dination geometry, but not in the domed coordination site of an N-substituted porphyrin).

The reduction potentials for copper complexes of N-substituted porphyrins (Table III) clearly show two trends: (1) a lack of a large difference between potentials for *N*-alkyl and *N*-aryl substituents and (2) a significant dependence of potentials in the nature of the axial ligand. A relatively hard ligand that binds well to Cu(II), Cl^-, gives rise to the least favorable potential shown. A poorly coordinating ligand, ClO_4^-, is more favorable for reduction, and a soft ligand expected to stabilize Cu(I), triphenylphosphine, provides the most favorable reduction potential. The visible absorption spectra of Cu(II) complexes with Cl^- and PPh_3 as axial ligands and the lack of any shift of potentials on addition of excess ligand shows the stoichiometry of these complexes to be 1:1. All of the cyclic voltammograms show reversible processes (i.e., the difference between cathodic and anodic peak potentials was within 10 mV of that for ferrocene under the same conditions), indicating that the coordination site geometry (and, hence, the presence or absence of covalently bound axial ligand) remains the same when the copper atom is reduced or oxidized.

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Contribution from the Department of Chemistry, University of Notre Dame, Notre Dame, Indiana 46556, and Department of Biochemistry and Microbiology, University of St. Andrews, Fife KY16 9AL, United Kingdom

Kinetics and Mechanisms of Reduction of Rusticyanin, a Blue Copper Protein from *Thiobacillus ferrooxidans*, by Inorganic Cations

A. GRAHAM LAPPIN,*[1] C. ANN LEWIS,[2] and W. JOHN INGLEDEW[2]

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Oxidation-reduction reactions of the blue copper protein rusticyanin from *Thiobacillus ferrooxidans* have been investigated. Cyclic voltammetry using a 4,4'-bipyridyl-modified carbon-paste electrode gives a reduction potential of 0.67 V (vs. NHE) for the protein, independent of pH in the range 1-3. Kinetic studies of the reduction of the copper(II) form of rusticyanin by iron(II) show anion effects. The dependence of reaction rate on iron(II) concentration is first order in chloride media but shows limiting zero-order kinetics in the presence of sulfate ion. The absence of inhibition by cobalt(II) ion suggests that this is due to a rate-limiting protein conformational change. Although the reaction with iron(II) is slow, it is not inconsistent with an interaction of physiological importance. In contrast, reduction by chromium(II) is rapid and comparable with reactions of other blue copper proteins.

Introduction

Thiobacillus ferrooxidans[3] is a bacterium capable of growth solely on the energy available from the oxidation of iron(II) to iron(III) by O_2 at pH 2 and in doing so fixes its own CO_2 and N_2 and also produces a blue copper protein, rusticyanin, RCu^{II}, that can constitute up to 5% of the total cell protein.[4] Rusticyanin comprises a single polypeptide chain with 159 residues (M_r 16300) and a single copper ion as prosthetic group. The oxidized form of the protein shows visible[5] (ϵ_{597} 2240 M^{-1} cm^{-1}) and EPR[6] spectroscopic parameters consistent with type 1 copper and has a relatively high reduction potential of 0.68 V (vs. NHE).[7] The protein has an isoelectric point at pH 9.1[8] and is unusually stable at low pH. It has been proposed[9] that rusticyanin is the initial electron acceptor from iron(II) in the bacterial electron-transport chain. An examination of the interactions and electron-transfer mechanisms between rusticyanin and iron(II) in solution is of considerable interest.

Electron-transfer reactions of blue copper electron-transfer proteins with low molecular weight inorganic complexes have been studied extensively.[10,11] Although these reactions are not physiologically important, they reveal a great deal about the mechanisms of electron transfer available to the proteins. No previous reactions of rusticyanin have been reported, though there are studies[12,13] of the reduction of a number of blue copper proteins by the chromium(II) ion that, like iron(II), is a labile metal cation. It would seem to be important to compare the reactivity of rusticyanin, a protein where the physiological partner is an inorganic complex, with similar proteins where the physiological redox partners are other metalloproteins.

Experimental Details

T. ferrooxidans (strain T.f.3), obtained from the Microbiological Research Establishment, Porton Down, Salisbury, Wilts, U.K., was grown and harvested as described by Cobley and Haddock.[4] Rusticyanin was isolated and purified by the method of Cox and Boxer[5] and yielded a single protein band by poly(acrylamide) gel electrophoresis. The protein was stored at -70 °C in 5×10^{-2} M β-alanine buffer, pH 3.5, until use. Protein that had not been covalently modified was recycled by reoxidation (Na_2IrCl_6, Aldrich) followed by dialysis.

After removal from storage, protein solutions were diluted and dialyzed for 4-12 h at 0 °C against the appropriate medium required for kinetic studies. In the chromium(II) experiments, air-free conditions were achieved by bubbling the dialyzing solution with N_2 gas.

Reduced protein was reoxidized with small amounts of Na_2IrCl_6 followed by dialysis. Attempts to modify the protein with chromium(III) were carried out by reduction of the protein with a small excess of chromium(II) in sulfate media, pH 2 and 0.5 M ionic strength, followed immediately by reoxidation using $IrCl_6^{2-}$ and dialysis (3 h) against 1×10^{-2} M H_2SO_4 at 0.5 M ionic strength (Na_2SO_4) or against 2×10^{-2} M HCl at 0.5 M ionic strength (NaCl).

The reagents H_2SO_4, HCl, Na_2SO_4, NaCl, $CoSO_4 \cdot 7H_2O$, $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, $FeSO_4 \cdot 7H_2O$, $FeCl_2 \cdot 4H_2O$, $FeNH_4(SO_4)_2 \cdot 12H_2O$, $Fe_2(SO_4)_3$, and $FeCl_3 \cdot 6H_2O$ (Fisher ACS and Baker "Analyzed") were used without further purification. Solutions containing iron(II) were stored under N_2 and were standarized by titration with $KMnO_4$.

Chromium(II) stock solutions were made by zinc amalgam reduction of chromium(III) chloride (Baker, "Analyzed") in 0.5 M HCl, under an N_2 atmosphere. Dilute solutions were prepared by adding aliquots of the stock to flasks containing deionized and N_2-purged water and sufficient HCl/NaCl or H_2SO_4/Na_2SO_4 to give the required pH and 0.5 M ionic strength. Transfers were achieved by syringes fitted with Teflon or

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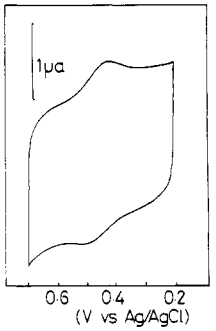

Figure 1. Cyclic voltammogram of rusticyanin (5×10^{-5} M) on a 4,4′-bipyridyl-modified carbon-paste electrode at pH 2.23, 25 °C, and 0.5 M ionic strength. Potentials are measured against Ag/AgCl (3 M NaCl).

stainless-steel needles. The chromium(II) concentrations were determined by reacting aliquots with excess standard $KMnO_4$ in 0.2 M H_2SO_4 and determining the residual MnO_4^- spectrophotometrically ($\epsilon_{545} = 2340$ M^{-1} cm^{-1}).[12]

All kinetic and electrochemical experiments were run at 0.5 M ionic strength. The pH of solutions was measured after reaction using a Beckman Selection 2000 ion analyzer with a Corning combination-glass electrode.

Reactions were monitored at 597 nm, the absorption maximum of RCu^{II}, under pseudo-first-order conditions, on a Durrum D-110 stopped-flow spectrophotometer equipped where necessary for O_2-free work and thermostated at 25.0 °C. Absorbance data were collected on a Nicolet 3091 digital oscilloscope and analyzed on an Apple II microcomputer.

Cyclic voltammetric behavior of rusticyanin was examined under N_2 at 25 °C in sulfuric acid solutions pH 1–3, ionic strength 0.5 M, with $[RCu^{II}] = 5 \times 10^{-5}$ M. A three-electrode system consisting of a carbon-paste working electrode, a platinum-wire auxiallary electrode, and a Ag/AgCl (3 M) reference electrode was used. Voltammograms were generated using a Princeton Applied Research Corp. 173 potentiostat in conjunction with a 175 universal programmer and 176 current to voltage converter and were recorded on a Houston Instruments Model 2000 X-Y recorder.

Carbon paste in Nujol and 4,4′-bipyridyl were used (3.5:1 solid volume) and hand ground as described by Cotton and co-workers.[14] The mixture was used to pack the carbon-paste electrodes (Bionalaytical Systems Inc.).

Results

(a) Cyclic Voltammetry. A 5×10^{-5} M solution of the blue copper protein rusticyanin at pH 2.23 and at 0.5 M ionic strength (Na_2SO_4) shows no cyclic voltammetric response at a carbon-paste electrode. However, when the carbon paste was mixed (3.5:1) with 4,4′-bipyridyl, well-defined oxidation and reduction peaks were detected (Figure 1) with a peak-to-peak separation of 70 mV and midpoint potential of 0.46 V (vs. Ag/AgCl, 3 M).[15] The voltammogram increased in intensity over a period of approximately 1 h, suggesting that the protein is absorbed at the carbon-paste surface. Confirmation of this comes from the observation that the cyclic voltammetry signal persists after the electrode was rinsed with deionized water and immersed in a solution at pH 2.23 and 0.5 M ionic strength (Na_2SO_4) but containing no rusticyanin.[14]

This protein absorption, while complicating the voltammetric behavior, also provides a convenient method for examining the effect of pH on the protein redox couple. The electrode, with protein absorbed, was immersed in solutions at 0.5 M ionic strength (Na_2SO_4) and different pH values between 1 and 3. Apart from a small (ca. 40 mV) increase at pH 1, no significant shift in midpoint potential was detected over this range. Peak-to-peak separations decreased at lower pH.

(b) Reduction of RCu^{II} by Iron(II) in Sulfate Media. The rate of reduction of RCu^{II} by iron(II) follows pseudo-first-order kinetics under conditions of excess [Fe(II)]. The dependence of the pseudo-first-order rate constat, k_{obsd}, on [Fe(II)] at pH 2.2 (Table I; Figure 2a) shows limiting behavior at high [Fe(II)] consistent with the eq 1, where the best fit parameters $a = 4.03 \pm 0.10$ M^{-1} s^{-1} and $b = 100 \pm 15$ M^{-1} yield the calculated curve shown in the figure.

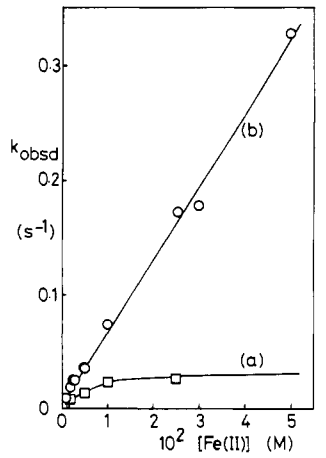

Figure 2. Plots of k_{obsd} against [Fe(II)] for the reduction of rusticyanin by iron(II) in sulfate medium, pH 2.2 (a), and in chloride medium, pH 1.6 (b), at 25 °C and 0.5 M ionic strength.

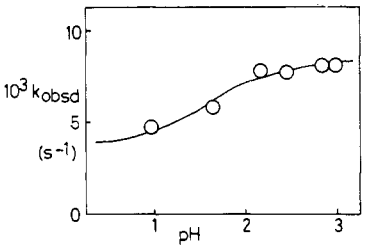

Figure 3. Dependence of k_{obsd} on pH for the reduction of rusticyanin by iron(II) (2.50×10^{-3} M) in sulfate medium at 25 °C and 0.5 M ionic strength.

$$k_{obsd} = a[Fe(II)]/(1 + b[Fe(II)]) \quad (1)$$

The reaction is slightly affected by changing the pH with a decrease around 50% below pH 2 at low [Fe(II)] where the reaction is substantially first order in [Fe(II)]. Under these conditions, the pH dependence can be described by eq 2 where

$$k_{obsd} = \frac{\{a_A[H^+] + K_a a_B\}[Fe(II)]}{\{[H^+] + K_a\}\{1 + b[Fe(II)]\}} \quad (2)$$

$a_A = 1.89 \pm 0.18$ M^{-1} s^{-1}, $a_B = 4.22 \pm 0.40$ M^{-1} s^{-1}, and $K_a = (2.3 \pm 0.3) \times 10^{-2}$ M yield the calculated curve. Addition of iron(III) to the reaction mixture has a slight (30%) inhibitory effect, but this is not large enough to cause interference with the kinetics in the absence of added iron(III) since amounts of iron(III) produced in the reaction are stoichiometric. Likewise, the presence of cobalt(II) in the reaction medium has little effect on the reaction kinetics.

(c) Reduction of RCu^{II} by Iron(II) in Chloride Media. The reduction of RCu^{II} by iron(II) in chloride media is pseudo first order under conditions of excess [Fe(II)]. The dependence of k_{obsd} on [Fe(II)] at pH 1.6 is shown in Figure 2b and is quite different from the dependence in sulfate media, with no major deviations from first-order behavior.

Least-squares analysis of Figure 2b gives a second-order rate constant in chloride media of 6.31 ± 0.15 M^{-1} s^{-1} with a negligible intercept of $(3.6 \pm 3.0) \times 10^{-3}$ s^{-1}. The reaction is almost independent of pH with a small (20%) difference between pH 0.3 and 2.4 and shows little effect of added [Fe(III)] although at higher [Fe(III)] there is incomplete reduction of RCu^{II} and the pseudo-first-order plots are not linear.

At high[Fe(II)] where differences in the behavior in chloride and sulfate media are most significant, the effect of $[SO_4^{2-}]$, added in the iron(II) solution, on the protein in chloride media was

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(15) Potential of Ag/AgCl (3 M NaCl) is 0.205 V vs. NHE.

Table I. Pseudo-First-Order Rate Constants for Reduction of RCu^{II} by Iron(II) at 25 °C and 0.5 M Ionic Strength

10^3[Fe(II)], M	10^3[Fe(III)], M	pH	$10^3 k_{obsd}$, s^{-1}
	a. Na_2SO_4 Medium		
2.50		0.97	4.67
2.50		1.65	5.79
1.00		2.17	3.65
2.00		2.18	6.94
2.50		2.16	7.84
2.51		2.17	9.76
2.51	11.0[a]	2.17	9.70
2.51	33.0[a]	2.17	8.16
2.51	55.0[a]	2.17	10.0
5.00		2.15	13.2
10.00		2.14	22.6
25.00		2.23	25.8
2.58		2.45	7.92
2.59		2.85	8.42
2.58		2.98	8.39
2.50	0.45	2.17	5.29
2.50	0.23	2.16	5.39
1.00[b]		2.43	13.9
2.00[b]		2.43	18.3
2.51[b]		2.15	41.1
5.00[b]		2.41	41.6
15.00[b]		2.43	54.8
25.00[b]		2.45	72.0
	b. NaCl Medium		
1.19		0.32	13.1
0.20		1.53	2.18
1.01		1.71	8.71
2.00		1.80	17.2
2.02		1.60	17.4
2.51		1.78	23.9
3.04		1.56	25.6
5.06		1.82	39.7
9.99		1.79	73.5
25.6		1.89	172
30.0		1.78	177
50.0		1.78	327
1.06		2.38	9.23
1.01	0.21	1.54	16.4[c]
1.01	0.62	1.53	15.6[c]
	1.30	1.61	21.2[d]
	2.60	1.52	51.6[d]
	6.50	1.42	127[d]
	c. 1:1 Na_2SO_4:NaCl Medium[e]		
2.51		2.02	15.7
25.0		1.89	100
	d. 1:3 Na_2SO_4:NaCl Medium[e]		
25.3		1.78	143

[a] Cobalt(II) concentration. [b] Chromium(III)-bound protein. [c] Estimates, reactions not first order. [d] Oxidation of RCu^{I} by iron(III). [e] Chloride ion dialyzed protein.

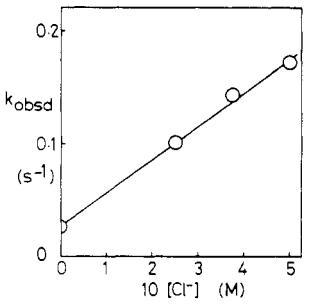

Figure 4. Plot of k_{obsd} against [Cl^-] for the reduction of rusticyanin by iron(II) (2.5×10^{-2} M) at 25 °C and 0.5 M ionic strength.

investigated. Rate enhancement is directly proportional to [Cl^-] (Figure 4).

The oxidation of RCu^{I} by iron(III) was investigated in chloride media at pH 1.5. Under pseudo-first-order conditions with an excess of [Fe(III)], a plot of k_{obsd} against [Fe(III)] is linear with a seond-order rate constant of 18.6 ± 2.3 M^{-1} s^{-1}.

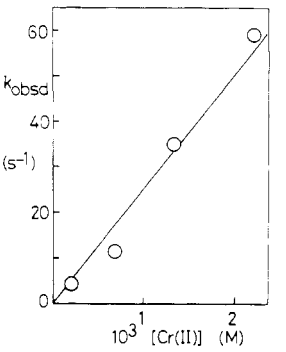

Figure 5. Plot of k_{obsd} against [Cr(II)] for the chromium(II) reduction of rusticyanin at pH 1.90, 25 °C, and 0.5 M ionic strength (Na_2SO_4).

(d) Reduction of RCu^{II} by Chromium(II) in Sulfate Media. The reduction of RCu^{II} by chromium(II) was examined under pseudo-first-order conditions with an excess of [Cr(II)]. Good first-order traces were obtained, and a plot of the observed first-order rate constants, k_{obsd}, against [Cr(II)] is shown in Figure 5. The reaction is first order in [Cr(II)] with a second-order rate constant of (2.5 ± 0.5) × 10^4 M^{-1} s^{-1} at pH 1.9.

Discussion

(a) Cyclic Voltammetry. The reduction potential of RCu^{II} adsorbed on a carbon-paste electrode is 0.67 V[15] (vs. NHE) in excellent agreement with the value obtained potentiometrically in titration with Fe(II)/Fe(III)[7] at pH 3.2. The potential is substantially independent of pH in the region 1–3.

In comparison of the reduction potential of rusticyanin to those of other small blue copper proteins, [10,16] the value is seen to be relatively high. However, the reduction potentials of plastocyanins are known to increase markedly below pH 5, an increase that is ascribed to a combination of protein conformational effects and ligand histidine protonation.[10] It is likely that the copper center in rusticyanin has similar coordination to that in the other blue proteins.[17,18] Amino acid analysis[5] shows the presence of five histidines, one cysteine, and three methionines.

(b) Kinetics of Reduction by Iron(II). The kinetics of reduction of RCu^{II} by iron(II) differ in chloride and sulfate media. This is not entirely surprising since both the protein and hexaaquairon(II) ion, Fe_{aq}^{2+}, are positively charged at pH values less than 4 and anion interactions are expected. Complexation of Cl^-[19] and SO_4^{2-}[20] with the substitution-labile Fe_{aq}^{2+} is well documented (eq 3 and 4). No reliable values for the stability constants at 0.5

$$Fe_{aq}^{2+} + Cl^- \rightleftharpoons FeCl(aq)^+ \qquad K_3 \qquad (3)$$

$$Fe_{aq}^{2+} + SO_4^{2-} \rightleftharpoons FeSO_4(aq) \qquad K_4 \qquad (4)$$

M ionic strength have been reported but reasonable estimates based on data[21] for similarly charged and sized cations are 0.5 M^{-1} for K_3 and 5 M^{-1} for K_4. Thus, under the conditions of the study, around 20% of the iron(II) in chloride ion solutions is present as $FeCl(aq)^+$ while around 45% of the iron(II) in sulfate ion solutions is present as $FeSO_4(aq)$.

Information on the binding of anions to RCu^{II} is not available. However, ^1H NMR spectra of both oxidized and reduced forms of rusticyanin dialyzed in DCl and D_2SO_4 solutions at pH 1.6 give

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information on the conformation of the protein in the different media. The spectra resemble those of other small blue copper proteins and are consistent with a compact globular structure. Although the spectra of RCu^{II} and RCu^{I} in chloride and sulfate media are broadly similar, they differ in detail showing changes in the relative intensities and positions of a number of peaks, particularly in the aliphatic region. This is considered to indicate that some conformational differences exist in the presence of different anions. The spectra also reveal conformational differences between oxidized and reduced forms of the protein.[22]

Differences in the kinetic parameters in the presence of different anions are of considerable interest since it is known[23,24] that *T. ferrooxidans* has a specific sulfate requirement for iron(II) oxidation. Lazaroff has suggested that $FeSO_4(aq)$ is the natural substrate for *T. ferrooxidans*.[23-25] This suggestion is based on the specific sulfate requirement for bacterial growth and the observation[25] that D_2O is excluded from the inner coordination sphere of iron(III) produced in the bacterial oxidation of $FeSO_4 \cdot 7H_2O$ in D_2O, an observation that is difficult to reconcile with the picture of Fe_{aq}^{2+} in sulfate media established by ultrasonic measurements.[26] Sulfate does have a thermodynamic effect on the reaction, stabilizing iron(III) and favoring reduction of the protein by iron(II), whereas this reduction in chloride media is slightly thermodynamically unfavorable.

In chloride media, reduction of RCu^{II} is apparently simpler than in sulfate media. The reduction is first order in both $[RCu^{II}]$ and [Fe(II)] with a second-order rate constant of 6.3 M^{-1} s^{-1}, largely independent of pH. There are small deviations from this behavior at very low [Fe(II)], resulting in an intercept in the plot of k_{obsd} against [Fe(II)] but this is almost within the experimental error and has not been interpreted. Deviations from simple kinetic behavior are expected at low[Fe(II)] where the driving force for the reaction is small. In chloride media, under comparable conditions, oxidation of RCu^{I} by iron(III) has a second-order rate constant of 18 ± 2 M^{-1} s^{-1} indicating that this reaction is thermodynamically favored.

Addition of sulfate ions to the reaction results in rate retardation, particularly at high iron(II) concentrations where the reaction order in [Fe(II)] also changes from first to zero order. This limiting kinetic behavior suggests that a more complex mechanism is operating in sulfate media.

A possible explanation for the limiting kinetic behavior, and the one favored in reactions of other blue copper electron-transfer proteins with inorganic oxidants and reductants,[27-29] involves rapid preequilibrium association between RCu^{II} and iron(II) (eq 5), followed by rate-limiting electron transfer (eq 6). The rate law (eq 7) is of the same form as eq 1 with $K_5 = 100 \pm 15$ M^{-1} and $k_6 = (4.0 \pm 0.5) \times 10^{-2}$ s^{-1}, at pH 2.2.

$$RCu^{II} + Fe(II) \rightleftharpoons [RCu^{II}, Fe(II)] \qquad K_5 \qquad (5)$$

$$[RCu^{II}, Fe(II)] \rightarrow RCu^{I} + Fe(III) \qquad k_6 \qquad (6)$$

$$k_{obsd} = \frac{K_5 k_6 [Fe(II)]}{1 + K_5[Fe(II)]} \qquad (7)$$

Despite this apparently strong binding interaction in sulfate media, the second-order rate constant at low [Fe(II)] derives no enhancement over the rate in chloride media. Binding between the protein and Fe(II) (eq 5) need not result in a complex that provides a favorable configuration for electron transfer. Such a "dead-end complex" mechanism involves an alternative pathway (eq 8) for electron transfer with $k_8 = 4.03 \pm 0.10$ M^{-1} s^{-1} at pH 2.2.

$$RCu^{II} + Fe(II) \rightarrow RCu^{I} + Fe(III) \qquad k_8 \qquad (8)$$

Although the observation that added iron(III) partially inhibits the reduction of RCu^{II} by iron(II) tends to support a mechanism in which protein–complex association takes place, addition of Co_{aq}^{2+}, a redox-inactive ion of similar size and charge compared with Fe_{aq}^{2+}, has no effect on the reaction kinetics over a concentration range where substantial competitive inhibition might be expected if binding of the divalent metal cation to the protein is kinetically important. Unless binding of iron(II) in the presence of sulfate is a very specific process, this result would tend to rule out a mechanism where the limiting kinetic behavior is the result of binding between iron(II) and the protein.

An alternative mechanism involving rate-determining protein activation (eq 9), followed by electron transfer (eq 10), gives rise

$$RCu^{II} \rightleftharpoons RCu^{II*} \qquad k_9, k_{-9} \qquad (9)$$

$$RCu^{II*} + Fe(II) \rightarrow RCu^{I} + Fe(III) \qquad k_{10} \qquad (10)$$

to the law (eq 11), which is identical with eq 1 with $k_9 = (4.0 \pm 0.5) \times 10^{-2}$ s^{-1} and $k_{-9}/k_{10} = (1.00 \pm 0.15) \times 10^{-2}$ M at pH 2.2. At first sight this mechanism would also seem unlikely since reduction of RCu^{II} should be limited by this rate, no matter the reductant. This is not the case since rate constants for reduction by chromium(II) in sulfate solutions and by iron(II) in chloride solutions exceed this value. However, reductions by the powerful reductant chromium(II) need not be subject to the same mechanistic restrictions as iron(II), and NMR experiments reveal that the protein conformation is anion dependent, suggesting that protein activation by a conformational change might differ in sulfate and chloride media. At present, this mechanism best fits the facts.

$$k_{obsd} = \frac{k_9 k_{10} [Fe(II)]}{k_{-9} + k_{10}[Fe(II)]} \qquad (11)$$

The reaction at low [Fe(II)] in sulfate solutions shows an increase in rate from 1.89 to 4.22 M^{-1} s^{-1} with increasing pH, suggesting the involvement of a species with pK_a around 1.64. This value is close to the pK_a of HSO_4^-, 1.32[30], under the conditions and the study, and this small pH dependence again suggests the importance of anions in the reaction. Further evidence that anions have a significant effect on the interaction of metal cations with rusticyanin comes from electron paramagnetic resonance experiments. With the paramagnetic exogenous Dy(III) ion as a probe, power-saturation profiles of the protein copper(II) signal differ in chloride and sulfate media, consistent with a closer interaction between the metal centers in the latter case.[31]

Rusticyanin is located in the periplasmic space near the inner membrane of *T. ferrooxidans*. It has been proposed[9] that the protein is the primary electron acceptor from iron(II) in the respiratory chain. It is of interest to view this proposal in light of the present study under nonphysiological conditions.

An important point is that the reaction between rusticyanin and iron(II) is slow, much slower than reactions of other blue copper proteins with their physiological reaction partners.[32-34] This does not necessarily rule out rusticyanin as the primary electron acceptor since growth of the bacterium is slow and rusticyanin is found in high concentrations in the periplasmic space. The iron(II) oxidase rates in whole cells of *T. ferrooxidans*[35] indicate a required turnover of rusticyanin of 1–5 s^{-1}, much higher than the limiting rate of 4.0×10^{-2} s^{-1} found in sulfate media and corresponding to an iron(II) concentration around 0.5 M in the absence of sulfate. However, the actual periplasmic conditions cannot be mimicked and protein conformation may be important.

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The most plausible electron acceptor from rusticyanin is a cytochrome c_{552} with a reduction potential of 0.64 V[7]. There is a report[36] that this cytochrome can be isolated, bound to rusticyanin, and that the copper protein is preferentially reduced by iron(II). Using data published by Imai and co-workers at pH 3.5[36] and assuming the reaction to be first order in iron(II) and cytochrome c_{552}(III)–rusticyanin(II) complex concentrations, a second-order rate constant around 16 M^{-1} s^{-1} for reduction of the rusticyanin component can be calculated. This is not significantly different from the value found in the present study and suggests that the presence of bound cytochrome c_{552}(III) has little effect on the reactivity of rusticyanin.

(c) Kinetics of Reduction by Chromium(II). Reduction of RCu^{II} by chromium(II) in sulfate media is much faster than the corresponding reaction with iron(II) and exhibits no limiting kinetic behavior. The second-order rate constant, $(2.5 \pm 0.5) \times 10^4$ M^{-1} s^{-1}, shows little sensitivity to small amounts ($<10^{-3}$ M) of chloride ion, and it may be that the electron transfer from this powerful reductant is less specific than with iron(II).

There are data available[12,13] for the reductions of other small blue copper proteins, azurins, plastocyanins, and stellacyanin by chromium(II) at pH 4.2. As with rusticyanin the reactions are first order in [Cr(II)] and the second-order rate constants are comparable in magnitude, varying between 10^4 and 10^5 M^{-1} s^{-1}. There does not appear to be any close correlation with reduction potentials.

In chromium(II) reductions of a number of electron-transfer proteins including the blue copper plastocyanins[37] and azurins,[38] some of the chromium(III) product has been shown to remain bound to the protein, labeling and blocking key reaction sites.[33] It is of some interest then to discover whether RCu^{II}, reduced in sulfate media by chromium(II) and reoxidized by $IrCl_6^{2-}$, can be reduced by iron(II) or whether chromium(III) is bound to the protein at the iron(II) reaction site.

Reaction of iron(II) with the chromium(II)-treated protein in sulfate media leads to no significant decrease in rate compared with the untreated protein. Indeed, the rates are slightly enhanced, and the rate law (eq 1) is followed with $a = 12 \pm 1$ M^{-1} s^{-1} and $b = 100 \pm 20$ M^{-1}. These experiments provide further evidence for a rate-limiting protein conformational change in the reduction of RCu^{II} by iron(II). It would appear that the conformational change is marginally facilitated by the binding of chromium(III).

(d) Conclusions. The blue copper protein, rusticyanin, has a reduction potential of 0.67 V (vs. NHE) independent of pH in the range 1–3. The reduction of the oxidized form of the protein by iron(II) is limited by a conformational change in sulfate media, but this limiting rate is absent in chloride media. Reduction of the protein by chromium(II) takes place at a rate comparable to the reductions of other small blue copper proteins by this reagent. Binding of chromium(III) to the protein has little effect on the rate of the conformational change.

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Contribution from the Department of Chemistry,
University of Wisconsin, Eau Claire, Wisconsin 54701

Kinetic Study of the Oxidation of Spinach Plastocyanin by Ferrocenium Ion Derivatives

J. R. PLADZIEWICZ,* M. S. BRENNER, D. A. RODEBERG, and M. D. LIKAR

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The oxidation of reduced spinach plastocyanin by four ferrocenium ion derivatives has been studied at pH 7.0 (0.020 M phos) and $\mu = 0.12$ (NaCl, sodium phosphate) at 25 °C. All four reactions are observed to obey a simple second-order rate law that is first order with respect to the concentration of protein and ferrocenium derivative. No rate saturation at high ferrocenium ion concentrations is observed for any of the reactions studied. The second-order rate constants for the four protein oxidations at 25 °C are 0.20 (±0.02) × 10^6, 1.02 (±0.04) × 10^6, 2.10 (±0.12) × 10^6, and 9.4 (±1.0) × 10^6 M^{-1} s^{-1} for 1,1′-dimethylferrocenium, ferrocenium, chloromercuriferrocenium, and phenylferrocenium, respectively. The protein oxidations have been studied as a function of temperature, and the enthalpies and entropies of activation are 6.7 ± 0.4, 5.5 ± 0.3, 5.0 ± 0.4, and 6.2 ± 0.5 kcal/mol and −11.7 ± 1.2, −12.6 ± 0.8, −13.1 ± 1.5, and −5.9 ± 1.7 cal/(mol K), respectively, for 1,1′-dimethylferrocenium, ferrocenium, chloromercuriferrocenium, and phenylferrocenium. Possible mechanisms for electron transfer are discussed, and the observed second-order rate constants are used to derive apparent protein-exchange rate constants by the Marcus equation.

We are interested in the mechanisms by which metalloproteins undergo electron transfer. An important aspect of this is identifying mechanistic features that diverse metalloproteins share in their electron-transfer reactions. Gray and co-workers[1-5] have shown that small octahedral coordination compounds with hydrophobic liands and ligands that allow delocalization of electron density in the complex through π bonding are more facile at electron transfer with metalloproteins than are small compounds without these features. We reasoned from these observations and related facts that ferrocene and its derivatives might be facile one-electron titrants of metalloproteins. Previous work[6,7] from our laboratory has shown this to be true for electron-transfer reactions of horse heart cytochrome *c*. This report describes our studies on the electron-transfer reactions of plastocyanin from spinach chloroplasts with four ferrocenium ion derivatives. Plastocyanin is a protein of molecular weight 10 500 containing one copper atom per molecule. It cycles between the copper I/II oxidation states and serves an electron-transport function in the photosynthetic pathways in a variety of algae and plants. Its electron-transfer reactions, both with other proteins and with inorganic reagents, have been extensively studied.[1,8] The X-ray crystal structure of plastocyanin from *Populus nigra* is known,[9]

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