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# Constraints of T conformation of carp azide hemoglobin on Fe site structure

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The iron site structure modifications induced by the transition from the quaternary R to T structure in ferric carp azide hemoglobin have been detected from analysis of multiple scattering resonances in the XANES (X-ray absorption near edge structure) spectra. High signal-to-noise XANES spectra measured at the Frascati 'wiggler' synchrotron radiation facility reveal that the forces on the Fe active site, due to the transition from the R to T quaternary conformation, only induce the tilting of the porphyrin plane and probably also of the proximal histidine. The variation of the Fe–N mean distance is not detected by XANES spectroscopy and therefore it is less than 0.01 Å.

*Hemoglobin* *Synchroton radiation* *XANES*

## 1. INTRODUCTION

The binding of ligands induces a transition from the tense (T) quaternary conformation of deoxyhemoglobin (deoxy-Hb) to the relaxed (R) conformation of ligated hemoglobins like in ferrous carbonmonoxy-Hb (HbCO), in oxy-Hb and in ferric azide-Hb ($HbN_3$). The hypothesis that there is a correlation between the quaternary structure and the position of the Fe atom out of or in the porphyrin plane has been advanced to explain the cooperativity [1]. The cooperative binding of ligands in hemoproteins has been interpreted as due to the triggering of the T to R transition by ligand binding only in some subunits of intermediate forms of hemoglobins. The experimental evidence for intermediate forms of hemoglobin has recently been found by time-resolved resonance Raman spectroscopy [2] and X-ray diffraction [3] using synchrotron radiation. The presence of allosteric effectors can induce the stabilization of ligated hemoglobin in the T conformation, as it has been found in the structure determined by X-ray diffraction [4] of ferric fluoromethemoglobin in the T conformation.

It has been demonstrated in carp hemoglobin, at acid pH (pH 5.6) and in the presence of allosteric effectors such as inositol hexaphosphate (IHP), that the transition from the T deoxy conformation (deoxy-carp-Hb) to the R conformation with ligand binding is inhibited [5–8], therefore carp hemoglobin presents the anomalous T conformation of ligated hemoglobin that we will call T*. This protein is a valuable model for studying the constraints of the T conformation of the protein on the local structure of the active site because it is possible to induce, by allosteric effectors, the R to T* transition in ferric carp-$HbN_3$. Magnetic susceptibility measurements have shown the dependence of spin-state equilibria on conformation of the quaternary structure of carp-HbCO and carp-$HbN_3$ [9–11]. The correlation between the magnetic moment and the Fe coordination has been established in a large series of chemical compounds [12] and therefore an effect of the quaternary structure on the Fe site can be expected.

In this work we have used XANES (X-ray absorption near edge structure) spectroscopy [13–17]

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to investigate the local structure changes around the Fe in the heme pocket induced by the R to T* transition in carp-$HbN_3$.

In the XANES energy range, some tens of eV above the absorption threshold, the absorption cross-section is modulated by scattering of the photoelectron, emitted at the absorbing atom (Fe), by neighbouring atoms. In XANES the photoelectron is in the multiple scattering regime. All the multiple scattering pathways with 2, 3, 4,... scattering events which begin and end on the Fe site at the origin contribute to XANES. It is via multiple scattering that XANES probes higher-order correlation functions of atomic spatial distribution, i.e. bonding angles. In fact we have been able to measure the displacement of the iron out of the porphyrin plane in deoxy hemoproteins [18,19] and the bonding angle of the ligands [20–22] by means of XANES.

## 2. MATERIALS AND METHODS

Carp blood was obtained as described [5,11]. The packed cells, after washing, were kept under liquid nitrogen. To achieve lysis 2.5 vols of 0.1 mM Tris, pH 8, were added. After 1 h 0.1 M NaCl was added to the lysate and the mixture was centrifuged at 15000 rpm in a Sorvall centrifuge for 20 min to remove the stroma. The samples were passed through a Sephadex G-25 column equilibrated with 1 mM Tris (pH 8) and then through a Dintzis deionizing column. To obtain carp $HbN_3$, the ferric hemoglobin was first prepared by allowing the hemolysate to incubate at 4°C with a 3-fold excess of $NaNO_3$. The ferric form so obtained was passed through a Sephadex G-25 column and a Dintzis type deionizing column. The 2 forms R and T* were obtained from the ferric form as follows. To obtain the carp $HbN_3$ in the R state, 0.075 ml Bistris buffer (pH 6.6) and 0.075 ml of 1 M $NaN_3$ were added to 0.6 ml of $Hb^{3+}$. The T* state was obtained by adding to 0.6 ml $Hb^{3+}$, 0.075 ml Bistris buffer (pH 4.9), 0.075 ml 1 M $NaN_3$, and 0.02 ml of 200 mM IHP. The samples were at equal concentrations (about 10 mM).

The XANES measurements were performed at the Frascati 'wiggler' facility using synchrotron radiation monochromatized with an Si(111) channel cut crystal. The spectra were recorded by the transmission method. The threshold energy of the Fe edge has been defined as the first maximum of the derivative of the metal Fe K edge. The absorption background due to excitations of lower binding energy levels was subtracted in all spectra by polynomial fitting of the pre-edge region. The obtained spectrum due to core excitations from the Fe(1s) level has been normalized to the atomic absorption coefficient at high energy beyond the absorption jump $\alpha/\alpha_{a\infty}$ obtained by linear fitting of the spectrum in the extended X-ray absorption fine structure (EXAFS) region. An accurate normalization procedure is necessary to extract unambiguous variations of relative intensities of the XANES peaks. The energy resolution was about 1 eV but the accuracy in determination of the energy positions was $\Delta E = 0.2$ eV. The high signal-to-noise ratio was obtained by reaching very good stability of the electron beam in the storage ring and in the wiggler.

## 3. RESULTS AND DISCUSSION

In fig.1 we show the X-ray absorption spectra of carp azidemethemoglobin in the R and T* states

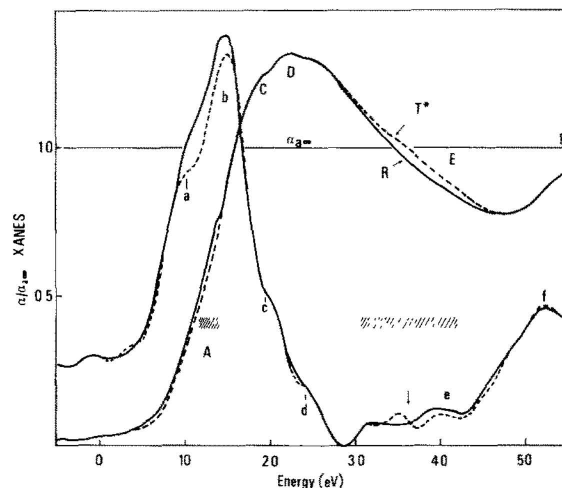


Fig.1. XANES spectra at the Fe K edge of carp azidemethemoglobin in the R (——) and T* quaternary conformation (---). The absorption coefficient has been normalized to the atomic absorption after the absorption jump $\alpha/\alpha_{a\infty}$. The spectral features A–F can be identified in the absorption spectra. The derivative spectra are superimposed and clearly show the corresponding structures a–f. The hatched areas indicate the energy regions where the transition of the quaternary structure induces changes in the XANES spectrum.

over the XANES range. The main differences between the spectra of the R and T* form are in the energy range indicated by the hatched areas in the figure. There is no shift of the spectral features within our sensitivity $\Delta E = 0.2$ eV.

A multiple scattering resonance at energy $E_r$ moves at higher energy with decreasing interatomic distance $d$ following the rule $(E_r - E_b)d^2$ = constant, according to multiple scattering theory of XANES [23], where $E_b$ is the energy of a localized atomic-like excitation at threshold in the Fe K-edge XANES spectra of hemoglobins the weak 1s–3d excitation at threshold, where the origin of the energy scale has been fixed in fig.1, can be used as reference energy. In human adult hemoglobin a contraction of 0.1 Å of the Fe–$N_p$ distance gives a blue shift of 1.8 eV of the multiple scattering resonances A, C and D at about 20 eV above threshold [24].

We do not observe any energy shift of XANES multiple scattering resonances A, C and D with the T to R transition in fig.1, therefore there is no contraction of interatomic distances larger than 0.01 Å induced by the transition of quaternary structure. This result should be compared with the contraction of about 0.07 Å of the average Fe–N distance in the transition from the deoxy to the ligated state of hemoglobins.

The observed variation of the XANES spectrum can therefore be associated only with doming of the porphyrin plane and/or bond angle variations either of the ligand or of the proximal histidine.

The effects of rotations of the ligand in XANES spectra have been studied by using the full multiple scattering theory [15,17]. In this approach the photoabsorption cross-section $W_c$ for one-electron transition from a core level at energy $E_c$ to a state on the continuum at energy $E = E_c + \hbar\omega$ is calculated in real space using the Green formalism

$$W_c(\hbar\omega) = -2\sum_{LL'} m_L(E)\, |m[\tau^{\infty}_{LL'}(E)] \times m^*_{L'}(E)\Theta(E - E_f)$$

where $E_f$ is the Fermi level, L stands for the pair $(l,m)$ angular momentum numbers, $m_L(E)$ is the dipole matrix element, which is an atomic quantity and $\tau^{\infty}_{LL'}$ is the dipole scattering matrix. This latter contains all the effects of the surrounding atoms. Therefore the measured absorption spectra can be considered to be the product of an atomic absorption cross-section by a factor due to the presence of neighbouring atoms which gives the modulation of the atomic part. In Green's function formalism $\tau^{\infty}_{LL}$ gives the sum of all scattering paths for a photoelectron of energy $E$ which begin and end on the Fe site.

XANES calculations have been performed for a cluster of 30 atoms with the Fe at the center including the porphyrin, the proximal histidine and the ligand. We have studied the effect of ligand rotation in the theoretical spectra. The rotation of the ligand induces changes mainly in the intensity of peak [20–22,25] and this effect is not observed in the experimental spectra in fig.1. The ligand contributes to the XANES also at about 35 eV where there is a second multiple scattering feature due to the ligand. A variation of the feature at 35 eV in fig.1, indicated by an arrow in the derivative spectrum, has been observed going from the R to the T* conformation. However, because we do not observe any change in the intensity of peak C, due to the ligand, we find that the variation of the Fe-N-N bonding angle is at maximum a few degrees.

Evidence for the tilted azide bonding can be obtained by comparing the absorption spectrum of carp azidemethemoglobin in the R state with the very similar spectrum of human oxy-Hb where the oxygen has been found to be tilted both by diffraction experiments in crystals [26] and by XANES experiments in solution [21,24].

XANES calculations have been performed to determine the effect of the tilting of the pyrroles of the heme plane towards the Fe (doming effect) with the transition from the R to the T* conformation. The calculations for a set of models have been reported in [27] where the Fe is moved out of the plane with tilting of the pyrroles of the porphyrin towards the Fe in such a way that Fe remains conplanar with each pyrrole keeping constant interatomic distances. The spectra are affected very little by this distortion of the heme and the sole energy region affected corresponds exactly with the hatched energy region in fig.1. The experimental increase of the absorption coefficient in this region, at about 30 eV, indicates increasing tilting of the heme plane in the T* conformation.

In conclusion the small variations of the XANES spectrum of carp azidemethemoglobin with the R to T* transition can be explained by modifications of the bonding angles of the Fe-

heme complex keeping constant interatomic distances. The tilting of the heme induced by the T quaternary structure is in good agreement with the findings of Brzozowski et al. [3]. Also, the rotation of the proximal histidine, keeping constant Fe–N distances, is in agreement with our findings.

This experiment shows that the constraints due to the T conformation in ligated hemoglobin are present and determine rotations of the heme-proximal histidine group, but Fe does not move closer to the nitrogen nearest neighbors. Therefore the protein conformation modulates the local Fe active site structure by subtle modifications. This result shows that the main part of the cooperative energy of hemoglobin is not localized at the Fe site but distributed over other bonds of the protein in agreement with recent studies [6,7,28].

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