XANES study of iron displacement in the haem of myoglobin

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The XANES (X-ray absorption near edge structure) spectra of deoxy human adult haemoglobin (HbA) and myoglobin (Mb) have been measured at the wiggler beam line of the Frascati synchrotron radiation facility. The XANES are interpreted by the multiple scattering cluster theory. The variations in the XANES between HbA and Mb are assigned to changes in the Fe-porphyrin geometry.

Myoglobin *Hemoglobin* *XANES* *X-ray absorption* *Synchrotron radiation* *Heme structure*

1. INTRODUCTION

The displacement of the iron atom out of the plane of porphyrin-nitrogens (Fe-Ct) in the haem of haemoglobin has attracted large interest since the work in [1]; Fe, out of plane in deoxygenated haemoglobin (deoxy-Hb), moves into the plane upon oxygenation. This movement has been considered to be the main pathway by which the information of O_2 bonding to Fe is transmitted to the rest of the protein and it is associated with the transition between the two states of haemoglobin (the low affinity, tense (T) state, and the high affinity, relaxed (R) state), first suggested by authors in [2] to provide a satisfactory description of the cooperative ligand binding in haemoglobin [3].

The distance of Fe from the plane of porphyrin nitrogens (Fe-Ct) in deoxy-Hb has been related to the affinity for ligand binding of haemoglobin in the state T [4]. Although many recent papers have pointed out that the ligand affinity in haemoglobin [3] and in synthetic porphyrin models [5–7] is a complex phenomenon, involving also atoms of the globin and the rest of the protein, the iron displacement in the deoxy form is one of the key parameters in the control of ligand affinity.

High energy electron storage rings provide intense synchrotron radiation in the X-ray range which makes possible measurements of X-ray absorption spectra of proteins in solution. The appealing aspect of X-ray absorption spectroscopy for molecular biology is that it can provide direct information on the local structure of proteins in solution and therefore makes feasible the studies of structural changes during the function of the proteins.

Above the continuum threshold for core (K level) excitation the absorption cross-section is modulated by diffraction from neighbour atoms of the photoelectron emitted in the continuum at the site of the absorbing atom. At the photoelectron kinetic energies (given by the photon energy less the energy of the absorption threshold) higher than 50 eV (EXAFS regime) the photoelectron is scattered only once from each neighbour atom (single scattering) and the interference effects modulate the absorption cross-section giving the EXAFS oscillations which give the interatomic distances and coordination numbers. At lower energies (XANES regime) the photoelectron is generally in the multiple scattering regime and the X-ray absorption near edge structure (XANES) can give

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more information on the local geometry: bonding angles and distortions of symmetry due to small atomic displacements [8–10]. EXAFS has been successfully used to measure the mean distance of Fe from the porphyrin nitrogen (Fe-Np) of haemoglobin in solution with an accuracy of ± 0.01 Å. Authors in [11,12] have found the same Fe-Np distance 2.055 Å in the low affinity human deoxy-Hb A (T), in the high affinity deoxy-Hb kempsey and in the deoxy 'picket-fence' porphyrin Fe(TpivPP)(N-MeIm). They reach the conclusion that the difference between the high and low affinity forms of Hb is not associated with a Fe-Np bond strain.

The iron displacement Fe-Ct cannot be measured directly from EXAFS because EXAFS data do not contain information on bonding angles, as pointed out in [13]. Therefore the conclusion of authors in [12] that the iron displacement in deoxy HbA is the same as in Fe(TpivPP)(N-MeIm), Fe-Ct = $0.2^{+0.1}_{-0.2}$ Å cannot convincingly be inferred from their similar EXAFS spectra.

Crystallographic data (see table 1) give different Fe-Ct displacements in deoxy-HbA, deoxy myoglobin [14] and in Fe(TpivPP)(N-MeIm) porphyrin [12,15] (see table 1). We have performed this experiment to show that XANES, because of multiple scattering, contains information on the bonding angles and therefore on variations of the Fe displacement.

2. EXPERIMENTAL

The XANES measurements were performed at the Frascati 'wiggler' beam line using synchrotron radiation monochromatized with a Si(220) channel-cut crystal and 0.5–1 mm exit slits were used. The absorption spectra were collected in transmission using solution samples of the proteins at concentrations ranging from 4 to 10 mM in haem. The zero of the energy scale was carefully fixed at the absorption threshold of the Fe metal K-edge defined as the first maximum of its derivative spectrum. This energy is very close to the absorption threshold of the deoxymyoglobin spectrum defined as the energy of the maximum of the first weak absorption peak. The pre-edge absorption background was subtracted in all spectra. The absorption coefficient was normalized in all spectra to α_0, the atomic absorption above the absorption jump obtained by extrapolation toward lower energy of the linear fitting of EXAFS oscillations in the range 50–150 eV above the absorption threshold. Because the Fe atomic absorption is the same for all spectra, this normalization procedure allows the quantitative comparison between different spectra. The spectra have been obtained both by adding up to three scans and by single scans with integration time of 10 s/point.

The experiment has been repeated in 3 different runs over a year using different samples. The energy resolution is about 1 eV and energy shifts

Table 1

Geometry of deoxy Fe-haem complexes from crystallographic data[a]

	Fe-Ct (Å)	Fe-Np (Å)[b]	Fe-Cp (Å)	Fe-N_ϵ (Å)
Deoxy-HbA	0.434	2.06 (2.055)[c]	0.54	2.12 (7)[c]
Deoxy-Mb (pH 5.75, 20°C)	0.42	2.03	0.47	2.22
Deoxy-Mb (pH 8.41, −12°C)	0.3	2.02 (5)	0.38	2.10
Deoxy Fe(TpivPP) N-MeIm	0.25	2.055[c]		

[a] Refined data communicated by G. Fermi and S.E. Phillips
[b] Mean distance
[c] From EXAFS data

Fe-Np, distance of Fe from the porphyrin nitrogen; Fe-N_ϵ, distance of Fe from the nitrogen of the proximal histidine F8 (His F8); Fe-Ct, distance of Fe from mean plane of porphyrin nitrogens, denotes displacement towards histidine F8 (His F8); Fe-Cp, distance of Fe from mean plane of porphyrin; deoxy, deoxygenated; HbA, human adult haemoglobin; Mb, myoglobin (sperm whale); TpivPP, meso-tetrakis{$\alpha,\alpha,\alpha,\alpha$[(-*O*-pivaloy)amido]phenyl}porphyrin; N-MeIm, *N*-methylimidazole

larger than 0.2 eV can be measured.

Deoxyhaemoglobin samples were prepared from human oxyhaemoglobin A flushing with nitrogen gas and adding dithionite before the beginning of the experimental run. Deoxymyoglobin (deoxy Mb) was prepared at pH 7.2 from sperm whale metmyoglobin from Sigma. Optical spectra of non-irradiated and irradiated samples were used to verify their quality. The samples, exposed to radiation in an inert He atmosphere, were kept at constant temperature (10°C).

3. RESULTS AND DISCUSSION

From figs 1 and 2 it appears that the features labeled A–E are present in all spectra and their relative intensity changes going from Mb to Hb while no significant changes are observed between Mb and the picket fence porphyrin. It is interesting to remark that while XANES spectra of Hb and of this porphyrin are different no differences were observed between their EXAFS spectra [12].

Fig.3 shows the results of theoretical calculations of XANES of a porphyrin cluster by the multiple scattering theory [17] which has been successful in the determination of small geometrical distortions in ferro- and ferricyanide complexes. We have used the same scattering amplitude for

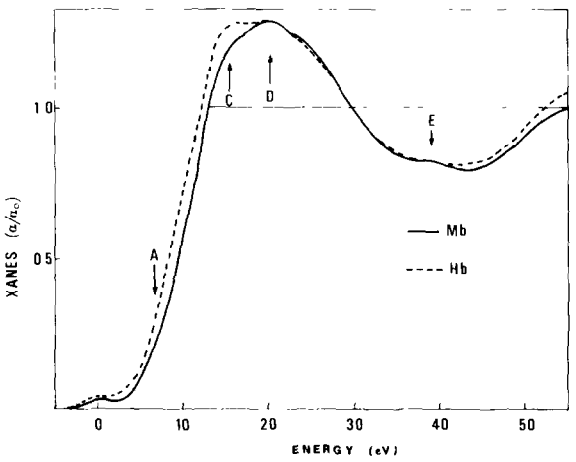

Fig.1. Experimental Fe K-XANES of sperm whale deoxymyoglobin (Mb) (——) and of human adult deoxyhaemoglobin (HbA) (– – –). The spectra are normalized at the atomic absorption and the zero of the energy is fixed at the localized Fe 3d resonance at threshold.

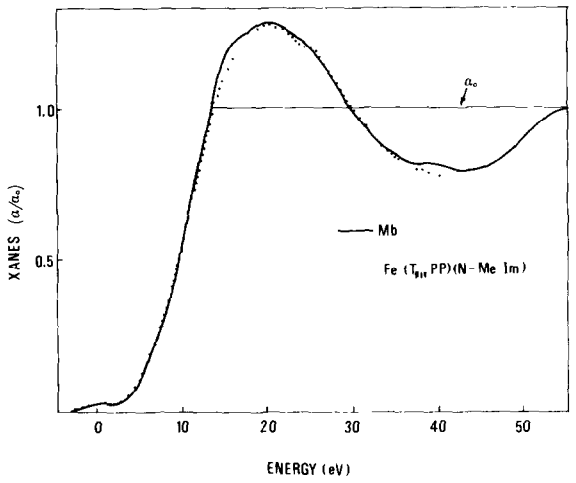

Fig.2. Experimental Fe K-XANES of deoxymyoglobin (——) and of picket fence porphyrin Fe(TpivPP)(N-MeIm) from [16] (···).

Fe, C and N atoms already tested in the analysis of XANES of $K_3Fe(CN)_6$ [18]. The size of the cluster of atoms determining the XANES is limited by non-elastic photoelectron scattering with valence electrons and it has been empirically determined by successive XANES calculations including successive shells of neighbour atoms. A cluster formed by the 24 atoms of the porphyrin and the 3 atoms of the proximal histidine F8 plus the central Fe atom was necessary to reproduce all XANES features from A to E of the experimental spectra. At the same time, the non-elastic scattering also gives rise to the large broadening of XANES, and it has been introduced into the calculations as an imaginary part in the energy. We found a good agreement [19] between the experimental spectrum of deoxy Hb and the averaged spectrum over the two polarizations of the photon beam (parallel to the normal to the porphyrin plane $\vec{n}$, and perpendicular to $\vec{n}$, where the photoelectron is scattered in the porphyrin plane) using the atomic positions previously determined for Hb [12,13,20]. The structure used in the calculations was idealised in that the porphyrin ring was taken to be planar and 4-fold symmetrical.

To relate the differences in the experimental XANES spectra to atomic displacements we have calculated the effect of Fe displacement out of the porphyrin plane by keeping the porphyrin planar.

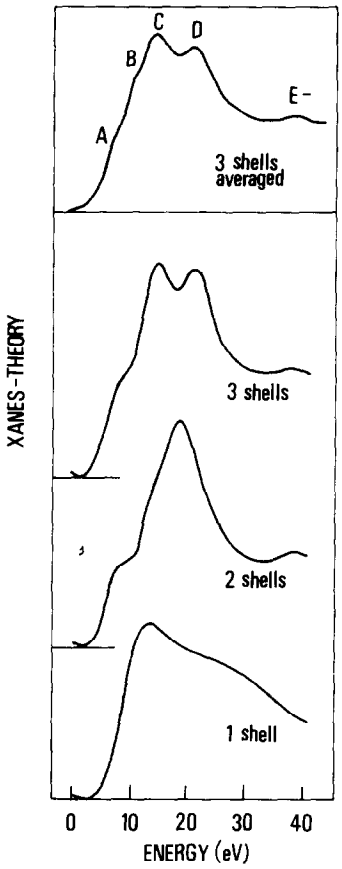

Fig.3. Theoretical Fe K-XANES of the haem cluster of deoxy haem-proteins. The unpolarized spectrum for a symmetrical idealized haem cluster including 27 neighbour atoms is shown in the upper panel. The curves in the lower panel show the effect on calculated XANES, of a cluster including Fe and the atoms in the haem plane, of the size of the cluster. The spectra for small clusters including only the first shell (5 atoms), 1st and second shells (21 atoms) is shown. The zero of the energy scale for the photoelectron kinetic energy is fixed at the energy of the atomic Fe 3d resonance which corresponds in the experimental spectra to the defined absorption threshold.

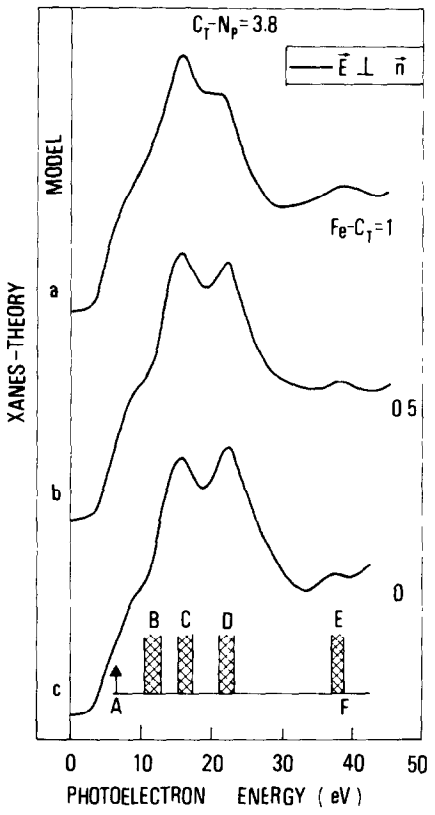

Fig.4. Theoretical XANES spectra for a cluster of 30 atoms formed by a planar porphyrin where the Fe is moved out of plane: model (a): Fe-Ct = 1 a.u. (0.53 Å), model (b): Fe-Ct = 0.5 a.u., model (c): Fe-Ct = 0 a.u., only the contribution of the scattering in the haem plane is shown.

Fig.4 shows that as the Fe atom goes out of the porphyrin plane (Fe-Ct from 0 to 0.53 Å = 1 a.u.) the intensity of peak C, due to multiple scattering of the photoelectron (E $\perp$ *n*) in the porphyrin plane, increases with the iron displacement (Fe-Ct). The agreement between the calculated XANES and the experimental spectra is very good for the energy positions of the spectral features.

The lineshape of the features depends on the energy dependent non-elastic scattering which is approximated in the theory by a constant imaginary part of the energy. Moreover, the porphyrin plane is expected to be distorted in the proteins with some doming effect which is not taken into account here. Therefore, only a qualitative agreement for the lineshape between the theoretical and experimental XANES is expected. However, the XANES calculations indicate that the physical effect of the iron displacement (Fe-Ct) (keeping all atomic positions of the porphyrin fixed) on the XANES is an increase of peak C. The theory shows that the XANES spectra of haemoproteins are determined by a large cluster of ~30 atoms but the XANES spectra features are only 5

which can shift in energy, mainly following the variation of interatomic distance [8,9] and change their shape and relative intensities following the bonding angles. In deducing the variation of the structure from XANES in this class of proteins it is therefore necessary to keep in mind that several geometrical distortions such as movement of carbon atoms in the periphery of the cluster as well as the tilt of pyrroles (the doming effect) and other distortions can affect the lineshape of XANES.

The experimental XANES spectra of deoxy HbA and deoxy Mb in fig.1 show variations in the energy region of peaks B and C. The XANES of deoxy human haemoglobin HbA exhibits a clear increase of the peak C in comparison with the XANES of myoglobin.

The variation between the XANES of deoxy Mb and deoxy HbA are in agreement with the predicted spectral variation arising from the iron displacement toward the haem plane in Mb, by 0.1 Å, with respect to the placement in HbA (~0.4 Å). Given the noise level of our data we estimate that we can appreciate the variation of 0.05 Å of the iron displacement. However, it is possible to use it only as a qualitative estimate of the error of the iron displacement in myoglobin (0.3 ± 0.05 Å) because other possible distortions of the haem can also affect the XANES in a similar way.

The similarity observed in fig.2 between the XANES of deoxy Mb and of the picket fence porphyrin where the Fe is ~0.3 out of plane and where the EXAFS spectra of both systems [11,12] give the same Fe-Np distance, seems to confirm a similar iron displacement in the two systems. However, while the different atomic arrangements between Mb and HbA are different only because of atomic positions, in the picket fence porphyrin there are different side molecular groups on the periphery of porphyrin plane which can affect the XANES, and therefore the comparison between the two XANES spectra cannot be significative of variation or similarity in the local structure.

4. CONCLUSION

Our data indicate a smaller Fe displacement in myoglobin than in haemoglobin. This result is in agreement with Mossbauer data [21], from which a Fe displacement 0.1 Å smaller in Mb than in HbA has been deduced, and with resonance Raman spectra [22]. Two diffraction studies of myoglobin at different temperatures and different pH have been carried out (see table 1) [14]. The diffraction results give two different Fe-Ct displacements 0.42 Å at pH 5.7 and 0.3 Å at pH 8.5. The XANES of deoxymyoglobin taken in solution at 10°C and pH 7.2 seem to be in agreement with diffraction data of Mb taken at pH 8.4.

It is interesting to note that the XANES spectra of deoxy α and β chains of haemoglobin measured in [23] show a weak difference in the energy range of peaks A and B which, if it is confirmed by better signal-to-noise spectra, would indicate an iron displacement larger in the α chain than in the β chain.

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