Sulfur K-edge X-ray absorption spectroscopy for determining the chemical speciation of sulfur in biological systems

Ingrid J. Pickering[a,*], Roger C. Prince[b], Thomas Divers[c], Graham N. George[a]

[a] *Stanford Synchrotron Radiation Laboratory, Stanford University, P.O. Box 4349, MS 69, Stanford, CA 94309, USA*
[b] *Exxon Research and Engineering Company, Annandale, NJ 08801, USA*
[c] *College of Veterinary Medicine, Cornell University, Ithaca, NY 14853, USA*

Received 10 August 1998; received in revised form 1 October 1998

Abstract Sulfur is an essential biological element, yet its biochemistry is only partially understood because there are so few tools for studying this element in biological systems. X-ray absorption spectroscopy provides a unique approach to determining the chemical speciation of sulfur in intact biological samples. Different biologically relevant sulfur compounds show distinctly different sulfur K-edge X-ray absorption spectra, and we show here, as an example, that this allows the deconvolution of the sulfur species in equine blood.

Key words: Sulfur; X-ray absorption spectroscopy

1. Introduction

Sulfur has been called a spectroscopically silent element, and as such there are few in situ measurements of its chemical forms. ^{33}S-NMR is of little use due to low natural abundance, and large linewidths with a small nuclear magnetic moment. Sulfur K-edge X-ray absorption spectroscopy provides a potential tool for determining the chemical forms of sulfur in intact biological samples. X-ray absorption spectra can be divided into two constituent parts: the near-edge spectrum and the extended X-ray absorption fine structure (commonly known as EXAFS). The near-edge region of the spectrum is dominated by dipole-allowed ($\Delta l = \pm 1$) bound-state transitions of the 1*s* electron (for a K-edge) to vacant molecular orbitals of substantial *p*-orbital character. The near-edge spectrum thus provides a sensitive probe of electronic structure and hence of chemical form. Sulfur exhibits particularly rich near-edge spectra, due to the relatively sharp linewidths and the large chemical shift range (of some 14 eV [1]) over its range of oxidation states (−2 to +6).

Sulfur K-edge X-ray absorption spectroscopy has been successfully used to investigate sulfur chemistry in fuels such as coals and oils [1–7] but to date there have been only a few applications in the biological sciences [8–13] and in other fields [14]. In the studies of fuels, extensive use of least-squares fitting to sums of model compound spectra has been used to quantitatively speciate the chemical forms of sulfur in the fuels [4]. In the present work we explore the utility of sulfur K-edge X-ray absorption spectroscopy as a probe of sulfur chemistry in intact biological systems, and show that it can, with appropriate analysis, be used to quantitatively speciate sulfur metabolites in biological tissues without any chemical manipulations of the specimen.

*Corresponding author. Fax: (1) (650) 926 4100.
E-mail: pickering@ssrl.slac.stanford.edu

2. Materials and methods

Sulfur K-edge X-ray absorption spectra were collected on beamline 6-2 at the Stanford Synchrotron Radiation Laboratory using a Si(111) double-crystal monochromator and a downstream Ni-coated harmonic rejection mirror. Incident intensity was monitored using a helium-filled ion chamber. Energy resolution was optimized by decreasing the vertical aperture upstream of the monochromator until no further sharpening of features of the near-edge spectrum of a sodium thiosulfate ($Na_2S_2O_3 \cdot 5H_2O$) standard were detected (we estimate an approximate resolution of 0.5 eV). The X-ray fluorescence was monitored using a Stern-Heald-Lytle fluorescent ion chamber detector and solid model compounds were measured using total electron yield. The energy scale was calibrated with reference to the lowest energy peak of the sodium thiosulfate standard which was assumed to be 2469.2 eV [15]. Residual undulator structure from the 54-pole insertion device was minimized by moving the aperture vertically to a position just off the peak of maximum intensity. All spectra were recorded at room temperature, and data were analyzed with the EXAFSPAK suite of programs [16]. Mixtures were deconvoluted by least squares fitting of the data to sums of reference compound spectra [4].

Blood was collected from horses into tubes containing EDTA to inhibit clotting. The widely-used anticoagulant heparin was not used as it contains sulfur. The erythrocytes were allowed to settle, and clear plasma and packed erythrocytes were collected. Oxidized and reduced glutathione, and the other model compounds, were obtained from Sigma or Aldrich Chemical Companies. *Clostridium pasteurianum* ferredoxin was a gift from R.E. Bare, Exxon.

3. Results and discussion

Fig. 1 shows the sulfur K-edge spectra of a series of biologically relevant model compounds. The spectra show a large chemical shift range with subtle but significant differences between similar forms. Near-edge spectra can be conveniently and quantitatively compared by normalization to the edge-step (e.g. [5]). Spectra of samples with more than one species of sulfur present can be simply represented by the sum of appropriate normalized model spectra weighted by the fraction of sulfur present in each component, and this is the basis of the curve-fitting analysis used below. The spectra in Fig. 1 show that, as expected, the energy of the dipole-allowed major peak of the near-edge spectrum generally increases with formal oxidation state, with a concomitant gain in intensity due to increased 3p holes. The spectra show considerable richness, with compounds as similar as methionine and cysteine having significantly different spectra. Deconvolution of the near-edge spectra (not illustrated) reveals that there are at least two transitions in the major peak of these compounds, which are expected to be $1s \rightarrow \sigma^*$(S-C) [17] and possibly $1s \rightarrow \pi^*(CH_2)$ [18]. These two features have the same separation (0.88 ± 0.03 eV) and ratio of intensities of higher energy to lower (0.43 ± 0.10) in cysteine and methionine (7 observations) but are shifted to 0.2 eV lower energy for cysteine. An identical energy shift between the major transitions of the

PII: S0014-5793(98)01402-1

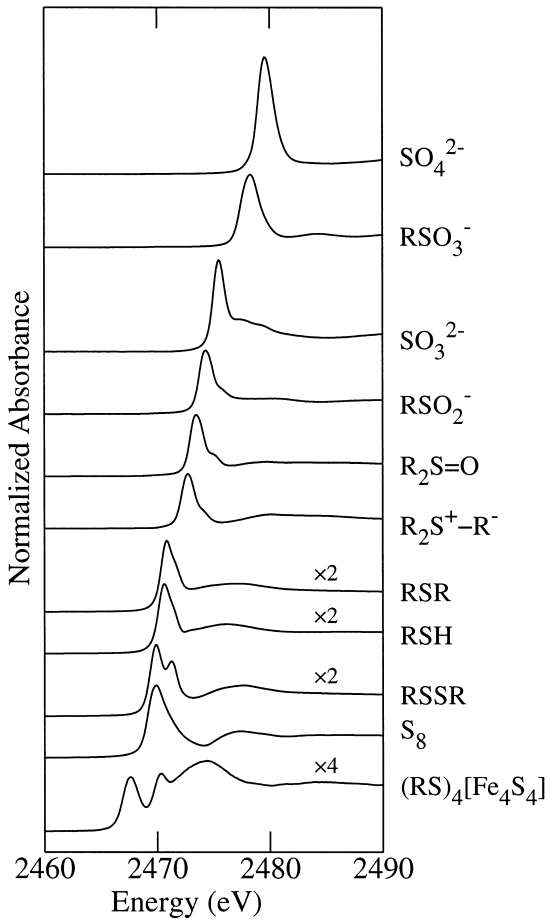

Fig. 1. Sulfur K-edge X-ray absorption near-edge spectra of a series of biologically relevant compounds. All compounds are in aqueous solution near to pH 7.0, except as noted below. From top to bottom the compounds are: sulfate pH 8.2, cysteic acid, sulfite, cysteine sulfinic acid, methioninesulfoxide, dimethylsulfoniopropionate pH 4.0, methionine, cysteine, oxidized glutathione, solid rhombic sulfur, and *Clostridium pasteurianum* ferredoxin. X-ray absorption was monitored using X-ray fluorescence except for the solid elemental sulfur, which was measured as total electron yield. In all cases data were normalized to the height of the edge jump after background removal.

spectra of methyl sulfide and methyl thiol has previously been reported by Dezenard et al. [17]. Interestingly, the intensities of the edge features of methionine and cysteine are similar, although very slightly (~2.5%) larger with methionine. Again, similar intensities have been observed for methyl sulfide and methyl thiol [17]. Cysteine and reduced glutathione have extremely similar major peaks but show some differences in the region above 2472 eV. The disulfides cystine and oxidized glutathione show two clearly resolved peaks, separated by some 1.5 eV, which have been assigned as $1s \rightarrow \sigma^*$(S-S) and $1s \rightarrow \sigma^*$(S-C), for the lower and higher energy peaks, respectively [19].

Fig. 2 shows some possible experimental pitfalls. Self-absorption is a well known problem with soft X-ray near-edge spectra [20]. It is significant when there are large changes in total X-ray absorption coefficient over the range of the spectrum, and causes attenuation of peaks in spectra, a phenomenon which can be clearly seen in Fig. 2A. Thus, samples that are either too concentrated or too thick (e.g. solid model compounds) will have distorted near-edge spectra when measured with fluorescence detection. Grinding with an inert diluent may be effective in minimizing self-absorption for solids of moderate sulfur content, but in many cases the particle size that must be obtained is so small that this method becomes impractical (e.g. < 1-μm particles are required for sulfate) and some other method of detection must be employed in these cases. Transmission X-ray absorption spectroscopy, which is generally used for concentrated samples at higher energies, is challenging at the sulfur K-edge due to the requirement of very thin samples. Fortunately, electron-yield detection provides a convenient alternative probe that does not suffer from thickness effects, since the path length of the X-ray is always much greater than that of the emitted electrons. A distortion to which electron yield is prone, due to sample charging, can be easily minimized by mixing the sample with a small quantity of graphite to render it more conductive.

Spectra of solid and solution samples are generally quite different (e.g. Fig. 2B). In general, the intensity of the major spectral features are smaller in the solid, probably because crystal packing forces cause a subtle lowering of symmetry, with otherwise degenerate orbitals becoming non-degenerate. All the solids investigated also had structure on the high energy side of the absorption edge which was not present in solution. This is expected from the long range order in the solids, which gives rise to multiple-scattering effects. Thus solid standard compounds provide inadequate models for analysis of solutions. In agreement with this, we note that

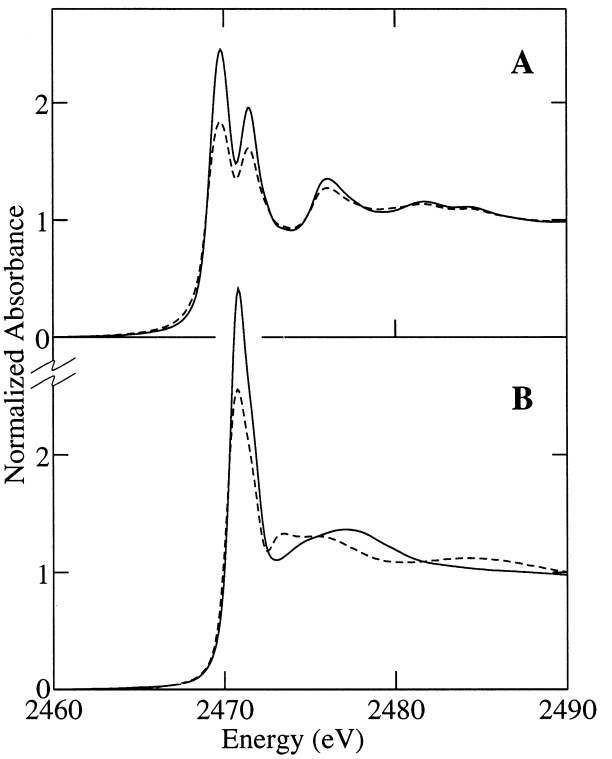

Fig. 2. Possible experimental pitfalls of sulfur K-edge X-ray absorption near-edge spectroscopy. Panel A compares spectra of solid cystine recorded using total electron yield (solid line) and X-ray fluorescence (broken line). Damping of the intense features of the fluorescence spectrum is clearly observed. Panel B compares the spectra of methionine in aqueous solution at pH 7.0 (solid line), and as a solid (broken line), recorded using X-ray fluorescence and total electron yield, respectively.

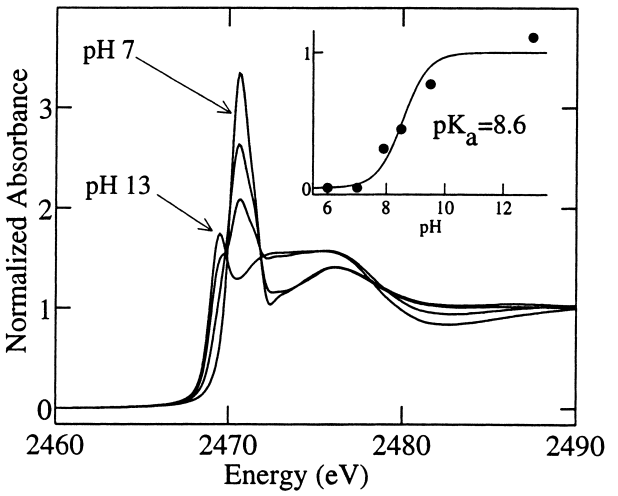

Fig. 3. Variation of the sulfur K-edge X-ray absorption near-edge spectrum of cysteine with pH. The spectra shown were collected by monitoring X-ray fluorescence of solution samples with pH values of 7.0, 7.9, 9.5 and 13.0. The inset shows a pK_a determination based on the near-edge data as discussed in the text.

the spectra of the amino acids cysteine and methionine in protein solutions resemble those of aqueous solutions (e.g. [12]).

Once in solution the spectra are generally dependent on pH (Fig. 3). While methionine shows only subtle sensitivity, the ionizable proton of the -SH group in cysteine confers a dramatic effect (Fig. 3). Deconvolution of the intermediate spectra by least squares fitting using the spectra recorded at pH 6 and 13 gave a pK_a of 8.6 ± 0.3, which compares well with the actual value of 8.33. We note that other ionizable groups in cysteine have pK_a values in the pH range of the titration shown in Fig. 3 and these may contribute more subtle changes to the spectrum. The prominent peak of the near-edge of the cys-S^- species is shifted to lower energy and is less intense relative to that of the cys-SH spectrum, rather resembling a sulfide (e.g. see Fig. 1).

Fig. 4 shows the sulfur K-edge spectra of equine erythrocytes and plasma, together with a curve-fitting analysis. Model compounds were chosen to be representative of the functional groups of sulfur which are likely to be present. These were aliphatic disulfides, thiols, thioethers and sulfoxides, represented by oxidized glutathione, cysteine, methionine and methionine sulfoxide, respectively. Inorganic sulfate, clearly indicated by its $\sim$2480 eV peak, was also required for adequate fits. Thus, for example, the use of oxidized glutathione as a disulfide model does not imply explicit quantification of that precise compound in the biological sample, but rather of total disulfide present, which would include both oxidized glutathione and protein disulfides. Additional components such as sulfonates and sulfones were tested but did not improve the fits. The best fit for the erythrocytes indicated 21.4% disulfide, 54.4% thiol, 21.3% thioether, 2.1% sulfoxide and 0.8% sulfate, whereas the best fit for the plasma yielded 76.5% disulfide, 20.6% thiol, 0% thioether, 0% sulfoxide and 2.9% sulfate. Accuracies for these numbers are difficult to estimate, principally because we cannot be certain that we are fitting appropriate model compounds to the biological spectrum. Precisions estimated from the diagonal elements of the covariance matrix are typically $< 0.1\%$, and we estimate that accuracies are close to $\pm 5\%$ of the values quoted. Similar spectra were obtained from a dozen horses, including samples from animals suffering from glutathione peroxidase deficiency. In all cases only subtle differences in the spectra and the curve-fitting analyses were observed.

Our results provide striking confirmation of the conventional wisdom regarding the chemical form of intra- and extracellular sulfur. In general extracellular cysteine is supposed to be mostly involved in disulfide bonds (cystine) while the reverse is supposed to be true inside the cell. In plasma we anticipate a considerable protein disulfide component, as crystalline horse serum albumin contains some 34 cystine disulfide sulfur atoms, with only one cysteine and no methionine residues [21].

While this paper was in preparation a paper by Klein and co-workers appeared addressing the same topic as the present work, and examining human blood [22]. They used a cryostat that maintained their samples at 140 K, which may have the advantage of minimizing radiation damage. Unfortunately their data seem to suffer from some of the experimental pitfalls discussed above, and they did not note the differences between the spectra of cysteine and methionine (shown in Fig. 1). In addition, they fit the spectra of blood with combinations of the spectra of solid rather than solution model compounds, and only fit a very limited part of the data. As we show in Fig. 2, the spectra of solid model compounds are

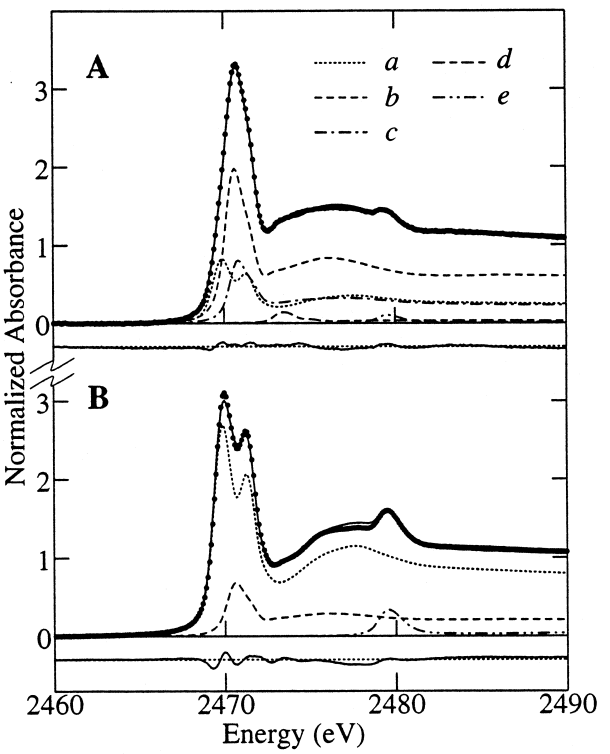

Fig. 4. Analysis of the chemical forms of sulfur in packed erythrocytes (A) and plasma (B) from horse blood. Spectra were fit to the sum of the spectra of five model compounds, all in aqueous solution at pH 7.0, representing the different chemical forms that are likely to be present, as discussed in the text: oxidized glutathione (a), cysteine (b), methionine (c), methionine sulfoxide (d), and sulfate (e). The points show the experimental data and the overlaid solid line the results of the fit, with the residual shown beneath. The individual components shown as broken lines have been scaled according to their fractions in the fits.

quite different from the spectra of the same compound in solution, and the quantitative aspects of their work may be somewhat suspect. Nevertheless, we concur that sulfur X-ray absorption spectroscopy will become a very useful tool for studying biological systems, as it continues to be for studying fuels and for other systems.

Acknowledgements: Part of this work was funded by the National Institutes of Health, Award R01 GM57375. The Stanford Synchrotron Radiation Laboratory is funded by the Department of Energy, Office of Basic Energy Sciences, Divisions of Chemical and Materials Science. The Biotechnology Program is supported by the National Institutes of Health, National Center for Research Resources, Biomedical Technology Program. Further support is provided by the Department of Energy, Office of Biological and Environmental Research. Parts of this work were presented at the 1995 SSRL Users Conference.

References

[1] George, G.N. and Gorbaty, M.L. (1989) J. Am. Chem. Soc. 111, 3182–3186.
[2] Spiro, C.L., Wong, J., Lytle, F.W., Greegor, R.B., Maylotte, D.H. and Lamson, S.H. (1984) Science 226, 48–50.
[3] Huffman, G.P., Huggins, F.E., Mitra, S., Shah, N., Pugmire, R.J., Davis, B., Lytle, F.W. and Greegor, R.B. (1989) Energy Fuels 3, 200–205.
[4] George, G.N., Gorbaty, M.L., Kelemen, S.R. and Sansone, M. (1991) Energy Fuels 5, 93–97.
[5] Waldo, G.S., Carlson, R.M.K., Moldowan, J.M., Peters, K.E. and Penner-Hahn, J.E. (1991) Geochim. Cosmochim. Acta 55, 801–814.
[6] Gorbaty, M.L., Keleman, S.R., George, G.N. and Kwiatek, P.J. (1992) Fuel 71, 1255–1264.
[7] Waldo, G.S., Mullins, O.C., Penner-Hahn, J.E. and Cramer, S.P. (1992) Fuel 71, 53–57.
[8] Frank, P., Hedman, B., Carlson, R.M.K., Tyson, T.A., Roe, A.L. and Hodgson, K.O. (1987) Biochemistry 26, 4975–4979.
[9] George, G.N., Byrd, J. and Winge, D.R. (1988) J. Biol. Chem. 263, 8199–8203.
[10] Shadle, S.E., Penner-Hahn, J.E., Schugar, H.J., Hedman, B., Hodgson, K.O. and Solomon, E.I. (1993) J. Am. Chem. Soc. 115, 767–776.
[11] Frank, P., Hedman, B., Carlson, R.M.K. and Hodgson, K.O. (1994) Inorg. Chem. 33, 3794–3803.
[12] George, G.N., Garrett, R.M., Prince, R.C. and Rajagopalan, K.V. (1996) J. Am. Chem. Soc. 118, 8588–8592.
[13] Williams, K.R., Gamelin, D.R., LaCroix, L.B., Houser, R.P., Tolman, W.B., Mulder, T.C., de Vries, S., Hedman, B., Hodgson, K.O. and Solomon, E.I. (1997) J. Am. Chem. Soc. 119, 613–614.
[14] Vairavamurthy, A., Zhou, W., Eglinton, T. and Manowitz, B. (1994) Geochim. Cosmochim. Acta 58, 4681–4687.
[15] Sekiyama, H., Kosugi, N., Kuroda, H. and Ohta, T. (1986) Bull. Chem. Soc. Jpn. 59, 575–579.
[16] http://ssrl.slac.stanford.edu/exafspak.html
[17] Dezarnaud, C., Tronc, M. and Hitchcock, A.P. (1990) Chem. Phys. 142, 455–462.
[18] Hitchcock, A.P., Horsely, J.A. and Ŝthr, J. (1986) J. Chem. Phys. 85, 4835–4847.
[19] Hitchock, A.P., Bodeur, S. and Tronc, M. (1989) Physica B 158, 257–258.
[20] Tröger, L., Arvantis, D., Baberschke, K., Michaelis, H., Grimm, U. and Zschech, E. (1992) Phys. Rev. B 46, 3283–3289.
[21] Ho, J.X., Holowachuk, E.W., Norton, E.J., Twigg, P.D. and Carter, D.C. (1993) Eur. J. Biochem. 215, 205–212.
[22] Rompel, A., Cinco, R.M., Latimer, M.J., McDermott, A.E., Guiles, R.D., Quintanilha, A., Krauss, R.M., Sauer, K., Yachandra, V.K. and Klein, M.P. (1998) Proc. Natl. Acad. Sci. USA 95, 6122–6127.