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EXAFS analysis of the pH dependence of the blue-copper site in amicyanin from *Thiobacillus versutus*

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The room temperature Cu K-edge EXAFS (extended X-ray absorption fine structure) spectrum of reduced and oxidized amicyanin, the blue copper protein from *Thiobacillus versutus*, was measured at low and high pH. The data interpretation was partly based on independent NMR evidence for the occurrence of a ligand histidine protonation at low pH (pK_a = 6.9) in the reduced protein. In the oxidized protein two nitrogen-donors (from two histidines; Cu-N distances 1.95-2.01 Å and 1.86-1.89 Å) and a sulfur-donor (from a cysteine; Cu-S distance 2.11–2.13 Å) were identified and the coordination appears independent of pH. Upon reduction at high pH the Cu-S bond and one of the Cu-N bonds lengthen slightly (from 2.11 to 2.19 Å and from 2.01 to 2.18 Å, respectively). Upon lowering of the pH one of the N-donors of the Cu in reduced amicyanin disappears from the Cu EXAFS and a second S-donor (from a methionine) becomes visible at 2.41 Å from the Cu. The Debye-Waller factors are compatible with a Cu-N vibrational stretch frequency in the range of 150–250 cm⁻¹ and one > 285 cm⁻¹, and a Cu-S vibrational stretch frequency of about 150 cm⁻¹ (Cu-Smet; reduced amicyanin at low pH) and one in the range of 230–800 cm⁻¹ (Cu-Scys).

Introduction

As more structural and mechanistic information becomes available on blue copper proteins, their phenotypical variety, which is so striking at first sight, is beginning to yield to attempts at systematic classification, a process leading in the end, hopefully, to a better understanding of the evolutionary relationships between these proteins and their modes of operation. Although blue copper proteins all function as electron carriers in photosynthetic or bacterial redox chains and although they all possess a single typed Cu site as their active centre, they do vary in other respects, like the value of their redox potential, their amino acid chain length, their occurrence in particular types of redox chain and the pH sensitivity of their Cu-site structure and their redox activity [1–5].

Until recently the only blue copper proteins known to undergo a reversible change in their Cu-site structure with pH were the plastocyanins [3,5,6]. At high pH the Cu in these proteins is coordinated by two nitrogens and two sulfurs (deriving from two histidines, a cysteine and a methionine), an N₂S₂' coordination which is commonly observed for type-I Cu sites. At low pH one of the histidines in reduced plastocyanin becomes protonated and dissociates from the Cu, with a concomitant loss of redox activity of the protein [5]. Plastocyanins have been found exclusively in the photosynthetic electron transport chains of green plants and algae. It was all the more surprising, therefore, when a similar Cu-site lability was observed in amicyanin, the blue-copper protein of *Thiobacillus versutus*, which is a non-photosynthetic micro-organism [7,8]. For this protein also a loss of redox activity was observed upon a lowering of the pH [8].

Amicyanins constitute a recently discovered sub-class of the blue-copper proteins [2,9–13]. Discerning features appear to be amino acid chain lengths of about 100–106 residues [2,14], an unusual tightness (compared to other blue copper proteins) of the loop containing three of the four Cu ligands (Cys-Thr-Pro-His-Pro-Phe-Met, Cu ligands underlined) [2,14] and occurrence as electron carriers in C₁-oxidizing redox chains [2,9–13].
The sensitivity of the Cu-site structure of amicyanin from *T. versutus* to changes in pH has been documented on the basis of spectroscopic (especially NMR) evidence [7,8]. Since detailed structural information on amicyanins is not available yet, it was considered worthwhile to investigate the local structure of the Cu site of *T. versutus* amicyanin by means of EXAFS (extended X-ray absorption fine structure) spectroscopy. In particular it was deemed important to probe the effects of oxidation state and pH on the structure of the Cu site. The results of such a study are reported here. A careful analysis of the experimental results provides for the Cu-ligand distances. Furthermore the EXAFS data appear consistent with previous conclusions that in reduced amicyanin at low pH one of the ligand histidines is protonated and dissociates from the Cu [8].

Materials and Methods

Protein sample preparation

Culture conditions and protein isolation procedures have been described elsewhere [7,8,10,15]. About 100 mg of purified protein in 100 mM phosphate buffer (pH 7.4) was concentrated to approx. 600 µl (23 mM), sufficient for at least four samples. Reduction of a sample was achieved by adding a small excess of sodium dithionite. To ensure full reduction during the experiment ascorbic acid was also added (2 mM end concentration). The pH of the samples was adjusted by adding small amounts of 1 M NaOH and HCl stock solutions. When done carefully no denaturation of the amicyanin appeared to occur during these adjustments. A microelectrode MI-412 combination pH probe and a radiometer PHM 84 research pH meter were used to measure the pH. Final protein concentrations of the EXAFS samples were in the range of 15 to 20 mM.

Check on decomposition

Before and after the EXAFS measurements the 1H-NMR spectra of the protein samples were measured on a Bruker WM 300 spectrometer. No difference between the spectra before and after the EXAFS measurements was observed.

Check on oxidation state

Reduced amicyanin solutions tend to oxidise back slowly when exposed to air. It was carefully checked, therefore, that no trace of the blue colour characteristic of the oxidized form of the amicyanin (absorbance maximum at 596 nm), was present immediately before and after the EXAFS experiments on the reduced samples. Further, during experiments on oxidized amicyanin the sample was checked after each scan by examining the near edge of the EXAFS. No trace of the sharp feature which is characteristic of reduced protein, was observed.

EXAFS measurements

EXAFS protein samples were made up by injecting the sample solution in liquid sample holders with a thickness of 0.4 mm ensuring good S/N in fluorescence mode. Copper phthalocyanin and zinc sulfide (from a freshly opened container) were used as reference compounds. (ZnS was preferred over CuS as a reference compound since contrary to CuS the first coordination sphere of ZnS is characterised by a single metal-S distance). Reference samples were mixed with boron nitride and pressed in the form of self-supporting wafers. Spectra of the reference compounds were recorded in absorption mode and in fluorescence mode. Amplitude differences were found to be less than 5% between spectra run in different modes. Since the data range is larger and the signal to noise ratio is better for data collected in transmission mode, transmission mode data of reference compounds were used as a reference for phase shifts and backscatterings amplitudes. The EXAFS spectra were recorded at the synchrotron radiation source (SRS) in Daresbury, U.K., on station 9.2 equipped with a Si[220] monochromator. The monochromator was detuned to 50% intensity in order to avoid the presence of higher harmonics. The storage ring was operated at 2.0 GeV at a ring current of 120–170 mA. All experiments were done at 293 K. In fluorescence mode scintillation counters were used in combination with an ion chamber for the incident intensity. In transmission mode two ion chambers were used. Spectra of oxidized amicyanin (pH 9.2, 20 mM, 6 scans; notation: HOA; pH 5.2, 20 mM, 6 scans; notation: LOA) and reduced amicyanin (pH 8.9, 17 mM, 7 scans; notation: HRA; pH 6.6, 15 mM, 9 scans; a file obtained after subtraction of 1/3 times the HRA file is designated LOWRE (vide infra) were recorded in fluorescence mode with counting times linearly increasing with k from 1 s to 3 s in the EXAFS region.

Data analysis

Data handling and analysis were done with programmes developed at the Eindhoven University of Technology. A comprehensive review of data analysis techniques is given in reference [16]. Standard procedures were used to extract the raw EXAFS-data from the measured spectrum. Background was removed by using cubic spline routines and spectra were normalized on the height of the edge [17,18]. The raw EXAFS data of the reference compounds and the protein are displayed in Fig. 1A and C, and Fig. 2A, C, E and G, respectively.

Teo and Lee [19] have shown that phase shifts and backscattering amplitudes of nearest neighbours (like Zn and Cu) in the periodic table hardly differ. The use
of phase shifts and backscattering amplitudes obtained from EXAFS data of reference compounds has been shown to be valid in cases when the chemical state and local structure of the reference and the sample are comparable. Teo and Lee [19] also showed that shifts in the phase function associated with changes in the electronic configuration or oxidation state can be largely compensated by an adjustment of the inner potential \( E_0 \). In the present analysis the inner potential was used as an adjustable parameter. Since the variations in \( E_0 \) observed in the analyses of the protein data were always small (\(< 10 \text{ eV}\) the assumptions concerning the phase transferability (Zn-S to Cu-S) were assumed to be correct.

The phase shifts and backscattering amplitudes for the Cu-N and the Cu-S absorber-backscatterer pairs were obtained by forward and inverse Fourier transforms (see Table I).

![Graphs of EXAFS data](image)

**Fig. 1.** Raw EXAFS data (\( k^2 \) weighted) and Fourier transforms (\( k^3 \) weighted) of ZnS (A,B) and Cu-phthalocyanine (C,D). (For Fourier transform ranges see Table I).

---

**TABLE I**

Structural parameters of the reference compounds and the Fourier transform ranges, \( \Delta k \) and \( \Delta R \), used to obtain the EXAFS reference data (\( k^2 \)-weighted Fourier transforms were used)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Shell</th>
<th>( N )</th>
<th>( R (\text{Å}) )</th>
<th>( \Delta k (\text{Å}^{-1}) )</th>
<th>( \Delta R (\text{Å}) )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cu-phthalocyanine</td>
<td>Cu-N</td>
<td>4</td>
<td>1.93</td>
<td>2.53–12.7</td>
<td>0.00–2.04</td>
</tr>
<tr>
<td>ZnS</td>
<td>Cu-S</td>
<td>4</td>
<td>2.35</td>
<td>3.53–11.8</td>
<td>0.00–2.54</td>
</tr>
</tbody>
</table>

*See Ref. 22.*

*See Ref. 23.*

---

The protein raw data were Fourier transformed over the largest possible range (range varying from \( \Delta k = 5.7 \) to \( \Delta k = 7.7 \text{ Å}^{-1} \)) for which the data were considered reliable (see Fig. 2). The main EXAFS peak (0–2.5 Å) in the transformed data was inverse Fourier transformed over a region starting at 0 Å and ending at a node between 2.3–2.7 Å. The choice of this node was made in such a way that errors due to truncation of the data were minimized. The resulting inverse Fourier transforms are shown in Fig. 2B, D, F and H, respectively.

Four parameters are needed to characterize an EXAFS-shell: the coordination number \( (N) \), the distance \( (R) \), the difference of the squared Debye-Waller factor \( \Delta \sigma^2 \) relative to the reference shell (which accounts for the change in disorder relative to the reference compound), and the inner potential correction \( \Delta E_0 \), which allows for a correction on the position of the absorption edge. When fitting the data \( N \) was always kept at a fixed, pre-chosen value for each shell. Moreover, the variation in \( \Delta E_0 \) was constrained to a narrow band of values (\( \pm 10 \text{ eV} \)). This effectively resulted in less than three free adjustable parameters per shell. Since in practice only 2- or 3-shell fits were used the actual number of free adjustable parameters amounted to \( \leq 6 \) (2-shell fits) and \( \leq 9 \) (3-shell fits). The theoretically allowed number of free adjustable parameters as given by the expression \( 2 \times \Delta k \times \Delta R / \pi \) [20], in which \( \Delta k \) and \( \Delta R \) are the ranges used in the forward and backward Fourier transforms, amounts to 9–11, sufficient, therefore, for the present data analysis.

Inverse Fourier transformed data were fitted using various models with different shell types and different occupation numbers. The final refinement was performed by fitting in an iterative manner [16]. For 2-shell fits \( k^2 \)-weighted data were first fitted with \( R \)'s and \( \Delta \sigma^2 \)'s as variables and \( \Delta E_0 \)'s as constants; subsequently \( k^3 \)-weighted data were fitted with \( \Delta E_0 \)'s as variables and \( R \)'s and \( \Delta \sigma^2 \)'s as constants. This cycle was repeated until no further significant changes were observed. For 3-shell fits an iteration cycle consisted of 4 steps. \( k^2 \)- and \( k^3 \)-weighted fits (as described above) were first executed on the first two shells while the third shell was not varied. The iteration cycle was then concluded by \( k^1 \)- and \( k^3 \)-weighted fits on the third shell, while the first two shells were held constant. Again this was repeated until no further change between two consecutive cycles was observed. By applying in the analysis a \( k^1 \)-weighted, as well as a \( k^2 \)-weighted fit (in \( k \)- and \( R \)-space) the statistical correlation between \( \Delta E_0 \) and \( N \) and \( R \) and \( \Delta \sigma^2 \), is minimised. This procedure led to a set of parameters which simulated equally well \( k^2 \) and \( k^3 \)-weighted data [21]. It was checked that all final fits were not in local minima. This was done by using a
Fig. 2. Raw EXAFS data ($k^2$ weighted) of A: HOA, C: LOA, E: HRA and G: LOWRE. Isolated EXAFS (solid lines) and fits (dotted lines) of B: HOA (isolation: $k^3$, $\Delta k = 3.0-10.7$ Å$^{-1}$; $\Delta R = 0-2.3$ Å), D: LOA (isolation: $k^3$, $\Delta k = 3.0-10.5$ Å$^{-1}$; $\Delta R = 0-2.3$ Å), F: HRA (isolation: $k^3$, $\Delta k = 3.1-8.8$ Å$^{-1}$; $\Delta R = 0-2.5$ Å) and H: LOWRE (isolation: $k^3$, $\Delta k = 3.1-8.8$ Å$^{-1}$; $\Delta R = 0-2.7$ Å). Fit range for B,D : 4-10 Å and F,H: 4-8 Å.

large variety of combinations of starting values for $R$ (varied over a range of $\pm 10\%$), $\Delta E_0$, ($\Delta E_0$ (varied over $\pm 5$ eV) and $\sigma^2$ (variation of $\pm 0.003 \AA^2$) and checking that the fits all converged to the same limit. No attempt to fit the higher shell region was made.

Absolute values for the $\sigma^2$'s were obtained by analyzing the reference compounds with theoretically obtained phase shifts and back scattering amplitudes. The theoretical reference files were constructed with the help of the McKale's tables by using equation (61) on page 45 of reference [16]. The EXAFS data of the reference compounds were then analyzed with the help of fixed values of $N = 4$, $S_0^2 = 0.80$, $\lambda = 5$ Å$^{-1}$ [16] while $\Delta E_0$, $\sigma^2$ and $R$ were allowed to vary. The data analysis resulted in a Cu-N distance of 1.95 and a Cu-S distance of 2.34 Å which favourably compare to the crystallographic distances of 1.93 Å [22] and 2.35 Å [23]. The values of $\sigma^2$ (quoted per individual atom) for the two reference compounds are reported in the next section (see Table IV).

Results

No unique analysis of the experimental EXAFS data appeared possible, unless use was made of previously acquired information about the Cu-site structure [7,8,10]. In oxidized amicyanin it is known that the Cu is coordinated, independent of pH, by 2 nitrogens from 2 histidines, a sulfur from a cysteine and a sulfur from a methionine [7,8,10]. Since the sulfur of the methionine group in oxidized type-I copper sites is usually present at approx. 2.7-3.1 Å (outside the range of the inverse Fourier transform) the EXAFS data were fitted by considering only one S and two N scatterers. One fit was performed with coordination numbers $N = 2$ for the Cu-N and $N = 1$ for Cu-S contributions. A second fit was performed by employing two independent nitrogens (with different coordination distances) and a single sulfur. The results of both fits are presented in Table II. The results of the 3-shell fits are shown in Fig. 2B and D and Fig. 3A, B, C and D.

In reduced amicyanin one of the ligand histidines is known to titrate with pH ($pK_a = 6.9$), this value was derived in the present study from $^1$H-NMR pH titration experiments at 293 K in 100 mM phosphate buffer in 95% $H_2O/5\% D_2O$ and is in agreement with previous experiments in 99.95% $D_2O$ [7, 8]. Moreover, the low-pH form occurs in two conformations corresponding to different conformations of the titrating histidine [8]. Since one of these conformations occurs only to an extent of 15% of the other one, the two conformations can be treated as one for the purpose of the present analysis without introducing unacceptably large errors. Thus only a single low- and a single high-pH form are distinguished for reduced amicyanin.

In the pH 8.9 EXAFS experiment on reduced amicyanin it is almost exclusively the high-pH form which is present in solution. The analysis could thus be performed in the same manner as the analysis of the oxidized amicyanin data (see above). The results are presented in Table II. The results of the 3-shell fits are shown in Fig. 2F and Fig. 3E and F. On the other hand, the low-pH measurements were made at pH 6.6, which
TABLE II
Parameters corresponding with best fits of EXAFS protein data at T = 293 K

<table>
<thead>
<tr>
<th>FIEX</th>
<th>N</th>
<th>R (Å)</th>
<th>Δσ² (Å²)</th>
<th>ΔE₀ (eV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LOA</td>
<td>a</td>
<td>2.00 N</td>
<td>1.91 (2)</td>
<td>-0.001 (1)</td>
</tr>
<tr>
<td></td>
<td>1.00 S</td>
<td>2.13 (2)</td>
<td>-0.001 (1)</td>
<td>-2 (1)</td>
</tr>
<tr>
<td></td>
<td>1.00 N</td>
<td>1.84 (2)</td>
<td>-0.004 (1)</td>
<td>-7 (1)</td>
</tr>
<tr>
<td></td>
<td>b</td>
<td>1.00 N</td>
<td>1.95 (2)</td>
<td>0.001 (1)</td>
</tr>
<tr>
<td></td>
<td>1.00 S</td>
<td>2.13 (2)</td>
<td>-0.004 (1)</td>
<td>6 (1)</td>
</tr>
<tr>
<td>HOA</td>
<td>a</td>
<td>2.00 N</td>
<td>1.95 (2)</td>
<td>0.012 (1)</td>
</tr>
<tr>
<td></td>
<td>1.00 S</td>
<td>2.10 (2)</td>
<td>-0.005 (1)</td>
<td>2 (1)</td>
</tr>
<tr>
<td></td>
<td>1.00 N</td>
<td>1.89 (2)</td>
<td>-0.003 (1)</td>
<td>-10 (1)</td>
</tr>
<tr>
<td></td>
<td>b</td>
<td>1.00 N</td>
<td>2.01 (2)</td>
<td>0.002 (1)</td>
</tr>
<tr>
<td></td>
<td>1.00 S</td>
<td>2.11 (2)</td>
<td>-0.005 (1)</td>
<td>4 (1)</td>
</tr>
<tr>
<td>HRA</td>
<td>a</td>
<td>2.00 N</td>
<td>1.91 (2)</td>
<td>0.010 (1)</td>
</tr>
<tr>
<td></td>
<td>1.00 S</td>
<td>2.10 (2)</td>
<td>0.004 (1)</td>
<td>-9 (1)</td>
</tr>
<tr>
<td></td>
<td>1.00 N</td>
<td>1.86 (2)</td>
<td>-0.004 (1)</td>
<td>-8 (1)</td>
</tr>
<tr>
<td></td>
<td>b</td>
<td>1.00 N</td>
<td>2.18 (2)</td>
<td>0.003 (1)</td>
</tr>
<tr>
<td></td>
<td>1.00 S</td>
<td>2.19 (2)</td>
<td>-0.004 (1)</td>
<td>-6 (1)</td>
</tr>
<tr>
<td>LOWRE</td>
<td>a</td>
<td>0.67 N</td>
<td>1.88 (2)</td>
<td>-0.004 (1)</td>
</tr>
<tr>
<td></td>
<td>0.67 S</td>
<td>2.18 (2)</td>
<td>0.004 (1)</td>
<td>-9 (1)</td>
</tr>
<tr>
<td></td>
<td>0.67 N</td>
<td>1.89 (2)</td>
<td>-0.003 (1)</td>
<td>-6 (1)</td>
</tr>
<tr>
<td></td>
<td>b</td>
<td>0.67 S</td>
<td>2.18 (2)</td>
<td>-0.003 (1)</td>
</tr>
<tr>
<td></td>
<td>0.67 S</td>
<td>2.41 (2)</td>
<td>0.006 (1)</td>
<td>7 (1)</td>
</tr>
</tbody>
</table>

a LOA: oxidized protein, pH 5.2.
b HOA: oxidized protein, pH 9.2.
c HRA: reduced protein, pH 8.9.
d LOWRE: reduced protein, pH 6.6; file obtained after subtraction of 1/3 times HRA from the experimental file (see text).
N: coordination number after mean free path correction.
f Sets a and b refer to alternative fits (2 shell and 3 shell respectively).

is only 0.3 units below the pKₐ of the titrating histidine. At this pH the sample consisted of a mixture of 1/3 of the high-pH form and 2/3 of the low-pH form. (Experiments at a pH below 6.6, although preferable, were prohibited by the instability of the protein below pH 6.6 [7,8]). The analysis of the experimental data was performed by subtracting first 1/3 of the high-pH spectrum from the experimental pH 6.6 spectrum. The resulting difference spectrum was used in the subsequent analysis. Since in reduced amicyanin one of the histidines dissociates from Cu at low pH, the Cu-N coordination number was set to one. For the Cu-S coordination two alternatives were tried: a) a single Cu-S contribution with a coordination number of one, and b) 2 independent Cu-S contributions (with different coordination distances), each with a coordination number of one. The results are presented in Table II (coordination numbers are given as 0.67 to account for the fact that 1/3 of the intensity had been removed from the spectra by the subtraction procedure described above). The 3-shell fits are shown in Fig. 2H and Fig. 3G and H. The results obtained on the basis of the 3-shell fits appear to be preferable to the results from the 2-shell fits (vide infra).

**Higher shell results**

In order to obtain information about the contribution of the higher shells to the EXAFS spectra, the calculated Cu-S and Cu-N contributions (from Table II) were subtracted from the original data. The resulting difference file was Fourier transformed (k⁴ weighted) in the range of k = 4 to k = 8 (see Fig. 4).

The main observation in Fig. 4 is that for the reduced species the higher shells have much less amplitude compared to the oxidized species (notice the difference in
the HOA file, 0.015 Å² for the HRA file) compared to the reference compound (0.005 Å²). This points to the presence of independent sub-shells, as employed in the b-set. The second consideration makes use of the inverse correlation between the length of a metal-ligand bond, \( R \), and the corresponding metal-ligand bond stretch frequency [24–27]. The frequency of the stretch vibration is directly related to the vibrational contribution to the absolute Debye-Waller factor through [28]

\[
\alpha_{\text{DW}}^2 = \frac{h}{8\pi^2 v^2} \coth \left( \frac{hv}{2kT} \right)
\]

in which \( T \) is the temperature, \( \mu \) is the reduced mass of the absorber-scatterer pair and \( v \) is the absorber-scatterer vibrational frequency. One thus expects the scatterer-pair distance, \( R \), to be correlated with the absolute Debye-Waller factor in the sense that a higher \( R \) corresponds to a larger absolute Debye-Waller factor.

It can be easily verified that the b-sets appear to conform better to this expectation than the a-sets. The subsequent analysis has therefore been based on the b-sets.

Copper coordination

When looking at the data for oxidized amicyanin (Table II) it appears that within the experimental uncertainty there is almost no effect of pH on the Cu-site structure. One nitrogen is found at 1.86–1.89 Å from the Cu, the second one at 1.95–2.01 Å and one sulfur is found at a distance of 2.11–2.13 Å. By analogy with type-I Cu sites which have been structurally characterized [3,4,29–31], the sulfur detected here is assigned to the coordinating cysteine. The distances reported here are in the range of values normally encountered for type-I Cu sites (see Table III), although a Cu-N distance of 1.86–1.89 Å is shorter than found in most cases (see Table III).

<table>
<thead>
<tr>
<th>TABLE III</th>
<th>Crystallographically determined Cu-ligand distances (Å) in blue-copper proteins</th>
</tr>
</thead>
<tbody>
<tr>
<td>ox a</td>
<td>ox b</td>
</tr>
<tr>
<td>N (His)</td>
<td>2.11</td>
</tr>
<tr>
<td>N (His)</td>
<td>2.09</td>
</tr>
<tr>
<td>S (Cys)</td>
<td>2.20</td>
</tr>
<tr>
<td>S (Met)</td>
<td>2.79</td>
</tr>
</tbody>
</table>

\( ^a \) Cu(II) azurin from *Pseudomonas aeruginosa* [29].
\( ^b \) Cu(II) azurin from *Alcaligenes denitrificans* [4].
\( ^c \) Cu(I) plastocyanin from *Populus nigra*, pH 3.8 [3].
\( ^d \) as \( ^c \), pH 7.8 [3].
\( ^e \) Cu(II) plastocyanin from *Populus nigra* [30].
\( ^f \) Cu(II) pseudoazurin from *Alcaligenes fasciatus* [31].
\( ^g \) Cu(II) plastocyanin from *Enteromorpha prolifera* [48].
A similar short Cu-N distance was obtained from the analysis of the Cu-EXAFS of _P. aeruginosa_ azurin [32], somewhat at variance with the crystallographic data on azurin (see Table III). Also for plastocyanin EXAFS distances appear to be slightly shorter than the crystallographically measured values [33]. The discrepancies between the EXAFS and crystallographic data may be due to a possible asymmetry in the pair distribution function of the distance between the central atom and the scatterer [34]. An asymmetric distribution in general will lead to apparent distances which are shorter than the true mean distance [34,35]. More theoretical efforts would be required to find out to what extent such an asymmetry might affect the EXAFS and crystallographic data analysis to a different extent. Alternatively, it is pointed out that the EXAFS and crystallographic data of azurin were obtained from samples measured under different conditions (solution vs. crystalline state). It is conceivable that the short Cu-N distance found here for amicyanin is not an artefact of the EXAFS analysis, but correctly represents the Cu-N distance.

Upon reduction at high pH the Cu site appears to basically maintain its geometry. The only change seems to be a lengthening of one of the Cu-N bonds to 2.18 Å and of the Cu-S bond to 2.19 Å. These numbers are quite acceptable for a type-I Cu site (see Table III). The small change in geometry upon reduction is also reflected in a change in the contribution from the higher shells to the EXAFS (vide infra).

A satisfactory fit of the EXAFS of the reduced amicyanin at low pH appears possible with a single N shell plus a split S shell. The resulting Cu-N and Cu-S distances of 1.89 and 2.18 Å are much like they are in the reduced high-pH species; the interesting observation, however, is that the second N-donor observed at high pH (at 2.18 Å from the Cu) can be replaced in a satisfactory manner by a S at 2.41 Å from the Cu. This second sulfur we ascribe to the coordinating methionine.

Analysis of Debye-Waller factors: The values of \( \sigma^2 \) for the two reference compounds are reported in Table IV. Absolute values of \( \sigma^2 \) for the amicyanin files were obtained from the \( \Delta \sigma^2 \) values reported in Table II by augmenting them with the absolute Debye-Waller factors of the reference compounds (Table IV). The Debye-Waller factor \( \sigma^2 \) contains contributions from structural disorder (non-equivalence of bond distances) and contributions due to the vibrational motion of the atoms in the metal-ligand bond, \( \sigma_{vib} \). There is no indication from NMR [7,8] for the occurrence of structural disorder, i.e. the presence of more than one stable conformation of the protein in solution (apart from the conformers of reduced amicyanin observed at low pH, which has already been accounted for in the analysis). There has also been no indication for such a disorder in any of the other type-I Cu sites studied up till now [3,4,30,31]. It is therefore assumed that the main contribution to the Debye-Waller factor derives from the vibrational motion of the atoms in the Cu-ligand bonds, as given by equation (1).

From equation (1) and the values of \( \sigma^2 \) with their quoted experimental uncertainties (see Table IV) the upper and lower limits for the vibrational frequency, \( \nu \), can be derived. In the case of the Cu-S bond the reduced mass is calculated with \( m_{Cu} = 63.5 \) and \( m_S = 32 \). The reduced mass for the Cu-N bond is calculated by replacing the nitrogen mass by the mass of a 'stiff' imidazole ring in accordance with literature data on metal-imidazole coordination compounds [36].

The values thus calculated for the vibrational frequencies \( \nu \) and the force constants \( f \) are reported in Table IV. The frequencies of the Cu-N stretch appear in the range of 150–250 cm\(^{-1}\) for the longer and > 285 cm\(^{-1}\) for the shorter Cu-N bond, in accordance with literature data. For instance, for the Cu-N stretch in Cu(II)(imidazole)\(_2\)Cl\(_2\), \( \nu = 307 \) cm\(^{-1}\) [36], for a similar mode in Cu(171)octaethylporphyrin \( \nu = 234 \) cm\(^{-1}\) [37] and \( \nu = 246 \) cm\(^{-1}\) for the Cu-N breathing mode in Cu-porphin [38] (compare to \( \nu = 193–243 \) cm\(^{-1}\) for Cu-phthalocyanin, see Table IV). Also the range of 230–820 cm\(^{-1}\) for the Cu-S (cysteine) stretch frequency is in accordance with other spectroscopic evidence relating to the frequency of this vibration. For instance, multiple intense peaks in the resonance Raman spectra...
of blue copper proteins are observed in the range of 350–420 cm\(^{-1}\) \cite{39-42} and have been ascribed to coupling of the Cu-S stretch mode (360 cm\(^{-1}\)) with internal ligand (imidazole) vibrational modes \cite{39}. Furthermore, a Debye-Waller factor of 0.008 \(\AA^2\) for a Zn-S bond as found here for ZnS (see Table IV) was also found for the Zn(cysteine)\(_4\) site of a zinc-finger protein \cite{43} (Zn-S distance 2.32 \(\AA\)).

The Debye-Waller factors thus appear compatible with Cu-N and Cu-S vibrational stretch frequencies of the right order of magnitude. It is useful here to consider the Debye-Waller factors of the S-shell in somewhat more detail. The contribution from the methionine sulfur to the EXAFS of Type-I Cu sites has been a point of recurrent discussion in the literature. Tullins et al. \cite{44} were one of the first to point out that this contribution is difficult to detect if present at all, in the EXAFS of \textit{P. aeruginosa} azurin. Groeneveld et al. \cite{3} in their study of the pH-dependence of the EXAFS of the same protein reached a similar conclusion concerning the long Cu-S distances. A similar conclusion was reached by Feiters et al. for stellacyanin \cite{45}. Again, for umecyanin Sykes and coworkers \cite{46} report that no fourth Cu-ligand (possibly an S-donor) is discernible in the EXAFS of the Cu-site. Finally, Scott et al. \cite{33} in their extensive EXAFS investigations of single crystals of plastocyanin suggest that the lack of correlated motion between the Cu and the methionine sulfur is responsible for the absence of a noticeable contribution from the methionine sulfur to the Cu EXAFS, a conclusion worked out further by Penner-Hahn et al. \cite{47}. It has been stated that for normal type-I Cu sites with an \(N_2SS'\) coordination the stretch frequency of the Cu-Smet bond will probably not exceed 50 cm\(^{-1}\) \cite{47}. According to what is found in the present study, a Cu-S vibrational frequency of 155 cm\(^{-1}\) already corresponds with a high Debye-Waller factor of 0.014 \(\AA^2\) (see Table IV) and a 50 cm\(^{-1}\) vibration may therefore be expected too lead to still higher Debye-Waller factors. Considering that the contribution from the S at 2.41 \(\AA\) to the Cu EXAFS is already weak in the LOWRE file, it is not unexpected that the methionine-sulfur contribution to the Cu EXAFS of amicyanin is difficult to identify.

**Higher shells**

The contributions to the EXAFS that remain after subtraction of the simulated main peak (data from Table II) are shown in Fig. 4. They arise from higher shells. In the region of 2.6–4.5 \(\AA\) they must at least contain contributions from two imidazole groups (histidines), a \(\beta\)-carbon of the cysteine and the methionine sulfur. The most conspicuous feature in Fig. 3 is the much smaller amplitude of the higher shells in the reduced than in the oxidized species. At least part of this can probably be ascribed to an increasing inequivalence of the 2 ligand histidines upon reduction of the Cu, either due to an increased difference in Cu-N bond lengths (high pH) or to the \(\alpha\)-coordination of one histidine from the Cu (low pH). The inequivalence would cause destructive interference of the contributions to the EXAFS from the higher shells. A model calculation (results not shown) of the EXAFS resulting from two 1N shells (with \(R\) approx. 3 \(\AA\)) shows that as the difference in Cu-N distance between the two shells increases from 0.0 to 0.3 \(\AA\), the amplitude of the combined EXAFS from the two shells drops by 70–80%. It is thought, therefore, that the observed lack of EXAFS intensity in the 2.3–5.0 \(\AA\) region in the spectrum of the reduced species is due, at least in part, to mutual cancellation of the contributions from the higher shells of the imidazole rings. It supports the previously stated conclusion that the difference in the two Cu-N distances increases upon reduction.

**Conclusion**

In the present study amicyanin has been investigated at four different sets of experimental conditions: oxidized at low and at high pH, and reduced at low and at high pH. In all cases contributions from three ligands to the Cu EXAFS could be identified: two N-donors and one S-donor in oxidized amicyanin (low and high pH) and reduced amicyanin at high pH, and one N- and two S-donors in reduced amicyanin at low pH. Best results were obtained in all cases with the use of split shells for donors of the same type.

In accordance with previous spectroscopic evidence the Cu coordination in the oxidized species appears relatively insensitive to changes in pH \cite{7,8,10}. Reduction of the oxidized protein at high pH results in a slight lengthening of one of the Cu-N bonds and of the single observable Cu-S bond, but otherwise the coordination appears to remain intact. The occurrence of slight changes in the Cu-site structure is also testified at by a smaller contribution from higher shells to the EXAFS of the reduced protein.

It is worthwhile at this point to consider what extraneous information has been used as input for the EXAFS analysis. For the low and high pH oxidised amicyanin and the high pH reduced amicyanin use was made of the knowledge that the coordination of the Cu consists of two N- and two S-donors \cite{7,8,10}. The EXAFS analysis is unique in that it provided for three of the four Cu-ligand distances in these cases. Moreover, the EXAFS data independently demonstrated the insensitivity of the Cu-environment to pH in the oxidised protein, thereby providing independent confirmation of the NMR experiments \cite{7,8}. For the analysis of the reduced low pH EXAFS data, a data file first had to be constructed on the basis of the known pK\(_a\) of the equilibrium between the low and high pH forms of the amicyanin. For the subsequent analysis in addition use
was made of the knowledge that only one N-donor coordinates to the Cu at low pH. EXAFS then provided again for unique Cu-ligand distance information and it could be shown that at low pH a second S-donor is probably present at 2.41 Å from the Cu. The loss of the N-donor from the Cu-coordination environment caused apparently is compensated for by a decrease in the Cu-S methionine bond length.

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