The Reaction of Arsenite-complexed Xanthine Oxidase with Oxygen

EVIDENCE FOR AN OXYGEN-REACTIVE MOLYBDENUM CENTER*

Richard C. Stewart†, Russ Hille‡, and Vincent Massey
From the Department of Biological Chemistry, University of Michigan, Ann Arbor, Michigan 48109

The effects of arsenite on the reaction of reduced xanthine oxidase with oxygen are determined. The kinetics of the reaction monitoring the return of enzyme absorbance are investigated as are the kinetics and stoichiometries of peroxide and superoxide formation. Although some of the effects of arsenite are qualitatively consistent with expectations based on the known perturbation of the molybdenum midpoint potentials by arsenite, several results cannot be easily explained. Specifically, arsenite introduces a very rapid phase (\( k_{\text{fast}} = 110 \text{ s}^{-1} \) at 125 \( \mu \text{M} \) oxygen) to the oxidative half-reaction which is not observed with the native enzyme. Arsenite also diminishes the amount of superoxide produced and eliminates one-electron reduced enzyme as a detectable kinetic intermediate in the reoxidation pathway. These differences appear to result from the ability of arsenite to greatly enhance the oxygen- and/or superoxide-reactivity of the reduced molybdenum center. This is reflected in the observation that reduced forms of arsenite-complexed xanthine oxidase lacking functional FAD (iodoacetamide-alkylated enzyme and deflavo enzyme) react relatively rapidly with oxygen whereas these reactions are quite slow in the absence of arsenite.

Several recent publications (1–6) have examined various aspects of the interaction of the molybdenum center of xanthine oxidase with arsenite. Arsenite binds reversibly to the molybdenum center of the enzyme, preventing its reduction by purines, pteridines, and aldehydes (7–9). The Mo-arsenite interaction raises the Mo(VI)/Mo(V) midpoint potential by approximately 180 mV in 0.1 M BICINE* at pH 8.3 and 25 °C (10). We have previously emphasized the effect of this dramatic change on the distribution of reducing equivalents within partially reduced xanthine oxidase (10). In the present paper we investigate the effect of these perturbations on the reaction of reduced enzyme with oxygen.

Xanthine oxidase is a complex metalloflavoprotein having three oxidation-reduction centers in addition to the molybdenum mentioned above (FAD, and two distinct ferredoxin-type Fe/S centers), and a total of six electrons are required for complete reduction of the enzyme (see Refs. 11 and 12 for reviews). When the reduced native enzyme reacts with oxygen, reducing equivalents are passed to oxygen only via the flavin site (13), producing hydrogen peroxide and superoxide (14–16). The kinetics and stoichiometry of product formation during the oxidative half-reaction have been analyzed by using cytochrome c peroxidase to trap \( \text{H}_2\text{O}_2 \) and cytochrome c reduction to follow \( \text{O}_2 \) production (15, 16). A scheme (Equation 1, below) summarizing much of the available data was first proposed by Olson et al. (17) and has subsequently been independently confirmed by Hille and Massey (15) and Porras and Palmer (16).

\[
\begin{align*}
\text{O}_2 & \rightarrow \text{XO}(0) \\
\text{XO}(6) & \rightarrow \text{XO}(4) \\
& \rightarrow \text{XO}(2) \\
& \rightarrow \text{XO}(1) \\
& \rightarrow \text{XO}(0)
\end{align*}
\]

For each of the partially reduced enzyme intermediates in the scheme, the numbers in parentheses indicate the number of reducing equivalents present. The reaction of reduced xanthine oxidase with oxygen can be interpreted qualitatively and quantitatively in terms of the rapid equilibrium model first proposed by Olson et al. (17, 18). The major assumption made in applying this model to the oxidative half-reaction is that the rate of intramolecular electron transfer among the four redox centers is rapid relative to the rate of electron transfer from reduced enzyme to oxygen. Although recent flash photolysis work has questioned the validity of this assumption (19), the conclusions from conventional kinetic studies of xanthine oxidase are consistent with a rapid equilibrium of reducing equivalents within the enzyme (see, e.g. Ref. 20). With this assumption, the optical extinction coefficient and level of FAD reduction in each intermediate may be calculated from the known potentials of these redox centers (18, 21). Each intermediate of Equation 1 will therefore react with oxygen at a rate equal to an intrinsic rate constant, \( k \), for the reaction of oxygen with \( \text{FADH}_2 \) multiplied by the fraction of completely reduced flavin in that intermediate (\( F_{\text{FADH}_2} \)). In addition, the reactivity of the flavin semiquinone...
toward oxygen must be taken into account with an intrinsic rate constant \( k' \), multiplied by the fraction of flavin semiquinone in that intermediate (\( f_{\text{FADH}} \)). Such calculations have been used as the basis for successful simulations of the red-ox reaction time course of completely reduced enzyme, two-electron reduced enzyme, and reduced alloxanthine-complexed enzyme (15).

In the presence of arsenite the distributions of reducing equivalents in partially reduced enzyme molecules are altered in a predictable manner due to the increased Mo(VI)/Mo(V) oxidation-reduction potential (10). In light of the anticipated effects of this perturbation of the oxidation-reduction equilibrium within the enzyme on the oxidative half-reaction, we have undertaken an investigation of the reaction of arsenite-complexed enzyme with oxygen. Our results indicate that the major effect of arsenite is not the perturbation of this equilibrium but rather the introduction of a new site in the enzyme capable of reacting with oxygen.

**MATERIALS AND METHODS**

Details of the experimental procedures used in these studies are given in the Miniprint Supplement (Refs. 22-33).

**RESULTS**

The Reaction of Arsenite-complexed Xanthine Oxidase with Oxygen—Previous studies (15-17) of the oxidative half-reaction of xanthine oxidase have established that Equation 1 accurately describes the kinetics and product stoichiometries observed with the native enzyme. The extinction coefficient changes and fractional levels of flavin reduction thus calculated for the native enzyme intermediates (10) yield the original low-temperature potentials for the several centers in the enzyme (17) as well as the more recently determined room temperature potentials (10, 34, 35) given in Table I. The major difference between the two sets of numbers lies in the levels of FADH\(_2\) and FADH\(^+\) for XO(2) and XO(1). In the present study, the latter set of potentials has been used throughout in the calculations. Significantly lower levels of FADH\(_2\) and higher levels of FADH\(^+\) are predicted for these two intermediates when the room temperature potentials are used. Also shown in Table I are the fractions of flavin present as FADH\(_2\) and FADH\(^+\) predicted for the intermediates of the re-oxidation of arsenite-complexed enzyme using the room temperature potentials for the arsenite-complexed enzyme. Compared with the native enzyme, arsenite-complexed enzyme has considerably lower levels of FADH\(_2\) and FADH\(^+\) in the XO(2) and XO(1) intermediates. If the reaction of reduced arsenite-complexed enzyme with oxygen occurs in an analogous fashion to the re-oxidation of native enzyme (Equation 1), then three phases are predicted for the re-oxidation of completely reduced arsenite-complexed enzyme (as depicted in Equation 2).

\[
\begin{align*}
\text{XO(6)} & \rightarrow \text{XO(4)} & \rightarrow \text{XO(2)} & \rightarrow \text{XO(1)} & \rightarrow \text{XO(0)} \\
\text{FADH}_2 \rightarrow \text{FADH}^+ & \rightarrow \text{FAD} & \rightarrow \text{FADH}_2 & \rightarrow \text{FADH}^+ & \rightarrow \text{FAD} \\
\end{align*}
\]

The first phase would be composed of two discrete steps of approximately the same rate, generating XO(2) from XO(6). Two-electron reduced enzyme in complex with arsenite would then be converted to one-electron reduced enzyme at a rate considerably less than that of the first two steps, and would constitute the second phase. The reaction of oxygen with one-electron reduced enzyme in complex with arsenite would then proceed in the third phase at a still slower rate because of the extremely low level of FADH\(^+\).

As shown in Fig. 1, when arsenite-complexed xanthine oxidase is completely reduced by dithionite (at approximately 3 mol of dithionite/mol of FAD) and then mixed with oxygenated buffer, a triphasic reaction is in fact observed. This can be seen more clearly in the semilog plots accompanying the observed absorbance changes at 370 nm (A-C), 450 nm (D-F), and 550 nm (G-I). The rates of the three phases comprising the re-oxidation of arsenite-complexed enzyme are notably more rapid than expected on the basis of the above discussion, however, and are in fact greater than the corresponding rates associated with oxidation of the native enzyme in the absence of arsenite. For example, at 62 \( \mu \)M oxygen and 550 nm rates of 110 s\(^{-1}\) (32% of \( \Delta A_{\text{max}} \)), 8 s\(^{-1}\) (50% of \( \Delta A_{\text{max}} \)), and 1.5 s\(^{-1}\) (18% of \( \Delta A_{\text{max}} \)) are observed with arsenite-complexed enzyme, compared with rates of 10 s\(^{-1}\) (65% of \( \Delta A_{\text{max}} \)) and 0.6 s\(^{-1}\) (37% of \( \Delta A_{\text{max}} \)) for the native enzyme under the same conditions. At all wavelengths (including 370 nm where the re-oxidation of the molybdenum-arsenate complex contributes appreciably to the observed spectral change; Ref. 10), all of the spectral change associated with re-oxidation was complete within 1 s, indicating complete re-oxidation of all centers, including the Mo-arsenate, during this interval. The rates of each of the three phases observed with arsenite-complexed enzyme appear to exhibit hyperbolic dependencies on oxygen concentration, as shown in the double reciprocal plots of Fig. 2. The observed rate constants and the relative spectral contributions of the three phases depend on the wavelength of observation. At 370 and 550 nm, three distinct phases are observable at all oxygen concentrations employed, and the relative contributions of these phases are essentially constant over this range of oxygen concentrations (Fig. 2). At 450 nm, however, the fastest phase is observable only at the lower oxygen concentrations (31 and 62 \( \mu \)M). At oxygen concentrations greater than 125 \( \mu \)M, the fastest phase cannot be readily resolved from the second phase at 450 nm (see top panel of Fig. 2A). As discussed below, this behavior...
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Table 1

| e/FAD | Native xanthine oxidase | Native xanthine oxidase | Xanthine oxidase-arsenite |
|-------|------------------------|------------------------|--------------------------|
|       | $f_{FADH_2}$ | $f_{FADH}$ | $\Delta f_{FAD}$ | $f_{FADH_2}$ | $f_{FADH}$ | $\Delta f_{FAD}$ | $f_{FADH_2}$ | $f_{FADH}$ | $\Delta f_{FAD}$ |
| 6     | 1.0        | 0          | 26.6            | 1.0        | 0          | 26.6            | 1.0        | 0          | 26.6            |
| 4     | 0.97       | 0.014      | 23.3            | 1.0        | 0.003      | 25.9            | 0.95       | 0.04       | 17.5            |
| 2     | 0.58       | 0.087      | 11.2            | 0.23       | 0.24       | 13.6            | 0.02       | 0.04       | 5.5             |
| 1     | 0          | 0.062      | 4.6             | 0          | 0.02       | 4.7             | 0          | 0.01       | 2.4             |

* For native xanthine oxidase, the relative redox potentials from Ref. 18 were used (obtained using a procedure that combined room temperature measurements with epr data collected at liquid nitrogen and helium temperatures).

* For native xanthine oxidase and xanthine oxidase-arsenite the room temperature midpoint potentials (10, 34, 35) were used in the calculations.

The hyperbolic dependences of the three phases suggest that each phase involves formation of a reduced enzyme-oxygen complex that proceeds to give a less reduced enzyme molecule and reduced forms of oxygen ($O_2$ or $H_2O_2$). The observed rate of each phase should therefore be given by the following equation:

$$k_{obs} = \frac{k[O_2]}{(K_d + [O_2])}$$

The fastest (first) phase has a limiting rate of approximately 150 s⁻¹ and a $K_d$ of about 50 μM. This phase accounts for 30–40% of the total spectral change at 370 nm and for 35–45% of the total spectral change at 550 nm. At 450 nm, 20% of the total spectral change occurs in the first phase at oxygen concentrations lower than 62 μM. At higher oxygen concentrations, however, the first phase cannot be resolved from the second phase, and the entire time course of re-oxidation at 450 nm is adequately described as the sum of two exponentials. The intermediate (second) phase has a limiting rate of approximately 50 s⁻¹ and a $K_d$ of 250 μM. This phase accounts...
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Effect of Superoxide Dismutase on the Re-oxidation Reaction—The time course for re-oxidation of native xanthine oxidase (no arsenite) is not noticeably altered by the presence of superoxide dismutase (17), indicating that the superoxide produced on oxidizing two-electron reduced enzyme is not readily utilized as an oxidant for one-electron reduced enzyme. With arsenite-complexed enzyme, however, the later stages of the oxidation reaction are markedly slower in the presence of superoxide dismutase, as shown in Fig. 3. Dismutase affects only the rate of the slowest phase: the relative contributions of the three phases to the overall absorbance changes are not altered significantly by the dismutase, nor are the rates of the first and second phases. It thus appears that arsenite-complexed enzyme can utilize superoxide as an electron acceptor.

Kinetics and Stoichiometry of Superoxide and Peroxide Production—We have monitored production of superoxide by using ferric cytochrome c as a trapping reagent, as described previously for the native enzyme (14-16). An experimentally determined operational extinction coefficient difference of \( \Delta \varepsilon_{552-565} = 20 \text{ mM}^{-1} \text{ cm}^{-1} \) was used as described in Ref. 15. The results of these experiments are summarized in Table II. As shown in Fig. 4, with arsenite-complexed enzyme approx-
imately 0.4 O$_2$/FAD were produced in a biphasic manner; considerably less than the 1.6 O$_2$/FAD observed with the native enzyme under the same conditions (16).

The reaction between cytochrome c peroxidase and H$_2$O$_2$ quantitatively produces a stable Compound I (CCP-I) (36, 37). Formation of this complex results in a characteristic spectral change and is rapid ($k = 3-4 \times 10^7$ M$^{-1}$ s$^{-1}$, Ref. 33), and this reaction has been used successfully to monitor H$_2$O$_2$ production during the re-oxidation of completely-reduced, native xanthine oxidase (16). Using similar conditions, we have compared peroxide production in the oxidative half-reaction of native and arsenite-complexed xanthine oxidase (Fig. 4). The results of these experiments are also summarized in Table II. Our experimental results with the native enzyme (Fig. 4) are in qualitative agreement with previous results (16). A total of approximately 3 H$_2$O$_2$/FAD is detected: 2.4 H$_2$O$_2$/FAD at a rate of 15 s$^{-1}$ and 0.6 H$_2$O$_2$/FAD at 0.9 s$^{-1}$. With arsenite-complexed enzyme (Fig. 4), approximately 1.8 H$_2$O$_2$/FAD are produced at a rate of 24 s$^{-1}$, 0.8 H$_2$O$_2$/FAD at a rate of 2 s$^{-1}$, and 0.2 H$_2$O$_2$/FAD at a rate of 0.2 s$^{-1}$. This indicates that less than 10% of the reducing equivalents initially present in the arsenite-complexed enzyme produce superoxide. It is therefore not surprising that the process giving rise to superoxide is difficult to observe in following the re-oxidation of enzyme. It should be noted that the superoxide dismutase and cytochrome c which scavenge the superoxide in these experiments also slow the third and slowest phase of the enzyme re-oxidation, as demonstrated in the previous section. The slow process (0.3 s$^{-1}$) producing O$_2$ may well be the result of superoxide itself reacting with enzyme in a prior step and may thus not be a part of the re-oxidation scheme for arsenite-complexed enzyme in the presence of dismutase or cytochrome c.

While the kinetics and stoichiometry of peroxide production which we observe for the native enzyme in the absence of arsenite agree with the results expected for the accepted pathway for the re-oxidation of xanthine oxidase (Equation 1), they differ somewhat from previously reported results (16). In this earlier work no slow phase for peroxide production was observed, for example. It was suggested that a slow reaction of CCP-I by partially reduced enzyme was responsible for eliminating the slow phase of H$_2$O$_2$ production, and such a process was demonstrated to take place with a $t_0$ of approximately 10 s (16). In our hands, a roughly similar $t_0$ of 30-40 s is observed.

There are several differences in our experimental conditions compared with those of the earlier work. First, we routinely used approximately half the xanthine oxidase and peroxidase concentrations, which would have the effect of slowing the second order reaction between CCP-I and partially re-oxidized xanthine oxidase. Second, most of our peroxide-monitoring experiments utilized the large spectral change at 563 nm associated with the formation of CCP-I. While the spectral change is larger at 424 nm, where the reaction was observed in the earlier work, the background absorbance was so great (at least in the instrument used in the present work) that poor signal to noise was observed. It is thus possible that the slow reaction was made more difficult to observe in the previous work for this reason as well. Finally, the xanthine oxidase used in the present studies was considerably more active than that in the previous study, having only trace amounts of the inactive desulfo form of the enzyme. Any complications arising from the inability of the desulfo molybdenum center to participate in the rapid equilibrium during the course of the oxidative half-reaction (17, 38) are thus avoided in the present work. Also, as the desulfo molybdenum center has an appreciably lower redox potential than the functional center, it is possible that this results in some indirect effect on the rate of peroxide production in the last stages of the enzyme re-oxidation when inactive enzyme is present.

![Fig. 3. Time course of absorbance change at 370 nm during the reaction of completely reduced arsenite-complexed enzyme with oxygen in the presence and absence of superoxide dismutase. 7.5 µM arsenite-complexed xanthine oxidase was completely reduced with sodium dithionite than mixed in the stopped flow instrument with 125 µM oxygen in the absence or presence of 25 µg/ml of superoxide dismutase. The absorbance changes in the presence of dismutase are shown on two different time scales in panel A: ---, 0.4 s full scale; ----, 16 s full scale. As shown in Fig. 2, A-D, the reaction in the absence of dismutase is triphasic. In the presence of dismutase, however, there are four phases observed at 370 nm. Panel B shows semilog plots of the data of panel A indicating that the slowest phase (k$_3$ days) in the presence of dismutase (---) is significantly slower than the slowest phase (k$_3$) in the absence of dismutase (----). Panel C shows a semilog plot of the remaining spectral change when the contribution of the slowest phase in the presence of dismutase is subtracted. The rate of the slowest phase obtained from this plot (k$_3$ days) is approximately the same as k$_3$, but this phase contributes significantly less of the total absorbance change in the presence of dismutase than its absence. When only the contributions of the first three phases are considered (panel D), the semilog plots show no difference between the traces in the presence (---) and absence (----) of dismutase.](image)
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FIG. 4. Kinetics of superoxide and hydrogen peroxide production by reduced arsenite-complexed and native xanthine oxidase. Panel A shows the absorbance change at 550 nm due solely to superoxide-mediated reduction of cytochrome c (see Ref. 15) when 8.8 μM reduced arsenite-complexed xanthine oxidase is mixed with air-equilibrated buffer containing 120 μM ferric cytochrome c. A stoichiometry of 0.4 O₂/FAD was determined as described in the text. The biphasic semilog plot (inset) of the ΔA₅₅₀ data (— — —) indicates that 0.2 O₂/FAD are produced at a rate of approximately 0.4 s⁻¹ and 0.2 O₂/FAD are produced at a rate of approximately 18 s⁻¹ (— —). Panel B shows the time course for hydrogen peroxide production during the reaction of 2.1 μM reduced native (no arsenite) xanthine oxidase with 125 μM oxygen. The trace (shown on two different time scales) represents the difference between traces taken at 563 nm in the presence and absence of 25 μM cytochrome c peroxidase (concentrations after mixing). An extinction coefficient change of 8 m⁻¹ cm⁻¹ at 563 nm was used for the CCP → CCP-I conversion in calculating the H₂O₂ stoichiometry (36, 37). The total ΔΔ₅₆₃ for this reaction indicates a stoichiometry of 3.0 H₂O₂/FAD. Analysis of the semilog plot of these data is shown in the inset and indicates two phases. The slower phase (0.9 s⁻¹) accounts for 20% of the total observed spectral change and therefore accounts for 0.6 H₂O₂/FAD. When the contribution of this slower phase is subtracted from the trace, a semilog plot of the remaining absorbance change (— — —; in the inset) indicates that the other 2.4 H₂O₂/FAD are produced at a rate of 16 s⁻¹. In Panel C is shown the time course (obtained as discussed above) for H₂O₂ production during the reaction of 2.1 μM reduced arsenite-complexed xanthine oxidase with 125 μM oxygen. The total ΔΔ₅₆₃ indicates that 2.8 H₂O₂/FAD are produced. A semilog plot of the data of Panel C is shown in Panel D (— — —) and indicates three phases. The contribution of the third (slowest) phase is quite small (8% of ΔΔ₅₆₃, i.e. 0.2 H₂O₂/FAD). When this small contribution is subtracted from the data, a biphasic semilog plot (— — —) of the remaining spectral change is obtained. This plot indicates that 0.8 H₂O₂/FAD are produced at a rate of 2 s⁻¹. When the contribution of this phase is subtracted, the semilog plot of the remaining absorbance change (— — —, Panel D) indicates that the remaining 1.8 H₂O₂ are produced in a single phase (kₘ₉ = 24 s⁻¹). This phase exhibits a gradually accelerating time course (or a noticeable lag) which may be due, in part, to the fact that two sequential steps of approximately the same rate comprise this phase (see Refs. 17 and 18).

H₂O₂/FAD. There is no peroxide produced at a rate comparable to the rate of the fastest phase (120 s⁻¹ at 125 μM oxygen) observed when monitoring the enzyme absorbance change. This observation is discussed further below.

Effect of Arsenite on Re-oxidation of Reduced Enzyme Containing Alkylated FAD—When completely reduced enzyme is
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TABLE II
Stoichiometry and kinetics of superoxide and hydrogen peroxide production during the reaction of oxygen with various forms of reduced xanthine oxidase

Reactions were carried out as described in the text with 125 μM oxygen in 0.1 M BICINE at pH 8.3 and 25 °C.

| Enzyme form | O₂ | H₂O₂ |
|-------------|----|------|
| | Total stoichiometry | Rate of phase | Total stoichiometry | Rate of phase |
| | 0.8/FAD | 28 s⁻¹ | 0.6/FAD | 0.9 s⁻¹ |
| Native xanthine oxidase | 1.8/FAD | 0.9 s⁻¹ | 2.4/FAD | 15 s⁻¹ |
| | (0.2/FAD) | (0.2 s⁻¹) | (0.2/FAD) | (0.2 s⁻¹) |
| Xanthine oxidase-arsenite | 0.2/FAD | 0.3 s⁻¹ | 0.2/FAD | 0.3 s⁻¹ |
| Desulfo xanthine oxidase + arsenite | 1.0/FAD | 1 s⁻¹ | ND* | ND |
| Xanthine oxidase-arsenite + salicylate | 0.3/FAD | 0.7 s⁻¹ | ND | ND |

*ND, not determined.

FIG. 5. Time course of the reaction of completely reduced, iodoacetamide-alkylated xanthine oxidase with 125 μM oxygen in the absence (A and B) and presence (C-E) of 2 mM arsenite. Panel A shows (on two different time scales) the absorbance change observed at 470 nm when 6.2 μM dithionite-reduced, alkylated enzyme (no arsenite) is mixed with 250 μM oxygen in the stopped flow spectrophotometer. There are three phases to this reaction. The rate of the third phase (0.0018 s⁻¹) can be obtained from the semilog plot of the data (——) shown in panel B. When the contribution of this phase is subtracted, the remaining spectral change has a biphasic semilog plot (—–) from which the rate of the second phase (0.016 s⁻¹) is obtained. When the contributions of the two slower phases are subtracted, the remaining spectral change can be accounted for by a single exponential phase of approximately 1 s⁻¹ (—). Panel C shows the absorbance change (on three different time scales) observed at 470 nm when 8 μM completely reduced, alkylated enzyme in complex with arsenite is mixed with 250 μM oxygen. This reaction is also triphasic. The semilog plot of the data shown in panel D (——) indicates a rate 0.0028 s⁻¹ for the slowest phase. When the contribution of this phase is subtracted, a biphasic semilog plot (—–) is obtained, and this indicates a rate of 7.8 s⁻¹ for the middle phase. The absorbance change due to the fastest phase is obtained by subtracting the contributions of the second and third phases. A semilog plot of the remaining absorbance change (panel E) indicates a rate of 110 s⁻¹.

Treated with iodoacetamide, the FAD is alkylated at the C(4a) position, rendering the reduced flavin redox-inactive and incapable of reducing oxygen (27): none of the other redox centers are affected by this treatment. Thus, although alkylated enzyme has less than 1% of the original xanthine:O₂ reductase activity, it retains its original xanthine:2,6-dichlo-
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When alkylated enzyme is reduced with approximately 2 eq of dithionite/active site and then is mixed with oxygenated buffer, the re-oxidation reaction proceeds very slowly and with multiple phases (Fig. 5, A and B). Neither the rates themselves nor their dependence on the oxygen concentration correspond to those observed for the oxidative half-reaction of native enzyme containing functional FAD. Because the rates observed for alkylated enzyme are so slow, the flavin-independent re-oxidation pathway appears to play no role in oxidation of reduced native enzyme. However, when arsenite-complexed alkylated xanthine oxidase is completely reduced and then reacted with oxygen, most of the absorbance change occurs relatively rapidly (Fig. 5, C–E). For example, in the presence of 2 mM arsenite and 125 μM oxygen, over 50% of the total absorbance change at 470 nm occurs during the first 80 ms, and almost 80% of the total absorbance change at 470 nm is complete within 0.8 s of mixing. In contrast with these relatively rapid rates are the results for alkylated enzyme in the absence of arsenite: at the same oxygen concentration, less than 1% of the total absorbance change at 470 nm occurs during the first 80 ms, and almost 40 min are required for the reaction to go to completion. The dependences of the rates of the three, well-resolved phases on oxygen concentration for alkylated, arsenite-complexed enzyme are given in Fig. 6. The rates of the first two phases are sufficiently fast that the flavin-independent re-oxidation pathway could well play a major role in the re-oxidation reaction of arsenite-complexed enzyme, even when the enzyme contains functional FAD. For example, the double reciprocal plot of Fig. 6A indicates a limiting rate of 180 s\(^{-1}\) and a \(K_v\) of 70 μM for the fastest phase of the reaction of reduced iodoacetamide-alkylated, arsenite-complexed enzyme with oxygen. These values are comparable to those observed for the fastest phase of the oxidation of arsenite-complexed enzyme containing functional FAD (Fig. 2). The rate of the second phase for the alkylated arsenite-complexed enzyme (7–8 s\(^{-1}\)) is also sufficiently rapid that it could play a role in the re-oxidation reaction even when functional FAD is present.

The insensitivity of the rate of the second phase in the reaction of alkylated arsenite-complexed xanthine oxidase with oxygen to the oxygen concentration suggests that it is due to utilization of \(O_2\) generated in the very rapid \(O_2\)-dependent first phase rather than \(O_2\) as an electron acceptor. This possibility is supported by the ability of superoxide dismutase to alter the time course of the re-oxidation occurring during the second phase of the re-oxidation of alkylated arsenite-complexed enzyme (Fig. 7A). In the absence of dismutase, the second phase contributes approximately one-third of the total observable spectral change at 470 nm. As shown in the semilog plots of Fig. 7, B–D, in the presence of dismutase the amplitude of the second phase is cut almost by half, although its rate is approximately the same. An additional slow phase (\(k_{obs} = 0.3\) s\(^{-1}\)) which is not present in the absence of dismutase accounts for the diminished amplitude of the second phase. Neither the rates nor the amplitudes of the first phase and the final phase are altered by dismutase.

**Reaction of Reduced Arsenite-complexed Deflavo Xanthine Oxidase with Oxygen**—We have also examined the effect of arsenite on the re-oxidation kinetics of another form of xanthine oxidase lacking functional FAD, the deflavo enzyme. In the absence of arsenite, the re-oxidation of dithionite-reduced deflavo enzyme is extremely slow, exhibiting a rate constant of about 0.02 s\(^{-1}\) at 125 μM oxygen in pyrophosphate buffer at pH 8.5 and 25 °C (13). As shown in Fig. 8, arsenite dramatically enhances the rate of re-oxidation of the reduced deflavo enzyme. The presence of a small amount of holoenzyme (5–10%) in the deflavo enzyme sample complicates the detailed analysis, but it is clear that the very rapid first phase observed with the alkylated arsenite-complexed enzyme (\(k_{obs} = 110–130\) s\(^{-1}\) at 125 μM oxygen) is also observed with the arsenite-complexed deflavo enzyme (\(k_{obs} = 110–120\) s\(^{-1}\) at 125 μM oxygen, see inset to Fig. 8). A double reciprocal plot (\(k_{obs}^{-1}\) versus [O\(_2\)]\(^{-1}\)) of the data for this phase with the arsenite-
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FIG. 7. Effect of superoxide dismutase on the re-oxidation reaction of reduced alkylated enzyme in complex with arsenite. 12.8 μM completely reduced alkylated enzyme in complex with arsenite was mixed with 1.25 mM oxygen in the presence and absence of 40 μg/ml of superoxide dismutase. The time course of the oxidation reaction at 470 nm is shown on two time scales in panel A. For the traces where full scale is 400 s ——, represents the data in the absence of dismutase while —— represents the data in the presence of dismutase. For the traces with 0.4 s full scale, --- indicates the data in the absence of dismutase while —— indicates the data in the presence of dismutase. The reaction in the presence of dismutase exhibits four distinct phases, while in the absence of dismutase three phases are observed (Fig. 8). Panel B shows the semilog plots of the data in the presence (—) and absence (-----) of dismutase. The approximate rate and spectral contribution of the slowest phase is not significantly altered by dismutase. When the contribution of the slowest phase is subtracted, a semilog plot of the remaining spectral change is triphasic in the presence of dismutase. The rate of the slowest phase of this reaction, obtained from the semilog plot of panel C, had no counterpart in the reaction observed in the absence of dismutase. The rates of the first two phases in the presence of dismutase agree reasonably well with the rates of the first two phases in the absence of dismutase (panel D), however, the contribution of the second phase to the total spectral change is significantly less in the presence of dismutase. Panel E shows the reaction observed when 8 μM alkylated enzyme in complex with arsenite is reduced to a level of two electrons/FAD, then mixed with 1.25 mM oxygen. The time course of the reaction, shown on two time scales in the figure, is not altered by the presence of superoxide dismutase.

complexed deflavo enzyme (not shown) indicates a limiting rate of 170 s⁻¹ and a Kₐ of 70 μM, in agreement with the values determined for alkylated arsenite-complexed enzyme (Fig. 6) and with the values for arsenite-complexed enzyme containing functional FAD (Fig. 24). As was observed with the alkylated enzyme in complex with arsenite, the deflavo arsenite-complexed enzyme must be completely reduced for the enhancement of oxygen reactivity to be observed. Further results are given in the Miniprint Supplement.

DISCUSSION

From a comparison of the rates of re-oxidation for alkylated or deflavo xanthine oxidase in the presence and absence of arsenite (Figs. 5 and 8, respectively), it is clear that the inorganic reagent enhances the oxygen reactivity of the enzyme. Arsenite does not appear to alter the properties of either Fe/S center (4–6), but there is abundant evidence that arsenite interacts directly with the molybdenum center of xanthine oxidase (2–10). Therefore, it is not unreasonable to propose that arsenite changes the oxidative half-reaction by making the molybdenum center capable of reducing oxygen and superoxide. Although there have not been many reports of reactions between reduced forms of molybdenum and oxygen, Wilshire et al. (40) have reported a mononuclear Mo(V) catechol compound capable of reducing O₂ to O₂⁻. In addition, the molybdenum center of sulfite oxidase appears to be capable of reducing oxygen (41) in a steady-state system, although the oxidative half-reaction with O₂ has not yet been well-characterized. The possibility that arsenite affects the oxidative half-reaction by altering the oxygen-reactivity of Fe/S I cannot be ruled out completely on the basis of work reported in this paper, although we regard this as unlikely in view of the ability of xanthine, salicylate, or nitrate (reagents all known to interact with xanthine oxidase at the molybdenum center) to block the rapid reaction of oxygen with the
These simulations used the rapid equilibrium predictions for the rates of oxygen reacting with XO(2)-arsenite via the FADH\(^+\) and gave the best fit to both time courses when a second order rate constant of approximately \(10^3\) M\(^{-1}\) s\(^{-1}\) was assigned to the reaction of superoxide with XO(1)-arsenite.

At 125 \(\mu\)M oxygen the first phase of the re-oxidation of arsenite-complexed xanthine oxidase proceeds at a rate of approximately 110 s\(^{-1}\), and the above discussion suggests that the reduced Mo-arsenite site participates in this reaction. There is, however, no superoxide or peroxide released from the enzyme at a rate corresponding to this phase. This process could involve a single electron transfer step generating bound superoxide that is subsequently reduced to H\(_2\)O\(_2\) in a slower reaction. It will be remembered, however, that very little spectral change is predicted for the XO(6)-arsenite to XO(5)-arsenite conversion because this primarily represents oxidation of the lowest potential site, Mo(IV)-arsenite (10). To explain the observed spectral change one would have to propose that bound superoxide raises the Mo(V)-arsenite/Mo(IV)-arsenite potential sufficiently to make electron transfer from the site of next lowest potential (Fe/S) thermodynamically favorable. Alternatively, the first phase could represent conversion of XO(6)-arsenite to XO(4)-arsenite producing H\(_2\)O\(_2\) which is then released into solution at a slower rate (comparable to that for the second phase of the reaction). Results with the native enzyme (no arsenite) presented in this paper and in previous publications (15, 16) have demonstrated that the rates of O\(_2\) and H\(_2\)O\(_2\) release from the flavin center correspond well with the rates determined by monitoring enzyme absorbance changes. However, the first phase of the oxidative half-reaction with arsenite-complexed xanthine oxidase appears to involve reduction of oxygen at the Mo-arsenite site, so there is no requirement that the rate constant for product release correlate with the rate constant describing enzyme absorbance change. The spectral change associated with the first phase in the re-oxidation of reduced arsenite-complexed enzyme (see Miniprint Supplement) is in fact quite close to that expected for the two-electron alternative based on rapid equilibrium considerations (see Miniprint Supplement).

The rate constant for the rapid phase of peroxide generation (24 s\(^{-1}\)), on the other hand, matches well the rate constant of the second phase of enzyme-arsenite re-oxidation (20 s\(^{-1}\)). Approximately 2 H\(_2\)O\(_2\)/FAD are produced in this phase, and the slightly accelerating time course shown in the semilog plot (Fig. 4, inset) is to be expected for two sequential reactions taking place at similar rates (approximately 30 s\(^{-1}\) on the basis of computer simulations), each generating a single peroxide molecule/flavin.

A qualitative model describing the re-oxidation of arsenite-complexed xanthine oxidase is presented in Equations 4 through 6.

\[
\begin{align*}
\text{XO(6)} + \text{O}_2 &\rightarrow \text{XO(6)} - \text{O}_2 \rightarrow \text{XO(4 or 5)} - (\text{H}_2\text{O}_2 \text{ or O}_2^-) & \quad (4) \\
\text{XO(4 or 5)} - (\text{H}_2\text{O}_2 \text{ or O}_2^-) &\rightarrow \text{XO(4)} & \quad (5)
\end{align*}
\]
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\[ \text{H}_2\text{O}_2 \xrightarrow{\text{O}_2} \text{XO(1):O}_2^+ \xrightarrow{\text{XO}(2):\text{O}_2} \text{XO(0)} \]

Equation 4 represents the first phase of this reaction; it exhibits a hyperbolic oxygen dependence and does not result in product release. This phase appears to involve \( \text{O}_2 \) reacting with the Mo(IV)-arsenite center. Equation 5 summarizes the reactions of the second phase which generate \( \text{XO}(2):\text{arsenite} \) and produce \( 2\text{H}_2\text{O}_2/\text{FAD} \). Re-oxidation may occur via both the FAD and the Mo-arsenite site in this phase. The two steps indicated for this phase are not necessarily sequential as indicated, since both the FADH\(_2\) and the flavin-independent re-oxidation site are presumably capable of functioning simultaneously. Equation 6 represents the third phase of the oxidative half-reaction, re-oxidation of two-electron reduced enzyme. In this phase, oxygen reacts with \( \text{XO}(2):\text{arsenite} \) (probably via the FADH\(_2\)) to generate superoxide. This superoxide is subsequently utilized relatively rapidly by \( \text{XO}(1):\text{arsenite} \) to generate completely oxidized arsenite-complexed enzyme. Some of the superoxide generated in the third phase is accessible to scavengers such as superoxide dismutase and cytochrome \( \text{c} \). Such a scheme would adequately account for the very low yield of superoxide anion in the reaction of arsenite-complexed enzyme with oxygen.

REFERENCES

1. Johnson, J. L., and Rajagopalan, K. V. (1978) *Bioinorg. Chem.* 8, 439–444
2. Hille, R., Stewart, R. C., and Massey, V. (1982) *Fed. Proc.* 41, 869.
3. Hille, R., Stewart, R. C., Fee, J. A., and Massey, V. (1982) in *Proceedings of the Fourth International Conference on the Chemistry and Uses of Molybdenum* (Barry H. F., and Mitchell, P. C. H., eds) pp. 169–172, Climax Molybdenum Co., Ann Arbor
4. Barber, M. J., and Siegel, L. M. (1983) *Biochemistry* 22, 618–624
5. George, G. N., and Bray, R. C. (1983) *Biochemistry* 22, 1013–1021
6. Hille, R., Stewart, R. C., Fee, J. A., and Massey, V. (1983) *J. Biol. Chem.* 258, 4049–4055
7. Mackler, B., Mahler, B., and Green, D. E. (1954) *J. Biol. Chem.* 210, 149–164
8. Peters, J. M., and Sanadi, D. R. (1961) *Arch. Biochem. Biophys.* 93, 312–313
9. Coughlan, M. P., Rajagopalan, K. V., and Handler, P. (1969) *J. Biol. Chem.* 244, 2659–2663
10. Stewart, R. C., Hille, R., and Massey, V. (1984) *J. Biol. Chem.* 259, 14426–14436
11. Massey, V. (1973) in *Iron-Sulfur Proteins* (Ehrenberg, A., ed) pp. 301–360, Academic Press, New York
12. Bray, R. C. (1975) in *The Enzymes* (Boyer, P. D., ed) Vol 12, pp. 299–419, Academic Press, New York
13. Komai, H., Massey, V., and Palmer, G. (1969) *J. Biol. Chem.* 244, 1692–1700
14. McCord, J. M., and Fridovich, I. (1968) *J. Biol. Chem.* 243, 5753–5760
15. Hille, R., and Massey, V. (1981) *J. Biol. Chem.* 256, 9090–9095
16. Fornas, A. G., Olson, J. S., and Palmer, G. (1981) *J. Biol. Chem.* 256, 9096–9103
17. Olson, J. S., Ballou, D. P., Palmer, G., and Massey, V. (1974) *J. Biol. Chem.* 249, 4350–4362
18. Olson, J. S., Ballou, D. P., Palmer, G., and Massey, V. (1974) *J. Biol. Chem.* 249, 4363–4362
19. Bhattacharyya, A., Tollin, G. D., Davis, M. D., and Edmondson, D. E. *Biochemistry* 22, 8933–8940
20. Edmondson, D., Ballou, D., Van Heuvelen, A., Palmer, G., and Massey, V. (1973) *J. Biol. Chem.* 248, 6135–6144
21. Hille, R., Fee, J. A., and Massey, V. (1981) *J. Biol. Chem.* 256, 8933–8940
22. Massey, V., Brumby, P. E., Komai, H., and Palmer, G. (1969) *J. Biol. Chem.* 244, 1682–1691
23. Morrison, M., and Hultquist, D. E. (1963) *J. Biol. Chem.* 238, 2847–2848
24. Massey, V., and Edmondson, D. (1970) *J. Biol. Chem.* 245, 2837–2844
25. Massey, V., Komai, H., Palmer, G., and Elion, G. B. (1970) *J. Biol. Chem.* 245, 2837–2844
26. Nishino, T., Nishino, T., and Tsushima, K. (1981) *FEBS Lett.* 131, 369–372
27. Komai, H., and Massey, V. (1971) in *Flavins and Flavoproteins* (Kainin, H., ed) pp. 399–415, University Park Press, Baltimore, MD
28. Beaty, N. B., and Ballou, D. P. (1981) *J. Biol. Chem.* 256, 4611–4618
29. Van Mastrigt, R. (1977) *Comput. Biol. Med.* 7, 231–247
30. Burleigh, B. D., Feast, G. P., and Williams, C. H., Jr. (1969) *Anal. Biochem.* 84, 75–81
31. Beinert, H., Orme-Johnson, W. H., and Palmer, G. (1978) *Methods Enzymol.* 54, 111–132
32. Nelson, C. E., Sitzman, E. V., Kong, C. H., and Margoliash, E. (1977) *Annu. Rev. Biochem.* 46, 622–631
33. Loo, S., and Ernson, J. E. (1975) *Biochemistry* 14, 3467–3470
34. Forras, A. G., and Palmer, G. (1983) *J. Biol. Chem.* 258, 11617–11626
35. Spence, J. T., Barber, M. J., and Siegel, L. M. (1982) *Biochemistry* 21, 1556–1561
36. Yonetani, T. (1965) *J. Biol. Chem.* 240, 4509–4514
37. Yonetani, T., and Ray, G. S. (1965) *J. Biol. Chem.* 240, 4503–4508
38. Bray, R. C., Barber, M. J., Lowe, D. J., Fox, R., and Cammack, R. (1975) *FEBS Lett.* 56, 159–172
39. Cammack, R., Barber, M. J., and Bray, R. C. (1976) *Biochem. J.* 157, 469–478
40. Wilshire, J. P., Leon, L., Bosserman, P., and Sawyer, D. T. (1980) in *Molybdenum Chemistry of Biological Significance* (Newton, W. E., and Otsuka, S., eds) pp. 277–344, Plenum Press, New York
41. Cohen, H. J., and Fridovich, I. (1971) *J. Biol. Chem.* 246, 359–366
Oxidative Half-reaction of Arsenite-complexed Xanthine Oxidase

Richard C. Nester, Russ Hills, and Vincent Nessey

Materials and Methods

Xanthine oxidase was purified using the procedure of Nessey et al. (23) for fresh, unpurified whole milk obtained from the dairy herd at Michigan State University. Occasionally, a small amount of contaminating lipase was present in the final stages of the purification and could be removed by passing the enzyme over a small column (column of Sephadex G-25 in 0.1 M sodium acetate buffer, pH 6.2) in the presence of 1 mM arsenite (23). The enzyme obtained by these procedures had an A260/A280 ratio of less than 0.9, and was approximately 70% active as indicated by an A280 value of 160. Standard assay conditions were: 11 mM sodium in air-saturated 0.1 M phosphate buffer containing 0.1 mM EDTA at pH 6.2 and 25°C. Complete oxidation of the enzyme was achieved using 0.1 M arsenite in 11 mM sodium phosphate buffer containing 0.1 mM EDTA at pH 6.2 and 25°C. Complete oxidation was considered to be achieved when the enzyme activity decreased to zero. The enzyme was then assayed using 0.1 M arsenite in 11 mM sodium phosphate buffer containing 0.1 mM EDTA at pH 6.2 and 25°C.

The arsenite-complexed enzyme was prepared by adding 0.1 M arsenite to the assay mixture. The enzyme was then assayed using 0.1 M arsenite in 11 mM sodium phosphate buffer containing 0.1 mM EDTA at pH 6.2 and 25°C.

Results

Figures 1 and 2 show the spectral changes observed during the oxidation and reduction of the enzyme, respectively. The spectral changes observed during the oxidation of the enzyme are shown in Figure 1. The enzyme was oxidized using 0.1 M arsenite in 11 mM sodium phosphate buffer containing 0.1 mM EDTA at pH 6.2 and 25°C. The enzyme was then assayed using 0.1 M arsenite in 11 mM sodium phosphate buffer containing 0.1 mM EDTA at pH 6.2 and 25°C.

Discussion

The results of this study indicate that the spectral changes observed during the oxidation and reduction of the enzyme are due to the presence of a single, redox-active chromophore. The spectral changes observed during the oxidation of the enzyme are consistent with the presence of a single, redox-active chromophore. The spectral changes observed during the reduction of the enzyme are consistent with the presence of a single, redox-active chromophore.

Conclusion

In conclusion, the results of this study suggest that the spectral changes observed during the oxidation and reduction of the enzyme are due to the presence of a single, redox-active chromophore. The spectral changes observed during the oxidation of the enzyme are consistent with the presence of a single, redox-active chromophore. The spectral changes observed during the reduction of the enzyme are consistent with the presence of a single, redox-active chromophore.

References

1. Nessey et al. (23) The J. Biol. Chem. 250, 4000-4006 (1975).
2. Nessey et al. (24) The J. Biol. Chem. 250, 4007-4012 (1975).
3. Nessey et al. (25) The J. Biol. Chem. 250, 4013-4018 (1975).
4. Nessey et al. (26) The J. Biol. Chem. 250, 4019-4024 (1975).
5. Nessey et al. (27) The J. Biol. Chem. 250, 4025-4030 (1975).

Figure 1: Spectral changes observed during the oxidation of the enzyme. The enzyme was oxidized using 0.1 M arsenite in 11 mM sodium phosphate buffer containing 0.1 mM EDTA at pH 6.2 and 25°C. The enzyme was then assayed using 0.1 M arsenite in 11 mM sodium phosphate buffer containing 0.1 mM EDTA at pH 6.2 and 25°C.

Figure 2: Spectral changes observed during the reduction of the enzyme. The enzyme was reduced using 0.1 M arsenite in 11 mM sodium phosphate buffer containing 0.1 mM EDTA at pH 6.2 and 25°C. The enzyme was then assayed using 0.1 M arsenite in 11 mM sodium phosphate buffer containing 0.1 mM EDTA at pH 6.2 and 25°C.
Oxidative Half-reaction of Arsenite-complexed Xanthine Oxidase

The Effect of Xanthine, Dihydroxyacetone, and Nitrite on the Reduction of Arsenite-complexed Xanthine Oxidase. Xanthine (12), dihydroxyacetone (12), and nitrite (46) are known to interact with the guanidino groups of xanthine oxidase. In order to confirm that the guanidino group of the arsenite-complexed enzyme was the site of the flavin-independent reduction, the reaction of reduced arsenite-complexed enzyme with oxygen was performed in the presence of each of these compounds. The timecourses for these reactions as monitored at 370 nm are shown in Figure 3, along with that for the reduction reaction in their absence. It may be seen that all three compounds decrease the rate of reaction of the arsenite-complexed enzyme with oxygen. At 125 mM oxygen, the observed rate constants for the reduction of arsenite-complexed alkylation xanthine oxidase in the presence of nitrite (250 mM), xanthine (370 mM), or salicylate (250 mM) are 1 x 10^-3, 4 x 10^-4, and 2 x 10^-4, respectively, compared with 22 x 10^-4 in the absence of these compounds. It is thus clear that molecules known to interact with xanthine oxidase at the guanidino group bring about a three- to tenfold decrease in the rate for the flavin-independent reduction of arsenite-complexed enzyme.