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Transient binding of plastocyanin to its physiological redox partners modifies the copper site geometry

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Abstract **The transient complexes of plastocyanin with cytochrome *f* and photosystem I are herein used as excellent model systems to investigate how the metal sites adapt to the changes in the protein matrix in transient complexes that are involved in redox reactions. Thus, both complexes from the cyanobacterium *Nostoc* sp. PCC 7119 (former *Anabaena* sp. PCC 7119) have been analysed by X-ray absorption spectroscopy. Our data are consistent with a significant distortion of the trigonal pyramidal geometry of the Cu coordination sphere when plastocyanin binds to cytochrome *f*, no matter their redox states are. The resulting tetrahedral geometry shows a shortening of the distance between Cu and the S_δ atom of its ligand Met-97, with respect to the crystallographic structure of free plastocyanin. On the other hand, when plastocyanin binds to photosystem I instead of cytochrome *f*, the geometric changes are not significant but a displacement in charge distribution around the metal centre can be observed. Noteworthy, the electronic density around the Cu atom increases or decreases when oxidised plastocyanin binds to cytochrome *f* or photosystem I, respectively, thus indicating that the protein matrix affects the electron transfer between the two partners during their transient interaction.**

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1. Introduction

The biological electron transfer (ET) processes – which enable photosynthesis and respiration, among others – involve the formation of transient protein–protein complexes with weak binding affinities (in the μM^{-1}–mM^{-1} range) and short lifetimes (on the ms time-scale) [1]. These features define high turnover systems, as the ET chains, which demand a continuous flow of electrons between redox partners [2].

When analysing the factors influencing the ET reactions of metal-containing proteins, the changes in the properties of the metal centre upon formation of the transient complex have to be greatly considered. Indeed, there are many examples in the literature on proteins which exhibit a different redox potential value upon binding to their respective physiological partner. Cytochrome c_{550}, for instance, increases its redox potential value in 160 mV upon binding to photosystem II [3,4], a finding that has been attributed to a decrease in the solvent accessibility of the heme group upon complex formation. In early studies, Malkin et al. [5] showed that the redox potential of plastocyanin (Pc) decreases in 30 mV upon binding to cytochrome *f* (C*f*), and further on Drepper et al. [6] reported that the redox potential of Pc increases ca. 50 mV upon binding to photosystem I (PSI) while that of the P700 cofactor in the photosynthetic reaction centre remains unchanged. As shown by perturbed angular correlation of γ-rays, the binding to PSI stabilises the Ag adducts of Pc but not the Cd ones [7]. This fact was ascribed to the stabilisation of the reduced form of Pc upon binding to PSI, thus explaining the differences between the two redox states of Pc regarding their affinity for and efficiency in ET to PSI. Noteworthy, it is known that three of the four Cu ligands of Pc are located on a loop, so giving rise to a structurally adaptable metal site [8]. However, the structural changes of the native Cu-containing Pc upon binding to its physiological partners remain veiled.

ET rates depend on the orbital coupling between donors and acceptors along alternative ET pathways, which rely on the number and strength of chemical bonds in each one, including those between the metal and its ligands [8–10]. In addition, ET rates are determined by the redox potential of donor and acceptor molecules and, also, by reorganisation phenomena that act as an activation barrier and may involve changes of the geometry in the metal first co-ordination sphere concomitant to ET [8]. In biological redox reactions, the polypeptide

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Abbreviations: C*f*, water-soluble fragment of cytochrome *f*; ET, electron transfer; EXAFS, extended x-ray absorption fine structure; FT, Fourier transform; HOMO, highest occupied molecular orbital; NIR, nitrite reductase; Pc, plastocyanin; 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; ΔE_0, internal potential correction; Δr, change in the distance between the metal and ligand atoms; σ, Debye–Waller parameter related to the system dynamic disorder

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chain plays a crucial role in maintaining the coordination sphere and spatial conformation of the metal centres while providing the intervening medium for a fast and efficient ET. In this context, it is highly interesting to investigate whether the protein matrix – by means of geometric and electronic perturbations around the metal centre – may accommodate the two redox partners within the transient complex and affect the ET process.

The thylakoid proteins Pc and its two physiological membrane partners (C*f* and PSI) thus represent an excellent biological model to study the ET reactions as they have been all extensively studied at the functional level [11–13]. In particular, the relevance of electrostatic and hydrophobic interactions in the formation of the transient Pc–C*f* and Pc–PSI complexes has been highlighted. Actually, the NMR solution structures [14–17], as well as a docking analysis taking into account mutagenesis data [18], of the Pc–C*f* complex from different organisms reveal a high variability in the electrostatic nature of the protein–protein interactions [16,19].

Despite the large amount of data concerning the factors influencing the binding between redox partners in transient complexes, little is known about the effects of complex formation on the metal cofactors and how they modulate both the affinity of such complexes and the ET process itself. Recently [20], we have shown that it is possible to detect tiny changes in the metal environment in short living complexes involving cytochromes by X-ray absorption spectroscopy (XAS) in solution, despite the stiffness of the heme moiety. To get a deeper insight into the role played by the protein matrix, we have extended our studies to analyse the copper centre perturbations in the transient Pc–C*f* and Pc–PSI complexes.

2. Materials and methods

2.1. Protein samples

Nostoc sp. PCC 7119 (former Anabaena sp. PCC 7119) Pc was purified from *Escherichia coli* cells transformed with the pEAP-WT plasmid [21]. Production and purification of the soluble domain of *Nostoc* sp. PCC 7119 C*f* were as previously described [22]. *Nostoc* sp. PCC 7119 Hg-containing PSI (PSI^{Hg}) samples, in which the metal atoms of the Fe–S centres are replaced by Hg ions, were obtained as described previously [23,24] with minor modifications, as explained in Supplementary Material. As PSI^{Hg} experiences a faster charge recombination than native PSI, the oxidation of Pc upon binding to PSI^{Hg} was negligible. Likewise, no evidence of changes in the interaction between Pc and PSI due to PSI treatment with $HgCl_2$ was observed.

For the XAS experiments, the Pc and C*f* samples were concentrated to the required volume by ultrafiltration methods, and exchanged into 10 mM sodium phosphate, pH 6.0. That is, 1 pH unit above the His-92 pK_a value, according to previous NMR [25] and functional [26] data on the same protein and at similar experimental conditions (buffer concentration and temperature). This makes protonation of the imidazol ring of His-92 rather improbable (see Supplementary Material). After concentration, stock solutions of 10 mM Pc and 4.5 mM C*f* were obtained. Protein concentration was determined by optical spectroscopy using an absorption coefficient of 4.5 $mM^{-1}\,cm^{-1}$ at 598 nm for oxidised Pc [21] and 31.5 $mM^{-1}\,cm^{-1}$ at 556 nm for reduced C*f* [22]. The 1.0 mM PSI^{Hg} samples were in 10 mM sodium phosphate, pH 6.0, buffer supplemented with 0.025% (w/v) β-dodecyl-maltoside.

Oxidised Pc (Pc^{II}) was prepared by adding an equimolecular amount of potassium ferricyanide, followed by gel filtration in a Pharmacia Superdex G75 column to remove ferrocyanide. Reduced Pc (Pc^{I}) was obtained upon addition of a 10-fold excess of sodium ascorbate to a resulting Pc^{II} sample. Oxidised C*f* (Cf^{III}) was obtained by addition of ferricyanide, as described for Pc. The oxidised complex (Pc^{II}–Cf^{III}) was prepared by adding an aliquot of a 10 mM Pc^{II} onto a Cf^{III} sample up to reach a final concentration of ca. 2 mM each. Under these conditions, the percentage of Pc bound to C*f* was estimated to be ca. 87%, according to the binding constant calculated from NMR data [19]. The reduced complex (Pc^{I}–Cf^{II}) was obtained upon addition of a 10-fold excess of sodium ascorbate to a Pc^{II}–Cf^{III} sample prepared as above. Similarly, the Pc^{II}–PSI^{Hg} samples were achieved by adding an aliquot of a 1 mM PSI^{Hg} solution onto Pc^{II} sample, up to ca. 0.8 mM of each partner, with 70% of Pc bound to PSI^{Hg} according to the binding constant calculated from laser flash absorption spectroscopy data [27]. The samples were kept in the dark to maintain PSI^{Hg} in its reduced state, and the reduced complex (Pc^{I}–PSI^{Hg}) was obtained upon addition of 5 mM sodium ascorbate and 10 mM sodium dithionite to a Pc^{II}–PSI^{Hg} sample prepared as above. Interaction studies were carried out using PSI^{Hg}, which experiences a faster charge recombination than native PSI [25,26], in order to prevent the oxidation of Pc.

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 Cu K-edge (8979 eV) was measured at beam line BM29 using a double crystal monochromator fitted with a pair of flat Si(3 1 1) crystals detuned to 50% fwhm of the maximum transmission, for the suppression of high energy harmonics.

All measurements were made at room temperature in fluorescence mode, using a 13-element Canberra solid-state germanium detector. The samples were contained in a PTFE cell equipped with two 12 μm Kapton foil windows.

Energy calibration was achieved by measuring a copper foil for the Cu edge. The spectra of the foil were measured simultaneously, with the data in transmission mode, to be used as an internal reference. Ionisation chambers filled with the appropriate mixture of gases were used as detectors.

For all measurements, each data point was collected for 4 s, and several scans were averaged to achieve a good signal-to-noise ratio. In no case protein photoreduction or damage was observed, even in those samples in which the measurements were made over several hours. For the oxidised forms of free and C*f*–bound Pc, the edge remained at fix energy position from the first to the last scan, thus confirming the absence of protein photoreduction (see Supplementary Material). The lack of radiation-induced changes in the samples was also confirmed by UV–Vis measurements before and after XAS measurements.

2.3. XAS data analysis

The background subtraction required to obtain the extended X-ray absorption fine structure (EXAFS) functions $\chi(k)$ from the measured X-ray absorption spectra were performed using the AUTOBK code from the University of Washington. E_0 was defined as the maximum of the first derivative of the absorption edge.

To analyse the EXAFS spectra, the theoretical phases and amplitudes were calculated using the FEFF 6.0 code [28]. The fit to the experimental Pc data was performed using the Artemis program (version 0.7.008) [29,30].

2.4. Visible spectrometry of the Pc^{II}–Cf^{III} complex

In order to compare the electronic spectrum of Pc^{II} bound to Cf^{III} with that of free Pc^{II}, a solution containing 200 μM C*f*, 3 mM Pc and 10 mM sodium phosphate, pH 6.0, was titrated into a control sample containing 200 μM C*f* in the same buffer, which was previously used as blank in order to eliminate the contribution of C*f* from the spectra. The Pc and C*f* samples were independently oxidised by potassium ferricyanide, washed and concentrated before the experiments. Measurements were performed on a 2 mm path length cuvette in a Varian Cary spectrophotometer operating in single beam mode.

3. Results

3.1. XANES region

The X-ray absorption near edge structure (XANES) region of the X-ray absorption spectra corresponding to Pc in its

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