The pH dependence and solvent isotope sensitivity of three discrete steps in the reductive half-reaction of xanthine oxidase have been investigated. The pH dependence of both $k_{cat}/K_m$ from steady-state experiments and $K_{red}/K_d$ from rapid reaction experiments with xanthine as substrate indicate that enzyme reacts preferentially with the neutral form of substrate and that an ionizable group in the active site having a $pK_a$ of 6.6 must be unprotonated for reaction to take place. The solvent kinetic isotope effect on $K_{cat}/K_d$ is 2.4, once a uniform shift on going to $D_2O$ of −1 unit for both $pK_a$ values is taken into account. The pH dependence of the formation and decay of $E_{red}$-P formed in the course the reaction of xanthine oxidase with lumazine has also been examined. Formation of this complex exhibits bell-shaped pH dependence, with $pK_a$ values of 6.5 and 7.8, consistent with the results obtained with xanthine. Decay of the $E_{red}$-P complex is base-catalyzed with a $pK_a > 11$ and exhibits a small solvent kinetic isotope effect of 2.0 and 3.5 at pH 10. The results are discussed in the context of a specific reaction mechanism for the reductive half-reaction of xanthine oxidase, in which discrete ionizations associated with the molybdenum center of the active site play critical roles in determining the magnitude of the rate constants by which the Mo(V)-P and Mo(V)-P intermediates form and decay.

By contrast to other biological systems catalyzing hydroxylation reactions, the molybdenum-containing hydroxylases utilize water rather than dioxygen as the source of the oxygen atom incorporated into product and generate rather than consume reducing equivalents in the course of turnover. As such they represent a fundamentally different solution to the chemistry of hydroxylation of carbon centers under physiological conditions. Xanthine oxidase is the best studied member of this class of enzyme, principally due to its ease of isolation in substantial quantities. The enzyme from cow’s milk is a homodimer of molecular weight 300,000, with each subunit possessing four prosthetic groups: one molybdenum center, two iron-sulfur centers of the spinach ferredoxin variety, and one equivalent of flavin adenine dinucleotide (1, 2). A variety of aromatic heterocycles and simple aldehydes are oxidatively hydroxylated at the molybdenum center of the enzyme (3), reducing the molybdenum from the Mo(VI) to the Mo(IV) oxidation state. These reducing equivalents are subsequently passed to the flavin center of the enzyme via intramolecular electron transfer; the reduced flavin in turn reacts with dioxygen to form either peroxide or superoxide, depending on the overall level of enzyme reduction.

Understanding of the chemical mechanism of xanthine oxidase and related enzymes has been greatly facilitated by studies of the chemistry of inorganic complexes that are likely mechanistic models for the active sites of these enzymes. Of the several mononuclear model compounds that have been prepared, those developed by Holm and co-workers (MoO$_2$(LNS)$_2$; $L = 2,6$-bis(2,2-diphenyl-2-mercaptoethyl)pyridine) (4–8) and Enemark and co-workers (Bpz$_3$MoO$_2$SR; Bpz$_3$ = hydrotris(3,5-dimethyl-1-pyrazolyl)borate) (9, 10) are the best understood. Both systems have been shown to cleanly catalyze the reversible transfer of an oxygen atom from a suitable oxo donor (e.g. dimethyl sulfoxide) to an acceptor (e.g. triphenylphosphine), in each case alternating between Mo(VI)O$_2$ and Mo(V)O species. In the case of the Bpz$_3$MoO$_2$SR complex it is particularly significant that in the presence of a suitable oxidant water is able to act as the oxo donor, reacting with the Mo(V)O complex to give the Mo(VI)O$_2$ species (10). A variety of oxo donors and acceptors have been studied, and turnover for a given donor: acceptor pair has been justified on thermodynamic grounds (11). The substantial negative activation entropy in the reaction of the Mo(VI)O$_2$ species with phosphine as oxo acceptor in the reductive limb of the catalytic cycle of these complexes is consistent with an associative reaction mechanism that proceeds by nucleophilic attack of the phosphine lone pair on the oxygen atom of one of the Mo(VI)$\pm$O groups, which results in simultaneous formation of the P-O bond of product and reduction of the molybdenum by two equivalents (6). Despite the known strength of the Mo–O bond (with an enthalpy of formation on the order of –45 kcal/mol; Ref. 11), it is clear from the chemistry of these complexes that the Mo–O moiety is sufficiently reactive to permit removal of the oxygen (either with its transient replacement by a solvent ligand or leaving a vacant ligand coordination position, depending on the model system) under the appropriate conditions.

Similar oxo-transfer chemistry is likely taking place in the reductive half-reaction of xanthine oxidase, although the enzyme possesses a MoO$_5$ unit in its active site rather than the MoO$_6$ unit of the above models (12). When enzyme is labeled with $^{18}$O by extensive turnover in H$_2^{16}$O and then transferred...
to H$_2^{16}$O and reacted with substoichiometric xanthine (single-turnover conditions). 92% of the uric acid recovered from the reaction mix is found to possess $^{18}$O at the 8-position as determined by mass spectral analysis of the product (12). When the converse experiment is done with $^{18}$O-labeled enzyme in H$_2^{16}$O, the uric acid is found to contain predominantly (~79%) $^{16}$O at the 8-position. Xanthine oxidase thus acts in such a way that oxygen is transferred from a catalytically labile site in the enzyme to substrate with each catalytic cycle in forming the hydroxyl group of product.

Kinetic studies of the reductive half-reaction of xanthine oxidase have been performed under single-turnover conditions with 2-hydroxy-6-methylpurine, a slow substrate for the enzyme that is hydroxylated to give 2,8-dihydroxy-6-methylpurine with a $k_{cat}$ of 0.1 s$^{-1}$ at pH 8.5, 25 °C (13, 14). Spectral changes observed in the course of the aerobic reaction of xanthine oxidase with stoichiometric 2-hydroxy-6-methylpurine in 0.1 m CAPS, pH 10, 25 °C provide clear evidence for two intermediates, the first exhibiting an absorption difference maximum (relative to oxidized enzyme) at 470 nm, and the second a difference maximum at 540 nm, with extinction changes of approximately 500 m$^{-1}$ cm$^{-1}$. The rate constants for formation and decay of the second intermediate correlate very well with those for the formation and decay of a particular paramagnetic species long thought to represent an authentic catalytic intermediate (that giving rise to the so-called “very rapid” EPR signal) under the same experimental conditions (14). Further, it has been demonstrated that this EPR-active species is formed by one-electron oxidation of a Mo(V) species that precedes it in the catalytic sequence rather than by direct reduction of Mo(VI) by substrate in a radical-based mechanism. In conjunction with the results cited above in support for oxy-

Figure 1. Structure for the signal-giving species given in Scheme 1 and indicated in Scheme 1. EPR studies of the very rapid signal using enzyme that has been labeled with $^{17}$O and $^{33}$S (20–22), in conjunction with studies of comparably labeled Mo(V) model compounds (23–25), have been interpreted in the context of the structure for the signal-giving species given in Scheme 1 and constitute the principal experimental evidence in support of the structure drawn.

Another substrate germane to the present studies is lumazine (2,6-dihydroxypteridine), which is converted by xanthine oxidase to violapterin (2,6,7-trihydroxypteridine). The Mo(IV)-violapterin complex generated transiently in the course of this reaction is distinctive in that it possesses distinctive long wavelength absorbance due to a charge-transfer interaction between the reduced metal and pterin and is thought to represent the initial intermediate of the catalytic sequence (26, 27). The identical complex is conveniently generated in a stable form by the addition of violapterin to dithionite-reduced enzyme under anaerobic conditions, and the long wavelength absorbance of the complex has been exploited to perform resonance Raman studies of the E$_{red}$-P complex (28). Consistent with the above EPR results concerning the Mo(V) species, this work has been interpreted in terms of an Mo(IV)-OR species, with nascent product coordinated to the active site molybdenum via the newly introduced hydroxyl group. While only a small amount of very rapid EPR signal is observed in the course of reaction of enzyme with lumazine (1–2% Ref. 13), the complex is nevertheless thought to decay via oxidation of the molybdenum center rather than by direct dissociation of product from the reduced molybdenum center (27); the small amount of very rapid EPR signal that accumulates in the course of turnover presumably is due to unfavorable relative rates of formation and decay for the signal-giving species. Independently, x-ray absorption spectroscopic studies have demonstrated that the complex of reduced enzyme with violapterin also possesses a Mo=O group (29), as does the corresponding complex of reduced enzyme with the inhibitor alloxanthine (29, 30). This requires that the Mo=O group be regenerated immediately upon formation of the C=O bond of product, either by hydroxide from solvent (as drawn in Scheme 1) or, alternatively, by deprotonation of a hydroxide ligand present in the molybdenum coordination sphere (for which there is some evidence).

The reaction mechanism shown in Scheme 1 incorporates protonation/deprotonation events at specific points in the course of the reaction. In the present work the pH dependence and solvent isotope sensitivity of the steady-state kinetic parameters observed with xanthine, as well as formation and decay of the Mo(IV)-violapterin complex and the species giving rise to the very rapid EPR signal observed in the course of the reaction of enzyme with 2-hydroxy-6-methylpurine, have been investigated in order to further elucidate the role of protonation/deprotonation events in catalysis. The results are consistent with the mechanism shown in Scheme 1, in which prototropic equilibria associated with each intermediate significantly influence the rate constants associated with their formation and decay.

**MATERIALS AND METHODS**

Xanthine oxidase was purified from unpasteurized cow’s milk using the procedure reported by Massey et al. (31). Sephacryl S-300 gel filtration and CM-52 ion exchange steps at the end of the procedure ensured removal of contaminating lactoperoxidase (32). The purified enzyme exhibited a ratio of absorbance at 276 and 450 nm of ~5.4 and 0.2.
was typically ~ 70% functional. The ~30% inactive enzyme found in conventional preparations of xanthine oxidase lacks a catalytically essential sulfur atom at the molybdenum center (33) and is unreactive in the experiments described here. Enzyme assays and the determination of specific activity were performed as described by Massey et al. (31). D$_2$O (99.9% enriched) was purchased from Cambridge Isotopes Laboratory. The pH readings of buffers solutions in D$_2$O were taken by adding water to the pH meter readings (34). D$_2$O solutions of enzyme were obtained by passing through a Sephadex G-25 column equilibrated with D$_2$O buffer of the desired pH. 2-Hydroxy-6-methylpurine was obtained from the Alfred Bader Division of Aldrich and further purified as described previously (14) prior to use. Xanthine was from Sigma, as was L-arginine. 3-Phosphoglycerate was purchased from Sigma and used without further purification. The Reductive Half-reaction of Xanthine Oxidase

The reductive half-reaction of xanthine oxidase at varying concentrations of xanthine and saturating concentrations of O$_2$ (1.25 mM) were obtained following the absorbance increase at 295 nm for the conversion of xanthine to uric acid. As shown in Fig. 1 (squares), the steady-state kinetic parameter $k_{cat}/K_m$, reflecting the second-order rate constant for the reaction enzyme with free substrate in the low substrate concentration regime, is found to exhibit a bell-shaped pH dependence with a maximum at pH 7.0. Fits of the profile to a double-ionization equation (see "Materials and Methods") indicate that an ionizable group having a $pK_a$ of 6.6 must be protonated and that one having a $pK_a$ of 7.4 must be protonated for reaction to take place. The latter $pK_a$ agrees well with that reported for the ionization of xanthine to the monoanion (36), and we conclude that substrate must be in the neutral form for catalysis to be initiated. The group having a $pK_a$ of 6.6 must correspond to a functional group in the active site of the enzyme, possibly that of the Mo=5 group of the molybdenum center itself or a metal-bound water molecule that has recently been established crystallographically (see below).

The pH dependence of $k_{cat}/K_m$, the ratio of the apparent limiting rate constant for the reductive half-reaction to the dissociation constant for xanthine as determined from rapid reaction studies, is also shown in Fig. 1 (circles). The pH dep-
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Fig. 1. pH-dependence of $k_{\text{cat}}/K_m$ (squares) and $k_{\text{red}}/K_d$ (circles) for the reaction of xanthine oxidase with xanthine. Experiments were performed as described under “Materials and Methods.” Data are from the present work (filled symbols) and Ref. 37 (open symbols), the latter data being corrected for the lower oxygen concentrations used (air-equilibrated (250 μM) rather than O$_2$-saturated (1.25 mM) as in the present work). The solid line represents a simulation to the data assuming a double-ionization mechanism (Equation 3, “Materials and Methods”), using $pK_a$ values of 6.6 and 7.4 and a theoretical maximum for $k_{\text{cat}}/K_m$ of $2.2 \times 10^5$ M$^{-1}$ s$^{-1}$. The identical $pK_a$ values were obtained in fits to the pH profile of $k_{\text{red}}/K_d$ using a theoretical maximum of $1.1 \times 10^5$ M$^{-1}$ s$^{-1}$ for this constant. The pH dependence of $K_{\text{red}}/K_d$ is also shown (triangles); fits to the data gave $pK_a$ values of 7.5 and 8.3 and a theoretical maximum of $4.6 \times 10^3$ M$^{-1}$ s$^{-1}$, yielding a solvent kinetic isotope effect on $k_{\text{red}}/K_d$ of 2.4. The value for $k_{\text{red}}/K_d$ determined at pH 7.0 in 9% sucrose (approximating the relative viscosity of D$_2$O) is indicated by the cross; the value corresponds to $5.9 \times 10^3$ M$^{-1}$ s$^{-1}$, which is indistinguishable from the value of $5.8 \times 10^3$ M$^{-1}$ s$^{-1}$ determined in the absence of sucrose.

The pH dependence is identical to that observed for $k_{\text{cat}}/K_m$, as expected given that the reductive half-reaction is known to be rate-limiting in the case of xanthine oxidase (37, 38). The numerical discrepancy between the observed values for $k_{\text{cat}}/K_m$ and $k_{\text{red}}/K_d$ at the optimal pH of 7.0 (1.2 $\times$ $10^5$ M$^{-1}$ s$^{-1}$ and 5.8 $\times$ $10^3$ M$^{-1}$ s$^{-1}$, respectively) is due to the well-established fact that $k_{\text{red}}$ determined from the reaction of enzyme with a pseudo-first-order excess of substrate underestimates the true $k_{\text{red}}$ by a factor of 2 (38) owing to the necessarily sequential nature of the reaction under these conditions (three successive equivalents of substrate are required to bring about full reduction of the enzyme given its complement of redox-active centers).

Given the above evidence for kinetically significant ionizations in the reaction of xanthine oxidase with substrate, it was of interest to determine whether the reaction exhibited a significant solvent kinetic isotope effect. In order to eliminate possible complications owing to any solvent isotope sensitivity of the oxidative half-reaction (which might have rendered this reaction partially rate-limiting), the study was carried out on the reductive half-reaction parameter $k_{\text{red}}/K_d$ rather than the steady-state term $k_{\text{red}}/K_m$. The dependence of $k_{\text{red}}/K_d$ on pH in 99% D$_2$O is shown in Fig. 1 (triangles), where two effects are evident. The first is a shift in the optimal pH/D value from 7.0 in H$_2$O to 7.9 in D$_2$O, as is frequently observed; the $pK_a$ values giving the best fit to the data using a double-ionization scheme are 7.5 and 8.3. The second effect is a decrease in the maximal value for $k_{\text{red}}/K_d$ observed at the optimal pH; a comparison of the values at pH 7.0 and pH 8.0 (5.8 $\times$ $10^3$ M$^{-1}$ s$^{-1}$ and 2.6 $\times$ $10^3$ M$^{-1}$ s$^{-1}$, respectively) indicate a similar kinetic isotope effect on $k_{\text{red}}/K_d$ of 2.4 (a comparison of the maximal theoretical values from the fits to the data yields a comparable value). It should be noted that since the two $pK_a$ values remain equally separated in H$_2$O to D$_2$O, there is no correction necessary for the fact that the maximal value for $k_{\text{red}}/K_d$ is not achieved in either case; in both cases the two $pK_a$ values are spaced so closely together that in each case the maximal observed value for $k_{\text{red}}/K_d$ represents approximately half of the theoretical maximum value were both ionizable groups completely in the appropriate ionization state. Small solvent kinetic isotope effects can in some circumstances be attributed entirely to the difference in solvent viscosity in going from H$_2$O to D$_2$O (39–41). In order to discount this trivial explanation for the present results, $k_{\text{cat}}/K_d$ was determined at pH 7.0 in 9% sucrose, which possesses a relative viscosity comparable with that of D$_2$O (42). The observed value was $5.9 \times 10^3$ M$^{-1}$ s$^{-1}$ (cross in Fig. 1), indicating that the observed solvent kinetic isotope effect was not due to a difference in solvent viscosity.

The pH dependence of $k_{\text{cat}}/K_m$ and $k_{\text{red}}/K_d$ with xanthine as substrate are given in Table I; while the data do not describe a well-behaved profile for a single ionization, it can be seen that there is a general trend toward higher values as the pH is increased from 6.0 to 10.0. One significant difficulty associated with a quantitative interpretation of the $k_{\text{red}}$ data is that the kinetic term is necessarily a composite one, given the overall complexity of the breakdown of the Michaelis complex and subsequent electron transfer to the iron-sulfur and flavin centers of the enzyme (whose reduction is used to follow the reaction of enzyme with xanthine). As discussed below, each step exhibits different pH dependence and solvent isotope sensitivity, making it impossible to quantitatively evaluate the kinetic behavior of the enzyme. It is possible, however, to take advantage of desirable features associated with the reaction of xanthine oxidase with lumazine (2,6-dihydroxypteridine) and 2-hydroxy-6-methylpurine in order to critically assess each of the discrete steps observed in the course of the reaction.

The pH Dependence and Solvent Kinetic Isotope Effect on the Formation and Decay of the Mo(VI)-Violapterin Complex—The reaction of xanthine oxidase with lumazine gives rise to transient absorbance in the 600–700-nm region that has been shown to be due to the Mo(VI)-product complex formed immediately from the Michaelis complex (26). We have taken advantage of this spectroscopic signature to directly monitor formation and decay of this complex as a function of pH. In the first experiment, the reaction of enzyme with varying pseudo-first-order concentrations of lumazine under aerobic conditions was followed at 650 nm in a stopped-flow apparatus. As shown in Fig. 2A, the rate constant for formation of the long wavelength absorbance attributable to the E$_{\text{red}}$-violapterin complex is found to exhibit hyperbolic dependence on the lumazine concentration (consistent with previous results; Ref. 26). $k_{\text{cat}}/K_d$ as determined from the reciprocal of the slope of a plot of 1/[E$_{\text{cat}}$] versus 1/[lumazine] (Fig. 2A, inset) is found to exhibit bell-shaped pH dependence as shown in Fig. 2B; a fit to the data (as described under “Materials and Methods”) gives $pK_a$ values of 6.5 and 7.8. The lower $pK_a$ agrees well with that of 6.6 seen with xanthine, consistent with involving an ionization on the enzyme rather than substrate. The $pK_a$ of 7.8 seen in the lumazine experiment is consistent with the observation that lumazine exhibits a $pK_a$ approximately 0.3 units higher than xanthine. Given the good agreement between the pH dependences of the reductive half-reaction with xanthine and lumazine, it is reasonable to assume that the latter exhibits a solvent isotope effect comparable to that of the former.

In another experiment, an attempt to determine the pH dependence of the reaction of one equivalent of lumazine with enzyme was followed at 650 nm, following the formation and decay of E$_{\text{red}}$-violapterin. While evidence suggesting a base-
catalyzed process was obtained (data not shown), it was found that the amount of absorbance accumulating transiently in the course of the reaction decreased significantly above pH 10 and below pH 6, making it impossible to follow the reaction outside this pH range. (In the case of the formation of the Mo(IV)-violapterin complex, this was not a serious problem given the overall pH dependence of this process and the fact that the Mo(IV)-violapterin complex persisted at the completion of the reaction when performed with a pseudo-first-order excess of violapterin.) In order to more reliably ascertain the pH dependence of the decay of the Mo(IV)-P—violapterin, At completion of the titration the sample exhibited the characteristic long wavelength absorption band arising from the complex of reduced enzyme with violapterin. The enzyme solution was mounted on a stopped-flow apparatus and mixed with concentrated O2-equilibrated, concentrated buffer solution containing concentrated buffer at the desired pH and incubated for a given period of time prior to freezing in liquid N2/acetone, as described under "Materials and Methods." Representative semilogarithmic plots of data obtained in this way are shown in Fig. 4A. A plot of $k_{\text{obs}}$ as a function of pH is shown in Fig. 4B and can be fitted to obtain a pK_a value associated with the break-down of the very rapid EPR signal. Given the poorly defined acid limb of the plot the fit provides only an estimate for the pK_a associated with the decay process, with a value <6. It is nevertheless clear that the decay of the species giving rise to the very rapid EPR signal is an acid-catalyzed process. The observed rate constant for the decay of the species giving rise to the very rapid signal at pH 10 in the present experiment (0.006 s$^{-1}$) is in good agreement with the reported value for $k_{\text{rate}}$ of 0.0085 s$^{-1}$ determined under identical reaction conditions by stopped-flow spectrophotometry.

The pH Dependence and Solvent Isotope Sensitivity of the Formation and Decay of the Very Rapid EPR Signal (Mo(V)-P)—The formation of the Mo(V) species giving rise to the very rapid EPR signal (by oxidation of Mo(IV)-P by one equivalent) and its subsequent decay is most clear-cut in the case of the reaction of enzyme with the substrate 2-hydroxy-6-methylpurine (also called 2-oxo-6-methylpurine). When xanthine oxidase reacts with one equivalent of 2-hydroxy-6-methylpurine, pH 10, at 4°C, approximately 80% of the enzyme molybdenum accumulates at 35 s as the species that gives the very rapid species; both formation and decay of this EPR-active state have been shown to be independent of the concentration of substrate (14). The long time scale of this reaction makes it possible to monitor the rate of decay of the species as a function of pH. In the present experiments, one equivalent of 2-hydroxy-6-methylpurine was mixed with a concentrated aliquot of xanthine oxidase in aerob P, pH 10, at 4°C. After 35 s the reaction mix was transferred via syringe to an EPR tube containing concentrated buffer at the desired pH and incubated for a given period of time prior to freezing in liquid N2/acetone, as described under "Materials and Methods." Representative semilogarithmic plots of data obtained in this way are shown in Fig. 4A. A plot of $k_{\text{obs}}$ as a function of pH is shown in Fig. 4B and can be fitted to obtain a pK_a value associated with the breakdown of the very rapid EPR signal. Given the poorly defined acid limb of the plot the fit provides only an estimate for the pK_a associated with the decay process, with a value <6. It is nevertheless clear that the decay of the species giving rise to the very rapid EPR signal is an acid-catalyzed process. The observed rate constant for the decay of the species giving rise to the very rapid signal at pH 10 in the present experiment (0.006 s$^{-1}$) is in good agreement with the reported value for $k_{\text{rate}}$ of 0.0085 s$^{-1}$ determined under identical reaction conditions by stopped-flow spectrophotometry.

Fig. 5 shows the appearance and disappearance of the very rapid EPR signal in the course of the reaction of 40 $\mu$m functional xanthine oxidase with 20 $\mu$m 2-hydroxy-6-methylpurine (before mixing) at pH 10.0 in H$_2$O and pD 10 in D$_2$O. The rate constants obtained from simulations of the data using the kinetic model $A \rightarrow B \rightarrow C$ (43) with B being the signal-giving species are 0.064 s$^{-1}$ and 0.0083 s$^{-1}$ for the formation and the decay, respectively, of the EPR signal, in good agreement with previously reported values 0.07 s$^{-1}$ and 0.0085 s$^{-1}$, respectively (Ref. 14 and see above). When the reaction is performed in D$_2$O under otherwise identical conditions, the observed rate constants for the formation and the decay of the very rapid EPR signal are 0.032 s$^{-1}$ and 0.0024 s$^{-1}$, indicating substantial solvent kinetic deuterium isotope effects of 2.0 and 3.5 for the formation and the decay of the signal-giving species, respec-
tively. We consider the former value to be in good agreement with the value of 1.7 obtained for decay of the $E_{\text{red}}$-violapterin complex determined above (Fig. 3). Our results indicate that both formation and decay of the signal-giving species involve the motion of solvent-exchangeable protons in the active site in the course of the reaction.

**DISCUSSION**

The present results can be understood in the context of the reaction mechanism given in Scheme 1, which involves formation of a Mo(IV)-P intermediate, its deprotonation concomitant with oxidation by one equivalent to give Mo(V)-P, followed by protonation accompanying the subsequent decay of this latter species. With xanthine as substrate, both $k_{\text{cat}}/K_m$ and $k_{\text{red}}/K_d$ exhibit the bell-shaped pH dependence expected for a double-ionization system, with only the singly ionized form being catalytically active. Fits of the two profiles using Equation 2 (see "Materials and Methods") give $pK_a$ values of 6.5 and 7.8 obtained, along with a theoretical maximum for $k_{\text{red}}/K_d$ of $4.0 \times 10^6$ s$^{-1}$ M$^{-1}$. C, kinetic transients observed for the decay of the preformed $E_{\text{red}}$-violapterin complex at various pH values, performed as described under "Materials and Methods." In each case a small absorbance increase at short time (reflecting the rapid reoxidation of the iron-sulfur centers under the experimental conditions) is followed by a large absorbance decrease due to oxidation of the $E_{\text{red}}$-violapterin complex. The data obtained were at pH 6.5, 7.5, 8.5, 9.0, 9.5, 10.0, 10.5, and 11.0 (slowest to fastest transients, respectively). Also shown are single-exponential fits to the slow phase of the reaction, illustrating the quality of fit to the data; the rate constants thus obtained were $0.31, 0.36, 0.39, 0.42, 0.42, 0.49, 0.75$, and $1.5$ s$^{-1}$, respectively. D, the pH dependence of the rate constants obtained by single-exponential fits to the data shown in Panel C. The solid line represents a fit to the pH profile using Equation 2 from "Materials and Methods"; the best fit was obtained using a $pK_a$ of 11.4, a limiting rate constant at high pH of $4.0$ s$^{-1}$, and a limiting rate constant at low pH of $0.39$ s$^{-1}$. The Reductive Half-reaction of Xanthine Oxidase

**FIG. 2.** Effect of pH on the observed rate constants for the formation and decay of the Mo(IV)-violapterin complex. A, representative rapid kinetic transients for formation of the complex of reduced xanthine oxidase with violapterin, as described under "Materials and Methods." This particular experiment was at pH 8.5, with a functional enzyme concentration of 6.0 $\mu$M. Inset, a double-reciprocal plot of the data giving a slope of 1/(9.9 $\times$ 10$^{-1}$ s$^{-1}$). B, the pH dependence of $k_{\text{cat}}/K_m$, determined from the reciprocal of the slope of a plot of 1/$[\text{lumazine}]$ versus 1/$k_{\text{cat}}$. C, the pH dependence of the Mo(IV)-violapterin complex with $pK_a$ of 7.5 (36), thought to arise from the ionization of its N-1 proton, that is in very good agreement with the observed value of 7.4. The implication is that the protonated (neutral) form of substrate is required for binding to enzyme. The requirement for the protonated form of substrate for catalysis is consistent with a reaction mechanism involving proton abstraction from C-8, as the negative charge of the ionized substrate would be expected to significantly destabilize the accumulating negative charge on C-8 in the course of deprotonation. As discussed below, it is possible that the ionizable group on the enzyme having a $pK_a$ of 6.6 is the Mo(VI)-S group of the oxidized enzyme or alternatively a metal-coordinated water.

On going to D$_2$O, both $pK_a$ values associated with $k_{\text{cat}}/K_m$ increase by approximately one pH unit, and the maximum observed value for $k_{\text{cat}}/K_m$ decreases by a factor of 2.4. This is a
The enzyme and lumazine concentrations correlate with that seen with xanthine (Fig. 1). The apparent primary kinetic isotope effect on a substantial solvent kinetic isotope effect, greater even than the (apparent) primary kinetic isotope effect on $k_{red}/K_m$ of 1.7 that is seen in reductive half-reaction studies using $[8-2H]$xanthine. The clear implication is that a solvent-exchangeable proton is in motion in the course of formation of the Mo(IV)-product complex (the first irreversible step in the catalytic sequence). The chemistry shown in Scheme 1 for the formation of this initial reaction intermediate is predicated on the assumption that the catalytically labile oxygen that is known to be transferred to substrate is the Mo=O. If this is the case, then the regeneration of the Mo=O group subsequent to formation of the C–O bond of product is the most likely process from which the isotope effect arises. The Mo=O must be regenerated (using solvent) in the course of formation of the Mo(IV)-P species, as it has been shown to possess a Mo=O group (29) and the original Mo=O oxygen is now present as a Mo=O–R moiety (28). As shown, this regeneration is envisioned as occurring by incorporation of hydroxide from water, whose deprotonation would reasonably give rise to the observed solvent kinetic isotope effect on formation of the Mo(IV)-product complex. We note, however, that the recent crystal structure of an aldehyde oxidoreductase from Desulfovibrio gigas, which exhibits considerable homology to xanthine oxidase, has provided evidence for a water ligand to the molybdenum (45), and it is possible that this group is directly involved in regeneration of the Mo=O rather than hydroxide from free solvent and/or is responsible for the ionization having a $pK_a$ of 6.6 that is associated with the first step of the overall reaction.

In Scheme 1, formation of the Mo(V)–product complex arises from deprotonation of the carbon to be hydroxylated (C-8 of xanthine or hydroxymethylpurine, C-7 of lumazine) by the Mo=O group, with subsequent (or concerted) nucleophilic attack on the Mo=O oxygen to create the C=O bond of product and simultaneously reduce the molybdenum from Mo(VI) to Mo(IV). This chemistry has precedent in relevant model systems (4-10) and is consistent with all known features of the reductive half-reaction of the enzyme, including evidence for negative charge accumulation in the purine ring in the course of the reaction (46). The basicity of the Mo=O group relative to Mo=O is well established, it being known from x-ray absorption studies that upon reduction of the molybdenum center the Mo=O bond of oxidized enzyme becomes protonated (as evidenced by the elongation of the Mo=O bond from 2.15 to 2.39 Å) in both xanthine dehydrogenase (47) and xanthine oxidase (48). Furthermore, it is known that the proton of the Mo(IV)-SH group is initially derived from substrate but exchanges rapidly with solvent (49, 50). This accounts not only for the observed sensitivity to isotopic label in solvent (present work) but also for the relatively small apparent primary isotope effect on the limiting rate constant for the reductive half-reaction (42). The above notwithstanding, in light of the crystallographic evidence for a water ligand to the molybdenum of the MoOS class of molybdenum enzymes (43), the possibility (first considered by Wedd and co-workers; Ref. 25) must be considered that it is the water ligand rather than the oxo group that represents the catalytically labile oxygen site of the enzyme. The most likely mechanism whereby this group could be incorporated into the hydroxyl group of product is by nucleophilic attack on C-8, followed by hydride transfer of the C8–H to the sulfur of the Mo=O group (resulting in reduction of the molybdenum). In this scenario, the origin of the solvent kinetic isotope effect on $k_{red}/K_d$ would arise from the deuteration of the Mo=O–H2 group required for the oxygen to be an effective nucleophile. Although we prefer the interpretation given in Scheme 1, our results do not rule out this mechanistic alternative for the first step of the catalytic sequence.

The pH dependence of $k_{red}/K_d$ for discrete formation of the Ered-violapterin complex generated transiently in the course of the reaction of enzyme with lumazine is bell-shaped and in good agreement with the above results with xanthine; considerations discussed above for the reaction of enzyme with xanthine can also be invoked in the case of lumazine. On the other hand, the pH dependence for the decay of the Mo(IV)-violapterin complex, as monitored directly at 650 nm, is quite distinct from that for its formation; the data indicate that the process is described by the equation $k_{red}/K_d = k_{red}/K_d^0 e^{-pK_a pH}$, where the rate constant has a slope of $-2.98$ and a $pK_a$ of 9.4. These results were interpreted as reflecting a reaction mechanism in which catalysis was initiated by nucleophilic attack on the carbon center to be hydroxylated, followed by hydride transfer to the enzyme. We note that the same dependence is to be expected if the reaction proceeds via proton abstraction, as the result is the same (negative charge accumulation on the heterocycle ring). We do not exclude the possibility of a mechanism based on nucleophilic attack rather than proton abstraction, and indeed the susceptibility of the C-8 position of purines to nucleophilic attack is well documented. In this work with a homologous series of substituted quinazolines, it was found that the dependence of $k_{red}/K_d$ on the $pK_a$ was linear with a slope of $-2.86$, above a $pK_a$ of 9.4. These results were interpreted as reflecting a reaction mechanism in which catalysis was initiated by nucleophilic attack on the carbon center to be hydroxylated, followed by hydride transfer to the enzyme. We note that the same dependence is to be expected if the reaction proceeds via proton abstraction, as the result is the same (negative charge accumulation on the heterocycle ring). We do not exclude the possibility of a mechanism based on nucleophilic attack rather than proton abstraction, and indeed the susceptibility of the C-8 position of purines to nucleophilic attack is well documented.
base-catalyzed with a pK_a > 11. While we cannot rule out the involvement of active site residue(s) in accounting for the observed pH dependence, a more likely interpretation is that the ionization is due to the Mo-SH group of the Mo(IV)-violapterin complex. A reasonable mechanism for the pH-dependent decay of this intermediate is shown in Scheme 2, in which ionization of the Mo-SH group is responsible for governing the rate of decay of the Mo(IV)-violapterin intermediate. This group is expected to be readily solvent-exchangeable (49, 50), thus accounting for the observed solvent kinetic isotope effect of 1.7 on this step. Because the very rapid EPR signal exhibited by lumazine (and all other substrates of xanthine oxidase) lacks strong magnetic coupling to solvent-exchangeable protons, deprotonation of the Mo(IV)-SH group of the first intermediate in Scheme 1 must accompany its one-electron oxidation to generate the species giving rise to the signal-giving species. The magnitude of the solvent kinetic isotope effect is consistent with such an obligatory deprotonation.

Due to the vanishing small K_m for 2-hydroxy-6-methylpurine (14), no determination of the solvent isotope effect on k_cat/K_m could be made with this substrate. The inability to determine K_m for 2-hydroxy-6-methylpurine is more than compensated for by the ability to follow the specific reaction intermediate that gives rise to the very rapid EPR signal seen with this substrate and also the pH dependence of its decay. The present work indicates that the decay of the Mo(VI)-P intermediate is acid-catalyzed, with an associated pK_a giving the best fit was 3.7, with low pH and high pH limiting rate constants of 7.4 s^{-1} and 0.002 s^{-1}, respectively.

![Figure 4: Effect of pH on the observed rate constant (k_{obs}) for the breakdown of the very rapid EPR signal.](image-url)
pH 10 (2.0) is also consistent with the mechanism shown in Scheme 3.

Below pH 6.0, we suggest that the imidazole portion of the bound heterocyclic product molecule is protonated at N-7 (either directly by solvent or in an acid-catalyzed process involving an active site residue), thereby facilitating product dissociation from the molybdenum coordination sphere. Re-release of the protonated 2,8-dihydroxy-6-methylpurine product gives the correct keto tautomeric form of product and is followed by rapid one-electron oxidation of the paramagnetic molybdenum center by electron transfer to other redox-active centers of the enzyme to give the EPR-silent Mo(VI) oxidation state (38). Dissociation of the neutral form of the heterocycle is expected to be more facile than dissociation of the unprotonated form to give the anionic enolate form of product. The finite rate of breakdown of the signal-giving species at high pH is presumably due to slow oxidation of the Mo(V)-product complex via intramolecular electron transfer rather than dissociation of product from Mo(VI).

In summary, the present work demonstrates that each of the catalytic steps for xanthine oxidase considered herein exhibits distinctive pH dependence. Although it is expected that the specific $pK_a$ values and rate constants obtained will vary from one substrate to another, the overall pH dependence, reflecting acid- or base-catalyzed chemistry for a given step in the reaction, should be the same for all substrates. The observations are entirely consistent with the reaction mechanism shown in Scheme 1, which involves successive deprotonation and re-protonation steps in the formation and decay of the species giving rise to the very rapid EPR signal. It has long been appreciated that protonation/deprotonation events in the coordination sphere of even the simplest molybdenum complexes are tightly coupled to changes in metal oxidation state (51, 52). Deprotonation of a Mo(VI)O(\(\text{OH}\))-viologen species in the course of its oxidation is consistent with this principle. Acid-catalyzed decay of the species giving rise to the very rapid signal observed with the substrate 2-hydroxy-6-methylpurine can be understood from the standpoint that protonation of the coordinated product should destabilize the complex, indirectly facilitating further oxidation of the metal subsequent to product dissociation. The present work demonstrates that there is a kinetic as well as thermodynamic aspect to the phenomenon of linked prototropic equilibria and oxidation-reduction behavior of biological molybdenum centers that in the case of xanthine oxidase profoundly influences the rate constants associated with breakdown of specific intermediates encountered in the course of the catalytic sequence.

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The Reductive Half-reaction of Xanthine Oxidase

The Reductive Half-reaction of Xanthine Oxidase: THE INVOLVEMENT OF PROTOTROPIC EQUILIBRIA IN THE COURSE OF THE CATALYTIC SEQUENCE
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