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Determination of the Stoichiometry of Electron Uptake and the Midpoint Reduction Potentials of Milk Xanthine Oxidase at 25 °C by Microcoulometry†

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ABSTRACT: Microcoulometric titrations of several forms of milk xanthine oxidase at 25 °C (bicine buffer, pH 7.7) showed that full reduction of the native enzyme (with a functional Mo center, FAD, and two Fe_2S_2 centers) required six electrons, while reduction of the desulfo enzyme (with FAD, two Fe_2S_2 centers, and a nonfunctional Mo center) required four electrons, and reduction of the desulfo enzyme with its flavin alkylated (with two Fe_2S_2 centers and both nonfunctional FAD and Mo) required two electrons. From these data, the following reduction potentials were determined: Mo^{VI}/Mo^{V}, -375 mV; Mo^{V}/Mo^{IV}, -405 mV; FAD/$FADH_2$, -280 mV; Fe/S $I_{ox/red}$, -320 mV; Fe/S $II_{ox/red}$, -230 mV. Each of these potentials was shifted significantly in a negative direction (25-75 mV) when native enzyme was titrated coulometrically at pH 8.9 (Ches buffer), in agreement with the pH dependence predicted for these potentials by the results of Barber & Siegel [Barber, M. J., & Siegel, L. M. (1982) *Biochemistry* (first paper of three in this issue)]. Pyrophosphate caused positive shifts in the Mo^{V}/Mo^{IV} and Fe/S $II_{ox/red}$ potentials from values found in zwitterionic buffers. The potentials for Mo^{V}/Mo^{IV} and Fe/S $II_{ox/red}$ determined by coulometric titration at 25 °C were 25-30 mV more negative and 25-45 mV more positive, respectively, than the values determined by EPR analysis of enzyme frozen after being poised at defined potentials at 25 °C, whereas the Mo^{VI}/Mo^{V}, FAD/$FADH_2$, and Fe/S $I_{ox/red}$ potentials obtained by the two methods were in good agreement. It can be concluded that the prosthetic groups of xanthine oxidase possess differing entropies of reduction [$dE_m/dT = \Delta S/(nF)$], and it is therefore not strictly valid to combine results obtained from EPR analysis of some groups in frozen samples with optical spectroscopic measurements of other groups at 25 °C in order to determine the reduction state of the various enzyme centers during titrations and during turnover.

Milk xanthine oxidase is a dimer of apparently identical M_r 140 000 subunits, each containing one FAD, one Mo center (with an associated pterin cofactor; Johnson et al., 1980), and two spectroscopically distinct Fe_2S_2 centers, termed Fe/S I and Fe/S II (Bray, 1975). There has been controversy in the literature as to the exact number of electrons which can be taken up by xanthine oxidase. If each Fe/S center takes up one electron and the FAD and Mo centers two each, then xanthine oxidase should take up six electrons per functional half-molecule. Edmondson et al. (1972) and Olson et al. (1974a) observed close to this stoichiometry in reductive ti-

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trations with dithionite when the end point was determined by measuring absorbance or EPR changes in the enzyme itself. However, when they followed the disappearance of dithionite, by means of its absorbance at 315 nm, these workers found consistent uptake of eight electrons per enzyme half-molecule. There has been speculation as to the nature of the enzyme group responsible for the extra uptake of 2 electrons: reduction of Mo beyond the Mo^{IV} state has been suggested (Coughlan et al., 1980), as has the reduction of a disulfide moiety (Bray, 1975). The interesting possibility must now be raised that the pterin cofactor of the Mo center can undergo oxidation and reduction (Johnson et al., 1980).

With the recent introduction of microcoulometry to the investigation of oxidative enzymes, it has seemed appropriate to reinvestigate the problem of the stoichiometry of electron uptake in xanthine oxidase. Microcoulometry utilizes a three electrode potentiostat to poise enzyme samples (containing a mixture of dye mediators to facilitate equilibration between enzyme and electrode) at controlled potentials, with the electron uptake associated with the enzyme at each potential being readily and precisely measured. No chemical reductant, such as dithionite, whose concentration may be difficult to determine due to its facile air oxidation or its ability to undergo side reactions, is required.

In addition, microcoulometry can be utilized to measure the reduction potentials of the centers taking up electrons. The measurements are performed at 25 °C, i.e., under conditions which can be readily correlated with catalytic function. Such measurements are important for xanthine oxidase, since Olson et al. (1974a,b) have provided strong evidence that internal electron transfer between the various centers of that enzyme is always faster than the rates of reduction and oxidation of the enzyme by substrates. Thus the oxidation states of the various enzyme centers during the catalytic cycle should be determined solely by their relative reduction potentials. Olson et al. (1974a) calculated such sets of relative potentials, and Cammack et al. (1976) and Barber & Siegel (1982) have determined absolute potentials for the various centers under a variety of experimental conditions. Both groups have utilized EPR spectroscopic analysis of samples frozen in liquid N_2 after reduction at 25 °C to follow reduction of most of the enzyme centers. Using such techniques, Barber & Siegel (1982) have reported that the Mo and Fe/S centers of xanthine oxidase, as well as the FAD, accept protons upon reduction, i.e., their potentials are pH dependent. They have reached conclusions as to the effects of ligand substitution on the Mo center on proton affinity vs. electron affinity of that center.

However, several reports (Williams-Smith et al., 1977; Orii & Morita, 1977) have shown that the process of freezing samples for EPR analysis can result in significant pH changes in the system, with the magnitude of the changes depending upon the nature of the buffer used. Palmer & Olson (1980) have pointed out the possibility of electron reequilibration in frozen samples rendering meaningless the potentials which are to be applied to an enzyme functioning at 25 °C. We have utilized microcoulometry of xanthine oxidase, together with certain of its derivatives which contain prosthetic groups modified so as to render them incapable of accepting electrons under the conditions of our experiments, to determine the reduction potentials of the enzyme centers at 25 °C. We have examined the effects of pH and possible anion binding on these potentials.

Experimental Procedures

Enzyme. Bovine milk xanthine oxidase was isolated from fresh cream by the modification of the procedure of Hart et al. (1970) described by Barber & Siegel (1982). Concentrations of native and desulfo enzyme were estimated from the A_{450} by using $E_{mM} = 72\ mM^{-1}\ cm^{-1}$ (Avis et al., 1956). Total active center concentration (functional plus nonfunctional Mo) was taken as twice the enzyme concentration. The proportion of functional sites in native enzyme was estimated from the activity/A_{450} ratio (23.5 °C), taking the limiting value of the ratio for fully functional enzyme given by McGartoll et al. (1970). The native enzyme preparation used in this work was 85% functional.

Desulfo xanthine oxidase was prepared as described by Massey & Edmondson (1970); the final enzyme preparation was less than 0.5% functional. Desulfo xanthine oxidase containing alkylated FAD was prepared by a modification of the method of McGartoll et al. (1970). Cyanide-treated enzyme, 17 μM in 50 mM Pipes–1 mM EDTA,[1] pH 6.5, was placed in a Thunberg tube and the solution made anaerobic by repeated evacuation and flushing with Ar. Solid $Na_2S_2O_4$ (final concentration 3 mM) and iodoacetamide (final concentration 4 mM) were added from the side arm and dissolved. The closed tube was incubated at 20 °C in the dark for 90 min, and solid cysteine hydrochloride was then added (final concentration 10.5 mM) to quench the reaction. The enzyme was exhaustively dialyzed vs. 50 mM bicine, pH 7.7, and the solution was clarified by centrifugation at 8000 rpm for 30 min at 4 °C. The enzyme was concentrated by ultrafiltration and stored under liquid N_2. The degree of flavin alkylation was determined by measurements of the absorbance at 450 and 550 nm, with the assumption that 100% alkylation results in a 37% decrease in the absorbance ratio compared to native or desulfo enzyme (McGartoll et al., 1970). The visible spectrum of the alkylated desulfo enzyme was identical with that previously described for the alkylated native enzyme.

Microcoulometry. The basic coulometric technique has been described by Watt (1979) and Watt et al. (1980). A three electrode cell of 3 mL capacity equipped with a Teflon magnetic stirrer, a rubber septum, and a working electrode prepared by electroplating gold onto a double coil of Pt gauze was used. A saturated calomel electrode, separated from the cell solution by a compartment with a Vicor disk, served as reference electrode, and a platinum gauze strip in a compartment separated from the cell solution by a Vicor disk served as counter electrode. A controlled potential was supplied by a PAR 173 potentiostat with an electrometer probe.

The enzyme, frozen in liquid N_2, was added to a small flask (1 mL capacity) equipped with a rubber septum and allowed to thaw under a stream of nitrogen which had been passed through Oxy-Trap (<1 ppm of O_2). The flask was evacuated and flushed several times with N_2 and then stirred gently at room temperature for 1 h. This procedure reduced the O_2 concentration to below the experimental error of the measurements.

The cell was filled with 3 mL of solution 0.1 M in KCl at the proper pH and buffer concentration. The solution was deaerated with N_2 and kept under a positive pressure of N_2. The mediators were added (5 μL of 30 mM solutions), and the proper potential was maintained with the potentiostat until the current dropped to a small but constant level (1–20 μA). A 5- or 10-μL portion of the enzyme was then transferred from the deaeration flask to the electrochemical cell with a gas-tight N_2-flushed syringe. The time–current curve was recorded and the coulombs required for reduction measured by an electronic

[1] Abbreviations: bicine, *N,N*-bis(2-hydroxyethyl)glycine; Ches, 2-(*N*-cyclohexylamino)ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; Pipes, piperazine-*N,N'*-bis(2-ethanesulfonic acid).

Table I: Midpoint Reduction Potentials for Xanthine Oxidase Obtained from Microcoulometric Titrations at 25 °C and from EPR Analysis of Frozen Samples

	50 mM bicine, pH 7.7 [a]		50 mM Ches, pH 8.9		55 mM sodium pyrophosphate, pH 8.2	
couple	coulometry [b] E_m [d]	EPR [c,e] E_m	coulometry E_m	EPR [e] E_m	coulometry E_m	EPR [f] E_m
Mo^{VI}/Mo^{V}	−375	−373	−450	−453	≤−365	−355
Mo^{V}/Mo^{IV}	−405	−377	−450	−427	≥−335	−355
$FAD/FADH_2$	−280	−283	−330	−340	−315	−294
Fe/S $I_{ox/red}$	−320	−310	−355	−350	−345	−343
Fe/S $II_{ox/red}$	−230	−255	−255	−285	−260	−303

[a] All buffers contained 1 mM EDTA. [b] Microcoulometric titrations were performed at 25 °C and midpoint potentials determined as described in the text. [c] Data were obtained for samples titrated potentiometrically in the indicated buffer at 25 °C and then analyzed by EPR spectroscopy of frozen samples. [d] All potentials are in millivolts and are expressed with reference to the standard hydrogen electrode. [e] Data of Barber & Siegel (1982). [f] Data of Cammack et al. (1976).

integrator in the circuit. Values of the current–time integral were obtained from the current–time curve by the use of a K&E planimeter. Because of small background current level changes, the planimeter method was found to be superior in precision and accuracy to the electronic integrator. After the current reached a small but constant level, the potential was stepped to the next value and the procedure repeated. Each point required a new 5- or 10-μL sample of enzyme.

The reference electrode was calibrated after each run against both a standard calomel electrode equipped with a Luggins capillary and a Ag/HCl electrode; it was found to be reproducible with an error of ±5 mV. The method was checked for both coulometric accuracy and potential with cytochrome *c* (Sigma, type III). The error was found to be ±25 μC, and E_{m7} (determined from a Nernst plot) agreed with published values within 10 mV. A mixture of five mediators was used in these experiments: anthraquinone-2-sulfonate (E_{m7} = −225 mV), safranine T (E_{m7} = −289 mV), benzylviologen (E_{m7} = −311 mV), methylviologen (E_{m7} = −440 mV), and triquat (E_{m7} = −540 mV).

Data Analysis. Midpoint potentials were obtained from the coulometric data as described under Results. Theoretical curves were plotted by using the Nernst equation for n = 1 or n = 2 reduction processes and a given set of potentials by means of a Hewlett-Packard 9825A computer interfaced to a 7225A graphics plotter.

Results

Native and Modified Xanthine Oxidases (pH 7.7). Figure 1 shows the results of microcoulometric titrations of native xanthine oxidase (85% functional) (curve A) and of two modified enzymes: desulfo xanthine oxidase, in which the terminal sulfur ligated to the Mo of functional enzyme has been replaced by an oxygen (Bordas et al., 1980) (curve B), and desulfo xanthine oxidase with its FAD alkylated so as to render the flavin incapable of oxidation and reduction (McGartoll et al., 1970) (curve C). All of the titrations were performed in 50 mM bicine, pH 7.7.

Electron addition to the desulfo enzyme with alkylated FAD occurs in a single wave with a midpoint potential of about −270 mV. Reduction is complete at potentials of −370 mV and involves uptake of 2.0 ± 0.2 electrons per half-molecule. On the basis of the potentials determined by Barber & Siegel (1982) at pH 7.7, using EPR analysis of frozen samples (see Table I) one would expect the electron uptake in this enzyme derivative in this range of potentials to be associated with reduction of Fe/S II and Fe/S I (the alkylated flavin cannot be reduced). There seems to be no electron uptake in the range of potentials expected for reduction of the desulfo Mo centers (average E_m for Mo^{VI}/Mo^{IV} = −405 mV at pH 7.7; Barber & Siegel, 1982). This result may indicate an inability of the desulfo Mo to be reduced by the mediator dyes used in the time periods used in these studies; previous experiments have utilized dithionite to reduce desulfo Mo centers, and even with this reductant electron transfer to this center is quite slow (Bray, 1975).

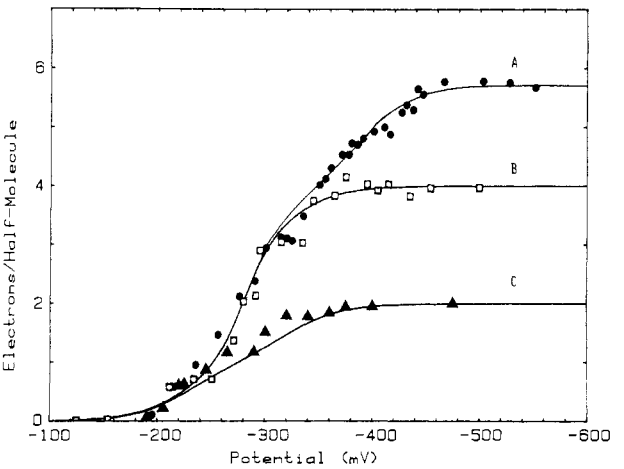

FIGURE 1: Microcoulometric titration of native xanthine oxidase (curve A), desulfo xanthine oxidase (curve B), and desulfo xanthine oxidase with alkylated FAD (curve C), in 50 mM bicine–1 mM EDTA, pH 7.7. Each experimental point represents the electron uptake following addition of the following amounts of xanthine oxidase half-molecules to the titration vessel: native enzyme, 2.72 nmol; desulfo enzyme, 2.08 nmol; desulfo enzyme with alkylated FAD, 1.10 nmol. Curves shown were constructed by using the midpoint potentials listed in Table I. For details see the text.

The desulfo enzyme, with intact FAD, takes up 4.0 ± 0.1 electrons per half-molecule. Reduction occurs in a single wave with a midpoint potential of about −275 mV. These results indicate that the FAD moiety of xanthine oxidase takes up two electrons with an average E_m for $FAD/FADH_2$ of about −280 mV (slightly more negative than the average E_m values for the two Fe/S centers), a result in good agreement with the potentials determined for the FAD at pH 7.7 by Barber & Siegel (1982) (Table I). Again, there is no electron uptake at potentials more negative than −370 mV, a result which indicates that the desulfo Mo centers are not being reduced.

Electron uptake by the native enzyme occurs in two waves: the first corresponds to the four electron reduction process seen with desulfo enzyme, with an indicated midpoint potential of about −275 mV, and the second involves addition of between 1.5 and 2 electrons to each enzyme half-molecule and exhibits an apparent midpoint potential of −390 mV. This potential is similar to (but not identical with) the average functional Mo^{VI}/Mo^{IV} E_m at pH 7.7 of −375 mV reported by Barber & Siegel (1982) (Table I). The total uptake of electrons into

the native enzyme preparation in two separate experiments (data combined in Figure 1A) was 5.7 ± 0.1 electrons per half-molecule, a result which indicates uptake of 2 electrons by each functional Mo center (5.7 − 4.0 = 1.7 electrons per 0.85 functional Mo).

The experimental data of Figure 1 were used to find a consistent set of midpoint potentials which would provide the simultaneous best fit to the data for all three enzyme forms, assuming one electron reduction process of Fe/S I and Fe/S II in all three enzyme forms, two consecutive one electron reduction processes for FAD in the native and desulfo enzymes, and two consecutive one electron reduction processes for the Mo center in the native enzyme.

The data points for the desulfo alkylated enzyme were constrained to fit

$$n_C = 1/[1 + 10^{(E-E_{Fe/S\,I})/59}] + 1/[1 + 10^{(E-E_{Fe/S\,II})/59}]$$

The data points for the desulfo enzyme were constrained to fit

$$n_B = n_C + 1/[1 + 10^{(E-E_{FAD,1})/59} + 10^{(E_{FAD,2}-E)/59}] + 2/[1 + 10^{(E-E_{FAD,2})/59} + 10^{(2E-E_{FAD,1}-E_{FAD,2})/59}]$$

The data points for the native enzyme were constrained to fit

$$n_A = n_B + n_C + f/[1 + 10^{(E-E_{Mo,1})/59} + 10^{(E_{Mo,2}-E)/59}] + 2f/[1 + 10^{(E-E_{Mo,2})/59} + 10^{(2E-E_{Mo,1}-E_{Mo,2})/59}]$$

where $E_{Fe/S\,I}$, $E_{Fe/S\,II}$, $E_{FAD,1}$, $E_{FAD,2}$, $E_{Mo,1}$, and $E_{Mo,2}$ are the midpoint potentials for the couples Fe/S I (ox/red), Fe/S II (ox/red), FAD (ox/semiquinone), FAD (semiquinone/hydroquinone), Mo(VI/V), and Mo(V/IV), respectively; f = the fraction of xanthine oxidase molecules which contain functional Mo centers (0.85 in Figure 1); n = the number of electrons taken up per half-molecule of xanthine oxidase at solution potential E.

All of the data points were used to find a set of midpoint potentials which yielded the minimum total variance between the data points and the respective theoretical curves calculated according to the above equations. The best fit to the data was obtained (using an IBM 1300 series computer with a statistical least-squares program provided by J. P. Chandler, Oklahoma State University) with the potentials indicated in Table I. The computer search indicated that E_m for FAD/FADH is much less than that for FAD/FADH$_2$ (it was found that all fits with $E_{FAD,2} - E_{FAD,1} > 30$ mV were essentially identical), so that FAD reduction behaves as a two electron process in the coulometric titrations. Thus, only the average E_m for the FAD/FADH$_2$ couple could be obtained with any accuracy. (This result is in good agreement with the EPR results of Barber & Siegel (1982), which show that the two potentials for FAD reduction differ by 130 mV at pH 7.7.) The maximum error in the coulometrically determined potentials was ±10 mV, as determined by the curve fitting process. Given the errors discussed under Experimental Procedures involved in the microcoulometric measurement itself, it is likely that the potentials of Table I are accurate to at least ±20 mV [an error similar to that involved in the potentiometric titration–EPR measurements; see Barber & Siegel (1982)].

It can be seen in Table I that the midpoint potentials obtained at 25 °C from the microcoulometric titration data at pH 7.7 are in good agreement with those obtained by EPR analysis of samples titrated potentiometrically at 25 °C and then frozen for EPR analysis with the following exceptions: the Mo^V/Mo^{IV} potential is shifted more negative by about 30 mV, and the Fe/S II$_{ox/red}$ potential is shifted more positive by about 25 mV in the frozen samples. Since these species represent the first and last centers to be reduced in the coulometric titrations and electron uptake by them is not greatly masked by uptake into other centers, we can perhaps be more confident with respect to the accuracy of our estimate for these two potentials than with respect to the potentials of the other centers.

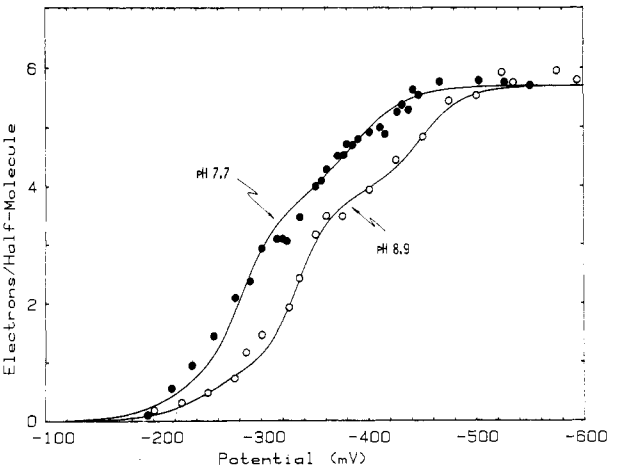

FIGURE 2: Microcoulometric titration of native xanthine oxidase as a function of pH. Titrations were performed, as described in the text, on enzyme samples in either 50 mM bicine–1 mM EDTA, pH 7.7, or 50 mM Ches–1 mM EDTA, pH 8.9. Each experimental point represents the electron uptake following addition of 2.72 nmol of enzyme half-molecules to the titration vessel. Curves shown were constructed by using the midpoint potentials listed in Table I. For details see the text.

Effect of pH on Midpoint Potentials. Barber & Siegel (1982), using EPR analysis of frozen samples, have indicated that the midpoint potential for each of the electron carrying centers in xanthine oxidase varies with pH. Williams-Smith et al. (1977) have documented changes in solution pH upon freezing samples in a number of buffers. Although Barber and Siegel used zwitterionic buffers (Good, 1966) to minimize this effect, as suggested by Williams-Smith et al. (1977), it seemed appropriate to see if the same pH effects on midpoint potential are observed if enzyme is both titrated and analyzed at 25 °C.

Figure 2 shows the results of a microcoulometric titration of native xanthine oxidase in 50 mM Ches, pH 8.9. The analogous data for native enzyme at pH 7.7 are shown for comparison. It is evident that all of the data points for which equivalent electron uptake occurs are located at more negative potentials at pH 8.9 compared to pH 7.7. The pH 8.9 titration curve, like that at pH 7.7, exhibits two distinct waves of electron uptake: one involving 4 electrons, with an apparent midpoint potential of −320 mV, and the second involving 1.5–2 electrons, with an indicated midpoint potential of −450 mV. Total electron uptake was again 5.7 ± 0.1 per enzyme half-molecule.

The potentials determined by EPR analysis of frozen samples are indicated for pH 8.9 and 7.7 in Table I. The EPR-derived potentials for the various centers differ at the two pHs as follows ($E_{m,8.9} - E_{m,7.7}$): Fe/S II, −30 mV; Fe/S I, −40 mV; FAD/FADH$_2$, −57 mV; Mo^{VI}/Mo^V, −80 mV; Mo^V/Mo^{IV}, −50 mV. If the pH dependence of the potentials determined by Barber and Siegel is correct, then one should be able to fit the coulometric titration data obtained at pH 8.9 by using the potentials obtained by coulometric titration at pH 7.7 together with the shifts in potential on changing pH estimated by EPR. The curve drawn through the points in the pH 8.2 titration of Figure 2 was calculated by using a set of potentials calcu-

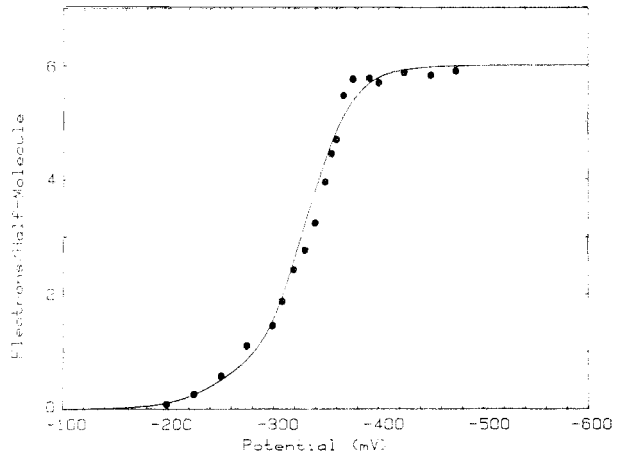

FIGURE 3: Microcoulometric titration of native xanthine oxidase in 55 mM sodium pyrophosphate–1 mM EDTA buffer, pH 8.2. Each experimental point represents the electron uptake following addition of 2.72 nmol of enzyme half-molecules to the titration vessel. The curve shown was constructed by using the midpoint potentials listed in Table I. For details see the text.

lated in just this fashion, with a permitted deviation of ±5 mV (the error in the coulometric measurements); these potentials are listed in Table I. It is evident that the calculated curve represents a good fit to the data. Thus the potentials for Mo, Fe/S I, and Fe/S II, as well as FAD, are pH dependent, as indicated by Barber and Siegel. The data also suggest that at pH 8.9, as at pH 7.7, the apparent midpoint potentials of Fe/S II and Mo^{V}/Mo^{IV} are altered upon freezing of the sample.

Effect of Pyrophosphate on Midpoint Potentials. Cammack et al. (1976) reported that the midpoint potentials for Mo reduction, measured in potentiometric titrations performed at pH 8.2, were shifted markedly in a positive direction when the titration was performed in pyrophosphate as opposed to Tris buffer. They suggested that pyrophosphate complexes to the Mo center of xanthine oxidase, and since then a number of anions have been reported to alter the EPR spectrum of Mo^{V} in the enzyme (Bray, 1980). They also suggested that the E_m for Fe/S II was shifted substantially (48 mV) in a negative direction in pyrophosphate as opposed to Tris buffer. Since the analyses were performed on frozen samples and Williams-Smith et al. (1977) showed that pyrophosphate buffers undergo a marked change in pH when frozen, the significance of these apparent anion-induced shifts in potential has been rendered uncertain.

In order to investigate this matter, we have performed a microcoulometric titration of native xanthine oxidase in pyrophosphate buffer, pH 8.2. The results are shown in Figure 3. It can be seen that the uptake of six electrons occurs in what is essentially a single wave with an indicated midpoint potential of approximately −320 mV. Best fits to this data required the electron uptake to involve two $n = 1$ processes, with $E_m = -260$ and −345 mV, respectively, and two $n = 2$ processes, with $E_m = -315$ and −350 mV, respectively. Since there appear in general to be little or no shifts in the midpoint potentials of Fe/S I and $FAD/FADH_2$ on freezing, we can probably associate the potentials at −345 and −315 mV with these respective species (see EPR data in Table I). The potential at −260 mV can then be associated with Fe/S II.

As with the samples examined in zwitterionic buffers at pH 7.7 and 8.9, the potential for Fe/S II determined by coulometry at 25 °C is significantly more positive (in this case by 43 mV) than that reported for Fe/S II in pyrophosphate for titrations analyzed by EPR of frozen samples (Cammack et al., 1976). The Fe/S II potential of the Fe/S II center is −255 mV (the same as that at pH 7.7) in zwitterionic buffer at pH 8.2 according to the EPR-based results of Cammack et al. (1976); thus, one would have expected a potential of −230 mV at pH 8.2 by microcoulometry in zwitterionic buffer. Thus it does appear that there is a pyrophosphate-induced negative shift in the Fe/S II potential at 25 °C.

Most striking is the finding that the Mo center must be assigned an average E_m of −350 mV on the basis of the coulometric data. According to the results of Barber & Siegel (1982), the two midpoint potentials for Mo reduction become more negative by −25 ± 3 mV on raising the pH from 7.2 to 8.2 in zwitterionic buffers; if one combines this potential shift with the coulometrically determined potentials at pH 7.7, the expected potentials for Mo^{VI}/Mo^{V} and Mo^{V}/Mo^{IV} in such buffers at pH 8.2 are −400 and −435 mV, respectively. Furthermore, although precise potentials for the individual Mo potentials could not be obtained from the coulometric data because Mo reduction behaved as an $n = 2$ process, that fact alone means that the Mo^{VI}/Mo^{V} potential must be at least 30 mV more negative than the Mo^{V}/Mo^{IV} potential in order to fit the data. Thus, although there may or may not be a shift in the Mo^{VI}/Mo^{V} potential on interaction of pyrophosphate with xanthine oxidase, there is certainly a significant positive shift in the Mo^{V}/Mo^{IV} potential (to ≥ -335 mV). We can conclude that pyrophosphate does indeed specifically affect one or both of the Mo potentials of xanthine oxidase at 25 °C.

Discussion

The results reported in this paper permit us to reach the following conclusions: (1) Xanthine oxidase can accept six electrons per functional half-molecule upon equilibration with the mediator dyes used in the present work: one electron in each of the Fe/S centers, two electrons in the FAD, and two electrons in the Mo center. There is therefore no need to postulate more reduced states of Mo than Mo^{IV} or involvement of other reducible groups (e.g., pterin or disulfide) in electron-accepting and -donating reactions of xanthine oxidase. Combination of the coulometrically derived midpoint potentials with those derived from EPR analysis of samples reduced under a common set of conditions demonstrates that the only potentials for this enzyme which need to be considered given the evidence available to date are those for Fe/S $I_{ox/red}$, Fe/S $II_{ox/red}$, FAD/FADH, $FADH/FADH_2$, Mo^{VI}/Mo^{V}, and Mo^{V}/Mo^{IV}.

However, since it is evident that there exist conditions in which groups not able to accept electrons in the microcoulometry experiments can in fact be made to accept electrons [e.g., slow reduction of the desulfo Mo center by dithionite; see Bray (1975)], we cannot exclude the possibility that electron-accepting groups more fastidious in their specificity for electron donors than the functional Mo, FAD, and Fe/S centers could exist in xanthine oxidase.

(2) The midpoint potentials determined for the Mo^{VI}/Mo^{V}, Fe/S $I_{ox/red}$, FAD/FADH, and $FADH/FADH_2$ couples of xanthine oxidase by EPR analysis of samples frozen after equilibration at a fixed potential at 25 °C are valid as measurements of the potential at 25 °C in at least the three different buffer systems examined in the present work.

In contrast, the midpoint potential of Fe/S $II_{ox/red}$ exhibited a distinct negative shift of 25–45 mV, while that of Mo^{V}/Mo^{IV} exhibited a positive shift of about 30 mV, in samples analyzed after freezing as compared to samples analyzed at 25 °C. In view of the relative constancy of the pH-dependent potentials for FAD, Mo^{VI}/Mo^{V}, and Fe/S I in the two types of measurement, it seems unlikely that changes in pH on freezing

can account for the variation observed with the Fe/S II and Mo^{V}/Mo^{IV} potentials. Such changes more likely represent differences in the entropy of reduction for the various centers [$dE_m/dT = \Delta S/(nF)$], as discussed by Palmer & Olsón (1980). Since one cannot strictly determine the temperature at which internal electron transfer between the centers of xanthine oxidase [these centers are relatively close together in space, as determined by studies of magnetic interactions between them reported by Lowe & Bray (1978) and Barber et al. (1982)] ceases on freezing of samples, one cannot readily estimate the expected magnitude of the potential shifts due to this entropic factor alone.

The finding of differential variation in potential of the centers on freezing renders suspect calculations of the internal electron distributions in xanthine oxidase in reductive titrations and catalytic experiments performed by Olson et al. (1974a,b). These calculations were based on EPR measurements of Mo^{V}, FADH, and reduced Fe/S center concentrations in frozen samples together with measurements of absorbance changes at room temperature which were combined with the EPR data to yield concentrations of $FADH_2$. They have been used as the experimental basis for the generally accepted model for electron transfer in xanthine oxidase (Palmer & Olson, 1980); this model may now be in need of reexamination.

(3) The midpoint potentials of the Mo, FAD, and Fe/S centers of xanthine oxidase are dependent on pH, with the variation with pH being in excellent agreement with that predicted by the data of Barber & Siegel (1982) obtained from EPR analysis of frozen samples. The shift in the Mo potentials, which together comprise a distinct wave of electron uptake at low potentials in xanthine oxidase in Good buffers at pH 7.7 and 8.9, is particularly evident when the pH is shifted (Figure 2). Thus, it is clear that protonation does indeed accompany electron uptake by the Mo centers in this enzyme, a result predicted by Stiefel (1973) in his general model of molybdenum hydroxylase function.

(4) The potentials of the Mo center, and probably the Fe/S II center as well, shift significantly at 25 °C when pyrophosphate is present during the titrations. The predominating effect of this anion would appear to be to evoke a large positive shift in the Mo^{V}/Mo^{IV} potential (the shift in the Mo^{VI}/Mo^{V} potential is uncertain). Such specific effects on the Mo^{V}/Mo^{IV} potential have been noted previously upon binding of uric acid to functional xanthine oxidase and on conversion of the enzyme Mo from the functional to the desulfo form by replacing a terminal sulfur ligand with an oxygen (Barber & Siegel, 1982). It is also of interest that it is this Mo potential which is most susceptible to the freezing induced shift, indicating the presence of a significant entropy change on reduction. These results suggest that a number of the effects of external agents on xanthine oxidase catalytic function (Bray, 1975) may be explained by binding to or changing the environment of the EPR-silent Mo^{IV} oxidation state of the enzyme.

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