Ultrafast energy transfer from photoexcited tryptophan to the haem in Cytochrome c

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Abstract:

We report femtosecond Fe K-edge absorption (XAS) and non-resonant X-ray emission (XES) spectra of ferric cytochrome C (Cyt c) upon excitation of the haem (> 300 nm) or mixed excitation of the haem and tryptophan (< 300 nm). The XAS and XES transients obtained in both excitation energy ranges show no evidence for electron transfer processes between photoexcited tryptophan (Trp) and the haem, but rather an ultrafast energy transfer, in agreement with previous ultrafast optical fluorescence and transient absorption studies.[1,2] The reported decay times of Trp fluorescence in ferrous (~350 fs) and ferric (~700 fs) Cyt c are among the shortest ever reported for Trp energy transfer in a protein. The observed timescales cannot be rationalised in terms of Förster or Dexter energy transfer mechanisms and call for a more thorough theoretical investigation.

I. Introduction:

Tryptophan (Trp) is an abundant amino acid residue in biological systems that has emerged as one of the most important fluorophores in terms of its use to investigate protein dynamics. Indeed, the high sensitivity of its spectral and photophysical properties to the environment makes it an important local probe of protein sites, [3] and of protein dynamics by detecting changes in its photophysical properties. [4–10]

Trp is also involved in energy and/or electron transfer processes in proteins. It has long been established that Trp is a donor (D) molecule that can undergo Electronic Energy Transfer (EET) to an acceptor (A) molecule. One of the most common mechanisms of EET is the so-called Fluorescence Resonance Energy Transfer (FRET) formalised by Förster. [11,12] It applies in the very weak coupling regime between the two chromophores and the conditions for this mechanism are most commonly met in coupled molecular systems. In FRET, molecules D and A are separated by a distance (R_{DA}) substantially larger than their dimensions and the coupling is approximated by the interaction energy of two electric dipoles localized on the chromophores. The EET rate is strongly sensitive to the distance between D and A as it varies as $(1/R_{DA})^6$ and depends on the relative orientations of the D and A dipoles and the spectral overlap of the D fluorescence with the A absorption. The distance at which EET is 50% efficient is called the Förster radius, and is determined by these three parameters (distance, orientation, overlap). Because of this high sensitivity to EET, Trp has emerged as the "molecular ruler" in FRET studies of protein dynamics. [13,14]

Another relaxation pathway involves Electron Transfer (ET), which is described by the Marcus theory. [15] Its rate depends exponentially on R_{DA}, with a decay factor of 1.1 $Å^{-1}$ describing most systems, [16] such that excited-state ET is significant for distances below 20 Å, [14] but multistep reactions can transport charges over distances of 30 Å or more. [17] Because of its low oxidation potential, Trp is involved in multistep electron transfer (ET) processes in proteins and DNA. [18–23] For example, in cytochrome c (Cyt c), shown in Figure 1a, the sole tryptophan (Trp59) of the protein serves as a bridge for the ET pathway from His39 to the haem. [24] This raises the question of the involvement of ET in the decay kinetics of photoexcited tryptophan (*Trp). Intraprotein tryptophan fluorescence quenching by peptide bonds [25,26] and various amino-acid residues [27] has been reported, and was attributed to electron transfer (ET) from the excited indole moiety to nearby electrophilic residue(s). A systematic study of these quenching processes was carried out by Qiu et al [8] using site-directed mutagenesis and femtosecond-resolved fluorescence spectroscopy. In all these studies, the typical time scales reported for EET and ET are on the order of 10 to 100 ps.

Myoglobin (Mb) or the Mb-like monomers of haemoglobin (Hb), contain two Trp residues: Trp7 that lies 21.2 Å (centre-to-centre) from the haem, and Trp14 that lies 15.2 Å. These distances are much larger than the individual sizes of the Trp and the haem. The fluorescence decay of photoexcited Trp (*Trp) in these systems has been attributed to EET to the haem. [9,10,28–32] However, recent studies using two-dimensional (2D) deep-ultraviolet (UV) Transient Absorption (TA) and UV pump/visible probe TA spectroscopy of ferric Cyano-Myoglobin (MbCN) and met-Myoglobin (metMb or MbH_2O) [33] and of ferrous deoxymyoglobin (deoxyMb) [34] have found that *Trp is also involved in ET processes. While it has been experimentally observed that Trp7 undergoes EET to the haem, a mechanism confirmed by calculations, [28–32] these experiments [33,34] revealed that *Trp14 decay undergoes both EET and ET to the haem porphyrin at comparable rates. This observation accounts for the deviations between the calculated EET rates and the measured *Trp14 fluorescence decay rates. [29,30] It was also suggested that ET proceeds in a multistep fashion via the Leucine 69 (Leu69) and Valine 68 (Val68) amino acid residues. [34] This was confirmed by theoretical modelling [35] based on TDDFT and DFT theory and on the pathway model by Beratan and co-workers. [36,37]

Therefore, the results obtained on the decay of Trp7 and Trp14 in Mb's fully fall within the trends predicted by the Förster and Marcus theories. [14] In this respect, the smaller the R_{DA}, the more efficient the ET, overwhelming the EET. This would in principle be the case with ferric and ferrous Cytochrome c (Cyt c), because their single Trp59 residue lies ~9 Å (centre-to-centre) from the haem and its centre-of-mass is located almost in the plane of the porphyrin, while the indole plane forms an angle of ~70-80° with the latter. The distance and orientation of Trp59 with respect to the haem are shown in Figures 1b for ferric Cyt c and figure S3 for both ferric and ferrous Cyt c.

Ultrafast fluorescence up-conversion studies with polychromatic detection showed that the Stokes-shifted (from its absorption maximum at 280 nm) Trp emission has its maximum around 370 nm and is very broad, spanning from ~325 to ~400 nm, in both ferric and ferrous Cyt c.[1] The decay of Trp fluorescence is significantly shorter in ferrous Cyt c (~350 fs) and ferric Cyt c (~700 fs) [1] than in Mb's, [29] and furthermore so compared to aqueous solutions (~3 ns). [4] These decay times reflect a Trp fluorescence quantum yield (QY) in Cyt c's of the order of 10^{-5} considering that the QY of Trp in water is 0.13. [38] Similar decay times were retrieved by visible-ultraviolet transient absorption (TA) of Cyt c upon excitation of Trp,[2] directly confirming the occurrence of a Trp-to-haem EET and ruling out the occurrence of an ET process, which would be detected via reduction of the haem in ferric Cyt c, as was the case with

ferric Mb's. [33] However, studies by Gu et al [39] pointed out to a photoreduction of the haem in hemoproteins, and specifically in Cyt c, whose yield increases with excitation energy. More recently, fs-TA studies by Kovalenko and co-workers also reported photoreduction of ferric Cyt c with a risetime of ~5 ps of the reduced species. Photoreduction quantum yields of 16 % and 8 % upon 403 nm and 266 nm excitation, respectively, were also reported. However, the reported energy dependence contradicts the trends shown by the results of Gu et al. [39]

In order to further clarify the issues of ET vs EET in Cyt c, here we use femtosecond (fs) X-ray absorption (XAS) and non-resonant X-ray emission (XES) spectroscopies. These are ideal tools for determining oxidation state changes of specific atoms, [40,41] in a wide variety of systems. [42–46] Indeed, if the iron ion is the recipient of the transferred electron in ferric haem proteins, [33] an increase of electron density on the haem would be reflected in a red shift of the Fe K-edge absorption. Furthermore, oxidation and spin state changes are detectable by X-ray emission spectroscopy (XES). [47] In a previous study using fs XAS and XES, we investigated the photodynamics of ferric Cyt c upon excitation of the haem porphyrin at 350-400 nm, [48] while a similar study had already investigated the case of ferrous Cyt c. [49,50] The conclusions of these studies are that a structural change of the porphyrin from planar to domed takes place, which is due to a population cascade through an intermediate spin state to the final high spin excited state of the haem. This is followed by relaxation back to the ground state. In the case of ferrous Cyt c, dissociation of the methionine ligand occurs in the early steps of the cascade, while this is not the case for ferric Cyt c. The entire relaxation process in both forms of Cyt c occurs in < 10 ps in very good agreement with optical TA studies.[2] A fairly similar picture of the relaxation processes emerged from a study of ferrous nitrosyl-myoglobin (MbNO) upon excitation of its haem. [51]

The absorption of Trp in Cyt c sets in at ~300 nm, while absorption at longer wavelengths only excites the haem (Figure S1). Here we investigate the response of ferric Cyt c to photoexcitation in the region <300 nm, which reaches both the Trp and the haem, and we compare it with the response of the system upon excitation of the haem only (>300 nm). We find that the rates of electronic relaxation agree with those found in optical studies [1,2] and we observe no evidence for a photoreduction, ruling out ET. In addition, the decay rate of *Trp to the haem cannot be explained in terms of dipole-dipole energy transfer (FRET).

II. Results:

We compare the fs-XAS and XES transients exciting both above (haem only) and below (Trp and haem) 300 nm. The Fe K-edge XAS and Fe K_α XES experiments were carried out at the

SwissFEL (Paul-Scherrer-Institute, Villigen), [52] while further K_α and K_β XES measurements were performed at the European XFEL (Eu-XFEL, Hamburg). Details of the experimental schemes are given in § S1. In the SwissFEL experiments, we used two pump wavelengths: 288 nm and 350 nm. At 288 nm, ~30% of the absorption is due to Trp and ~70% to the haem (Figure S1). [33] At 350 nm, excitation is 100% to the haem. The choice of this wavelength is motivated by the fact that it lies around the maximum of the Trp fluorescence in Cyt c. [1] As already mentioned above, the ultrafast optical TA and fluorescence studies pointed to an energy transfer from the emissive Trp energy. [1,2] At the Eu-XFEL, excitation wavelengths of 400 nm and 266 nm were used. The former is resonant with the Soret band of the porphyrin, while the latter excites both the Trp and the haem at almost identical proportions as at 288 nm. The experimental set-ups have been described in detail elsewhere [48] and are summarized in §S1. It is important to emphasize that the laser fluence was chosen such that the overall absorbance at the different wavelengths is very similar.

Figure 2 shows the steady-state Fe K-edge absorption spectrum of ferric Cyt c (black trace), which agrees with the literature. [53] It exhibits a pre-edge feature at 7112 eV, due to the Fe 1s-3d transition, followed by the edge at 7120 eV and the XANES modulations above. The transients at 500 fs delay for 288 nm (blue) and 350 nm (red) excitation are also shown. The signal of both transients is weak (S/N=2-3) and is identical within error bars. The transients are characterised by an absorption increase at the edge (7124 eV) followed by a negative signal between 7130 and 7160 eV. As discussed in ref. [48] for the 350 nm excitation, the XANES transient are explained in terms of haem doming, identical to the case of ferrous haem proteins, such as Myoglobin-CO, [54] Myoglobin-NO, [55] and ferrous Cyt c. [56] That the transient for 288 nm excitation is identical points to the fact that no reduction occurs at this excitation wavelength either. However, considering the poor S/N and in order to make the point, it is worth noting that as shown in ref. [48] and reproduced in figure S4, the 350 nm transient clearly deviates from the transient expected in the case of photoreduction, which we simulate by taking the difference between the red shifted ground state spectrum minus the ground state spectrum. This simple procedure generally accounts for the expected general trends. [42,44,57]

Further evidence ruling out a photoreduction comes from the transient $K_{\alpha 1}$ and $K_{\alpha 2}$ XES spectra under 266 nm and 400 nm excitation, shown in Figures 3 and S5. They show no noticeable difference within error bars, confirming the conclusions of the XAS transients.

Figure 4 compares the time traces of the XANES signal at 7123 eV (maximum in Figure 2) up to 25 ps time delay upon 288 and 350 nm excitation. The inset zooms into the first 5 ps. Both

signals appear promptly, within the cross-correlation of the experiment (~150 fs) but they show somewhat different decay profiles, which can best be fit using bi-exponential functions convoluted to the cross-correlation of the experiment, and having similar time constants, of typically ~600 fs and ~9-10 ps. The fit parameters are given in table 1 and are identical (within the error bars) to those of ultrafast optical experiments also given in this table. [1,2] Under 288 nm excitation, the short component is more prominent than the long one (the ratio of pre-exponential factors is $a_1/a_2 \approx 3$) compared to the 350 nm excitation ($a_1/a_2 \approx 2$). This can be rationalized on the basis of the ultrafast optical fluorescence and TA studies, [1,2] which show that the Trp fluorescence in ferric Cyt c decays in ca. 700 fs, feeding population to the haem. This component should appear as a rise time of the haem signal but because the initial intrahaem relaxation occurs in < 100 fs, [2,58] the ~700 fs component becomes rate-determining and therefore it shows up as a decay adding up to the component, on the same time scale, due to relaxation among spin excited states of haem. Indeed, in our previous fs-XAS study using 530 nm excitation, [48] we had identified a ~770 fs component as the relaxation from the S=3/2 to the S=5/2 spin states of the haem. In the event of an ET from *Trp (excluded for 350 nm excitation), its ~700 fs fluorescence decay should have shown up as a rise time of the newly formed reduced haem as was observed for the ferric metMb and MbCN species, [33] which is not the case here. Important for the EET from Trp to the haem is the short component observed in the X-ray and optical studies (~350 fs for ferrous and ~700 fs for ferric Cyt c) since these time scales reflect the decay of the Trp fluorescence. The other components (~700 fs and ~10 ps) are due to intramolecular processes. [2,48] In the following, we focus on the decay time of the Trp fluorescence.

In summary, we can rule out the occurrence of electron transfer from *Trp to the haem and conclude that an energy transfer occurs instead based on the combination of ultrafast optical TA and fluorescence studies and the present X-ray ones:

- Fluorescence up-conversion [1] establish that *Trp fluoresces from its Stokes-shifted energy of ~350 nm with lifetimes of 350 and 700 fs in the ferrous and ferric forms, respectively.
- Optical transient absorption studies [2] of Cyt c's under 266 nm show a response of the haem to Trp excitation recovering the same time scales as the *Trp fluorescence decay times. Furthermore, no signature of reduction due to electron transfer from *Trp is observed.
- the present results confirm those of optical TA in that EET from *Trp to haem occurs and no ET is detected.

III. Discussion:

The above results point to extremely fast decay times of *Trp in Cyt c's. Indeed, ultrafast *Trp fluorescence has been investigated in a large class of proteins such as myoglobins,[28–31,33,34] Thioredoxin, cutinase, nuclease and calmodulin, [8] subtilisin, [59] Monellin [60] and the IIA^{Glc} protein, [61] in metalloazurins, [62] The decay times were multipexponential with components spanning from a few ps to several tens or hundreds of ps. Thus, the decay times for *Trp in ferric and ferrous Cyt c are, to our knowledge, the shortest reported so far, and they reflect a strong interaction of the excited Trp with the haem. An even shorter time (~200 fs) was reported for Trp in bacteriorhodopsin, [63] but it needs to be confirmed as it was measured using optical TA rather than ultrafast fluorescence detection.

EET is usually discussed in the framework of the Fluorescence resonance energy transfer (FRET), [11] which relies on the Fermi Golden Rule and supposes an incoherent electric dipole-dipole interaction. It is also valid for D-A distance much larger than the individual sizes of the D and A molecules. This formalism also links the electronic matrix elements to the overlap of the D fluorescence and A absorption spectra. The distance at which the dipole approximation has been estimated to break down for FRET rate calculations is around 20 Å distance between the two coupled molecules.[14,66] In Cyt c, the distance is below this threshold: 9.6 Å and 9.9 Å in ferrous and ferric Cyt c, respectively, from the two molecular centres of masses and only 3.2 Å and 3.7 Å between the edges of the D and A molecules (Figure S3). In this case, the FRET calculations thus lead to much lower transfer rates than observed (see § S2 and Tables 1 and S1), while this violates the assumption that the distance between D and A (R_{DA}) to be much larger than the sizes of the D or A.

As mentioned above, observations of ferric and ferrous Cyt c XAS, XES and optical TA are not compatible with a change of the oxidation state of the iron, ruling out an ET relaxation pathway. Nevertheless, we did perform the calculations of the ET rates in Cyt c using the Marcus theory (§ S2). Here again, the decay rates are incompatible with the observed ones, although based on the considerations of *Trp decay in Mb's [33,34] would have implicated ET as the primary decay mechanism. Our calculations show that the ET yield is about 45% in ferric Cyt c and almost 100% in ferrous Cyt c, in clear contradiction with the optical TA studies. [2]

The main differences between the two forms of Cyt c arises from the edge-to-edge distance (R=3.16 Å vs 3.67 Å), and the slightly different angle between Trp and the porphyrin plane. The results of these calculations using FRET EET and ET are compared in Table 1 with the experimental results from both optical TA and X-ray studies. It should be stressed that in all of

the above calculations, we have used the crystallographic data, which of course, concerns the ground state. This excludes effects such as the structural relaxation in the excited state of both the donor and acceptor, which may also affect both distances and dipole orientations.

As the FRET model does not rationalise the present results, one may invoke the Dexter mechanism [65] which recognises that electron exchange interactions contribute to EET at small values of R_{DA}. The so-called Dexter EET mechanism can be viewed as a double electron transfer. [66] It requires overlap of D and A wave functions, which decays exponentially with distance. The short range of the interaction relegates exchange energy transfer, in most cases, to collisional processes. Of course, if operative this mechanism implies a double-electron process at time scale unobservable in our experiments due to a limited temporal resolution. However, this is very unlikely as with time scales of 350 or 700 fs, the occurrence of a reduced haem should have been transiently detected either by optical TA or by fs-XAS and XES, which is not the case.

Whichever the mechanism of energy transfer, with their high density of states porphyrins in general [58] and in Cyt c [2] in particular exhibit extremely efficient and fast intramolecular relaxation, so that they acts as a sink for the EET. The present energy transfer from *Trp to the haem occurs from the emissive state of the Trp donor, as the first event following its excitation is an ultrafast intramolecular relaxation [67] that causes a large Stokes shift of about 1.1 eV from 280 nm (absorption) to approximately 370 nm (emission). The emissive state partly overlaps the Soret band of the porphyrin, which has a very large transition dipole moment (2-4. 10^5 $M^{-1}cm^{-1}$).

We mentioned earlier the case of bacteriorhodopsin for which a Trp decay to the retinal chromophore of ~200 fs was reported in optical TA studies. [63] In this system, retinal is surrounded by 8 Trp's of which two (Trp86 and Trp182) are almost at contact distance to it. This vicinity results in an excitonic coupling observed in absorption, [68,69] and we are confident that all the ingredients are present for an efficient quenching of the Trp residues in bR.

In summary, we reported on X-ray absorption and X-ray emission studies of ferric Cyt c excited into the haem only (>300 nm) and into the region of tryptophan and haem absorption (<300 nm). We thus complemented earlier ultrafast optical fluorescence [1] and transient absorption [2] studies, confirming the conclusions that the Trp fluorescence decays due to an energy transfer to the haem, and ruling out any possible ET mechanism. The EET time scales in ferric (700 fs) and ferrous (350 fs) Cyt c are the fastest EET times ever reported for Trp as a donor. Surely, the results presented here call for a deep theoretical treatment that can rationalise

them and provide a mechanism for the efficient EET.

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Authors' contributions: CB, JRR, RAI, GFM, DK, OC, CC, GK, JS, FAL, SM, DO, GP, KK, DK, WG, ARF, MB, CB, CAA, PJMJ, CJM carried out the measurements. CB and JRR analysed the data. JRR and YZ performed the calculations, MC conceived the experiment. CB, JRR and MC wrote the paper.

Data Availability. Raw data were generated at SwissFEL and the European XFEL large-scale facilities, and due to the nature and quantity of data produced, are available upon request. All derived data supporting the findings of this study and the corresponding analysis scripts are available through the Open Science Repository: https://osf.io/a7xcm/?view_only=bb984adcdde1425ba3d15c837cd4f86b.

Table 1: Summary of the observed and computed decay timescales in Ferric and Ferrous Cyt c. The XAS data for ferric Cyt c is presented for 288 and 350 nm excitation, while for ferrous Cyt c, it is for 530 nm excitation. All entries are in ps.

Species	**$\lambda_{exc.}$ (nm)**	**X-ray absorption Time constant (pre-exponential factor)**	**Optical TA [2]**	**FRET-EET**	**Marcus ET**
Fe^{3+} Cyt c	288 350	0.54±0.2 (0.65) /10±4 (0.25)* 0.61±0.2 (0.7) / 9±4 (0.3) [48]	0.7 / 11	3.9	1.8
Fe^{2+} Cyt c	520	5.9 [49]	0.35 / 5.9	2.8	0.5

* This work

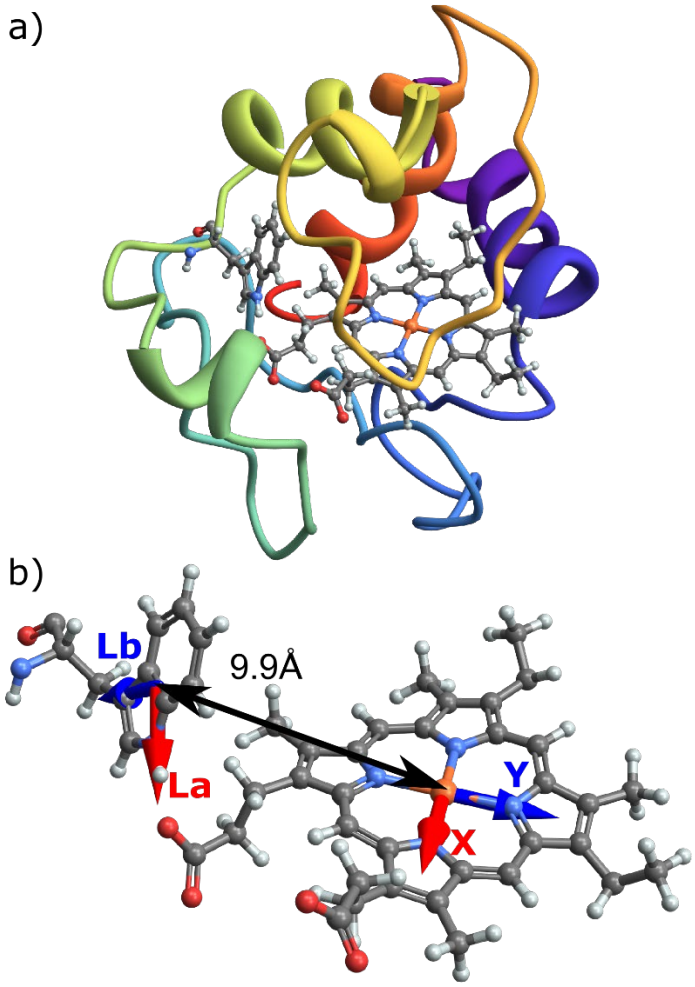

Figure 1: (a) Structure of ferric Cyt c. (b) Zoom into the haem and the tryptophan residue pair. The orientation of their relevant electric transition dipoles is highlighted with colours.

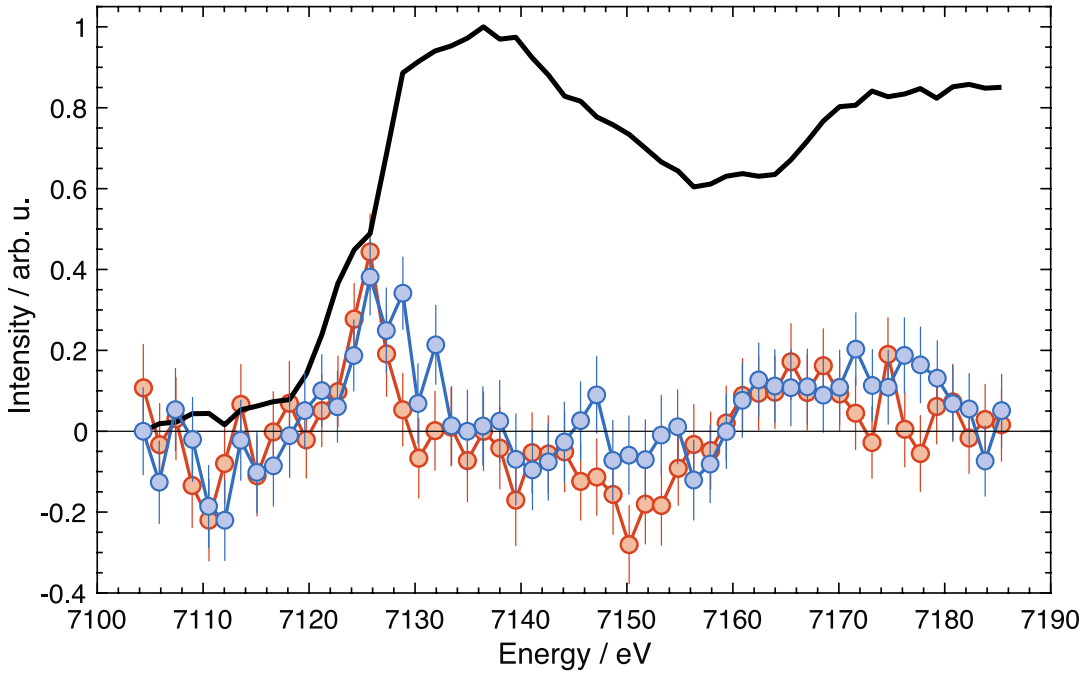

Figure 2: Steady state Fe K-edge absorption spectrum of ferric cytochrome c (black) and the transients (excited minus unexcited) at 500 fs time delay after excitation of the haem only at 350 nm (red) and of the haem and tryptophan at 288 nm (blue).

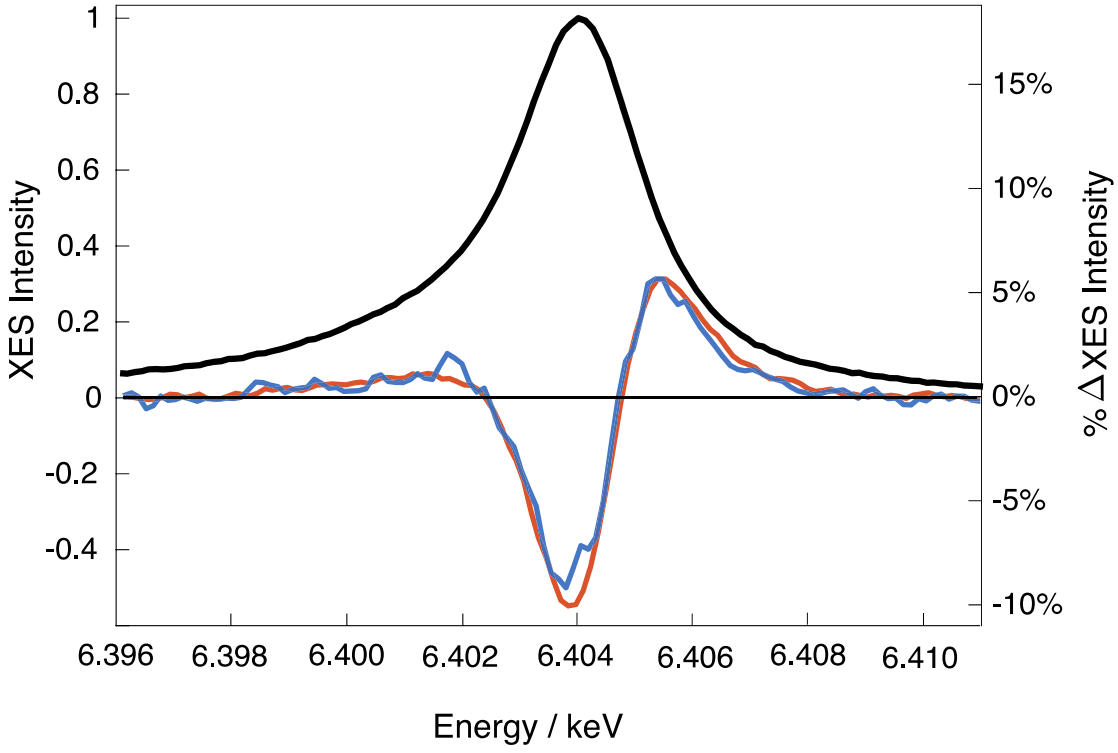

Figure 3: Steady-state (black) non-resonant Fe Kα X-ray emission spectra of ferric Cyt c and

the transients at 200 fs time delay, upon 400 nm excitation (red) and 266 nm excitation (blue).

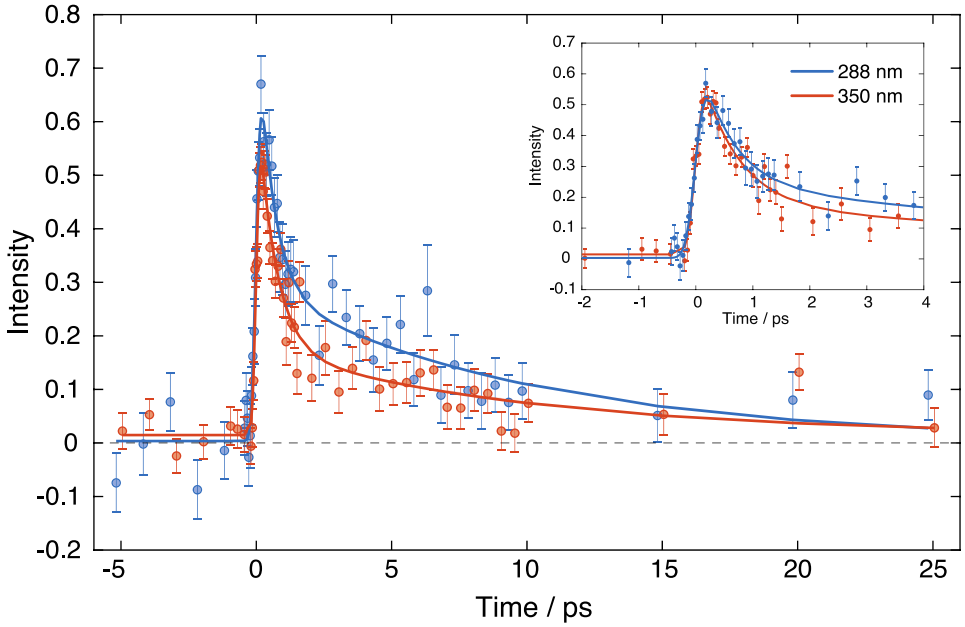

Figure 4: Kinetic traces of the XANES signal at 7.123 keV of ferric Cytochrome c for 288 nm and 350 nm excitation. The solid lines are fits using a biexponential function, whose parameters are given in Table 1.

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