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Detecting transient protein–protein interactions by X-ray absorption spectroscopy: The cytochrome c_6-photosystem I complex

Irene Díaz-Moreno[a], Antonio Díaz-Quintana[a], Gloria Subías[b], Trevor Mairs[c], Miguel A. De la Rosa[a,*], Sofía Díaz-Moreno[d]

[a] *Instituto de Bioquímica Vegetal y Fotosíntesis, Universidad de Sevilla y Consejo Superior de Investigaciones Científicas, Américo Vespucio 49, 41092 Sevilla, Spain*

[b] *Instituto de Ciencia de Materiales de Aragón, Universidad de Zaragoza y Consejo Superior de Investigaciones Científicas, Departamento de Física de la Materia Condensada, Plaza San Francisco s/n, 50009 Zaragoza, Spain*

[c] *European Synchrotron Radiation Facility (ESRF), 6 rue Jules Horowitz, BP220, 38043 Grenoble Cedex, France*

[d] *Diamond Light Source Ltd., Rutherford Appleton Laboratory, Chilton, Didcot, Oxfordshire OX1 0QX, United Kingdom*

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Abstract **Reliable analysis of the functionality of metalloproteins demands a highly accurate description of both the redox state and geometry of the metal centre, not only in the isolated metalloprotein but also in the transient complex with its target. Here, we demonstrate that the transient interaction between soluble cytochrome c_6 and membrane-embedded photosystem I involves subtle changes in the heme iron, as inferred by X-ray absorption spectroscopy (XAS). A slight shift to lower energies of the absorption edge of Fe^{2+} in cytochrome c_6 is observed upon interaction with photosystem I. This work constitutes a novel application of XAS to the analysis of weak complexes in solution.**

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1. Introduction

The function of many metalloproteins relies both on their ability to form weak complexes upon binding to protein targets and on the chemistry of the metal cofactor, which is very sensitive to changes in its environment. A detailed structural characterization of the metal sites is thus essential for understanding their role in the functionality of metalloproteins. X-ray diffraction (XRD) by itself seldom yields accurate structural data on the metal centre, mainly due to the masking of the ligands by the Fourier ripples generated by the strong electron density at the metal atom. Recently, X-ray absorption spectroscopy (XAS) applied to crystalline samples has been shown to be an ideal technique to complement XRD and get further data on the chemical state and local structure of the metal site [1,2].

Many reactions between metalloproteins are mediated by transient complexes that cannot be easily co-crystallized and thus makes it difficult to study the subtle changes that may occur upon binding by XRD. On the contrary, XAS can be applied to the study of in solution samples, and provides simultaneous information on the electronic structure of the metal and its coordination geometry during complex formation. In this paper, we use XAS to accurately analyze the electronic and structural changes in metal centres during binding in transient complexes between metalloproteins in solution at physiological pH. Thus cytochrome c_6 (Cc_6) and membrane-embedded photosystem I (PSI), which interact with each other by forming a transient complex, were used as a model system to study the effect of binding on the environment of the Cc_6 heme metal centre. Such a weak complex has never been co-crystallized, but the physiological reaction between Cc_6 and PSI has been extensively studied by NMR in solution [3].

Early comparative XAS studies on isolated *c*-type cytochromes have shown that the heme co-ordination geometry is highly conserved, independently of its redox state [4,5]. However, no data has been reported with regard to the changes in the heme moiety upon binding of a cytochrome to its targets. Here, we show that XAS is sensitive enough to detect subtle changes in the electronic structure of the metal centre of Cc_6 upon binding to PSI, even though no measurable changes in the co-ordination geometry occur.

2. Materials and methods

2.1. Protein samples

Nostoc sp. PCC 7119 Cc_6 was purified from *Escherichia coli* cells with the pEAC-WT plasmid [6]. In order to prevent Cc_6 oxidation and eliminate any other contribution apart from that of the Cc_6 heme group, the metal atoms of the Fe–S clusters in *Nostoc* sp. PCC 7119 PSI were replaced by Hg ions. Hg-containing PSI (PSI^{Hg}) experiences a faster charge recombination than native PSI. The PSI^{Hg} samples were obtained as described previously [7,8], with minor modifications (see *Supplementary Material*). No evidence of changes in the interaction between Cc_6 and PSI due to PSI treatment with $HgCl_2$ was observed.

For the X-ray absorption experiments, the Cc_6 sample was concentrated by ultrafiltration methods and exchanged into 10 mM sodium phosphate pH 6.0 solutions. After concentration, a stock solution of 2 mM for Cc_6 was obtained. Protein concentration was determined by optical spectroscopy using a value of 26.5 $mM^{-1}\,cm^{-1}$ for the

*Corresponding author. Fax: +34 954 460065.
E-mail address: marosa@us.es (M.A. De la Rosa).

Abbreviations: (Cc_6), Cytochrome c_6; EXAFS, extended X-ray absorption fine structure; FT, Fourier transform; PSI, photosystem I; PSI^{Hg}, Hg-containing photosystem I; XANES, X-ray absorption near edge structure; XAS, X-ray absorption spectroscopy; XRD, X-ray diffraction

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molar extinction coefficient at 553 nm [9]. A 1.0 mM PSI^{Hg} sample in 10 mM sodium phosphate pH 6.0, supplemented with 0.025% (w/v) β-dodecyl-maltoside, was used.

Oxidized Cc_6 (Cc_6^{III}) was obtained upon addition of an equimolecular amount of potassium hexachloroiridiate (K_3IrCl_6) to the stock solution. The oxidizing agent was kept in the samples with free Cc_6^{III}, but it was removed from the samples with the Cc_6^{III}-PSI^{Hg} complex because of its unknown effect on the complex interface. The oxidized complex (Cc_6^{III}-PSI^{Hg}) was thus obtained by adding small aliquots of a 1.0 mM PSI^{Hg} solution onto a K_3IrCl_6-free Cc_6^{III}, sample up to reach a final concentration of ca. 0.8 mM each. Under these conditions, the percentage of Cc_6 bound to PSI^{Hg} was estimated to be ca. 70%, according to the binding affinity constant calculated previously [9].

Reduced Cc_6 (Cc_6^{II}) was obtained upon addition of a tenfold excess of sodium ascorbate to the stock solution. The reduced complex (Cc_6^{II}-PSI^{Hg}) was obtained by adding 5 mM sodium ascorbate and 10 mM sodium dithionite to Cc_6^{III}-PSI^{Hg} samples, which were kept in the dark to maintain PSI^{Hg} in its reduced state. In both cases, the reducing agents were added in excess to guarantee total reduction of the heme protein, as also confirmed by recording their UV–Vis spectra.

2.2. XAS measurements

The X-ray absorption spectra were recorded at the European synchrotron radiation facility (ESRF) in Grenoble (France), which was operating with a ring current of 200 mA and energy of 6 GeV. The fluorescence spectra at the energy of the Fe K-edge (7112 eV) was measured at beam line BM29 [10] using a double crystal monochromator fitted with a pair of flat Si(1 1 1) crystals detuned to 50% fwhm of the maximum transmission, for the suppression of high energy harmonics. The vertical aperture of the pre-monochromator slit was 0.7 mm giving an estimated energy resolution for the spectrometer of $\Delta E/E \sim 1.4 \times 10^{-4}$.

All measurements were made at room temperature in fluorescence mode, using a Canberra 13-element solid-state germanium detector. The incident beam intensity was monitored by an ionization chamber placed before the sample filled with the appropriate mixture of gases in order to absorb 30% of the incident beam. Energy calibration was achieved by measuring the absorption spectrum of an iron foil simultaneously in transmission mode using ionization chambers filled with the appropriate mixture of gases to absorb 30% and 70% of the remaining beam that passed through the measured sample. The energy stability of the spectrometer throughout the entire experiment was better than 0.1 eV.

For all measurements, each data point was collected for 4 s, and on average 10 scans were collected to achieve a good signal-to-noise ratio. In no case radiation damage was observed, even in those samples in which the measurements were made over several hours. With oxidized Cc_6 (either free or bound to PSI^{Hg}), the edge remained at the same position from the first to the last scan, thus indicating the absence of protein photoreduction.

2.3. XAS data analysis

The average X-ray absorption near edge structure (XANES) and extended X-ray absorption fine structure (EXAFS) signals have been extracted from raw data following standard procedures [11]. The average XANES spectra were normalized to the absorption at high energies (ca. 80 eV above the absorption edge) after a linear background subtraction. EXAFS spectra were obtained by removing the smooth atomic absorption coefficient (μ_0) by means of a cubic spline fit. The Fourier transforms (FT) of the k-weighted EXAFS spectra was calculated using a Hanning window.

To analyze the EXAFS spectra, the theoretical phases and amplitudes were calculated using FEFF 6.0 code [12]. The fit to the experimental data was performed using the Artemis program (version 0.7.008) [13,14].

3. Results

3.1. XANES region

The XANES region of the spectra corresponding to free Cc_6 in its reduced and oxidized states is shown in Fig. 1a. The two spectra are very similar, including the pre-edge feature at ca. 7113 eV, which corresponds to the forbidden transition 1s–3d [5,15,16]. The intensity of the transition is consistent with the proposed octahedral geometry of the Fe centre in these metalloproteins. The main difference is the higher energy value at which the absorption edge of the oxidized form

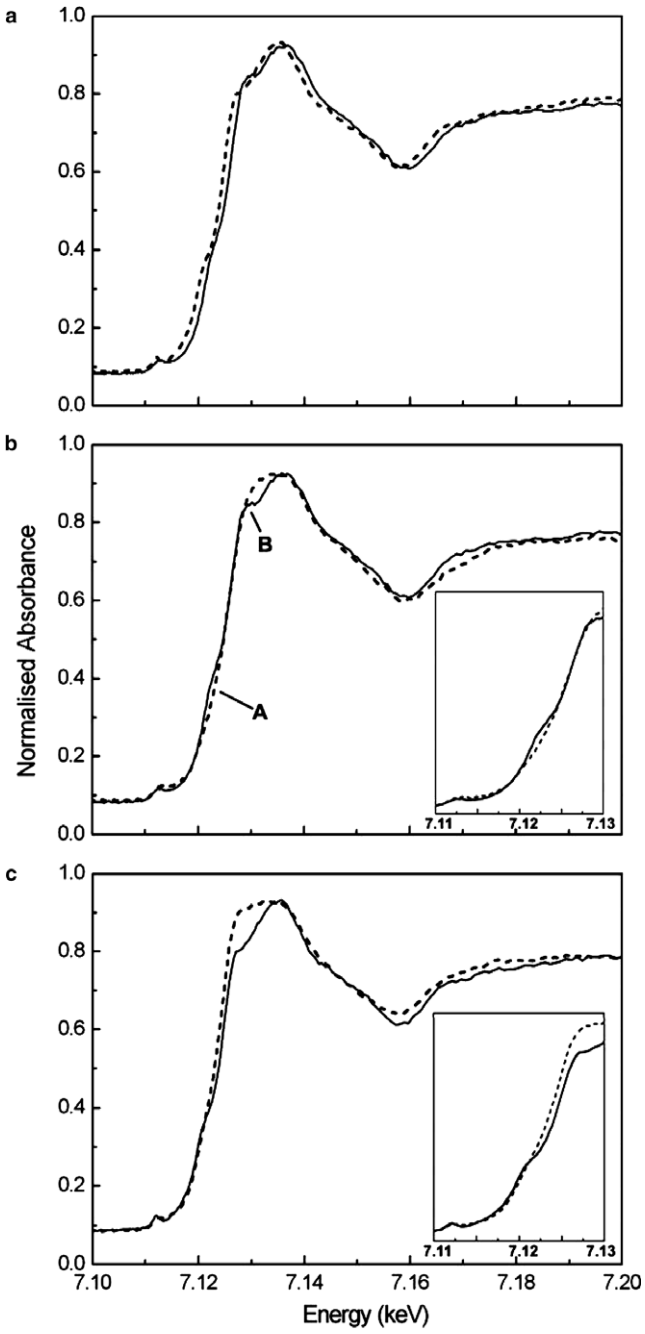

Fig. 1. XANES region of the absorption spectra at the Fe K-edge of free and PSI^{Hg}-bound Cc_6. (a) Experimental data for the oxidized and reduced forms of the free protein, namely Cc_6^{III} (continuous line) and Cc_6^{II} (dashed line). (b) Data for the oxidized species, namely free Cc_6^{III} (continuous line) and Cc_6^{III}-PSI^{Hg} complex (dashed line). (c) Data for the reduced species, namely free Cc_6^{II} (continuous line) and Cc_6^{II}-PSI^{Hg} complex (dashed line). See text for an explanation of features A and B in the middle panel. Enlarged absorption edge spectra from 7.11 to 7.13 keV are shown in the insets in panel b and c.

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