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# Kinetic Studies on 1:1 Electron-Transfer Reactions of Blue Copper Proteins. 13. Reactions of Rusticyanin from *Thiobacillus ferrooxidans* with Inorganic Redox Partners

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Kinetic information has been obtained (pH 2-6, $I$ = 0.10 M (NaCl)) for the redox reactions of six inorganic complexes with the single (type 1) blue Cu protein rusticyanin from *Thiobacillus ferrooxidans*. The protein is unusual in that it is normally functional at pH ~2, at which pH it has a reduction potential of 0.68 V. From the dependence of rate constants on pH in three cases protein acid dissociation p$K_a$ values have been determined. These are as follows: with $[Mn(Cydta)(H_2O)]^-$ as oxidant for $RCu^I$, 4.17 at 25 °C; with $[Ru(NH_3)_4(phen)]^{2+}$ as reductant for $RCu^{II}$, 4.06 at 15 °C; with $[Co(dipic)_2]^-$ as oxidant for $RCu^I$, 4.10 at 25 °C. The reduction potential of rusticyanin decreases to 0.62 V at pH 5.3. The behavior observed suggests that protonation is at neither the active site nor binding site(s) on the protein. No other p$K_a$'s have been detected in studies up to pH 6. When the ionic strength is adjusted with $Na_2SO_4$, rate constants (pH 3-6) for the $[Ru(NH_3)_4(phen)]^{2+}$ reduction are ~50% of those obtained with NaCl.

## Introduction

The blue Cu protein rusticyanin is a component of the respiratory chain of the bacterium *Thiobacillus ferrooxidans*.[1-4] This bacterium is capable of growth solely on the energy available from the oxidation of aqua Fe(II) to Fe(III) by $O_2$. It is found in acid mine leachings and is used commercially in the extraction of copper and uranium from their ores.[4]

Rusticyanin is located in the periplasmic space between the cell wall and the inner cell membrane. It is possible that the electron is transferred directly from the Fe(II) to rusticyanin[5] or with mediation across the cell wall by Fe(III) chelated to phospholipid "head" groups.[6] In the latter case rusticyanin picks up an electron from the inside of the cell wall. The electron is then transferred to a *c*-type cytochrome, also in the periplasm, which passes it on to a cytochrome $a/a_1$ complex in the membrane. A pH gradient is maintained across the membrane, the cytoplasm being at pH 6.5 while the periplasm and surrounding medium are at pH 2.

Rusticyanin consists of a single polypeptide chain of 159 amino acid residues (mol wt ~16 500) with a single Cu at the active site. In the oxidized form, $RCu^{II}$, it shows spectroscopic properties typical of blue (type 1) Cu proteins, with a strong absorbance at ~600 nm in the visible spectrum,[2] and a very small hyperfine coupling in the parallel region of the ESR spectrum.[7] It has a relatively high reduction potential of 0.68 V (vs. NHE),[3] has an isoelectric point of 9.1, and is unusual in being stable at pH 2 (the pH for optimum growth of *T. ferrooxidans*), whereas plastocyanin for example is denatured at pH <4.5. Properties of rusticyanin are compared with those of other representative blue copper proteins in Table I.

The active site in plastocyanin[9] and azurin[10] is known to have a distorted tetrahedral geometry about the copper. The ligating groups are two $N^\delta$ nitrogens from histidine residues, a thiolate sulfur from a cysteine, and a thioether sulfur from a methionine. The last has an exceptionally long bond (2.9 Å). In stellacyanin there is no methionine and in umecyanin no correctly positioned methionine, and it has been suggested that the fourth ligand may be a disulfide.[11,12] Recent sequence information[13] on rusticyanin has shown that a cysteine, one histidine, and a methionine are similarly positioned relative to each other to be ligands to the Cu (Cys127, His132, Met137) as in plastocyanin (Cys84, His87, Met92) and azurin (Cys112, His117, Met121). As far as an additional ligand or ligands are concerned, it is noted that there are four other histidines and two other methionines at earlier stages in the sequence. This composition is unusual. Differences in coordination of the Cu may explain the high reduction potential of rusticyanin. The absorption coefficient at ~600 nm (2240 $M^{-1}$ $cm^{-1}$) is about half that observed for plastocyanin and azurin.

Electron-transfer reactions of blue Cu proteins with small inorganic complexes have been extensively studied.[14-21] The effect of pH on rate constants has given information concerning active-site chemistry and binding sites on the protein surface. The reactions of rusticyanin with the labile metal ions $[Fe(H_2O)_6]^{2+}$ and $[Cr(H_2O)_6]^{2+}$ (hereafter $Fe^{2+}$ and $Cr^{2+}$) have been investigated.[22] Here we present results of studies with other ligated

**Table I.** Comparison of Properties of Single Blue (Type 1) Copper Proteins[a]

| protein | mol wt | λ (ε), nm ($M^{-1}$ $cm^{-1}$) | $E°$ (vs. NHE),[b] V | isoelectric point |
|---|---|---|---|---|
| plastocyanin | 10 500 | 597 (4500) | 0.370 | 4.2 |
| azurin | 14 000 | 625 (4800) | 0.330 | 5.4 |
| stellacyanin | 20 000[c] | 609 (4080) | 0.184 | 9.9 |
| umecyanin | 14 600 | 610 (3400) | 0.283 | 5.8 |
| rusticyanin | 16 500 | 597 (2240) | 0.680[d] | 9.1 |

[a] See, e.g., ref 8. [b] pH ~7 except as stated. [c] 40% carbohydrate. [d] pH 2.

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complexes that function as outer-sphere electron-transfer agents.

## Experimental Section

**Protein.** Rusticyanin was purified by the method of Cox and Boxer[2] and yielded a single band on polyacrylamide gel electrophoresis. Preparations frequently showed evidence for the presence of a small amount of cytochrome impurity, which showed up as a shoulder at 410 nm on the absorption band at 450 nm. A further CM-52 column generally sufficed to remove this. The latter column (3.0 × 0.5 cm for small amounts of protein) was equilibrated in 10 mM acetate at pH 5.4. Elution was initially with 0.10 M NaCl in the same buffer until the rusticyanin band reached the bottom of the column. The eluant was then changed to 0.20 M NaCl in buffer, and fractions in which no cytochrome was detected ($A_{450}/A_{410} > 1.5$, estimated <<0.5% cytochrome) were pooled and used for kinetics. The peak ratio $A_{287}/A_{597} = 2.3$ quoted by Cox and Boxer[2] was never attained in any of our studies. The UV maximum was found to be at 280 nm, and the ratio $A_{280}/A_{597}$ varied between 4.5 and 10, though it was generally 6.5–7. Wide variations in this ratio had no apparent effect on the kinetics.

The protein was stored frozen in 50 mM β-alanine at pH 3.5. On removal from storage it was frequently found to be partially reduced. Samples were oxidized by addition of $Na_2[IrCl_6]$ (Johnson Matthey Chemicals), or reduced with sodium ascorbate and then dialyzed at 4 °C against the appropriate buffer (two 100-fold volumes each for >3 h), before use in kinetics. It was found that oxidized protein tended to reduce partially during dialysis. For runs where $RCu^{II}$ was used, the dialyzed solution was about twice the desired concentration and the dialysis time as short as practicable; the $RCu^{II}$ concentration was checked and the solution diluted as required before use.

Protein was recovered from reacted solutions by extensive dialysis to remove the excess inorganic complex, followed by concentration on a small CM-52 column as above.

**Complexes.** These were prepared by literature methods and characterized from previously reported peak positions λ/nm ($\epsilon/M^{-1}$ cm$^{-1}$) as follows: potassium (1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetato)-manganate(III), K[Mn(Cydta)($H_2O$)], 510 (345) at pH 5–7[23] (the p$K_a$ of 8.11 indicates the presence of a coordinated $H_2O$); (1,10-phenanthroline)tetraammineruthenium(II) trifluoromethanesulfonate, $[Ru(NH_3)_4(phen)](CF_3SO_3)_2 \cdot H_2O$, 471 (6680);[24] ammonium bis(pyridine-2,6-dicarboxylato)cobaltate(III), $NH_4[Co(dipic)_2]$, 510 (680);[25] potassium (2,2′-bipyridine)tetracyanoferrate(II), $K_2[Fe(CN)_4(bpy)] \cdot 3H_2O$, 482 (2880);[26] the (1,10-phenanthroline)tetracyanoferrate complexes $K_2[Fe(CN)_4(phen)] \cdot 4H_2O$, 462 (4420), and $H[Fe(CN)_4(phen)] \cdot 2H_2O$, 506 (298);[26] and potassium octacyanomolybdate(IV), $K_4[Mo(CN)_8] \cdot 2H_2O$, 367 (170).[27] Solutions of [Mn(Cydta)($H_2O$)]$^-$ were used within 45 min of preparation; decomposition occurred at pH <1.5. Solutions containing $[Mo(CN)_8]^{3-}$ were obtained by electrolytic oxidation at a Pt electrode. Samples of potassium hexacyanoferrate(III), $K_3[Fe(CN)_6]$, 420 (1010), and potassium hexacyanoferrate(II), $K_4[Fe(CN)_6] \cdot 3H_2O$, 320 (320) (both BDH, Analar), were used without further purification. All other reagents were either Analar or the best available and were used without further purification. Relevant reduction potentials are indicated below in Table V.

**Buffers.** Buffers were made up to concentrations of 10 or 20 mM, and the ionic strength was adjusted to 0.10 M with NaCl or $Na_2SO_4$ as required. Solutions used: HCl/NaCl unbuffered (pH 1–2); chloroacetic acid/NaOH (pH 2–3.5); potassium hydrogen phthalate/HCl (pH 3.84); sodium acetate/HCl or $H_2SO_4$ (pH 3.8–5.5); Mes/NaOH (pH >5.5).

**Kinetics.** Kinetic runs were carried out on a Dionex D-110 stopped-flow instrument interfaced to a Commodore PET microcomputer, except in the case of the $[Co(dipic)_2]^-$ oxidation of $RCu^I$, where a conventional spectrometer (Perkin-Elmer 554) was used. Reactions were monitored at 597 nm. Runs were carried out with complex in >10-fold excess. First-order plots of ln ($A_\infty - A_t$) against time were linear to 3–4 half-lives, and rate constants $k_{obsd}$ were obtained from the slopes. Linear dependences of $k_{obsd}$ on concentration of complex were observed: (0.3–5.2) × $10^{-3}$ M in the case of [Mn(Cydta)($H_2O$)]$^-$, (0.9–3.0) × $10^{-4}$ M in the case of $[Ru(NH_3)_4(phen)]^{2+}$ (restricted by the rapidity of this reaction), and (0.4–1.9) × $10^{-3}$ M for $[Co(dipic)_2]^-$ (restricted by the need to confine the reaction to the conventional spectrophotometry time range). Second-order rate constants $k$ were obtained directly from $k_{obsd}$. The pH of mixed solutions was measured with a Radiometer PHM-62 pH meter fitted with a Russell combination electrode.

**Table II.** Pseudo-First-Order Rate Constants $k_{obsd}$(25 °C) for the [Mn(Cydta)($H_2O$)]$^-$ Oxidation of *T. ferrooxidans* Rusticyanin $RCu^I$ ((0.8–3.0) × $10^{-5}$ M, $I$ = 0.10 M (NaCl))

| pH | $10^3$[Mn(Cydta)-($H_2O$)]$^-$, M | $k_{obsd}$, $s^{-1}$ | pH | $10^3$[Mn(Cydta)-($H_2O$)]$^-$, M | $k_{obsd}$, $s^{-1}$ |
|---|---|---|---|---|---|
| 1.24 | 0.58 | 0.183 | 3.84 | 0.53 | 0.130 |
| | 0.94 | 0.277 | | 1.07 | 0.271 |
| 1.50 | 1.00 | 0.189 | | 1.37 | 0.34 |
| | 1.06 | 0.217 | | 3.14 | 0.78 |
| 2.00 | 0.68 | 0.120 | | 5.19 | 1.26 |
| | 1.02 | 0.157 | 4.20 | 0.37 | 0.123 |
| | 2.06 | 0.294 | | 0.74 | 0.24 |
| | 4.18 | 0.66 | 4.30 | 0.52 | 0.190 |
| 2.58 | 0.91 | 0.114 | | 0.81 | 0.286 |
| | 1.17 | 0.137 | 4.45 | 0.63 | 0.257 |
| 2.99 | 0.89 | 0.118 | 4.79 | 0.30 | 0.151 |
| | 1.17 | 0.155 | | 0.71 | 0.34 |
| 3.50 | 0.85 | 0.146 | 5.20 | 0.85 | 0.42 |
| | 1.30 | 0.239 | 5.23 | 0.86 | 0.44 |
| | | | 5.65[a] | 0.35 | 0.184 |
| | | | | 0.60 | 0.32 |

[a] Acetate buffer.

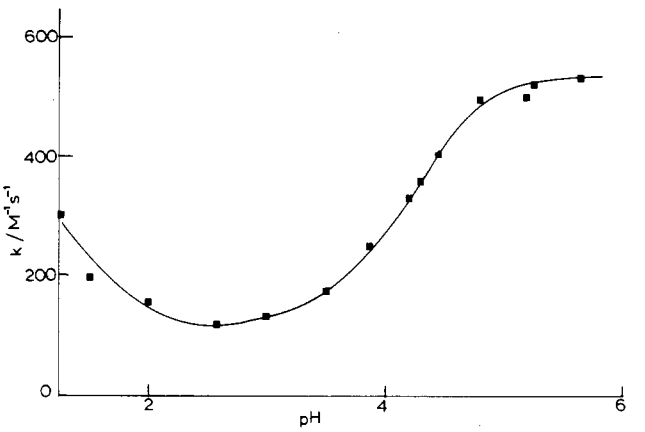


**Figure 1.** Variation of second-order rate constants, $k$(25 °C), with pH for the [Mn(Cydta)($H_2O$)]$^-$ oxidation of *T. ferrooxidans* rusticyanin, $RCu^I$, at $I$ = 0.10 M (NaCl).

**Reduction Potential of the Protein.** A solution of 4.5 × $10^{-5}$ M $RCu^I$ (1.1 mL) was dialyzed at pH 5.4, $I$ = 0.10 M (NaCl), and then titrated by addition of 0.01–0.02-mL aliquots of H[Fe(CN)$_4$(phen)] from a microsyringe. From the absorbance increase at 597 and 462 nm an $E°$ value of 620 ± 10 mV was determined. A similar experiment at pH 2.95 did not yield satisfactory data, though a significantly higher $E°$ was indicated, as expected.

## Results

**Oxidation of $RCu^I$ with [Mn(Cydta)($H_2O$)]$^-$.** Rate constants (Table II) give a pH profile as shown in Figure 1. The behavior observed corresponds to the reaction sequence

$$PH^+ \rightleftharpoons P + H^+ \quad (K_a) \tag{1}$$

$$PH^+ + C \xrightarrow{k_H} \text{products} \tag{2}$$

$$P + C \xrightarrow{k_o} \text{products} \tag{3}$$

which gives the expression

$$k = \frac{k_o K_a + K_H[H^+]}{K_a + [H^+]} \tag{4}$$

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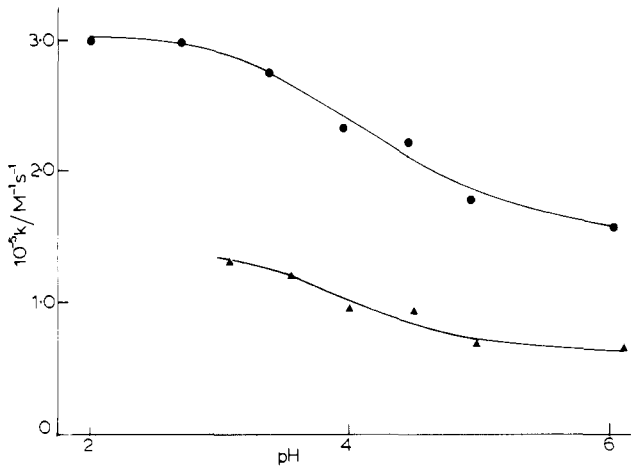


**Figure 2.** Variation of second-order rate constants, $k$(25 °C), with pH for the $[Ru(NH_3)_4(phen)]^{2+}$ reduction of *T. ferrooxidans* rusticyanin, $RCu^{II}$, at $I$ = 0.10 M (NaCl) (●) and $I$ = 0.10 M ($Na_2SO_4$) (▲).

**Table III.** Pseudo-First-Order Rate Constants $k_{obsd}$(15 °C) for the $[Ru(NH_3)_4(phen)]^{2+}$ Reduction of *T. ferrooxidans* Rusticyanin $RCu^{II}$ ((0.9–2.4) × $10^{-5}$ M)

| pH | $10^4[Ru(NH_3)_4(phen)^{2+}]$, M | $k_{obsd}$, $s^{-1}$ | pH | $10^4[Ru(NH_3)_4(phen)^{2+}]$, M | $k_{obsd}$, $s^{-1}$ |
|---|---|---|---|---|---|
| | | $I$ = 0.10 M (NaCl) | | | |
| 2.02 | 2.43 | 69 | 4.40 | 1.38 | 31 |
| 2.06 | 2.96 | 94 | 4.92 | 0.96 | 16.3 |
| 2.70 | 0.93 | 27.4 | | 1.40 | 26.3 |
| 3.36 | 0.99 | 27.0 | 6.02 | 1.88 | 30.5 |
| 3.38 | 1.46 | 40 | | 2.10 | 32.1 |
| 3.95 | 1.91 | 45 | | | |
| | 2.11 | 49 | | | |
| | | $I$ = 0.10 M ($Na_2SO_4$) | | | |
| 3.08 | 0.91 | 11.8 | 4.48 | 0.97 | 9.0 |
| | 1.24 | 16.0 | 5.47 | 1.02 | 7.0 |
| 3.55 | 1.37 | 16.1 | 6.10 | 1.59 | 10.7 |
| 3.57 | 0.92 | 11.3 | | 2.64 | 16.9 |
| 4.00 | 1.20 | 11.3 | | | |

From the fit of data at pH >2, $k_H$ = 104 ± 7 $M^{-1}$ $s^{-1}$, $k_o$ = 554 ± 7 $M^{-1}$ $s^{-1}$, and p$K_a$ = 4.17 ± 0.03.

At pH <2 rate constants exhibit a further variation. These data are subject to some uncertainty owing to rapid decomposition of $[Mn(Cydta)(H_2O)]^-$ at pH <1.5, and this effect has not been further investigated.

At pH 2.0 and 3.8, the range of Mn(III) concentrations was extended up to 4–5 mM, with retention of a first-order dependence, and no evidence for saturation kinetic behavior.

**Reduction of $RCu^{II}$ with $[Ru(NH_3)_4(phen)]^{2+}$.** This reaction was much faster than that of $[Mn(Cydta)(H_2O)]^-$ and had to be studied at a lower temperature (15.0 °C) to obtain satisfactory data. The pH profile of rate constants in Figure 2 was obtained. The best fit parameters to (4) obtained from the data in Table III are $k_H$ = (3.0 ± 0.07) × $10^5$ $M^{-1}$ $s^{-1}$, $k_o$ = (1.6 ± 0.07) × $10^5$ $M^{-1}$ $s^{-1}$, and p$K_a$ = 4.06 ± 0.12.

The effect of adjusting the ionic strength with sulfate instead of chloride was also investigated (Figure 2). At the same ionic strength, a similar pH profile was found (p$K_a$ = 4.02 ± 0.14), with rate constants ~45% of those in chloride.

**Oxidation of $RCu^I$ with $[Co(dipic)_2]^-$.** Rate constants (Table IV) give a pH profile similar to that found for $[Mn(Cydta)(H_2O)]^-$ (Figure 3). Best fit parameters are $k_H$ = 2.50 ± 0.22 $M^{-1}$ $s^{-1}$, $k_o$ = 11.4 ± 0.21 $M^{-1}$ $s^{-1}$, and p$K_a$ = 4.10 ± 0.05.

**Other Reactions.** The reaction of $[Fe(CN)_4(phen)]^{2-}$ with $RCu^{II}$ is rapid, and with 1:1 amounts at ~2.5 × $10^{-5}$ M the rate constant is >$10^7$ $M^{-1}$ $s^{-1}$ at 25 °C and pH 4.5. With $[Fe(CN)_6]^{4-}$ as reductant for $RCu^{II}$ and $[Mo(CN)_8]^{3-}$ as oxidant for $RCu^I$, absorbance changes were also too fast to be followed, and it is concluded that $k$ > $10^6$ $M^{-1}$ $s^{-1}$ at 25 °C and pH 4.5. The complex

**Table IV.** Pseudo-First-Order Rate Constants $k_{obsd}$(25 °C) for the $[Co(dipic)_2]^-$ Oxidation of *T. ferrooxidans* Rusticyanin $RCu^I$ (~4 × $10^{-5}$ M, $I$ = 0.10 M (NaCl))

| pH | $10^3[Co(dipic)_2]^-$, M | $10^3k_{obsd}$, $s^{-1}$ | pH | $10^3[Co(dipic)_2]^-$, M | $10^3k_{obsd}$, $s^{-1}$ |
|---|---|---|---|---|---|
| 1.88 | 0.40 | 0.97 | 4.30 | 0.32 | 2.28 |
| | 1.93 | 4.5 | | 0.58 | 4.5 |
| 1.92 | 0.88 | 2.16 | 4.41 | 0.29 | 2.6 |
| 2.91 | 0.44 | 1.43 | | 0.52 | 4.7 |
| | 0.90 | 2.86 | 4.89 | 0.35 | 3.6 |
| 3.32 | 0.37 | 1.41 | | 0.68 | 7.0 |
| | 0.69 | 2.66 | 5.56 | 0.33 | 3.8 |
| 3.95 | 0.46 | 2.89 | | 0.72 | 7.6 |
| | 0.84 | 5.1 | 6.00 | 0.50 | 5.7 |
| | | | | 0.52 | 5.7 |

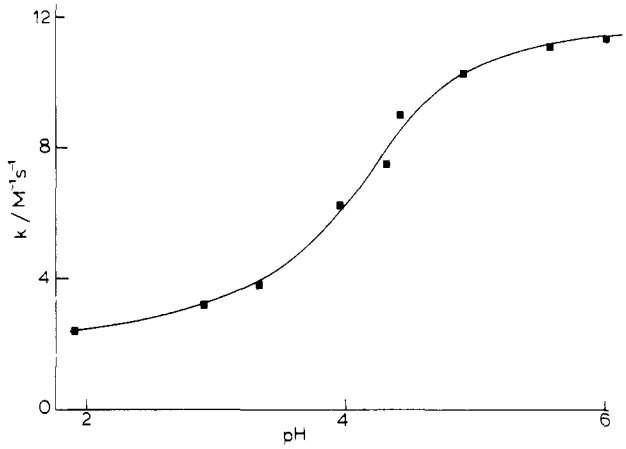


**Figure 3.** Variation of second-order rate constants, $k$(25 °C), with pH for the $[Co(dipic)_2]^-$ oxidation of *T. ferrooxidans* rusticyanin, $RCu^I$, at $I$ = 0.10 M (NaCl).

$[Fe(CN)_4(bpy)]^{2-}$ (0.54 V) interacted with both $RCu^{II}$ and $RCu^I$, giving an increase in absorbance and a flocculent orange precipitate on standing for ~1 h. This effect was not investigated further, though we note that rusticyanin could be recovered (~45%) by dialysis, oxidation, and chromatography in the usual manner. Precipitation was observed also with the phenanthroline complex, but only after standing overnight.

## Discussion

The high reduction potential for rusticyanin as compared to those for other blue (type 1) Cu proteins in Table I suggests that coordination at the Cu active site is different. An attractive possibility is that the $RCu^I$ form is 3-coordinate at pH ~2. It is known that the reduction potential for plastocyanin increases from 370 mV at pH 7.5 to >430 mV as the pH is decreased to below 5.[8] This is due to protonation and dissociation of the His87 of $PCu^I$ (p$K_a$ ~ 5), leaving the Cu(I) in a 3-coordinate (relatively) redox inactive form.[8,14,19] Clearly therefore, the effects of pH on reactivity of $RCu^I$ and $RCu^{II}$ are of considerable interest. With $Fe^{2+}$ and $Cr^{2+}$ as reductants for $RCu^{II}$ the pH was restricted to <3,[22] no doubt due to the tendency of the III-state products to undergo hydrolysis at higher pH. In the present work complexes selected enable a wider range of pHs to be studied. At pH >6 the protein has been reported to exhibit rapid irreversible loss of color, and we therefore took pH 6 as an upper limit for these studies.[2] A range of substitution-inert complexes with suitable reduction potentials (Table V) was selected. The three complexes $[Mn(Cydta)(H_2O)]^-$, $[Ru(NH_3)_4(phen)]^{2+}$, and $[Co(dipic)_2]^-$ gave measurable rates, but reactions with $[Fe(CN)_4(phen)]^{2-}$, $[Fe(CN)_6]^{4-}$, and $[Mo(CN)_8]^{3-}$ were too fast to monitor by the stopped-flow method, and $[Fe(CN)_4(bipy)]^{2-}$ gave spurious absorbance changes (followed by precipitation) on mixing with the protein.

For the three complexes studied the effects of pH on rate constants cannot be attributed to the influence of deprotonation/protonation on the electrostatics. Moreover, the p$K_a$'s

**Table V.** Summary of Rate Constants (25 °C) for Reaction of Rusticyanin at pH 2–3 ($k_H$) and pH 5–6 ($k_o$)[a]

| reaction | $k_H$, $M^{-1}$ $s^{-1}$ | $k_o$, $M^{-1}$ $s^{-1}$ | $E°$, V |
|---|---|---|---|
| $RCu^I + [Mo(CN)_8]^{3-}$ | | $>10^6$ [b] | 0.84[c] |
| $RCu^I + [Mn(Cydta)(H_2O)]^-$ | 104 | 554 | 0.76 |
| $RCu^I + [Co(dipic)_2]^-$ | 2.5 | 11.4 | 0.75 |
| $[Fe(H_2O)_6]^{2+} + RCu^{II}$ | 6.3[d] | | 0.77 |
| $[Ru(NH_3)_4(phen)]^{2+} + RCu^{II}$ | $3 \times 10^5$ [e] | $1.6 \times 10^5$ [e] | 0.53 |
| $[Fe(CN)_4(phen)]^{2-} + RCu^{II}$ | | $>10^7$ [b] | 0.57[f] |
| $[Fe(CN)_6]^{4-} + RCu^{II}$ | | $>10^6$ [b] | 0.42 |
| $[Cr(H_2O)_6]^{2+} + RCu^{II}$ | $2.5 \times 10^4$ [g] | | −0.41 |

[a] $I$ = 0.10 M (NaCl) unless otherwise stated. Reduction potentials ($E°$) are for the inorganic reactant. [b] pH ~4.5. [c] Kratochvil, B.; Diehl, H. *Talanta* **1960**, *3*, 346. Hartley, A. M.; Lingane, J. J. *Anal. Chim. Acta* **1955**, *13*, 183. [d] $I$ = 0.50 M (NaCl); ref 22. [e] 15 °C. [f] Determined in this laboratory. [g] $I$ = 0.5 M ($Na_2SO_4$), pH <3.0; ref 22. From results with $Fe^{2+}$ it is estimated that the $SO_4^{2-}$ decreases $k$ to a value ~50%.

for the protein in its two oxidation states are essentially the same, 4.06 (15 °C) for the reaction of $[Ru(NH_3)_4(phen)]^{2+}$ with $RCu^{II}$ and 4.10 and 4.17 (25 °C) for the reaction of $RCu^I$ with $[Co(dipic)_2]^-$ and $[Mn(Cydta)(H_2O)]^-$, respectively. This suggests that protonation is at a site not influenced by the oxidation state of the Cu. The $pK_a$ of 4.1 ± 0.1 in the present studies suggests involvement of a carboxylate group as the most likely site for protonation.

We have previously considered[17] the possibility that the $pK_a$ observed with $[Mn(Cydta)(H_2O)]^-$ is due in part to the protonation of a free carboxylate arm of the Cydta. However UV–visible spectra at 400–600 nm for solutions of pH 2.0–5.5 and pH titration experiments by ourselves and in the literature[23a] show no evidence for a $pK_a$ in this region, and we now believe that the $pK_a$ of 4.17 observed in the reaction of $[Mn(Cydta)(H_2O)]^-$ with $RCu^I$ is due solely to the protein. Neither $[Ru(NH_3)_4(phen)]^{2+}$ nor $[Co(dipic)_2]^-$ is susceptible to protonation in the range studied, so that the $pK_a$'s determined with these reagents can be assigned exclusively to the protein.

In experiments with the complex $[Fe(CN)_4(phen)]^{2-}$ the reduction potential of the $RCu^{II}/RCu^I$ couple was found to decrease to 620 mV at pH 5.3. Rate constants vary with pH in accordance with this decrease. The fact that the reduction potential remains high and only a small difference is observed from 680 mV at pH 2 is noted. Since no other $pK_a$ is observed in the range up to pH 6, it seems unlikely that rusticyanin exhibits the change in coordination number as observed for plastocyanin $PCu^I$. This does not exclude the possibility that both forms are three coordinate. More likely however an alternative explanation for the high reduction potential has to be sought. Recent studies[29] have suggested that the reduction potential can be fine tuned by the effects of polypeptide backbone structure on the Cu–S(Cys) and Cu–S(Met) bond distances and the Cu ligand field. In the light of these studies we would expect rusticyanin, with its high reduction potential, to have one or both Cu–S distances shorter than those in other single blue copper proteins.

Protonation/deprotonation effects that have been observed for single blue (type 1) Cu proteins can be summarized as follows:

(a) First, there are those involving a change in geometry at the active site as observed for plastocyanin $PCu^I$ but not $PCu^{II}$.[8,14,19] At present plastocyanin is the only example.

(b) Second, there are those occurring at or close to a binding site, as at the east face on $PCu^I$ and $PCu^{II}$, when $pK_a$ values differing by 0.3–0.8 unit for the two oxidation states are obtained.[18,19] The case is similar for azurin, when $pK_a$'s assigned to the uncoordinated His35 and His83 residues differ by up to 1 unit for $ACu^I$ and $ACu^{II}$.[30] Conformational changes are believed to be relevant.

(c) Third, there are those, as in the present case, that give about the same $pK_a$ for both oxidation states. Protonation is not directly at the active site, but the active site is affected as shown by the change in reduction potential. Neither is there any apparent effect of $[H^+]$ on the electrostatics of interaction with a redox partner at the binding site. A similar situation has been reported for umecyanin,[18] with $pK_a$'s of 9.7 for the $[Co(C_2O_4)_3]^{3-}$ oxidation of $UCu^I$ and 9.5 for the $[Ru(NH_3)_5py]^{2+}$ reduction of UCu(II). In the latter case rate constants for both reactions decrease with pH, and the effect is not due simply to a change in reduction potential.

The composition of rusticyanin, with four histidines and two methionines in addition to the histidine (His132) and methionine (Met137) assumed coordinated to the Cu, is unusual. It will also be interesting to see to just what extent these are involved in coordination to the Cu and in providing protection for the active site, which is not only stable but is normally functional at pH ~2.

*T. ferrooxidans* is known to have a specific sulfate requirement for growth,[4] and preliminary NMR experiments on rusticyanin have shown that the protein is affected by anions, small differences being detected in sulfate and chloride media.[22] In the kinetic study of the reduction of rusticyanin with ~1 mM $Fe^{2+}$, sulfate (used to adjust $I$ to 0.50 M) was found to inhibit the reaction, and rate constants (pH 2.2) were 42% of those in chloride (pH 1.6).[22] We find a similar inhibiting effect of sulfate on the $[Ru(NH_3)_4(phen)]^{2+}$ reduction of $RCu^{II}$ with rate constants about half those in $Cl^-$ over the pH 3–6 range investigated. This effect can be assigned to minor changes resulting from association of $SO_4^{2-}$ with the positively charged $RCu^{II}$. The effect is not to moderate the electrostatics, thereby making for faster reaction. No evidence for saturation kinetics was observed in the reduction of $RCu^{II}$ with $[Ru(NH_3)_4(phen)^{2+}$ (up to 1 mM) when the ionic strength (0.10 M) was adjusted with $Na_2SO_4$. In the corresponding reaction with $Fe^{2+}$, at higher reductant concentrations and with $I$ = 0.50 M ($Na_2SO_4$), association to give $FeSO_4$ is relevant.

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**Registry No.** $[Mn(Cydta)(H_2O)]^-$, 36444-08-3; $[Ru(NH_3)_4(phen)]^{2+}$, 69799-59-3; $[Co(dipic)_2]^-$, 71605-21-5; $[Fe(CN)_4(phen)]^{2-}$, 17455-55-9; $[Fe(CN)_6]^{4-}$, 13408-63-4; $[Fe(CN)_4(bpy)]^{2-}$, 17455-56-0; $[Mo(CN)_8]^{3-}$, 17845-99-7; $SO_4^{2-}$, 14808-79-8.

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