MECHANISMS OF ELECTRON TRANSFER IN CYTOCHROMES AND BLUE COPPER PROTEINS

Thesis by

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ABSTRACT

The mechanisms of electron transfer in cytochromes and blue copper proteins are investigated by examining the oxidation and reduction of horse heart cytochrome _c_, _Pseudomonas aeruginosa_ cytochrome $\underline{c}_{551}$, _Rhus vernicifera_ stellacyanin, _Phaseolus vulgaris_ plastocyanin and _Pseudomonas aeruginosa_ azurin by inorganic redox agents. The rates of electron transfer in these systems have been analyzed within the framework of the relative Marcus theory of outer sphere electron transfer. The activation parameters for these processes have also been examined.

The reduction of _Pseudomonas aeruginosa_ cytochrome $\underline{c}_{551}$, by $Fe(EDTA)^{2-}$ has been investigated. The electrostatics-corrected self-exchange rate constant is similar to that obtained for horse heart cytochrome _c_. The close correspondence indicates that the two proteins employ similar electron transfer mechanisms. It is proposed that this mechanism involves reagent contact and little protein conformational change at the partially exposed heme edge.

The oxidation of the blue copper proteins by tris complexes of 1,10-phenanthroline and its 5-chloro, 5,6-dimethyl, 4,7-dimethyl, and 4,7-diphenyl-4′-sulfonate derivatives with cobalt (III) has been performed. The reactivity order at 25°C (pH 7.0) is stellacyanin > plastocyanin > azurin for oxidation by $Co(phen)_3^{3+}$. This order matches that found previously for $Fe(EDTA)^{2-}$ reduction of the blue copper proteins. It is suggested that the activation parameters for

electron transfer from reduced plastocyanin and azurin to $Co(phen)_3^{3+}$ may be accounted for in terms of oxidant-induced protein structural changes which expose sites that are, by comparison with stellacyanin, inaccessible to reagent attack. Arguments based on blue protein reduction potentials, calculated protein self-exchange electron transfer rate constants, and isokinetic correlations among acitvation parameters for the series of cobalt (III) oxidants containing 1,10-phenthroline derivatives, are presented in support of this hypothesis.

The reductions of cytochromes and blue copper proteins by $Fe(HEDTA)^-$ have been investigated. Marcus theory analysis indicates that the reactivities of $Fe(HEDTA)^-$ and $Fe(EDTA)^{2-}$ are similar.

The anion effects on the reduction have also been examined. The data have been analyzed in terms of a mechanism of electron transfer involving $Fe(HEDTA)^-$ attack on a protein-anion complex. Equilibrium constants and rates of reduction have been presented. Rate enhancement was observed for all reactions in the presence of anions of π-symmetry, azide and thiocyanate, whereas no effect was apparent for anions of σ-symmetry, chloride and sulfate.

The oxidations of cytochromes and blue copper proteins by $Co(EDTA)^-$ and $Co(HEDTA)X^-$ complexes (where X = NO_2, Cl, Br) have been investigated. The application of Marcus theory indicates that similar activation processes are involved in the $Co(EDTA)^-$ oxidations and $Fe(EDTA)^{2-}$ reductions of these proteins. $Co(EDTA)^-$

is able to penetrate closer to the redox center in cytochrome $\underline{c}_{551}$ than $Fe(EDTA)^{2-}$ as evidenced by the increased cross reaction rate over the calculated value and more positive activation parameters. Azurin employs a more favorable mechanism of electron transfer in the oxidation by $Co(EDTA)^-$ than in the reduction by $Fe(EDTA)^{2-}$ characterized by low activation parameters generally associated with tunneling. The room temperature reactivity for $Co(HEDTA)X^-$ oxidation increases according to $Co(EDTA)^- < Co(HEDTA)NO_2^- < Co(HEDTA)Cl^- < Co(HEDTA)Br^-$. The rates of oxidation are enhanced upon substitution of ligands with more diffuse π systems.

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CHAPTER 1. INTRODUCTION

The kinetics of electron transfer reactions are currently under investigation in order to determine the mechanisms employed by small molecule reagents as well as complex metalloproteins.[1,2] The redox proteins adopt a wider variety of mechanisms compared to small reagents owing to the greater anisotropy of the proteins. Of particular interest are experiments designed to provide information concerning the symmetry and distance dependence of electron transport.

Electron transfer reactions of inroganic complexes have been classified as inner or outer sphere depending on the metal coordination geometry.[3] An outer sphere process is one in which the first coordination sphere of the metal remains intact while an inner sphere process involves a transition state in which the two metal ions are connected through a bridging ligand. The relative Marcus theory[4] has been developed to interpret the kinetics of outer sphere electron transfer reactions.

This thesis will be concerned with a determination of the mechanism of electron transfer in cytochromes and blue copper proteins. Specific experiments involve the measurement of cross reaction rates and activation parameters for electron transfer between small molecule reagents and metalloproteins. The modification of the inorganic complexes will be attempted in order to determine the effects of symmetry and distance on the reactions. Data will be analyzed within the framework of the relative Marcus theory for outer sphere electron transfer.

Marcus theory provides a method of correlating the cross reaction rate constant (k_{12}) with the self-exchange rate constants for

the two reactants (k_{11} and k_{22}) and the equilibrium constant for the reaction (K_{12}) as described in equation (1)[5]

$$k_{12} = (k_{11}k_{22}K_{12}f_{12})^{\frac{1}{2}} \quad (1)$$

where

$$\log f_{12} = (\log K_{12})^2/[4 \log(k_{11}k_{22}/Z^2)] \ .$$

The collision frequency (Z) is approximately equal to $10^{11}\ M^{-1}\ s^{-1}$. The factor f_{12} will be approximately equal to unity when either $(\log K_{12})^2$ or $k_{11}k_{22}$ is negligible, resulting in equation (2).

$$k_{12} \sim (k_{11}k_{22}K_{12})^{\frac{1}{2}} \quad (2)$$

There are many assumptions inherent in the Marcus theory which must be presented. It is an adiabatic theory of electron transfer which implies that, within the activated complex, the probability of electron transfer is unity.[6] It also assumes that the work terms for the self-exchange and cross reactions are the same.[7] The electron transfer reagents are treated as spherical reactants employing the same mechanism of electron transfer in the self exchange and cross reactions.[8] In deriving equations (1) and (2) it has been further assumed that no very rapid pre-equilibrium changes take place prior to the electron transfer step.[9] The motions of solvent and inner coordination shells are also considered to be harmonic in the oxidized and reduced states.[10]

This theory has been applied successfully in calculating the electron transfer cross reaction rate constants (k_{12}) of a number of inorganic systems.[11,12] More recently, Sutin and coworkers[13] have

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This theory has been applied successfully in calculating the electron transfer cross reaction rate constants (k_{12}) of a number of inorganic systems.[11,12] More recently, Sutin and coworkers[13] have

examined several outer sphere electron transfer reactions which are not consistent with Marcus theory owing to a breakdown in the assumptions.

Electrostatic interactions between the two reacting species have been ignored in deriving equations (1) and (2) due to the fact that most simple inorganic reactions involve electron transfer between reagents of similar charge and size.[14] This is not the case for protein reactions where the corrections for electrostatic interactions must be included.[15] The specific equations which will be used to calculate the electrostatic work terms will be presented in Appendix I.

Almost all organisms are dependent on electron transport chains for the production of adenosine triphosphate (ATP). Electron transport proteins may be classified as either isopotential electron carriers if they transfer electrons between the sites of energy conservation or non-isopotential if they are capable of conserving a change in the free energy as ATP.[16] The cytochromes c and blue copper proteins to be discussed here are all isopotential electron carriers.

Cytochromes c are a group of proteins containing an iron protoporphyrin IX redox center (Figure 1) which is covalently attached to the polypeptide backbone by at least one thioether bond to a cysteine residue.[17] This thesis will be concerned with a comparison of the reactivity of horse heart cytochrome c and Pseudomonas aeruginosa cytochrome c_{551}. Horse heart cytochrome c functions as the electron carrier between the membrane bound cytochrome c_1 and cytochrome oxidase proteins[18] (Figure 2). Cytochrome c_{551} serves as the physio-

Figure 1. The structural formula of heme c, showing attachment to the protein from rings 1 and 2.

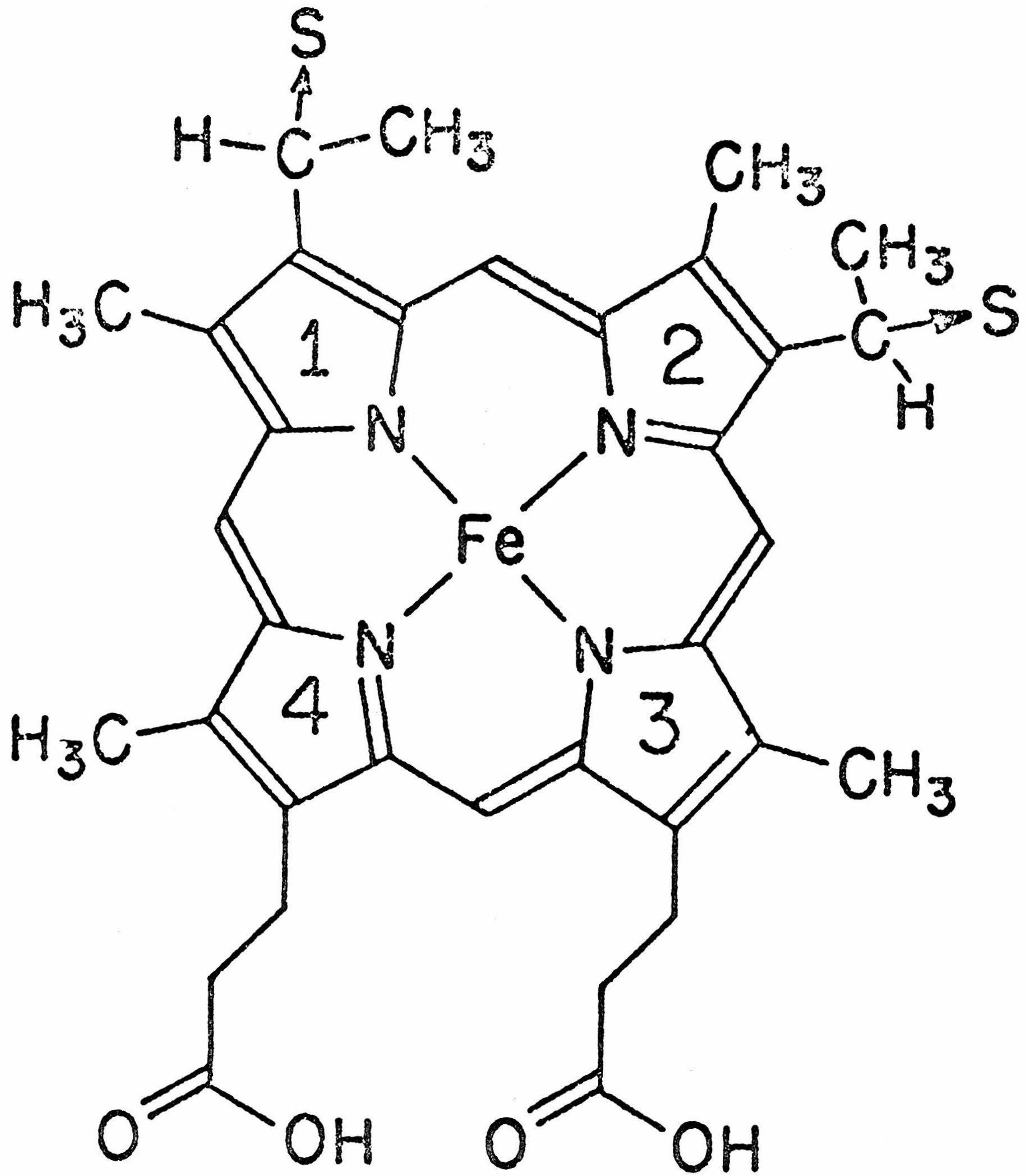
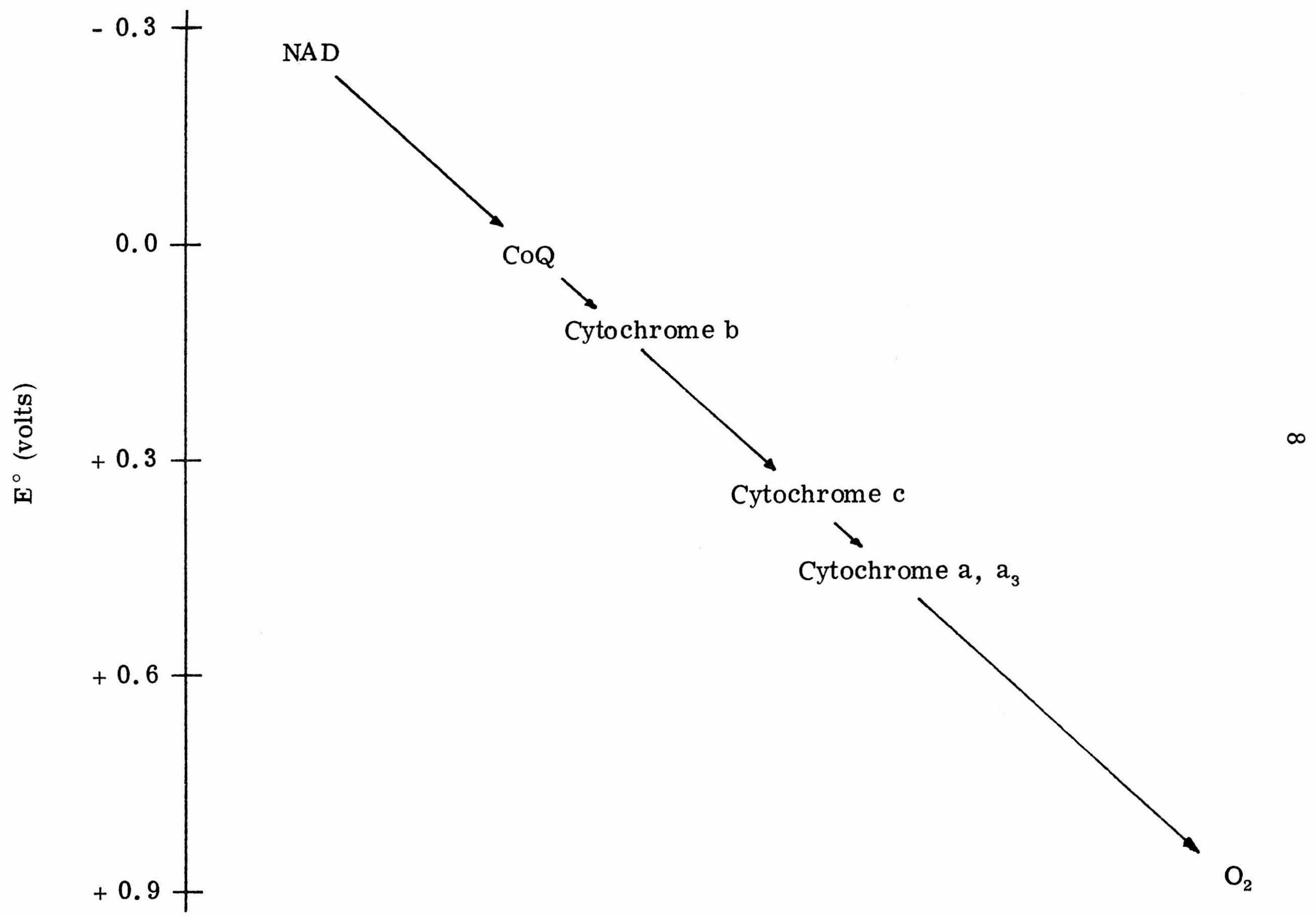

S
H
C
CH3
CH3
CH3
C
S
H
H3C
1
2
N
N
Fe
N
N
4
3
H3C
CH3
O
OH
O
OH

Figure 2. The position of cytochrome c in the respiratory chain.

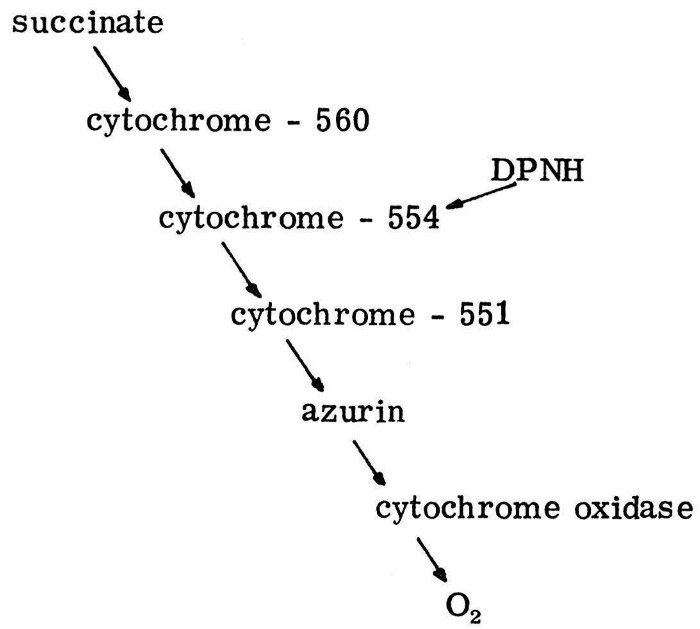

- 0.3
0.0
+ 0.3
+ 0.6
+ 0.9
E° (volts)
NAD
CoQ
Cytochrome b
Cytochrome c
Cytochrome a, a_3
O_2

logical reductant of azurin in the respiratory chain of Pseudonomas aeruginosa (Figure 3).[19]

The positively charged horse heart cytochrome c has the same reduction potential as the negatively charged bacterial protein.[20] The X-ray crystal structures of both of these proteins have been completed by Dickerson and coworkers.[21,22] The analysis reveals that the bacterial protein has approximately twenty fewer amino acids which correspond to the residues from 38 to 57 on the horse heart protein. However, the distance from the partially exposed heme edge to the surface does not vary appreciably in the two proteins.[23]

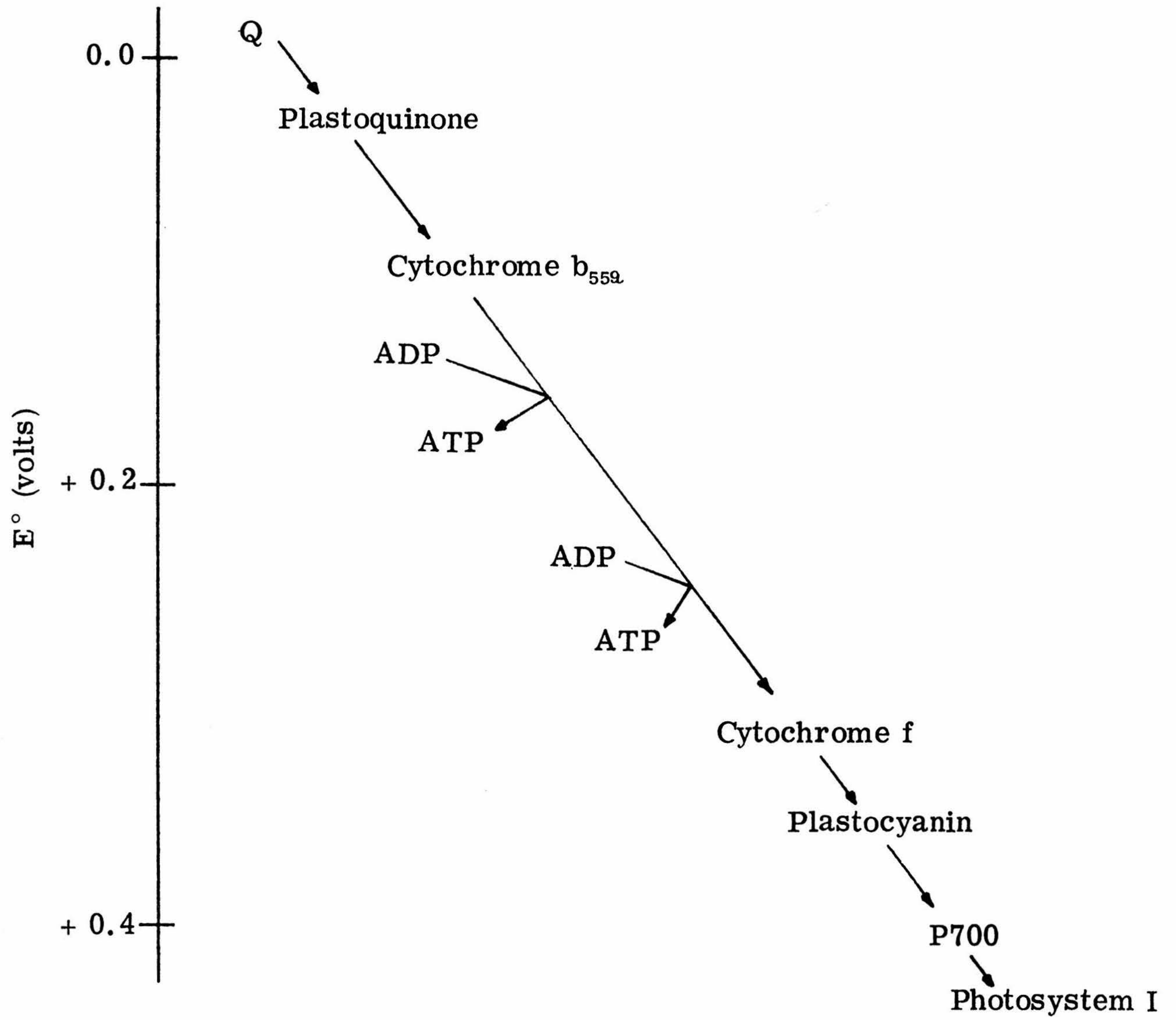
The physical properties and electron transfer reactions of horse heart cytochrome c have been extensively investigated owing to the availability and relative stability of the protein. The reduction by dithionite[24] and oxidation by ferricyanide[25] showed evidence of electrostatic binding of these reagents to the protein. The chromous ion reduction of cytochrome c involves inner sphere attack at the iron atom.[26] More recently, the electron transfer reactivities of cytochrome c with water soluble, outer sphere inorganic reductants and oxidants which are stable under physiological conditions have been examined and analyzed according to the relative Marcus theory.[27,28] The results are consistent with a mechanism involving reagent attack at the partially exposed heme edge.

In comparison, the mechanism of electron transfer of cytochrome c_{551} is not well understood. The dithionite reduction[29] and ferricyanide oxidation[30] have been reported. The outer sphere oxidation of the bacterial protein by Cobalt (III) (1,10 phenanthroline)$_3^{3+}$ indicates that



succinate
cytochrome - 560
DPNH
cytochrome - 554
cytochrome - 551
azurin
cytochrome oxidase
O_2

Figure 4. The position of plastocyanin in the redox chain between Photosystems I and II of green leaves.


E° (volts)
0.0
+ 0.2
+ 0.4
Q
Plastoquinone
Cytochrome b559
ADP
ATP
ADP
ATP
Cytochrome f
Plastocyanin
P700
Photosystem I

Resonance raman spectral data indicate a four[41] or five[42] coordinate model for the blue copper site composed of one sulfur and three or four nitrogen ligands. Nuclear magnetic resonance (NMR)[43] and electron-nuclear double resonance (ENDOR) studies[44] also suggest the presence of nitrogen ligands. A peptide nitrogen ligand has been proposed for bean plastocyanin as a result of infrared (IR) spectral data (Figure 5).[45]

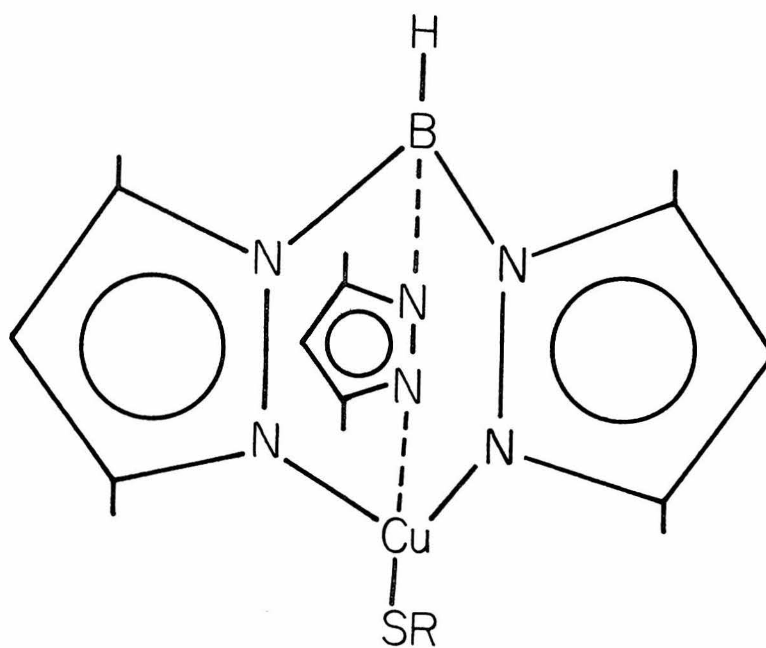
Inorganic active site analogs for the blue copper proteins have been difficult to prepare and characterize primarily due to the disproportionation of copper (I). The first well-defined synthetic analogs of the proposed binding sites have been reported recently.[46] They are of the form $CuN_3(SR)$ (Figure 6) where N_3 is the hydroxytris(3,5-dimethyl-1-pyrazolyl)borate ligand and RS is p-nitrobenzenethiolate or o-ethylcysteinate. The spectrochemical characteristics of the $Cu^{II}N_3(SR)$ species are very similar to those observed for the blue copper proteins.

The relative reactivity of the blue copper proteins as determined by the $Fe(EDTA)^{2-}$ reductions indicate that stellacyanin $>$ plastocyanin $>$ azurin.[47] This has been interpreted in terms of the accessibility of the redox center to inorganic reactants, with stellacyanin as the most accessible. This thesis will be concerned with the determination of the mechanism of oxidation of the blue copper proteins and the examination of the symmetry and distance dependence of electron transfer in these systems.

Figure 5. Structural model for the blue copper center in bean plastocyanin.

HN
N
38
N
Cu
HN
N
S
88
85

Figure 6. Structure of the $CuN_3(SR)$ Active Site Analogs.


H
B
N
N
N
N
N
N
Cu
SR

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CHAPTER 2. KINETICS STUDIES OF THE REDUCTION OF *PSEUDOMONAS AERUGINOSA* FERRICYTOCHROME *C*$_{551}$ BY $Fe(EDTA)^{2-}$

The kinetics of reduction of horse heart ferricytochrome c by $Fe(EDTA)^{2-}$ have been studied previously.[1] The measured second order rate constant of 2.6×10^4 M^{-1} s^{-1} [25°, μ 0.1 M, pH 7.0 (phosphate)] has been used to calculate an electrostatics-corrected, self-exchange rate constant, k_{11}^{corr}, for the protein of 6 M^{-1} s^{-1}.[2] This latter value is somewhat smaller than the experimental k_{11} (extrapolated to the same conditions) of 2.8×10^2 M^{-1}, which has led to the proposal that $Fe(EDTA)^{2-}$ has difficulty in penetrating the protein surface in the vicinity of the partially exposed heme edge.

As this proposal depends on the reliability of a calculation based on a cross reaction between a positively-charged[3] protein and a negatively-charged substrate, we have turned to negatively-charged[4,5] ferricytochrome c_{551} from Pseudomonas aeruginosa, whose three-dimensional structure is very similar to that of the horse heart protein.[3,6,7] Accordingly, we report herein the results of a kinetic study of the reduction of ferricytochrome c_{551} by $Fe(EDTA)^{2-}$. The mechanistic meaning of some striking similarities and differences in the reactivities of the two cytochromes c with $Fe(EDTA)^{2-}$ and $Co(phen)_3^{3+}$ is considered.

Methods: Cytochrome c_{551} from Pseudomonas aeruginosa was purified according to the method of Ambler and Wynn[5] to a ratio A_{551}/A_{280} of 1.2. Reagent grade chemicals and deionized-distilled water were used throughout. The nitrogen gas used for deoxygenation of solutions for kinetic studies was passed through two chromous scrubbing towers to eliminate impurity oxidants. Ferrous EDTA

solutions were prepared as previously described[8] with the concentration of EDTA in 20% excess over iron to insure complete formation of the $Fe(EDTA)^{2-}$ complex. The reduction of ferricytochrome $\underline{c}_{551}$ was followed at 551 nm. All data were analyzed as pseudo-first-order in protein with the reducing agent in 100- to 1000-fold excess.

All kinetic experiments were performed on a Durrum Model D-110 stopped-flow spectrometer. Solutions were allowed at least 20 minutes to come to temperature equilibrium prior to mixing. Temperature was controlled to ±0.2° by a Forma Scientific temperature bath. Data were sent to a Tektronix 564B storage oscilloscope and to an A/D converter. The A/D converter was used in conjunction with a PDP-10 computer to evaluate the observed rate constants. A Cary 17 spectrophotometer was used for all absorption spectral measurements and Brinkman pH 101 instrument was used for all pH determinations.

Results: First-order plots were linear for greater than 90% of the reduction of ferricytochrome $\underline{c}_{551}$ by $Fe(EDTA)^{2-}$. The dependence of observed rate constants on concentration of reductant at pH 7.0 is shown in Figure 1. The second-order rate constant is $4.2 \pm 0.1 \times 10^3$ $\underline{M}^{-1}$ s^{-1} [25°, μ 0.1 $\underline{M}$, pH 7.0 (phosphate)], and the activation parameters for the reduction of ferricytochrome $\underline{c}_{551}$ obtained from a standard Eyring plot (Figure 2) are as follows: $\Delta H^{\ddagger}$ 3.2 ± 0.1 kcal/mol and $\Delta S^{\ddagger}$ -30 ± 1 cal/mol-deg.

Discussion: The rate constant for reduction of ferricytochrome $\underline{c}_{551}$ by $Fe(EDTA)^{2-}$ is nearly a factor of ten smaller than that obtained

Figure 1. Concentration dependence of k_{obsd} for the reduction of ferricytochrome $\underline{c}_{551}$ by $Fe(EDTA)^{2-}$ [pH 7.0 (phosphate), $\mu = 0.1$ $\underline{M}$, T = 25°].

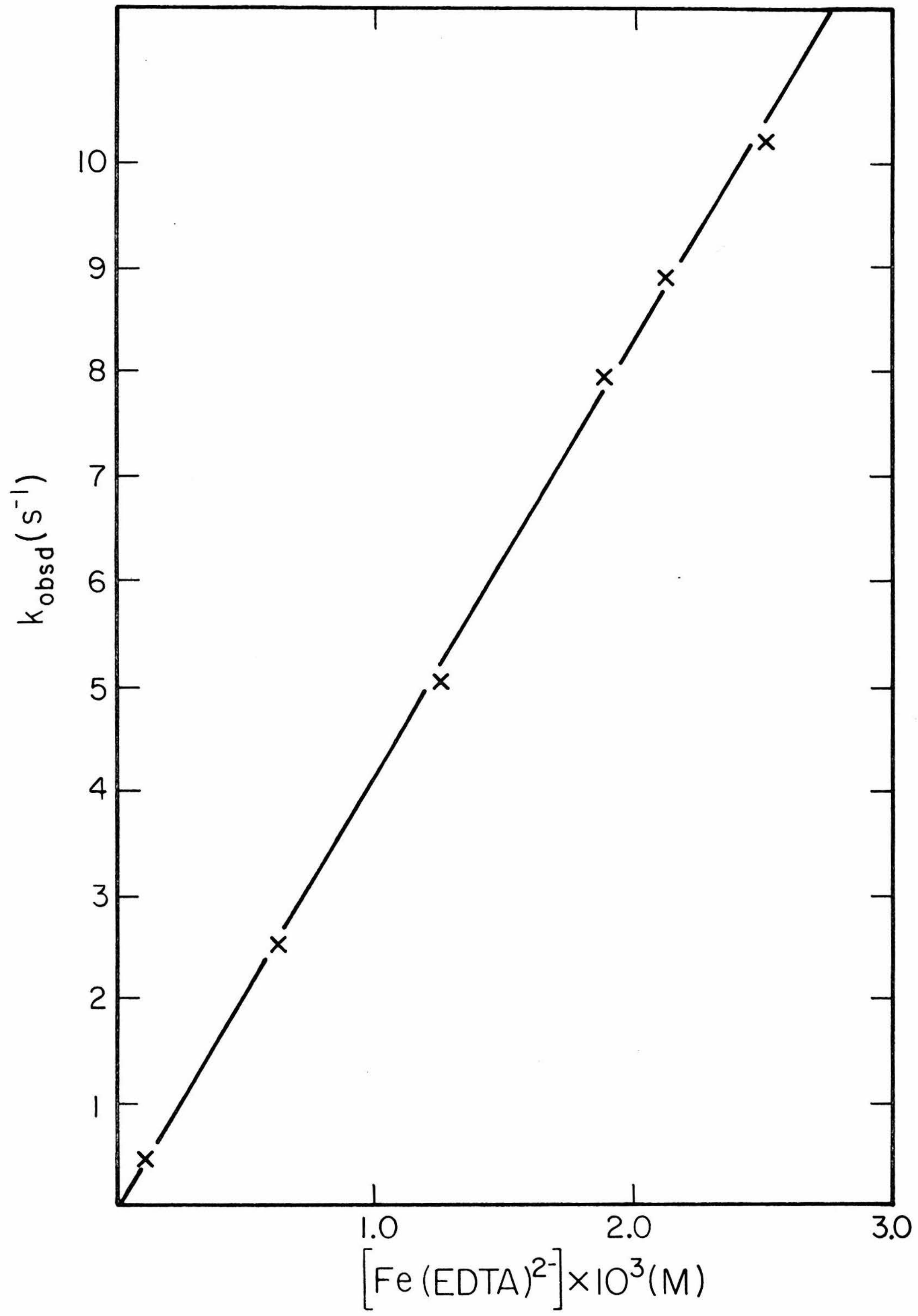
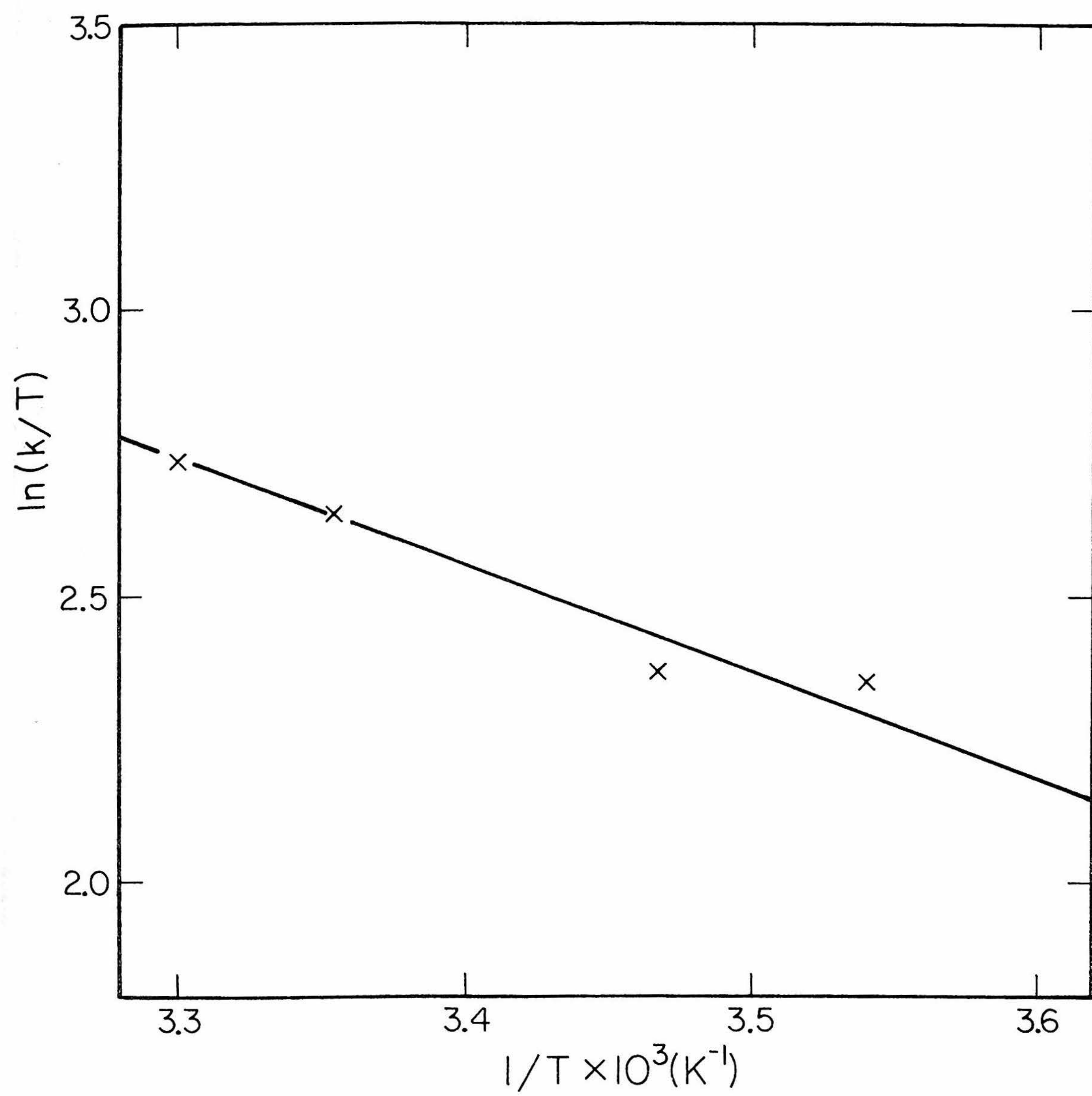

10
9
8
7
6
5
4
3
2
1
$k_{obsd}(s^{-1})$
1.0
2.0
3.0
$[Fe(EDTA)^{2-}] \times 10^3 (M)$

Figure 2. Eyring plot for the reduction of ferricytochrome $\underline{c}_{551}$ by $Fe(EDTA)^{2-}$ [pH 7.0 (phosphate), μ = 0.1 $\underline{M}$].


3.5
3.0
2.5
2.0
ln(k/T)
3.3
3.4
3.5
3.6
1/T ×10^3(K^{-1})

from the analogous horse heart protein reaction, which is not unexpected in view of the fact that the former pair of reactants are like-charged. The difference in rate in fact may well be entirely due to electrostatic charge effects, as both horse heart cytochrome c[3] and cytochrome c_{551}[9] have the same reduction potential (E 260 mV). To explore this point further, we have utilized a previously-developed model[2] to calculate the electrostatics-corrected self-exchange rate constant (k_{11}^{corr}) for cytochrome c_{551} based on the cross reaction with $Fe(EDTA)^{2-}$. The result is 2 M^{-1} s^{-1}, as compared to 6 M^{-1} s^{-1} for the corresponding k_{11}^{corr} for the horse heart protein. The parameters and work terms that were used in the cytochrome c_{551}-$Fe(EDTA)^{2-}$ calculation are as follows: $Fe(EDTA)^{-/2-}$, E 120 mV, k_{22} 3×10^4 M^{-1} s^{-1}, R 4 Å;[2] cytochrome c_{551}, E 260 mV, R 14.4 Å, Z -2/-3;[9] k_{12} 4.2×10^3 M^{-1} s^{-1}; w_{12} 0.194, w_{21} 0.146, w_{11} 0.079, w_{22} 0.493, $\Delta G_{11}{}^{*uncorr}$ 16.80, $\Delta G_{11}{}^{*corr}$ 17.03 kcal/mol.

The fact that the k_{11}^{corr} values for cytochrome c_{551} and horse heart cytochrome c based on $Fe(EDTA)^{2-}$ are virtually the same is entirely consistent with a mechanistic model in which the transition state for electron transfer features substrate contact with the protein at a point on the surface near the partially exposed heme edge. If the substrate were not able to penetrate the surface (and induce a conformational change) of the protein, the electrostatics-corrected electron transfer rate would simply be a function of the distance of closest approach to the heme edge. It may be concluded from our kinetic analysis that this closest approach distance is approximately the same

in the $Fe(EDTA)^{2-}$ reduction of the two cytochromes. In this connection, it is of interest to note that the X-ray structural studies have indicated that the distance from the surface to the partially exposed heme edge does not vary appreciably in the two proteins.[7] Apparently, the hydrophilic nature of most of the surface of $Fe(EDTA)^{2-}$ makes it a particularly poor substrate insofar as penetration into the hydrophobic interior of a protein is concerned.

The situation is markedly different in the $Co(phen)_3^{3+}$ reactions with ferrocytochrome $\underline{c}_{551}$ and horse heart ferrocytochrome $\underline{c}$.[10] Here the calculated k_{11}^{corr} for the bacterial protein (1.8×10^5 $\underline{M}^{-1}$ s^{-1}) is over 10^2 times larger than that (7.1×10^2 $\underline{M}^{-1}$ s^{-1}) for cytochrome $\underline{c}$.[9] As both of these k_{11}^{corr} values are substantially larger than the corresponding quantities based on the $Fe(EDTA)^{2-}$ reactions, it is likely that there are favorable nonelectrostatic $Co(phen)_3^{3+}$-protein interactions in the transition state for electron transfer. These interactions probably involve the phenanthroline rings and hydrophobic protein residues, as proposed previously.[10] As cytochrome $\underline{c}_{551}$ has a shorter polypeptide chain[4,5] than cytochrome $\underline{c}$[3] (82 amino-acid residues vs. 104), and that as a result both the composition and flexibility of the chain near the partially exposed heme edge are likely to be altered, it is hardly surprising, given a model including strong nonelectrostatic substrate-protein interactions, that the k_{11}^{corr} values are quite different. It could not have been predicted in advance that the k_{11}^{corr} for cytochrome $\underline{c}_{551}$ would have been larger than that for cytochrome $\underline{c}$, but the fact that it is allows us to conclude that $Co(phen)_3^{3+}$ is able to penetrate and contact the redox center in the bacterial protein quite effectively.

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CHAPTER 3. KINETICS STUDIES OF THE OXIDATION OF BLUE COPPER PROTEINS BY TRIS(1,10-PHENANTHROLINE)COBALT(III) IONS[1]

Copper proteins containing only type 1 (or blue) sites function as electron carriers.[2] Although X-ray crystallograhpic results are not presently available for any of the blue proteins, detailed structural models are emerging for the type 1 copper site. The intense absorption near 600 nm characteristic of blue proteins has been assigned as a cysteine sulfur to copper(II) charge transfer transition, and a number of physical studies have provided evidence that the metal atom occupies a slightly flattened tetrahedral binding site.[3-5] It should also be noted that magnetic resonance[6-11] and fluorescence[12] measurements strongly suggest that in at least some blue proteins the copper atom lies within a solvent-inaccessible hydrophobic environment.

Kinetic parameters for electron transfer from the nonphysiological reductant $Fe(EDTA)^{2-}$ to blue copper proteins have recently been reported.[13,14] Large second-order rate constants and minimal activation enthalpies for the reduction of stellacyanin ($k = 4.3 \times 10^5$ M^{-1} s^{-1}, 25 °C, pH 6.9, $\mu = 0.5$ M; $\Delta H^{\ddagger} = 3.0$ kcal/mol, $\Delta S^{\ddagger} = -21$ cal/(mol deg), pH 7.0, $\mu = 0.1$ M), bean plastocyanin ($k = 8.2 \times 10^4$ M^{-1} s^{-1}, 25 °C, pH 6.9, $\mu = 0.2$ M; $\Delta H^{\ddagger} = 2.1$ kcal/mol, $\Delta S^{\ddagger} = -29$ cal/(mol deg)), and Pseudomonas aeruginosa azurin ($k = 1.3 \times 10^3$ M^{-1} s^{-1}, 25 °C, pH 7.0, $\mu = 0.2$ M; $\Delta H^{\ddagger} = 2.0$ kcal/mol, $\Delta S^{\ddagger} = -37$ cal/(mol deg)) have been interpreted as an indication that there is little activation requirement for either protein or inner-sphere rearrangement.[2,13] It has been suggested that the wide range of electron transfer reactivity of the blue copper proteins reflects variations in the accessibility of the active site to outer sphere contact

with external redox agents, and that the large negative apparent entropies of activation may be attributable to some nonadiabaticity in the electron transfer from $Fe(EDTA)^{2-}$ to the Cu(II) centers.[2,14]

To explore the electron transfer reactivity of blue proteins further, we have investigated the oxidation of reduced stellacyanin, plastocyanin, and azurin by tris complexes of 1,10-phenanthroline and several of its derivatives with cobalt(III). By utilizing substitution in the 4, 5, 6, and 7 positions of the phenanthroline ring system, we have been able to evaluate the reactivity of the reduced blue proteins as influenced by systematic variations in the charge and molecular structure of the Co(III) oxidant complex.

Experimental Section

Reagent grade chemicals and deionized distilled water were used throughout. Nitrogen gas passed through two chromous scrubbing towers was used to deoxygenate kinetics solutions. *Rhus vernicifera* stellacyanin, bean plastocyanin, and *Pseudomonas aeruginosa* azurin were isolated and purified as previously described.[13,14]

Solutions of reduced plastocyanin and azurin were prepared by adding a 20-fold excess of $Fe(EDTA)^{2-}$ to deoxygenated, buffered solutions of the cupric proteins. The excess reductant was then removed by dialysis against deoxygenated buffer using a hollow fiber Dow beaker dialyzer obtained from Bio-Rad Laboratories. As reduced stellacyanin was readily oxidized by traces of O_2 which could not be excluded using this technique, solutions of cuprous stellacyanin were prepared by adding an equivalent amount of ascorbic acid to the cupric

protein without removing the dehydroascorbate product. Dehydroascorbate was shown to have no influence on the rate of oxidation of stellacyanin(I) by $Co(phen)_3^{3+}$ in concentrations up to 1×10^{-4} M (tenfold molar excess over protein concentration).

The various oxidant solutions were prepared by standard methods by J. V. McArdle.[15,16] Some kinetic runs with stellacyanin were performed using the perchlorate salt of $Co(phen)_3^{3+}$, prepared by the method of Schilt and Taylor.[17] Good agreement was found between observed rate constants obtained with the chloride and perchlorate salts. Sodium phosphate buffers were used throughout, and sodium chloride or ammonium sulfate was added to adjust the ionic strength to the desired level.

Kinetic data for the oxidation of stellacyanin were obtained by monitoring absorbance increases at 604 nm using a Durrum Model D-110 stopped flow spectrometer. Plastocyanin and azurin oxidations were monitored at 597 and 625 nm, respectively. Kinetic measurements were made under pseudo-first-order conditions by employing 10- to 100-fold excess of oxidant for protein concentrations of ca. 10 μM. Most data were assimilated in an analog to digital converter and transmitted directly to a PDP-10 computer for analysis. The data for the oxidation of stellacyanin by $Co(phen)_3^{3+}$ were collected by R. A. Holwerda as photographs of absorbance-time traces on a Tektronix Model 564 B storage oscilloscope. Observed rate constants (k_{obsd}) were evaluated as usual from the least-squares slopes of $\log(A_\infty - A_t)$ vs. time plots.

All spectral data were acquired on a Cary 17 UV-visible spectrophotometer. A Brinkman pH 101 instrument was used to make pH measurements.

Results

I. Oxidation of Cuprous Stellacyanin, Plastocyanin, and Azurin by $Co(phen)_3^{3+}$. First-order plots of absorbance-time data for the oxidation of azurin(I), plastocyanin(I), and stellacyanin(I) by $Co(phen)_3^{3+}$ were found to be linear for greater than 90% of the reactions. The dependence of observed rate constants on $[Co(phen)_3^{3+}]$ is illustrated in Figure 1 for data collected at 25 °C (pH 7.0). In each case the experimental results are compatible with the rate law:

$$\frac{d[\text{type 1 Cu(II)}]}{dt} = k[\text{type 1 Cu(I)}][Co(phen)_3^{3+}]$$

over the tenfold range in oxidant concentration accessible in phosphate media. Second-order rate constants obtained from the least-squares slopes of k_{obsd} vs. $[Co(phen)_3^{3+}]$ plots are summarized in Table I, along with activation parameters derived from linear Eyring plots of ln (k/T) vs. 1/T (Figure 2). Rate parameters evaluated for stellacyanin in acetate (μ = 0.5 M, pH 5.1) and in phosphate (μ = 0.5 M, pH 7.0; μ = 0.1 M, pH 7.0) buffers are in good agreement.

Data describing the dependence of the oxidation rate of the proteins on ionic strength at pH 7.0 are assembled in Table II. The rates of oxidation of stellacyanin and azurin are observed to be nearly independent of ionic strength over the interval 0.05-0.5 M, whereas

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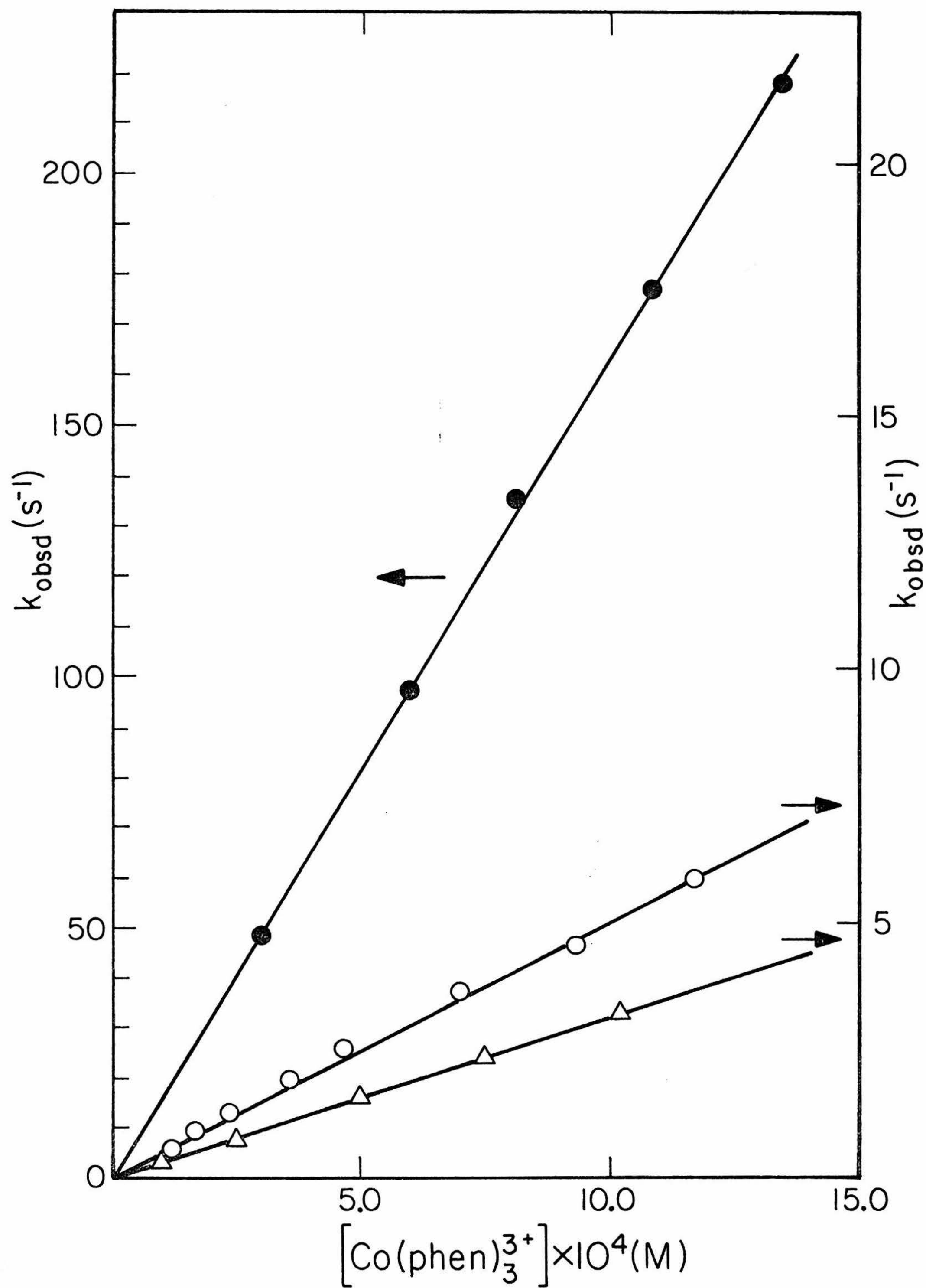
Figure 1. The dependences of observed rate constants on the concentration of $Co(phen)_3^{3+}$ at 25°, pH 7.0 (phosphate): stellacyanin, ● (μ = 0.5 M (phosphate)); plastocyanin, ○ (μ = 0.1 M ($(NH_4)_2SO_4$)); azurin △ (μ = 0.2 M (NaCl)).



200
150
100
50
0
20
15
10
5
$k_{obsd}(s^{-1})$
$k_{obsd}(s^{-1})$
5.0
10.0
15.0
$[Co(phen)_3^{3+}]\times10^4(M)$

Figure 2. Eyring plots of the rate data for oxidations by $Co(phen)_3^{3+}$ at pH 7.0 (phosphate): stellacyanin, ● (μ = 0.5 $\underline{M}$ (phosphate)); plastocyanin, ○ (μ = 0.1 $\underline{M}$ ($(NH_4)_2SO_4$)); azurin, △ (μ = 0.2 $\underline{M}$ (NaCl)).

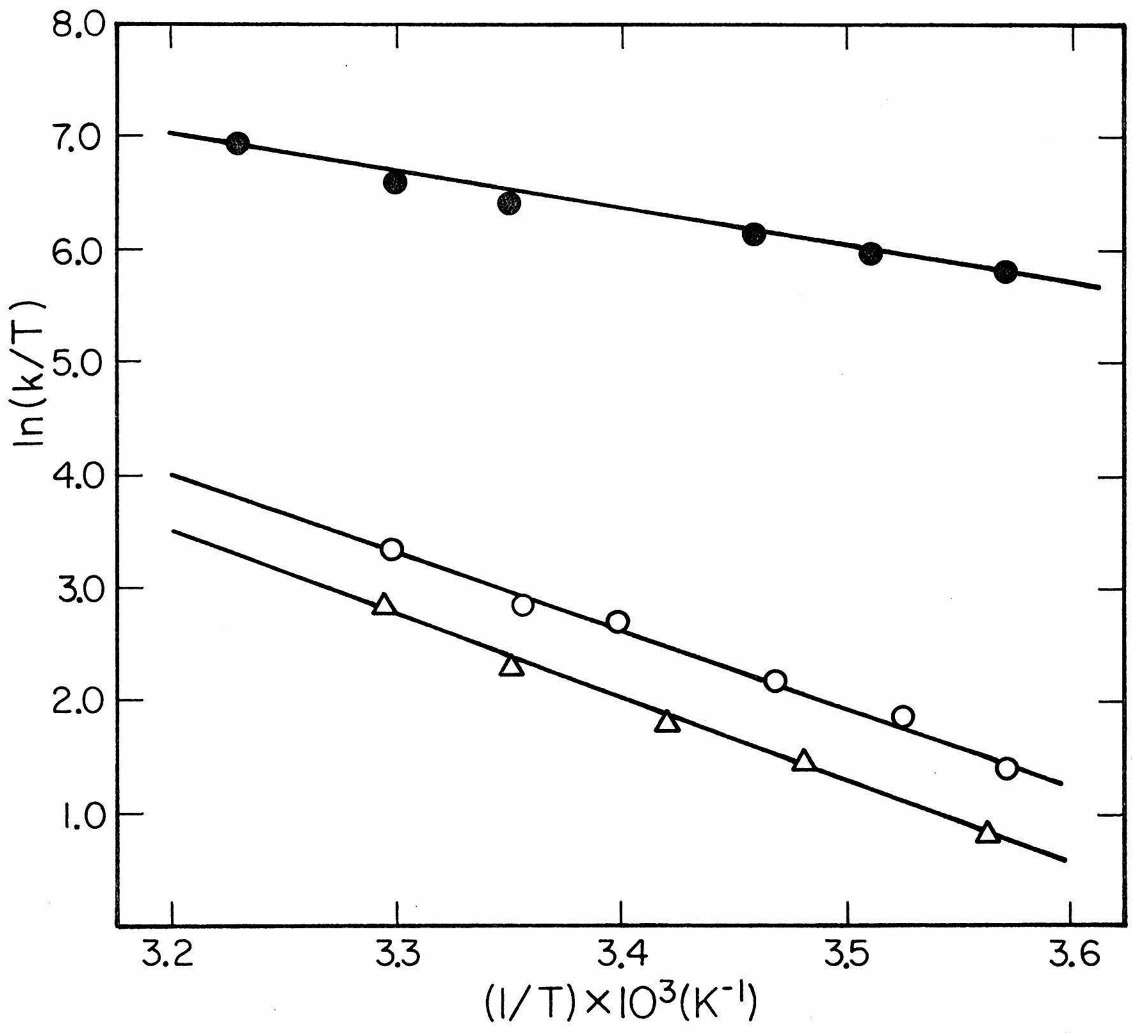

8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
ln(k/T)
3.2
3.3
3.4
3.5
3.6
(l/T)×10³(K⁻¹)

Table I. Kinetic Parameters for the Oxidation of Blue Copper Proteins at 25°C

Oxidizing agent	Stellacyanin[a]			Plastocyanin[e]			Azurin[f]		
	k M^{-1} s^{-1}	$\Delta H^{\ddagger}$, kcal/mol	$\Delta S^{\ddagger}$, cal/(mol deg)	k M^{-1} s^{-1}	$\Delta H^{\ddagger}$, kcal/mol	$\Delta S^{\ddagger}$, cal/mol deg)	k M^{-1} s^{-1}	$\Delta H^{\ddagger}$, kcal/mol	$\Delta S^{\ddagger}$, cal/(mol deg)
$Co(phen)_3^{3+}$	$1.80 (5) \times 10^5$	6.0 (2)	-13 (1)	$4.87 (5) \times 10^3$	14.0 (5)	5 (1)	$3.20 (5) \times 10^3$	14.3 (5)	5 (1)
	$1.8 (1) \times 10^5$ [b]	6.1 (2)	-14 (1)						
	$1.3 (1) \times 10^5$ [c]	5.9 (2)	-15 (1)						
$Co(5,6\text{-}Me_2phen)_3^{3+}$	$1.85 (5) \times 10^4$	9.5 (2)	-7 (1)	$7.97 (5) \times 10^3$	13.6 (5)	1 (1)	$1.54 (5) \times 10^3$	11.6 (5)	-5 (1)
$Co(5\text{-}Cl(phen))_3^{3+}$	d		-10 (1)	$6.96 (5) \times 10^2$	10.9 (5)	-8 (1)	$4.21 (5) \times 10^2$	8.9 (5)	-17 (1)
$Co[4,7\text{-}(PhSO_3)_2phen]_3^{3-}$	$2.31 (5) \times 10^6$			$2.59 (5) \times 10^1$	7.8 (5)	-26 (1)	d		
$Co(4,7\text{-}Me_2phen)_3^{3+}$	d			d			$8.41 (5) \times 10^1$	9.9 (5)	-17 (1)

[a] For pH 7.0 (phosphate), μ = 0.1 M (NaCl) unless otherwise specified.

[b] μ = 0.5 M (phosphate).

[c] pH 5.1 (acetate), μ = 0.5 M (acetate).

[d] Experimental determinations were not made.

[e] For pH 7.0 (phosphate), μ = 0.1 M ($(NH_4)_2SO_4$).

[f] For pH 7.0 (phosphate), μ = 0.2 M (NaCl).

Table II. Ionic Strength Dependences of $Co(phen)_3^{3+}$ Rate Parameters[a]

Protein	μ (M)	k ($M^{-1} s^{-1}$)	$10^3[Co(phen)_3^{3+}]$ (M)
Stellacyanin[b]	0.052	1.89×10^5	2.00
	0.194	1.89×10^5	2.00
	0.296	1.93×10^5	2.05
	0.390	1.92×10^5	1.50
	0.502	1.84×10^5	0.30-2.35
Plastocyanin[c]	0.05	2.49×10^0	0.56
	0.10	1.50×10^0	0.56
	0.20	8.11×10^{-1}	0.56
	0.30	5.95×10^{-1}	0.56
	0.50	4.75×10^{-1}	0.56
Azurin[c]	0.05	2.61×10^3	2.00
	0.08	2.20×10^3	2.00
	0.12	2.11×10^3	2.00
	0.16	2.11×10^3	2.00
	0.20	2.02×10^3	2.00
	0.05	2.43×10^3	1.00
	0.08	2.26×10^3	1.00
	0.12	2.08×10^3	1.00
	0.16	1.83×10^3	1.00
	0.20	1.89×10^3	1.00

[a] All values are the mean of at least two separate determinations.

[b] 25.3°C, pH 7.0 (phosphate).

[c] 25°C, pH 7.0 (phosphate).

the plastocyanin oxidation rate decreases slightly with increasing ionic strength.

II. Oxidations by Ring-Substituted Tris(1,10-phenanthroline)-cobalt(III) Ions. The rate law observed for the oxidation of reduced blue proteins by $Co(phen)_3^{3+}$ was found to pertain in most cases to the analogous reactions employing tris complexes of the 5-chloro, 4,7-dimethyl, 5,6-dimethyl, and 4,7-diphenyl-4′-sulfonate derivatives of 1,10-phenanthroline with Co(III) as oxidants. Kinetic parameters for oxidations by $Co(phen)_3^{3+}$ derivatives (Table I) exhibit striking variations in both second-order rate constants and activation parameters. Oxidation rate constants for the ring-substituted tris(1,10-phenanthroline)cobalt(III) ions are, with the exception of that for the stellacyanin(I)-$Co[4,7-(PhSO_3)_2\text{-phen}]_3^{3-}$ reaction, substantially smaller than the corresponding parameters for the unsubstituted oxidants.

Discussion

Comparison of kinetic parameters for the oxidation of reduced stellacyanin, plastocyanin, and azurin by $Co(phen)_3^{3+}$ reveals that the latter two proteins have similar reactivities, whereas stellacyanin falls into a distinctly different class. The room temperature reactivity sequence for $Co(phen)_3^{3+}$ oxidation is stellacyanin > plastocyanin > azurin, which matches that found[13] for the $Fe(EDTA)^{2-}$ reduction of the blue Cu(II) proteins. The azurin and plastocyanin oxidation pathways are characterized by abnormally large enthalpic activation requirements of ca. 14 kcal/mol, coupled with favorable entropies of activation amounting to 5 cal/(mol deg) in both cases. Stellacyanin, on the other

hand, prefers a pathway for which $\Delta H^{\ddagger}$ is smaller by ca. 8 kcal/mol and $\Delta S^{\ddagger}$ is more negative by ca. 20 cal/(mol deg). Activation parameters obtained for the reaction between $Co(phen)_3^{3+}$ and a low molecular weight reductant, $Co(terpy)_2^{2+}$ ($\Delta H^{\ddagger}$ = 6.6 kcal/mol, $\Delta S^{\ddagger}$ = -24 cal/mol deg); μ = 0.5 M = PH 7.0)[18] resemble those for the oxidation of stellacyanin much more closely than those for the oxidation of the other two blue proteins.

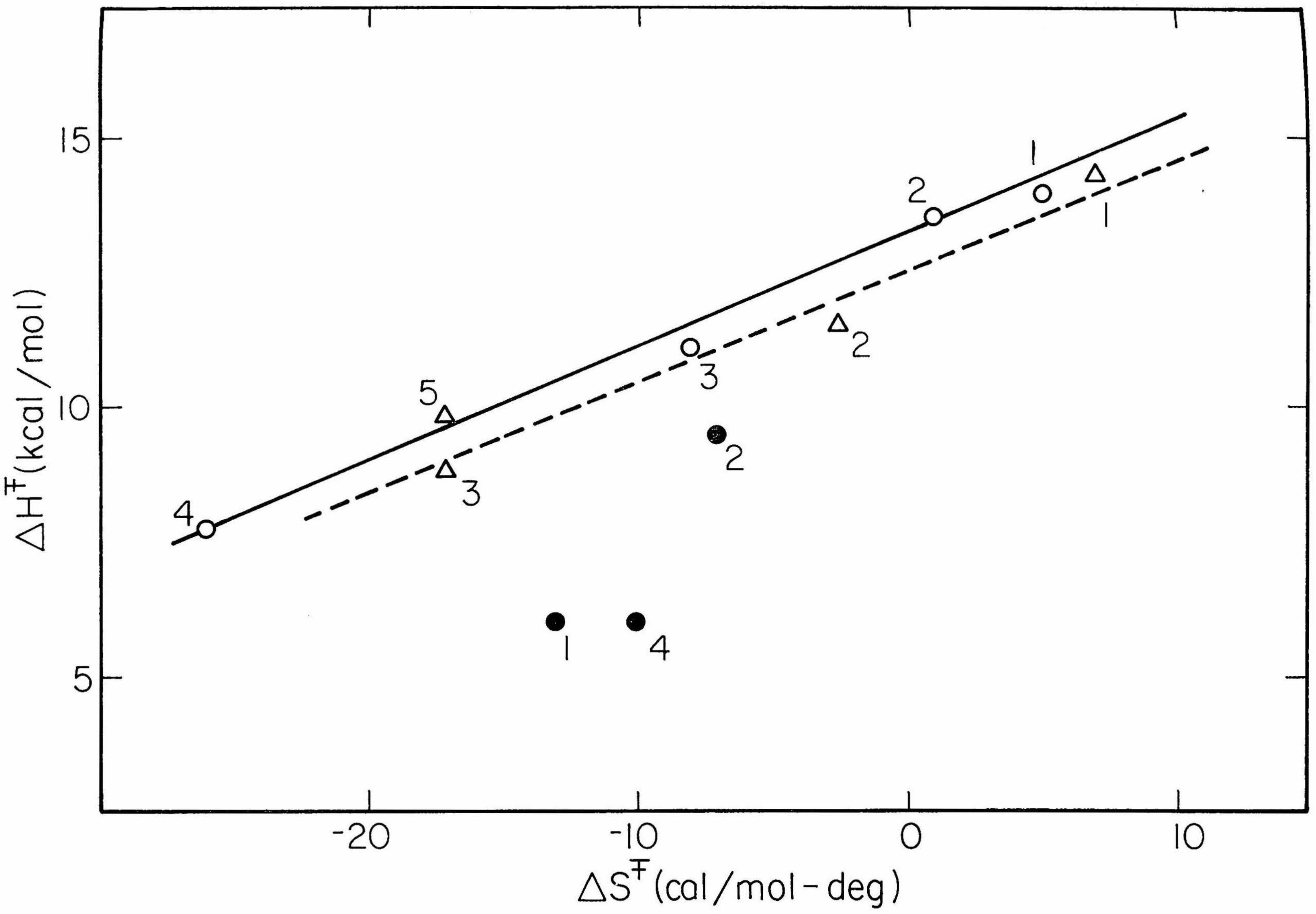
On the basis of the observed activation parameters we propose that the oxidation of stellacyanin by $Co(phen)_3^{3+}$ occurs by an adiabatic outer-sphere electron transfer mechanism. The entropy of activation (-13 cal/(mol deg)) is in good quantitative correspondence with expectations for the association contribution to $\Delta G^{\ddagger}$ resulting from the conversion of the separated reactants into a bimolecular collision complex.[19] Furthermore, the small difference in rate parameters for tris(1,10-phenanthroline)cobalt(III) and its 4,7-diphenyl-4′-sulfonate derivative suggests that electrostatic interactions between reduced stellacyanin and the external oxidant are of little importance in stabilizing the precursor complex for electron transfer. The insensitivity of the pH 7.0 oxidation rate to the charge carried by the oxidant is especially striking, as stellacyanin is a strongly basic metalloprotein (pI 9.86).[20] The fact that the stellacyanin oxidation rate varies very little over wide ranges in pH and ionic strength provides further support for the hypothesis that interactions between the oxidant and charged amino acid side chains are not an important feature of the electron transfer mechanism.

The activation parameters for the oxidation of plastocyanin and azurin may be accounted for in terms of structural changes in the reduced proteins which expose type 1 copper sites that are, by comparison with stellacyanin, inaccessible to attack by external redox agents. No conclusions based on kinetic results may be drawn regarding the position of the redox centers in the crystallized proteins; rather, the crucial factor apparently is the ability of $Co(phen)_3^{3+}$ and its derivatives to influence the protein conformation in such a way that overlap is established between donor and acceptor redox orbitals. A requirement for both normal Franck-Condon and substantial conformational activation is consistent with the unusually high experimental $\Delta H^{\ddagger}$ values. The entropic driving force for specific protein activation perhaps could be linked to hydrophobic interactions between the phenanthroline ligands and nonpolar amino acid side chains, which result in the disordering of water molecules held in the outer coordination sphere of $Co(phen)_3^{3+}$ as well as those on a portion of the surface of the reduced proteins. Activation parameters for the reduction of parsley plastocyanin by cytochrome f ($\Delta H^{\ddagger}$ = 10.5 kcal/mol, $\Delta S^{\ddagger}$ = 11 cal/(mol deg), pH 7.0, μ 0.1 M)[21] and for electron transfer between the physiological redox partners cytochrome $\underline{c}_{551}$ (II) and azurin(II) ($\Delta H^{\ddagger}$ = 10.6 kcal/mol, $\Delta S^{\ddagger}$ = 8.6 cal/(mol deg); pH 7.0, μ = 0.05 M phosphate)[22] are remarkably similar to those for the reactions of azurin(I) and plastocyanin(I) with $Co(phen)_3^{3+}$. In accord with our proposal, Wood has accounted for the positive entropy of activation for the cytochrome f(II)-plastocyanin (II) reaction in terms of unfolding of the proteins and breakage of ordered water structure.[21]

Plots of $\Delta H^{\ddagger}$ against $\Delta S^{\ddagger}$ for the oxidation of blue proteins by the series of tris(1,10-phenanthroline)cobalt(III) ions (Figure 3) reveal that good isokinetic correlations[23] exist among the activation parameters for azurin and plastocyanin but not for stellacyanin. Thus $\Delta H^{\ddagger}$ varies linearly with $\Delta S^{\ddagger}$, yielding β values (slopes) of 217 K for azurin (correlation coefficient 0.981) and 206 K for plastocyanin (correlation coefficient 0.992). Compensation in behavior has been documented for a number of protein unfolding or denaturation processes,[24,25] with β values typically falling somewhat higher (250-320 K)[24] than those reported here. That linear compensation plots are found for the oxidation of plastocyanin and azurin strongly suggests that the mechanism by which each of the reduced proteins transfers an electron to $Co(phen)_3^{3+}$ is not substantially altered upon introduction of substituents into the 1,10-phenanthroline ring system. Rather it seems more likely that in each case variations in activation parameters may be accounted for in terms of substituent-induced perturbations on a common interaction mechanism.

Introduction of substituents into the phenanthroline ligands probably limits the distance of closest approach between the cobalt(III) oxidant and the type 1 Cu(I) site, forcing electron tranfer to occur over a larger distance than is preferred by the unsubstituted oxidant. The conformational contribution to $\Delta H^{\ddagger}$ presumably will become smaller to the extent that ring substituents prevent close approach between the Cu(I) and Co(III) centers. At the same time, however, disordering of water molecules linked to extensive van der Waals attractions between

Figure 3. Compensation plot for the oxidation of blue copper proteins by tris(1,10-phenanthroline)cobalt(III) ions. Data from Table I: pH 7.0 (phosphate); stellacyanin, ●; plastocyanin, ○, — ; azurin, △, ----. Oxidant key: 1, $Co(phen)_3^{3+}$; 2, $Co(5,6\text{-}Me_2phen)_3^{3+}$; 3, $Co(5\text{-}Cl(phen))_3^{3+}$; 4, $Co[4,7\text{-}(PhSO_3)_2phen]_3^{3-}$; 5, $Co(4,7\text{-}Me_2phen)_3^{3+}$.



ΔH‡(kcal/mol)
15
10
5
ΔS‡(cal/mol-deg)
-20
-10
0
10

a reduced protein and the phenanthroline ligands of the oxidant will occur to a lesser extent, causing $\Delta S^{\ddagger}$ to become less favorable.

Looked at in another way, the isokinetic relationships between $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ for the oxidation of azurin and plastocyanin are consistent with the reaction acquiring nonadiabatic character to the extent that ring substituents block overlap between donor and acceptor redox orbitals on the two metal centers. Thus nonadiabaticity or tunneling is associated with minimal reorganizational activation requirements and large negative apparent entropies of activation, the latter a consequence of decreases in the transmission coefficient to values less than 1.[26] Nonadiabaticity is expected to become increasingly important as electron transfer is constrained to occur over larger and larger distances. Indeed, the values of both activation parameters consistently decrease with increasing size of the ring substituent. The correlation between the activation parameter trends and substituent size, however, also is compatible with an interpretation of the isokinetic relationships based solely on the extent of protein conformational rearrangement. Both factors may in fact contribute to the enthalpy-entropy compensation effect. On the basis of the data presently available, it is not possible to ascertain the relative importance of the two proposed substituent-induced perturbations.

The availability of only three points in the compensation plot for stellacyanin precludes our firmly ruling out the presence of an isokinetic relationship in this case. The available data, however, seemingly indicate that, although activation parameters are a sensitive function of the structure of the oxidant, they do not follow the syste-

matic compensation pattern exhibited by the other two blue proteins. Thus it may be concluded that ring substituents have a much larger effect on the stellacyanin oxidation mechanism than on the related processes for azurin and plastocyanin. In accord with this reasoning are comparisons of the ratio of rate constants for oxidation by $Co(phen)_3^{3+}$ to that for oxidation by the 5, 6-Me_2phen derivative. The ratio decreases in the order ten for stellacyanin, six for plastocyanin, and two for azurin. For plastocyanin or azurin the oxidation rate is less sensitive to substituent effects as the activation process appears to be primarily protein dependent. In addition, each protein apparently is able to compensate for steric hindrance by allowing electron transfer to occur over larger distances without drastically altering the overall activation free energy. Activation free energies and standard free energy changes for the redox reactions of interest are set out in Table III. Many of the derivatives exhibit activation free energies higher than those for $Co(phen)_3^{3+}$ by only about 1 kcal/mol, the maximum displacement being 3 kcal/mol for the $Co[4,7\text{-}(PhSO_3)_2phen]_3^{3-}$ plastocyanin(I) reaction. By contrast, variations in $\Delta H^{\ddagger}$ of over 6 kcal/mol and in $\Delta S^{\ddagger}$ of over 30 cal/(mol deg) may be noted in Table I.

As both plastocyanin[27] and azurin[28] are acidic metalloproteins with substantial net negative charges at pH 7.0, the possibility of electrostatic stabilization of precursor complexes for electron transfer to $Co(phen)_3^{3+}$ must be considered. Although the reactivity of the anionic 4, 7-diphenyl-4′-sulfonate derivative with plastocyanin(I) is a factor of 200 lower than that of $Co(phen)_3^{3+}$, activation parameter comparisons cast some doubt on the assignment of this difference to electrostatic

Table III. Free Energy Changes[a]

Protein	Oxidant	$\Delta G^{\ddagger}$ (kcal/mol)	$\Delta G°$ (kcal/mol)[b]
Stellacyanin	$Co(phen)_3^{3+}$	9.9	-4.3
	$Co(5,6\text{-}Me_2phen)_3^{3+}$	11.6	-5.7
	$Co[4,7\text{-}(PhSO_3)_2phen]_3^{3-}$	8.9	-3.4
Plastocyanin	$Co(phen)_3^{3+}$	12.5	-0.5
	$Co(5,6\text{-}Me_2phen)_3^{3+}$	13.3	-1.8
	$Co(5\text{-}Clphen)_3^{3+}$	13.3	-1.6
	$Co[4,7\text{-}(PhSO_3)_2phen]_3^{3-}$	15.6	0.5
Azurin	$Co(phen)_3^{3+}$	12.8	-1.0
	$Co(5,6\text{-}Me_2phen)_3^{3+}$	13.1	-2.4
	$Co(5\text{-}Clphen)_3^{3+}$	14.0	-2.1
	$Co(4,7\text{-}Me_2phen)_3^{3+}$	15.0	-0.3

[a]25 °C, μ = 0.1 M, pH 7.0 (phosphate).

[b]E (stellacyanin), 184 mV (pH 7.1, μ = 0.3 M), B. Reinhammar, Biochim. Biophys. Acta, 275, 245 (1972); E (bean plastocyanin), 350 mV (pH 6.6), N. Sailasuta, unpublished results; E (*Pseudomonas aeruginosa* azurin), 328 mV (pH 6.4), T. Yamanaka in "The Biochemistry of Copper", J. Peisach, P. Aisen, and W. E. Blumberg, Ed., Academic Press, New York, N.Y., 1966, p. 275; E ($Co(phen)_3^{3+}$), 370 mV, D. Cummins, unpublished results; E ($Co(5,6\text{-}Me_2phen)_3^{3+}$), 430 mV (0.05 M NaCl); E ($Co(4,7\text{-}Me_2phen)_3^{3+}$), 340 mV (0.05 M NaCl); E ($Co(5\text{-}Cl(phen)_3^{3+}$), 420 mV (0.05 M NaCl); E ($Co[4,7\text{-}(PhSO_3)_2phen]_3^{3-}$), 330 mV (0.05 M NaCl), A. R. Bowen, unpublished results.

effects. A tendency toward rate saturation with increasing oxidant concentration is expected in the presence of attractive forces between redox partners which enhance the equilibrium constant for precursor complex formation, yet no such tendency is evident in rate data for the azurin(I)- or plastocyanin(I)-$Co(phen)_3^{3+}$ reactions.

It might be expected that differences in the kinetic accessibility of type 1 Cu(I) among the blue electron carriers would be reflected in some of the physical properties exhibited by the metal site. Very few differences may be noted among the optical or the EPR spectra, or the composition and geometry of the copper coordination environment.[2,4,29] However, a main point of distinction among the blue proteins lies in their reduction potentials (Table III). The potential for stellacyanin (184 mV) is comparable with that for the cupric/cuprous couple in water (153 mV),[30] whereas E^0 values for plastocyanin and azurin lie above 300 mV, indicative of a markedly enhanced driving force for reduction of Cu(II) to Cu(I) (ΔG° more negative by 3-4 kcal/mol). Kassner has proposed[31] that the standard reduction potentials of high potential cytochromes may be accounted for in terms of a local heme environment of low dielectric constant. Thus the ferriheme unit carries a formal charge of +1, causing it to develop a larger tendency for reduction to the electrically neutral ferroheme analog as its immediate environment becomes more hydrophobic. An increase of 300 mV was noted between E^0 values for the reduction of a pyridine complex of ferrimesoheme dimethyl ester in water and in benzene solution.[31] Similar considerations may be applied toward understanding the potential differences among the blue proteins. Thus the relatively

low reduction potential of stellacyanin may be attributable to the fact that its blue Cu(II) site lies in a comparatively polar environment near the protein surface, whereas the azurin and plastocyanin type 1 copper atoms presumably are buried deep within the hydrophobic interior of the polypeptide structure.

It has been shown recently that self-exchange electron transfer rate constants calculated on the basis of relative Marcus theory provide a useful framework for comparisons of the reactivity of metalloproteins with a variety of reductants and oxidants.[2,13,14] Three distinctly different reactivity types have been identified for azurin, with kinetic access to the blue copper center decreasing according to cytochrome $\underline{c}_{551}$(II) $\gg$ cytochrome $\underline{c}$(II) $\gg$ $Fe(EDTA)^{2-}$.[14] The reactions that give the highest predicted self-exchange rates frequently are associated with relatively favorable activation entropies, whereas the more forbidden reactions proceed with minimal $\Delta H^{\ddagger}$ but highly unfavorable $\Delta S^{\ddagger}$ values.[2,14]

The Marcus equation[32]

$$\log k_{12} = 0.5[\log k_{11} + \log k_{22} + 16.9\,\Delta E^{0}]$$

was employed to acquire calculated protein self-exchange rates based on the present data. The self-exchange rate constants for the metalloprotein and its redox partner are k_{11} and k_{22}, respectively, the cross reaction rate constant is k_{12}, and the cell potential for the redox reaction is ΔE^{0}. Table IV provides a comparison of k_{11} values calculated for stellacyanin, plastocyanin, and azurin on the basis of

Table IV. Calculated Protein Self-Exchange Rate Constants[a]

Protein	$Fe(EDTA)^{2-}$ k_{12} (M^{-1} s^{-1})	$Fe(EDTA)^{2-}$ k_{11} (M^{-1} s^{-1})	$Co(phen)_3^{3+}$ k_{12} (M^{-1} s^{-1})	$Co(phen)_3^{3+}$ k_{11} (M^{-1} s^{-1})
Stellacyanin	1.0×10^6	3×10^6 [b]	1.8×10^5	5×10^5
Plastocyanin	5.3×10^4	1×10^1 [c]	4.9×10^3	2×10^5
Azurin	1.3×10^3	2×10^{-2} [c]	3.2×10^3 [d]	4×10^4

[a]For pH 7.0, $\mu = 0.1$ M. 25 °C, except when noted otherwise. Protein nd $Co(phen)_3^{3+}$ reduction potentials are given in Table III. For $Fe(EDTA)^{2-}$, E = 120 mV (G. Schwarzenbach and J. Heller, Helv. Chim. Acta, 34, 576 (1951). k_{22} ($Co(phen)_3^{3+/2+}$) = 4.5×10^1 M^{-1} s^{-1}, k_{22} (($Fe(EDTA)^{2-/-}$) = 3×10^4 M^{-1} s^{-1} (see S. Wherland and H. B. Gray, Proc. Natl. Acad. Sci. U.S.A., 73, 2950 (1976).

[b]From ref 13.

[c]From ref 14.

[d]For $\mu = 0.2$ M.

$Fe(EDTA)^{2-}$ reduction and $Co(phen)_3^{3+}$ oxidation rate constants.

It is immediately evident from the data in Table IV that the k_{11} estimates based on $Fe(EDTA)^{2-}$ and $Co(phen)_3^{3+}$ cross reactions agree closely in the case of stellacyanin, but differ by four and six orders of magnitude for plastocyanin and azurin, respectively. The former observation is consistent with the hypothesis that the type 1 copper atom in stellacyanin is readily accessible in aqueous solution to outer sphere contact with external redox agents and employs essentially similar mechanisms in donating and accepting an electron. As little in the way of protein conformational change appears to be required for electron transfer in the case of stellacyanin, we expect the calculated k_{11} value of about 10^6 M^{-1} s^{-1} based on $Fe(EDTA)^{2-}$ to accord with the actual protein self-exchange rate constant, although no experimental measurement is available to test this point.

The wide variation in calculated k_{11} values for plastocyanin and azurin based on the two redox agents is entirely consistent with the view that the copper center in each of these proteins is buried.[33] It is apparent that $Fe(EDTA)^{2-}$ has particularly poor access to the blue copper in these proteins and is forced to employ electron transfer mechanisms characterized by substantial nonadiabaticity. The relatively large calculated k_{11} values for plastocyanin and azurin based on $Co(phen)_3^{3+}$, however, accord with our earlier proposal that this redox agent evidently is able to gain intimate contact with the type 1 Cu(I) site, presumably by a mechanisms involving induced protein conformational change.

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CHAPTER 4. ANION EFFECTS ON THE KINETICS OF REDUCTION OF METALLOPROTEINS BY $Fe(HEDTA)^-$

Introduction

Studies of the mechanism of electron transfer between redox metalloproteins and inorganic reagents have resulted in a description of the kinetic accessibility of the reactive site.[1,2,3] Structural evidence[4] and kinetics data[5,6] have supported the suggestion that electron transfer from $Fe(EDTA)^{2-}$ to horse heart cytochrome *c* and *Pseudomonas aeruginosa* cytochrome c_{551} involves outer sphere attack at the partially exposed heme edge. Although X-ray crystal structures are not available for any of the blue copper proteins, spectroscopic evidence[7] has suggested a detailed structural model involving a distorted tetrahedral copper atom coordinated to one cysteine, one peptide nitrogen, and two histidine ligands. The kinetic accessibility of the blue copper centers to $Fe(EDTA)^{2-}$ has been investigated.[8]

The rates of reduction of the cytochromes c and blue copper proteins by $Fe(HEDTA)^{-}$ have been analyzed according to Marcus theory[9] in order to identify the contributions to the activation free energy from the reagent reactivity, electrostatic forces, and thermodynamic driving force for the reaction. As the sixth coordination position in $Fe(HEDTA)^{-}$ is occupied by a labile water molecule, the possibility of catalyzing electron transfer by means of reductant-anion-protein bridging exists. Azide and thiocyanate have been shown to catalyze certain outer sphere electron transfer reactions involving inorganic complexes, and some useful criteria for distinguishing outer and inner sphere reactions have been developed.[10,11] Accordingly,

we have measured the effects of anions on the kinetics of reduction of horse heart ferricytochrome c, Pseudomonas aeruginosa ferricytochrome c_{551} and azurin, bean plastocyanin, and Rhus vernicifera stellacyanin by $Fe(HEDTA)^-$. Rate enhancement was observed for all reactions in the presence of anions of π symmetry, azide and thiocyanate, whereas no effect was apparent for anions of σ symmetry, sulfate and chloride. Slight rate enhancement was also observed for the reduction of horse heart ferricytochrome c by $Fe(EDTA)^{2-}$ in the presence of azide.

Experimental Section

Deionized, distilled water and reagent grade chemicals were used in the preparation of solutions for kinetics measurements. Nitrogen gas, passed through two chromous scrubbing towers to remove oxidizing impurities, was used to deoxygenate solutions for kinetics. Phosphate buffer solutions at pH 7.0 were used in all kinetics experiments and the ionic strength was adjusted with ammonium sulfate. Protein and reducing agent solutions were deoxygenated in serum-capped bottles by purging with N_2. Nitrogen was first carefully bubbled through protein solutions for several minutes and then passed over the solution in order to avoid denaturation due to frothing.

Stellacyanin was prepared according to the method of Reinhammer.[12] and French bean plastocyanin was isolated and purified according to the procedure of Milne and Wells[13] to absorbance ratios A_{280}/A_{604} of 5.6 and A_{280}/A_{597} of 1.2, respectively. Pseudomonas aeruginosa were grown and cytochrome c_{551} and azurin were purified according to

the methods developed by Ambler et al.[14,15] to ratios A_{551}/A_{280} of 1.2 and A_{625}/A_{280} of 0.44, respectively. Horse heart cytochrome c was obtained from Sigma (Type VI) and was not further purified. The absorbance ratios are all in agreement with literature values.

Hydroxyethylenediaminetriacetic acid (HEDTA) stock solutions were prepared by combining the correct proportions of the acid and trisodium salt to give the desired pH and concentration. A twenty percent excess of ligand was present in this solution to insure complete formation of $Fe(HEDTA)^-$. A degassed stock solution of ferrous ammonium sulfate was prepared in water to avoid iron hydroxide formation in phosphate buffer at pH 7.0. As $Fe(HEDTA)^-$ is extremely oxygen sensitive, the ferrous ammonium sulfate was added under nitrogen to the ligand stock solution using Hamilton gas tight syringes. Minor pH adjustments were made using deoxygenated solutions of HCl and NaOH. The reductant concentration was always in pseudo-first order excess over the protein and was varied over as wide a range as possible. The final ionic strength of the solutions remained constant as sodium azide, sodium thiocyanate, and sodium chloride were substituted for ammonium sulfate in the anion dependence studies. The anions were added to the reductant solution in all cases studied, as changes occur in the tertiary structure of some of the proteins in the presence of azide and thiocyanate for long periods of time.

The reduction of stellacyanin was followed at 604 nm, ($\Delta\epsilon$ 4.1 $\times$ 10^3 $\underline{M}^{-1}$ cm^{-1}), plastocyanin at 597 nm ($\Delta\epsilon$ 4.5 $\times$ 10^3 $\underline{M}^{-1}$ cm^{-1}) and

azurin was followed at 625 nm ($\Delta\epsilon$ 5.7 × 10^3 $\underline{M}^{-1}$ cm^{-1}). Ferricytochrome c ($\Delta\epsilon$ 1.85 × 10^4 $\underline{M}^{-1}$ cm^{-1}) and ferricytochrome c_{551} ($\Delta\epsilon$ 1.90 × 10^4) reductions were monitored at 550 nm and 551 nm respectively. An absorbance change of 0.1 was measured for all reductions. A Durrum Model D-110 stopped-flow spectrophotometer equipped with a Forma Scientific temperature bath was used for all kinetics experiments. Data were sent to a Tektronix 564B storage oscillioscope and to an A/D converter. The A/D converter was used in conjunction with a PDP-10 computer to evaluate the observed rate constants. The pseudo-first-order rate constants (k_{obsd}) were obtained from the slope of the linear least-squares fit of log (A_t-A_∞) $\underline{vs.}$ time data. A Cary 17 spectrophotometer was used for all visible-UV spectral measurements and a Brinkman pH 101 instrument was used for all pH determinations.

Potential determinations were made using a Princeton Applied Research Model 173 Potentiostat with a gold working electrode, and standard calomel reference electrode. Cyclic voltamograms were obtained for $Fe(HEDTA)^-$ in phosphate buffer (pH 7.0, μ 0.2). The average of the peak potentials for the anodic, E_{PA}, and cathodic, E_{PC} currents were used to calculate the reduction potentials ($E_{\frac{1}{2}}$) of the complex. This differs from the formal electrode potential, E^0, by 30 log (D_{III}/D_{II}) mV, where D_{III} and D_{II} are diffusion coefficients of the metal (III) and metal (II) complexes, respectively.

Results and Discussion

Pseudo-first order kinetics were observed for the reduction of all the proteins by $Fe(HEDTA)^-$. A first order dependence of the observed rate constants on reductant concentration was found in all cases, as illustrated in Figure 1. The dependences of the observed rate constants on temperature is shown in Figure 2. Rate constants and activation parameters are presented in Table I.

The application of Marcus theory to the $Fe(HEDTA)^-$ reduction data has resulted in the calculation of electrostatics-corrected cross reaction rates, k_{12}^{corr}. This enables a comparison reactivity independent of differences in thermodynamic driving force, reagent reactivity, and electrostatic forces. The reagent and protein properties to be used in the calculation are listed in Table II. The protein charges have been determined from amino acid sequence data and the protein radii from known density and molecular weight values.

Although the $Fe(HEDTA)^-$ self-exchange rate has not been measured, it can be calculated based on the assumption that stellacyanin obeys Marcus theory. This has been shown to be a valid assumption, as the electrostatics corrected stellacyanin self-exchange rates, k_{11}^{corr}, vary less than a factor of two for all the small molecule reagents investigated.[3] The calculated $Fe(HEDTA)^-$ cross reaction rates, k_{12}^{corr}, are presented in Table II, together with the observed values. The agreement between the calculated and observed rate constants indicates that these reactions obey Marcus theory and that similar activation process are involved in the reactions of the proteins with $Fe(EDTA)^{2-}$ and $Fe(HEDTA)^-$.

Figure 1. The dependences of observed rate constants on the concentration of $Fe(HEDTA)^-$ at 25°, pH 7.0 (phosphate), μ = 0.2 M: stellacyanin, ●; plastocyanin, ○; azurin, △; cytochrome c, X; cytochrome c_{551}, □.

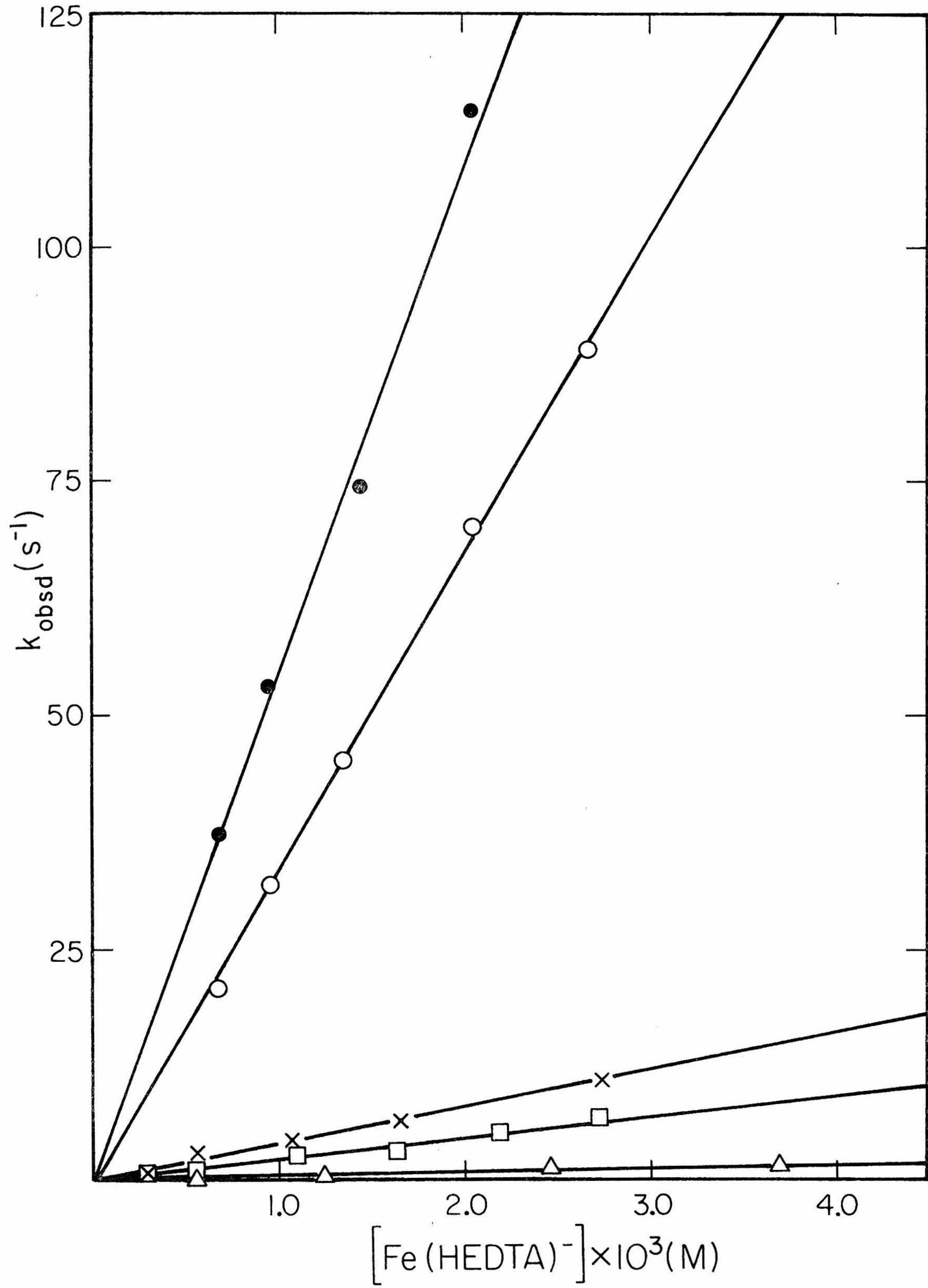
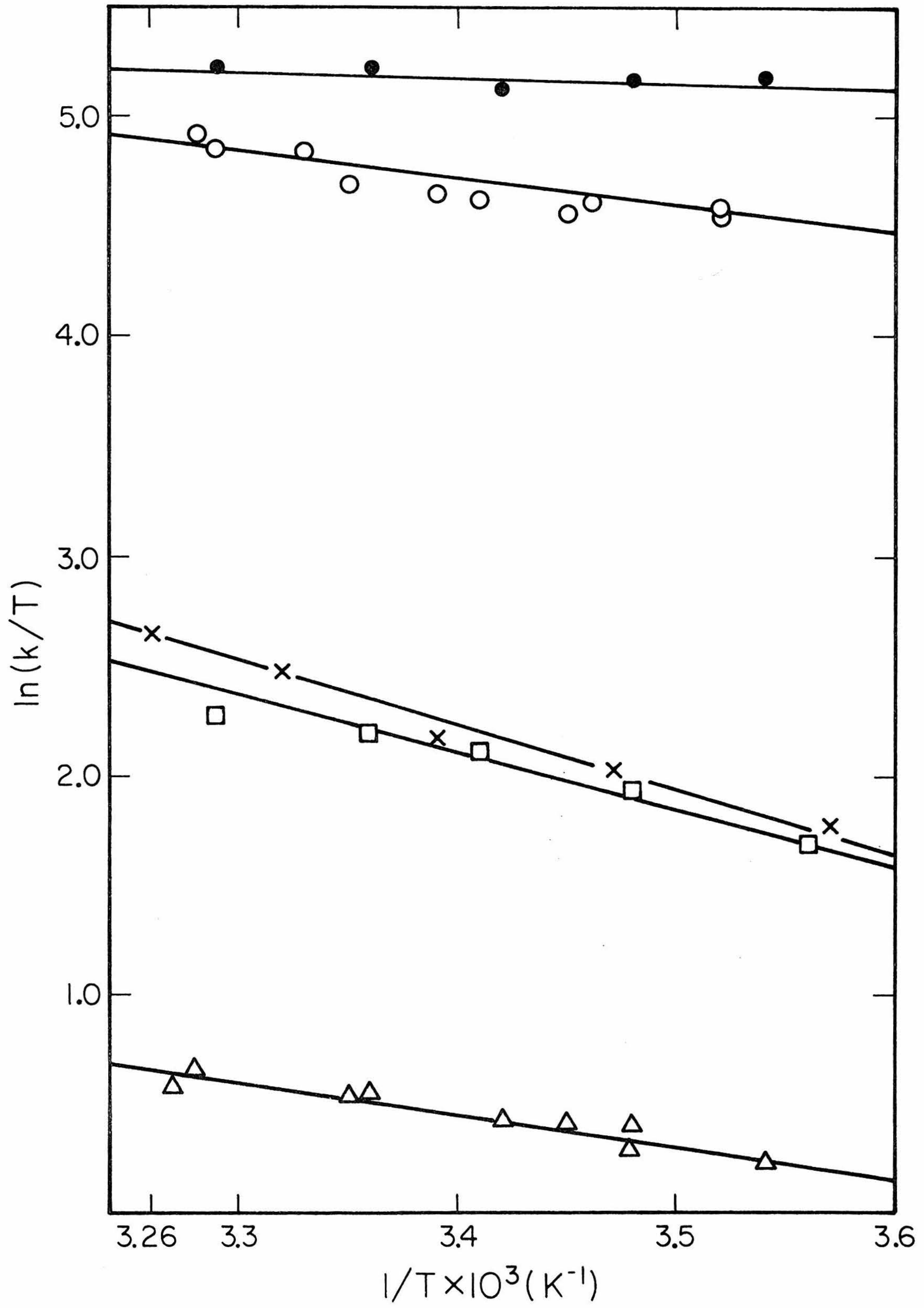

125
100
75
50
25
$k_{obsd}(s^{-1})$
1.0
2.0
3.0
4.0
$[Fe(HEDTA)^{-}] \times 10^{3} (M)$

Figure 2. Eyring plots of the rate data for reductions by $Fe(HEDTA)^-$ at pH 7.0 (phosphate) μ = 0.2 M: stellacyanin, ●; plastocyanin, ○; azurin, △; cytochrome c, X; cytochrome c_{551}, □.



5.0
4.0
3.0
2.0
1.0
ln(k/T)
3.26
3.3
3.4
3.5
3.6
$1/T \times 10^3 (K^{-1})$

Table I. Rate Constants and Activation Parameters for the Reduction of Proteins by $Fe(HEDTA)^-$ (pH 7.0 (phosphate), μ 0.2 M, T = 25 °C)

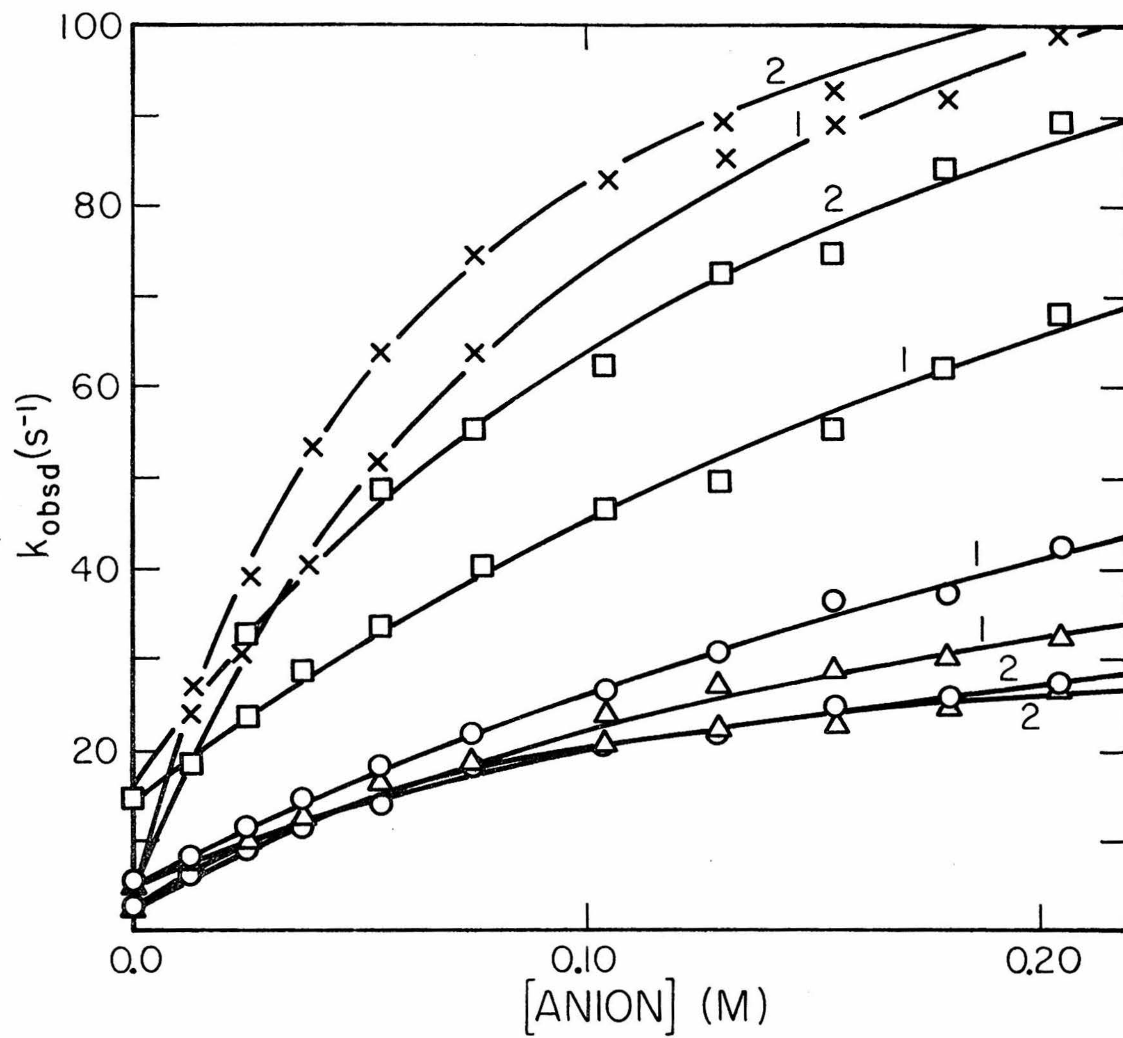
Protein	k (M^{-1} s^{-1})	$\Delta H^{\ddagger}$ (kcal/mol)	$\Delta S^{\ddagger}$ (cal/mol deg)
Rhus vernicifera stellacyanin	5.48×10^4	2.9	-27
Phaseclus vulgaris plastocyanin	3.38×10^4	2.3	-29
Horse heart cytochrome c	8.52×10^3	3.1	-26
Pseudomonas aeruginosa cytochrome c_{551}	4.92×10^3	5.2	-26
Pseudomonas aeruginosa azurin	4.42×10^2	3.4	-34

The protein reactivity calculated from the $Fe(EDTA)^{2-}$ cross-reaction varies by greater than seven orders of magnitude over the range of proteins investigated. This has been interpreted as resulting from the hydrophillic nature of the reagent and lack of π-orbitals for penetration and favorable electron transfer.[3] For these reasons, $Fe(EDTA)^{2-}$ has been shown to be particularly sensitive to the kinetic accessibility of the protein metal site. The cross reaction electron transfer rates for the reduction of the proteins by $Fe(EDTA)^{2-}$ decrease according to stellacyanin > plastocyanin > cytochrome c > cytochrome c_{551} > azurin. The same order of reactivity is also observed for the reduction by $Fe(HEDTA)^{-}$.

Similar activation parameters are observed for the $Fe(HEDTA)^{-}$ and $Fe(EDTA)^{2-}$ reactions with all the proteins indicating that the mechanism involves an adiabatic outer-sphere process. The activation enthalpy varies from 2.3 to 6.0 kcal/mol for the $Fe(EDTA)^{2-}$ reactions and from 2.1 to 5.2 kcal/mol for the corresponding $Fe(HEDTA)^{-}$ reactions. The activation entropy varies from -18 to -32 cal/mol deg for the $Fe(EDTA)^{2-}$ reactions and from -26 to -34 cal/mol deg for the analogous $Fe(HEDTA)^{-}$ reactions. The small changes in activation parameters would suggest that the major contribution to the activation enthalpy is due to Franck-Condon activation of the two redox centers and little, if any, enthalpic cost for protein penetration.

The dependences of the observed rate constants on concentration of anion exhibit saturation behavior as illustrated in Figure 3. The

Figure 3. The dependences of observed rate constants on anion concentration at 25°, pH 7.0 (phosphate), μ = 0.5 M: plastocyanin, ○; azurin, △; cytochrome c, X; cytochrome c_{551}, □; azide, 1; thiocyanate, 2. The lines are the least squares fit of data obtained using equation (1).

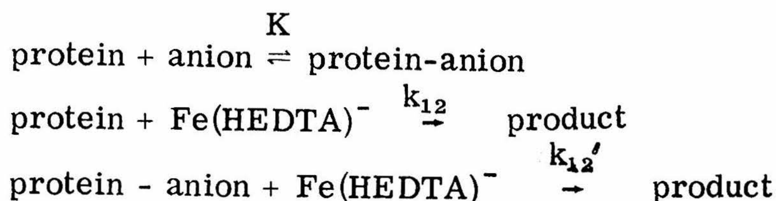

100
80
60
40
20
0.0
0.10
0.20
$k_{obsd}(s^{-1})$
[ANION] (M)
2
1
2
1
1
1
2
2

observed kinetic behavior in the presence of azide and thiocyanate can be fit to the following rate law:

$$\frac{dA}{dt} = \frac{k_{12} + k_{12}' K[\text{anion}]}{1 + K[\text{anion}]} [\text{FeHEDTA}^-] [\text{protein}]$$

$$\text{where} \quad \frac{k_{12} + k_{12}' K[\text{anion}]}{1 + K[\text{anion}]} = k_{obsd}$$

This rate law is consistent with the following mechanism:



An alternative mechanism involving the formation of a [Fe(HEDTA)-anion]$^{2-}$ complex may be ruled out by observing the different values of the stability constants, K, obtained in the reactions studied and presented in Table III. The anion binding constants are inversely proportional to the protein charge and range from 2.15 M^{-1} to 9.78 M^{-1} for azide and from 5.25 M^{-1} to 17.7 M^{-1} for thiocyanate. In comparison, the observed rate constants, k_{obsd}, for the anion-inhibited reduction of Co(EDTA)$^-$ by Fe(HEDTA)$^-$ do not saturate with anion concentrations up to 0.2 M, indicating that the binding constants for azide and thiocyanate to Co(EDTA)$^-$ are extremely small ($\ll 10^{-2}$ M^{-1}). The slight anion-assistance observed in the reduction of ferricytochrome *c* by Fe(EDTA)$^{2-}$ can be explained by the formation

Table III. Stability Constants for Protein-Anion Complexes and Electron Transfer Rate Constants (pH 7.0 (phosphate), $\mu = 0.5$ M, 25°C)

Protein	pI	K_{N_3} (M^{-1})	K_{SCN} (M^{-1})	k_{12} ($M^{-1}s^{-1}$)	k'_{12} ($M^{-1}s^{-1}$)	k_{SCN}/k_{Cl}	k_{N_3}/k_{SCN}
Cytochrome c	10.5[a]	9.78	17.7	1.08×10^3	1.04×10^4 (N_3) 9.41×10^3 (SCN)	87.1	1.11
Stellacyanin	9.9[b]	c	c	c	c	106[d]	5.5[d]
Azurin	5.2[b]	4.80	11.0	9.44×10^2	2.36×10^4 (N_3) 1.35×10^4 (SCN)	14.3	1.75
Cytochrome c_{551}	4.7[a]	2.61	5.65	5.70×10^3	6.05×10^4 (N_3) 5.50×10^4 (SCN)	9.65	1.10
Plastocyanin	< 6[b]	2.15	5.25	1.90×10^3	4.61×10^4 (N_3) 1.78×10^4 (SCN)	9.37	2.59

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[c] Reactions are too fast to measure.

[d] $\mu = 0.2$ M.

of a less stable protein-anion-reductant interaction. In all cases investigated, the thiocyanate complexes are more stable than the azide complexes.

The ratios of the rate constants for the anion-assisted reactions are also presented in Table III. The small kNNN/kSCN ratios and larger kSCN/kCl ratios are indicative of an outer sphere mechanism. Przystas and Sutin[10] have postulated that for anion-assisted outer sphere reactions involving two hard redox centers the kNNN/kSCN ratio is smaller and the kSCN/kCl ratio is larger than for inner sphere processes. The large kNNN/kSCN ratio for inner sphere reactions is based on the fact that for inner-sphere processes the symmetrical transition state (M-NNN-M′) is much more favorable than the non-symmetrical transition state (M-SCN-M′) where one of the hard metals must coordinate to a soft sulfur atom. Equal enhancement is observed for outer sphere processes due to the similarity of the π-systems. The smaller kSCN/kCl ratio for inner-sphere reactions is primarily a result of larger kCl rates, as chloride is more effective for inner sphere processes when directly bonded to the metals.

The most striking rate enhancement effects are observed for the reduction of horse heart ferricytochrome c. Previous workers have determined by thermodynamic and electrophoretic mobility studies that the oxidized protein binds anions readily, whereas the reduced protein preferentially binds cations.[16-18] Stellwagen and Cass[19] have more recently presented evidence to show that electro-

static binding of at least two iron hexacyanides to horse heart ferricytochrome c occurs. Studies of chemically-modified proteins resulted in the conclusion that one of these anion binding sites was either lysine 79 or lysine 13[20] which are both located near the partially exposed heme edge and are presumably in a position to affect the mechanism of electron transfer. The π symmetry of the azide and thiocyanate anions would be expected to overlap favorably with the porphyrin edge in the protein increasing the rate of electron transfer. Rate enhancement of the oxidation of ferrocytochrome c by $Co(phen)_3^{3+}$ was not observed in the presence of anions presumably owing to the fact that ferricytochrome c preferentially binds cations.

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CHAPTER 5. KINETICS STUDIES OF THE OXIDATION OF CYTOCHROMES AND BLUE COPPER PROTEINS BY $Co(EDTA)^-$ AND $Co(HEDTA)X^-$ COMPLEXES

Introduction

Kinetic studies of the reductions of the cytochromes c and blue copper proteins by $Fe(EDTA)^{2-}$ are consistent with a mechanism involving outer sphere attack of the reductant with little protein-reagent interaction in the transition state.[1-3] These reactions are characterized by low enthalpies and entropies of activation. Similar studies of the corresponding oxidations by $Co(phen)_3^{3+}$ indicate that oxidant-induced protein structural changes are responsible for the observed deviations from Marcus theory.[4,5] Increased enthalpies and more positive entropies of activation compared to the $Fe(EDTA)^{2-}$ reductions are consistent with this interpretation. We have now studied the oxidations of these proteins by $Co(EDTA)^{-}$, in an attempt to determine whether the two mechanisms are a result of different symmetries of the redox orbitals of the reagents or different sites of oxidation and reduction.

The potential of $Co(EDTA)^{-}$ is approximately the same as $Co(phen)_3^{3+}$,[6] however, the self-exchange rate is more than eight orders of magnitude slower.[7] As a result, the cross reaction rates for oxidation by $Co(EDTA)^{-}$ are slower than the corresponding $Co(phen)_3^{3+}$ oxidations. The application of Marcus theory[8] will enable the evaluation of electron transfer reactivity while taking into account differences in driving force, reagent reactivity and charge.

As the symmetry of the reactive site on the protein is important in determining the redox mechanism, the oxidations by several $Co(HEDTA)X^{-}$ complexes (where X = NO_2, Cl, and Br) will be examined.

Anions have been shown to assist the outer sphere reduction of the cytochromes c and blue copper proteins by $Fe(HEDTA)^-$.[9] The mechanism for this enhancement involves pre-equilibrium binding of anions to the proteins followed by reductant attack. The oxidation reactions to be discussed here differ from the $Fe(HEDTA)^-$ reductions in that the $Co(HEDTA)X^-$ complexes are isolated and characterized prior to the kinetic studies. For comparison to the protein results, the oxidation of $Fe(EDTA)^{2-}$ by each of the cobalt complexes was also investigated.

Experimental

Deionized, distilled water and reagent grade chemicals were used throughout. Nitrogen gas was passed through two vanadous scrubbing towers to remove oxidizing impurities. Argon was purified on a manganese oxide column. Phosphate buffer solutions at pH 7.0 were used in all kinetics experiments with the final ionic strength adjusted to μ 0.2 with ammonium sulfate. A Brinkman pH 101 instrument was used for pH determinations.

Stellacyanin was prepared according to the method of Reinhammer[10] and French bean plastocyanin was isolated and purified according to the procedure of Milne and Wells[11] to absorbance ratios A_{280}/A_{604} of 5.6 and A_{280}/A_{597} of 1.2, respectively. Pseudomonas aeruginosa were grown and cytochrome c_{551} and azurin were purified according to the developed by Ambler et al.,[12] to ratios A_{551}/A_{280} of 1.2 and A_{625}/A_{280} of 0.44, respectively. Horse heart cytochrome c was obtained

from Sigma (Type VI) and was not further purified. The absorbance ratios are all in agreement with literature values. Polyacrylamide gel electrophoresis of stellacyanin, azurin, and cytochrome $\underline{c}_{551}$ resulted in a single band. Purified plastocyanin was not available at the time of the electrophoresis experiments.

Potassium (ethylenediaminetetraacetato)cobaltate(II) 2 hydrate, $K[Co(EDTA)] \cdot 2H_2O$, was prepared by treating cobalt(II) carbonate with ethylenediaminetetraacetic acid and oxidizing with 3% hydrogen peroxide.[13] The resulting purple complex was characterized by visible absorption spectroscopy and elemental analysis. The Na[Co(HEDTA)X] complexes were prepared according to the procedures described by Busch[14] and Schwarzenbach.[15] A DEAE A-50 sephadex ion exchange column was used to purify $Co(HEDTA)Cl^-$ before analysis and kinetics use. These complexes were also characterized by visible absorption spectra and elemental analysis. All the cobalt complexes were very hygroscopic and care was taken to store them in a vacuum dessicator. $Fe(EDTA)^{2-}$ solutions were prepared as previously described.[1]

Solutions of reduced cytochrome $\underline{c}$, cytochrome $\underline{c}_{551}$, plastocyanin and azurin were prepared by adding a twenty-fold excess of $Fe(EDTA)^{2-}$ to deoxygenated, buffered solutions of the oxidized proteins. The excess reductant was removed from the blue copper proteins by dialysis against deoxygenated buffer using a hollow fiber Dow beaker dialyzer obtained from Bio-Rad Laboratories. The excess reductant was removed from

the cytochromes by using a Sephadex G-25 gel filtration column. As reduced stellacyanin was readily oxidized by traces of O_2 which could not be excluded using these techniques, solutions of cuprous stellacyanin were prepared by adding an equivalent amount of ascorbic acid to the cupric protein without removing the dehydroascorbate product.[4]

The oxidation of stellacyanin was followed at 604 nm, ($\Delta\epsilon$ 4.1 × 10^3 $\underline{M}^{-1}$ cm^{-1}), plastocyanin at 597 nm, ($\Delta\epsilon$ 4.5 × 10^3 $\underline{M}^{-1}$ cm^{-1}) and azurin was followed at 625 nm ($\Delta\epsilon$ 5.7 × 10^3 $\underline{M}^{-1}$ cm^{-1}). Ferrocytochrome c ($\Delta\epsilon$ 1.85 × 10^4 $\underline{M}^{-1}$ cm^{-1}) and ferrocytochrome c_{551} ($\Delta\epsilon$ 1.90 × 10^4 $\underline{M}^{-1}$ cm^{-1}) oxidations were monitored at 550 and 551 nm, respectively. An absorbance change of 0.1 was measured for all reactions investigated.

All oxidations of stellacyanin and $Fe(EDTA)^{-2}$ in addition to all oxidations by $Co(HEDTA)Br^-$ and $Co(HEDTA)Cl^-$ were sufficiently fast to monitor using a Durrum Model D-110 stopped-flow spectrophotometer equipped with a Forma Scientific temperature bath. Data were collected as previously described.[9] The cobalt(III) complexes were stored in serum-capped round bottom flasks fitted with a nitrogen inlet and luer-lock fitting. All solutions were nitrogen-saturated for at least fifteen minutes prior to introduction into the stopped-flow.

The slower reactions of plastocyanin, azurin, cytochrome $\underline{c}$, and cytochrome c_{551} were monitored using a Cary 15 or Cary 17 spectrophotometer. A quartz 1 cm cell with two side chambers for freeze-pump degassing was used in these experiments. Both reagents were freeze-pump degassed three times for the cytochrome reactions, however, the

blue copper proteins showed signs of denaturation under these conditions. To avoid this problem, these proteins were saturated with nitrogen as previously described and introduced into the cell under a positive flow of argon after the oxidant was freeze-pump degassed.

The O_2 oxidations were much slower than the cobalt oxidations and did not interfere with the kinetics in all reactions studied. Solutions were equilibrated to the correct temperature at least fifteen minutes before mixed and placed in a thermostated cell holder. The Guggenheim method[16] was used to analyze the kinetics of the slow reactions.

Results

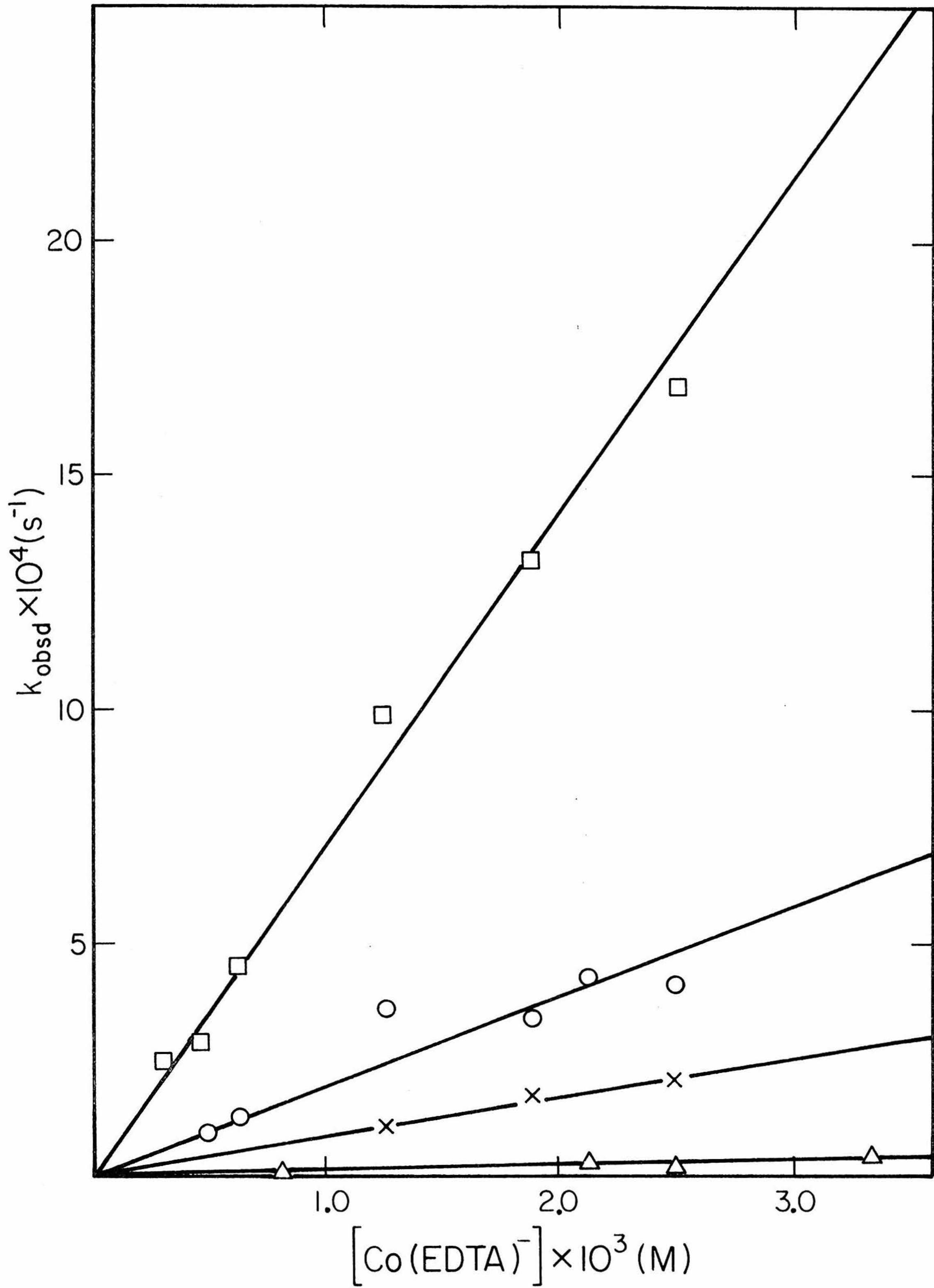
First order plots of the absorbance-time data observed for the oxidations were found to be linear for greater than 90% of the reactions. The dependences of observed rate constants on the concentration of $Co(EDTA)^-$ are illustrated in Figure 1. The experimental results are consistent with rate law (1) over a tenfold range in oxidant concentration.

$$\frac{d[\text{Prot}]_{ox}}{dt} = k[\text{Prot}]_{red}[\text{Co(EDTA)}^-] \qquad (1)$$

The dependences of the observed rate constants on temperature are shown in Figure 2. Second order rate constants and activation parameters are presented in Table I.

The rate law observed for the $Co(EDTA)^-$ oxidations was found to pertain in most cases to the analogous reactions of the proteins with $Co(HEDTA)X^-$ complexes. The second order rate constants and activation

Figure 1. The dependences of observed rate constants on the concentration of $Co(EDTA)^-$ at 25°, pH 7.0 (phosphate), μ = 0.2 M: plastocyanin, ○; azurin, Δ; cytochrome c, X ; cytochrome c_{551}, □.



20
15
10
5
$k_{obsd} \times 10^4 (s^{-1})$
1.0
2.0
3.0
$[Co(EDTA)^-] \times 10^3$ (M)

Figure 2. Eyring plots of the rate data for oxidations by $Co(EDTA)^-$ at pH 7.0 (phosphate), μ = 0.2 M: plastocyanin, ○; azurin, Δ; cytochrome c, X; cytochrome c_{551}, □.

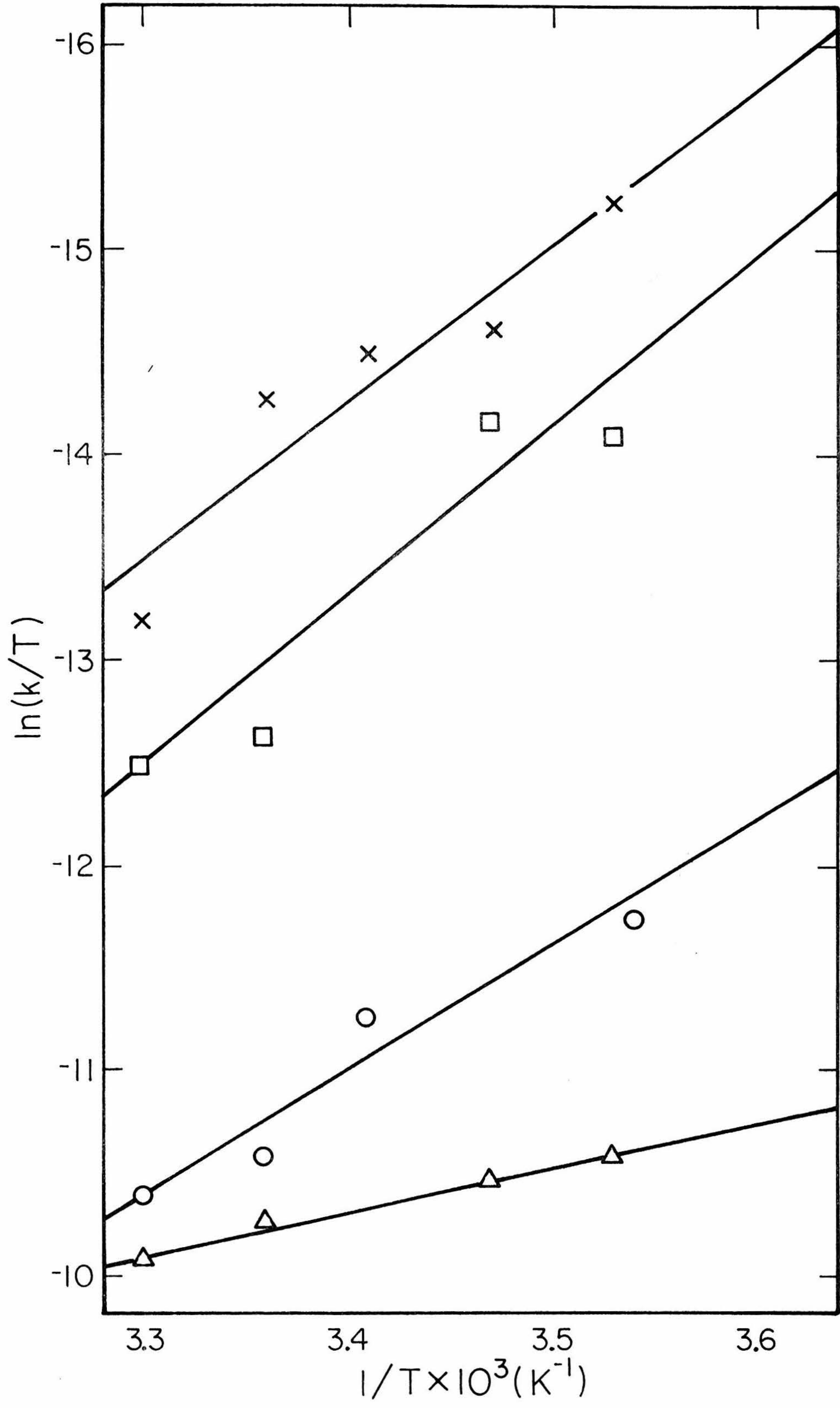

-16
-15
-14
-13
-12
-11
-10
ln(k/T)
3.3
3.4
3.5
3.6
1/T×10³(K⁻¹)

Table I: Second Order Rate Constants and Activation Parameters for Oxidations by $Co(EDTA)^-$ and $Co(HEDTA)X^-$. (pH 7.0 (Phosphate), $\mu = 0.2$)

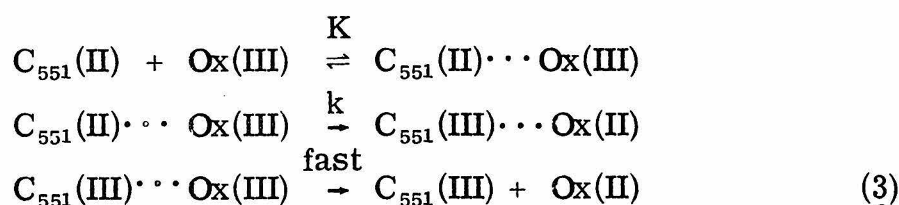
Reductant	Oxidant	$k(\underline{M}^{-1}s^{-1})$	$\Delta H^{\ddagger}$(kcal/mol)	$\Delta S^{\ddagger}$(cal/mol-deg)
$Fe(EDTA)^{2-}$	$Co(EDTA)^-$	6.57	7.3	-30
	$Co(HEDTA)NO_2^-$	1.98×10^{1}	7.6	-27
	$Co(HEDTA)Cl^-$	2.56×10^{2}	5.7	-28
	$Co(HEDTA)Br^-$	3.74×10^{2}	5.5	-29
Rhus vernicifera stellacyanin	$Co(EDTA)^-$	2.52×10^{1}	1.8	-56
	$Co(HEDTA)NO_2^-$	6.74×10^{2}	3.1	-35
	$Co(HEDTA)Cl^-$	2.71×10^{3}	5.2	-26
	$Co(HEDTA)Br^-$	1.59×10^{4}	6.5	-17
Phaseolus vulgaris plastocyanin	$Co(EDTA)^-$	6.33×10^{-3}	12.8	-26
	$Co(HEDTA)NO_2^-$	1.15×10^{-2}	9.3	-36
	$Co(HEDTA)Cl^-$	9.66×10^{-1}	13.4	-14
	$Co(HEDTA)Br^-$	5.02	9.9	-21
Horse heart cytochrome *c*	$Co(EDTA)^-$	6.94×10^{-2}	14.2	-15
	$Co(HEDTA)NO_2^-$	1.59×10^{-1}	16.3	- 7
	$Co(HEDTA)Cl^-$	8.04	6.7	-32
	$Co(HEDTA)Br^-$	7.66×10^{1}	8.4	-22
Pseudomonas aeruginosa cytochrome c_{551}	$Co(EDTA)^-$	6.27×10^{-1}	15.0	-10
	$Co(HEDTA)NO_2^-$	1.98×10^{-1}	11.3	-24
	$Co(HEDTA)Cl^-$	2.61×10^{1}	10.9	-29
	$Co(HEDTA)Br^-$	1.23×10^{2}	9.4	-34
Pseudomonas aeruginosa azurin	$Co(EDTA)^-$	1.28×10^{-2}	4.1	-54
	$Co(HEDTA)NO_2^-$	9.90×10^{-3}	11.1	-31
	$Co(HEDTA)Cl^-$	1.45×10^{-1}	7.8	-35
	$Co(HEDTA)Br^-$	2.65	5.5	-37

parameters for the reactions with the substituted oxidants are also presented in Table I. Rate constants for the $Co(HEDTA)X^-$ reactions are, with the exception of that for azurin-$Co(HEDTA)NO_2^-$, substantially larger than the corresponding parameters for $Co(EDTA)^-$.

The oxidations of cytochrome $\underline{c}_{551}$ by $Co(HEDTA)Cl^-$ and $Co(HEDTA)Br^-$ are characterized by linear first order plots of absorbance-time data; however, saturation behavior is observed in the concentration dependence, as illustrated in Figure 3. The data are consistent with rate law (2), which can be derived from a mechanism involving rapid pre-equilibrium

$$\frac{d[C_{551}]_{TOT}}{dt} = \frac{kK[Ox]}{1 + K[Ox]}[C_{551}]_{TOT} \qquad (2)$$

protein-oxidant complex formation followed by rate limiting intramolecular electron transfer as described in (3). The resulting precursor complex



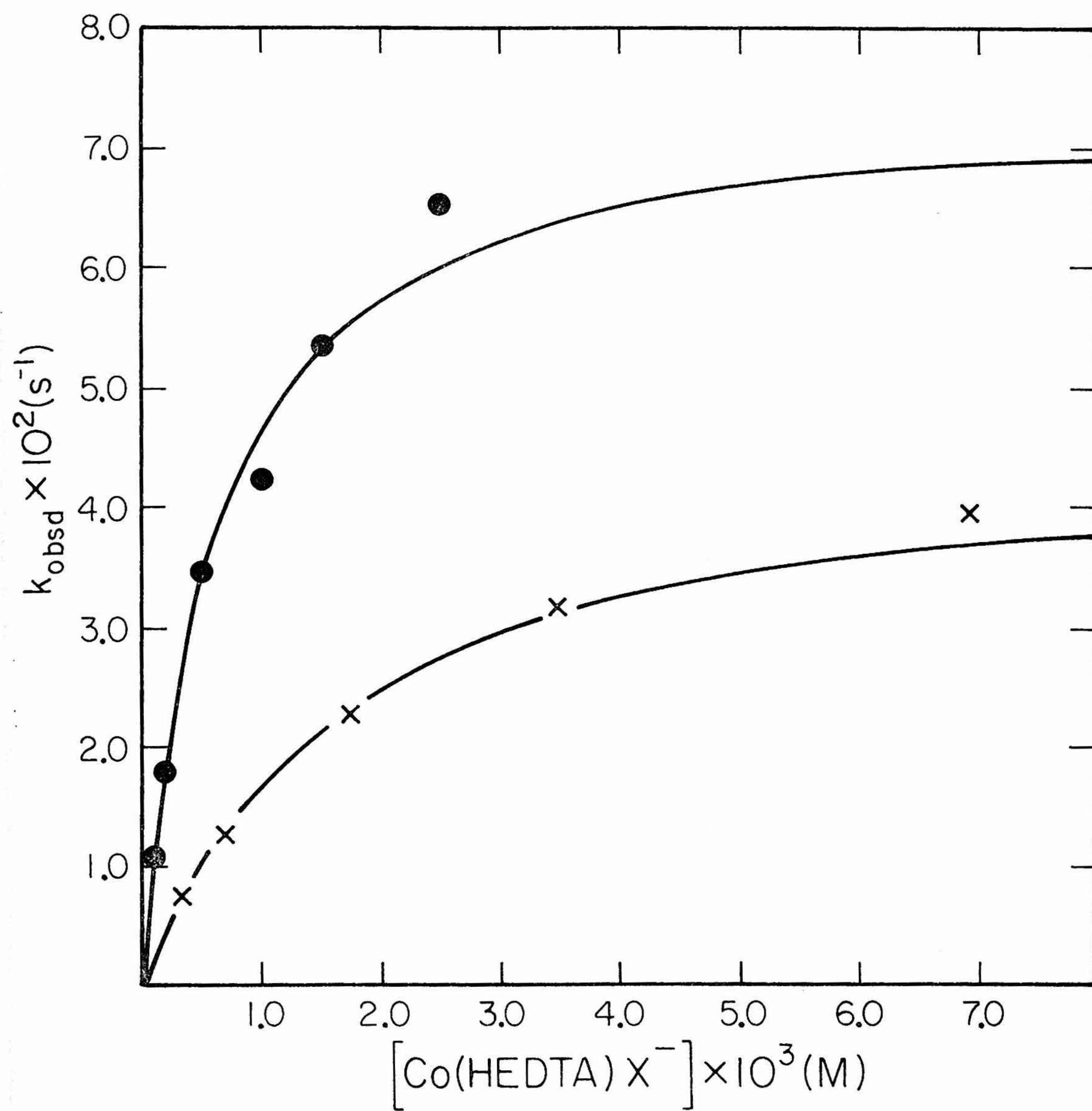
$$\begin{aligned} C_{551}(II) + Ox(III) &\overset{K}{\rightleftharpoons} C_{551}(II)\cdots Ox(III) \\ C_{551}(II)\cdots Ox(III) &\overset{k}{\rightarrow} C_{551}(III)\cdots Ox(II) \\ C_{551}(III)\cdots Ox(III) &\overset{fast}{\rightarrow} C_{551}(III) + Ox(II) \end{aligned} \qquad (3)$$

formation constants are $5.5 \times 10^2\ \underline{M}^{-1}$ and $1.7 \times 10^3\ \underline{M}^{-1}$ and electron transfer rate constants are $4.7 \times 10^{-2}\ s^{-1}$ and $7.5 \times 10^{-2}\ s^{-1}$ for $Co(HEDTA)Cl^-$ and $Co(HEDTA)Br^-$, respectively.

Discussion

The application of Marcus theory to the $Co(EDTA)^-$ oxidation data has resulted in the calculation of electrostatics-corrected protein self-exchange rates, k_{11}^{corr}.[17] This enables a comparison of protein

Figure 3. The dependences of observed rate constants on the concentration of $Co(HEDTA)X^-$ for the oxidation of cytochrome $\underline{c}_{551}$ at 25°, pH 7.0 (phosphate), μ = 0.2 $\underline{M}$ $Co(HEDTA)Br^-$, ●; $Co(HEDTA)Cl^-$, X. The lines are the least squares fit of data obtained using Equation (2).



8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
$k_{obsd} \times 10^2 (s^{-1})$
1.0
2.0
3.0
4.0
5.0
6.0
7.0
$[Co(HEDTA)X^-] \times 10^3 (M)$

reactivity independent of differences in thermodynamic driving force, reagent reactivity, and electrostatic interactions. The reagent and protein properties to be used in the calculations are presented in Table II. The protein charges have been determined from amino acid sequence data and the protein radii from known density and molecular weight values.[18]

The self-exchange rate for $Co(EDTA)^-$ has been measured by Busch[7] to be 3×10^{-7} $\underline{M}^{-1}$ s^{-1}. The cross reactions of $Fe(EDTA)^{2-}$ with the $Co(HEDTA)X^-$ complexes will be used to calculate the oxidant self-exchange rates, as these rates have not been independently measured. The standard reduction potential of $Co(EDTA)^-$ is 380 mv. The potentials of the other $Co(HEDTA)X^-$ complexes have not been evaluated, owing to the irreversibility of the electrode reactions caused by dissociation of the cobalt(II) species.[19] For the purposes of these calculations, it will be assumed that all the cobalt complexes have the same potential. This assumption will only affect the values of the reagent self-exchange and not change the calculated protein self-exchange rates.

The reactivity order for the blue copper proteins as determined by the calculated protein self-exchange rates is stellacyanin > plastocyanin > azurin, which matches that found for the $Fe(EDTA)^{2-}$ reductions and $Co(phen)_3^{3+}$ oxidations of these proteins. Cytochrome $\underline{c}_{551}$ appears more kinetically accessible than cytochrome $\underline{c}$, which agrees with the reactivity pattern observed for the $Co(phen)_3^{3+}$ oxidations.

Table II. Calculated Electrostatics-Corrected $Co(EDTA)^-$ Cross Reaction Rates. [pH 7.0 (phosphate), μ = 0.2 M, 25°C]

Protein	Reagent	k_{12} [a]	E^0_{prot} [b]	E^0_{rgt} [b]	k_{22}	Z_1/Z_1'	Z_2/Z_2'	R_1 [c]	R_2 [c]	W_{12} [d]	W_{21} [d]	W_{11} [d]	W_{22} [d]	k_{11} corr [a]	k_{11} Fe(EDTA) [a]	k_{12} calc [a]	$k_{12}^{obs}/k_{12}^{calc}$
Rhus vernicifera stellacyanin	$Co(EDTA)^-$	2.52×10^{1}	0.180	0.380	3×10^{-7}	10/11	-1/-2	19.5	4	-0.1615	-0.3554	0.1753	0.3701	1.9×10^{5}	2.3×10^{5}	2.9×10^{1}	0.862
	$Co(HEDTA)NO_2^-$	6.74×10^{2}			5×10^{-7}									8.7×10^{7}			
	$Co(HEDTA)Cl^-$	2.71×10^{3}			9×10^{-5}									7.6×10^{6}			
	$Co(HEDTA)Br^-$	1.59×10^{4}			2×10^{-4}									1.2×10^{8}			
Phaseolus vulgaris plastocyanin	$Co(EDTA)^-$	6.33×10^{-3}	0.350	0.380	3×10^{-7}	-10/-9	-1/-2	15.8	4	0.2245	0.4401	0.3551	0.3701	3.8×10^{1}	3.4×10^{1}	9.4×10^{-3}	0.680
	$Co(HEDTA)NO_2^-$	1.15×10^{-2}			5×10^{-7}									7.4×10^{1}			
	$Co(HEDTA)Cl^-$	9.66×10^{-1}			9×10^{-5}									2.9×10^{3}			
	$Co(HEDTA)Br^-$	5.02			2×10^{-4}									3.5×10^{4}			
Horse heart cytochrome c	$Co(EDTA)^-$	6.94×10^{-2}	0.260	0.380	3×10^{-7}	6.5/7.5	-1/-2	16.6	4	0.0876	0.1168	0.0340	0.3701	9.3×10^{1}	6.2	4.2×10^{-2}	1.65
	$Co(HEDTA)NO_2^-$	1.59×10^{-1}			5×10^{-7}									2.8×10^{2}			
	$Co(HEDTA)Cl^-$	8.04			9×10^{-5}									4.1×10^{3}			
	$Co(HEDTA)Br^-$	7.66×10^{1}			2×10^{-4}									1.7×10^{5}			
Pseudomonas aeruginosa cytochrome *c*$_{551}$	$Co(EDTA)^-$	6.27×10^{-1}	0.260	0.380	3×10^{-7}	-3/-2	-1/-2	14.4	4	-0.1444	0.3332	0.1572	0.3701	9.8×10^{3}	2.0	1.0×10^{-2}	62
	$Co(HEDTA)NO_2^-$	1.98×10^{-1}			5×10^{-7}									2.2×10^{5}			
	$Co(HEDTA)Cl^-$	2.61×10^{1}			9×10^{-5}									5.5×10^{4}			
	$Co(HEDTA)Br^-$	1.23×10^{2}			2×10^{-4}									5.6×10^{5}			
Pseudomonas aeruginosa azurin	$Co(EDTA)^-$	1.28×10^{-2}	0.330	0.380	3×10^{-7}	-2/-1	-1/-2	13.9	4	0.0624	0.0624	0.01295	0.3701	5.1×10^{1}	1.2×10^{-2}	2.0×10^{-4}	64
	$Co(HEDTA)NO_2^-$	9.90×10^{-3}			5×10^{-7}									1.8×10^{1}			
	$Co(HEDTA)Cl^-$	1.45×10^{-1}			9×10^{-5}									2.2×10^{1}			
	$Co(HEDTA)Br^-$	2.65			2×10^{-4}									3.3×10^{3}			

[a] In $M^{-1} s^{-1}$.
[b] In V.
[c] In Å.
[d] In kcal/mol.

The cross reaction rates calculated according to Marcus theory for the $Co(EDTA)^-$ oxidations of stellacyanin, plastocyanin, and cytochrome c are within a factor of two of the measured values (Table II). This indicates that the $Co(EDTA)^-$ oxidations and $Fe(EDTA)^{2-}$ reductions of these proteins involve similar protein activation processes. The rates of electron transfer of stellacyanin with all inorganic outer sphere redox agents investigated have been shown to obey Marcus theory. As this protein is the most accessible of all the metalloproteins investigated, it is reasonable to expect that the activation processes for the cross reactions closely resemble those observed for the corresponding self-exchange reactions.

The calculated cross reaction rates for cytochrome c_{551} and azurin are approximately sixty times slower than the measured values. The reactivity of these proteins more closely resembles that observed for the $Co(phen)_3^{3+}$ oxidations. The reactivity order for the reactants is $Fe(EDTA)^{2-} < Co(EDTA)^- < Co(phen)_3^{3+}$ with all the proteins investigated. In general, $Co(EDTA)^-$ is a better reagent than $Fe(EDTA)^{2-}$, but not as good as $Co(phen)_3^{3+}$.

The activation parameters for the oxidation of plastocyanin and cytochrome c, by $Co(EDTA)^-$ are characterized by large enthalpic activation requirements of 13 to 14 kcal/mol coupled with activation entropies of -15 to -26 cal/mol-deg. These values are very similar to the activation enthalpy of 20 kcal/mol and entropy of -21 cal/mol-deg observed for the $Co(EDTA)^-$ self-exchange reaction.[7] This would indicate the same type of outer sphere mechanism as the cobalt self-

exchange with little protein-oxidant interaction in the transition state for the cross reactions. The higher activation enthalpy and more positive activation entropy observed for the oxidation of cytochrome c_{551} are consistent with a mechanism involving $Co(EDTA)^-$ penetration in the transition state.

Reduced azurin prefers a mechanism for which $\Delta H^{\ddagger}$ is smaller (4.1 kcal/mol) and $\Delta S^{\ddagger}$ is more negative (-54 cal/mol-deg). These parameters are consistent with a mechanism involving long distance outer sphere electron transfer. It has been suggested that the blue copper center in azurin is the most inaccessible of the proteins investigated.[20] Nonadiabaticity is expected to become increasingly important as the distance between redox centers increases. The interpretation presented here is consistent with this, as the small $\Delta H^{\ddagger}$ and very negative $\Delta S^{\ddagger}$ for azurin would be expected if the reaction involved long distance electron transfer.

The unusually low activation entropy and enthalpy indicate that the $Co(EDTA)^-$ activation in the cross reaction with azurin is not similar to the activation in the self-exchange. This activation process differs from that involved in the $Fe(EDTA)^{2-}$ reduction of azurin in that the $Fe(EDTA)^{2-}$ reaction proceeds by an adiabatic outer sphere process in accordance with Marcus theory. The $Co(EDTA)^-$ oxidation does not resemble the $Co(phen)_3^{3+}$ oxidation as $Co(phen)_3^{3+}$ is able to induce a conformational change resulting in more favorable overlap for electron transfer. Although a conformational change is apparent, as evidenced by the unusually low activation entropy, the small activation enthalpy indicates that very little redox orbital overlap results

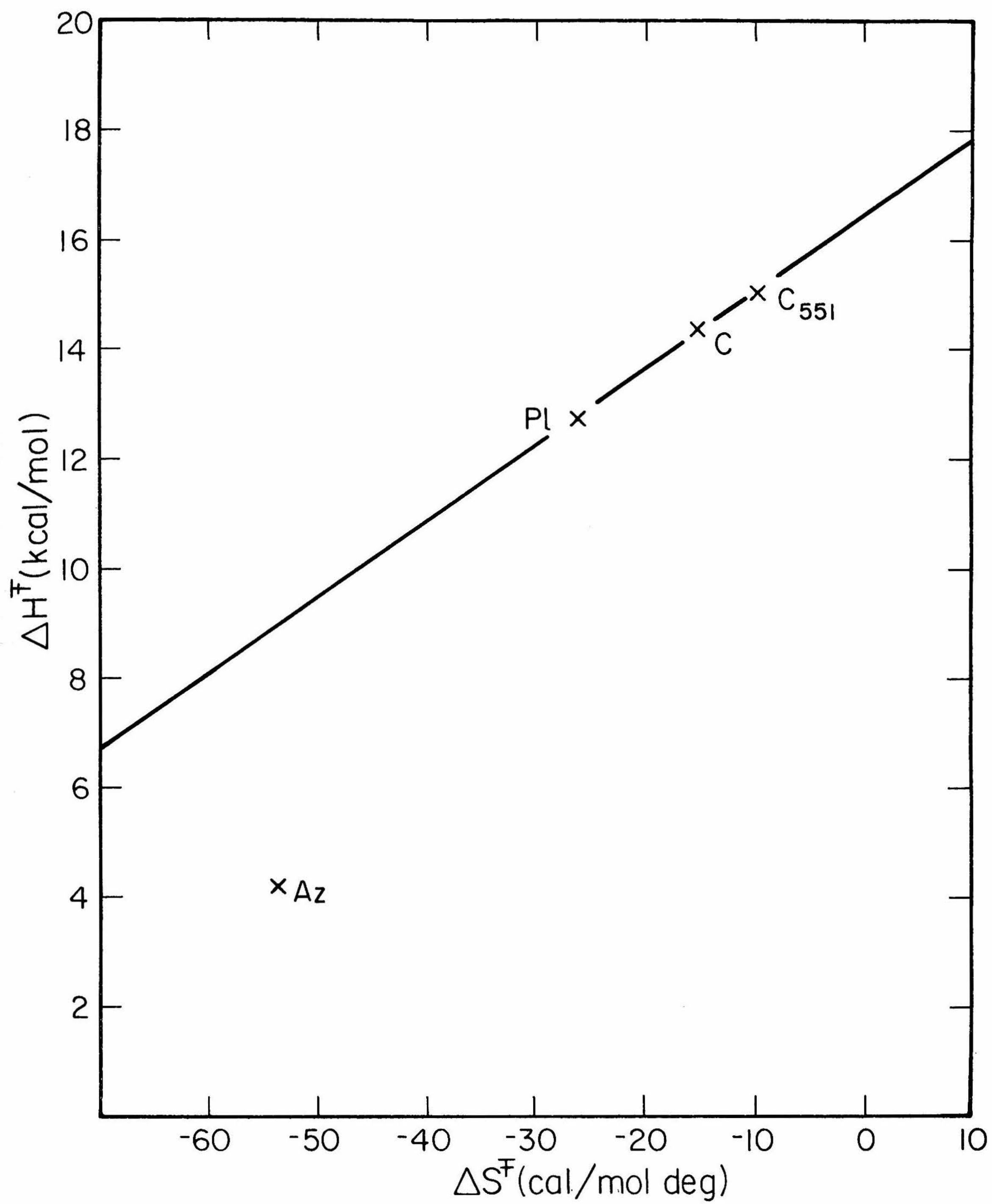
and non adiabatic long distance electron transfer is involved.

A linear compensation plot is obtained for the oxidation of plastocyanin, cytochrome c, and cytochrome c_{551} by $Co(EDTA)^-$ with a β (value) slope of 136K and correlation coefficient of 0.994 as illustrated in Figure 4. This suggests that the mechanisms by which each of these proteins transfer an electron to $Co(EDTA)^-$ are similar.[21] The variations in activation parameters may be accounted for in terms of protein-induced perturbations on a common mechanism. The inclusion of azurin in this plot changes the β value to 252K and the correlation coefficient to 0.986.

The activation parameters for the oxidation of stellacyanin by $Co(EDTA)^-$ cannot be compared directly to those obtained for the other proteins as the mechanism for stellacyanin involves the formation of a pre-equilibrium complex followed by intramolecular electron transfer similar to the mechanism described in (3).[22] The reported enthalpy and entropy correspond to the first order electron transfer step. The fact that the electrostatics-corrected stellacyanin self-exchange rate calculated from the $Fe(EDTA)^{2-}$, $Co(phen)_3^{3+}$, and $Co(EDTA)^-$ cross reactions all agree within a factor of two, suggests that the electron transfer pathway is similar.

The room temperature reactivity for $Co(HEDTA)X^-$ oxidation increases according to $Co(EDTA)^- < Co(HEDTA)NO_2^- < Co(HEDTA)Cl^- < Co(HEDTA)Br^-$ for most reactions investigated, except for the oxidations of cytochrome c_{551} and azurin by $Co(HEDTA)NO_2^-$ where the rates are approximately the same as the corresponding $Co(EDTA)^-$ oxidations. The enhancement is similar to the anion-assisted outer sphere mechanism observed for the reduction by $Fe(HEDTA)^-$. The main

Figure 4. Compensation plot for the oxidation of metalloproteins by $Co(EDTA)^-$. Data from Table I [pH 7.0 (phosphate), $\mu = 0.2$].


20
18
16
14
12
10
8
6
4
2
$\Delta H^{\ddagger}$(kcal/mol)
C551
C
Pl
Az
-60
-50
-40
-30
-20
-10
0
10
$\Delta S^{\ddagger}$(cal/mol deg)

distinction is that the anion is in the inner coordination sphere of the cobalt prior to the electron transfer. These differ from the effects observed for the substituted $Co(phen)_3^{3+}$ ions, as the $Co(phen)_3^{3+}$ complexes involved placing methyl groups on the phenanthroline rings which essentially disrupts the π system available for facile electron transfer in $Co(phen)_3^{3+}$.

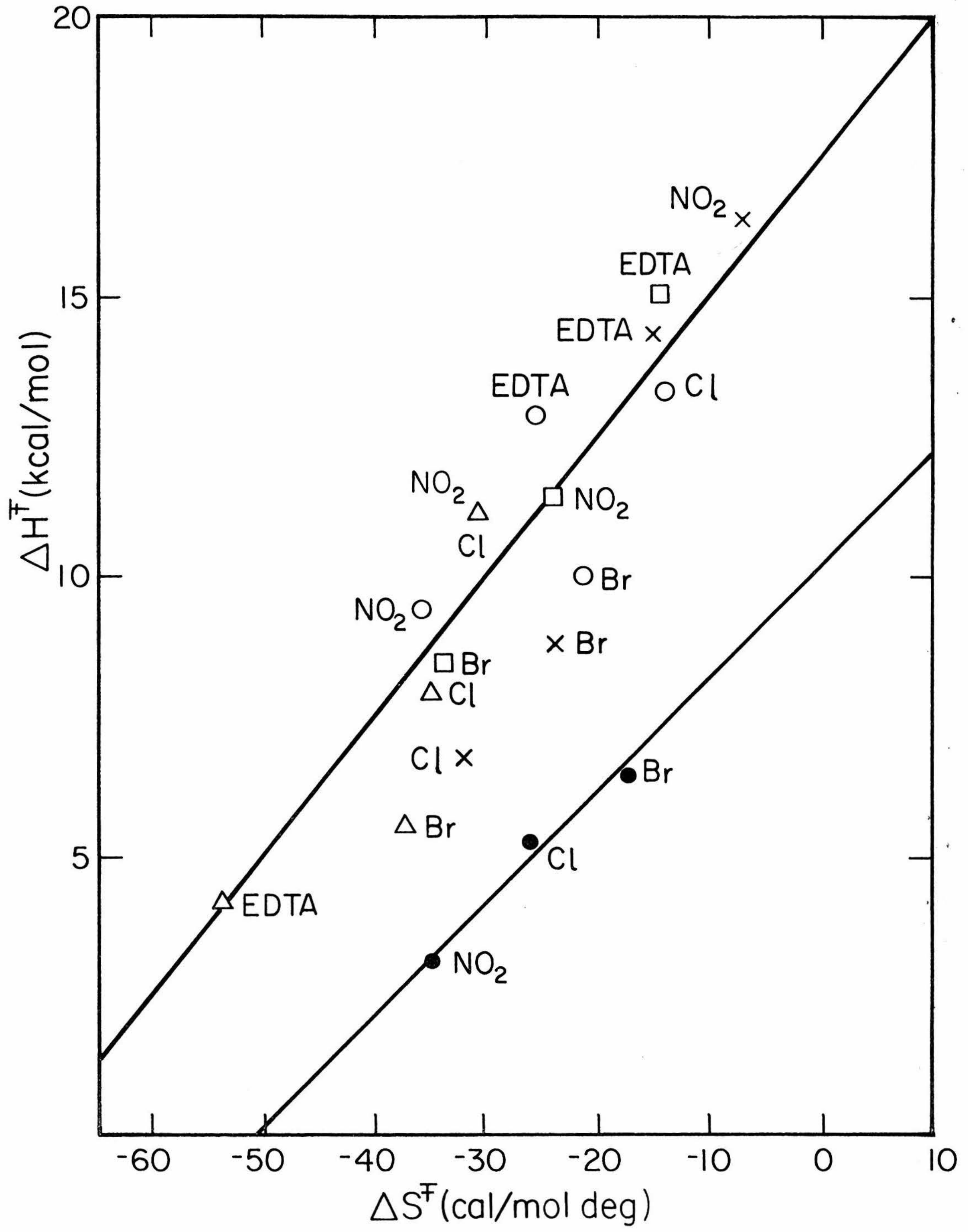
The ratio of electrostatics-corrected protein self-exchange rate constants for the oxidation by $Co(HEDTA)NO_2^-$ compared to $Co(EDTA)^-$ decreases in the order stellacyanin (458) > plastocyanin (1.9) > azurin (0.4) for the blue copper proteins and cytochrome $\underline{c}_{551}$ (22) > cytochrome c (3) (Table III). The substitution has a greater effect on the more kinetically accessible protein sites, as was the case for the substituted $Co(phen)_3^{3+}$ reagents as well. The rate of oxidation by $Co(HEDTA)Cl^-$ is approximately sixty times the $Co(EDTA)^-$ rate for stellacyanin and plastocyanin, with a much smaller effect on azurin oxidation. More dramatic enhancement is observed for the oxidation of the blue copper proteins by $Co(HEDTA)Br^-$ where the rate of oxidation of stellacyanin and plastocyanin are increased seven hundred fold. The rate of oxidation of azurin by $Co(HEDTA)Br^-$ is ninety times the corresponding oxidation by $Co(EDTA)^-$.

The activation parameters for the oxidation of the cytochromes, plastocyanin, and azurin by the substituted $Co(HEDTA)X^-$ complexes are all in one region of the compensation plot with a β value (slope) of 255 K (Figure 5). This indicates that the substituents have similar effects on the oxidation mechanisms for these proteins.[23] The compensation plot for stellacyanin has a β value of 189 K, which indicates

Table III. Comparison of Protein Self-Exchange Rates Calculated from from $Co(HEDTA)X^-$ Cross Reactions

Protein	$k_{11}(NO_2)/k_{11}(EDTA)$	$k_{11}(Cl)/k_{11}(EDTA)$	$k_{11}(Br)/k_{11}(EDTA)$
Rhus vernicifera stellacyanin	4.6×10^{2}	4.0×10^{1}	7.0×10^{2}
Phaseolus vulgaris plastocyanin	1.9	7.6×10^{1}	9.2×10^{2}
Pseudomonas aeruginosa azurin	0.35	0.43	8.9×10^{1}
Horse heart cytochrome c	3.0	4.4×10^{1}	6.5×10^{2}
Pseudomonas aeruginosa cytochrome c_{551}	2.2×10^{1}	5.0	6.4×10^{1}

Figure 5. Compensation plot for the oxidation of metalloproteins by $Co(HEDTA)X^-$ complexes. Data from Table I [pH 7.0 (phosphate), $\mu = 0.2$]: stellacyanin, ●; plastocyanin, O; azurin, Δ; cytochrome c, X; cytochrome c_{551}, □.



20
15
10
5
-60
-50
-40
-30
-20
-10
0
10
ΔH‡ (kcal/mol)
ΔS‡ (cal/mol deg)
NO2
EDTA
EDTA
EDTA
Cl
NO2
NO2
Cl
Br
NO2
Br
Br
Cl
Cl
Br
Br
Cl
EDTA
NO2

a different type of interaction mechanism. Similar results were obtained for the oxidation of the blue copper proteins by modified $Co(phen)_3^{3+}$ ions, where the mechanisms employed by plastocyanin and azurin involve considerable reagent induced protein conformational changes.

In conclusion, the $Co(EDTA)^-$ oxidations and $Fe(EDTA)^{2-}$ reductions of the cytochromes and blue copper proteins involve similar activation processes. $Co(EDTA)^-$ is able to penetrate closer to the redox center in cytochrome $\underline{c}_{551}$ than $Fe(EDTA)^{2-}$ as evidenced by the increased cross reaction rate over the calculated value and more positive activation parameters. Azurin employs a more favorable mechanism of electron transfer in the oxidation by $Co(EDTA)^-$ than in the reduction by $Fe(EDTA)^{2-}$ characterized by low activation parameters generally associated with tunneling. The rate of oxidation is enhanced upon substitution of ligands with more diffuse π systems for electron transfer.

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CHAPTER 6. SUMMARY

A tremendous amount of data have been accumulated over the past few years concerning the electron transfer reactivity of metalloproteins which must be placed in perspective. The relative Marcus theory has been used to compare protein reactivities as the reactions discussed involve outer sphere reagents.

The reduction of metalloproteins by $Fe(EDTA)^{2-}$ provides considerable information on the distance dependence of electron transfer. Previous studies have indicated that although the blue copper site in these proteins is similar, the reactivity decreases according to stellacyanin > plastocyanin > azurin. This has been interpreted in terms of redox center accessibility. The exposed nature of the active site and kinetic accessibility can not be equated for reactions with all redox agents as reagent penetration and protein conformation may also be involved in the mechanism. An examination of the activation parameters and symmetry requirements for electron transfer in the metalloproteins investigated indicates that the reactivity with $Fe(EDTA)^{2-}$ can be interpreted as an indication of the distance of the redox center from the surface of the protein.

This thesis has assisted in formulating this theory by a comparison of the $Fe(EDTA)^{2-}$ reactivities and structures of cytochrome *c* and cytochrome c_{551}. The electrostatics-corrected protein self-exchange rate constants calculated from the cross reactions with $Fe(EDTA)^{2-}$ are similar for the two proteins as described in Chapter 2

and the distance of the heme edge from the surface of the proteins is approximately 2.5 Å and does not vary appreciably. The only other redox metalloprotein investigated whose structure is known is the high potential iron sulfur protein (HIPIP) where the distance of the redox center from the surface of the protein is approximately 4.5 Å and the protein self-exchange rate calculated from the $Fe(EDTA)^{2-}$ cross reaction is 1.3×10^{-2}. The electrostatics-corrected protein self-exchange rates calculated are presented in Table I from the $Fe(EDTA)^{2-}$ cross reactions and the distance dependence of electron transfer illustrated in Figure 1 for all redox metalloproteins investigated.

Similar results were obtained for the oxidation of cytochromes and blue copper proteins by $Co(EDTA)^{-}$ indicating that both oxidation and reduction proceed by similar mechanisms at the same site on the proteins as described in Chapter 5. An examination of the reduction of metalloproteins by $Fe(EDTA)^{2-}$ and oxidation by $Co(EDTA)^{-}$ can provide information concerning the position of the redox center in the protein and nature of the oxidation and reduction sites.

Information concerning the symmetry of the reactive sites of the proteins has been obtained by examining the anion effects on the reduction by $Fe(HEDTA)^{-}$ as described in Chapter 4. These results indicate that electron transfer is enhanced by anions of π symmetry while no effect is observed with anions of σ symmetry. Similar results

Table I. Distance Dependence of Electron Transfer

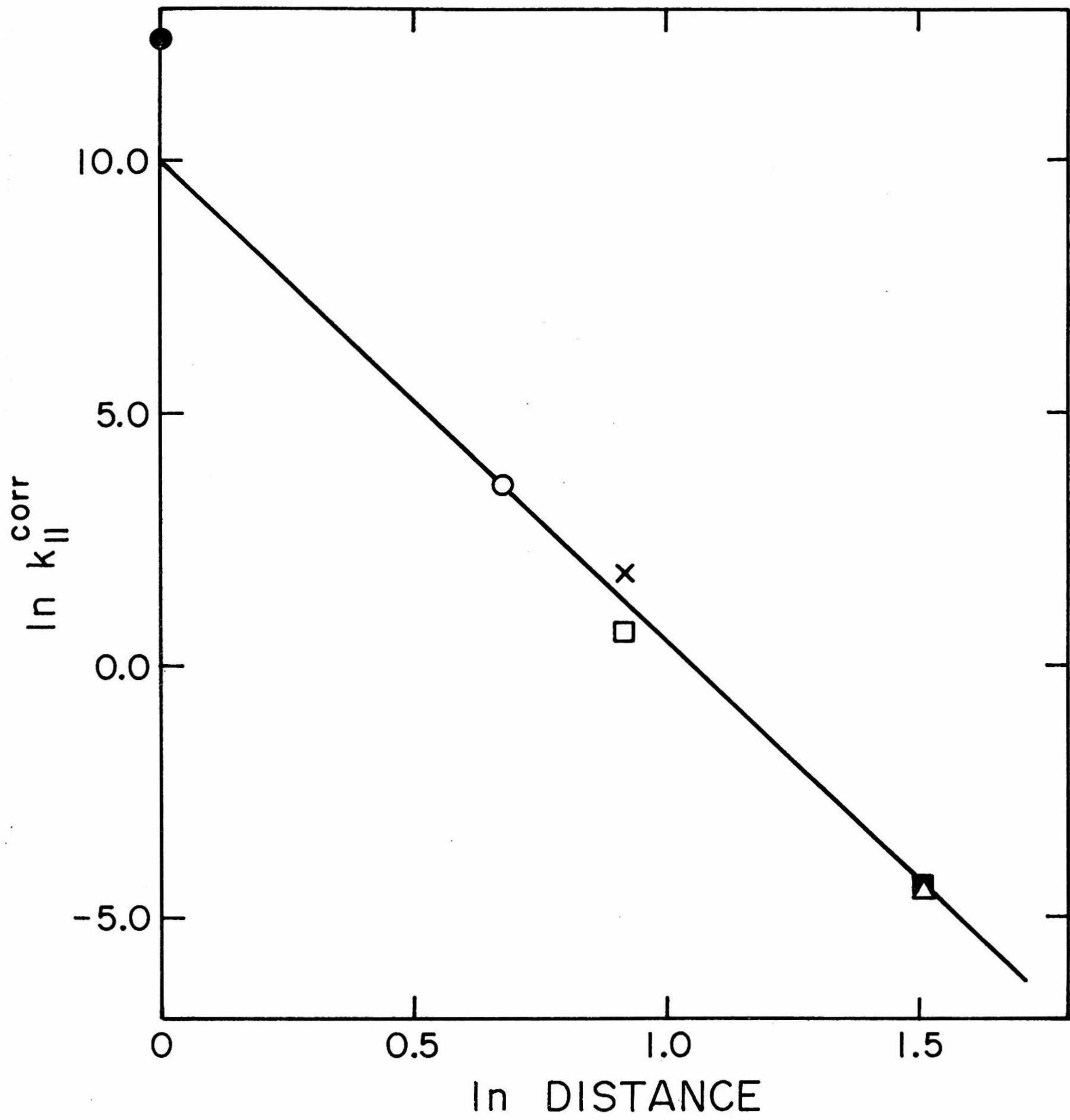
Protein	k_{12}^{corr} (M^{-1} s^{-1})	Distance (Å)
Stellacyanin (*Rhus vernicifera*)	2.3×10^{5} [b]	a
Plastocyanin (*Phaseolus vulgaris*)	3.4×10^{1} [b]	a
Cytochrome c (Horse heart)	6.2 [b]	2.5
Cytochrome c_{551} (*Pseudomonas aeruginosa*)	2.0 [c]	2.5
HiPIP (*Chromatum vinosum*)	1.3×10^{-2} [b]	a
Azurin (*Pseudomonas aeruginosa*)	1.2×10^{-2} [b]	4.5

[a] Structural information not available.

[b] S. Wherland and H. B. Gray, "Biological Aspects of Inorganic Chemistry", D. Dolphin, ed., John Wiley and Sons, Inc., 1977.

[c] Chapter 2 of this thesis.

Figure 1. Distance dependence of electron transfer. Plot of $\ln k_{11}^{corr}$ vs. ln distance. Line is least squares fit of data for which distances are known. Stellacyanin, ●; plastocyanin, ○; cytochrome c, X; cytochrome c_{551}, □; HiPIP, □; azurin, △.



10.0
5.0
0.0
-5.0
ln k_{II}^{corr}
0
0.5
1.0
1.5
ln DISTANCE

were obtained for the oxidation of the blue copper proteins by $Co(phen)_3^{3+}$ where electron transfer was greatly enhanced by the hydrophobic π system of the phenanthroline ligands as described in Chapter 3. The rates of oxidation of the cytochromes and blue copper proteins by $Co(HEDTA)X^-$ complexes are also enhanced by the more diffuse π systems of the oxidant as described in Chapter 5.

This thesis has been concerned with the determination of the mechanisms of oxidation and reduction of the cytochromes and blue copper proteins. Techniques have been developed to examine the symmetry and distance dependence of oxidation and reduction of redox metalloproteins in general.

APPENDIX I. A SUMMARY OF THE RELATIVE MARCUS THEORY ANALYSIS INCLUDING ELECTROSTATIC INTERACTIONS

In order to correct for the electrostatic contributions to the protein self-exchange rates, the free energy for the cross reaction, ΔG^0_{12}, can be expressed as the sum of an electrostatic term, W^0, and a term which is independent of electrostatic interactions, ΔG^0_r, as presented in (1).[1,2] Furthermore, the free energy of the cross

$$\Delta G^0_{12} = \Delta G^0_r + W^0 \quad (1)$$

reaction, ΔG^0_{12} can be related to the change in potential, ΔE^0 by equation (2),

$$\Delta G^0_{12} = -nF\Delta E^0 = -23.06\ \Delta E^0 \text{ kcal/mol} \quad (2)$$

where

F = Faraday's constant.

The electrostatic terms can be expressed as the difference between the work to bring the reactants together, W_{12}, and the work to bring the products together, W_{21}. Substituting into expression (1), we obtain (3).

$$\Delta G^0_r = \Delta G^0_{12} - W_{12} + W_{21} \quad (3)$$

Activation free energies may also be separated into two terms corresponding to equation (1) as seen below:

$$\Delta G^{**}_{11} = \Delta G^*_{11} - W_{11} \quad \text{(for the protein)} \quad (4)$$

$$\Delta G^{**}_{22} = \Delta G^*_{22} - W_{22} \quad \text{(for the reagent)} \quad (5)$$

$$\Delta G^{**}_{12} = \Delta G^*_{12} - W_{12} \quad \text{(for the cross reaction)} \quad (6)$$

where

$$\Delta G_{12}^{**} = \tfrac{1}{2}(\Delta G_{11}^{**} + \Delta G_{22}^{**} + (1 + \alpha^{**})\Delta G_r^0 \qquad (7)$$

and

$$\alpha^{**} = \Delta G_r^0/4\ (\Delta G_{11}^{**} + \Delta G_{22}^{**}) \qquad (8)$$

The values of ΔG_{ab}^* can be calculated from the reaction rates, k_{ab} according to equation (9).[3]

$$k_{ab} = k_B T \exp\left(\frac{-\Delta G_{ab}^*}{RT}\right) = 6.21\times 10^{12} \exp(-\Delta G_{ab}^*/0.5921) \qquad (9)$$

@ 298 K

where

k_B = Boltzmann's constant

T = temperature (K)

h = Planck's constant.

Work terms to be calculated are the work required to bring the protein and reagent together from infinite dilution to their positions in the activated complex. For the purposes of this discussion the two reagents will be considered to be spheres with totally symmetric charge distributions. In this way the actual detailed charge distribution is not required and the work terms may be expressed according to equation (10).[4,5]

$$W = 2.1175 \frac{Z_a Z_b}{(R_a + R_b)} \left[\frac{e^{-\kappa R_b}}{1 + \kappa R_a} + \frac{e^{-\kappa R_a}}{1 + \kappa R_b} \right] \tag{10}$$

where

	Z_a	Z_b	R_a	R_b
W_{12}	Z_1	Z_2	R_1	R_2
W_{21}	Z_2'	Z_1'	R_2	R_1
W_{11}	Z_1	Z_1'	R_1	R_1
W_{22}	Z_2	Z_2'	R_2	R_2

and

$\kappa = 0.329\ \mu^{\frac{1}{2}}\ \text{Å}^{-1}$

Z_1/Z_1' = charge of protein (reactant/product)

Z_2/Z_2' = charge of reagent (reactant/product)

R_1 = radius of protein

R_2 = radius of reagent.

The values of protein and reagent radii[6] were estimated from crystal structure data. In several cases, however, the X-ray crystal structure analysis has not been completed and molecular weight and density data were used to determine the radii. The protein charges were obtained from amino acid sequences.[7]

The activation free energy of the protein, ΔG_{11}^{**}, can be calculated using the quadratic equation described in (11).

$$A(\Delta G_{11}^{**})^2 + B(\Delta G_{11}^{**}) + C = 0 \tag{11}$$

where

$$A = 4$$

$$B = 8\Delta G_{22}^{**} + 4\Delta G_r^0 - 8\Delta G_{12}^{**}$$

$$C = 4\Delta G_{22}^{**}(\Delta G_{22}^{**} + \Delta G_r^0 - 2\Delta G_{11}^{**}) + (\Delta G_r^0)^2.$$

By applying equations (4) and (9) we can obtain the electrostatics-corrected protein self-exchange rates, k_{11}^{corr}

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APPENDIX II. SUPPLEMENTARY MATERIAL

I. Supplementary Data for the Reduction of Pseudomonas aeruginosa cytochrome $\underline{c}_{551}$ by $Fe(EDTA)^{2-}$.

Concentration Dependence

	$[Fe(EDTA)^{2-}] \times 10^3$ (M)	k_{obsd} (s^{-1})	μ
Pseudomonas aeruginosa cytochrome $\underline{c}_{551}$	2.50	10.4	0.2
	2.13	8.88	
	1.88	7.91	
	1.25	5.04	
	0.625	2.52	
	0.125	0.494	
	2.50	13.9	0.1
	2.00	11.5	
	1.50	8.72	
	1.00	5.68	
	0.500	2.87	
	0.250	1.36	

Temperature dependence

ln(k/T)	1/T × 10^3 (K^{-1})	μ
10.11	3.54	0.2
10.75	3.47	
12.62	3.42	
13.53	3.36	
15.64	3.30	

Ionic Strength Dependence

μ	k_{obsd} (s^{-1})
0.05	3.27
0.10	3.86
0.20	4.15
0.30	4.88
0.50	5.70

II. Supplementary Data for the Oxidation of Blue Copper Proteins by $Co(phen)_3^{3+}$.

Concentration dependence

	$[Co(phen)_3^{3+}]$ $\times 10^3$ (M)	k_{obsd} (s^{-1})
Stellacyanin[a]	0.30	48.0
	0.60	97.0
	0.815	134.4
	1.10	175.6
	1.35	216.7
	1.60	324.1
	1.85	359.4
	2.10	390.6
	2.35	414.7
Plastocyanin[b]	0.117	0.572
	0.164	0.918
	0.234	1.29
	0.351	1.99
	0.468	2.54
	0.700	3.69
	0.935	4.56
	1.170	5.84

	$[Co(phen)_3^{3+}]$ $\times 10^3$ (M)	k_{obsd} (s^{-1})
Azurin[c]	0.100	0.34
	0.250	0.78
	0.500	1.56
	0.750	2.39
	1.020	3.27

a. pH 7.0 (phosphate) $\mu = 0.1$, T = 25°

b. pH 7.0 (phosphate) $\mu = 0.1$, T = 25°

c. pH 7.0 (phosphate) $\mu = 0.2$, T = 25°

Temperature Dependence

	ln(k/T)	1/T $\times 10^3$ (K^{-1})
Stellacyanin[a]	5.817	3.57
	5.945	3.51
	6.146	3.46
	6.409	3.35
	6.506	3.30
	6.928	3.23
Plastocyanin[b]	1.393	3.57
	1.835	3.52
	2.161	3.47
	2.687	3.39
	2.818	3.36
	3.302	3.29
Azurin[c]	0.829	3.56
	1.463	3.48
	1.784	3.42
	2.348	3.35
	2.836	3.30

a. pH 7.0 (phosphate), $\mu = 0.5$

b. pH 7.0 (phosphate), $\mu = 0.1$

c. pH 7.0 (phosphate), $\mu = 0.2$

III. Supplementary Data for the Reduction of Metalloproteins by $Fe(HEDTA)^-$ [pH 7.0 (phosphate), μ = 0.2).

Concentration dependence @ 25°

	$[Fe(HEDTA)^-] \times 10^3$ (M)	k_{obsd} (s^{-1})
Stellacyanin	2.71	148
	2.04	114
	1.36	74.0
	0.950	52.9
	0.678	37.2
Plastocyanin	2.71	88.9
	2.04	70.0
	1.36	44.9
	0.950	31.8
	0.678	20.6
Azurin	6.15	2.59
	4.91	2.18
	3.68	1.66
	2.46	1.15
	1.23	0.573
	0.615	0.277
Cytochrome c	2.71	10.97
	1.63	6.90
	1.09	4.31
	0.540	2.06
	0.271	0.549
Cytochrome c_{551}	2.71	6.74
	2.17	5.36
	1.63	4.07
	1.09	2.76
	0.540	1.45
	0.271	0.714

Temperature Dependence

	$[Fe(HEDTA)^-] \times 10^3$ (M)	T (K)	k_{obsd} (s^{-1})	ln(k/T)	$1/T \times 10^3 (K^{-1})$
Stellacyanin	1.36	303.5	76.1	5.22	3.29
		298.0	74.0	5.21	3.36
		292.7	66.4	5.12	3.42
		287.5	67.8	5.16	3.48
		282.4	67.5	5.17	3.54
Plastocyanin	1.36	283.9	37.1	4.57	3.52
		289.5	37.3	4.55	3.45
		295.0	41.4	4.64	3.39
		300.2	46.7	4.74	3.33
		305.1	56.8	4.92	3.28
	2.71	284.2	72.0	4.54	3.52
		289.2	77.5	4.59	3.46
		293.3	80.0	4.61	3.41
		298.3	87.2	4.68	3.35
		303.7	105.0	4.85	3.29
Azurin	1.90	282.5	0.679	0.235	3.54
		287.4	0.817	0.403	3.48
		290.0	0.833	0.413	3.45
		297.7	0.978	0.548	3.36
		305.7	1.04	0.583	3.27
	0.815	286.9	0.312	0.288	3.49
		291.4	0.363	0.424	3.43
		298.1	0.417	0.540	3.35
		305.7	0.483	0.662	3.27
Cytochrome <u>c</u>	2.71	280.5	4.45	1.77	3.57
		288.2	5.92	2.03	3.47
		295.1	6.96	2.16	3.39
		301.3	9.67	2.47	3.32
		306.6	11.79	2.65	3.26
Cytochrome c_{551}	2.71	281.2	4.14	1.69	3.56
		287.5	5.48	1.95	3.48
		293.1	6.63	2.12	3.41
		298.0	7.22	2.19	3.36
		304.2	8.00	2.27	3.29
	1.36	284.9	1.99	1.64	3.51
		290.8	2.29	1.76	3.44
		297.7	3.31	2.10	3.36

Anion dependence*

	$[Fe(HEDTA)^-]$ $\times 10^3$ (M)	[Anion] (M)	k_{obsd} (NNN) (s^{-1})	k_{obsd} (SCN) (s^{-1})
Plastocyanin	0.271	0.204	42.2	27.2
		0.179	37.6	25.7
		0.154	36.7	24.8
		0.129	30.9	22.9
		0.104	26.6	20.2
		0.075	22.0	17.6
		0.054	18.1	14.3
		0.038	14.5	12.1
		0.025	11.5	10.1
		0.013	7.75	7.34
		0.0	5.44	5.44
Azurin	2.71	0.204	32.3	26.7
		0.179	30.2	26.8
		0.154	28.4	22.8
		0.129	27.8	21.3
		0.104	24.1	20.9
		0.075	18.1	18.2
		0.054	13.9	16.8
		0.038	11.5	13.0
		0.025	8.57	9.39
		0.013	6.06	6.74
		0.0	2.84	2.84

	$[Fe(HEDTA)^{-}]$ $\times 10^{3}$ (M)	[Anion] (M)	k_{obsd}(NNN) (s^{-1})	k_{obsd} (SCN) (s^{-1})
Cytochrome c	2.71	0.204	186	187
		0.179	179	187
		0.154	167	192
		0.129	163	184
		0.104	153	180
		0.075	125	156
		0.054	98.2	130
		0.038	76.2	96.5
		0.025	53.7	67.6
		0.013	30.7	43.0
		0.0	8.26	8.26
Cytochrome c_{551}	2.71	0.204	68.4	89.1
		0.179	62.4	84.5
		0.154	55.1	74.8
		0.129	49.5	72.5
		0.104	47.4	62.6
		0.075	40.2	55.4
		0.054	33.8	49.0
		0.038	28.9	40.7
		0.025	23.9	32.9
		0.013	18.5	27.0
		0.0	14.0	14.0

*pH 7.0 (phosphate), μ = 0.5, T = 25°

IV. Supplementary Data for the Reduction of Metalloproteins by $Co(EDTA)^-$

[pH 7.0 (phosphate), $\mu = 0.2$]

Concentration dependence @ 25°

	$[Co(EDTA)^-] \times 10^3$ (M)	$k_{obsd} \times 10^4$ (s^{-1})
Plastocyanin	2.50	4.12
	2.13	4.26
	1.88	3.40
	1.25	3.60
	0.625	1.25
Azurin	3.34	0.429
	2.50	0.216
	2.13	0.218
	0.830	0.0865
Cytochrome *c*	2.50	2.15
	1.88	1.72
	1.25	1.04
	0.500	0.943
	0.125	0.383
Cytochrome c_{551}	2.50	16.8
	1.88	13.1
	1.25	9.85
	0.625	4.45
	0.468	2.85
	0.313	2.41

Temperature dependence

	ln(k/T)	1/T $\times 10^3 (K^{-1})$
Plastocyanin	-11.73	3.54
	-11.25	3.41
	-10.57	3.36
	-10.38	3.30
Azurin	-10.57	3.53
	-10.45	3.47
	-10.26	3.36
	-10.07	3.30
Cytochrome c	-15.24	3.53
	-14.59	3.47
	-14.49	3.41
	-14.27	3.36
	-13.18	3.30
Cytochrome c_{551}	-14.09	3.53
	-14.16	3.47
	-12.62	3.36
	-12.47	3.30

Oxidation by $Co(HEDTA)X^-$ Complexes [pH 7.0 (phosphate),

$\mu = 0.2$, T = 25°]

	$[Co(HEDTA)Br^-] \times 10^3$ (M)	k_{obsd} (s^{-1})
Stellacyanin	2.5	38.4
	2.0	34.4
	1.0	17.1
	0.5	9.68
	0.2	

	$[Co(HEDTA)Cl^-] \times 10^3$ (M)	k_{obsd} (s^{-1})
	4.12	11.2
	3.29	8.64
	1.65	4.98
	0.83	1.92
	0.329	0.843
	0.165	0.413

	$[Co(HEDTA)NO_2^-] \times 10^3$ (M)	k_{obsd} (s^{-1})
	1.25	0.902
	1.00	0.705
	0.50	0.415
	0.25	0.216
	0.10	0.122
	0.05	0.0773

	$[Co(HEDTA)Br^-] \times 10^3$ (M)	$k_{obsd} \times 10^2$ (s^{-1})
Plastocyanin	2.50	1.31
	2.00	1.09
	1.00	0.524
	0.50	0.307
	0.20	0.199
	0.10	0.0979

	$[Co(HEDTA)Cl^-] \times 10^3$ (M)	$k_{obsd} \times 10^3$ (s^{-1})
	2.14	2.23
	1.71	1.44
	0.856	0.833
	0.428	0.417
	0.171	0.204

	$[Co(HEDTA)NO_2^-] \times 10^3$ (M)	$k_{obsd} \times 10^5$ (s^{-1})
	3.33	3.81
	2.50	4.89
	1.88	2.58
	1.25	1.47
	0.625	1.12

	$[Co(HEDTA)Br^-] \times 10^3$ (M)	$k_{obsd} \times 10^3$ (s^{-1})
Azurin	2.50	6.85
	1.50	3.89
	1.00	2.60
	0.50	1.25
	0.20	0.684
	0.10	0.490

	$[Co(HEDTA)Cl^-] \times 10^3$ (M)	$k_{obsd} \times 10^3$ (s^{-1})
	2.88	2.09
	2.30	0.569
	1.15	0.336
	0.806	0.375
	0.576	0.310

	$[Co(HEDTA)NO_2^-] \times 10^3$ (M)	$k_{obsd} \times 10^5$ (s^{-1})
	2.50	2.05
	1.67	0.972
	1.26	0.691
	0.830	0.415

	$[Co(HEDTA)Br^-] \times 10^3$ (M)	k_{obsd} (s^{-1})
Cytochrome *c*	2.50	0.192
	2.00	0.151
	1.00	0.0736
	0.50	0.0362
	0.20	0.0150
	0.10	0.00809

$[Co(HEDTA)Cl^-] \times 10^3$ (M)	$k_{obsd} \times 10^2$ s^{-1})
1.67	1.54
1.33	1.30
1.00	0.975
0.667	0.709
0.333	0.493

$[Co(HEDTA)NO_2^-] \times 10^3$ (M)	$k_{obsd} \times 10^4$ (s^{-1})
3.33	5.25
2.50	4.59
1.67	2.38
0.833	1.56

	$[Co(HEDTA)Br^-] \times 10^3$ (M)	$k_{obsd} \times 10^2$ (s^{-1})
Cytochrome c_{551}	2.50	6.55
	1.50	5.35
	1.00	4.22
	0.50	3.47
	0.20	1.78
	0.10	10.7

$[Co(HEDTA)Cl^-] \times 10^3$ (M)	$k_{obsd} \times 10^2$ (s^{-1})
3.46	3.30
1.73	3.17
0.865	3.13
0.346	1.13
0.173	0.706

$[Co(HEDTA)NO_2^-] \times 10^3$ (M)	$k_{obsd} \times 10^4$ (s^{-1})
1.88	4.35
1.25	2.64
0.625	1.24
0.313	1.45

PROPOSITION I. THE DETERMINATION OF THE SITE OF ELECTRON TRANSPORT IN PROTEINS BY MAPPING THE SURFACE REACTIVITY

This proposal is concerned with the attachment of a probe reagent to a specific site on the polypeptide chain of a protein through some chemical modification and the attempt to measure kinetic parameters for intramolecular electron transfer between the protein redox center and the captive redox agent. It is recognized[1,2] that such intramolecular kinetic data can provide a more detailed characterization of electron transfer than do corresponding intermolecular results. The first part of the experiment is designed to provide important new information on the rates of electron transfer between redox units that are separated by rather long distances (7 - 14Å),[3] and to test the possibility that special mechanisms may exist that allow rapid electron transfer at such distances. In the second part of the experiment, derivatives will be synthesized that allow the captive redox agents to contact the protein redox center, and intramolecular electron transfer rates will be compared to those obtained previously. Activation parameters will be determined in all cases, in order to characterize further the nature of the electron transfer process.

Initial experiments will involve horse heart cytochrome c, as here there is a solid foundation of structural and solution kinetic data. There have been three proposed mechanisms for electron transfer in cytochrome c. The first,[4] and most widely accepted, is transfer through the partially exposed porphyrin edge. The second mechanism[5] involves the reorganization of aromatic side groups so as to form a low energy pathway. The third mechanism[6] involves electron tunneling over fairly long distances.

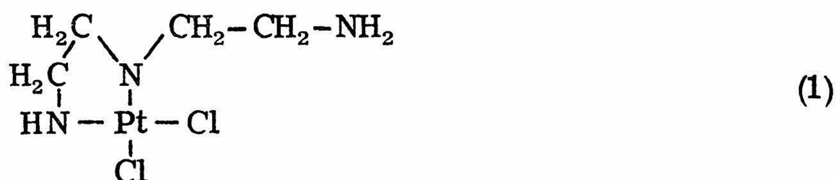
Although the X-ray crystal structures[3] of these proteins are extremely important to the understanding of the mechanisms involved, they serve to propose possible mechanisms rather than provide conclusive evidence in support of a specific mechanism. The reactions of these redox proteins with other proteins in the respiratory chain[7] and with inorganic reagents[8] also provide considerable information concerning the type of interaction involved. It would be desirable, however, to be able to map out the surface of a redox protein with regard to reactivity toward a specific reagent and hence determine the exact site of the most favorable interaction as well as the possibility of long distance transfer in proteins.

One such way to map the redox capabilities of the protein surface would be to synthesize polypeptide chains of various lengths. One end of this polypeptide chain must be attached to a specific site on the protein surface in such a way that the original conformation of the protein is not disturbed. The other end must contain a suitable probe of the protein reactivity, hence, an oxidant or reductant which is inert to substitution in both oxidation states under consideration. As the length of the chain increases the reactivity of the protein toward the reagent should increase until the reagent is in the most favorable position for interaction with the protein. The problem is then divided into three categories. The first is to find a labeling technique which will tag the protein in one and only one specific place. The second is to prepare polypeptide chains of various lengths. The third is to find a redox agent which will remain attached to the chain in both oxidation states.

Heavy metal derivatives of proteins have been extensively utilized by X-ray crystallographers[9] in order to obtain isomorphous protein crystals which are invaluable in determining the final structure of the protein. These derivatives would be more useful for the purposes of this experiment than those which modify the amino acid side groups as heavy metals coordinate to the surface without altering the tertiary structure of the molecule. Heavy metal derivatives of platinum and uranium with cytochrome c have been characterized by R. E. Dickerson and coworkers.[10]

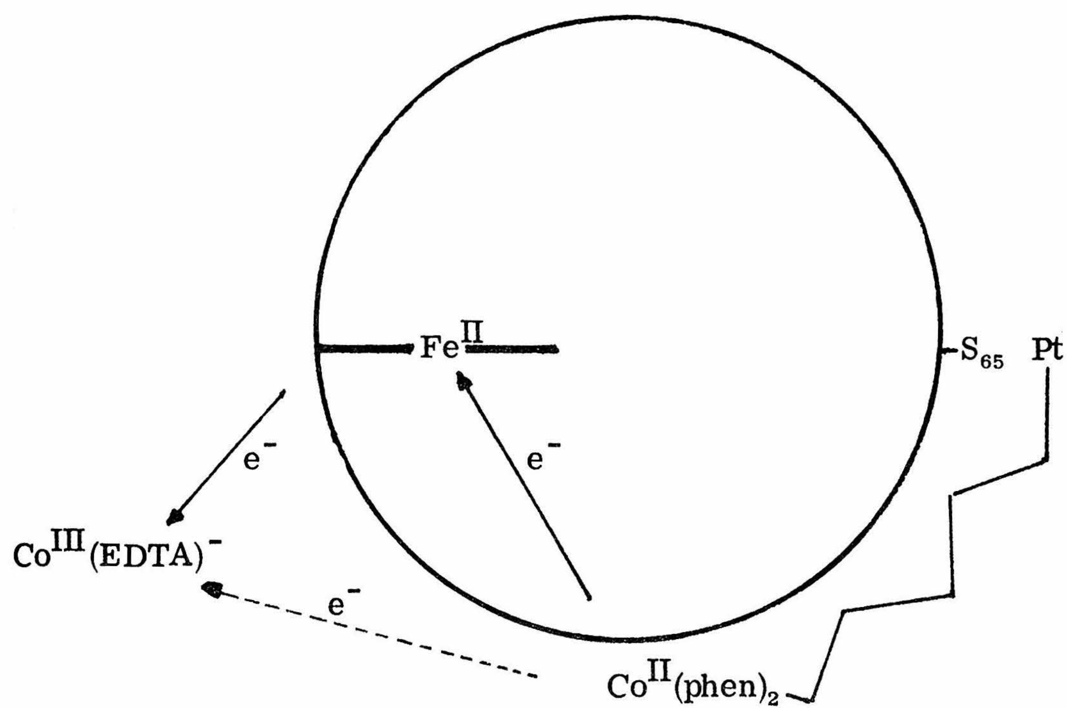
The platinum derivatives are of considerable important to this experiment since they are relatively stable under physiological conditions and form a one-to-one complex with horse heart cytochrome c. The X-ray crystal structure of the K_2PtCl_4 derivative indicates that the platinum is bound to the methionine 65 sulfur which is on the surface of the protein directly opposite the partially exposed heme edge as shown schematically in Figure 1.

Preliminary experiments also indicate that a similar protein-labeled complex can be formed with other more complex platinum derivatives such as $Pt(dien)Cl_2$ shown in (1). Therefore, a platinum



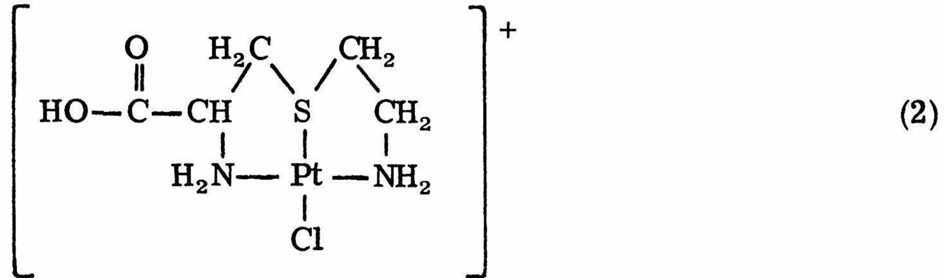
complex capable of being attached to one end of a long polypeptide chain would be suitable for one end of this proposed molecule. One

Figure 1. Schematic representation of the reactions involved in the final experiment. [⟶ indicates slow electron transfer and ---→ indicates fast electron transfer.] Due to the slow reaction of $Co(EDTA)^-$ the different electron transfer steps can be separated.

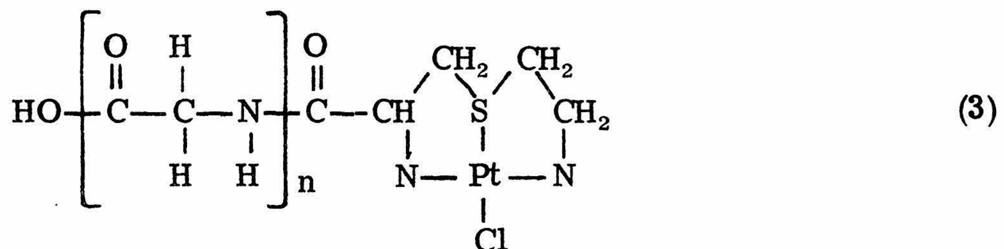


Fe^II
S65
Pt
e⁻
e⁻
e⁻
Co^III(EDTA)⁻
Co^II(phen)2

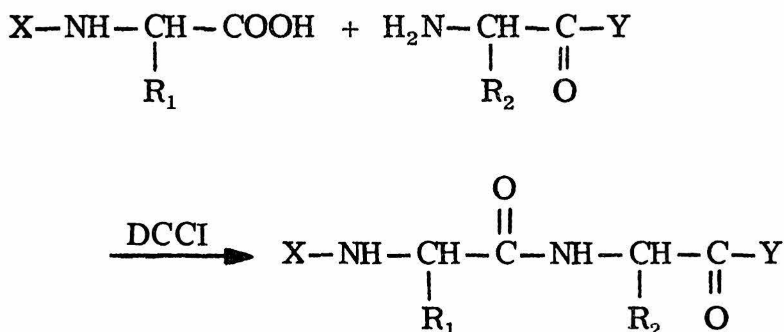
such complex is Pt(thialysine)Cl^+ (2) which can be prepared[11] by

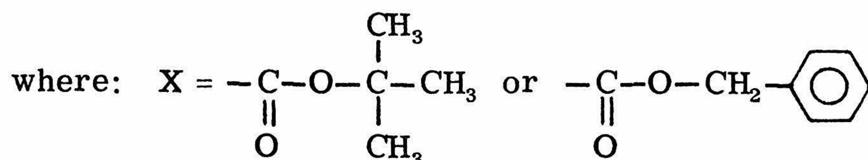


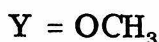
combining equimolar quantities of thialysine and K_2PtCl_4 in 0.03N HCl. After stirring for one hour the solution is alkanized to pH = 5.0 with 1N NaOH. The pale yellow filtrate can be obtained by evaporating the solution at room temperature and recrystallizing from ethanol/water. Presumably this same procedure may be applied to prepare the corresponding platinum complex of a long polypeptide chain with a thialysine ligand on the end as described in (3).



The peptides can be synthesized according to the literature preparations[12] outlined below:

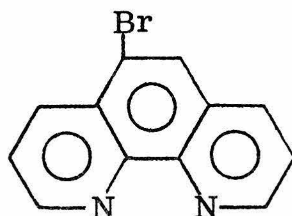






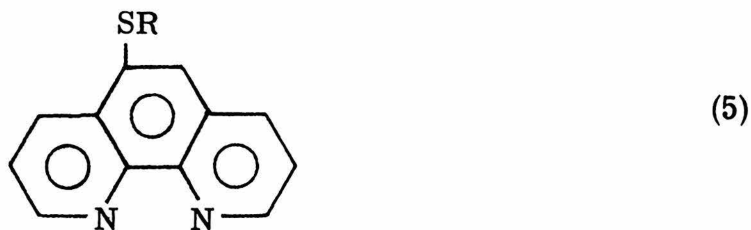
The remaining portion of the molecule to be discussed involves the selection of a redox agent which will serve as the probe of reactivity for the protein surface. A suitable reagent for this purpose is a tris Cobalt (III) complex of 1,10-phenanthroline. This complex is capable of oxidizing cytochrome c and is also inert in both oxidation states.

The polypeptide chain can be attached to the cobalt complex by modifying one of the phenthroline ligands. $[Co(phen)_2(5\text{-}Br\text{-}phen)]^{3+}$ can be prepared by combining equimolar quantities of $[Co(phen)_2Cl_2]^+$ and the ligand 5-Br-1,10-phenanthroline (4). This ligand can be



(4)

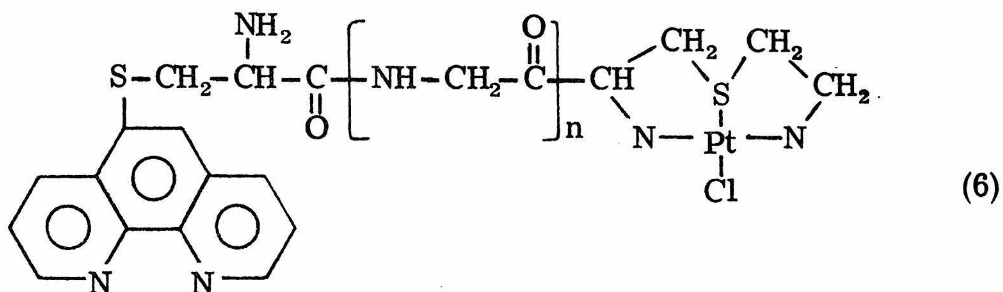
modified further by reacting it with cysteine to form 5-Cys-1,10-phenanthroline (5). Presumably, the cobalt (III) complex of the



(5)

cysteine modified phenanthroline can be prepared as previously described from $[Co(phen)_2Cl_2]^+$.

The strategy for this portion of the experiment is as follows: (1) prepare polypeptide chains of various lengths with thislysine on one end and cysteine on the other, (2) react the cysteine with 5-Br-phenanthroline, (3) react the thialysine with $[PtCl_4]^{2-}$, and (4) react this modified phenanthroline ligand illustrated in (6) with $[Co(phen)_2Cl_2]^+$. The platinum end of this complex should coordinate



(6)

to the methionine 65 of horse heart cytochrome c and the length of the chain can be varied.

A variety of techniques can be used to initiate electron transfer. As the platinum (II) labeling of methionine would be expected to be very fast ($\sim 10^8\ M^{-1}s^{-1}$) the cytochrome complex can be prepared

with iron (II) heme and cobalt (III) oxidant. The slower electron transfer process can be monitored spectrally after fast platinum labeling. If this reaction is not as fast as expected, the protein complex can be prepared in the fully reduced form and the $Co(phen)_3^{3+}$ complex oxidized by adding one equivalent of $Co(EDTA)^-$ as illustrated in Figure 1. The advantage of this external oxidant is that it reacts very fast with $Co(phen)_3^{3+}$ and very slowly with cytochrome c. These experiments should provide important information on the kinetics of long distance electron transfer and, more specifically, the mechanism of electron transfer in cytochrome c.

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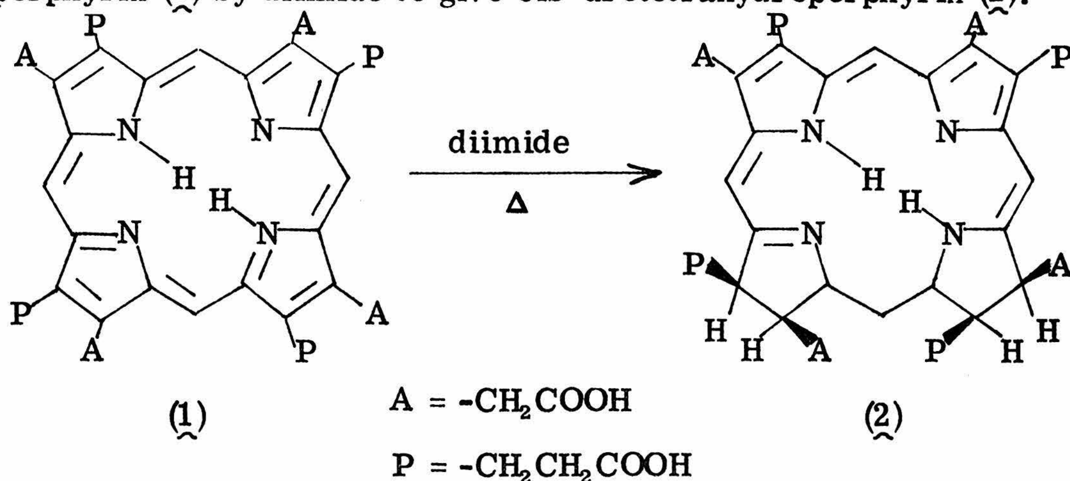
PROPOSITION II. THE STRUCTURE AND MECHANISM OF CATALYSIS OF NITRITE AND SULFITE REDUCTASES

This proposal is concerned with elucidating the mechanism of catalysis of nitrite and sulfite reductases by: 1) the preparation and characterization of synthetic analogs of the seroheme prosthetic group to determine the substrate binding site; 2) utilization of the techniques of iron-sulfur core extrusion to identify and characterize the iron-sulfur component; and 3) determination of the mechanism of electron transfer in these enzymes with inorganic reagents. As most of the available nitrogen and sulfur in the biosphere is oxidized[1] (N_2, NO_3^-, NO_2^-, SO_4^{2-}, SO_3^{2-}) and most animals require and are unable to reduce these compounds, they must rely on plants and microorganisms that are capable of reducing and incorporating the resulting NH_3 and H_2S into organic linkages. If we are able to understand the catalytic mechanism involved, it might be possible to apply the results toward optimization of the reductase activity and synthesis of inorganic reagents designed to treat diseases resulting from malfunctions in this process and to improve the nutritional value of our food products.

Recent evidence strongly suggests that spinach ferredoxin-nitrite reductase (EC 1.7.7.1; ammonia:ferredoxin oxidoreductase) and Escherichia coli sulfite reductase (EC 1.8.1.2; hydrogen sulfide: $NADP^+$ oxidoreductase) contain similar iron tetrahydroporphyrin prosthetic groups.[2] The specific experiments proposed for preparing

a synthetic analog would involve the stereoselective reduction of uroporphyrin (1) by diimide to give cis-urotetrahydroporphyrin (2).[3]



This procedure has been shown to selectively cis-hydrogenate octaethylporphyrin and tetraphenylporphyrin in very high yields (72-98%).[4]

Once the iron complex of (2) has been prepared,[5] a determination of the nitrite and sulfite binding sites can be attempted and equilibrium constants evaluated. As carbon monoxide and cyanide are reductase inhibitors,[6] it would also be interesting to examine the relative stability of these complexes. The visible absorption spectra of porphyrin complexes vary significantly depending upon the nature of the sixth metal ligand[7] and this can be utilized effectively to determine stability constants if the binding site is the iron atom. The ^{13}C nuclear magnetic resonance (NMR) spectroscopy of labeled cyanide and carbon monoxide complexes would also be useful in determining whether the site of interaction is at the porphryin edge or the iron atom.[8] In addition to the spectroscopic studies, part of

this work will be devoted to determining if such model complexes can effect $SO_3^{2-} \rightarrow S^{2-}$ and $NO_2^- \rightarrow NH_3$ reductions in the presence of reducing agents.

The nitrite and sulfite reductases to be considered also contain an iron-sulfur component.[9] The exact composition and structure of this site is not known. Holm and coworkers[10] have recently developed a method for iron-sulfur core extrusion which would facilitate the identification and characterization of the iron-sulfur site in these proteins and will be attempted.

The next seires of experiments proposes to examine the mechanism of electron transfer of these enzymes by inorganic redox agents. Although the exact potentials of these proteins have not been determined, they are estimated to be less than -0.45 volts.[11] A suitable reductant for these enzymes would be $V(EDTA)^{2-}$, as it is reasonably stable under physiological conditions, has an estimated potential of less than -1.0 volt, and its visible spectrum (and that of $V(EDTA)^-$) should not interfere with observations of the reductase bands.[12] The mechanism of oxidation of these proteins by $Fe(EDTA)^-$ can also be monitored as the potential of the iron complex is +0.12 volts.[13] These experiments should enable the determination of the mechanism of reduction and oxidation of these proteins by inorganic reagents in addition to providing information concerning the accessibility of the redox centers and the rate of intramolecular electron transfer in these enzymes.[14,15]

In summary, I propose a series of experiments directed toward understanding the mechanism of catalysis of nitrite and sulfite reductases through the characterization of the iron redox centers, identification of the substrate binding site, and examination of the electron transfer properties of these enzymes with inorganic reagents.

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PROPOSITION III. THE KINETICS OF REDUCTION OF FERRIFERIOXIMINE B BY SPINACH FERREDOXIN

Siderochromes are red-brown, iron-containing metabolites involved in iron transport with a characteristic absorption band at 420-500 nm.[1] There are two general types of siderochromes,[2] the phenolates and the hydroxamates. The structures of these compounds are illustrated in Figures 1 and 2. The exclusive presence of oxygen as the coordinating atoms result in a high iron (III)/iron (II) stability ratio.

Deferriferrioximine B is the iron-free, linear, trihydroxamate siderochrome which is useful in removing pathological deposits of iron and in neutralizing accidentally ingested quantities of iron. One proposed mechanism[3] of iron transport in this system involves the formation of iron (III) complexes outside the cell membrane, followed by active transport of the complex inside the cell, and subsequent release of iron to be used in the synthesis of iron enzymes and proteins. One difficulty inherent in this scheme is that ferrous ion is required by the microbe for incorporation into the various enzymes and proteins, whereas ferric ion is the most abundant source of iron outside the membrane. This implies that once the iron (III) complex has been transferred inside the cell, a mechanism must exist whereby the iron is released from the complex and reduced to iron (III).

Since ferrousferrioximine B has not been detected inside the cells of microbes, a more recent proposed mechanism[4] suggests that the mode of transport involves the reduction of the iron (III) complex accompanied by the dissociation of the complex as soon as it enters the interior of the microbe. It is therefore extremely important to understand the mechanism of reduction of the ferrioximine B complex.

Figure 1. The structure of the hydroxamate siderochromes.

For ferrioximine B, R = H, and R′ = CH_3.

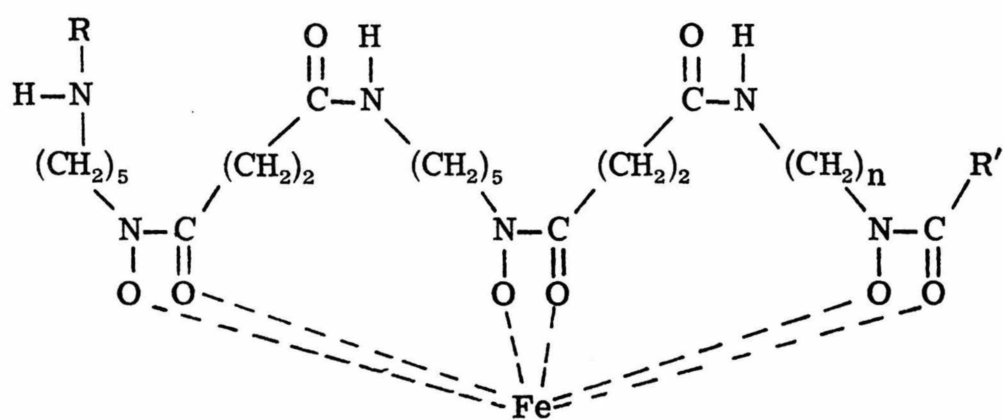
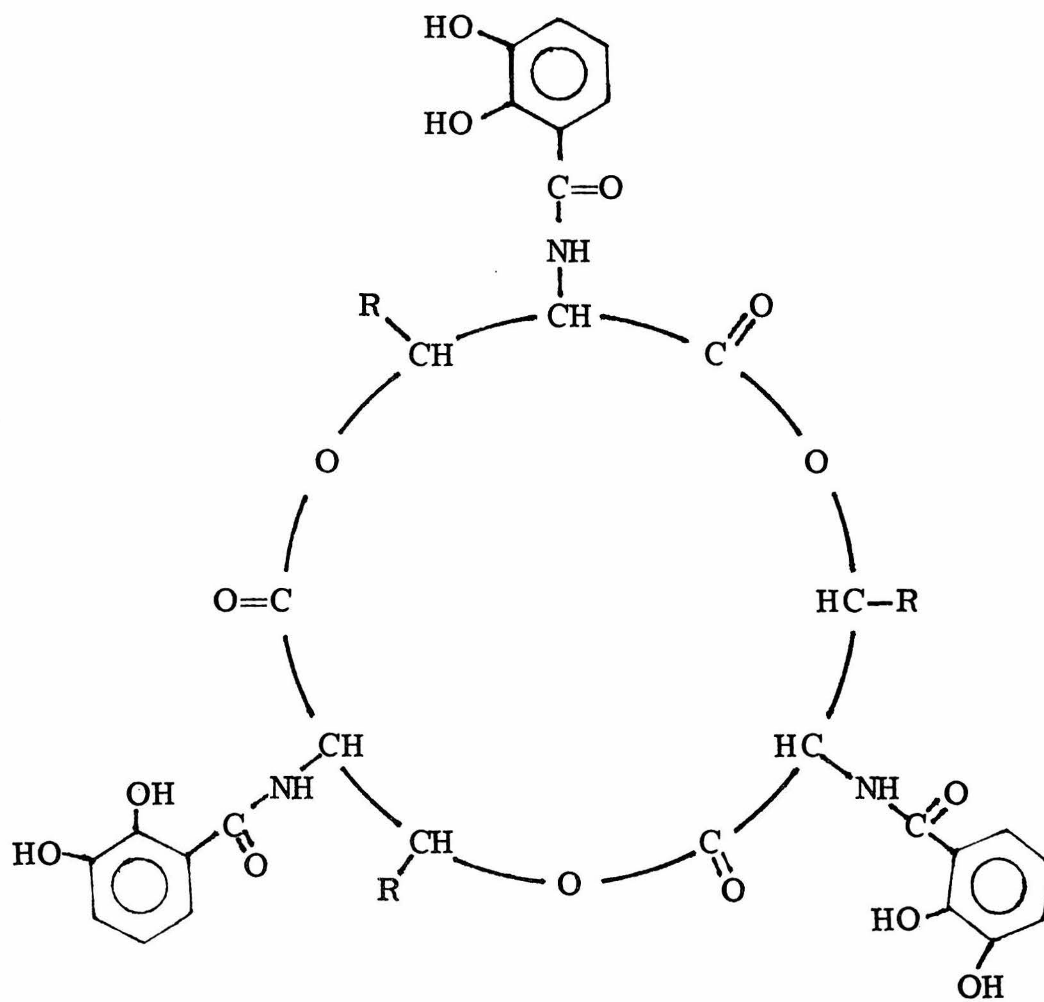
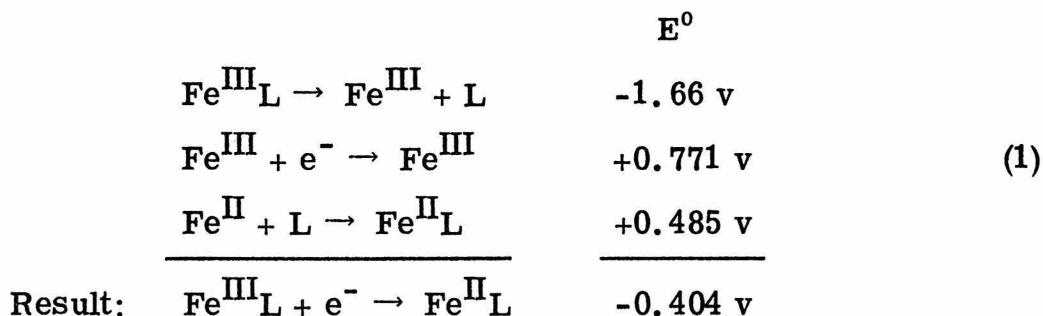

R
H—N
(CH2)5
N—C
O
O
O H
C—N
(CH2)2
(CH2)5
N—C
O
O
O H
C—N
(CH2)2
(CH2)n
R′
N—C
O
O
Fe

Figure 2. The structure of the phenolate siderochromes.


HO
HO
C=O
NH
R
CH
CH
C
O
O
O
O=C
HC—R
CH
HC
OH
NH
NH
O
HO
C
O
CH
R
O
C
O
C
HO
OH

The standard reduction potential of the iron (III) complex at pH 7.0 can be calculated from stability constants[1,2] using the cycle outlined in (1) together with the simple relationship described in (2).



		E^0	
	$Fe^{III}L \rightarrow Fe^{III} + L$	-1.66 v	
	$Fe^{III} + e^- \rightarrow Fe^{III}$	+0.771 v	(1)
	$Fe^{II} + L \rightarrow Fe^{II}L$	+0.485 v	
Result:	$Fe^{III}L + e^- \rightarrow Fe^{II}L$	-0.404 v	

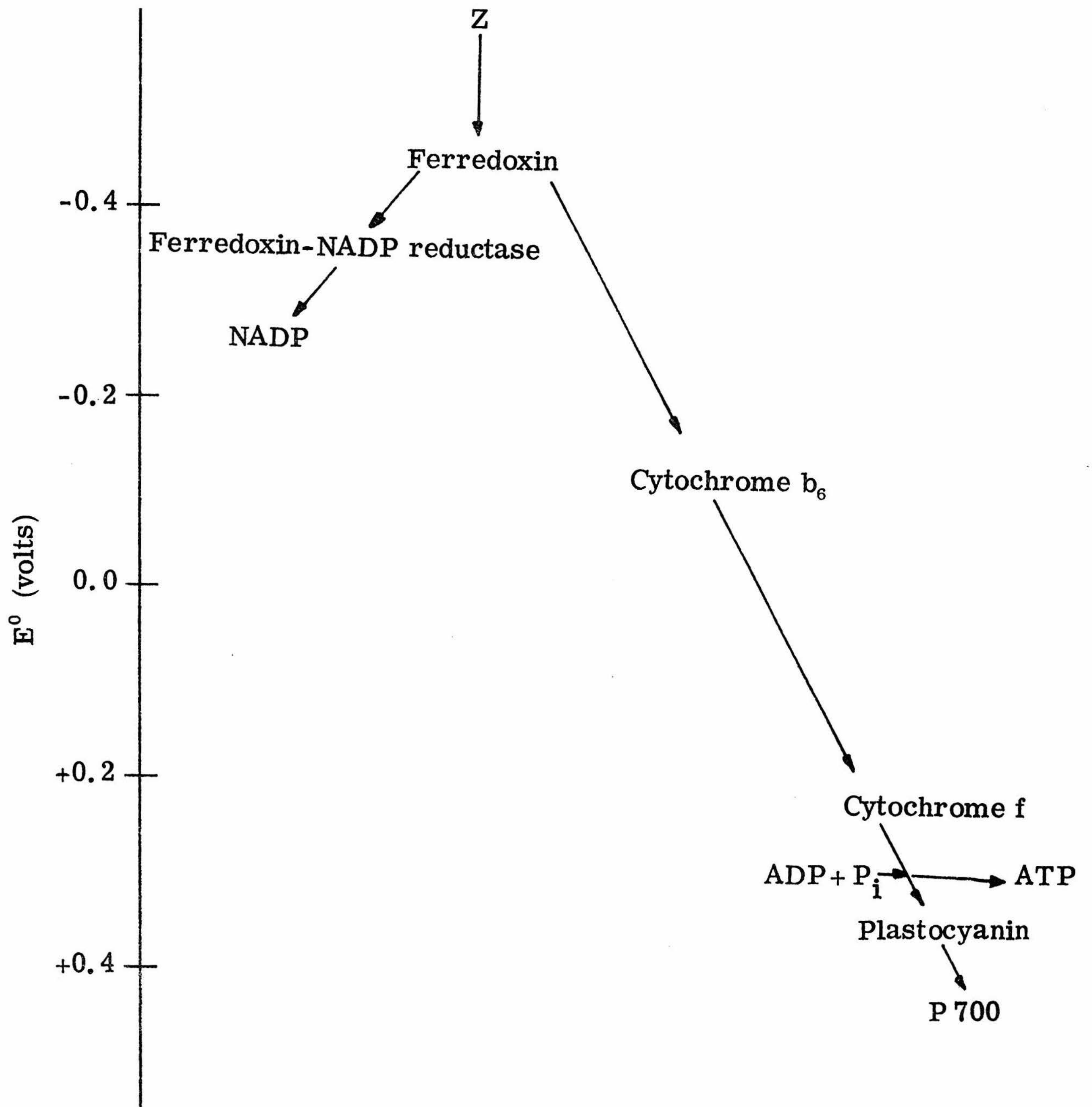
$$\Delta G^0 = -RT\ \ln k = -nf\Delta E^0 \qquad (2)$$

Since the reduction potential of ferrioximine B is -0.404 v, it is difficult to find a suitable reagent which is capable of reducing this complex, and stable at pH 7.0.

The ferredoxins are a group of proteins containing non-heme iron and labile sulfur which are present in certain anaerobic bacteria and in all photosynthetic organisms.[5] Spinach ferredoxin, a two iron-two sulfur protein isolated from the photosynthetic apparatus, has a standard reduction potential of -0.430 volts[6] and is therefore capable of reducing the ferrioximine B complex. Under physiological conditions, spinach ferredoxin acts as a one electron carrier between the photoreduction system of the chloroplast and pyridine nucleotide[7] as illustrated in Figure 3.

The electronic nature of the 2Fe:2S ferredoxins has been extensively studied by proton magnetic resonance (PMR),[8] electron

Figure 3. The position of ferredoxin in the photosynthetic pathway.


Z
Ferredoxin
-0.4
Ferredoxin-NADP reductase
NADP
-0.2
Cytochrome b_6
E^0 (volts)
0.0
+0.2
Cytochrome f
ADP + P_i
ATP
Plastocyanin
+0.4
P 700

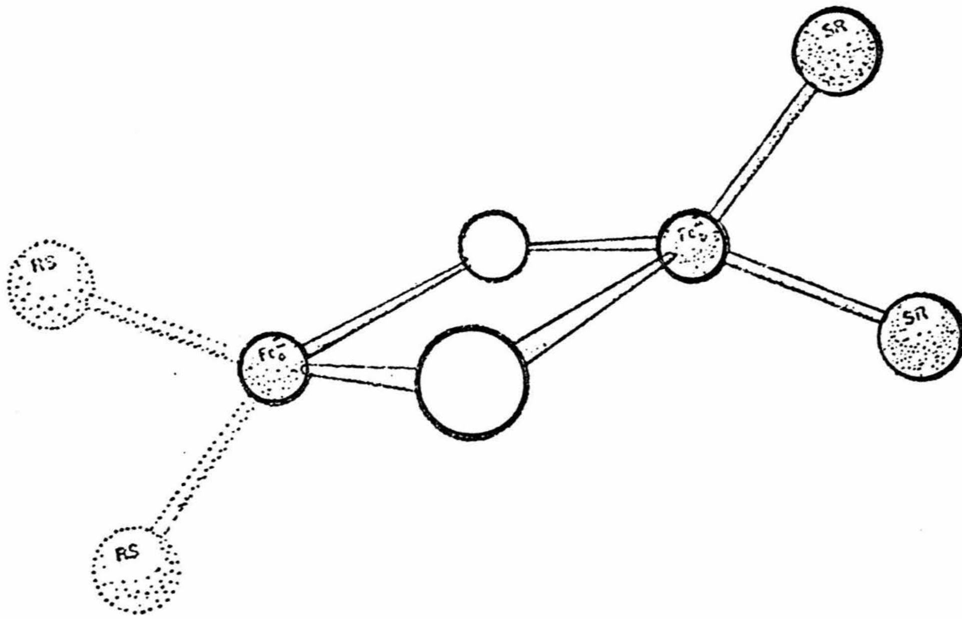
paramagnetic resonance (EPR),[9] magnetic susceptibility,[10] near infrared circular dichroism,[11] electron nuclear double resonance,[9] Mossbauer,[11] and ultraviolet-visible[12] absorption spectroscopies. The conclusion of all these investigations indicate that the two high spin iron atoms are non-equivalent and antiferromagnetically coupled. Both iron atoms are ferric in the oxidized state with one of them becoming ferrous upon reduction.

Although the X-ray crystal structure analysis has not been completed on any plant ferredoxin, the structure and properties[13] of a large number of synthetic analogs of the active site have been determined. The structure[14] of the synthetic 2Fe-2S analogs consists of a planar bridged iron-sulfur unit in which the iron atoms are in a distorted tetrahedral environment as indicated in Figure 4.

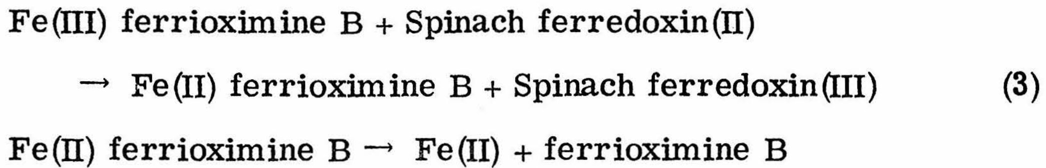
The reduction of this protein by sodium dithionite[15] as well as the oxidation by $Fe(EDTA)^{2-}$ have been previously investigated. An attempt has been made to study the redox kinetics by differential pulse polarography;[16] however, oxidative denaturation due to adsorption of the spinach ferredoxin on the electrode surface complicated the analysis.

The experiment to be proposed involves the determination of the mechanism involved in the reduction of ferriferrioximine B by spinach ferredoxin. By monitoring the absorption band of the ferriferrioximine B complex it will be possible to determine if the complex does indeed dissociate once the reduced species is formed. Since this is a key factor in the iron transport mechanism previously

Figure 4. Proposed active center of spinach ferredoxin. (Figure taken from Palmer.)[17]


SR
RS
Fe
SR
Fe
RS

mentioned, this experiment will either disprove that mechanism, if dissociation does not occur upon reduction, or support it. If the ferrousferrioximine B complex does dissociate, as shown in (3), dissociation would be expected to be slower than the electron transfer



$$\begin{aligned} &\text{Fe(III) ferrioximine B + Spinach ferredoxin(II)} \\ &\quad \rightarrow \text{Fe(II) ferrioximine B + Spinach ferredoxin(III)} \qquad (3) \\ &\text{Fe(II) ferrioximine B} \rightarrow \text{Fe(II) + ferrioximine B} \end{aligned}$$

reaction and parallel first order kinetics would be observed. Once the iron (II) is released in to the pH 7.0 medium, precipitation of the iron hydroxide complex can be monitored independently. If the ferrousferrioximine B complex does not dissociate readily, pseudo-first order kinetics would be observed.

Since the proposed mechanism for iron transport across microbial membranes requires the reduction of ferriferrioximine B to be accompanied by dissociation of the reduced complex, it is important to determine if these processes are indeed related.

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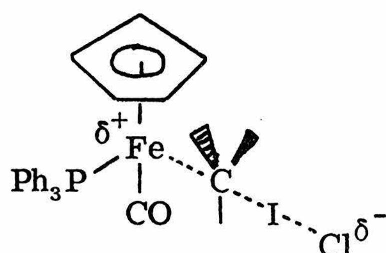
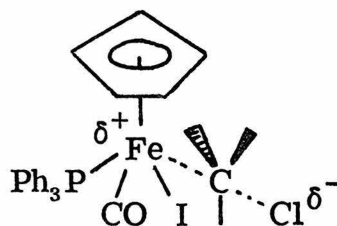
PROPOSITION IV. THE DETERMINATION OF THE MECHANISM OF METAL-CARBON BOND CLEAVAGE

$CpFe(CO)(PPh_3)I$ via transition state II. As $CpFe(CO)(PPh_3)Cl$ is stable to methyl iodide, the fact that only the metal iodide was formed[7] argues for mechanism (iii) or possibly (iv).

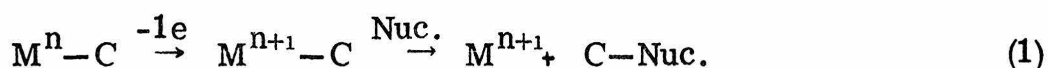


(I)

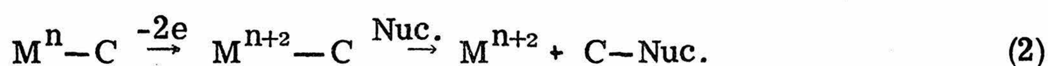


(II)

Mechanisms (iii) and (iv) involve oxidation of the metal by one and two electrons respectively, thereby weakening the metal-carbon bond, followed by nucleophilic attack at the carbon as illustrated in Equations (1) and (2). The evidence in support of an electron transfer



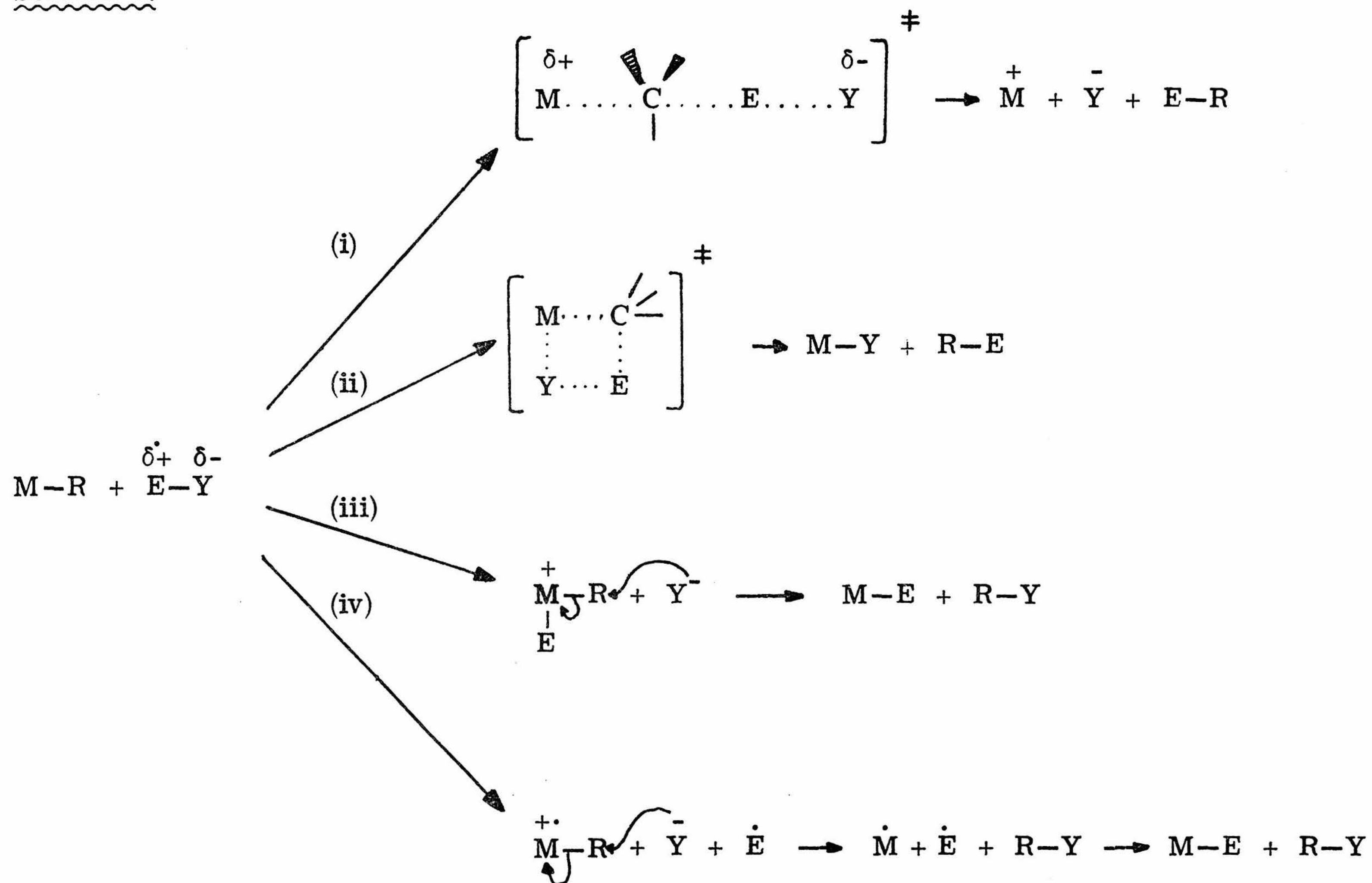
$$M^{n}-C \xrightarrow{-1e} M^{n+1}-C \xrightarrow{Nuc.} M^{n+1} + C-Nuc. \quad (1)$$



$$M^{n}-C \xrightarrow{-2e} M^{n+2}-C \xrightarrow{Nuc.} M^{n+2} + C-Nuc. \quad (2)$$

mechanism involves the use of the outer sphere oxidants $IrCl_6^{2-}$ and Ce(IV). Johnson[8] has reported that various alkyl-Co(III) complexes reacted with $IrCl_6^{2-}$ in the presence of external nucleophiles to give products resulting from the cleavage of cobalt-carbon bonds. Similar results were obtained when various alkyl Mo(II), W(II), Fe(II) and Co(I) complexes were treated with Ce(IV).[9] It is important to note that these complexes did not react with the external nucleophiles in the absence of oxidants.

Scheme 1:



The mechanism of cleavage of transition metal-carbon bonds by electrophilic reagents is an area of considerable current interest[1,2] owing to the synthetic usefulness of this process in organic chemistry[3] and its relationship to the mechanism of Vitamin B_{12}[4] action. Several proposed cleavage mechanisms are presented in Scheme 1. This proposal will attempt to examine evidence which is inconsistent with the first two of these mechanisms and outline experiments designed to distinguish between the two remaining pathways.

Preliminary evidence concerning the stereochemistry of metal-carbon cleavage by halogens has shown that the reaction proceeds with inversion at carbon for d^6 Fe(II)[5] and Co(III)[6] complexes. As the classical S_E2 closed pathway (ii) would predict the retention of configuration at carbon, this mechanism is inconsistent with the experimental results for these systems.

An investigation of the stereochemistry at the metal has revealed that the cleavage of the resolved chiral complexes of $CpFe(CO)(PPh_3)R$ (where R = Me, Et, $-CH_2CO_2$, $-CH_2O$) by I_2, ICl and HgI_2 proceeds with net retention of configuration at iron,[7] but stereospecificity is not high and is insensitive to the nature of the alkyl group. The fact that starting chiral iron complexes are recovered partially racemized[7] would rule out path (i) if it could be shown that the racemizing intermediate is on the mechanistic pathway to cleavage. In this respect, the experiment using ICl is important as mechanism (i) would be expected to proceed through transition state (I) resulting in the formation of $CpFe(CO)(PPh_3)Cl$, whereas mechanism (iii) would produce

Halpern[10] has reported the oxidation of alkyl [Co(III)(dimethyl-glyoximato)$_2$] complexes by Ce(IV) using cyclic voltametric techniques. Levitin[11] has observed that similar oxidized cobalt alkyl intermediates react with added nucleophiles. These results strongly suggest the participation of electron transfer in the mechanism of transition metal-carbon bond cleavage.

This proposal is concerned with elucidating the mechanism of iron-carbon bond cleavage in the CpFe(CO)(PPh_3)R systems[12] (where R = Me, Et, -CH_2CO_2, -CH_2O) by cyclic voltametry. These specific iron complexes were chosen because there has been extensive mechanistic work on determining the stereochemistry at the carbon and iron centers.[13,14] The electrochemical techniques of cyclic voltametry[15] will allow determination of the number of electrons involved in the reaction,[16] thereby distinguishing between mechanism (iii) and (iv). Once the oxidized intermediates have been identified and characterized, an investigation of their reactivity toward different added nucleophiles can be determined and products analyzed by conventional techniques (i.e., gas chromatography, mass spectrometry and nuclear magnetic resonance). The stereochemistry of the products of the electrochemical oxidation are expected to be the same as that obtained for the halogen cleavage provided the same intermediates are involved in both reactions.

The oxidation of the alkyl iron complexes by outer sphere reagents such as $IrCl_6^{2-}$ and Ce(IV) is also proposed. An analysis of the products as previously described for the electrochemical studies

will be conducted. It would also be interesting to compare the differences in reactivity toward nucleophiles between the electrochemically and chemically generated species.

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16. For conditions usually employed with hanging mercury drop and the micro-platinum wire electrode,

$$E_p = E_{\frac{1}{2}} + \frac{0.0285}{n} \text{ (volts)}$$

for a reversible oxidation. E_p is the peak potential, $E_{\frac{1}{2}}$ is the polarographic half-wave potential and n is the number of electrons involved.

PROPOSITION V. THE DETERMINATION OF THE REACTIVITY OF QUADRUPLY BONDED SPECIES BY ION CYCLOTRON RESONANCE SPECTROSCOPY

Ion cyclotron resonance (icr) spectroscopy is a relatively new experimental technique which is particularly useful for investigating ion-molecule reactions from which the thermochemical properties of the species, the structure of the ions, and the rates of ion-molecule reactions can be obtained in the absence of complicating solvent effects. The principles of ion cyclotron resonance spectroscopy and the instrumental design have been reviewed previously.[1,2] A pulsed double resonance experiment can be used to identify the ion-molecule reaction of interest. For the general reaction,

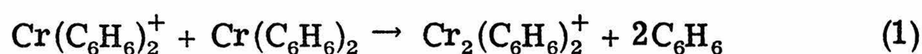
$$A^+ + B \rightarrow C^+$$

the observation of C^+ in a fixed magnetic field is achieved by utilizing a marginal oscillator detector. The application of a second radio-frequency electric field equal to the cyclotron frequency of A^+, will cause an increase in the ion energy of A^+, which, in turn, leads to a change in the amount of C^+ present, as the rate constant is dependent on the ion energy. In this manner, the specific ion-molecule reaction of interest can be identified.

The ion-molecule reaction rate constants may be obtained by using trapped-ion techniques developed by McIver.[3] Ions are formed by pulsing the electron beam for a short time compared to the time between collisions. In this way, the ions can be trapped for up to several seconds. The ion composition can therefore be monitored as a function of time and the reaction rates determined. Although icr

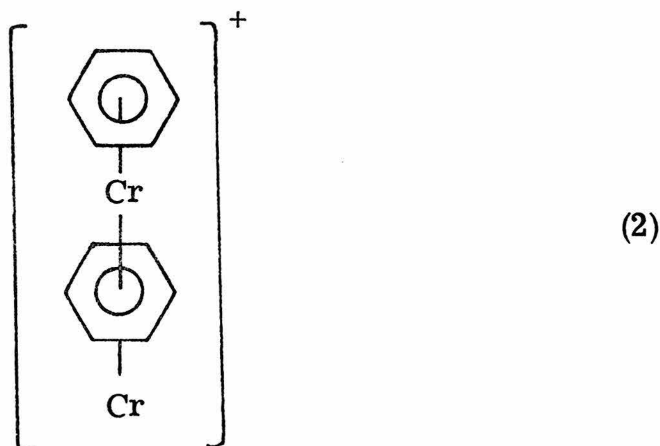
was first applied to the study of organic species,[4,5] it is becoming increasingly useful in the study of inorganic[6] and organometallic[7] compounds. As a result, the ion-molecule reactions of a number of metal carbonyls[8] and metallocenes[9] have been investigated.

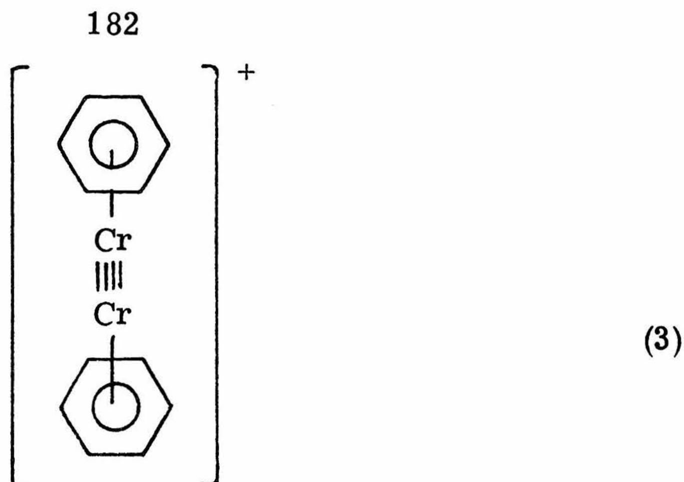
The ion-molecule reactions of organometallic complexes can be characterized either by the formation of new metal-ligand bonds or the formation of bimetallic ions. This study will be primarily concerned with the formation and structure of the bimetallic species. Of particular interest is the reaction described in (1) of bis-π-benzene



$$Cr(C_6H_6)_2^+ + Cr(C_6H_6)_2 \rightarrow Cr_2(C_6H_6)_2^+ + 2C_6H_6 \quad (1)$$

chromium.[10] The structure of this bimetallic product ion cannot be determined from this reaction alone since the only information available is merely the mass of the product ion and the rate of its formation from the reactant indicated. Two possible structures for this species are illustrated in (2) and (3).





(3)

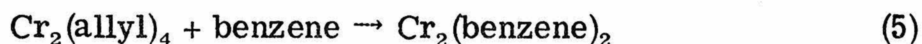
Although there is considerable evidence[11] for species of the type described in (2) for the products observed in the ion-molecule reactions of ferrocene and nicolocene, it is quite resonable to expect bis-π-benzene chromium to react differently considering the electronic structure of chromium and the stability of the structure described in (3)

One method which has been used to determine the structure of organic isomers which are products of ion-molecule reactions is to independently isolate one of the two possible structures and compare the chemistry of the known compound to the chemistry of the unknown species.[12] If the reactions of these two compounds are the same and the rates of the reactions are comparable, then it is reasonable to assume that the two compounds are indeed the same.

As the tetraallyldichromium (4) complex has been prepared and crystal structure completed,[13] the analogous benzene complex

(4)

should also be stable. There are several methods which could be proposed to prepare this compound; however, in order to avoid the formation of the stable bis-π-benzene chromium species it would be advantageous to begin with the quadruple bond already formed. The thermal chemistry of many of these binuclear species, studied primarily by F. A. Cotton et al.,[14-20] has shown the quadruple bond to be surprisingly inert, while ligand substitution is quite facile. The mass spectra of several of the di-molybdenum compounds[21] has also shown the metal-metal bond to remain intact. The proposed ligand substitution reaction is that described in (5). Once this product



$$Cr_2(allyl)_4 + benzene \rightarrow Cr_2(benzene)_2 \quad (5)$$

is isolated, it can be identified by nmr, ir, uv-vis spectra, and mass spectrometry.

The ion-molecule reactions of this linear quadruply bonded species can now be investigated. It will be extremely interesting to determine whether the metal-metal bond will remain intact under a variety of conditions. Several proposed ion-molecule reactions that would be interesting are presented in Figures 1 and 2.

Figure 1. The reaction of $\{Cr_2(benzene)_2\}^+$ with $Mo_2(benzene)_2$ and some of the expected products.

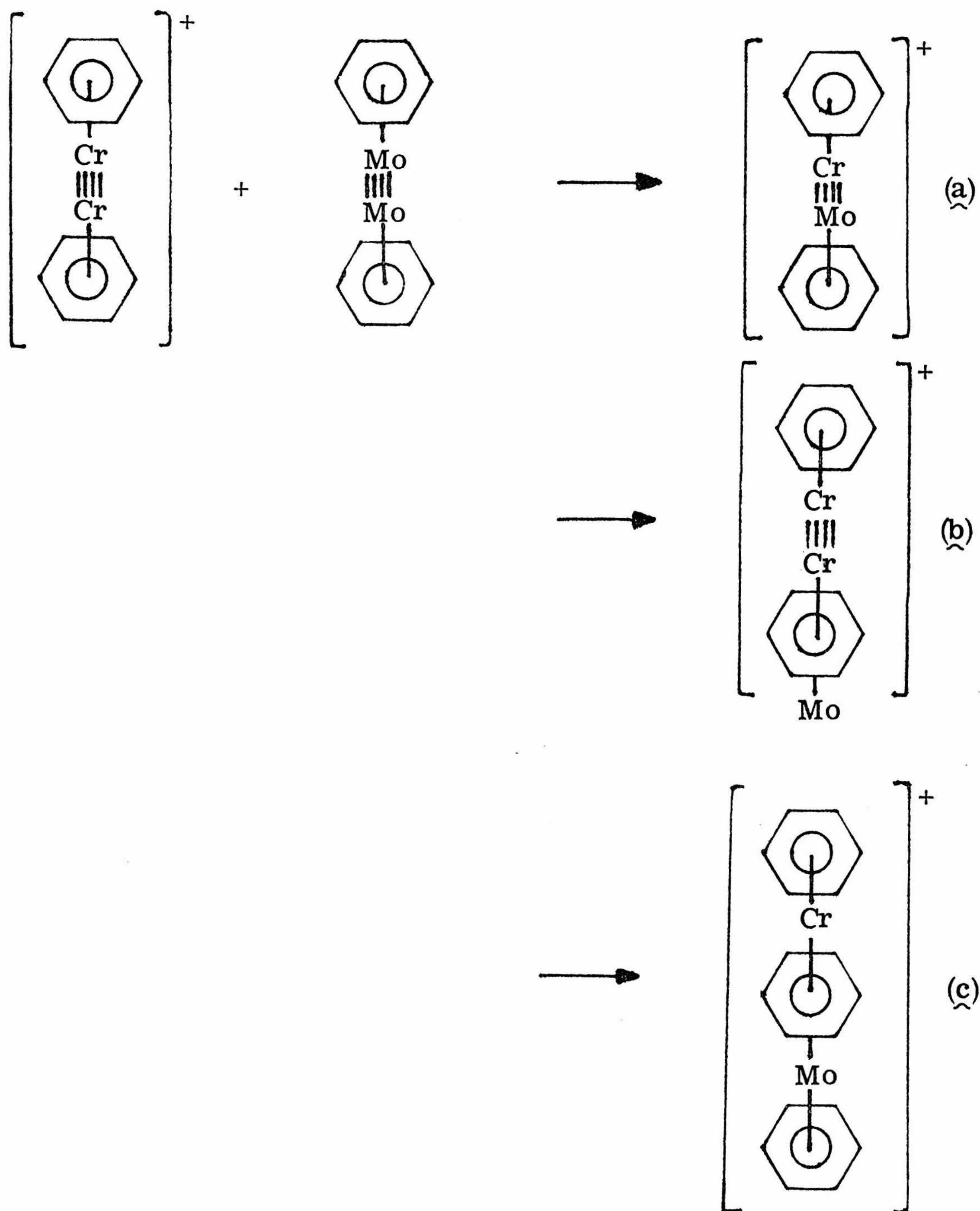
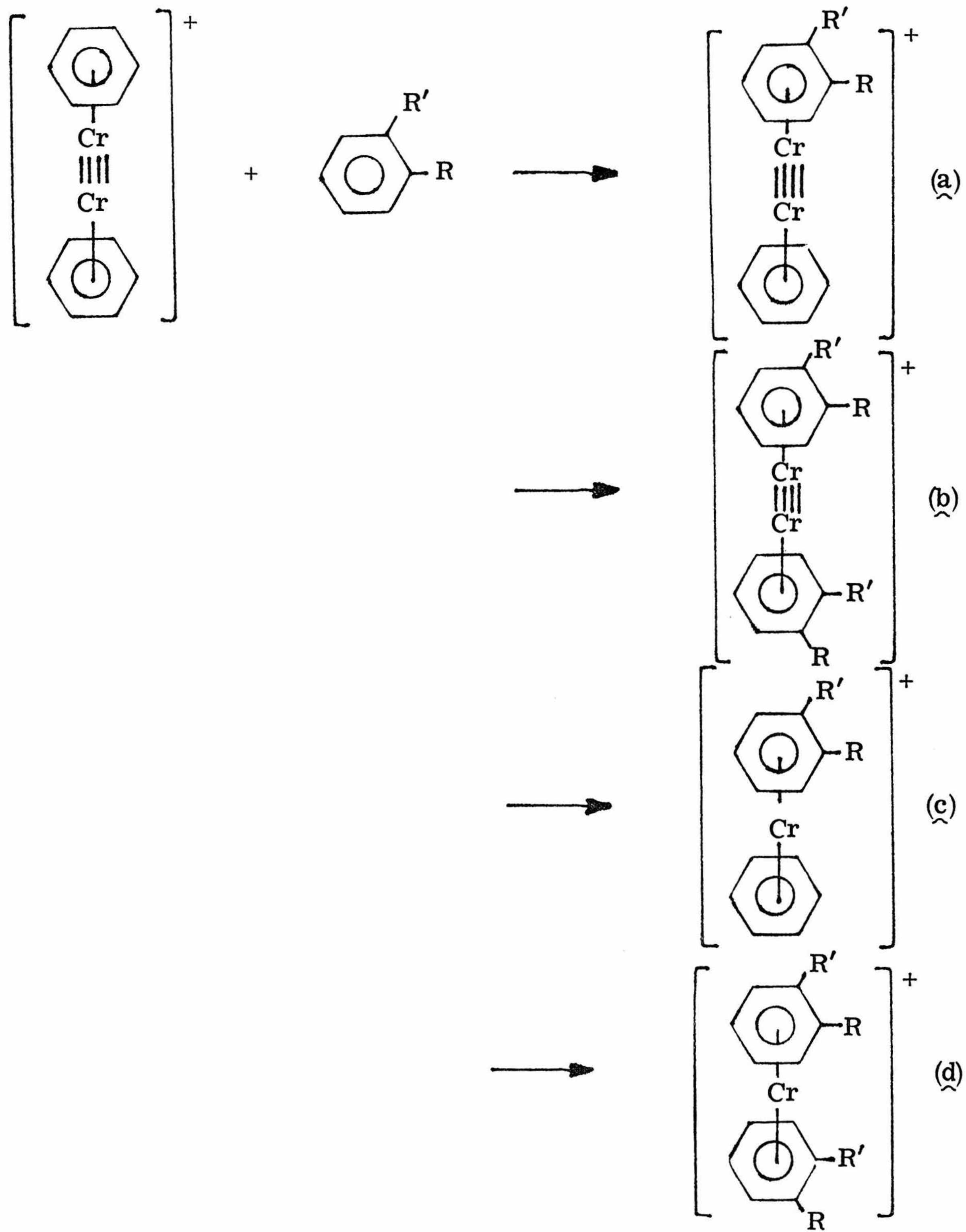

+
Cr
Cr
+
Mo
Mo
+
Cr
Mo
(a)
+
Cr
Cr
Mo
(b)
+
Cr
Mo
(c)

Figure 2. The reaction of $\{Cr_2(benzene)_2\}^+$ with substituted benzene and some probable products where R = Br, Cl, No_2, CH_3, $-C(CH_3)_3$, etc.


+
Cr
Cr
+
R′
R
R′
R
Cr
Cr
+
(a)
R′
R
Cr
Cr
R′
R
+
(b)
R′
R
Cr
+
(c)
R′
R
Cr
R′
R
+
(d)

If the reaction described in Figure 1 proceeds to product (a), it would be the first evidence for a mixed-metal quadruple bond. Should the triple decker sandwich product (c) be formed it would involve the rupture of the very strong quadruple bonds in the reactants. Since the chromium atoms are formally sixteen electron systems in the Cr_2(benzene)$_2$ complex,[22] they should favor the formation of complexes which donate more electron density to the metals. In this regard it would be interesting to determine the relative stability of the products with regard to electron withdrawing and donating substituents on the benzene ring as described in Figure 2.

The metal-metal bond in Cr_2(allyl)$_4$ is the shortest metal bond ever reported,[23] and it is believed to be one of the strongest. It is therefore expected that the Cr_2(benzene)$_2$ complex should have a similar structure and metal-metal bond strength. It would also be intersting to perform the same series of experiments on the corresponding molybdenum complex described in (8). In this way,

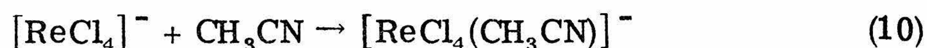
correlations can be drawn between the characteristics of the metal-metal bond and the reactivity of the species in the absence of complicating solvent effects.

The techniques for investigating photochemical processes involving ionic species in the gas phase have recently been developed.[24,25] The particular advantage of icr is that the photochemically excited ions can be trapped for several minutes. Of particular importance in the context of this proposal is the photodissociation of quadruply bonded species.

The metal-metal bond of $[Re_2Cl_8]^{2-}$ has been shown to be thermochemically inert;[14] however, the photochemical dissocation has been reported with irradiation at 366 nm.[26] These reactions were carried out in the presence of acetonitrile, which coordinates to the mononuclear species. The specific reaction to be investigated is illustrated in (9). Once the dissociation has been characterized

$$[Re_2Cl_8]^{2-} \xrightarrow[366\,nm]{hr} 2[ReCl_4]^- \qquad (9)$$

by noting ion intensities in the presence and absence of radiation and substantiated using icr double resonance techniques, the ion-molecule reactions described in (10) and (11) can be examined. The reaction



$$[ReCl_4]^- + CH_3CN \rightarrow [ReCl_4(CH_3CN)]^- \qquad (10)$$

$$[ReCl_4(CH_3CN)]^- + CH_3CN \rightarrow [ReCl_4(CH_3CN)_2]^- \qquad (11)$$

rates for these 'solvation' processes can be determined by using trapped ion techniques as previously described.[3] Variations in the metal complexes and reacting ligands will provide information not available from solution studies concerning the characteristics and reactivity of quadruply bonded species.

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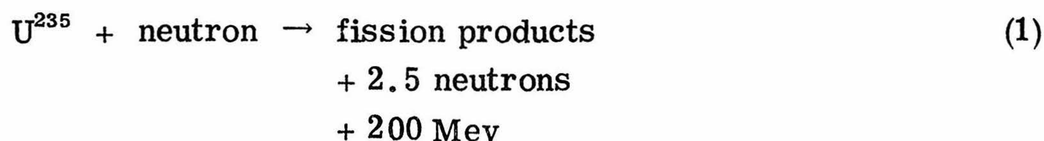
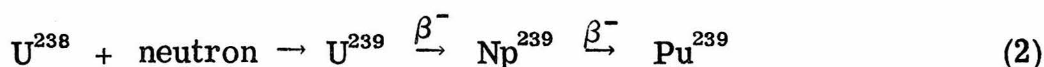
PROPOSITION VI. THE DESIGN AND SYNTHESIS OF PLUTONIUM CHELATING AGENTS

This proposal is concerned with the design and synthesis of plutonium sequestering agents. The most important isotope of plutonium is Pu^{239} which is a product of the 'pile reactions' described by equations (1) and (2) for the processing of nuclear fuels and



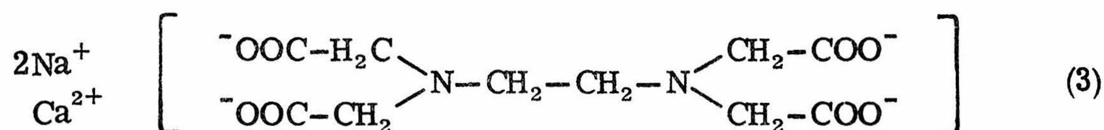
$$U^{235} + \text{neutron} \rightarrow \text{fission products} + 2.5 \text{ neutrons} + 200 \text{ Mev} \quad (1)$$



$$U^{238} + \text{neutron} \rightarrow U^{239} \xrightarrow{\beta^-} Np^{239} \xrightarrow{\beta^-} Pu^{239} \quad (2)$$

weapons.[1] Although elaborate precautions have been taken to prevent the Pu^{239} from entering the environment,[2] there have been several reported cases of poisoning in man, animals and vegetation.[3] The development of nuclear fuels as an alternative form of energy, and the construction of several new atomic energy plants has focused attention on the problems of dealing with radioactive leaks and accidents resulting from an increased number of people working with these materials. This proposal will focus on the treatment of plutonium poisoning in humans.

The present therapy for plutonium poisoning involves the administration of $CaNa_2EDTA$ (3). This treatment has been shown to be



$$\begin{matrix} 2Na^+ \\ Ca^{2+} \end{matrix} \left[\begin{matrix} ^-OOC-H_2C \\ ^-OOC-CH_2 \end{matrix} > N-CH_2-CH_2-N < \begin{matrix} CH_2-COO^- \\ CH_2-COO^- \end{matrix} \right] \quad (3)$$

twenty-four percent (24%) effective in the removal of plutonium when treatment was administered five days after exposure and essentially ineffective when treatment was initiated thirty days after exposure.[4]

As the plutonium apparently becomes incorporated into the tissues and bone to a significant extent thirty days after exposure,[5] it is therefore inaccessible to chelating agents in the blood.

Diethylenetriaminepentacetic acid, DTPA, (4) has been shown to

$$\begin{array}{l} HOOC-H_2C \\ HOOC-H_2C \end{array} \!\!>\! N-CH_2-CH_2-\underset{\underset{COOH}{|}}{\underset{CH_2}{\underset{|}{N}}}-CH_2-CH_2-N \!<\! \begin{array}{l} CH_2-COOH \\ CH_2-COOH \end{array} \qquad (4)$$

be more effective in preventing the deposition of plutonium if treatment is initiated within eight hours after exposure.[6] The advantage of this reagent lies in its ability to form eight coordinate complexes with actinide metals rather than the six coordinate complexes of EDTA.[7] The DPTA therapy administered twenty days after plutonium exposure, however, showed little effect in reducing the levels of plutonium in the bone, liver, spleen and kidneys of rats.[8]

There are several factors to consider in the design of an effective chelating agent for plutonium. As plutonium is considered to be a hard acid,[9] it would presumably favor coordination to hard ligands such as nitrogen and oxygen. The actinide elements from stable eight-coordinate complexes, however, the other necessary trace metals in the body do not. The chelating agent must be water soluble, able to retain its chelating ability at physiological pH's, and exhibit low toxicity. Once the radioactive element has been incoporated into the animal tissues, an effective chelating agent must be able to transfer across lipid membranes and into the hydrophobic interior of the tissues

to sequester the metal.

Diethylenetriaminepentacetic acid, DTPA, is an octadentate, water soluble, hard chelating agent which is stable at physiological pH.[10] The calcium salt has been shown to be innocuous to mammals.[11] The one problem which remains is to modify this reagent to enable it to penetrate into hydrophobic environments.

The preparation and characterization of phenylethylenediamine-tetraacetic acid (5) has been described.[12] The procedure is outlined

$$\begin{array}{l} HOOC-H_2C \\ HOOC-H_2C \end{array} \!\!> N-\underset{\overset{|}{C_6H_5}}{CH}-CH_2-N <\!\! \begin{array}{l} CH_2-COOH \\ CH_2-COOH \end{array} \qquad (5)$$

in Scheme 1. This ligand has been shown to form stable complexes with a variety of metals.[13]

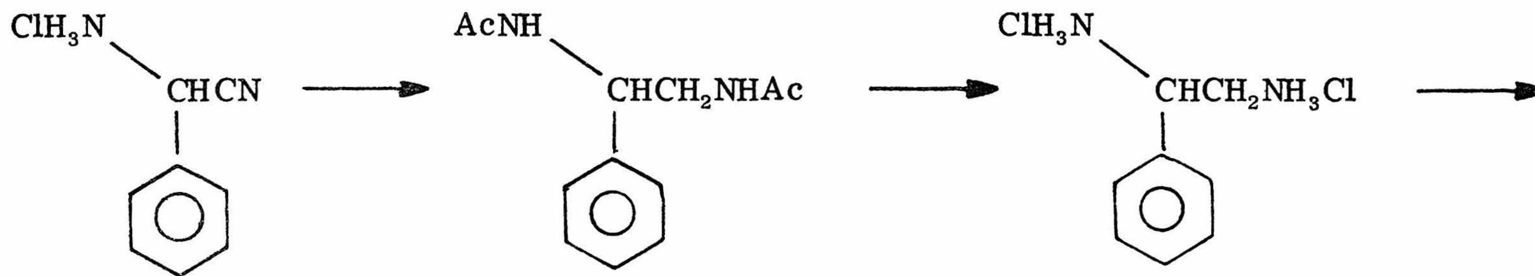
Similar techniques can be applied to the preparation of phenyl-diethylenetriaminepentaacetic acid (ϕ-DTPA) (6) as described in

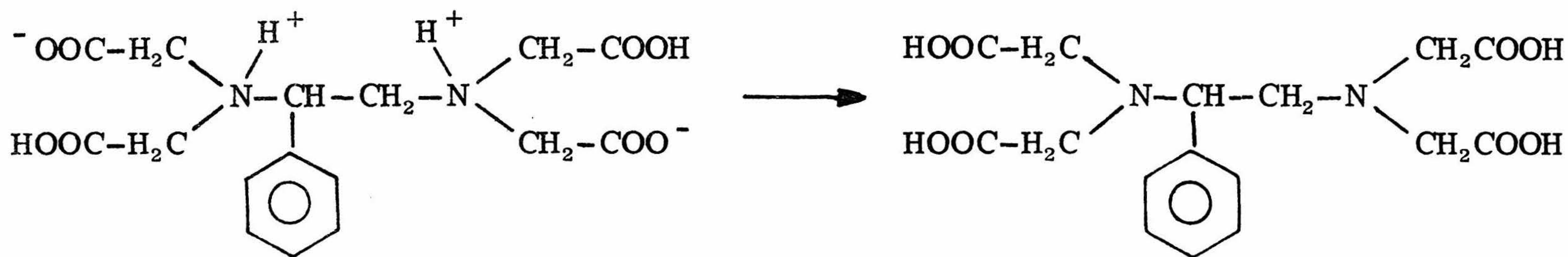
$$\begin{array}{l} HOOC-H_2C \\ HOOC-H_2C \end{array} \!\!> N-\underset{\overset{|}{C_6H_5}}{CH}-CH_2-\underset{\underset{COOH}{\underset{|}{CH_2}}}{N}-\underset{\overset{|}{C_6H_5}}{CH}-CH_2-N <\!\! \begin{array}{l} CH_2-COOH \\ CH_2-COOH \end{array} \qquad (6)$$

Scheme 2. The phenyl groups on the surface should allow penetration of the complex into hydrophobic environments while not restricting the eight-coordinate complex formation.

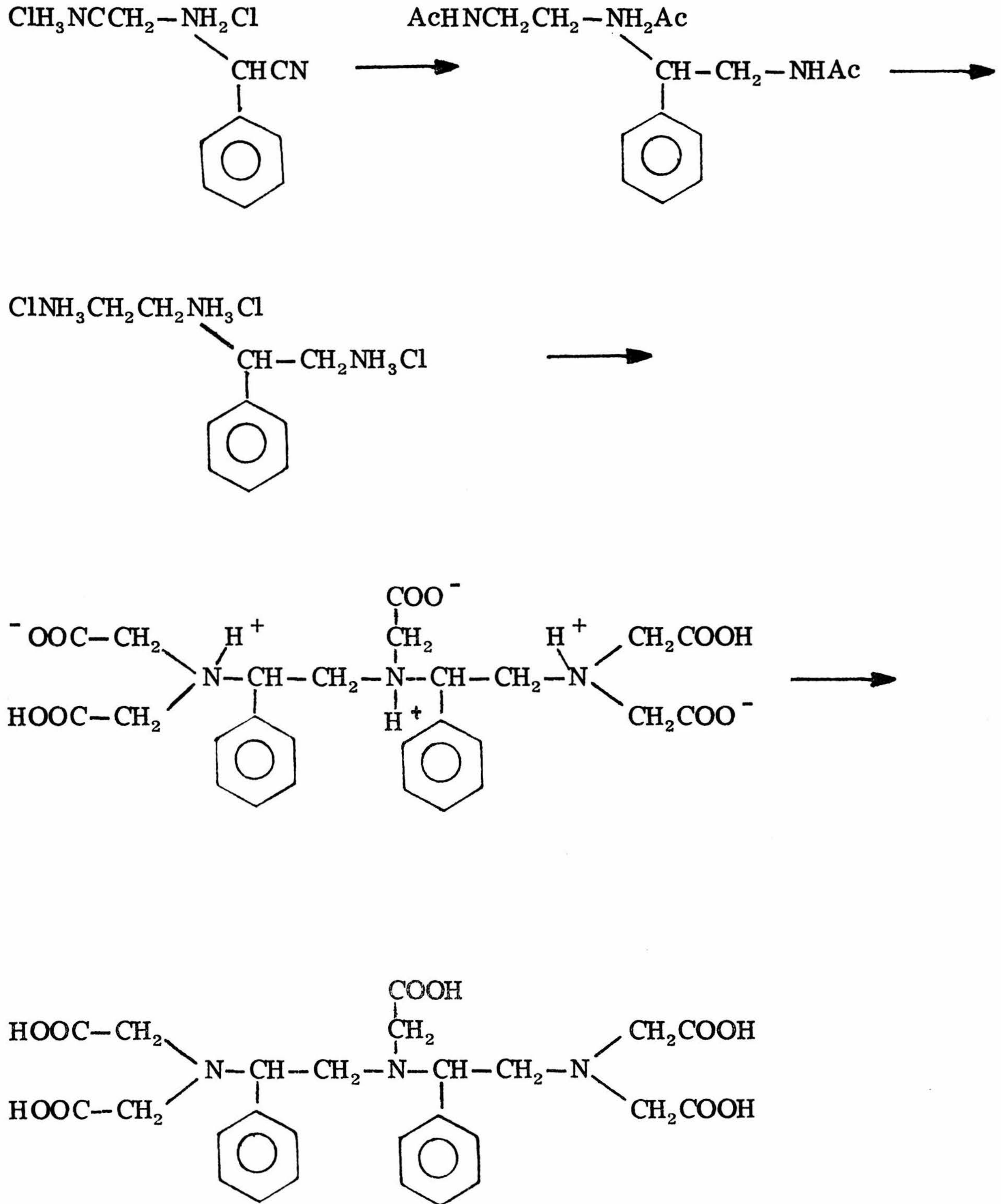
Although DTPA has been shown to be effective in preventing the deposition of plutonium, it has not been useful in reducing the levels

Scheme 1:





Scheme 2:



of plutonium after the metal has been deposited in the various organs owing to the hydrophillic nature of the ligand. It is suggested that ϕ-DPTA will be more effective in removing plutonium from the tissues and organs, as it is more hydrophobic.

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