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EXAFS of the type-1 copper site of rusticyanin

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Extended X-ray absorption fine structure (EXAFS) spectra at the Cu K-edge have been recorded of the oxidized and reduced form at pH 3.5 of rusticyanin, the type-1 or 'blue'-copper protein from *Thiobacillus ferrooxidans*. The EXAFS of oxidized rusticyanin is well simulated with models assuming a ligand set of 2 N(His) and 1 S(Cys) at 1.99 and 2.16 Å, respectively. Upon reduction, the average Cu-N ligand distance increases by approx. 0.08Å. For both redox states studied, the fit by the simulation is significantly improved by including a contribution of an additional sulfur ligand at approx. 2.8 Å. From comparison with structural data of other blue-copper proteins, it is concluded that the copper coordination environment is relatively rigid, which may be a clue to its high redox potential.

Rusticyanin; EXAFS; Blue-copper protein; Redox potential; *Thiobacillus ferrooxidans*

1. INTRODUCTION

Thiobacillus ferrooxidans is a member of the *Thiobacillus* group of sulfur bacteria. It is able to derive the energy required for growth from the oxidation of reduced sulfur compounds to, ultimately, sulfuric acid, and from the oxidation of Fe(II) to Fe(III) using oxygen as an oxidant [1]. The bacterium is a dominant organism in the process of ore extraction by microbial leaching of pyritic ores. The product of the oxidations is an acidic solution of Fe(III).

Rusticyanin is a small (M_r 16,300) blue-copper protein which is present at relatively high concentrations, up to 5%, of the cell protein [2,3], and is located in the periplasm of the bacterium [4]. It has optical spectra similar to other type-1 or 'blue'-copper proteins, although with a lower extinction coefficient at 590 nm [3], and electron paramagnetic resonance spectra similar to the type-1 protein stellacyanin [5,6]. At +680 mV, the mid-point potential of rusticyanin is considerably higher [7] than that of other blue-copper proteins, which range from approx. + 180 to + 370 mV [8]. The value is pH independent between pH 2 and 6.5, with an indication of pH dependence below pH 2, as determined by cyclic voltammetry and redox potentiometry [8,9] (A.G. Lappin and W.J. Ingledew, unpublished results). Proton nuclear magnetic resonance (^{1}H-NMR) spectra show the protein to be intact and globular in structure even at pH 1.0 (H.A.O. Hill and W.J. Ingledew, unpublished results). The protein has been shown to be reducible by Fe(II) when pure [3], and to undergo rapid oxidation, concomitantly with a cytochrome *c*, in kinetic experiments [2]. The reduction of rusticyanin Cu(II) by Fe(II), the putative substrate, in different media has been studied by stopped-flow spectrophotometry [9].

Rusticyanin does not contain detectable amounts of carbohydrate, and its amino acid sequence has been determined (R. Ambler, personal communication). When compared to other blue-copper proteins it is interesting that near the C-terminal end of the protein a Cys, a His and a Met residue are conserved. This is known to be part of the copper binding site in plastocyanin in which a second His close to a centrally located Asn completes the copper coordination sphere. In rusticyanin the Asn is not present in precisely the same position relative to a His but instead a number of Asn and His residues are close to the copper binding site. ^{1}H-NMR studies on reduced rusticyanin indicate that two of the 5 His residues are ligated to the copper (H.A.O. Hill and W.J. Ingledew, unpublished results). The purpose of the present study was to try and establish the nature of the copper ion coordination sphere in rusticyanin and perhaps offer an explanation for its considerably high midpoint potential compared to those of other type-1 copper proteins.

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Abbreviations: EXAFS, extended x-ray absorption fine structure; ^{1}H-NMR, proton nuclear magnetic resonance

2. MATERIALS AND METHODS

T. ferrooxidans (strain NC1B 8455) was grown in a medium of 180 mM $FeSO_4$, 1 mM $(NH_4)_2SO_4$, 0.2 mM KH_2PO_4, 2 ml per litre 98% H_2SO_4 and 0.2 ml of a salts solution (Ingledew, 1982). Cells were grown at 22°C in a chemostat with a working volume of 45 l with a

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Table I

Parameters used to simulate the EXAFS associated with the copper K-edge of rusticyanin in its oxidized (cf. Fig. 1a) and reduced (Fig. 1b) forms

			Oxidized form		Reduced form	
Shell no.	Atom type	No.	$2\sigma^2$ (Å^2)	R (Å)	$2\sigma^2$ (Å^2)	R (Å)
1	N	2	0.006	1.987	0.003	2.066
2	S	1	0.012	2.160	0.009	2.168
3	S	1	0.004	2.836	0.007	2.795
4	C	4	0.004	2.909	0.017	2.862
5	N	2	0.011	3.803	0.007	3.823
6	C	2	0.013	4.195	0.003	4.342
ΔE_o (eV)			28.09		26.14	
Fit index (k^2-weighting)			0.272		0.271	

R denotes the EXAFS distances of atoms from the central metal ion; $2\sigma^2$ is a Debye–Waller type factor, accounting for thermal and static disorder.

dilution rate of 0.024 h^{-1} and vigorous aeration. The effluent was centrifuged using an MSE continuous flow rotor (18 000 rpm with a flow rate of 300 ml·min^{-1}) to give a cell paste (5 g net weight per 50 l media). The cells were washed by resuspension and recentrifugation three times in 10 mM H_2SO_4 plus 50 mM Na_2SO_4 and once in 50 mM Na_2SO_4.

T. ferrooxidans cells were broken by French Press and rusticyanin was purified as described previously [3], but with the addition of a repeat of the final ion-exchange step. Only batches free of spectroscopically detectable cytochrome-*c* were used. The protein was concentrated against 50 mM β-alanine/SO_4^{2-}, pH 3.5, and concentrated by ammonium sulfate precipitation (95% saturation). Oxidized and reduced forms of the proteins were obtained by using K_2IrCl_6 as oxidant and Na_2SO_4 als reductant.

X-ray absorption spectra at the Cu K-edge were recorded at the SERC Synchroton Radiation Source at Daresbury Laboratory, using a Si 220 double crystal monochromator, using the set-up with fluorescence detectors described earlier [10]. The spectra were recorded on the rusticyanin as an ammonium sulfate paste of 3–4 mM concentration at ambient temperature as fluorescence excitation spectra. Copper foil was used to calibrate the X-ray photon energy. The reported spectra are the sum of 5 (reduced form) and 8 (oxidized form) scans, each scan having taken 90 min. The protein samples were monitored for radiation damage and redox change by spectroscopic measurement before and after the scans. No change in protein or redox state was discernable. In addition, the EXAFS spectra were compared after each scan and found to be identical.

The EXAFS was only analyzed out to 400 eV above the Cu edge because of the relatively poor signal to noise ratio of the data at higher energy. The background subtracted data were transformed into k space, with k given by $[(E-E_0)2m]^{1/2}/\hbar$, where k is the photoelectron wave vector, E the incident beam energy and E_0 the energy of the copper edge. The phase shifts and backscattering factors were derived from ab initio calculations using Clementi-Roetti wave functions for the neutral scattering atoms. This implies that the values found for the Debye-Waller type factors, accounting for thermal and static disorder, are absolute, rather than relative to a reference compound, and, neglecting thermal contributions, can be considered equivalent to ΔR^2 r.m.s. The excited copper atom was simulated by neutral zinc with one 1s electron removed. The phaseshifts were tested on model compounds [11] and it was found that a charge of +2 on the central atom in the phaseshift calculations gave the closest agreement with crystallographic distances. The EXAFS spectra were simulated using the fast curved-wave simulation and fitting programme EXCURVE [12,13]. The quality of fit was assessed by comparison of the experimental and simulated EXAFS and their Fourier transforms and by the fit index, calculated as $_i\Sigma^N[x(i)_{experimental}-x(i)_{simulation}]^2/N$ over all data points x_i, in k^2-weighting, with N the number of data points. All shells were analyzed after Fourier filtering, mainly in order to identify the atom types involved. Final refinements were carried out on non-Fourier filtered data. The data were weighted by k^2 and in each cycle of iteration, distances, Debye-Waller-type factors and ΔE_o were allowed to refine, whereas occupancies were varied in steps.

3. RESULTS

The background-subtracted EXAFS spectra of oxidized and reduced rusticyanin at pH 3.5 and their Fourier transforms are shown in Figs. 1a and 1b, respectively, together with their simulations. The parameters used to model the experimental data are given in Table I. The EXAFS spectra were simulated on the assumption that the coordination sphere of the copper consisted of two imidazole nitrogens (N(His)) and a cysteine sulfur (S(Cys)). As a possible fourth ligand, a methionine sulfur (S(Met)), as in plastocyanin and azurin, was considered, in agreement witht the spectral parameters and amino acid content of rusticyanin. From Fig. 1a and 1b it can be seen that the amplitude and phase show little change in going from oxidized to the reduced form of the protein. This indicates that the coordination number of the copper ion does not change and that if there are changes in metal ligand bond lengths then they are necessarily small (Table I).

3.1. *Oxidized rusticyanin*

The major shell could be satisfactorily simulated by a subshell of two nitrogen atoms and a subshell of one sulfur atom, at bond distances of 1.99 and 2.16 Å respectively, in agreement with the postulated N and S ligation. The peak in the Fourier transform at approximately 3 Å was Fourier-filtered and well simulated with a sulfur at 2.84 Å. The fit was improved by including a 4-carbon subshell, whereas simulations by one shell of 4 carbon atoms alone, or by two subshells, each of two carbon atoms, without sulfur, led to high values for the Debye-Walter-type factors which were physically unrealistic. To fit the peak at approximately 4 Å it was necessary to use two shells, one of two carbons and one of two nitrogens, with one of the distances (3.8 Å) apparently shortened relative to that expected for outer shell imidazole atoms (4.1–4.3 Å). Such effects have been found in the simulation of the EXAFS of other histidine ligated metalloproteins [14–16], and are due to

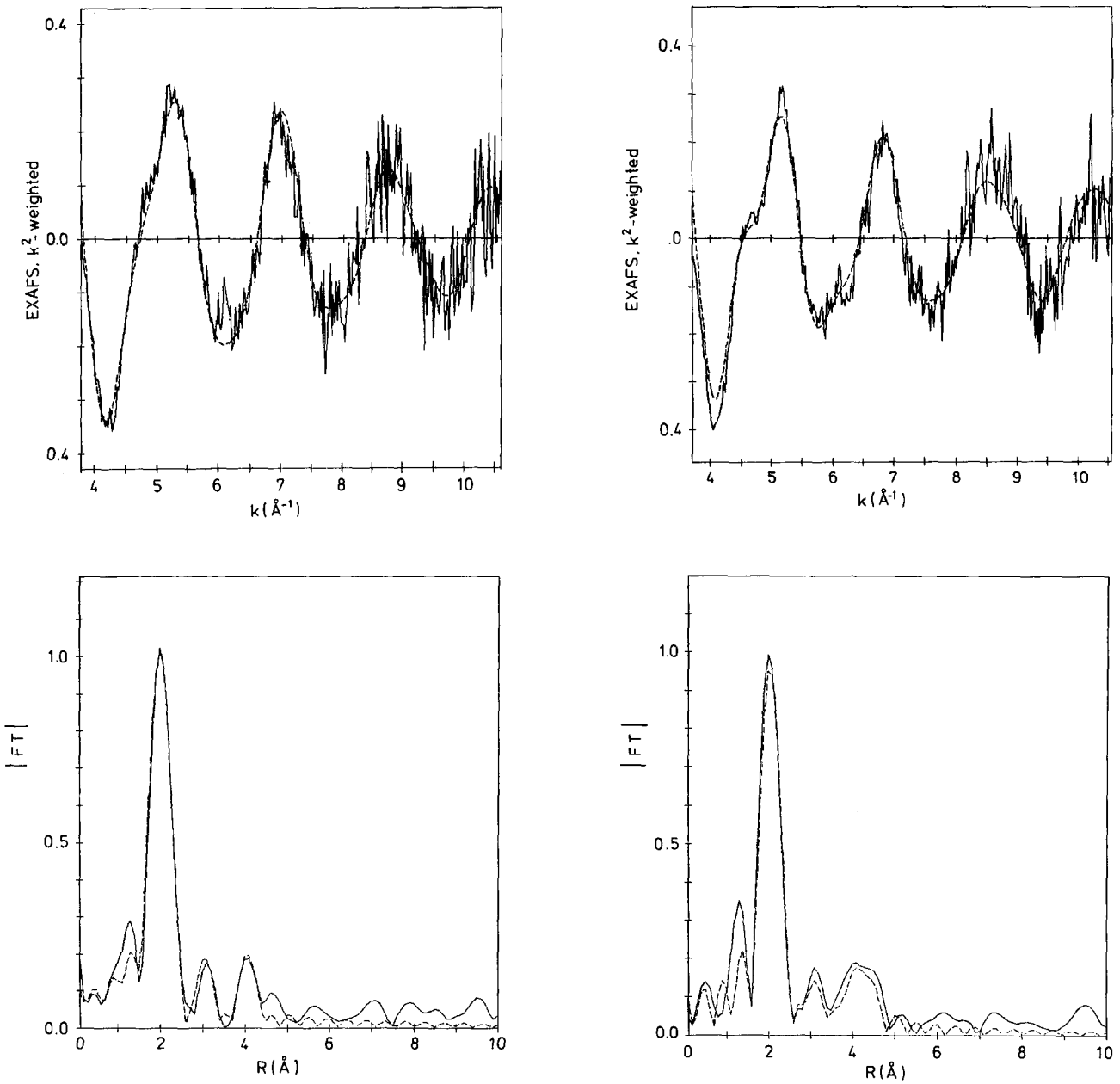

Fig. 1. k^2-weighted EXAFS (upper panels) and phase-corrected Fourier transform (lower panels) of oxidized (a) and reduced (b) rusticyanin. Solid lines, experimental; dashed lines, simulations with the parameters given in Table I.

the presence of multiple scattering in addition to the single scattering to which EXAFS analysis is usually restricted [17–19]. Such effects can now be simulated with EXCURVE [13], but the application of multiple scattering simulations to imidazole systems for analytical purposes gives misleading results [18] because of the many parameters involved [19]. It is possible to make reliable structure predictions from EXAFS analysis of such systems without simulation of the multiple scattering, e.g. for the zinc coordination in transcription factor TF IIIA [20], as recently corroborated by ^{1}H-NMR studies on one of the zinc coordinating peptide parts [21]. As the phase of only the shell of atoms at approx. 4 Å is affected by multiple scattering [19], neglect of the effect does not impair the validity of our conclusions with regard to the presence of both carbon and sulfur contributions in the shell at approx. 3 Å. For the closest shells, the error in the distance values derived from the refined simulations is not larger than ±0.03 Å [11].

3.2. *Reduced rusticyanin*

The major shell was again well simulated by a subshell of two nitrogen atoms and a single sulfur subshell, but at 2.07 Å the Cu-N distance is slightly larger than the corresponding distance in the oxidized protein. It

was again found necessary to simulate the peak at approx. 3 Å by a subshell of four carbon atoms and a subshell of one sulfur atom.

4. DISCUSSION

Early EXAFS studies on blue-copper proteins [22–24] detected 2 N(His) and 1 S(Cys) ligands to Cu, but no additional sulfur ligands, and indicated little change in the coordination sphere upon oxidation/reduction. In recent EXAFS studies [11,25,26], additional sulfur ligands at approx. 2.7 Å in the reduced forms of azurin and stellacyanin have been detected, as well as a spread, as concluded from the increased value for the Debye-Waller type factor, in the two Cu-N(His) distances, and a slight increase in the average Cu-N(His) distance, upon reduction. It was concluded that in oxidized azurin, the additional sulfur ligand has moved out to a distance where interference of its EXAFS contribution with those of the imidazole carbons occurs.

Comparing the present results with those studies (Table II), and others on stellacyanin [29] and umecyanin [30] (for a compilation of blue-copper ligand distances derived from EXAFS and crystallographic studies, cf. [24]), the most striking features are the increase in the Cu-N(His) distances upon reduction, combined with the relatively small spread in it, as judged from the values of the Debye-Waller-type factor, and the invariability of the Cu-S distances. The rusticyanin copper site appears to be more rigid than that of other blue-copper proteins. The closest Cu-S interaction, of 2.16–2.17 Å, is probably a Cu-S(Cys) interaction, as crystallographic studies of the blue-copper proteins plastocyanin [31,32] and azurin [33-35] give similar distances for Cu-S(Cys). In those crystal structures, the Cu-S(Met) distance is much longer (2.7-3.1 Å), and hence the long Cu-S distance found in the present study could be a Cu-S(Met) interaction. The fact that the Met ligand of blue-copper proteins is conserved in rusticyanin makes it unlikely that the long Cu-S distance represents a relatively long Cu-S(Cys), or Cu-S(Cys-Cys-disulfide bridge) interaction, as has been proposed for stellacyanin [36,37], which lacks methionine [38]. The detection of a distant sulfur atom by EXAFS must be treated with some circumspection. In EXAFS studies on the model compound Cu(bbdhp) (where bbdhp = 1,7-bis(2-benzimidazole-2,6-dithiaheptane) [11,25] strong correlations were found between the contributions of a distant S and carbon atoms in the imidazole ring, which are exactly out of phase if they are at the same distance from Cu, as is the case in the crystal structure [37]. In a polarized EXAFS study on plastocyanin single crystals, no contribution of the distant sulfur of the coordinating methionine was observed, even with the optimum orientation of the crystal in the beam, indicating that the motions of copper and methionine sulfur are highly uncorrelated, as is typical for weakly bonded or non-bonded atoms [24]. In the study on oxidized azurin [11], the distant S, if included, refined to a distance of approx. 3.45 Å, and its contribution was not considered significant. In the present rusticyanin study, however, it refines to a distance around 2.8 Å in both oxidized and reduced protein, which gives us confidence that its contribution is real. All decreases in fit indices observed upon inclusion of additional shells in the simulations in the present work are considered significant based on criteria derived from statistical tests [40].

This information about the distant sulfur is important when we try to account for the high midpoint potential of rusticyanin [7–9,32]. In the blue-copper proteins, the protein forces upon the metal a coordination geometry, viz. distorted tetrahedral, and a ligand set, viz. a mixture of sulfurs and nitrogens, that are in between those preferred by the Cu(I) and Cu(II) ion, as shown by the crystallographic studies on oxidized and reduced plastocyanin [31,32], presenting a nice illustration of the concept of the 'entatic state' [41]. While this feature accounts for the facile electron-transfer dynamics of the blue-copper proteins in general, the differences in redox potential from one blue-copper protein to another are more difficult to explain. It has been suggested that variation in π back bonding from

Table II

'Blue'-copper ligand distances, in Å, as derived from recent EXAFS studies on rusticyanin from *Thiobacillus ferrooxidans* (this work), azurin from *Pseudomonas aeruginosa* [25], and stellacyanin from *Rhus vernicifera* [26]

Protein/redox potential (mV)	2 N(His)	1 S(Cys)	1 Apical S	
Rusticyanin	oxidized	1.99 (.006)	2.16 (.012)	2.84 (.004)
+ 680 [7,8]	reduced	2.07 (.003)	2.17 (.009)	2.80 (.007)
Azurin	oxidized, pH 9.1	1.95 (.0025)	2.22 (.012)	
+ 300 [27]	pH 4.1	1.95 (.005)	2.18 (.006)	
	reduced, pH 9.2	1.96 (.024)	2.23 (.009)	2.73 (.035)
	pH 5.5	1.97 (.016)	2.25 (.0065)	2.70 (.016)
Stellacyanin	oxidized	1.93 (.003)	2.21 (.002)	
+ 180 [28]	reduced	1.98 (.007)	2.25 (.014)	2.66 (.015)

Debye-Waller-type factors are given in parentheses as $2\sigma^2$, and can be considered equivalent to ΔR^2 r.m.s. (root mean square).

Cu to the distant S can provide a tuning mechanism [42], a strong π back bonding ligand providing additional stabilization to the Cu(I) state, leading to a higher redox potential. As judged from the ligand distances and Debye-Waller factors in Table II, Cu(I) rusticyanin does not appear to have any additional stabilization as compared to Cu(I) azurin and Cu(I) stellacyanin. There appears to be no simple correlation between structural features like Cu(I)-(distant S)-distance, or spread in the Cu(I)-N(His) distances, and redox potential. This does not exclude that geometrical features like bond angles, which cannot be derived from the EXAFS, are important in tuning the redox potential. From the data presented here, the copper site of rusticyanin appears to be rigid as compared to that of other blue copper proteins. Destabilization of the Cu(II) state due to the relatively close additional sulfur may provide an explanation for the high redox potential in the case of rusticyanin rather than stabilization of the Cu(I) state.

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