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# An investigation by iron K-edge spectroscopy of the oxidation state of iron in hemoglobin and its subunits

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*Hemoglobin* *Iron charge* *K edge positions*

## 1. INTRODUCTION

Although the change in hemoglobin affinity for oxygen (the cooperative effects [1]) is known to be accompanied by some modifications in protein conformation [2], the processes which control the hemoglobin iron affinity for the ligands are still poorly understood. A variety of chemical [3–5] and spectroscopic studies [6–8] on hemoglobin have not yet attributed the formal charge of the iron bound to the oxygen molecule. Likewise, the oxidation state and degree of covalency of the iron in other oxygenated complexes have not yet been ascertained. Experimental data on the electronic states of iron in hemoglobin and its isolated subunits are scarce. We report here an approach to this problem using 'X-ray absorption near-edge structures' (XANES) [9] spectroscopy in the neighborhood of the $K_\alpha$ absorption edge of iron. Precise energy X-ray absorption edge values of the Fe centers in oxidized, oxygenated and reduced isolated subunits and hemoglobin have been obtained with synchrotron radiation from the electron storage ring at Lure (Orsay). Comparative analysis of K absorption edge positions for the oxidized and oxygenated hemoproteins suggests strongly that the iron in the $Fe{-}O_2$ complex of these hemoproteins carries a formal charge of + 3. Absorption edge features of hemoglobin spectra have also been compared with those of each isolated subunit and with the mean value of the $\alpha$ plus $\beta$ spectra to see whether or not the oligomeric association [10,11] dramatically affects the electronic repartition of the iron of each protomere.

## 2. MATERIALS AND METHODS

Hemoglobin [12] and isolated subunits [13] were prepared from fresh adult blood. Samples were concentrated by vacuum dialysis up to 13 mM in heme. Reduced proteins were obtained by dissolving a small excess of solid sodium dithionite in oxygenated species. Oxidized forms were obtained with sodium nitrite [14] reacting on the protein mixed with 1 M glycine [15] (pH 7.6). All solutions were suspended in 10 mM bis–Tris buffer (pH 6) and the final pH-value of the sample with the protein was adjusted to pH 7.4 with 0.5 M Tris solution (pH 12.8). Just before the experiment, the sample was centrifuged and X-ray measurements were performed on the supernatent.

Control procedure on possible radiation damage was made by visible absorption measurements on the irradiated samples (with the appropriate dilution 5/1000) before and after their X-ray exposition. The absorption spectra (from 450–650 nm) were identical for the unirradiated and irradiated proteins.

X-ray absorption K-edge measurements were made at room temperature by the fluorescence intensity [16] of the iron as a function of incident X-ray energy. An hyperpure germanium Ortec detector placed at 90° to the incident beam recorded the fluorescent intensity from the sample. The counting rate of the intensities was typically 20 000 cps, and each spectrum represents the sum of 6 or more scans (140 pt/6 min). Since fluorescence intensity is proportional to the photoabsorption cross-section, the raw data represent the ratio of this fluo-

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rescence intensity to the incident intensity [17] as a function of X-ray energy. The residual elastic scattering pre-edge background was removed by subtracting a straight line from the data [18]. Electronic repartition and effective charge number of the iron were extracted from the XANES spectra* using shape and shift of its principal peaks.

## 3. RESULTS AND DISCUSSION

Comparison of the spectra of the oxidized, the oxygenated and reduced proteins are shown in fig.1. The X-ray absorption spectra of the deoxygenated and oxygenated hemoglobin agree globally with respect to the positions of all the maxima with the ones obtained in [17]. For the 1*s*–4*p* transition a similar large chemical shift between deoxy- and oxy-hemoglobin is observed. Furthermore, one notices for the same 1*s*–4*p* transition a shift of equal distance between the oxidized and reduced forms. For the 1*s*–3*d* forbidden transition it is not possible to choose a clear peak position and to measure seriously any shift length.

The extremely weak intensity of the forbidden electronic transition 1*s*–3*d* is often useful in the study of the site symmetry of the metal center. In the coordinated compounds the 1*s*–3*d* transition reflects more particularly the distances between the metal and its nearest neighbours [19]. In general, the absorbance intensity of the 1*s*–3*d* transition increases as the metal surroundings become more compact. In going from the deoxy protein to its analogous oxy-form, the magnitude of this absorbance increases somewhat as expected, but in going from the oxidized to the reduced form, the 1*s*–3*d* absorbance also increases. This data is not consistent with crystallographic X-ray results. One must realize however, that the observed band involves, in this case, a 3*d*–4*p* mixing and that a systematic relationship between the absorbance amplitude and the iron–nitrogen distances does not exist. For this reason, an analysis of the shapes of the band for the electronic distribution around the iron remain qualitative and phenomenological. Our quantitative analysis of the formal charge on the iron is thus limited to the changes in the K edge position.

It is difficult from the spectra to determine visually the positions of the 1*s*–3*d*, 1*s*–4*s* and 1*s*–4*p* transitions. To know by how much the position of the K-absorption edge shifts between the oxidized, oxygenated and reduced hemoproteins an internal and arbitrary energy reference must be used. This information has been determined by examining the position of the peaks from the derivatives of the intensities *vs* the energies. The shift energy can then be determined with a precision of ± 0.5 eV. The K-absorption edge derivatives for all the compounds are presented in fig.2. The principal peak, situated between the 1*s*–4*s* and 1*s*–4*p* transitions, reflects the more important inflection of the absorption edge and is correlated with the positions of both these transitions. Unligated proteins and oxidized forms exhibit the expected ferrous to ferric energy shift of the 1*s*–4*p* and 1*s*–4*s* transitions. These transitions shift 5.5 eV between the deoxy- and oxy (or met) forms of both hemoglobin and $\beta$ proteins, but only 4 eV for the $\alpha$ chain. The de oxygenated iron of this protein appears to have a more positive ferrous charge (see table 1).

The position of the 1*s*–4*p* transition depends on the combined effects of covalency, ionic charge and iron site symmetry as well as on the nature of the environment [19]. Since the protein conformations [20] of the oxygenated forms are indistinguishable from those of the oxidized forms and since there are no consistent indications of any difference in energy for the K-shell absorption edge between the two XANES spectra, the formal charge of the iron is + 3 in both cases. However, the slight inflection changes between the edge spectra of these two compounds show a lack of equivalence in electron repartition around the iron nucleus.

Some experiments (such as magnetic susceptibility, electron paramagnetic resonance, or chemical reactions) seemed to show that the ionic state of the iron in the oxidized and the oxygenated hemoglobin was different. In fact, all these experiments do not split the coupling between the iron and its environment, and the effective charge distribution of the iron is not collected.

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*The K absorption edge of coordinated ions exhibits a fine structure (XANES) showing that the atomic description is not adequate for these compounds. However, the principal transitions using atomic notations (1*s*–4*s*), (1*s*–4*p*) correspond to a transfer (inside the ion) of one electron from the 1*s* electronic level to the 4*s* (or 4*p*) level

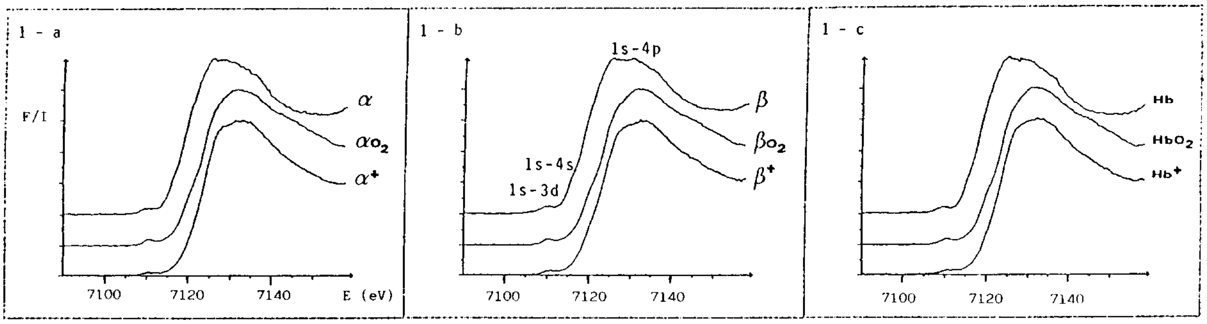

Fig.1. Iron K-absorption edges. Ratio of the iron $K_\alpha$ fluorescence intensity (*F*) to the incident intensity (*I*) as a function of incident X-ray energy (*E*). The unit-energy is the electron-volt (eV). Spectrum from: (a) α chain, in deoxy (α), oxy ($\alpha O_2$) and met ($\alpha^+$) forms; (b) β chain, in deoxy (β), oxy ($\beta O_2$) and met ($\beta^+$) forms; (c) Hb, in deoxy (Hb), oxy ($HbO_2$) and met ($Hb^+$) forms.

For a long time it was also supposed that the conformational change between $HbO_2$ and Hb [10] was induced by a low-spin to high-spin transition of a ferrous iron. Movement of the iron II atom and reorganization of the electronic cloud that accompany a low-spin to high-spin transition may displace the $K_\alpha$ edge position of the metal. This was certainly the major reason why the previous authors [17] who had only observed the shift between deoxy- and oxy-hemoglobin K-edge have not concluded that the state of the iron is + 3 in the oxygenated form. Detailed examinations to identify how much a change in the state of the spin of the iron in a fixed oxidation state can move the position of the K-edge are in progress. The key point of our experiments is the comparison of the oxidized and oxygenated hemoproteins. Comparing the energy shift of the K-edge position, it is clear that the effective charge number on the iron ion is the same. Comparing the shape of the main peak, it is obvious that the cloudy distribution of the electrons around the iron nucleus between these 2 forms is different.

Although the ionic state of the iron ($Fe^{2+}$ or $Fe^{3+}$) is independent of the protein part, the transition behaviour depends on the protein. Indeed analysis of the transition shape indicates some small difference between the electronic states of the iron for each protein (fig.3). In the simplest terms, the electronic repartition of the iron is

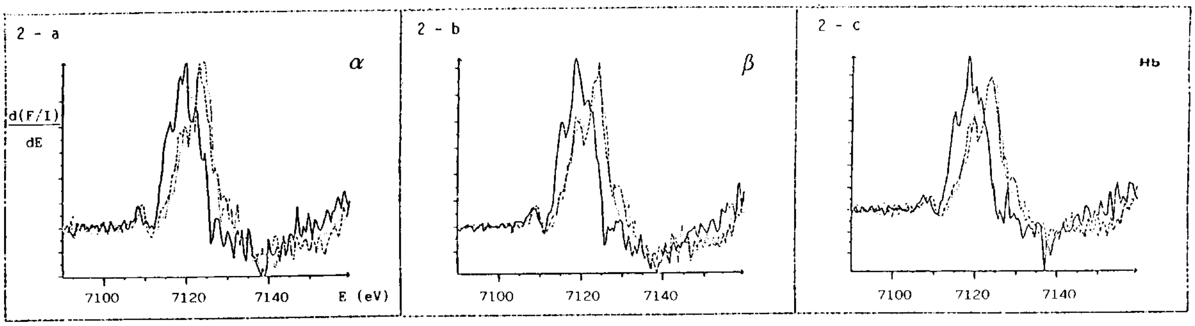

Fig.2. Iron K-absorption edge derivatives. The *Y*-axis of each spectrum is the derivative of the ratio *F*/*I* (of the fluorescence intensity *F* of the iron over the incident X-ray intensity *I*) *vs* the incident X-ray energy *E*: d(*F*/*I*)/d*E*. The X-axis represents the energy *E* given in electron-volt (eV). K-absorption edge derivative positions for: (a) α chain; (b) β chain; (c) Hb; (···) oxidized form; (---) oxygenated form; (——) reduced form.

Table 1

Principal peak positions of the derivative K-absorption edge (in eV ± 0.5 eV)

| Protein | Unligated | Oxygenated | Oxidized |
|---|---|---|---|
| $\alpha$ | 7120 | 7124 | 7124 |
| $\beta$ | 7118.5 | 7124 | 7124 |
| Hb | 7118 | 7123.5 | 7123.5 |

largely affected by the particular protein environment around the iron complex (fig.3$_I$). Shaanan [21], studying the geometry of the $N^\varepsilon$ (of the His E7) with the oxygen in $HbO_2$, showed different sets of $N^\varepsilon$–$O_2$ hydrogen bonds in the $\alpha$ and $\beta$ subunits. Thus these local interactions may also influence the distribution or electronic states of the iron in the heme–oxygen complexes and affect the $K_\alpha$ transition shapes.

It is also clear from these results that for the oxidized and oxygenated forms, oligomeric association of $\alpha$ and $\beta$ does not change the electronic repartition of the iron in either subunit. In the deoxygenated case, the small apparent difference between Hb and $(\alpha+\beta)/2$ (see fig.3b$_{II}$) could reflect a heme–heme interaction but this small difference could also be due to experimental conditions. With the precision obtained here, the results do not show that the electronic states of the iron are sensitive to the overall quaternary structure of the protein.

The essential finding of this work is that after the departure of oxygen the iron picks up an electron, changing its formal charge from + 3 to + 2. As yet, we do not know if this electron comes from the oxygen ligand [5] or from the porphyrin ring. Regardless of the origin of the electron however, the deoxygenation process represents a real reduction for the iron. The reactivity of the iron may thus be studied by the electronic repartition around the iron; such studies may help to elucidate the mechanism of cooperate binding in hemoglobin.

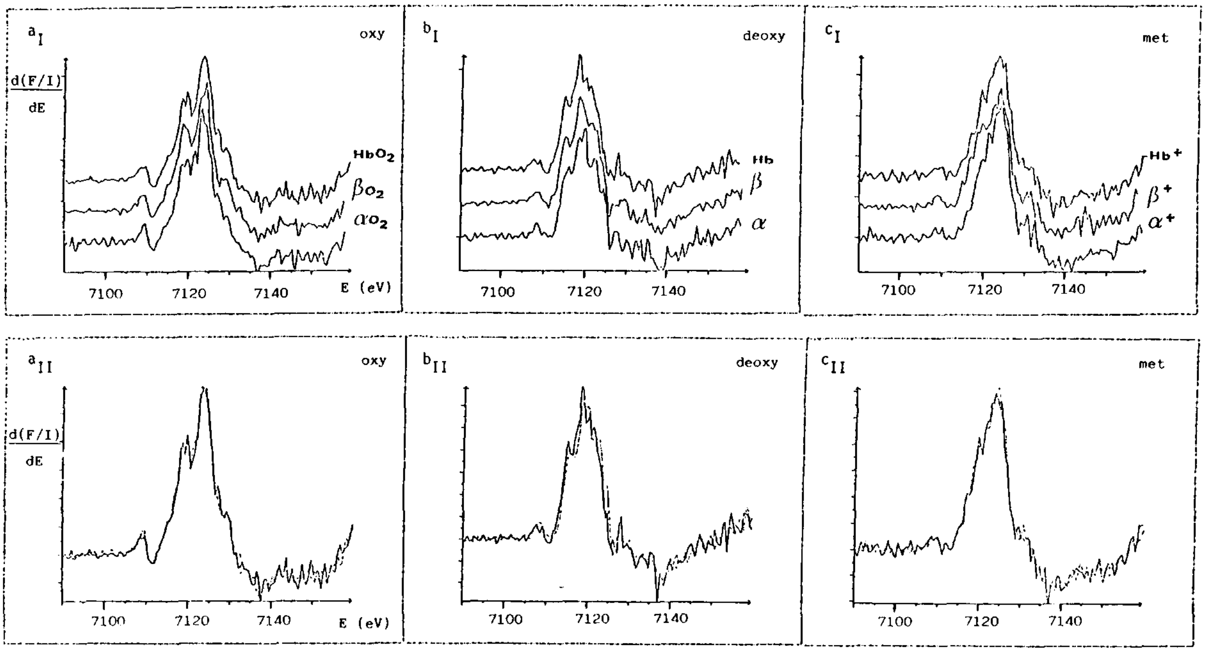

Fig.3. Shapes of K-edge derivatives. K-absorption edge behaviour for the same iron ionic state in different protein environments: (I) without spectral superposition; (II) with spectral superposition of Hb (–) and $(\alpha+\beta)/2$ (---); (a) oxygenated forms; (b) reduced forms; (c) oxidized forms.

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