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OXYGEN BONDING IN HUMAN HEMOGLOBIN AND ITS ISOLATED SUBUNITS : A XANES STUDY

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The X-ray absorption near edge structure (XANES) spectra of the human adult and foetal hemoglobin, of the isolated α and β chains, in the oxygenated forms, and of the oxymyoglobin and carp oxyhemoglobin have been measured at the wiggler beam line of the Frascati Synchrotron radiation facility.The bonding angle of oxygen molecule at the iron site in these hemoproteins in solution , has been measured using the multiple scattering theory for data analysis.

The experimental determination of the bonding geometry of the diatomic molecules O_2, CO and CN in hemoproteins in solution is still an open problem in spite of extensive experimental research. Diffraction experiments are limited to crystallized proteins, on the contrary x-ray absorption near edge structure (XANES) spectroscopy provides a new tool for determination of local structure of active site in metalloproteins in solution.(1-8).The oxygen binding structure, concerning the determination of Fe-O-O bonding angle and the electronic structure of the iron-oxygen bonding in hemoproteins, has been object of many theoretical and experimental works for understanding the reversible binding of O_2.

Here we report the results of an extensive investigation of the structure of the oxygen binding site to test how the protein structure can modulate the local chemical bonding. We have compared the XANES spectra of the human hemoglobin (foetal and adult) and the separated α and β subunits, in the oxygenated forms, with the XANES spectra of the oxy-myoglobin, the oxy carp-hemoglobin and of a porphyrin model compound.

EXPERIMENTAL

The XANES measurements were performed at the Frascati synchrotron radiation facility using the Wiggler beam line. The synchrotron radiation was monochromatized by a Si (111) channel cut single crystal. The absorption spectra were collected in transmission . The absorption coefficient in the spectra was normalized to α_0 defined

0006-291X/87 $1.50

as the atomic absorption above the absorption jump obtained by linear fitting of EXAFS oscillations in the range 50-150 eV.

The ligated forms of the hemoproteins have been prepared by standard methods(9). The subunits of the HbA were separated according to the method of Geraci et al.(10). The purity of the chains was determined by isoelectrofocusing (11).

RESULTS and DISCUSSION

In fig.1 the experimental XANES spectra of oxygenated proteins: human foetal hemoglobin ($HbFO_2$), human adult hemoglobin ($HbAO_2$), carp hemoglobin (carp-HbO_2) and myoglobin (MbO_2) are reported. In fig. 2 their derivative spectra are shown.

The zero of the energy scale has been fixed at the absorption threshold of the iron metal K-edge. All proteins show a weak structure at threshold due to transitions to unoccupied molecular Fe-3d derived states. The features above 10 eV are very similar for MbO_2, carp-HbO_2, and adult human $HbAO_2$. The spectrum of foetal hemoglobin shows some differences concerning the lineshape of the spectrum at about 18 eV. In fig. 3 the spectrum of the oxygenated porphyrin model compound Fe O_2TpivPP (1-MeIm)reported by Cramer in his thesis (12) is shown because its structure is well known from x-ray diffraction (13).

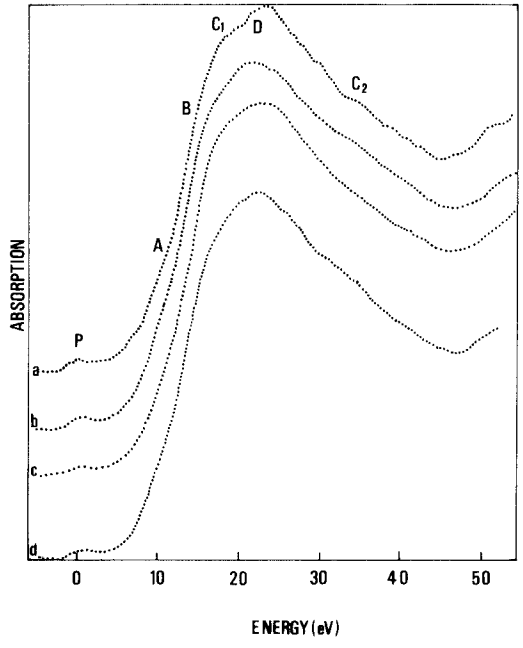

Fig. 1. Iron K-edge XANES spectra of oxygenated hemoproteins : a) human foetal hemoglobin ,b) human adult hemoglobin, c) carp hemoglobin and d) myoglobin.

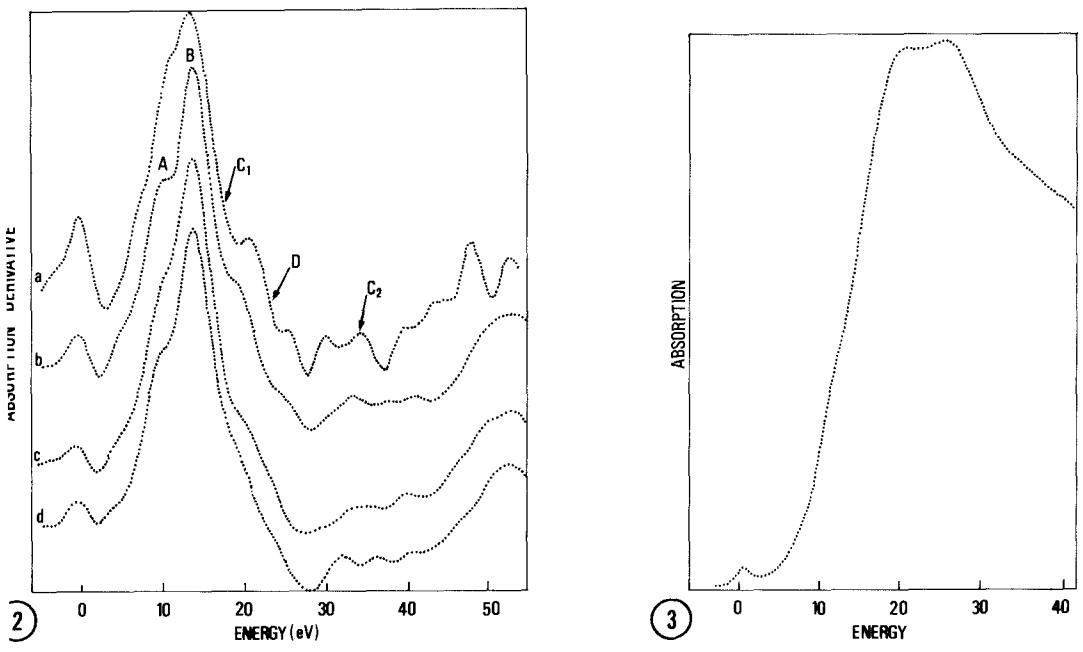

Fig. 2. Derivative spectra of the XANES : a) human foetal hemoglobin, b) human adult hemoglobin c) carp hemoglobin and d) myoglobin.

Fig. 3. XANES spectrum of the porphyrin model compound FeTpivPP(1-MeIm)O_2 from the Cramer thesis ref. 11.

The interpretation of XANES spectra has been demostrated to be feasible on the basis of the full multiple scattering theory for a cluster of about 30 atoms (2). In the first shell 4 nitrogens of the porphyrin on the x,y plane and one nitrogen N_ε of the proximal histidine F8 on the z axis are present. In the second shell the cluster includes 12 carbons of the porphyrin and 2 carbons of the proximal histidine F8. In the third shell the cluster includes 8 carbons of the porphyrin. The structure of the heme was taken to be planar and four fold symmetrical. This is an idealized model for the porphyrin where the heme is expected to be distorted with ruffling and doming. These effects play a minor role in XANES as showed by model calculations (14). Figure 4a shows several theoretical angular resolved XANES spectra of HbO_2 for two polarization of the photoelectron : the photon beam perpendicular to the normal of the porphyrin plane (solid line), the photon beam parallel to the normal of the porphyrin plane (dashed line).Three calculations for different bonding angle Fe-O-O $\theta = 115^0$, 156^0, 180^0 ,were performed.The dots indicate the calculated points using the CRAY computer.In order to save the computer time the spectra for $\theta = 156^0$ and 115^0 have been calculated with a larger space between points; therefore these last spectra are calculated with a resolution lower than that of the spectra for $\theta = 180^0$. Peaks C_1 and C_2 appear (fig.4a) in the XANES spectra with electric field polarization in the direction of the heme normal ; therefore they are due to the multiple scattering of the electron

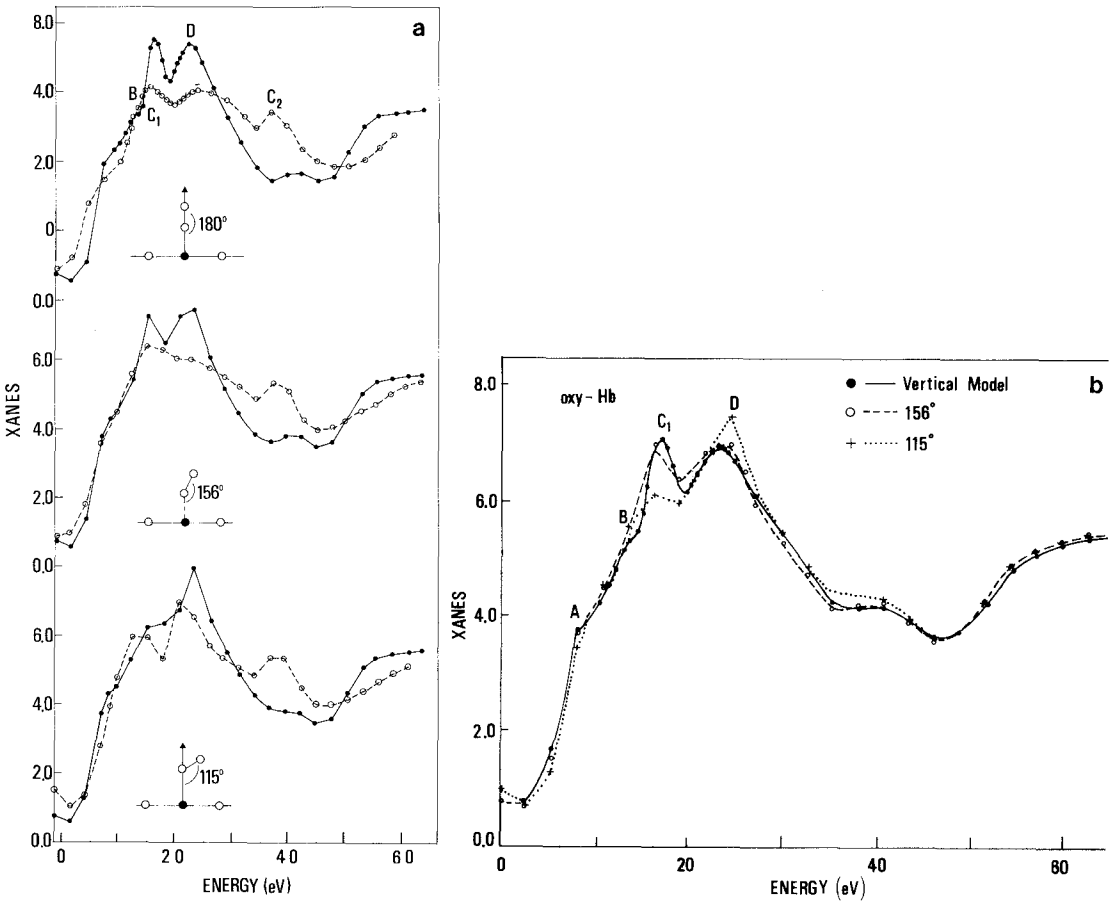

Fig.4a. Calculated Fe K XANES spectra for two polarization of the photoelectron (the photon beam perpendicular , solid line,and parallel, dashed line, to the normal of the porphyrin plane) with different Fe-O-O bonding angles, indicated by the arrows: 180^0 upper curve, 156^0 middle curve and 115^0lower curve.

Fig.4b. Calculated Fe K -XANES spectra of the cluster :the iron ion at center of the porphyrin plane,the proximal histidine and the oxygen molecule with different Fe-O-O bonding angles (180^0,156^0 and 115^0)

ejected in the z direction and interacting with the oxygen molecule. These peaks are observed in the experimental spectra at the predicted energies. From the variation of the intensity of the peak C_1 in the experimental spectra of the different proteins and model compounds it is possible to detect variations of the Fe-O-O bonding angle. The main peak D , which is due to multiple scattering in the porphyrin plane, is not affected by the oxygen orientation in the z direction. In fig.4a the variation of the peak C_1 with decreasing the Fe-O-O angle is much lower than for the case of Fe-C-O bond angle reported in ref.6. This might indicate a limitation of the XANES sensitivity to oxygen

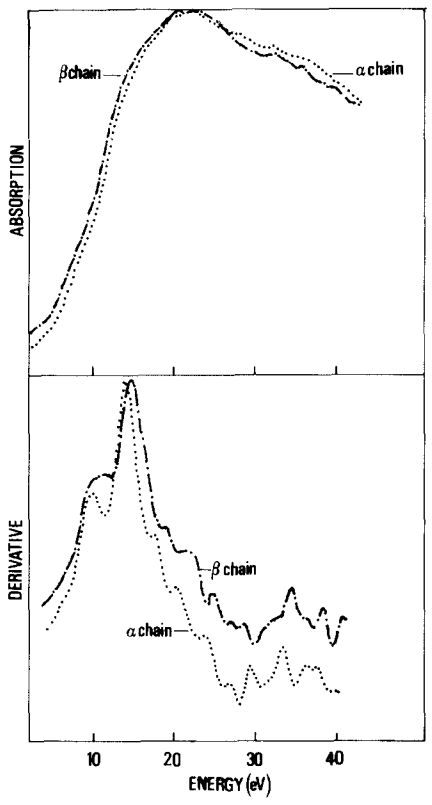

Fig. 5. Fe k-XANES of α and β chains in the oxygenated forms and their first derivative.

bonding angle as compared with the case of CO. In fig.4b the calculated unpolarized XANES spectra for different bonding angles are reported .

It is evident that 1) all the experimental features, due to the scattering in the haem plane are predicted by theory and 2) the main effect of rotating the bonding angle Fe-O-O is the variation of the intensity ratio between the peak C_1 and D.

The intensity ratio between the C_1 and D peaks in MbO_2 is in good agreement with the calculated spectra in fig.4b for Fe-O-O bond angle $\theta=115^o$ according to diffraction data for myoglobin (15). The intensity ratio C_1/D in the porphyrin in fig. 3 indicates that the Fe-O-O configuration is bent but with an angle larger than in oxymyoglobin, in agreement with crystallographic results(13) giving an angle of 131°. These results confirm the validity of the theoretical calculations.

The intensity ratio between the C_1 and D in the spectrum of adult oxy-hemoglobin (HbO_2) is the same as in MbO_2 indicating a similar bonding angle (fig. 1). This result for HbO_2 in solution is in opposition with the diffraction of hemoglobin in the crystal phase giving 156°(16,17) . It is interesting to observe that the bonding angle values in the ligated forms of the hemoglobin obtained by XANES in solution are different from that obtained by X-ray diffraction in crystals for both oxy and carboxy-Hb. In fact the

values obtained in HbO_2 in solution and in crystal are respectively 115^0 and 156^0, and in HbCO (6 ,18) 165^0 and 136^0 .On the contrary the same value of the bonding angle have been calculated for the MbO_2 and MbCO in solution and in crystal (6,15) .This fact could indicate that the local structure is modified by the crystal phase more easily in a more complex molecule as the hemoglobin than in myoglobin .

The intensity ratio C_1/D in the XANES spectrum of the foetal hemoglobin is smaller than in the adult hemoglobin. Therefore oxygen bonding angle in the foetal hemoglobin is deduced to be smaller than in adult hemoglobin. This effect can be assigned to the substitution of the β chains with the γ chains which affects the local structure of the heme as it has been reported in the deoxy form (4).

A possible variation of the Fe-O-O bonding angle in different chains has been tested by studying the separated α and β chains. We have measured the XANES spectra of α and β subunits of human hemoglobin in the oxygenated form. The results are shown in fig. 5 with their derivatives. These results are in good agreement with previous studies by Pin et al.(7) .

The bonding angle Fe-O-O was found by Shaanan in HbO_2 crystal to be 153^0 and 159^0 for the α and β chain respectively. Brzozowski et al.(19) have found a bonding angle of 160^0 in the α chains of a crystal of partially oxygenated HbO_2 (α-oxy, β-deoxy) in the T conformation. Moreover the contraction of either the Fe-N_ε distance (going from 2.07 Å in β chains to 1.94 Å in α–chains) and the Fe-O distance (going from 1.87 Å in β chains to 1.66 Å in α chains) has been found by Shaanan (16,17). This contraction in the Fe coordination distances in z axis is compensated by the elongation of the Fe-N_p distances in the porphyrin plane. Mössbauer experiments on isolated subunits have shown that the quadrupole splittings of oxy-β chains are smaller than that of oxy-α chains (20,21). On the contrary there is no difference either in quadrupole splitting and in isomer shift between HbO_2 and isolated oxy-α chains. These results can be correlated by a different Fe-O_2 bonding.

It is also reported that in the absence of organic phosphates there is no preferential oxygen binding to the β-chains or α-chains (22). On the contrary in the presence of organic phosphates the α hemes have higher affinity for oxygen as compared with the β hemes as it is in the hemoglobin tetramer where the α-chains are the first to bind the oxygen (22-24). The phosphates in our samples of oxy-β chains, oxy-α chains and HbO_2 were removed by passage through a column of Sephadex G-25 equilibrated with Tris HCl buffer.

Comparing the XANES spectra of the isolated subunits it not possible to observe large difference in the energy region of the peaks C_1 and D in agreement with the fact that the variation of the Fe-O-O bonding angle is expected to be small. We have focused our interest on the energy region of the peaks A and B on the absorption rising edge. We observe that in the derivative spectra the two peaks are better separated in the α-chains spectrum which exhibits a clear minimum between A and B. The absorption spectra show that there is a shift of the rising edge toward higher energy of the

spectrum in the α–chain. In agreement with the calculation reported in ref.25 this effect is interpreted as due to the movement of the proximal histidine toward the Fe in the α-chain. This structural change of the local structure of the active site should be correlated with the Mössbauer results. Work is in progress to study also the site structure of the oxygenated γ -chain.

In conclusion this work points out i) the possible difference between the oxygen bonding configuration in human adult hemoglobin going from the solution to the crystal phase; ii) the small difference between the active sites in separated oxy α and β-chain assigned to the movement of the proximal histidine; iii) the existence of a difference between the oxygen binding geometry in the foetal hemoglobin and that of the adult hemoglobin, indicating that the sequence changes induces variations of the local structure in the oxygenated form as well in the deoxygenated form (4).

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