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MODEL STUDIES FOR MOLYBDENUM ENZYMES. THE REDUCTION OF
CYTOCHROME c BY MOLYBDENUM(V)-CYSTEINE COMPLEXES

by

Glen D. Lawrence

A dissertation submitted in partial fulfillment
of the requirements for the degree

of

DOCTOR OF PHILOSOPHY

in

Biochemistry

UTAH STATE UNIVERSITY
Logan, Utah

1976

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Glen D. Lawrence

TABLE OF CONTENTS

	Page
ACKNOWLEDGMENTS	ii
LIST OF TABLES	
LIST OF FIGURES	
ABSTRACT	
INTRODUCTION	1
LITERATURE REVIEW	3
General Survey of Molybdenum in Biochemical Systems	3
Model Complexes for Molybdenum Enzymes	5
Molybdenum Cofactors in Enzymes	8
Nitrate Reductase	9
Sulfite Oxidase	11
Formate Dehydrogenase	12
Structure and Physical Properties of Cytochrome c	13
Spectral Properties of Cytochrome c	19
Interaction of Cytochrome c ^{III} with Iron Binding Ligands	21
Redox Reactions with Cytochrome c	23
Studies with Modified Cytochrome c Species	32
EXPERIMENTAL PROCEDURES	38
Materials	38
Di- μ -oxo-bis[oxo(L-cysteinato)molybdate(V)]	38
μ -oxo-bi [oxodihydroxo(L-cysteinato)molybdate(V)]	39
Mo(V)-penicillamine complexes	39
Cytochrome c and derivatives	40
Methods	47
Reduction of cyt c ^{III} by complex I	47
Binding studies with cyt c ^{III} and complex I	48
Reduction of cyt c ^{III} by complex II	49
Potentiometric titration of complex II	51
Potentiometric titration of native and ligand bound cyt c ^{III}	53
Electron spin resonance studies	55

TABLE OF CONTENTS (Continued)

	Page
Treatment of Data	57
Determination of rate constants	57
RESULTS	62
Reduction of Cyt c ^{III} by Complex I	62
Reduction of Cyt c ^{III} by Complex II	78
Reduction of Ligand Bound Cyt c ^{III} by Complex II	85
Potentiometric Titration	100
Reduction of PLP Modified Cyt c ^{III}	109
Reduction of Nitrotyrosyl Derivatives of Cyt c ^{III}	115
Reduction of Formyl-Cyt c ^{III} by Complex I	120
Reduction of Formyl-Cyt c ^{III} by Complex II	123
Reduction of Cyt c ^{III} by Mo(V)-Penicillamine Complexes	126
DISCUSSION	133
Reduction of Cyt c ^{III} by Complex I	133
Reduction of Cyt c ^{III} by Complex II	139
Reduction of Ligand Bound Cyt c ^{III} by Complex II	140
Reduction of Prydoxal Phosphate Modified Cyt c ^{III}	151
Reduction of Nitrated Cyt c ^{III}	153
Reduction of Formyl-Cyt c ^{III}	156
Reduction of Cyt c ^{III} by Mo(V)-Penicillamine Complexes	160
CONCLUSIONS	161
REFERENCES	165
VITA	173

LIST OF TABLES

Table	Page
1. Rate and equilibrium constants for ligand binding to cytochrome c ^{III}	23
2. Apparent rate constants for oxidation and reduction of cytochrome c by iron hexacyanides	27
3. Physicochemical properties and activity of native and various modified preparations of cytochrome c at neutral pH	46
4. Reduction of cyt c ^{III} by complex I in 0.10 M phosphate buffer, pH 7.5, $\mu = 0.50$ with NaCl, 20.0° C, $[\text{cyt c}^{\text{III}}]_0 = 3.35\text{--}3.78 \times 10^{-5} \text{ M}$	63
5. Rate constants for the reduction of cyt c ^{III} by complex I with varying concentrations of I	64
6. Reduction of cyt c ^{III} by complex I following absorbance at 695 nm in 0.10 M phosphate buffer, pH 7.5, $\mu = 0.50$ with NaCl, at 20.0° C, $[\text{cyt c}^{\text{III}}] = 1.75\text{--}1.95 \times 10^{-4} \text{ M}$	65
7. Effect of pH on the reduction of cyt c ^{III} by complex I in 0.10 M phosphate buffer, $\mu = 0.50$ adjusted with NaCl, 20.0° C, $[\text{cyt c}^{\text{III}}]_0 = 3.26\text{--}3.71 \times 10^{-5} \text{ M}$	67
8. Effect of pH on the rate constants for the reduction of cyt c ^{III} by complex I	68
9. Effect of temperature on the reduction of cyt c ^{III} by complex I	69
10. Effect of temperature on the rate constants for the reduction of cyt c ^{III} by complex I	71
11. Reduction of cyt c ^{III} by complex I in 10 mM phosphate buffer, pH 7.5, $\mu = 0.026$, $T = 20.0^\circ \text{ C}$, $[\text{cyt c}^{\text{III}}]_0 = 1.80\text{--}1.85 \times 10^{-5} \text{ M}$	73
12. Reduction of cyt c ^{III} by complex I in 20 mM tris-cacodylate buffer, pH 7.0, $\mu = 0.018$, at 20.0° C, $[\text{cyt c}^{\text{III}}]_0 = 3.75\text{--}3.80 \times 10^{-5} \text{ M}$	74
13. Reduction of cyt c ^{III} by complex I in 10 mM HEPES buffer, pH 7.5 $\mu = 0.0050$, at 20.0° C, $[\text{cyt c}^{\text{III}}]_0 = 3.64\text{--}3.70 \times 10^{-5} \text{ M}$	75

LIST OF TABLES (Continued)

Table	Page
14. Reduction of cyt c ^{III} by complex I in 10 mM HEPES buffer, pH 7.5, $\mu = 0.0050$, at 20.0° C, [cyt c ^{III}] ₀ = 1.92-198 x 10 ⁻⁵ M	76
15. Reduction of cyt c ^{III} by complex I in 10 mM HEPES buffer, pH 7.5, $\mu = 0.015$, at 20.0° C, [cyt c ^{III}] ₀ = 1.90-2.00 x 10 ⁻⁵ M	76
16. Observed rate constants for the reduction of cyt c ^{III} by complex I in different buffers	77
17. Reduction of cyt c ^{III} by complex II in 0.20 M phosphate buffer, pH 7.0, $\mu = 0.40$, at 20.0° C and 25.0° C, [cyt c ^{III}] ₀ = 9.10-9.90 x 10 ⁻⁶ M	80
18. Effect of temperature on the reduction of cyt c ^{III} by complex II in 0.20 M phosphate buffer, pH 7.0, $\mu = 0.40$, [cyt c ^{III}] ₀ = 0.970-1.02 x 10 ⁻⁵ M, [II] ₀	81
19. Observed rate constants for the reduction of cyt c ^{III} by complex II	83
20. Reduction of N ₃ -cyt c ^{III} by complex II on 0.20 M phosphate buffer, 0.25 M NaN ₃ , pH 7.0, $\mu = 0.60$, 25.0° C, [cyt c ^{III}] ₀ (total) = 2.00 x 10 ⁻⁵ M, [N ₃ -cyt c ^{III}] ₀ = 1.1 x 10 ⁻⁵ M	86
21. Reduction of CN-cyt c ^{III} by complex II in 0.20 M phosphate buffer, 1.1 x 10 ⁻² M KCN, pH 7.0, $\mu = 0.40$, 25.0° C, [cyt c ^{III}] ₀ (total) = 1.95 x 10 ⁻⁵ M, [CN-cyt c ^{III}] ₀ = 1.90 x 10 ⁻⁵ M, [II] ₀ = 1.00 x 10 ⁻³ M	86
22. Reduction of cyt c ^{III} by complex II in the presence of N-acetylmethionine	87
23. Observed rate constants for the reduction of cyt c ^{III} by complex II in the presence of heme ligands	88
24. Reduction of Im-cyt c ^{III} by complex II with both cyt c ^{III} and complex II in 0.20 M phosphate 0.50 M imidazole buffer, pH 7.0, $\mu = 0.65$ prior to mixing	91
25. Reduction of Im-cyt c ^{III} by complex II with cyt c ^{III} in 0.20 M phosphate-0.50 M imidazole buffer, pH 7.0, $\mu = 0.65$ and II in 0.20 M phosphate buffer, pH 7.0, $\mu = 0.65$ (adjusted with NaCl), T = 25.0° C, [cyt c ^{III}] ₀ (total) = 2.10-2.15 x 10 ⁻⁵ M, [Im-cyt c ^{III}] ₀ = 1.85-1.89 x 10 ⁻⁵ M	92

LIST OF TABLES (Continued)

Table	Page
26. Reduction of Im-cyt c^{III} at high concentrations of complex II	94
27. Reduction of Im-cyt c^{III} by complex II in 0.20 M phosphate-0.50M imidazole buffer, pH 8.1, $\mu = 0.60$, 20.0°C , $[\text{cyt } c^{III}]_0$ (total) = $1.80\text{--}1.90 \times 10^{-5} \text{ M}$, $[\text{Im-cyt } c^{III}]_0 = 1.69\text{--}1.79 \times 10^{-5} \text{ M}$	97
28. Rate constants for the slow phase of reduction of Im-cyt c^{III} by complex II in the presence of imidazole	98
29. Reduction of cyt c^{III} by complex II in the presence of imidazole at pH 10.0	99
30. Observed rate constants for the reduction of cyt c^{III} by complex II in the presence of imidazole at pH 10	100
31. Midpoint potentials for the oxidative titration of complex II	104
32. Reduction of $\text{PLP}_2\text{-cyt } c^{III}$ by complex I in 0.10 M phosphate buffer, pH 7.0, $\mu = 0.50$, $T = 20.0^\circ \text{C}$, $[\text{PLP}_2\text{-cyt } c^{III}]_0 = 5.76 \times 10^{-6} \text{ M}$	110
33. Reduction of $\text{PLP}_1\text{-cyt } c^{III}$ by complex I in 0.10 M phosphate buffer, pH 7.0, $\mu = 0.50$, $T = 20.0^\circ \text{C}$, $[\text{PLP}_1\text{-cyt } c^{III}]_0 = 1.40 \times 10^{-5} \text{ M}$	110
34. Rate constants for the reduction of PLP derivatives of cyt c^{III} by complex I	112
35. Reduction of $\text{PLP}_2\text{-cyt } c^{III}$ by complex II in 0.10 M phosphate buffer, pH 7.0, $\mu = 0.50$ with NaCl, 20.0°C , $[\text{PLP}_2\text{-cyt } c^{III}]_0 = 3.80 \times 10^{-6} \text{ M}$	113
36. Reduction of $\text{PLP}_1\text{-cyt } c^{III}$ by complex II in 0.10 M phosphate buffer, pH 7.0, $\mu = 0.50$ with NaCl, 20.0°C , $[\text{PLP}_1\text{-cyt } c^{III}]_0 = 9.04 \times 10^{-6} \text{ M}$	113
37. Rate constants for the reduction of PLP derivatives of cyt c^{III} by complex II	115
38. Reduction of mononitromonotyrosyl (MNMT)-cyt c^{III} by complex I in 0.20 M phosphate buffer, pH 7.0, $\mu = 0.50$, at 20.0°C , $[\text{MNMT-cyt } c^{III}]_0 = 4.30 \times 10^{-6} \text{ M}$	116

LIST OF TABLES (Continued)

Table	Page
39. Observed rate constants for the reduction of MNMT-cyt c^{III} by compl x I	116
40. Reduction of mononitromonotyrosyl (MNMT)-cyt c^{III} by complex II in 0.20 M phosphate buffer, pH 7.0, $\mu = 0.50$ with NaCl, 20.0° C, $[MNMT-cyt\ c^{III}]_0 = 2.20 \times 10^{-6}$ M	118
41. Observed rate constants for the reduction of MNMT-cyt c^{III} by complex II	118
42. Reduction of formyl-cyt c^{III} by complex I in 0.40 M phosphate buffer, pH 7.5, $\mu = 1.01$, T = 20.0° C, $[formyl-cyt\ c^{III}] = 2.40 \times 10^{-5}$ M	121
43. Rate constants for the reduction of formyl-cyt c^{III} by complex I	123
44. Reduction of formyl-cyt c^{III} by complex II on 0.40 M phosphate buffer, pH 7.5, $\mu = 1.01$ 20.0° C, $[formyl-cyt\ c^{III}]_0 = 1.88 \times 10^{-5}$ M	125
45. Rate constants for the slow phase of reduction of formyl-cyt c^{III} by complex II	128
46. Reduction of cyt c^{III} by D,L-penicillamine in 0.10 M phosphate buffer, pH 7.0, $\mu = 0.50$, T = 20.0° C, $[cyt\ c^{III}]_0 = 1.91 \times 10^{-5}$ M	129
47. Reduction of cyt c^{III} by the dioxobridged Mo(V)-penicillamine dimer (I-pen)	130
48. Observed rate constants for the reduction of cyt c^{III} by the dioxobridged Mo(V)-penicillamine complex (I-pen)	131
49. Reduction of cyt c^{III} by the monoxobridged Mo(V)-penicillamine dimer (II-pen) in 0.20 M phosphate buffer, pH 7.0, $\mu = 0.40$, T = 20.0° C, $[cyt\ c^{III}]_0 = 1.95 \times 10^{-5}$ M	132
50. Rate constants for reactions 18-22 which were used for the computer simulation of the reduction of Im-cyt c^{III} by complex II	145

LIST OF FIGURES

Figure	Page
1. The amino acid sequence of horse heart cytochrome c	15
2. The structure of oxidized horse heart cytochrome c as determined by X-ray crystallography by Dickerson and coworkers (reproduced from reference 46)	17
3. Elution profile for chromatographic purification of PLP-cyt c ^{III} derivatives on Amberlite CG-50	43
4. Elution profile for chromatographic purification of nitrated cytochrome c on Amberlite CG-50	45
5. Potentiometric titration apparatus (potentiograph not shown)	52
6. First order plots for the reduction of cyt c ^{III} by complex I	66
7. Eyring plot for the reduction of cyt c ^{III} by complex I	70
8. Oscilloscope tracings for the reduction of cyt c ^{III} by complex II	79
9. Second order plots for the reduction of cyt c ^{III} by complex II	82
10. Eyring plot for the reduction of cyt c ^{III} by complex II	84
11. Oscilloscope tracings for the reduction of Im-cyt c ^{III} by complex II	89
12. Second order plot for the reduction of Im-cyt c ^{III} by Mo(V) monomer	95
13. First order plots for the reduction of Im-cyt c ^{III} by complex II	96
14. The experimental potentiometric titration curve for the oxidative titration of complex II by thionine	102
15. Potentiometric titration curve for the oxidative titration of complex II by thionine	103

LIST OF FIGURES (Continued)

Figure	Page
16. The experimental potentiometric titration curve for the oxidative and reductive titrations of Im-cyt c	105
17. The experimental potentiometric titration curves for the oxidative and reductive titrations of N ₃ -cyt c	106
18. Potentiometric titration curve for the oxidative and reductive titration of Im-cyt c	107
19. Potentiometric titration curves for the oxidative and reductive titration of N ₃ -cyt c	108
20. First order plots for the reduction of PLP modified cyt c ^{III} by complex I	111
21. Second order plots for the reduction of PLP modified cyt c ^{III} by complex II	114
22. First order plot for the reduction of MNMT-cyt c ^{III} by complex I	117
23. Pseudo first order plot for the reduction of MNMT-cyt c ^{III} by complex II	119
24. First order plot for the reduction of formyl-cyt c ^{III} by complex I	122
25. Oscilloscope tracings for the reduction of formyl-cyt c ^{III} by complex II	124
26. First order plots for the reduction of formyl-cyt c ^{III} by complex II	127
27. The experimental vs. computer simulated production of cyt c ^{II} in the reduction of Im-cyt c ^{III} by a low concentration of complex II	146
28. The experimental vs. computer simulated production of cyt c ^{II} in the reduction of Im-cyt c ^{III} by an intermediate concentration of complex II	147
29. The experimental vs. computer simulated production of cyt c ^{II} in the reduction of Im-cyt c ^{III} by a high concentration of complex II	148

ABSTRACT

Model Studies for Molybdenum Enzymes. The Reduction of
Cytochrome c By Molybdenum(V)-Cysteine Complexes

by

Glen D. Lawrence, Doctor of Philosophy

Utah State University, 1976

Major Professor: Dr. Jack T. Spence
Department: Chemistry and Biochemistry

The reduction of ferricytochrome c ($\text{cyt } c^{\text{III}}$), as well as some iron ligand complexes and chemically modified derivatives of $\text{cyt } c^{\text{III}}$, by two molybdenum(V)-cysteine complexes has been investigated as a model for electron transfer in molybdenum enzymes. The reduction of $\text{cyt } c^{\text{III}}$ by di- μ -oxo-bis[oxo(L-cysteinato)molybdate(V)] (I) is first order in $\text{cyt } c^{\text{III}}$ and zero order in I over a wide range in concentration of I at neutral pH. The reduction of $\text{cyt } c^{\text{III}}$ by μ -oxo-bis[oxodihydroxo(L-cysteinato)molybdate(V)] (II) is first order in both reactants and has a rate several orders of magnitude greater than the rate of reduction by I. Activation parameters have been determined for both reactions.

The reduction of two pyridoxal phosphate derivatives of $\text{cyt } c^{\text{III}}$ by I proceeds at the same rate as the reduction of native $\text{cyt } c^{\text{III}}$, while the mononitromonotyrosyl derivative is reduced somewhat faster. The reduction of the N-formyltryptophyl derivative of $\text{cyt } c^{\text{III}}$ by I is biphasic, with the fast phase proceeding with a rate similar to the

native cyt c^{III} and the slow phase being somewhat slower. The reduction by I of all derivatives studied is first order in cyt c^{III} and zero order in I, suggesting the mechanism does not change with these chemically modified forms of cyt c^{III}.

The pyridoxal phosphate and mononitromonotyrosyl derivatives of cyt c^{III} are reduced by II with rates similar to the native cyt c^{III} and by the same mechanism. The reduction of the N-formyltryptophyl derivative by II has complicated kinetics which suggest the presence of at least two formyl-cyt c^{III} species.

The reduction of the cyanide, azide and imidazole complexes of cyt c^{III} by II occurs at a greatly reduced rate and by a different mechanism than the reduction of native cyt c^{III}. The rate of reduction of the cyanide and azide complexes is dependent on the rate of dissociation of the ligand cyt c^{III} complex and independent of the concentration of II. The reduction of the imidazole complex by II is much more complicated, with the rate independent of II at very low concentrations but shows a dependence on the concentration of II at higher concentrations.

Rate constants have been determined for the reactions and possible mechanisms have been developed. The results have been discussed with regard to electron transfer in molybdenum enzymes and electron transfer to cyt c^{III}. This study is especially valuable as a model for the hepatic sulfite oxidase enzyme.

INTRODUCTION

Transition metal ions play a very important role in biological oxidation-reduction reactions. Many of the elements of the first transition series have been shown to be involved in biochemical systems. Molybdenum is unique among the elements of the second and third transition series, however, in being essential in several enzymatic systems. Two molybdenum enzymes which are ultimately important to all living organisms are nitrogenase and nitrate reductase (1), which are involved in reducing inorganic nitrogen so it may be incorporated into amino acids, nucleic acids and other nitrogenous cell constituents. Other enzymatic systems which contain molybdenum include xanthine oxidase, xanthine dehydrogenase, aldehyde oxidase, sulfite oxidase and formate dehydrogenase (1,2). All of these molybdenum enzymes also contain iron, either as heme or in iron-sulfur clusters, and some contain additional prosthetic groups such as flavin and possibly quinone (1,2).

Iron is rather ubiquitous in nature and is probably the most studied transition metal with respect to its function in biological systems. Iron exists predominantly in two general forms when it is involved in the catalysis of biological electron transfers: in the heme prosthetic group or in iron-sulfur proteins. Proteins containing the heme group, which are involved in electron transfer reactions, are called cytochromes. The most extensively studied of the cytochromes is cytochrome c, the X-ray crystal structure having been determined for oxidized horse heart cytochrome c (3) and reduced tuna heart cytochrome c (4).

Enzymes which have been shown to contain both molybdenum and a heme moiety include nitrate reductase from Neurospora crassa (5) and Escherichia coli (6), hepatic sulfite oxidase (7) and formate dehydrogenase (2). Knowledge of the ligand environment of molybdenum in enzymatic systems is limited, at best. Some evidence for sulfur coordination in xanthine oxidase has been obtained from comparison of electron spin resonance parameters of the enzyme with those of molybdenum complexes containing sulfur ligands (1,8).

The purpose of this study is to gain a better understanding of the roles of molybdenum and the heme moiety in biological electron transfers, as well as a better understanding of their interactions with each other. Molybdenum(V)-cysteine complexes were used in this study as a model for the molybdenum moiety in proteins. The reduction of horse heart cytochrome c by these Mo(V)-cysteine complexes has been investigated as a model for electron transfer in the molybdenum-heme enzyme systems.

It is obvious that the results of such a study are not only valuable for the development of a model for molybdenum-heme iron interactions, but may also provide some insight into the mechanism of electron transfer in cytochrome c. For that reason, modified cytochrome c species were also used in this study as an approach to elucidating sites within the molecule which are important in the electron transfer process.

LITERATURE REVIEW

General Survey of Molybdenum in Biochemical Systems

The importance of molybdenum in biological systems has been known for some time. Bortels (9) first demonstrated a molybdenum requirement in Azotobacter chroococcum in 1930. Burk (10) later showed that molybdenum was a specific requirement for nitrogen fixation in several species of Azotobacter. Molybdenum has since been shown to be an essential constituent of several enzymes from a wide variety of organisms. These enzymes include nitrogenase, nitrate reductase and formate dehydrogenase from plants and microorganisms and xanthine oxidase, aldehyde oxidase, sulfite oxidase and xanthine dehydrogenase generally isolated from animal tissue (several of the latter enzymes are also found in plants and microorganisms) (1,2,8). In addition to molybdenum, all of these enzymes contain iron, either as heme iron or an iron-sulfur moiety, and some also contain flavin. The enzyme formate dehydrogenase has been shown to contain selenium as a cofactor (2).

The oxidation state of molybdenum in enzymes is generally accepted to be 6+ and 5+, probably alternating between these two states during the catalytic cycle. The possibility of lower oxidation states cannot, however, be ruled out. Because Mo(V) is a d^1 ion, it is especially amenable to studies by electron spin resonance spectroscopy (esr), and, in fact, that has been the most extensively used tool in studying molybdenum enzymes. The Mo(V) esr signal is observed in several

enzymes upon the addition of reducing substrate, which suggests the metal is in the 6+ oxidation state in the resting enzyme. The possibility of the addition of substrate causing spin decoupling of Mo(V) in the resting enzyme cannot, however, be ignored (11). Xanthine dehydrogenase from Micrococcus lactilyticus exhibits a large Mo(V) esr signal in the resting enzyme which changes and decreases slightly upon addition of reducing substrate, suggesting the molybdenum may remain as Mo(V) throughout the catalytic cycle (12).

Although the Mo(III) ion is paramagnetic, its esr signal has never been observed in biochemical systems. This does not, however, indicate that it is not present in these systems since its being a d^3 ion would cause considerable line broadening in the esr spectrum, making it undetectable in the concentrations of enzyme which are normally studied (13). Furthermore, arguments concerning the reduction potential of the Mo(V)/Mo(III) couple, estimated to be about -0.6 to -1.0 at neutral pH (14), are not conclusive since the potential is strongly dependent on the ligands (13). Some relatively stable complexes of Mo(IV) are known, but are generally considered unlikely to be present in biological systems (13).

The ligand environment of molybdenum in these enzymes is, at best poorly defined. Some work involving inhibition studies with sulfhydryl binding agents has implicated cysteine as a ligand (15). Further evidence for sulfur ligands has come from esr data for xanthine oxidase (8): high g_{av} values and low molybdenum hyperfine couplings in signals from the enzyme are analogous to those in Mo(V)-thiol complexes.

Model Complexes for Molybdenum Enzymes

Much attention has been concentrated on cysteine as a ligand for molybdenum in model systems of molybdenum enzymes. Complexes of Mo(V) and Mo(VI) with L-cysteine and related ligands have been prepared (16). The X-ray crystal structure for the dioxobridged Mo(V)-cysteine dimer (di- μ -oxo-bis[oxo(L-cysteinato)molybdate(V)]) (I) has been determined (17). The Mo atoms in the dianion are in a distorted octahedral environment with cysteine acting as a tridentate ligand:

The bond length of the carboxyl oxygen-molybdenum bond is very long, however, and the bond is thought to be displaced in solution by solvent molecules (17). Spence and Chang (18) found that Mo(VI) forms complexes with cysteine with Mo:cysteine ratios of 1:1 to 1:3 in the pH range 4 to 6.5; however, there is no detectable complex formation at higher pH.

Since it has been suggested that cysteine may be a ligand for molybdenum in enzymes and the binuclear dioxobridged Mo(V)-cysteine complex (I) has been well characterized, this complex has been the subject of some very extensive investigation as a model system. The complex is diamagnetic and exhibits an intense absorption band at 306 nm with a molar extinction of 12,500 (19). A solution of the complex

is converted from a yellow color to blue upon standing at pH 8-11 and a weak esr signal and weak absorption band near 600 nm are observed. Both the esr signal and the visible absorption were initially attributed to a small amount of paramagnetic monomer in equilibrium with the dimer under alkaline conditions (20). Further investigation of this system indicated that in the presence of excess cysteine at pH > 9.0 the absorption band at 306 nm completely disappears, although the esr signal still accounts for less than 1 percent of the total molybdenum (19). The weak absorption band near 600 nm ($\epsilon = 100$) is therefore due to another species which is believed to be the monoxobridged dimer (μ -oxo-bis[oxodihydroxo-(L-cysteinato)molybdate(V)] (II).

When the dibridged complex is allowed to stand under alkaline conditions without excess cysteine added, the monoxobridged cysteine complex disappears yielding another species with broad absorption maxima near 575 and 750 nm (21), the intensity of the absorption bands being pH dependent. It was found that cysteine is released from the complex during the formation of this latter species, suggesting that this species is a monoxobridged dimer with the cysteine ligands hydrolyzed. The following scheme was proposed (19):

where $X = \text{OH}^-$, H_2O or buffer ions.

Further studies showed that complex II readily reduces flavin mononucleotide (22). In the course of the reaction large intermediate esr signals were observed which were attributed to both flavosemiquinone and Mo(V) monomer. This strongly suggested that the initial fast reaction involved a one electron transfer from complex II to FMN. This system serves as an interesting model for xanthine oxidase, since the proposed scheme for electron transfer in that enzyme involves the transfer of electrons from reducing substrate through molybdenum to flavin and oxidizing substrate (8):

Molybdenum Cofactors in Enzymes

Nason and coworkers (23-25) have obtained interesting results concerning subunit relationships in nitrate reductase from Neurospora crassa which may have important implications for all molybdenum containing enzymes. An active assimilatory nitrate reductase can be formed in vitro by incubation of extracts of a nitrate-induced N. crassa mutant, nit-1 (which lacks nitrate reductase activity), with other acid-treated enzymes known to contain molybdenum. The implication is that a low molecular weight molybdenum containing subunit, common to all molybdenum enzymes, is responsible for imparting nitrate reductase activity in the mutant extract.

Cell free extracts of a selected group of bacteria, capable of metabolizing dinitrogen and/or nitrate, were found to contain a soluble form of constitutive component which is active in the in vitro formation of NADPH-nitrate reductase activity when mixed with extracts of the N. crassa nit-1 mutant (26). This constitutive component was dialyzable and postulated to be a low molecular weight cofactor or polypeptide presumed to contain molybdenum. Several other reports have appeared in the literature which lend support to such a low molecular weight molybdenum subunit common to molybdenum enzymes (27-31).

Ganelin et al. (28) isolated a molybdenum containing peptide from the molybdoferredoxin component of the nitrogenase enzyme from Azotobacter vinelandii. About 50% of the molybdenum in this molybdoferredoxin component was removed from the protein by high ionic strength in the form of a low molecular weight (~1000) peptide complex, the other 50% of the molybdenum remained tightly bound to the protein but could, however,

be removed under alkaline conditions. The isolated Mo-peptide complex exhibited an esr signal at approximately $g = 2.00$ and was tentatively attributed to an Mo(V) complex. The esr signal disappeared upon reduction with dithionite, presumably due to reduction of Mo(V) to a lower oxidation state. Zumft (31) isolated several low molecular weight components from acid-treated molybdoferredoxin from Clostridium pasteurianum nitrogenase, one of which had a molecular weight of less than 1300 and contained molybdenum. This molybdenum component has an absorption spectrum with narrow bands at 311 and 394 nm and was able to restore nitrate reductase activity to extracts of N. crassa mutant, nit-1.

Nitrate Reductase

Several studies, mostly utilizing esr techniques, have revealed the components of nitrate reductase systems from various sources. Garrett and Nason (32, 33) have shown that assimilatory nitrate reductase from N. crassa contains a b-type cytochrome, cyt b_{557} , which shows the same flavin requirement as NADPH-nitrate reductase activity and its reduction is insensitive to cyanide, although cyanide blocks its reoxidation by nitrate. Kinsky and McElroy (34) showed the NADPH-cytochrome c reductase and NADPH-nitrate reductase could be induced concomitantly by nitrate. These activities have so far proved inseparable. Garrett and Nason (33) proposed the following electron transfer scheme for the Neurospora enzyme:

The NADPH-cytochrome c reductase activity is inhibited by o-phenanthroline and 8-hydroxyquinoline but not by azide, cyanide or thiourea. The NADPH-nitrate reductase activity is inhibited by all of these reagents. FADH and reduced methylviologen can replace NADPH as electron donors to give nitrate reductase activities. The FADH- and reduced methylviologen-nitrate reductase activities are inhibited by cyanide, azide and thiourea but not by the chelating ligands. It was suggested that the cyt b_{557} would not likely be influenced by metal binding agents, such as o-phenanthroline and 8-hydroxyquinoline, and therefore another metal must be present to account for such observations. The identification of this metal has not yet been reported.

Several anaerobic organisms synthesize dissimilatory (or respiratory) nitrate reductase which utilizes nitrate as a terminal electron acceptor for metabolic processes. The enzyme from Pseudomonas aeruginosa was shown to contain cytochrome c (34,35). The proposed electron transfer scheme for this enzyme is (36):

A study of the dissimilatory nitrate reductase from Micrococcus denitrificans revealed the presence of molybdenum and non-heme iron (iron and acid labile sulfide) in this enzyme, but it did not contain heme or flavin (36). It is obvious that nitrate reductases can be obtained from diverse sources with widely varying properties, all containing molybdenum, however, which has been proposed to be essential for binding nitrate.

Sulfite Oxidase

Sulfite oxidase has been isolated in a high state of purity from mammalian (7,37,38) and avian (38) liver. Its role appears to be the oxidative detoxification of sulfur compounds in mammals, especially the oxidation of sulfur dioxide and sulfite to sulfate. The importance of sulfite oxidase in humans was demonstrated in a report of a child whose liver, kidney and brain were completely deficient in this enzyme (39). Large amounts of cysteine-S-sulfate, sulfite and thiosulfate, but not sulfate, were observed in the child's urine. The patient died at 2.5 years with severe neurological disorders and other abnormalities. Cysteine-S-sulfate, like glutamate and similar acidic amino acids have been shown to have both neuroexcitatory and neurotoxic properties, causing rapid destruction of neurons in the retina and hypothalamus (40).

The presence of molybdenum in bovine hepatic sulfite oxidase was discovered, more or less by accident, in esr experiments designed to study the heme prosthetic group (41). Sulfite oxidase from various other sources, including chicken liver, human liver, wheat germ and Thiobacillus thioparus, has been shown to contain molybdenum by esr investigation (38). A Mo(V) esr signal is observed in the enzyme from all the above mentioned sources upon addition of sulfite. The mammalian enzyme contains a b-type cytochrome component which is reduced in the steady state (in the presence of sulfite and oxygen), but oxidized if ferricyanide replaces oxygen as electron acceptor (42). Cytochrome c could also function as electron acceptor, presumably at the same site as

ferricyanide, since it is also reduced more rapidly than cyt b of the enzyme. Furthermore, the reduction of ferricyanide and cytochrome c was inhibited by anions, whereas the reduction of oxygen was not, indicating the active sites for these two classes of electron acceptor are different (41,42). The following electron transfer scheme was proposed (41):

Hepatic sulfite oxidase, from both avian and mammalian sources, has a molecular weight of about 110,000 and has two similar, if not identical, subunits (7,38). The enzyme contains two molybdenum and two heme per molecule, further suggesting identical subunits. Kinetic studies, however, showed that half of the heme is reduced very rapidly upon addition of sulfite and the other half reduced more slowly (42). These results were interpreted to mean the two subunits possessed different kinetic behavior, probably due to conformational differences.

Formate Dehydrogenase

Formate dehydrogenase has recently been purified from Escherichia coli and found to contain molybdenum, heme, selenium and non-heme iron in the relative proportions of 1:1:1:14 (2). The enzyme has a molecular weight of approximately 590,000 with 3 different polypeptide subunits. The heme content corresponds to an equivalent weight of 154,000,

suggesting a tetrameric structure. The absorbance spectrum of the enzyme indicates the presence of a b-type cytochrome which can be completely reduced by formate.

Kinetic studies have not been reported on this enzyme to determine the sequence of intramolecular electron transfer or involvement of the prosthetic groups. A proposed function for formate dehydrogenase in E. coli is that it is involved in electron transfer from formate to nitrate and is probably linked to nitrate reductase via a membrane electron carrier (e.g. quinone) (2).

Structure and Physical Properties of Cytochrome c

Cytochrome c is a small electron transfer protein found in mitochondria of all aerobic organisms. It is part of the terminal respiratory electron transport chain, which completes the breakdown of foods to carbon dioxide and water and couples the energy liberated to phosphorylation of ADP. It is a soluble hemoprotein made up of one heme group and a single polypeptide chain, whereas the other cytochrome components of the chain are part of multimolecular lipoprotein complexes. The iron atom of the heme group alternates between the 2+ and 3+ oxidation states as cytochrome c interacts with cytochrome c reductase and cytochrome c oxidase. An extensive review of structure and function in cytochrome c has been presented by Margoliash and Schejter (43).

The unusual stability and ease of isolation of cytochrome c has resulted in the accumulation of a vast amount of information on

structure and reactivity of this protein since it was first isolated from baker's yeast by Keilin in 1930 (44). The primary structures of cytochrome c from over 30 species are known, covering a spectrum of organisms from mammals to yeast and fungi (45). Dickerson (46) has recently discussed similarities in the structure of cytochrome c from many eucaryotic organisms and the curious invariance of the primary sequence. Since horse heart cytochrome c is commercially available, and the majority of investigations, including this one, use this source, this survey will be concerned primarily with this protein.

Horse heart cytochrome c contains 104 amino acid residues, with the N-terminal glycine residue acetylated. The amino acid sequence is shown in Figure 1. The heme group is covalently bound to the protein by thioether linkages to cysteinyl residues 14 and 17. The silver salt method of thioether bond cleavage has allowed the unequivocal identification of the linkage of the protoheme through the α -carbon of the vinyl side chains to the sulfur of the cysteinyl residues (47). Four of the octahedral ligands of the iron are contributed by the porphyrin ring and the fifth and sixth coordination sites are occupied by the imidazole nitrogen of histidine-18 and the sulfur of methionine-80 (3). Such a closed structure for the heme environment makes the iron relatively inaccessible to iron binding ligands, in comparison with the iron of hemoglobin and myoglobin.

The X-ray crystal structures of oxidized cytochrome c from horse and bonito (3) and the reduced cytochrome c of tuna (4) have been determined.

CH ₃ CO-NH-GLY-asp-val-glu-lys-GLY-lys-lys-ilu-PHE-val-gln-	12
-lys-[CYS]-ala-gln-[CYS]-HIS*-thr-val-glu-lys-gly-	23
-gly-lys-his-LYS-thr-GLY-PRO-asn-LEU-his-GLY-leu-	35
-phe-gly-ARG-lys-thr-GLY-gln-ala-pro-GLY-phe-thr-	47
-TYR-thr-asp-ALA-ASN-lys-asn-lys-gly-ilu-thr-TRP-	59
-lys-glu-glu-thr-leu-met-glu-TYR-LEU-glu-ASN-PRO-	71
-LYS-LYS-TYR-ILU-PRO-GLY-THR-LYS-MET*-ilu-PHE-ala-	83
-GLY-ilu-lys-LYS-lys-thr-glu-ARG-glu-asp-leu-ilu-	95
*ala-tyr-leu-lys-lys-ala-thr-asn-glu-COOH	104

Figure 1. The amino acid sequence of horse heart cytochrome c. [CYS] are the cysteine residues involved in the thioether linkage to the porphyrin side chains. (*) denotes the residues which provide the fifth and sixth ligands to the heme iron. Amino acid residues which are invariant in all species studied are shown by capital letters.

Figure 2 shows the structure of oxidized horse heart cytochrome c as determined by X-ray crystallography by Dickerson and coworkers (3). This figure has been color coded for clarity. Notice that the hydrophilic amino acids are located on the exterior of the protein in contact with the polar environment and the interior of the protein is very hydrophobic.

One of the more peculiar characteristics of the protein is its high lysine content; the horse protein contains 19 lysine residues (45). It also has 2 arginines and only 12 acidic residues. This results in a basic protein with an isoelectric point near pH 10 (48). It is of interest that both arginine and 5 of the lysine residues are totally invariant in all cytochromes c studied (46). Note in Figure 2 that most of the lysine residues form clusters on the surface of the protein (e.g. lysyl residues 86-88 form a cluster in the upper left portion of the protein and residues 5, 7, and 8 form another cluster in the upper right portion). Wada and Okunuki (49) have shown that trinitrophenylation of lysine-13, located at the outer edge of the heme crevice, causes a large decrease in the reactivity of the protein with cytochrome c oxidase. Trinitrophenylation of other lysine residues has a much smaller effect. Acetylation of lysine residues also inhibits oxidase activity, with activity decreasing linearly with the number of lysyl residues acetylated (50).

Another feature of the molecule worthy of note is the hydrophobic nature of the left heme crevice. Tyrosine residues 67 and 74 and tryptophan-59 have been suggested to be involved in electron transfer

to cytochrome c (51). Such electron transfer has been proposed to proceed via π -electron cloud interactions of these residues with one another and with the porphyrin ring.

Comparison of the structures of oxidized and reduced proteins indicates that the peptide chain from residues 80 to 83 swings to the right upon reduction (refer to Figure 2), and phenylalanine-82 becomes tucked into the heme crevice nearly parallel to the heme plane (46). This conformational change presumably displaces an anion from the heme crevice, which has been proposed to stabilize the Fe(III) charge, and the anion in turn is stabilized by lysine-13 (52). The result is a much more compact and stable protein in the reduced state (4). A recent nmr study has shown that the aromatic residues do undergo changes in orientation and interactions with other aromatic residues in the course of oxidation and reduction in solution (53).

In general, speculation has favored two separate paths for the electron in the reactions with the oxidase and reductase enzymes. A purified, single-site antibody for cytochrome c, prepared in rabbit, blocked reaction of human cytochrome c with cytochrome c oxidase without affecting its activity with cytochrome c reductase (54). Other modifications have resulted in specific inhibition of either oxidase or reductase activities (vide infra). The cytochrome c oxidase has been proposed to interact at the top of the molecule (as shown in Figure 2), binding through the lysyl residues, while the reductase has been proposed to interact either in the vicinity of tyrosine-74 or the exposed heme edge (3).

Spectral Properties of Cytochrome c

Theorell and Åkesson (55) investigated the effects of pH on the electronic spectrum of cyt c^{III}, observing five spectrally distinct species in the pH range 0 to 14. These species were designated I-V, for simplicity, in order of increasing pH. Type I exists only at pH < 1, has a spectrum which closely resembles acid hemin and was proposed to have neither protein ligand coordinated to the iron. Cyt c^{III} has never been completely converted to this form; some type II exists even in 3 N HCl.

Type II predominates in the pH range 1 to 2.5 and was proposed to have one of the protein ligands bound to the iron. It is characterized by an absorption band around 620 nm, the α -band is shifted about 5 nm to the blue (to 525 nm) and is decreased in intensity and the Soret band is also shifted to the blue by about 14 nm (to 395 nm) with an increase in intensity (relative to native cyt c^{III}). The 620 nm absorption band in hemoprotein spectra has been demonstrated to be characteristic of a high-spin ferrihemoprotein complex (56).

Type III is native cyt c^{III} and predominates in the pH range 2.5 to 9.3. This form has both protein ligands bound to the iron and is characterized by a weak absorption band at 695 nm, and maxima at 530, 408 and 365 nm, with a weak shoulder at 565 nm. The weak absorption band at 695 nm has been the subject of much investigation. Schejter and George (57) suggested this band was an indication of the conformational integrity of the protein due to its sensitivity to temperature and denaturing agents and was found to be absent in polymeric forms of cyt c^{III} (58). It was later suggested that this 695 nm absorption band

was a consequence of ligation of a methionine-sulfur to the heme iron, since a similar absorption band was observed when thioethers, including N-acetylmethionine, were added to solutions of a heme peptide obtained from cytochrome c (59). Eaton and Hochstrasser (60) have shown by examining single crystal spectra of cyt c^{III} that the 695 nm band is z-polarized and attributed it to a charge transfer transition. The methionine-80 sulfur has since been confirmed as the sixth ligand by X-ray crystallography (3). Wilson and Greenwood (61) have studied the correlation between reducibility and the presence of the 695 nm absorption band in cyt c^{III}.

Type IV cyt c^{III} exhibits absorption maxima at 537, 408 and 355 nm with a shoulder at 565 nm. The major spectral difference between this form and native cyt c^{III} is the lack of the 695 nm band. Using high resolution nmr spectroscopy, Gupta and Koenig (62) found the broad high field absorption near 24 ppm (assigned to the methyl protons of methionine-80 when it is bound to the heme iron) disappears with a pK $\sim$ 9. Further investigation of this alkaline isomerization indicated an ionizing group with pK 11.0 binds the iron with an equilibrium constant of $\sim$ 125, resulting in an overall observed pK of $\sim$ 9 (63). Furthermore, the reduction potential of cyt c^{III} shifts from +0.26V at pH 7 to +0.12V at pH 10, and suggests the ionization of a group with an apparent pK of 9.3 (64). The lysyl-79 residue has been proposed as the most likely ligand to displace the methionine sulfur under alkaline conditions (62,63,65).

The final spectral transition occurs with pK ~ 11 to give type V cyt c^{III}. It has been suggested that OH⁻ ion displaces one of the ligands, since the spectrum of this species closely resembles alkaline methemoglobin (55). This transition is probably not due to dissociation of the histidine-18 nitrogen from its axial coordination position, since there is no change in the CN-cyt c^{III} complex (presumed to have histidine-18 as one axial ligand and CN⁻ as the other axial ligand) with a similar pK (62).

It is interesting to note that cyt c^{II} shows the same spectrum throughout this pH range. This is due to the fact that Fe(II) is low spin and in octahedral symmetry, which is the crucial factor in hemo-chromogen spectra (66).

Interaction of Cytochrome c^{III} with Iron Binding Ligands

As a result of their spectroscopic observations, Keilin and Hartree (67,68) concluded that cyt c^{III} did not form a cyanide complex, in contrast to other ferriheme proteins. This conclusion was generally accepted until Potter (69) reported a decrease in the amount of reducible cyt c^{III} by the succinic dehydrogenase system in the presence of cyanide. Potter also noted a slight shift from 530 to 535 nm in the absorption maximum of cyt c^{III} in the presence of cyanide, further suggesting complex formation. Horecker and Kornberg (70) investigated this complex and found that it was indeed stable and, furthermore, caused a large decrease in the 695 nm absorption band. Several investigations since have shown that azide and imidazole form complexes with cyt c^{III} as well.

George and coworkers (71-73) have studied the reaction of cyt c with various ligands quite extensively. Cyt c^{III} reacts with both HCN and CN⁻ in the physiological pH range, although the reaction with HCN is insignificant compared with that of CN⁻ (71). (See Table 1 for rate and equilibrium constants). Cyt c^{II}, on the other hand, forms complexes with iron binding ligands only at extremely alkaline pH and dissociation of the CN⁻ ligand was observed upon neutralization of the solution.

Azide (73) and imidazole (74,75) have been shown to interact with cyt c^{III}, and furthermore, an extensive study of rate and equilibrium constants for ligand binding to cyt c^{III} by Sutin and Yandell (76) has resulted in a unifying mechanism for complex formation. Since the rate of formation of ligand-cyt c^{III} complexes fall within a limited range (see Table 1), the proposed mechanism for complex formation involved a rate limiting heme crevice opening or loosening of the methionine-80 sulfur-iron bond (reaction 1). The first order rate constant for this rate limiting process is 60 sec⁻¹. The implications of this proposed rate limiting crevice opening will be discussed further below. The ligand then binds to the heme iron (reaction 2) and the two equilibrium reactions represented as (76):

Table 1. Rate and equilibrium constants for ligand binding to cytochrome c^{III} . Values for azide and imidazole are from reference 76 and values for cyanide are from reference 71. The value of K_X is the observed equilibrium constant, K_X' is the equilibrium constant calculated for proton dissociated ligand at pH 7.0; $K_X' = K_X(1 + [H^+]/K_a)$. The rate constants are determined from the mechanism shown in reactions 1 and 2. All measurements made at 25° C. a- 40 mM lutidine buffer, pH 7.0, $\mu = 1.0$, b- 75 mM phosphate buffer, pH 7.1, $\mu = 0.16$, c- 75 mM phosphate buffer, pH 7.4, d- this value was estimated by Sutin and Yandell (76).

Ligand	K_X	K_X'	k_1	k_2/k_{-1}	k_{-2}
	M^{-1}	M^{-1}	sec^{-1}	M^{-1}	sec^{-1}
Azide ^a	4.5	4.5	31±10	0.82	5.2
Imidazole ^a	15.3	32	60±15	1.3	2.4
Cyanide ^b	4.3×10^3	1.2×10^6	15.2 ^c	0.5 ^d	1.3×10^{-5}

where $cyt\ c^{III*}$ is proposed to be a conformational isomer of $cyt\ c^{III}$ with the methionine-80 sulfur-iron bond broken, and X represents ligand.

A high resolution nmr study of $cyt\ c^{III}$ (62) showed that azide does not complex to the alkaline form of $cyt\ c^{III}$ and the azide complex dissociates with a $pK \sim 9$, indicating the presence of a different, more tightly bound ligand under alkaline conditions (proposed to be the ϵ -amino group of lysine-79). An esr investigation of the azide and imidazole complexes indicated that both are in the purely low spin state between -253 and 23° C (77).

Redox Reactions with Cytochrome c

Since the literature pertaining to redox reactions with cytochrome c is quite extensive, this survey will be restricted to those studies which are most pertinent to this investigation.

The rate of reduction of cyt c^{III} by its physiological reductant, cyt c_1^{II} , was found to be slower than expected, giving a second order rate constant of $3.3 \times 10^6 \text{ M}^{-1} \text{ sec}^{-1}$ at 10° C (78). The rate constant for the reverse reaction was $1.0 \times 10^6 \text{ M}^{-1} \text{ sec}^{-1}$ under the same conditions (78), giving a value for the equilibrium constant of 3.3, which is in good agreement with spectroscopic measurements of K_{eq} (79). The equilibrium constant was independent of temperature and ionic strength but dependent on pH, whereas the rate constants were a function of all three properties.

The oxidation of cyt c^{II} by its physiological oxidant, cytochrome c oxidase, under pre-steady state conditions is complex and depends on the cyt c:cytochrome oxidase ratio (80). The second order rate constant for reduction of the cytochrome a component in the oxidase is 10^6 - $10^7 \text{ M}^{-1} \text{ sec}^{-1}$, depending on the cytochrome oxidase preparation. Van Buuren et al. (81) determined the rate constant of the reaction to be $6 \times 10^7 \text{ M}^{-1} \text{ sec}^{-1}$ at 22° C and pH 7.2, utilizing a pulse radiolysis technique.

Rates of electron transfer from bacterial (*Pseudomonas fluorescens*) redox proteins, cyt c_{551} and azurin (a copper protein) to cyt c^{III} have been investigated (82). Second order rate constants for the two reactions are $8 \times 10^4 \text{ M}^{-1} \text{ sec}^{-1}$ for cyt c_{551} and $1.1 \times 10^3 \text{ M}^{-1} \text{ sec}^{-1}$ for azurin, both at 20° C .

The reduction of cyt c^{III} by sodium dithionite ($\text{Na}_2\text{S}_2\text{O}_4$) has been studied quite extensively by a number of workers. All of these studies indicated that at least two reactions were involved with different rates. Creutz and Sutin (83) proposed that $\text{S}_2\text{O}_4^{=}$ reacts with cyt c^{III}

by two reaction pathways, one being remote attack at the periphery of the cytochrome molecule (possibly at the exposed heme edge) and the second being adjacent attack at the heme iron which would have a rate limited by the rate of opening of the heme crevice ($k = 30 \text{ sec}^{-1}$). The rate constant for the remote attack pathway was $1.17 \times 10^4 \text{ M}^{-1} \text{ sec}^{-1}$.

Lambeth and Palmer (84) interpreted the complexity of the reaction as being due to the reduction of $\text{cyt } c^{\text{III}}$ by both $\text{S}_2\text{O}_4^{=}$ and SO_2^- , which are in equilibrium in solution. The equilibrium constant, determined by esr spectroscopy, had a value of $1.4 \times 10^{-9} \text{ M}$. The rate constants for reduction of $\text{cyt } c^{\text{III}}$ by $\text{S}_2\text{O}_4^{=}$ and SO_2^- were 1.5×10^4 and $3.9 \times 10^7 \text{ M}^{-1} \text{ sec}^{-1}$, respectively (84). Further evidence for the significance of the reaction with SO_2^- was obtained by reduction of other redox components by dithionite (e.g. ferredoxin, lumiflavin-3-acetate, plastocyanin and ferricyanide) (84). Miller and Cusanovich (85) reported rate constants for the reduction of $\text{cyt } c^{\text{III}}$ of 2.8×10^8 and $6 \times 10^5 \text{ M}^{-1} \text{ sec}^{-1}$ for SO_2^- and $\text{S}_2\text{O}_4^{=}$, respectively, at infinite dilution and pH 7.0.

Lambeth et al. (86) studied the reduction of $\text{cyt } c^{\text{III}}$ by dithionite at pH 10.7 and found that a transient $\text{cyt } c^{\text{II}}$ species is formed with more intense absorption characteristics in the visible region. This transient form reverted to the more stable native $\text{cyt } c^{\text{II}}$ with a rate constant of 2 to 8 sec^{-1} , depending on pH. In addition, they studied the rapid oxidation of $\text{cyt } c^{\text{II}}$ by ferricyanide at pH 10.5 and found a transient $\text{cyt } c^{\text{III}}$ species is formed at this pH which has the 695 nm absorption band and an esr spectrum characteristic of neutral $\text{cyt } c^{\text{III}}$. This species is converted to the normal alkaline form of $\text{cyt } c^{\text{III}}$, lacking the 695 nm band, with a rate constant of 0.8 sec^{-1} .

The interaction of iron hexacyanides with cytochrome c in both oxidation states has been studied quite extensively. Table 2 shows the rate constants reported by several workers for oxidation of cyt c^{II} by ferricyanide and reduction of cyt c^{III} by ferrocyanide. The equilibrium for this system has been studied under a variety of conditions by several techniques. Brandt et al. (87) found that the rate of reduction of cyt c^{III} by ferrocyanide was not affected in the neutral to slightly alkaline pH range, although the equilibrium did shift, due to the formation of alkaline cyt c^{III}. Stellwagen and Shulman (88), using nmr spectroscopy, found that both ferri- and ferrocyanide form complexes with both oxidation states of cyt c. They proposed a mechanism involving complex formation between the iron hexacyanides and cyt c prior to electron transfer:

where Fe(III) and Fe(II) represent ferri- and ferrocyanide, respectively. Their value for K_1 was 400 M^{-1} , the values for k_2 and k_{-2} were 208 sec^{-1} and $2.08 \times 10^4 \text{ sec}^{-1}$, respectively. The value for k_{-2} was determined assuming a value of 400 M^{-1} for K_3^{-1} (or $K_3^{-1} = K_1$). Complex formation was further supported by the findings of Miller and Cusanovich (85), although their values for the rate and equilibrium constants differed quite significantly. Their value for K_1 was $2.4 \times 10^3 \text{ M}^{-1}$, with a rate of formation of the cyt c^{III}-Fe(II) complex being $1.7 \times 10^4 \text{ M}^{-1} \text{ sec}^{-1}$.

Table 2. Apparent rate constants for oxidation and reduction of cytochrome c by iron hexacyanides. All measurements were made in the physiological pH range (6.0 to 8.0) and at 20 to 25° C. k_{ox} is the apparent rate constant for oxidation of cyt c^{II} by ferricyanide and k_{red} for the reduction of cyt c^{III} by ferrocyanide.

k_{ox} ($\times 10^{-6}$)	k_{red} ($\times 10^{-4}$)	Reference
$M^{-1} \text{ sec}^{-1}$	$M^{-1} \text{ sec}^{-1}$	
8.7	2.4	87
12.0	1.56	91
16.0	-	92
2.5	-	93
6.5	2.39	94
2.1	-	88

Their value for k_2 was $1.5 \times 10^3 \text{ sec}^{-1}$ and the largest discrepancy between their work and that of Stellwagen and Shulman was in the value for K_3 which they found to be $1.5 \times 10^{-5} M^{-1}$, indicating the complex between cyt c^{II} and ferricyanide was stronger than the complex between cyt c^{III} and ferrocyanide.

Equilibrium dialysis measurements have indicated that there are at least two binding sites on cyt c for the iron hexacyanides (89). Tri-fluoracetylation of all of the lysyl residues of cyt c prevents binding, indicating that the interaction is primarily electrostatic. Both binding sites are eliminated by raising the pH to 10 and one of the binding sites is eliminated by carboxymethylation of methionine-80, which presumably causes ligation of a lysyl ϵ -amino group to the heme iron (90). It was therefore concluded that lysine-79 was involved in

binding the iron hexacyanides, which suggested electron transfer through the exposed heme edge, since it is in such close proximity to the lysyl-79 side chain (89).

The reduction of $\text{cyt } c^{\text{III}}$ by transition metal ions has been studied by several workers. Kowalsky (95) showed that Cr(II) ion reduces $\text{cyt } c^{\text{III}}$ rapidly and the chromium becomes bound to the protein, in an approximately 0.5 molar ratio of chromium to $\text{cyt } c$, during the course of the reaction. Addition of phosphate to the medium resulted in an increase in the ratio of bound chromium to nearly 1.0 and phosphate is also bound with a similar ratio. A similar, phosphate specific activation has been observed in the reduction of $\text{cyt } c^{\text{III}}$ by Fe(II) ion (96-98). Grimes et al. (99) were able to effect a 1:1 $\text{Cr(III)-cyt } c^{\text{II}}$ complex in the absence of phosphate. Hydrolysis of the complex by trypsin and subsequent chromatography yielded two fragments containing chromium, which indicated the Cr(III) ion had crosslinked two residues of the peptide chain. Examination of a model of $\text{cyt } c^{\text{III}}$ indicated that only two residues of the crosslinked fragments were sufficiently close to allow crosslinking to take place. These residues were tyrosine-67 and asparagine-52. It was therefore concluded that tyrosine-67 participates in the reduction of $\text{cyt } c^{\text{III}}$ by Cr(II) and it was suggested that this residue may participate in the reduction mechanism in vivo.

Dawson et al. (100) determined the rate constant for reduction of $\text{cyt } c^{\text{III}}$ by Cr(II) in NaCl medium ($\mu = 0.1$) at pH 4.8 and 25°C , and obtained a value of $1.2 \times 10^4 \text{ M}^{-1} \text{ sec}^{-1}$. Yandell et al. (101)

investigated the rate of reduction of cyt c^{III} by Cr(II) ion more extensively and found the reaction is catalyzed by various anions, with the order of catalytic activity being $\text{Cl}^- < \text{I}^- < \text{N}_3^- \sim \text{SCN}^-$. Furthermore, it was found that the rates were not directly proportional to the concentration of Cr(II), but tend to a limiting value at high concentrations of Cr(II). Two mechanisms were suggested to account for such behavior; one involving a conformational change in the cyt c^{III}, which becomes rate limiting at high Cr(II) concentrations, the second involves complex formation between cyt c^{III} and Cr(II) and subsequent electron transfer, which also becomes rate limiting at high Cr(II) concentrations. The order of the catalytic activity and the nature of the anions involved suggested that electron transfer proceeds via remote attack, probably at the exposed heme edge or through the aromatic residues (101).

The reduction of cyt c^{III} by $\text{Fe}(\text{EDTA})^{2-}$ is first order in both reactants over a wide range in concentration of reductant with a rate constant of $2.57 \times 10^4 \text{ M}^{-1} \text{ sec}^{-1}$ in pH 7.0 phosphate buffer, $\mu = 0.10$ with NaCl and at 25° C (102). Kinetic studies at pH 9 indicated this reductant reacts with two slowly interconverting forms of the protein (102). The rate constant for the reduction of the alkaline form was $26.7 \text{ M}^{-1} \text{ sec}^{-1}$. These kinetic results suggested an outer sphere mechanism with the probable site of attack at the exposed heme edge.

Ewall and Bennett (103) studied the reduction of cyt c^{III} by $\text{Ru}(\text{NH}_3)_6^{2+}$ ion which is classified as an outer sphere reductant. The reaction was first order in both reactants over a wide range in concentration of reductant. Although the rate law included a hydrogen ion

dependent term, this was insignificant at neutral pH and the rate constant obtained was $3.78 \times 10^4 \text{ M}^{-1} \text{ sec}^{-1}$. Creutz and Sutin (104) found that the pentaamminebenzimidazolruthenium(II) ion reduced $\text{cyt c}^{\text{III}}$ much faster than the hexaammine complex with a rate constant of $4.7 \times 10^5 \text{ M}^{-1} \text{ sec}^{-1}$ at pH 6.1 and $\mu = 1.0$.

Greenwood and Palmer (74) studied the reduction of $\text{cyt c}^{\text{III}}$ at varying pH on the alkaline side of neutrality and provided further evidence for the existence of two functionally distinct forms of cyt c in this pH region. They found that ascorbate and tetrachlorohydroquinone react with only the native form of $\text{cyt c}^{\text{III}}$ and were able to determine the rate of interconversion of these two forms. The rate constants for the reaction with these two reductants were very different at pH 8.0 in this buffer, varying by several orders of magnitude. The values of the rate constants were 7.5×10^2 and $6 \times 10^6 \text{ M}^{-1} \text{ sec}^{-1}$ for ascorbate and tetrachlorohydroquinone, respectively. The rate constant for conversion from native to alkaline $\text{cyt c}^{\text{III}}$ at pH 10.0 was 0.188 sec^{-1} and the rate constant for conversion back to the native form at pH 7.6 was 0.048 sec^{-1} , both at 21° C (74).

The reduction of $\text{cyt c}^{\text{III}}$ by hydroquinone is much slower than the rate with tetrachlorohydroquinone. The rate constant for hydroquinone reduction is $3.7 \text{ M}^{-1} \text{ sec}^{-1}$ (105), with the semiquinone radical formed in the reaction causing autocatalysis, indicating its reaction with $\text{cyt c}^{\text{III}}$ is much faster (a rate constant was not determined).

Some of the fastest reactions reported for cyt c are its reduction by radical species produced in aqueous solution by pulse radiolysis. Several groups have studied the reduction of cyt c^{III} by the hydrated electron produced by pulse radiolysis and the rate constants are in reasonable agreement, varying from $2-10 \times 10^{10} \text{ M}^{-1} \text{ sec}^{-1}$ at neutral pH and 20° C (106-109). As expected for two oppositely charged species, the rate is dependent on the ionic strength of the medium, the lower values observed in 0.1 M ionic strength (106,107) and the larger values observed in distilled water (108,109).

The reduction of cyt c^{III} by the hydrogen atom is also very rapid, but the rate is independent of ionic strength. The rate constant determined for this reaction is $1.0 \times 10^{10} \text{ M}^{-1} \text{ sec}^{-1}$, and is the same for hydrogen atoms produced by pulse radiolysis (110) or by an external hydrogen generator (111).

Wilting et al. (106) found that at high concentrations of cyt c^{III} (>20 μM) the rate of reduction by the hydrated electron becomes limiting by a first order process. The following mechanism was proposed for the reaction in 3.3 mM phosphate buffer (pH 7.2, 21° C):

where $k_1 = 4.5 \times 10^{10} \text{ M}^{-1} \text{ sec}^{-1}$, $k_{-1} < 10^4 \text{ sec}^{-1}$, $k_2 = 5 \times 10^6 \text{ sec}^{-1}$ and $k_3 = 1.3 \times 10^5 \text{ sec}^{-1}$. According to this mechanism, a transient complex is formed initially between the hydrated electron (e_{aq}^{-}) and cyt c^{III} which undergoes intramolecular electron transfer to form cyt c^{II*}, which is an unstable form of reduced cyt c. This reduced species then

undergoes a conformational change to form the stable cyt c^{II} species. This mechanism argues in favor of electron transfer to cyt c^{III} involving the aromatic amino acid residues, such as tyrosine-67 (106).

Land and Swallow (108) have reported the rate constants for the reduction of cyt c^{III} by various radicals produced by pulse radiolysis. The CO₂⁻ radical is produced from formate and gives a rate constant for reduction of cyt c^{III} of $5 \times 10^8 \text{ M}^{-1} \text{ sec}^{-1}$. The rate increases by nearly a factor of 10 below pH 4, which was attributed to protonation of the radical, forming the more reactive COOH radical. The superoxide anion radical reduces cyt c^{III} with a rate constant of $1.1 \times 10^5 \text{ M}^{-1} \text{ sec}^{-1}$ at pH 8.5. The protonated form of superoxide, HO₂, is relatively unreactive with cyt c^{III}.

Studies with Modified Cytochrome c Species

Since the three dimensional structure of cytochrome c has been elucidated in both the oxidized (3) and reduced (4) states, this protein has been especially useful in the study of structure and function relationships. One approach in such studies has been in the selective modification of amino acid residues within the protein. This approach has been simplified by the advancement of techniques designed to measure cytochrome c reactivities with cytochrome c oxidase (112) and NADH-cytochrome c reductase (113) systems independently as well as measuring activities in cytochrome c depleted mitochondria (114,115). Naturally the more interesting results have occurred by modification of the amino acid residues in and around the heme crevice.

Modification of the single tryptophan residue in cyt c (trp-59) has been accomplished using a variety of group specific reagents. Aviram and Schejter (115) first described the formylation of the indole nitrogen using formic acid in the presence of HCl. The modification was reversed by incubating the formyl-cyt c at pH 11 for 30 min. Formyl-cyt c^{III} lacks the 695 nm absorption band, has the Soret maximum shifted 3 nm to the blue (406 nm) and is not reduced by ascorbate at pH 7, but is reduced by dithionite, although much more slowly than the native protein (115). The reduced protein is autoxidizable and binds CO at neutral pH (115). These characteristics indicated that methionine-80 is not bound to the heme iron in this modified protein even though the modification procedure does not chemically alter the methionine. Since tryptophan-59 is involved in hydrogen bonding with one of the propionic acid side chains of the porphyrin, formylation of the indole nitrogen would necessarily break this hydrogen bond, apparently causing gross conformational changes to take place in the protein (115). Thermal denaturation studies, circular dichroism and electronic absorption spectra have indicated that formyl-cyt c^{III} not only has an altered coordination configuration of the metal but produced a grossly deranged molecular conformation (116).

Erecinska (117) found that a single species of formyl-cyt c could be isolated by ion exchange chromatography, which had a midpoint potential of 0.085 V at pH 7.0. (Potential measurements of the formyl-cyt c preparation prior to purification, exhibited a sigmoid titration curve characteristic of two components with resolved midpoint potentials of

0.090 and -0.055 V). This purified preparation was reduced by ascorbate (in contrast to the preparation of Aviram and Schejter), was reactive with cytochrome c oxidase (although the K_m was 6 fold greater than for native cytochrome c and the apparent V was smaller) but did not restore respiratory activity to cytochrome c depleted mitochondria. The formyl-cyt c^{III} was not reactive with purified mitochondrial NADH-cytochrome c reductase, which is not surprising since the reaction is not thermodynamically favorable; it was, however, reactive with purified microsomal NADPH-cytochrome c reductase which is known to have a lower reduction potential, although the rate is approximately 20% that observed with native cyt c^{III} (117).

Brittain and Greenwood (118) have reported a kinetic investigation of the reduction of formyl-cyt c^{III} by Cr(II) ion as well as the rates of ligand binding with CO and NO as ligands. At pH 6.3, the reduction is biphasic with one phase dependent on the concentration of Cr(II) ($k = 4 \times 10^4 \text{ M}^{-1} \text{ sec}^{-1}$) and one phase independent ($k = 5.0 \text{ sec}^{-1}$). Similarly the reaction of NO with formyl-cyt c^{III} is biphasic with one phase dependent and the other independent of NO concentration with rate constants of $2.8 \times 10^3 \text{ M}^{-1} \text{ sec}^{-1}$ and 0.2 sec^{-1} , respectively. The reaction of formyl-cyt c^{II} with CO is also biphasic; both phases however, are dependent on the concentration of CO with rate constants of $1.8 \times 10^2 \text{ M}^{-1} \text{ sec}^{-1}$ and $1.6 \times 10^3 \text{ M}^{-1} \text{ sec}^{-1}$. Dissociation of the CO by photolysis and subsequent recombination resulted in a simple second order reaction with a rate constant of $1.9 \times 10^2 \text{ M}^{-1} \text{ sec}^{-1}$. A scheme was proposed to account for such kinetic behavior which involves a slow

equilibrium between two forms of the formyl-cyt c^{II} , both of which react with CO to form a single formyl-cyt c^{II} -CO complex which dissociates upon photolysis to give only one of the forms (the slower reacting one), and a subsequent recombination reaction which is fast in comparison with the equilibrium. Although no data was presented for the reactions with formyl-cyt c^{III} (Cr(II) reduction or NO binding), the authors suggested that the reduction of formyl-cyt c^{III} indicates a reductive step and subsequent conformational change. It should be pointed out, however, that their preparation procedure was essentially that of Aviram and Schejter (115) which would not have separated the two components of different reduction potential reported by Erecinska (117).

Sokolovsky et al. (119) found that cytochrome c could be nitrated with tetranitromethane at pH 8.0 and a protein modified specifically at the tyrosyl-67 residue could be purified. This nitro derivative is unable to restore respiratory activity to cytochrome c depleted mitochondria (119). The purified nitro-cyt c^{III} was found to be in the purely high spin state in acid medium and in the low spin state in alkaline medium; at intermediate pH values, two forms exist, one with high spin and the other with low spin characteristics (120). The low spin form is ascorbate reducible, but has a heme linked pK of 5.9 instead of the pK 9.0 of the native protein. The other form is not reduced by ascorbate and remains in the high spin state up to pH 8.6. Both forms are autoxidizable and bind CO.

A more rigorous purification procedure allowed the separation of a mononitromonotyrosyl-cytochrome c derivative (MNMT-cyt c) with modification of tyrosine-67, and a mononitrodityrosyl-cytochrome c (MNMT-cyt c)

with the additional modification of tyrosine-48, from the tetranitromethane reaction mixture (121). The MNMT-cyt c^{III} has spectral characteristics very similar to the native cyt c^{III}; the 695 nm absorption band, however, has an extinction coefficient, at pH 4.5, approximately 60% that of the native cytochrome and the 695 nm band disappears with a pK of 6.2, in contrast to the native protein which loses its 695 nm band with a pK of about 9.0 (121). The MNMT-cyt c^{III} does not exhibit the 695 nm band at all. The MNMT-cyt c is ascorbate reducible, shows about 24% activity with NADH-cytochrome c reductase, about 82% activity with cytochrome c oxidase and is slowly autoxidized. The MNMT derivative on the other hand, is not reduced by ascorbate, shows little or no activity with NADH-cytochrome c reductase and is autoxidized too rapidly to determine its activity with cytochrome c oxidase (121). Circular dichroism spectra indicated that the MNMT-cyt c^{III} is conformationally very similar to the native protein, although at neutral pH it resembled the alkaline form of cyt c^{III} quite closely. The MNMT-cyt c^{III} at neutral pH represents a form with altered iron coordination as well as deranged protein conformation (121).

Aviram and Schejter (122) found that pyridoxal phosphate could be covalently bound to cytochrome c by borohydride reduction, giving two derivatives which could be separated by ion exchange chromatography. These two derivatives were found to have one or two residues of pyridoxal phosphate covalently bound and were designated PLP₁- and PLP₂-cyt c, respectively. Both modified proteins show cytochrome c oxidase activity nearly identical with native cyt c^{II} but the activities with

cytochrome c reductase are about 25% and 10% of the native for PLP₁- and PLP₂- cyt c^{III}, respectively (122). Proteolytic digestion of these cyt c derivatives and subsequent purification of the fragments indicate pyridoxal phosphate is covalently bound to three peptide fragments corresponding to the partial sequences: 1-10, 68-74 and 83-94 (122). Since the amino terminal end of cyt c is acetylated, only the lysine residues would be susceptible to modification with pyridoxal phosphate and borohydride. The PLP-bound peptides 1-10 and 83-94 have three lysyl residues each, which define a positive region at the top portion of the molecule (see Figure 2) and the peptide 68-74 has two lysyl residues which are located in a different section of the molecule. It was concluded that these two regions of the molecule are possible sites of interaction with the reductase system, but must not be involved in the reaction with cytochrome c oxidase. Further studies of PLP-modified cytochrome c have not appeared in the literature.

Several other derivatives of cytochrome c have been prepared (e.g. see 51); limited space and their irrelevancy to this study, however, forbids the description of each of their properties.

EXPERIMENTAL PROCEDURES

MaterialsDi- μ -oxo-bis[oxo(L-cysteinato)-molybdate(V)]

The synthesis of di- μ -oxo-bis[oxo(L-cysteinato)molybdate(V)] (complex I) has been reported by Kay and Mitchell (16). L-Cysteine hydrochloride (3.2 g) in 5 ml of water was added to sodium molybdate (5.1 g) in 8.3 ml of water. While stirring with a magnetic stirrer, sodium dithionite (1.5 g) in 6.5 ml of water was added and the mixture stirred for 15 min. It was then vacuum filtered in a buchner funnel and washed once with 1:1 (v/v) ethanol-water. The residue was dried by suction of prepurified N₂ through the crystals. The complex was recrystallized once prior to use by dissolving the crystalline solid in a minimum amount of hot water and adding ethanol until a precipitate just formed. The solution was heated to boiling and then cooled. The precipitate was vacuum filtered, washed twice with 1:1 (v/v) ethanol-water and dried by suction of prepurified N₂ through the crystals. The crystals were further dried in an oven at 60° C for 3 to 4 hr.

The purity of the complex was checked by weighing some of the dried solid and dissolving in pH 7.0 phosphate buffer which had been deaerated with prepurified N₂. The absorption spectrum has a maximum at 306 nm with an extinction coefficient of 12,500 M⁻¹ cm⁻¹ (19).

μ -oxo-bis[oxodihydroxo(L-cysteinato)-molybdate(V)]

The μ -oxo-bis[oxodihydroxo(L-cysteinato)molybdate(V)] (complex II) was prepared in situ from complex I in pH 10 borate buffer in the presence of a 20 fold excess of L-cysteine unless noted otherwise. Under these conditions (with a 20 fold excess of L-cysteine), the bridge opening goes essentially to completion in 3 hr (21). Due to the sensitivity of complex II to air oxidation, solutions deaerated with prepurified N_2 were prepared immediately prior to use (within 2 to 3 hr) and kept under prepurified N_2 .

Mo(V)-penicillamine complexes

The Mo(V)-penicillamine complex (I-pen), analogous to complex I, was prepared in the same way as complex I, substituting D,L-penicillamine for L-cysteine. D,L-penicillamine (0.80 g) was dissolved in a minimum amount of pH 2 aqueous HCl (ca. 10 ml) to which was added 1.3 g sodium molybdate and the mixture stirred with a magnetic stirrer. After mixing, 0.35 g sodium dithionite was added and the mixture stirred for 15 min. The complex did not precipitate until ethanol was added. The solution was cooled to 0° C and filtered in vacuo.

The penicillamine complex was recrystallized by dissolving it in a minimum amount of 0.1 N HCl. Ethanol was added until a precipitate just persisted and the mixture heated to boiling and cooled. The complex was vacuum filtered and dried by suction of prepurified nitrogen through the crystals. The solid was further dried in an oven at 60° C for 3 to 4 hr. There is always a small amount of free penicillamine present in

the crystallized complex as will be shown in the results, although the residual free penicillamine was generally less than 2 %.

The monoxobridged Mo(V)-penicillamine complex (II-pen), analogous to complex II, was prepared by dissolving the sodium salt of the dibridged complex in 0.25 M borate buffer at pH 10. The concentration of the dibridged complex initially was 2.0×10^{-3} M. The solution was allowed to stand at room temperature for 24 hr in the presence of excess L-cysteine under prepurified N_2 . Approximately 80% of the dibridged species disappeared as evidenced by the disappearance of its polarographic wave. The addition of flavin mononucleotide to this solution shows that only 60% of the molybdenum added initially is reactive with flavin (123). A different preparation under identical conditions showed less than 30% of the total molybdenum present to be reactive with cytochrome c^{III} in a rapid reaction. The concentration of active molybdenum (assumed to be monoxobridged Mo(V)-penicillamine) was determined from the amount of cyt c^{III} rapidly reduced in the presence of less than one equivalent of the active species.

Cytochrome c and derivatives

Horse heart cytochrome c (types III and VI) was obtained from Sigma Chemical Co. (St. Louis, Mo.) and used without further purification. Type III cytochrome c was used in all modification procedures. Kinetic results were the same with either type III or type VI cytochrome c^{III} and the two were used interchangeably. The concentration of native cytochrome c was determined from the molar absorptivity at 550 nm after complete reduction, using an extinction coefficient of 27,700 (124).

N-formyltryptophyl-cytochrome c. Cytochrome c was formylated at the tryptophan-59 residue by the method of Aviram and Schejter (115). Cytochrome c (50 mg) was dissolved in 2.0 ml formic acid (97+ %) to which 2.0 ml formic acid saturated with HCl was added. The mixture stood at room temperature for 90 min and the mother liquor was removed in vacuo. The protein residue was then dissolved in a minimum amount of pH 3 aqueous HCl and chromatographed on a Sephadex G-25 column (2.5 x 15 cm) to remove residual reagent. The original procedure recommends lyophilization at this stage; the lyophilized protein, however, is not very soluble and the preparative procedure was modified. The eluant from the gel filtration was dialyzed against 0.02 M phosphate buffer, pH 7.5. The dialyzed protein was then adsorbed on an Amberlite CG-50 ion exchange column (1.5 x 20 cm), washed with 2 to 3 vols of 0.02 M phosphate buffer, pH 7.5 and eluted with 0.40 M phosphate buffer, pH 7.5. The single protein band which was eluted was characterized as formyl-cyt c^{III} by its characteristic absorption spectrum, which lacks the 695 nm absorption band and has the Soret maximum at 406 nm.

The concentration of formyl-cytochrome c solutions was determined by the absorbance of the fully reduced cytochrome at 550 nm using an extinction coefficient of 21,300 (117). The stock solution of formyl-cyt c^{III} was stored at ca. 4° C until used.

Pyridoxal-5'-phosphate modification of cytochrome c. Cytochrome c was modified with pyridoxal-5'-phosphate (PLP) by the procedure of Aviram and Schejter (122). Cytochrome c (50 mg) and 4.5 mg PLP were dissolved in 10 ml of 0.05 M tris-cacodylate buffer, pH 8.0. After 7 min in solution, 0.7 ml of 0.40 M NaBH_4 in 0.02 M NaOH was added to give a final concentration of NaBH_4 of ca. 25 mM. After 15 min the mixture was diluted to 60 ml and 25 mg $\text{K}_3\text{Fe}(\text{CN})_6$ added to oxidize the cytochrome c. The solution was placed under vacuum for 10 min to remove excess hydrogen. The protein solution was then adsorbed on an Amberlite CG-50 column (2.5 x 12 cm), equilibrated with 0.02 M phosphate buffer, pH 8.0. After all of the protein was on the resin, the column was washed with 4 to 5 volumes of the equilibrating buffer and then eluted with a 0 to 0.6 M NaCl gradient in 0.02 M phosphate buffer, pH 8.0. Fractions were collected (5 ml each) and the absorbance of each fraction measured at 530 and 320 nm. The elution profile was plotted for both wavelengths as well as the ratio $A_{320}:A_{530}$ vs fraction number (Figure 3). Aviram and Schejter (122) found by amino acid analysis that the protein fractions with an $A_{320}:A_{530}$ ratio of 2.5 contained 2 PLP groups per cytochrome c molecule and the protein with a ratio of 2.0 contained 1 PLP group per cytochrome c molecule. The $A_{320}:A_{530}$ ratio for native cytochrome c is approximately 1.4. The fractions with $A_{320}:A_{530}$ ratios near 2.5 were taken to be $\text{PLP}_2\text{-cyt } c^{\text{III}}$ and fractions with ratios near 2.0 were taken to be $\text{PLP}_1\text{-cyt } c^{\text{III}}$ without subjecting them to amino acid analysis. Because the PLP derivatives had very little effect on the kinetics with either complex I or complex II, the actual site of covalent binding was not determined.

Figure 3. Elution profile for chromatographic purification of PLP-cyt c^{III} derivatives on Amberlite CG-50. $\blacksquare$ -Absorbance at 320 nm, $\bullet$ -absorbance at 530 nm, $\blacktriangle$ -the ratio of A_{320}/A_{530} .

All PLP-cyt c^{III} solutions were 0.10 M phosphate buffer, pH 7.0, $\mu = 0.50$ (adjusted with NaCl).

Nitration of cytochrome c. The mononitromonotyrosyl derivative of cytochrome c^{III} (MNMT-cyt c^{III}) was prepared according to the method of Sokolovsky et al. (119). Cytochrome c (50 mg) was dissolved in 20 ml of 0.10 M tris-HCl buffer, pH 8.0. Tetranitromethane (0.023 ml, 31.4 mg) was dissolved in 0.5 ml ethanol and this solution was added to the cytochrome c solution and allowed to stand at room temperature for 35 to 40 min. The mixture was chromatographed on Sephadex G-25, which had been equilibrated with 0.02 M phosphate buffer, pH 7.5, to stop the reaction and remove excess reagent. The eluant containing most of the protein was diluted to 50 ml and adsorbed on an Amberlite CG-50 column (2.5 x 15 cm) equilibrated with the same buffer as the Sephadex column. The column was washed with 3 to 4 volumes of equilibrating buffer and eluted with a 0 to 0.3 M NaCl gradient in 0.02 M phosphate buffer, pH 7.5. Fractions (11 ml each) were collected and their absorbance measured at 530 nm. The elution profile is shown in Figure 5. The absorption spectra were recorded for fractions at the maxima in the elution profile.

The spectral and chemical properties of fraction C are given in Table 3 and are consistent with the properties of MNMT-cyt c^{III} reported by Pal et al. (121). The spectral characteristics are not easily distinguishable from native cytochrome c; its chemical characteristics, however, indicate that this fraction is MNMT-cyt c^{III} as reported by Pat et al. (121). The cytochrome is >90% reduced by ascorbate.

Figure 4. Elution profile for chromatographic purification of nitrated cytochrome c on Amberlite CG-50.

Table 3. Physicochemical properties and activity of native and various modified preparations of cytochrome c at neutral pH

Property	Native ^a	Formyl ^a	PLP ₁ ^b	PLP ₂ ^b	MNMT ^c
Spectrum					
cyt c ^{III}					
Soret max (nm)	409	406	409	409	409
ϵ (Soret, mM)	104	94	ND	ND	ND
695 nm band	Present	NP	Present	Present	Present
cyt c ^{II}					
Soret max (nm)	416	417	416	416	416
ϵ (Soret, mM)	138	118	ND	ND	167
ϵ_{550} (mM)	27.6	21.3 ^d	ND	ND	26.5
ϵ_{520} (mM)	16.0	13.0 ^d	ND	ND	17.3
Ascorbate reducibility	Fast	None	Fast	Fast	Fast
Autoxidizability	None	Fast	None	None	Slow
Cytochrome c reductase activity (%)	100	10	25	10	24
Cytochrome c oxidase activity (%)	100	50	90	90	82

a--reference 116; b--reference 122; c--reference 121; d--reference 117.

Complete reduction can be attained with sodium dithionite and the completely reduced cytochrome is autoxidizable, which is a characteristic native cytochrome c lacks. The oxidation is extremely slow with an estimated first order rate constant of $1.3 \times 10^{-4} \text{ sec}^{-1}$ in a solution saturated with O_2 . The autoxidation goes to >85% completion. These results indicate the MNMT-cyt c^{III} fraction contains approximately 10% impurity.

All chemicals used in the preparation of molybdenum complexes and buffers were reagent grade. Penicillamine was 99+% and tetranitromethane was 98%, both purchased from Aldrich Chemical Co., L-cysteine (free base) was purchased from Nutritional Biochemicals Corp. and pyridoxal-5'-phosphate was purchased from Sigma Chemical Co. The water used in all preparations and kinetic studies was deionized distilled water.

Methods

Reduction of cyt c^{III} by complex I

Stock solutions of the reactants were prepared with deaerated buffer and maintained anaerobic by continuously passing prepurified N₂ through them. Prepurified N₂ was generally passed over the cyt c^{III} solution and bubbling kept to a minimum to prevent denaturation of the protein. The stock solutions were kept in a constant temperature bath and appropriate amounts transferred to a stoppered, deaerated spectrophotometer cell with gas tight syringes. The spectrophotometer cell was maintained at constant temperature with a water jacketed cell holder. Absorbance at 550 and 695 nm was recorded as a function of time on a Varian Techtron 635 spectrophotometer equipped with a Hewlett-Packard X-Y recorder. The reduction of modified cyt c^{III} species by complex I was followed in a similar manner. The reduction of cyt c^{III} by the dibridged Mo(V)-penicillamine complex was followed similarly.

The reduction of cyt c^{III} by penicillamine was followed on the stopped flow spectrometer equipped with an X-Y recorder. The instrument

was equipped in the same way as with the studies of the reduction by complex II (vide infra).

Binding studies with cyt c^{III}
and complex I

A Sephadex G-25 column (2.5 x 20 cm) was equilibrated with 0.20 M phosphate buffer, pH 7.0, $\mu = 0.53$ with NaCl, placed in the cold room and about 10 volumes of deaerated buffer, kept under prepurified N₂, was passed through the column to remove oxygen. The reactants were mixed to give final concentration of 1.9×10^{-4} M for both cyt c^{III} and complex I in the equilibrating buffer. All procedures were done in deaerated buffer and fractions were collected and their absorption spectra recorded. The cytochrome c fraction eluted in <30 ml and there was no indication of complex I being present in the cyt c^{III} fraction. Less than 30% reduction of cyt c^{III} took place on the column. Complex I was eluted in 50 to 90 ml of eluant, and approximately 85% of the total complex I added to the column was recovered in these fractions.

A technique known as the Hummel-Dryer method (125) was also employed. A Sephadex G-25 column (1.0 x 10 cm) was equilibrated with a deaerated, 4×10^{-4} M solution of complex I in 0.02 M phosphate buffer, pH 7.0, by passing several volumes of the solution through the column. The column outlet was connected directly to a 1.00 mm flow spectrophotometer cell in the Varian model 635 spectrophotometer to monitor the absorbance of the eluant at 305 nm (the absorbance maximum for complex I). If complex I binds to cyt c^{III}, the absorbance should show a peak where the cyt c^{III} passes through the cell, followed by a trough due to a decrease

in the concentration of I in its wake. A peak was observed for the cyt c^{III}, due to its absorbance in the 305 nm region; however, no trough followed, indicating lack of binding.

Electronic absorption difference spectra were recorded for cyt c^{II} and complex I, mixed vs separated. Compartmented quartz cells with 1.00 cm pathlength per compartment, 2.00 cm pathlength overall, were used in this study. The final concentrations for I and cyt c^{II} were both 4.00×10^{-5} M in each cell. The two were mixed in one compartment of the sample cell with buffer in the second compartment. The two were placed in separate compartments in the reference cell and the difference spectrum recorded. There was no significant deviation from the baseline in the difference spectrum.

Reduction of cyt c^{III} by complex II

The stock solution of complex II was prepared as previously described (19). The cyt c^{III} stock solution was prepared with deaerated buffer and both stock solutions were kept in a constant temperature bath under prepurified N₂. The absorbance at 550 nm was followed on an Aminco-Morrow stopped flow spectrometer, equipped with a constant temperature circulating system. The loading syringes were maintained anaerobic by constantly passing prepurified N₂ through them and nitrogen was continuously passed around the mixing chamber compartment. The spectrometer was flushed several times with deaerated buffer prior to use to remove oxygen.

The proper amounts of deaerated stock solutions and buffer were transferred to the loading syringes with gas tight syringes and measurements were made as rapidly as possible (1 to 2 min). At least three runs were recorded at each concentration and the superimposed oscilloscope tracings were photographed. The dead time of the instrument is 3 to 4 msec.

Complex II was prepared from I in the presence of added cysteine (20 fold excess to catalyze the bridge opening and inhibit hydrolysis) (19) for most runs. Cysteine itself reduces $\text{cyt } c^{\text{III}}$ slowly, with a rate constant of ca. $0.2 \text{ M}^{-1} \text{ sec}^{-1}$ at pH 7.45 (126). The reduction of $\text{cyt } c^{\text{III}}$ by concentrations of cysteine that would be found in complex II solutions was checked and found to be much too slow to be significant with respect to the reduction by II. Complex II, prepared in the absence of cysteine, was found to reduce $\text{cyt } c^{\text{III}}$ at the same rate as II prepared in its presence.

Complex II is in a slow, pH dependent equilibrium with complex I (21). The rate of conversion of II to I was determined at pH 7.0 by monitoring the absorbance change at 305 nm (due to appearance of I). The observed rate constant ($k = 1.2(\pm 0.2) \times 10^{-4} \text{ sec}^{-1}$) indicates the amount of II converted to I, from the time II is diluted in the loading syringe until the reaction is recorded (1-2 min), is negligible. The reduction of ligand bound and modified $\text{cyt } c^{\text{III}}$ species by complex II was followed in the same way as the reduction of native $\text{cyt } c^{\text{III}}$. The reduction of $\text{cyt } c^{\text{III}}$ by the monobridged Mo(V)-penicillamine complex was followed similarly.

Potentiometric titration of complex II

Janus Green B in 0.02 M borate buffer, pH 8.0, $\mu = 0.10$ (adjusted with NaCl) was placed in the potentiometer cell and deaerated with argon. An appropriate amount of stock solution of II was transferred to the potentiometer cell in a gas tight syringe to give a final volume of 5.0 ml. The final concentration of Janus Green B was 5.0×10^{-6} M and the final concentration of II was 1.00×10^{-4} M prior to titrating.

The oxidant solutions were placed in the reservoir of the automatic titrator (Figure 5) and deaerated with argon. The automatic titrator was flushed several times with the deaerated oxidant solution, to remove oxygen, prior to connecting it to the potentiometric cell containing complex II solution.

The potentiometric titration was recorded on a Metrohm model E336A potentiograph equipped with a model E436D automatic titrator. A Brinkmann EA234 combination platinum silver-silver chloride electrode was employed as the measuring/reference electrode. The automatic titrator added 1.00 ml of oxidant solution at a rate of 0.50 ml/min or 0.25 ml/min.

Three redox dyes were used as oxidants: methylene blue, pyocyanine and thionine. These three dyes were chosen because they are reduced by complex II very rapidly but show no detectable reduction in the presence of cysteine alone, thereby avoiding any complication which would be caused by the excess cysteine present in the complex II solution. Janus Green B was selected as a mediator since it was found to be only partially reduced by a slight excess of complex II. Titrations were also performed in the absence of any mediator and gave similar results.

Figure 5. Potentiometric titration apparatus (potentiograph not shown).

Potentiometric titration of native
and ligand bound cyt c^{III}

The potentiometric titration of native cyt c^{III} was studied in the absence of any heme iron binding ligands to insure the accuracy of the method and apparatus being used. The same general procedure was used for measuring the redox potential of the cytochrome c system that was used for complex II potential measurements (see Figure 5).

Cyt c^{III} was dissolved in 0.02 M phosphate buffer, pH 7.0, $\mu = 0.50$ (adjusted with NaCl) which had been deaerated with argon. 2,6-Dichlorophenolindophenol (DCIP) was added to the cyt c^{III} solution as a mediator to give a final concentration of DCIP of less than 5% the final concentration of cyt c^{III}. The reductive titration was accomplished with either reduced phthiocol or ascorbate as reductant in the same buffer system which had been deaerated with argon. The cyt c^{III} solution obtained in the reductive titration was then back titrated with $K_3Fe(CN)_6$ as oxidant. The concentration of cyt c and DCIP mediator were 15-20% less in the back titration due to the dilution caused by the initial reductive titration. The amount of reductant added in the initial titration was such that there was not a large excess of reductant present at the beginning of the back titration.

The redox potential of ligand bound cyt c^{III} was measured in the same way as the redox potential of native cyt c^{III}. The redox potential of the imidazole-cyt c^{III} complex (Im-cyt c^{III}) was determined in 0.02 M phosphate buffer which was 0.50 M in imidazole, pH 7.0, $\mu = 0.35$. The concentration of cyt c^{III} was $7.5-15.0 \times 10^{-5}$ M with 2.0×10^{-6} M DCIP

added as mediator. The redox potential of the azide-cyt c^{III} complex (N_3 -cyt c^{III}) was determined in 0.02 M phosphate buffer which was 0.50 M in NaN_3 , pH 7.0, $\mu = 0.60$. The concentration of cyt c^{III} was 7.0 – 7.7×10^{-5} M with 2.0×10^{-6} M DCIP added as mediator. The reductive titration of both the imidazole and azide complexes was accomplished with reduced phthiocol as titrant.

Phthiocol was reduced electrolytically just prior to titration. A 5.0 mM solution of oxidized phthiocol in 0.02 M phosphate buffer, pH 7.0, $\mu = 0.10$ (with NaCl) was deaerated in the coulometer cell. The potential was supplied by a Princeton Applied Research model 173 coulometer at -0.650 V vs a silver-silver chloride reference electrode. The number of coulombs used to reduce the phthiocol was used to accurately determine its concentration and was generally within 2 or 3% of the concentration determined from the amount of phthiocol added. The proper amount of reduced phthiocol solution was then transferred with a gas tight syringe to the titrant reservoir of the automatic titrator. The diluting buffer in the titrant reservoir was the same as the buffer system for cyt c^{III} , including ligand, and was deaerated with argon.

The redox potential of the cyanide-cyt c^{III} complex (CN-cyt c^{III}) was not determined by potentiometric titration because no redox dye was found to reduce the complex rapidly. The redox potential of CN-cyt c^{III} has been estimated at ca. -0.40 V (72), since it is reduced by sodium dithionite ($E_o' = -0.46$ V) but not by cysteine ($E_o' = -0.34$ V).

Electron spin resonance studies

The fast, second order phase in the reduction of Im-cyt c^{III} by complex II suggested that a Mo(V) monomeric species may be involved in the reaction, since the fast phase was noticeable only at higher concentrations of II. A previous esr investigation of complex II (19) indicated less than 1% esr active monomeric Mo(V) at pH >8.0, with the maximum esr signal observed above pH 11.0. If a Mo(V) monomeric species is involved in the rapid reduction of Im-cyt c^{III} , its concentration would have to be >1% to account for the fast phase of the reaction. It was therefore suspected that imidazole may stabilize the Mo(V) monomer.

A solution of $K_3Mo(CN)_8$ in 1 N H_2SO_4 was used as a standard for the determination of Mo(V) monomer concentration. The $Mo(CN)_8^{3-}$ was prepared in situ from $K_4Mo(CN)_8 \cdot 2H_2O$ in 1 N H_2SO_4 by oxidation with 0.10 M ceric ammonium sulfate (127). The method for determination of concentration of monomer is based on the fact that the area under the esr absorption curve is proportional to the concentration of unpaired electrons. Since $K_3Mo(CN)_8$ is present as 100% monomer in solution (128), a comparison of the area under the absorption curve for a known concentration of $Mo(CN)_8^{3-}$ with the area under the absorption curve for an unknown concentration of Mo(V) monomer permits the estimation of the unknown concentration. Caution was taken in keeping the $K_3Mo(CN)_8$ solution in the dark, due to its photosensitivity.

The esr spectrum of a 1.0×10^{-5} M $K_3Mo(CN)_8$ solution, frozen in liquid N_2 , was recorded and the area under the first derivative

absorption curve was determined by double integration. The concentration of the unknown species was calculated from the equation:

$$C_{\text{unk}} = \frac{C_{\text{std}} \times A_{\text{unk}} \times SL_{\text{std}}}{A_{\text{std}} \times SL_{\text{unk}}}$$

where C_{unk} = molar concentration of the unknown sample,

C_{std} = molar concentration of the standard sample,

A_{unk} = area under the absorption curve of the unknown sample
in arbitrary units,

A_{std} = area under the absorption curve of the standard sample in
arbitrary units,

SL_{unk} = signal level for the esr measurement of the unknown and

SL_{std} = signal level for the esr measurement of the standard.

The determination of g values was not necessary for this study.

A solution of 1.0×10^{-3} M complex II in 0.16 M phosphate, 0.40 M imidazole buffer, pH 7.1, was added to the esr tube under anaerobic conditions and frozen in liquid N_2 1 min after mixing (the approximate time for II in imidazole solutions for kinetic runs). The esr spectrum was recorded and the area under the first derivative curve determined in the same way as for the standard.

The esr spectrum of a mixture of cyt c^{III} and complex I was recorded to see if any radical intermediates could be detected in the reaction. A solution of 2.0×10^{-3} M cyt c^{III} and 1.0×10^{-2} M I in 0.10 M phosphate buffer, pH 7.4, was mixed in a serum bottle and kept anaerobic. Aliquots were transferred to an esr tube and frozen in

liquid N_2 at 1 min and 5 min after mixing. The esr spectra of these samples were recorded at liquid N_2 temperature ($77^\circ K$). Room temperature spectra were recorded in a flat quartz cell (Varian Associates). The cyt c^{III} - complex I solution was added to the deaerated cell, sealed with a rubber septum and the esr spectrum recorded periodically throughout the reaction.

All esr spectra were recorded on a Varian V-4500 spectrometer.

Treatment of Data

Determination of rate constants

The preliminary data obtained for the reduction of cyt c^{III} by I indicated the reaction was zero order in I and probably first order in cyt c^{III} . A first order dependence on cyt c^{III} yields the following rate expression:

$$\frac{d[\text{cyt } c^{II}]}{dt} = k_{\text{obs}}[\text{cyt } c^{III}]$$

Since $[\text{cyt } c^{III}]$ at any given time is equal to $[\text{cyt } c^{III}]_0 - [\text{cyt } c^{II}]$, integration of this rate expression gives:

$$\ln([\text{cyt } c^{III}]_0 - [\text{cyt } c^{II}]) = k_{\text{obs}} t + C$$

Letting $[\text{cyt } c^{III}]_0 = (A_f - A_o)/(\epsilon_{\text{red}} - \epsilon_{\text{ox}})$ and $[\text{cyt } c^{II}] = (A - A_o)/(\epsilon_{\text{red}} - \epsilon_{\text{ox}})$, where A is absorbance at any given time, A_f is final absorbance, A_o is initial absorbance and ϵ_{red} and ϵ_{ox} are the extinction coefficients for the reduced and oxidized species, respectively, this expression becomes:

$$\ln(A_f - A) = k_{\text{obs}} t + C'$$

where C' contains the term $\ln(\epsilon_{\text{red}} - \epsilon_{\text{ox}})$. A plot of $\ln(A_f - A)$ vs t should be linear for a reaction which is first order in $\text{cyt } c^{\text{III}}$ and zero order in reductant (or under pseudo first order conditions in a reaction which is first order in reductant and the reductant is in a large excess). The slope of such a plot yields the observed first order rate constant directly.

The first order plots for the reduction of $\text{cyt } c^{\text{III}}$ by I deviated from linearity after about 2 half-lives, so the first order rate constants were determined from the linear portion of the plots. The observed autocatalytic effect is probably due to the production of cysteine, which also reduces $\text{cyt } c^{\text{III}}$.

The reduction of $\text{cyt } c^{\text{III}}$ by II was found to be second order, first order in each reactant. Since II is a two electron reductant and $\text{cyt } c^{\text{III}}$ is a one electron oxidant, the stoichiometry of the reaction must be accounted for in the rate expression, although the rate has a first order dependence on both reactants:

$$\frac{d[\text{cyt } c^{\text{II}}]}{dt} = k[\text{cyt } c^{\text{III}}][\text{II}]$$

Letting $[\text{cyt } c^{\text{II}}] = X$, this expression becomes:

$$\frac{dX}{dt} = k([\text{cyt } c^{\text{III}}]_0 - X)([\text{II}]_0 - 1/2 X)$$

Integration of this rate expression gives:

$$\frac{1}{[\text{II}]_0 - [\text{cyt } c^{\text{III}}]_0/2} \ln \frac{([\text{II}]_0 - 1/2 X)}{([\text{cyt } c^{\text{III}}]_0 - X)} = k_{\text{obs}} t + C$$

A plot of the left side of this integrated expression vs t should be linear for a second order reaction and the observed second order rate constant is obtained directly from the slope. The rate constants for second order reactions were determined from an appropriate least squares program on a PDP-8 computer. The second order plots for such reactions were generally linear for greater than 3 half-lives, with standard deviations generally less than 10%.

In cases where the reaction was biphasic (e.g. with formyl-cyt c^{III}), rate constants for the initial phase were determined by the Guggenheim method (129). According to this method the following relationship holds:

$$\ln(A_{t+\Delta t} - A_t) = \text{constant} - k(t - t_0)$$

where A_t is absorbance at time t , $A_{t+\Delta t}$ is absorbance at some increment of time, Δt , later and k is the first order rate constant. Using a constant increment of time, Δt , for a given run and plotting the left side of this expression against $(t - t_0)$ should produce a straight line with slope k . One drawback with this method is the large standard deviations obtained in the plots. This is due to the limited accuracy in measuring absorbance and the rather small differences in $A_{t+\Delta t}$ and A_t which tend to magnify the errors. First order or pseudo first order conditions prevailed when this method was employed.

The reduction of Im-cyt c^{III} by II is complex and, on first inspection, appeared to be biphasic. A very rapid phase, the amount of which depended on the concentration of II, was found to be best explained as

a reduction of Im-cyt c^{III} by Mo(V) monomer. The concentration of monomer was estimated by the fraction of Im-cyt c^{III} reduced in the fast phase and an equilibrium constant determined for the reaction:

The values for the equilibrium constant determined in this way, is estimated at 4×10^{-7} M. Since the Mo(V) monomer is a one electron reductant, the second order rate expression for the reaction of Mo(V) monomer with cyt c^{III} is:

$$\frac{dX}{dt} = k([\text{cyt } c^{III}]_0 - X)([\text{Mo(V)}]_0 - X)$$

where $X = [\text{cyt } c^{II}]$, and integration gives:

$$\frac{1}{[\text{Mo(V)}]_0 - [\text{cyt } c^{III}]_0} \ln \frac{([\text{Mo(V)}]_0 - X)}{([\text{cyt } c^{III}]_0 - X)} = kt + C$$

A plot of the left side of this expression vs t should be linear and the slope = k_{obs} . A least squares treatment of the data, where $[\text{Mo(V)}]_0 > [\text{cyt } c^{III}]_0$, gave linear second order plots with standard deviations generally less than 10%. The second order rate constants could only be determined for cases where $[\text{Mo(V)}]_0 > [\text{Im-cyt } c^{III}]_0$, since at lower initial concentrations of Mo(V) monomer, other reactions interfered and resulted in nonlinear plots.

The mechanism proposed for the reduction of Im-cyt c^{III} by complex II yields a very complicated rate law which is not readily integrable. The mechanism could, however, be tested, using a computer program which

generates simulated data for the overall mechanism. The computer method, developed by Moore and coworkers (130,131), is based on the fact that each chemical component has a time dependent rate expression (the first derivative) for its appearance and disappearance, which can be differentiated again. The second derivative of this component can be expressed in terms of the first derivatives and concentrations of the other involved components. This operation can be performed for all of the chemical species involved in the mechanism. Since the first and higher derivatives for each component can be obtained, a Taylor Series expansion can be used, which gives the time dependent concentration of each component as a function of the differential expressions and concentrations of the other involved components. A convergence test can be built into the program to determine a suitable time increment, and the series inherently conserves mass. The accuracy of the numerical solution is related to the stringency of the convergence test.

Each step of the chemical mechanism must be fed into the computer program, along with the rate constants for each step and the initial concentration of each species involved. The computer printout gives the concentration of each component for any time interval desired. Comparison of the computer simulated data with the experimentally observed data should indicate the accuracy of the proposed mechanism. The main disadvantage of this approach is that all of the rate constants and the initial concentrations of all species must be known and the accuracy of the method is strongly dependent on the accuracy of the values used for these parameters.

RESULTS

Reduction of Cyt c^{III} by Complex I

Complex I reduces cyt c^{III} very slowly; the reduction is very interesting, however, in that the rate of reduction is independent of the concentration of I over a very wide concentration range (Table 4). The rate was found to be first order in cyt c^{III} and followed a first order equation, except for an autocatalytic effect observed beyond one half-life, which is probably due to the production of cysteine which also reacts with cyt c^{III}. Table 6 shows the observed rate constants (k_{obs}) for the reduction of cyt c^{III} by I in phosphate buffer; the mean value for k_{obs} in 0.10 M phosphate buffer, pH 7.5, $\mu = 0.50$, at 20.0° C is $1.09(\pm 0.10) \times 10^{-3} \text{ sec}^{-1}$. Similar rate constants were obtained when the disappearance in absorbance at 695 nm was followed (Table 6). The first order plots are shown in Figure 6.

The effect of pH on the reduction of cyt c^{III} by I is shown in Table 7. The value of k_{obs} increased approximately two fold in going from pH 6.0 to 7.5, but increased by a factor of about 2.5 between pH 7.5 and 8.0 (Table 8). Such a large increase in the rate constant between pH 7.5 and 8.0 can be attributed to the formation of complex II, which results from the equilibrium between I and II in basic solution (pH >7.5) (19). At pH >8.0 the rate increased even more and deviated significantly from first order.

Table 4. Reduction of cyt c^{III} by complex I in 0.10 M phosphate buffer, pH 7.5, μ = 0.50 with NaCl, 20.0° C, [cyt c^{III}]₀ = 3.35-3.78 x 10⁻⁵ M. [I]₀: (1) 2.00 x 10⁻⁵ M; (2) 4.00 x 10⁻⁵ M; (3) 8.00 x 10⁻⁵ M; (4) 1.60 x 10⁻⁴ M; (5) 4.00 x 10⁻⁴ M; (6) 8.00 x 10⁻⁴ M; (7) 2.00 x 10⁻³ M; (8) 4.00 x 10⁻³ M; (9) 4.00 x 10⁻⁴ M; (10) 8.00 x 10⁻⁴ M; (11) 2.00 x 10⁻⁴ M; (12) 8.00 x 10⁻⁴ M. a 0.28 M phosphate buffer, pH 7.5, μ = 0.74, b 0.01 M phosphate buffer, pH 7.5, μ = 0.50 with NaCl.

t(sec)	Absorbance (550 nm)											
	1	2	3	4	5	6	7	8	9 ^a	10 ^a	11 ^b	12 ^b
0	.338	.355	.350	.350	.334	.363	.330	.355	.373	.349	.350	.365
100	.393	.388	.405	.415	.404	.419	.400	.388	.436	.406	.420	.431
200	.446	.426	.456	.469	.459	.467	.461	.426	.487	.456	.480	.471
300	.497	.473	.504	.520	.507	.510	.514	.473	.532	.505	.540	.547
400	.548	.515	.548	.565	.550	.550	.561	.515	.575	.550	.593	.600
500	.593	.555	.590	.610	.590	.587	.604	.555	.616	.595	.641	.648
600	.634	.592	.630	.644	.627	.624	.642	.592	.654	.636	.685	.691
800	.710	.659	.699	.708	.693	.689	.708	.659	.723	.710	.760	.767
1000	.774	.714	.759	.765	.747	.746	.760	.714	.781	.773	.820	.826
1200	.825	.760	.810	.808	.792	.797	.800	.760	.830	.825	.864	.875
1400	.870	.796	.855	.845	.828	.840	.838	.796	.870	.867	.898	.911
1600	.905	.824	.890	.875	.856	.879	.858	.824	.900	.900	.920	.939
1800	.932	.846	.920	.897	.878	.910	.875	.846	.923	.926	.937	.959
A _f	1.026	.923	1.026	.957	.930	1.042	.935	.923	.965	.980	.980	1.028

Table 5. Rate constants for the reduction of cyt c^{III} by complex I with varying concentrations of I. $[cyt\ c^{III}]_0 = 3.35-3.78 \times 10^{-5}$ M, $\mu = 0.50$ adjusted with NaCl, pH 7.5, 20.0° C. a $\mu = 0.74$, b rate constants obtained from the decrease in absorbance at 695 nm, conditions were as described in Table 6. $k_{av} = 1.09 (\pm 0.10) \times 10^{-3} \text{ sec}^{-1}$.

$[I]_0$ ($M \times 10^4$)	[phos] (M)	k_{obs} ($\text{sec}^{-1} \times 10^3$)
0.200	0.10	0.87
0.400	0.10	1.02
0.800	0.10	1.05
1.60	0.10	1.15
4.00	0.10	1.18
8.00	0.10	0.99
20.0	0.10	1.02
40.0	0.10	1.09
4.00	0.28 ^a	1.17
8.00	0.28 ^a	1.12
2.00	0.01	1.27
8.00	0.01	1.13
4.00 ^b	0.10	1.06
20.0 ^b	0.10	1.02
40.0 ^b	0.10	1.09

Table 6. Reduction of cyt c^{III} by complex I following absorbance at 695 nm in 0.10 M phosphate buffer, pH 7.5, $\mu = 0.50$ with NaCl, at 20.0° C, [cyt c^{III}] = $1.75-1.95 \times 10^{-4}$ M. (1) $[I]_0 = 4.00 \times 10^{-4}$ M; (2) $[I]_0 = 2.00 \times 10^{-3}$ M; (3) $[I]_0 = 4.00 \times 10^{-3}$ M.

t(sec)	Absorbance (695 nm)		
	1	2	3
0	.147	.161	.142
100	.138	.153	.132
200	.130	.145	.123
300	.122	.138	.116
400	.114	.134	.108
500	.107	.125	.102
600	.100	.118	.095
800	.088	.107	.084
1000	.079	.097	.075
1200	.071	.088	.067
1400	.064	.082	.061
1600	.059	.076	.056
1800	.055	.071	.052
<hr/> A _f	<hr/> .050	<hr/> .067	<hr/> .047

Figure 6. First order plots for the reduction of cyt c^{III} by complex I. Reduction of cyt c^{III} was monitored by increase in absorbance at 550 nm (left) or decrease in absorbance at 695 nm (right).

Table 7. Effect of pH on the reduction of cyt c^{III} by complex I in 0.10 M phosphate buffer, $\mu = 0.50$ adjusted with NaCl, 20.0° C, [cyt c^{III}]₀ = 3.26-3.71 x 10⁻⁵ M. (1) [I]₀ = 4.00 x 10⁻⁴ M; (2) [I]₀ = 8.00 x 10⁻⁴ M.

t(sec)	Absorbance (550 nm)									
	pH 6.0		pH 6.4		pH 6.0		pH 7.5		pH 8.0	
	1	2	1	2	1	2	1	2	1	2
0	.338	.340	.360	.370	.340	.358	.334	.363	.323	.328
100	.375	.411	.425	.459	.387	.418	.404	.419	.456	.445
200	.400	.448	.464	.515	.421	.463	.459	.467	.556	.538
300	.424	.478	.498	.562	.450	.504	.507	.510	.635	.613
400	.448	.505	.528	.604	.479	.542	.550	.550	.697	.673
500	.470	.529	.556	.642	.506	.577	.590	.587	.745	.722
600	.492	.550	.584	.675	.531	.610	.627	.624	.784	.762
800	.532	.592	.635	.735	.578	.670	.693	.689	.834	.818
1000	.570	.628	.680	.783	.621	.720	.747	.746	.862	.851
1200	.604	.661	.720	.823	.660	.764	.792	.797	.876	.870
1400	.637	.690	.758	.857	.696	.800	.828	.840	.884	.878
1600	.667	.718	.790	.884	.728	.832	.856	.879	-	.883
1800	.695	.743	.821	.906	.757	.858	.878	.910	-	-
2400	.768	.804	.894	.946	.825	.911				
3000	.825	.850	.943	.960	.874	.937				
3600	.870	.883	.973	.964	.907	.949				
A _f	.977	.987	1.023	.973	.955	.962	.930	1.042	.900	.898

Table 8. Effect of pH on the rate constants for the reduction of cyt c^{III} by complex I. 0.10 M phosphate buffer, $\mu = 0.50$ adjusted with NaCl, 20.0° C, [cyt c^{III}]₀ = 3.26-3.71 x 10⁻⁵ M.

[I] ₀ (M x 10 ⁴)	pH	k _{obs} (sec ⁻¹ x 10 ³)
4.00	6.0	0.45
8.00	6.0	0.53
4.00	6.4	0.53
8.00	6.4	0.83
4.00	6.9	0.62
8.00	6.9	0.83
4.00	7.5	1.18
8.00	7.5	0.99
4.00	8.0	2.72
8.00	8.0	2.38

The rate also increased with increasing temperature over the temperature range studied (Table 9). Table 10 shows the effect of temperature on k_{obs}: at temperatures above ca. 35° C, the rate decreases and the reaction becomes complex, probably due to heat denaturation of the protein.

Activation parameters were calculated from an Eyring plot, $\ln(k_{\text{obs}}/T)$ vs $1/T$ (Figure 7), of the data in Table 10, using a least squares program for the best fit of the data. The enthalpy of activation, $\Delta H^\ddagger$, is 22.8(±2.8) kcal/mol and the entropy of activation, $\Delta S^\ddagger$, is +6.8 (±6.2) eu.

Such a zero order dependence on complex I in the reduction of cyt c^{III} suggests a possible mechanism involving an initial rapid complex formation between cyt c^{III} and I and a subsequent slow electron transfer (see discussion). To test such a mechanism, an attempt was made to

Table 9. Effect of temperature on the reduction of cyt c^{III} by complex I. 0.10 M phosphate buffer, pH 7.5, $\mu = 0.50$ with NaCl, $[\text{cyt } c^{III}]_0 = 3.44\text{--}3.92 \times 10^{-5} \text{ M}$, $[\text{I}]_0 = 8.00 \times 10^{-4} \text{ M}$.

t(sec)	Absorbance (550 nm)											
	10.0° C	15.2° C	20.0° C	25.0° C	30.0° C							
0	.338	.360	.402	.334	.365	.370	.360	.367	.387	.372	.375	.385
50	—	—	—	.371	.405	.402	.459	.452	.460	.480	.481	.533
100	.365	.435	.502	.404	.440	.433	.541	.525	.525	.574	.574	.645
150	—	—	—	.433	.474	.462	.610	.591	.584	.654	.651	.733
200	.385	.492	.572	.459	.504	.490	.668	.647	.636	.720	.716	.798
250	—	—	—	—	—	—	.718	.695	.685	.775	.769	.845
300	.403	.541	.632	.507	.559	.544	.758	.738	.729	.820	.813	.880
400	.421	.586	.685	.550	.609	.592	.820	.807	.803	.885	.878	.922
500	.439	.627	.732	.590	.654	.638	.864	.858	.864	.928	.920	.944
600	.459	.665	.778	.627	.696	.680	.892	.895	.912	.953	.947	.951
700	—	—	—	—	—	—	—	—	—	.969	.962	—
800	.493	.730	.855	.693	.766	.754	.922	.942	.980	—	—	—
1000	.526	.786	.916	.747	.821	.815	.934	.964	1.020	—	—	—
1200	.557	.830	.961	.792	.863	.865	—	.973	1.044	—	—	—
1400	.589	.865	1.000	.828	.893	.902	—	—	—	—	—	—
1600	.619	.893	1.029	.856	.913	.930	—	—	—	—	—	—
1800	.649	.915	1.050	.878	.930	.951	—	—	—	—	—	—
2400	.725	—	—	—	—	—	—	—	—	—	—	—
3000	.791	—	—	—	—	—	—	—	—	—	—	—
3600	.844	—	—	—	—	—	—	—	—	—	—	—
A_f	1.013	.977	1.090	.930	.948	1.010	.947	.990	1.082	.993	.990	.961

Figure 7. Eyring plot for the reduction of $\text{cyt } c^{\text{III}}$ by complex I. Conditions are as described in Table 10. $\Delta H^{\ddagger} = 22.8 (\pm 2.8) \text{ kcal/mol}$, $\Delta S^{\ddagger} = 6.8 (\pm 6.2) \text{ eu}$.

Table 10. Effect of temperature on the rate constants for the reduction of cyt c^{III} by complex I. 0.10 M phosphate buffer, pH 7.5, $\mu = 0.50$, $[\text{cyt c}^{\text{III}}]_0 = 3.44\text{--}3.92 \times 10^{-5} \text{ M}$, $[\text{I}]_0 = 8.00 \times 10^{-4} \text{ M}$.

T (°C)	k_{obs} ($\text{sec}^{-1} \times 10^3$)
10.0	0.33
15.0	1.14
20.0	1.38
25.0	3.15
30.0	5.76

detect such a cyt c^{III}-I complex. Electronic absorption spectra, recorded immediately after mixing the two reactants, showed no difference in absorption in the visible or near ultraviolet regions of the spectrum. Addition of cyt c^{III} to a solution of complex I caused no immediate shift in the polarographic reduction potential of complex I. Furthermore, there was no evidence of complex formation between cyt c^{II} and I by either spectral or polarographic techniques.

Attempts were also made to show complex formation between complex I and either oxidized or reduced cyt c by chromatography on Sephadex G-25 gel columns. Mixing the two reactants together and chromatographing the mixture immediately on a Sephadex G-25 column gave no evidence for binding. The Hummel-Dryer method (see methods section) for determining ligand binding to proteins also gave negative results.

In order to obtain evidence for possible intermediates in the reaction, esr spectra were recorded at room temperature and liquid N₂ temperature at various times during the reaction. No evidence for

appreciable amounts of esr active Mo(V) monomer was obtained. This is not surprising since the Mo(V) monomer would probably be much more reactive than complex I and would therefore not accumulate. Cyt c^{III} shows no appreciable esr absorption under these conditions due to unfavorable characteristics of its spin lattice relaxation (132) and signals are only observed for cyt c^{III} at very low temperatures (liquid He temperatures).

Schejter and Margalit (133) found that chloride and phosphate ions have a significant effect on the thermodynamic properties of cyt c^{III}. Tris-cacodylate buffer was used in that study as a nonbinding medium for comparison. The nonbinding medium caused a decrease in the observed redox potential with increasing ionic strength, whereas a binding medium (chloride) caused an increase in the observed redox potential with increasing ionic strength. Increasing the concentration of chloride in the nonbinding medium at constant ionic strength caused a decrease in the observed redox potential, thus favoring the oxidized form of cyt c.

Other buffer systems were used in this study to determine what effect phosphate may have on the kinetic properties of the reaction between cyt c^{III} and complex I (see Tables 11-15 for data). Table 16 gives the observed rate constants for the reduction of cyt c^{III} by I in 10 mM phosphate buffer, 20 mM tris-cacodylate buffer, 10 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) buffer, and 10 mM HEPES buffer with 10 mM NaCl added. The observed first order rate constants increase with increasing concentration of I in phosphate and HEPES buffers, but are affected very little by the concentration of I in tris-cacodylate buffer ($k = 9.6(\pm 1.0) \times 10^{-3} \text{ sec}^{-1}$). The first order

Table 11. Reduction of cyt c^{III} by complex I in 10 mM phosphate buffer, pH 7.5, $\mu = 0.026$, $T = 20.0^\circ \text{C}$, $[\text{cyt } c^{III}]_0 = 1.80\text{--}1.85 \times 10^{-5} \text{ M}$. (1) $[\text{I}]_0 = 1.10 \times 10^{-5} \text{ M}$; (2) $[\text{I}]_0 = 2.20 \times 10^{-5} \text{ M}$; (3) $[\text{I}]_0 = 4.40 \times 10^{-5} \text{ M}$; (4) $[\text{I}]_0 = 8.80 \times 10^{-5} \text{ M}$; (5) $[\text{I}]_0 = 2.20 \times 10^{-4} \text{ M}$; (6) $[\text{I}]_0 = 4.40 \times 10^{-4} \text{ M}$; (7) $[\text{I}]_0 = 1.10 \times 10^{-3} \text{ M}$.

t(sec)	Absorbance (550 nm)						
	1	2	3	4	5	6	7
0	.165	.170	.180	.182	.185	.200	.225
10				.207	.214	.236	.276
20				.228	.236	.262	.305
30				.248	.257	.287	.330
40				.268	.278	.308	.352
50	.228	.253	.270				
60				.304	.315	.348	.389
80				.337	.348	.381	.419
100	.278	.322	.347	.365	.377	.408	.443
120				.390	.402	.430	.462
140				.412	.423	.449	.478
150	.320	.376	.403				
160				.435	.440	.463	.489
180	.			.445	.455	.475	.499
200	.355	.416	.442				
250	.383	.446	.468				
300	.406	.466	.482				
350	.425	.477	.490				
400	.439	.484	.494				
500	.460	.490					
600	.473						
A_f	.495	.495	.500	.500	.500	.510	.528

Table 12. Reduction of $\text{cyt } c^{\text{III}}$ by complex I in 20 mM tris-cacodylate buffer, pH 7.0, $\mu = 0.018$, at 20.0° C, $[\text{cyt } c^{\text{III}}]_0 = 3.75\text{--}3.80 \times 10^{-5} \text{ M}$. $[\text{I}]_0$: (1) $2.00 \times 10^{-5} \text{ M}$; (2) $4.00 \times 10^{-5} \text{ M}$; (3) $6.00 \times 10^{-5} \text{ M}$; (4) $8.00 \times 10^{-5} \text{ M}$; (5) $1.00 \times 10^{-4} \text{ M}$; (6) $2.00 \times 10^{-4} \text{ M}$; (7) $5.00 \times 10^{-4} \text{ M}$; (8) $1.00 \times 10^{-3} \text{ M}$; (9) $2.00 \times 10^{-3} \text{ M}$.

t(sec)	Absorbance (550 nm)								
	1	2	3	4	5	6	7	8	9
0	.363	.375	.368	.365	.365	.368	.380	.390	.430
10	.435	.430	.428	.423	.428	.424	.435	.450	.486
20	.494	.488	.488	.480	.488	.464	.482	.497	.538
30	.552	.543	.543	.535	.543	.522	.525	.540	.575
40	.608	.600	.597	.585	.595	.565	.566	.579	.616
60	.707	.700	.692	.677	.685	.644	.640	.650	.688
80	.785	.784	.772	.755	.761	.712	.702	.712	.751
100	.847	.850	.836	.818	.822	.767	.755	.765	.806
120						.815	.800	.812	.855
140	.932	.943	.925	.908	.908	.855	.840	.850	.895
160						.888	.872	.885	.930
180	.984	.994	.978	.961	.962	.915	.900	.912	.960
A_f	1.038	1.038	1.038	1.038	1.038	1.040	1.043	1.045	1.090

Table 13. Reduction of cyt c^{III} by complex I in 10 mM HEPES buffer, pH 7.5, $\mu = 0.0050$, at 20.0° C, [cyt c^{III}]₀ = 3.64-3.70 x 10⁻⁵ M. [I]₀: (1) 2.00 x 10⁻⁵ M; (2) 5.00 x 10⁻⁵ M; (3) 1.00 x 10⁻⁴ M; (4) 2.00 x 10⁻⁴ M; (5) 5.00 x 10⁻⁴ M; (6) 1.00 x 10⁻³ M; (7) 2.00 x 10⁻³ M.

t(sec)	Absorbance (550 nm)						
	1	2	3	4	5	6	7
0	.344	.347	.340	.345	.360	.388	.460
25	.388	.425	.415	.458	.468	.480	.560
50	.430	.501	.494	.558	.560	.560	.640
75	.470	.569	.560	.643	.638	.627	.708
100	.507	.629	.623	.712	.704	.685	.763
125	-	.683	.677	.772	.760	.735	.810
150	.574	.730	.725	.822	.806	.780	.852
175	-	.774	.772	.863	.847	.818	.885
200	.636	.813	.810	.897	.880	.852	.915
250	.693	.878	.875	.947	.930	.904	.960
300	.744	.930	.925	.977	.965	.946	.990
350	.792	.967	.960	.992	.985	.975	1.011
400	.835	.988	.983	.997	.998	.998	1.024
500	.907	-	-	-	-	-	-
600	.958	-	-	-	-	-	-
A _f	1.020	1.012	1.012	1.004	1.010	1.036	1.047

Table 14. Reduction of cyt c^{III} by complex I in 10 mM HEPES buffer, pH 7.5, $\mu = 0.0050$, at 20.0°C , $[\text{cyt } c^{III}]_0 = 1.92\text{--}1.98 \times 10^{-5} \text{ M}$. $[\text{I}]_0$: (1) $1.0 \times 10^{-5} \text{ M}$; (2) $2.00 \times 10^{-5} \text{ M}$; (3) $5.00 \times 10^{-5} \text{ M}$; (4) $1.00 \times 10^{-4} \text{ M}$.

t(sec)	Absorbance (550 nm)			
	1	2	3	4
0	.165	.190	.195	.205
10	.229	.250	.261	.272
20	.262	.292	.312	.329
40	.316	.358	.391	.415
60	.359	.409	.447	.472
80	.394	.448	.486	.508
100	.423	.479	.512	.529
120	-	-	-	.537
140	.468	.518	.534	-
180	.500	.532	-	-
A_f	.530	.538	.544	.548

Table 15. Reduction of cyt c^{III} by complex I in 10 mM HEPES buffer, pH 7.5, $\mu = 0.015$, at 20.0°C , $[\text{cyt } c^{III}]_0 = 1.90\text{--}2.00 \times 10^{-5} \text{ M}$. $[\text{I}]_0$: (1) $1.00 \times 10^{-5} \text{ M}$; (2) $2.00 \times 10^{-5} \text{ M}$; (3) $5.00 \times 10^{-5} \text{ M}$; (4) $1.00 \times 10^{-4} \text{ M}$.

t(sec)	Absorbance (550 nm)			
	1	2	3	4
0	.188	.190	.203	.213
20	-	-	-	.264
40	-	-	-	.310
50	.270	.266	.303	-
60	-	-	-	.351
80	-	-	-	.388
100	.305	.330	.386	.420
140	-	-	-	.472
150	.379	.385	.449	-
180	-	-	-	.509
200	.419	.430	.494	-
250	.451	.467	.521	-
300	.477	.496	.534	-
350	.496	.515	-	-
400	.511	.526	-	-
500	.526	-	-	-
A_f	.534	.536	.541	.550

Table 16. Observed rate constants for the reduction of cyt c^{III} by complex I in different buffers. a. 10 mM phosphate buffer, pH 7.6, $\mu = 0.026$, $[\text{cyt c}^{\text{III}}]_0 = 1.80 \times 10^{-5} \text{ M}$; b. 20 mM tris-cacodylate buffer, pH 7.0, $\mu = 0.018$, $[\text{cyt c}^{\text{III}}]_0 = 3.76 \times 10^{-5} \text{ M}$; c. 10 mM HEPES buffer, pH 7.5, $\mu = 0.0050$, $[\text{cyt c}^{\text{III}}]_0 = 1.95 \times 10^{-5} \text{ M}$; d. 10 mM HEPES buffer, pH 7.5, $\mu = 0.0050$, $[\text{cyt c}^{\text{III}}]_0 = 3.70 \times 10^{-5} \text{ M}$; e. 10 mM HEPES buffer, 10 mM NaCl, pH 7.5, $\mu = 0.15$, $[\text{cyt c}^{\text{III}}]_0 = 1.95 \times 10^{-5} \text{ M}$; $T = 20.0^\circ \text{ C}$ for all runs.

$[\text{I}]_0$ ($\text{M} \times 10^5$)	k_{obs} ($\text{sec}^{-1} \times 10^3$)				
	Phos ^a	T-C ^b	HEPES ^c	HEPES ^d	HEPES-NaCl ^e
1.00	4.5	-	11.3	-	5.48
2.00	6.7	10.9	15.6	2.98	5.62
4.00	8.0	10.4	-	-	-
5.00	-	-	20.2	5.72	7.53
6.00	-	10.5	-	-	-
8.00	9.1	9.9	-	-	-
10.0	-	10.5	23.1	6.87	9.33
20.0	9.9	9.0	-	8.58	-
40.0	11.3	-	-	-	-
50.0	-	8.4	-	7.73	-
100	12.1	8.6	-	6.19	-
200	-	8.6	-	7.35	-

plots at such low ionic strength indicate the reaction is much more autocatalytic (a positive deviation in the first order plot) than at higher ionic strength, probably due to a faster reduction of cyt c^{III} by cysteine (released in the reaction) at these lower ionic strengths.

The rate constants observed in HEPES buffer are interesting; by increasing the initial concentration of cyt c^{III}, the rate constant decreases. This was very puzzling at first, but considering the cyt c^{III} has a net charge of 7 or 8+ at this pH, and the ionic strength of the HEPES buffer is only 5 mM, the concentration of cyt c^{III} would affect

the ionic strength of the medium significantly; therefore, 10 mM NaCl was added to the HEPES buffer to see how drastic the ionic effect would be. It is apparent the ionic effect is quite strong under these conditions; addition of 10 mM NaCl caused a >50% decrease in the observed rate constants (Table 16). The slight decrease in rate with increasing concentration of I in tris-cacodylate buffer, although hardly beyond the limits of experimental error, may also be due to an ionic strength effect.

Reduction of Cyt c^{III} by Complex II

Complex II reduces cyt c^{III} very rapidly, several orders of magnitude faster than does complex I. The rate of reduction is in fact of the same order of magnitude as the enzymatic reduction of cyt c^{III} by NADH-cytochrome c reductase (cyt c_1^{II}) (78), and is the most rapid rate reported for the reduction of cyt c^{III} by a transition metal complex. The reduction by complex II also differs from the reduction by complex I in that the rate is first order in both reactants. Representative oscilloscope tracings from the stopped flow data are shown in Figure 8 and Tables 17 and 18 have the absorbance data tabulated for dependence on concentration of II and temperature dependence, respectively.

Typical second order plots for the reduction of cyt c^{III} by complex II are shown in Figure 9 and the observed second order rate constants for the reaction are given in Table 19. It is necessary to set a lower limit on the concentration of II (ca. 5×10^{-6} M) due to oxidation of II by residual oxygen in the system. It is necessary to emphasize that

Figure 8. Oscilloscope tracings for the reduction of $\text{cyt } c^{\text{III}}$ by complex II. Experimental conditions were: 0.20 M phosphate buffer, pH 7.0, $\mu = 0.40$, 25.0°C , $[\text{cyt } c^{\text{III}}]_0 = 1.00 \times 10^{-5} \text{ M}$, $[\text{II}]_0$: (a) $3.00 \times 10^{-6} \text{ M}$; (b) $6.70 \times 10^{-6} \text{ M}$; (c) $9.00 \times 10^{-6} \text{ M}$; (d) $1.90 \times 10^{-5} \text{ M}$.

Table 17. Reduction of cyt c^{III} by complex II in 0.20 M phosphate buffer, pH 7.0, $\mu = 0.40$, at 20.0° C and 25.0° C, [cyt c^{III}]₀ = 9.10-9.90 x 10⁻⁶ M. [II]₀: (1) 6.70 x 10⁻⁶ M; (2) 9.00 x 10⁻⁶ M; (3) 1.34 x 10⁻⁵ M; (4) 1.90 x 10⁻⁵ M; (5) 6.70 x 10⁻⁶ M; (6) 1.34 x 10⁻⁵ M; (7) 3.00 x 10⁻⁶ M; (8) 6.70 x 10⁻⁶ M; (9) 8.00 x 10⁻⁶ M; (10) 9.00 x 10⁻⁶ M; (11) 1.90 x 10⁻⁵ M. $\mu = 1.35$ with NaCl.

Absorbance (550 nm)					Absorbance (550 nm)				
t(msec)	1	2	3	4	t(msec)	5 ^a	6 ^a		
20.0° C									
0	.085	.085	.085	.085	0	.097	.097		
4	.178	.183	.220	.220	5	-	.177		
5	-	-	-	.228	10	.167	.215		
6	.193	.198	.237	.233	15	-	.233		
8	.205	.211	.250	.245	20	.195	.245		
10	.214	.222	.256	-	25	-	.253		
14	-	.233	-	-	30	.210	-		
<hr/>									
7		8		9		10		11	
t(msec)	A ₅₅₀	t(msec)	A ₅₅₀	t(msec)	A ₅₅₀	t(msec)	A ₅₅₀	t(msec)	A ₅₅₀
0	.085	0	.090	0	.085	0	.085	0	.085
20	.182	4	.195	4	.200	4	.183	4	.220
40	.193	6	.210	6	.212	6	.200	5	.228
60	.196	8	.225	8	.223	8	.212	6	.233
80	.199	10	.233	10	.233	10	.220	8	.242
100	.201	12	.240	12	.238	14	.230		
120	.204			14	.242	20	.239		
140	.206								

Table 18. Effect of temperature on the reduction of cyt c^{III} by complex II in 0.20 M phosphate buffer, pH 7.0, $\mu = 0.40$, $[\text{cyt c}^{\text{III}}]_0 = 0.970\text{--}1.02 \times 10^{-5} \text{ M}$, $[\text{II}]_0$: (1) $6.70 \times 10^{-6} \text{ M}$; (2) $1.34 \times 10^{-5} \text{ M}$.

t (msec)	Absorbance (550 nm)					
	11.0° C		15.0° C		30.0° C	
	1	2	1	2	1	2
0	.120	.120	.095	.095	.093	.093
4	-	.220	-	.208	.185	.234
5	.190	-	.175	-	-	.240
6	-	.237	-	.223	.200	.248
8	-	.249	.196	.238	.212	-
10	.217	.258	.207	2.48	.223	-
14	-	.264	-	-	.232	-
15	.232	-	.222	-	-	-
20	.242	-	.235	-	.243	-
25	.250	-	.243	-	.243	-
30	.255	-	-	-	-	-

under these conditions the half-life of the reaction is between 1.6 and 8.4 msec. Since the dead time of the stopped flow instrument is 3 to 4 msec, the rate constants had to be evaluated from the last 30 to 50% of the reaction. The linearity of the second order plots (Figure 9) and the agreement of the rate constants for varying concentrations of II indicate that the results are reasonable for such rapid reactions.

Figure 9. Second order plots for the reduction of cyt c^{III} by complex II. 0.20 M phosphate buffer, pH 7.0, $\mu = 0.40$, 20.0°C , $[\text{cyt } c^{III}] = 9.80 \times 10^{-6} \text{ M}$. $[\text{II}] = 1.34 \times 10^{-5} \text{ M}$, $o\text{-}[\text{II}] = 6.70 \times 10^{-6} \text{ M}$. $C_r = [\text{cyt } c^{II}]$ and M_0 and C_0 are the initial concentrations of II and cyt c^{III} , respectively.

Table 19. Observed rate constants for the reduction of cyt c^{III} by complex II. 0.20 M phosphate buffer, pH 7.0, $\mu = 0.40$, $[\text{cyt c}^{\text{III}}]_0 = 1.00 \times 10^{-5} \text{ M}$. $*\mu = 1.35$ with NaCl.

$[\text{II}]_0$ ($\text{M} \times 10^5$)	T ($^{\circ}\text{C}$)	k_{obs} ($\text{M}^{-1} \text{ sec}^{-1} \times 10^{-7}$)
0.670	11.0	1.61
1.34	11.0	1.43
0.670	15.0	1.91
1.34	15.0	1.98
0.670	20.0	2.40
0.900	20.0	2.00
1.34	20.0	2.75
1.90	20.0	2.43
0.300	25.0	2.63
0.670	25.0	3.83
0.800	25.0	2.08
0.900	25.0	2.26
1.90	25.0	2.10
0.670	30.0	2.82
1.34	30.0	2.96
0.670*	20.0	0.59
1.34*	20.0	0.79

The activation parameters were calculated from a least squares program from the Eyring plot ($\ln(k_{\text{obs}}/T)$ vs. $1/T$), shown in Figure 10, of the data in Table 19. The values obtained are: $\Delta H^{\ddagger} = 4.8 (\pm 1.3)$ kcal/mol and $\Delta S^{\ddagger} = -8.6 (\pm 4.4)$ eu in 0.20 M phosphate buffer, pH 7.0, $\mu = 0.40$.

The rate decreased very significantly with an increase in ionic strength (the last two entries in Table 19), which is expected for two oppositely charged interacting species.

Figure 10. Eyring plot for the reduction of cyt c^{III} by complex II. Conditions are as described in Table 19. $\Delta H^{\ddagger} = 4.8$ (± 1.3) kcal/mol, $\Delta S^{\ddagger} = -8.6$ (± 4.4) eu.

Reduction of Ligand Bound Cyt c^{III} by Complex II

The reduction of N₃-cyt c^{III} by II is much slower than the reduction of native cyt c^{III} and the rate is independent of the concentration of II (Table 20). The observed first order rate constant for the reaction is 5.4(±0.2) sec⁻¹. The rate of reduction of CN-cyt c^{III} by II (Table 21) is extremely slow and also independent of the concentration of II, with an observed first order rate constant of 1.6 x 10⁻⁵ sec⁻¹. The observed rate constants for these reactions as well as the reduction of cyt c^{III} in the presence of N-acetylmethionine (NAM) (Table 22) are given in Table 23. NAM has no significant effect on the rate of reduction of cyt c^{III} by II and is assumed to not interact with the heme iron. It is possible that NAM does bind to the heme iron giving the same properties as the native protein; this does not seem likely, however, since the bulk of the NAM would be expected to produce significant conformational changes which would affect the kinetic and thermodynamic properties of the cyt c molecule.

The rate constants for reduction of the CN⁻ and N₃⁻ complexes of cyt c^{III} (Table 23) are nearly identical to the rate constants for dissociation of these ligands from the complex. This strongly suggests that II does not react with these complexes of cyt c^{III}, but does react very rapidly with the free cyt c^{III} produced by ligand dissociation. The observed rate constants for dissociation of N₃⁻ and CN-cyt c^{III} are 5.4 sec⁻¹ and 1.3 x 10⁻⁵ sec⁻¹, respectively (76), in very close agreement with the observed rate of reduction of these complexes by II.

Table 20. Reduction of $\text{N}_3\text{-cyt c}^{\text{III}}$ by complex II in 0.20 M phosphate buffer, $0.25 \text{ M } \text{Na}_3\text{NaN}_2$, pH 7.0, $\mu = 0.60$, 25.0°C , $[\text{cyt c}^{\text{III}}]_0$ (total) = $2.00 \times 10^{-5} \text{ M}$, $[\text{N}_3\text{-cyt c}^{\text{III}}]_0 = 1.1 \times 10^{-5} \text{ M}$. $[\text{II}]_0$: (1) $4.00 \times 10^{-5} \text{ M}$; (2) $8.00 \times 10^{-5} \text{ M}$; (3) $2.00 \times 10^{-4} \text{ M}$; (4) $1.00 \times 10^{-3} \text{ M}$.

t(msec)	1	2	3	4
50	.403	.405	.410	.450
100	.430	.440	.450	.490
150	.460	.462	.480	.510
200	.475	.483	.500	.527
250	.490	.500	.510	.543
300	.500	.508	.522	.555
400	.518	.522	.537	.573
500	.526	.535	.543	.589
A_f	.540	.550	.558	.593

Table 21. Reduction of $\text{CN-cyt c}^{\text{III}}$ by complex II in 0.20 M phosphate buffer, $1.1 \times 10^{-2} \text{ M KCN}$, pH 7.0, $\mu = 0.40$, 25.0°C , $[\text{cyt c}^{\text{III}}]_0$ (total) = $1.95 \times 10^{-5} \text{ M}$, $[\text{CN-cyt c}^{\text{III}}]_0 = 1.90 \times 10^{-5} \text{ M}$, $[\text{II}]_0 = 1.00 \times 10^{-3} \text{ M}$.

t(sec)	A_{550}	t(sec)	A_{550}
0	.230	6,250	.430
500	.246	7,500	.457
1000	.268	10,000	.493
1500	.288	12,500	.518
2000	.307	15,000	.530
2500	.324	17,500	.539
3750	.366	20,000	.543
5000	.402	A_f	.547

Table 22. Reduction of $\text{cyt } c^{\text{III}}$ by complex II in the presence of N-acetylmethionine. 0.10 M phosphate buffer, 0.20 M N-acetylmethionine, $[\text{cyt } c^{\text{III}}]_0 = 9.40 \times 10^{-6} \text{ M}$, (1) $[\text{II}]_0 = 9.00 \times 10^{-6} \text{ M}$, (2) $[\text{II}]_0 = 1.80 \times 10^{-5} \text{ M}$.

1		2	
t(msec)	A_{550}	t(msec)	A_{550}
0	.105	0	.105
8	.190	6	.225
10	.203	8	.234
15	.217	10	.243
20	.227	12	.250
30	.240		
40	.245		
50	.248		

0.05 M phosphate buffer, 0.30 M N-acetylmethionine, $[\text{cyt } c^{\text{III}}]_0 = 7.00 \times 10^{-6} \text{ M}$, (3) $[\text{II}]_0 = 9.00 \times 10^{-6} \text{ M}$, (4) $[\text{II}]_0 = 1.80 \times 10^{-5} \text{ M}$. All runs at pH 7.0, $\mu = 0.40$, 25.0° C .

3		4	
t(msec)	A_{550}	t(msec)	A_{550}
0	.055	0	.055
5	.130	5	.150
10	.149	8	.164
15	.163	10	.173
20	.170	14	.181
25	.177		
35	.182		

Reduction of the imidazole- $\text{cyt } c^{\text{III}}$ complex ($\text{Im-cyt } c^{\text{III}}$) is a much more complex reaction. At low concentrations of II (approximately equal molar with $\text{cyt } c^{\text{III}}$) the rate of reduction is nearly zero order in II with a rate very nearly that of dissociation of the $\text{Im-cyt } c^{\text{III}}$ complex ($k_{\text{obs}} \approx 1.9 \text{ sec}^{-1}$ for reduction of $\text{Im-cyt } c^{\text{III}}$ by II and $k_d = 2.4 \text{ sec}^{-1}$ for dissociation of the $\text{Im-cyt } c^{\text{III}}$ complex (76)).

Table 23. Observed rate constants for the reduction of cyt c^{III} by complex II in the presence of heme ligands. a. 0.20 M phosphate buffer, 0.25 M NaN₃, pH 7.0, $\mu = 0.65$, giving 50% ligand bound cyt c^{III}. The initial rapid reduction of free cyt c^{III} was taken into account in determining the rates. b. 0.20 M phosphate buffer, 0.11 M KCN, pH 7.0, $\mu = 0.41$, giving ca. 98% ligand bound cyt c^{III}. c. 0.10 M phosphate buffer, 0.20 M N-acetylmethionine, pH 7.0, $\mu = 0.40$. d. 0.05 M phosphate buffer, 0.30 M N-acetylmethionine, pH 7.0, $\mu = 0.40$.

Ligand	$[II]_0$ (M x 10 ⁴)	[cyt c ^{III}] ₀ (M x 10 ⁵)	k_{obs} (sec ⁻¹)
N ₃ ^{-a}	0.400 0.800 2.00 10.0	2.00 2.00 2.00 2.00	5.4 5.3 5.7 5.3
CN ^{-b}	5.00 10.0	2.00 2.00	(sec ⁻¹) ₅ 1.5 x 10 ⁻⁵ 1.6 x 10 ⁻⁵
N-acetyl- methionine	0.090 ^c 0.180 ^c 0.090 ^d 0.180 ^d	0.940 0.940 0.690 0.710	M ⁻¹ sec ⁻¹ 1.70 x 10 ⁷ 1.90 x 10 ⁷ 1.20 x 10 ⁷ 0.96 x 10 ⁷

At higher concentrations of complex II, however, the reaction becomes biphasic, with the amount of fast phase being dependent on the concentration of II. At very high concentrations of II (>100 fold excess), the reaction becomes monophasic again. First order plots of this single phase were not linear and suggested a second order reaction was in effect. Figure 11 shows the oscilloscope tracings obtained from the stopped flow spectrometer and the complex II concentration dependence on the reduction of Im-cyt c^{III}.

Figure 11. Oscilloscope tracings for the reduction of Im-cyt c^{III} by complex II. Experimental conditions were: 0.20 M phosphate-0.50 M imidazole buffer, pH 7.0, $\mu = 0.65$, 25.0° C, [cyt c^{III}]₀ (total) = 2.15×10^{-5} M, [Im-cyt c^{III}]₀ = 1.88×10^{-5} M. Complex II was diluted in the loading syringe with 0.20 M phosphate buffer, pH 7.0, $\mu = 0.65$ (adjusted with NaCl), [II]₀: (a) 2.50×10^{-5} M; (b) 5.00×10^{-5} M; (c) 1.00×10^{-4} M; (d) 2.50×10^{-4} M; (e) 5.00×10^{-4} M; (f) 1.00×10^{-3} M.

Since complex II was initially diluted in the loading syringe of the stopped flow instrument with pH 7.0 buffer containing imidazole (Table 24), it was suspected that imidazole may affect complex II in such a way as to cause the rapid reaction taking place. Complex II was therefore diluted with pH 7.0 buffer in the absence of imidazole (Table 25); however, the rates were identical with the rates observed in the presence of imidazole (within experimental error). Complex II was also diluted in the loading syringe with 0.04 M borate buffer, pH 9.8, which again resulted in very similar rates.

The dependence of the amount of fast phase on the concentration of II suggested Mo(V) monomer may be causing the fast phase. The amount of Mo(V) monomer in solutions of complex II was therefore estimated by esr spectroscopy, using $K_3Mo(CN)_8$ as a standard. The esr spectra were recorded at liquid N_2 temperature at pH 7.0 and 10.0 and a double integration procedure was used to estimate the amount of Mo(V) monomer in a 1.00×10^{-3} M solution of complex II in either the presence or absence of imidazole. The concentration of esr active monomer in the presence of imidazole was found to be approximately 2×10^{-5} M and the concentration of Mo(V) monomer in the absence of imidazole was approximately 5×10^{-6} M. These results suggest that imidazole has an effect on the concentration of monomer in solutions of complex II. The estimated equilibrium constants for the equilibrium between II and Mo(V) monomer are $K = 4 \times 10^{-7}$ M in the presence of 0.50 M imidazole and $K = 2 \times 10^{-8}$ M in the absence of imidazole, with the pH having little effect between pH 7.0 and 10.0.

Table 24. Reduction of Im-cyt c^{III} by complex II with both cyt c^{III} and complex II in 0.20 M phosphate 0.50 M imidazole buffer, pH 7.0, $\mu = 0.65$ prior to mixing. For runs 1-3, T = 25.0° C, [cyt c^{III}]₀ (total) = 2.12-2.18 x 10⁻⁵ M, [Im-cyt c^{III}]₀ = 1.85-1.90 x 10⁻⁵ M, [II]₀: (1) 5.00 x 10⁻⁵ M; (2) 2.50 x 10⁻⁴ M; (3) 1.00 x 10⁻³ M. For runs 4-6, T = 20.0° C, [cyt c^{III}]₀ (total) = 1.96-2.02 x 10⁻⁵ M, [Im-cyt c^{III}]₀ = 1.73-1.78 x 10⁻⁵ M, [II]₀ (4) 1.00 x 10⁻⁴ M; (5) 2.00 x 10⁻⁴ M; (6) 5.00 x 10⁻⁴ M.

1		2		3		4		5		6	
t(msec)	A ₅₅₀	t(msec)	A ₅₅₀	t(msec)	A ₅₅₀	t(msec)	A ₅₅₀	t(msec)	A ₅₅₀	t(msec)	A ₅₅₀
10	.265	7	.338	6	.420	5	.270	5	.303	5	.390
35	.302	10	.360	8	.453	10	.295	10	.332	10	.440
50	.330	20	.410	10	.485	15	.305	15	.355	15	.480
100	.363	30	.442	12	.513	20	.317	20	.370	20	.503
200	.410	40	.460	14	.530	25	.327	25	.385	26	.520
300	.443	50	.475	16	.545	50	.360	50	.415	35	.540
400	.478	80	.500	18	.560	100	.390	100	.440	50	.545
500	.500	100	.510			200	.423	150	.460	100	.555
600	.515	150	.533			300	.450	200	.475	150	.562
700	.525	200	.553			400	.473	250	.487	250	.575
800	.537	250	.570			500	.490	300	.501		
						600	.503	350	.510		
						700	.512	450	.523		
						900	.523				
A _f	.585	A _f	.590	A _f	.590	A _f	.548	A _f	.548	A _f	.585

Table 25. Reduction of Im-cyt c^{III} by complex II with cyt c^{III} in 0.20 M phosphate-0.50 M imidazole buffer, pH 7.0, $\mu = 0.65$ and II in 0.20 M phosphate buffer, pH 7.0, $\mu = 0.65$ (adjusted with NaCl), $T = 25.0^\circ \text{C}$, $[\text{cyt c}^{\text{III}}]_0 (\text{total}) = 2.10\text{--}2.15 \times 10^{-5} \text{ M}$, $[\text{Im-cyt c}^{\text{III}}]_0 = 1.84\text{--}1.89 \times 10^{-5} \text{ M}$. $[\text{II}]_0$: (1) $2.50 \times 10^{-5} \text{ M}$; (2) $5.00 \times 10^{-5} \text{ M}$; (3) $1.00 \times 10^{-4} \text{ M}$; (4) $2.50 \times 10^{-4} \text{ M}$; (5) $5.00 \times 10^{-4} \text{ M}$; (6) $1.00 \times 10^{-3} \text{ M}$.

1		2		3		4		5		6	
t(msec)	A ₅₅₀	t(msec)	A ₅₅₀	t(msec)	A ₅₅₀	t(msec)	A ₅₅₀	t(msec)	A ₅₅₀	t(msec)	A ₅₅₀
5	.245	5	.245	10	.278	10	.360	7	.425	6	.445
25	.282	25	.285	50	.365	20	.410	10	.455	8	.483
50	.300	50	.312	100	.405	30	.433	20	.520	10	.513
100	.320	100	.350	200	.465	50	.456	30	.545	12	.530
200	.360	200	.400	300	.500	100	.495	40	.560	14	.540
400	.440	300	.440	400	.523	200	.533	50	.566	16	.550
600	.485	400	.470	500	.545	300	.556	80	.580		
800	.515	500	.492	600	.555	400	.563				
1000	.538	600	.510	700	.562						
1200	.550	700	.525								
1400	.560	800	.535								
A _f	.587	A _f	.590	A _f	.580	A _f	.590	A _f	.590	A _f	.590

The reduction of Im-cyt c^{III} by Mo(V) monomer, at concentrations of complex II which were large enough to produce sufficient monomer to completely reduce the Im-cyt c^{III} complex, was plotted as a second order reaction assuming a value of $K_{eq} = 4.0 \times 10^{-7}$ M for the equilibrium between complex II and Mo(V) monomer:

A second order plot of the data in Table 26 was quite linear at 2.00×10^{-3} M complex II, as shown in Figure 12, and gave a second order rate constant of ca. $1 \times 10^7 \text{ M}^{-1} \text{ sec}^{-1}$.

A first order plot of the slow phase is linear for the concentrations where the slow phase could be studied (as shown in Figure 13). The observed first order rate constants showed little dependence on the concentration of II at low concentrations ($< 5 \times 10^{-5}$ M), but showed a much more significant dependence at higher concentrations of II (Table 28). Such results suggest complex II, as well as Mo(V) monomer, is reacting with Im-cyt c^{III} . A second order rate constant was estimated for the reduction of Im-cyt c^{III} by II from the following relationship:

$$k_{II} = \frac{k_{obs} - k_d}{[II]}$$

where k_{II} is the second order rate constant for reduction of Im-cyt c^{III} by II, k_{obs} is the observed first order rate constant and k_d is the dissociation rate constant for Im-cyt c^{III} , for which a value of 1.6 sec^{-1} was estimated at the lowest concentrations of II (this

Table 26. Reduction of Im-cyt c^{III} at high concentrations of complex II. $[cyt\ c^{III}]_0 = 1.90 \times 10^{-5}$ M in 0.20 M phosphate buffer, 0.50 M imidazole, pH 7.0, $\mu = 0.65$. Complex II was in 0.02 M borate buffer, pH 9.8, prior to mixing. $[II]_0$: (1) 1.00×10^{-3} M; (2) 2.00×10^{-3} M.

1		2	
t(msec)	A_{550}	t(msec)	A_{550}
0	.240	0	.335
5	.475	4	.595
10	.515	6	.617
15	.530	8	.630
20	.539	10	.642
25	.543	14	.655
35	.550		
A_f	.580	A_f	.675

relationship is only valid for pseudo first order conditions where $[II] \gg [Im-cyt\ c^{III}]$. The estimated second order rate constant for the reduction of Im-cyt c^{III} by II is $1.4 \times 10^4\ M^{-1}\ sec^{-1}$ (see Table 28).

The reduction of Im-cyt c^{III} by complex II was followed at pH 8.1 (Table 27). At this pH, 95% of the imidazole is in the free base form (proton dissociated) and ca. 94% of the $cyt\ c^{III}$ is ligand bound (as opposed to ca. 88% ligand bound at pH 7.0 and the same concentration of imidazole). The results were similar; the observed rate constants for the slow phase are given in Table 28, with the values for k_{II} (the calculated second order rate constant for the reduction of Im-cyt c^{III} by II). It appears that the rate of dissociation of the Im-cyt c^{III} complex is somewhat slower at this pH, however, and a value of $k_d = 1.2\ sec^{-1}$ is estimated.

Figure 12. Second order plot for the reduction of Im-cyt c^{III} by Mo(V) monomer. 0.20 M phosphate buffer, 0.50 M imidazole, pH 7.0, $\mu = 0.65$, $[\text{cyt } c^{\text{III}}]_0 = 1.90 \times 10^{-5} \text{ M}$, using a value of $2.82 \times 10^{-5} \text{ M}$ Mo(V) monomer in a $2.00 \times 10^{-3} \text{ M}$ solution of II. Complex II was in 0.02 M borate buffer, pH 9.8, prior to mixing. $T = 20.0^\circ \text{ C}$. M_0 and C_0 are the initial concentrations of Mo(V) and cyt c^{III} , respectively and $C_r = [\text{cyt } c^{\text{II}}]$.

Figure 13. First order plots for the reduction of Im-cyt c^{III} by complex II. 0.20 M phosphate buffer, 0.50 M imidazole, pH 7.0, $\mu = 0.65$, 20.0° C, [cyt c^{III}]₀ (total) = 1.98×10^{-5} M, with 88% imidazole bound. $-[II]_0 = 2.50 \times 10^{-5}$ M, $\Delta-[II]_0 = 5.00 \times 10^{-5}$ M, $o-[II]_0 = 1.00 \times 10^{-4}$ M.

Table 27. Reduction of Im-cyt c^{III} by complex II in 0.20 M phosphate-0.50 M imidazole buffer, pH 8.1.
 $\mu = 0.60$, 20.0°C , $[\text{cyt c}^{\text{III}}]_0 (\text{total}) = 1.80\text{--}1.90 \times 10^{-5} \text{ M}$, $[\text{Im-cyt c}^{\text{III}}]_0 = 1.69\text{--}1.79 \times 10^{-5} \text{ M}$. $[\text{II}]_0$: (1) $2.00 \times 10^{-5} \text{ M}$; (2) $5.00 \times 10^{-5} \text{ M}$; (3) $1.00 \times 10^{-4} \text{ M}$; (4) $2.00 \times 10^{-4} \text{ M}$; (5) $5.00 \times 10^{-4} \text{ M}$; (6) $1.00 \times 10^{-3} \text{ M}$.

1		2		3		4		5		6	
t(msec)	A ₅₅₀	t(msec)	A ₅₅₀	t(msec)	A ₅₅₀	t(msec)	A ₅₅₀	t(msec)	A ₅₅₀	t(msec)	A ₅₅₀
0	.160	0	.160	0	.160	0	.170	0	.205	0	.280
100	.244	10	.217	5	.235	5	.268	5	.367	5	.478
200	.280	20	.235	15	.272	10	.302	10	.423	6	.496
400	.325	40	.260	25	.298	15	.325	15	.463	8	.523
600	.363	60	.280	35	.317	20	.345	20	.495	10	.545
800	.397	80	.295	45	.325	26	.362	25	.513	12	.563
1000	.416	100	.302	100	.368	35	.383	30	.523	14	.581
1200	.430	200	.342	200	.415	45	.400	35	.530	16	.598
1400	.440	400	.400	300	.440	100	.443			18	.604
1800	.458	600	.433	400	.460	150	.463				
		800	.455	500	.477	200	.478				
		1000	.472	600	.484	250	.488				
		1400	.483	800	.497	350	.500				
A _f	.500	A _f	.500	A _f	.508	A _f	.517	A _f	.547	A _f	.623

Table 28. Rate constants for the slow phase of reduction of Im-cyt c^{III} by complex II in the presence of imidazole. 0.20 M phosphate buffer, 0.50 M imidazole. a. k_{II} is the second order rate constant for the reduction of the Im-cyt c^{III} complex by II which was calculated as described in the text. b. Conditions are the same as described in Table 23. c. Conditions are the same as described in Table 24. d. Same conditions as c except $[cyt\ c^{III}]_0 = 2.00 \times 10^{-5}$ M, 20.0° C. e. Conditions are the same as described in the legend for Table 26

pH	$[II]_0$ (M x 10^4)	k_{obs} (sec $^{-1}$)	k_{II}^a (M $^{-1}$ sec $^{-1}$ x 10^{-4})
7.0 ^b	0.500	2.2	1.6
	2.50	7.2	2.2
7.0 ^c	0.250	1.9	1.2
	0.500	2.0	0.80
	1.00	3.8	2.2
	2.50	5.6	1.6
	5.00	11.3	1.9
7.0 ^d	1.00	2.5	0.87
	2.00	4.2	1.3
	5.00	5.5	0.78
8.1 ^e	0.200	1.2	-
	0.500	2.2	1.8
	1.00	3.6	2.4
	2.00	6.2	2.5

The reduction of Im-cyt c^{III} by complex II was also studied at pH 10 (Table 29). The reaction is a single phase over a wide concentration range at this pH and showed a first order dependence on the concentration of II throughout the concentration range studied. The observed second order rate constants are shown in Table 30. The second order rate constant ($k = 6.2(\pm 1.1) \times 10^4$ M $^{-1}$ sec $^{-1}$) is very nearly the same as the rate constant observed for the reduction of the high pH

Table 29. Reduction of cyt c^{III} by complex II in the presence of imidazole at pH 10.0. 0.10 M borate buffer, pH 10.0, $\mu = 0.50$, 25.0° C, [cyt c^{III}]₀ = 0.950-1.05 x 10⁻⁵ M. For runs 1-5 [imidazole] = 0.50 M, and for runs 6-12 [imidazole] = 0.10 M. [II]₀: (1) 1.00 x 10⁻⁵ M; (2) 4.00 x 10⁻⁵ M; (3) 1.00 x 10⁻⁴ M; (4) 5.00 x 10⁻⁴ M; (5) 1.00 x 10⁻³ M; (6) 9.00 x 10⁻⁶ M; (7) 1.90 x 10⁻⁵ M; (8) 3.60 x 10⁻⁵ M; (9) 9.00 x 10⁻⁵ M; (10) 1.00 x 10⁻⁴ M; (11) 5.00 x 10⁻⁴ M; (12) 1.00 x 10⁻³ M.

1		2		3		4		5		6	
t(sec)	A ₅₅₀	t(sec)	A ₅₅₀	t(sec)	A ₅₅₀	t(sec)	A ₅₅₀	t(sec)	A ₅₅₀	t(sec)	A ₅₅₀
0	.065	0	.080	0	.083	0	.145	0	.195	0	.073
.05	.078	.10	.128	.05	.112	.005	.160	.005	.237	.2	.119
.10	.088	.20	.158	.10	.140	.010	.185	.010	.270	.5	.147
.20	.110	.40	.200	.15	.165	.015	.207	.015	.300	1.0	.174
.50	.142	.60	.225	.20	.182	.020	.223	.020	.320	1.5	.192
1.0	.180	.80	.242	.30	.207	.030	.255	.025	.333	2.0	.206
1.5	.203	1.0	.255	.40	.221	.040	.278	.025	.333	2.0	.206
2.0	.220	1.2	.263	.50	.230	.050	.293	.030	.343	2.5	.215
2.5	.232	1.4	.270	.60	.237	.060	.302	.035	.349	3.5	.225
4.0	.250	1.8	.278	.70	.241			.040	.353		
A _f	.273	A _f	.287	A _f	.250	A _f	.315	A _f	.365	A _f	.250

7		8		9		10		11		12	
t(sec)	A ₅₅₀	t(sec)	A ₅₅₀	t(sec)	A ₅₅₀	t(sec)	A ₅₅₀	t(sec)	A ₅₅₀	t(sec)	A ₅₅₀
0	.073	0	.073	0	.075	0	.090	0	.125	0	.145
.1	.109	.1	.128	.05	.141	.01	.120	.005	.185	.005	.257
.2	.123	.2	.150	.10	.170	.03	.152	.010	.208	.008	.273
.4	.151	.4	.172	.20	.201	.05	.175	.015	.224	.010	.285
.6	.169	.6	.203	.30	.220	.08	.200	.020	.240	.015	.315
.8	.183	.8	.216	.40	.231	.10	.210	.030	.263	.020	.327
1.0	.195	1.0	.227	.50	.239	.15	.232	.050	.283	.025	.342
1.4	.210	1.4	.233	.60	.242	.20	.245	.050	.300		
1.8	.220	1.8	.241			.30	.263	.060	.315		
						.40	.273	.070	.320		
A _f	.245	A _f	.248	A _f	.250	A _f	.293	A _f	.333	A _f	.357

form of cyt c^{III} by II ($k = 7.2(\pm 4) \times 10^4 \text{ M}^{-1} \text{ sec}^{-1}$) (134). This suggests that imidazole is not bound to cyt c^{III} at this pH.

Table 30. Observed rate constants for the reduction of cyt c^{III} by complex II in the presence of imidazole at pH 10. 0.10 M borate buffer, pH 10.0, $\mu = 0.50$, 25.0° C , $[\text{cyt } c^{III}]_0 = 0.950\text{--}1.05 \times 10^{-5} \text{ M}$. a. 0.50 M imidazole; b. 0.10 M imidazole, $k_{av} = 6.2 \pm 1.1 \times 10^4 \text{ M}^{-1} \text{ sec}^{-1}$.

$[\text{II}]_0$ ($\text{M} \times 10^4$)	k_{obs} ($\text{M}^{-1} \text{ sec}^{-1} \times 10^{-4}$)	k_{obs} (sec^{-1})	$k_{\text{obs}}/[\text{II}]_0$ ($\text{M}^{-1} \text{ sec}^{-1} \times 10^{-4}$)
<u>a</u>			
0.100	8.3	-	-
0.400	4.6	-	-
1.00	-	4.7	4.7
5.00	-	36.0	7.2
10.0	-	69.2	6.9
<u>b</u>			
0.090	7.0	-	-
0.190	5.8	-	-
0.360	5.4	-	-
0.900	5.5	-	-
1.00	-	5.4	5.4
5.00	-	30.4	6.1
10.0	-	70.2	7.0

Potentiometric Titration

Since complex II reduces Im-cyt c^{III} but not N_3^- or CN-cyt c^{III} , it was necessary to determine whether this observation was due to the thermodynamics of the reaction between II and these ligand bound cyt c^{III} species or whether the inability of II to reduce N_3^- and CN-cyt

c^{III} is a kinetic effect. The redox potentials of complex II and ligand bound cyt c^{III} were therefore determined.

The experimental potentiometric titration curve for the oxidative titration of complex II by thionine is shown in Figure 14. According to the Nernst equation:

$$E = E_o + \frac{.059}{n} \log \frac{[ox]}{[red]}$$

where [ox] = concentration of oxidized species of a redox couple and [red] = concentration of reduced species of that couple. A plot of E vs $\log ([ox]/[red])$ should produce a straight line with a slope of $.059/n$ for a reversible process. The plot of E vs $\log([ox]/[red])$ is shown in Figure 15 and it is obvious that the oxidation of II by thionine is not a reversible process. The midpoint potentials obtained from the oxidative titration curves for complex II with various oxidants are given in Table 31. The mean value obtained for the midpoint potential of complex II is -153 ± 16 mV.

The potentiometric titration of native cyt c^{III} in phosphate buffer, with ascorbate as reductant, gave a midpoint potential at pH 7.0 of +251 mV, in excellent agreement with the published value of $E_o' = +254$ mV (135). Back titration of the cyt c^{II} -ascorbate solution with $K_3Fe(CN)_6$ as oxidant gave a midpoint potential of +235 mV.

The potentiometric titration of cyt c^{III} in the presence of 0.50 M imidazole or 0.50 M NaN_3 at pH 7.0 was accomplished using electrolytically reduced phthiocol as reductant and $K_3Fe(CN)_6$ as oxidant; the experimental titration curves are shown in Figures 16 and 17 for

Figure 14. The experimental potentiometric titration curve for the oxidative titration of complex II by thionine. E vs silver-silver chloride electrode.

Figure 15. Potentiometric titration curve for the oxidative titration of complex II by thionine. E vs NHE.

Table 31. Midpoint potentials for the oxidative titration of complex II. $E_{m8.0}$ is the midpoint potential at pH 8.0 vs the standard hydrogen electrode.

Oxidant	$E_{m8.0}$ (mV)
Methylene Blue	-135
Pyocyanine	-170
Thionine	-154
$K_3Fe(CN)_6$	-138
Average	-135 ± 16

imidazole and azide, respectively. The midpoint potential for the reductive titration of Im-cyt c^{III} is $+151 \pm 8$ mV and of N_3 -cyt c^{III} is 183 ± 5 mV. The midpoint potentials for the oxidative titration of both species were about 10 mV more negative. The plot of E vs $\log([ox]/[red])$ for Im-cyt c is shown in Figure 18 and for N_3 -cyt c is shown in Figure 19. The nonlinearity of these plots and the large deviation of the slopes from the theoretical value of 0.059 (for a one electron process) indicate these two redox processes are irreversible, which is not unexpected, since neither imidazole nor azide bind to cyt c^{II} .

The thermodynamic midpoint potentials were calculated for the Im- and N_3 -cyt c couples using equilibrium constants for ligand binding. Using the Nernst equation and the following equations:

$$[cyt\ c^{III}] \text{ (total)} = [cyt\ c^{III}] + [X-cyt\ c^{III}]$$

and

$$k_{eq} = \frac{[X-cyt\ c^{III}]}{[cyt\ c^{III}][X]}$$

Figure 16. The experimental potentiometric titration curve for the oxidative and reductive titrations of Im-cyt c. (a) Reductive titration with phthiocol as reductant. (b) Oxidative titration with $K_3Fe(CN)_6$ as oxidant. E vs silver-silver chloride electrode.

Figure 17. The experimental potentiometric titration curves for the oxidative and reductive titrations of N_3 -cyt c. (a) Reductive titration with phthiocol as reductant. (b) Oxidative titration with $K_3Fe(CN)_6$ as oxidant. E vs silver-silver chloride electrode.

Figure 18. Potentiometric titration curve for the oxidative and reductive titration of Im-cyt c. Data taken from Figure 16. $\blacksquare$ -Reductive titration, $\circ$ -oxidative titration. E vs NHE.

Figure 19. Potentiometric titration curves for the oxidative and reductive titration of N_3 -cyt c. Data taken from Figure 17. -reductive titration, o-oxidative titration. E vs NHE.

where X is ligand and X-cyt c^{III} is ligand bound cyt c^{III}, one obtains the relationship

$$E = E^0 - .059 \log(1 + K_{eq}[X])$$

at the midpoint (E^0 is the standard midpoint potential of native cyt c). The thermodynamic midpoint potentials of Im-cyt c and N₃-cyt c, calculated from this expression are +198 mV and +224 mV, respectively.

The large differences in the observed and calculated values for the midpoint potentials, as well as the indication of nonreversibility in the potentiometric titration curve suggests that ligand binding causes significant alterations in the cytochrome c molecule, which will be discussed later.

Reduction of PLP Modified Cyt c^{III}

The reduction of both PLP₁-cyt c^{III} and PLP₂-cyt c^{III} by complex I are similar to the reduction of native cyt c^{III} by that complex. The data are shown in Tables 32 and 33. The rate is zero order in I and first order in cyt c^{III}. The first order plots ($\ln(A_f - A)$ vs t) deviate slightly beyond one half-life, similar to the results with native cyt c^{III} as shown in Figure 20. Table 34 gives the observed first order rate constants for the reaction. The mean value for k_{obs} for the reduction of PLP₁-cyt c^{III} is $7.07 (\pm 1.20) \times 10^{-4} \text{ sec}^{-1}$ and for the reduction of PLP₂-cyt c^{III} is $10.0 (\pm 2.6) \times 10^{-4} \text{ sec}^{-1}$. The observed rate constant for reduction of native cyt c^{III} under similar conditions is $7.3 \times 10^{-4} \text{ sec}^{-1}$. It is obvious the introduction of PLP groups has little effect on the rate of reduction by complex I, and furthermore, has no apparent effect on the mechanism.

Table 32. Reduction of PLP₂-cyt c^{III} by complex I in 0.10 M phosphate buffer, pH 7.0, $\mu = 0.50$, $T = 20.0^\circ \text{C}$, $[\text{PLP}_2\text{-cyt c}^{\text{III}}]_0 = 5.76 \times 10^{-6} \text{ M}$. $[\text{I}]_0$: (1) $2.00 \times 10^{-5} \text{ M}$; (2) $8.00 \times 10^{-5} \text{ M}$; (3) $4.00 \times 10^{-4} \text{ M}$.

t(sec)	Absorbance (550 nm)		
	1	2	3
0	.053	.060	.088
50	.057	.065	.094
100	.061	.069	.099
200	.068	.076	.106
400	.081	.089	.120
600	.092	.101	.131
800	.102	.111	.140
1000	.100	.119	.147
1500	.127	.134	1.60
2000	.139	.145	.166
2500	.146	.151	-
3000	.150	.154	-
3500	.153	.156	-
A _f	.159	.161	.169

Table 33. Reduction of PLP₁-cyt c^{III} by complex I in 0.10 M phosphate buffer, pH 7.0, $\mu = 0.50$, $T = 20.0^\circ \text{C}$, $[\text{PLP}_1\text{-cyt c}^{\text{III}}]_0 = 1.40 \times 10^{-5} \text{ M}$. $[\text{I}]_0$: (1) $1.00 \times 10^{-5} \text{ M}$; (2) $2.00 \times 10^{-5} \text{ M}$; (4) $4.00 \times 10^{-4} \text{ M}$.

t(sec)	Absorbance (550 nm)			
	1	2	3	4
0	.123	.123	.123	.145
100	.142	.144	.139	.164
200	.156	.162	.152	.179
400	.184	.195	.178	.205
600	.209	.226	.202	.229
800	.232	.254	.224	.250
1000	.253	.278	.243	.269
1500	.295	.325	.285	.308
2000	.328	.355	.319	.337
2500	.349	.369	.343	.358
3000	.361	.375	.359	.372
3500	.370	.378	.370	.382
A _f	.388	.388	.389	.398

Figure 20. First order plots for the reduction of PLP modified cyt c^{III} by complex I. 0.10 M phosphate buffer, pH 7.0, $\mu = 0.50$, 20.0°C , $[\text{I}]_0 = 2.00 \times 10^{-5} \text{ M}$. $-\text{[PLP}_1\text{-cyt } c^{\text{III}}]_0 = 1.40 \times 10^{-5} \text{ M}$; $o\text{-[PLP}_2\text{-cyt } c^{\text{III}}]_0 = 5.76 \times 10^{-6} \text{ M}$.

Table 34. Rate constants for the reduction of PLP derivatives of cyt c^{III} by complex I. 0.10 M phosphate buffer, pH 7.0, $\mu = 0.50$ with NaCl, $[\text{PLP}_1\text{-cyt c}^{\text{III}}]_0 = 1.40 \times 10^{-5}$ M, $[\text{PLP}_2\text{-cyt c}^{\text{III}}]_0 = 5.80 \times 10^{-6}$ M, 20.0° C.

$[\text{I}]_0$ ($\text{M} \times 10^5$)	k_{obs} ($\text{sec}^{-1} \times 10^4$)	
	PLP ₁ -cyt c	PLP ₂ -cyt c
1.00	6.74	-
2.00	8.79	8.22
8.00	6.00	8.90
40.0	6.74	13.0

The reduction of PLP₁-cyt c^{III} and PLP₂-cyt c^{III} by complex II was also very similar to the reduction of native cyt c^{III} by that complex, the data are given in Tables 35 and 36. A representative second order plot is shown in Figure 21 and the observed second order rate constants for the reaction are given in Table 37. The observed rate constant for the reduction of PLP₁-cyt c^{III} by II is $1.12 (\pm 0.20) \times 10^7 \text{ M}^{-1} \text{ sec}^{-1}$ and for the reduction of PLP₂-cyt c^{III} by II is $1.04 (\pm 0.27) \times 10^7 \text{ M}^{-1} \text{ sec}^{-1}$. The observed rate constant for the reduction of native cyt c^{III} under similar conditions is $2.40 (\pm 0.31) \times 10^7 \text{ M}^{-1} \text{ sec}^{-1}$.

The introduction of pyridoxal phosphate in the ratio of either 1 or 2 PLP groups per cyt c^{III} molecule has only a small effect on the rate of the reaction with II. The rate with I is not affected to any appreciable extent and the rate with II is decreased by a factor of about 0.5.

Table 35. Reduction of PLP₂-cyt c^{III} by complex II in 0.10 M phosphate buffer, pH 7.0, $\mu = 0.50$ with NaCl, 20.0° C, [PLP₂-cyt c^{III}]₀ = 3.80×10^{-6} M. [II]₀: (1) 4.00×10^{-6} M; (2) 1.00×10^{-5} M; (3) 2.00×10^{-5} M.

1		2		3	
t(msec)	A ₅₅₀	t(msec)	A ₅₅₀	t(msec)	A ₅₅₀
0	.040	0	.040	0	.040
5	.058	5	.065	4	.085
10	.063	10	.078	6	.091
20	.073	15	.084	10	.099
30	.080	20	.088	12	.101
40	.085	25	.092		
50	.090				
A _f	.103	A _f	.100	A _f	.105

Table 36. Reduction of PLP₁-cyt c^{III} by complex II in 0.10 M phosphate buffer, pH 7.0, $\mu = 0.50$ with NaCl, 20.0° C, [PLP₁-cyt c^{III}]₀ = 9.04×10^{-6} M. [II]₀: (1) 3.00×10^{-6} M; (2) 1.00×10^{-5} M; (3) 2.00×10^{-5} M.

1		2		3	
t(msec)	A ₅₅₀	t(msec)	A ₅₅₀	t(msec)	A ₅₅₀
0	.087	0	.087	0	.087
5	.125	5	.162	4	.197
10	.138	10	.187	6	.210
15	.148	15	.202	8	.221
20	.156	20	.212	10	.229
25	.160	25	.220	12	.235
35	.165	35	.229	16	.243
A _f	.175	A _f	.247	A _f	.250

Figure 21. Second order plots for the reduction of PLP modified cyt cIII by complex II. 0.10 M phosphate buffer, pH 7.0, $\mu = 0.50$, 20.0° C, $[II]_0 = 1.00 \times 10^{-5}$ M. $\diamond$ $[PLP1\text{-}cyt\ cIII]_0 = 9.04 \times 10^{-6}$ M; $\circ$ $[PLP2\text{-}cyt\ cIII]_0 = 3.80 \times 10^{-6}$ M. $C_r = [cyt\ cII]$ and M_0 and C_0 are the initial concentrations of II and PLP-cyt cIII, respectively.

Table 37. Rate constants for the reduction of PLP derivatives of cyt c^{III} by complex II. 0.10 M phosphate buffer, pH 7.0, $\mu = 0.50$ with NaCl, 20.0° C, $[\text{PLP}_1\text{-cyt c}^{\text{III}}]_0 = 9.00 \times 10^{-6}$ M, $[\text{PLP}_2\text{-cyt c}^{\text{III}}]_0 = 3.80 \times 10^{-6}$ M.

$[\text{II}]_0$ (M x 10 ⁶)	k_{obs} (M ⁻¹ sec ⁻¹ x 10 ⁻⁶)	
	PLP -cyt c	PLP ₂ -cyt c
3.00	13.1	-
4.00	-	12.2
10.0	9.12	7.22
20.0	11.4	11.7

Reduction of Nitrotyrosyl Derivatives of Cyt c^{III}

The reduction of the mononitromonotyrosyl derivative of cyt c^{III} (MNMT-cyt c^{III}) by complex I is very similar to the reduction of the native cyt c^{III}. The data are shown in Table 38 and a representative first order plot is shown in Figure 22. The observed first order rate constants for the reaction are given in Table 39 and the value for k_{obs} is $2.75 (\pm 0.12) \times 10^{-3} \text{ sec}^{-1}$ compared with $0.73 \times 10^{-3} \text{ sec}^{-1}$ for native cyt c^{III} under similar conditions. This is about a 3.5 fold increase in rate as a result of introducing the nitro group on one of the tyrosyl residues. The reaction does, however, maintain a zero order dependence on the reductant.

The reduction of MNMT-cyt c^{III} by complex II is similar to the reduction of native cyt c^{III} by II. The data are given in Table 40. A representative psuedo-first order plot for the reaction is shown in Figure 23 and rate constants are shown in Table 41. The observed rate

Table 38. Reduction of mononitromonotyrosyl (MNMT)-cyt c^{III} by complex I in 0.20 M phosphate buffer, pH 7.0, $\mu = 0.50$, at 20.0° C, $[MNMT\text{-}cyt\ c^{III}]_0 = 4.30 \times 10^{-6}$ M. $[I]_0$: (1) 1.60×10^{-5} M; (2) 7.80×10^{-5} M; (3) 1.50×10^{-4} M.

t(sec)	Absorbance (550 nm)		
	1	2	3
0	.058	.055	.051
50	—	.059	.056
100	.067	.062	.059
200	.074	.069	.067
300	.078	.075	.074
400	.081	.079	.079
500	—	.082	.082
600	.089	—	—
700	—	.084	.086
1000	.091	.086	.087
A_f	.098	.096	.097

Table 39. Observed rate constants for the reduction of MNMT-cyt c^{III} by complex I. 0.20 M phosphate buffer, pH 7.0, $\mu = 0.50$ with NaCl, 20.0° C, $[MNMT\text{-}cyt\ c^{III}]_0 = 4.3 \times 10^{-6}$ M. Rate constants are obtained assuming 90% purity (see text).

$[I]_0$ (M $\times 10^5$)	k_{obs} (sec $^{-1}$ $\times 10^3$)
1.60	2.8
7.80	2.6
15.0	2.8

Figure 22. First order plot for the reduction of MNMT-cyt c^{III} by complex I. 0.20 M phosphate buffer, pH 7.0, $\mu = 0.50$, 20.0°C , $[\text{MNMT-cyt } c^{\text{III}}]_0 = 4.30 \times 10^{-6} \text{ M}$, $[\text{I}]_0 = 1.50 \times 10^{-4} \text{ M}$.

Table 40. Reduction of mononitromonotyrosyl (MNMT)-cyt c^{III} by complex II in 0.20 M phosphate buffer, pH 7.0, $\mu = 0.50$ with NaCl, 20.0° C, [MNMT-cyt c^{III}]₀ = 2.20×10^{-6} M. Absorbance changes measured at 416 nm. [II]₀: (1) 1.00×10^{-5} M; (2) 2.00×10^{-5} M.

1		2	
t(msec)	A ₄₁₆	t(msec)	A ₄₁₆
0	.160	0	.160
5	.184	4	.198
10	.198	6	.203
15	.204	8	.208
20	.209	10	.210
A _f	.214	A _f	.214

Table 41. Observed rate constants for the reduction of MNMT-cyt c^{III} by complex II. 0.20 M phosphate buffer, pH 7.0, $\mu = 0.50$ with NaCl, 20.0° C, [MNMT-cyt c^{III}]₀ = 2.2×10^{-6} M. Rate constants were obtained assuming 90% purity (see text).

[II] ₀ (M x 10 ⁵)	k _{obs} (sec ⁻¹ x 10 ⁻⁷)
1.00	1.2
2.00	1.3

Figure 23. Pseudo first order plot for the reduction of MNMT-cyt c^{III} by complex II. 0.20 M phosphate buffer, pH 7.0, $\mu = 0.50$, 20.0°C , $[\text{MNMT-cyt } c^{\text{III}}]_0 = 2.20 \times 10^{-6} \text{ M}$, $[\text{II}]_0 = 2.00 \times 10^{-5} \text{ M}$.

constant is $1.23 (\pm 0.05) \times 10^7 \text{ M}^{-1} \text{ sec}^{-1}$ compared with $2.40 (\pm 0.31) \times 10^7 \text{ M}^{-1} \text{ sec}^{-1}$ for native cyt c^{III} under similar conditions. The same mechanism obtains for the reduction of MNMT-cyt c^{III} as for native cyt c^{III} . The introduction of the nitro group on one of the tyrosyl residues causes only a small decrease in the rate of reaction with II.

The more highly substituted nitro derivatives of cyt c^{III} could not be purified on ion exchange columns to give a kinetically homogeneous species. Fraction II which was eluted just ahead of MNMT-cyt c^{III} was rechromatographed once and two fractions isolated. The major component in both fractions reacted much more slowly with II than MNMT-cyt c^{III} . The observed rate constant for the reaction of this species is $1.42 (\pm 0.62) \times 10^4 \text{ M}^{-1} \text{ sec}^{-1}$. The major component in this fraction is presumed to be mononitrodityrosyl-cyt c^{III} (MNDT-cyt c^{III}) since it possesses the same spectral and chromatographic properties as the MNDT-cyt c described by Pal et al. (121). The major contaminant in both fractions reacted very rapidly with II and is presumed to be MNMT-cyt c^{III} .

The rate of reduction of this fraction with I is not significantly different from the rate of reduction of MNMT-cyt c^{III} . The observed first order rate constant is $2.3 (\pm 0.4) \times 10^{-3} \text{ sec}^{-1}$. Again this reaction is somewhat faster than the reduction of native cyt c^{III} by I.

Reduction of Formyl-Cyt c^{III} by Complex I

The reduction of formyl-cyt c^{III} by complex I is biphasic. The absorbance data are given in Table 42 and a typical first order plot is shown in Figure 24. The rate constant for the slow phase was determined

Table 42. Reduction of formyl-cyt c^{III} by complex I in 0.40 M phosphate buffer, pH 7.5, $\mu = 1.01$, $T = 20.0^\circ \text{C}$, [formyl-cyt c^{III}] = $2.40 \times 10^{-5} \text{ M}$. $[I]_0$: (1) $8.00 \times 10^{-5} \text{ M}$; (2) $5.00 \times 10^{-4} \text{ M}$.

t(sec)	Absorbance (550 nm)	
	1	2
0	.212	.228
100	.243	.260
200	.267	.287
300	.389	.311
400	.308	.332
500	.325	.351
600	.339	.367
800	.362	.393
1000	.378	.411
1500	.405	.442
2000	.421	.463
2500	.434	.480
3000	.448	.494
3500	.460	.505
4000	.471	.516
4500	.479	.524
5000	.486	.532
A_f	.560	.580

from the linear portion of the first order plot beyond the point of inflection (i.e. near the end of the reaction). The rate constant for the fast phase of the reaction was determined by the Guggenheim method, as described in the experimental section. The values of the rate constants for the fast and slow phases of the reaction are given in Table 43. The observed rate constant for the slow phase is $2.6 (\pm 0.6) \times 10^{-4} \text{ sec}^{-1}$ and for the fast phase, $1.5 (\pm 0.2) \times 10^{-3} \text{ sec}^{-1}$.

Figure 24. First order plot for the reduction of formyl-cyt c^{III} by complex I. Conditions are as described in Table 42 with $[I]_0 = 5.00 \times 10^{-4} \text{ M}$.

Table 43. Rate constants for the reduction of formyl-cyt c^{III} by complex I. 0.40 M phosphate buffer, pH 7.5, $\mu = 1.01$, 20.0° C, [formyl-cyt c^{III}]₀ = 2.40×10^{-5} .

$[I]_0$ (M x 10 ⁴)	k_{fast} (sec ⁻¹ x 10 ³)	k_{slow} (sec ⁻¹ x 10 ⁴)
0.80	1.59	1.37
5.00	1.38	1.08

Reduction of Formyl-Cyt c^{III} by Complex II

The reduction of formyl-cyt c^{III} by II is a very complex reaction which appears to be more than biphasic. The oscilloscope tracings from the stopped flow instrument, for the reduction of formyl-cyt c^{III} by complex II are shown in Figure 25 and the data tabulated in Table 44. Some comments can be made about the reaction, however, which are valuable to the understanding of the properties of this modified cytochrome.

The first characteristic of this reaction is a very fast phase, which is complete in the dead time of the stopped flow instrument at concentrations of II $> 5.0 \times 10^{-5}$ M. A second order rate constant was estimated for this fast phase at a concentration of II approximately equimolar with formyl-cyt c^{III}, using the half-life method. When both formyl-cyt c^{III} and complex II are about 1.9×10^{-5} M, the half-life of the fast phase is ca. 5 msec. Using the relationship

$$k = \frac{1}{(a_0 t_{1/2})}$$

where a_0 is the initial concentration of the fast component and $t_{1/2}$ is the half-life, a value for the second order rate constant is estimated

Figure 25. Oscilloscope tracings for the reduction of formyl-cyt c^{III} by complex II. Experimental conditions were: 0.40 M phosphate buffer, pH 7.5, $\mu = 1.01$, 20.0° C, [formyl-cyt c^{III}]₀ = 1.88×10^{-5} M. [II]₀: (a) 1.90×10^{-5} M; (b) 5.00×10^{-5} M; (c) 1.00×10^{-4} M; (d) 2.00×10^{-4} M; (e) 5.00×10^{-4} M; (f) 1.00×10^{-3} M.

Table 44. Reduction of formyl-cyt c^{III} by complex II on 0.40 M phosphate buffer, pH 7.5, $\mu = 1.01$, 20.0° C, [formyl-cyt c^{III}]₀ = 1.88×10^{-5} M. [II]₀: (1) 1.90×10^{-5} M; (3) 1.00×10^{-4} M; (4) 2.00×10^{-4} M; (5) 5.00×10^{-4} M; (6) 1.00×10^{-3} M.

1		2		3		4		5		6	
t(msec)	A ₅₅₀	t(msec)	A ₅₅₀	t(msec)	A ₅₅₀	t(msec)	A ₅₅₀	t(msec)	A ₅₅₀	t(msec)	A ₅₅₀
0	.150	0	.150	0	.155	0	.170	0	.215	0	.270
10	.220	10	.226	10	.250	5	.258	5	.310	5	.368
30	.232	30	.245	30	.267	10	.278	10	.340	10	.399
50	.239	50	.255	50	.280	30	.304	30	.378	20	.428
250	.255	250	.285	70	.288	50	.316	50	.395	25	.438
500	.272	500	.312	100	.292	100	.327	70	.400	50	.455
1000	.300	1000	.340	200	.305	200	.350	100	.406	100	.473
1500	.318	1500	.360	400	.335	300	.365	200	.427	150	.485
2000	.329	2000	.370	600	.350	400	.378	300	.440	200	.495
2500	.338	2500	.378	800	.362	500	.385	400	.448	300	.502
3500	.344			1000	.373	700	.398	500	.454		
				1200	.380	1000	.404	700	.462		
A _f	.365	A _f	.393	A _f	.405	A _f	.423	A _f	.473	A _f	.518

at $4 \times 10^6 \text{ M}^{-1} \text{ sec}^{-1}$. The similarity between this rate and the rate of reduction of native cyt c^{III} by complex II, under similar conditions, suggests there may be some residual native cyt c^{III} present in this fraction. There is, however, no other evidence for the presence of native cyt c^{III} in this fraction as judged by the lack of 695 nm absorbance and complete autoxidation in air. The maximum amount of native cyt c^{III} in this fraction would be ca. 5%, which is much too small to account for the approximately 30%, or more, fast reaction.

A first order plot of this reaction for pseudo-first order conditions is linear for the last 40-50% of the reaction, as shown in Figure 26. The second order rate constants for this phase, obtained by dividing the observed pseudo-first order rate constants by the concentration of II, are given in Table 45 and were reasonably consistent at the higher concentrations of II. The second order rate constant does, however, decrease by a factor of 2 over a 10 fold increase in concentration of II. The larger rate constants at the lower concentrations (Table 45) may be due to Mo(V) monomer produced by the fast phase of this reaction. The value of the second order rate constant for this slow phase is estimated, from the highest concentrations of II, to be $6.7 \times 10^3 \text{ M}^{-1} \text{ sec}^{-1}$.

Reduction of Cyt c^{III} by Mo(V)-Penicillamine Complexes

The reduction of cyt c^{III} by the dioxobridged Mo(V)-penicillamine complex (I-pen) was complicated by the presence of free penicillamine.

Figure 26. First order plots for the reduction of formyl-cyt c^{III} by complex II. Experimental conditions were as described in table 43. ■-[II]₀ = 1.00×10^{-4} M, ○-[II]₀ = 2.00×10^{-4} M, ▲-[II]₀ = 5.00×10^{-4} M, ●-[II]₀ = 1.00×10^{-3} M.

Table 45. Rate constants for the slow phase of reduction of formyl-cyt c^{III} by complex II. 0.40 M phosphate buffer, pH 7.5, $\mu = 1.01$, 20.0° C, [formyl-cyt c^{III}]₀ = 1.88×10^{-5} M. k_{obs} is the observed pseudo-first order rate constant and $k_{\text{obs}}/[\text{II}]$ is the second order rate constant.

$[\text{II}]_0$ ($\text{M} \times 10^4$)	k_{obs} (sec^{-1})	$\frac{k_{\text{obs}}}{[\text{II}]_0}$ ($\text{M}^{-1} \text{sec}^{-1} \times 10^{-3}$)
1.00	1.37	13.7
2.00	2.25	11.2
5.00	3.19	6.38
10.0	6.94	6.94

Penicillamine reduces cyt c^{III} much more rapidly than L-cysteine and in fact is even faster than the rate with I-pen at equivalent concentrations. The reduction of cyt c^{III} by penicillamine was therefore studied in order to better understand the reduction of cyt c^{III} by I-pen; absorbance data are given in Table 46. The kinetic data for the reaction of cyt c^{III} with penicillamine were not as reproducible as one would like. The reason probably being that penicillamine, like most commercially obtained thiols, is not pure to begin with and is also very unstable to air oxidation. The small amount of impurities may cause some very unpredictable results. In the most carefully controlled study of this reaction, at low concentrations of penicillamine, the observed second order rate constant for the reaction is ca. $5 \times 10^2 \text{ M}^{-1} \text{sec}^{-1}$.

Table 46. Reduction of $\text{cyt } c^{\text{III}}$ by D,L-penicillamine in 0.10 M phosphate buffer, pH 7.0, $\mu = 0.50$, $T = 20.0^\circ \text{C}$, $[\text{cyt } c^{\text{III}}]_0 = 1.91 \times 10^{-5} \text{ M}$; $[\text{penicillamine}]_0$: (1) $1.50 \times 10^{-5} \text{ M}$; (2) $2.00 \times 10^{-5} \text{ M}$; (3) $5.00 \times 10^{-5} \text{ M}$; (4) $1.00 \times 10^{-4} \text{ M}$.

t(sec)	Absorbance (550 nm)			
	1	2	3	4
0	.217	.217	.225	.208
10	.261	.261	.276	.279
20	.295	.295	.318	.332
30	.325	.325	.352	.375
40	.350	.350	.380	.409
50	.367	.372	.405	.435
60	.383	.391	.425	.457
80	.406	.421	.458	.485
100	.421	.444	.479	.502
140	.437	.474	.506	.518
180	.443	.491	.518	.522
A_f	.451	.523	.529	.529

I-pen reduced $\text{cyt } c^{\text{III}}$ at a rate comparable to the rate of reduction by complex I; absorbance data are given in Table 47. The rate of the reaction did, however, increase with increasing concentration of I-pen, the observed rate constants are given in Table 48. A close inspection of the kinetic behavior of this reaction showed that the data fit a first order plot very well, with an autocatalytic effect very similar to the reaction with I. The increase in rate with increasing concentration of I-pen is probably due to the presence of residual free ligand, since the dependence of the rate on concentration of reductant varied with the I-pen preparation. The autocatalytic

Table 47. Reduction of cyt c^{III} by the dioxobridged Mo(V)-penicillamine dimer (I-pen). 0.10 M phosphate buffer, pH 7.0, $\mu = 0.50$, 20.0°C [cyt c^{III}] $_0 = 4.05\text{--}4.15 \times 10^{-5} \text{ M}$. [I-pen] $_0$: (1) $3.00 \times 10^{-5} \text{ M}$; (2) $9.00 \times 10^{-5} \text{ M}$; (3) $1.50 \times 10^{-4} \text{ M}$; (4) 5.00×10^{-4} ; (5) $7.50 \times 10^{-4} \text{ M}$; (6) $1.00 \times 10^{-3} \text{ M}$; (7) $2.00 \times 10^{-3} \text{ M}$; (8) $4.00 \times 10^{-3} \text{ M}$.

t(sec)	Absorbance (550 nm)							
	1	2	3	4	5	6	7	8
0	.350	.360	.340	.425	.400	.497	.505	.538
50	.375	.393	.370	.494	.472	.600	.577	.620
100	.392	.420	.400	.557	.529	.687	.643	.690
200	.425	.472	.462	.665	.634	.824	.763	.815
300	-	-	-	-	-	.925	.860	.918
400	.488	.564	.569	.840	.803	1.003	.940	1.000
500	-	-	-	-	-	1.060	1.010	1.065
600	.545	.645	.660	.967	.923	1.102	1.065	1.120
800	.599	.718	.740	1.057	1.007	1.152	1.147	1.195
1000	.650	.785	.807	1.116	1.062	-	1.200	1.202
1200	-	-	-	1.153	-	-	-	-
1500	.769	.915	.941	1.180	1.123	-	1.255	-
2000	.866	1.004	1.027	-	-	-	-	-
2500	.944	1.055	1.072	-	-	-	-	-
3000	1.005	1.080	1.097	-	-	-	-	-
3500	1.047	1.095	-	-	-	-	-	-
A_f	1.125	1.115	1.120	1.195	1.148	1.195	1.270	1.290

Table 48. Observed rate constants for the reduction of cyt c^{III} by the dioxobridged Mo(V)-penicillamine complex (I-pen). 0.10 M phosphate buffer, pH 7.0, $\mu = 0.50$ with NaCl, 20.0° C. $[\text{cyt c}^{\text{III}}]_0 = 4.05\text{--}4.15 \times 10^{-5}$ M. All runs were made with the same recrystallized preparation of Mo(V)-penicillamine complex. $k_{\text{av}} = 1.6 (\pm 0.7) \times 10^{-3} \text{ sec}^{-1}$.

$\frac{[\text{I-pen}]_0}{(\text{M} \times 10^5)}$	$\frac{k_{\text{obs}}}{(\text{sec}^{-1} \times 10^3)}$
3.00	0.49
9.00	0.83
15.0	0.92
50.0	1.94
75.0	1.97
100	2.12
200	2.20
400	2.48

effect would be due to the production of penicillamine in the reaction.

Because it is difficult to estimate the amount of free penicillamine present in solutions of the I-pen complex, only a very rough estimate can be made of the rate constant for the reaction. The most reasonable estimate would come from the lowest concentrations of I-pen used, which gives a value of $k_{\text{obs}} = 4\text{--}10 \times 10^{-4} \text{ sec}^{-1}$. This value is approximately the same as the observed rate constant for complex I (ca. $7 \times 10^{-4} \text{ sec}^{-1}$).

The reduction of cyt c^{III} by the monoxobridged Mo(V)-penicillamine complex (II-pen) was not easy to study either. The bridge opening reaction was much slower than the bridge opening of I to form II. The bridge opening also did not go to completion at pH 10 in the presence

of either added penicillamine or added cysteine. L-cysteine was found to catalyze the ring opening more efficiently than penicillamine (123). As mentioned in the experimental section, less than 30% of the total molybdenum was reactive with cyt c^{III}. The absorbance data for the reduction of cyt c^{III} by II-pen are given in Table 49. If it is assumed that the active molybdenum is the monoxobridged dimer, an estimated rate constant for the reaction is $9 \times 10^7 \text{ M}^{-1} \text{ sec}^{-1}$. The rate is therefore 3 to 4 times faster than the rate of reduction by complex II. It may be, however, that the active molybdenum species is something other than the monoxobridged Mo(V)-penicillamine complex. These results should therefore be interpreted with much caution.

It should be pointed out that the purpose of the kinetic studies with the penicillamine complexes was to compare them with the analogous cysteine complexes. It is obvious that the penicillamine complexes will not lend themselves to as rigorous a kinetic investigation as the cysteine complexes; the results, however, do suggest that they behave similarly.

Table 49. Reduction of cyt c^{III} by the monoxobridged Mo(V)-penicillamine dimer (II-pen) in 0.20 M phosphate buffer, pH 7.0, $\mu = 0.40$, $T = 20.0^\circ \text{ C}$, $[\text{cyt c}^{\text{III}}]_0 = 1.95 \times 10^{-5} \text{ M}$.

$[\text{II-pen}]_0 = 6.0 \times 10^{-6} \text{ M}$		$[\text{II-pen}]_0 = 9.1 \times 10^{-6} \text{ M}$	
t(msec)	A ₅₅₀	t(msec)	A ₅₅₀
0	.185	0	.185
4	.358	5	.423
6	.370	10	.462
8	.380	15	.483
10	.388	20	.497
14	.400	25	.500

DISCUSSION

Reduction of Cyt c^{III} by Complex I

Complex I reduces cyt c^{III} slowly, with a rate which is independent of the concentration of I over a wide range in concentration. Several mechanisms can be proposed which would be in agreement with the kinetic data for the reduction of cyt c^{III} by complex I. The first mechanism involves the rapid formation of a preliminary complex between cyt c^{III} and I (reaction 3) and a subsequent rate controlling electron transfer (reaction 4) to give products:

where L is cysteine. The Mo(V) species produced in reaction (4) by the one electron oxidation of I by cyt c^{III} is proposed to be $\text{MoO}_2(\text{OH})\text{L}^{2-}$. This reactive monomer then rapidly reduces another cyt c^{III}-I complex (reaction 5). In the reduction of flavins by II, under similar conditions, Mo(V) monomer was observed (22). No such signal was observed in this study; therefore, reaction (5) would have to be much faster than (4). The Mo(VI) product is MoO_4^{2-} , since Mo(VI) does not complex with cysteine to any appreciable extent at pH >6.0 (18).

The rate expression obtained from this mechanism is:

$$\frac{d[\text{cyt } c^{\text{II}}]}{dt} = \frac{2k_4 K[\text{I}][\text{cyt } c^{\text{III}}]}{1 + K[\text{I}]}$$

where $K = k_3/k_{-3}$. If $K[\text{I}] \gg 1$, this expression simplifies to the experimental rate law:

$$\frac{d[\text{cyt } c^{\text{II}}]}{dt} = 2k_4[\text{cyt } c^{\text{III}}]$$

Since the rate is independent of the concentration of I at the lowest concentration used, the minimum value of K is ca. 10^6 . This indicates that the $\text{cyt } c^{\text{III}}\text{-I}$ complex would have to be quite strong.

It should be pointed out that no changes were observed in the absorption spectrum of $\text{cyt } c^{\text{III}}$ immediately after adding I, no change was observed in the polarographic reduction wave of I immediately after adding $\text{cyt } c^{\text{III}}$ and two chromatographic techniques used to detect binding of I to $\text{cyt } c^{\text{III}}$ were negative. So, although this mechanism is consistent with the kinetic data, the negative results in the binding studies leave it open to much criticism.

A second mechanism involves the rapid formation of a "dead end" complex (136) (reaction 6) which does not undergo electron transfer. I may also react with $\text{cyt } c^{\text{III}}$ to give products (reaction 7).

As in the first mechanism, the Mo(V) monomer produced in reaction (7) must be very reactive and would reduce either the $\text{cyt } c^{\text{III}}$ or $\text{cyt } c^{\text{III}}\text{-I}$ complex rapidly.

Assuming reaction (7) is rate limiting, the following expression obtains:

$$\frac{d[\text{cyt } c^{\text{II}}]}{dt} = k_7[\text{I}][\text{cyt } c^{\text{III}}]$$

Applying the steady state approximation to $\text{cyt } c^{\text{III}}$ gives the observed rate law:

$$\frac{d[\text{cyt } c^{\text{II}}]}{dt} = \frac{k_{-6}}{k_6 + k_7} [\text{cyt } c^{\text{III}}\text{-I}]$$

Although this mechanism is also in agreement with the kinetic data, it requires that $\text{cyt } c^{\text{III}}$ and I form a strong unreactive complex, for which there is also no other evidence.

A third mechanism involves a slow conformational change as the rate controlling step (reaction 10) followed by a fast interaction of I with the active conformer of $\text{cyt } c^{\text{III}}$ ($\text{cyt } c^{*\text{III}}$) to give products (reaction 11):

Reaction (12) in this mechanism is analogous to reactions (5) and (8) in the other two mechanisms. The same Mo(V) monomer is proposed and is expected to be very reactive; it would react with either $\text{cyt } c^{\text{III}}$ or $\text{cyt } c^{*\text{III}}$ (reaction 13).

A similar mechanism was proposed for the reduction of $\text{cyt } c^{\text{III}}$ by Cr(II) ion in aqueous chloride media (101). The rate of reduction of $\text{cyt } c^{\text{III}}$ becomes independent of the concentration of Cr(II) at high concentrations. Yandell et al. (101) proposed a mechanism involving the breaking of the methionine-80 sulfur-iron bond as the rate determining step and subsequent bridging of the Cr(II) and Fe(III) ions by chloride to effect electron transfer. The rate limiting step in that reaction has a first order rate constant of 60 sec^{-1} , which is much faster than the rate of reduction of $\text{cyt } c^{\text{III}}$ by I. The possibility of a slower conformational change cannot, however, be eliminated.

This mechanism gives a similar rate expression:

$$\frac{d[\text{cyt } c^{\text{II}}]}{dt} = \frac{k_{10}k_{11}[\text{I}][\text{cyt } c^{\text{III}}]}{k_{-10} + k_{11}[\text{I}]}$$

which reduces to the observed experimental rate law,

$$\frac{d[\text{cyt } c^{\text{II}}]}{dt} = k_{10}[\text{cyt } c^{\text{III}}]$$

if $k_{11}[\text{I}] \gg k_{-10}$. Although there is no evidence for such a slow conformational change, there is also no evidence against it.

Looking at the kinetic data in low ionic strength media, it is difficult to explain the rather large increase in observed rate constants with increasing concentration of I in phosphate and HEPES buffers.

The increase in rate is, however, far from being large enough to suggest a first order dependence on complex I.

If mechanism three (reactions 10-13) is to be in effect for the reduction of cyt c^{III} by I, the ionic strength of the medium would have to have a very significant effect on the slow, rate controlling conformational change. Salemmé et al. (52) have proposed that a stabilizing anion is located in the heme crevice in the oxidized form of cyt c and is displaced by phenylalanine-82, which swings into the heme crevice on binding of cytochrome c reductase. If such an anion (a buffer ion or Cl⁻) has to be displaced for interaction of I with cyt c^{III}, the ionic strength of the medium should have the same effect as observed. This does not necessarily imply that complex I replaces the anion, but rather that the rate controlling conformational change occurs more slowly in the presence of a higher concentration of anions.

The increase in rate with increasing concentration of I in phosphate and HEPES buffers suggests that complex I may catalyze such a conformational change. Such a catalytic effect can be visualized as a weak interaction of complex I with the lysine rich region of cyt c^{III} in the vicinity of phenylalanine-82 (see Figure 2) resulting in the phenylalanine side chain favoring a position nearer the heme crevice, due to steric factors, consequently favoring displacement of the bound anion in the heme crevice. In the presence of high concentrations of anion, as in the case with high ionic strength buffers, the interaction of I with lysyl residues would probably be less important, due to the competition with the other anions in solution.

The evidence accumulated for the reduction of $\text{cyt } c^{\text{III}}$ by complex I favors the third mechanism (reactions 10-13) involving a rate limiting conformational change. Such a slow conformational change would appear at first to be unimportant in the very rapid physiological reduction of $\text{cyt } c^{\text{III}}$ by cytochrome c reductase. Vanderkooi and Erecinska (137) found that the redox potential of cytochrome c is lowered ca. 40 mV by the presence of cytochrome c reductase, suggesting a strong binding interaction between the two proteins. Since the results of the present study indicate that complex I does not bind to $\text{cyt } c^{\text{III}}$, any contiguous interaction between these two species must be very weak. Therefore, if electron transfer to $\text{cyt } c^{\text{III}}$ is contingent on a conformational change with certain reducing agents (such as cytochrome c reductase or complex I) and conformational isomerism caused by reductase binding to $\text{cyt } c^{\text{III}}$ is the driving force for electron transfer in vivo, as suggested by Salemm et al. (52), a large difference in binding constants for cytochrome c reductase and complex I binding to $\text{cyt } c^{\text{III}}$ could account for the large difference in the rate of electron transfer to $\text{cyt } c^{\text{III}}$ from these species. The mechanism for the reduction of $\text{cyt } c^{\text{III}}$ by complex I cannot, therefore, be totally disregarded as a model for the in vivo reduction by cytochrome c reductase simply because of the large difference in rates.

Consideration of the activation parameters observed for the reduction of $\text{cyt } c^{\text{III}}$ by I suggests that bond breaking may be taking place (due to the large value of $\Delta H^\ddagger$) in the rate limiting step. Such a value for $\Delta H^\ddagger$ may also arise from a conformational change taking place

in a large molecule such as cyt c. This would also argue in favor of the third mechanism.

Reduction of Cyt c^{III} by Complex II

Complex II reduces cyt c^{III} very rapidly and the reaction is first order in both reactants over the concentration range studied. A mechanism which is in agreement with the kinetic data involves an initial one electron oxidation of II by cyt c^{III} (reaction 14) and a faster reaction between cyt c^{III} and the Mo(V) monomer which is produced in reaction 14 (reaction 15):

where L is cysteine and the Mo(V) and Mo(VI) species are those proposed in the previous mechanisms for oxidation of I. This mechanism leads directly to the observed rate law:

$$\frac{d[\text{cyt c}^{\text{II}}]}{dt} = 2k_{14}[\text{II}][\text{cyt c}^{\text{III}}]$$

The very fast rate of this reaction restricted the limits of the concentration ranges of the reactants. This made it impossible to study the reaction at 695 nm, since the concentration of cyt c^{III} would have to be such that the reaction would be essentially complete in the dead time of the stopped flow instrument.

The reduction of cyt c^{III} by a number of nonphysiological reductants has led to at least two general mechanisms (83,101). One mechanism involves a rate limiting heme crevice opening with a first order rate constant of 60 sec^{-1} , and a subsequent rapid reduction of the heme iron. The second mechanism involves an outer sphere electron transfer through the exposed heme edge or interaction at the periphery of the protein, followed by electron transfer via π -cloud interactions of aromatic residues and the porphyrin ring. Reductions proceeding with a rate more rapid than the heme crevice opening would presumably follow the remote pathway.

The activation parameters for the reduction of cyt c^{III} by II are in keeping with an outer sphere mechanism (102,103), i.e. small values for $\Delta H^\ddagger$ (4.8 kcal/mol) and negative values for $\Delta S^\ddagger$ (-9 eu). Since II reduces cyt c^{III} at a rate as fast or faster than the natural reductant, cyt c_1^{II} , in vitro ($k = 3.3 \times 10^6 \text{ M}^{-1} \text{ sec}^{-1}$, pH 7.4, 10° C) (78), a similar mechanism would be feasible for the in vivo reduction by cytochrome c reductase.

Reduction of Ligand Bound Cyt c^{III} by Complex II

The reduction of cyt c^{III} by II in the presence of the heme iron binding ligands, azide and cyanide, suggests that II does not react with the ligand bound form of cyt c^{III} , but instead reduces free cyt c^{III} rapidly as the ligand dissociates. Sutin and Yandel (76) studied ligand binding in cytochrome c and found the reaction of cyt c^{III} with iron binding ligands was in agreement with a mechanism involving a rate limiting heme crevice opening and/or breaking of the methionine-80

sulfur-iron bond (see literature review). The first order rate constant for this rate limiting step was estimated as 60 sec^{-1} . The rate constants for dissociation of cyanide, azide and imidazole are 1.3×10^{-5} , 5.2 and 2.4 sec^{-1} , respectively.

A mechanism for the reduction of N_3^- and $\text{CN-cyt c}^{\text{III}}$ by II, which is in agreement with the kinetic data, involves the dissociation reaction for ligand-cyt c^{III} complex (reaction 16) and the rapid reduction of free cyt c^{III} by II (reaction 17):

where X represents ligand. Although Mo(V) monomer is probably also produced in reaction 17, its reaction with $\text{cyt c}^{\text{III}}$ is not shown since it does not enter into the rate expression. If Mo(V) monomer were to react with $\text{X-cyt c}^{\text{III}}$, the observed rate constant for reduction of $\text{X-cyt c}^{\text{III}}$ by II would be about twice the rate of dissociation of the ligand-cyt c^{III} complex, assuming Mo(V) monomer reacts more rapidly with $\text{X-cyt c}^{\text{III}}$ than its rate of dissociation. The observed rate constants are, however, in excellent agreement with the rate constants for ligand dissociation, which suggests Mo(V) monomer does not react with $\text{X-cyt c}^{\text{III}}$, or reacts with a rate much slower than the rate of ligand dissociation. The rate constants for the reduction of $\text{cyt c}^{\text{III}}$ by II in the presence of azide ($k_{\text{obs}} = 5.4 \text{ sec}^{-1}$) and cyanide ($k_{\text{obs}} = 1.6 \times 10^{-5} \text{ sec}^{-1}$) are in excellent agreement with such a mechanism, i.e. the rate limiting

step being dissociation of the ligand-cyt c^{III} complex. The experimental rate law is obtained from this mechanism by applying the steady state approximation to $[cyt\ c^{III}]$:

$$\frac{d[cyt\ c^{II}]}{dt} = k_{-16}[X-cyt\ c^{III}]$$

The reduction of $cyt\ c^{III}$ by complex II in the presence of imidazole is much more complicated. At low concentrations first order plots are linear giving a first order rate constant of ca. $2.0\ sec^{-1}$, which is in good agreement with the rate of dissociation of the Im-cyt c^{III} complex and suggests the above mechanism (reactions 16 and 17) may be in effect. When the concentration of II is at a 5 to 50 fold excess, however, the reaction becomes biphasic and the amount of reaction which occurs in the fast phase is dependent on the concentration of II. The entire reduction of $2.0 \times 10^{-5}\ M\ Im-cyt\ c^{III}$ occurs in the fast phase when the concentration of II is ca. $1.0 \times 10^{-3}\ M$. The most plausible explanation for this fast phase is that Mo(V) monomer, which is in equilibrium with complex II in solution, is reducing the Im-cyt c^{III} complex. The esr data indicate that the amount of Mo(V) monomer in a $10^{-3}\ M$ solution of II would be sufficient to completely reduce ca. $2.0 \times 10^{-5}\ M\ Im-cyt\ c^{III}$.

The slow phase of the reduction is nearly independent of the concentration of complex II at low concentrations of II (as shown in Table 28), but shows a rather strong dependence on the concentration of II at the higher concentrations, where the slow phase can still be observed. This is probably due to the reduction of Im-cyt c^{III} by

complex II, as was shown in the results, since the second order rate constants calculated for such a reaction are reasonably consistent (see Table 28).

A mechanism which is reasonably consistent with the data is:

where Im is free imidazole, L is cysteine and Mo(V) and Mo(VI) are Mo(V) monomer and MoO_4^{2-} , the species proposed in the previous mechanisms. The only steady state approximation that would be valid in this mechanism, over the entire concentration range of II studied, is for $[\text{cyt } c^{\text{III}}]$. The rate expression obtained, cannot, therefore, be put in a form which can be integrated and plotted to obtain rate constants or ratios of rate constants.

A computer method, developed by Moore and coworkers (130,131), was used, which develops integrated, time dependent expressions for the differential rate laws, which are derived from the chemical mechanism (see experimental section). The rate constants for reactions 18-22

have been estimated (see results), and the values for these rate constants which were used in the computer simulation are given in Table 50. The initial concentrations of all species are known, with the possible exception of Mo(V) monomer. The concentration of Mo(V) monomer was calculated using a value of $K_{eq} = 4.0 \times 10^{-7}$ M for the equilibrium between complex II and Mo(V) monomer. Supplying the computer program with the rate constants for each step in the mechanism and the initial concentration of each species, simulated concentrations for each species are generated as a function of time. The simulated concentrations for cyt c^{II}, at given times, were compared with the observed concentrations of cyt c^{II} at the corresponding times for several sets of initial conditions. Figures 27-29 illustrate the observed vs. computer simulated concentrations of cyt c^{II} as a function of time. It is apparent that the simulated data is in good agreement with the observed data, considering the uncertainty in determining the rate constants and initial concentrations.

Upon close inspection of Figures 27-29, one may deduce that the initial concentration of Mo(V) monomer used at the two lower concentrations of II may have been too large. Since these concentrations of Mo(V) monomer were calculated from the equilibrium constant which was estimated at the high concentration of II, one may suggest that another species may enter into the equilibrium expression for the monomer-dimer relationship. It is reasonably certain that cysteine does enter into the equilibrium expression in the absence of imidazole (138); however, imidazole was found to have a monomer stabilizing effect, suggesting it too enters into the equilibrium expression.

Table 50. Rate constants for reactions 18-22 which were used for the computer simulation of the reduction of Im-cyt c^{III} by complex II

$k_{18} = 60 \text{ M}^{-1} \text{ sec}^{-1}$	$k_{-18} = 0.75 \text{ sec}^{-1}$
$k_{19} = 1.8 \times 10^7 \text{ M}^{-1} \text{ sec}^{-1}$	$k_{20} = 1.0 \times 10^4 \text{ M}^{-1} \text{ sec}^{-1}$
$k_{21} = 1.5 \times 10^8 \text{ M}^{-1} \text{ sec}^{-1}$	$k_{22} = 1.0 \times 10^7 \text{ M}^{-1} \text{ sec}^{-1}$

Lowering the initial concentration of monomer in Figures 27 and 28, at the two lower concentrations of II, would give a better correlation between the observed data and computer simulated data.

The data obtained for the reduction of cyt c^{III} by complex II in the presence of imidazole at pH 10 have some interesting implications. The observed second order rate constant for the reaction is $6.2 (\pm 1.1) \times 10^4 \text{ M}^{-1} \text{ sec}^{-1}$, which is in close agreement with the observed rate constant for the reduction of the high pH form of cyt c^{III} by complex II ($k = 7.2 (\pm 0.4) \times 10^4 \text{ M}^{-1} \text{ sec}^{-1}$) (134). This suggests that imidazole does not bind to cyt c^{III} at this pH and the reaction taking place is the reduction of the high pH form of cyt c^{III} by II. This is further supported by the fact that the reaction occurs in a single phase over a very wide range in concentration of II.

The high pH form of cyt c^{III} is proposed to have the ϵ -amino group of lysine-79 as the sixth ligand of the heme iron, in place of the methionine-80 sulfur (62,63). The equilibrium constant for the conformational change involving displacement of the methionine-80 sulfur ligand by the lysine-79 amino group has been estimated at ca. 125 (63),

Figure 27. The experimental vs. computer simulated production of cyt c^{II} in the reduction of Im-cyt c^{III} by a low concentration of complex II. Experimental data was taken from Table 25, [II]₀ = 2.50 x 10⁻⁵ M. The rate constants and initial concentrations used for the computer simulated data were as described in Table 50 and the text [Im-cyt c^{III}]₀ = 1.88 x 10⁻⁵ M, [cyt c^{III}]₀ = 2.51 x 10⁻⁶ M. Experimental data, o computer simulated data.

Figure 28. The experimental vs. computer simulated production of cyt c^{II} in the reduction of Im-cyt c^{III} by an intermediate concentration of complex II. Experimental data was taken from Table 25, $[II]_0 = 2.50 \times 10^{-4}$ M. The rate constants and initial concentrations used for the computer simulated data were as described in Table 50 and the text, $[Im-cyt\ c^{III}]_0 = 1.88 \times 10^{-5}$ M, $[cyt\ c^{III}]_0 = 2.51 \times 10^{-6}$ M. Experimental data, o computer simulated data.

Figure 29. The experimental vs. computer simulated production of cyt c^{II} in the reduction of Im-cyt c^{III} by a high concentration of complex II. Experimental data was taken from Table 25, $[II]_0 = 1.00 \times 10^{-3}$ M. The rate constants and initial concentrations used for the computer simulated data were as described in Table 50 and the text, $[Im-cyt\ c^{III}]_0 = 1.88 \times 10^{-5}$ M, $[cyt\ c^{III}]_0 = 2.51 \times 10^{-6}$ M. Experimental data, o computer simulated data.

approximately 4 times the value for the binding constant for imidazole (obtained at neutral pH) (76). It is therefore reasonable to expect the cyt c^{III} to be predominantly in the high pH form (lysine-79 bound) in the presence of imidazole at pH 10.

The kinetic behavior at pH 10 implies that Mo(V) monomer does not react with the high pH form of cyt c^{III} or reacts much more slowly than with Im-cyt c^{III} . The redox potential for cyt c^{III} at pH 10 is +0.12 V (64), which is only slightly lower than the redox potential of +0.15 V for Im-cyt c^{III} found in this study. The lack of activity of Mo(V) monomer with the high pH form of cyt c^{III} is probably not thermodynamic. Although the redox potential of the Mo(VI)/Mo(V) couple is not known at pH 10, it must be sufficiently negative, since Mo(V) monomer was found to reduce flavin (22). It may be that the rate of reduction of the high pH form of cyt c^{III} by Mo(V) monomer is much slower than the rate of reduction of Im-cyt c^{III} by that species or, there may be only a pH effect on the rate. Since the concentration of Mo(V) monomer in a 1.0×10^{-3} M solution of II is estimated to be 2.0×10^{-5} M, the second order rate constant for the reduction of high pH cyt c^{III} by Mo(V) monomer must be less than $ca. 5 \times 10^5 \text{ M}^{-1} \text{ sec}^{-1}$, which is considerably smaller than the estimated rate constant for the reduction of Im-cyt c^{III} by Mo(V) monomer.

The potentiometric titration studies indicate the reduction of N_3 -cyt c^{III} by complex II is thermodynamically favorable and the apparent lack of activity must be purely kinetic. Since the rate of reduction of N_3 -cyt c^{III} was not affected by concentrations of II up to

1.0×10^{-3} M, the second order rate constant for reduction of this ligand bound species by II would have to be less than $\text{ca. } 10^2 \text{ M}^{-1} \text{ sec}^{-1}$ and the rate constant for reduction by Mo(V) monomer less than $\text{ca. } 10^4 \text{ M}^{-1} \text{ sec}^{-1}$, if these reactions occur at all.

The potentiometric titration of complex II indicates a redox potential of -0.15 V; however, complex II reduces flavin mononucleotide (22) for which $E_o' = -0.22$ V (135). Complex II does not, however, reduce CN-cyt c^{III} , for which $E_o' = -0.40$ V (72), which may be a kinetic phenomenon as in the case with $\text{N}_3\text{-cyt } c^{\text{III}}$, but because of the extremely slow rate of dissociation of the CN-cyt c^{III} complex, is probably thermodynamic. If the reason for lack of activity with CN-cyt c^{III} is thermodynamic, that would suggest the value of E_o' for complex II is approximately -0.30 to -0.35 V.

The kinetic limitations with $\text{N}_3\text{-cyt } c^{\text{III}}$ must lie in the structure and/or electronic properties of the $\text{N}_3\text{-cyt } c^{\text{III}}$ complex. Ikeda-Saito and Iizuka (77) found the azide and imidazole complexes of cyt c^{III} are purely in the low spin state at room temperature. They did find, however, from esr data, that the axial and rhombic distortions for the azide complex are greater than for the imidazole complex. The greater basicity of imidazole, with respect to azide, is expected to decrease the electron density on the iron (as well as decrease electron density in the porphyrin ring) which would make the Im-cyt c^{III} a better electron sink than $\text{N}_3\text{-cyt } c^{\text{III}}$. This argument has much to recommend it in the difference in the kinetic properties of $\text{N}_3\text{-}$ and Im-cyt c^{III} . Furthermore, this would suggest that the mechanism for electron transfer may very well involve the π -cloud of the porphyrin ring.

It should be pointed out that high concentrations of N-acetyl-methionine had no effect on the electronic absorption spectrum of cyt c^{III} nor did it affect the kinetics with either I or II. These results suggest NAM does not bind to the heme iron of cyt c^{III} .

Reduction of Prydoxal Phosphate Modified Cyt c^{III}

Aviram and Schejter (122) showed that 1 or 2 PLP groups are covalently bound to cyt c^{III} upon modification with PLP and borohydride. Enzymatic digestion of the modified cyt c and subsequent purification of the peptide fragments showed that 3 regions of the peptide may be modified. These three regions include the amino acid sequences 1-10, 68-74 and 83-94. Since the amino terminal glycine in horse heart cyt c^{III} is acetylated, only lysine residues are subject to modification in this procedure. The peptide sequence 1-10 contains 3 lysine residues at positions 5, 7 and 8 and the sequence 83-94 also has 3 lysine residues at positions 86, 87 and 88. The peptide sequence 68-74 has 2 lysine residues at positions 72 and 73. The exact residues modified were not determined, but it is known that all of the lysine residues mentioned lie on the periphery of the cytochrome c molecule (3), removed from the close proximity of the heme, with lysine residues 5, 7 and 8 and 86-88 in one region of the molecule and residues 72 and 73 in another region (see Figure 2). Although three regions of the molecule may be modified, a maximum of 2 PLP groups is probably bound.

The introduction of either 1 or 2 PLP groups per cyt c molecule had essentially no effect on the kinetic behavior with I and only

slightly inhibited the reaction with II. The mechanism for reduction of the modified cyt c^{III} in both cases appears to be the same as for the native cyt c^{III} (vide supra). Since PLP modification does not affect the absorption bands of the heme prosthetic group of cyt c, one would expect that such modification has little effect on the thermodynamic properties of the protein (i.e. redox potential). PLP modification was found to have no effect on cytochrome c oxidase activity but does inhibit NADH-cytochrome c reductase activity by 75% in PLP₁-cyt c^{III} and 90% in PLP₂-cyt c^{III} (122).

The 50% decrease in rate of reduction of cyt c^{III} by II upon PLP modification may be due simply to difference in overall charge on the cyt c^{III} molecule. Lysine residues 72 and 73 do, however, lie in close proximity to the hydrophobic heme crevice and tyrosyl-74, which has been suggested to be involved in electron transfer by π -cloud interaction of aromatic groups in the protein (116,139). Since PLP₁- and PLP₂-cyt c^{III} have similar rates of reduction by II and both are ca. 1/2 the rate observed with native cyt c^{III}, it seems likely that the first modification causes inhibition of the reaction and the second modification has very little effect on reduction by II. The reduction of PLP₁- and PLP₂-cyt c^{III} by cytochrome c reductase indicates that each modification shows a progressive inhibition of activity with the reductase enzyme. Such contradiction in the results with II and reductase may lie in the fact that the reductase is much larger than II and the introduction of the second PLP group at a distance somewhat removed from the first modification could affect interaction with reductase and have no effect on interaction with II.

Reduction of Nitrated Cyt c^{III}

The reduction of mononitromonotyrosyl-cyt c^{III} (MNMT-cyt c^{III}) by complex I indicates the modification causes a 3.5 fold increase in the rate of reaction; this observation is interesting since all other modifications of the protein caused inhibition or no change in the rate of reduction by I. The rate of reduction of mononitrodityrosyl-cyt c^{III} (MNMT-cyt c^{III}) by I was nearly the same as the rate of the MNMT derivative. Structural and functional studies of nitrated cyt c (121) indicated the MNMT derivative is modified at tyrosyl-67 and the MNMT derivative has the additional modification at tyrosyl-48. The physico-chemical characteristics of MNMT-cyt c^{III} suggest a loosening of the heme crevice and weakening of the methionine-80 sulfur-iron bond at physiological pH (in comparison with native cyt c^{III}). MNMT-cyt c^{III} exhibited properties indicative of gross conformational change as well as change in the iron coordination sphere. These two nitrated derivatives of cyt c also exhibited very different chemical behavior, MNMT-cyt c^{III} was reduced by ascorbate, had 24% activity with NADH-cytochrome c reductase and the reduced form was autoxidizable (although very slowly) and had ca. 100% activity with cytochrome c oxidase. The MNMT derivative, on the other hand, was not reduced by ascorbate, had essentially no activity with NADH-cytochrome c reductase and was autoxidized very rapidly.

It is interesting to note that although the two nitrated derivatives exhibit such gross differences in physical and chemical properties,

their rate of reduction by I is nearly identical, and significantly faster than the rate of reduction of native cyt c^{III} by I. The increase in rate with the two nitrated derivatives may be taken as evidence in favor of complex formation of I at tyrosyl-67, since nitration would cause a decrease in the pK_a and would make the tyrosyl residue a better ligand for molybdenum. The increase in rate may also be taken as evidence in favor of a slow conformational change in cyt c^{III} as the rate controlling step in reduction by I. If, in fact, the electron transfer from complex I to cyt c^{III} is contingent on a conformational change in the region of the heme crevice, loosening of the heme crevice by nitration of tyrosyl-67 may favor such a conformational change and account for the increase in the rate of reduction of MNMT-cyt c^{III} . The additional modification of tyrosyl-48 to form MNDT-cyt c^{III} , causes gross conformational changes in the protein, evidenced by its physicochemical properties (121), with little effect on its kinetic properties with complex I. Such results are not necessarily inconsistent with the proposed mechanism for cyt c^{III} reduction by I involving a rate limiting conformational change.

It should also be pointed out that the breaking of the met-80 sulfur-iron bond exhibits an apparent pK of 9.2 in the native cyt c^{III} but has an apparent pK of 6.2 in MNMT-cyt c^{III} (121) (MNDT-cyt c^{III} apparently does not have the met-80 sulfur-iron bond at any pH). The rate of reduction of native cyt c^{III} by I is observed to increase rapidly with increasing pH above 7.5 (vide supra). These results were

interpreted as due to the conversion of I to II under these conditions; it is possible, however, that the increased rate is partially due to loosening of the heme crevice under alkaline conditions. It is not possible to test such a mechanism where most of the cyt c^{III} would have the met-80 sulfur-iron bond broken (i.e. pH > 9), since the rate of conversion of I to II under those conditions is relatively rapid.

The reduction of cyt c^{III} by II is, however, inhibited by the nitration of tyrosyl residues. The rate of reduction of MNMT-cyt c^{III} by II is ca. 1/2 the rate observed with native cyt c^{III} . Such a small change in the rate of this reaction suggests that tyr-67 is not directly involved in the electron transfer (i.e. by π -cloud interaction) since introduction of the nitro group should alter the electron density in the π -electron cloud by a very substantial amount. The slight decrease in rate may be due to either weakening of the met-80 sulfur-iron bond or merely to ionic interactions (nitration lowers the pK_a of the tyrosyl residue and would thus cause a decrease in the net positive charge of the protein).

Nitration of a second tyrosyl residue (presumably tyr-48), giving MNDT-cyt c^{III} , results in a very large decrease in the rate of reduction by II. The observed rate constant for reduction of MNDT-cyt c^{III} by II is two orders of magnitude slower than the reduction of MNMT-cyt c^{III} . Tyrosyl-48 is believed to be hydrogen bonded to one of the propionic acid side chains of the porphyrin in native cyt $c(3)$. Nitration of this residue is expected to lower its pK_a by ca. 3 units, thus greatly decreasing its hydrogen bonding capabilities at physiological pH. The MNDT-cyt c^{III} exhibits gross conformational changes in the protein as

well as changes in the iron coordination sphere (121); such gross changes in the structure of the cyt c molecule obviously have a very significant effect on its activity. It is possible that the lack of activity of MNMT-cyt c^{III} with NADH-cytochrome c reductase and ascorbate is thermodynamic, rather than kinetic, in nature. If this is true, the redox potential of MNMT-cyt c^{III} must be less than -50 mV, since the potential of ascorbate (deprotonated) is -12 mV (135). Such a thermodynamic argument lends further support to the conformational change mechanism (reactions 10-13) for reduction of cyt c^{III} by I since the lowered redox potential would not necessarily affect the rate of a conformational change, but should affect the rate of electron transfer (as in the binding mechanisms, reactions 3-9).

Reduction of Formyl-Cyt c^{III}

The reduction of formyl-cyt c^{III} by I suggests that this cyt c^{III} derivative exists as two species. Both phases of the reaction are first order in formyl-cyt c^{III} and zero order in I, suggesting a mechanism similar to that proposed for the reduction of the native cyt c^{III}. The difference in the rate constants between the fast and slow phases of the reaction is less than one order of magnitude, indicating the two phases are probably due to two independent species. The amount of reaction occurring in the fast phase is constant and about 40% of the overall reaction over a wide range in concentration of I, which further suggests the formyl-cyt c^{III} exists as two independent species. If the two species are in equilibrium, however, their rate of conversion would need to be much slower than the rate of reduction by I.

The reduction of formyl-cyt c^{III} by II is much more complex. There is a very rapid initial phase in the reaction which accounts for approximately 30% of the overall reaction. Although this phase has an estimated rate constant very nearly that for the reduction of native cyt c^{III} by II, it is probably not due to residual native cyt c^{III} since there is no additional evidence for any substantial amount of native cyt c^{III} in this fraction. This phase is probably not due to Mo(V) monomer either, since the concentration of Mo(V) monomer is much too low at the lowest concentrations of complex II. The amount of reaction which occurs in the fast phase, as in the case with complex I, was reasonably constant and about 30-35% of the overall reaction.

The slow phase of the reduction of formyl-cyt c^{III} by II does show a dependence on the concentration of II, although somewhat less than a first order dependence. Under pseudo-first order conditions, the observed first order rate constant increases by a factor of 5 over a 10 fold increase in the concentration of II (see Table 45). Such behavior suggests there may be a rate limiting first order reaction, independent of the concentration of II, as well as a second order reaction which is dependent on the concentration of II.

A possible mechanism involves two independent formyl-cyt c^{III} species, as suggested for the reduction of formyl-cyt c^{III} by I. The more reactive component, A, reacts with complex II (reaction 23) producing formyl-cyt c^{II} and Mo(V) monomer, which would react rapidly with another formyl-cyt c^{III} . The less reactive component, B, reacts with II to produce B' (reaction 25) and Mo(V) monomer, where B' may be a

B-II complex or a reduced form of B with a lower extinction coefficient. Mo(V) monomer also reacts with B to produce B' (reaction 26). B' is then converted to formyl-cyt c^{II} by a first order process (reaction 27).

This mechanism, like the mechanism for the reduction of Im-cyt c^{III} by II, gives a nonintegrable rate expression, since the steady state approximation would not be valid over a wide range in concentration of II. The mechanism was tested using the computer method described for the reduction of Im-cyt c^{III} by II; however, the computer simulated data did not correlate well with the experimental data. The problem arises in the computer method of treating B'. It is not likely that B' is a complex between B and II, which undergoes a rate limiting electron transfer, since the results for reduction of other forms of cyt c^{III} by II point up to an outer sphere mechanism. If B' is a form of formyl-cyt c^{II} with a lower extinction coefficient than the final reduced product, there is no way to treat such a species in the computer method without knowing the relative extinction coefficients of the two species.

Physicochemical studies of formyl-cyt c^{III} (116) indicate it is significantly different from the native protein in that it has an altered iron coordination sphere as well as gross perturbations in the protein conformation. The lack of the 695 nm band suggests the methionine-80 sulfur is not coordinated to the iron (116). The absorption spectrum does, however, indicate the iron is low spin at neutral pH, by the absence of the 620 nm band (typical of high spin cyt c^{III}), which suggests a strong field ligand is coordinated to the iron. Studies to date have not indicated what the sixth ligand may be.

The reduction of formyl cyt c^{III} by Cr(II) ion at pH 6.5 shows two phases (118), one phase dependent on the concentration of Cr(II) ion ($k = 4 \times 10^4 \text{ M}^{-1} \text{ sec}^{-1}$) and the second phase independent of Cr(II) concentration ($k = 5.0 \text{ sec}^{-1}$). These results were interpreted to mean Cr(II) reduces an active form of formyl-cyt c^{III} which is in equilibrium with an inactive form of the cytochrome. A similar interpretation is not in keeping with the results of the reduction by complex II, since the rate of the slow phase of the reaction was dependent on the concentration of II.

Another possible interpretation of these results is that a third component is present in the formyl-cyt c^{III} fraction, which may react with II directly, or may be in equilibrium with either the fast or slow component.

The kinetic results of the reaction between II and formyl-cyt c^{III} do indicate this derivative is not a homogeneous cytochrome species, but rather a mixture. A possible explanation of the complex kinetic

behavior is the existence of formyl-cyt c^{III} dimers (or possibly oligomers) which are in equilibrium with the monomer. This would account for the relative insolubility of formyl-cyt c^{III} and the large amount of protein which is strongly bound at the top of ion exchange columns during chromatographic purification. One must use considerable caution interpreting these results and most of the interpretation is purely conjectural until more information can be gathered.

Reduction of Cyt c^{III} by Mo(V)-Penicillamine Complexes

The reduction of cyt c^{III} by the dioxobridged penicillamine complex is very similar to the reduction by I. The similar rate strongly favors the mechanism involving conformational change in the protein as the rate controlling step (reactions 10-13).

Interpretation of the kinetic results with the monoxobridged Mo(V)-penicillamine complex is highly speculative at best since it is difficult, if not impossible, to assess the nature of the molybdenum species present. It is obvious that the active molybdenum species is capable of extremely rapid electron transfer. The only conclusions which can be drawn at this time are that the active Mo(V) species reacts with cyt c^{III} even faster than complex II, and would probably react in a similar manner, i.e., by the same mechanism (reactions 14 and 15).

Although the behavior of the Mo(V)-penicillamine complexes is much more complicated than that of the Mo(V)-cysteine complexes, their interaction with cyt c^{III} is very similar. Such results suggest that the structure and ligands of the Mo(V) coordination sphere strongly affect its kinetic behavior.

CONCLUSIONS

It is obvious from the results presented here, that complex I and complex II have very different kinetic properties. The reduction of $\text{cyt } c^{\text{III}}$ by complex I is very slow and the evidence accumulated points to a slow conformational change in the $\text{cyt } c^{\text{III}}$ molecule as the rate controlling step. Such a slow conformational change and its dependence on the reduction of $\text{cyt } c^{\text{III}}$ have not appeared in the literature and suggests that complex I is indeed, an unusual reductant. Complex II, on the other hand, reduces $\text{cyt } c^{\text{III}}$ very rapidly; in fact, the rate is the fastest reported reduction of $\text{cyt } c^{\text{III}}$ by a transition metal complex. The reaction between $\text{cyt } c^{\text{III}}$ and II is also first order in both reactants. Brown and coworkers (140,141) have indicated, by theoretical calculations of dioxobridged and monoxobridged Mo(V) dimers, that the electron density in the Mo-Mo , $d_{\sigma}-d_{\sigma}$ bond is considerably higher for dioxobridged Mo(V) dimers, which would produce a less favorable overlap with oxidant orbitals.

The reduction of $\text{cyt } c^{\text{III}}$ by complex II appears to occur by an outer sphere mechanism. The rapid rate of electron transfer ($k_{\text{obs}} = 2.6 \times 10^7 \text{ M}^{-1} \text{ sec}^{-1}$), the small value for the enthalpy of activation ($\Delta H^{\ddagger} = 4.8 \text{ kcal/mol}$) and a negative entropy of activation ($\Delta S^{\ddagger} = -8.6 \text{ eu}$) are in keeping with other reductions of $\text{cyt } c^{\text{III}}$ by outer sphere metal complexes (102,103). Two proposed sites of entry for the electron in outer sphere reductions of $\text{cyt } c^{\text{III}}$ are the exposed heme

edge (102) or an aromatic residue at the periphery of the protein molecule (probably tyrosyl-74), with subsequent electron transfer, by π -electron cloud interaction, to the heme (142).

The results of the studies with ligand bound cyt c^{III} have some very important implications with respect to electron transfer in sulfite oxidase and cytochromes in general. It is obvious that the nature of the heme iron ligands has a very pronounced effect on the reactivity of the cytochrome molecule. Although the thermodynamic properties of N_3 - and Im-cyt c^{III} are quite similar and not greatly different from native or alkaline cyt c^{III} , the kinetic properties of these species are grossly different. The fact that cyt c^{III} is a kinetically more active electron acceptor than the endogenous cyt b of sulfite oxidase may be attributed to the difference in the iron ligands. Although such arguments are not new, the results presented in this study provide further support of the idea that the nature and symmetry of the iron ligands strongly influences the reactivity of the cytochrome molecule.

The fact that complex II reduces native cyt c^{III} much more rapidly (>2 orders of magnitude faster) than the other forms of cyt c^{III} studied, strongly recommends it as a model for the molybdenum moiety in the sulfite oxidase enzyme, since kinetic evidence suggests the molybdenum in sulfite oxidase transfers electrons to the endogenous cyt b, and may transfer them even more rapidly to cyt c^{III} (42). Furthermore, kinetic studies with sulfite oxidase indicate one heme group of the enzyme is reduced more rapidly than the other (42). Such results were interpreted as differences in the heme prosthetic group.

The difference in rates of reduction may, in fact, be inherent in the nature of the Mo(V) species donating the electrons, since the results of this study imply the Mo(V) monomer, present in solutions of complex II, is more reactive with native and Im-cyt c^{III} but apparently less reactive with alkaline or N₃-cyt c^{III}.

Most information concerning the structure of the molybdenum site in enzymes has been obtained from esr studies. Comparison of the esr signals observed in xanthine oxidase and sulfite oxidase with known molybdenum complexes indicates sulfur is a very likely ligand in the enzymes. The results of this study indicate complex II may be a good model for the molybdenum center in sulfite oxidase, considering its rate of electron transfer to cyt c. One apparent discrepancy, however, is the fact that the fully reduced enzyme exhibits an esr signal which accounts for approximately 50% of the total molybdenum (41). This suggests that either half of the molybdenum is antiferromagnetically coupled or that only half of the molybdenum is in the 5+ oxidation state. Quantitation of the esr signal for xanthine oxidase has shown that only about 37% of the molybdenum is esr detectible (11), suggesting stronger magnetic interactions between metal centers in that enzyme. Clearly more work must be done to identify the molybdenum site in enzymes. If the molybdenum centers are, in fact, spin coupled, the results of the present study lend strong support to complex II as a model for the molybdenum moiety in enzymes.

Finally, the mechanism for electron transfer to $\text{cyt } c^{\text{III}}$ from various reductants appears to vary widely. The results of this study indicate that, although complexes I and II are physically quite similar, their mechanism of electron transfer to $\text{cyt } c^{\text{III}}$ are quite different. The overall evaluation of the data accumulated in this study, especially the work with modified forms of $\text{cyt } c^{\text{III}}$, suggests that electron transfer to $\text{cyt } c^{\text{III}}$ from complex II, and possibly complex I, probably occurs through the exposed heme edge. Since the nitration of tyrosyl-67 has such a small effect on the reduction of $\text{cyt } c^{\text{III}}$ by complex II, it may be concluded that this aromatic residue is probably not involved in electron transfer via π -electron cloud interactions with this particular reductant.

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