Studies on the Oxidative Half-reaction of Xanthine Oxidase*

Russ Hille‡ and Vincent Massey
From the Department of Biological Chemistry, The University of Michigan, Ann Arbor, Michigan 48109

The oxidative half-reaction of xanthine oxidase is re-examined with regard to the generation of the superoxide anion. By using cytochrome c reduction to monitor superoxide, it is found that the stoichiometry of superoxide produced to enzyme reoxidized is 2:1, significantly greater than previously reported (Olson, J. S., Ballou, D. P., Palmer, G., and Massey, V. (1974) J. Biol. Chem. 249, 4350-4362). Furthermore, the kinetics of superoxide-dependent cytochrome c reduction exhibits a pronounced lag during the rapid phase of enzyme reoxidation and a limiting rate identical with that of the slow phase of enzyme reoxidation. This indicates that superoxide is generated only in the last steps of the sequential removal of reducing equivalents from the enzyme by molecular oxygen. Experiments with the two-electron-reduced enzyme indicate that it too produces two superoxide ions for each molecule of enzyme reoxidized, demonstrating that it is the last two electrons to leave the enzyme in the course of reoxidation that form superoxide. The sequential scheme for the oxidative half-reaction must therefore involve some sort of sequential removal of reducing equivalents by more than one oxygen molecule. It has been inferred that superoxide is produced late in the course of enzyme oxidation (6). For example, under pseudo-first order conditions, the reoxidation reaction is markedly biphasic (6). The spectral characteristics of the slower phase suggest that it is due to the reoxidation of the one-electron-reduced form of the enzyme, a process which necessarily involves the production of superoxide. Furthermore, the two phases exhibit different behavior as a function of oxygen concentration (6), demonstrating that they are due to fundamentally different processes. The rate constant of the faster phase shows a hyperbolic dependence on oxygen concentration, indicating the existence of a preequilibrium step prior to the reoxidation event, while that of the slower phase remains directly proportional to oxygen concentration up to 625 μM.

In an effort to describe further the reoxidation reaction in terms of the generation of the superoxide anion, experiments have been undertaken that take advantage of the well established reduction of cytochrome c by superoxide (1) and the inhibition of this reduction by superoxide dismutase (7). The results demonstrate that the superoxide is indeed produced only in the final steps of the reoxidation reaction.

MATERIALS AND METHODS

Xanthine oxidase was isolated from fresh milk by the method of Massey et al. (8). The enzyme used had activity flavin ratio values (see Ref. 8) in the range of 140-150, i.e. it contained 67-72% functional active sites (see Ref. 11). All enzyme concentrations quoted refer to those of the molybdenum or flavin cofactors of which there are 2/ enzyme molecule (see Ref. 8). Kinetic experiments were performed with a stopped flow spectrophotometer designed and built by Dr. David P. Ballou of the Department of Biological Chemistry, University of Michigan, and interfaced with a Data General Nova 2 minicomputer (9). The instrument was designed so that the wavelength could be automatically scanned over a preselected 400-nm range with a total scanning time of 10 s to allow spectra to be recorded. Concentrations of the reagents reported in kinetic experiments are those before mixing.

Reduced enzyme was prepared by titration with sodium dithionite in an anaerobic tonometer equipped with a side arm covered. Reaction was monitored by optical spectroscopy in the 300-700-nm range. Spectra were recorded 20 min after each dithionite addition and carefully watched for increases in absorbance at 318 nm indicating the accumulation of excess dithionite. By ending the titration at the first sign of such absorbance increases, the concentration of excess reducing equivalents could be held to within 5% of the concentration of reducing equivalents in the enzyme.

Bovine heart cytochrome c (type V) was purchased from Sigma. Bovine liver superoxide dismutase was purchased from Diagnostic Data, Inc., Mountainview, CA. Unless otherwise stated, experiments were performed in 0.1 M sodium pyrophosphate buffer, pH 8.5, with 0.3 mM EDTA present at 25 °C.

Computer simulations were performed with a Nova 2 minicomputer using a fourth order Runge-Kutta (10) routine in the Fortran programming language. Extinction coefficients for the intermediate oxidation states of xanthine oxidase were those calculated by Olson et al. (6). Fits to the data were obtained by manually adjusting the

During turnover with xanthine and molecular oxygen, xanthine oxidase generates significant amounts of the superoxide anion in addition to hydrogen peroxide (1-3). The oxidative half-reaction of the catalytic cycle takes place at the flavin site, and the two iron-sulfur centers and molybdenum site of the enzyme do not appear to be directly involved (4). Instead, those sites become reoxidized by transferring their reducing equivalents to the flavin site for reaction with oxygen. This internal electron transfer appears to involve a rapid oxidation-reduction equilibrium among the various sites of the enzyme, with reducing equivalents being transferred to the flavin site because of its high oxidation-reduction potential relative to those of the other sites (5).

In the course of the reaction of fully reduced xanthine oxidase with oxygen, the steps involved in the production of superoxide are not known. Fully reduced enzyme contains a total of six electrons (two each at the flavin and molybdenum sites, and one each at the two iron-sulfur centers) and reoxidation must therefore involve some sort of sequential removal of reducing equivalents by more than one oxygen molecule. It has been inferred that superoxide is produced late in the course of enzyme oxidation (6). For example, under pseudo-first order conditions, the reoxidation reaction is markedly biphasic (6). The spectral characteristics of the slower phase suggest that it is due to the reoxidation of the one-electron-reduced form of the enzyme, a process which necessarily involves the production of superoxide. Furthermore, the two phases exhibit different behavior as a function of oxygen concentration (6), demonstrating that they are due to fundamentally different processes. The rate constant of the faster phase shows a hyperbolic dependence on oxygen concentration, indicating the existence of a preequilibrium step prior to the reoxidation event, while that of the slower phase remains directly proportional to oxygen concentration up to 625 μM.

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appropriate rate constants in a sequential scheme until the kinetic
time course of the reoxidation was adequately described. In simulating
the cytochrome c kinetic data, a second order rate constant of $1.8 \times 10^{-10}$ M$^{-1}$ s$^{-1}$ for the reduction of cytochrome c by O$_2$ was used (3).

**RESULTS AND DISCUSSION**

**Oxidation of Reduced Xanthine Oxidase with Molecular Oxygen.**—The optical absorbance change at 450 nm on mixing reduced xanthine oxidase with oxygen is shown in Fig. 1 where it can be seen that the excursion is markedly biphasic. Furthermore, after subtracting out the slow phase and expanding the time scale, it is found that the fast phase is an exponential process only after a pronounced lag phase. Plots of the observed rate constants for the fast and slow phases as functions of oxygen concentration are shown in Fig. 2, A and B, respectively. The fast phase exhibits hyperbolic dependence on oxygen concentration and from the x and y intercepts of a double reciprocal plot, a $K_a$ of $5 \times 10^{-4}$ M and limiting rate, $k_{	ext{lim}}$, of 125 s$^{-1}$, can be determined. These results are in reasonable agreement with those of Olson et al. (6) who obtained values of $8.3 \times 10^{-4}$ M and 120 s$^{-1}$ for $K_a$ and $k_{	ext{lim}}$, respectively. The rate constant for the slow phase is directly proportional to oxygen concentration giving a second order rate constant of $1.0 \times 10^{-4}$ M$^{-1}$ s$^{-1}$, in good agreement with the results of Olson et al. (6). These workers concluded from the wavelength dependence of the two phases that the faster involved oxidation of both flavin and iron-sulfur centers whereas the slower involved oxidation only of the iron-sulfur centers. The fact that the two phases exhibit different behavior when the oxygen concentration is varied indicates that two different mechanisms of reoxidation must be operative.

**The Reaction of Reduced Xanthine Oxidase with Oxygen in the Presence of Cytochrome c**—In an effort to determine which, if either, of the two phases of the oxidative half-reaction was responsible for the generation of superoxide, the oxidation of reduced xanthine oxidase by oxygen was carried out in the presence of oxidized cytochrome c, which is known to be reduced by superoxide (1,3). To eliminate complications from absorbance changes due to the reoxidation of the enzyme itself, the reaction was carried out first in the absence and then in the presence of a catalytic amount of superoxide dismutase, and the two traces subtracted from one another to give the kinetics of superoxide-dependent reduction of cytochrome c. Initially there was some concern that H$_2$O$_2$ also produced in the reaction would interfere by reoxidizing the cytochrome c reduced by superoxide, but control experiments demonstrated that at the conditions under which the reaction was carried out, no H$_2$O$_2$ was detectable. The absorbance change at 450 nm on mixing reduced xanthine oxidase with oxygen (determined from the peak at 553 nm and the trough at 585 nm in the difference spectrum between oxidized and reduced cytochrome c) is not profoundly sensitive to cytochrome c concentrations above 10 $\mu$M. The only effect of increasing the cytochrome c concentration from 10 to 60 $\mu$M (after mixing) is a slight increase in the rate of superoxide-dependent cytochrome c reduction immediately after the lag phase (data not shown). The limiting rate of the reaction is largely unaffected.

Because of the extremely narrow spectral band width of the reduced cytochrome c spectrum at 550 nm, spectrophotometric determination of superoxide absorbance changes is not a very reliable way to quantitate the amount of superoxide-dependent cytochrome c reduction. Instead, spectra were recorded in the stopped flow spectrophotometer after shots taken in the absence and presence of superoxide dismutase. For these quantitative determinations, an experimentally determined $e_{505-585}$ of 18.9 mm$^{-1}$ cm$^{-1}$ was used, representing the difference between the peak at 553 nm and the trough at 585 nm in the difference spectrum between oxidized and reduced cytochrome c. This operational extinction coefficient was obtained by measuring the difference spectrum for a known concentration of cytochrome c before and after addition of sodium dithionite in the stopped flow spectrophotometer. The stoichiometry of cytochrome c reduced/xanthine oxidase oxidized (determined from the $\Delta A_{450}$ nm on mixing reduced enzyme with oxygen in

![Fig. 1. Semilogarithmic plot of the absorbance change at 450 nm observed on reaction of fully reduced xanthine oxidase with 2.0 $\times 10^{-4}$ M oxygen. Enzyme concentration was 6.5 $\times 10^{-6}$ M. O, plot of the actual excursion; □, plot of the fast phase on an expanded time scale after having subtracted out the contribution of the slow phase. The rate constants thus determined for the fast and slow phases are 17.5 and 0.9 s$^{-1}$, respectively. Lines represent a simulation of the data as described in the text.](image)
Oxidative Half-reaction of Xanthine Oxidase

The time course for the reoxidation of two-electron-reduced xanthine oxidase by 200 μM oxygen is shown in Fig. 4A. It can be seen that the reaction is distinctly biphasic, with the slow phase contributing about 40% of the absorbance change. The rate constant for the slow phase, 1.0 s⁻¹, agrees very well with that for the slow phase in the oxidation of the fully reduced enzyme (0.9 s⁻¹). Significantly, when the slow phase of the reaction was subtracted out and the time scale was expanded, the fast phase did not exhibit a lag (Fig. 4A, squares). That the slow phase of the reaction is due to the reoxidation of one-electron-reduced enzyme is demonstrated in Fig. 5, which shows a series of time courses taken at various times after the preparation of the two-electron-reduced species. It is readily seen that with time the faster of the two phases in the reaction disappears without effect on the rate of the slower phase. This experiment is directly analogous to the spectral experiment of Olson et al. (6) establishing that on prolonged incubation of the two-electron-reduced enzyme, comproportionation with oxidized enzyme takes place to form a population of the thermodynamically stable one-electron-reduced enzyme.

The dependence of the rate constants for the two phases on oxygen concentration is shown in Fig. 6. It can be seen that, as was the case for the reoxidation of fully reduced xanthine oxidase, the slow phase in the time course for reoxidation of the two-electron-reduced enzyme is directly dependent on oxygen concentration. The slope of that plot in Fig. 6B gives a second order rate constant of 1.0 x 10⁻⁴ M⁻¹ s⁻¹ with fully reduced enzyme. The faster phase in reoxidation of the two-electron-reduced enzyme exhibits saturating behavior with K_d and limiting rate equal to 4.7 x 10⁻⁴ M and 160 s⁻¹, respectively (Fig. 6A). Considering the small values of the x and y intercepts in the double reciprocal plot, these values are in reasonable agreement with the corresponding values of 5.0 x 10⁻⁴ M and 125 s⁻¹ with fully reduced xanthine oxidase.

Cytochrome c Experiments with Two-electron-reduced Xanthine Oxidase—Because it seemed clear that one-electron-reduced xanthine oxidase was an intermediate in the

**Fig. 3. Kinetic traces at 550 nm for the reaction of fully reduced xanthine oxidase (2.3 x 10⁻⁴ M) with 2.0 x 10⁻⁴ M oxygen and 6.3 x 10⁻³ M oxidized cytochrome c. A, absorbance changes in the absence and presence of 5 x 10⁻⁴ M/S of superoxide dismutase. B, semilogarithmic plot of the difference between the two traces in A. The limiting rate is identical with that of the slow phase of enzyme reoxidation, 0.9 s⁻¹. The solid line represents a simulation to the data as described in the text.**
reaction whereas in the latter only
plex with Alloxanthine—Because
the oxidized minus-reduced difference spectrum. Because two
thine oxidase identically and there is therefore no change in
superoxide generation in the reaction of two-electron-reduced
molybdenum site to form an inhibitory complex that is air
xanthine oxidase with oxygen.

The kinetics of superoxide-dependent reduction of cyto-
chrome c on mixing the two-electron-reduced enzyme with
200 μM oxygen and 60 μM cytochrome c (before mixing) is
shown in Fig. 4B, for comparison with the time course for
enzyme reoxidation in the absence of cytochrome c in Fig. 4A.
Like enzyme reoxidation, cytochrome c reduction is biphasic,
and the faster phase exhibits no lag. The stoichiometry of
cytochrome c reduced/two-electron-reduced enzyme oxidized
(the latter determined from the absorbance change at 450 nm
using a ΔA450 for two-electron-reduced enzyme of 11,200 M−1
cm−1, Ref. 5) was found to be 2:1. The amount of cytochrome
c reduced did not vary with time as the two-electron-reduced
enzyme comproportionated to give a population of the one-
electron-reduced species. This observation provides strong
support for the conclusion that both electrons in the two-
electron-reduced enzyme react to form superoxide in the
reoxidation reaction. Thus, all of the superoxide
generated in the reaction of fully (six-electron-) reduced xan-
thise oxidase with oxygen is accounted for in the reoxidation
of the two-electron-reduced enzyme.

Reoxidation of Fully Reduced Xanthine Oxidase in
Complex with Alloxanthine—Because the fully (six-electron-)
reduced xanthine oxidase is reoxidized to the two-electron-reduced
enzyme without producing superoxide, two two-electron steps
must be involved with the four-electron-reduced enzyme as an intermediate. Four-electron-reduced enzyme cannot be generated quantitatively for kinetic examination, but as far as the oxidative half-reaction is concerned, the complex of fully reduced enzyme with alloxanthine should react in very nearly the same way. Massey et al. have shown that alloxanthine binds very tightly to the fully reduced molybdenum site to form an inhibitory complex that is air stable (11). Furthermore, these workers established that alloxanthine perturbs the spectra of reduced and oxidized xan-
thise oxidase identically and there is therefore no change in
the oxidized minus-reduced difference spectrum. Because two
electrons must remain behind at the molybdenum site in the
course of reoxidation, the overall process involves the removal
of four electrons. The only difference between this reaction and the reoxidation of four-electron-reduced enzyme is that,
in the former, 100% of the flavin is reduced at the onset of the reaction whereas in the latter only 96% of the flavin is reduced. Clearly, the effects of this discrepancy should be minimal, and

the reoxidation of fully reduced xanthine oxidase in complex
with alloxanthine should closely emulate the reoxidation of four-electron-reduced enzyme. The reduced xanthine oxidase-
alloxanthine complex can be conveniently generated by treat-
ing anaerobic enzyme with an excess of allopurinol, which is
converted to alloxanthine by active enzyme, which becomes
reduced. It has been shown that this reaction is rapid com-
pared to the formation of the reduced enzyme-alloxanthine
complex, and the active enzyme becomes fully reduced (11).
In the course of the reoxidation reaction, the inhibitory com-
pact prevents turnover with the remaining allopurinol present.
The results of such an experiment are shown in Fig. 7A. It
can be seen that as in the case of the fully reduced enzyme
(minus alloxanthine) the reoxidation of reduced xanthine
oxidase in complex with alloxanthine is markedly biphasic. In
contrast to the case with the uncomplexed enzyme, however,
the fast phase in the reoxidation of the alloxanthine complex

Fig. 5. Semilogarithmic plots for the absorbance changes at
450 nm observed on reaction of two-electron-reduced xanthine
oxidase with 2.0 × 10−4 M oxygen at various times after for-
formation of the partially reduced enzyme. Time after generating
the two-electron-reduced enzyme: A, 2 min; B, 18 min; C, 45 min; D,
90 min.

Fig. 6. Dependence of the rate constants for fast and slow
phases in the reoxidation of two-electron-reduced xanthine
oxidase on oxygen concentration. A, a double reciprocal plot of
1/kfast versus 1/(O2), giving a Kf of 4.7 × 10−4 M and a limiting rate of
180 s−1. B, plot of kslow versus (O2) giving a second order rate constant
of 1.0 × 10−4 M−1 s−1.

Fig. 7. Oxidation of the reduced xanthine oxidase-alloxan-
thine complex. Semilogarithmic plot of the absorbance change at
450 nm observed on reaction of fully reduced xanthine oxidase in
complex with alloxanthine with 2.0 × 10−4 M oxygen (A) and com-
comitant superoxide-dependent cytochrome c reduction (B). The enzyme
complex was prepared by treating the anaerobic enzyme (1.0 × 10−4
M) with a 100-fold excess of allopurinol, followed by a 5-min incuba-
tion. Other conditions were as described in Fig. 3. Solid lines represent
simulations to the data, as described in the text.
intermediate levels of reduction. The data described in the reaction of several oxygen molecules with enzyme at various +

previous sections on the behavior of two- and four-electron-

thine oxidase is necessarily a sequential process involving the

is produced in the last two. Olson et al. (6) showed that such

a scheme could be used to simulate the absorbance changes

observed on reoxidation very well using calculated extinction

changes for each intermediate, but they were unable to ac-

count for the discrepancy between the small amounts of

superoxide they observed and the 20-fold greater amounts

predicted by the above scheme. Our results using cytochrome

c reduction to monitor superoxide generation indicate that
two superoxide ions are indeed formed for each enzyme mol-

cule reoxidized, as predicted by the scheme.

Simulations of the kinetics for superoxide-dependent cyto-

crome c reduction and enzyme reoxidation demonstrate that

the first one-electron step in the reoxidation scheme is the

reaction of the two-electron-reduced intermediate with oxy-

gen. The time courses expected from the above scheme for

both enzyme reoxidation and superoxide generation are shown

as the solid lines in Figs. 1 and 3. As observed by Olson et al. (6), the rate constants required to adequately fit the enzyme

reoxidation data in the fast phase were approximately twice

as with the six-electron- and two-electron-reduced enzyme.

These were the rates used in the simulations.

Comparison of the fraction of reduced flavin in each of the kinetic

intermediates in the reaction of reduced xanthine oxidase with oxygen and the rate of reaction expected with 2.9 x 10^{-4} M oxygen

alloxanthine complex as does the reoxidation of uncomplexed reduced enzyme, as expected if one of the steps prior to

superoxide production is eliminated in the former case. The

stoichiometry of cytochrome c reduced to enzyme reoxidized

remains 2:1, as with the six-electron- and two-electron-reduced enzyme.

Scheme for the Reaction of Fully Reduced Xanthine Oxidase with Oxygen—The removal of six electrons from xan-

thine oxidase is necessarily a sequential process involving the

reaction of several oxygen molecules with enzyme at various

intermediate levels of reduction. The data described in the

previous sections on the behavior of two- and four-electron-

reduced enzyme suggest that the correct sequence is 6 → 4

→ 2 → 1 → 0, where the numbers represent the number of

electrons in each intermediate enzyme species. Hydrogen per-

oxide is produced in the first two steps and superoxide anion

is produced in the last two. Olson et al. (6) showed that such

a scheme could be used to simulate the absorbance changes

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reoxidation data in the fast phase were approximately twice

the rate constants only make the fit to the superoxide

reoxidation data poorer (data not shown). In any case, such a

scheme could not easily be reconciled with the results from

experiments with two-electron-reduced enzyme, indicating that

on oxidation both electrons in this intermediate produce

superoxide.

The kinetics for reoxidation of the two-electron-reduced

enzyme and the fully reduced enzyme complexed with allo-

xanthine can be submitted to the same type of kinetic analysis

as described above. In each case, using the same set of rate

constants as in the simulations of fully reduced enzyme, the

fits to the data are excellent (Figs. 4 and 7, solid lines). In the

case of the two-electron-reduced enzyme, the faster phase is

simulated accurately by an exponential process with a rate

constant of 20 s^{-1}, precisely 58% of the intrinsic rate (35 s^{-1})
as expected from the fraction of reduced flavin in the two-

electron-reduced species (Fig. 4A). The fraction of slow phase

(40%) agrees well with the ratio of extinction changes for two-

and one-electron-reduced enzyme from Olson et al. (6). The

kinetics of cytochrome c reduction in the presence of alloxan-

thine are also accurately simulated, with half of the absorbance change in each of the two

kinetic phases.

With the fully reduced enzyme complexed with alloxan-

thine, both the more exponential behavior and somewhat

faster rate observed in the fast phase of reoxidation (relative
to the case with fully reduced enzyme in the absence of

alloxanthine) are fit well by the simulation (Fig. 7A). In

addition, the less extensive lag in cytochrome c reduction in

the presence of alloxanthine is accurately reproduced in the

simulation. These results argue strongly that the fully reduced

enzyme in complex with alloxanthine behaves like the four-

electron-reduced enzyme during reoxidation, as expected from

previous work (11) and that the four-electron-reduced enzyme

is an intermediate in the reoxidation of the fully reduced

enzyme.

CONCLUSIONS

The reduction of cytochrome c by superoxide anion has been successfully utilized to study the generation of the latter in the reaction of reduced xanthine oxidase with molecular oxygen. In agreement with expectations from a considerable amount of indirect evidence (5, 6), it is observed that superoxide is generated only in the last steps of enzyme reoxidation, with a stoichiometry of two superoxide ions produced for each enzyme molecule reoxidized. The overall sequence for the reoxidation of fully (six-electron-) reduced xanthine oxidase is 6 → 4 → 2 → 1 → 0, where the numbers represent the number of electrons in each enzyme intermediate. The first two steps represent two-electron oxidations to form hydrogen peroxide, and the last two steps represent one-electron oxidations to form superoxide. Our results resolve the earlier discrepancy between results predicted by such a scheme and the very low amounts of superoxide actually detected (6). Superoxide was quantitated in this earlier work by observation of its electron paramagnetic resonance signal, which was collected by the rapid freeze technique. This method could suffer from appreciable dismutation of superoxide, even at the relatively high pH at which the experiment was performed. The cytochrome c technique, on the other hand, maintains the free superoxide concentration at a sufficiently low steady state concentration to preclude dismutation. The determinations of stoichiometry appear to be accurate within 5%. Furthermore, the steady
state superoxide levels remain so low that cytochrome c reduction can be analyzed kinetically to obtain rates that accurately reflect superoxide generation.

In good agreement with the previous work (5), the individual rate constants for the four-step reoxidation scheme that best fit both enzyme reoxidation and cytochrome c data (reacting with atmospheric oxygen) were 35 s\(^{-1}\), 33 s\(^{-1}\), 20 s\(^{-1}\), and 0.9 s\(^{-1}\) for steps 1 through 4, respectively. These values accurately reflect the fractions of reduced flavin expected to be found in the six-, four-, two-, and one-electron-reduced enzyme based on a rapid equilibrium model (Table I). Each step but the last involves the reaction of oxygen with FADH\(_2\), a relatively rapid process. The last step is slow not only because of the small amount of electron density actually residing at the flavin site but also because the reaction necessarily involves the blue neutral semiquinone, a species generally found to be relatively unreactive toward oxygen (13).

The reason for the different behavior of two-electron-reduced xanthine oxidase compared to the six- or four-electron-reduced enzyme is unclear. With respect to the reoxidation at the flavin site, the enzyme formally changes from an oxidase to an electron transferase (14). Olson et al. (6) proposed that the transfer of reducing equivalents takes place in one-electron steps and that rapid re-equilibration of electron density at the flavin nucleus in the nascent FADH\(_2\)····O\(_2\) to other parts of the enzyme (particularly the iron-sulfur centers) prevents further reduction of oxygen in the case of two-electron-reduced enzyme. Clearly, the oxidation state of the other sites plays an important role as this is the only difference between the six- and four-electron-reduced enzyme and that fraction of the two-electron-reduced enzyme population capable of reacting with oxygen (i.e. the 60% containing FADH\(_2\)), but in view of the current understanding of flavin activation of molecular oxygen an alternative explanation must be sought. It is likely that a covalent flavin-oxygen intermediate, similar or identical with the 4a-peroxyflavin formed with flavoprotein hydroxylases (9), is formed on reaction of FADH\(_2\) with oxygen. The very similar dependence of the oxidation of six- and two-electron-reduced enzyme on oxygen concentration argues that the same intermediate is formed in both cases and that only its subsequent breakdown is different in the two situations. In the six- and four-electron-reduced enzyme, the iron-sulfur centers would be reduced and the intermediate would be able to break down into oxidized flavin and H\(_2\)O\(_2\) as in the case of simple flavoprotein oxidases. In the two-electron-reduced enzyme, however, the oxidized iron-sulfur centers could perhaps exert a sufficient influence on the electronic structure of the flavin hydroperoxide to cause it to undergo a homolytic rather than a heterolytic cleavage. The immediate products of the reaction would be flavin semiquinone and O\(_2\), with the reducing equivalent of the semiquinone rapidly equilibrating away from the flavin site. The subsequent slow reaction of flavin semiquinone with O\(_2\), typical of electron transferases, would account for the second equivalent of O\(_2\) produced. Alternatively, structural changes around the flavin might occur in going from the four-electron-reduced enzyme to the two-electron-reduced form, bringing about sufficiently large changes in the hydrogen-bonding arrangement between the protein and isoalloxazine ring to cause the flavin to alter its chemical behavior (i.e. switch from a strict oxidase to an electron transferase; Ref. 14). While these concepts are clearly speculative, they are more in line with current ideas on the mechanism of oxygen activation by flavins.

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