Counting the number of disulfides and thiol groups in proteins and a novel approach for determining the local pK_a for cysteine groups in proteins *in vivo*

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X-ray Absorption Spectroscopy (XAS) is a powerful tool to investigate sulfur in biological molecules. The spectral features are sensitive to the local electronic and geometric environment of the atom; thus, they constitute a fingerprint of the different chemical forms in which the sulfur is present. This allows straightforward detection of the ratio between free thiols and disulfides. Intra- or inter-molecular disulfide bond formation between residues plays an important role in structural and conformational changes in proteins, and such changes can be investigated using sulfur XAS. Also, a thiolate-disulfide equilibrium is involved in the regulation of the redox potential in the cells by means of modulating the concentrations of the reduced (thiolate) and oxidized (disulfide) form of the tripeptide glutathione. Thus, we can monitor the redox state of a cell by means of sulfur XAS. Thiols also exhibit an acid-base equilibrium, and sulfur XAS can be used to determine the local pK_a of the -SH group. Here we report examples of how sulfur XAS has been used for these applications.

Keywords: sulfur K-edge, XANES, thiol, disulfide.

1. Introduction

Thiol groups (R-S-H) in biology are ubiquitous and play different roles. As part of the side chain of cysteine, they are involved in protein folding through the formation of disulfide bonds, and are involved in several enzymatic activities like those of the families of cysteine proteases, oxido-reductases, and transferases. In addition to proteins, thiols are also present in cellular factors like coenzyme A and glutathione.

Despite the huge variety of functions of the thiol group, it is not easy to characterize its "actual" chemical status. This group exhibits high sensitivity to the redox potential changes in the environment, which influences the ratio between the reduced form R-SH and the oxidized disulfide form (R-S-S-R). In addition to the redox equilibrium, thiols exhibit an acid/base equilibrium. The corresponding pK_a may depend on several factors, like the type of solvent, solvent accessibility, and the nature of the chemical groups in close proximity to the thiol via hydrogen bonding.

In addition to cysteine, methionine is the other common amino acid containing sulfur, normally present as a thioether functional group (R-S-CH_3). There is evidence that this group participates in the defense from oxidative attack as this is one of the easiest groups to oxidize in proteins. Oxidizing agents in fact may react preferentially with it (usually with formation of methionine sulfoxide), preventing the oxidation of other residues more critical for the normal function of proteins (Levine *et al.*, 1996).

An accurate determination of the chemical status of biological sulfur atoms using spectroscopic techniques requires minimal laboratory preparation procedures and minimizes alteration of the conditions present *in vivo*.

Encouraging results have been obtained with Raman spectroscopy, which identifies thiols by the characteristic S-H stretching vibration. X-ray diffraction contributes enormously in the discrimination between free thiols and disulfide bonds in proteins. Yet, each of these techniques presents limits.

Sulfur K-edge X-ray absorption spectroscopy is a powerful tool for the determination of the chemical speciation of sulfur in biological samples. Since the electronic levels involved in the K-edge transition are influenced by the chemical state of sulfur atom (oxidation state and the type of atom ligated to it), the shape of its absorption bands constitute a unique fingerprint that allows its recognition (Rompel *et al.*, 1998; Pickering *et al.*, 1998). Our aim is to achieve a general and non-invasive method for the discrimination between R-S-H, R-S^-, R-S-S-R and R-S-CH_3 (and other less common forms of biological sulfur).

We present an XAS study on papain, α-amylase and human serum albumin (HSA) and the corresponding deconvolution into contributing forms of sulfur for each protein. With HSA, we have also tried to monitor the reduction of disulfides bonds to thiols using a disulfide specific reducing agent.

2. Materials and Methods

Cysteine, reduced and oxidized glutathione, methionine and tris (2-carboxyethyl) phospine hydrochloride (TCEP) were purchased from Sigma (reagent grade) and used without any further purification. Papain (EC 3.4.22.2) from Papaya Latex, α-Amylase (EC 3.2.1.1), type II-A from Bacillus Species and Human Serum Albumin (HSA, fat and globulin free preparation) were also purchased from Sigma and washed and concentrated (except HSA) with Millipore Ultrafree centrifuge tubes (5000 MW cut off), using 0.2 M Tris buffer, pH 7.6. α-Amylase was also purified previously with Sephadex G-100 Chromatography (Tris buffer 0.2 M, pH 7.6 as eluent). To each of the concentrated protein solutions (except HSA), an equal volume of glycerol was added as cryoprotectant, to have a final buffer concentration of 0.1 M Tris in 50% water/glycerol. The final concentration of papain and α-amylase was in the range 1-5 mM.

All the operations with papain and α-amylase were carried out at 5°C, and soon after the samples were frozen in liquid nitrogen. The XAS measurements were taken at 90 K to minimize radiation damage. HSA concentration was 3 mM (the commercial preparation was directly dissolved in 1.2 M Tris buffer). The reduced HSA solution was prepared by adding a concentrated TCEP solution (also in 1.2 M Tris buffer) such as to have a molar excess of 34 with respect to the protein. All the operations with HSA and reduced HSA, including the XAS measurements, were done at room temperature.

To allow a qualitative and quantitative estimation of the different types of sulfur atoms, our approach has been to perform empirical fittings of the spectra taken on protein samples, using linear combinations of suitable model spectra. These model spectra have been chosen as follows: 1) reduced glutathione at pH 6 to simulate thiolic groups in the neutral form; 2) reduced glutathione at pH 10 to simulate thiolic groups in the anionic form; 3) oxidized glutathione for the simulation of disulfide bonds; 4) amino acid methionine for the simulation of peptidic methionine. The spectra of

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the frozen solutions of the model compounds (25 mM in a 50% glycerol/buffer) were taken at 90 K.

The XAS measurements were performed at beamline 6-2 of the Stanford Synchrotron Radiation Laboratory. The absorption was measured as a fluorescence excitation scan between 2400-2740 eV (2465-2485 eV at 0.1 eV steps). The spectra were calibrated using as a reference the position of the maximum of the first edge feature in the XAS spectrum of thiosulfate (placed as a thin layer of powder on polypropylene film), which occurs at 2472.02 eV. All the spectra were baseline subtracted using a linear fit in the flat pre-edge region 2461-2467 eV. Each of the model spectra was fitted with a cubic polynomial in the region 2490-2740 eV; this function was then assigned the value of 1 at 2480 eV and the spectra were scaled accordingly. The protein spectra were fit with a linear combination of the normalized model spectra, and the percentage of each component was determined from the corresponding weighting factors. The second derivatives of the absorption spectra where calculated over intervals of three energy points centered at the energy of interest. All the spectra were analyzed without applying any smoothing. Data analysis was performed using a suite of Microsoft Excel spreadsheets and Visual Basic programs written by Dr. Emanuele Bellacchio at UC-Berkeley.

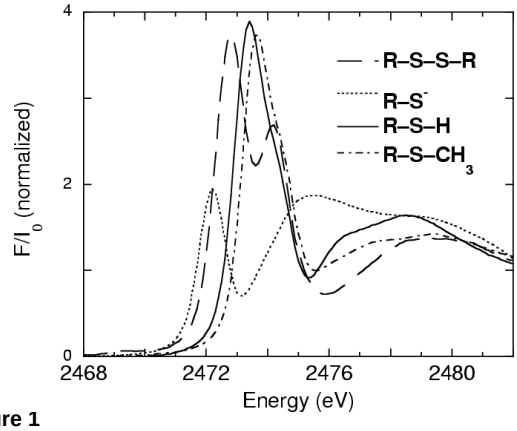

Figure 1
Sulfur XAS spectra of the various components in solution: R-S-H, R-S^-, R-S-S-R and R-S-CH_3. (see text for conditions)

3. Results and Discussion

The model spectra are reported in Figure 1. They are quite well distinguished from each other. Although the R-S-H group shows a similar absorption band to the R-S-CH_3 group, the maximum of the latter species occurs at significantly higher energy.

The proteolytic site of papain involves Cys 25 (McGrath, 1999), which acts as a strong nucleophile toward the carbonyl carbon due to activation by proximal groups. According to crystallographic data (Brookhaven code 9PAP), in addition to the active cysteine, this enzyme contains three other pairs of cysteines as disulfide bridges, and no methionine is present.

Figure 2 shows the experimental and the simulated spectra, as well as the corresponding 2nd derivatives. Papain is not completely soluble in water and our sample was in the form of a suspension. Despite this, the analysis gave excellent results; the sulfur species found with our least squares analysis reproduces those obtained with crystallography. Furthermore, we have been able to resolve the thiol and thiolate of the free cysteine. We calculated that the percentages of cysteines in the form of thiols and thiolates were of 65 and 35 respectively at pH 7.6. Since papain does not dissolve completely in water, these values do not reflect protonation equilibrium, and so we could not use them to calculate thiols pK_a in this case. To achieve this goal, we are planning experiments with other proteins that form homogeneous solutions.

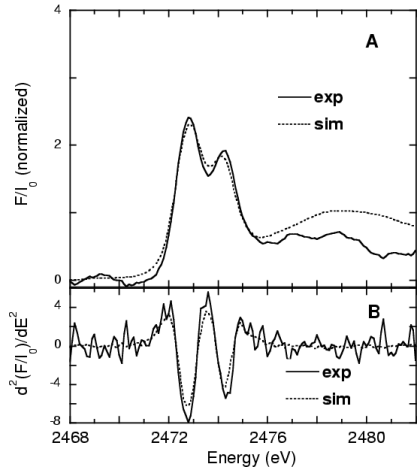

Figure 2
Experimental and simulated sulfur K-edge spectra of papain (A) and corresponding 2nd derivatives (B). The fit was obtained using the following components: 4.9 % R-S^-, 9.4 % R-S-H, 85.7 % R-S-S-R, and 0 % R-SCH_3. The greater noise level in the experimental spectra compared to the simulated ones reflects mainly the lower concentration of the absorbing units (sulfur atoms and disulfides) present in the protein sample compared to that of the model compounds. The fitting range was 2471.6–2476.5 eV.

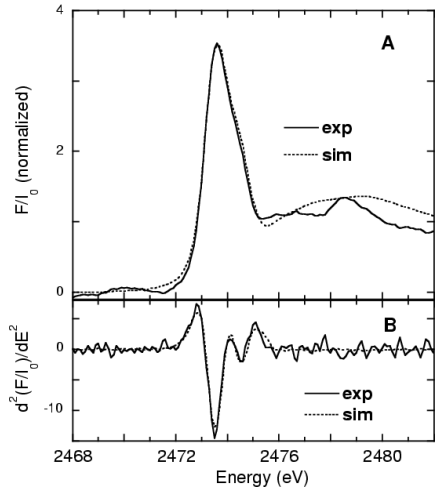

Figure 3
Experimental and simulated sulfur K-edge spectra of α-amylase (A) and their 2nd derivatives (B). The fit was obtained weighting the model components as follows: R-S-H 12.5 %, R-S^- 0%, R-S-S-R 0 %, R-S-CH_3 87.5 %. The fitting was 2471.6–2475.6 eV.

Figure 3 shows the experimental and simulated spectra of α-amylase and their 2nd derivatives. Our analysis suggests that no deprotonated cysteine residues are present in the enzyme.

Presently, a comparison with crystallographic data is unavailable; however, due to the relative similarity of R-S-H and R-S-CH_3 spectra, it could be argued that some small fraction of free thiols could contribute to the spectrum. Should this be the case, they must be present exclusively in the neutral form, since the spectrum of the anionic form is very different and easily resolvable. Indeed, at

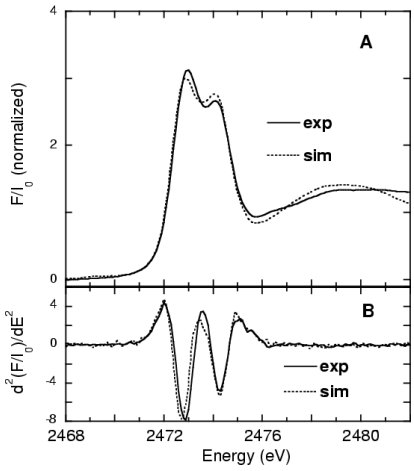

Figure 4
Experimental and simulated sulfur K-edge spectra of human serum albumin (A) and their 2nd derivatives (B). The percentage of sulfur as R-S-H, R-S$^-$, R-S-S-R and R-S-CH_3 used for the fit were 1.4, 1.1, 82.9 and 14.6 %, respectively. The fitting range was 2471.3– 2477.0 eV.

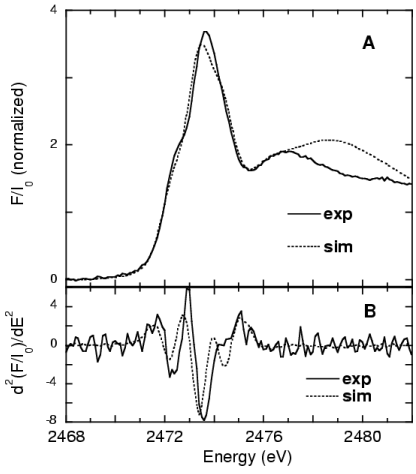

Figure 5
Experimental and simulated sulfur K-edge spectra of reduced human serum albumin (A) and their 2nd derivatives plots (B). The percentage of sulfur as R-S-H, R-S$^-$, R-S-S-R and R-S-CH_3 used for the fit were 32.9, 27.8, 24.7 and 14.6 %, respectively. The fitting range was 2471.3–2476.9 eV.

the pH of the experiment, if free thiols were present, a significant number would be present as thiolates, unless they were located in a micro-environment that strongly favors the equilibrium toward the protonated side.

Figure 4 shows the experimental spectrum of human serum albumin and its simulation, plus the related 2nd derivatives. For this protein, the crystallographic structure indicates that the ratio between cysteine, cystine and methionine is 1:17:6. The simulation was done adopting the same ratio of the R-S-H, R-S-S-R and R-S-CH_3 normalized model spectra. Although there is a good agreement between our simulation and the experimental spectra, it is worth mentioning that in HSA, as well in other proteins containing multiple cysteines, the ratio between thiols and disulfides may be different depending upon the redox conditions. Recent statistical studies on HSA samples taken from blood samples of different persons have shown that there is a correlation between the above mentioned ratio and the age of the individuals (Era *et al.*, 1995).

As a further exploration of the possibilities of sulfur XAS, we have reduced a solution of HSA with TCEP and observed the spectral changes. To a 1 mM solution of HSA in 0.5 M Tris buffer, pH 7.6, we added the reducing agent to a concentration of 100 mM. The spectrum of HSA changed dramatically and the characteristic features of disulfides bonds decreased, while those typical of free thiols increased, as expected. The spectrum was taken on the solution about 15 minutes after the addition of TCEP.

Figure 5 shows this spectrum together with the simulation performed keeping the percentage of sulfur as methionine the same (15 %) as in the non reduced HSA and letting the disulfides and free thiols components vary in a complementary fashion. The 2nd derivatives of the experimental and the simulated spectra are also reported. Going from untreated to reduced HSA, the percentage of sulfur in the form of total free cysteines (neutral thiols plus thiolates) increased from 2 % to 61 %, while that as disulfides decreased from 83 % to 24 %.

4. Conclusions

One of the main achievements with sulfur XAS applied to biology is the possibility of determining the number of disulfide bonds in proteins. There are several chemical methods that count the total number of disulfide bonds, but they require conditions where the redox equilibrium between R-S-H and R-S-S-R is likely to be perturbed. Thus, what is measured may be different from what is "in vivo."

In addition, the technique has allowed us also to discriminate between the neutral and the deprotonated form of the thiolic groups and this confers the additional advantage of determining the pK_a of cysteine thiols in intact biomolecules.

Because of the high specificity of sulfur K-edge XAS, this technique can be applied to the investigation of the changes that occur at the thiolic groups that constitute part of catalytic sites in enzymes. Intermediate species with bond formation or cleavage at the sulfur atom can be detected. These experiments are preliminary, however the potential for use in research in biology is enormous.

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