The kinetics of xanthine oxidase has been investigated with the aim of addressing several outstanding questions concerning the reaction mechanism of the enzyme. Steady-state and rapid kinetic studies with the substrate 2,5-dihydroxybenzaldehyde demonstrated that \( k_{cat}/K_m^{red} \) and \( k_{red}/K_d \) exhibit comparable bell-shaped pH dependence with pKa values of 6.4 ± 0.2 and 8.4 ± 0.2, with the lower pKa assigned to an active-site residue of xanthine oxidase (possibly Glu-1261, by analogy to Glu-869 in the crystallographically known aldehyde oxidase from Desulfovibrio gigas) and the higher pKa to substrate. Early steps in the catalytic sequence have been investigated by following the reaction of the oxidized enzyme with a second aldehyde substrate, 2-aminopteridine-6-aldehyde. The absence of a well-defined acid limb in this pH profile and other data indicate that this complex represents an \( E_{ox}\) rather than \( E_{red}\) complex (i.e., no chemistry requiring the active-site base has taken place in forming the long wavelength-absorbing complex seen with this substrate). It appears that xanthine oxidase (and by inference, the closely related aldehyde oxidases) hydroxylates both aromatic heterocycles and aldehydes by a mechanism involving base-assisted catalysis. Single-turnover experiments following incorporation of \( ^{17}\)O into the molybdenum center of the enzyme demonstrated that a single oxygen atom is incorporated at a site that gives rise to strong hyperfine coupling to the unpaired electron spin of the metal in the MoV oxidation state. By analogy to the hyperfine interactions seen in a homologous series of molybdenum model compounds, we conclude that this strongly coupled, catalytically labile site represents a metal-coordinated hydroxide rather than the Mo=O group and that this Mo–OH represents the oxygen that is incorporated into product in the course of catalysis.

Xanthine oxidase from cow’s milk is a homodimer with a molecular mass of 300 kDa, with each catalytically independent subunit possessing four prosthetic groups: one molybdenum center, one FAD, and two \( Fe_{2}S_{2}(Cys)_{4} \) iron-sulfur centers. Physiologically, the enzyme catalyzes the oxidative hydroxylation of hypoxanthine to xanthine and the subsequent hydroxylation of xanthine to uric acid, the final two steps of purine metabolism in mammals. Substrate hydroxylation takes place at the molybdenum center of the enzyme, which becomes reduced from MoVI to MoIV in the process (1). The reducing equivalents introduced at the molybdenum center are subsequently passed via intramolecular electron transfer to the flavin center, where reaction with \( O_2 \) takes place to give peroxide or superoxide, depending on the level of enzyme reduction (2, 3).

Xanthine oxidase exhibits an unusually broad specificity toward reducing substrates. It can hydroxylate a wide variety of purines, pteridines, and related aromatic heterocycles and also a range of both aliphatic and aromatic aldehydes, taking these to the corresponding carboxylic acid. Indeed, xanthine oxidase belongs to the same family of mononuclear molybdenum enzymes (the molybdenum hydroxylases) as do the aldehyde oxidases. These enzymes have similar cofactor constitutions and amino acid sequences and also have substantially overlapping specificity for reducing substrates (4). The specificity of xanthine oxidase toward aldehyde substrates has been examined by Morpeth (5), who has found that biogenic aldehydes such as acetaldheyde, indole-3-aldehyde, pyridine-2-aldehyde, etc. are reasonable substrates for the enzyme. To investigate the mechanism of aldehyde hydroxylation by xanthine oxidase and related molybdenum hydroxylases, the reaction of the enzyme with 2,5-dihydroxybenzaldehyde and 2-aminopteridine-6-aldehyde (PTA1) has been examined. The pH dependence of the kinetics for these reactions indicates that, as with purine hydroxylation, aldehyde substrates take place via a base-catalyzed mechanism and substrate must be protonated for hydroxylation to occur.

The reaction catalyzed by the molybdenum hydroxylases is unusual in comparison with that of other biological hydroxylating systems in that water rather than dioxygen is the ultimate source of the oxygen atom incorporated into product (4), and the reaction generates rather than consumes reducing equivalents. Although the oxygen atom incorporated into product is ultimately derived from water, it has been shown for some time that the proximal oxygen atom donor is a catalytically labile site on the enzyme, which, in the course of a single turnover, transfers its oxygen to substrate, to be regenerated subsequently by oxygen derived from solvent prior to a second turnover (6). In light of the known precedence for oxo transfer in the literature of small inorganic complexes of molybdenum (7–16), it was originally thought most likely that the catalytically labile site of the enzyme was the Mo=O group. More recently, it has been suggested that the catalytically labile site might instead be a metal-coordinated hydroxide (17–19). Given the crystallographic demonstration that water/hydroxide is a li-
The reductive half-reaction of xanthine oxidase.  

**Scheme 1. Alternate reaction mechanisms for xanthine oxidase.**

A: 

B: 

O₂-free argon for at least 15 min.

Reversed-phase HPLC separation of 2,5-dihydroxybenzaldehyde and 2,5-dihydroxybenzoic acid was carried out using a Beckman HPLC system equipped with an ODS Hypersil 200 × 46-mm column (5-μm pore size; Hewlett-Packard), eluting with a degassed 70% methanol and 30% double-distilled water mixture as the mobile phase; the flow rate was 1 ml/min, and the eluate was monitored at 350 nm. The product of the enzyme reaction with 2,5-dihydroxybenzaldehyde was obtained by incubation of the enzyme with 2,5-dihydroxybenzaldehyde for 1.5 h, followed by centrifugation through a 50-kDa cutoff membrane to remove the enzyme and dilution with 70:30 (v/v) MeOH/H₂O. Samples were analyzed by the HPLC system and compared with authentic 2,5-dihydroxybenzaldehyde and 2,5-dihydroxybenzoic acid to verify the product of the enzyme reaction.

The pKa values for 2,5-dihydroxybenzaldehyde and PTA were independently determined spectrophotometrically. The protonated (neutral) form of 2,5-dihydroxybenzaldehyde exhibits a maximum absorbance at 358 nm, which shifts upon deprotonation to 410 nm. The pKa was obtained by fitting the absorbance at 410 nm as a function of pH to the equation \( A = (A_{HA}[H^+] + A_K[H^+] + K_a) \), where \( A_{HA} \) is the absorbance of protonated substrate, \( A_K \) is the absorbance of the deprotonated form, and \( K_a \) is the acid dissociation constant. The absorption spectrum of PTA is also pH-dependent, exhibiting absorbance maxima at 280 and 370 nm at high pH, shifting upon deprotonation to 312 nm. The pKa value for PTA was obtained as described above following this absorbance change.

Steady-state kinetic measurements with 2,5-dihydroxybenzaldehyde as substrate were performed from pH 5.5 to 9.4 at 25 °C following the absorbance increase at 520 nm for the conversion of the aldehyde to the corresponding acid. Since both the substrate and product exhibit pH-dependent spectra, it was necessary to independently determine the extinction change for oxidation at each pH. The Δε values at 320 nm ranged from 2.8 mm⁻¹ cm⁻¹ at pH 5.5 to 4.0 mm⁻¹ cm⁻¹ at pH 9.4. The concentration of functional enzyme active sites ranged from 13 to 26 nM in these experiments, and the substrate concentration ranged from 20 to 2000 μM, corresponding to a range at least between 0.3 and 3 times the \( K_m \) values at each pH. The observed catalytic velocities were fit to the standard hyperbolic equation (Equation 1), where each term has its conventional definition.

\[
\frac{y}{[E]} = \frac{k_{cat} [S]}{K_m + [S]} 
\]  

(Eq. 1)

pH profiles were fit by Kaleidograph software using Equation 2 for a single-ionization process or Equation 3 for double ionization.

\[
\frac{k_{iial}[K_a]}{K_m} = \frac{(\frac{[H]}{K_a})_{max} - K_a}{K_m + [H]} 
\]  

(Eq. 2)

\[
\frac{k_{iial}/K_a}{[K_a]} = \frac{(\frac{[H]}{K_a})_{max} - 1}{[H] + K_a + [H]} 
\]  

(Eq. 3)

In these equations, \( K_a \) represents the dissociation constant for enzyme or substrate, and \( K_m \) and \( K_a \) represent the true acid dissociation constants for a double-ionization process (\( E + H^+ \rightleftharpoons EH^+ \)), where only the ionization state \( EH^+ \) is catalytically active. In the application of Equation 3 to the reductive half-reaction with 2,5-dihydroxybenzaldehyde, \( k_{iial}/K_a \) is substituted for \( k_{cat}/K_a \).
using a double-ionization equation (see “Materials and Methods”) to yield two $pK_a$ values that govern catalysis. The lower $pK_a$ of 6.3 ± 0.1 obtained from this analysis agrees well with that determined previously for an active-site residue essential in the hydroxylation of xanthine and lumazine (2,4-dihydroxypteridine) by xanthine oxidase ($pK_a \sim 6.5$). The higher $pK_a$ of 8.6 ± 0.1 from this work agrees well with that of the substrate 2,5-dihydroxybenzaldehyde, which has been independently determined spectrophotometrically ($pK_a = 8.4$) (Fig. 1, inset) and by acid-base titration ($pK_a 8.5$) (data not shown). The implication is that it is the neutral rather than the ionized form of substrate that is acted upon by the enzyme. The overall pH dependence of the reaction is comparable to that of enzymatic action on both xanthine and lumazine (26), suggesting strongly that xanthine oxidase hydroxylates aromatic heterocycles and aldehydes by the same principal mechanism, one involving base-assisted catalysis on an unionized (neutral) substrate.

**Reductive Half-reaction Kinetics with 2,5-Dihydroxybenzaldehyde—**Studies of the reductive half-reaction of xanthine oxidase with 2,5-dihydroxybenzaldehyde were carried out at 10 °C in a stopped-flow apparatus following the loss of absorption at 460 nm due to reduction of the flavin and iron-sulfur centers of the enzyme under anaerobic conditions. It is known that full reduction of xanthine oxidase requires 3 eq of substrate and that this reaction goes essentially to completion in the case of xanthine. On the other hand, xanthine is unable to fully reduce xanthine dehydrogenase, owing to the establishment of unfavorable internal redox equilibria with this enzyme (27). To determine whether the reaction of the aldehyde substrate with the oxidase also goes to completion under the present experimental conditions, the total absorbance change elicited by a 3-fold stoichiometric excess of 2,5-dihydroxybenzaldehyde was compared with that induced by a comparable amount of xanthine when the enzyme concentration and other conditions were the same. It was found that on addition of 2,5-dihydroxybenzaldehyde under anaerobic conditions, the level of xanthine oxidase reduction was the same as that by xanthine (data not shown), indicating that xanthine oxidase is indeed completely reduced by 2,5-dihydroxybenzaldehyde.

The reaction of xanthine oxidase with a pseudo first-order excess of 2,5-dihydroxybenzaldehyde under anaerobic conditions was found to exhibit two well resolved kinetic phases. The rate constant for the faster phase of the reaction exhibits hyperbolic dependence on substrate concentration, yielding values for the limiting rate constant ($k_{lim}$) and substrate dissociation constant ($K_D$) that are pH-dependent. $k_{lim}/K_D$ for this faster phase exhibits a bell-shaped pH dependence with a lower $pK_a$ of 6.5 ± 0.2 and a higher $pK_a$ of 8.3 ± 0.2 (Fig. 2), comparable to that seen in the steady-state experiments above and consistent with the reductive half-reaction being principally rate-limiting during turnover, as is the case for turnover with xanthine (25). The results again implicate an active-site residue that must be deprotonated for the reaction to occur.

The above results are consistent with a reaction mechanism involving nucleophilic attack on the substrate carbonyl by a Mo–OH group, with base-assisted proton abstraction from the attacking hydroxyl by a residue in the active site of the enzyme (Scheme 1B). Recently, the crystal structure of aldehyde oxidase from *Desulfocovibrio gigas* was solved at 1.8 Å, and on the basis of this structure, a mechanism of aldehyde hydroxylation was proposed that involves similar chemistry (19).3 Specifi-
cally, it was proposed that Glu-869 at the active site abstracts a proton from molybdenum-bound water/hydroxide and thereby facilitates nucleophilic attack on the substrate carbonyl to generate, after hydride transfer, a MoIV complex with product coordinated to the metal via the newly formed -OH. Xanthine oxidase and aldehyde oxidase share a highly homologous amino acid sequence in the vicinity of their molybdenum centers that includes this glutamate residue (VG\textsubscript{E}869LPL for the \textit{D. gigas} protein versus VG\textsubscript{E}1261PPL for the bovine enzyme). We thus suggest that Glu-1261 of xanthine oxidase plays a role analogous to that proposed for Glu-869 of aldehyde oxidase, in which case the \( pK_a \) associated with the enzyme is most likely to be associated with this residue. Although the observed \( pK_a \) of \( \approx 6.4 \) is rather high for a glutamate, it is not unreasonable: the \( pK_a \) for Glu-35 in lysozyme has been reported to be \( 6.5 \) in the absence of substrate and to shift to \( \approx 8.2 \) on binding glycol chitin (31). We note that it is unlikely that the ionization governing the acid limb of the \( k_{\text{cat}}/K_m \) versus pH profiles is due to the Mo–OH group itself, as x-ray absorption studies of xanthine oxidase have demonstrated quite unambiguously that at \( \text{pH} \approx 8.5 \) (well above the \( pK_a \) for the active-site residue), the molybdenum center possesses only a single Mo\textsuperscript{V}O group rather than the two that would be expected were the Mo–OH to deprotonate (32).

The slower phase of the reaction of xanthine oxidase with 2,5-dihydroxybenzaldehyde, which accounts for only 10–20% of...
the total observed spectral change at high substrate concentrations, has observed rate constants between 0.1 and 0.2 s\(^{-1}\) that are not strikingly dependent on substrate concentration. The rate constant associated with the slower phase of the reaction is too large to reflect the slow reduction of nonfunctional enzyme that is known to occur with xanthine oxidase (25, 33), and it appears that the slower phase most likely involves processes taking place during the reaction of the enzyme with a second or (more likely) third eq of substrate under the present conditions of pseudo first-order excess of substrate. Such kinetic complexity has also been observed in the reaction of xanthine with xanthine dehydrogenase (34). To test this idea, a single-turnover experiment was performed in which the enzyme rather than substrate was present in excess. When excess xanthine oxidase was reacted with 2,5-dihydroxybenzaldehyde (5:1 xanthine oxidase/substrate), only a single kinetic phase was observed, corresponding in rate to the fast phase of the reaction seen with excess substrate under comparable concentrations of substrate. This indicates that the slower phase of the reaction seen under conditions of excess substrate involves the reaction of the second or third eq of substrate with the enzyme, possibly rate-limited by product release from the molybdenum center in a prior catalytic sequence. As this slower phase of the reaction does not reflect the intrinsic initial reactivity of substrate with the molybdenum center of the enzyme and given that it contributes relatively little to the overall absorbance change seen in the course of the reaction, it has not been investigated further here.

**Reaction Kinetics with 2-Aminopteridine-6-aldehyde**—The above work indicates that the reaction of xanthine oxidase with an aldehyde substrate, through the first irreversible step of the reaction as traced by \(k_{\text{obs}}/K_m\) and \(k_{\text{lim}}/K_d\), involves base-assisted attack on neutral substrate. A series of reductive half-reaction and steady-state studies with 2-hydroxy-6-methylpurine, xanthine, and lumazine as substrate have been examined previously, with the evidence obtained indicating that the hydroxylation proceeds through two intermediates, corresponding to Mo\(^{IV}\)-product and Mo\(^{V}\)-product complexes (21, 26). To investigate early steps in the catalytic sequence seen with aldehyde substrates that are upstream from formation of the Mo\(^{V}\)-product intermediate, experiments following the reaction of xanthine oxidase with PTA have been performed. PTA is a slow substrate for xanthine oxidase that is hydroxylated to the corresponding carboxylic acid by the enzyme (35). This substrate is interesting in that its reaction with the oxidized enzyme gives rise to a transient long-wavelength absorbance (Fig. 3A) (35). At pH 8.5, the rate constant associated with formation of this species exhibits hyperbolic dependence on substrate concentration, indicating that formation of the long wavelength-absorbing species involves two distinct kinetic steps: \(E_{\text{ox}} + S \rightleftharpoons E_{\text{ox}}S \rightleftharpoons E_{\text{ox}}S^*\), where \(E_{\text{ox}}S^*\) represents the long wavelength-absorbing oxidized enzyme-substrate complex. We have examined the pH dependence of this reaction following \((k_{\text{lim}}/K_d)_{\text{app}}\) obtained from hyperbolic fits to plots of the observed rate constant versus PTA concentration as a function of pH. In contrast to the above work with 2,5-dihydroxybenzaldehyde as substrate, the plot of \((k_{\text{lim}}/K_d)_{\text{app}}\) as a function of pH is sigmoidal, indicating only a single pK\(_a\) with a value of 7.6 ± 0.2 (Fig. 3B). This is in fair agreement with the substrate pK\(_a\) of 7.2 ± 0.1, and we consider the observed pK\(_a\) to be that of the substrate. The implication, as observed, is that it is the neutral rather than the monoanionic form of substrate that binds to the enzyme. The distinct difference in pH profile for \((k_{\text{lim}}/K_d)_{\text{app}}\) for formation of \(E_{\text{ox}}S^*\) in the reaction with PTA (i.e. sigmoidal) as compared with that for \(k_{\text{lim}}/K_d\) for the reductive half-reaction with 2,5-dihydroxybenzaldehyde or xanthine (i.e. bell-shaped) indicates that the catalytically essential active-site base is not required for formation of the long wavelength-absorbing intermediate accumulating in the course of the reaction with PTA. The implication is that the chemistry of hydroxylation, which requires the active-site base, has not taken place in forming the long wavelength-absorbing species, consistent with this species being formulated as Mo\(^{V}\)S species rather than Mo\(^{IV}\)P. By way of confirming this conclusion, we find that addition of pterin-6-carboxylic acid to dithionite-reduced xanthine oxidase does not give rise to the observed long wavelength-absorance increase. There is in fact no discernible spectral change observed on addition of pterin-6-carboxylic acid to the reduced enzyme. In contrast, the reaction of xanthine oxidase with lumazine gives rise to a transient absorbance in the 600–700 nm region that has been shown to be due to the Mo\(^{IV}\)-violapterin (2,4,7-trihydroxypteridine) complex, and the identical complex can be conveniently generated by addition of the product violapterin to dithionite-reduced enzyme under anaerobic conditions (26).
Taken together, these results demonstrate that the observed long-wavelength absorbance increase seen with PTA is not due to a MoV-product complex, but is instead a MoVI-substrate complex in which the chemistry leading to substrate hydroxylation has not yet taken place.

**Binding of 2,5-Dihydroxybenzaldehyde and 2,5-Dihydroxybenzoic Acid to Oxidized and Reduced Xanthine Oxidase**—In previous work, it has been shown that a number of enzyme products and product analogs bind to reduced xanthine oxidase with reasonably high affinity ($K_d \approx 18 \mu M$) and elicit a small spectral change at the molybdenum center upon binding (37). To determine whether 2,5-dihydroxybenzoic acid interacts with the enzyme in the same way, titrations of both oxidized and reduced enzyme with 2,5-dihydroxybenzoic acid were performed. We find that 2,5-dihydroxybenzoic acid binds the oxidized enzyme and produces a spectral change similar to that of 8-hydroxyxanthine, a spectral change known to be associated with the enzyme flavin rather than its molybdenum center (37). The $K_d$ for 2,5-dihydroxybenzoic acid binding is large, $\sim 8$ mM. Titration of dithionite-reduced xanthine oxidase with 2,5-dihydroxybenzoic acid and 2,5-dihydroxybenzaldehyde has also been attempted, but no obvious spectral change is observed (data not shown).

Xanthine oxidase frequently exhibits excess substrate inhibition (37, 38), a phenomenon thought to be associated with substrate binding to reduced rather than oxidized forms of the enzyme generated in the steady state (37, 38). In this work, no obvious excess substrate inhibition was observed with 2,5-dihydroxybenzaldehyde, although product inhibition by the corresponding carboxylic acid did exist. To investigate how product binds to the oxidized molybdenum center (37). The $K_d$ for 2,5-dihydroxybenzoic acid binding is large, $\sim 8$ mM. Titration of dithionite-reduced xanthine oxidase with 2,5-dihydroxybenzoic acid and 2,5-dihydroxybenzaldehyde has also been attempted, but no obvious spectral change is observed (data not shown).

**Identification of the Catalytically Labile Oxygen in the Active Site of Xanthine Oxidase**—To establish whether the catalytically labile oxygen of the active site of xanthine oxidase is the Mo=O or Mo–OH moiety known to be present in the molybdenum coordination sphere, we have investigated the exchange of $^{17}O$ from labeled solvent into the molybdenum center in the course of a single turnover. After an initial turnover event, the site of the catalytically labile oxygen will be regenerated with oxygen derived from solvent, and the isotopically labeled oxygen from solvent can be used as a probe of the active site. This approach takes advantage of previous observations that after exhaustive exchange with solvent, several of the Mo$^V$ EPR signals of xanthine oxidase exhibit magnetic coupling to one strongly coupled $^{17}O$ nucleus and frequently a second much more weakly coupled one (17, 39–41). It has been demonstrated in suitable model compounds that Mo–OH groups couple strongly to the unpaired electron spin ($g_{av} \sim 6.5$ G; Mo–OH groups are much more weakly coupled ($g_{av} \sim 2.2$ G) (17). EPR thus represents a uniquely appropriate tool whereby exchange into the Mo–OH and/or Mo–O site under single-turnover conditions can be examined on a catalytic time scale.

Fig. 4 (spectrum A) shows the EPR spectrum observed when 150 $\mu M$ deflavoxanthine oxidase is reacted with 500 $\mu M$ 1-methylxanthine in unlabeled water. The incubation time prior to freezing was 0.5 s. Spectrum B, the signal observed using the same protocol but with $H_2^{16}O$ rather than $H_2^{18}O$ (49% enrichment). Spectrum C, spectrum B corrected for the $5\%^{18}O$ signal found in the sample (with a 4-fold expansion of the y axis). Spectrum D, a simulation of the spectrum based on the following parameters: $g_{1,2,3} = 1.986, 1.968$, and $1.966; a_{1,2,3} = 12, 12$, and $13$ G; $a_{17}^{17}O = 4, 6, 1.7$, and $1.6$ G; and $\alpha_{1,2,3} = 2, 3$, and $16$ G. The line spectrum based on the simulation (with $6 \times 2 \times 2$ hyperfine splitting) is also shown. In the simulation, the isotropic artifact at $g \sim 2$, frequently observed in the freeze-quenched enzyme, has been ignored. Spectrum E, the rapid Type 2 signal observed upon partial reduction of the enzyme with sodium dithionite in the presence of 200 $\mu M$ 8-bromo-xanthine. The incubation time prior to freezing was 5 s, and the reduced signal-to-noise level compared with that observed in spectra A–C is due to a modest level of over-reduction of the enzyme molybdenum (to the EPR-silent (IV) oxidation state). Instrument settings were as follows: 9.458-GHz microwave frequency, 10-milliwatt microwave power, 100-kHz modulation frequency, 2.0-G modulation amplitude, and 150 K temperature.
Type 1 £ form typically seen with this substrate, exhibiting a doublet-of-doublets on the low-field gz feature (due to the presence of two inequivalent protons in the signal-giving species (41)). Spectrum B shows the spectrum observed when the experiment is repeated in water that was 49% enriched in 17O. The spectrum is quite distinct from spectrum A, particularly in the gz region, where the spectral resolution is apparently poorer, and in the central portion of the spectrum, where the strong positive feature in the H216O sample is replaced by a considerably weaker doublet in H217O. Although hyperfine splitting due to the I = 5/2 nucleus of 17O is not evident in spectrum B, the observed spectrum is essentially identical to that observed when the enzyme is exhaustively exchanged with H217O prior to generation of the EPR signal (40). This signal has been demonstrated in computer simulations to arise from a species possessing a single strongly coupled 17O nucleus, with aav £ 7 G. The hyperfine structure arising from the nuclear spin of 17O is more evident when spectrum B is corrected for the 51% 16O present in the sample, as shown in spectrum C. The dashed line (spectrum D) shows a simulation of the spectrum using a1,2,3 = 16, 3, and 2 G (aav = 7 G), and the fit to the data is seen to be quite good. These values are in excellent agreement with simulations of spectra obtained in exhaustively labeled enzyme (17, 39–41) and are clearly in the range of splittings observed for Mo–OH groups in model compounds (17). In particular, the good fit to the high field features (527 G) indicates the presence of a strongly coupled oxygen atom.

On the time scale of the present experiment, the reaction subsequent to the initial reduction of the enzyme by the first eq of substrate and incorporation of 17O into the active site has three possible fates. 1) A portion of the enzyme will not have time to react with a second eq of substrate and will be reduced only to the level of 2 electron eq. Under the present reaction conditions (0.1 M pyrophosphate buffer, pH 8.5), the electron distribution within this 2-electron-reduced enzyme is heavily in favor of reduction of the two iron-sulfur centers over that of the molybdenum (25), and as a result, this enzyme population will be EPR-silent at 150 K. 2) A second portion of the enzyme, having been reduced to the level of 2 electron eq in the first turnover and possessing MoV after transfer of the reducing equivalents obtained from substrate to the iron-sulfur centers, will have reacted with a second eq of substrate to give fully reduced, 4-electron deflavoxanthine oxidase; this enzyme population possesses MoV and must be EPR-silent at 150 K. 3) The remainder of the enzyme, having also been reduced to the level of 2 electron eq in the course of the first turnover, will also have bound substrate, but not yet have advanced into a second catalytic sequence. To the extent that this enzyme population possesses MoV or MoIV rather than MoVI, it will be unable to react with substrate. The effect of substrate binding on the reduction potentials of the molybdenum center is in fact such that the MoV oxidation state is significantly stabilized by substrate binding to the active site of partially reduced enzyme (34, 39), a fact that accounts for the observed inhibition of the enzyme during turnover under conditions of high substrate concentration (5). In the present experiment, the net result is that £25% of the functional enzyme in the reaction mixture is trapped after the first turnover in a MoV–S state that specifically gives rise to the rapid Type 1 EPR signal when 1-methylxanthine is used as substrate. The clear implication from the above experimental result is that after a single turnover, the oxygen atom is incorporated into the molybdenum center at a strongly coupled site (aav £ 7 G) and, on the basis of previous work with model compounds (17), is unquestionably the Mo–OH group rather than Mo–O. The results are consistent with an overall sequence of events taking place in the enzyme active site as indicated in Scheme 2.

It is known that reduction of the molybdenum center gives rise to much more rapid noncatalytic exchange of solvent oxygen into the molybdenum coordination sphere (40, 41), and it is conceivable that under the present reaction conditions, the observed incorporation of 17O into the molybdenum center might be due to a rapid noncatalytic exchange into the site rather than to turnover per se. To establish that this is not the case on the time scale of the present experiment, unlabeled deflavoxanthine oxidase in H217O was partially reduced by reaction with sodium dithionite in an EPR tube in the presence of the substrate analog 8-bromoxanthine, followed by freezing after £5 s. 8-Bromoxanthine is not hydroxylated by xanthine oxidase, but does bind to the active site of the partially reduced enzyme to elicit a “rapid Type 2” MoV EPR signal, as reflected in a characteristic 1:2:1 triplet feature (40, 41). In particular, the 1:2:1 triplet of gz feature of the signal (37). The spectrum observed is shown in Fig. 4 (spectrum E) and is indistinguishable from that seen in H217O; in particular, the 1:2:1 triplet of gz is readily evident. This result indicates that no detectable solvent exchange has occurred in the much longer time interval between reduction of the enzyme and freezing than was used in the experiment with 1-methylxanthine; the exchange observed in the experiment with 1-methylxanthine must therefore be due to catalytic turnover.

CONCLUSIONS

In this work, we have investigated the mechanism of aldehyde hydroxylation by xanthine oxidase by both steady-state and reductive half-reaction kinetics and examined the nature of the catalytically labile oxygen in the enzyme active site by EPR. On the basis of the similarity of the bell-shaped pH profiles for aldehyde and heterocycle hydroxylation as catalyzed by xanthine oxidase (this work and Ref. 26, respectively), it is extremely likely that the mechanism of hydroxylation of both aldehydes and heterocycles by xanthine oxidase is essentially the same, involving base-assisted nucleophilic attack on substrate. In light of the crystal structure of the related aldehyde oxidoreductase from D. gigas and the proposed role of Glu-869 of this enzyme in catalysis (19), we suggest that the
homologous residue in xanthine oxidase, Glu-1261, represents the active-site base of xanthine oxidase. (Ultimately, proof of this will require development of a suitable expression system followed by site-directed mutagenesis of this residue.) The pH dependence of the reaction of enzyme with 2-aminopteridine-6-aldehyde indicates that the long wavelength-absorbing species formed at the completion of the reaction represents an $E_{ac}S$ rather than an $E_{red}P$ complex.

The $^{17}$O experiments described here demonstrate that it is the Mo–OH rather than the Mo=O group that is catalytically labile and support a mechanism proceeding as indicated in Scheme 1B, in which hydroxylation occurs via (base-assisted) nucleophilic attack of an enzyme Mo–OH group on substrate. Such a mechanism is consistent with the structure of the molybdenum center of xanthine oxidase as inferred from the crystal structure of the closely related enzyme aldehyde oxidoreductase from D. gigas and has been considered previously in light of the structural data (19). Similarly, Wedd and co-workers (17) suggested earlier that a metal-coordinated hydroxide rather than Mo=O might represent the catalytically labile oxygen site of xanthine oxidase on the basis of EPR studies of model compounds. Recently, Bray and co-workers (18) inferred from an ENDOR study of the “very rapid” EPR signal that the Mo=O group does not exchange with solvent in the course of catalysis (on the basis of their inability to detect a second, weakly coupled $^{17}$O nucleus in addition to the strongly coupled $^{17}$O of bound product) and concluded that Mo–OH rather than Mo=O is the catalytically labile oxygen site. The failure to detect a weakly coupled oxygen in this experiment is not surprising, however, given the intrinsically weak spectral signature of even a strongly coupled oxygen and the difficulty of identifying a second, more weakly coupled nucleus in the presence of a more strongly coupled one. Thus, the possibility cannot be excluded that a second oxygen site had indeed exchanged in the signal-giving species, but had escaped detection. The present work, unambiguously demonstrating that oxygen exchanges into a strongly coupled site of the enzyme in the course of a single turnover, provides concrete and positive support of the Mo–OH group being the catalytically labile oxygen site in the molybdenum center of xanthine oxidase.

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