Model Studies for Molybdenum Enzymes. Reduction of Cytochrome *c* Complexes by μ-Oxo-bis[oxodihydroxo(L-cysteinato)molybdate(V)]†

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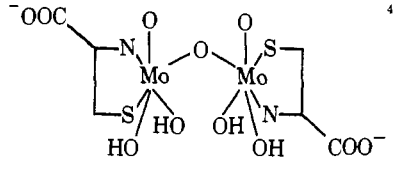
ABSTRACT: The reduction by μ-oxo-bis[oxodihydroxo(L-cysteinato)molybdate(V)] (I) of cytochrome *c* complexes in which the methionine-80 ligand has been replaced by N_3^-, CN^-, or imidazole has been investigated. The N_3^- and CN^- substituted species are reduced by I in a first-order reaction that appears to proceed via a slow dissociation of N_3^- or CN^-, followed by rapid reduction of native cytochrome *c*. At low concentrations of I, reduction of the imidazole-cytochrome complex occurs by the same mechanism, while, at higher concentrations of I, direct reduction by I and by a monomeric Mo(V) complex in equilibrium with I appears to occur, although at much lower rates than with native cytochrome *c*. Potentiometric measurements of $E°$ for the cytochrome *c* complexes with N_3^- and imidazole indicate the lack of reducibility, or reduction in rate relative to native cytochrome *c*, is not due to thermodynamic reasons. In the case of the CN^- complex, $E°$ may be too low for direct reduction by I. The effects on the reduction rates are attributed to a conformational change accompanying the replacement of the methionine-80 ligand, which makes the exposed heme edge less available to attack by outer sphere reductants.

As a model system for intramolecular electron transfer between the molybdenum and heme components of the enzyme hepatic sulfite oxidase (Cohen et al., 1971), we recently reported the results of a study of the reduction of native cytochrome *c* (cyt c^{III}) by molybdenum(V) cysteine complexes (Lawrence and Spence, 1975). In order to obtain more information concerning the mechanism of this reaction, we have extended this study to the reduction by the monoxobridged Mo(V)-cysteine complex, μ-oxo-bis[oxodihydroxo(L-cysteinato)molybdate(V)] (I) of the imidazole, azide, and cyanide complexes of cyt c^{III}. I reduces native cyt c^{III} extremely rapidly



I

in a second-order reaction with a rate constant of $(2.6 \pm 0.7) \times 10^7$ M^{-1} s^{-1} at pH 7.0 and 25 °C (Lawrence and Spence, 1975). The results were interpreted in terms of an outer sphere electron transfer mechanism, probably involving the exposed heme edge of the cyt c^{III} molecule.

Imidazole (Im),[1] azide (N_3^-), and cyanide (CN^-) are known to displace the methionine-80 ligand in the sixth coordination site of the heme iron of cyt c^{III}, with significant effects on the conformation and reactivity of the molecule (Schejter and Aviram, 1969; Sutin and Yandell, 1972; George and Tsou, 1952). It seemed of importance, as a mechanistic probe, to determine the effects of this displacement on the reduction by I.

Experimental Section

Horse heart ferricytochrome *c* (Sigma type III) was used without further purification. Its purity was determined by measuring the absorbance at 550 nm and comparing the values with published molar absorptivities (Margoliash and Frohwirt, 1959). Stock solutions of I were prepared and standardized as previously described (Lawrence and Spence, 1975). The cyt c^{III}-X complexes were prepared in situ by addition of the required amount of the appropriate ligand (NaN_3, NaCN, or imidazole) to a stock solution of cyt c^{III}.

All reductions were followed at 550 nm on an Aminco-Morrow stopped-flow spectrometer, as previously described for the reduction of cyt c^{III} (Lawrence and Spence, 1975). In all cases, corrections for the amount of native cyt c^{III} present were necessary. This amount was calculated from the known formation constants of the complexes and the amount of ligand added; it was also determined experimentally from the oscilloscope traces since the reduction of free cyt c^{III} by I is rapid with respect to the reduction of the complexes.

Reductive potentiometric titrations of cyt c^{III}-N_3 and cyt c^{III}-Im were performed in deaerated 0.02 M phosphate buffer, at pH 7.00, using reduced phthiocol or ascorbate as reductant and 2,6-dichlorophenolindophenol (DCIP) as mediator under argon. The reduced cyt c^{II} was back titrated with $K_3Fe(CN)_6$ as oxidant. All titrations were performed with a Metrohm potentiograph equipped with an automatic titrator. A combination platinum-Ag/AgCl electrode was used as the indicator-reference electrode. Phthiocol was reduced electrolytically in the buffer solution at a mercury cathode just prior to use, using a PAR coulometer system, and the proper amount was transferred with a gas-tight syringe to the titrant reservoir of the titrator.

The concentration of monomeric Mo(V) was estimated by ESR spectrometry at room temperature using a Varian V-4500 spectrometer. As a standard, $K_3Mo(CN)_8$ was prepared in situ by oxidation of $K_4Mo(CN)_8$ with $KMnO_4$ in 1 N H_2SO_4, being particularly careful to protect the solutions from light ($K_3Mo(CN)_8$ is extremely photosensitive). The concentration of spins was determined by double integration of the area under the first derivative curve and compared with the doubly integrated area of the standard.

All kinetic data were treated with appropriate programs on a PDP-8 computer to obtain rate constants.

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[1]Abbreviations used: Im, imidazole; DCIP, 2,6-dichlorophenolindophenol; ESR, electron spin resonance.

TABLE I: Properties of cyt c^{III} Complexes.

Ligand	K_f (M^{-1})	k_{dis} (s^{-1})	$E°$ (calcd)[a] (V)	$E°$(exptl)[b] (V)
CN^-	4.3×10^3 [c]	1.3×10^{-5} [c]	0.055	−0.40[e]
N_3^-	4.5[d]	5.2[d]	0.224	0.183 ± 0.005
Im	15[d]	2.4[d]	0.198	0.151 ± 0.008
Native				0.251 ± 0.005[5]

[a]pH 7.00, 25 °C. [b]pH 7.00, μ = 0.50 M, 25 °C. [c]George and Tsou (1952). [d]Sutin and Yandell (1972). [e]George and Schejter (1964).

TABLE II: Rates of Reduction of cyt c^{III} Complexes by I.[a]

Ligand	k
CN^-	$(1.6 \pm 0.2) \times 10^{-5}$ s^{-1}
N_3^-	(5.4 ± 0.5) s^{-1}
Im	(1.9 ± 0.2) s^{-1} [b]
Native	$(2.6 \pm 0.7) \times 10^7$ M^{-1} s^{-1} [c]

[a]pH 7.00, μ = 0.65 M, 25 °C. [b]At concentrations of I ≤ 2.50×10^{-5} M. [c]Lawrence and Spence (1975).

Results

The reactions of Im, N_3^-, and CN^- with cyt c^{III} have been previously investigated and the rate and equilibrium constants for their displacement of the methionine-80 ligand of cyt c^{III} determined (Sutin and Yandell, 1972; George and Tsou, 1952) (Table I).

The reductions of the azide (cyt c^{III}-N_3) and cyanide (cyt c^{III}-CN) complexes of cyt c^{III} by I are much slower than the reduction by I of native cyt c^{III}, and their rates are first-order in cytochrome complex and independent of the concentration of I. The observed first-order rate constants are nearly identical with the reported dissociation rates of these complexes, suggesting I reacts extremely slowly or not at all with cyt c^{III}-N_3 and cyt c^{III}-CN, but reacts rapidly with native cyt c^{III} as it dissociates from the complex (Table II).

The reduction of the imidazole complex (cyt c^{III}-Im) by I is much more complicated and its rate cannot be represented by a simple expression. At low (approximately equal molar) concentrations of I, the rate is nearly independent of the concentration of I and is essentially the same as the rate of dissociation of cyt c^{III}-Im, as with cyt c^{III}-N_3 and cyt c^{III}-CN. At higher concentrations of I, however, the rate increases dramatically and also becomes biphasic, with the relative amounts of the two phases dependent on the concentration of I. At the highest concentrations of I used (100× excess), the rate again becomes monophasic, but first-order plots are not linear.

The departure from first-order plots of the reduction of cyt c^{III}-Im at the highest concentrations of I and the dependence of the rate on the concentration of I at these concentrations suggested the possibility that a reactive Mo(V) monomer species, in equilibrium with the dimer I, might be involved in the reduction. In order to check this, the amount of monomeric Mo(V) present at the highest concentrations of I was estimated by ESR spectrometry. Using a double integration procedure, $\sim 2 \times 10^{-5}$ M monomer was found to be present in 1.00×10^{-3} M solutions of I in the presence of 0.5 M Im at pH 7.0 and 25 °C. From these data, a value of the equilibrium constant for the formation of monomer was estimated:

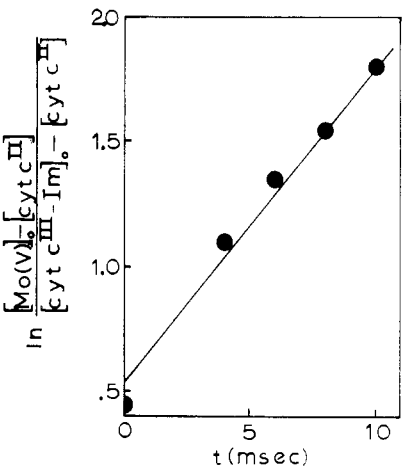

FIGURE 1: Second-order plot of the reduction of cyt c^{III}-Im by Mo(V) monomer. $[Mo(V)]_0 = 2.82 \times 10^{-5}$ M; $[\text{cyt } c^{III}\text{-Im}]_0 = 1.68 \times 10^{-5}$ M; 0.50 M imidazole; 0.20 M phosphate, pH 7.00; μ = 0.65 M; 25 °C.

$$I \rightleftarrows [Mo(V)]^2$$

$$K_1 = \frac{[Mo(V)]^2}{[I]} = (4.0 \pm 1.0) \times 10^{-7}\ M^{-1} \qquad (1)$$

Using this value of the equilibrium constant to calculate Mo(V) monomer concentration, the rate data at the highest concentrations of I were treated using a second-order rate expression, first-order in cyt c^{III}-Im and first-order in Mo(V) monomer. The results gave an excellent straight line to >90% reaction with a rate constant of $1.4 \pm 0.2 \times 10^7$ M^{-1} s^{-1} (Figure 1) (this treatment assumes equilibrium 1 is slow relative to the reduction of cyt c^{III}-Im by monomer).[2]

To determine if the inability of I to reduce cyt c^{III}-N_3 and cyt c^{III}-CN and the considerably reduced rate of reduction of cyt c^{III}-Im relative to native cyt c^{III} are due to thermodynamic considerations, $E°$ for cyt c^{III}-N_3 and cyt c^{III}-Im was measured by potentiometric titration. $E°$ for cyt c^{III}-CN was impossible to measure because of a lack of a suitable mediator (it has been estimated to be ~−0.4 V (George and Schejter, 1964)). The value for native cyt c^{III} was also measured as a check on the method. The titration curves for the reduction of cyt c^{III}-N_3 and cyt c^{III}-Im and the oxidation of the corresponding reduced species show some hysteresis, and plots of E vs. log [cyt c^{III}-X]/[cyt c^{II}], while reasonably linear, have slopes deviating significantly from the theoretical value for a one-electron reduction (Figure 2), indicating a lack of reversibility of the systems. The theoretical values for $E°$, calculated from the formation constants for cyt c^{III}-N_3 and cyt c^{III}-Im, and the experimental results are found in Table I.

Discussion

The data indicate cyt c^{III}-N_3, cyt c^{III}-CN, and cyt c^{III}-Im (the latter at low concentrations of I) are not reduced directly by I; their reduction probably proceeds via a rate-controlling dissociation of the ligand, followed by the known (Lawrence and Spence, 1975) fast reduction of native cyt c^{III} by I:

$$\text{cyt } c^{III}\text{-X} \rightleftarrows \text{cyt } c^{III} + X \qquad (2)$$

$$\text{cyt } c^{III} + I \rightarrow \text{cyt } c^{II} + MoO(OH)_3L^{2-} + MoO_4^{2-} + L \qquad (3)$$

[2]Because of the high reaction rate, k_9 had to be obtained from the last 25–35% of the reaction, which accounts for the large uncertainty in the value (at initial concentrations of 2.8×10^{-5} M monomer and 1.90×10^{-5} M cyt c^{III}-Im, $t_{1/2} \cong 2$ ms; the dead time of the instrument is 3–4 ms).

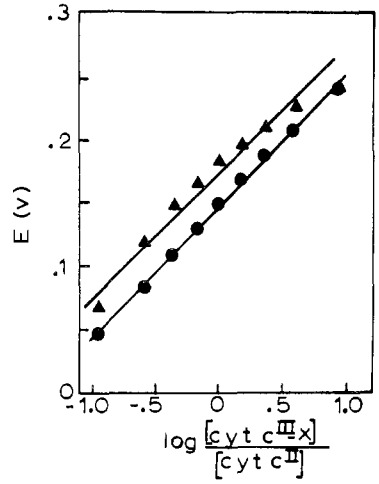

FIGURE 2: Potentiometric titration of cyt c^{III}-X with reduced phthiocol. E vs. NHE (normal hydrogen electrode) is plotted against log ([cyt c^{III}-X]/[cyt c^{II}]). (●) [cyt c^{III}-Im] = 1.30×10^{-4} M; 0.5 M imidazole. (▲) [cyt c^{III}-N_3] = 7.70×10^{-5} M, 0.50 M NaN_3, 0.02 M phosphate, pH 7.00; μ = 0.65 M; 25 °C. DCIP used as mediator.

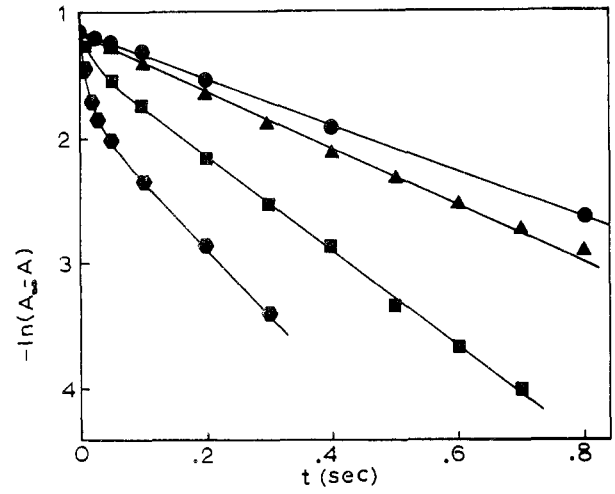

FIGURE 3: First-order plots for reduction of cyt c^{III}-Im. (●) $[I]_0 = 2.50 \times 10^{-5}$ M; (▲) $[I]_0 = 5.00 \times 10^{-5}$ M; (■) $[I]_0 = 1.00 \times 10^{-4}$ M; (●) $[I]_0 = 2.50 \times 10^{-4}$ M. [cyt c^{III}-Im]$_0$ = 1.90×10^{-5} M; 0.20 M phosphate, pH 7.00; μ = 0.65 M; 25 °C. Absorbance measured at 550 nm.

$$\text{cyt } c^{III} + MoO(OH)_3L^{2-} \rightarrow \text{cyt } c^{II} + MoO_4^{2-} + L \quad (4)$$

(L = cysteine; $MoO(OH)_3L^{2-}$ = postulated Mo(V) monomer; X = N_3^-, CN^-, or Im). The independence of the reduction rate on the concentration of I and the values of the observed first-order rate constants support this mechanism (Tables I and II).

In order to account for the complexity of the reduction of cyt c^{III}-Im at higher concentrations of I, the following mechanism is proposed:

$$\text{cyt } c^{III}\text{-Im} \underset{k_{-5}}{\overset{k_5}{\rightleftharpoons}} \text{cyt } c^{III} + \text{Im} \quad (5)$$

$$\text{cyt } c^{III} + I \xrightarrow{k_6} \text{cyt } c^{II} + MoO(OH)_3L^{2-} + MoO_4^{2-} + L \quad (6)$$

$$\text{cyt } c^{III} + MoO(OH)_3L^{2-} \xrightarrow{k_7} \text{cyt } c^{II} + MoO_4^{2-} + L \quad (7)$$

$$\text{cyt } c^{III}\text{-Im} + I \xrightarrow{k_8} \text{cyt } c^{II} + MoO(OH)_3L^{2-} + MoO_4^{2-} + L \quad (8)$$

$$\text{cyt } c^{III}\text{-Im} + MoO(OH)_3L^{2-} \xrightarrow{k_9} \text{cyt } c^{II} + MoO_4^{2-} + L \quad (9)$$

Reactions 5, 6, and 7 account for the slow, first-order reduction observed at the lowest concentrations of I and correspond to the mechanism of the N_3^- and CN^- reductions postulated above. As the concentration of I is raised, reactions 8 and 9 become important. At the highest concentrations of I, reaction 9 becomes rate controlling since the amount of monomer, dependent on the amount of I initially present, becomes sufficient to completely reduce cyt c^{III}-Im (if reactions 8 and 9 are rapid with respect to equilibrium 1, the amount of monomer available to reduce cyt c^{II}-Im is fixed by the initial concentration of I). At moderate concentrations of I (1×10^{-4} to 5×10^{-4} M), the reaction will be biphasic, with both a rapid pseudo-first-order (eq 8) and a more rapid second-order phase (eq 9), as is in fact observed.

As mentioned, k_9 was estimated from the second-order plot

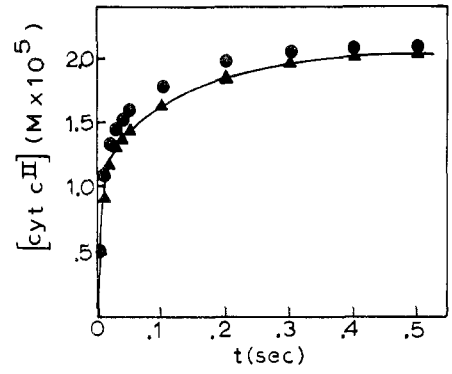

FIGURE 4: Reduction of cyt c^{III}-Im by I. (▲) Experimental data. (●) Computer simulated data. [cyt c^{III}-Im]$_0$ = 2.00×10^{-5} M. $[I]_0$ = 2.50×10^{-4} M. 0.20 M phosphate, pH 7.00; μ = 0.65 M; 25 °C.

at the highest concentrations of I (Figure 1). The value of k_5 was obtained from the first-order plots at the lowest concentrations of I, while k_{-5}, k_6, and a lower limit of k_7 are known from previous work (Lawrence and Spence, 1975; Sutin and Yandell, 1972). The value of k_8 was estimated from the first-order phase of the plots at moderate concentrations of I (Figure 3).[3]

Because of its complexity, an integrated rate expression for the proposed mechanism cannot be obtained. A computer method, which generates time–concentration curves for cyt c^{II}, using the estimated values for the rate constants and K_1 was used. By varying these values systematically over small ranges, the program was used to obtain the best fit of the data (Figure 4). The values used for generating these curves are found in Table III. Clearly, the results are reasonably satisfactory, considering the complexity of the mechanism and the difficulties in estimating the rate constants and K_1. If this mechanism is correct, it indicates cyt c^{III}-Im is reduced by I via three different routes, depending on the concentration of I.

The potentiometric titration measurements indicate the lack

[3]The first-order part of the plots consists of the first-order reaction (eq 5) and the pseudo-first-order reaction (eq 8). Since k_5 is known, $k_8 = (k_{obsd} - k_5)/2[I]$, where k_{obsd} = observed first-order constant. $k_8 = (9.2 \pm 0.9) \times 10^3$ M^{-1} s^{-1}.

TABLE III: Values of Constants Used for Computer Simulation of Data for Reduction of cyt c^{III}-Im by I.[a]

$k_5 = 1.50\ s^{-1}$	$k_8 = 1.0 \times 10^4\ M^{-1}\ s^{-1}$
$k_{-5} = 60\ M^{-1}\ s^{-1}$ [b]	$k_9 = 1.0 \times 10^7\ M^{-1}\ s^{-1}$
$k_6 = 1.8 \times 10^7\ M^{-1}\ s^{-1}$ [c]	$K_1 = 3.4 \times 10^{-7}\ M^{-1}$
$k_7 = 1.5 \times 10^8\ M^{-1}\ s^{-1}$ [c]	

[a] pH 7.00, μ = 0.65 M, 25 °C, except for k_{-5}, k_6, and k_7. [b] pH 7.00, μ = 1.0 M, 25 °C. Sutin and Yandell (1972). [c] pH 7.00, 25 °C, estimated values at μ = 0.65 M. Lawrence and Spence (1975).

of reactivity of cyt c^{III}-N_3 and cyt c^{III}-Im (at low concentrations of I in the latter case) is not due to thermodynamic reasons since the measured $E°$ values are well within the range for complete reduction by I.[4] Clearly, displacement of the methionine-80 ligand by N_3^- and Im is accompanied by conformation changes that drastically alter the reducibility by I. The differences between the observed and calculated $E°$ values of the complexes and the evidence of irreversibility from the titration curves support this conclusion. Furthermore, the estimated rate constant for the reduction of cyt c^{III}-Im by I at moderate concentrations of I is much lower than predicted by the relative Marcus theory, considering the differences in $E°$ between native cyt c^{III} and cyt c^{III}-Im (Bennett, 1973).

In the case of cyt c^{III}-CN, the estimated $E°$ (−0.4 V) is probably too low for reduction by I, although this is not entirely certain, since the lower limit of $E°$ for the Mo(VI)/I couple is unknown.[5]

Clearly, then, the nature of the ligand bound at the sixth coordination site of the heme iron of cyt c^{III} has a profound effect on its rate of reduction by I. In addition to the evidence presented here, it is of interest to note the high pH form of native cyt c^{III}, in which the methionine-80 has been replaced by another ligand (possibly the amino group of lysine-79), is reduced considerably more slowly than native cyt c^{III}, both by I (Lawrence and Spence, 1975) and the outer sphere reducing agent $Fe(EDTA)^{2-}$ (Hodges et al., 1974) and in both cases the differences in rates are much greater than predicted by the relative Marcus theory (Lawrence and Spence, 1975; Hodges et al., 1974). A limited conformation change has been proposed to accompany this change in ligand of cyt c^{III} with pH (Wilson and Greenwood, 1971). Significantly, a lowering of $E°$ to 0.184 V for cyt c^{III} in which methionine-80 has been selectively oxidized to the sulfoxide (but apparently remains ligated) has been interpreted as a loosening of the heme crevice (Ivanetich et al., 1976). Other workers have previously concluded, on the basis of spectral evidence, replacement of methionine-80 by CN^- and N_3^- produces a small conformational change around the heme (Sreenathan and Taylor, 1971).

We conclude this conformational change produced by the replacement of methionine-80 by N_3^-, CN^-, and Im makes the exposed heme edge less available for electron transfer by outer sphere reducing agents. In this regard, recent work indicates the rate of reaction of cyt c with a series of outer sphere reagents is dependent on the degree of penetration by the reagent of the hydrophobic region near the exposed heme edge and the degree of π orbital overlap of the reagent and the heme (Wherland and Gray, 1976). A conformational change that makes either of these factors less favorable should reduce the rate of reduction by I. In addition, in the cases of cyt c^{III}-N_3 and cyt c^{III}-CN, the additional negative charge contributed by the ligand may have an increased inhibitory effect.

Acknowledgment

We are indebted to Professor William M. Moore of this department for the development and use of the computer program to obtain the time-concentration curves for the reduction of cyt c^{III}-Im (Moore and Eccles, 1974).

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[4] I completely reduces flavin mononucleotide (FMN) at pH 8.0 (Kroneck and Spence, 1973); $E°$ for FMN at this pH is −0.25 V (Clark, 1960). $E°$ for the Mo(VI)/I couple is estimated to be < −0.25 V at pH 7.0 (Spence and Lawrence, 1976).

[5] The reported value for native cyt c^{III} is +0.261 V (Margalit and Schejter, 1970).