XANES spectroscopy of carp hemoglobin-iron in correlation with the affinity changes of the protein for ligand

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The strong variation of ligand-binding properties with pH for carp hemoglobin is not reflected in the electronic distribution of the heme-iron. Thus, we can suppose that hemoglobin affinity is directly controlled by the protein and not by some particular changes of the iron atom.

Heme-protein *Iron reactivity* *X-ray absorption*

1. INTRODUCTION

Ligand-binding processes in heme-proteins involve the iron reactivity for the ligand and the conformational organization of the protein. Structural differences between deoxygenated and liganded hemoglobins are now completely established [1–3] and intermediate forms of hemoglobin have been recently found [4].

Perutz [5,6] has correlated the position of the iron atom with respect to the porphyrin plane with the different affinities of the hemoglobin for ligand, thus offering a possible explanation for the observed cooperativity. Indeed heme-iron movement should be accompanied by reorganization of the electron distribution of the iron nucleus and could reasonably produce a change in iron reactivity for ligand. Thus, in an attempt to understand how the switch of the quaternary structure of the protein modulates the response of the iron-binding site, the iron K-edge spectrum (measured by XANES spectroscopy) was used to investigate the iron structure of hemoglobin.

XANES spectroscopy (X-ray absorption near edge structure), which reveals the electronic transitions from the 1s level to the lowest unoccupied atomic states [7], is a powerful tool to investigate the electronic repartition of the iron atom in the porphyrin [8,9]. Comparative XANES studies, previously used on human hemoglobin [10], were performed on carp hemoglobin which presents the largest affinity change ever observed with pH and allosteric effectors [11–14]. These experiments did not indicate that the iron atoms exist in different electronic states regardless of the affinity changes observed. Thus, the molecular properties responsible for the ligand affinity changes have yet to be elucidated.

2. MATERIALS AND METHODS

Carp hemoglobin was prepared as in [11,12]. Stripped hemoglobin was obtained by passing the hemoglobin solution through a mixed-bed ion-exchange resin (AG 501, Bio-Rad, Richmond, CA). Methemoglobin (Hb^+) was prepared by ox-

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idizing the hemoglobin solution with sodium nitrite [15]. Excess nitrite was removed by dialysis against distilled water. Deoxyhemoglobin was obtained by dissolution of sodium dithionite in a buffered methemoglobin solution [16].

Solutions were concentrated by vacuum dialysis up to 5–6 mM in heme. Samples were buffered by 300 mM Bis-Tris (pH 6 and 6.5) or Tris (pH 8.5). Inositol hexaphosphate (IHP), when required, was added to the hemoglobin solution in a 10-fold molar to tetramer excess and pH was readjusted.

X-rays were produced by synchrotron radiation from the DCI electron storage ring of LURE (Orsay, France) and experiments were carried out on the EXAFS II set-up (SI 311 monochromator and NaI detector). Iron K-edge measurements were obtained by the total fluorescence emission of the iron as a function of the incident X-ray energy. The maximum counting rate of the fluorescence intensity was limited to 80 000 cps and the XANES spectrum was the sum of at least four scans (140 points/7 min). The normalized ratio of the iron fluorescence to the X-ray incident intensity and the subtraction of the scattering pre-edge background were obtained using the computer analysis of Michalowicz [17]. The standard deviation of each XANES spectrum was evaluated as being half of the experimental dispersion as described in [10]. The sensitivity of the method was determined by the amount of contaminating species which disturb the XANES spectra [10]. We found that a slight fraction of 1% is easily detectable.

The iron K-edge spectra of carp deoxyhemoglobin were recorded at pH 6.5 and 8.5 in the presence or absence of IHP. Spectra of methemoglobin were taken at pH 6 without or with IHP.

3. RESULTS

The electronic repartition of the heme-iron was investigated by the lineshape of the iron K-shell X-ray absorption edge (XANES) as previously described [10].

3.1. *Hemoglobin affinity changes and ferrous iron reactivity*

Carp deoxyhemoglobin exhibits an affinity change for oxygen of approx. 200-fold between pH 8.5 in the absence of the allosteric effector, IHP, and pH 6.5 in its presence [11–14]. Iron K-edge spectra, however, taken under these conditions revealed no changes in the electronic distribution of the iron atom (fig.1). This indicates the presence of a unique deoxyferrous species, despite the large changes in affinity of the whole protein. Therefore, these affinity changes cannot be correlated with a reactivity change of the active site of ferrous iron.

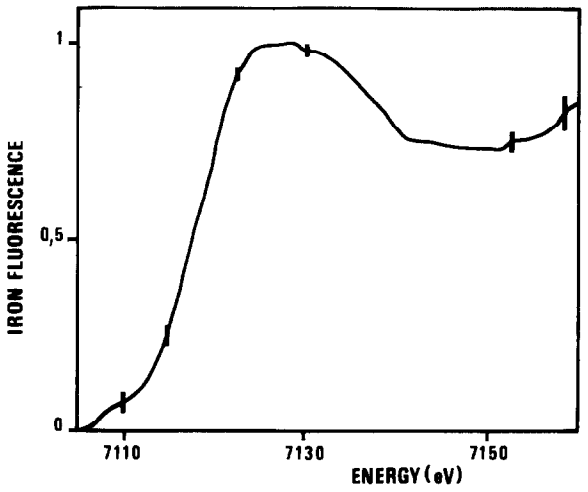

Fig.1. Normalized X-ray absorption spectra of carp deoxyhemoglobin in low- and high-affinity forms. Low affinities have been obtained by stripped deoxyhemoglobin in Bis-Tris buffer, pH 6.5, without or with IHP (2.5 IHP equivalents per heme). High affinity was obtained in Tris buffer, pH 8.5. Hemoglobin concentration: 6 mM in heme. Experimental dispersions (I) of all spectra are within the standard deviation of XANES spectroscopy.

3.2. *Conformational switch and ferric iron electronic distribution*

It is also interesting to consider the six-coordinated iron in methemoglobin in relation to the iron movement out of the heme plane when the protein binds IHP [18]. At pH 6, IHP induces a change in the R-T populations and no appreciable modification in the spin state equilibrium is produced [19]. Under these conditions the effect of a supposed considerable strain by IHP [2] is again not detected in the electronic distribution of the ferric iron of carp aquomethemoglobin (fig.2). These data, consistent with previous measurements on human hemoglobin [10], demonstrate that the binding of IHP has no effect on the electron densi-

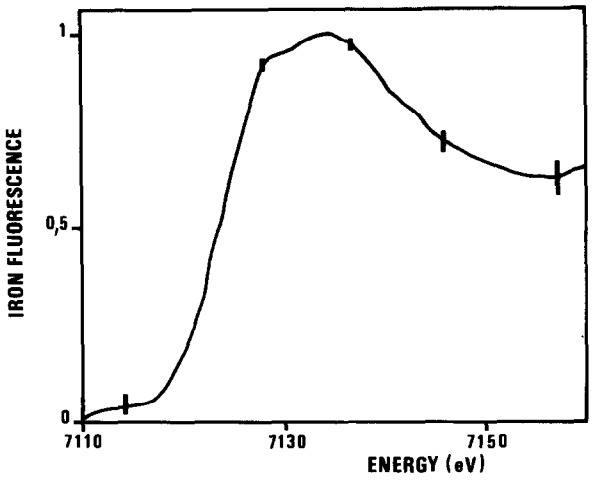

Fig.2. Normalized X-ray absorption spectra of carp aquomethemoglobin in the R and T states: R state was obtained by stripped methemoglobin in Bis-Tris buffer, pH 6. T state was obtained in Bis-Tris, pH 6, with IHP (2.5 IHP equivalents per heme). Hemoglobin concentration: 5 mM in heme. Experimental dispersion (I) of each spectrum is within the standard deviation of XANES spectroscopy.

ty of the iron. There is no apparent correlation between the electronic properties of the ferric iron atom and local protein deformation induced by the allosteric effector.

4. DISCUSSION

4.1. *Affinity changes and iron reactivity*

According to Perutz [5,6] the increase in affinity of carp deoxyhemoglobin for oxygen at high pH should be accompanied by a movement of the iron atom toward the heme, resulting in a stereochemical and electronic restructuration of the heme-iron. However, although the binding affinity of carp hemoglobin for oxygen is considerably altered by pH (= 200-fold), the electronic distribution of the heme-iron remains the same. We must note here that two types of heme (α and β) are being averaged. However the XANES spectrum of hemoglobin appears to be the rigorous superposition of the α and β spectra [8]. Thus, changes in affinity for the iron of the α- or β-subunit should be associated with some electronic reorganization of this iron. This kind of effect is not revealed in the XANES spectrum behavior. Consequently, the intrinsic reactivity of the iron for the ligand does not change significantly when the heme-protein affinity changes drastically. It is therefore clear that heme-protein affinity for ligand is not directly related to a repositioning of the iron atom. For carp hemoglobin the binding process involves a unique chemical iron reactivity for the ligand as has been previously determined for human hemoglobin [10].

4.2. *Affinity changes and the R-T switch*

It has been demonstrated that carp hemoglobin has a low affinity for ligand at acid pH (pH 6.5) in the presence of allosteric effectors such as IHP (P_{50} = 110 mmHg), and a high affinity at basic pH (pH 8.5) (P_{50} = 0.63 mmHg) [14]. In the framework of the MWC two-state model [20], when the oxygen pressure at half-saturation (P_{50}) shifts, the concentration ratio (L) of T to R states in the absence of oxygen also changes. One has the allosteric relation [21]:

$$P_{50} = L^{1/4} K_R \text{ (when } K_R > K_T)$$

Since the allosteric effector and pH do not affect K_R, P_{50} depends only upon the initial R-T population in deoxyhemoglobin. For a very large change in the affinity index (P_{50}), such as in the case of carp hemoglobin, it has been postulated that the deoxy form switches from T to R upon raising the pH [11-14]. At pH 6.5 and 8, the values of L were estimated at oxygen pressure equilibrium to be 1160 and 0.38, respectively [22]. Therefore, carp deoxyhemoglobin is a valuable model for studying the constraints of the T (pH 6.5 + IHP) and R (pH 8.5) conformations of the protein on the structure of the active iron site.

Our X-ray absorption spectra of carp deoxyhemoglobin at pH 8.5 and pH 6.5 + IHP were identical within the standard deviation of the XANES spectra. Thus, we were not able to detect any measurable deviation in the shape of the band and in the K-edge position. It seems from our results that the T-R protein conformations do not modulate the local structure of the iron active site.

As in the case of our XANES results, no modifications in the UV circular dichroism (CD) spectra of carp deoxyhemoglobin were observed when the protein switched from low affinity (pH 6) to high affinity (pH 9) [23]. This is in contrast to a large negative ellipticity at 287 nm observed

under different conditions which has been attributed to the T state and a slight positive ellipticity in this region attributed to the R state [24]. Although the binding affinity of carp hemoglobin is considerably altered by pH, no structural variation can be also detected by Resonance Raman of the $\nu_{Fe\text{-}His}$ band [25]. Carp deoxyhemoglobin remains in a T-like quaternary structure at high pH. Perhaps the protein conformation is not always directly correlated with affinity. The protein contribution expected in the binding variation could reside in the dynamics of the macromolecule. Some change in the mobility of the backbone of the apoprotein could be produced by pH without affecting the quaternary structure of hemoglobin. NMR spectra show an exchangeable proton (9.1 ppm) at low pH which disappears at high pH [26]. According to this result we may conclude that the protein flexibility varies with the pH. Conformational changes between the constrained (T) and relaxed (R) states could in some cases be disconnected from the cooperative affinity change. Although this absence of connection was not expected, we must note that this possibility has already been demonstrated on another allosteric protein, aspartate transcarbamylase [27].

4.3. *Iron electronic distribution and iron position*

We must remember that the electron cloud around the heme-iron is sensitive to the ligand [10] and the apoprotein nature [8]. Therefore, for two different proteins, there is no simple correlation between the stereochemistry of the heme-iron and its electronic organization. For example, in human oxyhemoglobin and sperm whale oxymyoglobin the Fe geometry is different (0.12 and 0.18 Å out of plane, respectively) but the iron electronic repartitions are the same [9]. The iron electronic distributions of deoxyhemoglobin and deoxymyoglobin are distinct [9] as their geometry (0.37 and 0.42 Å out of plane, respectively). The difference observed in the deoxy forms (0.05 Å) is equal to that observed for the oxy forms. So, the response of the heme-iron to globin structural changes must be subsequently studied on the same protein. In this case, the lineshapes of XANES reveal the variations in iron symmetry induced by the protein. Thus in carp hemoglobin, the affinity changes induced by pH and allosteric effector cannot be due to some protein tensions controlling the iron geometry. However, any iron displacement which produces local constraints but keeps constant the iron–nitrogen interatomic distances without perturbing the iron symmetry will not perturb the XANES spectrum. So, there is no direct correlation between the iron position and its electronic properties.

4.4. *Iron displacement and the R-T switch*

In aquomethemoglobin, the heme irons are sexta-coordinated and the R quaternary structure is dominant. Organic phosphates such as IHP and DPG bind to methemoglobin in the same site as to deoxyhemoglobin [18] and induce a change in the CD ellipticity in the UV region [19] and an increase in intensity of Raman lines near 340 and 380 cm^{-1} [19]. Perutz et al. [18] and Henry et al. [19] suggest that this high-spin form ($Hb^{+}H_2O$) switches from the R state to the T state upon addition of IHP. Our XANES results show that the electronic repartition of the heme-iron is not perturbed by the addition of IHP. Our data are consistent with recent observations [19] that the position of the iron atom with respect to the heme plane does not vary upon addition of IHP. Thus, R-T protein structure changes are not systematically correlated with out-of-plane displacement of the iron.

Recent XANES work on carp azidomethemoglobin [28] also shows that in this compound the iron remains coplanar with the four pyrrole nitrogens although the R to T transition does induce the tilting of the pyrroles of the heme plane and a rotation of the heme proximal histidine. This study and those of Brzozowski et al. [4] demonstrate that the heme proximal histidine group can rotate while the interatomic distance between the iron and the pyrrole nitrogens remains constant.

4.5. *Iron electronic distribution and the R-T switch*

The most important implication of the XANES data is that the Raman frequency of the iron-proximal histidine stretching mode can vary (from 210 to 224 cm^{-1}) with the T-R quaternary switch in deoxyhemoglobin [29–33], without perturbing the electron configuration of the heme iron. Optical absorption of methemoglobin upon addition of IHP also exhibits significant changes [19,34], certainly because the effector molecule, inducing the

tilt of the heme plane, perturbs the specific molecular interactions between the heme pocket and the heme periphery without affecting the iron atom. We must note here that the XANES technique describes the electron distribution of the iron and only this repartition. Any Fe stereochemistry changes and any modifications in the Fe-N_ϵ or Fe-L vibrations which do not perturb the electronic cloud of the iron nucleus are not revealed in the XANES spectra. Thus, all heme-protein reorganization which does not affect the XANES spectrum cannot involve a change in the chemical reactivity of the iron atom since its electronic cloud is not modified.

4.6. *Affinity changes and protein dynamics*

Contrary to the hypothesis [5] correlating heme-protein affinity for oxygen and the distance between the iron atom and the heme plane, the origin of the affinity changes is not localized at the iron site. The mechanism of heme-protein affinity changes may instead involve to a large degree the protein organization as was suggested many years ago by Wyman [35], Weber [36] and Hopfield [37]. Moreover, experiments by Resonance Raman of photodissociated hemoglobins provide direct evidence that a fraction of the free energy of the affinity changes is located away from the heme [38]. The affinity changes in heme-proteins for their ligands could result (at least in part) from variations in protein fluctuations which would distribute the free energy over many bonds of the apoprotein [36]. Ligand binding would be simultaneously controlled by the interaction of the ligand with iron and the motions of the ligand through the protein [39]. Thus, ligand combination in hemoglobin would be governed more by diffusion process inside the protein matrix [39] than by the chemical reactivity of the iron.

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