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

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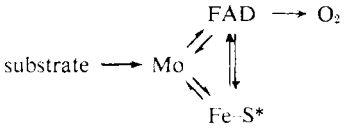
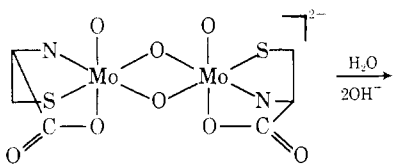
ABSTRACT: The reduction of flavine mononucleotide (FMN) by μ-oxo-bis[oxodihydroxo(L-cysteinato)molybdate(V)] in basic solution, pH 8.0–11.0, has been investigated as a model for Mo(V)–flavine electron transfer reactions in molybdenum flavoenzymes. The reaction, which is remarkably rapid for reductions by Mo(V) complexes, is overall second order, first order in each reactant. Esr studies indicate the reaction proceeds partially at least by an initial one-electron step, followed by disproportionation of the flavosemiquinone into reduced and oxidized FMN. It was found that the reaction rate increases with increasing pH, and is catalyzed by cysteine. A mechanism to explain the cysteine catalysis involving the formation of a reactive flavine–cysteine intermediate complex is proposed. The implications for electron transfer in molybdenum flavoenzymes are discussed.

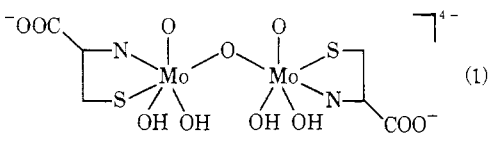
Molybdenum is known to be a necessary redox active cofactor of several complex nonheme iron flavoenzymes such as xanthine oxidase, xanthine dehydrogenase, and aldehyde oxidase (Bray and Swann, 1972). Considerable effort has been expended in attempting to elucidate the respective roles of the molybdenum, flavine, and iron components in reactions catalyzed by xanthine oxidase. Recently, a pooled system with molybdenum as the entrance point for reducing equivalents has been proposed for electron transfer in this enzyme (Bray and Swann, 1972):



Considerable evidence indicates the site of metal binding in xanthine oxidase is the mercapto group of a cysteine residue (Bray and Swann, 1972). Consequently, molybdenum sulfhydryl complexes have been of great interest as models for the active site (Knox and Prout, 1969; Huang and Haight, 1970). In the first two parts of this series, we showed the dioxo bridged molybdenum(V)–cysteine complex, di-μ-oxo-bis[oxo(L-cysteinato)molybdate(V)] (I), undergoes bridge cleavage in basic solution (pH 8–11) to give the monooxo bridged complex, μ-oxo-bis[oxodihydroxo(L-cysteinato)molybdate(V)] (II) (Kroneck and Spence, 1973a,b). Complex II, in contrast to I, was found to reduce flavine mononucleotide (FMN) rapidly (Kroneck and Spence, 1973a), thus serving as an interesting model for electron transfer between molybdenum and flavine in xanthine oxidase.



I



II (1)

We report here the results of a kinetic investigation of the reduction of FMN by complex II.

Experimental Section

Solutions of complex II, μ-oxo-bis[oxodihydroxo(L-cysteinato)molybdate(V)], were prepared as described previously (Kroneck and Spence, 1973b). Flavine mononucleotide and L-cysteine were obtained from Nutritional Biochemicals Co., and were used without further purification. All buffers were made from reagent grade chemicals.

The kinetics for the reduction of FMN by complex II were followed by measuring the height of the polarographic reduction wave of FMN at −0.80 V *vs.* AgCl electrode. Reactions were initiated by adding the proper amount of deareated FMN stock solution with a gas-tight syringe to deareated solutions of complex II in a thermostated polarographic cell. Rapid mixing was ahiceved by the gas stream, using 99.995% pure nitrogen or argon from Matheson and Co.

Continuous flow electron spin resonance (esr) experiments were carried out with the Varian V-4549 liquid flow mixing chamber accessory (2 ml/sec flow rate). Spectra at 77°K were obtained by removing samples from the reaction under nitrogen or argon with a gas-tight syringe and freezing immediately in liquid nitrogen. Spectra were recorded on a Varian V-4500 spectrometer using 100-kHz modulation. 2,2′-Diphenyldipicrylhydrazyl was used as a standard for determining *g* values, and spin concentrations were determined using $K_3Mo(CN)_8$ as a standard for comparison.

Electronic spectra were obtained with a Beckman Acta-V spectrophotometer and polarographic data with a Metrohm E-261 polarograph.

All rate constants were obtained from appropriate plots

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TABLE I: Rate Constants.

Run	pH	$[II]_0$ (M × 10^3)	$[Fl_{ox}]_0$ (M × 10^3)	[Cysteine] (M × 10^2)	k_2[a] (M^{-1} sec^{-1})
1	8.05	0.289	1.17		4.74
2	9.15	0.816	1.51		5.49
3	10.00	1.34	1.59		7.80
4	11.05	0.658	1.11		13.97
5	8.45	0.743	1.13	3.85	8.15
6	8.65	0.946	0.955	3.85	8.57
7	9.00	1.39	1.01	3.85	9.40
8	9.50	1.06	1.41	3.85	11.40
9	10.00	0.539	1.04	3.85	34.14[b]
10	10.00	0.985	1.49	0.610	11.32
11	10.00	0.932	1.49	0.672	13.00
12	10.00	0.999	1.52	1.13	16.21
13	10.00	1.03	1.46	1.78	18.12
14	10.00	0.973	1.46	3.00	22.73

[a] k_2 = observed rate constant (k_{obsd}) at 25° in 0.50 M borate buffer; run 4 in 0.50 M phosphate buffer; for all runs, k_2 is the average value for duplicates. [b] k_2 = 34.14 ± 3.38 M^{-1} sec^{-1} (eight runs).

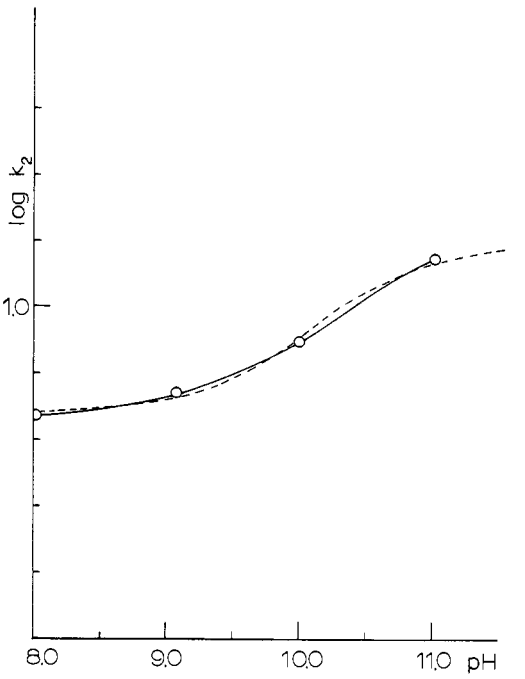


FIGURE 1: pH dependence of observed rate constant, log k_2, of uncatalyzed reaction (2): 0.50 M borate; 25°; (- - -) calculated curve (see text).

using least squares and quadratic programs on a Digital Corp. PDP-8 computer.

The pK_a of the N-3 proton in oxidized FMN was determined by pH titration with standard base using a Metrohm E336A potentiograph.

Results

When FMN is added to anaerobic solutions of II at pH 8–11, both the electronic spectrum and the polarographic reduction wave characteristic of oxidized FMN rapidly disappear, giving the spectrum and oxidation wave of fully reduced FMN. When less than 1 equiv of FMN is used, the resulting visible spectrum is that of unreacted II (λ_{max} 635 nm); Kroneck and Spence, 1973a) and the remaining absorbance indicates a 1:1 stoichiometry.

$$FMN_{ox} + II \longrightarrow 2MoO_4^{2-} + 2cysteine + FMN_{red} \quad (2)$$

It was found that addition of cysteine increases the rate of reaction 2. Recent work (Gibian *et al.*, 1972) has indicated a very slow reduction of FMN by cysteine occurs, but its rate is insignificant compared to the rate of reduction by complex II. It was, therefore, concluded added cysteine catalyzes the reaction. A similar catalysis was observed in the conversion of I to II (Kroneck and Spence, 1973b).

Kinetics. The kinetics of reaction 2 were determined by following the disppearance of FMN polarographically. The reaction was found to be second order overall, first order in FMN, and first order in II. Second-order plots gave good straight lines to 90–95% reaction over a pH range of 8.0–11.0, and consistent rate constants were obtained from the data (Table I).

The rate of the uncatalyzed reaction (no cysteine added) was found to increase with increasing pH, but a plot of log k_2 *vs.* pH gave a curve (Figure 1), indicating a complex pH dependence.

In order to investigate the catalytic effect of cysteine, the rate of the reaction was determined as a function of cysteine concentration at pH 10.0. Again reaction 2 was found to be second order, but a plot of log k_2 *vs.* [cysteine] did not give a straight line, indicating both the catalyzed and uncatalyzed reactions contribute to the overall rate. The complete rate expression is

$$-d[FMN]/dt = k_2'[II][FMN] + k_2''[II][FMN][cysteine]$$

$$k_2 = k_2' + k_2''[cysteine]$$

In accordance with this expression, a plot of k_2 *vs.* [cysteine] gave a straight line, with intercept k_2' = 8.00 M^{-1} sec^{-1}, and slope k_2'' = 600 M^{-1} sec^{-1}. The value of k_2' thus obtained is seen to be in good agreement with the value of k_2 found for the uncatalyzed reaction at this pH (Table I).

Again, as in the uncatalyzed reaction, the effect of pH on the rate in the presence of added cysteine was found to be complex (Figure 2).

Addition of the product, Na_2MoO_4, was found to have no effect on the reaction rate.

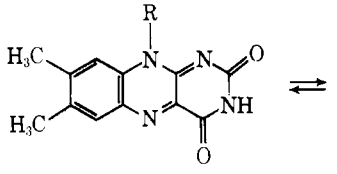
Esr Studies. In order to determine if the reaction proceeds by a one- or two-electron step, esr spectra of the reaction mixture were obtained at room temperature and 77°K. Samples were withdrawn and immediately frozen in liquid nitrogen, and, for room temperature spectra, a flow system in which the reactants were passed through a four-jet mixing chamber was used. In both cases, Mo(V) monomer (g 1.974) and flavosemiquinone (g 2.003) signals were found (Figure 3), while solutions of II or FMN alone gave no signal under these conditions. The Mo(V) monomer signal gives the same g value and hyperfine splitting reported earlier for the Mo(V)–cysteine complex (Kroneck and Spence, 1973a; Huang and Haight, 1970) (Figure 3). The flavine radical concentration was found to be approximately equivalent to that observed with solutions containing the same amounts of oxidized and reduced FMN. Due to the rapidity of the reaction, the first sample for esr measurement at 77°K

could not be obtained before ~25 sec. At this time, as seen in Figure 4, the monomer concentration has already reached or passed its maximum (2.2% total Mo). Assuming this first point is the maximum concentration, the rate constant for monomer formation was estimated from the initial rate, obtained by measuring the slope of the line drawn through this point and the origin of Figure 4, giving a value of 1.62 M^{-1} sec^{-1}. Clearly, this is a minimum value since this procedure ignores the reaction by which the monomer disappears, and would undoubtedly be greater if data for the first few seconds of the reaction could be obtained.

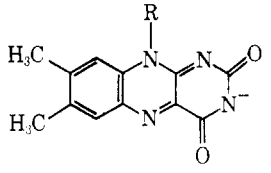
Discussion

The results indicate that although cysteine is released during the uncatalyzed reaction, the amount is not sufficient to markedly influence the rate. As is seen from the values of k_2' and k_2'', the rate of the catalyzed reaction due to released cysteine at 90% reaction is only about 10% of the rate of the uncatalyzed reaction at pH 10.0. Below pH 9.5 the relative rate of the catalyzed reaction is even smaller, due to protonation of released cysteine.

In order to explain the increase of k_2 for the uncatalyzed reaction with increasing pH (Figure 1), the acidic properties of the N-3 proton of the FMN nucleus must be considered (Hemmerich and Spence, 1966).



$Fl_{ox}H$



Fl_{ox}^-

$pK_a = 10.28$

$25°, \mu = 0.10$

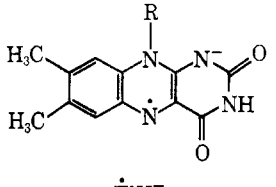
$R = -CH_2(CHOH)CH_2OPO_3^{2-}$

Assuming that both FMN ($Fl_{ox}H$) and its anion (Fl_{ox}^-) react with II, the following reactions may be written.[1]

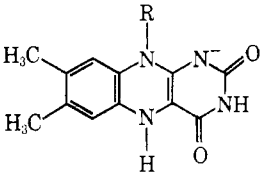
$$Fl_{ox}H + II \xrightarrow{k_3} Fl_{red}H_2^- + 2MoO_4^{2-} + 2cysteine \quad (3)$$

$$Fl_{ox}^- + II \xrightarrow{k_4} Fl_{red}H_2^- + 2MoO_4^{2-} + 2cysteine \quad (4)$$

[1] Because of their acidic properties, FlH^- and $Fl_{red}H_2^-$ are the forms of FMN semiquinone and fully reduced FMN present, respectively, in basic solution (Hemmerich and Spence, 1966).



$\dot{F}lH^-$



$Fl_{red}H_2^-$

$R = -CH_2(CHOH)_3CH_2OPO_3^{2-}$

This gives the rate expression

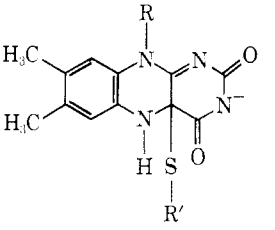
$$-d[II]/dt = -d[Fl_{ox}]/dt = k_3[Fl_{ox}H][II] + k_4[Fl_{ox}^-][II]$$

Substituting from the expression for K_a and rearranging give

$$k_2([H^+] + K_a) = k_3[H^+] + k_4K_a$$

By plotting the left side of this last equation *vs.* $[H^+]$ a straight line with slope k_3 and intercept k_4K_a was obtained, giving $k_3 = 4.68$ M^{-1} sec^{-1} and $k_4 = 15.24$ M^{-1} sec^{-1}. These values were used to calculate the dashed curve of Figure 1, which is in good agreement with the experimental results.

A reasonable explanation of the catalysis by cysteine is the formation of a reactive intermediate complex between cysteine and FMN. Because of its low thermodynamic stability, it is not possible to prove the existence of such a complex by physical methods, but considering well-established properties of the flavine molecule, it may involve a covalent addition of cysteine to the 4a,5-azomethine bond of the flavine nucleus (Brown and Hamilton, 1970).

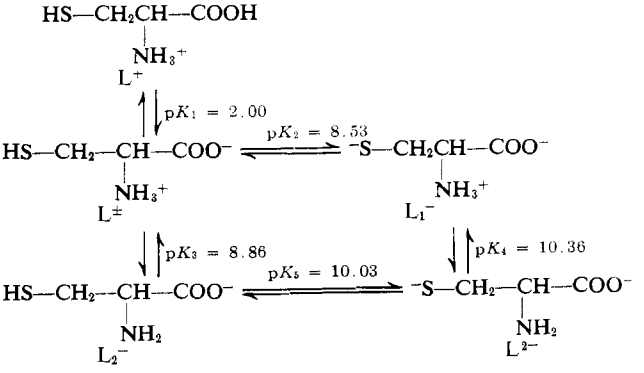


$R = -CH_2(CHOH)_3CH_2OPO_3^{2-}$

$R' = -CH_2CH(NH_3^+)COO^-$ or $-CH_2CH(NH_2)COO^-$

Such an intermediate has been proposed for the reduction of flavines by sulfhydryl compounds (Gibian *et al.*, 1972). Since the reduction of Fl_{ox} involves a change from an essentially planar system to a "butterfly" structure (Hemmerich *et al.*, 1971) the formation of such an intermediate flavine–cysteine complex, which should be considerably distorted from planarity, might accelerate the reaction. Alternatively, the presence of sulfur at this position may provide a low energy path for electron transfer from II to the flavine nucleus.

In order to explain the pH dependence of the rate of the catalyzed reaction, the acidic properties of both FMN and cysteine must be taken into account. Cysteine exists in five forms in solution, depending on pH (Benesch and Benesch, 1955)



Assuming intermediate complexes are formed between the L_1^- and L^{2-} forms of cysteine and both $Fl_{ox}H$ and Fl_{ox}^- leads to the following reactions

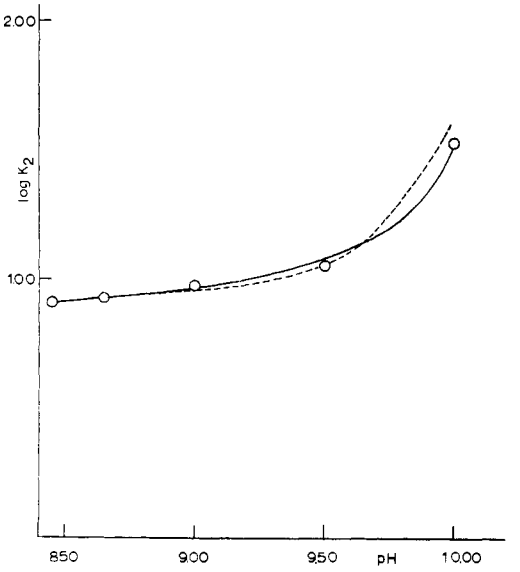


FIGURE 2: pH dependence of observed rate constant, log k_2, for catalyzed reaction in presence of added cysteine: 0.0385 M cysteine, 0.50 M borate; 25°; (- - -) calculated curve (see text).

$$Fl_{ox}H + L_1^- \underset{k_{-5}}{\overset{k_5}{\rightleftharpoons}} C_1 \qquad k_5/k_{-5} = K_{C_1} \qquad (5)$$

$$C_1 + II \xrightarrow{k_6} Fl_{red}H_2^- + 2MoO_4^{2-} + 3\text{cysteine} \qquad (6)$$

$$Fl_{ox}H + L^{2-} \underset{k_{-7}}{\overset{k_7}{\rightleftharpoons}} C_2 \qquad k_7/k_{-7} = K_{C_2} \qquad (7)$$

$$C_2 + II \xrightarrow{k_8} Fl_{red}H_2^- + 2MoO_4^{2-} + 3\text{cysteine} \qquad (8)$$

$$Fl_{ox}^- + L_1^- \underset{k_{-9}}{\overset{k_9}{\rightleftharpoons}} C_3 \qquad k_9/k_{-9} = K_{C_3} \qquad (9)$$

$$C_3 + II \xrightarrow{k_{10}} Fl_{red}H_2^- + 2MoO_4^{2-} + 3\text{cysteine} \qquad (10)$$

$$Fl_{ox}^- + L^{2-} \underset{k_{-11}}{\overset{k_{11}}{\rightleftharpoons}} C_4 \qquad k_{11}/k_{-11} = K_{C_4} \qquad (11)$$

$$C_4 + II \xrightarrow{k_{12}} Fl_{red}H_2^- + 2MoO_4^{2-} + 3\text{cysteine} \qquad (12)$$

Combination with the rate expression for the uncatalyzed reaction (reactions 3 and 4), applying the steady-state treatment to complexes C_1–C_4, and assuming the dissociation reactions of the complexes are much faster than the reactions of C_1–C_4 with II ($k_{-5} \gg k_6$, *e.g.*), the following expression is obtained.

$$-d[II]/dt = k_3[Fl_{ox}H][II] + k_4[Fl_{ox}^-][II] + k_6K_{C_1}[Fl_{ox}H][II][L_1^-] + k_8K_{C_2}[Fl_{ox}H][II][L^{2-}] + k_{10}K_{C_3}[Fl_{ox}^-][II][L_1^-] + k_{12}K_{C_4}[Fl_{ox}^-][II][L^{2-}]$$

Substitution for $[L_1^-]$, $[L^{2-}]$, $[Fl_{ox}H]$, and $[Fl_{ox}^-]$, from the appropriate expressions, gives the rate equation

$$-d[Fl_{ox}]/dt = \frac{[Fl_{ox}][II]}{[H^+] + K_a}\left[k_3[H^+] + k_4K_a + \frac{k_6K_{C_1}K_2K_5C_L[H^+]^2 + k_8K_{C_2}K_2K_4K_5C_L[H^+] + k_{10}K_{C_3}K_2K_5K_aC_L[H^+] + k_{12}K_{C_4}K_2K_4K_5K_aC_L}{[H^+]^2K_5 + [H^+]K_2(K_4 + K_5) + K_2K_4K_5}\right]$$

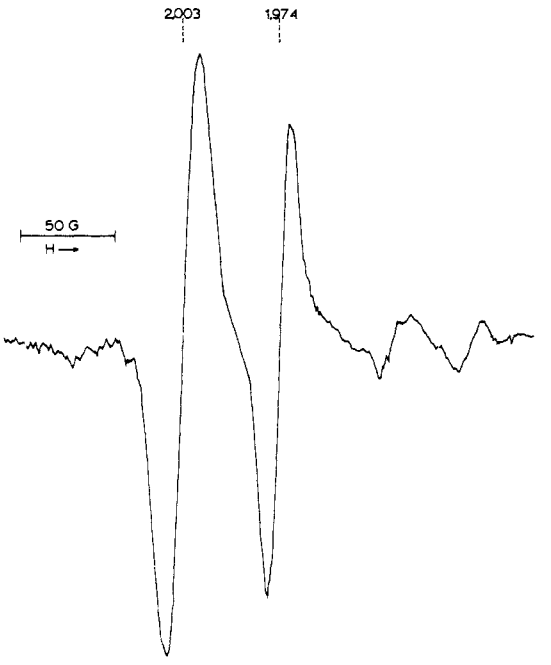


FIGURE 3: Esr spectra of Fl_{ox}–complex II reaction mixture (continuous flow): 0.040 M cysteine, 5.0×10^{-3} M Fl_{ox}, 1.25×10^{-3} M II (pH 10.00), 0.50 M borate, 25°.

At constant pH and C_L (total cysteine), this is identical with the experimental rate expression

$$-d[Fl_{ox}]/dt = k_2[Fl_{ox}][II]$$

Thus

$$(k_2([H^+] + K_a) - k_3[H^+] - k_4K_a)([H^+]^2K_5 + [H^+]K_2(K_4 + K_5) + K_2K_4K_5) = k_6K_{C_1}K_2K_5C_L[H^+]^2 + k_8K_{C_2}K_2K_4K_5C_L[H^+] + k_{10}K_{C_3}K_2K_5K_aC_L[H^+] + k_{12}K_{C_4}K_2K_4K_5K_aC_L$$

At constant C_L, this is an equation of the form $y = a[H^+]^2 + b[H^+] + c$, with a, b, and c being combinations of the various rate and equilibrium constants. Since the value of the left-hand side of this equation is known at each pH, the data could be treated with a computer program to obtain the best values of the constants a, b, and c. Using these values, the dashed curve of Figure 2 was calculated, giving good agreement with the experiment. It is not possible to obtain the values of the individual rate constants for reactions 5–12 occurring in the a, b, and c terms from this treatment. The results show, however, that each term is important, indicating all the reactions contribute to the overall rate in the pH range used.

Previous work in this laboratory has shown the reduction of FMN by Mo(V) in tartrate buffer (pH 2–5), which is considerably slower than the reaction reported here, proceeds by a two-electron step involving Mo(IV) as an intermediate (Colovos and Spence, 1972). The kinetic results in the present case are compatible with either a one- or two-electron reduction. The flavosemiquinone radical (Figure 4) could also arise from either a one- or two-electron step, since its rapid disproportionation is well known (Barman and Tollin, 1972).

$$2\dot{F}lH^- \rightleftharpoons Fl_{ox} + Fl_{red}H_2^- \qquad (13)$$

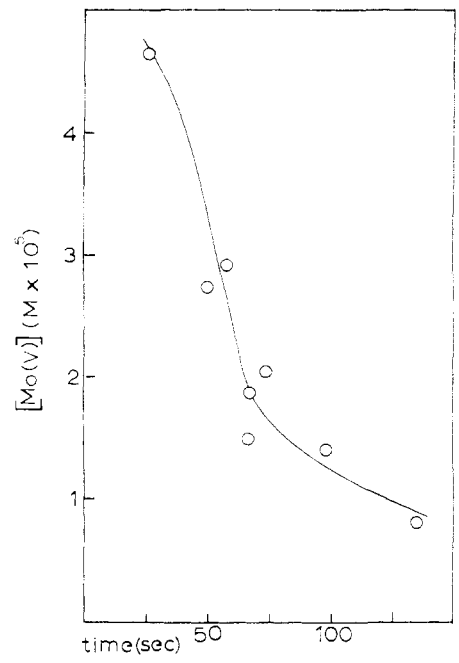


FIGURE 4: Mo(V) monomer concentration during reaction, as determined by esr spectrometry: $[Fl_{ox}]_0 = 1.37 \times 10^{-3}$ M; $[II]_0 = 1.08 \times 10^{-3}$ M; 0.0385 M borate (pH 9.00); 25°.

The formation of an intermediate Mo(V) monomer, however, must be due to a one-electron transfer, since there is no other reasonable way in which it could arise (a two-electron step would result in oxidation of both Mo(V) ions of complex II to Mo(VI) species). This result is most easily rationalized by the following mechanism for the uncatalyzed reaction.

$$Fl_{ox} + II \longrightarrow \dot{F}lH^- + Mo^VO(OH)_2(cysteine)^- + MoO_4^{2-} + cysteine \quad (14)$$

$$2Mo^VO(OH)_2(cysteine)^- \longrightarrow II \quad (15)$$

$$Mo^VO(OH)_2(cysteine)^- + Fl_{ox} \longrightarrow \dot{F}lH^- + MoO_4^{2-} + cysteine \quad (16)$$

The rate-controlling step (reaction 14) is a one-electron transfer in which the Mo(V) monomer species and flavosemiquinone are produced. The monomer may then dimerize (reaction 15), or react with a second Fl_{ox} molecule (reaction 16). The flavosemiquinone formed disproportionates rapidly (reaction 13). Assuming reactions 15 and 16 are fast compared to reaction 14, and applying the steady-state treatment to both Mo(V) monomer and flavosemiquinone, a rate expression identical with the experimental expression is readily obtained.

$$-d[Fl_{ox}]/dt = k_{14}/2[Fl_{ox}][II] \quad \text{(using reaction 15)}$$

$$-d[Fl_{ox}]/dt = 3k_{14}/2[Fl_{ox}][II] \quad \text{(using reaction 16)}$$

Similar treatment for the catalyzed reaction also leads directly to the corresponding experimental expressions.

Clearly, the steady-state approximation for Mo(V) monomer is not strictly valid, since the esr results show ~2% of total molybderum is present as the monomer at its highest level (Figure 4). This does not appear to affect the second-order plots of the data, which are essentially linear throughout the reaction.

The question arises as to whether the one-electron mechanism is the major reaction pathway or a relatively minor competing reaction with the reaction proceeding mainly by a two electron mechanism. If the former is true, rate constants of 18.8 M^{-1} sec^{-1} (eq 15, $2k_{obsd}$) or 6.3 M^{-1} sec^{-1} (eq 16, $2k_{obsd}/3$) should be obtained for the formation of the Mo(V) monomer. The estimated value, 1.6 M^{-1} sec^{-1}, is considerably smaller than either. Certainly, as explained earlier, this is a minimum estimate only, and the actual value must be somewhat larger. This suggests the one-electron transfer is a significant, and possibly the exclusive, pathway for the reaction.

In the presence of substrate, esr signals for both Mo(V) monomer and flavosemiquinone are observed with xanthine oxidase (Bray and Swann, 1972). Although these results have been interpreted in terms of two monomeric noninteracting Mo(V) sites in the enzyme, the data reported here indicate substantial Mo(V) esr signals, without evidence of metal–metal interaction, can arise from one-electron oxidation of esr inactive Mo(V) dimers. Thus, the presence of such dimeric structures in the enzyme should not be ruled out. Furthermore, the results suggest a possible alternative explanation for the origin of the enzymatic Mo(V) esr signals. Instead of resulting from the reduction of Mo(VI) by substrate, they might arise from a one-electron oxidation of a Mo(V) dimer by the flavine component.

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