LIGAND BINDING PROCESSES IN HEMOGLOBIN

Chemical Reactivity of Iron Studied by XANES Spectroscopy

S. PIN, P. VALAT, R. CORTES, A. MICHALOWICZ, AND B. ALPERT
Laboratoire de Biologie Physico-Chimique, Université Paris VII, Tour 43-42, 75251 Paris, France; and Laboratoire pour l'Utilisation du Rayonnement Electromagnétique (CNRS), associé à l'Université Paris-Sud, Orsay, France

ABSTRACT K-absorption edge of coordinated ions exhibits a fine structure (through the use of XANES, or x-ray absorption near edge structures) that reflects the electronic repartition and the chemical reactivity of these ions. Comparative analysis of iron K-absorption–edge shape for hemoglobin derivatives with different ligand affinity suggests strongly that in hemoglobin, iron-forms with high and low affinity are highly improbable.

INTRODUCTION

The mechanism responsible for the different ligand affinities of hemoglobin is not yet fully understood. The influence of some heterotropic substances on ligand affinity of hemoglobin was for a long time interpreted as evidence for a change in the heme iron affinity for the ligand. This change was supposedly the result of the reversible transition of the protein resulting in iron with high (R) or low (T) ligand affinity (1). However, many researchers have shown that the energies responsible for the change of ligand affinity can not be localized at the heme-iron, (2, 3, 4). The contradiction between the classical allosteric model and refined spectroscopic data (5, 6, 7) strongly suggested that the origin of the S-shaped curve of hemoglobin oxygenation involved greater complexities than a simple heme deformation or a simple iron heme distance change (8).

To better understand the modulation of the hemoglobin ligand binding, we have examined—in the presence and in the absence of heterotropic effectors—the electronic state of the iron which must reflect the real chemical reactivity of the hemoglobin for the ligand. From the K absorption edge investigation (XANES) (9), it appeared that iron-forms with high and low affinity are highly improbable. Hence the change in hemoglobin affinity for the ligands does not come from specific iron states.

Dr. Valat's present address is Laboratoire de Photochimie, Centre Nationale Recherche Scientifique (CNRS), 94320 Thiais, France.

Dr. Cortes' present address is Groupe de Recherche no4 (CNRS) "Physique des Liquides et Electrochimie," associé à l'Université Pierre et Marie Curie, 75230 Paris Cedex 05, France.

Dr. Michalowicz's present address is Laboratoire de Physico-Chimie Structurale, Université Paris XII, UER de Sciences 94010 Créteil Cedex, France.

MATERIALS AND METHODS

Human hemoglobin was extracted from fresh adult blood (10). Stripped hemoglobin was obtained by passing hemoglobin desalted solution through a 20–50 mesh mixed-bed ion-exchange resin (AG 501; Bio-Rad Laboratories, Richmond, CA) (11).

Samples were concentrated by vacuum dialysis up to 13 mM in heme. Reduced protein was obtained by dissolving a small excess of solid sodium dithionite in oxygenated hemoglobin (12). CO forms were obtained by dissolving CO gas in the oxygenated hemoglobin solution until total oxygen displacement occurred. The met form was obtained with sodium nitrite reacting on the protein buffered at pH 7 (13).

Solutions were buffered in 0.3 M in Bis-Tris, Tris, or phosphate K/K_2 buffer at pH 6, 7, 8, and 9. Some experiments were also made in water pH 7. Inositol hexaphosphate (IHP) solutions were prepared with ten equivalents of IHP per hemoglobin tetramer. The quality of each hemoglobin preparation was verified by electrophoresis on cellulose acetate and by measuring the absorption spectra on a Cary model 219 spectrophotometer.

X-ray absorption K-edge values of the Fe centers have been obtained with synchrotron radiation from the electron storage ring at LURE (Orsay-France). Measurements were performed on EXAFS II experimental set up. X-ray absorption measurements were made by the total fluorescence emission of the iron as a function of incident x-ray energy. In our experimental conditions, the monochromator slit gave a resolution of ~3 eV. Measurements were made at room temperature (20°C ± 1) on a thin layer of sample (20 × 5 mm^2). The maximum counting rate of the fluorescence intensity was limited to 80,000 cps by using a NaI scintillator and each K-edge spectrum is the sum of four (or more) scans (140 points/9 mn 20 s).

RESULTS

X-ray absorption near edge structures (XANES) spectroscopy data represent the ratio of the iron fluorescence intensity to the incident intensity beam as a function of x-ray energy, where elastic scattering pre-edge background was removed. Small changes in the orientation between the sample compartment and the detector can give slight distortions in the collected XANES spectra. To evaluate the magnitude of such effects, we obtained near K-edge structures of the same methemoglobin sample

collected in two separate experiments with very similar geometrical conditions; results are given in Fig. 1. Thus, standard deviation of the XANES spectra has been evaluated to the half of the experimental dispersion shown in Fig. 1.

Comparison of the XANES spectra for hemoglobin in different forms is shown in Fig. 2. Spectra of the deoxygenated and oxygenated hemoglobin agree globally with respect to the positions of all maxima with previously obtained spectra (5, 14, 15). No modification in the shapes of the band or in the K-edge position was detected with the pH variation (Fig. 3 *a*) although an affinity change of the hemoglobin for the ligands exists as a function of pH (Bohr effect) (16).

It is also known that the Bohr effect is still present in salt free hemoglobin solution. This dependence of the salt concentration (17) on the Bohr effect has been investigated using the K-edge spectra. The proton and all the other ions which interact with oxy, carboxy, or deoxyhemoglobin do not affect the XANES spectra (Fig. 3 *a*), and thus do not affect the electronic distribution of the iron from the hemoglobin.

The situation is analogous for human hemoglobin in the presence and absence of the allosteric IHP effector (Fig. 3 *b*) (hemoglobin has higher ligand affinity in the absence of IHP than in its presence).

The general shapes of these spectra are very similar to each other; only the beginning of the EXAFS—after the main peak of Fe edge—showed very slight deviations. Lineshape of XANES spectra exhibit a structure due to the existence of different peaks distributed along the K-absorption edge. Energy positions and relative intensities of these peaks are correlated with the atomic arrangements of each hemoglobin derivative (18). Thus, comparison between the position of the K-absorption edge of different hemoglobin derivatives is not sufficiently significant. However, to relate the differences in the experimental XANES spectra, Table I shows positions of the iron K-edge maximum of the different hemoglobin derivatives.

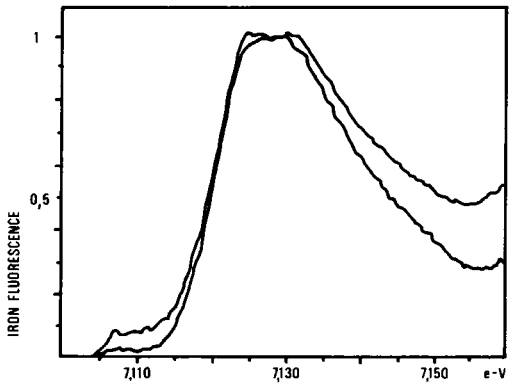

FIGURE 1 Standard deviation of the iron K-edge structure. Distortion in the Hb^+ H_2O XANES spectrum came from slight geometrical change between the detector and the sample compartment.

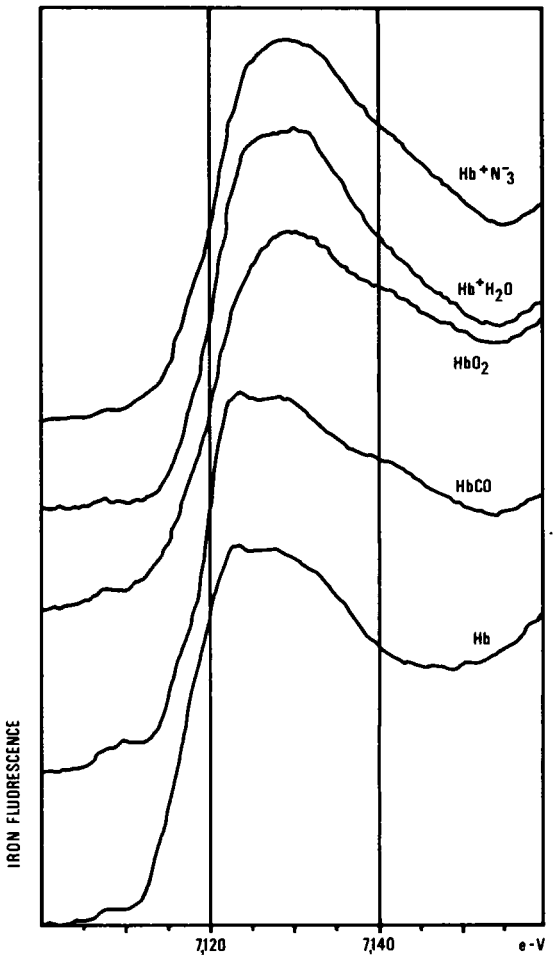

FIGURE 2 Effect of the ligand on shapes of the iron K-edge spectrum.

The energy positions of the K-edge maxima depend upon the ligand nature, perhaps on their electronegativity or on an induced displacement of the iron toward the heme plane (18), or both. So the differences in the experimental XANES spectra of hemoglobin come only from the iron ligand.

These results demonstrate that the electron cloud around the iron nucleus is largely affected by the ligand bound to the iron and yet is not sensitive to allosteric effectors.

In the limitations of this experimental technique, it appears that the hemoglobin ligand affinity might be different, but the iron K-edge has the same spectral features. These observations suggest that all allosteric effectors have no effect on the state of the iron in hemoglobin. To control these findings and test whether a small amount of contaminating species can disturb the XANES spectra, we have observed deoxyhemoglobin containing 20%, 10%, and 1% of oxyhemoglobin. Under these conditions, the XANES spectrum change can be safely attributed to a mixed solution (see Fig. 4). This result confirms the validity of our unexpected and interesting observations.

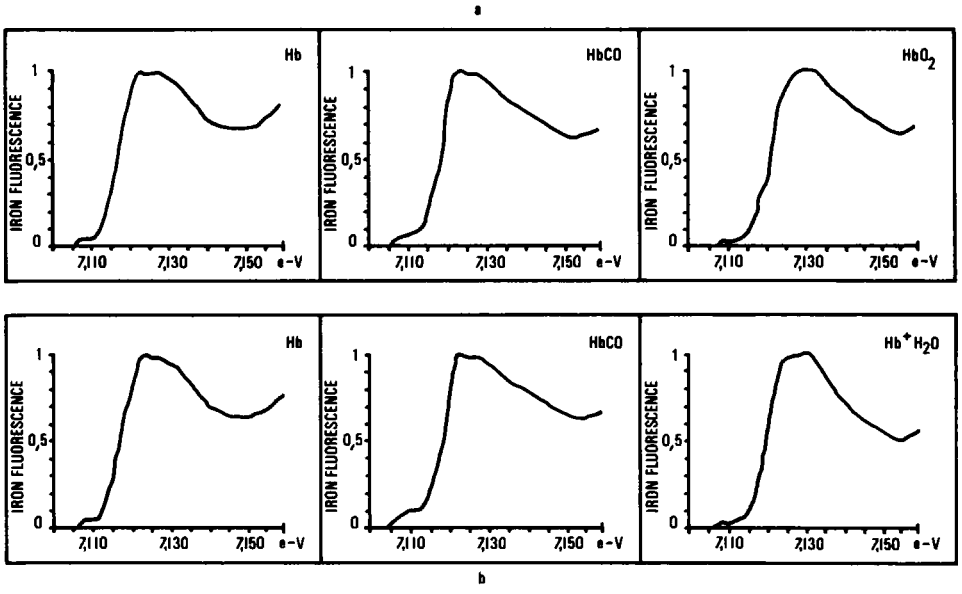

FIGURE 3 XANES spectra for hemoglobin derivates in different conditions represented with their standard deviations: (*a*) Hb, HbCO, HbO_2 with pH between 6 to 9 (buffer solutions: bis-tris, tris, phosphate or pure water). (*b*) Hb, HbCO and $Hb^+ H_2O$ without and with IHP (at pH6.)

DISCUSSION

Lineshapes of XANES are expected to be sensitive to distortion or tilt of pyrroles, movement of carbon atoms of porphyrin cluster and iron displacement. All variation in iron symmetry affects the behavior of the XANES spectra. So the differences between the iron spectra due to different ligands could reflect displacement of heme-iron position or doming effect changes. Consequently, the electronic repartition of the iron is highly dependent on the specific ligand involved. But for the same ligand, the transition shape of the main K_α peak is not altered by the heterotropic effectors (protons, salt nature and concentration, IHP). These data are in agreement with previous work indicating that the electron cloud around the iron nucleus is insensitive to the overall protein quaternary structure (14). Any iron symmetry changes can be correlated with the effects of the heterotropic effectors. Thus, iron affinity changes in hemoglobin do not come from iron symmetry modification and cannot be a reflection of some perturbation in the "tension" produced by the proximal histidine on the heme-iron (19).

TABLE I
ENERGY OF THE IRON K-EDGE MAXIMUM FOR DIFFERENT HEMOGLOBIN DERIVATIVES

Hemoglobin derivatives	Hb	HbCO	$Hb^+ N_3^-$	HbO_2	$Hb^+ H_2O$
iron K-edge positions (in electron volts).	7123	7124	7128.5	7129	7130.5

Energy positions are given with an error of ±0.5 e.V.

Movement of the iron is always accompanied by reorganization of the electron repartition around the iron nucleus. In simplest terms, the heterotropic effectors—which can perturb some amino-acids ionisation or apoprotein conformation—do not influence the electronic distribution of the hemoglobin-iron. Although the iron K-edge spectra do not change with the allosteric effectors, there is

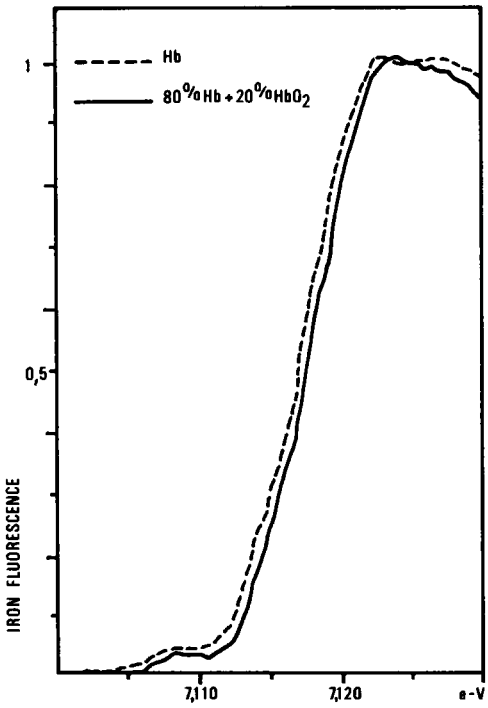

FIGURE 4 XANES spectra of pure deoxyhemoglobin (- - - -) and a mixture containing 20% of oxyhemoglobin (——).

no doubt that hemoglobin affinities for the ligand do change with these effectors. Thus, full discussion of ligand-hemoglobin reaction is possible only after investigations of each elementary step of the ligand binding process. In this process the rate of acquisition of the ligand by the apoprotein and the diffusion rate of the ligand in the protein interior are the two initial steps (20, 21). When the ligand molecules are sufficiently close to the heme, they interact with it and can bind the iron. At this step, only the chemical reactivity of both partners determines the reaction. During successive iron-ligand collisions, a resonance process is established between the ligand-free molecule and the iron atom that results in binding. The resonance phenomenon between the iron and the ligand depends strictly on the electron configurations of the iron. So, it is clear that the reactivity of the iron for the ligands in hemoproteins can be revealed by its electronic repartition and thus investigated by the lineshape of the iron K-edge features.

We must remember here that the K-shell absorption edge of hemoglobin is the superposition of the α and β K-edge (14). Regardless of this complexity, the electronic repartition around the analyzed iron represents the mean reactivity of the iron atoms in either subunits.

In each hemoglobin derivative, the heterotropic effectors do not influence the reactivity (or the electronic distribution) of the iron for the ligand. So modifications in protein conformations are not the processes that control the iron reactivity for the ligands. The concept of Monod-Wyman-Changeux (22) involving an all-or-none transition between two apoprotein conformations having different iron affinities for ligand is in total disagreement with the XANES experiment. In the M-W-C model, there are two allosteric parameters, L and c. L represents the ratio between the concentrations of the high and low affinity forms, and c represents the ratio of the binding affinity in the R state to that in the T state. The overall binding affinity (for example, expressed as P_{50}) depends on both parameters. However, a given allosteric effector may only influence one parameter. L may be altered by the addition of an allosteric effector that changes the relative equilibrium between the R and T forms but this same effector need not affect c and therefore K_T and K_R will not be changed. And if L is changing, then the ratio of hemoglobin iron-forms with high (R) and low (T) affinity also change, and properties of the iron K-shell absorption edge need change. For deoxyhemoglobin L is very large making the concentration of T state molecules orders of magnitude larger than R state molecules. And nobody expects to see any difference in the XANES spectra when deoxyhemoglobin is stripped or liganded with IHP. But for aquomethemoglobin the conditions between R and T are exactly the opposite, and the XANES spectrum would change significantly. The fact that for all derivatives the same iron K-edge exists in stripped hemoglobin as in hemoglobin complexed with IHP (or salt and protons) demonstrates the absence of iron species having low or high ligand affinities.

This evidence was already demonstrated by calorimetric studies of hemoglobin ligand binding. Intrinsic heats of hemoblogin ligandation with oxygen or carbon monoxide were found respectively to be: 14.1 (23) and 23.2 kcal/mol (24). These values, constant for partially or fully liganded hemoglobin, are totally independent of the buffer, pH, or the salt-solution concentration. Knowledge of these values showed that chemical processes did not have individual elementary reactions. The ligand binding energies—which are correlated with the degree of iron reactivity for the ligands—were a good probe to demonstrate that iron having different reactivities was not realistic.

Since no particular property of hemoglobin iron seems implicated in the hemoglobin affinity changes, how may we explain the differences in hemoglobin ligand affinity?

The constancy of the intrinsic heat of ligandation of the saturation curve, as well as its independance of the pH (from 5 to 10) demands that homotropic and heterotropic interactions in hemoglobin are entropic in nature (25, 4, 26). On the other hand, the different affinities of hemoblobin involving protons, organic phosphates, or some ions cannot be attributed to the chemical difference of the active iron-site. The differences observed involve mainly the protein organization. The observable binding properties for ligand and protons must be correlated with the macromolecular system. X-ray analysis of crystal structures that correspond to the unligated and fully ligated states (27, 28) are not disparate with our remarks. We know now that the interactions at the subunit contacts are not propagated to unliganded heme in a manner that increases or decreases the iron ligand affinity. It is possible that the structural changes (tertiary and quaternary) cause important modification in partition coefficient for the ligand between the protein and solvent phases (21). The breaking of salt bridges, which stabilized the deoxy structure, can also perturb the migration of the ligand into the protein interior. Also, the steric environment on the distal side of heme can be more or less restrictive to the ligand association (1, 8).

We do not exclude also the dynamic character of the protein (29), which could perturb the flow and the distribution (30) of molecule ligand throughout the apoprotein ensemble.

A change in hemoglobin affinity for one ligand (produced by allosteric effector or during the ligandation process) cannot be due to some electronic reorganization of the iron. For each ligand, the binding process involves always the same and permanent iron reactivity. The changes in hemoglobin affinity do not depend upon the state of the iron. Thus, it appears that the change in hemoglobin affinity for one ligand must be associated with apoprotein phase. Two processes interconnected could govern the ligand binding of hemoglobin : a diffusionnal modulation of the ligand by the protein matrix—which

should be sensitive to the allosteric effectors—and a chemical reaction between the ligand and the iron having the same iron reactivity in the presence as in the absence of heterotropic effectors.

We are very much indebted to technicians and engineers of LURE and of the Orsay Linear Accelerator Laboratory (Orsay, France) for running DCI for synchrotron radiation.

Received for publication 1 November 1984 and in final form 5 August 1985.

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