Structure and dynamics of heme proteins using X-ray Absorption Spectroscopy

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Abstract. Experimental investigations on protein dynamics are usually carried out in solution; however, in most cases, the determination of protein structure is done by crystallography that relies on the diffraction properties of the protein arranged in a three-dimensional lattice. X-ray Absorption Spectroscopy (XAS) is an ideal tool to investigate the deep relationships between structure and dynamics, on a wide class of metal containing proteins, including heme proteins. In fact, this technique can be used either as a structural refinement of X-ray diffraction on the same crystal, or as an independent tool to study structural dynamic properties both in crystal and solution. Different examples will be presented and discussed, with particular emphasis onto the original contribution due to the application of the MXAN software package to the analysis of XANES spectra.

1. Introduction

The intricate nature of biochemical reactions taking place in living cells demands these reactions to be highly specific. Usually, proteins achieve such specificity through geometrical and chemical precision in molecular interactions. As a consequence a detailed determination of protein structure is a critical step in modern biology. On the other hand since the seminal work on myoglobin (Mb) published more than 30 years ago by Austin et al. [1], the role of protein dynamics in controlling function emerged. In fact, the concept of conformational substates of a protein and the description of its energetics in terms of a complex landscape [2, 3] are milestones in our understanding of the relationship between structure, dynamics and function in proteins.

X-ray Absorption Spectroscopy (XAS) is a powerful technique able to investigate both local structure and dynamics, on a wide class of metal containing protein, such as heme protein. In fact, XAS can reveal structural parameters around metal centers with a very high precision, comparable to that of small-molecule crystallography. Such detailed structural data can be essential for elucidating
the function of hemeprotein [4], as structural changes to the metal coordination during redox or substrate-binding reactions are generally <0.1 Å and hence remain unnoticed in standard protein crystallography. We have previously demonstrated the feasibility of a structural study combining polarized XAS and X-ray diffraction (XRD) on the same protein crystal of the ferric cyanomet derivative of sperm whale Mb (Mb+CN). Working at a MAD beamline equipped on this specific purpose, we were able to determine both the 1.4 Å X-ray structure of the protein and, by XANES, the local structure around the Fe ion with enhanced resolution [5], comparable to that of atomic resolution XRD.

In this work we show a wide application of XAS technique, both in crystal and solution, to Neuroglobin (Ngb), a hemeprotein recently discovered that is expressed in the brain and that has been suggested to be involved in protection from ischemic damage [6, 7]. This protein belongs to the globin family and is able to bind gaseous ligands such as NO, CO and O2. A peculiarity of Ngb’s active site is the direct binding of the proximal and the distal histidines to the heme iron, yielding a hexacoordinate complex both in the ferric and ferrous oxidation state; thus binding kinetics with these diatomic molecules is associated with distal histidine release and to a repositioning of the heme that has been characterised by means of XRD (see figure 1A). Our purpose is to demonstrate that XAS can be a valuable tool for structural and functional studies as it can be used to refine XRD data, as well as to investigate the dynamical behavior of the hemeprotein of interest.

2. Methods
The XAS spectra in solution have been measured in fluorescence mode at 15K at the European Synchrotron Radiation Facility in Grenoble, ESRF-BM30-B Fame beamline. The polarized XANES spectra (in fluorescence mode) and the X-ray Diffraction data (at 1.7 Å resolution) of protein single crystals have been measured at Daresbury, CCLRC MAD-10 beam line. XAS data have been analyzed by means of MXAN software package [8] for the low energy range and by GNXAS [9, 10] for the high energy range.

3. Results and Discussion
3.1. Polarised XAS: a local probe of the structure of the heme sites
It has been recently demonstrated that polarized XAS on metallprotein crystals, can be a valuable tool to elucidate at atomic resolution the local structure of the metal site under investigation [5, 11]. In such a context, the application of this method to hemeproteins is of particular interest, as it allows in some cases to directly probe the axial ligand coordination to the iron by suppressing the contribution due to the porphyrin ring. One example is the P21 space group of sperm-whale myoglobin where the favorable orientation of the two hemes in the unit cell, allows the polarization axis of the X-ray beam ε, to be oriented as parallel to the c* axis of the reciprocal lattice (nearly parallel to the heme normal) and with ε parallel to the a axis (in the heme plane). The XANES signal collected in these two orientations are largely different. Moreover, it is possible to determine the (ε || heme_normal) polarized XANES spectrum as a linear combination of the two orientations collected, ε || c* and ε || a as previously demonstrated [12]; this reconstructed spectrum is extremely sensitive to the type and binding geometry of the heme axial ligand, providing the fine structural details of the coordination of various molecules to the Fe-heme, like proximal histidine, CN, CO, NO and H2O.

The possibility to combine XRD and polarized XAS onto the same crystal of hemeprotein, is not limited to the P21 space group as demonstrated by the example of Neuroglobin (Ngb). This protein is arranged in the orthorhombic R32 space group. The unit cell containing 18 protein monomers is shown in Figure 1B (only the heme groups are shown). As can be seen, the molecules of the prosthetic group are still oriented approximately parallel or antiparallel to each other, so that polarized signal parallel and orthogonal to the heme plane, can be easily extracted in a properly equipped beam line together with the XRD pattern. Thus, if the crystal structure of a heme protein is available together
with the XAS spectra both in crystal and solution, a state of the art analysis on the local structure around the metal center is possible.

Figure 1. Panel A: XRD structure of Ngb in the oxidised state (magenta) and in the CO bound state (red). Panel B: Sketch of the unit cell of Neuroglobin crystals; only the position of the heme groups for the 18 symmetry related protein molecules are represented. Space group: R32 $a = b$ (green) 87.92 Å; $c$ (blue) = 113.65 Å;

The comparison between the structural data obtained with the same technique onto different states of the protein (solution and crystal) was required since at room temperature crystals of reduced Ngb tend to crack following CO soaking. Moreover, the structure of NgbCO deposited onto the Protein Data Bank (code 1W92), that was obtained by flash freezing the soaked crystal, was found to undergo a substantial conformational change compared to the ferric form, involving a sliding motion of the heme falling into the proximal cavity (see figure 1A). Since crystal cracking upon CO binding at room temperature suggests further relaxation, we have applied XAS to NgbCO both in solution and in crystal to determine the parameters of the Fe-heme-CO geometry in the two different conditions applying the same experimental technique. In figure 2A it is shown the result of a MXAN best fit procedure over the solution spectrum of NgbCO species together with (panel B of the same picture) the result of the EXAFS analysis via the GNXAS package; XANES polarised spectra collected onto NgbCO crystals along the heme normal and along the heme plane are also reported in panel C and D of the same figure together with the best fitted spectrum found using the MXAN package. The relevant structural parameters obtained by all these measurements are reported in Table 1 and compared with results from 1.7 Å resolution XRD.

As can be seen by inspection of Table 1, there is a good agreement between XAS data in crystal and solution even if the MXAN analysis of the polarized spectra is more accurate than in solution. Nevertheless, the crystal structure of the NgbCO species by XRD, determined onto a sample obtained by flash-freezing the soaked crystal, is consistent with the local structure around the Fe-ion determined by XAS both in crystal and solution. This result reinforces the model proposed to explain the functional transition of the protein when an exogenous ligand displaces the distal histidine in the sixth position. In fact, the sliding motion of the entire heme group into a different cavity is induced by CO binding and is able to trigger the tertiary rearrangement of the protein.
3.2. X-ray induced dynamics of hemeprotein

A specific aim for XANES applied to hemeproteins, is the structural dynamics investigation of low temperature intermediate states. This goal was pursued using different strategies. A first example was the study both in crystal and solution of aquomet-myoglobin to follow the transition from the ligated ferric form, His-Fe(III)-H₂O, to the unligated ferrous form His-Fe(II) [13]. This structural dynamics was induced by photoreduction of the metal centre by prolonged X-ray irradiation at low temperature and the XANES analysis via the MXAN package was used to identify the structural parameters of the transition intermediate, a ligated ferrous form Fe(II)-H₂O, trapped at T<140K. In another work, we have exploited the sensitivity of XANES to study two light induced photoproduct intermediates of MbCO at low temperature which were prepared by different photolysis protocols [14]. For both photoproducts, we subsequently monitored a temperature-dependent relaxation process while increasing the temperature at a constant rate, revealing the different thermodynamic properties of the two intermediate states. Recently we have found that Fe-CO bond breaking in hemeprotein can be directly induced by prolonged irradiation at low temperature, so that the X-ray beam can be used both as a pump and a probe on the same protein system [15].

Figure 2. Panel A, Fe K-edge XANES spectrum of NgbCO in solution (open circles). Best fit obtained by the MXAN procedure (solid line). Panel B, EXAFS spectrum of the same sample (dots), and best fit obtained by the GNXAS procedure (solid line). Panel C, Fe K-edge ε/∥ heme-normal polarised XANES spectrum of NgbCO (open circles). Best fit obtained by MXAN procedure (solid line). Panel D, Fe K-edge ε/∥ heme plane polarised XANES spectrum of NgbCO (open circles). Best fit obtained by MXAN procedure (solid line).
### Table 1. NgbCO structural determination.

| Experimental technique | Protein state | Fe-Np (Å) | Fe-His (Å) | Fe-CO (Å) | Bend (°) | C-O (Å) |
|------------------------|---------------|-----------|------------|-----------|---------|---------|
| XRD crystal            |               | 2.00(15)  | 2.11(18)   | 1.81(14)  | 157     | 1.03(15)|
| Polarised XANES crystal|               | 1.97(2)   | 2.06(2)    | 1.86(2)   | 161(5)  | 1.23(2) |
| EXAFS solution         |               | 1.99(1)   | 2.10(4)    | 1.81(2)   | 173(15) | 1.08(2) |
| XANES solution         |               | 2.02(2)   | 1.96(7)    | 1.86(5)   | 179(16) | 1.10 F  |

* Numbers in parentheses represent the statistical error of the last digit.

### Figure 3. Panel A: XANES spectra of NgbCO (dotted line) at 9K and Ngb*CO at 9K after prolonged irradiation (solid line). In the inset the difference spectrum Ngb*CO-NgbCO is shown and the energy corresponding to the maximum difference is highlighted; panel B: open circles, time course of the formation of the Ngb*CO adduct under X-ray irradiation at 9K; panel C: closed circles, rebinding profile under a Temperature gradient of 1 K/min.
Here we show in figure 3 a summarizing picture of the experiment performed onto NgbCO. In panel A, the spectra corresponding to the starting species, the NgbCO adduct, and the final species, the pentacoordinate Ngb*CO are reported, together with the difference spectrum (in the inset) that is diagnostic of the formation of this intermediate under X-ray irradiation [14,15]. In panel B of the same picture it is reported the time course of the X-ray Absorption variation recorded at T = 9K at the maximum of the difference spectrum (7124 eV) and normalised in the range [0-1], according to the following algorithm \[(X(t)-X(t=0))/[X(t=\infty)-X(t=0)]\]. In panel C, a temperature dependent rebinding profile under a temperature gradient of 1 K/min is followed at the same Energy value and normalised in the same range according to the following algorithm \[(X(T)-X(80K))/[X(9K)-X(80K)]\]. The spectrum of the CO bound species at T= 80 K may be overlapped (data not shown) with the first spectrum acquired at low temperature. This fact demonstrates that the X-ray induced lysis of the CO molecule is a fully reversible process and consequently the rebinding curve give insights in the energy profile of the protein. This fact paves the way to the study of different protocols of X-ray induced lysis of hemeprotein that can be used in the same way of the light induced protocols ([16] and Della Longa et al. in the present issue) to try to trap a specific intermediate state.

4. Conclusion

The possibility to determine the local structure around the metal centre by XAS may be coupled, under opportune experimental conditions, to the possibility to follow the dynamical transition from the initial state to a functionally relevant intermediate state and viceversa. Moreover, the trapped state local structure that is usual elusive for an EXAFS investigation and even more for an XRD study, may be determined by applying the MXAN package to the full XANES spectrum both in solution and crystalline state.

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