Model Studies for Molybdenum Enzymes. The Reduction of Cytochrome *c* by Molybdenum(V)–Cysteine Complexes[†]

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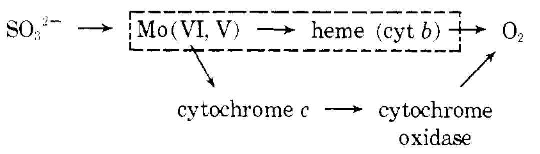
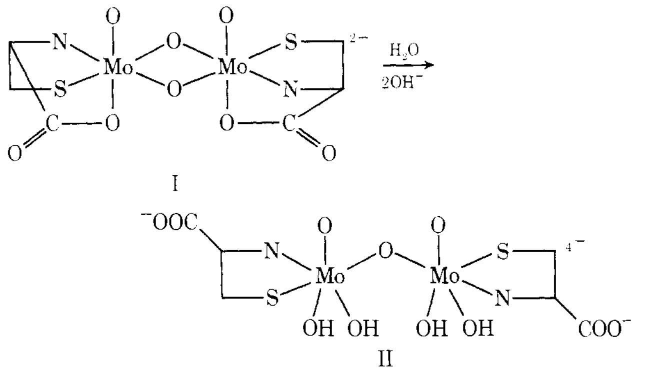
ABSTRACT: The reduction of ferricytochrome *c* by two molybdenum(V)–cysteine complexes has been investigated as a model for electron transfer in the molybdenum enzymes sulfite oxidase and nitrate reductase. The reduction by the dioxo-bridged Mo(V)–cysteine complex, di-μ-oxo-bis-[oxo(L-cysteinato)molybdate(V)] (I), is relatively slow and its rate is first order in cyt c^{III} and zero order in I ($k = (1.09 \pm 0.10) \times 10^{-3}$ sec^{-1}, pH 7.5, 20°). The reduction by the monoxo-bridged complex, μ-oxo-bis[oxodihydroxo(L-cysteinato)molybdate(V)] (II), is extremely rapid and its rate is first order in both reactants ($k = (2.6 \pm 0.7) \times 10^7$ M^{-1} sec^{-1}, pH 7.0, 25°). Above pH 7.5, the reduction by II follows biphasic kinetics due to the fast reduction of a low pH form of cyt c^{III} and a slower reduction of a high pH form (at pH 10.0, 25°, $k = 2.9 \times 10^6$ M^{-1} sec^{-1} for the low pH form and $k = 7.2 \times 10^4$ M^{-1} sec^{-1} for the high pH form). Reaction mechanisms for reductions by both I and II are proposed and the biological implications of the results, both for sulfite oxidase and mechanisms of electron transfer to cytochrome *c*, are discussed.

The enzymes hepatic sulfite oxidase (Cohen et al., 1971) and nitrate reductase of *Neurospora crassa* (Garrett and Nason, 1969) contain both heme iron and molybdenum, and it is generally thought that electrons are transferred intramolecularly between these components during catalysis. Furthermore, in the case of sulfite oxidase, cytochrome *c* appears to be a natural electron acceptor for the enzyme, being reduced more rapidly than the heme iron cofactor, and providing an alternative pathway to oxygen via cytochrome oxidase (Cohen et al., 1972):



The dioxo-bridged complex I (di-μ-oxo-bis[oxo(L-cysteinato)molybdate(V)]) is a well-characterized species, the structure of which has been determined by X-ray crystallography (Knox and Prout, 1969). In basic solution (pH 7–11) I is slowly converted to the monoxo-bridged complex II (μ-oxo-bis[oxodihydroxo(L-cysteinato)molybdate(V)] (Kroneck and Spence, 1973a,b):



I

II

Since enzymatic molybdenum appears to be bound to a cysteine side chain of the protein (Bray and Swann, 1972), both I and II have been used in a number of model studies. Recently, complex II has been shown to be much more reactive toward oxidation by flavines than complex I (Kroneck and Spence, 1973c).

As a model system for electron transfer in these enzymes, the reduction of cytochrome *c* (cyt c^{III}) by these molybdenum(V)–cysteine complexes has been investigated. In addition, the results are of value with respect to mechanisms of electron transfer by cytochrome *c*, a research subject of great current interest (Ewall and Bennett, 1974; Bennett, 1973; Yandell et al., 1973).

Experimental Section

Horse heart ferricytochrome *c* (Sigma types III and VI) was used without further purification. Their purity was determined by measuring the absorbance at 550 nm and comparing the values with published molar absorptivities (Margoliash and Frohwirt, 1959). The synthesis of complex I has been reported (Kay and Mitchell, 1970), and stock solutions of complex II were prepared in situ from complex I in pH 10.0 borate buffer, both in the presence and absence of added cysteine (Kroneck and Spence, 1973b). The concentration of complex II was determined by the absorbance at 635 nm and of complex I by the polarographic wave height at −1.4 V vs. AgCl electrode. Due to the sensitivity of II to air oxidation, solutions deaerated with prepurified nitrogen were prepared immediately before use and kept under prepurified nitrogen. All buffers were made from reagent grade chemicals.

Reduction of cyt c^{III} by I was studied by mixing the proper amounts of deaerated stock solutions of the reactants in a stoppered spectrophotometer cell under nitrogen. The stock solutions were kept in a constant temperature bath under nitrogen, and the spectrophotometer cell was maintained at constant temperature by a water jacketed cell holder. Absorbance at 550 and 695 nm was recorded as a function of time on a Varian Techtron 635 spectrometer equipped with an X–Y recorder.

Reduction of cyt c^{III} by II was followed at 550 nm on an

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Aminco-Morrow stopped-flow spectrometer, equipped with a constant temperature circulating system. The proper amounts of deaerated reactant stock solutions and buffer, kept in the constant temperature bath under nitrogen, were transferred with gas-tight syringes to the loading syringes under nitrogen and measurements were made as rapidly as possible (1-2 min). The amount of stock solution of II in pH 10.0 buffer used was such that the pH of the buffer solution in the loading syringes was not affected. Each run at a given concentration was repeated at least three times, and the superimposed tracings on the oscilloscope were photographed. Before use, the spectrometer was flushed repeatedly with deaerated buffer solution, and prepurified nitrogen was continuously passed around the mixing chamber. The dead time of the instrument is approximately 3-4 msec.

The rate data from the recorder or the oscilloscope photographs were treated with appropriate least-squares programs on a PDP-8 computer to obtain rate constants and activation parameters.

For most runs, II was prepared from I in the presence of added cysteine (20-fold excess in order to increase the rate of formation of II and to inhibit its hydrolysis (Kroneck and Spence, 1973)). Cysteine itself is known to reduce cyt c^{III} slowly (Levander et al., 1973), with a rate constant (calculated from the data of Levander, et al.) of $\sim 2 \times 10^{-1}$ M^{-1} sec^{-1} at pH 7.45. The reduction of cyt c^{III} by cysteine was checked over the pH range of interest and found to be far too slow to be of any significance with respect to the reduction by II. Complex II prepared in the absence of cysteine was found to reduce cyt c^{III} at the same rate as complex II prepared in the presence of cysteine, thereby demonstrating cysteine has no effect on the reaction rate.

Complex II is in a slow, pH dependent equilibrium with I (Kroneck and Spence, 1973b). To determine if appreciable amounts of I are formed from II during the time between mixing of II (at pH 10.0) with the desired buffer and the kinetic measurements (1-2 min) the rate of conversion of II to I at pH 7.0 was measured by monitoring the absorbance at 305 nm (I). The results ($k = 1.2 \pm 0.2) \times 10^{-4}$ sec^{-1}) indicate the amount of I formed during this time interval is negligible.

Reductions of both Sigma types III and VI cyt c^{III} were found to proceed with identical rates within experimental error; type III was therefore used in this work.

In order to obtain evidence for complex formation between cyt c^{III} and I, a solution containing equivalent amounts of cyt c^{III} and I at ~10° (cold room) was added to a column of Sephadex G-25 under N_2. The cytochrome passed through the column and was collected and its absorbance at 550 nm measured, indicating about ⅓ of the cyt c^{III} had been reduced. No further reduction occurred, however, upon standing, indicating I remained on the column. Similarly, when a solution of cyt c^{II} + I was added to the column, I was quantitatively retained while cyt c^{II} passed through.

Results

Reduction of Cyt c^{III} by Complex I. In the pH range 6.0-8.0 complex I reduces cyt c^{III} rather slowly, as compared with many other reductants. Kinetic studies, made by following the absorbance change at 550 and 695 nm, indicate the reaction is first order in cyt c^{III} and, surprisingly, zero order in I over a wide concentration range (Table I). Such a zero order dependence on reductant appears to be unusual for cyt c^{III} reductions. The activation parameters for the reaction, obtained from the usual plot of ln (k/T) vs. $1/T$ are 22.8 ± 2.8 kcal for $\Delta H^{\ddagger}$ and 6.8 ± 6.2 cal for $\Delta S^{\ddagger}$. No studies could be made at pH >8.0 because of the rapid conversion of I to II. Addition of MoO_4^{2-} was without effect on the rate.

Table I: Rate Constants for the Reduction of Cyt c^{III} by Complex I.

$[I]_0$ ($M \times 10^4$)	pH	Temp (°C)	[Buffer] (M)	k_{obsd} ($\text{sec}^{-1} \times 10^3$)
4.00	6.00	20.0	0.10	0.45
8.00	6.00	20.0	0.10	0.53
4.00	6.40	20.0	0.10	0.53
8.00	6.40	20.0	0.10	0.83
4.00	6.90	20.0	0.10	0.62
8.00	6.90	20.0	0.10	0.83
0.200	7.50	20.0	0.10	0.87
0.400	7.50	20.0	0.10	1.02
0.800	7.50	20.0	0.10	1.05
1.60	7.50	20.0	0.10	1.15
4.00	7.50	20.0	0.10	1.18
8.00	7.50	20.0	0.10	0.99
20.00	7.50	20.0	0.10	1.02
40.00	7.50	20.0	0.10	1.09
4.00	8.00	20.0	0.10	2.72
8.00	8.00	20.0	0.10	2.38
2.00	7.50	20.0	0.01	1.13
4.00	7.50	20.0	0.01	1.27
4.00	7.50	20.0	0.28	1.17
8.00	7.50	20.0	0.28	1.12
4.00	7.50	10.0	0.10	0.33
4.00	7.50	15.0	0.10	1.14
4.00	7.50	20.0	0.10	1.38
4.00	7.50	25.0	0.10	3.15
4.00	7.50	30.0	0.10	5.76

[a] $[\text{Cyt } c^{III}]_0 = 4.00 \times 10^{-5}$ M; $\mu = 0.50$ (adjusted with NaCl), phosphate buffer; $\Delta H^{\ddagger} = 22.8 \pm 2.8$ kcal; $\Delta S^{\ddagger} = 6.8 \pm 6.2$ cal, 25° pH 7.5; $k_{obsd} = (1.09 \pm 0.10) \times 10^{-3}$ sec^{-1}, pH 7.5, 20°.

Since the zero order dependence of the rate on the reductant suggests a preliminary complex formation between I and cyt c^{III}, attempts were made to obtain evidence for such a species. Electronic spectra taken immediately after mixing of reactants showed no difference in absorption, nor was there any change in the polarographic reduction potential for I upon the addition of cyt c^{III}. Furthermore, no spectral or polarographic evidence for complex formation was obtained from solutions of I and reduced cytochrome c (cyt c^{II}). Attempts to demonstrate complex formation between cyt c and I by the use of chromatography on Sephadex also gave negative results.

To obtain evidence for possible intermediates in the reaction, ESR spectra at room temperature and liquid nitrogen temperature were obtained by sampling the reaction at various times. No evidence for appreciable concentrations of molybdenum(V) monomer (ESR active) was obtained, in contrast to studies of the reduction of flavines by II (Kroneck and Spence, 1973c).

Reduction of Cyt c^{III} by Complex II. The reduction of cyt c^{III} by complex II differs considerably from the reduction by complex I. First, the rate is several orders of magnitude greater and, second, it follows a rate law first order in both reactants (Table II). It is, in fact, the most rapid rate reported for reduction of cyt c^{III} by a metal complex (Bennett, 1973), and approaches the limit of the stopped-flow apparatus under the conditions (a lower limit to concentrations of about 10^{-5} M for II is imposed because of the difficulty of removal of the last traces of O_2, with which it reacts fairly rapidly). It should be pointed out that for the

Table II: Rate Constants for the Reduction of Cyt c^{III} by Complex II.

$[\text{Cyt } c^{III}]_0$ ($M \times 10^5$)	$[\text{II}]_0$ ($M \times 10^5$)	Temp (°C)	pH	μ (M)	k_{obsd} (sec^{-1})	k'_{obsd} (M^{-1} sec^{-1} $\times 10^{-6}$)	$k_{obsd}/[\text{II}]_0$ (M^{-1} sec^{-1} $\times 10^{-4}$)
1.95	2.55	25	6.0	0.76		8.8	
1.95	2.02	25	6.0	0.76		6.7	
2.01	2.01	25	8.0	0.76		5.6	
1.99	2.51	25	8.0	0.76		7.4	
1.75	4.13	25	10.0[a]	0.76		2.9[a]	
1.75	2.06	25	10.0[a]	0.76		2.9[a]	
1.65	10.3	25	10.0	0.76	7.8		7.6
1.37	20.6	25	10.0	0.76	14.9		7.2
1.22	30.9	25	10.0	0.76	22.8		7.4
1.31	51.6	25	10.0	0.76	34.0		6.6
1.00	0.67	11	7.0	0.40		16.1	
1.00	1.34	11	7.0	0.40		14.3	
1.00	0.67	15	7.0	0.40		19.1	
1.00	1.34	15	7.0	0.40		19.8	
1.00	0.67	20	7.0	0.40		24.0	
1.00	0.90	20	7.0	0.40		20.0	
1.00	1.34	20	7.0	0.40		27.5	
1.00	1.90	20	7.0	0.40		24.3	
1.00	0.30	25	7.0	0.40		26.3	
1.00	0.67	25	7.0	0.40		38.3	
1.00	0.80	25	7.0	0.40		22.6	
1.00	0.90	25	7.0	0.40		20.8	
1.00	1.90	25	7.0	0.40		21.0	
1.00	0.67	30	7.0	0.40		28.2	
1.00	1.34	30	7.0	0.40		29.6	
1.00	0.67	25	7.0	1.35		5.9	
1.00	1.34	25	7.0	1.35		7.9	

[a] pH jump: cyt c^{III} in weak pH 7.0 buffer, mixed with II in strong pH 10.0 buffer. pH 6.0, 8.0, 0.1 M phosphate; pH 10.0, 0.5 M borate. μ adjusted with NaCl. All rate constants are averages of two or more values. $k'_{obsd} = (2.6 \pm 0.7) \times 10^7 M^{-1} sec^{-1}$, 25°, pH 7.0, μ 0.40. $\Delta H^{\ddagger} = 4.8 \pm 1.3$ kcal/mol, $\Delta S^{\ddagger} = -8.6 \pm 4.4$ cal, 25°, pH 7.0.

most rapid group of runs (pH 7.0, μ 0.40, temperature, 11-30°) the half-life of the reaction is between 1.6 and 8.4 msec. Since the dead time of the instrument is 3-4 msec, many of the data were obtained only for the last 30-50% of reaction. The results, therefore, must be interpreted somewhat cautiously. The linearity of the plots (for greater than 90% reaction), the general agreement of the rate constants for different concentrations of II (Table II), and the uncertainty values in the rate parameters indicate the results are quite reasonable for such rapid reactions.

Above pH 7.5, the reduction gives biphasic kinetics due to the presence of two forms of cyt c^{III}, both of which are reduced by II, but at different rates. The presence of two species of cyt c^{III} at basic pH is well known and the relatively slow equilibrium between them has been studied (Wilson and Greenwood, 1971). In order to demonstrate this, and to obtain rate constants for the reduction of the low pH form at high pH, pH-jump experiments were performed. Under these conditions, in which cyt c^{III}, weakly buffered at pH 7.0, was mixed with II, strongly buffered at pH 10.0, no biphasic kinetics were observed, due to the fact that conversion of the low pH form to the high pH form is much slower than the reduction by II. It was found that the rate of reduction of the low pH form decreases with increase of pH (Table II). Similar biphasic kinetics have recently been reported for the reduction of cyt c^{III} by Fe(EDTA)$^{2-}$ (Hodges et al., 1974). No data were obtained below pH 6.0 because of the equilibrium between I and II. Activation parameters ($\Delta H^{\ddagger} = 4.8 \pm 1.3$ kcal, $\Delta S^{\ddagger} = -8.6 \pm 4.4$ cal) for the reduction of the low pH form were obtained from the plot of ln (k/T) vs. $1/T$. As expected for two species of opposite charge, increasing ionic strength reduces the rate considerably (Table II).

Discussion

The slow first-order reduction of cyt c^{III} by complex I is in marked contrast to the extremely rapid second-order reduction by complex II. Clearly, different mechanisms are most likely involved in the two cases.

Reduction of Cyt c^{III} by Complex I. There are at least three mechanisms that are in agreement with the kinetic data for the reduction of cyt c^{III} by I. The first involves the preliminary formation of a complex between I and cyt c^{III} (reaction 1) which reacts subsequently in a rate-controlling step (reaction 2) to give products:

$$\text{cyt } c^{III} + \text{I} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{cyt } c^{III}\text{–I} \tag{1}$$

$$\text{cyt } c^{III}\text{–I} \xrightarrow{k_2} \text{cyt } c^{II} + \text{MoO}_2(\text{OH})\text{L}^{2-} + \text{MoO}_4^{\ 2-} + \text{L} \tag{2}$$

$$\text{cyt } c^{III}\text{–I} + \text{MoO}_2(\text{OH})\text{L}^{2-} \xrightarrow{k_3} \text{cyt } c^{II} + \text{MoO}_4^{\ 2-} + \text{L} + \text{I} \tag{3}$$

In this mechanism, $\text{MoO}_2(\text{OH})\text{L}^{2-}$ is presumed to be the structure of the Mo(V) monomer formed by the one-electron oxidation of I by cyt c^{III}. This reactive monomer then rapidly reduces another cyt c^{III}–I complex (reaction 3). In the reduction of flavines by II under similar conditions a large intermediate ESR signal due to Mo(V) was observed (Kroneck and Spence, 1973c). Since no such signal was found in this study, reaction 3 would have to be considerably faster than 2. The Mo(VI) product is MoO_4^{2-}, since

Mo(VI) does not complex appreciably with cysteine at pH >6.0. This mechanism gives the rate expression:

$$\mathrm{d}[\text{cyt } c^{\mathrm{II}}]/\mathrm{d}t = \frac{2k_2K[\mathrm{I}][\text{cyt } c^{\mathrm{III}}]}{1 + K[\mathrm{I}]}$$

$$K = k_1/k_{-1}$$

If $K[\mathrm{I}] \gg 1$, this reduces to the experimental rate law:

$$\mathrm{d}[\text{cyt } c^{\mathrm{II}}]/\mathrm{d}t = 2k_2[\text{cyt } c^{\mathrm{III}}]$$

Since the rate is independent of the concentration of I at the lowest concentration used (2.00×10^{-5} M), the minimum value of K is $\sim 10^6$, which indicates the cyt c^{III}-I complex would have to be quite strong. Furthermore, since no changes in the spectrum of cyt c^{III} were observed immediately after the addition of I, and the rate of reduction when following the 695-nm absorption (generally associated with the integrity of the methione-80 sulfur-iron bond (Wilson and Greenwood, 1971)) is the same as when the 550-nm absorption is followed, the site of complexing of I with cyt c^{III} would not likely be at the heme iron.

A second mechanism involves the preliminary formation of a "dead end" complex (Wilkins, 1974):

$$\mathrm{I} + \text{cyt } c^{\mathrm{III}} \rightleftarrows \text{cyt } c^{\mathrm{III}}\text{-I}$$

$$\mathrm{I} + \text{cyt } c^{\mathrm{III}} \longrightarrow \text{products}$$

This leads to the same rate expression as the first mechanism and cannot be distinguished kinetically from it.

In the reduction of cyt c^{III} by Cr(II) in chloride media the rate becomes independent of Cr(II) concentration at high Cr(II) concentrations (Yandell et al., 1973). In that case a third type of mechanism involving the opening of the heme crevice and breaking of the methionine-80 sulfur-iron bond as the rate-determining step was proposed. That reaction has a first-order rate constant of 60 sec^{-1}, however, which eliminates it from consideration in the reduction by I. A much slower conformation change as a rate-controlling step, however, cannot be eliminated:

$$\text{cyt } c^{\mathrm{III}} \rightleftarrows \text{cyt } c^{\mathrm{III}'} \qquad \text{(slow)}$$

$$\text{cyt } c^{\mathrm{III}'} + \mathrm{I} \longrightarrow \text{products} \qquad \text{(fast)}$$

Such a mechanism also leads to the same kinetic expressions as those involving complex formation, but again no evidence for such a slow conformational change is available. Clearly, no choice can be made at this time between these mechanisms.

Reduction of Cyt c^{III} by Complex II. The reduction of Cyt c^{III} by complex II is first order in each reactant over the range of concentrations studied. Because of the rapidity of the reaction, however, this range is necessarily limited; furthermore, the absorbance changes at 695 nm could not be followed, nor could attempts to detect reactive intermediates be made (as in the case of reduction by I). The following mechanism, involving an initial one-electron transfer between cyt c^{III} and II as the rate-controlling step (reaction 4), followed by a faster reaction between cyt c^{III} and $\mathrm{MoO_2(OH)L^{2-}}$ monomer, is in agreement with the data:

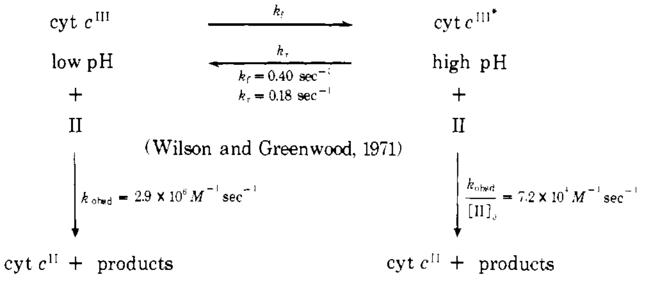
$$\text{cyt } c^{\mathrm{III}} + \mathrm{II} \xrightarrow{k_4} \text{cyt } c^{\mathrm{II}} + \mathrm{MoO_2(OH)L^{2-}} + \mathrm{MoO_4{}^{2-}} + \mathrm{L} \qquad (4)$$

$$\text{cyt } c^{\mathrm{III}} + \mathrm{MoO_2(OH)L^{2-}} \xrightarrow{k_5} \text{cyt } c^{\mathrm{II}} + \mathrm{MoO_4{}^{2-}} + \mathrm{L} \qquad (5)$$

This mechanism leads directly to the observed rate law:

$$\mathrm{d}[\text{cyt } c^{\mathrm{II}}]/\mathrm{d}t = 2k_4[\text{cyt } c^{\mathrm{III}}][\mathrm{II}]$$

Scheme I



pH 10, 25°

The biphasic kinetics observed at pH >7.5 are explained by Scheme I. The results of the pH-jump experiments, in which the low pH form is reduced before it can be converted to the high pH form, are in agreement with this scheme. Similar biphasic kinetics with rate constants approximately three orders of magnitude lower have been reported for the reduction of cyt c^{III} by $\mathrm{Fe(EDTA)^{2-}}$ (Hodges et al., 1974). Biphasic reductions have also been observed with ascorbate (Wilson and Greenwood, 1971) and ferrocyanide (Brandt et al., 1966), but in these cases the high pH form does not react with the reductant, the slow phase being due to the conversion of the high pH form to the low pH form, which is then reduced. Again as with $\mathrm{Fe(EDTA)^{2-}}$ (Hodges et al., 1974), the difference in reduction rates for the two pH forms is somewhat greater than that predicted by the relative Marcus theory, suggesting some factors other than the difference in E^0 values for the two species of cyt c^{III} are responsible for the difference in reduction rates of the two forms.

Reduction of cyt c^{III} by a number of species has recently been interpreted in terms of two general mechanisms (Yandell et al., 1973; Creutz and Sutin, 1973). In one case, the rate-determining step is a relatively slow heme crevice opening ($k = 60$ sec^{-1}), followed by a rapid reduction of the heme iron. The second mechanism involves a remote attack at the exposed heme edge or elsewhere on the periphery of the protein. Reductions proceeding with a rate greater than that of the heme crevice opening presumably follow a remote pathway.

Recently, work by Grimes et al. (Grimes et al., 1974) on the reduction of cyt c^{III} by Cr(II) implicates tyrosine-67 as the site of attack by this ion, in support of the crevice opening mechanism (tyrosine-67 forms part of the crevice lining). The electron is then proposed to be transferred to the heme via overlap of the π clouds of the tyrosine and the heme. Similar mechanisms have been proposed and discussed previously (Takano et al., 1973; Salemme et al., 1973). An alternative interpretation of the Cr(II) work has been proposed by Creutz and Sutin (Creutz and Sutin, 1974).

As discussed above, reduction by I may proceed by initial complex formation with cyt c^{III} at some site other than the heme iron. This may possibly be at tyrosine-67, although a comparison of models of I with the various distances reported for the cyt c^{II}-Cr(III) complex (Grimes et al., 1974) suggests I is probably too large to bind at this position in the crevice.

The reduction of cyt c^{III} by complex II appears to occur by the remote pathway. In the case of $\mathrm{Fe(EDTA)^{2-}}$, the ability to reduce both forms of cyt c^{III} was interpreted in terms of an outer sphere mechanism, probably at the exposed edge of the heme (Hodges et al., 1974). Similar arguments may be applied to the reduction by II. The value of

$\Delta H^{\ddagger}$ (4.8 kcal) is in keeping with an outer sphere mechanism and the entropy term is not greatly different than for other reductions of cyt c^{III} by outer sphere metal complexes (Ewall and Bennett, 1974; Hodges et al., 1974). If this reduction by complex II does in fact proceed via an outer sphere mechanism at the exposed heme edge, it indicates such a mechanism can be very rapid indeed. The second-order rate constant for the reduction by II is about twice as large as that for the reduction in vitro by the natural reductant, cyt c_1^{II} (3.3×10^6 M^{-1} sec^{-1} at pH 7.4 and 10°) (Yu et al., 1973). Thus, a similar mechanism has much to recommend it in considering the in vivo reaction (Ewall and Bennet, 1974).

In addition to the above considerations with regards to the mechanism of reduction of cyt c^{III}, the biological implications with respect to the enzyme sulfite oxidase are of considerable interest. This enzyme contains two molybdenum centers per molecule, both of which appear to be reduced from oxidation state +6 to +5 by substrate (Cohen et al., 1971). As indicated above, in the presence of cyt c^{III}, electrons are not transferred to the heme iron of the enzyme until all the cyt c^{III} is reduced, cyt c^{III} apparently being a natural electron acceptor for the enzyme (Cohen et al., 1972). Furthermore, the rate-controlling step for the enzymatic oxidation of sulfite is reported to be the reoxidation of reduced enzyme by the electron acceptor (Cohen et al., 1972). Using the published data for the rate of reduction of cyt c^{III} by reduced sulfite oxidase (of ~88% purity) (Cohen and Fridovich, 1971), a second-order rate constant of 4×10^6 M^{-1} sec^{-1} at pH 8.5 and 25° can be estimated. Clearly, the rate of reduction of cyt c^{III} by complex II is as fast or faster than that of the reduced enzyme, making it an excellent kinetic model for the enzymatic reaction. This suggests that similar monoxo-bridged Mo(V) dimers, with the molybdenum ions coordinated to cysteine residues of the protein, may be present at the molybdenum center of the enzyme. The large Mo(V) monomer signals observed during catalysis by the enzyme (Cohen et al., 1971) might then be due to transient Mo(V) monomers or mixed valence Mo(V)-Mo(VI) species produced by one-electron oxidations of these dimers by the electron acceptors (ESR kinetic studies using the fast freezing method of Bray would be interesting in the present investigation; such equipment is not currently available in this laboratory). In any case, the results indicate such monoxo-bridged dimers are certainly compatible with the kinetic characteristics of sulfite oxidase.

Finally, the results point up the enormous difference in reactivity between complexes I and II (which in this case also appears to involve an entirely different mechanism), two compounds with very similar structures. As was also found in the reduction of flavines by the two species (Kroneck and Spence, 1973c), the dioxo bridge confers a certain inertness toward oxidation, although the reason for this is not certain. Recent theoretical studies of both monoxo- and dioxo-bridged Mo(V) dimers (Blues et al., 1974; Brown et al., 1972) indicate a considerably higher electronic density in the Mo-Mo, d_σ-d_σ bond in the dioxo-bridged structure, producing a less favorable overlap with oxidant orbitals. It may be of interest in this respect that the disulfur bridged analog of I (Kay and Mitchell, 1970) is quite stable in basic solution, not undergoing bridge opening to give mono-bridged complex nor oxidation of flavines (Spence, 1973). Further studies of the reactions of both mono- and dibridged Mo(V) complexes are underway to clarify the relationship between structure and reactivity of these interesting compounds.

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