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X-Ray-Induced Lysis of the Fe−CO Bond in Carbonmonoxy-Myoglobin

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By using X-ray absorption near edge structure (XANES) spectroscopy, we show that under prolonged exposure to Synchrotron X-rays, at $T < 10$ K, the Fe-heme in carbonmonoxy-myoglobin (MbCO) undergoes a slow two-state transition process. The final spectrum is nearly identical to that of the classical photoproduct (Mb*CO) obtained by UV−visible light illumination at 15 K. By increasing the temperature, the starting spectrum of MbCO is recovered at $T > 100$ K, demonstrating that the process is reversible and no damage occurred at the heme site in the time course of the experiment. Thus, the overall X-ray-induced process at low temperature is identical to the well-known (light-induced) photolysis of CO-hemeproteins.

Introduction

Our knowledge of the protein structure and structure−function relationships comes predominantly from protein crystallography, based on X-ray diffraction (XRD) experiments that are increasingly carried out at Synchrotron Radiation facilities. In a typical XRD experiment, the elastic (and inelastic) scattering of ∼1 Å X-rays accounts for about 20% of the radiation−matter interaction events, whereas the main part is accounted for by X-ray absorption events from any atom of the sample, via the photoelectric effect. After the creation of a photoelectron, the core-hole of the absorbing atom is refilled by an outer-shell electron, giving either X-ray fluorescence or emission of a second (Auger) electron. The latter effect is predominant for light atoms (N, C, O). Electron emission from water results in the dissociation (radiolysis) of the molecule in the highly reactive hydrogen and hydroxyl radicals (giving in turn hydrogen peroxides), able to penetrate in the protein matrix and induce further reactions. Water radiolysis is the main concern for Synchrotron X-ray studies of proteins both in a water solution and in crystals, as protein crystals contain 50−75% water. The damage due to diffusion of radicals into the protein matrix can be considerably reduced by using radical scavengers and by working at very low temperatures; however, the thermalized electrons produced by the radiolysis of water have a mean-free-path large enough (about 100 Å) to reach any inner reducible site of the protein.[1−4] Metal sites of proteins,[5] including hemeproteins,[6] can be "photo-reduced" this way at low temperatures, the protein structure remaining frozen in its initial conformation.

Besides photoreduction effects, cleavage of specific covalent bonds, like disulfide bridges, has been observed, so that X-ray and γ-ray irradiation has been recognized as an alternative way to generate intermediate states of the proteins,[1,4] able to trigger protein dynamics processes.[7,8] More recently, X-ray cryo-radiolysis of water has been deliberately used in EPR[9] and XRD[10] experiments to provide the electrons needed to drive the catalytic cycle of Cyt-P450 (a hemeprotein of the oxidative metabolism), including cleavage of the O−O bond of the iron ligated oxygen, so that the intermediate state of Cyt-P450, with activated oxygen species, has been successfully trapped and studied.

In the present work, we show that X-rays can be used to both induce and probe, by XANES, the Fe−CO bond lysis in carbonmonoxy-myoglobin (MbCO) at very low temperatures.

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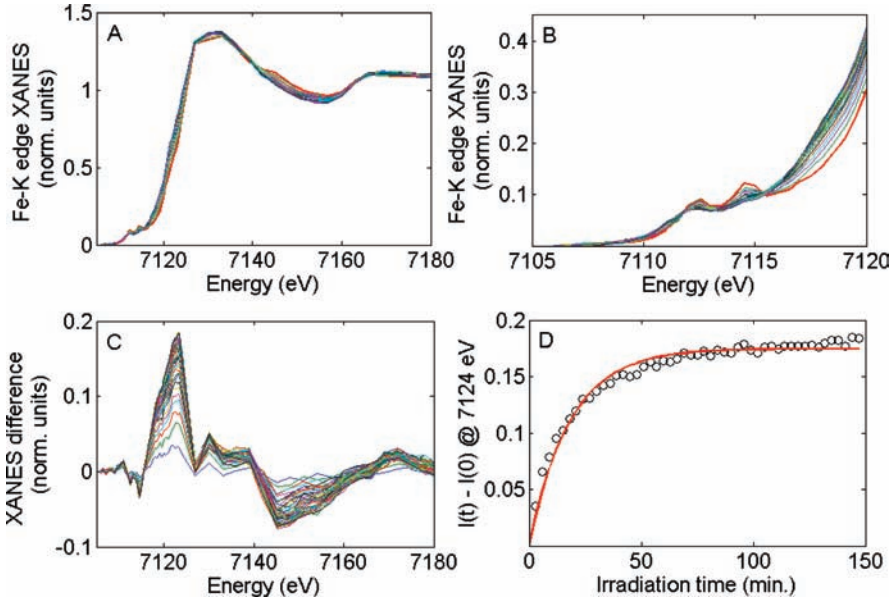

Figure 1. Evolution of the Fe K-edge XANES spectrum of MbCO under X-ray irradiation at 6 K. The first spectrum acquired is in red. The process is followed for 150 min. Fast (2.5 min) spectra at low energy resolution (0.5 eV up to 7130 eV; 2.0 eV above) are normalized to a unitary absorption jump. (A) Absolute spectra; (B) same spectra as panel A, with a magnification of the pre-edge and rising edge region; (C) difference spectra $X(t) - X(t = 0)$; (D) intensity $I(t) - I(t = 0)$ at 7124 eV as a function of the irradiation time.

We have observed the long-lasting CO dissociation process at $T < 15$ K and CO recombination by increasing temperature, as well as in classical photolysis experiments,[11,12] but without using an external source of visible light.

In all repetitions of the experiment, the overall process, lasting less than 5 h under irradiation, has been shown to be reversible, the XANES spectrum of the CO form being restored at $T > 100$ K, demonstrating that no relevant protein damage occurs at least inside the heme pocket.

Methods

Sperm whale myoglobin was obtained by SIGMA-ALDRICH and used without further purification. The Fe K-edge XANES spectra of 5 mM MbCO in solution, with 32 mM phosphate buffer, at pH 7.4, 240 mM NaCl, and 20% glycerol, have been collected in the fluorescence mode at ESRF-BM30B, Grenoble, by using a 30-element ultrapure Ge detector and a Si(2 2 0) double crystal monochromator.

We have evaluated the absorbed X-ray dose D expressed in Grays (Gy), in our experiment, according to the following formula:[5,13]

$$D = \frac{(\mu/\rho)nteE}{A}10^{11} \qquad (1)$$

In this equation, μ/ρ (cm^2/g) $= 14$ is the mass absorption coefficient of the sample at $E = 7112$ eV, calculated by using the program XCOM;[14] n (photons/s) $= 10^{11}$ is the estimated photon flux, averaged as the synchrotron beam flux decreases in time, and considering absorption by the cryostat and vacuum system windows; t (s) $= 1.5 \times 10^4$ is the total time required for a complete CO dissociation−reassociation cycle; e (J/eV) $= 1.6 \times 10^{-19}$ is the elementary charge; and A (μm^2) $= 3 \times 10^4$ is the irradiation area.

Using these values, the absorbed dose by our solution sample after an overall dissociation−reassociation cycle is $D = 9$ MGy, a value still below the maximum recommended dose to a protein crystal, that is 30 MGy.[15]

The sample was maintained in an open cycle liquid helium cryostat. The sample was in a helium atmosphere, the temperature of the sample holder being monitored by a platinum/carbon resistor with 0.1 K accuracy. Due to the He atmosphere, no temperature gradient was present between the position of the sample and the temperature sensor in the sample holder. The spectra were calibrated by assigning the first inflection point of the Fe foil spectrum to 7112 eV. The energy stability of each spectrum was carefully assessed by checking the position of a glitch in the I_0 at 7220 eV.

Alignment of the beam on each sample was done at $T = 120$ K. Then, the temperature was lowered at 6 K, and the time zero of irradiation at this temperature coincides with the first point of the first XAS spectrum collected. The entire experimental hutch of the Synchrotron beamline was in the dark during the experimental runs. After saturation of the X-ray-induced effects, the temperature was raised with a rate of 1 K/min, and XANES spectra were acquired from 6 to 100 K. By repeating the irradiation cycles, we acquired both fast (2.5 min) XANES spectra at low energy resolution and slow (21 min) spectra at higher energy resolution, as indicated in the Figures 1, 2, and 3. In one repetition of the experiment, the MbCO sample has been maintained at $T < 10$ K for more than 4 h in the sample holder, whereas other samples were exposed to X-rays. Thereafter, the very first, fast XANES spectrum exhibited all the typical features of MbCO species. Thus, irradiation of the near environment did not determine

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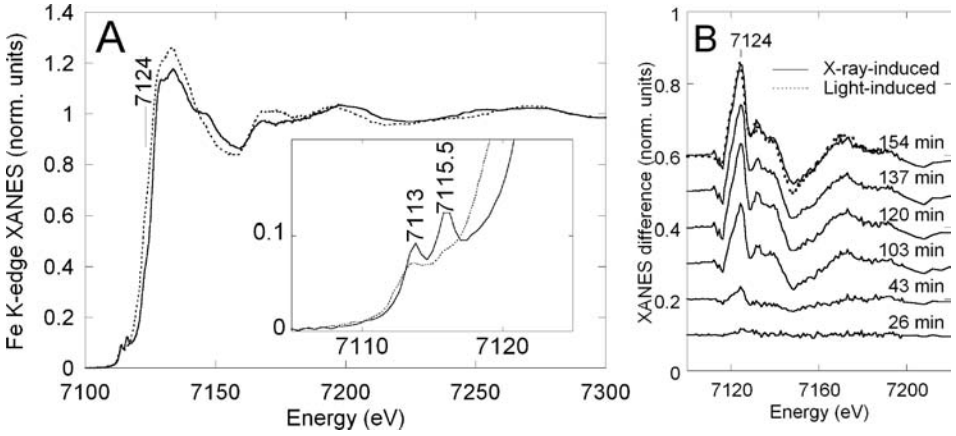

Figure 2. Left frame: MbCO Fe K-edge XANES spectrum at $t = 0$ (solid line) and after $t = 154$ min (dashed line) under X-ray exposure at 6 K. Spectra at high energy resolution (0.5 eV) were acquired in 17 min. In the inset, a magnification of the pre-edge peaks. Right frame: Time course of the difference spectrum $X(t) - X(t = 0)$ (solid lines). The spectrum obtained after conventional photolysis[12] is the dotted line.

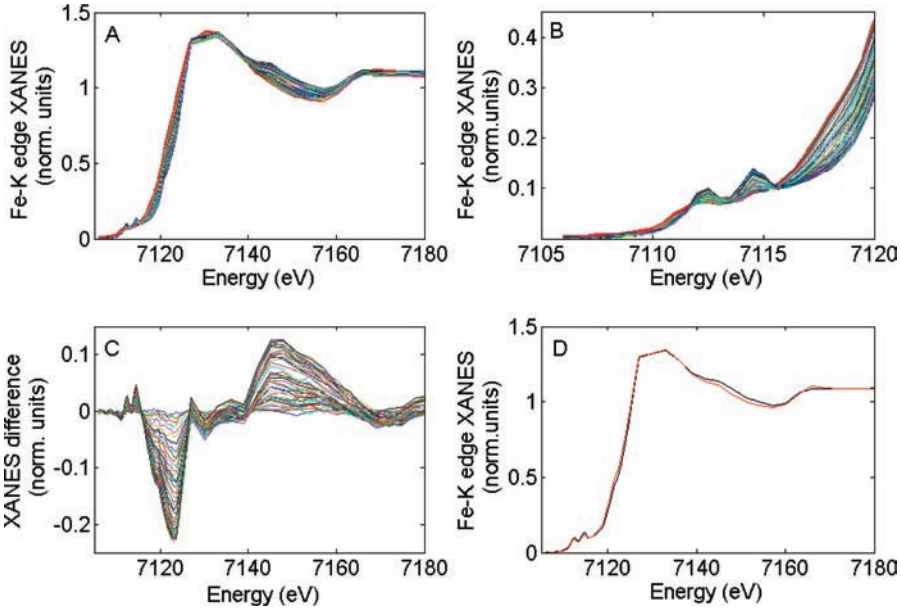

Figure 3. Evolution of the Fe K-edge XANES spectrum of MbCO under a temperature gradient of 1 K/min. The process is followed from 6 to 100 K. The first spectrum acquired is in red. Fast (2.5 min) spectra at low energy resolution (0.5 eV up to 7130 eV; 2.0 eV above) are normalized to a unitary absorption jump. (A) Absolute spectra; (B) same spectra as panel A, with a magnification of the pre-edge and rising edge region; (C) difference spectra $X(T) - X(6\ \mathrm{K})$, and (D) comparison between the first XANES spectrum at 6 K (red line) and the last spectrum acquired after the 6 K→100 K temperature gradient (black line).

any effect on the XANES spectrum, ruling out the hypothesis that secondary radiation events (a weak isotropic luminescence by either some metallic phases of the experimental setup or the kapton window of the cryostat) can induce at $T < 10$ K the observed spectral changes. Finally, the presence of even a small percentage of UV−visible radiation on the sample due to the synchrotron beam is excluded by the presence along the beamline of various berillium windows between the storage ring and the sample, for a total thickness of 740 μm, corresponding to a transmission factor, for photons below 1000 eV, lower than 10^{-37}.

Results and Discussion

In Figure 1A, the evolution of the Fe K-edge XANES spectrum of sperm whale MbCO at 6 K under irradiation is shown. The first spectrum collected is displayed in red. Fifty spectra at low energy resolution (energy step is 0.5 eV at the rising edge, 2 eV above), each lasting 150 s, are represented in the plots. A magnification of the pre-edge region is shown in Figure 1B, whereas the XANES difference (obtained by subtracting the first one from each spectrum) is displayed in Figure 1C. The spectra under continuous, prolonged X-ray irradiation evolve, reaching saturation of the effect in about 150 min, as shown by Figure 1D, displaying the maximum of the XANES difference (at 7124 eV). A relevant red-shift of the edge, and a variation of all the relevant edge features included within at least 5 isosbestic points, put in evidence the existence of a two-state process, characterized by the single exponential curve of Figure 1D.

Then, the experiment was repeated at a higher energy resolution and a more extended energy interval. In Figure 2, left frame, the spectra of the initial state (MbCO, solid line) and the final state (after saturation, dotted line) are displayed. The inset contains a magnification of the pre-edge region.

The XANES difference spectra $X(t) - X(t = 0)$ are shown in the right frame for t = 26, 43, 103, 126, and 154 min after starting irradiation (bottom to top, solid lines). The last difference spectrum is superimposed on the XANES difference spectrum previously obtained by conventional photolysis of MbCO under visible light at 15 K.[12] This latter difference spectrum was collected at LURE, a second generation Synchrotron source. A complete superimposition of the difference XANES spectra of light-induced and X-ray-induced Fe−CO bond lysis is obtained by multiplying the light-induced spectrum by 0.85.

After rescaling, the two difference spectra look identical; i.e., during collection of XANES of MbCO under the high photon flux of ESRF, the spectrum evolves toward the same species observed at LURE after photolysis of the same sample by visible light. As mentioned in the Methods, the X-ray flux density measured at ESRF is about 10^{11} photons/s/100 mA at 7−8 KeV, this value being about 2 orders of magnitude higher than what is available at LURE due to lower brilliance of the source. Using, from eq 1 of the Methods section, values consistent with those of the LURE beamline station, i.e., a photon flux n of 10^9 photons/s and an irradiated area A of 6×10^6 μm^2, the absorbed dose calculated for the same experiment turns out to be only 400 Gy, i.e., approximately the dose absorbed at ESRF in 1 s. It explains why the effect was unnoticed in the past while now it is observed growing so fast. Thus, we conclude that the Fe−CO bond of MbCO was broken, and a photoproduct Mb*CO has been obtained at ESRF by prolonged X-ray irradiation only. Similar observations have already been reported for carbonmonoxy-neuroglobin,[16] a globin expressed in the brain of vertebrates.

Figure 3A displays the effect of increasing temperature after saturation of the X-ray photodissociation at 6 K. Under a temperature gradient of 1 K/min from 6 K to 100 K, the first Fe K-edge XANES spectrum (red line), coincident with the last one of Figure 1A (Mb*CO), reconverts to that of MbCO at 100 K. The magnification of the pre-edge region and the difference spectra $X(T) - X(T = 6\,\mathrm{K})$ as a function of temperature T are shown in Figure 3B and C, respectively. The evolution observed is the inverse of that observed at 6 K, demonstrating the reversibility of the process and the feasibility of XAS experiments along dissociation−reassociation cycles. Actually, the observed maximum intensity change is slightly bigger during reassociation (−0.23 norm. units) than during dissociation (0.18 norm. units). As demonstrated in Figure 3D by the direct comparison of the first spectrum at 6 K (red line) and the last spectrum at the end of the dissociation/reassociation cycle, after the 6 K→ 100 K temperature ramp (black line), this small difference is due to a not negligible fraction of CO that dissociates during the scan of the very first XANES spectrum at 6 K.

The overall dynamical process observed under X-rays looks identical to the well-known light-induced low-temperature Fe−CO photolysis of carbonmonoxy-hemeproteins. We already observed[16] X-ray-induced lysis of the Fe−CO bond in CO-neuroglobin (a globin expressed in the brain of vertebrates), and also occasionally in nitrosyl-myoglobin and nitrosyl-hemoglobin (data not shown). The primary event leading to the breaking of the Fe−sixth ligand bond in light-induced photolysis experiments, according to iterative extended Huckel theory,[17,18] is due to a porphyrin ring $\pi \rightarrow \pi^*$ electronic transition, due to light absorption, followed by a radiationless decay into a state $d\pi \rightarrow d_{z^2}{}^*$ or its triplet partner (which is presumed to weaken the Fe−sixth ligand bond). The $d_{z^2}{}^*$ state is better described as an Fe−CO σ antibonding orbital $[\sigma(CO) + d_{z^2}(Fe)]^*$[19] that is empty in MbCO and, according to XAS theory and measurements of polarized XAS spectra,[19,20] is probed as a 1s→3d dipole-forbidden transition giving the prepeak at 7113 eV (Figure 3).

As specified above, in our X-ray absorption experiment, the main event is the photoelectric effect determining the ejection of primary photoelectrons and Auger electrons from light atoms of both the protein and the solvent. The observed ligand bond rupture could be due to either a direct 1s→ 3d photoelectron transition or later decay events of the photoelectron and/or the free Auger electrons from water, populating the same antibonding $d_{z^2}{}^*$ state. Each of these processes would have low probability, becoming non-negligible only at high X-ray doses. This topic could be addressed by comparing the Fe−CO dissociation process using two X-ray energies (below and above the Fe K-pre-edge peak, using the same X-ray dose). It would require an accurate measure of the absorbed dose that varies in time, energy, and focalization conditions, along the various experimental runs; an independent probe (like "in situ" optical spectroscopy) would be an optimal choice in such an experiment to avoid interferences between the pump and the probe.

Conclusions

X-ray-induced bond lysis of the Fe−sixth ligand bond seems to be common in hemeproteins, a bond cleavage event similar to those reported by other investigators.[10] It could be deliberately used allowing alternative sample conditions and experimental setup requirements for X-ray diffraction studies of hemeprotein ligand binding using Synchrotron radiation; moreover novel X-ray experiments could be planned, aimed at a dynamical characterization of pentacoordinate ferrous states in hemeproteins, the species involved in binding external ligands.

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