The Reaction of Xanthine Oxidase with Molecular Oxygen*

(Received for publication, November 16, 1973)

JOHN S. OLSON,‡ DAVID P. BALLOU, GRAHAM PALMER,‡ AND VINCENT MASSEY
From the Department of Biological Chemistry and Biophysics Research Division, Institute of Science and Technology, University of Michigan, Ann Arbor, Michigan 48105

SUMMARY

The reaction of reduced xanthine oxidase with oxygen occurs in two distinct kinetic phases with the rate of the fast phase being about 10 times greater than that of the slow phase. Comparison of transient state and steady state kinetics demonstrates that only the fast phase of oxidation is catalytically significant. The time course of oxidation is quantitatively independent of the following factors: (a) the activity to flavin ratio; (b) presence of competitive inhibitors; (c) pretreatment of enzyme with either cyanide or allopurinol; and (d) addition of superoxide dismutase. Removal of flavin eliminates both oxidase activity and the reactivity of the reduced enzyme with molecular oxygen (KOMAI, H., MASSEY, V., AND PALMER, G. (1969) J. Biol. Chem. 244, 1692–1700).

Both optical and EPR kinetic data support the interpretation that 5 electrons are consumed in the fast phase and 1 in the slow phase. The spectral properties of the slowly reacting species suggest that the residual electron is present principally as reduced iron-sulfur center II at pH 8.5. The simplest interpretation of the kinetic data requires that rapid reaction of the enzyme with oxygen occurs via fully reduced flavin and that the slow phase observed for 1-electron reduced enzyme is a consequence of both the poor reactivity and small equilibrium concentration of flavin semiquinone. The absence of any effect of superoxide dismutase on both the optical and EPR data and the small amount of superoxide generated indicates that hydrogen peroxide is the major product formed during oxidation of fully reduced xanthine oxidase.

Although milk xanthine oxidase is one of the most widely investigated enzymes, few direct studies of the reaction of reduced enzyme with oxygen have been reported. Most previous investigations have dealt either with steady state kinetics at fixed oxygen concentrations or with the appearance and decay of reduced molybdenum, iron, and flavin signals during anaerobic reduction. It has long been assumed that the reduction of xanthine oxidase is the rate-limiting step in catalysis, and this does appear to be the case when saturating concentrations of oxygen and reducing substrate are present (1, 2).

However, at lower oxygen concentrations the rate of oxidation must approach that of reduction since the $K_m$ values for oxygen are relatively large (about $5 \times 10^{-5}$ M at pH 8.5 (2)) and substantial amounts of reduced enzyme are observed in the steady state during turnover with xanthine. For example, during the initial part of the steady state, xanthine oxidase is about 50% reduced when the initial concentrations of xanthine and oxygen are $2.5 \times 10^{-4}$ and $1.27 \times 10^{-4}$ M, respectively (2), suggesting that the rate of oxidation in air-saturated buffers is no more than about twice the rate of reduction by xanthine, and therefore should be readily measurable by ordinary rapid kinetic techniques.

The primary purpose of the present communication is to examine directly the reaction of reduced xanthine oxidase with molecular oxygen and to compare the results obtained with those predicted from steady state data and previous anaerobic reduction studies. Evidence is presented which indicates that the reduced states of enzyme-bound flavin react directly with oxygen and that superoxide is not the main product of oxidation but rather that the first few steps in the over-all reaction result directly in the formation of $H_2O_2$.

MATERIALS AND METHODS

Xanthine oxidase was isolated from freshly churned buttermilk by the method of Massey et al. (2). Prior to use in either steady state or rapid kinetic experiments, enzyme solutions were freed of salicylate by dialysis against several changes of the appropriate buffer; all buffers contained $3 \times 10^{-4}$ M EDTA. Xanthine oxidase activity was measured spectrophotometrically at 265 nm following the conversion of xanthine to uric acid; extinction differences were determined separately for each pH and wavelength examined. The amount of active enzyme was determined from either the extent of the rapid phase in anaerobic reduction experiments with xanthine or from the activity to flavin ratio (3). AFR values were obtained by dividing the absorbance change per min at 295 nm by the absorbance at 450 nm of the xanthine oxidase used in catalytic assays in 0.1 M pyrophosphate, pH 8.5, at 25°. Fully active enzyme preparations exhibit an AFR value equal to 210 under these conditions (3). Enzyme concentrations were determined spectrophotometrically using a value of 37,309 M$^{-1}$ cm$^{-1}$ for the molar absorbance per enzyme-bound FAD at 450 nm (2). Cyanide-inactivated enzyme was prepared according to the procedure of Massey and Edmondson (4).

Xanthine and allopurinol were purchased from Nutritional Biochemicals Corporation. Rapid mixing experiments were performed with a Gibson-Milnes stopped flow spectrophotometer.

*This work was supported by National Institutes of Health Research Grants GM12176 and GM01106 and by National Institutes of Health Postdoctoral Fellowship GM53052 (J. O.).

‡Present address, Department of Biochemistry, Rice University, Houston, Texas 77001.

1 The abbreviations used are: AFR, activity to flavin ratio; Fe/S, non-heme iron-sulfur center.
et al. (2) reportred values of $K_1$ for oxygen, and $K_2$ for xanthine is in excess. The slower phase reflects changes in the steady state condition and is followed by a much slower increase rapid decrease in absorbance represents the approach to the steady state. Rapid freeze experiments were performed with a modified Bray apparatus (6). EPR spectra were recorded using the conditions described by Edmondson et al. (7). Edmondson et al. (7) assumed that the signal at $g = 2.12$ represented iron center II and the signal at $g = 1.90$ represented iron center I. However, from the results of Lowe et al. (8) and those in Fig. 12, it is clear that the latter assignment is not correct. A comparison of the resolved EPR spectra of Fe/S I and II suggests that Fe/S II contributes about 50% of the signal measured at $g = 1.90$. This result is particularly evident from kinetic experiments involving the oxidation of reduced xanthine oxidase (Fig. 12). A better measure of Fe/S II appears to be the amplitude at $g = 1.95$ where Fe/S II has a derivative of approximately zero.

RESULTS

Steady State Measurements—A series of experiments in which xanthine oxidase was mixed with $10^{-4}$ M xanthine at several different oxygen concentrations is shown in Fig. 1. The very rapid decrease in absorbance represents the approach to the steady state condition and is followed by a much slower increase or decrease in absorbance depending on whether oxygen or xanthine is in excess. The slower phase reflects changes in the amount of reduced enzyme as the substrate molecules are consumed. If one substrate is in excess, the area circumscribed by the curve in the slow phase and a line horizontal to the final absorbance reading is proportional to the absolute initial concentration of the limiting substrate. The rate of change of this area at a given time after mixing is proportional to the turnover number of the enzyme at a substrate concentration defined by the remaining area under the curve. Using an analysis of this type, Massey et al. (2) reported values of $V_{\text{max}}$, $K_m$ for oxygen, and $K_m$ for xanthine equal to 850 min$^{-1}$, $5 \times 10^{-3}$ M, and $1.2 \times 10^{-3}$ M, respectively.

In addition to steady state parameters, the results of Fig. 1 also contain more specific information concerning the individual half-reactions. Previous steady state analyses (2, 9) have shown that the xanthine-oxygen reaction exhibits a kinetic pattern which is consistent with a simple binary complex mechanism and can be described accurately by the following formulation:

$$
E_{\text{ox}} + X \overset{k_1}{\underset{k_{-1}}{\rightleftharpoons}} E_{\text{ox}}X \overset{k_2}{\underset{k_{-2}}{\rightarrow}} E_{\text{red}} + U
$$

where $E_{\text{ox}}$ and $E_{\text{red}}$ represent oxidized and reduced enzyme and $X$ and $U$, xanthine and uric acid, respectively. Assuming the simplest possible behavior, the initial steady state fraction of oxidized enzyme defined by the ordinate in Fig. 1A is given by $k_{10}/(k_{10} + k_{o2})$, where $k_{10}$ and $k_{o2}$ are the effective reduction and oxidation rates and are equal to $k_d[X]/(k_{-1}/k_1 + [X])$ and $k_d[O_2]/(k_{-2}/k_2 + [O_2])$, respectively. From the linear portion of the top curve in Fig. 1B, the rate of reduction by $10^{-4}$ M xanthine was estimated to be 885 min$^{-1}$. This value coupled with the initial fractions of oxidized enzyme in the steady portions of the curves given in Fig. 1A allows a rough calculation of the rate of oxidation at the various oxygen concentrations examined. From these results, the value of $K_1$ was estimated to be about 3000 min$^{-1}$ and the ratio of $k_{-1}/k_1$ of the order of $5 \times 10^{-4}$ M. Similar values were obtained by analyzing the dependence of the rate of approach to the steady state on oxygen concentration. Assuming $k_2$ equals 885 min$^{-1}$, the value of $V_{\text{max}}$ calculated from this simple pre-steady state analysis is 680 min$^{-1}$. This low value suggests that either or both $k_2$ and $k_4$ are underestimated.

A more systematic examination of the steady state parameters was carried out by measuring uric acid formation in the presence of low concentrations of enzyme in the stopped flow apparatus at five different oxygen and xanthine concentrations (Fig. 2). In agreement with previous work (2, 9), parallel lines were observed in the double reciprocal plots (Fig. 2). This pattern of parallel lines is still observed in the presence of salicylate, a potent competitive inhibitor (2), indicating that a ternary complex is not formed. The various steady state parameters for the simple binary complex mechanism (Equation 1) are given in Equation 2.

$$
V_{\text{max}} = \frac{k_2}{k_2 + k_4} ; K_m = \frac{k_4}{(k_2 + k_4)} ; \frac{k_{-1} + k_2}{k_1} ; \frac{k_4}{(k_2 + k_4)}
$$

The numerical values for $V_{\text{max}}, K_1$, and $K_2$ obtained from Fig. 2 are 1100 min$^{-1}, 9.4 \times 10^{-4}$ M, and $5.3 \times 10^{-4}$ M, respectively. Virtually identical results were obtained when the entire experiment was repeated with another enzyme preparation of higher specific activity. It is important to note that the deviation from linearity with high xanthine concentration shown in Fig. 2 is a reflection of excess substrate inhibition, a well documented property of xanthine oxidase-catalyzed reactions at intermediate and low pH values.\(^3\)

\(^3\) Olson and Massey (unpublished results) have shown that at pH 8.5 concentrations of xanthine greater than $10^{-4}$ M appear to inhibit both the catalytic and anaerobic reduction reactions non-competitively, exhibiting a $K_I$ of about $10^{-4}$ M. Both the extent and type of inhibition was found to be very similar to that reported by Hofstee (10).
to oxygen and is independent of the wavelength of observation. The rate of the slow phase is accurately second order with respect to concentration is shown in Fig. 4. In all cases the concentration of oxygen was kept much larger than that of xanthine oxidase. About 40 to 50% at wavelengths greater than 500 nm.

The dependence of the observed rates of the fast and slow phases (k1 and k2) and the relative amplitude of the slow phase on oxygen concentration is shown in Fig. 3. The amplitude of the slow phase is dependent on the wavelength of observation and is greater at longer wavelengths, particularly at 600 nm. The simplest interpretation of this latter behavior is to assume that oxygen binds to xanthine oxidase to form a reduced enzyme-O2 complex which subsequently decays at a much slower rate producing partially oxidized enzyme and reduced forms of oxygen. For such a case, the observed rapid oxidation rate would equal k1 [O2]/(Kd + [O2]), as before. Therefore, a plot of 1/kf versus 1/O2 should yield a straight line with a y intercept equal to k1 and an x intercept equal to -1/Kd. The dependence of kf on oxygen concentration does indeed appear to be described by a simple hyperbolic function, and values of Kd and kf were estimated to be 8.3 x 10^-4 M and 7.2 x 10^6 M^-1 cm^-1, respectively. The inset shows time courses for the fast phases which were obtained graphically by subtracting absorbance changes due to the slow phase from the over-all observed changes.

These values of Kd and Kx are almost identical with those obtained by Massey et al. (2) from turnover experiments in which changes in enzyme absorbance were measured, and we find the extrapolated turnover number to be in good agreement with the previous value when this is corrected for the per cent functional active sites in the enzyme used previously. The observed value for Vmax measured directly by product formation is slightly greater than the rate of anaerobic reduction by 10^-4 M xanthine (7). This apparent discrepancy between steady state and reduction experiments is not limited to oxidation of xanthine at pH 8.5 and has been observed with several other substrates and at other pH values. This point will be discussed later and more completely in the accompanying paper (11).

**Reaction of Reduced Enzyme with Oxygen** When xanthine oxidase is reduced with approximately 3 moles of dithionite per mole of FAD and then mixed with oxygen, a markedly biphasic reaction occurs, with the rate of the fast phase roughly 10 times greater than that of the slow phase (Fig. 3). The amplitude of the slow phase is dependent on the wavelength of observation and contributes about 20% of the absorbance change at 450 nm and about 40 to 50% at wavelengths greater than 500 nm.

The simplest and most straightforward treatment of the oxidation reaction is to analyze the observed time course graphically in terms of two exponentials with amplitudes reflecting the spectral weights of the rapidly and slowly decaying kinetic components. The dependence of the observed rates of the fast and slow phases (k1 and k2) and the relative amplitude of the slow phase on oxygen concentration is shown in Fig. 4. In all cases the concentration of oxygen was kept much larger than that of xanthine oxidase. The rate of the slow phase is accurately second order with respect to oxygen and is independent of the wavelength of observation (Fig. 4A). In contrast, the rate of the fast phase appears to approach a limiting value at high oxygen concentrations and is greater at longer wavelengths, particularly at 600 nm. The simplest interpretation of this latter behavior is to assume that oxygen binds to xanthine oxidase to form a reduced enzyme-O2 complex which subsequently decays at a much slower rate producing partially oxidized enzyme and reduced forms of oxygen. For such a case, the observed rapid oxidation rate would equal k1 [O2]/(Kd + [O2]), as before. Therefore, a plot of 1/kf versus 1/O2 should yield a straight line with a y intercept equal to k1 and an x intercept equal to -1/Kd. The dependence of kf on oxygen concentration does indeed appear to be described by a simple hyperbolic function, and values of Kd and kf were estimated to be 8.3 x 10^-4 M and 7.2 x 10^6 M^-1 cm^-1, respectively, for measurements at 450 nm (Fig. 4C). The absolute values of kf are consistently greater when measured at 600 nm and extrapolate to a value of about 10,000 min^-1 at infinite oxygen concentration, but the value of Kd is independent of wavelength.

In contrast to the fast and slow rates, the amplitudes of the two kinetic components do not vary with oxygen concentration and are a function only of the wavelength of observation (when the dead time of the stopped flow apparatus is taken into account) (Fig. 4C). Thus, other than the decay of the postulated reduced enzyme-oxygen complex, there does not appear to be any slow first order intramolecular process which limits the rate of oxidation of xanthine oxidase. If this were not true, the relative amplitudes of the two kinetic phases (and therefore the shapes of the time courses) would be expected to vary as the absolute velocity of the over-all reaction is increased by raising the oxygen concentration.

**Effects of Inhibitors and Chemical Modification**—Implicit in the idea of a binary complex mechanism is the assumption that the two half reactions are physically separate steps. The clearcut...
FIG. 4. Dependence of the time course of oxidation on oxygen concentration. The rates and relative proportions of the two phases shown in Fig. 3 were examined as a function of oxygen concentration. Reactions were carried out as described in Fig. 3 at the five $O_2$ concentrations shown. Wavelengths of observations were: 450 nm (C); 550 nm ($\bullet$); and 600 nm ($\Delta$). $A$, rate of the slow kinetic component versus oxygen concentration. $B$, rate of the fast kinetic component versus oxygen concentration. $C$, percentage of slow phase versus oxygen concentration and the reciprocal of the rate of the fast component versus the reciprocal oxygen concentration.

example of the separation of the oxidation and reduction reactions has been presented by Komai et al. (12) in their studies of the reversible removal of enzyme-bound FAD. The deflavoenzyme was shown to exhibit no oxidase activity but could be reduced by xanthine to the same extent as both native and reconstituted xanthine oxidase. In fact, the rate of reduction of the deflavoprotein was observed to be some 40% higher than that of the native enzyme. In contrast, the rate of oxidation of reduced deflavoenzyme was found to be some 60 times smaller than the rate observed for the slow phase in Fig. 3. This result indicates strongly, if not conclusively, that FAD is the prosthetic group which reacts with oxygen and further that the presence of the flavin is not a requirement for reduction by xanthine.

A large body of evidence, chemical, spectral, and kinetic, has indicated that most reducing substrates react directly with molybdenum which donates electrons to the iron-sulfur and flavin chromophores (13). The major exception to this rule is NADH which donates electrons through oxidized FAD (12). Thus, diaphorase activity would be expected to correspond closely to its kinetic pattern to that of the oxidation reaction with molecular oxygen. For example, the removal of FAD abolishes the capability of the enzyme to react with both NADH and oxygen. As a corollary, modification of the molybdenum site would not be expected to alter either the $O_2$ or NADH reactivity of xanthine oxidase. In the case of NADH, both complexes the molybdenum in a lower reduction state by alloxanthine and cyanoxylysis of the active center persulfide have little or no effect on diaphorase activity whereas the same treatment completely abolishes both xanthine oxidase activity and anaerobic reduction by xanthine (4, 14). Therefore, neither of these treatments would be expected to affect greatly the oxidation reaction.

Both the rates and the over all features of the reaction of reduced xanthine oxidase with oxygen are indeed independent of the activity to flavin ratio of the enzyme preparation (Fig. 5 and Table I). Treatment with cyanide which abolishes all enzymatic activity with xanthine and cyanalysis of the active center persulfide have little or no effect on diaphorase activity whereas the same treatment completely abolishes both xanthine oxidase activity and anaerobic reduction by xanthine (4, 14). Therefore, neither of these treatments would be expected to affect greatly the oxidation reaction.

Both the rates and the over all features of the reaction of reduced xanthine oxidase with oxygen are indeed independent of the activity to flavin ratio of the enzyme preparation (Fig. 5 and Table I). Treatment with cyanide which abolishes all enzymatic activity with xanthine produces little or no effect on reoxidation. This is true for both the rates and amplitudes of the fast and slow phases. Similarly, enzyme which is reduced with excess allopurinol is reoxidized at rates almost identical with those of native enzyme. The product of this reaction, alloxanthine, binds extremely tightly to a lower reduction state of molybdenum, and this complex (which is responsible for the inhibition of enzymatic activity) is stable for long periods of time, even in the presence of oxidizing agents (14). Therefore, even though the molybdenum is complexed in a lower reduction state, presumably Mo(IV), the reoxidation of the remaining iron-sulfur and FAD chromophores occurs via a mechanism essentially identical with that of reduced native enzyme (Fig. 5 and Table I).

Neither 10 mM salicylate, a powerful competitive inhibitor of both anaerobic reduction and catalytic activity, nor the product of xanthine oxidation, uric acid, produce any marked effects on the oxidation reaction (Fig. 5A). The reoxidation reaction of
Reactions were carried out in the stopped flow apparatus and measured at 450 nm. Time courses were analyzed graphically in terms of two independent first order reactions. The extent of reduction prior to mixing is given in the second column. The rate of the slow phase, \( k_s \), is second order with respect to oxygen concentration. The oxygen dissociation constant, \( K_d \), and the limiting first order rate, \( k_i \), were obtained from reciprocal plots of the rate of the fast phase as shown in Fig. 4.

<table>
<thead>
<tr>
<th>Enzyme sample</th>
<th>Reducing agent</th>
<th>( k_s ) (min(^{-1}))</th>
<th>( k_i ) (min(^{-1}))</th>
<th>( K_d ) (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native, AFR = 138</td>
<td>Xanthine (2 moles/total FAD)</td>
<td>( 8.00 \times 10^6 )</td>
<td>8300</td>
<td>0.72</td>
</tr>
<tr>
<td>Native, AFR = 131</td>
<td>Dithionite (2 moles/total FAD)</td>
<td>( 8.40 \times 10^6 )</td>
<td>7200</td>
<td>0.67</td>
</tr>
<tr>
<td>Native, AFR = 127</td>
<td>Dithionite (3 moles/total FAD)</td>
<td>( 7.00 \times 10^6 )</td>
<td>7200</td>
<td>0.83</td>
</tr>
<tr>
<td>Native, AFR = 170</td>
<td>Dithionite (3.5 moles/total FAD)</td>
<td>( 5.30 \times 10^6 )</td>
<td>6800</td>
<td>0.67</td>
</tr>
<tr>
<td>CN-inactivated, AFR = 0.08</td>
<td>Dithionite (3 moles/total FAD)</td>
<td>( 6.20 \times 10^6 )</td>
<td>6700</td>
<td>0.71</td>
</tr>
<tr>
<td>Allopurinol (excess)</td>
<td></td>
<td>( 8.30 \times 10^6 )</td>
<td>6700</td>
<td>0.63</td>
</tr>
<tr>
<td>Average</td>
<td></td>
<td>( 7.20 \times 10^6 )</td>
<td>7150</td>
<td>0.705</td>
</tr>
</tbody>
</table>

**Fig. 6.** Spectral properties of the fast and slow components. Xanthine oxidase (AFR = 138) was reduced with an amount of xanthine equal to 3 times the concentration of active FAD units and reacted with air-equilibrated 0.1 M pyrophosphate, pH 8.5, at 25°. Approximately 66% of the total FAD (0.0078 mM after mixing) was reduced. The amounts of absorbance change in the fast and slow phases were obtained graphically. A, oxidized minus reduced difference spectra for the total, fast, and slow enzyme reduced with either dithionite or xanthine gave similar results, and the small differences that were observed are readily accounted for by the differences in the extents of reduction prior to mixing with oxygen.

The shape of the time course of reoxidation was also not dependent on the absolute enzyme concentration as long as pseudo-first order conditions were maintained (viz. the concentration of oxygen was kept at least 10 times greater than that of enzyme-bound FAD). The addition of superoxide dismutase produced little effect on the time course of reoxidation. This result is in marked contrast to studies with free flavins in which effects as large as a 9-fold decrease in the rate of oxygen reaction are observed in the presence of superoxide dismutase (15). Both the lack of effect of enzyme concentration and added superoxide dismutase and the second order dependence on oxygen concentration of the slow phase indicate strongly that if \( O_2^- \) is generated in the early stages of oxidation it is not affecting the later stages of the reaction. Similar arguments apply for the lack of an effect of hydrogen peroxide generated during the reaction on the later portions of the oxidation time course.

**Properties of Two Kinetic Components—**Perhaps the most striking feature of the reaction of reduced xanthine oxidase with oxygen is the presence of two distinct kinetic components exhibiting widely different rates. These two phases are not the result of active and inactive enzyme molecules in the classical sense (Table I). For example, in anaerobic reduction experiments with xanthine, a rapid phase is observed followed by a very much slower decrease in absorbance which occurs with half-times of the order of several hours. The extent of bleaching during the fast phase of reduction is directly proportional to the amount of active enzyme molecules (3), i.e. to the activity to flavin ratio of the enzyme preparation. In contrast, there is no dependence of the amount of rapid phase of oxidation on specific activity, suggesting that both phases of reoxidation are an intrinsic property of all of the enzyme molecules present.

In an effort to elucidate the chemical identity of the two kinetic components, the wavelength dependence of the amplitudes of the two phases was examined (Fig. 6A). It is clear that the spectral characteristics of the slower kinetic component are markedly different from those of either the rapid phase or the over-all reaction (Fig. 6A). The over-all reaction has a difference spectrum with a broad maximum between 450 and 470 nm whereas the spectral properties of the fast and slow components. Xanthine oxidase (AFR = 138) was reduced with an amount of xanthine equal to 3 times the concentration of active FAD units and reacted with air-equilibrated 0.1 M pyrophosphate, pH 8.5, at 25°. Approximately 66% of the total FAD (0.0078 mM after mixing) was reduced. The amounts of absorbance change in the fast and slow phases were obtained graphically. A, oxidized minus reduced difference spectra for the total, fast, and slow enzyme reduced with either dithionite or xanthine gave similar results, and the small differences that were observed are readily accounted for by the differences in the extents of reduction prior to mixing with oxygen.
slower component exhibits a shoulder at 440 nm and a relatively sharp peak at 470 nm. In addition, the slow component exhibits proportionately larger changes at wavelengths greater than 500 nm than either the over-all reaction or the fast component. For example, the ratio of the changes at 450 nm to those at 550 nm are 4.2, 7.0, and 2.2 for the over-all reaction, the fast phase, and the slow phase, respectively. Assuming that the absorbance changes at 550 nm reflect primarily changes in the oxidation states of the iron-sulfur centers, these ratios alone suggest that the slow kinetic component represents the oxidation of enzyme molecules in which only reduced iron-sulfur groups are present. Similarly, the changes in the rapid phase of the reaction would then reflect primarily the oxidation of fully reduced FAD. However, some oxidation of reduced iron-sulfur centers is also occurring in the rapid phase since rapid absorbance changes are observed at wavelengths greater than 500 nm. This is particularly true when the enzyme is fully reduced with 3 eq of dithionite since under these conditions about 60% of the absorbance change at 550 nm is rapid (Fig. 3). However, even when fully reduced, the ratio of the amplitude of the slow phase at 450 nm to that at 550 nm is still about 2.0, again suggesting that the slow kinetic component represents the oxidation of iron-sulfur centers.

The striking similarity between the difference spectrum of the deflavo-enzyme and the spectrum of the slow component observed in oxidation, particularly the peaks at 440 and 470 nm (Fig. 6B), adds further support to the idea that the slow phase represents the oxidation of iron-sulfur groups. In addition, the difference spectrum between deflavo- and native enzyme (which is similar to that of FAD) is quite similar to that of the fast component in oxidation (Fig. 6B), indicating that reduced FAD is completely oxidized during the rapid phase. It should be emphasized that when xanthine oxidase is reduced with 6 electron eq per FAD, both the total amount of reduced flavin and a substantial portion of the reduced iron-sulfur centers are reoxidized in the rapid phase of the oxygen reaction.

When enzyme partially reduced with dithionite is mixed with oxygen, most of the absorbance change is slow, even at 450 nm (Fig. 7 and Table II). As more reducing equivalents are added, the amount of rapid oxidation measured at 450 nm increases much more than that measured at 550 nm (inset in Fig. 7). At all concentrations the rate of the slow phase is, to a very good approximation, unchanged and independent of the wavelength of observation (Table II). In contrast, the rate of the fast phase appears to decrease as the enzyme becomes more reduced. When the enzyme is completely reduced (6 electron eq per enzyme FAD (11)), the rate of the fast phase measured at 550 nm is slightly greater than that observed at 450 nm. It is also apparent from the upper time course in Fig. 7 that the fast phase for the reoxidation of fully reduced xanthine oxidase cannot be described by a single exponential process. Rather, when the slower component is subtracted from the observed changes, the logarithmic plot of the resultant absorbance changes curves downward. The rates given in Table II were taken from the linear portions of the curve which exist after an initial lag. This procedure is clearly subjective, and a more detailed discussion of this type of behavior is presented in the accompanying paper (11). However, this empirical analysis is sufficient for the present qualitative description of the oxidation reaction. If it is assumed that the removal of each electron from the enzyme is accompanied by an equivalent spectral change at 450 nm, the percentage of slow component at this wavelength should give a rough estimate of the number of electrons in the slowly reacting xanthine oxidase molecules. The

![Fig. 7](http://www.jbc.org/)

**Fig. 7.** Reaction of oxygen with xanthine oxidase reduced with various amounts of dithionite. Reactions were carried out in 0.1 M pyrophosphate, pH 8.5, at 25°C and observed at 450 nm. Concentrations of total enzyme FAD and oxygen after mixing were 9.4 × 10⁻⁴ and 1.25 × 10⁻⁴ M, respectively. Going from the lower to the upper curves, the ratios of added dithionite to FAD were approximately 0.2, 0.4, 1.3, and 5.0, respectively. The inset shows the dependence of the percentage of slow phase at 550 nm (C, △) and 450 nm (○, △) on the percent of reduced enzyme present initially. ○, △: two separate experiments in which enzyme was reduced with varying amounts of dithionite (see Table II). △, △: an experiment in which enzyme was reduced with xanthine and varying amounts of oxygen were added prior to mixing with air-equilibrated buffer.

**Table II**

Kinetic parameters for oxidation of xanthine oxidase reduced with varying amounts of dithionite

Reactions were carried out in 0.1 M pyrophosphate at pH 8.5 and 25°C. Time courses were analyzed in terms of two independent first order reactions. The results represent two separate experiments. Concentrations after mixing were 10⁻⁴ M total enzyme FAD and 125 μM O₂. The per cent reduction was taken as the average of values calculated at 450 and 550 nm. The dithionite:FAD ratios are approximate.

<table>
<thead>
<tr>
<th>Reduction</th>
<th>Dithionite:FAD</th>
<th>λ nm</th>
<th>Slow phase %</th>
<th>kₙ⁰</th>
<th>kₙ⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6.6</td>
<td>0.2</td>
<td>450</td>
<td>86.5</td>
<td>1200</td>
<td>69</td>
</tr>
<tr>
<td>15.9</td>
<td>0.3</td>
<td>450</td>
<td>73.7</td>
<td>1200</td>
<td>69</td>
</tr>
<tr>
<td>17.8</td>
<td>0.4</td>
<td>450</td>
<td>61.3</td>
<td>1340</td>
<td>68</td>
</tr>
<tr>
<td>47.4</td>
<td>1.2</td>
<td>450</td>
<td>42.2</td>
<td>1340</td>
<td>75</td>
</tr>
<tr>
<td>51.8</td>
<td>1.5</td>
<td>450</td>
<td>29.5</td>
<td>1340</td>
<td>68</td>
</tr>
<tr>
<td>65.2</td>
<td>1.9</td>
<td>450</td>
<td>29.5</td>
<td>1040</td>
<td>65</td>
</tr>
<tr>
<td>100</td>
<td>3.0</td>
<td>450</td>
<td>20.6</td>
<td>849</td>
<td>68</td>
</tr>
<tr>
<td>100</td>
<td>3.0</td>
<td>550</td>
<td>35.0</td>
<td>967</td>
<td>64</td>
</tr>
<tr>
<td>100</td>
<td>3.0</td>
<td>550</td>
<td>21.0</td>
<td>683</td>
<td>60</td>
</tr>
</tbody>
</table>

Downloaded from http://www.jbc.org/ by guest on August 15, 2017
observed value of 20% suggests that 5 electrons are removed in the rapid phase and the remaining electron in the slow phase. This result, coupled with those in Fig. 6, further suggests that the remaining 1 electron resides in one of the iron-sulfur centers.

Thus, the following qualitative picture of the oxidation reaction emerges. When a small amount of reducing equivalents is added anaerobically a population of 1-electron-reduced and fully oxidized xanthine oxidase molecules is generated. In the case of 1-electron-reduced enzyme molecules, this electron primarily resides in the Fe/S centers and is not readily available to molecular oxygen. As more electrons are added they begin to populate the reduced states of the flavin, primarily FADH₂, which is capable of reacting rapidly with oxygen. This accounts for the sharper decrease with over-all reduction of the relative amplitude of slow phase measured at 450 nm as compared to that measured at 550 nm. Finally the last few electrons populate the remaining reduction states of the iron-sulfur and molybdenum centers and are also readily removed by reactions mediated through fully reduced FAD. Thus, the key to understanding the reaction with oxygen appears to be the observation that as long as FADH₂ can be formed, i.e. the number of electrons present in the enzyme molecule is greater than or equal to 2, the removal of electrons occurs rapidly. One-electron-reduced enzyme molecules are only moderately reactive toward oxygen, presumably because FADH₂ cannot be formed.

Effects of pH on Oxidation and Reduction Reactions—A limited attempt was made to examine the pH dependence of the time course of oxidation, hoping that this study would shed further light on the chemical identity of the two kinetic components and the mechanism of the reaction. First, the steady state kinetics of xanthine oxidation was examined in air-equilibrated buffers ranging in pH from 5 to 10. In all cases, linear double reciprocal plots were observed and the y and x axis intercepts are given in Table III. The observed values of the apparent Kₘ for xanthine (Kₓ) are in excellent agreement with those previously reported by Fridovich (17). The pH dependence of Kₓ in the range of 5 to 9 has been interpreted to reflect the inability of un-ionized and doubly ionized xanthine to bind to the active site of the enzyme, an idea consistent with the observed minimum at pH 7.0 (17). The large increase in Kₘ at pH 10.0 is thought to be due to the deprotonation of a basic amino acid at or near the active site of reduction (18). In contrast to the Kₓ values, the values of Vₘₐₓ do not vary greatly with pH, the value at pH 5.0 being only 1/4 that observed at pH 10.0 or 8.5. This observation does not support the idea that the rate-limiting step in catalysis is the attack of hydroxide on the persulfide-xanthine bond which has been postulated to be formed during reduction (3).

A comparison of the steady state parameters with those of the individual half-reactions at pH values of 6.0, 8.5, and 10.0 is also given in Table III. Again, it was assumed that these reactions could be described by the simple formulation of Equation 1 and that the values of the substrate-binding rate constants were much faster than those describing the decay of the two Michaelis complexes. In general, there is reasonable agreement between the steady state constants and those predicted from the rate and equilibrium constants determined from investigations of the individual half-reactions. For example, the upper limits to Vₘₐₓ at infinite xanthine in air-equilibrated buffer predicted from the stopped flow results are 320, 680, and 650 min⁻¹ at pH 6, 8.5, and 10.0, respectively. These compare with steady state values of 323, 885, and 870 min⁻¹ (Table III).

At each pH value the slow phase in oxidation was accurately second order with respect to oxygen and comprised 20 to 25% of the total absorbance change at 450 nm. As shown in Table III, the rate of the fast phase observed in the oxidation experiments increases with increasing pH, whereas the rate of the slow phase decreases. The rate of the rapid phase at pH 10.0 is nearly second order with respect to oxygen over the concentration ranges examined, so that the observed first order rate constant at a given oxygen concentration at this pH is only slightly greater than that observed under similar conditions at pH 8.5. Perhaps the most striking feature of the results presented in Table III is the observation that only the Michaelis constant for xanthine appears to exhibit a marked dependence on pH and even then significant changes are only observed at high pH.

Although the kinetic parameters do not vary widely, there are significant spectral differences between the individual reduction and oxidation half-reactions measured at high and low pH. A series of anaerobic reduction experiments is shown in Fig. 8. At pH 6.0, reduction by xanthine occurs in at least two catalytically

### Table III

<table>
<thead>
<tr>
<th>Buffer</th>
<th>pH</th>
<th>Steady state</th>
<th>Reduction</th>
<th>Oxidation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Vₘₐₓ/min⁻¹</td>
<td>Kₓ M⁻¹</td>
<td>Kₓ M⁻¹</td>
</tr>
<tr>
<td>0.1 M Sodium acetate</td>
<td>5.0</td>
<td>220</td>
<td>5.0 x 10⁻⁴</td>
<td>462</td>
</tr>
<tr>
<td>0.1 M Potassium phosphate</td>
<td>6.0</td>
<td>323</td>
<td>1.4 x 10⁻⁴</td>
<td>1050</td>
</tr>
<tr>
<td>0.1 M Sodium pyrophosphate</td>
<td>8.5</td>
<td>885</td>
<td>9.0 x 10⁻⁴</td>
<td>1030</td>
</tr>
<tr>
<td>0.1 M Tris-HCl</td>
<td>8.5</td>
<td>870</td>
<td>1.3 x 10⁻⁴</td>
<td>1030</td>
</tr>
<tr>
<td>0.1 M Glycine NaOH</td>
<td>10.0</td>
<td>870</td>
<td>1.3 x 10⁻⁴</td>
<td>1030</td>
</tr>
</tbody>
</table>

* The Michaelis constants were derived from the integrated form of the steady state equation.
* Little or no dependence of the rate of reduction on xanthine concentration was observed at pH 6.0. The value shown is an approximate upper limit.
* Values for Vₘₐₓ and Kₓ at infinite xanthine and infinite oxygen concentrations are given in the text.
* Average values taken from Table II.
significant phases. There is a rapid appearance of flavin semiquinone which is evident from the initial increase in absorbance at long wavelengths, particularly 600 nm (Fig. 8, left panel). Assuming an extinction coefficient of 4.5 × 10^4 M⁻¹ cm⁻¹ for the neutral radical at 600 nm (19), the observed increase in absorbance suggests that about 20% of the total flavin is in the semiquinone form (100 ms after mixing). This appearance of large amounts of flavin radical at low pH is not simply a kinetic phenomenon. When xanthine oxidase was mixed with approximately 1 mole of xanthine per active FAD at pH 6.0, an exponential decrease in absorbance at 450 nm was observed. However, the absorbance at 600 nm increased and reached a final level equal to that observed transiently in the over-all reduction reaction. This latter result suggests that the semiquinone form of FAD is markedly stabilized at lower pH. Similar partial reduction experiments carried out at pH 8.5 and 10.0 failed to show an increase in absorbance at 600 nm. At pH 8.5 a marked lag is observed in the over-all time course of reduction measured at 600 nm and presumably represents formation of a small amount of flavin radical. However, the amount of semiquinone generated must be at least 1/4 to 1/3 that produced during reduction at pH 6.0. In agreement with earlier EPR results (7), no evidence for the formation of flavin semiquinone is observed during reduction at pH 10.0.

At pH 8.5, a small lag is observed in the initial portions of the absorbance changes at 450 nm, and is more pronounced at 550 nm. In contrast, at pH 10.0 there is no lag at 450 nm and the initial rate of the absorbance change at 550 nm is greater than that at 450 nm. This latter result suggests that at pH 10.0, the Fe/S centers are being reduced before FAD.

A corresponding set of oxidation experiments at pH 6.0, 8.5, and 10.0 is shown in Fig. 9. As expected from anaerobic reduction studies, large amounts of flavin radical were observed during the reoxidation of fully reduced xanthine oxidase at pH 6.0. This was expressed by an “overshoot” of the absorbance changes at wavelengths greater than about 550 nm. At 600 nm, the absorbance increased to a value greater than the final reading by an amount roughly equal to the initial increase seen in the corresponding reduction experiment. In contrast, at pH 8.5 and 10.0 little or no flavin radical appears to be generated during the reaction with oxygen (Fig. 9).

A comparison of the oxidized minus reduced difference spectra for the various kinetic phases seen during oxidation (Fig. 10) shows that at pH 6.0 the spectrum of the slow component resembles the difference spectrum of neutral semiquinone minus oxidized flavin, the most characteristic feature being the negative difference in wavelength regions greater than 500 nm. However, the difference spectrum of the slow component observed at pH 10.0 resembles that of deflavoxanthine oxidase and is quite similar to the corresponding difference spectrum measured at pH 8.5 (Fig. 6).

A comparison of the rates of the fast phases of reoxidation measured at pH 6.0 and 10.0 is shown in Fig. 11. At pH 6.0, the rates measured at 550 and 600 nm are consistently greater than at 450 nm, which is again consistent with the rapid formation of flavin semiquinone. In contrast, at pH 10.0 the rates measured at 550 and 600 nm are less than those observed at 450 nm. This latter result suggests that fully reduced flavin is being oxidized more rapidly than are the iron-sulfur centers. This order appears to be inverted when reduction with xanthine is examined since the initial rates of reduction observed at 550 nm are greater than those observed at 450 nm (Fig. 11).

If it is again assumed that 5 electrons are removed during the rapid phase of oxidation, the spectral and kinetic results given in Figs. 9, 10, and 11 suggest that at pH 6.0 the remaining electron is present to a much greater extent in the flavin prosthetic group than at either pH 8.5 or 10.0. This result coupled with the greater rate of oxidation during the slow phase at pH 6.0 suggests that the neutral semiquinone is the species of flavin present in 1-electron-reduced enzyme which reacts directly with oxygen. If it is assumed that at pH 6.0 the remaining 1 electron is equally partitioned among flavin radical, the two Fe/S centers, and molybdenum, but only the semiquinone is capable of reacting with oxygen, the apparent second order rate constant for the reaction of radical with O₂ would be 4 times the observed rate for the slow phase, viz. 7 × 10⁴ M⁻¹ s⁻¹. This value is close to the reported value of 3 × 10⁴ M⁻¹ s⁻¹ for the reaction of neutral radical of free flavins (20).

Rapid Freeze EPR Measurements—A more direct method of investigating the chemical nature of the two phases observed in the reaction of reduced xanthine oxidase with oxygen is to examine the kinetics of the appearance and decay of the various electron paramagnetic resonance (EPR) signals. A comparison of the oxidized minus reduced difference spectra for the various kinetic phases seen during oxidation (Fig. 10) shows that at pH 6.0 the spectrum of the slow component resembles the difference spectrum of neutral semiquinone minus oxidized flavin, the most characteristic feature being the negative difference in wavelength regions greater than 500 nm. However, the difference spectrum of the slow component observed at pH 10.0 resembles that of deflavoxanthine oxidase and is quite similar to the corresponding difference spectrum measured at pH 8.5 (Fig. 6).

Rapid Freeze EPR Measurements—A more direct method of investigating the chemical nature of the two phases observed in the reaction of reduced xanthine oxidase with oxygen is to examine the kinetics of the appearance and decay of the various electron paramagnetic resonance (EPR) signals. A comparison of the oxidized minus reduced difference spectra for the various kinetic phases seen during oxidation (Fig. 10) shows that at pH 6.0 the spectrum of the slow component resembles the difference spectrum of neutral semiquinone minus oxidized flavin, the most characteristic feature being the negative difference in wavelength regions greater than 500 nm. However, the difference spectrum of the slow component observed at pH 10.0 resembles that of deflavoxanthine oxidase and is quite similar to the corresponding difference spectrum measured at pH 8.5 (Fig. 6).
Fig. 9. Effect of pH on the time course of oxidation. Reactions were carried out in 0.1 M buffers at 25° and observed at: 450 nm (○); 550 nm (□); and 600 nm (△). In all cases, enzyme was reduced with 3 moles of dithionite per total FAD and reacted with air-equilibrated buffer. Left panel, reactions at pH 6.0 (acetate) and 3.9 μM FAD units after mixing. Middle panel, reactions at pH 8.5 (pyrophosphate) and 4.0 μM FAD after mixing. Right panel, reactions at pH 10.0 (glycine NaOH) and 5.1 μM FAD after mixing.

Fig. 10. Spectral properties of the fast and slow components observed during oxidation at pH 6.0 and 10.0. The reaction conditions were identical with those given in Fig. 9. Symbols: △, total absorbance change; ○, absorbance change in fast phase; and ◇, absorbance change in the slow phase. At pH 6.0, the absorbance changes occurring after 300 ms were attributed to the slow kinetic component. At pH 10.0, the amplitudes of the two phases were obtained by analyzing the reaction traces graphically in terms of two independently reacting components.

Fig. 11. Dependence of the rate of the fast phase on oxygen concentration at pH 6.0 and 10.0. Reaction conditions were identical with those given in Fig. 9 and observations were made at: 450 nm (○); 550 nm (□); and 600 nm (△). The inset in the upper panel shows the dependence of the initial rate of anaerobic reduction at pH 10.0 on substrate concentration (the reaction conditions are given in Fig. 8).
marily the observed rates and not the amplitudes of the two kinetic components. The simplest experiment, and the one tried first, was to reduce xanthine oxidase with excess allopurinol and then mix the reduced protein with oxygen in the rapid freezing apparatus. In this case, the molybdenum is trapped in the IV valence state by virtue of binding the product of the reaction, alloxanthine (14). Therefore, no molybdenum signals were observed and only the time courses of the flavin radical and the reduced Fe/S centers could be examined. The results for the iron signals measured at liquid helium temperatures are shown in Fig. 12A.

The oxidation of Fe/S II monitored at $g = 2.12$ occurs entirely in the slow phase of the reaction when the enzyme is reduced with allopurinol, whereas 60% of the $g = 1.90$ signal decays at the slower rate. If the $g = 1.90$ resonance were due solely to Fe/S I, these results predict that in a corresponding stopped flow absorbance measurement a percentage of slow change measured at 550 nm should be at least 60%. However, the amount of slow phase measured in a comparable stopped flow experiment (Fig. 12B) is only about 45% at 550 nm, which is too small even if Fe/S II exhibits no spectral change at this wavelength. Thus the signal at $g = 1.90$ cannot represent Fe/S I alone, but rather must contain contributions from both iron signals. The results of Lowe et al. (8) indicate that a better measure of Fe/S I is the positive derivative peak at $g = 1.95$ since the resolved spectrum of Fe/S II gives a derivative approximately equal to zero at this $g$ value. When the time course of the disappearance of the $g = 1.95$ signal is examined, the amplitude of the slow phase is 26% of the total which is much more comparable with the stopped flow absorbance measurement (Fig. 12). However, it is still clear that Fe/S II contributes less absorbance change than Fe/S I at all wavelengths. Similar results were obtained by Edmondson et al. (7) from a comparison of the results of rapid freeze and stopped flow reduction experiments at high pH.

A clear demonstration of the separation in rates of the reoxidation of the two iron centers can be seen in the EPR spectra given in Fig. 12. In this experiment xanthine oxidase was reduced with an amount of xanthine equal to 3 times the amount of active FAD units. The zero time point, recorded by mixing the sample of reduced enzyme with anaerobic buffer, shows a ratio of about 3.5 for the peak heights of the signals at $g = 1.95$ and 2.12 (Fig. 13, top). This value decreases markedly during the rapid phase of the reaction and equals about 1.3 at 210 ms after mixing with 6.25 × 10^{-4} M oxygen (Fig. 13, bottom). This change in the ratio of the two signals during oxidation is the opposite of that observed in either kinetic or static reduction experiments in which the normalized signal at $g = 2.12$ is proportionately greater than the $g = 1.95$ signal at low levels of reduction (1, 3). Thus both oxidation and reduction experiments suggest that Fe/S II has a greater affinity for electrons than Fe/S I.

Oxidation time courses for the appearance and decay of all the EPR signals of xanthine-reduced enzyme are shown in Fig. 14. The behavior of the iron signals was the same as that observed during the oxidation of allopurinol-reduced xanthine oxidase (Fig. 12). In both cases complete reduction was not achieved, due to the presence of inactive enzyme molecules which are not readily reduced by ordinary substrates. The integrated intensities are reported as the number of electrons per total FAD, including both active and inactive molecules. The combined integrated intensities of the iron signals were 1.85 electrons per FAD. Since Fe/S II has been shown to exhibit a significantly higher reduction potential (8, 11) it was assigned 1 electron per FAD, the remaining intensity being assigned to Fe/S I. Again most of the $g = 2.12$ signal decayed at a slow rate while most of the $g = 1.95$ signal decreased at a much faster rate.

The addition of superoxide dismutase produced no measurable effect on the time courses of the iron and molybdenum signals (Fig. 14) which is in agreement with our stopped flow absorbance measurements (Fig. 5). Both the flavin semiquinone and molybdenum signals increase slightly at the beginning of the reaction and then decay rapidly during the fast phase of the reaction. Only a small proportion of these signals is present in the slow phase. The amount of flavin radical observed during oxidation is consistently smaller (by a factor of about 2) than that previously reported for reduction experiments (7). When limiting amounts of xanthine were used to reduce the enzyme the only molybdenum signal observed was that which Bray and Swann have designated *"rapid."* (15) Under no conditions was a signal

![Fig. 12](https://example.com/fig12.png)

**Fig. 12.** Time course for the iron-sulfur signals during the oxidation of allopurinol-reduced xanthine oxidase. Reactions were carried out in 0.1 M pyrophosphate, pH 8.5. A, rapid freeze EPR results. Enzyme was reduced with excess allopurinol and then mixed with oxygen. Conditions after mixing: 0.1 mm total FAD units (AFR = 138; therefore only 0.065 mm FAD unit was reduced), 0.43 mm allopurinol, and 0.625 mm oxygen. EPR spectra were recorded as described under "Materials and Methods" and the derivative signals were measured at the $g$ values shown beside each time course. B, stopped flow absorbance results. Enzyme was reduced with excess allopurinol prior to mixing with air-equilibrated buffer. Wavelengths of observation are given beside each curve. Conditions after mixing: 0.0051 mm total FAD units; (AFR = 138; therefore only 0.0034 mm FAD unit was reduced), 0.1 mm allopurinol, and 0.125 mm O$_2$.

![Fig. 13](https://example.com/fig13.png)

**Fig. 13.** EPR spectra of iron-sulfur centers during oxidation. Xanthine oxidase (AFR = 107) was reduced with an amount of xanthine equal to 3 times the concentration of active FAD units and then reacted with oxygen in 0.1 M pyrophosphate, pH 8.5, at 25°. Conditions after mixing: 0.100 mm total FAD units (80% reduced) and 0.625 mm O$_2$. Superoxide dismutase (2.5 × 10^{-4} M) was added to remove O$_2$- signal. EPR spectra were recorded as described under "Materials and Methods." Time after mixing and the relative gain used are given beside each curve.
Oxygen in the rapid freeze apparatus. As shown in Fig. 15, the moles of dithionite per enzyme-bound FAD and then mixed with enzyme, a preparation of xanthine oxidase was reduced with 3.2 moles of dithionite with oxygen, the time course of its disappearance of FADH and reaches a maximum value of about 0.10 electron per FAD. A comparison of the observed spectrum with dithionite titration data (11) suggested that the amounts of “slow” and “rapid” molybdenum were roughly equal. As expected from previous work (13), no rapid phase in the decay of the “slow” molybdenum signal is seen. Again, a slight increase followed by a rapid decrease was observed for the flavin radical and “rapid” molybdenum signals, and only a small proportion of these signals is present in the slow phase of the oxidation reaction.

**DISCUSSION**

Previous anaerobic titrations with dithionite have suggested that both active and inactive xanthine oxidase molecules are capable of consuming 3.5 to 4.0 moles of dithionite per mole of enzyme-bound FAD. However, the results of Edmondson et al. (3) have shown that all enzyme absorbance changes are complete after 3 moles of dithionite per mole of FAD have been added. The addition of more reducing equivalents produces no increase in absorbance at 315 nm due to excess dithionite until after a total of about 4 moles of dithionite per mole of FAD has been achieved. This observation has been confirmed and the results are presented in the accompanying paper (11). The chemical nature of the group(s) which accept the 7th and 8th electrons remains somewhat of a mystery; it may be due to a reduction of molybdenum to valence states lower than IV (23).

For the present discussion, the simplifying assumption is made that, during catalysis, xanthine oxidase is capable of accepting 6 electrons per FAD, 1 for each of the two iron-sulfur centers and 2 each for FAD and molybdenum. This does appear to be the case for ordinary substrates such as xanthine and only with dithionite is further reduction observed (2, 3, 11). This suggests that the reduction potentials of the groups which accept the additional electrons are more negative than that of uric acid.

A further simplifying assumption is also implicit in the following discussion. For lack of evidence to the contrary, it is assumed that there are two independent active centers per molecule of protein, each containing 1 atom of molybdenum, Fe/S I and II, and 1 molecule of FAD. This idea follows from the observation that the molecular weight determined from ultracentrifugation analysis appears to be about 300,000, whereas the minimum molecular weight per FAD based on amino acid analysis is about 190,000, and the fact that the ratio of Mo:FAD:Fe/S is 1:1:4. If these active sites were not independent, that is, if electrons could freely distribute among all the oxidation-reduction groups within the 380,000 molecular weight protein molecule, it should be possible to generate fully reduced flavin with very low levels of added reducing equivalents. For example, each enzyme molecule could be considered to accept 12 electrons, and only in the case of 1-electron reduction is the formation of FADH₂ prohibited. Similarly, if the removal of each electron caused an equivalent spectral change at 450 nm, the amount of slow phase observed in the reoxidation of fully reduced xanthine oxidase would be expected to be 1/2 of the total or about 8%, which is considerably less than the observed value of 90%.

Finally, most of the results presented represent reactions carried out in 0.1 M pyrophosphate, pH 8.5. Under these conditions, very little flavin semiquinone accumulates during either reduction or oxidation. From studies with model systems, molybdenum is not expected to contribute much absorbance to either the oxidized or reduced optical spectrum of xanthine oxidase (13, 14), and even when changes in the spectrum due to molybdenum-substrate complexes or to modification of the active site are observed, they are quite small in comparison to those observed during the oxidation and reduction reactions. Therefore, to a very good approximation, absorbance measurements at 450 nm reflect changes from oxidized to fully reduced flavin and changes from oxidized to reduced iron-sulfur centers, whereas those at 550 nm reflect only changes in the states of the iron-sulfur centers. At 450 nm the proportion of the total absorbance change between oxidized and reduced enzyme due to flavin is given by the differences between the spectral properties of deflavoxanthine oxidase (Fig. 6). The rapid freezing results in a difference spectrum of the slow component and that of the native enzyme and is equal to about 46% of the total (Fig. 6B).

The reaction of reduced xanthine oxidase with oxygen occurs in two distinct kinetic phases. The rates involved in the fast phase are about 10 times greater than those observed in the slow phase. The relative amplitudes of the two kinetic components observed with fully reduced enzyme suggest that the first 5 electrons are rapidly removed by oxygen. The remaining electron is removed much more slowly and at pH 8.5 appears to reside primarily in the Fe/S centers by virtue of the similarity between the difference spectrum of the slow component and that of deflavoxanthine oxidase (Fig. 6). The rapid freezing results (Figs. 12, 14, and 15) are consistent with this idea and further indicate that the remaining electron is partitioned between Fe/S II and I in a ratio of about 3:1, with a small fraction being present in the Mo(V) state and flavin radical. The amount of spin label measured at 550 nm with fully reduced enzyme is about 40% of the total absorbance change. Since the Fe/S centers contribute about 50% of the total change at 450 nm, the observed value of 20% for the amplitude of the slow phase measured at 450 nm indicates that FADH2 is completely oxidized during the slow phase of the reaction. This result, coupled with the inability of reduced deflavoxynzyme to react rapidly with oxygen, suggests very strongly that oxygen reacts directly with flavin and not with Fe/S I and II. The removal of electrons from the other centers is mediated by fully reduced flavin. This idea is consistent with the observation that when 1 electron is left in the enzyme the rate of oxidation is markedly decreased, presumably because FADH2 can no longer be formed.

The results presented in Table III suggest that flavin is the site of oxygen attack even for the case of 1-electron-reduced enzyme molecules. At low pH substantial quantities of flavin semiquinone are generated during the fast phase of the oxidation reaction, and there is an apparent correspondence between the greater amount of radical present and the increased rate observed in the slow phase. Thus, although at higher pH values the removal electron resides primarily in the iron centers, its removal occurs by the reaction of oxygen with the small fraction of semiquinone present. Since the fraction of semiquinone present in 1-electron-reduced enzyme molecules decreases with increasing pH, the second order rate of the slow phase in oxidation would also be expected to decrease with increasing pH by an amount proportional to the fraction of flavin radical present.

If, indeed, the reaction of flavin semiquinone with oxygen does account for the slow phase, the observed second order dependence of the slow rate suggests that the rates of intramolecular transfer from the iron centers and molybdenum to flavin are very rapid with respect to the rate of the oxygen reaction. Similarly, if the hyperbolic dependence of the fast rate on oxygen concentration is simply a reflection of the formation and decay of a reduced flavin-oxygen complex, these rates of intramolecular electron transfer between the various electron-accepting groups must be quite large, at least 100 s⁻¹ or greater at pH 8.5 and 25°C. This idea that the rates of intramolecular electron transfer are not rate-limiting for either catalysis or the individual half-reactions is supported by the pH jump experiments of Edmondson et al. (7).

When xanthine oxidase partially reduced at pH 6.6 was mixed with a high concentration of glycine buffer at pH 10.0, the large amount of flavin radical characteristic of low pH values vanished within the mixing time of rapid freeze apparatus. Within the same time period, the expected amount of "very rapid" molybdenum signal which is characteristic of high pH values was observed. The corresponding rapid appearance and disappearance of flavin and molybdenum signals were also seen when the pH was dropped from 10.0 to 6.8 (7). Thus, it seems that the fractional amount of electrons present in the various oxidation-reduction groups is not kinetically determined but rather is a reflection of the relative reduction potentials of the groups. This would explain the observation that Fe/S II is oxidized more slowly but reduced more rapidly than Fe/S I (Figs. 12, 14, and 15 and Ref. 7), an order which is consistent with the relative reduction potentials of these groups (8, 11).

Several workers have shown that electron paramagnetic signals from Fe/S I and II exhibit markedly different temperature sensitivities, which suggests that these two groups are physically separated from each other and can be considered as chemically distinct iron-sulfur pairs (8, 24). The kinetic differentiation of the g = 2.12 and 1.90 signals during anaerobic reduction at high pH led Edmondson et al. (7) to postulate that Fe/S II contributes a much smaller proportion of the absorbance change observed by stopped flow techniques than Fe/S I. The results reported in Fig. 12 confirm their idea and allow a more quantitative estimate of the extinction change due to the two iron centers. It appears that Fe/S II contributes about 20% of the total possible absorbance change at 550 nm and 10% at 450 nm (Fig. 12). Fe/S I contributes 80% of the change at 550 nm and about 40% at 450 nm, with flavin contributing the remaining 50% at 450 nm. These results suggest that both iron centers differ markedly in their absorbance properties from that of spinach ferredoxin. For example, the total extinction change between oxidized and reduced xanthine oxidase is 26,600 M⁻¹ cm⁻¹ at 450 nm and 6,900 M⁻¹ cm⁻¹ at 550 nm (11). Our present best estimates of the extinction changes for iron center I at 450 and 550 nm are 11,600 and 5,600 M⁻¹ cm⁻¹, respectively, and those for iron center II at the same wavelengths are 2,800 and 1,300 M⁻¹ cm⁻¹, respectively. In contrast, most ferredoxins exhibit extinction changes of about 4,500 M⁻¹ cm⁻¹ at 450 nm and 2,000 M⁻¹ cm⁻¹ at 550 nm (25). Thus Fe/S I yields greater absorbance changes and Fe/S II smaller absorbance changes than those produced from more simple iron-sulfur proteins, but the total changes due to both iron centers are roughly equal to the amount expected for two simple ferredoxin iron-sulfur pairs. Unfortunately, there is at present no structural information which allows a physical interpretation of these various differences between the optical properties of Fe/S I and II. However, it is important to re-emphasize that both the widely different temperature sensitivities of the EPR signals assigned to the two iron-sulfur centers and the fact that these signals do not appear to be derived from each other during either oxidation, reduction, or static titration experiments argue strongly in favor of the idea that Fe/
S I and II are physically and chemically distinct iron sulfur pairs. One of the most intriguing results is the relatively small amount of $O_2^-$ that is generated during the reaction of reduced xanthine oxidase with oxygen (Fig. 13). If electrons were removed 1 at a time from fully reduced enzyme, 5 moles of $O_2^-$ per FAD would be produced in the fast phase. However, the observed amount is only 0.1 mole of $O_2^-$ per FAD. Even when this amount is corrected for the rate of disproportionation at pH 8.5 (2 $\times$ $10^4$ M$^{-1}$ s$^{-1}$ (23)) and for the fact that total reduction by xanthine was not achieved (Fig. 13), the amount expected if oxygen removes electrons 1 at a time is still an order of magnitude greater than that observed. Furthermore, this small amount of $O_2^-$ is not easily explained in terms of the oxidation of partially reduced enzyme molecules with free superoxide anions since no effect of superoxide dismutase was observed on either the time course of the absorbance changes at low enzyme concentration or on the EPR signals at very high enzyme concentration. In the case of the oxidation of free flavins, the reaction of $O_2^-$ with the reduced forms of flavin does play an important role in the over-all reaction with oxygen, and for these compounds, there is a marked slowing of the over-all rate by the addition of superoxide dismutase. Thus, it appears that the primary pathway in the oxidation of xanthine oxidase during the rapid phase of the reaction is a 2-electron transfer to form hydrogen peroxide. Since the slow phase of the enzyme reaction is thought to reflect the oxidation of 1-electron-reduced xanthine oxidase molecules, superoxide anions must be generated during this step. However, the absolute rate of production of $O_2^-$ during the slow phase is somewhat less than the rate of disproportionation of the oxygen radicals at pH 8.5, and therefore an increase in the amount of EPR signal is neither expected nor observed.

The small amount of $O_2^-$ observed during oxidation at pH 8.5 is not in conflict with previous observations. Knobels et al. (22) and Orme-Johnson and Beinert (21) also reported rather small amounts of oxygen radical signal in turnover experiments at pH values around 8. Similarly, if the specific activity of cytochrome c reduction by xanthine in the presence of excess cytochrome c is equated with the amount of $O_2^-$ production, the observed rates at pH 8.5 suggest that the amount of 2-electron reduction of oxygen is about 4 times greater than that of 1-electron reduction (12, 26). A more detailed discussion of the question of 1-electron versus 2-electron reduction of oxygen is given in the accompanying paper (11). At pH 8.5, the reaction of oxygen with reduced xanthine oxidase appears to be independent of both the specific activity of the enzyme preparation and the type of reagent used for reduction. This relationship is true not only of the over-all rates, but also for the more detailed spectral features of the reaction. Only when FAD is removed from the enzyme is the oxygen reaction markedly affected, and in that case it does not occur to any catalytically significant extent (12). This result points out the clear delineation of the prothestic groups which are involved in the over-all catalytic oxidation of xanthine by the enzyme. Previous work has shown unequivocally that xanthine reacts directly with molybdenum and that electrons are donated through this group to Fe/S centers and FAD (4, 7, 13). The most reasonable, if not the only, interpretation of the studies of Komai et al. (12) with deflavoenzyme and those presented here is that oxygen removes electrons from the enzyme by reaction with the flavin moiety. The iron-sulfur centers may be considered to act as an electron sink which connects the molybdenum and flavin groups and serve to regenerate fully reduced FAD (during catalysis) for reaction with oxygen.

The separate chemical identity of the sites for oxidation and reduction adds more convincing evidence for a binary complex mechanism which was first devised from an analysis of steady state data. The rates observed for the individual half-reactions are also in reasonable agreement with those required by this simple mechanism. At pH 8.5, the observed values of $V_{max}$, $X$ and $K_{O_2}$ are 1100 min$^{-1}$, 9.4 $\times$ $10^{-4}$ M, and 8.3 $\times$ $10^{-4}$ M, respectively. The predicted value of $V_{max}$ calculated from the rates of the fast phases observed in oxidation and reduction experiments is 916 min$^{-1}$. Assuming that the rates involved in the binding of either oxygen or xanthine are much greater than the rates of decay of the corresponding enzyme-substrate complexes, the calculated values of $K_X$ and $K_{O_2}$ are 11 $\times$ $10^{-4}$ and 9 $\times$ $10^{-4}$ M, respectively. This correspondence between the observed and calculated values again indicates a degree of kinetic simplicity which would not necessarily be expected from an enzyme which contains such a large number of electron-accepting groups. A further refinement of this comparison between observed and predicted kinetic constants is given in the accompanying paper (11).

REFERENCES

1. GUTFREUND, H., and STURTEVANT, J. M. (1959) Biochim. J. 70, 1–0

Downloaded from http://www.jbc.org/ by guest on August 15, 2017
The Reaction of Xanthine Oxidase with Molecular Oxygen
John S. Olson, David P. Ballou, Graham Palmer and Vincent Massey