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Energy transfer and relaxation mechanisms in Cytochrome *c*

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ABSTRACT

Using broadband UV–vis femtosecond transient absorption and fluorescence up-conversion, we investigate the interaction between the haem moiety of ferric and ferrous Horse heart Cytochrome *c* and its single Tryptophan (Trp) residue and the energy dissipation mechanisms upon excitation at various wavelengths in the visible and the UV. Varying the amount of energy deposited in the haem does not affect the relaxation and cooling processes. Differences are observed between the cooling time-scales of the two redox states, which are attributed to different haem–protein couplings. While energy transfer from the Trp to the haem is observed in the decay of Trp and the response of the haem, excitation of the latter does not induce a clear response of the former. This suggests that for Cytochrome *c*, Trp is not a good marker of the protein response, probably due to its orientation with respect to the haem plane.

1. Introduction

Understanding the signaling through a protein is fundamental for describing its functionality [1]. Among different possible long-range interaction mechanisms, the redistribution of the localized excess of energy generated by biological functions plays an important role, as it induces conformational changes of the entire protein [2].

Optical methods offer a clean way to deposit a controlled amount of energy on a specific protein site and to monitor the following processes. The occurrence of global protein dynamics triggered by localized excess of vibrational energy (EVE) was invoked for haem proteins [2,3], where ligand photolysis by visible light induces a sub-100 fs rise of the haem temperature by up to 300 K [4]. The issue of how, and how fast, energy is redistributed and dissipated in this family of proteins has been widely addressed by experiments [5–7] and molecular dynamics (MD) simulations [4,8–10].

Horse heart Cytochrome *c* (hh Cyt *c*, Cyt *c* in the following) is a relatively small membrane-bound haem protein which plays an important role in many electron transfer processes. Its cofactor is a 6-coordinated iron porphyrin, where the iron is axially bound to a proximal Histidine (His18) and a distal Methionine (Met80). The protein exists in both ferrous and ferric redox states. Upon photoexcitation, the ferrous state undergoes Met80 detachment, followed by a fast biphasic recombination on time scales of 5 and 16 ps. On the contrary, no signatures of photodissociation have been observed in the ferric state [5]. Cyt *c* contains a single Tryptophan residue at 9 Å from the haem and its fluorescence is strongly quenched by Fluorescence Resonance Energy Transfer (FRET) to the haem group [11,12]. The center of mass of the Trp is located almost in the plane of the porphyrin and its indole plane forms an angle of ~70°–80° with the latter.

Cyt *c* is a very promising system to investigate energy redistribution in proteins, since molecular dynamics (MD) simulations have pointed to a very weak influence of the solvent on the energy relaxation [13]. In fact, in contrast to other hemoproteins such as Myoglobin, where the cofactor is almost in contact with the solvent, the haem-pocket of Cyt *c* is buried well inside the protein. The thermal bath of the cofactor is therefore the protein itself. MD simulations determined the Cyt *c* thermal relaxation to be mono-exponential on a ~7 ps time scale, independent of the redox states [13]. Time-resolved resonance Raman (TR^3) results showed instead a two-phase peak position shift of the ν_4^* band in both compounds, on a sub-2 ps and a ~7 ps time scales [5]. These results speak for a more complex temperature relaxation of the haem. Moreover, the authors suggested that the time scales of the intramolecular vibrational energy redistribution are longer in the ferrous moiety, which they attributed to the haem doming induced by Met80 photolysis. The 7 ps temperature relaxation does not appear in transient absorption measurements in the visible range, while pump-probe studies on the two redox species reported different cooling time scales, generally longer for the ferric form [3,5,14]. The extent to which the cooling time scales are affected by the excess of vibrational energy is still not clarified.

The use of naturally occurring amino acids (Tryptophan, Tyrosine), which absorb below 300 nm, as probes of local protein dynamics has been demonstrated both in ultrafast transient absorption measurements of retinal [15,16] and haem proteins

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[3], as well as in ps UV-resonance Raman experiments on retinal [17,18] and haem [19] proteins. Dartigalongue et al. [20] also performed ultrafast transient absorption and circular dichroism (CD) on Mb-CO in the 220–360 nm region, concluding that the time-resolved response is mainly due to the haem, while the amino-acids do not contribute significantly. On the other hand, they reported a strong CD change at 230 nm, which they suggested may be due to a transient deformation of the α-helices in the protein. Zhong and co-workers [3] also reported a possible global conformational dynamics following dissociation of the ligand in ferrous Cyt *c*.

Here we report on UV–Vis pump/white-light probe transient absorption measurements of ferric and ferrous Cyt *c* excited at 530 nm and 288 nm. In the former case the lowest haem optical transition (the so-called Q band) is accessed, while the latter corresponds to excitation of both the porphyrin and the Trp. The main novelties of our approach are: (i) we use an ultra-broadband fs probe covering the 670–270 nm range, thus accessing both the Trp absorption [21] and the less exploited porphyrin related absorption transitions [22–25]; (ii) by moving the excitation from 530 nm to 400 nm and to 288 nm, almost 1.2 and 2 eV, respectively, of additional EVE are deposited impulsively in the haem group [26] which, according to the MD simulations [4,13], correspond to a temperature increase of up to ~400 K; (iii) the comparison of the two redox states allows us to disentangle cooling from structural ligand detachment dynamics.

2. Experimental methods

2.1. Sample preparation

Horse heart Cyt *c* was purchased from Sigma Aldrich. The compound was dissolved without any further purification in pH 7 phosphate buffer (30 mM/l KH_2PO_4 + NaOH). No oxidant was added to the solution with ferric Cyt *c*. Ferrous Cyt *c* was prepared by reduction of ferric Cyt *c* in pH 7 buffer by addiction of sodium dithionite ($Na_2S_2O_4$). The salt was then removed by means of gel-filtration desalting columns. Static UV–visible absorption (see Fig. 1) was used to estimate the content of the two redox states and the quality of the sample before and after the measurements. In this way we verified the almost unitary content of the desired state and a negligible content of photo-damaged molecules after laser exposure.

For TA measurements, the concentration of all the samples was adjusted to 0.39 OD at 290 nm in a 0.5 mm cell when probing in the UV, corresponding to a concentration of 0.41 mM. When probing in the visible range, the concentration was reduced to ~0.29 mM. For the visible pump – visible probe experiments on the ferric sample, the concentration was further reduced to ~0.10 mM.

2.2. Transient absorption setup

The experimental setup was already described elsewhere [27]. Briefly, the output of a Regenerative Amplifier (typically 650 μJ at 1 kHz) is used to pump two home-made NOPAs, which produce tunable broadband visible pulses in the range 500–750 nm with a typical length of 40 fs. One of the NOPAs is used to produce either the 530 ± 20 nm broadband pump beam or the UV 288 nm pulse after frequency doubling in a 250 μm β-BBO crystal. The other NOPA is used to produce the broadband UV probe pulse through achromatic doubling in a 150 μm β-BBO crystal. The visible probe is obtained through continuum generation by focusing the 800 nm pulse in a CaF_2 crystal. The pump and probe beams are typically focused into 110 and 90 μm diameter spots, respectively, at the sample position. Typical pump energies are 130 nJ for UV excitation and 160 nJ for visible excitation. The sample was circulated in a 0.5 mm thick UV-grade quartz cell to avoid photo-damage; the flow speed was adjusted to have refresh the sample after each excitation pulse, to avoid re-excitations of the same molecules or heating effects. The pump-probe cross-correlation time, estimated from cross-phase modulation measurements in pure water, was ~160 fs.

The TA signal at each delay time are calculated accordingly to the formula

$$\Delta A = 2 \ln\left(\frac{S_p - S_u}{S_p + S_u}\right) \quad (1)$$

where S_p and S_u are the spectra of the pumped and unpumped sample, respectively. The polarization of the pump with respect to the probe pulse was controlled with a half-wave plate and was set at magic angle (MA).

The TA signals in the spectral region between 400 and 420 nm are distorted because of the high optical density (OD) of the sample. Indeed, when the sample content is adjusted to have a typical value of 0.3–0.4 OD at the pump wavelength, the optical density in the Soret band region (see Fig. 1 and next paragraph) is 1.5 or more. This causes ⩾95% of the probe photons to be absorbed and the TA signal is thus calculated on a small number of counts on the detector. In this situation, small unavoidable contamination due to scattered light, usually negligible, increases the amplitude

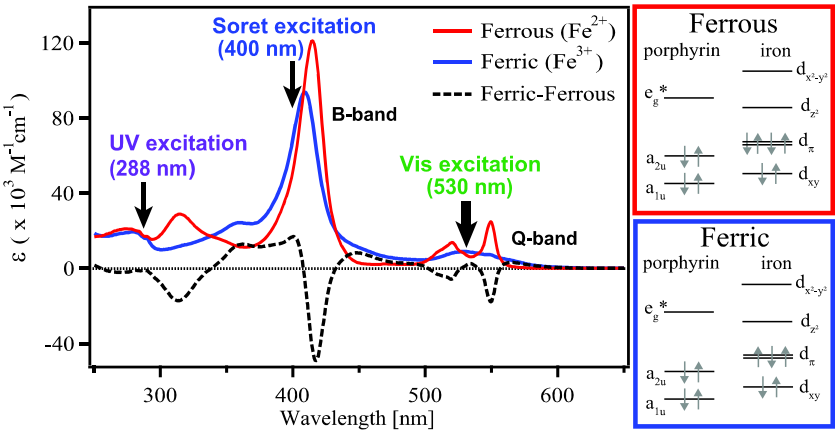

Fig. 1. Static electronic absorption spectra of ferrous and ferric forms of Cyt *c*. Arrows show the pump wavelengths used here. The difference spectrum of the ferric minus the ferrous form is shown as a dashed line. The panels on the right show a qualitative sketch of the ground state orbitals and their occupancy for ferrous and ferric Cyt *c*.