Probing the Electronic Structure of the Hemoglobin Active Center in Physiological Solutions

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Soft-x-ray absorption spectroscopy at the $L_{2,3}$ edge of the iron center in bovine hemoglobin and hemin under physiological conditions is reported for the first time. Spectra of the same compounds in solid form are presented for comparison. Striking differences in the electronic structure of the metalloporphyrin are observed between the liquid and solid compounds. We unambiguously show that hemoglobin and hemin are in a high-spin ferric state in solution, and that the $2p$ spin-orbit coupling decreases for hemin compared to the hemoglobin, while this is not the case in solids. The spectra were simulated using ligand field multiplet theory, in good agreement with the experiment, allowing quantification of the amount of charge transfer between the porphyrin and Fe^{3+} ion in hemoglobin and in hemin.

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Hemoglobin (Hb) is the respiratory protein of the red blood cells which carries oxygen from the lungs to the tissues in order to maintain the viability of cells. The active center of Hb is the heme group [Fig. 1(a), inset], which is located at the center of the protein and consists of a flat porphyrin ring molecule with an Fe^{2+} or Fe^{3+} ion center. Hemin is the salt form of the heme group with a Cl coaxial ligand instead of the imidazole group of histidine in Hb [Fig. 1(b), inset]. Describing and understanding the process by which heme proteins discriminate between ligands, in particular, diatomics, that bind to the iron atom has been the subject of intense studies for several decades [1–6].

The study of the active metal center by optical spectroscopy is difficult because the intense $\pi \rightarrow \pi^*$ porphyrin transitions obscure the (forbidden) transitions to d orbitals of the metal, which are involved in the binding process. To circumvent this problem, techniques such as nuclear magnetic resonance (NMR) [7], electron paramagnetic resonance (EPR) [8], and Mössbauer spectroscopy [9] have been employed. Although NMR and EPR delivered detailed pictures about the splitting of the $d\pi$ orbitals, they do not provide direct information about the nature of the bonding between the Fe ion and the ligand. Moreover, neither EPR nor Mössbauer spectroscopy have to our knowledge been applied to heme proteins in physiological solutions.

X-ray absorption spectroscopy (XAS) is ideal for probing the electronic and geometric structure of the active site. Hard-x-ray absorption spectroscopy studies have been carried out at the K edge of iron [10,11]. While much insight was gained about the local geometric structure around the iron atom, these do not deliver information about the electronic structure. For this purpose, soft-x-ray $L_{2,3}$ edge spectroscopy (due to $2p_{1/2,3/2} \rightarrow 3d$ transitions, respectively) for first row ($3d$) transition metals is much more appropriate, since: (a) The smaller intrinsic core hole lifetime width (0.5 eV) of p orbitals results in sharper spectral features than K edge ($1s \rightarrow 3d$) ones; (b) $2p_{1/2,3/2} \rightarrow 3d$ transitions are dipole-allowed, yielding more intense and more structured spectra than the dipole-forbidden K edge transitions; (c) $L_{2,3}$ edge features are directly proportional to the amount of d character of unoccupied or partially occupied valence orbitals of the metal; (d) ligand field multiplet calculations are well-established tools to interpret L edge spectra [12], delivering a detailed description of metalloprotein electronic structures. However, due to the large absorption cross section of solvents and air (e.g., in the Fe L edge energy range the x-ray path length in water is only 1–2 μm), and to their very low metal concentration (100–1000 ppm), soft-x-ray absorption spectroscopy of physiological solutions has so far not been possible. In order to circumvent these difficulties, dried frozen films of concentrated solutions of heme proteins or room temperature powders coupled to a fluorescence detection were successfully used [13,14]. However, a critical issue with solid samples concerns radiation damage, which can be minimized by different strategies (low temperatures, frequent sample changes, etc.) but never completely avoided. Recent developments at synchrotron light facilities have made soft-x-ray spectroscopic studies of high vapor pressure samples possible, either with cells equipped with silicon nitride windows, or with high pressure liquid microjets in vacuum [15]. Extending these techniques to investigate proteins in physiological media is obvious, and is the subject of this report.

Two issues are addressed here: (i) the spin state of the Fe-porphyrin system plays a crucial role in the dissociation and binding of small diatomic ligands to the Fe atom, and

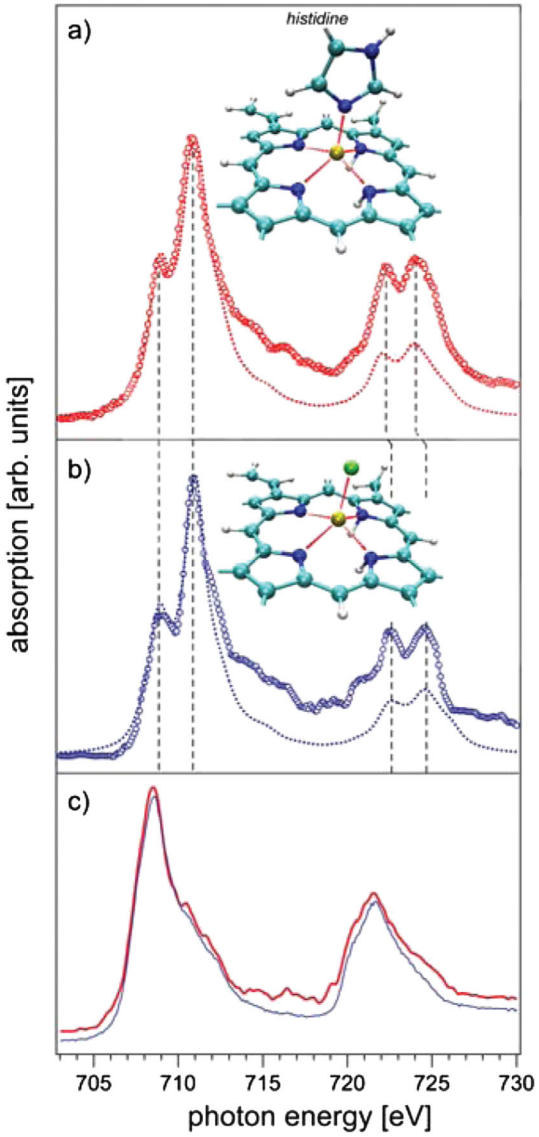

FIG. 1 (color). Experimental Fe $L_{2,3}$ edge XA spectra of hemoglobin (a), and hemin (b) in solution (3 mM), recorded with 200 meV resolution, along with the molecular structure of the metalloporphyrin. The L_3 edge lies in the ~708–716 eV region, while the L_2-edge lies in the ~720–726 eV region. Panel (c) presents the experimental spectra for hemoglobin (red thick line) and hemin (blue line) in powder form. The dotted lines in (a) and (b) represent the simulated Fe $L_{2,3}$ edge spectra calculated using the ligand field multiplet (LMF) theory (see text and supplementary material [19] for details).

in cooperativity and allostery in hemoglobin [6,16]. While it is assumed from a large body of indirect evidence (optical, Raman and infrared spectroscopies, low temperature EPR and Mössbauer spectroscopies, and x-ray crystallography studies) that the system is in the high spin state, there has been much debate that it may be in a mixed spin state. [17]; (ii) the relation between protein chemistry and electronic structure of the metalloporphyrin is still not clearly established. Metal-heme displacements, as well as the tilt of the proximal histidine play a role in the affinity of heme proteins to specific ligands. These factors are determined by the porphyrin-metal (back)donation and the interaction with the coaxial ligands, which are key to understand the specificity of the Hb function [17,18]. Soft-x-ray studies in physiological media can deliver unambiguous and quantitative information about the spin state of the heme and about the degree of charge transfer therein, as demonstrated here for the first time.

Here, the physiological sample circulates in direct contact with the 150 nm-thick Si_3N_4 membrane entrance window of the cell, inside a high vacuum chamber. The XA spectra are recorded by sampling the total fluorescence yield (TFY) using a GaAsP photodiode. The Fe^{3+} $L_{2,3}$ edge spectra were measured with a resolution of 200 meV at a concentration of ~3 mM Hb in water. For comparison, we also recorded the spectra for hemin in ethanol at the same concentration (as it is not soluble in water; see the supplementary material [19]). This allows us to identify the effect of coaxial ligand interchange (Cl vs imidazole). Key to the success of this experiment is the use of Si_3N_4 windows, allowing us to continuously renew the sample by flowing the solutions. The lack thereof resulted in spectra that differ (Fig. S1) from the ones presented here, and change with irradiation time, emphasizing the importance of sample damage under nonrenewable conditions. Finally, in order to make contact with the literature, we also measured hemin and Hb in the solid phase. Further experimental details are given in the supplementary material [19].

The Fe $L_{2,3}$ edge spectra of Hb and hemin in physiological solution are presented in Figs. 1(a) and 1(b), respectively. In Fig. 1(c), we also show the spectra of Hb and hemin in powder form. The former is in agreement with the low-spin (LS) ferrous Heme spectrum of Ref. [20], while the latter with spectra of (ferric) hemin on metal surfaces, which are considered to be reduced by the substrate [21]. The L_3 edges of the Hb and hemin in solution exhibit two main peaks at the same energies, with the first (at 708.8 eV) being slightly more intense in Hb compared with hemin. The second peak (710.9 eV) exhibits some broadening on the low energy side for Hb, relative to hemin. The L_2 edge in Hb exhibits a preedge peak at approximately 720.3 eV followed by two peaks at 722.2 and 724 eV, which lie 300 and 500 meV lower in energy, respectively, compared with hemin. The preedge feature is broader and lies at lower energy in hemin compared to Hb, though it is hard to estimate its energy accurately. Interestingly, the overall spectra of Hb and hemin in solution look close to those of $Fe_2(salmp)_2$, where salmp is bis(salicylidenamino)-2-methylphenolate(3-) [22], Fe_2O_3 [23], $LaFeO_3$ [24], $Fe(acac)_3$, where acac is acetyl acetonate, and $(FeCl_6)^{3-}$ [25], which are high-spin (HS) ferric complexes. On the other hand, they differ from those of Fig. 1(c), which both

point to a ferrous state. We believe this to be a consequence of reduction due to exposure to the soft-x-ray radiation. The spectra of Figs. 1(a) and 1(b) also differ from those of the LS ferric heme compound [Fe(tpp)(ImH)$_2$] (where tpp is tetraphenylporphyrin and ImH stands for histidine imidazole) [20]. This is direct experimental evidence that Hb and hemin in solution are in the HS state. Presumably, this is due to the fact that no strong forces are exerted by the coaxial ligand on the Fe^{3+} ion, as is the case in solid samples, which favor the LS state. Obviously, the LS solid samples are not sensitive to the interchange of the coaxial ligand [Fig. 1(c)]. Upon dissolving the iron-porphyrin group in solution, it switches to the HS state causing the L edge to exhibit high sensitivity to the coaxial ligand. As a matter of fact, the L_3-L_2 splitting decreases for Hb in solution, compared with hemin. The fact that Hb was measured in water while hemin in ethanol cannot explain that the p orbital spin-orbit splitting is reduced in the former case compared to the latter, since the L_3-L_2 splitting of Fe^{3+} ions in solutions has been demonstrated to be solvent-independent [26]. Consequently, we conclude that the differences between Hb and hemin in solution are mainly due to the binding of the imidazole ligand in Hb, which is clearly connected to its HS character, as supported below by our calculations.

Quantitative information about bonding, backbonding, charge transfer, and spin character can be obtained by modeling the spectra using the so-called ligand field multiplet theory developed by Thole *et al.* [27], and improved to include charge transfer effects [12]. The theory takes into account all Coulomb interactions as well as the spin-orbit coupling between the atomic orbitals, and treats the geometric environment of the absorbing atom through a crystal field potential. To model the ligand-to-metal-charge-transfer (LMCT) effects, the ground state of a $3d^n$ ion is taken as a linear combination of two appropriate configurations, $3d^n$ and $3d^{n+1}\underline{L}$, where $3d^{n+1}\underline{L}$ denotes a configuration with an extra $3d$ electron coming from the ligands and the corresponding hole on a ligand orbital. Details on the fit and its parameters are given in the supplementary material [19].

The dotted lines in Fig. 1 show the simulated XA spectra, normalized to the main L_3 edge peak of the experimental ones. The agreement between the simulated and experimental XA spectra is very satisfactory. The configurations best reproducing the experimental data are $64\%d^5 + 36\%d^6\underline{L}$ for Hb, and $63\%d^5 + 37\%d^6\underline{L}$ for hemin. This reveals a significant σ and π donation as already observed in other heme compounds [20]. In particular, the metal character of the b_{1g} orbital of the Fe^{3+} increases compared with nonheme complexes due to the increased σ donation from the porphyrin. The metal character of the e_g orbitals increases as well, reflecting a substantial π donation from both the porphyrin and the axial ligand.

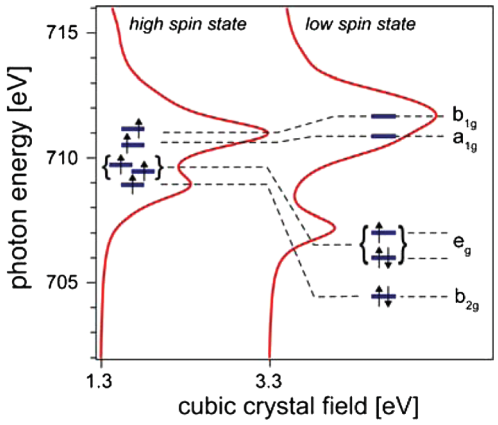

FIG. 2 (color). Calculated Fe L_3-edge spectra in the HS (left side, hemoglobin case) and the LS case [right side, compare with Fig. 1(b) of Ref. [20]], as a function of the cubic crystal field strength. The Fe d orbital energy levels are also shown for both cases. In the LS state, the b_{2g}, and the e_g orbital of lower energy are completely filled and the hole is localized in the other e_g orbital due to a symmetry lowering induced by the π-donor ligands [20]. The transition from the $2p_{3/2}$ core level to the half-filled e_g orbital leads to the peak at about 707 eV. The empty a_{1g} and b_{1g} orbitals yield the main peak and its doublet structure at about 711 and 712 eV. In the HS state, all the orbitals are filled by one electron and can thus contribute to the XA spectrum. The b_{2g} and e_g orbitals lead to the peak at about 709 eV and the a_{1g} and b_{1g} orbitals to the main peak at 711 eV.

Figure 2 shows two simulated XA spectra in D_{4h} symmetry, which illustrates the differences between the HS state ($10Dq = 1.3$ eV) and the LS state ($10Dq = 3.3$ eV), accompanied by the corresponding energy diagram of the pure d^5 configuration (without LMCT). Since the former simulation agrees much better with our experimental data, while the latter agrees with that of Ref. [20], we can safely conclude that Hb and heme in solution are both in a HS state. This confirms our experimental observation and explains the origin of the strong effect caused by the coaxial ligand in solution compare to the solid phase.

This difference in spin state also suppresses π backbonding as reported earlier in LS heme compounds [20]. Indeed, the authors inferred that introducing 2%–3% of backbonding could improve the agreement with the experimental data, which is not the case in our present investigation. This difference stems from the fact that the electronic density in the b_{2g} and e_g levels is lower in the HS state ($b_{2g}{}^1 e_g{}^2 a_{1g}{}^1 b_{1g}{}^1$) than in the LS state ($b_{2g}{}^2 e_g{}^3$), implying that π backbonding becomes much less effective in HS heme complexes. It is also worth mentioning that previous simulations of HS ferric complexes with similar XA properties [22], only accounted for the covalency of chemical bonds using a 65% reduction factor of Slater-Condon integrals. In our case, the reduction factor is kept at the free ion value of 80% and LMCT is considered

(supplementary material [19], Table S1), which allows for a better understanding of the origin of the covalency.

The 300–500 meV shift to lower energies of the L_3-L_2 splitting of Hb relative to hemin is ascribed to an increase by 0.30 eV of the $2p$ spin-orbit coupling ζ_{2p} in hemin. Since the same ζ_{2p} constant (8.2 eV) is used for Hb, and for the LS powder compounds [20], the shift can only be explained by considering the imidazole coaxial ligand in Hb. The Cl ion in hemin induces a stronger distortion at the Fe^{3+} site than the imidazole coaxial ligand of histidine in Hb. This distortion is the origin of the smaller first-to-second peak intensity ratio at the L_3 edge in the hemin spectrum compare to the Hb one (Fig. 1). While a slightly distorted symmetry is considered to model the spectrum of Hb ($10Dq = 1.3$ eV, $Dt = 0.05$ eV, and $Ds = 0$), a much bigger effect needs to be taken into account in hemin ($10Dq = 1.3$ eV, $Dt = 0.06$ eV, $Ds = -0.13$ eV). In hemin, the Fe^{3+} ion is indeed out of the porphyrin plane [17], whereas in Hb the imidazole (due to its π and σ bonding) tends to push it back into the plane, thus reducing the distortion. This is in accord with previous observations in LS heme compounds [20], where the distortion was even found to be weaker ($Dt = 0.028$ eV) due to the presence of two imidazole coaxial ligands, as well as due to their spin state. The Fe-N bonds are $\sim$0.2 Å shorter in LS heme complexes than in HS ones because the a_{1g} and b_{1g} antibonding orbitals are populated in the HS state [17]. This should allow the LS Fe^{3+} metal center to be potentially more located in the middle of the porphyrin ligand than the HS center.

On the contrary, σ donation from the coaxial ligands to the a_{1g} metal orbitals does not seem to be affected since even a tiny variation would clearly be visible on the calculated spectra (supplementary material [19], Fig. S2). This reveals that the imidazole ligand in Hb and the chloride anion in hemin give a similar contribution to the σ donation. In addition, the π donation of the single imidazole ring to the metal e_g orbitals in Hb is lower than what is observed in LS heme compounds connected with two imidazoles (supplementary material [19], Table S1 and Fig. S3) [20]. This suggests that imidazole is not only acting as a strong σ-donor but also a strong π-donor.

In summary, we have directly probed the electronic and spin states of metalloproteins under physiological conditions using soft x rays at the Fe $L_{2,3}$ edges. We show that both Hb and hemin are in a HS ferric state. Quantitative details about the amount of charge transfer between the porphyrin and the Fe^{3+} ion is obtained by simulating the spectra using ligand field multiplet theory, in good agreement with the experiment. The present work opens a whole new approach to the detailed description of function in respiratory and photosynthetic proteins.

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