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Oxidation–Reduction Potentials of Molybdenum, Flavin, and Iron–Sulfur Centers in Milk Xanthine Oxidase: Variation with pH[†]

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ABSTRACT: Potentiometric titrations of native and desulfo milk xanthine oxidase were performed at 25 °C as a function of pH in the range 5.7–10.9. Concentrations of Mo^{V}, FAD semiquinone, and reduced Fe/S were determined by EPR spectroscopy of enzyme frozen after equilibration at each potential. Proton addition was found to accompany reduction of each enzyme center. The following standard reduction potentials were obtained (for the *specific* relative protonation states indicated): native and desulfo $Mo^{VI}/Mo^{V}\cdot H$, −337 and −349 mV (pH 7.0), respectively; native and desulfo $Mo^{V}\cdot H/Mo^{IV}\cdot H_2$, −351 and −368 mV (pH 7.0), respectively; $FAD/FAD^{-}\cdot$, −410 mV; $FADH\cdot/FADH_2$, −235 mV; Fe/S $I_{ox/red}$, −363 mV; Fe/S $II_{ox/red}$, −297 mV. The following proton dissociation reactions were found (p*K* in parentheses): native $Mo^{VI}\cdot H^+ \rightarrow Mo^{VI} + H^+$ (6.2; tentative); native $Mo^{IV}\cdot H_2 \rightarrow Me^{IV}\cdot H^- + H^+$ (8.5); desulfo $Mo^{IV}\cdot H_3^+ \rightarrow Mo^{IV}\cdot H_2 + H^+$ (7.6); $FADH\cdot \rightarrow FAD^{-}\cdot + H^+$ (8.8; this change was accompanied by a decrease in radical EPR line width from 19 to 14 G); $FADH_2 \rightarrow FADH^- + H^+$ (6.7); $Fe/S^{n}\cdot H \rightarrow Fe/S^{n-1} + H^+$ (7.0 and 8.5 for oxidized and reduced Fe/S I, respectively; 8.2 and 9.0 for oxidized and reduced Fe/S II, respectively). We can conclude that, as predicted by Stiefel [Stiefel, E. I. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 988–992], protonation does accompany Mo^{VI} reduction to both the Mo^{V} and Mo^{IV} states. $Mo^{V}\cdot H$ remains protonated from pH 5.7 to 10.9. The intrinsic electron affinity of the Mo center is not significantly altered when a terminal S ligand of the Mo is replaced by an oxygen, even though the enzyme is rendered nonfunctional. However, the affinity for protons of the Mo^{IV} state is substantially different in the two forms; it is this effect which makes the observed Mo^{V}/Mo^{IV} potential so negative at alkaline pH in the desulfo enzyme. Evidence was also obtained for a specifically tight binding of uric acid (the product of xanthine oxidation) to the Mo^{IV} oxidation state of native enzyme. The reported effects can be used to experimentally vary the order of appearance of electrons in the various enzyme centers on reaction with appropriate reductants and thereby rigorously test current models [Olson, J. S., Ballou, D. P., Palmer, G., & Massey, V. (1974) *J. Biol. Chem. 249*, 4350–4362] for the mechanism of electron transfer in xanthine oxidase.

Milk xanthine oxidase is a dimer of identical subunits, each of which contains 1 mol of FAD, two iron–sulfur centers (termed Fe/S I and Fe/S II) with differing spectroscopic properties, and a Mo center containing a recently described pterin cofactor (Bray, 1975; Johnson et al., 1980). EXAFS studies have shown that, in both native xanthine oxidase (Bordas et al., 1980) and the related enzyme xanthine dehydrogenase (Cramer et al., 1981), the Mo atom in the oxidized (Mo^{VI}) enzyme contains one terminal sulfur (Mo═S) and one terminal oxygen (Mo═O) ligand, as well as several Mo–SR ligands. Cyanide treatment of xanthine oxidase (or dehydrogenase) causes the terminal sulfur to be removed as SCN^- (Massey & Edmondson, 1970; Coughlan, 1980) and to be replaced by a terminal oxygen (Bordas et al., 1980; Cramer et al., 1981). Enzyme so treated is termed "desulfo" xanthine oxidase and is inactive in catalyzing oxidation of xanthine (Bray, 1975). A minor proportion of xanthine oxidase as normally prepared consists of desulfo enzyme; since such enzyme is not active, yet it contains the normal complement of FAD and Fe/S prosthetic groups, the only centers which absorb significantly at 450 nm, the amount of functional enzyme in any xanthine oxidase preparation can be readily estimated from the activity/A_{450} ratio [see Bray (1975)].

The Mo^{VI} centers of both native and desulfo enzymes can be reduced (e.g., by dithionite) to the Mo^{V} and Mo^{IV} states. The Mo^{V} oxidation state gives rise to characteristic EPR signals termed "rapid" and "slow" for the dithionite-treated native and desulfo enzymes, respectively. [For a recent review of the EPR spectra of Mo^{V} in xanthine oxidase, see Bray (1980a,b)]. Examination of the splittings of the EPR signals in H_2O vs. D_2O has shown that protons (probably two, at least one of which is always strongly coupled to the Mo) are bound in close proximity to the Mo^{V} atom, probably as Mo–XH. There is no firm evidence as to the nature of X, although speculation has centered on the terminal sulfur and oxygen ligands of the oxidized enzyme (Gutteridge et al., 1979; Malthouse & Bray, 1980). EXAFS studies of reduced xanthine dehydrogenase do indeed suggest that in this enzyme the Mo═S and one of the Mo═O ligands of oxidized native and desulfo enzymes, respectively, are reduced to Mo–SR and Mo–OR in the Mo^{IV} oxidation state (Cramer et al., 1981).

Stiefel (1973) has proposed a general scheme for the function of the Mo center in catalysis by Mo hydroxylases. In this scheme, based on studies with model Mo compounds, the Mo is postulated to function simultaneously as a proton and electron accepting group [thus facilitating, for example, replacement of C(8) H by C(8) OH in the xanthine molecule]. Gutteridge et al. (1978a) have demonstrated direct transfer of hydrogen from C-8 of xanthine-related substrates to the Mo center of xanthine oxidase. There has been speculation as to which of the known Mo ligands serves as "proton acceptor" vs. "hydroxyl carrier" in xanthine oxidase (Bray et al., 1979; Gutteridge & Bray, 1980), although as yet there have been no systematic studies of the changes in overall protonation state of the xanthine oxidase Mo center accompanying its reduction. One method of observing such changes would be to study the pH dependence of the reduction potentials of the xanthine oxidase Mo center. Such a study, for both native and desulfo

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enzymes, is reported in the present work.

Olson et al. (1974a,b) concluded that, under a variety of experimental conditions, internal electron transfer between the various reducible centers of xanthine oxidase is not rate limiting to catalysis; i.e., the enzyme can be successfully modeled as a site for binding of and electron abstraction from xanthine (with accompanying exchange of hydroxyl for proton) at the Mo center and a site for binding of and electron donation to O_2 (the FAD moiety), with these centers connected by an internally equilibrated pool of electrons. The electron distribution among the various enzyme centers could at all observed times be determined from a knowledge of the total number of electrons in the enzyme and the relative *equilibrium* reduction potentials of the individual centers. The reduction potentials of the Mo center had to be modified upon binding of xanthine (and/or uric acid) in order to account for the observed electron distributions during catalysis. Olson et al. (1974a) predicted substantial positive shifts in the Mo potentials consequent to such binding events. Cammack et al. (1976) examined the reduction potentials of the various centers of xanthine oxidase at pH 8.2; their results were in good agreement with those of Olson et al. (1974a). We have in the present work examined the potentials of the FAD and Fe/S centers, as well as the Mo center, of xanthine oxidase as a function of pH. In addition, we have examined the effect of uric acid on the reduction potentials of the Mo center. These studies show that the order of reduction of the various centers can be altered by manipulation of pH and that uric acid can significantly shift the potential for the enzymic Mo^V/Mo^{IV} couple while not affecting that for the Mo^{VI}/Mo^V couple.

Experimental Procedures

Enzyme Preparation. Bovine milk xanthine oxidase was isolated from unpasteurized raw cream (Long Meadow Dairies, Durham, NC). Buttermilk was prepared by diluting the fresh cream with 0.5 volume of cold distilled water and churning the solution at 9 °C in a Waring Blendor at high speed until phase separation had occurred (3–5 min). The butter was removed by filtration through cheesecloth, and the following components were added to the buttermilk (per liter): 40 mL of a stabilizing solution containing 0.5 M EDTA[1] and 0.125 M sodium salicylate, pH 7.0, 17 g of sodium bicarbonate, 0.33 g of L-cysteine hydrochloride, and 1.6 g of pancreatin. Xanthine oxidase was purified from this material by the method of Hart et al. (1970), except that the salicylate denaturation and Sephadex G-200 column chromatography steps were omitted. The purified enzyme was concentrated by ultrafiltration and then dialyzed vs. several changes of 5 mM bicine, pH 8.0, containing 0.1 mM EDTA. The concentrated enzyme was stored over liquid N_2 in the form of frozen beads of 20–30 μL volume, and a minimal amount was thawed as required.

The xanthine oxidase preparation used for the experiments involving native enzyme exhibited a ratio of activity/A_{450} of 131. Activity was measured at 23.5 °C as described by Avis et al. (1955). The concentration of enzyme was estimated from the absorbance at 450 nm by using $E_{mM} = 72\ mM^{-1}\ cm^{-1}$ (Avis et al., 1956). The total concentration of enzyme active centers (functional and nonfunctional) was taken as twice the enzyme concentration. The proportion of functional sites was estimated from the activity/A_{450} ratio by taking the limiting value of the ratio for fully active enzyme as 197 (McGartoll et al., 1970).

Desulfo xanthine oxidase was prepared as described by Massey & Edmondson (1970).

EPR Spectroscopy. EPR spectra were recorded on a Varian E9 spectrometer equipped with a variable temperature accessory and using 100-kHz modulation. Measurements at temperatures of less than 80 K were made by using a liquid helium cryostat (Air Products). Mo^V and FAD semiquinone spectra were recorded at 173 K, 5 mW of power, and 2.5-G modulation. Spectra of reduced Fe/S centers were recorded at 21 K, 1 mW of power, and 10-G modulation. The g_1 feature of reduced Fe/S II was also examined at 21 K and 100 mW of power.

Double integrations of the experimental spectra were performed as described by Wyard (1965); CuEDTA served as standard. Signals, if showing sign of slow relaxation, were recorded at a series of powers and corrected to nonsaturating conditions. Correction for the failure to integrate all of the $I = {}^5/_2$ Mo hyperfine lines was as described by Barber et al. (1976). Correction for the difference in g-value transition probability between the standard and the unknown was as described by Aasa & Vänngård (1975).

Observed Mo^V spectra were analyzed in terms of the rapid and slow species [corresponding to native and desulfo enzyme, respectively (Bray, 1975)] by measurement of the β and γ peak heights (Palmer et al., 1964) and comparison with computer simulated spectra prepared by adding pure experimental rapid and slow Mo^V spectra in varying proportions (Barber, 1976). A similar procedure was used to quantitate the relative amounts of reduced Fe/S I and Fe/S II. The measured peak heights of the g_1 (for Fe/S II) and g_3 (for Fe/S I and II) features were used (Barber, 1976). To confirm that changes in pH or buffer medium did not affect the analysis of experimental spectra in terms of individual Fe/S centers when the computer simulated spectra were used, the amount of reduced Fe/S II was determined independently by measurement of the height of the g_1 feature in spectra recorded at high power, when there is little or no contribution from the reduced Fe/S I center. The amount of reduced Fe/S I could be determined independently by measurement of the height of the g_2 feature above the base line in spectra recorded at low power. All methods gave nearly identical results for quantitation of the various centers.

Calculation of the number of electrons per active center was achieved by dividing the integrated signal intensity of the various centers (corrected as indicated above) by the appropriate active-center concentration. Reduced Fe/S centers and FAD semiquinone in both native and desulfo enzymes and Mo^V slow signals in desulfo enzyme were expressed relative to the total concentration of functional plus nonfunctional active centers. Mo analyses of the native and desulfo xanthine oxidase preparations used in the present work by atomic absorption spectroscopy showed >0.9 Mo per FAD in all cases. Mo^V rapid signals for native enzyme were expressed relative to the concentration of functional active centers.

Buffers. Since the recording of EPR spectra for the various enzyme centers requires low temperatures and frozen samples, it was necessary to select conditions which would minimize changes in the pH of the enzyme samples as the temperature is lowered from that utilized for the potentiometric titrations (298 K) to that utilized for EPR measurement (173 K or less).

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[1] Abbreviations: bicine, *N*,*N*-bis(2-hydroxyethyl)glycine; Caps, (cyclohexylamino)propanesulfonic acid; Ches, 2-(*N*-cyclohexylamino)ethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; EXAFS, extended X-ray absorption fine structure; EPR, electron paramagnetic resonance.

Table I: Potentials for Maximum Mo^V and FAD Semiquinone Formation in Xanthine Oxidase as a Function of pH[a]

pH	solution	FAD semiquinone		native enzyme Mo^V rapid		desulfo enzyme Mo^V slow	
		E_{max}	max fraction of FAD	E_{max}	max fraction of Mo	E_{max}	max fraction of Mo
5.7	Mes[b]	−192	0.18	−288	0.24		
6.0	Mes					−240	0.09
6.1	Mes	−215	0.21	−301	0.32		
6.5	Pipes	−232	0.31	−324	0.30		
6.6	Pipes					−300	0.20
7.1	Pipes	−262	0.20	−356	0.34		
7.7	bicine	−283	0.07	−375	0.36	−405	0.46
7.7	bicine + 2 mM uric acid	−283	0.05	−320	0.08		
8.2	Tris[c]	−301	0.03	−401	0.38		
8.7	bicine					−460	0.42
8.9	Ches	−340	0.03	−440	0.23		
9.3	Ches	−350	0.05	−450	0.18		
9.7	Ches					−500	0.42
9.8	Ches	−349	0.06	−472	0.12		
10.7	Caps					−590	0.30
10.9	Caps	−350	0.05	−505	0.05		

[a] Potentiometric titrations were performed as described in the text, and values given represent computer best fits to the experimental points. Potentials are expressed in millivolts relative to the standard hydrogen electrode. [b] All buffers were 50 mM and contained 1 mM EDTA. [c] Data of Cammack et al. (1976).

Williams-Smith et al. (1977) and Orii & Morita (1977) have investigated the effects of freezing on the pH of a variety of buffer systems. They concluded that pH variations are minimized when zwitterionic buffers of the type described by Good (1966) are used. We therefore utilized such buffers (always at 50 mM and containing 1 mM EDTA) in the present work; the buffers used are listed in Table I.

Potentiometric Titrations. Potentiometric titrations were carried out at 25 °C in an anaerobic glass cell of the type described by Dutton (1971), modified by addition of an extra microelectrode to allow continuous monitoring of pH. The cell was fitted with a gold foil indicating electrode and a calomel electrode (Radiometer K401) calibrated vs. a standard solution of quinhydrone. Enzyme concentrations of 20–100 μM were used. The potential of the system was adjusted by addition of small volumes of 0.1 M $Na_2S_2O_4$ (in 0.1 M Tris-HCl, pH 8.5) or 0.2 M $K_3Fe(CN)_6$. Equilibration between enzyme and the indicating electrode was facilitated by the use of a mixture of mediator dyes (generally present at 20–30 μM each compound, except in titrations of desulfo enzyme, when at least 100 μM of each mediator was used). Mediator concentrations were varied in a number of experiments to ensure that equilibrium was achieved. See Barber et al. (1980) for a list of the two groups of mediator dyes used and their reduction potentials. All potentials in this paper are referred to the standard hydrogen electrode.

After equilibration was achieved at each potential, enzyme samples were removed with a gas-tight Hamilton syringe fitted with a 30-cm stainless steel needle, transferred to an Ar-flushed EPR tube, and quickly frozen in liquid N_2 for subsequent recording of EPR spectra. Samples were stored at 77 K. Equilibration times of 5–10 min were found to be sufficient to permit achievement of equilibrium for all EPR-detectable species in redox titrations involving native enzyme. Equilibration times were extended to 30–45 min in titrations involving desulfo xanthine oxidase. In all of the titrations reported in this work, a series of potentials was achieved by addition of $S_2O_4^{2-}$ to oxidized enzyme and a second overlapping series of potentials by addition of $Fe(CN)_6^{3-}$ to the reduced enzyme. In all cases the experimental points fit on a common titration curve, a result which demonstrates reversibility of the potentiometric titrations.

Plots of amount of individual enzyme center oxidation states, detected by EPR, vs. applied potential were generated by using a Hewlett-Packard 9825A computer. Theoretical curves representing the behavior of species either (a) stably formed as a result of single electron addition (the Fe/S centers) or (b) transiently formed as the intermediate species in two consecutive single electron addition processes (FAD semiquinone, Mo^V) were calculated as described by Clark (1960) and Barber & Salerno (1980), and parameters were adjusted until a best fit to the experimental data was obtained. As discussed by Cammack et al. (1976), errors in redox potentials derived from the type of analysis used in the present work are expected to be ±15 mV. Theoretical curves for variation of midpoint potentials derived in this fashion with pH were calculated and computer-derived best fits to the data obtained.

Results

Molybdenum. (*A*) *Native Enzyme.* Figure 1A shows the results of potentiometric titrations of native xanthine oxidase performed at pH 6.1, 7.7, and 8.9 for the Mo^V rapid EPR signal. The curves fitted to the experimental points correspond to two consecutive one-electron ($n = 1$) reduction steps representing the couples Mo^{VI}/Mo^V (with potential E_{m1}) and Mo^V/Mo^{IV} (with potential E_{m2}).[2] As shown in Table I, the maximum integrated Mo^V signal intensity, as well as the potential at which this maximum signal is produced, varies markedly with pH in the range 5.7–10.9. The line shape of the Mo^V rapid signal was constant at all potentials and all pH values examined (although mixtures of rapid and slow Mo^V species were encountered at at least some potentials in all the titrations). E_{m1} and E_{m2} values, calculated from the experi-

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[2] The greater width of the Mo^V vs. potential curves than would be expected from ideal $n = 1$ behavior, seen in the data of Figure 1A at pH 6.1 and 7.7, is quite reproducible and has been found in all potentiometric titrations of both native xanthine oxidase and desulfo xanthine oxidase at pH values < 8.0. The Mo^V vs. potential curves obtained at pH > 8.0 show nearly ideal Nernstian behavior. We have no explanation for this phenomenon at present. Because of this unusual behavior of the Mo^V titration curves, which is particularly marked between pH 6 and 7, as well as the limited number of experimental points involved, the authors suggest that the $pK_0 = 6.2$ determined for the $Mo^{VI}{\cdot}H^+$ species of native xanthine oxidase should be viewed with some caution.

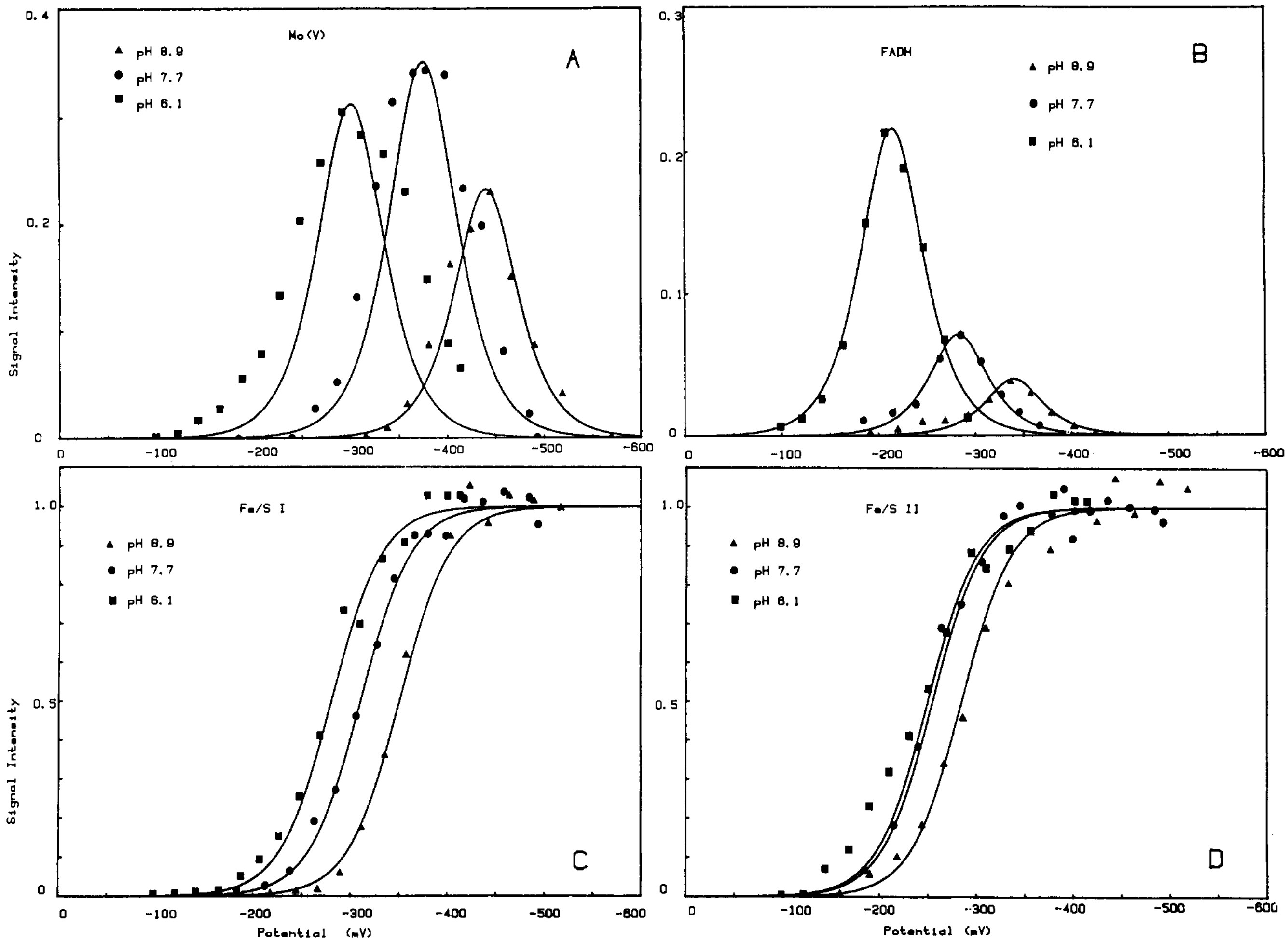

FIGURE 1: Behavior of the Mo^V rapid, FAD semiquinone, reduced Fe/S I, and reduced Fe/S II EPR signals of native xanthine oxidase in potentiometric titration at pH 6.1, 7.7, and 8.9. Signal intensity is plotted as a function of potential (with respect to the standard hydrogen electrode). The experiments were performed as indicated in the text in 50 mM Mes, pH 6.1 (■), bicine, pH 7.7 (●), and Ches, pH 8.9 (▲). The intensity of the Mo^V rapid signal (A) is expressed relative to the concentration of functional active centers, while the intensities of the FAD semiquinone (B), Fe/S I (C), and Fe/S II (D) signals are expressed relative to the concentration of total active centers. Theoretical curves for (A) and (B) represent the amount of intermediate species in two consecutive $n = 1$ reduction processes with midpoint potentials E_{m1} and E_{m2}. Theoretical curves for (C) and (D) represent the amount of reduced species formed in a single $n = 1$ reduction process with midpoint potential E_m. Best fit values for the midpoint potentials are given in Table II.

Table II: Midpoint Reduction Potentials of Xanthine Oxidase Prosthetic Groups as a Function of pH[a]

pH	solution	native Mo E_{m1}	native Mo E_{m2}	FAD E_{m1}	FAD E_{m2}	Fe/S I E_m	Fe/S II E_m	desulfo Mo E_{m1}	desulfo Mo E_{m2}
5.7	Mes[b]	−300	−276	−214	−170	−260	−250		
6.0	Mes							−283	−197
6.1	Mes	−303	−298	−230	−199	−280	−250		
6.5	Pipes	−327	−321	−235	−229	−290	−245		
6.6	Pipes							−318	−282
7.1	Pipes	−355	−356	−280	−244	−300	−255		
7.7	bicine	−373	−377	−332	−234	−310	−255	−392	−418
7.7	bicine + 2 mM uric acid	−369	−271	−343	−223	−320	−270		
8.2	Tris[c]	−397	−405	−378	−223	−330	−255		
8.7	bicine							−452	−468
8.9	Ches	−453	−427	−405	−275	−350	−285		
9.3	Ches	−472	−428	−410	−290	−360	−290		
9.7	Ches							−490	−506
9.8	Ches	−505	−439	−402	−296	−365	−282		
10.7	Caps							−595	−585
10.9	Caps	−605	−405	−407	−293	−370	−320		

[a] Potentiometric titrations were performed as described in the text, and values given represent computer best fits to the experimental points. Potentials are expressed in millivolts relative to the standard hydrogen electrode. [b] All buffers were 50 mM and contained 1 mM EDTA. [c] Data of Cammack et al. (1976).

mental data of Table I, are listed for each pH in Table II. Plots of E_{m1} and E_{m2} for native xanthine oxidase (curves marked rapid) as a function of pH are presented in parts A and B of Figure 2, respectively.

E_{m1} appears to be independent of pH at the lowest pH values examined.[2] As the pH is raised, E_{m1} is seen to decrease by approximately 60 mV for each unit increase in pH. Behavior of this type has been analyzed by Clark (1960) in terms of

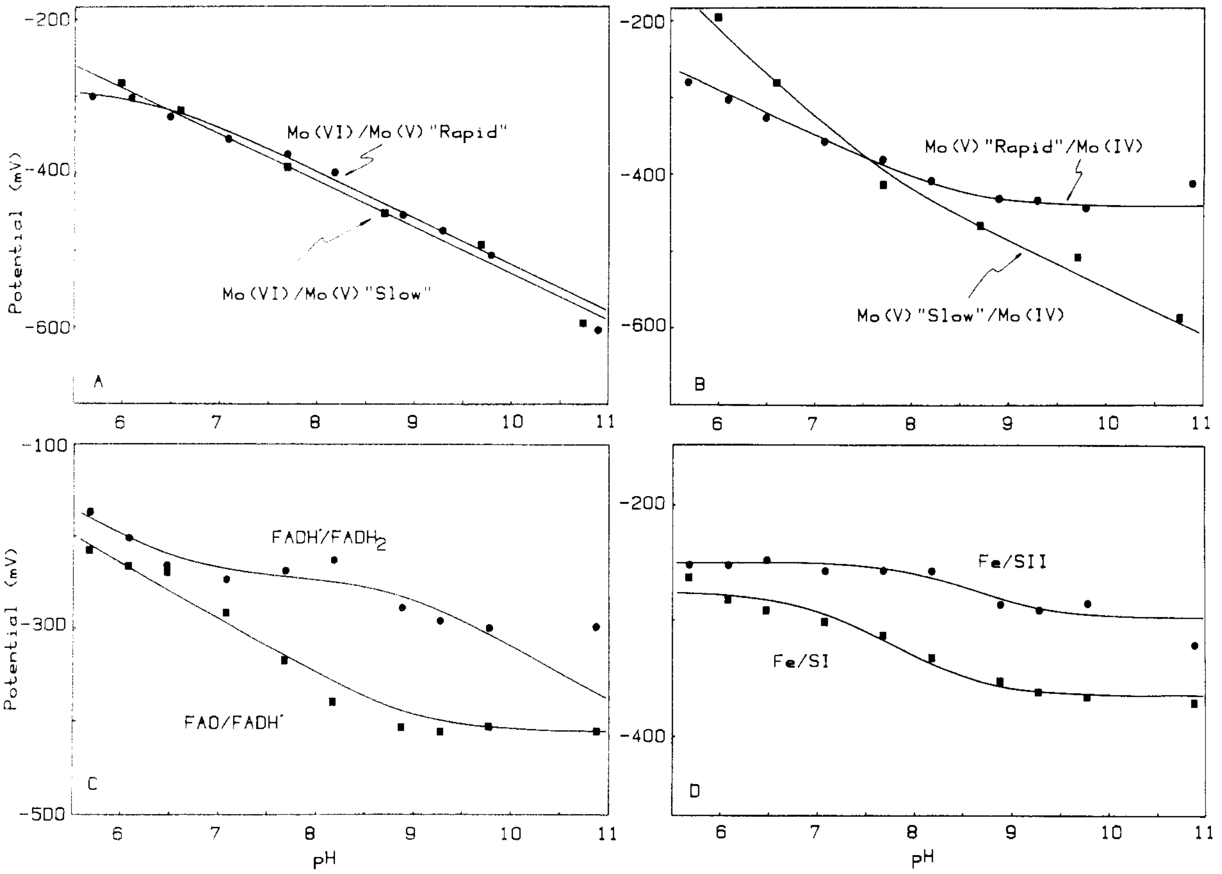

FIGURE 2: Variation of the midpoint potentials for xanthine oxidase prosthetic groups with pH. Midpoint potentials were derived from titrations such as those shown in Figure 1; values are given in Table II. Theoretical curves follow equations and utilize the parameters described in the text. (A) Potentials for Mo^{VI}/Mo^{V} "Rapid" in native enzyme (●) and Mo^{VI}/Mo^{V} "Slow" in desulfo enzyme (■). (B) Potentials for Mo^{V} "Rapid"/Mo^{IV} in native enzyme (●) and Mo^{V} "Slow"/Mo^{IV} in desulfo enzyme (■). (C) Potentials for FAD oxidized/semiquinone (■) and semiquinone/hydroquinone (●). The terms FADH and $FADH_2$ used in the figure represent relative oxidation states and are not meant to indicate specific protonation states. (D) Potentials for Fe/S I (■) and Fe/S II (●) oxidized/reduced.

ionization of a single proton from the more oxidized species (arbitrarily assigned the designation[3] $Mo^{VI}\cdot H^+$; see Scheme I) with a dissociation constant K_0. The experimental points can be fit (Figure 2A) by an equation of the form

$$E_{m1} = E_1 - 59 \log (1 + 10^{pH-pK_0}) \qquad (1)$$

where E_{m1} is the pH-independent midpoint potential for the $Mo^{VI}\cdot H^+/Mo^{V}\cdot H$ couple (see Scheme I). Best fits were obtained with $E_1 = -290$ mV and $pK_0 = 6.2$. The Mo^{VI}/Mo^{V} potential for native enzyme at pH 7.0 is calculated to be −342 mV.

In contrast, E_{m2}, the potential of the Mo^{V}/Mo^{IV} couple, decreases by approximately 60 mV per unit increase in pH in the pH region from 5.7 to 8.5. At more alkaline pH values, the potential becomes independent of pH. Behavior of this type has been analyzed by Clark (1960) in terms of ionization of a single proton from the more reduced species of the couple (Mo^{IV}) with a dissociation constant K_r. The experimental points can be fit (Figure 2B) by an equation of the form

Scheme I

$$Mo^{IV}\cdot H_2 \xrightleftharpoons[-H^+]{pK_r\,=\,8.5} Mo^{IV}\cdot H^- \xrightleftharpoons[e^-]{E_2\,=\,-440\text{ mV}}$$

$$Mo^{V}\cdot H \xrightleftharpoons[e^-]{E_1\,=\,-290\text{ mV}} Mo^{VI}\cdot H^+ \xrightleftharpoons[-H^+]{pK_0\,=\,6.2} Mo^{VI}$$

$$E_{m2} = E_2 + 59 \log (1 + 10^{pK_r-pH}) \qquad (2)$$

where E_2 is the pH-independent midpoint potential for the $Mo^{V}\cdot H/Mo^{IV}\cdot H^-$ couple (see Scheme I). Best fits were obtained with $E_2 = -440$ mV and $pK_r = 8.5$. The Mo^{V}/Mo^{IV} potential for native enzyme at pH 7.0 is calculated to be −349 mV. The results are summarized in Scheme I.

(*B*) *Desulfo Enzyme*. The molybdenum center of desulfo xanthine oxidase differs from that of the native enzyme in that a terminal oxygen has replaced a terminal sulfur ligand (Bordas et al., 1980). In order to investigate the effect of this replacement on the potentials and protonation states of the Mo center, we performed a series of potentiometric titrations on the desulfo enzyme at a number of pH values, with the amount of Mo^{V} slow species formed at each potential estimated by integration of the EPR spectra. The line shape of the Mo^{V} slow signal was constant at all potentials and all pH values examined, and bell-shaped curves of Mo^{V} slow signal intensity as a function of potential, analogous to those in Figure 1A, were obtained. The maximum amount of Mo^{V} slow signal observed and the potential at which this maximum signal

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[3] All protonated species in this paper are designated relative to the least protonated form of the most oxidized species found in the pH range 5.7–10.9. Thus, the actual number of protons associated with the species termed "$Mo^{VI}\cdot H^+$" is not known, but this species does in fact contain one more proton than the species termed "Mo^{VI}", and it is the pK for dissociation of this proton which is observed in the present work. This convention has been used for FAD and the Fe/S centers, as well as the Mo. Its use may cause some confusion with respect to the flavin protonation states, because the absolute numbers of protons associated with various species of oxidized and reduced free flavins are known. Thus, the species which we term "FAD" does in fact contain one potentially dissociable proton (whose dissociation pK in xanthine oxidase is obviously not in the pH range examined in the present work); this species would be designated as FADH in the flavin literature [see Muller et al. (1970)]. Similarly, the neutral flavin semiquinone and hydroquinone, which we term "FADH·" and "$FADH_2$", respectively, would conventionally be designated as $FADH_2$ and $FADH_3$, respectively.

Scheme II

$$\mathrm{Mo^{VI}} \xrightleftharpoons[\mathrm{H^+ + e^-}]{E_1 = +64\ \mathrm{mV}} \mathrm{Mo^{V}\cdot H} \xrightleftharpoons[\mathrm{2H^+ + e^-}]{E_2 = +493\ \mathrm{mV}} \mathrm{Mo^{IV}\cdot H_3^+} \xrightleftharpoons[\mathrm{p}K_r = 7.6]{-\mathrm{H^+}} \mathrm{Mo^{IV}\cdot H_2}$$

intensity occurs are tabulated for a number of pH values in Table I. The E_{m1} and E_{m2} values for desulfo enzyme calculated from these data are listed for each pH in Table II, and plots of these potentials as a function of pH are presented in parts A and B of Figure 2, respectively (curves marked "Slow").

Figure 2A shows that the midpoint potential E_{m1} for the Mo^{VI}/Mo^{V} couple is nearly identical at all pH values for the native and desulfo enzymes. (The pK_0 at 6.2 associated with the native Mo^{VI} species is not, however, seen with the desulfo enzyme.) Thus, replacement of terminal sulfur by oxygen on the Mo does not markedly affect the reducibility of the Mo^{VI}/Mo^{V} couple. As with native enzyme, Mo^{V} in desulfo xanthine oxidase is protonated (and shows no pKs for proton dissociation) throughout the entire pH range studied. The data for Mo^{V} "Slow" of Figure 2A obey the equation $E_{m1} = E_1 - 59(\mathrm{pH})$, where E_1 is the standard potential at unit $[H^+]$. The data are best fit with E_1 = 64 mV. The E_{m1} calculated at pH 7.0 is then −350 mV.

In contrast, the behavior of the midpoint potential E_{m2} for the Mo^{V}/Mo^{IV} couple differs markedly in the native and desulfo enzymes. In the pH range 6.0–7.7, the E_{m2} for desulfo xanthine oxidase exhibits a decrease of approximately 120 mV per unit increase in pH. At more alkaline pH values, the slope shifts to −60 mV/pH. This behavior is indicative of a process in which reduction of Mo^{V} to Mo^{IV} is accompanied by addition of two H^+, with one of these protons on the reduced species exhibiting a dissociation constant K_r. (Note that K_r refers to proton dissociation from Mo^{IV} species with differing numbers of protons in the native and desulfo enzymes.) The equation governing this process is

$$E_{m2} = E_2 - 118(\mathrm{pH}) + 59 \log (1 + 10^{\mathrm{pH}-\mathrm{p}K_r}) \qquad (3)$$

where E_2 is the standard potential (at unit $[H^+]$) for the $Mo^{V}{\cdot}H/Mo^{IV}{\cdot}H_3^+$ couple. (Note that the values of E_1 and E_2 for the desulfo enzyme Mo center are not directly comparable to the values obtained for the native enzyme Mo, since the potentials in the latter case refer to proton-independent reduction reactions.) Best fits to the data were obtained with E_2 = +493 mV and pK_r = 7.6. The Mo^{V}/Mo^{IV} potential for the desulfo enzyme at pH 7.0 is calculated to be −327 mV. The results are summarized in Scheme II.

Although the reference potentials E_1 and E_2 are not directly comparable for the Mo of the native and desulfo enzymes, it is possible to compare potentials for reduction reactions of the two types of Mo which involve comparable changes in protonation upon electron addition. Thus, the standard potentials (at unit $[H^+]$) for the $Mo^{VI} + e^- + H^+ \rightarrow Mo^{V}{\cdot}H$ reaction are +76 mV $[E_1 + 59(pK_0)]$ for the native enzyme and +64 mV (E_1) for the desulfo enzyme. For the $Mo^{V}{\cdot}H + e^- + H^+ \rightarrow Mo^{IV}{\cdot}H_2$ reaction the analogous standard potentials are +62 mV $[E_2 + 59(pK_r)]$ and +45 mV $[E_2 - 59(pK_r)]$ for native and desulfo enzymes, respectively. For calculation of the midpoint potentials for these reactions at pH 7.0, 59(pH) mV must be added to each of these standard potentials. Thus the potentials of $Mo^{VI}/Mo^{V}{\cdot}H$ at pH 7.0 are −337 and −349 mV, and the potentials of $Mo^{V}{\cdot}H/Mo^{IV}{\cdot}H_2$ at pH 7.0 are −351 and −368 mV for native and desulfo enzymes, respectively. It is evident from these considerations that the conversion of a terminal sulfur to a terminal oxygen ligand has little effect on the intrinsic electron affinities of the various Mo oxidation states. The primary effect of the ligand conversion is to markedly alter the protonation behavior of the Mo^{IV} state. It is this difference in protonation of the fully reduced enzymes which causes the observed Mo^{V}/Mo^{IV} potential of the desulfo enzyme to be so different from that of native enzyme at relatively acid and alkaline pH values (Figure 2B).

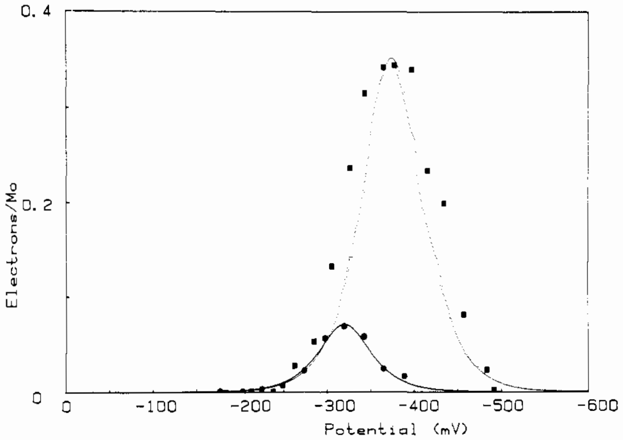

FIGURE 3: Behavior of the Mo^{V} rapid EPR signal of native xanthine oxidase in the presence (●) and absence (■) of 2 mM uric acid. Signal intensity is plotted as a function of potential. The experiments were performed in 50 mM bicine, pH 7.7, at 25 °C. The lines present best fit theoretical curves for the amounts of intermediate species formed in two consecutive $n = 1$ reduction processes. E_{m1} and E_{m2} values used for these curves are indicated in Table II.

(*C*) *Effect of Uric Acid.* Figure 3 shows the results of potentiometric titrations of native xanthine oxidase Mo centers in 50 mM bicine, pH 7.7, performed in the presence and absence of 2 mM uric acid, a compound known to bind to the native enzyme Mo center. It is apparent from Figure 3 and from Table I that uric acid both increases the potential at which maximum Mo^{V} occurs and significantly lowers the maximum intensity of the Mo^{V} EPR signal. E_{m1} and E_{m2} values calculated from these data are presented in Table II. E_{m1} (ca. −370 mV) is not appreciably affected by the presence or absence of 2 mM uric acid. In contrast, E_{m2} is made more positive by over 100 mV in the presence of uric acid (from −377 to −271 mV). These results indicate preferential binding of uric acid to the Mo^{IV} oxidation state of the enzyme. [Note that there is no significant effect of uric acid on the midpoint potentials of the FAD or iron–sulfur centers of the enzyme (Table II).]

FAD. The behavior of the flavin semiquinone EPR signal of native xanthine oxidase as a function of applied potential is shown in Figure 1B for titrations performed at pH 6.1, 7.7, and 8.9. The curves fitted to the experimental data correspond to two consecutive one-electron ($n = 1$) reduction steps representing the couples $FAD_{\mathrm{oxidized}}/FAD_{\mathrm{semiquinone}}$, with potential E_{m1}, and $FAD_{\mathrm{semiquinone}}/FAD_{\mathrm{hydroquinone}}$, with potential E_{m2}. As observed with the Mo^{V} signals, both the maximum integrated FAD semiquinone EPR signal intensity and the potential at which this maximum intensity was produced varied markedly with pH (Table I). E_{m1} and E_{m2} values for the enzyme FAD, calculated from the experimental data of Table I, are listed for each pH in Table II. Plots of E_{m1} and E_{m2} as a function of pH are shown in Figure 2C.[4]

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[4] Xanthine oxidase was somewhat unstable at pH 10.9 over the period of time required to perform the potentiometric titration. No such instability, measured as loss in xanthine/O_2 activity, was detected in enzyme aliquots removed from titrations performed at lower pH values. Thus the data obtained at pH 10.9 should be considered less reliable than those obtained at lower pH. The deviation of the experimentally determined potentials from the theoretical curves shown in Figure 2 are particularly marked for the FAD semiquinone/hydroquinone couple at pH 10.9.

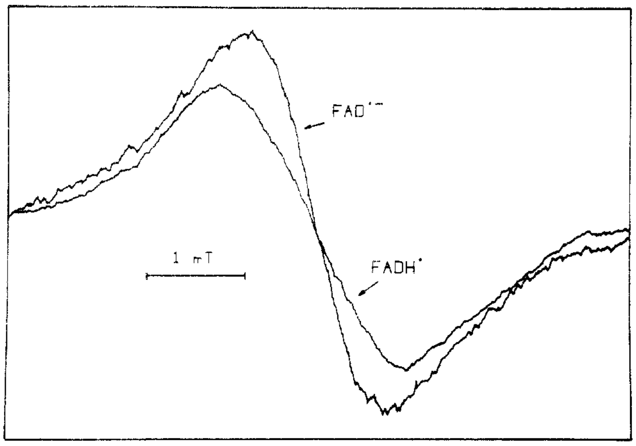


FIGURE 4: EPR spectra of FAD semiquinone species produced at pH 5.7 and 10.9. The samples represented the maximal amounts of FAD semiquinone formed in potentiometric titrations performed in 50 mM Mes, pH 5.7 (FADH·), and 50 mM Caps, pH 10.9 (FAD^-·). Spectra were recorded at 173 K with 5 mW of power and 2.5-G modulation. Peak to trough line widths are 19 G for the FADH· spectrum and 14 G for the FAD^-· spectrum.

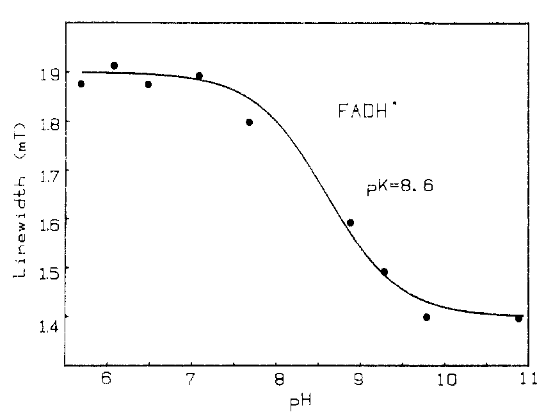


FIGURE 5: Variation in the line width of the FAD semiquinone EPR signal as a function of pH. EPR spectra were recorded, as described in Figure 4, on samples representing the maximal amount of FAD semiquinone formed in potentiometric titrations performed at the pH values indicated (for buffers, see the text). The theoretical curve was produced by using eq 5 in the text with a pK_s of 8.6.

E_{m1} shows a variation of approximately −60 mV/pH in the pH range 5.7–9.0. At more alkaline pH, the potential becomes independent of pH. As was the case for the Mo^V/Mo^{IV} couple of native enzyme, this behavior indicates dissociation of a single proton from the more reduced species (in this case the FAD semiquinone) at high pH. If the dissociation constant for the protonated semiquinone species is termed K_s, then the experimental points can be fit (Figure 2C) by

$$E_{m1} = E_1 + 59 \log (1 + 10^{pK_s - pH}) \qquad (4)$$

where E_1 is the midpoint potential (for the FAD/FAD^-· couple; see Scheme III) in the pH-independent region. Best fits were obtained with $E_1 = -410$ mV and $pK_s = 9.0$. The midpoint potential for the oxidized/semiquinone flavin couple at pH 7.0 was calculated to be −290 mV.

pK_s for the protonated flavin semiquinone could also be estimated by a nonpotentiometric method. During the series of potentiometric titrations, it was found that the line width of FAD semiquinone EPR signal varied with pH (although at any one pH, the line width was independent of the solution potential), decreasing from 19 G at pH 5.7 to 14 G at pH 10.9. EPR spectra of the FAD semiquinone produced at the two extremes of pH are shown in Figure 4, and the variation of line width with pH is shown in Figure 5. The points are well fit by a theoretical curve for ionization of a single proton, with a pK_s of 8.6, from the FAD semiquinone. This curve was calculated from

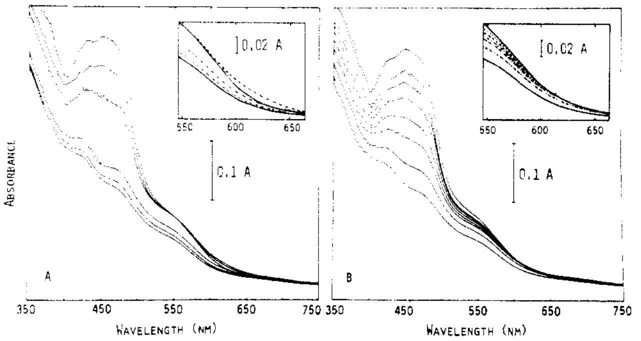


FIGURE 6: Optical spectra obtained upon photoreduction of native xanthine oxidase at pH 6.5 and 7.7. A solution containing 6 μM enzyme, 2 mM EDTA, and 1 μM 5′-deazaflavin in either 50 mM Pipes, pH 6.5 (A), or 50 mM bicine, pH 7.7 (B), was placed in a sealed cuvette, made anaerobic by repeated evacuation and flushing with Ar, and subjected to short periods (10–30 s) of illumination from a 200-W spot lamp placed 25 cm from the cuvette, with the intervening space being filled by a glass tank containing a water–ice mixture. After each illumination period, absorption spectra were measured with an Aminco DW-2 spectrophotometer. The spectra shown were recorded after all absorbance changes had ceased after each period of illumination. At 450 and 550 nm, the absorbance decreased monotonically with increasing time of illumination at both pH values; the greatest absorbance represents the most oxidized sample. The inset to (A) shows that the absorbance in the region 580–660 nm increased for the first and second periods of illumination and then decreased. The inset to (B) shows that no increased absorbance is seen in this wavelength region at any successive periods of illumination. In both insets, the spectra of fully oxidized and reduced enzymes are represented by solid lines, whereas the spectra of intermediate species are represented by broken or dotted lines.

$$\text{radical line width (G)} = 14 + 5/(1 + 10^{pH - pK_s}) \qquad (5)$$

Massey & Palmer (1966) have previously shown that such a decrease in EPR line width is characteristic of the conversion of flavin semiquinones from the neutral to the anionic form.

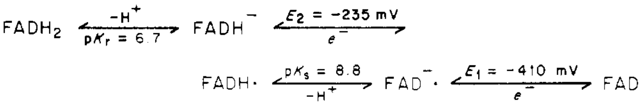
The pH dependence of the flavin semiquinone/hydroquinone couple potential, E_{m2}, shown in Figure 2C, could be fit if it were assumed that in addition to the dissociable proton present on the semiquinone (with dissociation constant K_s), there is an ionizable proton present on the hydroquinone, with dissociation constant K_r. The latter ionization accounts for the change in variation of E_{m2} with pH from a −60 mV/pH dependence in the region of pH 5.7–7.0 to a near independence of pH in the region pH 7.0–9.0. The data can be fit by

$$E_{m2} = E_2 + 59 \log (1 + 10^{pK_r - pH}) - 59 \log (1 + 10^{pH - pK_s}) \qquad (6)$$

where E_2 is the pH-independent midpoint potential for the $FADH·/FADH^-$ couple (see Scheme III). Best fits were obtained with $E_2 = -235$ mV, $pK_s = 8.8$, and $pK_r = 6.7$. The calculated midpoint potential for the FAD semiquinone/hydroquinone couple at pH 7.0 is −235 mV. Note that the K_s derived by three distinct methods is 8.8 ± 0.2. The results obtained with the FAD moiety of xanthine oxidase are summarized in Scheme III.

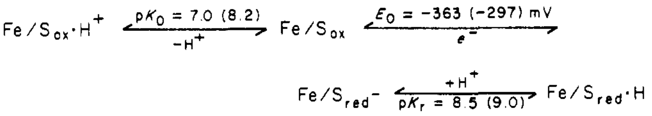
Parts A and B of Figure 6 show the results of optical spectra obtained upon reduction of native xanthine oxidase at pH 6.5

Scheme III



$$FADH_2 \underset{pK_r = 6.7}{\overset{-H^+}{\rightleftarrows}} FADH^- \underset{e^-}{\overset{E_2 = -235\ mV}{\rightleftarrows}} FADH\cdot \underset{-H^+}{\overset{pK_s = 8.8}{\rightleftarrows}} FAD^-\cdot \underset{e^-}{\overset{E_1 = -410\ mV}{\rightleftarrows}} FAD$$

Scheme IV



and 7.7, respectively. The enzyme was reduced anaerobically by illumination of samples containing 5′-deazaflavin and EDTA. No mediator dyes were present. After each illumination period, spectra were recorded only after all absorbance changes had ceased [see Olson et al. (1974a)], in order to allow achievement of solution equilibrium. The spectra show the characteristic bleaching of the 450-nm absorbance due to reduction of the FAD and iron–sulfur centers, with this bleaching increasing as the time of illumination is increased in both the pH 6.5 and 7.7 samples. However, the spectra for the two samples show a characteristic difference at longer wavelengths at early stages of reduction. At pH 7.7, there was a decrease in absorbance with increasing illumination time at all wavelengths. At pH 6.5, in contrast, there was an increase in absorbance in the 580–660-nm region during the early stages of photoreduction. The absorbance in this region then decreased to less than the starting absorbance upon further reduction of the sample. This behavior is consistent with (a) the presence of a neutral ("blue") FAD semiquinone species at pH 6.5 and (b) the prediction from the potentiometric data of Table I that considerably more FAD semiquinone is formed in xanthine oxidase at pH 6.5 than at pH 7.7.

Iron–Sulfur Centers. The behavior of the Fe/S I and Fe/S II centers of native xanthine oxidase as a function of applied potential is shown in parts C and D of Figure 1, respectively, for titrations performed at pH 6.1, 7.7, and 8.9. Both centers exhibited $n = 1$ reduction behavior, with full reduction corresponding to one electron per center. Both centers showed a small but reproducible decrease in midpoint potential with increasing pH in the pH range 6–9, indicating involvement of protons in the reduction processes. The E_m values measured for all pH values examined are tabulated in Table I and plotted as a function of pH in Figure 2D.

The data for each center are best fit by an equation involving two proton dissociation processes, one associated with the oxidized Fe/S center (with dissociation constant K_0) and the other associated with the reduced Fe/S center (with dissociation constant K_r):

$$E_m = E_0 + 59 \log (1 + 10^{pK_r - pH}) - 59 \log (1 + 10^{pK_0 - pH}) \tag{7}$$

where E_0 is the pH-independent midpoint potential for the nonprotonated Fe/S$_{oxidized}$/Fe/S$_{reduced}$ couple (see Scheme IV). For Fe/S I, best fits to the data were obtained with $E_0 = -363$ mV, $pK_0 = 7.0$, and $pK_r = 8.5$. For Fe/S II, $E_0 = -297$ mV, $pK_0 = 8.2$, and $pK_r = 9.0$. Since the difference between pK_0 and pK_r is so small for Fe/S II, the variation of potential with pH for this center would be expected to be considerably less than that observed for Fe/S I. The calculated midpoint potentials at pH 7.0 are −292 and −251 mV for Fe/S I and Fe/S II, respectively. The potentiometric titration results for the Fe/S centers of xanthine oxidase are summarized by Scheme IV (values for Fe/S II are in parentheses).

Discussion

Molybdenum. The results presented in this work strikingly confirm, for milk xanthine oxidase, the prediction by Stiefel (1973) that Mo reduction would be accompanied by protonation of the Mo center. The curves of potential vs. pH in Figure 2A,B show that, over a wide range of pH, proton addition accompanies both conversion of Mo^{VI} to Mo^{V} and Mo^{V} to Mo^{IV}. Limited studies with chicken liver xanthine dehydrogenase conducted at two different pH values (Barber et al., 1980) have suggested that protonation accompanies Mo reduction in this enzyme as well.

Previous work [for review, see Bray (1980a,b)] has shown that one or more protons are closely associated with the Mo^{V} species, giving rise to the rapid and slow EPR signals in both native xanthine oxidase and desulfo xanthine oxidase. Gutteridge et al. (1978b) studied the rates of exchange of a proton tightly coupled to the Mo^{V} center in both enzyme forms with solvent ^2H_2O. They postulated that such exchange occurs by proton dissociation from the center, followed by deuteron addition, with the rate of the latter process being diffusion controlled. They then derived apparent pK values for dissociation of an H$^+$ from the Mo^{V} center by combining the observed proton-exchange rate constants with reasonable values for the diffusion-controlled "on" rate constants and concluded that the Mo^{V} rapid species of native enzyme should exhibit a pK for proton dissociation of 7.8, while the Mo^{V} slow species of the desulfo enzyme should exhibit a pK of 10.1. These differences in pK, it was suggested, represent the differing proton affinities of the bound sulfur vs. oxygen ligand. We have found, in the present work, that the Mo^{V} centers of both native xanthine oxidase and desulfo xanthine oxidase remain protonated throughout the pH range 6.0–10.9. There is no evidence from the potentiometric titration curves for any pK for proton dissociation from the Mo^{V} redox state in this pH range. Furthermore, we have found, in agreement with Gutteridge et al. (1978a), no evidence for any change in the EPR line shape or line width of the Mo^{V} centers at any of the pH values studied. Such changes in Mo^{V} EPR signals have been observed as a function of pH in other molybdenum enzymes, notably sulfite oxidase (Cohen et al., 1971) and nitrate reductase (Vincent & Bray, 1978). We must conclude that the results of Gutteridge et al. (1978b) do not reflect true pK values for the Mo^{V} oxidation state of either native or desulfo xanthine oxidase.

Although the Mo^{V} redox state does not appear to exist in a multiplicity of protonation states in xanthine oxidase, the proton equilibria involving the Mo^{IV} state seem to be complex. The reduced Mo center in the desulfo enzyme apparently has a higher affinity for protons than the native enzyme Mo^{IV}, with the reduction of Mo^{V} actually involving uptake of two protons in the former enzyme at pH values below 7.0, as opposed to one proton in the latter enzyme form. It seems fruitless at present to speculate on the basis for this complex behavior, since added protons may reside not only on the oxygen or sulfur ligands of the Mo, as suggested by the recent EXAFS results of Cramer et al. (1981), but also on the pterin cofactor associated with the Mo center in the molybdenum hydroxylases (Johnson et al., 1980) or on the protein moiety itself. Any proposal offered for the structure of the oxidized and reduced Mo centers in xanthine oxidase must, however, accommodate the observed proton associations with the various Mo oxidation states.

One of the most striking findings of the present work is the near independence of the Mo^{VI}/Mo^{V} potentials of the nature of the terminal ligand (oxygen vs. sulfur) of enzyme-bound Mo. This independence was observed throughout the pH range 6.0–10.9. Although the uric acid complex of xanthine oxidase was studied only at pH 7.7, this enzyme species exhibited the same E_{m1} as the uncomplexed enzyme Mo.

Although the Mo^V/Mo^{IV} standard potentials are also insensitive to the substitution of terminal oxygen for sulfur when reduction reactions involving analogous proton additions are compared, the observed midpoint potentials E_{m2} for the two types of Mo centers can differ markedly due to the different proton equilibria for the Mo^{IV} oxidation state in the two enzyme forms. In contrast to previous assertions derived from measurements performed at a single pH, 8.2 (Cammack et al., 1976), it is now clear that the E_{m2} for desulfo enzyme Mo need not be more negative than that of native enzyme. At acidic pH values the desulfo enzyme actually exhibits a considerably more positive E_{m2} than native enzyme.

The present work also clearly demonstrates that the Mo^{VI}/Mo^V and Mo^V/Mo^{IV} potentials need not be nearly equivalent in molybdenum enzymes, as suggested by Bray (1980a). Although the potentials E_{m1} and E_{m2} in native xanthine oxidase diverge at alkaline pH so that very little Mo^V rapid EPR signal can be observed at pH 10.9 as compared to pH 7.7, the effect of uric acid, the product of enzyme-catalyzed xanthine oxidation, on E_{m2} may in fact be of more significance to the mechanism of catalysis. Thus, the presence of 2 mM uric acid preferentially increases E_{m2} by over 100 mV but has little or no effect on E_{m1}. The Mo^{IV} redox state is clearly stabilized by the presence of product. Olson et al. (1974a), in attempting to explain the enhancement of the level of Mo reduction (as compared to reduction of the FAD and Fe/S centers) in enzyme reacted with xanthine instead of dithionite, proposed large effects of xanthine (and perhaps product) binding on the Mo center reduction potentials. Bray et al. (1978) have presented EPR spectra demonstrating complexation between Mo^V in xanthine oxidase and uric acid. Cammack et al. (1976) suggested that uric acid complexation increased both E_{m1} and E_{m2} for Mo by as much as 55 mV at pH 8.2. Barber et al. (1977) found that 0.67 mM uric acid in pyrophosphate buffer caused a larger increase in E_{m2} (+73 mV) than in E_{m1} (+17 mV). The present findings indicate that the effects of substrate-related compounds, such as uric acid, may indeed be both larger in magnitude and more specific as to favored oxidation state than previous results had indicated.

FAD. Potentiometric titrations of avian xanthine dehydrogenases (Barber et al., 1977, 1980) at pH 8 have shown that considerably more FAD semiquinone is formed in these enzymes than in milk xanthine oxidase at that pH. Conversion of rat liver xanthine dehydrogenase to a xanthine oxidase was correlated with a dramatic decrease in the amount of FAD semiquinone which could be produced upon reduction of the enzyme (Waud & Rajagopalan, 1976). Inability to stabilize the FAD semiquinone state has come to be considered a characteristic property of xanthine oxidases (which have a high turnover number for xanthine oxidation with O_2 as electron acceptor and a very low turnover number with NAD^+ as acceptor) as opposed to the possibly more native dehydrogenases (which have a high turnover number with NAD^+ and a much lower turnover number with O_2) (Bray, 1975; Coughlan, 1980).

Previous work by Edmondson et al. (1973) and Olson et al. (1974b) has suggested that more FAD semiquinone can be formed in xanthine oxidase at pH 6.0 than at pH 8.5 or 10.0. We have found that at pH values of 7.0 and below 20–30% of the enzyme FAD can exist in the semiquinone state. This semiquinone has been detected both by EPR and by its characteristic absorbance in the 580–660-nm region of the optical spectrum. In agreement with the previous reports, we found that only minimal amounts of semiquinone are detected (by EPR) in the pH region 8–9, where the FAD potentials E_{m1} and E_{m2} are maximally divergent. We have performed studies of the turnover of xanthine oxidase with O_2 and NAD^+ as acceptors (extrapolating to saturating concentrations of both xanthine and acceptor) as a function of pH and have found no correlation between the amount of FAD semiquinone which can be stabilized at a given pH and the amount of either absolute or relative activities with O_2 vs. NAD^+ (M. J. Barber and L. M. Siegel, unpublished results). Thus it is not the ability to stabilize FAD in the one-electron oxidation state which is the basis for the differences in oxidase and dehydrogenase specificity.

Xanthine oxidase is one of a small number of flavoproteins in which interconvertability between neutral and anionic flavin semiquinone species can be demonstrated (Massey & Palmer, 1966). It is of interest that both the pK_s and pK_r values found for the FAD moiety of xanthine oxidase are nearly identical with the pK values reported for free flavin semiquinones (pK_s = 8.3–8.6; Ehrenberg et al., 1967; Draper & Ingraham, 1968; Land & Swallow, 1969) and hydroquinones (pK_r = 6.7; Muller et al., 1970) in aqueous solution.

Iron–Sulfur Centers. Both of the Fe/S centers of xanthine oxidase exhibit a significant dependence of E_m on pH, although in both cases the maximum slope of the E_m vs. pH plot was considerably less steep than −60 mV per pH unit. E_m for both centers was independent of pH at both high and low pH values (Figure 2D). This behavior is readily explained if there is a difference in the affinity of a single proton for the enzyme depending on whether a specific Fe/S center is oxidized or reduced (Scheme IV). The proton binds more tightly to the enzyme containing the reduced center than to the enzyme in which the Fe/S center is oxidized. The pK for dissociation of this proton is increased by less than 1 unit on electron addition to Fe/S II; thus the variation with pH of E_m for reduction of this center is relatively slight. The pK for proton dissociation is increased by 1.5 units on reduction of Fe/S I, accounting for the somewhat greater variation with pH of E_m for reduction of this center. Such small pH dependences of Fe/S reduction potentials have previously been observed with parsley ferredoxin (Fee et al., 1971) and putidaredoxin (Wilson et al., 1973), as well as with certain centers of mitochondrial NADH dehydrogenase (Ingledew & Ohnishi, 1980). The proton whose dissociation gives rise to the pH dependence of the potentials may be bound to the Fe/S center itself, as suggested by Magliozzo & Sweeny (1980) for *Clostridium pasteurianum* ferredoxin, or it may be bound to a protein residue near the center. It should be mentioned that in the present work no changes in line width or line shape of the EPR signal of either Fe/S center of xanthine oxidase was detected at any pH or any potential examined. Thus there is no evidence to support the idea that the Fe/S centers of xanthine oxidase are themselves protonated.

The variation of the flavin and Fe/S center reduction potentials with pH leads to the fact that the equilibrium sequence of reduction of these centers upon addition of small successive amounts of reductant can be expected to change as the pH is varied. Thus, at pH 6.1, for example, FAD reduction should largely precede reduction of either Fe/S center, while at pH 8.9, Fe/S II should be largely reduced prior to either FAD or Fe/S I.

The difference in pH dependence of the potentials of the various enzyme centers has been utilized to study the relaxation properties of the EPR signal of the FAD semiquinone of xanthine oxidase in the presence of different ratios of reduced (paramagnetic) Fe/S I and Fe/S II; this procedure has permitted estimation of the degree of magnetic interaction (and calculation of approximate distances) between the FAD and the individual Fe/S centers (Barber et al., 1982).

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