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EXAFS Studies on the c-Heme Environment in Native and Chemically Modified Cytochromes

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The chemical environment of the c heme in low-molecular weight and water soluble cytochromes like cytochrome c is well known thanks to the X-ray diffraction studies carried out by Dickerson and coworkers (Ref.1) for both the oxidation states of the molecule and for a number of different species. Their conclusions, summarized in Table 1 as far as the interatomic distances of the first five shells surrounding the chromophores are concerned, have been confirmed and substantiated by various physical and biochemical methods, including XAS (see, for example, Ref.2).

Table 1. The chemical environment of heme-iron in heart cytochrome c (from Ref.1)

Shell	occupancy	Interatomic distance
1 N	(His 18)	1.97 (Å)
4 N	(Heme)	2.00
1 S	(Meth 80)	2.32
8 Cα	(Heme)	3.05
4 Cγ	(Heme)	3.43
8 Cβ	(Heme)	4.25

The present paper refers to a series of EXAFS measurements carried out on the carboxymethylated form of cyt c (CM cyt c) as well as on the d-heme depleted form of cytochrome oxidasefrom Pseudomonas Aeruginosa taking advantage of: i) parallel investigations carried out on the native cytochrome c (NAT cyt c) and ii) extensive use of simulation techniques.

The carboxymethylated cytochrome c prepared according to Schejter and George (Ref.3) has been characterized spectroscopically and functionally by Brunori et al. (Ref.4). The latter authors suggested a reaction mechanism accounting for the kinetic and equilibrium behaviour of its reduced form in the reaction with CO and based on a proton induced shift between the alkaline and the acidic state of an amino acid residue in the heme pocket: at acidic pH (6) the prevailing conformation would have a pentacoordinated iron since Lys 79 (replacing Meth 80 as a distal ligand in CM cyt c) is only able to bind the iron in the dissociated state.

This is in good agreement with the results from our EXAFS spectra (Fig.1), in which the relevant differences between reduced NAT and CM cyt c are: 1) a narrower first peak in Fourier transform of the CM cyt c at pH 4.5 as compared to the NAT cyt c at pH 7.0, reflecting a higher level of chemical and structural homogeneity in the scatterers; 2) the presence, in the case of CM cyt c, of a new peak between those assigned to the heme nitrogens and Cα, which might be due to the S atom of Meth 80, here shifted at a longer distance from the iron than the 2.32 Å in the native form.

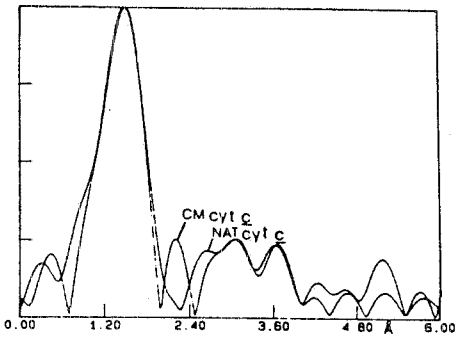

Fig.1. Fourier transforms of the EXAFS oscillations for native (NAT) and carboxymethylated (CM) cyt. c. The experimental data represented the average of at least three spectra run under identical conditions. The samples were prepared by addition to the lyophylized powder of NAT and CM cyt c of the appropiate buffer (K-Pho 0.1 M, pH = 7.0 and NaAcetate 0.1 M, pH = 4.5, respectively) up to a final concentration of 10 mM. The completely reduced state of the proteins was obtained by addition of minute amounts of dithionite. The EXAFS fluorescence spectra were recorded at T = 25°C and current intensity between 80 and 25 mA, using four photomultipliers in a semi-octahedral arrangement around a sample holder of three mm thickness. During the time required to run a single spectrum (between 60 and 80 min) no change occurred in the sample due to radiation damage, as checked by absorption spectroscopy in the VIS/UV region. Data analysis was carried out with the help of a software package developed by one of the authors (S. Mobilio). The Fourier transforms have been performed in the range 2.5-9.5 $Å^{-1}$ with a K^2 weight and a Hanning window function of 2 $Å^{-1}$ width

Figure 2 shows the Fourier transforms obtained after simulating the EXAFS spectra of NAT cyt c by the method described in Refs. 5-6 and on the basis of the interatomic distances listed in Table 1. The satisfactory matching in the peak position and shape of the Fourier transforms between experimental and simulated spectra in the case of NAT cyt c enhances our confidence in the experimental and analytical procedures followed, while comparison between Figs. 1 and 2 provides, as a minimum estimate for the Fe-S distance in CM cyt c, the value of 2.7 Å.

Figure 3 shows the results of the Fourier analysis carried out over the sum of eight EXAFS spectra relative to the c heme environment in Pseudomonas

cytochrome oxidase. This enzyme is characterized by the presence of two different hemes, of the c and of the d type, in two different (although unknown) environments, since the c heme only is covalently bound to the polypeptide chain (see Ref. 7 for a review). Taking advantage of this latter feature, selective and reversible splitting of the d heme has been achieved using a mild procedure which leaves unaltered the spectra properties of the c heme in the VIS/UV regions.

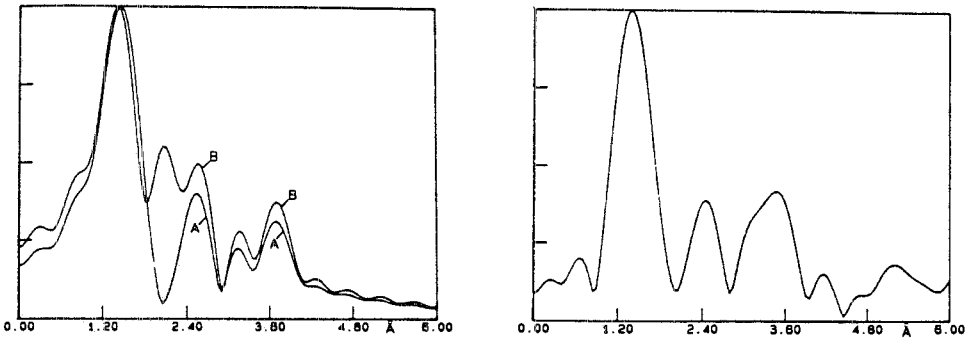

Fig.2. Fourier transforms of simulated EXAFS spectra for cytochrome c. EXAFS were simulated using the theoretical parameter of Refs. 5-6, on the basis of: the bond lengths listed in Tab. 1 (*A*); the same lengths above but a value of 2.7 Å for the Fe-S distance (*B*). The Fourier transforms have been calculated following the identical procedure described in Fig. 1.

Fig.3. Fourier transform of the EXAFS oscillations for the d Heme depleted form of Ps. cytochrome oxidase. Immediate after splitting of the d heme, the enzyme was centrifuged for 10 hs at $T = 0^{\circ}C$ and 30×10^3 g, and the pellet used to fill the sample holder. All the other experimental conditions and technical precautions as those in Fig. 1.

The average distances of at least the first two shells can be defined, on the basis of our data, the relative figures being 2.05 ± 0.06 Å and 3.12 ± 0.06 Å. The significant difference in the latter one as compared to that assigned to the $C\alpha$ in NAT cyt c (3.05 Å) indicates, in the case of Ps. oxidase, the possible contribution of some other scatterer situated at a noticeably longer distance. This is even more true for the 3rd peak, whose maximum is located around 4.05 Å and which is of clearly composite nature corresponding, as a first approximation, to the coalescence of the 4 $C\gamma$ and the 8 $C\beta$.

The chemical information avalaible up to now on Ps. cytochrome oxidase is limited to the amino acid composition and the sequence of the first 60 residues, the c heme-binding peptide. Assignment of the 6th coordination position of the iron to Meth 60, given by Meyer and Kamen (Ref. 8) on this basis alone, should be probably revised in the light of our data, although unequivocal definition of the chemical environment of the heme demands extensive use of

suitable model compounds. This is a central topic in our present experimental plans.

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