Notes

Preparation and Characterization of Cobalt(II)-Substituted Rusticyanin

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Rusticyanin is a blue copper protein found in the bacterium *Thiobacillus ferrooxidans*. Like the other blue copper proteins,[1,2] rusticyanin apparently functions as an electron-transfer protein,[3] and it exhibits a relatively strong ($\epsilon = 2240\ M^{-1}\ cm^{-1}$) absorbance band near 600 nm.[4] Rusticyanin also displays a number of unique and rather remarkable properties that distinguish it from the other blue copper proteins. The bacterium in which rusticyanin is found thrives in dilute sulfuric acid solution (pH ~2) and obtains energy from the oxidation of iron(II) to iron(III). Rusticyanin functions in the low-pH environment between the cell wall and the plasma membrane; it has been proposed as the initial electron acceptor for iron(II),[3] although this role has been disputed.[5] Other blue copper proteins are less stable at low pH; for example, when the pH of plastocyanin drops below 4, one of the ligands on copper is protonated, and the copper becomes redox-inactive.[6] Another significant difference between rusticyanin and other blue copper proteins lies in rusticyanin's redox potential of 680 mV vs NHE.[7,8] This is significantly higher than the 180–420-mV range of redox potentials observed for other blue copper proteins,[1,2] indicating that there may be a substantial difference in copper environment between rusticyanin and the other proteins.

On the basis of comparisons of the spectroscopic properties and amino acid sequence of rusticyanin with other blue copper proteins, it is likely that the copper coordination sphere in rusticyanin includes a histidine, a cysteine, and a methionine.[4,9-12] A second histidine and an aspartic acid have been proposed as fourth ligands;[9,12] a fifth possibility would be a carbonyl oxygen from the protein backbone, as found in azurin.[13] As with the more well-characterized blue copper proteins, a thorough understanding of the copper site in rusticyanin will require information from a variety of spectroscopic and structural investigations. This work describes the removal of copper from rusticyanin and the replacement of copper by cobalt(II). The absorbance spectrum of the cobalt derivative is compared with the spectra of other cobalt-substituted blue copper proteins.

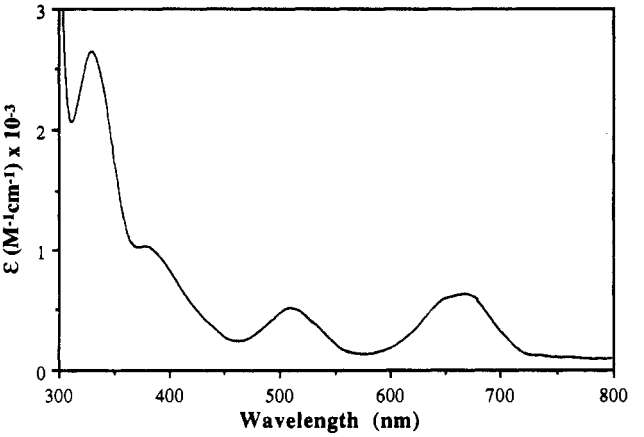

Figure 1. Absorbance spectrum of Co(II)-rusticyanin in 0.1 M sodium acetate, pH 5.5.

Experimental Section

Thiobacillus ferrooxidans (ATCC 23270) was grown in batch cultures on the medium described by Tuovinen and Kelly[14] with the addition of 1.6 mM copper(II) sulfate.[5] Cells were harvested by tangential flow ultrafiltration (Millipore Minitan). The isolation and purification of rusticyanin were based on published procedures.[4,5] Final purification was by cation-exchange chromatography on a Mono S column (Pharmacia), using 0.05 M sodium acetate, pH 4.0, with a sodium chloride gradient. This procedure yielded 0.2–0.4 mg of rusticyanin/L of culture with an absorbance ratio (A_{280}/A_{596}) ≤ 7.5.

Copper was removed from rusticyanin by anaerobic dialysis for 18 h at 4 °C against 0.5 M sodium thiocyanate in 0.1 M sodium bisulfate, pH 2.0. The protein was reduced prior to dialysis by the addition of sodium ascorbate. Dialysis against 0.2 M thiourea under similar conditions also removed the copper from rusticyanin, but the preparations were somewhat variable; the results described below were obtained using sodium thiocyanate. Metal removal was confirmed by the lack of absorbance at 596 nm on addition of the oxidant potassium hexachloroiridate(IV).

Rusticyanin was reconstituted by the addition of a 10-fold excess of Cu(II) to a 0.2–0.4 mM solution of the apoprotein in 0.1 M sodium acetate, pH 5.5. Co(II)-rusticyanin was prepared by addition of Co(II) to the apoprotein under similar conditions or by dialyzing the apoprotein against a solution of Co(II) with [Co(II)] = 10[apoprotein]. Excess cobalt was removed by ultrafiltration, using at least three washes with buffer. Atomic absorption standard solutions were used as the sources of Cu(II) and Co(II). Cobalt concentrations in the proteins were measured by atomic absorption spectroscopy. Protein concentrations were calculated on the basis of the absorbance at 280 nm, using a value of ϵ_{280} (15 700 $M^{-1}\ cm^{-1}$) determined for the native protein. The molar absorptivities of the d → d and charge-transfer bands in the Co(II)-rusticyanin spectrum were calculated on the basis of the cobalt concentration.

Nanopure (Barnstead) water was used throughout, with the exception of the culture media. Buffers for use with the apoprotein were passed through a column of Chelex 100 resin (Bio-Rad).

Results and Discussion

Preparation of Co(II)-Rusticyanin. Copper can be removed from rusticyanin by treatment with sodium thiocyanate. The milder conditions for metal removal reported for azurin[15] did not completely remove the copper from rusticyanin.

Reconstitution of rusticyanin by addition of Cu(II) to the apoprotein requires 25–30 min at room temperature under the conditions described above. The UV–visible spectrum of the reconstituted protein is indistinguishable from that of the native

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Table 1. UV–Visible Data for Cobalt-Substituted Blue Copper Proteins (λ, nm)

rusticyanin[a]	azurin[b]	plastocyanin[c]	stellacyanin[d]	pseudoazurin[e]	CBP[f]	assgnt
328	330	333	310 (sh)	335	331	S^-(Cys) → Co(II)
375 (sh)	375 (sh)	385 (sh)	365 (sh)	390 (sh)	390 (sh)	S^-(Cys) → Co(II)
	405 (sh)	430 (sh)		440 (sh)		S^-(Cys) → Co(II)
505	522	508	540	505	510	d → d
640 (sh)	638	650 (sh)	625 (sh)	640 (sh)	640 (sh)	d → d
666	645 (sh)	673	655	673	676	d → d

[a] *Thiobacillus ferrooxidans*; this work. [b] *Pseudomonas aeruginosa* azurin; refs 16,17. [c] *Phaseolus vulgaris*; ref 16. [d] *Rhus vernicifera*; ref 16. [e] *Achromobacter cycloclastes*; ref 23. [f] Cucumber basic protein; ref 22.

protein. Native and reconstituted rusticyanin have identical electrophoretic mobilities on a nondenaturing polyacrylamide gel at pH 3.8, providing evidence that the reconstituted protein regains the native structure.

Aporusticyanin reacts much more slowly with Co(II) than with Cu(II). The spectrum of the cobalt protein grows in over a period of $3^1/_2$ days at room temperature under the conditions described above; higher cobalt concentrations (up to 50×) do not appreciably shorten the time required for cobalt insertion. In order to assess the stability of the apoprotein under the conditions for cobalt insertion, aporusticyanin was stored at room temperature for 3 days in 0.1 M NaOAc, pH 5.5. On addition of a 10-fold excess of Cu(II), rusticyanin was reconstituted fully in 25–30 min, indicating no apparent damage to the copper binding site.

The cobalt-to-protein ratio was determined to be 0.57 ± 0.09, on the basis of five separate preparations of cobalt rusticyanin. At pH 2, the cobalt protein is significantly less stable than the native protein, losing cobalt over a period of several hours at 4 °C. Reasonable stability has been observed over the pH range from 4 to 7, although the intensity of the visible absorption bands begins to decrease within 24 h. The protein showed partial loss of cobalt during attempts to purify it by cation-exchange chromatography at pH 4.0.

Comparison with Other Blue Copper Proteins. The UV–visible spectrum of Co(II)-rusticyanin is shown in Figure 1. Comparison with the spectra of other cobalt-substituted blue copper proteins (see Table 1) provides strong evidence that the cobalt is bound in the copper-binding site. By analogy to band assignments in the other proteins,[16–18] the band at 328 nm ($\epsilon = 2.6 \times 10^3$ M^{-1} cm^{-1}) and the shoulder at 375 nm ($\epsilon = 1.1 \times 10^3$ M^{-1} cm^{-1}) can be assigned as S^-(Cys) → Co(II) charge transfer, and the bands at 505 nm ($\epsilon = 5.8 \times 10^2$ M^{-1} cm^{-1}) and 666 nm ($\epsilon = 6.4 \times 10^2$ M^{-1} cm^{-1}) can be assigned as d → d transitions. The positions of the charge-transfer bands in the Co(II)-rusticyanin spectrum are very similar to those for Co(II)-azurin, while the d → d region of the spectrum closely resembles that of the cobalt derivative of plastocyanin.[16–19] In the native forms of both azurin and plastocyanin, the copper forms three relatively short bonds to two histidines and a cysteine in a distorted trigonal planar arrangement, with an axial bond to a methionine and, in the case of azurin, a peptide carbonyl.[13] Thus, the strong similarity between the absorbance spectrum of Co(II)-rusticyanin and the spectra of the cobalt derivatives of azurin and plastocyanin lends support to the proposal[9] that the coordination sphere in rusticyanin contains two histidines, a methionine, and a cysteine. Co(II)-stellacyanin exhibits a markedly different spectrum, with blue-shifted charge-transfer bands.[16] Stellacyanin contains no methionine[20] and thus does not share the copper–methionine coordination. The intensities of the d → d bands in the Co(II)-rusticyanin spectrum tend to support a distorted tetrahedral or trigonal pyramidal ligand geometry, as in plastocyanin, rather than a distorted trigonal bipyramidal arrangement, as in azurin. Four-coordinate Co(II) model complexes generally exhibit more intense d → d transitions than five-coordinate complexes ($\epsilon <$ 250 M^{-1} cm^{-1}),[21] although the difference between the intensities in Co(II)-azurin and Co(II)-plastocyanin is small.

The Co(II)-rusticyanin absorbance spectrum bears a particularly strong resemblance to the spectra of the cobalt derivatives of pseudoazurin and cucumber basic protein.[22,23] In their native forms, all three of these proteins exhibit rhombic EPR spectra[11,23,24] and relatively intense (ϵ = 1000–2000 M^{-1} cm^{-1}) absorptions near 450 nm.[4,25] These spectral features have been interpreted[18,23,26,27] as indicating a stronger Cu(II)-methionine interaction than in azurin and plastocyanin, which exhibit axial EPR spectra and weaker absorption bands near 450 nm.[28,29] The Cu–methionine bond length in pseudoazurin from *Alcaligenes faecalis* S-6 is 2.69 Å,[30] while the bond length in cucumber basic protein is 2.62 Å;[31] the analogous bond lengths in azurin (*Alcaligenes denitrificans*)[13c] and plastocyanin (poplar)[6b] are 3.11 and 2.90 Å, respectively. This comparison suggests that the copper site in rusticyanin may include a relatively short Cu–methionine bond; such an interaction may also contribute to the high reduction potential of rusticyanin.[32] We note, however, that stellacyanin, with no methionine residues, also exhibits a rhombic EPR spectrum and a strong absorption near 450 nm.[33]

A model of the secondary structure of rusticyanin has been proposed, on the basis of circular dichroism data and computational analyses of the amino acid sequence.[12] In this model, aspartic acid serves as the fourth copper ligand rather than a histidine. Recently, a mutant of *P. aeruginosa* azurin was prepared in which the first histidine ligand was replaced by aspartic

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acid.[34] The charge-transfer bands in the mutant occur at significantly higher energy than in Co(II)-rusticyanin. Thus, the current work provides evidence against aspartic acid coordination in rusticyanin.

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