Mixed Ligand Complexes of Iron with Cyanide and Phenanthroline as New Probes of Metalloprotein Electron Transfer Reactivity

ANALYSIS OF REACTIONS INVOLVING RUSTICYANIN FROM THIOBACILLUS FERROOXIDANS*

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A family of 12 different mixed ligand complexes of iron with cyanide and substituted 1,10-phenanthroline was prepared. The electron transfer properties of each reagent were systematically manipulated by varying the substituent(s) on the aromatic ring system and the stoichiometry of the two types of ligands in the complex. Values for the standard reduction potentials of each member of this family of electron transfer reagents were determined and spanned from 500 to 900 mV. The one-electron transfer reactions between each of these substitution-inert reagents and the high potential blue copper protein, rusticyanin, from *Thiobacillus ferrooxidans* were studied by stopped flow spectrophotometry under acidic conditions. For comparison with the protein results, the kinetics of electron transfer between each of these reagents and sulfatoiron were also investigated. The Marcus theory of electron transfer was successfully applied to this set of kinetic data to demonstrate that 10 of the 12 reagents had equal kinetic access to the redox center of the rusticyanin and utilized the same reaction pathway for electron transfer. The utility of these synthetic electron transfer reagents in characterizing the electron transfer properties of very high potential, redox-active metalloproteins is illustrated.

Extensive kinetic data are available on the electron transfer reactions of selected inorganic and small organometallic complexes with isolated electron transport proteins, especially the cytochromes (1-4), type I blue copper proteins (5-8), and various iron sulfur proteins (9-12). One goal of such research has been the elucidation of reaction pathways available to the proteins and the manner in which the polypeptide envelopes have modified the inherent reactivity of the metal center. In particular, the Marcus theory of electron transfer has been widely employed to deduce, from the relevant kinetic and thermodynamic data, the factors governing the rates of outer sphere electron transfer reactions between small organometallic complexes and metalloproteins (13-15, and references therein). A wide assortment of well characterized outer sphere electron transfer reagents varying in charge, reduction potential, and ligand structure are available to systematically probe the reactive sites of metalloproteins that have reduction potentials in the approximate range of 0-400 mV.

Rusticyanin is a 16,3-kilodalton, type I blue copper protein isolated from *Thiobacillus ferrooxidans*, an acidophilic chemolithotroph that grows autotrophically on ferrous ions (16-19). The protein is thought to be expressed into the periplasmic space of this Gram-negative bacterium and to play an important, although as yet undefined, role in the iron respiratory electron transport chain. The rusticyanin possesses two functional characteristics that distinguish it from other comparably sized type I copper proteins: it is redox-active only in acidic solutions, down to pH 0.2; and its reduction potential is 680 mV (20), a value much higher than that of the average value of 210 mV (21) attributed to other members of this arbitrary class of copper metalloproteins. Previous kinetic studies on the electron transfer properties of purified rusticyanin utilized sulfatoiron, an inorganic complex of physiological significance to the intact organism (22-24). A principal barrier to further probes of the rusticyanin’s electron transfer reactivity is that relatively few well characterized inorganic or small organometallic complexes are available with the appropriate electrochemical, solubility, and structural properties that are required to probe a redox center of such high reduction potential under strongly acidic conditions.

The present paper describes the synthesis, characterization, and electron transfer properties of a series of mixed ligand complexes of Fe(II) and Fe(III) with cyanide and 1,10-phenanthroline. By introducing different substituents on the aromatic diimine and varying the stoichiometry of the substituted phenanthroline in the final organometallic complex, a series of substitution-inert electron transfer reagents were constructed with reduction potentials from 500 to 900 mV. Kinetic and thermodynamic studies on the electron transfer reactions of each of these substitution-inert iron complexes with both sulfatoiron and rusticyanin are presented. The family of electron transfer reagents described herein could prove useful in probing the electron transfer reactivity of this novel class of high potential respiratory proteins.

EXPERIMENTAL PROCEDURES AND RESULTS

Absorbance Properties—Both the redox potential and the electron transfer reactivity of the ferrous/ferric couple are profoundly influenced by the ligands coordinated to the iron cation. Hexaquoiron has a reduction potential of 770 mV and reacts relatively slowly in its electron transfer reactions both with itself (self-exchange electron transfer) and with other electron transfer reagents. The conjugate base of hydro-
gen cyanide binds much more strongly to ferrous ions than it
does to ferrous ions. This discriminatory binding serves to
make the ferrous ion more reducing; hexacyanoferrate thus
has a much lower reduction potential, 410 mV, than does
hexoquoiron. Conversely, the aromatic diimine 1,10-phen-
nanthroline binds much more strongly to the ferrous ion than
to the ferrous ions, with the consequence that triis(1,10-
phenanthroline)iron has a much higher reduction potential,
1,060 mV, than does hexoquoiron.

As part of an effort to devise substitution-inert electron
transfer reagents with reduction potentials intermediate be-
tween those of 410 and 1,060 mV, a series of mixed ligand
complexes of iron with cyanide and 1,10-phenanthroline were
constructed by varying the stoichiometry of the two types of
ligands in the final six-coordinate organoiron complex. The
visible absorbance spectra of a family of such complexes is
shown in Fig. 1. As illustrated in the figure, the complex
between Fe(II) and 1,10-phenanthroline exhibits a red color
whose intensity depends on the number of phenanthrolines
in the complex. Curves a, b, and c represent cyano(1,10-
phenanthroline)iron(II) complexes containing 3, 2, and 1
equivalents of 1,10-phenanthroline, respectively. Hexacyano-
ferrate(II), containing no phenanthroline ligand, has negli-
gible absorbance over the same range of wavelengths (data not
shown). A series of five additional families of cyano(1,10-
phenanthroline)iron complexes were obtained using 1,10-phe-
nanthroline derivatives that contained electron donating or
withdrawing substituents on the aromatic rings. The wave-
lengths of maximum absorbance in the visible region, along
with the corresponding molar absorption coefficients, of the
ferrous forms of each of the 12 cyano(1,10-phenanthro-
line)iron complexes synthesized here are summarized in the
first two columns of Table I (Miniprint). The visible absorb-
ance spectrum of each compound was substantially bleached
upon the one-electron oxidation of the ferrous complex. The
absorbance difference between the reduced and the oxidized
forms of each cyano(1,10-phenanthroline)iron complex thus
constitutes an intrinsic spectrophotometric probe whereby
the transient changes in the redox state of the population of
molecules can be monitored with great sensitivity.

Electron Transfer with Sulfatoiron—The object of these
experiments was to obtain detailed kinetic data on the one-
electron transfer reactions between cyano(1,10-phenan-
throline)iron and soluble iron in acidic solutions in the presence
of an excess of sulfate. Under these experimental conditions,
the principal form of the iron in solution is the soluble
sulfatoiron complex (29). When each of the 12 cyano(1,10-
phenanthroline)iron derivatives in Table I was mixed in a
stopped flow spectrophotometer with a 4-fold or greater molar
excess of soluble sulfatoiron (pseudo-first order conditions),
each kinetic trace of the change in absorbance could be
-described mathematically as a single exponential function of
time. Accurate values of both the amplitude and the pseudo-
first order rate constant for each absorbance change were
obtained from each kinetic trace as described previously (22).
As long as pseudo-first order conditions were maintained, a
10-fold variation in the concentration of the particular
cyano(1,10-phenanthroline)iron complex affected only the
amplitudes of the observed spectral changes, not the values
of the corresponding pseudo-first order rate constants. The
amplitudes were directly proportional to the concentration of
the cyano(1,10-phenanthroline)iron.

Representative examples of the dependence of the pseudo-
first order rate constants for the oxidation of selected di-
cyano(1,10-phenanthroline)iron(II) complexes upon the
total concentration of Fe(II) are presented in Fig. 2A (Min-
print). A typical kinetic study for the oxidation of these
organoiron(II) derivatives employed at least five different
concentrations of soluble Fe(III) that evenly spanned a 16-
fold range in concentrations. The dependence of the pseudo-
first order rate constants for the reduction of the correspond-
ing dicyano(1,10-phenanthroline)iron(II) complexes upon
the total concentration of Fe(II) is presented in Fig. 2B. A
typical kinetic study for the reduction of these organoiron(II)
derivatives employed at least four different concentrations of
soluble Fe(II) that spanned a 5-fold range in concentrations.
Regardless of the direction of electron flow, values of the
pseudo-first order rate constants for each electron transfer
reaction were directly proportional to the concentration of
excess soluble iron in all cases; no convincing evidence for
rate saturation was obtained in any of these studies. The
value of the slope of each line in Fig. 2 represents an apparent
second order rate constant for the electron transfer reaction
between the organoiron derivative and the soluble iron. Values
of these second order rate constants were obtained for both
the Fe(II)-dependent oxidation and the Fe(II)-dependent
reduction of each of the 12 cyano(1,10-phenanthroline)iron
compounds investigated and are summarized in Table II
(Miniprint).

The kinetic data summarized in Table II were exploited to
estimate the reduction potential of each cyano(1,10-phe-
nanthroline)iron complex. Each substitution-inert organoiron
complex featured in Table II would be expected to transfer
an electron by an outer sphere bimolecular reaction. An outer
sphere electron transfer reaction is one in which the two
reactants do not share a common atom or group, or more
generally, one in which the interaction of the relevant elec-
tronic orbitals of the two centers is weak. Extensive experi-
mental data on a variety of known outer sphere electron
transfer reactions have established that the kinetic and ther-
modynamic properties of the reaction partners may frequently
be correlated by the "cross-reaction" equation developed by
Marcus (13-15):

$$k_{XY} = \sqrt{k_{XX} k_{YY} K_{XY}}$$

where $k_{XY}$ is the second order rate constant for the transfer
of an electron from X to Y, $k_{XX}$ and $k_{YY}$ are the two self-
exchange rate constants (e.g. for the transfer of an electron
from one molecule of X to another molecule of X), and $K_{XY}$

![Fig. 1. Absorbance spectra of tris(1,10-phenanthro-
line)iron(II) (a), dicyano(1,10-phenanthroline)iron(II) (b),
and tetracyano(1,10-phenanthroline)ferrate(II) (c). Spectra
were recorded at 25 °C in 0.01 N sulfuric acid, pH 2.0. Each absorption
coefficient is the average of four determinations.](image-url)
is the equilibrium constant for the electron transfer. Each self-exchange rate constant is a measure of the intrinsic reactivity of each reactant and is related to the energy barrier created by the internal and solvent nuclear rearrangements that must occur immediately prior to actual electron transfer. Equation 1 is a much simplified version of the theoretical treatment developed by Marcus for outer sphere electron transfer reactions, but it has nonetheless been shown to correlate a large body of kinetic and thermodynamic data, particularly in those electron transfer reactions of an acceptor with a series of related donors (or vice versa). For the reverse reaction represented by Equation 1, the transfer of an electron from Y to X, it follows that

\[ k_{xy} = \frac{1}{k_{yx}} \]

where \( k_{xy} \) is the second order rate constant for the transfer of an electron from Y to X, \( k_{yx} \) is the corresponding equilibrium constant, and \( k_{xx} \) and \( k_{yy} \) are defined above. Recognizing that \( K_{xy} = 1/k_{yx} \), Equation 1 may be divided by Equation 2 and the dividend rearranged to yield

\[ K_{xy} = \frac{k_{xy}}{k_{yx}} \]

It is satisfactory, and indeed necessary, that the Marcus expressions for the forward and backward electron transfer reactions should satisfy the thermodynamics of the system! The kinetic data in Table II were used in accordance with Equation 3 to calculate an equilibrium constant for the electron transfer between sulfatoiron and each cyano(1,10-phenanthroline)iron complex investigated. Sulfate binds preferentially to the ferric form of soluble iron, thereby effectively lowering the value of the reduction potential for the ferric/ferrous couple. Under the solution conditions imposed on the kinetic experiments summarized in Table II, the effective reduction potential of the sulfatoiron in each case was calculated to be 710 mV (22). Using this potential of the sulfatoiron as a point of reference, the reduction potential of each cyano(1,10-phenanthroline)iron compound in Table II was then determined.

The value of each reduction potential determined from the kinetic studies described above was then verified by independent equilibrium experiments that employed soluble iron and the cyano(1,10-phenanthroline)iron complex of interest. A representative example of such an equilibrium experiment is illustrated in Fig. 3. Each absorbance spectrum in Fig. 3 was taken after a small concentration of dicyanobis(1,10-phenanthroline)iron(II) was introduced into a solution that contained an excess of soluble sulfatoiron. The concentrations of the two reagents were chosen to ensure that any net electron transfer reactions to or from the limiting dicyanobis(1,10-phenanthroline)iron would have a negligible effect on the relative concentrations of the ferrous and ferric forms of the excess sulfatoiron. Each spectrum in Fig. 3 provided a sensitive means of quantifying the fractions of the total population of dicyanobis(1,10-phenanthroline)iron molecules that were either oxidized or reduced. By systematically varying the ratio of sulfatoiron(II)/sulfatoiron(III), the redox state of the population of dicyanobis(1,10-phenanthroline)iron molecules was systematically manipulated to yield the standard Nernst plot shown in the inset in Fig. 3. A value of 800 mV for the reduction potential of dicyanobis(1,10-phenanthroline)iron was obtained from the abscessa intercept of the Nernst plot. Similar equilibrium experiments were performed on each of the 12 cyanobis(1,10-phenanthroline)iron compounds investigated. Acceptable agreement (within 5–15 mV) was observed between the value of each reduction potential obtained by this equilibrium method compared with that obtained by the kinetic method described above. The mean values of the reduction potentials obtained by the two methods are listed in the last column of Table I. Although very little data on the redox properties of these compounds are available for comparison from the literature, the reduction potential of tetracyano(1,10-phenanthroline)iron has been reported by two independent laboratories to be 570 (30) and 560 mV (31), respectively, in agreement with the value determined here.

**Electron Transfer with Rusticyanin**—The object of these experiments was to obtain detailed kinetic data on the one-electron transfer reactions between cyano(1,10-phenanthroline)iron and purified rusticyanin in the acidic sulfate solutions usually employed for functional studies of the purified protein. The visible absorbance spectrum of oxidized rusticyanin exhibits a prominent peak at around 600 nm that disappears upon the one-electron reduction of the protein (22). The redox-dependent absorbance properties of the rusticyanin thus permit transient changes in the redox state of the protein to be monitored with satisfactory sensitivity. When oxidized or reduced rusticyanin was mixed in a stopped flow spectrophotometer with a molar excess of a cyano(1,10-phenanthroline)iron(II) or corresponding cyano(1,10-phenanthroline)iron(III) compound, respectively, each kinetic trace of the change in absorbance at 597 nm could be described mathematically as a single exponential function of time. Representative kinetic traces of the tetracyano(5-nitro-1,10-phenanthroline)ferrate(II)-dependent reduction of rusticyanin are shown in Fig. 4 (Miniprint). These data represent the fastest electron transfer reactions that were monitored in direct mixing experiments for the current study. Examples of the dependence of the pseudo-first order rate constants for the reduction and oxidation of rusticyanin upon the concentration of selected cyano(1,10-phenanthroline) complexes of iron are presented in Fig. 5, A and B, respectively (Miniprint). Values of the pseudo-first order rate constants for each electron...
transfer reaction were directly proportional to the concentration of excess organoiron reagent in all circumstances. Values for the second order rate constants for each electron transfer reaction were obtained from the slopes of linear plots such as those illustrated in Fig. 5 and are summarized in Table III (Miniprint).

Many of the bimolecular electron transfer reactions between rusticyanin and individual cyano(1,10-phenanthroline)iron complexes were too rapid to be accurately investigated by direct mixing experiments in the stopped flow spectrophotometer. Noting that the bimolecular electron transfer reactions of cyano(1,10-phenanthroline)iron with either soluble iron (Fig. 2) or rusticyanin (Fig. 5) were reasonably rapid, limiting concentrations of selected cyano(1,10-phenanthroline)iron complexes were exploited to catalyze the relatively sluggish electron transfer reactions between soluble iron and purified rusticyanin. The examples in Fig. 6 (Miniprint) illustrate the approach adopted to estimate the second order rate constants for these extremely rapid electron transfer reactions. Acceptable agreement, within ±10%, was obtained between those values of electron transfer rate constants determined by direct stopped flow spectrophotometric measurements and the corresponding values determined by the indirect steady-state kinetic approach outlined in the Miniprint. The latter steady-state approach was therefore applied to each of the bimolecular electron transfer reactions between rusticyanin and individual cyano(1,10-phenanthroline)iron complexes that was too rapid to be investigated by direct mixing experiments in the stopped flow spectrophotometer. The values of the second order rate constants obtained by the steady-state approach are included in Table III.

The kinetic data summarized in Table III were exploited to estimate the reduction potential of the rusticyanin. It is evident from Equation 3 that

\[
\frac{k_{ox}}{k_{red}} = K_{RCu(I)CPI(III)} = \frac{[CPI(III)] \cdot [RCu(II)]}{[CPI(II)] \cdot [RCu(I)]}
\]

where \(k_{ox}\) and \(k_{red}\) are the second order rate constants for the cyano(1,10-phenanthroline)iron-dependent oxidation and reduction, respectively, of the rusticyanin, CPI(II) and CPI(III) represent the ferrous and ferric forms, respectively, of a cyano(1,10-phenanthroline)iron complex, RCu(II) and RCu(I) are oxidized and reduced rusticyanin, respectively, and \(K_{RCu(I)CPI(III)}\) is the equilibrium constant for the transfer of an electron from reduced rusticyanin to oxidized cyano(1,10-phenanthroline)iron. Equation 9 may be rearranged to

\[
(RT/F) \ln \left( \frac{k_{ox}}{k_{red}} \right) = E_{Cu} - E_{Fe}
\]

where \(R\) is the gas constant, \(T\) is the absolute temperature, \(F\) is Faraday’s constant, and \(E_{Cu}\) and \(E_{Fe}\) are the reduction potentials for the cyano(1,10-phenanthroline)iron and the rusticyanin, respectively. Values for the reduction potentials in Table I and the kinetic constants in Table III were used to construct a linear plot according to Equation 10, as illustrated in Fig. 7. The reduction potential of the rusticyanin was determined to be 680 mV from the abscissa intercept of the line in Fig. 7, a value in agreement with that reported from independent potentiometric experiments (20).

The reduction potential of the rusticyanin was also determined by equilibrium experiments analogous to those described above in Fig. 3. The rusticyanin was introduced into solutions of soluble iron composed of different ratios of the ferrous and ferric forms of sulfatoiron. A catalytic concentration of an appropriate cyano(1,10-phenanthroline)iron complex was included in each mixture to facilitate rapid electron transfer between the rusticyanin and the soluble iron and thus permit rapid equilibration of the available electrons among the individual redox partners. The concentrations of the three reaction partners were chosen to ensure that any net electron transfer reactions to or from the excess soluble iron would have a negligible effect on the relevant concentrations of the ferrous and ferric forms. The results of a representative titration are shown in Fig. 8. Each absorbance spectrum in Fig. 8 was recorded after the spectrum of the rusticyanin had come to equilibrium, usually within 10 s of the addition of rusticyanin to complete the reaction mixture. Each spectrum in Fig. 8 provided a sensitive means of quantifying the fractions of the total population of rusticyanin molecules that were either oxidized or reduced. By systematically varying the ratio of sulfatoiron(II) to sulfatoiron(III), the redox state of the population of rusticyanin molecules was systematically manipulated to yield the standard Nernst plot shown in the inset in Fig. 8. The data plotted in the inset were obtained using two different cyano(1,10-phenanthroline)iron complexes. The close correspondence between the two data sets demonstrated that the identity of the catalyst had no appreciable effect on the quantitative outcome of the results. A value of 677 mV for the reduction potential of rusticyanin was obtained from the abscissa intercept of the Nernst plot. These equilibrium experiments provided yet another independent verification of the value of the reduction potential of the rusticyanin.

**DISCUSSION**

Previous kinetic studies on the electron transfer reactions between sulfatoiron and purified rusticyanin indicated that the rates of reaction were far too slow to support the hypothesis that rusticyanin is the primary oxidant of ferrous ions in the iron-dependent respiratory electron transport chain of *T. ferrooxidans* (22). Indeed, the second order rate constants for
Electron Transfer between Rusticyanin and Cyano(phenanthroline)iron

FIG. 8. Absorbance spectra of rusticyanin in different mixtures of sulfateiron(II) and sulfateiron(III). Final concentrations: rusticyanin, 250 μM; sulfate, 0.1 M; total soluble iron, 20 mM; and dicyanobis(1,10-phenanthroline)iron, 1.0 μM. The ratios of sulfateiron(II)/sulfateiron(III) were 0.0, 1.2, 2.1, 3.0, 4.5, 6.0, 12, and ∞ in experiments a–h, respectively. Inset, a standard Nernst plot of the reductive titration of rusticyanin. RCu(ZI) and RCr(u) are the concentrations of rusticyanin in the oxidized and reduced states, respectively. The open and closed circles represent data collected in the presence of dicyanobis(1,10-phenanthroline)iron, and tetracyano(1,10-phenanthroline)ferrate, respectively. $E_{\text{ta}}$, is the total system reduction potential. The ordinate intercept and slope were determined by a linear regression analysis.

### Table IV

| Mixed ligand complex | Self-exchange rate constant $\text{mm}^{-1} \text{s}^{-1}$ |
|----------------------|------------------------------------------------------|
| (CN)$_2$(phenanthroline)iron | |
| Unsubstituted | 46 |
| 4-Methyl | 21 |
| 5-Methyl | 44 |
| 5-Chloro | 29 |
| 5-Nitro | 66 |
| 4,7-Diphenylsulfonic acid derivative | 0.035 |
| (CN)$_2$(phenanthroline)iron | |
| Unsubstituted | 76 |
| 4-Methyl | 130 |
| 5-Methyl | 96 |
| 5-Chloro | 66 |
| 5-Nitro | 120 |
| 4,7-Diphenylsulfonic acid derivative | 0.44 |

Using Equation 11, the kinetic constants in Table II, and a value for the self-exchange rate constant of sulfateiron of 8.7 $\text{m}^{-1} \text{s}^{-1}$ (32), a value for the apparent self-exchange rate constant of each of the 12 cyano(1,10-phenanthroline)iron compounds listed in Table II was obtained. These calculated self-exchange rate constants, along with the kinetic constants in Table III, were subsequently used to calculate 12 different apparent self-exchange rate constants for the rusticyanin. The values of these calculated constants are listed in Table IV. Recognizing that each value in Table IV is derived from the multiplicant of five individual experimental observations, each with its own level of experimental error, the close correspondence among 10 of the 12 values in Table IV is exceptional. One can conclude that these 10 cyano(1,10-phenanthroline)iron compounds have equal kinetic access to the redox center of the rusticyanin and utilize the same reaction pathway for electron transfer. One physical interpretation of these kinetic data is that the electron transfer reactivity of the rusticyanin is enhanced when the reaction partner possesses hydrophobic, π-conjugated ligands that can penetrate into the interior of the protein, thereby facilitating orbital overlap with the protein redox center. The 4,7-diphenylsulfonic acid derivatives are sterically hindered from penetrating as deeply into the protein interior and demonstrate a lower reactivity with the rusticyanin. The hydrophilic sulfateiron, which would not be expected to penetrate the protein interior at all, displays by far the slowest electron transfer reactivity with the rusticyanin. Kinetic studies such as these are very useful in defining the electron transfer reaction pathways that are possible in individual redox-active proteins.

A current interest of this laboratory includes the identification, isolation, and characterization of the electron transfer proteins responsible for aerobic respiration on soluble iron. Respiratory iron represents a principal metabolic activity in certain chemolithotrophic organisms that inhabit iron-bearing geological formations exposed to the atmosphere. Energy is derived from oxidative phosphorylation coupled to respiratory electron transfer. Since sulfate is the dominant anion both in the bacterium's natural habitat and in the laboratory culture media widely employed, the energy-yielding electron transfer reactions are initiated by electron donation from sulfateiron(II) at a standard reduction potential of, at its lowest, 650 mV. Preliminary studies in this laboratory suggest that exceptional diversity exists in the types of electron transfer proteins that are expressed by individual members of the diverse group of microorganisms that respire on iron (33). An entire class of very high potential electron transfer proteins has begun to emerge from these studies. It is anticipated that the synthetic electron transfer reagents characterized here will be of use in investigating the electron transfer reactivity of these high potential redox proteins.

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**Experimental Procedures**

All of the dicyano(1,10-phenanthroline)iron(I) and tetracyano(1,10-phenanthroline)ferrate(II) derivatives examined in the present study were obtained using the described procedure. For the preparation of these complexes, the reaction of the acidified solutions of the substituted 1,10-phenanthroline, or 1,10-phenanthroline itself, with concentrated sulfuric acid in aqueous solution was found to yield the desired products in high yields. The identity of each compound was determined by elemental analysis, and by the results of the magnetic susceptibility measurements. Each of these solutions was adjusted to pH 2.0 using concentrated sulfuric acid.

**Preparation of Dicyano(1,10-phenanthroline)iron(I)**

A mixture of 0.5 g of dicyano(1,10-phenanthroline)iron(III) and 200 ml of an aqueous solution of sodium chloride was treated with a 10% solution of sodium dithionite. The mixture was then treated with a 10% solution of sodium dithionite. The mixture was allowed to react for 1 hour, and the resulting solution was filtered. The filtrate was concentrated to a small volume, and the precipitate was collected and washed with ether. The product was then dried in vacuo.

**Preparation of Tetracyano(1,10-phenanthroline)ferrate(II)**

Each compound was obtained by the following procedure:

1. Preparation of a 10% solution of sodium dithionite in water.
2. Addition of the dicyano(1,10-phenanthroline)iron(III) compound to the sodium dithionite solution.
3. Heating the mixture to 80°C for 1 hour.
4. Filtration of the mixture to remove the un-reduced iron(II) compound.
5. Concentration of the filtrate to a small volume.
6. Recrystallization from water.
7. Drying of the product in vacuo.

**Preparation of Rusticyanin**

Large scale growth of Rusticyanin was achieved by incubating the culture of *R. rubrum* at 30°C in a fermenter. The cells were harvested by centrifugation and the supernatant was concentrated to a small volume. The concentrated solution was then dialyzed against distilled water.

**Kinetic Experiments**

Kinetic experiments were performed using the following procedure:

1. Preparation of a 10% solution of sodium dithionite in water.
2. Addition of the rusticyanin to the sodium dithionite solution.
3. Heating the mixture to 80°C for 1 hour.
4. Filtration of the mixture to remove the un-reduced rusticyanin.
5. Determination of the absorbance at 560 nm.
6. Calculation of the rate of oxidation of rusticyanin.

**Stability of Rusticyanin Preparation**

The stability of the rusticyanin preparation was determined by the following procedure:

1. Preparation of a 10% solution of sodium dithionite in water.
2. Addition of the rusticyanin to the sodium dithionite solution.
3. Heating the mixture to 80°C for 1 hour.
4. Filtration of the mixture to remove the un-reduced rusticyanin.
5. Determination of the absorbance at 560 nm.
6. Calculation of the rate of oxidation of rusticyanin.

**Results and Discussion**

The results of the kinetic experiments showed that the rusticyanin preparation was stable for at least 24 hours. The rate of oxidation of rusticyanin was found to be dependent on the concentration of sodium dithionite and the temperature of the reaction mixture. The results of the stability experiments showed that the rusticyanin preparation was stable for at least 24 hours. The rate of oxidation of rusticyanin was found to be dependent on the concentration of sodium dithionite and the temperature of the reaction mixture.
Electron Transfer between Rusticyanin and Cyano(phenanthroline)iron

Thermodynamic experiments were prepared fresh daily as a precautionary measure. Acidic solutions of the dicyanobis(1,10-phenanthroline) complexes of iron(III) were considerably less stable than those of the corresponding tetracyano derivatives. Consequently, experimental observations on the kinetic or thermodynamic properties of the dicyanobis(1,10-phenanthroline)iron complexes were limited to solutions utilized within 90 min of their preparation.

RESULTS

Spectral and electrochemical properties of cyano(1,10-phenanthroline)iron compounds

| Mixed ligand complex | Absorbance maximum of reduced species, nm | Reduction potential, mv |
|----------------------|------------------------------------------|------------------------|
| (CN)₂(phen)₂iron     |                                          |                        |
| unsubstituted        | 516                                      | 4.65                   |
| 4-methyl             | 567                                      | 4.13                   |
| 5-methyl             | 515                                      | 3.76                   |
| 5-chloro             | 522                                      | 4.40                   |
| 5-nitro              | 530                                      | 5.26                   |
| batho                | 537                                      | 4.42                   |
| (CN)₄(phen)iron      |                                          |                        |
| unsubstituted        | 450                                      | 1.85                   |
| 4-methyl             | 463                                      | 1.66                   |
| 5-methyl             | 437                                      | 1.30                   |
| 5-chloro             | 480                                      | 1.75                   |
| 5-nitro              | 521                                      | 2.09                   |
| batho                | 536                                      | 1.76                   |

* 4,7-diphenylsulfonic acid

Second order rate constants for electron transfer between soluble iron and cyano(1,10-phenanthroline)iron

| Mixed ligand complex | Rate constant for oxidation by Fe(III), mM⁻¹ s⁻¹ | Rate constant for reduction of Ru(II), mM⁻¹ s⁻¹ |
|----------------------|-----------------------------------------------|-----------------------------------------------|
| (CN)₂(phen)₂iron     |                                              |                                              |
| unsubstituted        | 1.2                                          | 60                                           |
| 4-methyl             | 2.3                                          | 22                                           |
| 5-methyl             | 1.4                                          | 40                                           |
| 5-chloro             | 0.53                                         | 120                                          |
| 5-nitro              | 0.29                                         | 240                                          |
| batho                | 16                                           | 88                                           |
| (CN)₄(phen)iron      |                                              |                                              |
| unsubstituted        | 1200                                         | 5.5                                          |
| 4-methyl             | 2700                                         | 1.6                                          |
| 5-methyl             | 850                                          | 6.0                                          |
| 5-chloro             | 550                                          | 29                                           |
| 5-nitro              | 130                                          | 37                                           |
| batho                | 1000                                         | 4.4                                          |

* 4,7-diphenylsulfonic acid

TABLE III

Second order rate constants for electron transfer between cyano(1,10-phenanthroline)iron and rusticyanin

| Mixed ligand complex | Rate constant for reduction of Ru(II), mM⁻¹ s⁻¹ | Rate constant for oxidation of Ru(II), mM⁻¹ s⁻¹ |
|----------------------|-----------------------------------------------|-----------------------------------------------|
| (CN)₂(phen)₂iron     |                                              |                                              |
| unsubstituted        | 0.10                                          | 4.8                                          |
| 4-methyl             | 0.20                                          | 4.3                                          |
| 5-methyl             | 0.12                                          | 5.6                                          |
| 5-chloro             | 0.032                                         | 24                                           |
| 5-nitro              | 0.079                                         | 66                                           |
| batho                | 0.073                                         | 1.2                                          |
| (CN)₄(phen)iron      |                                              |                                              |
| unsubstituted        | 0.06                                         | 0.67                                         |
| 4-methyl             | 0.13                                          | 0.39                                         |
| 5-methyl             | 0.42                                          | 0.50                                         |
| 5-chloro             | 7.4                                           | 3.6                                          |
| 5-nitro              | 5.4                                           | 6.2                                          |
| batho                | 6.3                                           | 0.018                                        |

* 4,7-diphenylsulfonic acid

Fig. 2: Dependence of the pseudo-first order rate constant, kₚₑₒₓ, for the Fe(III)-dependent oxidation (a) or the Fe(II)-dependent reduction (g) of selected dicyanobis(1,10-phenanthroline)iron derivatives on the concentration of soluble iron. The 1,10-phenanthroline ligands contained substituents as follows: g, 4-methyl-; h, unsubstituted; i, 5-chloro-; d, 5-nitro-. The sulfate was 0.1 M; the pH was 3.0.
Electron Transfer between Rusticyanin and Cyano(phenanthroline)iron

Fig. 1. Time course of the absorbance changes at 597 nm when oxidized rusticyanin was mixed with different concentrations of tetraacyano(5-nitro-1,10-phenanthroline)ferrate(II). Final concentrations after mixing were: rusticyanin, 15 μM; and Fe(III) 1.0 mM. Concentrations of rusticyanin and ferrate(II) in experiments a, b, and c, respectively, were 0.1 M, 0.05 M, and 0.02 M, respectively. The time-dependent absorbance changes were sufficient to permit only about 25, 50, and 75% of the anticipated changes in absorbance to actually be observed after flow was stopped in experiments a, b, and c, respectively. Since all three experimental traces exhibited pseudo-first order kinetic behavior, the rate of each reaction that occurred before the cessation of flow was reconstructed and is represented by the shaded portion of each curve in the figure. All three extrapolated curves intersected at a consistent point, which agreed with the total change in absorbance anticipated for the reaction. A value of 2.0 ± 0.3 msec was determined for the deadtime of the stopped flow spectrophotometer using data such as those included herein.

Fig. 2. Representative examples of the cyan(5,10-phenanthroline)iron-catalyzed transfer of electrons between rusticyanin and mobile iron. A. time course of the tetracyano(5-nitro-1,10-phenanthroline)ferrate-catalyzed, Fe(III)-dependent reduction of the rusticyanin. Final concentrations after mixing were: sulfate, 0.1 M; Fe(III), 1.0 mM; rusticyanin, 15 μM; and tetracyano(5-nitro-1,10-phenanthroline)ferrate, 0.71 μM. B. A linear plot according to Equation 9, below, of the integrated steady-state rate equation that describes the protein oxidation reaction. C. time course of the tetracyano(5-chloro-1,10-phenanthroline)ferrate-catalyzed, Fe(III)-dependent oxidation of the rusticyanin. Final concentrations after mixing were: dicyanobis(4-methyl-1,10-phenanthroline)iron(II), 0.1 M; rusticyanin, 15 μM; and tetracyano(5-chloro-1,10-phenanthroline)ferrate, 0.71 μM. D. A linear plot according to Equation 8, below, of the integrated steady-state rate equation that describes the protein reduction reaction.

Fig. 3. Dependence of the pseudo-first order rate constants for the reduction (A) and oxidation (B) of rusticyanin upon the concentration of selected mixed ligand complexes of iron. B. reducing agents were: a. tetracyanodicyanobis(4-methyl-1,10-phenanthroline)iron(II); b. tetracyano(5-chloro-1,10-phenanthroline)ferrate(II); c. tetracyano(5-chloro-1,10-phenanthroline)ferrate(II), and d. tetracyano(5-nitro-1,10-phenanthroline)ferrate(II). C. oxidizing agents were: a. dicyanobis(4,7-diphenylsulfonate-1,10-phenanthroline)iron(II); b. tetracyano(5-nitro-1,10-phenanthroline)ferrate(II); and c. tetracyano(5-chloro-1,10-phenanthroline)ferrate(II).
Electron Transfer between Rusticyanin and Cyanophenanthrolineiron

Steady-state kinetic studies - The sequence of reactions in each catalytic cycle of the cyano(1,10-phenanthroline)iron-catalyzed, Fe(II)-dependent reduction of the rusticyanin is illustrated by the following mechanism:

\[
\begin{align*}
\text{Fe}^{2+} & \rightarrow \text{RCu}^{2+} + \text{phenanthroline}^{2-} \\
\text{Fe}^{2+} + \text{RCu}^{2+} & \rightarrow \text{Fe}^{3+} + \text{RCu}^{3+}
\end{align*}
\]

where \( \text{RCu}^{2+} \) and \( \text{RCu}^{3+} \) are oxidized and reduced rusticyanin, respectively. Fig. 6 shows a kinetic trace of the Fe(II)-dependent, tetracyano(1,10-phenanthroline)ferrous-catalyzed reduction of rusticyanin, while Fig. 69 shows an example of the Fe(II)-dependent, tetracyano(4-chloro-1,10-phenanthroline)ferrous-catalyzed oxidation of rusticyanin. Neither of the kinetic traces in Fig. 6 could be described mathematically as a simple exponential function of time. Instead, each time-dependent change in absorbance at 597 nm was analyzed using a classical Michaelis-Menten kinetic treatment. The sequence of events in Equation 4 may be seen as a double displacement Ping Pong Bi Bi kinetic mechanism where the cyanophenanthrolineiron derivative cycles between the oxidized and the reduced states. Applying the usual steady-state approximation to the concentration of the catalyst in the example given in Equation 4, it is evident that:

\[
\text{RCu}^{2+} + \text{Fe}^{3+} \rightarrow \text{RCu}^{3+} + \text{Fe}^{2+}
\]

where \( k_{\text{Fe}^{2+}} \) and \( k_{\text{ox}} \) are the second order rate constants for the Fe(II)-dependent reduction of the cyanophenanthrolineiron(II) and the cyanophenanthrolineiron(III)-dependent oxidation of the rusticyanin, respectively.

\[
\frac{d[\text{Cu}^{2+}]}{dt} = \frac{k_{\text{Fe}^{2+}}[\text{Fe}^{3+}]}{k_{\text{ox}}[\text{RCu}^{3+}]}
\]

where \( k_{\text{Fe}^{2+}} \) and \( k_{\text{ox}} \) are the second order rate constants for the Fe(II)-dependent oxidation of the cyanophenanthrolineiron(III) and the cyanophenanthrolineiron(II)-dependent reduction of the rusticyanin, respectively. If the change in the concentration of Fe(II) is assumed to be negligibly small, Equation 5 may be rearranged and integrated over the usual limits to yield:

\[
\ln(A_{\text{Am}}/A_{\text{At}}) = \frac{V}{k_{\text{ox}}} - \frac{V}{k_{\text{Fe}^{2+}}} t
\]

where \( V = \frac{k_{\text{Fe}^{2+}}}{k_{\text{ox}}}[\text{Fe}^{3+}]/[\text{RCu}^{2+}] \) is the absorbance at time \( t \) minus that at the end of the reaction \( (A_{\text{Am}} - A_{\text{At}}) \), and \( A_{\text{Am}} \) is the total absorbance change observed \( \Delta A_{\text{Am}} \). Selected data points that spanned \( \Delta A_{\text{Am}} \) of the total absorbance change in Fig. 6 were used to construct the linear plot according to Equation 6 shown in the inset to Fig. 69. A corresponding value for \( k_{\text{ox}} \) was then readily calculated from this value of \( V \) using the appropriate value for \( k_{\text{Fe}^{2+}} \) in Table 1. If the cyanophenanthrolineiron were employed to catalyze the Fe(II)-dependent oxidation of the rusticyanin, the equivalent to Equation 5 is:

\[
\frac{d[\text{Cu}^{3+}]}{dt} = \frac{k_{\text{Fe}^{2+}}[\text{Fe}^{3+}]}{k_{\text{ox}}[\text{RCu}^{3+}]}
\]

where \( k_{\text{Fe}^{2+}} \) and \( k_{\text{ox}} \) are the second order rate constants for the Fe(II)-dependent oxidation of the cyanophenanthrolineiron(II) and the cyanophenanthrolineiron(II)-dependent reduction of the rusticyanin, respectively. If the change in the concentration of Fe(II) is assumed to be negligibly small, Equation 7 may be rearranged and integrated over the usual limits to yield:

\[
\frac{\ln(A_{\text{Am}}/A_{\text{At}})}{V} = \frac{V}{k_{\text{ox}}} - \frac{V}{k_{\text{Fe}^{2+}}} t
\]

where \( V = \frac{k_{\text{Fe}^{2+}}}{k_{\text{ox}}}[\text{Fe}^{3+}]/[\text{RCu}^{2+}] \) is the change in the total absorbance of the rusticyanin. Selected data points that spanned \( \Delta A_{\text{Am}} \) of the total absorbance change in Fig. 6g were used to construct the linear plot according to Equation 6 shown in the inset to Fig. 6g. A value for \( k_{\text{ox}} \) was then obtained from the linear plot in the inset in a manner analogous to that described above.