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XANES spectroscopy sensitivity to small electronic changes

Case of carp azidomethemoglobin

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Spin states equilibrium of hemoglobin-iron varies with external conditions: pH, allosteric effectors, temperature. The small electronic reorganization of the iron caused by the spin state changes has been detected by X-ray absorption near edge structure (XANES) spectroscopy at room temperature. The iron K-edge region which is sensitive to spin state is located in 7110–7130 eV. Here are presented the 100% high spin and 100% low spin XANES spectra of carp azido ferric hemoglobin.

Iron K-edge spectrum; Spin equilibrium; Hemoglobin

1. INTRODUCTION

The XANES (X-ray absorption near edge structure) spectroscopic data on hemoglobin [1–3] being inconsistent in Perutz's model [4], the credibility of this technique is often questioned. Indeed, one wonders whether the sensitivity of this spectroscopy is suitable to detect subtle iron electronic differences. In order to answer this question, we have decided to investigate the carp azidomethemoglobin ($Hb^+N_3^-$) compound which exhibits, at room temperature, different iron magnetic properties [5]. This ferric hemeprotein has its iron atom in equilibrium between two close electronic configurations, characteristic of 5- and 1-unpaired d electrons [6], and thus exhibits a thermal spin equilibrium. At room temperature, this equilibrium varies significantly with the external conditions (pH and the presence of allosteric effectors) [5,7].

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In many high spin compounds, the iron position is out of the mean heme plane [8], whereas in low spin derivatives it is expected to be in the heme plane [9]. In carp azidomethemoglobin, the iron spin change is not induced by a significant out-of-plane iron motion [10] but by a reorientation of the proximal histidine [10,11]. Therefore, in this heme-protein derivative, the iron d-eletron redistribution occurs without appreciable heme-iron geometric distortion [2,10,11]. Hence the iron electronic rearrangement and spin state changes are linked, i.e. the iron K-edge spectra can be related to intermediate paramagnetic susceptibilities of carp azidomethemoglobin.

2. MATERIALS AND METHODS

Carp hemoglobin was prepared according to the previously described method [12,13] and stripped on an AG 501 (BioRad) resin [14]. Iron oxidation was produced by sodium nitrite [15]. Azido derivative was obtained with an excess of sodium azide. $Hb^+N_3^-$ solutions (9 mM in heme) were buffered in 200 mM bis-Tris, pH 6.10 and 6.85. When specified, 2.5 eq/heme of inositol hexaphosphate (IHP) were added and final pH values were 6.25 and 6.90. The quality of each sample was controlled

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before and after X-ray irradiation by measuring the absorption spectra on a Cary model 219 spectrophotometer.

X-ray absorption iron K-edge measurements were made with synchrotron radiation from the LURE DCI electron storage ring (Orsay-France). The experiments were performed on EXAFS II set up with a SI 311 monochromator and a NaI detector (in front of which an Mn filter was set) placed at 90° from the incident beam. Iron K-edge measurements represent the total iron fluorescence emission as a function of incident X-ray energy [16]. Experiments were made at room temperature (20 ± 1°C) on a thin layer sample (20 × 5 mm^2). Each K-edge spectrum was the average of five scans. Each scan was collected with a 0.2 eV step and a 5 s integration time. Subtraction of the scattering background was made before normalization of fluorescence to X-ray incident intensity.

3. RESULTS

It has been reported by Messana et al. [7] that the addition of IHP to carp $Hb^+N_3^-$ rises the paramagnetic susceptibility (χ) of the protein-iron. This compound also exhibits a pH-dependent magnetic susceptibility [5]. Iron magnetic property changes are interpreted as a simple variation of the spin equilibrium between the high ($S = 5/2$) and low ($S = 1/2$) spin populations [17]. Fraction (α) of high spin in the spin mixture [7,10,17] can be deduced from the effective magnetic moment μ_e ($\mu_e = 2.828 \cdot \sqrt{\chi \cdot T} \cdot \mu_B$, where T is the absolute temperature and μ_B the Bohr magneton) by the relation:

$$\alpha = (\mu_e^2 - 3)/32$$

according to Henry et al. [10]. The iron electronic configurations ($^2T_2$ and $^6A_1$) are respectively associated with the low and high spin states. Thus, if S_n is the XANES spectrum of the superposition of the 2 iron electronic populations in equilibrium, the relationship between the experimental XANES spectrum and the iron magnetic state is given by:

$$S_n = \alpha_n H + (1 - \alpha_n)L$$

where H and L are the pure high and low spin spectra and α_n is the corresponding high spin fraction. Electronic states of the iron atom from $Hb^+N_3^-$ were examined for protein solutions without IHP (pH 6.10 and 6.85) or with IHP (pH 6.25 and 6.90). Typical iron XANES spectra of carp $Hb^+N_3^-$ pH 6.10 without IHP and pH 6.25 with IHP are presented in fig.1. The tilt angle of the azide molecule [18] has an influence on the XANES spectrum after reaching the edge [19,20]. The value

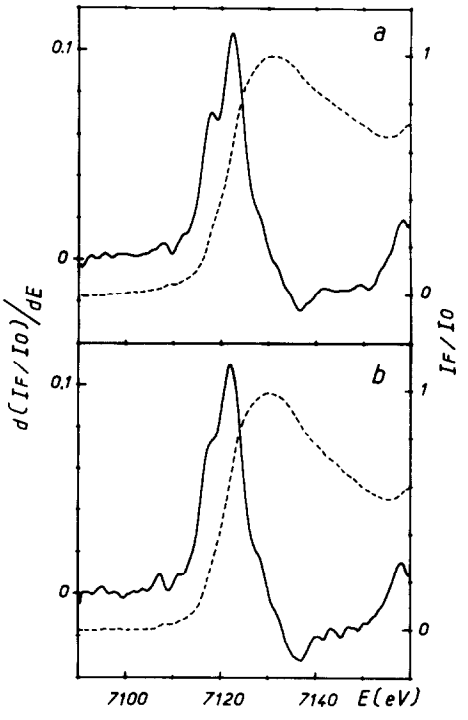

Fig.1. Iron K-edge data of 9 mM carp azidomethemoglobin obtained at room temperature (20 ± 1°C). Experimental XANES spectra (– – –); derivatized spectra (——). The right vertical axis is for the XANES spectra obtained from the ratio of the iron fluorescence intensity (I_F) to the incident intensity I_0) plotted as a function of the incident X-ray energy ($E = hc/\lambda$); the left axis is for the derivatives ($d(I_F/I_0)/dE$) obtained from the XANES spectra. (a) Stripped azidomethemoglobin in 200 mM bis-Tris buffer, pH 6.10; (b) azidomethemoglobin in the same buffer with 2.5 equivalents IHP per heme, pH 6.25.

of this angle was not searched for in this work. So, to keep out of the effect of azide bond length, the analysed spectra in this work were limited to a region below 7130 eV.

The pure L and H spectra can be extracted from any two $Hb^+N_3^-$ solutions containing different α_n high spin fractions: α_p and α_q. From the 4 experimental S_n spectra, the 6 paired combinations allow a good average. Fig.2a reports the averaged L and H spectra using the α_p and α_q values determined by Noble et al. [5]:

$$L = \frac{\sum_{p,q} (\alpha_q S_p - \alpha_p S_q)}{\sum_{p,q} (\alpha_q - \alpha_p)},$$

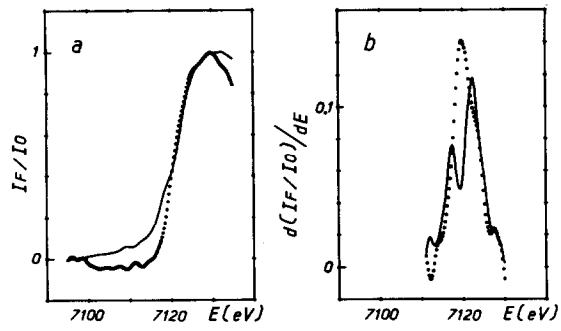

Fig.2. Spectra and derivatives of 100% low (——) and high (···) spin forms of carp azidomethemoglobin. (a) Iron K-edge absorption spectra, (b) derivatives obtained from spectra displayed in panel a.

$$\text{and } H = \frac{\sum_{p,q} [(1-\alpha_q)S_p - (1-\alpha_p)S_q]}{\sum_{p,q} (\alpha_p - \alpha_q)}.$$

Fig.2b shows the first derivatives of iron XANES spectra in pure low and high spin states. Several different features are clearly observed in these derivatives. These characteristic features are quite similar to those observed on high to low spin transition in hydroxy metmyoglobin induced by a temperature decrease [21]. In this case, the iron has a large displacement from the heme-plane. So these latter experiments reveal also the distortion of the octahedral ligand field caused by the iron displacement [21].

Fig.3 reports two comparisons between experimental and calculated spectra derivatives obtained by linear combination of pure low and pure high spin forms. Since experimental and calculated derivatives fit very well together, it can be assumed that the XANES technique is suitable to detect electronic differences as subtle as spin distribution.

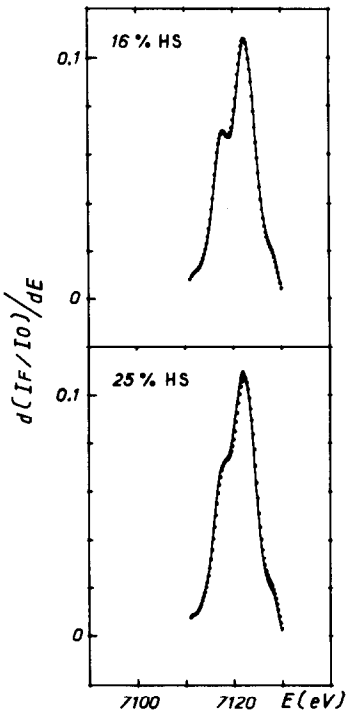

Fig.3. Sensitivity of the methodology. The experimental spectra derivatives (——) are compared with those calculated (···) by linear combination of pure low and high spin forms. (a) Comparison for 16% high spin, (b) comparison for 25% high spin.

4. DISCUSSION

Our data show that the iron XANES spectroscopy is such an accurate technique that it allows distinction – at room temperature – of the electronic difference between the low and high spin states in the same ligated ferric hemoglobin. From our data and those of Oyanagi et al. [21], it is likely that the spin state sensitive region is restricted to 7110–7130 eV. Above 7130 eV, the spectral pattern is sensitive to both geometry and spin state.

Iron electronic reorganization, subsequent to spin redistribution, can be observed from its effects on the XANES spectrum. Hence, this work reinforces the conclusions of previous XANES analysis. The chemical shifts for the 1 s core level which are observed in the oxygenated hemoglobin [1,22,23] and myoglobin [23] come as a consequence of the effective charge change of the iron during the oxygenation process. Indeed, it appears that the iron charge and spin state depending on the hemoglobin derivatives are perceptible in the XANES spectra [1]. The deoxygenated hemoglobin (Hb) ($S = 2$) has its iron K-edge position at 7123 eV, the carboxy-hemoglobin (HbCO) ($S = 0$) has its K-edge at 7124 eV, the oxy-hemoglobin (HbO_2) ($S = 0$) exhibits its K-edge at 7129 eV. Thus, HbCO and HbO_2 (which present no significant difference in the deviation of their iron atom from the heme-plane [24,25]) differ in their effective charge. We must also note that the allosteric effectors do not influence the iron elec-

tronic organization of the deoxygenated and ligated derivatives of the human and carp hemoglobins [1–3]. The contradiction between Perutz's model and XANES data strongly suggests that the hemoglobin oxygenation properties involve greater complexities than a simple iron-heme distance change.

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