The Reaction of Reduced Xanthine Oxidase with Oxygen

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Product formation during the oxidation of xanthine oxidase has been examined directly by using cytochrome c peroxidase as a trapping agent for hydrogen peroxide and the reduction of cytochrome c as a measure of superoxide formation. When fully reduced enzyme is mixed with high concentrations of oxygen, 2 molecules of H₂O₂/flavin are produced rapidly, while 1 molecule of O₂⁻/flavin is produced rapidly and another produced much more slowly. Time courses for superoxide formation and those for the absorbance changes due to enzyme oxidation were fitted successfully to the mechanism proposed earlier (Olson, J. S., Bailou, D. P., Palmer, G., and Massey, V. (1974) J. Biol. Chem. 249, 4363-4382). In this scheme, each oxidative step is initiated by the very rapid and reversible formation of an oxygen-FADH₂ complex (the apparent Kᵦ = 2.2 × 10⁻⁴ M at 20 °C, pH 8.3). In the cases of 6- and 4-electron-reduced enzyme, 2 electrons are transferred rapidly (kₑ = 60 s⁻¹) to generate hydrogen peroxide and partially oxidized xanthine oxidase. In the case of the 2-electron-reduced enzyme, only 1 electron is transferred rapidly and superoxide is produced. The remaining electron remains in the iron-sulfur centers and is removed slowly by a second order process (kᵦ = 1 × 10⁵ M⁻¹ s⁻¹). When the pH is decreased from 9.9 to 6.2, both the apparent Kᵦ for oxygen binding and the rapid rate of electron transfer are decreased about 20-fold. This result is suggestive of uncompetitive inhibition and implies that proton binding to the enzyme-flavin active site affects primarily the rate of electron transfer, not the formation of the initial oxygen complex.

The reaction of reduced xanthine oxidase with oxygen exhibits a markedly biphasic time course, with the rate of the fast phase being approximately 10 times that of the slow phase (1, 2). The relative amplitudes of these two phases are dependent on the wavelength of observation. The absorbance changes observed in the slow phase are very similar to the difference spectrum associated with the oxidation of the iron-sulfur centers, whereas the changes associated with the fast phase resemble a combination of flavin and iron-sulfur center difference spectra.

To explain these observations, Olson et al. (2) proposed a scheme in which the reoxidation occurs by way of a combination of 1- and 2-electron transfer steps to give both hydrogen peroxide and superoxide as products.

![Scheme 1](image)

**Scheme 1**

XO(n) represents a reduced enzyme species containing n electrons. In each of the steps except the last, oxygen binds rapidly to fully reduced flavin, and then an electron is transferred to generate a flavin semiquinone-superoxide complex. When the enzyme still contains 2 or more electrons, FADH₂ is regenerated very rapidly by intramolecular transfer from the reduced iron-sulfur centers and molybdenum. The rate of the latter process is postulated to be greater than the rate of superoxide diffusion out of the active site. Consequently, a 2nd electron is transferred to O₂⁻ to produce hydrogen peroxide.

As indicated in Scheme 1, this 2-electron oxidation process occurs until XO(2) is produced. At this stage, oxygen again binds rapidly, but in the resultant complex, fully reduced flavin cannot be regenerated after the 1st electron transfer to oxygen nor does the remaining electron stay in the flavin because the two iron-sulfur centers exhibit 4–10-fold greater affinities for electrons than does oxidized flavin (2). Thus, in 1-electron-reduced enzyme, almost all of the reducing equivalents are present in the iron atoms, and the superoxide molecule is released. The removal of the last electron from xanthine oxidase also produces superoxide. The rate of this last step is considerably slower than that of the previous ones since no fully reduced flavin can be present, the amount of semiquinone is quite small (2), and oxygen reacts extremely slowly, if at all, with the iron-sulfur centers (3).

Scheme 1 predicts that complete oxidation of 6-electron-reduced enzyme should produce 2 molecules of hydrogen peroxide and 1 of superoxide during the fast phase and 1 molecule of superoxide in the slow phase. Olson et al. (1, 2) proposed this sequence and stoichiometry to account for the 5:1 ratio of the fast and slow phase as measured by absorbance techniques at 450 nm. However, attempts to quantitate the production of superoxide by rapid freezing techniques yielded values no greater than 0.1 eq of O₂⁻/FAD for the complete oxidation of fully reduced enzyme. In addition to being inconsistent with Scheme 1, this result contradicts other work...
which has suggested that \( O_2^- \) is the initial product of the oxidative steps and that \( H_2O_2 \) is only produced by dismutation (4). In order to resolve this problem, we have measured directly the production of \( H_2O_2 \) and \( O_2^- \) during the oxidation of xanthine oxidase under a wide variety of conditions. Cytochrome \( c \) peroxidase was used as a trapping agent for \( H_2O_2 \), and the reduction of cytochrome \( c \) was used to monitor the formation of superoxide.

### MATERIALS AND METHODS

Xanthine oxidase was prepared from fresh unpasteurized butter milk according to the method of Massey et al. (5). The ratio of \( A_{340} / A_{600} \) was \( \approx 5.5 \). Before use, the enzyme was transferred to the appropriate buffer by desalting on a Bio-Gel P-6 column and was then diluted to the desired concentration. The concentration of xanthine oxidase is expressed in terms of total flavin sites. Cytochrome \( c \) peroxidase was prepared according to the method of Nelson et al. (6) from commercial yeast obtained from Universal Foods Corp. (Red Star). The final product had a value of 1.6-1.65 for the ratio of \( A_{340} / A_{600} \). Sodium dithionite was obtained from Virginia Smelting Co., and the buffer reagents and cytochrome \( c \) (Type VI) were all purchased from Sigma.

Rapid mixing experiments were performed using a Gibson-Durrum stopped flow spectrophotometer interfaced to a Nova computer by means of a high speed 12-bit A/D converter. The data were collected as voltage readings from the photomultiplier tube and converted to absorbance changes by means of software. In order to increase the signal-to-noise ratio, each experimental record consisted of the average of 4-10 consecutive measurements.

The following buffers were used: 0.1 M potassium 2-(N-morpholino)ethanesulfonic acid, pH 6.15; 0.1 M potassium 3-(N-morpholino)propanesulfonic acid, pH 7.2; 0.1 M potassium bicarbonate, pH 8.5; 0.1 M 2-(N-cyclohexylamino)ethanesulfonic acid for pH 9.2 and 0.1 M potassium glycinate for pH 9.9. Standard oxygen solutions were prepared by bubbling syringes containing the appropriate buffer with either air or pure \( O_2 \) in open glass vessels at room temperature for about 30 min. An aerobic buffer was prepared in the same way except that it contained 99.99% nitrogen. Solutions at other \( O_2 \) concentrations were prepared by diluting the standard oxygen solutions with the anaerobic buffer in the gas-tight syringes.

Reduced xanthine oxidase was prepared in a glass testosmeter equipped with a standard taper joint to accept a syringe containing the reductant and a 10-mm optical cuvette for direct spectrophotometry. The enzyme solution was made anaerobic by equilibration with an argon atmosphere containing less than 0.2 ppm of oxygen (7). Reduction was carried out by titrating to the desired level with a 29 mM solution of sodium dithionite (0.1 M pyrophosphate, pH 8.5) while monitoring the absorbance change at 450 nm (\( \Delta A = 26.600 \) M\(^{-1}\) cm\(^{-1}\)) in a Cary 17 spectrophotometer.

### RESULTS

Yonetani (8) and Yonetani and Ray (9) have reported that cytochrome \( c \) peroxidase reacts quantitatively and rapidly with hydrogen peroxide, producing large absorbance changes in the Soret region. The difference spectrum between the peroxidase and its hydroperoxide adduct has a maximum at 424 nm and an isosbestic point at 544 nm. This latter wavelength coincides closely with that of the maximum absorbance of xanthine oxidase. Thus, it appeared that binding to cytochrome \( c \) peroxidase could be used to monitor the formation of \( H_2O_2 \) during the oxidation of reduced xanthine oxidase.

Loo and Erman (10) have reported a bimolecular rate constant of \( 4.5 \times 10^7 \) M\(^{-1}\) s\(^{-1}\) for the binding of \( H_2O_2 \) to cytochrome \( c \) peroxidase. They assumed this process to be irreversible and reported that the enzyme was unstable above pH 8. We have repeated their work and have obtained results which are quite similar. Data were collected at 424 nm under second order conditions and subsequently fit to a reversible second order reaction. The association rate was independent of pH and equal to \( 1 \times 10^7 \) M\(^{-1}\) s\(^{-1}\). This value is somewhat smaller than that reported by Loo and Erman (10) but still sufficiently fast for the reaction to serve as a probe for the production of hydrogen peroxide by xanthine oxidase.

No reaction could be detected when cytochrome \( c \) peroxidase was reacted with reduced xanthine oxidase anaerobically. More importantly, the kinetics of reoxidation of reduced xanthine oxidase observed at 454 nm were unaffected by addition of cytochrome \( c \) peroxidase to the oxygen solution (Fig. 1). Thus, the peroxidase in the Fe(III) oxidation state does not accept electrons directly from xanthine oxidase, nor does it perturb the normal reoxidation reaction.

The production of \( H_2O_2 \) during the reaction of xanthine oxidase with \( O_2 \) was followed as the difference in the absorbance changes at 424 nm between reaction mixtures with and without added peroxidase. This difference was calculated from the results of two successive experiments, the kinetic traces from each having been manipulated and preserved by means of the on-line data system. As shown in Fig. 2, the time course for the appearance of the \( H_2O_2 \)-peroxidase complex at pH 8.3 exhibits a single phase which roughly parallels the fast phase observed for the reoxidation of xanthine oxidase monitored at 454 nm (see Fig. 4). Addition of superoxide dismutase to the reaction mixture had no significant effect on the data.

Scheme 1 predicts the formation of 2 molecules of \( H_2O_2 \) per flavin in the fast phase and 1 molecule of \( O_2^- / \) flavin in both the fast and slow phases of reoxidation. Since each superoxide is expected to dismute rapidly to produce hydrogen peroxide and oxygen, the absorbance change expected in this experiment should be consistent with formation of 2.5 molecules of the peroxidase-\( H_2O_2 \) complex during the fast phase and an additional 0.5 molecule during the slow phase. However, only a single fast phase is observed experimentally. This discrepancy appears to be due to a reaction of the heme-\( H_2O_2 \) compound with reduced xanthine oxidase.

In contrast to the experiment with untreated cytochrome \( c \) peroxidase, a slow (\( t_{1/2} \approx 10 \) s) decrease in absorbance at 424 nm is observed when 30 \( \mu \)M peroxidase-\( H_2O_2 \) complex is mixed anaerobically with 5 \( \mu \)M reduced xanthine oxidase (Fig. 2B). We interpret this absorbance change to represent direct electron transfer from the flavoprotein to the heme protein, since a similar decrease in absorbance accompanies the reduction of the cytochrome \( c \) peroxidase-\( H_2O_2 \) complex with dithionite.
Peroxisome and Superoxide from Xanthine Oxidase

In addition, an absorbance increase at 454 nm occurs, which indicates that anaerobic reoxidation of xanthine oxidase is taking place. Because this competing reaction is slow, it only affects the peroxidase absorbance changes toward the end of the reaction of O³⁻ with xanthine oxidase, when the concentration of the heme-peroxidase complex is relatively high. Nevertheless, it does complicate the interpretation of the results by eliminating the slow phase of H²O₂ production and by reducing the amount of H²O₂ detected during the fast phase. Quantitation of the extinction change at 424 nm yielded values of 2.3 mol of H²O₂/flavin at pH 8.3 and 2.0 mol of H²O₂/flavin at pH 6.15. These numbers agree reasonably well with the values of 2.5 mol predicted for the fast phase of reoxidation of totally reduced xanthine oxidase (Scheme 1).

Superoxide Formation—The reduction of cytochrome c by O³⁻ has often been used as a method of detection of superoxide in solution (11, 12). This reaction proceeds quite rapidly with a bimolecular rate constant of 10⁹ M⁻¹ s⁻¹ at pH 7.2 (12). At this pH, the cytochrome c reaction competes favorably with superoxide dismutation (2.1 × 10⁸ M⁻¹ s⁻¹ (13)). Cytochrome c reduction was followed at 600 nm to optimize the signal-to-noise ratio since high concentrations of protein were required (15 μM xanthine oxidase, 0.5 mM cytochrome c). This wavelength afforded the largest extinction change for cytochrome c reduction while still maintaining a total absorbance of less than 2.0. We routinely employed 0.5 mM cytochrome c as the superoxide scavenging system at all the pH values studied. In each case, controls using 0.125 and 0.25 mM cytochrome c were performed, and the experimental traces were always found to be independent of cytochrome c concentration.

The time courses observed for the production of reduced cytochrome c during the reaction of xanthine oxidase with O³⁻ are complex (Fig. 3A). There are several reasons for this complexity. First, the oxidation of xanthine oxidase and the reduction of cytochrome c proceed with different time dependencies and yield absorbance changes of opposite signs at 600 nm. Second, there is a direct electron transfer from reduced xanthine oxidase to cytochrome c. As shown in Fig. 3C, an absorbance decrease occurs at 600 nm when cytochrome c is mixed with reduced xanthine oxidase anaerobically. Fortunately, this reaction is quite slow compared to the reoxidation of the enzyme (see Figs. 3C and 4). Third, hydrogen peroxide appears to destroy cytochrome c. Incubation of ferricytochrome c with levels of H²O₂ comparable to those produced in the overall reoxidation of xanthine oxidase causes bleaching of the heme c absorbance at a rate of 10⁵ M⁻¹ s⁻¹. Addition of catalase to the reaction mixture eliminates some of the absorbance decrease at 600 nm. Since oxidation of c²⁺ leads to an absorbance increase at this wavelength, the hydrogen peroxide reaction does not represent simple reoxidation of newly formed reduced cytochrome. Even though the addition of catalasé modifies slightly the time courses obtained in the presence and absence of superoxide, the time course has little effect on the difference between these traces. This indicates that the reaction of H²O₂ with cytochrome c is essentially the same in both cases.

These complications can be overcome by performing the reaction both in the presence and in the absence of superoxide dismutase. Since all the interfering processes should remain unaffected, the differences in the kinetic data between the two experiments yield absorbance changes which are due exclusively to the interaction of cytochrome c²⁺ with superoxide (Fig. 3B). These data are independent of cytochrome c concentration in the range 0.125–0.50 mM and of superoxide dismutase concentration in the range 1–10 μM. As shown in Fig. 3B, the time course for superoxide formation at pH 8.3 consists of two distinct phases of equal amplitude. This result is representative of data obtained at the other pH values and agrees with the idea that 1 molecule of O³⁻ is produced in the rapid phase of reoxidation and one is produced in the slow phase. Effects of the Degree of Reduction of Xanthine Oxidase—Scheme 1 predicts that superoxide is generated only when the last 2 electrons are removed from xanthine oxidase. Thus, the amount of O³⁻ produced during reoxidation should be inde-

![Fig. 2. Time courses for the interactions of cytochrome c peroxidase with xanthine oxidase. A, time course for hydrogen peroxidase production during the reoxidation of xanthine oxidase. The reaction was carried out in 0.1 M Bicine, pH 8.3, at 25 °C. The following concentrations after mixing were used: 5 μM xanthine oxidase, 37.5 μM cytochrome c peroxidase, 125 μM oxygen. The squares represent the difference between traces taken at 424 nm in the presence and absence of peroxidase. (This difference represents the absorbance change due to the formation of the peroxidase-H₂O₂ complex.) The continuous line is the expected production of H₂O₂ as predicted by Scheme 1. The discrepancy between the two traces is explained in the text. Quantitation of the absorbance change of cytochrome c peroxidase indicated that 2.3 molecules of H₂O₂ were detected during the oxidation of fully reduced xanthine oxidase. B, time course for the interaction of reduced xanthine oxidase with cytochrome c peroxidase-H₂O₂ complex. The reaction was performed in the absence of oxygen in 0.1 M Bicine buffer, pH 8.3, at 25 °C. Concentrations after mixing were 10 μM xanthine oxidase, 15 μM cytochrome c peroxidase. The peroxidase-H₂O₂ complex was prepared by titrating anaerobic cytochrome c peroxidase with a stock solution containing approximately 5 mM hydrogen peroxide. The time course was followed at 424 nm. The small and rapid increase in absorbance observed initially is due to contamination of the stock peroxidase solution with oxygen, which oxidizes a small fraction of the xanthine oxidase. The large decrease in absorbance is due to a breakdown of the cytochrome c peroxidase-H₂O₂ complex which occurs much more slowly.
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pendent of the level of reduction of the enzyme once 2 electrons have been added. This idea was tested directly by mixing partially reduced xanthine oxidase with oxygen solutions containing cytochrome c. As shown in Fig. 4, the amount of superoxide generated increases by only 20% in going from 2- to 5-electron-reduced enzyme. In agreement with Scheme 1, there is also a marked lag in the time courses of cytochrome c reduction when oxygen is reacted with enzyme containing 4.5 and 6 electrons/flavin (Fig. 4B, upper curves).

FIG. 4. Oxidation of partially reduced xanthine oxidase. All reactions were carried out in 0.1 M Bicine, pH 8.3, at 25 °C. The concentrations after mixing were 15 μM xanthine oxidase, 0.5 mM cytochrome c^3+, 125 μM oxygen. The levels of reduction of the xanthine oxidase solutions before mixing were 2 electrons/flavin (○), 4.5 electrons/flavin (△), and 6 electrons/flavin (□). A, absorbance change for xanthine oxidase oxidation measured at 454 nm. B, absorbance change for cytochrome c^3+ reduction measured at 600 nm. The data in B were obtained as described in Fig. 2E and represent the difference between absorbance changes measured in the absence and in the presence of superoxide dismutase (5 μM after mixing). The data were collected at two time scales, 0-0.2 and 0.2-2.2 s. The symbols represent experimental data which are expressed as a percentage of the total absorbance change observed with fully reduced xanthine oxidase. The lines are the time courses predicted by Scheme 1 and Equation 2 for the different levels of reduction.

FIG. 5. Oxidation of fully reduced xanthine oxidase by various levels of O_2. The data at 454 and 600 nm were obtained as described in Figs. 2B and 4. The reactions were carried out in 0.1 M Bicine, pH 8.3, at 25 °C. The protein concentrations after mixing were 10 μM xanthine oxidase, 0.5 mM cytochrome c. The reaction was followed at 600 nm.
The small decrease in superoxide production which is observed for 2-electron-reduced xanthine oxidase (Fig. 4B, lower curve) is a result of the distribution of enzyme species which are present in this partially reduced sample. Olson et al. (2) have shown that small but significant amounts of XO(3) and XO(1) will be present at equilibrium when 2 electrons/flavin are added to xanthine oxidase. As shown in Scheme 1, oxidation of XO(3) and XO(1) produces only 1 molecule of O$_2^-$ whereas the oxidation of XO(2) produces 2. Thus, enzyme which is reacted with only 1 equivalent of dithionite is both expected and observed to exhibit less O$_2^-$ production than when it is fully reduced.

Dependence of Superoxide Production on Oxygen Concentration—A second test was designed to verify the reaction mechanism shown in Scheme 1. Xanthine oxidase was reacted with varying amounts of oxygen. When fully reduced enzyme is mixed with only 1 equivalent of O$_2$ (Fig. 5), little or no superoxide is produced as evidenced by the lack of absorbance change at 600 nm. This observation is consistent with Scheme 1 since oxidation of XO(6) to XO(4) is postulated to yield H$_2$O$_2$ exclusively. As a control, the enzyme solution was reacted with a 10-fold excess of oxygen and the typical result was obtained. In contrast, when the enzyme sample was reacted with 2 equivalents of O$_2$, the absorbance stayed essentially constant for 100 ms and then increased slowly (Fig. 5, lower curve).

Although unexpected, this result can also be explained by Scheme 1. The reaction of 6-electron-reduced xanthine oxidase with 2 molecules of O$_2$ is not synchronous for all molecules. Small fractions of enzyme are oxidized to the level of XO(1) and XO(0) leading to the formation of small amounts of O$_2^-$. This superoxide is scavenged by the cytochrome c$^{+}$ regenerating O$_2$. As a result, the amount of oxidizing equivalents available under these conditions exceeds the original oxygen concentration. In the presence of excess superoxide dismutase, this catalytic reduction of cytochrome c by O$_2^-$ is eliminated and the total oxidizing capacity is fixed by the amount of oxygen present. Thus, in the absence of dismutase, xanthine oxidase is oxidized to a greater extent than in its

![Fig. 6. pH dependence of enzyme oxidation and superoxide production.](image)

**Fig. 6.** pH dependence of enzyme oxidation and superoxide production. The reactions were carried out at 25 °C, using the following concentrations after mixing: 15 μM xanthine oxidase, 0.5 mM cytochrome c, 125 μM oxygen. The symbols represent the absorbance changes of xanthine oxidase at 454 nm □ and those of cytochrome c at 600 nm ▲ obtained as described in Fig. 2. The data are expressed as a percentage of the total absorbance change observed for each trace. The lines represent theoretical curves computed using Equation 1. The buffer systems used are given under “Materials and Methods.”

| pH | $K_{app}$ | $k_e$ | O$_2$/flavin | $k_e$ | O$_2$/flavin |
|----|----------|------|-------------|------|-------------|
| 6.15 | 7.7 × 10$^{-4}$ | 13 | 0.4 | 8 × 10$^{-3}$ | 0.4 |
| 7.2 | 1.1 × 10$^{-4}$ | 25 | 0.3 | 13 × 10$^{-3}$ | 0.3 |
| 8.3 | 2.2 × 10$^{-4}$ | 60 | 0.7 | 10 × 10$^{-3}$ | 0.7 |
| 9.2 | 1.4 × 10$^{-4}$ | 350 | 0.9 | 6.3 × 10$^{-3}$ | 0.9 |
| 9.9 | 1.4 × 10$^{-3}$ | 350 | 0.8 | 6 × 10$^{-3}$ | 0.8 |

**Table I**

Reaction constants for reoxidation of xanthine oxidase

Rate constants for the fast steps of oxidation (Equation 1) and the second order rate constants for the slow phase were obtained from an analysis of the data in Fig. 7 as described in the text. The quantity of superoxide produced was obtained from the magnitude of the absorbance change at 600 nm due to O$_2^-$ reduction of cytochrome c ($\Delta$e for cytochrome c reduction at 600 nm = $-1000 \times$ cm$^{-1}$).
presence. During oxidation, the absorbance of xanthine oxidase at 600 nm increases, while reduction of cytochrome c is accompanied by a decrease in absorbance. Since the absorbance change of xanthine oxidase/electron is 50% larger than that of cytochrome c, a net increase in absorbance at 600 nm is observed when the time courses in the presence and absence of superoxide dismutase are subtracted (Fig. 5, lower curve).

Effect of pH on Superoxide Production—Samples of fully reduced xanthine oxidase at pH values ranging from 6.15–9.9 were reacted with 125 μM oxygen. The resulting kinetic traces for reoxidation of the enzyme and for superoxide production are shown in Fig. 6. The cytochrome c absorbance changes associated with the O$_2^-$ formation were quantitated, and the results are presented in Table I.

**DISCUSSION**

**Quantitative Analysis**—Scheme 1 has been investigated numerically on the simplifying assumption that the rate constants for all the reoxidation steps except the last one are identical. Each oxidative stage is assumed to be a two step process composed of a rapid binding step followed by electron transfer

$$\text{XO}(n) + O_2 \rightarrow \text{XO}(n-2) + O_2$$

**SCHEME 2**

The concentration of the oxygenated intermediate is assumed to be small and to remain relatively constant throughout the reaction. Under these conditions, the observed rate is given by

$$k_1 = \frac{k_4[O_2]}{[O_2] + k_r + k_i}$$

(1)

Since the data to be fitted were obtained under pseudo-first order conditions, the oxygen concentration remains essentially constant throughout the reaction, $k_1$, for the first oxidative stage is given by Equation 1. The last step, XO(1)$\rightarrow$ XO(0), is defined as a simple second order process with the pseudo-first order rate, $k_2$, equal to $k_i[O_2]$ (1). The time courses for the disappearance of the reacting species, using all the assumptions above, are given by

$$\text{XO}(6) = \text{XO}(6); e^{-kt}$$

$$\text{XO}(5) = \text{XO}(5); e^{-kt}$$

$$\text{XO}(4) = \text{XO}(4); k_1te^{-kt}$$

$$\text{XO}(3) = \text{XO}(5); k_1te^{-kt}$$

$$\text{XO}(2) = \text{XO}(6); \frac{1}{2}k_r^2t^2e^{-kt}$$

$$\text{XO}(1) = \text{XO}(6); k_1^2\left[\frac{t^2}{2(k_1 - k_2)} - \frac{t}{(k_1 - k_2)^2} \right]$$

$$+ \text{XO}(5); k_1^2\left[\frac{t^2}{(k_1 - k_3)} - \frac{1}{(k_1 - k_3)^2} \right]$$

(2)

where XO(n) represents the concentration of n-electron-reduced enzyme at time t and XO(6); and XO(5); represent the initial concentrations of 5- and 6-electron-reduced enzyme. It should be noted that when fully reduced enzyme is used, XO(6); = 0, no XO(3) is produced, and the expression for X0(1) reduces to the first group of terms. More complicated expressions are required when partially reduced enzyme is reacted with oxygen since in that case the initial concentrations of all the reduction states must be considered. However, the form of these equations is similar to those of the equations given above. Absorbance changes at 454 nm were computed by combining the time courses of the various intermediates using the spectral weights reported by Olson et al. (2). The production of H$_2$O$_2$ is associated with the disappearance of XO(6), XO(5), XO(4), and XO(3), and the production of O$_2^-$ is associated with the disappearance of XO(2) and XO(1).

The observed time courses for the absorbance changes at 454 nm were fitted to Equation 1 using standard nonlinear least squares methods; only $k_1$ and $k_2$ were allowed to vary. The fitted values of $k_1$ and $k_2$ were then used to predict the time course for formation of H$_2$O$_2$ and O$_2^-$. Typical results of this analysis are shown in Figs. 4 and 6. It should be noted that, at each pH, 5 different oxygen concentrations were examined (63, 125, 312, 375, and 625 μM) and the resulting traces were fitted to Equation 1. For the most part, the observed and calculated curves are in very close agreement. The poorest fits were obtained for the time courses of cytochrome c reduction at pH 6.15 and 7.2. This is primarily a result of the small absorbance changes observed at these pH values and of the close similarity between the fast and slow rates.

The dependence of the pseudo-first order rates, $k_1$, and $k_2$, on oxygen concentration and pH is shown in Fig. 7. In agree-
ment with earlier work (1), both rates vary with pH. The rate of the fast phase, \( k_1 \), exhibits a hyperbolic dependence on oxygen concentration which is consistent with Scheme 2. The intrinsic electron transfer rate \( k \) was obtained from the y intercept of the double reciprocal plots shown in Fig. 7, while the apparent \( K_D \) for the enzyme-O₂ binary complex, \( (k_1 + k_2)/k_2 \), was obtained from the slope. As shown in Table I, the apparent affinity of reduced flavin for O₂ increases 20-fold as the pH is lowered from 9.9 to 6.2, whereas \( k_2 \) decreases by 25-fold over the same range. Since pH affects both the electron transfer rate and the apparent \( K_D \) to about the same extent, the double reciprocal plots are parallel and therefore suggestive of uncompetitive inhibition (i.e. proton binding to the enzyme-O₂ complex inhibits electron transfer and release of H₂O₂).

The rate of the slow phase, \( k_2 \), is plotted with respect to oxygen concentrations (in Fig. 7B). A large amount of scatter is observed at pH 6.15 and 7.2. This is due to the difficulty of determining the rate with precision since the amplitude of the slow phase at these pH values is only about 5% of the total absorption change (cf. Table I). In general, however, the rate of the slow phase appears to be directly proportional to the O₂ concentration, and, with the possible exception of the lowest pH, there is no evidence for rate saturation at the highest oxygen concentrations. This agrees with the earlier results of Olson et al. (1). Clearly, the complex between O₂ and 1-electron-reduced enzyme must be rather weak, if it exists at all.

Hydrogen Peroxide and Superoxide Production—The release of H₂O₂ is confined to the fast phase of reoxidation (Fig. 2) while superoxide production (Figs. 4–6) occurs only toward the close of the fast phase and during the subsequent slow phase. This behavior is precisely that postulated by Olson et al. (2) as outlined in Scheme 1. The production of H₂O₂ occurs exclusively during the steps XO(1) → XO(4) and XO(4) → XO(2); superoxide production is confined to the steps XO(2) → XO(1) and XO(1) → XO(0). Thus, 2 molecules of peroxide and 2 molecules of superoxide are the anticipated yield from the reoxidation of fully reduced enzyme. The superoxide should subsequently dismutate to yield 1 mol of H₂O₂, half of which should be formed rapidly with the remainder formed more slowly.

Our data are not completely consistent with this expectation. Typically, we find 2.0–2.3 mol of H₂O₂ formed rapidly at the different pH values examined with a time course essentially identical with that for the fast phase of reoxidation of the enzyme (Fig. 2). No production of peroxide was observed during the slow phase of reoxidation. This discrepancy is attributed to an unanticipated reaction of the cytochrome c peroxidase-H₂O₂ adduct with reduced xanthine oxidase. Fortunately, this process is only significant during the closing stages of reoxidation when substantial quantities of the peroxidase-H₂O₂ complex have accumulated. In view of this complication, the close to theoretical yields of H₂O₂ observed during the fast phase are rather satisfactory.

Quantitation of superoxide release is also complicated by the direct reaction of cytochrome c⁺ with reduced xanthine oxidase. The use of superoxide dismutase to resolve the direct and the superoxide-mediated reduction processes revealed that O₂⁻ was only released late in reoxidation. The yields of superoxide varied significantly, from 0.6 mol at pH 7.2 to 1.8 mol at pH 9.2. This observation, together with the fact that the value of \( k_2 \) decreases with pH, would suggest that, at lower pH, increased stability of the enzyme-oxygen adduct permits the existence of a competing enzyme-oxygen adduct-mediated reaction. E(2) → E(0), with the release of hydrogen peroxide. At all pH values studied, identical amounts of superoxide were trapped in the fast and slow phases of reoxidation (Table I). The postulated pathway of reoxidation is also supported by the observations that levels of O₂⁻ adequate to remove only 2 electrons from fully reduced xanthine oxidase did not yield any superoxide (Fig. 5) and that the yield of superoxide did not increase significantly in going from 2- to 6-electron-reduced enzyme (Fig. 4).

As previously discussed by Olson et al. (2), Scheme 1 explains observations concerning the reduction of cytochrome c during catalysis. Fridovich (4) has reported that the superoxide-mediated reduction of cytochrome c increases whenever the concentration of xanthine is lowered or the concentration of oxygen is raised. He also reported a far higher level of superoxide production at pH 10.0 than at pH 7.0. When the concentration of oxygen is high and that of xanthine is low, the enzyme cycles mainly between 2-electron-reduced and oxidized states (2). As indicated in Scheme 1, oxidation of XO(1) and XO(2) occurs largely by 1-electron transfer steps so that O₂⁻ is the principal product in agreement with the results of Fridovich (4). On the other hand, when the concentration of O₂⁻ is low and that of xanthine is high, the enzyme is largely reduced and little superoxide is produced in the oxidative steps. The dependence of superoxide production on pH is also explainable in terms of the results presented here. First, there is less production of O₂⁻ at pH 7 during the oxidation of reduced enzyme than at pH 10 (Table I). Second, the overall oxidation rate increases with increasing pH so that even at high concentrations of xanthine the fraction of the enzyme which is in the 1- or 2-electron-reduced state increases with pH at a fixed oxygen concentration. As a result, at a high pH more O₂⁻ is expected to be produced at pH 10 than at pH 7 in agreement with the results of Fridovich (4).

The rates of both phases of oxidation vary with pH. Double reciprocal plots of the rates of the fast phase suggest that the pH effect arises from uncompetitive inhibition by protons. We have observed that the flavin radicals produced by partial reduction of xanthine oxidase exhibit EPR line widths typical of the neutral radical at low pH (14) and of the anion radical at pH 9.9. Neutral and anionic radicals have been correlated with dehydrogenase and oxidase activities (15). Thus, the inhibition of the electron transfer step in Equation 1 is probably asserted through changes in the state of protonation at the flavin oxidation-reduction system. This suggestion is consistent with the proposal by Massey and Hemmerich (16) that oxidase activity is conferred upon flavin by protein stabilization of the N₁-O' anionic locus which is believed to favor formation of the C₁0(α) peroxide adduct. Subsequent breakdown of this adduct to H₂O₂ and oxidized flavin is presumed to be promoted when the negative charge is localized at the N' position. Lowering the pH will lead to protonation of N' with attendant reduction in the rate of electron transfer to the oxygen atoms and the subsequent release of peroxide.

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