shown in figure A are plotted against CmSos1 concentration. The data points are an average of observed rate constants obtained from three pushes in the stopped-flow apparatus. The solid line is a linear fit to the data with a gradient of $8 \times 10^{-4} \mu M^{-1}s^{-1}$ and intercept of $1.8 \times 10^{-3} s^{-1}$.
Figure 5.6

A

Relative Intensity

0 50 100 150 200

Time (sec)

0 0.08 0.06 0.04 0.02 0

[CmSosl] (nM)

165

B

$\kappa_{obs} (s^{-1})$

0 20 40 60 80 100

[CmSosl] (µM)
the kinetics of the release of mdGDP from N-Ras.mdGDP by CmSos1 in the presence of GTP and to compare the results to those in the presence of free GDP.

N-Ras.mdGDP (0.5 μM) was rapidly mixed with increasing concentrations of CmSos1 (5 to 50 μM) containing GTP (100 μM) in the Stopped-flow apparatus. Fluorescence intensity measurements were made in the L-format. The slit widths were set as small as possible to minimise photobleaching as described previously. It can be seen from figure 5.7a that the intensity decreased approximately 2.6 fold as expected but at low CmSos1 concentrations (5 and 10 μM) a definite lag is apparent. For comparison 0.5 μM N-Ras.mdGDP was mixed with 10 μM CmSos1 containing 100 μM GDP and the difference in the fluorescence intensity recorded is shown in figure 5.7b. It can be clearly seen that in the presence of GTP the fluorescence intensity decrease is faster and there is more than one process being observed. This result is discussed at the conclusion of this chapter.

5.3: The interaction of CmSos1 with the GTP bound form of N-Ras

There has been some debate as to whether GEFs act more specifically on the GDP bound form of Ras than the GTP bound form or more generally act to catalyse the release of nucleotide and it is the greater concentration of GTP in the cell which governs the predominant formation of Ras.GTP. Jacquet et al. (1995) suggested that the rate of nucleotide exchange on H-Ras, catalysed by the catalytic domain of Cdc25^Mm (RasGRF), was dependent on the nature of the bound nucleotide. They observed a 6 fold faster rate of exchange for H-Ras.GDP than for H-Ras.GTP. Increased activity towards the GTP bound form of the yeast Ras2 by SDC25 (Poulet et al., 1995) and by CDC25 (Lai et al., 1993) has also been published. However,
Figure 5.7

The interaction of CmSos1 with N-Ras.mdGDP in the presence of excess GTP

A N-Ras.mdGDP (0.5 μM) was rapidly mixed with 100 μM GTP in the presence of; buffer A ( ), 5 μM CmSos1 ( ), 10 μM CmSos1 ( ), 20 μM CmSos1 ( ) and 50 μM CmSos1 ( ). Measurements were recorded in the L-format in the stopped-flow apparatus in buffer A at 30°C.

B A comparison of the observed decrease in intensity on mixing 0.5 μM N-Ras.mdGDP with 10 μM CmSos1 and; 100μM GDP ( ) or 100μM GTP ( ). Both experiments were performed in the stopped-flow apparatus. Measurements were performed in the L-format using buffer A at 30°C.
Chapter 5  The interaction of CmSos1 with N-Ras.mdGDP and N-Ras.mdGTP

Figure 5.7

A

B
Klebe et al. (1995) proposed that the exchange factor RCC1 does not discriminate between GDP and GTP bound forms of Ran. Also Haney & Broach (1994) despite observing a 3 fold greater activity of CDC25 on Ras2.GTP than Ras2.GDP, suggested that the function of CDC25 was to promote the nucleotide free state and the greater pool of GTP in the cell was the driving force for the accumulation of Ras.GTP in vivo.

The aim of this section is to describe the interaction of CmSos1 with the GTP bound form of N-Ras and to compare these results with those obtained for the interaction with N-Ras.GDP earlier in this chapter.

5.3.1: Stopped-flow analysis of the interaction of CmSos1 with N-Ras/mdGTP

A 1:1 complex of N-Ras/mdGTP was prepared exactly as for N-Ras/mdGDP but using mdGTP. However as N-Ras hydrolyses mdGTP to mdGDP slowly the final sample was pipetted immediately after elution from the PD-10 column into 20 μl aliquots on dry ice and stored at -80°C. The percentage of mdGTP bound to N-Ras after one freeze thaw cycle was measured by HPLC (section 2.3.2) and found to be 93%.

N-Ras/mdGTP (0.5 μM) was rapidly mixed against excess CmSos1 (5-150 μM) using the stopped-flow apparatus in buffer A at 30°C exactly as performed with N-Ras/mdGDP in section 5.1. A fast increase in anisotropy and intensity occurred in the dead time of the instrument followed by a slower decrease in intensity (with no decrease in anisotropy). These processes are discussed in detail in the following sections and compared with the observations made when CmSos1 was mixed with N-Ras/mdGDP.
Chapter 5  The interaction of CmSosl with N-Ras.mdGDP and N-Ras.mdGTP

**The fast phase - an anisotropy and intensity increase**

As was observed on rapid mixing of increasing CmSosl concentrations against N-Ras.mdGDP, a fast increase in anisotropy was recorded (figure 5.8a). Again this occurred in the dead time of the instrument at 30°C. However, on observing the anisotropy of this process and the dependence of CmSosl concentration, an apparent binding isotherm is observed over the concentration range of the above experiment (figure 5.8b). This could be fitted to an anisotropy binding curve to estimate a $K_d$ for the process of $17.0 \pm 1.3 \mu$M.

The anisotropy data described above was converted into intensity measurements and, unlike as was observed with the interaction of CmSosl with N-Ras.mdGDP, a fast intensity increase was observed. As with the anisotropy process, this occurred within the dead time of the instrument. Figure 5.8c shows the average intensity value over 1 second plotted against CmSosl concentration (relative to the intensity of 0.5 $\mu$M N-Ras.mdGTP mixed with buffer A, set to 1). The data is fitted to a hyperbola, but the data shows considerable fluctuation, probably due to changes in the lamp intensity through the course of the experiment. This does though suggest an intensity increase of approximately 50% on association of N-Ras.mdGTP to CmSosl (hence the $Q$ value (section 3.2) of 1.5 for the fitting of the anisotropy data to the anisotropy binding curve as shown in figure 5.8b)

**The slow phase - an intensity decrease**

The subsequent slower intensity decrease observed on interaction of CmSosl with N-Ras.mdGDP (figure 5.3a) was also observed in this experiment on interaction of CmSosl with N-Ras.mdGTP. The data at each CmSosl concentration (5-150 $\mu$M)
Chapter 5 The interaction of CmSosl with N-Ras mdGDP and N-Ras mdGTP

Figure 5.8

The interaction of CmSosl with N-Ras mdGTP (1).

A Stopped-flow data on mixing 0.5 μM N-Ras mdGTP with; buffer A ( ), 5 μM CmSosl ( ), 10 μM CmSosl ( ), 20 μM CmSosl ( ), 50 μM CmSosl ( ), 100 μM CmSosl ( ) and 150 μM CmSosl ( ). Measurements were made in the T-format in buffer A at 30°C. Anisotropy was calculated as described in section 2.4. Each anisotropy data set is an average of 3 or 4 pushes.

B The average anisotropy value of each of the above traces is plotted against CmSosl concentration. The error bars are the standard deviation of these values. The solid line is a fit to the anisotropy binding equation as described in section 3.4. This gives an apparent Kd of 17.0 ± 1.3 μM as described in the text.

C The data described in figure A, above, was converted into fluorescence intensity data (section 2.4) and the starting intensity value was plotted against CmSosl concentration. The intensity is normalised to the starting intensity after mixing 0.5 μM N-Ras mdGDP against buffer A of 1. The solid line is a hyperbola with a half-maximal value of 0.9 μM and limiting intensity value of 1.49 units.
Figure 5.8

A

Anisotropy

Time (sec)

B

Anisotropy

[CmSos1] (µM)

C

Relative Intensity

[CmSos1] (µM)
shown in figure 5.8a were converted into intensity and the result is shown in figure 5.9a. Each data set could be fitted to a single exponential function. This is shown in figure 5.9b, the data on mixing 0.5 μM N-Ras.mdGTP with 100 μM CmSos1 is fitted to a single exponential with an observed rate constant of 0.22 s\(^{-1}\) an amplitude of 0.018 ± 0.002 units (a 1.8% intensity decrease). The amplitude and observed rate constant of each decrease were plotted against CmSos1 concentration (figures 5.9c and 5.9d). For the reasons explained in section 5.1.3 this decrease in intensity can be assumed to be release of a small percentage of the mdGTP after association of CmSos1 to N-Ras.mdGTP to form the ternary complex N-Ras.mdGTP.CmSos1 as shown in scheme 5.2.

Scheme 5.2

\[
\begin{align*}
N\text{-Ras.mdGTP} + \text{CmSos1} & \rightleftharpoons K_1 \quad N\text{-Ras.mdGTP}\cdot\text{CmSos1} \rightleftharpoons K_2 \quad N\text{-Ras.CmSos1} + \text{mdGTP}
\end{align*}
\]

The differences between the interaction of CmSos1 with GDP and GTP bound forms of N-Ras are highlighted in the next section.

**5.3.2: Comparison of the interaction of CmSos1 with N-Ras.mdGDP and N-Ras.mdGTP**

Figure 5.10 highlights the differences between the interaction of CmSos1 with N-Ras.mdGDP and N-Ras.mdGTP under identical conditions (buffer A and 30°C). It can be seen from figure 5.10a that the apparent affinity of CmSos1 for N-Ras.mdGTP (17 μM) is at least 10 fold tighter than for N-Ras.mdGDP (>150 μM). Also, the subsequent release of nucleotide is slower and to a lesser extent from the ternary N-
Figure 5.9

The interaction of CmSos1 with N-Ras.mdGTP (2).

A  The intensity decrease observed of mixing 0.5 μM N-Ras.mdGTP with buffer A (-----), 10 μM CmSos1 (--), 50 μM CmSos1 (---), 100 μM CmSos1 (----) and 150 μM CmSos1 (-----). Measurements were recorded in the T-format in the stopped-flow apparatus using buffer A at 30°C. The intensity value has been normalised to a starting value of 1 for each data to enable comparison of the intensity decreases.

B  The data set recorded on mixing 0.5 μM N-Ras.mdGTP with 100 μM CmSos1, as described in A above, is shown. The solid line is a fit to a single exponential function. This fit gives an observed rate constant of 0.22 s⁻¹ and an amplitude of 0.018 ± 0.002 units (1.8% intensity decrease).

C  The observed rate constants obtained from a single exponential fit to the data as described in A and B are plotted against CmSos1 concentration. The solid line is a fit to a hyperbolic function with a half-maximal value of 32.6 ± 10 μM, a limiting value of 0.32 ± 0.04 s⁻¹ and an intercept of 0.049 ± 0.002 s⁻¹.

D  The amplitude of the intensity decrease obtained from a single exponential fit to the data as described in A and B are plotted against CmSos1 concentration. The solid line is a fit to a hyperbolic function with a half-maximal value of 17.4 ± 5.7 μM, a limiting value of 0.0186 ± 0.001 units (1.86% intensity decrease) and an intercept of 0.006 ± 0.001 units (0.16%).
Figure 5.9

A

Relative Intensity

0 4 8 12 16 20
Time (sec)

B

Relative Intensity

0 4 8 12 16 20
Time (sec)

C

$k_{off}$ (s$^{-1}$)

0 40 80 120 160
[CMos1] (µM)

D

Amplitude

0 40 80 120 160
[CMos1] (µM)
Figure 5.10

Comparison of the interaction of CmSos1 with N-Ras.mdGDP and N-Ras.mdGTP

A The increase in anisotropy on rapid mixing of CmSos1 with N-Ras.mdGDP (○) and N-Ras.mdGTP (●) in the stopped flow apparatus is shown. The data is taken from figures 5.2b and 5.8b. The anisotropy of data set is shown relative to an anisotropy in the absence of CmSos1 of 1. The increased apparent affinity of CmSos1 for N-Ras.mdGTP (17 μM) over N-Ras.mdGDP (>150μM) can be clearly seen.

B The observed rate constant for the intensity decrease on rapid mixing of CmSos1 with N-Ras.mdGDP (○) and N-Ras.mdGTP (●) in the stopped flow apparatus is shown. The data is taken from figures 5.3b and 5.9c. It can be seen that the decrease in intensity occurs at a faster rate with N-Ras.mdGDP than with N-Ras.mdGTP (approximately three-fold faster).

C A comparison of the amplitude of the intensity decrease observed on rapid mixing of CmSos1 with N-Ras.mdGDP (○) and N-Ras.mdGTP (●) in the stopped flow apparatus. The data is as shown in figures 5.3c and 5.9d.
Figure 5.10

A

B

C

Relative Anisotropy

$[\text{CmSos1}] \text{ (\mu M)}$

$k_{on} \text{ (s}^{-1}\text{)}$

$[\text{CmSos1}] \text{ (\mu M)}$

Amplitude

$[\text{CmSos1}] \text{ (\mu M)}$
Ras.mdGTP.CmSosl complex than from N-Ras.mdGDP as seen in figures 5.10b and 5.10c.

5.4: The interaction of CmSosl with N-Ras.mantGMPPNP

The interaction of CmSosl with the GTP bound form of N-Ras was studied further by observing the intensity and anisotropy changes on titration of CmSosl (2.5-100 μM) into (0.5 μM) N-Ras.mantGMPPNP. GMPPNP is a non-hydrolysable analogue of GTP that has been used extensively in G-protein characterisation. This analogue was used to counter the problem of the intrinsic GTP hydrolysis properties of N-Ras. A 1:1 complex of N-Ras.mantGMPPNP was formed as described in section 2. It must be noted that the mantGMPPNP used was a mix of isomers not just the 2'deoxy isomer as in the case of mdGDP and mdGTP.

N-Ras.mGMPPNP (0.5 μM) was incubated at 30°C in the SLM spectrofluorimeter. Anisotropy measurements were recorded on titration of CmSosl into N-Ras.mGMPPNP. A binding isotherm giving a K_d of 12 μM was observed (figure 5.11a), in good agreement with the endpoint anisotropy recorded on mixing N-Ras.mdGTP with CmSosl in the stopped-flow apparatus (17 μM) as shown in figure 5.11b. Also shown in figure 5.11b is a titration of CmSosl into 0.5 μM N-Ras.mdGDP, followed in the same fashion with the SLM spectrofluorimeter, which correlates very well to the data obtained using the stopped-flow apparatus.
Chapter 5  The interaction of CmSos1 with N-Ras.mdGDP and N-Ras.mdGTP

Figure 5.11

The interaction of CmSos1 with N-Ras.mGMPPNP

A  CmSos1 was titrated into 0.5 μM N-Ras.mGMPPNP in buffer A at 30°C. The reaction was followed using the SLM spectrofluorimeter in the T-format (excitation at 360nm, emission at 440nm) and recording anisotropy after each addition of CmSos1. The data is fitted to an anisotropy binding equation (equation 3.4) with a maximum anisotropy of 0.263 ± 0.002 units an apparent K_d of 12 ± 0.6 μM.

B  A comparison of the anisotropy on titration of CmSos1 into N-Ras.mGMPPNP (●) as described above and the anisotropy on rapid mixing of CmSos1 with N-Ras.mdGTP (○) in the stopped-flow apparatus as described in figure 5.8b. It can be seen that there is a good correlation between the maximum anisotropy and apparent K_d for each of the association processes. Anisotropy values shown are relative to an anisotropy of 1 in the absence of CmSos1.

Also shown is a titration of CmSos1 into 0.5 μM N-Ras.mdGDP (▲) performed exactly as for the titration described in figure 5.11a above. This is compared with the results from the rapid mixing of CmSos1 with 0.5 μM N-Ras.mdGDP (▲) in the stopped flow apparatus as described in figure 5.2b. It can be seen that a similar linear relationship is observed up to 100 μM CmSos1. As above, anisotropy values are shown relative to an anisotropy of 1 in the absence of CmSos1.
Figure 5.11

A

Anisotropy

[B]mSosl (μM)

B

Relative Anisotropy

[B]mSosl (μM)
5.4.1 The interaction of CmSos1 with N-Ras.mGMPPPNP in the presence of excess unlabelled nucleotide

The catalysis of the rate of release of mGMPPPNP from N-Ras.mGMPPPNP by CmSos1 in the presence of excess competing nucleotide was also studied. N-Ras.mGMPPPNP (0.5 μM) was incubated at 30°C in the SLM spectrofluorimeter. CmSos1 (2 to 40 μM) was added followed by 100 μM unlabelled GDP and fluorescence anisotropy and intensity recorded. Figure 5.12a shows the decrease in anisotropy on addition of free nucleotide (the increasing starting anisotropy with increasing CmSos1 concentration is due to the association of CmSos1 with N-Ras.mGMPPPNP) towards that of mGMPPPNP as expected. This data was converted to intensity measurements and the observed rate constants (fit to a single exponential function) were plotted against CmSos1 concentration (figure 5.12b). These are compared to the rates of mdGDP release obtained on addition of CmSos1 to N-Ras.mdGDP in the presence of excess GDP in figure 5.12c. It can be seen that CmSos1 clearly enhances the release of GDP from N-Ras to a greater extent than the GTP analogue GMPPNP.

5.5: The effect of CmSos1 on the hydrolysis rate of N-Ras.mdGTP to N-Ras.mdGDP.

As in this work CmSos1 had been observed to bind more tightly to the GTP bound form of N-Ras, it was decided to test whether this binding had any effect on the rate of hydrolysis of GTP to GDP by N-Ras. Therefore, 0.5 μM N-Ras.mdGTP was incubated in buffer A or with a saturating concentration of CmSos1 (50 μM) at 30°C. As can be seen in figure 5.13a, the hydrolysis of GTP to GDP was followed over time by HPLC as described in section 2.3.2. On plotting the percentage of nucleotide
Figure 5.12

The interaction of CmSosl with N-Ras.mGMPPNP in the presence of excess GDP

A  N-Ras.mGMPPNP (0.5 μM) was incubated at 30°C in buffer A and fluorescence anisotropy and intensity measurements recorded with the SLM spectrofluorimeter in the T-format. CmSosl (2 μM , 5 μM, 10 μM , 20 μM , and 40 μM ) was added before the addition of 100 μM GDP at time zero. Shown is the anisotropy data highlighting the increase in anisotropy on addition of CmSosl (X marks the anisotropy of N-Ras.mGMPPNP), followed by the decrease on addition of GDP to the reaction mix.

B  The data described in figure 5.12a above were converted into intensity measurements and fitted to a single exponential function. The observed rate constants of these fits are plotted against the CmSosl concentration and fitted to a linear equation with a slope of 5.4 x 10^-5 μM^-1 s^-1.

C  The effect of CmSosl concentration on the observed rate constant for the release of nucleotide from N-Ras.mGMPPNP (●) and N-Ras.mdGDP (○) in the presence of excess GDP is compared. Data taken from figures 5.12b and 5.6b respectively.
Figure 5.12

A

[B](mM)

Time (sec)

0 400 800 1200 1600

0.04 0.02 0

0 10 20 30 40 50

[CmSos1] (μM)

B

k_{obs} (s^{-1})

0.006

0.004

0.002

0

0 10 20 30 40 50

[CmSos1] (μM)

C

k_{obs} (s^{-1})

0.04

0.02

0

0 20 40

[CmSos1] (μM)
**The Effect of CmSos1 on the GTP hydrolysis rate of N-Ras**

A  N-Ras.mdGDP (0.5 μM) was incubated with either buffer A or 50 μM CmSos1 at 30°C. Samples were removed at different time intervals and analysed by HPLC (section 2.3.2). Shown in this figure are the elution profiles after 0 (a), 60 (b), 210 (c) and 400 (d) minutes in the presence of CmSos1. The hydrolysis of GTP (retention time of 9 minutes) to GDP (retention time of 13 minutes) can be clearly seen.

B  The area under the eluted peaks (above) were calculated and plotted as the percentage of the total nucleotide present as mdGDP against time. The solid lines are fits to a single exponential equation with observed rate constants of $1.4 \times 10^{-4} \text{s}^{-1}$ in the absence of CmSos1 (•) and $1.8 \times 10^{-4} \text{s}^{-1}$ in the presence of 50 μM CmSos1 (○).
Figure 5.13

A

B

mdGTP

mdGDP

(a)

(b)

(c)

(d)

Time

mdGDP (% of total nucleotide)

0 10 20 30 40 50 60 70 80 90 100

Time (min)

0 100 200 300 400 500
present as GDP it can be seen from figure 5.13b that the binding of CmSosl to N-Ras.mdGTP does appear to increase the rate of GTP hydrolysis from $1.4 \times 10^{-4} \text{ s}^{-1}$ to $1.8 \times 10^{-4} \text{ s}^{-1}$, however this could well be within experimental error.

5.6: Conclusions

Weak binding of CmSosl to N-Ras.mdGDP ($K_d > 150 \mu\text{M}$) has been shown to occur by analysis of an increase in anisotropy on mixing. The rate constants of this process have not been able to be measured as the reaction is too fast, being $>95\%$ complete within 6 ms at 30°C. Following this, a small percentage of nucleotide release has been observed and quantified in the absence of excess unlabelled nucleotide. This in itself suggests that a sequential mechanism does not occur. Competition experiments in the presence of excess unlabelled nucleotide were unable to define a limiting rate of nucleotide release from N-Ras in the presence of CmSosl but at least a 1900 fold increase over the intrinsic release rate was observed.

At least a ten fold tighter binding of CmSosl to the GTP bound form of N-Ras was observed (17 \muM), but the rate of release of GTP from N-Ras.GTP in the presence of CmSosl was slower than for GDP in the presence and absence of unlabelled nucleotide.

A more quantitative conclusion can be drawn from these results with the help of computer simulation. However, it has also been possible to purify the N-Ras.CmSosl nucleotide free binary complex on a large scale for kinetic and equilibrium studies. These experiments are described in the next chapter and all the data then used to deduce rate constants and equilibrium constants for each step in the reaction.
Chapter 6

The binary, nucleotide-free, N-Ras.CmSosl complex

Before the start of this work, several groups had reported being able to show the existence of nucleotide free small G-protein.GEF complexes. Hart & Powers (1995) developed an in vitro co-precipitation assay which shows association of the catalytic domain of Cdc25 to nucleotide depleted Ras proteins but not in the presence of GDP or GTP. Similar results have been shown with Sos and RasGRF. Jacquet et al. (1995) were the first group to report the purification of a Ras.GEF nucleotide free complex with their work on the interaction of the catalytic domain of Cdc25\(^{Mm}\) with H-Ras. They were able to perform \[^{3}H\]GDP binding studies over nitrocellulose membranes.

This work has already obtained information about for the association of CmSos1 to the GDP and GTP bound forms of N-Ras that has not before been observed. In this chapter the association of CmSos1 with the nucleotide free form of N-Ras is discussed and also the association kinetics of nucleotide binding to this binary complex are addressed, enabling rate and equilibrium constants for the reverse reaction (scheme 6.1) to be investigated.

**Scheme 6.1**

\[
\begin{align*}
N-Ras.CmSosl + GXP & \rightleftharpoons_{k_{-1}}^{k_{+1}} N-Ras.mdGXP.CmSosl \\
& \rightleftharpoons_{k_{-2}}^{k_{+2}} N-Ras.mdGXP + CmSosl
\end{align*}
\]
Firstly, several methods for the purification of the binary complex are described followed by characterisation of the complex.

6.1: **Purification and characterisation of the nucleotide free N-Ras.CmSos1 complex**

The predominant theory is that GEFs form a stable complex with the nucleotide free form of small G-proteins. From the experiments described in the earlier chapters it was apparent that simply incubating N-Ras.GDP and CmSos1 together in buffer A would not induce formation of nucleotide free complex in the presence of high Mg\(^{2+}\) concentrations as only partial release of nucleotide occurs (up to approximately 6%). It has also been shown that high MgCl\(_2\) concentration affects the stability of some of these nucleotide free complexes (Beraud-Dufour *et al.*, 1998), decreasing the affinity of the small G-protein for the GEF.

To induce association of CmSos1 to N-Ras, it was decided to incubate CmSos1 and N-Ras.GDP proteins at high concentration in the presence of the metal ion chelator EDTA. If this solution was dialysed using a 10 kD cut off membrane, free nucleotide should be removed, moving the equilibrium toward N-Ras.CmSos1 formation. This could then be passed over a gel filtration column to remove any non-complexed N-Ras or CmSos1 protein. As it would be easier to separate a 21 kD protein (N-Ras) than a 59 kD protein (CmSos1) from an 80 kD complex (N-Ras.CmSos1), N-Ras was incubated at a 5 fold molar excess over CmSos1 in the hope that all the CmSos1 would form a complex with N-Ras.
Therefore, 1.25ml of N-Ras.GDP (750 μM) and CmSosl (150μM) was dialysed against 50 mM Tris.HCl, 100 mM NaCl, 40 mM EDTA, and 1 mM DTT, using a 10 kD molecular weight cut off slide-a-lyzer dialysis cassette (Pierce). This was performed overnight with one change buffer after 2 hours. The solution was then passed over a Superdex-75 gel filtration column (HiLoad 26/60, Amersham Pharmacia) equilibrated with 20 mM Tris.HCl pH 7.5, 100 mM NaCl, 1 mM EDTA and 1 mM DTT at 1ml.min⁻¹ and fractions were collected every 3 minutes. Three peaks were observed and analysed by SDS-PAGE (figure 6.1). The first peak eluted (fractions 47-50 pooled) can be seen to be N-Ras.CmSosl complex, the second peak (fractions 54-56 pooled) to be free CmSosl and the third peak (fractions 66-73) free N-Ras protein. Therefore, despite using a 5 fold molar excess of N-Ras.GDP, some of the CmSosl protein remained free in solution. An absorbance scan from 220 to 320nm of the N-Ras peak showed a characteristic profile for N-Ras.GDP with absorption maxima at 280 and 260 nm, showing the free N-Ras remained bound to GDP despite dialysis overnight with EDTA and CmSosl. Attempts were made to shift this equilibrium towards N-Ras.CmSosl formation such as adding alkaline phosphatase to degrade GDP present (John et al., 1990). This was performed in a MgCl₂ and EDTA free buffer as alkaline phosphatase requires a Zn²⁺ as cofactor. However, no significant improvement was achieved. Therefore, the N-Ras.CmSosl protein was produced as described above and only the leading edge fractions were pooled so as not to include any free CmSosl protein.

6.1.1: Gel filtration of the purified N-Ras.CmSosl complex

The integrity of the N-Ras.CmSosl complex on addition of MgCl₂ to the buffer and after one freeze-thaw cycle was examined by analytical gel filtration chromatography.
Figure 6.1

Purification of the N-Ras.CmSos1 complex

A Elution profile, monitoring absorbance at 280 nm, from the S-75 gel filtration column as described in the text. The sample was loaded at 1ml.min⁻¹ and fractions collected every 3 minutes.

B SDS-PAGE analysis of fractions eluted from the S-75 column. 5 µl of each fraction was prepared as described in section 2.2.3 and 5µl loaded onto a 20 % acrylamide gel. This clearly shows the first peak to be N-Ras.CmSos1, peak 2 to be free CmSos1 and peak 3 to be free N-Ras (N-Ras.GDP).
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Figure 6.1

A

Absorbance (280 nm)

Time

B

CmSos1

N-Ras

191
A Superdex 200 analytical gel filtration column (Amersham Pharmacia, HR 10/30) was equilibrated in 20 mM Tris.HCl pH 7.5, 100 mM NaCl, 1 mM EDTA and 1 mM DTT at 1 ml.min\(^{-1}\) using a Beckman Gold HPLC system. N-Ras.GDP and CmSos1 standards were applied to the column (100 µl of 20 µM of each sample) before 100 µl of 20 µM N-Ras.CmSos1 protein. The elution profiles of these samples are shown in figure 6.2a. It can be seen that there is some dissociation of the complex, a small percentage of free N-Ras protein eluting after 40 minutes. However, the retention time of the major peak (32.5 minutes) suggested this was stable N-Ras.CmSos1 complex when compared with the retention time of the CmSos1 standard (34 minutes). This dissociation could be due to disruption of the complex during the freeze-thaw cycle or to the dilution effect because of the equilibrium constant of the complex.

In order to test whether the stability of the complex was affected by the absence of Mg\(^{2+}\) ions, it was decided to repeat the gel filtration of the complex with the column equilibrated in buffer A (no EDTA and 2 mM MgCl\(_2\)). The sample was also diluted to 1.5 µM to examine the effect of further dilution on the dissociation of the complex over the column. A SMART system (Superdex-75 column, Amersham Pharmacia) was used due to a greater sensitivity of detection and smaller volumes can be loaded. The column was equilibrated in buffer A and samples loaded at 50 µl.min\(^{-1}\). Molecular weight markers (115, 29 and 15 kD, Amersham Pharmacia) and N-Ras.GDP and CmSos1 standards were applied to the column before applying 20 µl of 20 µM N-Ras.CmSos1 and then 20 µl of 1.5 µM N-Ras.CmSos1. The elution profiles are shown in figure 6.2b (1 and 2).
Analytical gel filtration of the N-Ras.CmSos1 complex

A Elution profiles from an S-75 analytical gel filtration column. The column was equilibrated in 20mM Tris.HCl pH 7.5, 100 mM NaCl, 1 mM EDTA and 1mM DTT at 1 ml.min$^{-1}$ (room temperature). The profiles are the absorbance traces at 280 nm of the elution of;

1 100 µl of 20 µM CmSos1
2 100 µl of 20 µM N-Ras.CmSos1

B Elution profiles from a SMART S-75 column (as described in the text). The column was equilibrated in buffer A at room temperature, and samples were run at 50 µl.min$^{-1}$. The profiles ($A_{280nm}$) are of the elution of;

1 10 µl of 20 µM N-Ras.CmSos1
2 10 µl of 1.5 µM N-Ras.CmSos1
Figure 6.2

A

[Graph showing absorbance (280nm) over time for CmSos1.N-Ras and N-Ras.]

B

[Graph showing absorbance units (280 nm) over time for peak 1 and 2.]
The presence of Mg\(^{2+}\) had little effect on the dissociation of the complex, whereas dilution of the complex to 1.5 \(\mu\)M increased the dissociation of the complex. It is possible that some of this dissociation was due to the process of gel filtration. However, it does suggest that the binding constant of CmSos1 to N-Ras is in the range of \(\mu\)M.

### 6.2: Association of nucleotide to the N-Ras.CmSos1 complex.

The association of both mdGDP and mdGTP to the nucleotide free N-Ras.CmSos1 complex was studied in order to gain equilibrium and rate constants for the process and to observe any isomerisations and conformation changes that may occur.

#### 6.2.1 Titration of mdGDP into N-Ras.CmSos1

Equilibrium analysis of the binding of mdGDP to the N-Ras.CmSos1 complex was performed by titration of mdGDP into different concentrations of N-Ras.CmSos1 followed by fluorescence intensity. Measurements were made in the SLM spectrofluorimeter in the L-format in buffer A at 30°C. Shown in figure 6.3a is the titration of 0.5-10 \(\mu\)M mdGDP into 5 \(\mu\)M N-Ras.CmSos1 complex. A control titration of 0.5-10 \(\mu\)M mdGDP into buffer A was performed simultaneously and subtracted from the experimental data in order to account for background emission from free mdGDP. It can be seen that intensity increased linearly with mdGDP concentration up to approximately 4.6 \(\mu\)M mdGDP and to a 2.6 fold greater intensity than that of free mdGDP. This suggests that the experiment was performed at concentrations above the \(K_d\) of the process and that approximately 92 % of the N-Ras.CmSos1 complex (and free N-Ras) was able to bind nucleotide. The experiment was also performed at 0.5 and 0.05 \(\mu\)M N-Ras.CmSos1. Shown in figure 6.3b is a titration of mdGDP into 0.5
**Figure 6.3**

*Titration of mdGDP into the N-Ras.CmSos1 complex*

**A**  
(1) Plot of intensity against mdGDP concentration. mdGDP (0.5-20 μM) was titrated into 5μM N-Ras.CmSos1 (●) and buffer A (○). Titrations were performed in buffer A at 30°C and intensity measurements recorded in the L-format (excitation at 366 nm, emission at 440 nm) on the SLM spectrofluorimeter. (2) The background mdGDP fluorescence has been subtracted from the intensity from the titration of mdGDP into 5 μM N-Ras.CmSos1. It can be seen that the intensity increases linearly to a concentration of 4.6 μM mdGDP.

**B**  
(1) Titration of mdGDP into 0.5 μM N-Ras.CmSos1 (●) and buffer A (○) performed exactly as A above. (2) The buffer readings were subtracted showing a linear increase in fluorescence intensity up to 0.3 μM mdGDP before the intensity values began to decrease.
Figure 6.3

A

1

B

1
μM N-Ras.CmSosl. It can be seen that interpretation of the results were complicated by apparent dissociation of nucleotide after an initial linear binding of mdGDP up to 0.3 μM. This could be due to dissociation of N-Ras.CmSosl at lower concentrations (as observed by gel filtration, section 6.1.1). The experiment was attempted at lower N-Ras.CmSosl concentrations (50 nM) but the error due to the sensitivity of the fluorimeter was too great at such low concentrations. However, these experiments do show that nucleotide bind the N-Ras.CmSosl complex giving a fluorescence increase, therefore the kinetics of the association process were studied in the stopped-flow apparatus.

6.3: Kinetics of the interaction of GDP and GTP with N-Ras.CmSosl

The association of mdGDP with N-Ras.CmSosl was followed over a fast time scale using the stopped-flow apparatus. The aim was to deduce rate constants for the binding of nucleotide to the binary complex and to observe any difference in the rates of association and binding affinities of mdGDP and mdGTP. This was achieved by mixing N-Ras.CmSosl with excess nucleotide and observing the dependence of the rate of binding on nucleotide concentration. The fluorescence of the mdGDP/mdGTP analogue was used, as on binding to the complex there is a 2.6 fold enhancement in fluorescence intensity. Because of the background fluorescence of the mdGDP/mdGTP when observing the binding of excess mdGDP/mdGTP over N-Ras.CmSosl, the ratio of mdGDP/mdGTP over N-Ras.CmSosl could not be too high as the signal to noise ratio became too small. In order to maintain pseudo first order conditions, the mdGDP/mdGTP was always at least five fold in excess over N-Ras.CmSosl. The concentration of N-Ras.CmSosl was varied over the range 0.5 to 2 μM allowing the concentration of mdGDP/mdGTP to be varied between 2.5 and 35...
μM. Traces at different concentrations of N-Ras.CmSos1 and identical concentrations of mdGDP/mdGTP gave comparable results showing that this approach was valid.

For each experiment, the appropriate concentration of mdGDP was mixed with buffer in the stopped-flow apparatus and the data for the reaction of N-Ras.CmSos1 with this concentration of mdGDP normalised against the value that this control experiment gave.

6.3.1: Stopped-flow analysis of mdGDP association to N-Ras.CmSos1

Figure 6.4 shows a record of the fluorescence intensity when 0.5 μM N-Ras.CmSos1 was mixed with 5 μM mdGDP. Fluorescence intensity changes were measured in the L-format at 30°C using the stopped-flow apparatus. It can be seen that fluorescence intensity increased exponentially from that of 5 μM mdGDP (set to 1) to a value of 1.058. Figure 6.4 shows the data fitted to a single exponential function with an observed rate constant of 25 ± 0.2 s⁻¹. It can be seen that the data is not a perfect fit to a single exponential. The data has therefore been analysed also using observed rate constants from a double exponential fit and the residual plots of the fits have been compared in figure 6.4 (constants are described later, showing that the data is better fitted to a double exponential than a single exponential). N-Ras.CmSos1 (0.5 μM) was also mixed with 2.5, 3.5, 6.5, 8 and 10 μM mdGDP. At a concentration of 10 μM mdGDP the amplitude of the intensity increase was only 0.02 and at the limit of detection of the apparatus. To follow the association of greater concentrations of mdGDP to N-Ras.CmSos1, the concentration of N-Ras.CmSos1 was increased to 1.0 and 2.0 μM. 1.0 μM N-Ras.CmSos1 was mixed with 5, 8, 10, 12.5 and 15 μM mdGDP and 2.0 μM N-Ras.CmSos1 was mixed with 15, 20, 25, 30 and 35 μM.
Figure 6.4

Stopped-flow analysis of the association of mdGDP to N-Ras.CmSos1

Data to show the rapid mixing of 0.5 μM N-Ras.CmSos1 to 5 μM mdGDP in the stopped-flow apparatus. The reaction was performed in buffer A at 30°C. Intensity was recorded in the L- format. A fluorescence intensity of 1 corresponds to that of 5 μM mdGDP. The data shown is an average of 4 pushes. The solid line is a fit to a single exponential function with a $k_{obs}$ of 25.3 ± 0.2 s⁻¹.

Shown below are the residual plots of fits of the above data to a single exponential (blue) and a double exponential (red) equation.
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Figure 6.4
The data were fitted to a single exponential function and the observed rate constants obtained were plotted against mdGDP concentration (figure 6.5a). The data could be fitted well to a straight line with a gradient of $4.2 \pm 0.2 \, \mu \text{M}^{-1} \text{s}^{-1}$ and an intercept of $1.05 \pm 3.1 \, \text{s}^{-1}$. This suggests an association rate constant of $4.2 \pm 0.2 \, \mu \text{M}^{-1} \text{s}^{-1}$ and a dissociation rate constant is too small to be defined reliably from the intercept on the y-axis.

The data could be better fitted to a function having two exponential increases as stated above. Figure 6.5b shows the observed rate constants obtained from fitting the data from the mixing of 0.5 μM N-Ras.mdGDP with 2.5, 3.5, 5, 6.5, 8 and 10 μM mdGDP to a double exponential function. At high concentrations of mdGDP (6.5, 8 and 10 μM) the amplitude of the signal increase was too small to resolve two processes. The $k_{\text{obs}}$ for the fast phase is fitted to a straight line of gradient $6.94 \, \mu \text{M}^{-1} \text{s}^{-1}$, the slow phase fitted to a straight line of gradient $1.14 \, \mu \text{M}^{-1} \text{s}^{-1}$. The $k_{\text{obs}}$ for the fits to a single exponential function are shown for comparison in figure 6.5a. The amplitude of the fast and slow process were consistent, the slow process having an amplitude of 17.7, 18.9 and 16.3% of the total amplitude for the experiments mixing 2.5, 3.5 and 5 μM mdGDP respectively.

6.3.2: Analysis of the association of mdGDP to N-Ras.CmSos1 in the presence of excess CmSos1

It has already been shown that there is some dissociation of the N-Ras.CmSos1 complex (gel filtration studies). Therefore, a possible explanation for the appearance of two phases of fluorescence increase on mixing of N-Ras.CmSos1 with mdGDP is
**Figure 6.5**

*Stopped-flow analysis of the association of mdGDP to N-Ras.CmSos1 – derivative plots*

**A** The fluorescence intensity increase observed due to the association of mdGDP to 0.5 μM (●), 1.0 μM (▲) and 2.0 μM (■) N-Ras.CmSos1 were fitted to a single exponential function and the observed rate constants plotted against mdGDP concentration. The solid line is a fit of the data to a straight line of gradient of 4.2 ± 0.2 μM⁻¹s⁻¹ and an intercept of 1.05 ± 3.1 s⁻¹.

**B** The observed rate constants of the fast phase (▲) and slow phase (■) of intensity increase is plotted against mdGDP concentration and compared with the observed rate constants of fits to single exponential increase (○). Solid lines are linear fits to the data, giving apparent association rate constants as quoted in the text.
Figure 6.5

**A**

![Graph A](image)

**B**

![Graph B](image)
the different rates of association of nucleotide to nucleotide free N-Ras and to the N-
Ras.CmSos complex. This was investigated by repeating the above experiment but
adding excess CmSosl to the 0.5 μM N-Ras.CmSosl before mixing with mdGDP. If
the reason for the double exponential process observed is as hypothesised then the
excess CmSosl would bind to the free N-Ras and therefore only association of
mdGDP to N-Ras.CmSosl would be measured.

CmSosl (10μM) was added to 0.5 μM N-Ras.CmSosl in the stopped-flow apparatus
before mixing against 2.5, 3.5 and 5 μM mdGDP as above. An exponential increase in
intensity was again observed as expected. The data was analysed by fitting to a single
and double exponential as in the absence of 10μM CmSosl.

The $k_{\text{obs}}$ obtained from a fit to a single exponential function were plotted against
mdGDP concentration and when fitted to a straight line gave a gradient of 5.7 μM$^{-1}$s$^{-1}$
compared with a gradient of 5.1 μM$^{-1}$s$^{-1}$ for the identical experiment in the absence of
10 μM CmSosl (figure 6.6a). However, again the data could be fitted more closely to
a function of two exponential processes. The $k_{\text{obs}}$ for the fast phase and slow phase
were plotted against mdGDP concentration and compared against the experiment in
the absence of 10μM CmSosl (figure 6.6a). It can be seen that the fast and slow
processes have similar observed rate constants as in the absence of excess CmSosl.
The gradient of a linear fit to the $k_{\text{obs}}$ values gave 7.01 μM$^{-1}$s$^{-1}$ (compared to 6.93 μM$^{-1}$s$^{-1}$)
for the fast phase and 1.11μM$^{-1}$s$^{-1}$ (compared to 1.14 μM$^{-1}$s$^{-1}$) for the slow phase.
However the amplitudes of the two process were found to be different. The amplitude
of the slow process was 8.1, 7.8 and 10.0% of the total amplitude, for the experiments
using 2.5, 3.5, and 5 μM mdGDP respectively. This is compared with the results in
Figure 6.6

**Stopped-flow analysis of the interaction of mdGDP with N-Ras.CmSos1 in the presence of excess CmSos1.**

A  N-Ras.CmSos1 (0.5 μM) was rapidly mixed with 2.5, 3.5 and 5 μM mdGDP in the presence of 10 μM CmSos1. The observed rate constants for the fast phase (▲) and slow phase (△) of intensity increase from a double exponential fit and from a single exponential fit (○) for each mdGDP concentration. Solid lines are linear fits to the data. Dashed lines are the fits to the fast phase (blue), slow phase (green) and single exponential (red) observed rate constants for the identical experiment performed in the absence of excess CmSos1 (figure 6.5b).

B  A bar chart to represent the increase in ratio of the fast phase to the slow phase of intensity increase on mixing mdGDP with 0.5 μM N-Ras.CmSos1 in the presence of 10 μM CmSos1. The amplitude of the slow phase as a percentage of the total amplitude of the reaction in the absence (green) and presence (red) of excess CmSos1 for each mdGDP concentration is shown.
Figure 6.6

A

![Graph showing a linear relationship between mdGDP concentration (µM) and the rate constant $k_{obs}$ (s⁻¹).]

B

![Bar chart showing the amplitude of slow phase (% of total amplitude) at different mdGDP concentrations (µM).]
the absence of excess CmSosl in figure 6.6b. It can be seen that the contribution of the slow phase to the total amplitude of the observed process has approximately halved. This decrease in contribution of the slow phase (or increase of the fast phase) explains the observed increase in the $k_{\text{obs}}$ obtained from the single exponential fits to the data. Therefore, although with the addition of 10 μM CmSosl to the binary complex, a double exponential process was still observed, the percentage of the slow phase decreased. This suggests that the additional CmSosl, which would be expected to bind free N-Ras in the solution forming a higher proportion of N-Ras.CmSosl in solution (if not all binary complex), has caused an increase in the proportion of the fast phase of intensity increase. This therefore suggests that the fast phase corresponds to the association of mdGDP to N-Ras.CmSosl complex (scheme 6.2) and the slow phase to the association of mdGDP to N-Ras (scheme 6.3).

**Scheme 6.2**

\[
\begin{align*}
N-\text{Ras}.Cm\text{Sosl} + \text{mdGDP} & \xrightleftharpoons[k_{-1}]{k_{+1}} N-\text{Ras}.\text{mdGDP}.Cm\text{Sosl} \xrightleftharpoons[k_{+2}]{k_{-2}} N-\text{Ras}.\text{mdGDP} + \text{CmSosl}
\end{align*}
\]

**Scheme 6.3**

\[
\begin{align*}
N-\text{Ras} + \text{mdGDP} & \xrightleftharpoons[k_{-1}]{k_{+1}} N-\text{Ras}.\text{mdGDP}
\end{align*}
\]

The gradients of the linear fits to these data give apparent association rate constants of mdGDP to N-Ras.CmSosl of 6.94 μM$^{-1}$s$^{-1}$ ($k_{+1}$, scheme 6.2) and of mdGDP to N-Ras 1.14 μM$^{-1}$s$^{-1}$ ($k_{+1}$, scheme 6.3). This latter rate constant is similar to that reported by John *et al.* (1990) who studied the association of mdGDP to nucleotide free H-Ras and observed an apparent association rate constant of 1.1 μM$^{-1}$s$^{-1}$ at 25°C. This was
obtained from a linear plot of the observed rate constants against mdGDP concentration.

6.3.3: *Stopped-flow analysis of the association of mdGTP to N-Ras.CmSos1*

The association of mdGTP to N-Ras.CmSos1 has also been examined and the rate constants obtained compared with those of the association of mdGDP to N-Ras.CmSos1.

The interaction of N-Ras.CmSos1 with mdGTP was investigated in the same way as described for the interaction of N-Ras.CmSos1 with mdGDP in the above section. Shown in figure 6.7a is the data from the stopped-flow apparatus on rapidly mixing 0.25 μM N-Ras.CmSos1 against 5 μM mdGTP in buffer A at 30°C. The intensity of 5 μM mdGTP has been normalised to a solution of 5 μM mdGTP was mixed with buffer. The data is fitted to a single exponential function in figure 6.7a with a $k_{obs}$ of $10.4 \pm 0.2$ s$^{-1}$ and amplitude $0.053 \pm 0.2$ intensity units. Again it can be seen that the data did not fit perfectly to a single exponential function. The experiment was repeated by mixing 0.5 μM N-Ras.CmSos1 with 7.5, 10 and 12.5 μM mdGTP and also mixing 0.25 μM N-Ras.CmSos1 with 1.25, 2.5, 5 and 7.5 μM mdGTP and mixing 1.0 μM N-Ras.CmSos1 with 10, 12.5, 15 and 20 μM mdGTP. The $k_{obs}$ obtained from fitting each of these traces to a single exponential function were plotted against mdGTP concentration as shown in figure 6.7b. The solid line is a linear fit to the data points with a gradient of $1.53 \pm 0.06$ μM$^{-1}$s$^{-1}$ and an intercept of close to zero.

The rate constants for the fast and slow phases obtained from a fit to two exponential processes were plotted as a function of mdGTP concentration (as for the experiment
Figure 6.7

**Stopped-flow analysis of the interaction of mdGTP with N-Ras.CmSosl**

A Raw data from the mixing of N-Ras.CmSosl (0.5 μM) with 5 μM mdGTP in the stopped-flow apparatus (buffer A at 30°C). An intensity reading of 1 corresponds to the intensity of 5 μM mdGTP (on rapid mixing with buffer A) and this trace is the average of 3 pushes. The solid line is the best fit to a single exponential function with a $k_{obs}$ of $10.4 \pm 0.2$ s$^{-1}$ and amplitude $0.053 \pm 0.2$ intensity units.

B The fluorescence intensity increase observed due to the association of mdGTP to 0.25 μM (●), 0.5 μM (▲) and 1.0 μM (▲•) N-Ras.CmSosl were fitted to a single exponential function and the observed rate constants plotted against mdGTP concentration. The solid line is a fit of the data to a straight line of gradient of $1.53 \pm 0.06$ μM$^{-1}$s$^{-1}$ and an intercept of $0.74 \pm 0.5$ s$^{-1}$.

C The observed rate constants of the fast phase (●) and slow phase (■) of intensity increase is plotted against mdGTP concentration and compared with the observed rate constants of fits to single exponential increase (○). Solid lines are linear fits to the data, giving apparent association rate constants as quoted in the text.
Figure 6.7

A

Relative Intensity

Time (sec)

B

$k_{on} (s^{-1})$

mdGTP concentration (μM)

C

$k_{on} (s^{-1})$

mdGTP concentration (μM)
with mdGDP above) and shown in figure 6.7c. Double exponential fits could only be made for those experiments with a 10 fold excess of mdGTP or less over N-Ras.CmSosl (0.25 μM N-Ras.CmSosl mixed with 1.25 and 2.5 μM mdGTP, 0.5 μM N-Ras.CmSosl mixed with 5 μM mdGDP and 1.0 μM N-Ras.CmSosl mixed with 10 μM mdGTP). The gradient of a linear fit to the \( k_{\text{obs}} \) of the fast phase is 1.87 μM\(^{-1}\)s\(^{-1}\) and to the \( k_{\text{obs}} \) of the slow phase is 0.14 μM\(^{-1}\)s\(^{-1}\). The percentage of the amplitude of the slow phase of the total amplitude was between 11 and 14%, similar to that recorded for the mdGDP association experiments. It could be assumed that the fast phase corresponded to association of mdGTP to N-Ras.CmSosl and the slow phase to association of mdGTP to N-Ras. This data therefore suggests association rate constants of 1.87 μM\(^{-1}\)s\(^{-1}\) and 0.14 μM\(^{-1}\)s\(^{-1}\) for mdGTP to N-Ras.CmSosl and to N-Ras respectively. Again the intercepts were too small to be reliably defined.

It must be noted that it is not always reliable to separate two such exponential processes in this way. The analysis of the results from the double exponential fits to this data has been reported here because the observed rate constants to follow a distinct trend and the amplitudes remain constant. However another reason for the deviation from the single exponential fit could be that true pseudo first order conditions were not achieved as less than a ten-fold excess of nucleotide over binary complex was used. The results from the addition of excess CmSosl to the reaction do suggest however that the association rate constant for the association of nucleotide to N-Ras.CmSosl is lower than the association rate constant of nucleotide to N-Ras.
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6.4: Conclusions

The binary nucleotide-free N-Ras.CmSosI complex has been purified and characterised. Gel filtration has shown that the N-Ras.CmSosI complex is not affected by the presence of Mg2+ ions but at μM concentrations some dissociation of the complex was apparent.

Tight apparent nucleotide binding to N-Ras.CmSos1 was observed on titration of mdGDP into N-Ras.CmSos1. The association rate constant of mdGDP to N-Ras.CmSos1 was measured to be 6.93 μM⁻¹s⁻¹ and the association rate constant for the binding of mdGTP to N-Ras.CmSos1 was found to be 1.86 μM⁻¹s⁻¹. The dissociation rate constants of both processes were close to zero.

This data could be added to what was known about the forward reaction from chapters 4 and 5, enabling the simulation of the data to deduce those constants that were unattainable. These computer simulation experiments are described in the next chapter.
Results have been described for the association of CmSos1 to N-Ras.mdGDP and N-Ras.mdGTP and the subsequent catalysis of the release of mdGDP and mdGTP in chapter 5. Also characterised in chapter 6 was the purification of the nucleotide free N-Ras.CmSos1 complex and the ‘reverse reaction’ of nucleotide association to this complex demonstrated. Some constants were not measurable so computer simulation of the data was undertaken to estimate these constants and verify that the experimentally obtained constants could be reconciled with a substituted mechanism of nucleotide exchange.

The results using two data simulation programs are discussed here. The program KSIM (N.C.Millar) was used to simulate the kinetics of the interaction and the program SCIENTIST (MicroMath) used to simulate the equilibrium constants of the scheme. Table 7.1 summarises the constants derived experimentally and the restrictions on the unknown constants from the experimental data. Using computer simulation the missing constants have been estimated as described below.

7.1: The interaction of CmSos1 with N-Ras.mdGDP

The simulation of the data involving the interaction of CmSos1 with N-Ras.mdGDP is described in detail in this section, those involving N-Ras.mdGTP are summarised later.
Figure 7.1

Substituted mechanism of nucleotide exchange by CmSos1 on N-Ras

A scheme to show the most simplistic substituted mechanism for the exchange of GDP for GTP on the N-Ras protein catalysed by CmSos1. CmSos1 binds to N-Ras.mdGDP to form the N-Ras.mdGDP.CmSos1 ternary complex (K₁) followed by release of mdGDP to leave the nucleotide-free N-Ras.CmSos1 binary complex (K₂). Nucleotide then rebinds (mdGTP, K₃) and CmSos1 dissociates (K₄) to leave the ‘active’ N-Ras.mdGTP complex.

Table 7.1

Constants and restrictions for computer simulation from experimental data

A table to show the constants deduced experimentally and those constraints that could be placed on the computer simulation from the experimental data. The sections from which these data have been taken are shown below.

a. Section 5.1
b. Section 5.1
c. Section 6.3.2
d. Section 6.3.3
e. Section 5.3.1
f. Section 5.3.1
Scheme 7.1

\[
\begin{align*}
N\text{-Ras.mdGDP} + \text{CmSosl} & \xrightarrow{k_{s1}} N\text{-Ras.mdGDP.CmSosl} \xrightarrow{k_{s2}} N\text{-Ras.CmSosl} + \text{mdGDP} \\
N\text{-Ras.CmSosl} + \text{mdGTP} & \xrightarrow{k_{s3}} N\text{-Ras.mdGTP.CmSosl} \xrightarrow{k_{s4}} N\text{-Ras.mdGTP} + \text{CmSosl}
\end{align*}
\]

Table 7.1

<table>
<thead>
<tr>
<th>Constant</th>
<th>Experimental</th>
<th>Restrictions</th>
</tr>
</thead>
<tbody>
<tr>
<td>(K_1)</td>
<td></td>
<td>(K_{\text{obs}} &gt; 150 \mu\text{M}) (a)</td>
</tr>
<tr>
<td>(k_{s1})</td>
<td></td>
<td>(\text{Reaction} &gt; 95% \text{ complete within} 6\text{ms}) (b)</td>
</tr>
<tr>
<td>(k_{s2})</td>
<td></td>
<td>(k_{s2}/k_2)</td>
</tr>
<tr>
<td>(k_{s3})</td>
<td>6.93 (\mu\text{M}^{-1}\text{s}^{-1}) (c)</td>
<td>(\text{Small} (&lt;5\text{s}^{-1})) (c)</td>
</tr>
<tr>
<td>(k_{s4})</td>
<td></td>
<td>(k_{s2}/k_2)</td>
</tr>
<tr>
<td>(k_{s5})</td>
<td>1.86 (\mu\text{M}^{-1}\text{s}^{-1}) (d)</td>
<td>(\text{Small} (&lt;5\text{s}^{-1})) (d)</td>
</tr>
<tr>
<td>(K_4)</td>
<td>17 (\mu\text{M}) (e)</td>
<td>(\text{Reaction} &gt; 95% \text{ complete within} 6\text{ms}) (e)</td>
</tr>
<tr>
<td>(k_{s4})</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
7.1.1: Simulation of kinetic parameters using KSIM

Constants were simulated to fit a simple substituted enzyme mechanism as shown in figure 7.1. The values and restrictions shown in table 7.1 were used in the following ways;

1. No rate constants were able to be assigned to $k_{+1}$ and $k_{-1}$ as the association of CmSos1 to N-Ras.mdGDP was too fast to measure, but a dissociation constant ($K_d$) of $> 150 \mu$M had to result (figure 5.2). Therefore a secondary rate constant of $10 \mu$M$^{-1}$s$^{-1}$ (a standard diffusion controlled association constant (Fersht, 1995)) and a $k_{-1}$ of 1500 s$^{-1}$ were used as starting values in the simulations.

2. The association rate constant for the binding of mdGDP to N-Ras.CmSos1 ($k_2$) was fixed at 6.93 $\mu$M$^{-1}$s$^{-1}$ (from figure 6.3) and the dissociation rate constant ($k_{-2}$) varied to find a good fit to the data. The only restriction on $k_{-2}$ was that it was small ($< 2$ s$^{-1}$ from figure 6.3).

It was also assumed that the association of nucleotide to N-Ras was too tight to have an impact on these results (i.e. no free N-Ras present). This is addressed in section 7.2.

It was found that constants based on these starting values could simulate the experimental data well as shown in figures 7.3a, 7.3b and 7.3c. The way these constants were simulated is described in the figure legends and the constants derived shown in figure 7.2. Many permutations based upon the restrictions were attempted but the constants shown gave the closest match to the experimental data. However,
Figure 7.2

The same scheme as shown in figure 7.1

Table 7.2

*Constants derived from computer simulation of the experimental data using KSIM*

The constants and restrictions from the experimental data were used as shown in figure 7.1 were simulated as described in the text and in figure 7.3 to give the constants shown in this table.
Figure 7.2

\[
\begin{align*}
    & N-Ras.mGDP + CmSosl & \xrightarrow{k_{-1}} & N-Ras.mGDP.CmSosl & \xrightarrow{k_{+1}} & N-Ras.CmSosl + mGDP \\
    & & \xrightarrow{k_{-2}} & & \xrightarrow{k_{+2}} & \\
    & N-Ras.CmSosl + mGTP & \xrightarrow{k_{-3}} & N-Ras.mGTP.CmSosl & \xrightarrow{k_{+3}} & N-Ras.mGTP + CmSosl \\
    & & & \xrightarrow{k_{-4}} & & \xrightarrow{k_{+4}} \\
\end{align*}
\]

Table 7.2

<table>
<thead>
<tr>
<th>Constant</th>
<th>Simulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>( K_1 )</td>
<td>300 ( \mu \text{M} )</td>
</tr>
<tr>
<td>( k_{+1} )</td>
<td>10 ( \mu \text{M}^{-1}\text{s}^{-1} )</td>
</tr>
<tr>
<td>( k_{-1} )</td>
<td>3000 ( \text{s}^{-1} )</td>
</tr>
<tr>
<td>( K_2 )</td>
<td>43 nM</td>
</tr>
<tr>
<td>( k_{+2} )</td>
<td>0.3 ( \text{s}^{-1} )</td>
</tr>
<tr>
<td>( k_{-2} )</td>
<td>6.93 ( \mu \text{M}^{-1}\text{s}^{-1} )</td>
</tr>
<tr>
<td>( K_3 )</td>
<td>3.8 nM</td>
</tr>
<tr>
<td>( k_{+3} )</td>
<td>1.86 ( \mu \text{M}^{-1}\text{s}^{-1} )</td>
</tr>
<tr>
<td>( k_{-3} )</td>
<td>0.007 ( \text{s}^{-1} )</td>
</tr>
<tr>
<td>( K_4 )</td>
<td>13.5 ( \mu \text{M} )</td>
</tr>
<tr>
<td>( k_{+4} )</td>
<td>135 ( \text{s}^{-1} )</td>
</tr>
<tr>
<td>( k_{-4} )</td>
<td>10 ( \mu \text{M}^{-1}\text{s}^{-1} )</td>
</tr>
</tbody>
</table>
Figure 7.3

Comparison of experimental and simulated data

A The constants as shown in table 7.2 were used to simulate scheme 7.1 using the computer simulation programme 'KSIM'. A starting concentration of N-Ras.mdGDP of 0.5 μM was used with increasing concentrations of CmSos1 from 0.5 to 150 μM and a starting concentration of competing nucleotide of 100 μM. Changes in fluorescence intensity were simulated by dividing free mdGDP by 2.6 and adding 1 equivalent of N-Ras.mdGDP and N-Ras.mdGDP. This was due to the 2.6 fold decrease in intensity observed on release of mdGDP from N-Ras.mdGDP and no intensity change on association of CmSos1 to N-Ras.mdGDP. The simulated intensity changes at each CmSos1 concentration were fitted to single exponential equations and the rate constants plotted against CmSos1 concentration. This simulated data is shown compared to the experimental data (as described in figure 5.5).

B Simulation was performed as described in figure 7.3a above except that the starting concentration of competing nucleotide was set to zero. The simulated intensity change at each CmSos1 concentration was fitted to a single exponential equation and the rate constant plotted against CmSos1 concentration. The simulated data (○) is shown compared to the experimentally derived data (●, figure 5.3b). Both data sets are fitted to a hyperbolic equation.
Figure 7.3

A

B

\[
\begin{align*}
\text{k}_{\text{obs}} (s^{-1}) & \\
\text{[CmSos1]} (\mu\text{M}) & \\
0 & 20 \ 40 \ 60 \ 80 \ 100
\end{align*}
\]

\[
\begin{align*}
\text{k}_{\text{obs}} (s^{-1}) & \\
\text{[CmSos1]} (\mu\text{M}) & \\
0 & 40 \ 80 \ 120 \ 160
\end{align*}
\]
fixing $k_{+1}$ at 10 $\mu$M$^{-1}$s$^{-1}$ and increasing $k_{-1}$ from 300 to 600 s$^{-1}$ (i.e. increasing $K_{d1}$ from 300 to 600 $\mu$M had little effect on the simulated rate constants. These simulations suggest a maximum rate of release of mdGDP from N-Ras.mdGDP.CmSosl of 0.3 s$^{-1}$. The amount of nucleotide released is governed by the relative dissociation constants obtained ($K_1$ of 300 $\mu$M and $K_2$ of 43 nM). These values were simulated in the program ‘SCIENTIST’ that allows the equilibrium constants of the whole system to be analysed together.

7.1.2: Simulation of equilibrium constants using ‘SCIENTIST’.

The model used is shown and described in figure 7.4. The parameters shown in figure 7.2 (and highlighted in the figure legend of 7.4a) were used. As no work has been performed on the dissociation constant of the interaction of nucleotide with free Ras protein in this work, a figure of 10 pM was used from the literature (John et al., 1993). It was found that increasing this dissociation constant to up to 1 nM had no effect on the concentrations of ternary complex and free nucleotide at equilibrium.

Figure 7.5a displays the concentrations of all the species at equilibrium, on mixing 0.5 $\mu$M N-Ras.mdGDP with increasing concentrations of CmSosl (4 to 1000 $\mu$M). This figure shows that, using these equilibrium constants, nucleotide release from the ternary complex is saturating at 150 $\mu$M CmSosl whereas the formation of ternary N-Ras.mdGDP.CmSosl is still approximately in the linear phase (as highlighted by the dashed line). The amount of nucleotide released at each concentration is compared with the experimental data in figure 7.5b. It can be seen that a similar trend is observed in the data, but importantly as described above the release of nucleotide saturates in the concentration range of Cmsos1 attempted experimentally with a similar apparent $K_d$ (50 $\mu$M simulated, 47 $\mu$M experimentally).
Figure 7.4

Simulations using the program ‘SCIENTIST’

A The reaction scheme shown was simulated in the programme ‘Scientist’ as described in B below.

B The coupled equilibria model (J.Hutchinson, N.I.M.R), shown, was used to simulate the formation of ternary complex and release of free nucleotide on interaction of 0.5 μM N-Ras.mdGDP with increasing concentrations of CmSos1 (4-1000 μM). The parameters (K₁, K₂ and K₃), calculated from the experimental data, were entered and the starting concentrations of N-Ras.mdGDP (0.5 μM) and CmSos1 (4-1000 μM) were entered as the total concentrations.

Key

ST  Total CmSos1 concentration
RMT Total N-Ras.mdGDP concentration
R   N-Ras
RM  N-Ras.mdGDP
RMS N-Ras.mdGDP.CmSos1
RS  N-Ras.CmSos1
S   CmSos1
M   mdGDP
Figure 7.4

A

\[ N\text{-Ras} + \text{GDP} + \text{CmSosl} \rightleftharpoons N\text{-Ras.GDP} + \text{CmSosl} \]

\[ K_{d1} \]

\[ N\text{-Ras.CmSosl} + \text{GDP} \rightleftharpoons N\text{-Ras.GDP.CmSosl} \]

\[ K_{d2} \]

\[ K_{d3} \]

\[ K_{d4} \]

B

// Exchange factor coupled equilibria
IndVars: ST, RMT
DepVars: R,M,RMS,RM,RS
params:K1,K3,K4
RM=(R*M)/K1
RS=(R*S)/((K1*K3)/K4)
RMS=(RM*S)/K3
RMS=(RS*M)/K4
RMT=R+RM+RS+RMS
RMT=M+RM+RMS
ST=S+RS+RMS
0<R<RMT
0<M<RMT
0<S<ST
0<RM<RMT
0<RS<RMT
0<RMS<RMT
***
Figure 7.5

'Scientist' simulation of the equilibrium constants for the interaction of CmSos1 with N-Ras and mdGDP.

A The scheme shown in figure 7.4a was simulated as described in figure 7.4b, with the equilibrium constants as described in the text and shown below;

<table>
<thead>
<tr>
<th>$K_{d1}$</th>
<th>10 pM</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_{d3}$</td>
<td>300 μM</td>
</tr>
<tr>
<td>$K_{d4}$</td>
<td>43 nM</td>
</tr>
</tbody>
</table>

Starting concentrations of 0.5 μM N-Ras.mdGDP and 4-1000 μM CmSos1 were entered. The concentrations of the ternary complex N-Ras.mdGDP.CmSos1 (blue), free nucleotide (black) and N-Ras.mdGDP (red) at equilibrium at each CmSos1 concentration was plotted against CmSos1 concentration. The dashed line at 150 μM CmSos1 shows the limit of the experimental data, at which point the amount of free nucleotide released is saturating whereas the ternary complex formation is still in the linear phase.

B The simulated release of nucleotide as described in A above is compared to the experimental data on addition of 0.5 μM N-Ras.mdGDP to increasing concentrations of CmSos1 in the absence of excess nucleotide (figure 5.3c). The decrease in intensity attributed to a release of nucleotide in figure 5.3c has been converted into free nucleotide present at equilibrium assuming a 2.6 fold decrease in intensity of free mdGDP as compared to N-Ras.mdGDP and no effect on the intensity of the fluorophore on CmSos1 binding to N-Ras.mdGDP. Both data sets are fitted to a hyperbolic equation.
Figure 7.5

A.

![Graph A](image1)

B.

![Graph B](image2)
There is one data set that cannot be fitted to this scheme (and the associated kinetic and equilibrium constants) and therefore as yet cannot be explained. This is the increased rate of release of mdGDP from N-Ras.mdGDP.CmSos1 in the presence of GTP as opposed to GDP as described in section 5.2.1. The scheme described above should be entirely independent of the nature of the incoming nucleotide, the rate-limiting step being the release of nucleotide from the ternary complex. This would suggest that the nucleotide association to the binary N-Ras.CmSosl complex was having a rate limiting effect. As these have been measured, this appears very unlikely. Alternatively, the mechanism could be complicated by a further step such as a GTP associating to form a quaternary complex and increasing the rate of nucleotide release. However, there is no other evidence for this mechanism in the literature and it is therefore highly unlikely. More experiments are required to probe this very interesting anomaly and before this is explained the mechanism cannot be confirmed.

7.2: The interaction of CmSos1 with N-Ras.mdGTP

The interaction of CmSos1 with N-Ras.mdGTP was also simulated in the same way as described above for the interaction of CmSos1 with N-Ras.mdGDP. To simulate the association of CmSos1 to N-Ras.mdGTP and subsequent release of mdGTP, the dissociation constant $K_4$ was fixed at 17.0 $\mu$M (i.e. $k_{+4}$ and $k_{-4}$ varied but ensuring that $K_{d4}$ remained 17 $\mu$M, starting values being $k_{+4}$ 10 $\mu$M$^{-1}$s$^{-1}$ and $k_{-4}$ 170 s$^{-1}$) (from figure 5.7). The association rate constant $k_3$ for mdGTP and N-Ras.CmSos1 was fixed at 1.86 $\mu$M$^{-1}$s$^{-1}$ and $k_{+3}$ had to be small ($< 2$ s$^{-1}$) from figure 6.5. The constants obtained after computer simulation are shown in table 7.2. It can be seen that the maximum rate of release of mdGTP from N-Ras.mdGTP.CmSos1 is 0.007 s$^{-1}$ and values for the equilibrium constants are a $K_4$ of 17 $\mu$M (formation of the ternary
complex) and a $K_3$ of 3.8 nM (affinity of mdGTP for the ternary complex). As for N-Ras.mdGDP, a dissociation constant for N-Ras.mdGTP of 10 pM was used.

### 7.3: Conclusions

These results have suggested that rate constant for nucleotide dissociation from the ternary step is the limiting factor in the reaction. For the release of mdGDP from N-Ras.mdGDP.CmSosl this has been shown to be $\sim 0.3 \, \text{s}^{-1}$ and for the release of mdGTP for N-Ras.mdGTP.CmSosl to be $\sim 0.007 \, \text{s}^{-1}$. This equates to a 7200 fold increase over the intrinsic release of mdGDP from N-Ras.mdGDP. The intrinsic GTP release rate has not been measured in this work, but Neal et al. (1990) showed that there was less than a two fold difference between the mantGDP and mantGDP release rates from N-Ras.mantGDP and N-Ras.mantGTP respectively. This therefore suggests a lower catalytic effect of CmSosl on the GTP bound form than GDP bound form of the N-Ras protein.

Klebe et al. (1998) claimed to find evidence for a conformational change in the ternary complex H-Ras.GDP.Cdc25 before release of nucleotide. No direct evidence has been seen in this work to give any justification for including a conformational change into this scheme, but this possibility can certainly not be discounted.

The weak association of CmSosl to N-Ras.mdGDP ($K_d \, 300 \, \mu\text{M}$) decreases the affinity of N-Ras and nucleotide by at least one order of magnitude, the $K_d$ increasing from $\sim 10 \, \text{pM}$ in the absence of CmSosl to $\sim 40 \, \text{nM}$ in the presence of CmSosl. The 15 fold tighter association of CmSosl to N-Ras.mdGTP ($K_d \, 17 \, \mu\text{M}$) does not induce
greater release of nucleotide, but less due to the greater affinity of mdGTP for the N-Ras.CmSos1 complex \( (K_d \sim 3.8 \text{ nM}) \) 10 fold higher than that of mdGDP \( (K_d \sim 43 \text{ nM}) \).
Chapter 8

The interaction of PIX with Rac1

The Dbl family of guanine nucleotide exchange factors are proposed to catalyse the exchange of GDP for GTP on the Rho family of small G-proteins (Whitehead et al., 1997). A newly discovered protein, PIX, has been assigned to this family due to sequence homology (DH domain) and from in vivo studies as a putative exchange factor for Rac proteins. GST-fusion PIX proteins containing DH and PH domains, were shown to catalyse the incorporation of GTPγS into Rac1, CDC42 and RhoA in vitro (Manser et al., 1998).

8.1: The intrinsic rate of nucleotide release from Rac1 and the catalytic activity of αPIX

The intrinsic release of mantGDP from Rac1.mantGDP was observed by fluorescence intensity exactly as for N-Ras. The detail of these experimental procedures has therefore been covered for N-Ras (chapter 3). A 1:1 complex of Rac1.mantGDP was prepared as for N-Ras.mantGDP and 0.5 μM Rac1.mantGDP was incubated in buffer B at 30°C. Excess unlabelled GDP (100 μM) was added and the fluorescence intensity of the fluorophore monitored over time in the SLM spectrofluorimeter. As expected the intensity value decreased exponentially over time. This could be fitted to a single exponential equation to give an observed rate constant of $6.1 \times 10^{-5}$s$^{-1}$. The intensity was observed to decrease 2.2 fold (figure 8.1).
Figure 8.1

*The effect of PIX-DP on the release of mantGDP from Rac1.mantGDP*

The graph shows the decrease in fluorescence intensity observed when excess GDP (100 μM) is added to 0.5 μM Rac1.mantGDP in the presence (white) and absence (black) of 5 μM PIX-DP. The solid lines are fits of the data to a single exponential function. An observed rate constant of 6.17 x 10⁻⁵ s⁻¹ was obtained for the intrinsic release of mantGDP from Rac1. It can be seen that the addition of PIX-DP produced the same rate constant within experimental error.

Figure 8.2

*The effect of PIX-DP on the incorporation of GTPγS into Rac1*

The incorporation of GTPγS into 0.2 μM Rac1.GDP in the presence (white) and absence (black) of 0.067 μM PIX-DP. Both data sets are fitted to a single exponential. The rate constant for the intrinsic incorporation of GTPγS into Rac1 was 5.2 x 10⁻⁵ s⁻¹, comparable to the nucleotide exchange rate implied by the mant assay. The rate constant in the presence of PIX-DP was 2.1 x 10⁻⁴ s⁻¹, a four fold enhancement over the intrinsic rate constant.
Chapter 8

The interaction of PIX with Rac1

Figure 8.1

![Graph showing relative intensity over time (min)]

Figure 8.2

![Graph showing GTPaS incorporation (% of final bound) over time (min)]
This was repeated but with the addition of 5 μM PIX-DP. As can be seen from figure 8.1 little, if any, increase in the rate of release of fluorescent nucleotide (over the intrinsic rate) was recorded on addition of 5 μM PIX-DP to 0.5 μM Rac1.mantGDP in the presence of excess (100 μM) GDP. This was repeated with concentrations PIX-DP up to 30μM with the same result.

As little to no exchange activity was observed using the mant assay the in vitro experiments, published by Manser et al. (1998), were repeated. The extent of nucleotide exchange was measured by the incorporation of [35S]GTPγS into 0.2 μM Rac1.GDP. The experimental procedures are described in section 2.5.2. A four-fold enhancement of nucleotide incorporation was observed in the presence of 0.067 μM PIX-DP (figure 8.2). This was in agreement with Manser et al. (1998). Although this is not a great increase in activity, it is a reproducible effect. The reason for observing exchange activity by incorporation of GTPγS and not using the mant assay is not clear at present and will be discussed later.

8.2: Complex formation between Rac1.mantGDP and PIX-DP.

Although PIX-DP did not promote the release of mantGDP from Rac1.mantGDP as shown above, the possibility that PIX-DP could bind Rac1.mantGDP without nucleotide release was investigated.

PIX-DP was added to 0.5 μM Rac1.mantGDP, in concentrations from 0.5 - 30 μM, in the presence and absence of excess (100 μM) free GDP and followed by fluorescence anisotropy. No effect was seen on the anisotropy of Rac1.mantGDP (0.198) on
addition of PIX-DP in the presence or absence of free GDP (data not shown), suggesting no interaction of Rac1.mantGDP with PIX-DP under these conditions.

8.3: Binary Rac1.PIX-DP complex formation

It has been shown that exchange factors form stable complexes with small G-proteins in the nucleotide free state. To investigate whether PIX bound the nucleotide free state of Rac1, 100 μM Rac1.GDP and 50 μM PIX-DP was dialysed against 50 mM Tris.HCl pH 7.5, 50 mM NaCl, 1 mM DTT and 40 mM EDTA overnight at 4°C with one change of buffer. In theory, removal of Mg²⁺ from nucleotide binding site by EDTA should favour formation of the nucleotide-free state as nucleotide is dialysed away, inducing binding of PIX-DP to Rac1 (as was shown with the N-Ras/CmSos1 system in chapter 6). The reaction mix was passed down a Superdex-75 analytical gel filtration column (Amersham Pharmacia) at 0.4 ml.min⁻¹ by HPLC (Beckman Gold) and the eluant monitored by absorbance at 280 nm (also by SDS PAGE). The elution profile was compared to Rac1 and PIX-DP standards (figure 8.3). Any complex formed would elute ahead of PIX-DP. As can be seen there was no sign of complex formation. Weak complexes with a K_d of more than 1 μM would dissociate on the gel filtration column, however stable GEF.p21 complexes have been purified by this method in this lab (CmSos1.N-Ras, section 6.1) as well as by others (Klebe et al., 1995).

8.4: The interaction of PIX-DP with Rac1.mGMPPNP

A personal communication from E.Manser suggested that binding to the GTP bound form of Rac1 by PIX had been observed. Therefore Rac1 was complexed with the non-hydrolysable fluorescent analogue of GTP, mantGMPPNP. PIX-DP was titrated
Figure 8.3

**Attempted purification of a PIX-DP.Rac1 binary complex by gel filtration.**

A The elution profile at 280 nm from the Superdex-75 gel filtration apparatus when 300 µl of a 50 µM PIX-DP solution was loaded onto the column. Conditions were as described in the text. It can be seen that PIX-DP eluted after 23.2 minutes.

B Elution profile of 300 µl of a 100 µM Rac1 solution as a standard. The Rac1 had not been dialysed against EDTA, so is still in a complex with GDP (no GDP elution peak). Rac1.GDP elutes after 27.4 minutes.

C The elution profile of the 100 µM Rac1.GDP and 50 µM PIX-DP mix (300 µl, dialysed overnight in EDTA buffer). Peaks were observed at 23.2 minutes (PIX-DP) and at 27.7 minutes (nucleotide free Rac1). Some sample was lost on loading onto the gel filtration column, explaining the lower than expected absorbance values.
Chapter 8  The interaction of PIX with Rac1

Figure 8.3

Absorbance units (280nm)

Time (min)
Chapter 8  The interaction of PIX with Racl

(0.1 – 30 μM ) into 0.5 μM Racl.mantGMPPNP in the absence and presence of excess free GDP (100 μM). Fluorescence anisotropy measurements were taken, but no change in anisotropy was observed in either case (results not shown), suggesting that no interaction between PIX-DP and Rac1mGMPPNP was occurring under these conditions.

8.5: Sequence alignment with, and relation of PIX to other DH family members

As little exchange activity had been detected with PIX-DP on Racl.GDP it was decided to check the alignment of α and β PIX with other DH domains as specific conserved residues could be missing and thus a possible reason for the apparent low exchange activity of PIX. An alignment was created using the domains and constraints of Whitehead et al. (1997) but adding the α and β PIX sequences. No obvious deviance from the other DH sequences was observed by eye in the α and β PIX sequences. A guide tree used to construct these alignments, produced using the gcg programme ‘Distances’ (figure 8.4), showed that PIX was closely related to the well characterised exchange factor Dbl and the proposed Rac1 exchange factor Tiam1 (Michiels et al., 1995). This work was performed with the help of J.Saldanha (Mathematical Biology, N.I.M.R.).

8.6: Conclusions

A clone for the putative Rac1 exchange factor αPIX has been obtained and a protein containing the DH and PH domains purified. Using the mant assay no exchange activity on or binding to Rac1 has been observed by PIX-DP. A four-fold enhancement of GTPγS incorporation into Rac1.GDP by PIX-DP has been observed
Figure 8.4

Guide tree from the sequence homology of the Dbl family proteins

DH domain sequences (see below for accession numbers) were aligned as by Whitehead et al. (1997). Gaps in the sequence homology or additional sequences of no homology present in sequences were removed and the resulting sequences translated into the guide tree using the ‘distances’ programme. It can be seen that PIX is closely related to the Rac1 exchange factor Tiam1 and the most characterised exchange factor of the group, Dbl.

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Figure 8.4
(in agreement with the work of Manser *et al.* 1998). No evidence for binary or ternary complex formation has been found as yet. Sequence homology to other Dbl family exchange factors is good and PIX is closely related to the Rac1 exchange factor Tiam1 and the most characterised exchange factor of the family, Dbl.
Chapter 9

Discussion

This investigation has reached conclusions in three main areas; the production of a high expression CmSosl clone, the mechanism of action of CmSosl with N-Ras and in the study of the interaction of αPIX with Rac1. A summary of the conclusions made are presented and discussed in the following sections.

9.1: Codon optimisation of the CmSosl gene

A 60-120 fold increase in the yield of soluble, active CmSosl has been achieved by the process of optimising the codon usage of the CmSosl gene. The highly expressing clone produced, QEc, codes for the same amino acid sequence as the original poorly expressing clone, the only difference being the silent codon changes made. This certainly suggests that ‘poor’ codon usage of the CmSosl for expression in E.coli was the reason for low recombinant protein expression levels. Other factors could have been altered during construction of the synthetic gene (such as disruption a particular sequence in the gene that hinders expression (Li et al., 1996) or the resulting mRNA stability and folding properties). However, from this study so far, it appears that codon usage of heterologous genes can have a dramatic effect on protein expression levels.

The PCR method used for the reconstruction of the gene is a recently developed technique (Stemmer et al., 1994). It has proved to be, in this case, a reproducible and
reliable way of creating the desired DNA sequence and is recommended if attempting to optimise expression or to just create a clone from a known sequence.

The other issue highlighted by this work is the minimal domain of mSos1 required for exchange activity on Ras proteins. Certainly it appears that the minimal domain for \textit{in vitro} stability of the protein is greater than 613-1076 (AE), assuming that the GST-tag did not affect the folding of the protein. Also, however, the soluble AE protein recovered was inactive, suggesting that the additional residues 577-613 are required for activity in some manner. Recently published data has supported this observation, Kim \textit{et al.} (1998) reporting exchange activity of a mSos1 fragment of residues 584-1088, but no activity on H-Ras by residues 618-1088. There is no sequence homology with other RasGEF’s in this region (577-618), so it could be important for correct folding or stability and is unlikely to be directly involved in the exchange mechanism. This theory has also been supported by the recently published H-Ras.hSos1 crystal structure that is discussed in section 9.3.

9.2: The mechanism of nucleotide exchange by CmSos1 on N-Ras

This large increase in CmSos1 expression enabled the purification of enough CmSos1 protein to study the interaction with N-Ras.mdGDP over rapid time scales and the purification of N-Ras.CmSos1 nucleotide free complex for characterisation.

The formation of the weak ternary complex, N-Ras.mdGDP.CmSos1, has been observed and estimated to have a $K_d$ of 300 $\mu$M and a fast dissociation rate constant of $\sim 3000 \text{ s}^{-1}$ in buffer A at 30°C. The binding of CmSos1 to N-Ras.mdGDP lowers the affinity of N-Ras for mdGDP by over an order of magnitude, the dissociation
constant of mdGDP for the ternary complex having been simulated to be 43 nM (the 
$K_d$ of N-Ras for nucleotide being in the pM range). This is how it is proposed that 
CmSosl catalyses the release of mdGDP from N-Ras.mdGDP, a maximum 7200 fold 
increase over the intrinsic release rate having been predicted.

CmSosl has been shown to bind more tightly to the GTP bound form of N-Ras, the 
N-Ras.mdGTP complex, with a $K_d$ of 17 µM under the same conditions. However, the 
$K_d$ of mdGTP for this ternary complex of 3.8 nM (compared to 43 nM for mdGDP 
above) means that CmSosl catalyses the release of mdGTP from N-Ras.mdGTP at a 
slower rate than mdGDP from N-Ras.mdGDP as observed experimentally.

No evidence for any conformational changes was found in this work, although several 
groups have proposed this idea in the mechanism of action of various exchange 
factors. Lenzen et al. (1998) demonstrated a two-step binding of mdGDP to the 
Ras.Cdc25$^{Mn}$ complex. They also suggested that the association of Cdc25$^{Mn}$ to 
Ras.mdGDP was a multi-step process, but were unable to show this, suggesting that 
an isomerisation to a state of low nucleotide affinity being the rate-limiting step in the 
mechanism. Eccleston et al. (1988) showed that there is a conformational change in 
the EF-Tu.GDP.EF-Ts ternary complex before release of nucleotide and Klebe et al. 
(1995) suggested the same for the Ran/RCC1 system. Therefore although no evidence 
for conformational changes was observed it is possible that they occurred but were 
not reported by fluorescence changes.

Lenzen et al. (1998), in their a kinetic analysis of the interaction between the catalytic 
domain of cdc25$^{Mn}$ (Ccdc25$^{Mn}$) and H-Ras, were able to perform experiments using
Plasmon Surface Resonance measurements (Biacore system) for a determination of the affinity between Ccdc25^™ and nucleotide free N-Ras. They recorded an association rate constant of \(3.3 \times 10^5\) M\(^{-1}\)s\(^{-1}\) and dissociation rate constant of \(1.0 \times 10^3\) s\(^{-1}\), giving a \(K_d\) of 3.3nM (4.6nM from global analysis of data), a constant which has not been able to be directly measured in this work. Although CmSosl is homologous to Ccdc25^™ this constant cannot be assumed to be similar for CmSosl and N-Ras. However a similar approach could be used to study the dissociation constant. To study this interaction nucleotide free N-Ras would need to be purified. An alternative approach would be to use a technique such as Isothermal Calorimetry (ITC).

9.3: The crystal structure of the Ras.Sos binary complex

Towards the end of this work, Boriack-Sjodin et al. (1998) solved the crystal structure of the complex between nucleotide free H-Ras (residues 1-166) and the catalytic domain of Human Sos1, ChSosl, protein (residues 564-1049, corresponding to residues 581-1066 of mSosl). The structure they reported is shown in figure 9.1. The structure of ChSosl consists of two distinct \(\alpha\) helical domains, the N-terminal and C-terminal domains. The N-terminal domain (N-domain), residues 568-741 (corresponding to 585-759 of mSosl) does not interact directly with H-Ras. It appears to play a purely structural role by stabilising a helical hairpin (\(\alpha H\)) that protrudes out of the core of the ChSosl domain and looks to play a crucial role in the exchange mechanism (explained later). This explains why the smaller fragments of CmSosl that were expressed from the optimised CmSosl gene were all found to be highly insoluble. The N-terminal domain had been removed entirely from RE and RK which, from analysis of this structure, would have made the domain very unstable. The AE and AK fragments however contained the conserved region SCR 0 (highlighted in
Figure 9.1

The crystal structure of the Ras.Sos binary complex

Taken from Boriack-Sjodin et al. (1998)

A Ribbon diagram of the crystal structure of human H-Ras with ChSos1.
N-domain of hSos1 (residues 568-741) is purple
The ‘catalytic domain’ of hSos1 (residues 752-1044) is green
Conserved regions among Ras GEFs (SCR) are cyan
Disordered regions of hSos1 are shown as dotted lines
Switch 1 and switch 2 regions of H-Ras are Orange
The P-loop of H-Ras is Red

B The Ras.Sos structure is shown with the ‘catalytic domain’ (752-1044) as a molecular surface. The conserved residues Ile 956 and Phe 958 in the catalytic domain that form a hydrophobic interface with the N-domain are labelled.
Figure 9.1

(a) Schematic representation of the interaction between Ras and Sos.

(b) Detailed view showing the Switch 1 and Switch 2 regions, with key residues Ile 956 and Phe 958.
cyan in figure 9.1b) of the N-terminal domain which contacts \( \alpha \)H, yet was still very insoluble. The soluble protein that was purified of AE protein was inactive on the N-Ras.GDP protein. This suggests that the \( \alpha \) helices N-terminal of this SCR 0 region have a structural role in stabilising the SCR 0 region that in turn stabilises the \( \alpha \)H protrusion from the core domain and so affects exchange activity. This additional region has been shown not to be required for \textit{in vitro} activity of Cdc25\textsuperscript{Mm} (Jacquet \textit{et al.}, 1995)

Figure 9.3 illustrates the implications for nucleotide binding to H-Ras of the insertion of helix \( \alpha \)H from ChSos1. It shows ChSos1 opening the nucleotide-binding pocket of H-Ras, removing switch 1 from the nucleotide-binding site like the opening of a jaw. Phe 28, at the N-terminus of switch 1 moves 9.6 Å. This interrupts the network of direct and water mediated interactions between switch 1 and the nucleotide but does not occlude the guanine and ribose binding sites. This is important because it would allow rebinding of nucleotide.

Two residues of the helix \( \alpha \)H of ChSos1 appear to interfere directly with nucleotide and magnesium binding, Glu 942 and Leu 938. The major contact residues of ChSos1 with H-Ras are compared with the contact residues of H-Ras with GTP in figure 9.2. The charged carboxylate group of Glu 942 is positioned where the \( \alpha \)-phosphate of GTP or GDP is found in the structures of nucleotide bound H-Ras. This residue also hydrogen bonds with Ser-17 of H-Ras, usually a ligand for Mg\textsuperscript{2+}. Leu 938 also appears to disrupt Mg\textsuperscript{2+} binding, increasing the hydrophobicity at the Mg\textsuperscript{2+} binding site.
**Figure 9.2**

*The crystal structure of the Ras.Sos binary complex 2*

Taken from Boriack-Sjodin *et al.* (1998)

This schematic representation highlights the differences in the switch 2 region and the \( \text{Mg}^{2+} \) binding site of Ras in the Ras.GTP (A) and Ras.Sos (B) crystal structures.

Ras residues are coloured orange and Sos residues coloured green. Selected hydrophobic interactions are shown as solid arcs and polar interactions shown as broken lines. The disruption of the \( \text{Mg}^{2+} \) and phosphate binding sites can be clearly seen on binding of Sos. Also the extensive interaction of Sos with the switch 2 domain is apparent.

**Figure 9.3**

*The crystal structure of the Ras.Sos binary complex 3*

Taken from Boriack-Sjodin *et al.* (1998)

The nucleotide-binding site on the surface of H-Ras is shown with a GTP analogue bound (left). The surface of H-Ras in the Ras.Sos complex (right), shown with the backbone of Sos (with the N-domain deleted), displays very clearly the effect of the \( \alpha \text{H} \) of Sos on the nucleotide binding site of Ras. This has been compared to the ‘opening of a jaw’, causing the nucleotide to be released and enabling nucleotide to rebind.
Figure 9.2

Figure 9.3
The overall interface between H-Ras and ChSos1 is extensive. It is primarily hydrophilic, except for three hydrophobic residues of H-Ras (Tyr 64, Met 67 and Tyr 71) that are buried in a hydrophobic core of ChSos1 (Phe 929, Ile 825, Leu 872 and Tyr 912). Almost every other external switch 2 side chain is co-ordinated by ChSos1 making its conformation well defined in this complex (poorly ordered in the GTP bound structure, Pai et al., 1989, and very poorly in the GDP bound structure Tong et al., 1991). This interface was examined in search of a rationale for the difference in affinity of the GTP and GDP bound forms of N-Ras for CmSosl (chapter 5), but firm conclusions were complicated by the poorly ordered nature of the switch regions in the GDP and GTP bound structures.

9.3.1: Comparison with other crystal structures

Like the Cdc25 homology domain of hSos, the Dbl homology domains of hSos (solved by Soisson et al., 1998) and Trio (Lui et al., 1998) are highly helical. Although the overall structures are quite different, the solved structure of these domains together with biochemical data, suggest that a conserved helix found in DH domains may be a Rho or Rac binding site similar to the α H of Sos.

The crystal structures of Sos.Ras, Gea2.Arf1 and EF-Tu.EF-Ts have been compared by Sprang & Coleman (1998) as shown in figure 9.4. Despite being structurally different, Sprang & Coleman suggest that there does seem to be a common theme recurring from biochemical studies and structural evidence from these G-protein.GEF binary complexes. In all cases switch I is pulled away from switch II and the P-loop exposing the active site. Switch II is restructured affecting the γ-phosphate binding site. Residues either from the GEF or G-protein are positioned to occupy or block the
Comparison of Ras, EF-Tu and ARF1 in their GDP, GEF and GTP-bound complexes

Taken from Sprang & Coleman (1998).

Shown are the crystal structures of the G-proteins H-Ras, EF-Tu, and ARF1 in their GDP, GEF (hSos1, EF-Ts and Gea2 respectively) and GTP bound complexes.

Key
G-proteins are coloured light grey
Switch 1 and switch 2 are coloured magenta
P-loop regions are coloured blue
Guanine nucleotide and Mg\(^{2+}\) are coloured yellow
GEF derived structures are green
Disordered regions are indicated by dashed lines
phosphate and Mg\textsuperscript{2+}- binding sites. The purine-binding site, however, remains easily accessed, enabling GTP rebinding.

9.3.2: Discussion of results in the light of the crystal structures
As has been shown in this work with the interaction of CmSos1 and N-Ras, the affinities Ras.GTP and Ras.GDP are in the picomolar range, Ras.GEF in the nanomolar range or below and the ternary complex with either nucleotide (Ras.GXP.GEF) is micromolar (also Klebe et al., 1995, Lenzen et al., 1998). This is the next structural challenge, to characterise the structural changes that accompany the formation of the ternary complex. As was shown in this work with the concentrations of protein Sos and Ras proteins now available, this is now a possibility.

The docking site of the ChSosl protein on the switch II region of Ras and the interaction with the switch 1 domain had been predicted by several groups who carried out mutational studies on Ras proteins. (Verroti et al., 1992, Segal et al., 1993, Mosteller et al., 1994, Quilliam et al., 1996). No information exists as to how these mutations actually affect the molecular interaction with Sos. With the ability now to probe the affinity of Sos with Ras.GDP, Ras GTP, and nucleotide free Sos and the affinities of nucleotide and rates of association and dissociation, a detailed study of these mutations is now possible.

Of particular interest also is the effect of some Sos mutants, especially Glu 942 and Leu 938, as they have been proposed to directly interrupt phosphate and Mg\textsuperscript{2+} binding. It has been suggested that substitution of only one of these residues has little
effect on the activity of CmSosl, (Bar-Sagi, D. unpublished data). It will be very interesting to observe the results of such mutations using this system.

9.4: Cellular significance of the kinetic and equilibrium constants described

Obviously the implications of the results described here for the \textit{in vivo} nucleotide dissociation rates depend on the local concentrations of the Ras and Sos proteins and free nucleotide (both GDP and GTP). These will vary between cell types. What can be said from these results, however is that the catalytic domain of the CmSosl protein would either have to be in a very high excess over Ras.GDP or be brought into close proximity in order for significant nucleotide exchange to occur (due to the very weak $K_d$ of CmSosl and N-Ras.GDP). As discussed in the introduction Sos is brought to the membrane (vicinity of the membrane bound Ras protein) by the adaptor protein Grb2 on phosphorylation of a receptor, but the weak affinity of CmSosl for N-Ras.GDP shown in this work may suggest that this could need to be more specific. Recently membrane proteins such as N-Ras have been shown to be found in distinct areas of the plasma membrane or ‘rafts’ (Simons & Ikonen, 1997). It could be that other proteins on the membrane bring the Sos protein specifically into contact with Ras, possibly via the N-terminal PH domain of Sos. Several groups have suggested a protein or lipid binding role for PH domains in membrane association (reviewed by Lemmon \textit{et al.}, 1997).

9.4.1: Implications for drug design

As mentioned in the introduction major interest began in the Ras superfamily of proteins when it was discovered that oncogenic Ras mutants were found in approximately 30% of all human tumours. A lot of research has been performed
towards the discovery of anti-proliferative drugs around those proteins involved in the Ras GTPase cycle (Prendergast & Gibb, 1993). Obviously the major problem in this field is that these signal transduction processes are essential to the normal function of the cell so is it possible to attain the specificity necessary for anti-tumour agents to be useful without inhibiting cell proliferation? (Levitski, 1996, and Saltiel & Sawyer, 1996, for reviews). Work on the inhibition of the prenylation of Ras by farnesyltransferase inhibitors (FTIs) has shown that these inhibitors can indeed selectively block Ras-transformed cell growth without inhibiting general mitogenic activity (Cox & Der, 1997, for review).

Cussac et al. (1999) designed a Sos derived peptidimer which bound the Grb2 SH3 domains from the crystal structure of Grb2 (Maignan et al., 1995) and NMR structure of a peptide bound to an SH3 domain of Grb2 (Goudreau et al., 1994). This was found to display antiproliferative behaviour. Boriack Sjodin et al. (1998) suggested, from the crystal structure of Ras.Sos, that hydrophobic compounds that bound to the Ras binding site in Sos may efficiently inhibit Sos action. With the Ras.Sos crystal structure and biochemical assays developed in this work, design and analysis of such compounds is now possible.

9.5: Future work

It is possible that the interaction of the catalytic domain of mSos1 (CmSos1) with Ras is affected by the other domains of mSos1 in vitro. It would be of obvious interest to obtain the full-length mSos1 in order to compare the activity and mechanism of action with N-Ras. The size of the protein is an obvious hindrance, but low yields have been obtained by Frech et al. (1995) from Baculovirus. Attempts in our laboratory have
been unsuccessful (K.Nurmohamed, unpublished data). It could be possible to either incorporate the optimised gene fragment into the full-length clone or repeat the optimisation on the other domains of the mSos1 gene to see if that enabled expression of the full-length protein. In the light of recent data on the role of the DH domain as an exchange factor for Rac1 (Nimnual et al., 1998) it would be interesting to see if a dual function for mSos1 could be observed in vitro.

Initial experiments using Pressure-Jump apparatus to measure the rate constants for the association and dissociation of CmSos1 and N-Ras.mdGDP (and N-Ras.mdGTP) has shown promising results. This enables the reaction to be followed over even faster time scales than the stopped-flow apparatus. A precise measurement of these figures would enable a tighter simulation of the results to be performed. Small signals were also obtained on titration of CmSos1 into N-Ras.mGMPPNP following heat changes using Isothermal Calorimetry apparatus in an attempt to confirm the anisotropy data of section 5.4. Such a technique could be used to measure the equilibrium constants not obtained in this work; for nucleotide free N-Ras to GDP/GTP and nucleotide free N-Ras to CmSos1. Alternatively similar experiments as Lenzen et al. (1998) performed with H-Ras and Cdc25Mm could be tried using equipment such as the Biacore (discussed earlier) to measure those constants.

The role of Mg\(^{2+}\) in the interaction between CmSos1 and N-Ras also needs to be investigated. Lenzen et al. (1998) in their study of the action of Ccdc25 on H-Ras showed that Ccdc25 is still able to increase the GDP dissociation rate from H-Ras in the presence of 15 mM EDTA. This suggested that the mechanism of action is, in part, independent of the Mg\(^{2+}\) binding site. This is as expected from the crystal
structure of Ras.Sos. From the studies in this thesis, it appears that the Mg$^{2+}$ ion does not greatly affect the stability of the Ras.Sos complex. However, it is necessary to study the effect of Mg$^{2+}$ on the catalysis of CmSos1 and binding constants of the CmSos1 and Ras interaction.

The crystal structures of the ternary complexes (both N-Ras.GDP.CmSos1 and N-Ras.GTP.CmSos1) would be very useful for further investigation of the mechanism. Simulation of the data in this work has shown that significant populations of these species should be obtainable with the highly concentrated N-Ras.GDP and CmSos1 now available.
Appendices

Appendix 1

Oligonucleotides used for the synthesis of the optimised CmSos1 gene. Oligonucleotides 1-37 cover the top strand (5'→3'), 38 - 75 the bottom strand (also 5'→3').

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<tr>
<td>34</td>
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<tr>
<td>37</td>
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Appendices

Appendix 2

Oligonucleotide primers for amplification of optimised gene fragments (QE,QK,AE,AK,RE and RK)

Top strand

<table>
<thead>
<tr>
<th>EcoRI</th>
<th>NdeI</th>
<th>BamHI</th>
<th>Q E E K</th>
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<tr>
<td>76</td>
<td>CGGGAAATTCATATGGGATCGCGCCCGGGCCATATCGAAACGTTC</td>
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<td></td>
</tr>
<tr>
<td>77</td>
<td>CGGGAAATTCATATGGGATCCCGCCGGGCCATATCGAAACGTTC</td>
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Bottom strand

<table>
<thead>
<tr>
<th>EcoRI</th>
<th>HindIII</th>
<th>PstI</th>
<th>XhoI</th>
<th>Ter</th>
<th>Ter</th>
<th>E Q Q</th>
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<tr>
<td>81</td>
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<td></td>
<td></td>
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