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INCREASE OF THE Fe EFFECTIVE CHARGE IN HEMOPROTEINS DURING OXYGENATION PROCESS

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The x-ray absorption near edge structure (XANES) spectra of hemoglobin and myoglobin have been measured at the wiggler beam line of the Frascati Synchrotron Radiation Facility. The energy shifts of the iron absorption jump edge and the chemical shifts of the bound excited state at threshold of 1*s* core excitations, going from deoxygenated to oxygenated form, are interpreted as evidence of some increase of the positive effective charge on the iron atom upon oxygenation.

Oxygen bonding in hemoproteins has been object of many theoretical and experimental works (1), because essential for understanding the reversible binding of O_2. Studies of the of the iron-oxygen bond in hemoproteins (2) have shown a bent $Fe\text{-}O_2$ configuration according with the first proposal of an end-on angular bond by Pauling (3) and by Weiss (4). Some controversy remains on the electronic structure of the $Fe\text{-}O_2$ bond.

The oxygen molecule has 12 valence electrons which in the ground state populate seven molecular orbitals: five pairs go into the five lower lying orbitals and one electron each goes into the two $1\pi_g^*$ antibonding orbitals. According to Pauling's model some transfer of charge is correlated to the hybridization between the π orbitals of the oxygen and the d_{xz} and d_{yz} orbitals of iron which is essentially ferrous ($Fe^{2+}O_2$), while Weiss suggested that the bond might be ionic with a net transfer of charge from the iron to oxygen ($Fe^{3+}O_2^-$). Experimental techniques (magnetic susceptibility, Mössbauer spectrometry, EPR, NMR, and others methods) have been used to confirm the different theoretical hypothesis (5,6). From the analysis of experimental data till now published we can deduce that the emphasis on the ($Fe^{2+}O_2$) vs ($Fe^{3+}O_2^-$) dichotomy in the discussion on the nature of the iron-oxygen bond in oxyhemoglobin is semplicistic because molecular bonding orbitals are hybridized and delocalized over the $Fe\text{-}porphirin\text{-}O_2$ complex. Therefore different experimental methods, probing different aspects of electronic structure, give different results.

In this paper we report the results obtained by using the new XANES (X-ray Absorption Near Edge Structure) method (7), which has been recently shown to give unique structural informations on the geometry of the site in ferrohexacyanides (7), in hemoproteins (8,9) and in calcium binding proteins (10). At threshold XANES spectra are determined by excitations of core Fe 1*s* electrons to the lowest unoccupied states at the Fe site, therefore it is a local probe which can give information on the effective charge on the Fe site via variation of the binding energy of 1*s* core electron.

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EXPERIMENTAL

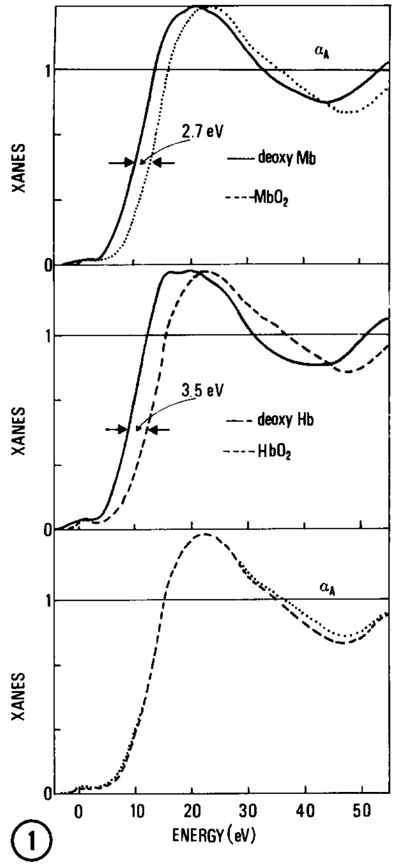
High energy electron storage rings provide intense synchrotron radiation in the x-ray range which makes possible measurements of x-ray absorption spectra of proteins in solution. The XANES measurements were performed at the synchrotron radiation facility of Frascati (Italy) using the "wiggler" beam line. The synchrotron radiation was monochromatized with a Si (220) channel-cut crystal. The absorption spectra were collected in transmission using solution samples of the proteins at concentrations ranging from 4 to 8 mM in haem. The high signal to noise spectra have been obtained by adding up to three scans with integration time of $10s/point$. The pre-edge continuum background has been substracted in all spectra by fitting the pre-edge part of the spectra. The high energy smooth atomic absorption α_A, due to core transition from the 1s core level to continuum p-states for the single Fe atom, has been obtained by linear fitting of the absorption spectra in the range 50-150eV above the absorption threshold. Because the Fe atomic absorption α_A is the same for all the spectra, they can be normalized to α_A and this allows the comparison between the intensity of different XANES spectra. Energy shifts down to $\simeq 0.2eV$ can be measured.

Hemoglobin in the oxy and deoxy form has been prepared from fresh human blood and myoglobin from sperm whale met-myoglobin from Sigma, by standard methods described elsewhere (11).

RESULTS AND DISCUSSION

Fig.1 shows the experimental shift of the Fe K-XANES upon oxygenation. In the panel a) the comparison between the XANES of O_2 and *deoxy-Mb* shows a shift of the rising absorption jump edge of $\simeq 2.7eV$. In panel b) a similar comparison between the XANES of HbO_2 and *deoxy-Hb* is shown: in this case a larger shift of $\simeq 3.5eV$ is obtained, in agreement with previous experiments on hemoglobin (12-14). In c) we see that the large rises of the principal peak in the XANES spectra for the oxygenated samples are superimposed. From these figures we may infer also a shift of $\simeq 0.8eV$ between the two deoxygenated forms of Mb and Hb which has been shown to be due to a different displacement of Fe out of the porphirin plane (9). Because the experimental XANES spectra for HbO_2 and MbO_2 are identical as shown in fig.1, the difference in the energy shift of the absorption jump rising edge between Hb and Mb is due only to different site structure of the deoxy forms.

We have calculated, by the multiple scattering theory, Fe K-XANES spectra of heme clusters including 24 atoms of the porphirin plane and three atoms of the proximal histidine F8. We succeded in reproducing all the principal XANES features of the experimental spectra. We have already demonstrated in the case of FeII and FeIII hexacyanides (7) that the energy shift of the XANES multiple scattering features can be due to two different possible effects: 1) a contraction in the interatomic distance, and/or 2) a different binding energy of the 1s core initial state. We calculated the XANES spectra for several models both in the deoxygenated and the oxygenated forms, the $Fe\text{-}N_p$ distance ranging from 2.06Å for *deoxy-Mb* to 1.96Å for MbO_2 (1.98Å for HbO_2), and the orientation of the O_2 molecule ranging from 180° (Fe-O-O bond parallel to the normal to the porphirin plane) to 115°, to analyze the combinated effects of $Fe\text{-}N_p$ contraction and O_2 orientation on the shift of the rising absorption edge and XANES features. We have found that geometrical distorsions of the Fe site give very small shifts of the absorption rising edge; on the contrary, the contraction of the $Fe\text{-}N_p$ distance determines large energy shifts. We have used crystallographic coordinates for the calculation of the XANES of *deoxy-Mb* and MbO_2 assuming the same Fe(1s) binding energy and the same Fe effective charge. The energy shift of $\simeq 1.8eV$ calculated for the XANES rising edge shown in fig.2 going from Mb to MbO_2 is essentially due



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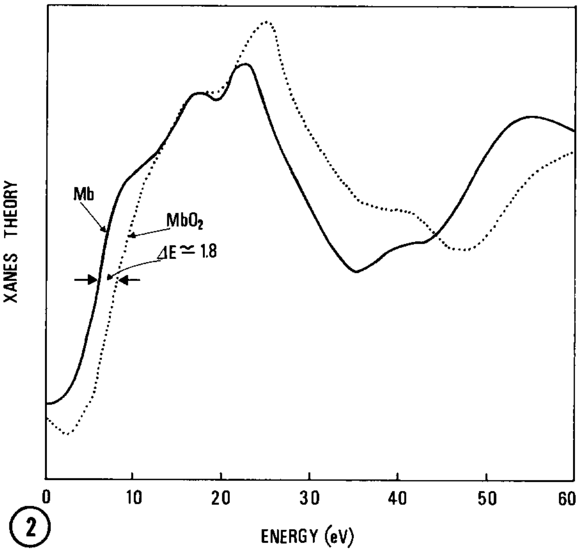

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Fig.1: Experimental XANES spectra of oxy and deoxy forms of hemoproteins are compared.
Panel a): MbO_2 and *deoxy-Mb*. Panel b): Hbo_2 and *deoxy-Hb*.
Panel c): The principal peaks of XANES spectra for the *oxy-Mb* and *oxy-Hb* are superimposed.

Fig.2: Theoretical XANES spectra of MbO_2 and *deoxy-Mb*. The energy shift of $\simeq 1.8eV$ is shown.

to the contraction of $\simeq 0.1\AA$ of Fe-N_p distance following O_2 bonding. This is the maximum calculated energy shift due only to atomic displacement. Therefore we were not able in no way to reproduce the $2.7eV$ energy shift between the XANES of oxy and deoxy myoglobin, which is $\simeq 1.0eV$ larger than that predicted for variations of interatomic distances and coordination geometry. Therefore there is an additional contribution to the energy shift of the XANES features due to the larger binding energy of the $1s$ core level both in the HbO_2 and MbO_2. This binding energy is controlled by the electronic charge of the occupied valence bonding orbitals in the atomic spatial region of Fe site.

Than we have addressed our attention to the weak excitonic peak that appears before the rising absorption jump edge. In all the experimental spectra the zero of the energy scale was carefully fixed at the absorption threshold of Fe metal K-edge defined as the first maximum of its derivative spectrum, shown in the upper part of fig.3. The half width at half height of this peak is $\simeq 1.4eV$ and is due partly to intrinsic width of $Fe(1s)$ core level and partly to instrumental

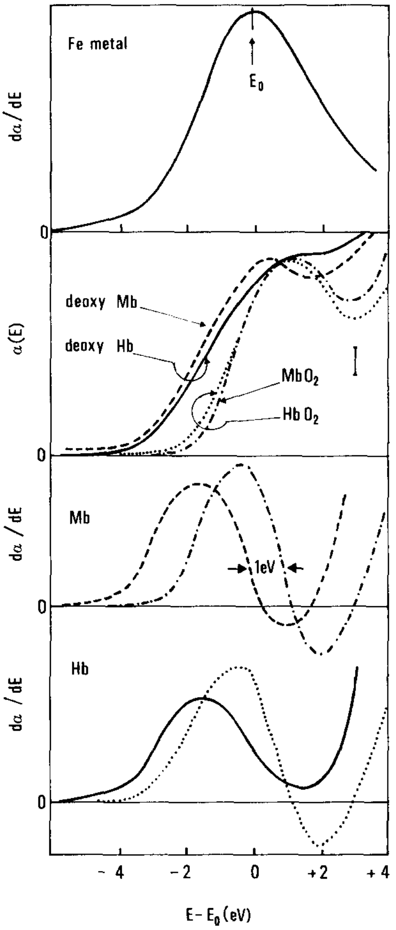

Fig.3: The derivative spectrum of absorption threshold of Fe K-edge is shown in the upper part. The 1*s* ↦ 3*d* peaks of experimental XANES spectra of HbO_2 and MbO_2, of *deoxy-Hb* and *deoxy-Mb* are compared in panel b). The shifts of the derivative spectra of these peaks are shown in panel c) for *oxy* and *deoxy-Mb*, and in panel d) for *oxy* and *deoxy-Hb*.

resolution. In fig.3b we show a comparison between the 1*s* ↦ 3*d* peaks in HbO_2 and MbO_2 and the *deoxy-Hb* and *deoxy-Mb* spectra.

In fig. 3c and 3d we can observe shifts of $\simeq 1.0eV$ between MbO_2 and *deoxy-Mb* and between HbO_2 and *deoxy-Hb* derivative spectra. This shift can be assigned to the variation of the core 1s core binding energy.

In fact the final states are the lowest unoccupied states which in both *Hb* and HbO_2 is a molecular orbital due to mixing between Fe(3d) and porphirin π^* orbital and it remains at the same energy going from *Hb* to HbO_2 (15). Because of core hole-electron attraction, the first excited state in molecules is a localized bound state and its energy shift follows the effective charge of the iron (15-17).

In conclusion we obtain a chemical shift of $\simeq 1eV$ for the 1*s* core level both in MbO_2 and HbO_2 due to change of the effective charge on Fe atom in hemoproteins during oxygenation process.

both from the shift of the multiple scattering XANES in the continuum and from the localzed excitations at threshold. A shift of $\simeq 1.0eV$ for the core levels of Fe in inorganic compounds in photoelectron spectroscopy (ESCA) is characteristic of transition from "formally" Fe^{2+} to Fe^{3+} compounds.

Our results are in agreement with Mössbauer (19) and infrared (20) spectroscopies which show evidence of electronic charge transfer from Fe to O_2 upon oxygenation which can be due to formation of delocalized molecular orbitals with larger electron density on the oxygen.

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