Figure A shows the iron K near-edge spectra of SOR in oxidized, dithionite-reduced, and (incompletely) photo-reduced forms. Oxidized SOR is unusually sensitive to photo-reduction, showing detectable changes in the near-edge spectrum after only one 37 min. scan; the spectrum in Fig. A is the result of a 4 ½ hour exposure to the X-ray beam. In contrast, the spectrum of dithionite reduced SOR did not change on exposure to the X-ray beam (not illustrated). Fitting a linear combination of the oxidized and dithionite reduced near-edge spectra reproduced the photo-reduced data sets exactly (not illustrated), and gave first-order kinetics with $k = 2.17 \times 10^{-2}$ min.$^{-1}$ (Fig. A) and indicated that photo-reduced and dithionite reduced SOR are essentially identical. For the near-edge spectra single scans provide adequate signal to noise for our purposes, but for EXAFS spectra averaging is required. The oxidized EXAFS data set was collected by moving the sample in the X-ray beam so as to interrogate a fresh spot with each scan. The near-edge and EXAFS spectra of all individual scans were compared, and were found to be identical within the noise. In this way, an average XAS data set for oxidized SOR containing no more than 10% photo-reduced enzyme was accumulated.

The EXAFS spectra of oxidized and reduced SOR, together with the EXAFS Fourier transforms are shown in Fig. B. The results of curve-fitting analysis is also shown in Fig. B, and the parameters for the best fits given in Table I. Based on the crystal structure, the first shell was modeled with four nitrogen (histidine) ligands and one oxygen, plus a single sulfur. We note that EXAFS analysis cannot readily distinguish between backscatterers of similar atomic number, such nitrogen and oxygen. The outer shell Fourier transform peaks between 3 and 4 Å are consistent with the presence of four histidine ligands in both oxidized and reduced data sets, and these were included in the analysis [A1].

Curve-fitting analysis of the EXAFS of the oxidized sample thus indicated five nitrogen and oxygen ligands at 2.12 Å, and one sulfur at 2.36 Å. Attempts to resolve Fe−N and

Fe−O ligands did not converge to significantly different bond-lengths. The Debye-Waller factor for the Fe−N/O ligand is unusually large at 0.0094 $Å^2$. The Debye-Waller factor is comprised of both static and vibrational components: $\sigma^2 = \sigma^2_{stat} + \sigma^2_{vib.}$ Assuming a Fe−N stretch of 220 cm^{-1} we can compute $\sigma^2_{vib} = 0.0049$ $Å^2$ [A1], and the EXAFS-derived Debye-Waller factor thus indicates a significant distribution in Fe−N and Fe−O bond-lengths. The low-temperature high resolution crystal structure shows two different sites which resemble the oxidized EXAFS data (sites A and C) and using the Fe−N and Fe−O bond-lengths of these we compute σ^2_{stat}= 0.0031, which combine to give a σ^2 value of 0.008 $Å^2$, which is reasonably close to the EXAFS-derived value of 0.0094±0.004 $Å^2$ (Table I). Modeling the Fe−N/O EXAFS using three different components was attempted, using two different sets of two Fe−N interactions, plus an Fe−O interaction. The two different Fe−N components were constrained to differ by at least 0.1 Å and at most 0.15 Å, and the σ^2 values were linked to the individual bond-lengths. This resulted in a fit that was not significantly better or worse than using a single Fe−N/O component (fit-errors of 0.335 and 0.334, respectively), indicating that the data are at least not inconsistent with a model of the active site which shows axial compression along one N−Fe−N axis, although direct support from EXAFS is not available.

As noted above, the low-temperature (high resolution) crystal-structure of oxidized SOR shows four sites, two of which (sites A and C) are six coordinate, while the other two (B and D) are five coordinate. The EXAFS-derived Fe−N/O bond-length is very close to the crystallographic average bond-length for sites A and C of 2.10 Å, but the Fe−S bond-length of 2.36 Å is significantly shorter than the crystallographically-derived value of 2.46 Å (for sites A and C). A search of the Cambridge Structure Database [A2] for compounds with $Fe(N/O)_5S_1$ coordination, and excluding entries where the sulfur bridges between metals, gave an average Fe−S bond-length of 2.34 Å in excellent agreement with the EXAFS-derived value. The crystal structure of sites B and D give an Fe−S bond-length of 2.67 Å. A search of the Cambridge

Structure Database [A2] for compounds with Fe−S− bond-lengths greater than 2.6 Å (restricted to two coordinate sulfur) revealed only six entries, all of which were ferrous species. Thus, while the Fe−S bond-length for sites B and D is unexpected it is not without chemical precedence.

The reduced EXAFS data fits best to a first-shell coordination of $Fe(N/O)_4S_1$ with Fe−N bond-lengths of 2.15 and 2.37 Å, respectively. This is in agreement with the crystallographic analysis, which indicated a 5 coordinate species with four histidine and one cysteine coordinated to the metal [A3]. For the reduced data set the Fe−N Debye-Waller factor is smaller that for the oxidized at 0.0045 $Å^2$, and is close to the vibrational value discussed above. This indicates much less static disorder in individual Fe−N distances in the reduced site than the oxidized site. As for the oxidized enzyme, the crystallographic analysis of sites A and C are in reasonable agreement with the EXAFS, while C and D appear anomalous. Thus, for A and B the average crystallographic Fe−N and Fe−S distances are 2.12 and 2.42 Å, respectively, while for C and D the Fe−N and Fe−S bond-lengths are 2.4 and 2.7 Å, respectively. Two of the Fe−N distances for site C are at the very long distance of 2.7 Å. Fe−N coordination at this length is very unusual, and while it has been reported with high metal coordination numbers (*e.g.* eight-coordinated ferrous [A4]) we consider the presence of such long bonds in reduced SOR unlikely. The Fe−S distances for sites B and D are also very long at 2.7 Å, and are similar to the oxidized Fe−S value for B and D of 2.68 Å, which we have discussed above.

We conclude that the EXAFS of both oxidized and reduced enzyme confirms the crystal structures for sites A and C, but that the crystal structures determined for sites B and D are chemically unexpected, and are not representative of the active site structure in frozen solution. Crystallographic data collection from the oxidized sample was conducted on SSRL's beamline 9-1, and involved approximately 45 min. of exposure to the X-ray beam. Beamline

9-1 has approximately 200 fold greater photon flux at the sample than beamline 7-3 (where the XAS data were collected), although a photon energy with approximately 6-fold lower absorption cross section was used for the crystallography. The effective radiation dose rate in the crystallography experiment was thus about 30 times that of the XAS, but for a fifth of the time. Furthermore, the crystallography was conducted at the relatively high temperature of 90 K. All of these factors will contribute towards X-ray photo-reduction and/or photo-damage of the metal site, and it seems very likely that X-ray induced changes in the iron site might have occurred in the crystallographic study of both oxidized and reduced proteins. Based on a comparison with the EXAFS-derived bond-lengths these appear to be most pronounced in sites B and D, and minimal in sites A and C.

References

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Figure Captions

Figure A. *Top:* Iron K near-edge spectra of SOR in oxidized, dithionite-reduced and 4.5 hour photo-reduced forms. *Bottom:* kinetics of X-ray induced photo-reduction estimated by curve-fitting of individual scans to linear combination of oxidized (5 min. beam exposure) and dithionite reduced spectra. The ordinate scale is logarithmic. The beam current varied from 80 to 82 mA during the experiment.

Figure B. EXAFS oscillations (top) and EXAFS Fourier transforms (bottom) of oxidized and reduced SOR. The Fourier transforms are phase-corrected for Fe−N backscattering. The solid lines show experimental data while the broken lines show the results of curve-fitting analysis (Table I).

Table I: EXAFS curve-fitting results for oxidized and reduced SOR.[a]

		Fe−N/O			Fe−S				
		N	*R* (Å)	σ^2 (Å^2)	*N*	*R* (Å)	σ^2 (Å^2)	ΔE_0	*F*
Oxidized	Fe−N/O	5	2.124(3)	0.0094(4)	1	2.361(5)	0.0065(5)	-9.8(1)	0.334
Oxidized	Fe−N	2	2.072(9)[c]	0.0067(3)[d]	1	2.361(5)	0.0066(8)	-9.8(1)	0.335
		2	2.172[c]	0.0070[d]					
	Fe−O	1	2.150(11)	0.0069[d]					
Reduced	Fe−N/O	4	2.146(2)	0.0045(2)	1	2.368(2)	0.0026(2)	-9.8(1)	0.239

Footnotes for Table.

a. *N* are the coordination numbers, *R* the interatomic distances in Å and σ^2 the mean-square deviation in *R* (the Debye-Waller factor) in Å^2. The values in parenthesis are the estimated standard deviations (precisions) obtained from the diagonal elements of the covariance matrix. We note that the accuracies will always be larger than and related to the precisions; *R* is expected to be better than ±0.02 Å.

b. The fit-error *F* is defined as $\sum\left[k^6(\chi_{\text{exptl}}-\chi_{\text{calcd}})^2/\sum k^6\chi_{\text{exptl}}{}^2\right]^{½}$.

c. Bond-lengths were constrained to differ by at least 0.1 Å and at most 0.15 Å.

d. The Fe−N and Fe−O σ^2 values were constrained to be proportional to the individual bond-lengths *R*.