XAS of dilute biological samples

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The experimental setup of beamline ID26 at ESRF (Grenoble) has been successfully exploited to obtain high-quality XAS (X-ray absorption spectroscopy) data from a biological sample where the metal concentration is about 100 μ*M*. The sample consists of the adenosine monophosphate deaminase (AMPD) histidine proline rich glycoprotein (HPRG) complex that contains 3–4 Zn(II) ions per dimer of approximately 320 kDa molecular weight. The experiment shows that third-generation X-ray sources equipped with insertion devices and appropriate optics and detectors allow the investigation of complex biological systems where the metal concentration is intrinsically low. The availability of such experimental setups makes possible a completely new set of experiments in biological XAS.

Keywords: X-ray absorption spectroscopy (XAS); biological systems.

1. Introduction

X-ray absorption spectroscopy (XAS) is a precious tool for structural biology. The technique is unique in its ability to provide the local structure of a metal ion embedded in a biological system in terms of accurate bond lengths, metal-site geometry and coordination number and to couple this with invaluable information about the metal oxidation state and electronic structure.

The potential applications of this technique to biological systems are limited by

(*a*) low signal-to-noise ratio owing to the intrinsically low concentration of a metal ion bound to a large biomolecule that lies in the μ*M* to a few m*M* range;

(*b*) damping of the EXAFS oscillations owing to the structural disorder present in the metal-ion coordination sphere.

These factors typically limit the extension of the measurable EXAFS data to 10–14 $Å^{-1}$ in *k* space depending on the local disorder of the sample. From these data, it is only possible to safely determine the metal-ion first coordination shell and to quantitatively estimate the number of rigid ligands, like histidine rings, where multiple-scattering effects enhance the signal. In favorable cases, it is possible to appreciate the presence of outer-shell heavy atoms up to a maximum of about 3.5 Å (Riggs-Gelasco *et al.*, 1995).

On the other hand, metalloproteins are abundant (30–50% of all proteins present in living organisms), and many of them contain a single metal ion for every 60–80 kDa of molecular weight. Frequently, the metal ion is involved in protein–protein interactions (*e.g.* metallo-chaperones) and, in order to understand its chemistry, it is necessary to appreciate the finer structural details and how they change along the pathway of the biochemical reaction in these even larger protein–protein adducts. Finally and most importantly, it is often observed that metalloproteins tend to form non-functional aggregates as their concentration increases to values useful for standard XAS data collection, leading to artifacts in the metal coordination sphere.

From the above considerations, it is evident that the need exists to study systems where the metal concentration is very low (in the μ*M* range) and to be able to increase the span of the measurable EXAFS data.

While the limitation indicated in (*b*) cannot be surmounted, that of point (*a*) can be challenged by taking advantage of the advances in X-ray synchrotron sources, beamline optics and detectors.

The difficulties in designing and making operative a beamline specifically built to collect XAS spectra on dilute systems have been addressed and recently overcome at ESRF. The EXAFS ID26 beamline at ESRF has been designed and successfully tested for this purpose (Gauthier *et al.*, 1999; Solé *et al.*, 1999).

The capabilities of the ID26 beamline are perfectly suited to tackling the study of a complex system like the zinc enzyme adenosine monophosphate deaminase from rabbit skeletal muscle, where the combination of high molecular weight and aggregation phenomena prevent millimolar concentration of the metal from being reached.

2. Experimental

Skeletal muscle adenosine monophosphate deaminase (AMPD) is a zinc-containing metalloenzyme that has been known for some time. Two separate studies of AMPD interaction with chelating agents have provided evidence that zinc is a firmly bound component essential for AMPD activity, although a different zinc content was determined in rat (Raggi *et al.*, 1970) and rabbit (Zielke & Suelter, 1971) enzymes, 2.0 and 2.6 g atoms per mole of enzyme, respectively.

Some of us have recently demonstrated the presence in purified rabbit skeletal muscle AMPD of a novel peptide with a striking similarity to rabbit plasma histidine proline rich glycoprotein (HPRG), a protein with a high affinity for zinc. Structural and immunohistochemical studies suggest that the HPRG-like peptide could participate in the structure of muscle AMPD (Ranieri-Raggi *et al.*, 1997; Sabbatini *et al.*, 1999). The isolated AMPD protein migrated as a single band with an apparent molecular weight of 90 kDa on SDS–PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) while the HPRG-AMPD adduct has a molecular weight of about 160 kDa. The HPRG-AMPD complex further dimerizes to give a holoprotein of 320 kDa. We have also observed that alignment of the four amino-acid residues supposed to bind zinc at the catalytic site of yeast AMPD with the deduced amino-acid sequence of muscle enzyme shows that one of the histidines is replaced by a glycine residue. This observation provides interest to a study to establish the zinc coordination in muscle AMPD as a promising approach to understanding the interactions of AMP substrate at the catalytic site. Up to now, attempts to obtain a stable enzyme solution suitable for XAS or EXAFS studies have been unsuccessful, because at high protein concentration a loss of catalytic activity is observed due to the formation of unsoluble aggregates. By ammonium sulfate precipitation in the presence of phosphate, we have obtained a stable active enzyme preparation that contains approximately 100–200 μ*M* of zinc ion. This concentration is one order of magnitude lower than that needed to obtain useful data from a conventional XAS spectrometer.

The AMPD-HPRG complex from rabbit skeletal muscle has been prepared as previously reported (Ranieri-Raggi *et al.*, 1997). Among the samples prepared for recording XAS spectra, the one discussed below was prepared as follows.

Holo AMPD-HPRG complex was dissolved in 5 m*M* of phosphate buffer and 0.2 *M* of KCl at pH 7.0. The maximum concentration of the complex attainable before aggregation and precipitation occurred was about 10 mg ml^{-1}. This resulted in an overall Zn(II) concentra-

tion of 170 μ*M* in the sample as indicated by atomic absorption data, consistent with the presence of four Zn(II) ions per AMPD-HPRG dimer.

Measurements on samples where metal concentrations are in the 100 μ*M* region entail careful control of experimental conditions because of the possible contamination of the sample and/or the cryostat with exogenous metal. This is particularly true with zinc that is ubiquitous. The experimental setup has been carefully checked for the presence of any trace metal. Energy scans of the cryostat and of the empty sample holder have been performed to confirm the presence of trace metals in the experimental setup. The sample holder and the kapton used to cover its windows were washed in a 50 m*M* sodium EDTA solution and then carefully rinsed with ultrapure water. The samples were prepared without addition of cryoprotectants (*e.g.* sucrose or ethylene glycol), even though it is known that preparation of glassy samples might improve the data quality. For our experiments, we prefer to use aqueous solutions of our proteins since it has been shown by X-ray crystallography that cryoprotectants might enter into the metal binding site and interfere with the metal coordination sphere (Ferraroni *et al.*, 2002; Benning *et al.*, 2001). The samples were rapidly frozen by immersion in liquid nitrogen under agitation. All the spectra were collected at 20 K using a liquid helium flow cryostat. X-ray absorption measurements were performed at the ESRF ID26 beamline (Grenoble), which uses a 1.65 m-long undulator source able to deliver more than 10^{13} photons s^{-1} on the sample as already described (Gauthier *et al.*, 1999). A PIN photodiode detector was used to measure the sample's fluorescence without any filter. Depending on the zinc content of the sample, the PIN diode measured between 70000 and 100000 counts s^{-1}. Before every measurement, the sample fluorescence was monitored by using a 13-element solid-state Ge detector located on the opposite side of the sample with respect to the PIN diode. In each case, it was found that zinc was the only metal present in the samples. An Si(220) double-crystal monochromator was used throughout the measurements. The first monochromator crystal was detuned to 50% of its peak intensity in order to reject higher harmonics. The monochromator angle was converted to an absolute energy scale by using a copper foil for calibration. The data were collected at the zinc edge (~9665 eV) from 9560 to 10307 eV with variable step widths. In the XANES and EXAFS regions, steps of 0.5 and 0.9–2.0 eV were used, respectively. Series of 40–120 scans for each sample were collected and averaged. Each scan took about 20 min to be completed. All the spectra were truncated at $k = 12$ Å^{-1}. The experimental data were affected by a few single-point glitches that could be removed by interpolation. An example of the quality of the data collected is given in Figs. 1 and 2, which show the edge and the EXAFS spectra of sample (1), respectively. The protein zinc sites did not experience any radiation damage during the data collection as judged by the invariance of the edge and of the EXAFS spectrum in the different scans.

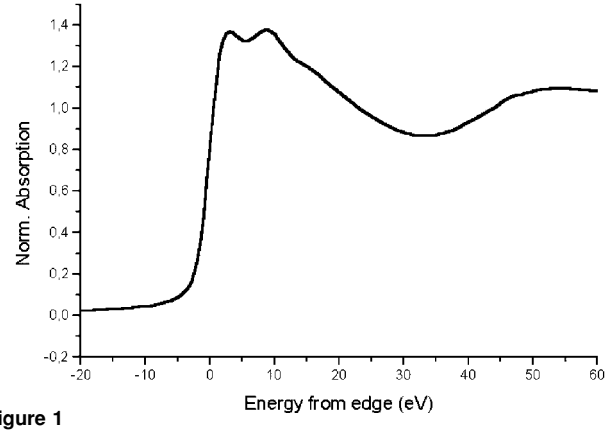

Figure 1
Zn *K*-edge XANES spectrum of the AMPD-HPRG complex containing 135 μ*M* of Zn(II).

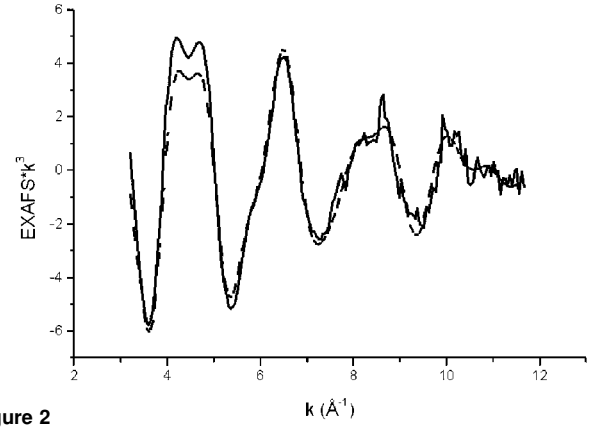

Figure 2
Zn *K*-edge EXAFS spectrum of the AMPD-HPRG complex containing 135 μ*M* of Zn(II) (continuous line) compared with its simulation (dashed line).

3. Conclusions

The use of a third-generation X-ray source coupled with optimal beamline design and performance can lead to routine measurements of XAS fluorescence data from very dilute biological samples.

The availability of such a beamline opens the way for a completely new set of experiments of biological X-ray absorption spectroscopy by allowing us to tackle complex systems where the metal concentration is very low. At present, the only beamline available in Europe for such studies is the ESRF ID26. We consider it extremely important for the biophysical community to have access to facilities like ID26, especially in view of the structural post-genomic projects where XAS may play an important role by accurately determining the metal environment in a complementary way to NMR and X-ray crystallography.

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