Charge Transfer Complexes between Pteridine Substrates and the Active Center Molybdenum of Xanthine Oxidase*

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An aerobic addition of lumazine (2,4-dihydroxypteridine) to xanthine oxidase leads to a rapid bleaching of the enzyme with concomitant formation of a long wavelength species with maximum absorbance at 650 nm. At the conclusion of the reaction there is a net increase in absorbance at wavelengths greater than 550 nm. A spectrally similar species is obtained when violapertin is added to dithionite-reduced enzyme. This long wavelength absorbance is not accompanied by an EPR signal characteristic of flavin neutral radical and it is produced equally well in flavin-free xanthine oxidase. Its production is eliminated by the inhibitors, cyanide and allopurinol, which react at the molybdenum center. We conclude that this new species is a charge transfer complex between Mo(IV) and the product of the reaction, violapertin (2,4,7-trihydroxypteridine). A similar, though less intense, absorbance at 650 nm is also obtained when lumazine is bound to the reduced enzyme. These Mo(IV)-pteridine charge transfer compounds are optically active and exhibit intense circular dichroism centered at 630 nm.

The mechanism whereby xanthine oxidase catalyzes the hydroxylation of purines and other heterocyclic compounds has been investigated extensively over the past 25 years and a comprehensive proposal for the sequence of events occurring during the catalytic hydroxylation of xanthine has been published (1). In this sequence xanthine is bound at or near the molybdenum center and converted to uric acid with attendant transfer of a pair of electrons to the metal center which is reduced to Mo(IV). These reducing equivalents are redistributed very rapidly among the Mo, the flavin, and two iron-sulfur centers present in the enzyme (2) according to the redox potentials of these four centers. Product is released, and the enzyme reoxidized by O2 reacting with the reduced flavin.

In this scheme it is proposed that Mo functions as the initial electron acceptor and also participates in the hydroxylation event. Both processes imply the formation of a discrete complex between the substrate and this metal and there are several pieces of data in support of the formation of such a complex: (i) the EPR properties of catalytically significant Mo(V) can vary depending upon the identity of the reducing substrate (3); (ii) reduction of the enzyme with 8-deuteroluxanthine results in the collapse of a proton hyperfine splitting in the EPR spectrum of "fast" Mo(V) (2); (iii) enzyme reduced with allopurinol or other pyrazolo[3,4-d]pyrimidines is activated and new absorbance bands attributed to complexes between Mo(IV) and product are found between 350-500 nm (4).

We now report the formation of intense long wavelength absorbance during the reduction of xanthine oxidase by lumazine (2,4-dihydroxypteridine) and present direct evidence that this band is due to a catalytically important charge-transfer complex between Mo(IV) and violapertin (2,4,7-trihydroxypteridine) i.e. a reduced enzyme-product intermediate.

MATERIALS AND METHODS

Milk xanthine oxidase was prepared from fresh raw cream obtained from Carnation Dairy, Houston, TX. The cream was permitted to warm to 17 °C and 2.5-gallon aliquots churned at this temperature for 1 h using an electric churn (Sears & Roebuck). The butter was removed and the buttermilk (typically 10 liters) processed by the method of Massey et al. (5) modified as follows: occasionally turbidity was present; after the second ammonium sulfate fractionation, this was removed by ultracentrifugation for 40 min at 105,000 g. When the product from the calcium phosphate gel column exhibited a ratio of A350/A440 greater than 5.4, the column chromatography was repeated. Enzyme was stored at 77 °K in 0.1 M sodium pyrophosphate, pH 8.3, containing 0.3 mM EDTA and 1.0 mM sodium salicylate. Prior to use, the salicylate was removed by passing the enzyme down a Bio-Gel P-6 column or by dialysis.

The final product had the value of 5.1-5.4 for A350/A440 and an activity to flavin ratio varying between 80-120. Based on a molar absorbance of 37,000/FAD, the preparation contained 1.07 g atoms of Mo, 4.1 g atoms of Fe, and 4.4 mol of S7. Thus, this preparation contains about 50% inactive enzyme and the normal complement of prosthetic groups. The concentration of native enzyme is given in terms of active sites throughout. Molybdenum was assayed using the published procedure (10) and had no detectable activity with lumazine as substrate.

Deflavo-xanthine oxidase was prepared by the method of Kanda et al. (9) using an all glass apparatus designed to allow the addition of a small excess of dithionite from a syringe to maintain anaerobic conditions throughout. The manipulations were performed in minimal light and the reagents kept on ice. After a 45-min incubation, the flask was opened and the protein solution chromatographed as rapidly as possible on a Bio-Gel P-6 column (24 X 21 cm) using 0.1 M pyrophosphate, pH 8.3, containing 0.3 mM EDTA as the eluant. The pink-brown protein eluate displayed an A440/A350 ratio of 1.9 and conformed to the published spectrum of deflavo enzyme (10). The enzyme oxidase activity was decreased to less than 1% of the starting value. The preparation was quite unstable but could be maintained at 0 °C for 6 h before significant turbidity developed; it could also be stored in liquid nitrogen for 2-3 weeks with only minimal denaturation. Cyanide-inactivated enzyme was prepared using the published procedure (10) and had no detectable activity with lumazine as substrate.

Lumazine was obtained from Aldrich and further purified by the method of Albert et al. (11). It appeared pure in three different chromatographic systems and exhibited the correct elemental analysis, decomposition temperature, ultraviolet, infrared, NMR, and mass spectra. The molar absorbance at 340 nm was determined to be 5750
Viologliptalin was synthesized as follows: 50 mg of purified lumazine was dissolved in 3 liters of 0.1 M triethylamine, pH 8.3, and passed twice down a column (1 x 10 cm) of immobilized xanthine oxidase (13). This process took 2 days. Spectrophotometric measurements established that complete conversion of the lumazine to violapterin occurred. The volume was decreased by rotor evaporation and was dissolved in Butanol (12). This is 15% larger than that reported by Albert et al. These systems and exhibited the correct infrared and mass spectra, and the material lyophilized. The off-white product was recrystallized twice down a column (1 x 10 cm) of immobilized xanthine oxidase is predominantly monophotic when observed at 450, 550 and 610 nm although a slight lag can be observed at long wavelengths (Fig. 1, left). The rate constant of this bleaching is about 14 s⁻¹ and is rapid enough to account for the steady state kinetics (1). By contrast, the anaerobic reduction of the enzyme with lumazine is markedly biphasic when observed at the same three wavelengths (Fig. 1, right). These two phases can be reasonably described by two exponentials with rate constants of 30 s⁻¹ and 0.3 s⁻¹ (Fig. 1, right, inset).

Stoichiometric reagent an hydroxypteridine was of the highest quality obtainable from commercial sources. Stopped flow data were obtained either with a homemade apparatus interfaced to a Nicolet 1060 digital oscilloscope or on line to an Interdata minicomputer, or with a Durrum stopped flow system equipped with the OLIS data acquisition system. The anaerobic reduction of xanthine oxidase by lumazine was monitored for 25 s to minimize the contribution from the reduction of inactive enzyme which has a rate constant of about 0.002 s⁻¹ at pH 8.3, at 25 °C. Stopped flow data were expressed as the sum of either two or three exponentials with the aid of the Marquardt curve fitting procedure detailed by Bevington (15).

EPR samples were obtained using the rapid freeze apparatus of Ballou and Palmer (16); EPR spectra were recorded using a Varian E-6 (X-band) spectrometer using standard conditions (2). Double integrations were performed using an on-line data system with nitrosoyl disulfonate as the standard for molybdenum and flavin radicals, and spinach ferredoxin as the standard for the iron-sulfur centers. Optical spectra were obtained with a Cary 17 spectrophotometer and CD spectra were measured using a Jasco J500C spectropolarimeter equipped with a model DP-400 data processor.

RESULTS

When xanthine oxidase is reacted anaerobically with excess xanthine the bleaching of the enzyme is predominantly monophotic when observed at 450, 550 and 610 nm although a slight lag can be observed at long wavelengths (Fig. 1, left). The rate constant of this bleaching is about 14 s⁻¹ and is rapid enough to account for the steady state kinetics (1). By contrast, the anaerobic reduction of the enzyme with lumazine is markedly biphasic when observed at the same three wavelengths (Fig. 1, right). These two phases can be reasonably described by two exponentials with rate constants of 30 s⁻¹ and 0.3 s⁻¹ (Fig. 1, right, inset).

The sign and amplitude of these two phases are wavelength-dependent (Fig. 1, right). At 450 nm, about one-fourth of the absorbance decrease occurs during the fast phase with the remaining three-fourths decreasing slowly. At 550 nm, there is a small rapid increase in absorbance which is followed by a much larger decrease. At 610 nm, a large increase in absorbance occurs during the fast phase; this is followed by a slow decrease of comparable magnitude yielding almost no net color change for the overall reaction. The oxidized minus reduced difference spectrum for the fast phase is shown in Fig. 2. The maximum changes are observed at 455 nm (Δε = −6500 M⁻¹ cm⁻¹) and 640 nm (Δε = 6400 M⁻¹ cm⁻¹). The difference spectrum for the overall reaction exhibits an absorbance decrease in the range of 400 to 605 nm and only a slight increase.

**FIG. 1.** Comparison of the time course of reduction of xanthine oxidase by xanthine and lumazine. Left, 50 μM xanthine is reacted with 5.4 μM active xanthine oxidase; right, 51 μM lumazine is reacted with 4.7 μM active enzyme. The reactions were carried out anaerobically in 0.1 M pyrophosphate-HCl, pH 8.3, at 25 °C and observations made at 450 (○), 550 (△), and 610 (□) nm. The insets show the data from 450 nm replotted semilogarithmically on appropriate time scales. For the reaction with lumazine, the solid lines represent two exponential fits to the data.
at longer wavelengths. The maximum decrease is at 460 nm ($\Delta e = -27,000$ m$^{-1}$ cm$^{-1}$); the weak long wavelength band ($\Delta e = 1,900$ m$^{-1}$ cm$^{-1}$) is centered at 650 nm.

The $V_{\text{max}}$ of the enzyme with lumazine is 0.7 s$^{-1}$; this value should be compared with the $V_{\text{max}}$ of about 12 s$^{-1}$ obtained with xanthine under identical conditions.$^1$

**The Long Wavelength Band does not Correlate with Flavin Radical**—One striking difference in the reaction of xanthine oxidase with these two substrates is the large increase in absorbance between 600-750 nm which occurs very rapidly with lumazine. By contrast, only a brief lag phase is observed on reaction with xanthine (Fig. 1). The lag observed with xanthine has been interpreted as the consequence of compensating absorbance changes associated with the formation of small amounts of the blue, neutral flavin semiquinone and the partial reduction of the two iron-sulfur centers (5). The balance of the reaction is then taken to be the conversion of the semiquinone to the colorless FADH$_2$ along with the continued reduction of the iron-sulfur centers. This interpretation was supported by EPR data which showed the appearance and disappearance of flavin radical in the expected amounts.

If a similar rationale is applied to the reaction with lumazine, the observed increase in absorbance at 640 nm would require the complete conversion of flavin to the semiquinone during the fast phase, assuming typical extinction coefficients for neutral blue flavosemiquinones (4000-6500 m$^{-1}$ cm$^{-1}$) (17). Rapid freeze EPR experiments (not shown) confirm the formation of flavin radical during the reaction with lumazine and the kinetics of radical formation and decay correlate with the absorbance change at 610 nm. However, integrations of the EPR signals establish that the maximum amount of flavin radical formed is less than 0.06 eq/FAD, which is very much less than that required to account for the absorbance changes observed at 640 nm when lumazine is used as reductant.

This result is supported by experiments in which excess lumazine was reacted with deflavo-xanthine oxidase. The changes in absorbance are again biphasic (Fig. 3) and can again be described by two exponentials, with rate constants of about 60 and 0.45 s$^{-1}$ (Fig. 3, inset). At 450 nm, the fast phase accounts for 40% of the total change with the remainder decreasing at the slower rate. At 650 nm, the fast phase is associated with a large increase in absorbance; this is followed by a slightly smaller decrease during the slow phase so that the final absorbance is a little greater than the initial value.

These observations demonstrate conclusively that the long wavelength absorbance band produced rapidly on reaction of lumazine with xanthine oxidase is not due to flavin. A molybdenum-pteridine charge-transfer band is the most likely alternative cause for the long wavelength absorbance. Molybdenum charge-transfer complexes have previously been implicated to account for new spectral species observed when xanthine oxidase is reacted with alloxanthine and several of its analogs (4, 18) while the iron-sulfur centers are believed to be inaccessible to substrate (1).

**The Charge-Transfer Band Requires Active Molybdenum**—Spectral changes which occur on addition of 1.6 and 3.2 molar equivalents of lumazine to xanthine oxidase are shown in Fig. 4. Immediately following the first addition, there is a large decrease in absorbance at 450-585 nm and an increase in absorbance beyond 585 nm. Over the next several hours the reaction equilibrates and the absorbance decreases uniformly at all wavelengths. The second addition of lumazine causes an immediate decrease in absorbance between 400-530 nm with a concomitant increase between 550-750 nm; again the absorbance decreases uniformly at all wavelengths during the subsequent slow equilibration.

These observations can be interpreted by assuming the charge-transfer band is only formed by enzymatically active, reduced molybdenum. Thus, the first addition is sufficient to fully reduce all of the active enzyme. This produces the large decrease in absorbance between 400-585 nm due to reduction of the flavin and iron-sulfur centers while the increase observed beyond 585 nm is consistent with the formation of the charge-transfer complex. During the subsequent slow phase, electrons equilibrate between the reduced, active enzyme and the oxidized, inactive species with the qualitative effect of oxidizing the active molybdenum and reducing the FAD and FeS components of the inactive enzyme. Consequently, additional bleaching is observed at 450 nm and the charge transfer band is lost. The second addition repeats this series of events yielding a further decrease in absorbance at 450 nm and the initial formation of extra absorbance in the near infrared, part of which disappears during the slow phase.

These results imply that it is the product, violapterin, that interacts with molybdenum(IV) to yield the charge transfer band.

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$^1$ M. Davis, J. S. Olson, and G. Palmer, manuscript in preparation.
band. This idea was tested by examining the reaction of violapeterin with enzyme reduced with excess dithionite; previous titration data (1, 5) imply that the molybdenum is at the Mo(IV) oxidation state in the presence of excess dithionite. When chemically reduced enzyme is titrated with violapeterin, increasing amounts of the long wavelength charge-transfer band are observed with essentially no changes in absorbance in the region of 450 nm (Fig. 5). The difference spectrum produced by the addition of violapeterin is shown in Fig. 6; the absorbance is maximum at 650 nm.

The end-point of the titration was obtained by extrapolating the spectral changes to infinite lumazine concentration. This extrapolation yielded a value of 7400–8600 M⁻¹ cm⁻¹ (Fig. 6) for the total extinction change, the upper limit being set by the 14% loss in activity that occurred during the titration. The data are not readily fit to a simple equilibrium binding process. Although Scatchard plots are linear, the stoichiometry deduced from the graph is close to zero and calculated dissociation constants appear to increase from 2.5–10 μM over the range of violapeterin concentrations employed. This behavior implies that the reaction is complex, but it is difficult to exclude the possibility of a slight contamination with oxygen toward the end of the experiment.

Addition of either lumazine or violapeterin to reduced, cyanide-inactivated enzyme did not produce any changes in the visible spectrum, implying that no charge-transfer complex is formed (Fig. 7a). Cyanide inactivates xanthine oxidase by removal of sulfur which is believed to be in the coordination sphere of the molybdenum. Thus, the lack of any spectral change in this experiment is good evidence that active molybdenum is required for the formation of the charge-transfer absorption.

In a complementary experiment, xanthine oxidase was treated anaerobically with an excess of allopurinol. The resulting optical spectrum was equal to that of partially reduced enzyme complexed with alloxanthine (3). Addition of either lumazine or violapeterin produced no further changes in the visible spectrum (Fig. 7b). On exposure to air, the absorbance increased at all wavelengths, and the spectrum was the same as that previously published for the allopurinol-oxidized-enzyme complex. Subsequent addition of dithionite bleached the enzyme solution and the resultant spectrum was typical of allopurinol-inactivated enzyme with no evidence of additional long-wavelength absorbance. These data again demonstrate that reagents believed to inactivate the molybdenum center eliminate the formation of the charge transfer complex.

**The Kinetics of Reaction of Reduced Enzyme with Violapeterin**—When a large excess of violapeterin is reacted with chemically reduced enzyme in a stopped flow spectrometer, the increase in absorbance at 650 nm cannot be adequately described by a single exponential. A fast phase occurs with a rate constant of 56 s⁻¹ and represents 25% of the total absorbance change while the remaining increase occurs with a rate constant of 3.5 s⁻¹ (Fig. 8).

The rate of the slow phase exhibits very little dependence on the violapeterin concentration even when its concentration approaches that of the enzyme. The proportion of the absor-

![Fig. 6. The difference spectrum obtained after addition of violapeterin to dithionite-reduced xanthine oxidase. The data is that of Fig. 5 and the difference spectrum was obtained by manually subtracting the spectrum of dithionite-reduced enzyme from the final spectrum of the titration after correction for dilution. The absorbance is expressed/mol of active flavin after extrapolation of the absorbance changes to infinite violapeterin concentration.](image)

![Fig. 5. Titration of dithionite-reduced xanthine oxidase with violapeterin under anaerobic conditions. 21 μM total xanthine oxidase was first reduced by the incremental addition of microliter quantities of a strong dithionite solution. When the spectrophotometric changes at 450 nm were complete, the syringe containing dithionite was replaced by a syringe containing 2.6 mM violapeterin. Small increments of violapeterin were then added to the enzyme solution. (The pH of the viapeterin solution had been raised to 12 to ensure solubility and 0.2 mM dithionite included to assist anaerobiosis). The data to the right of the figure was obtained with a 5-fold increase in sensitivity. The inset shows the relative spectral changes at 550 (C), 600 (D), 630 (I), and 750 nm (X) with respect to the changes at 650 nm. The enzyme lost 17% of its activity over the time course of this experiment.](image)
Fig. 7. Effect of violapterin on inactivated xanthine oxidase. 

(a) 18.9 μM cyanide-inactivated enzyme was reduced by the addition of the minimal amount of dithionite solution and violapterin added to a final concentration of 90 μM. Subsequently, the reaction mixture was exposed to air. The absorbance change occurring during the fast phase increases with increasing concentration of violapterin; however, in no case does it exceed 25% of the total change. The binding of violapterin with reduced xanthine oxidase can also be demonstrated by fluorescence measurements (Fig. 8b). Because of its strong intrinsic fluorescence, only low concentrations of violapterin can be used and consequently the signal-to-noise ratio is small. Nevertheless, there is a clear correlation between the spectrophotometric and fluorimetric results. The decrease in fluorescence which occurs when violapterin binds to the reduced enzyme demonstrates that Mo(IV) quenches the fluorescence of this pteridine.

The observation that the rate of binding of violapterin to reduced enzyme is independent of reactant concentration above 30 μM, implies a two-step process. The initial bimolecular step is presumably much more rapid than the subsequent first order transition which exhibits the greatest spectroscopic changes.

The Charge-Transfer Complex Formed between Reduced Xanthine Oxidase and Lumazine—Addition of lumazine to dithionite-reduced xanthine oxidase leads to an increase in absorbance between 500-800 nm with little or no absorbance changes at shorter wavelengths (Fig. 9). This increase in absorbance takes about 20 min to develop fully, but the reaction is stoichiometric implying that the dissociation constant for the reaction is very much less than 10 μM. The resulting difference spectrum has a maximum amplitude at about 650 nm, but the absorption band is narrower than that observed with violapterin. The species responsible for this long wavelength band is not stable and the spectrum reverts to that of the fully reduced enzyme over a period of 2 days. The maximum increase in absorbance observed at 650 nm is small (Δε = 1400 M⁻¹ cm⁻¹).

Optical Activity of the Pteridine-reduced Xanthine Oxidase Charge-Transfer Complex—The circular dichroism spectra of resting and fully reduced xanthine oxidase is dominated by the optical activity of the iron-sulfur centers, there being no obvious difference in the CD spectra before or after removal of flavin from the enzyme (10). The intensity and shape of these spectra are consistent with the presence of two 2Fe-2S centers. Addition of either lumazine or violapterin to reduced enzyme produces marked changes in the CD spectrum (Fig. 10). With violapterin in particular, the changes are largest in the 600-700 nm region with an extremum at 630 nm close to the maximum in the absorbance difference spectrum.
The only well documented optical species previously assigned to molybdenum is observed in the interaction of Mo(IV) with alloxanthine (4). The ensuing absorbance bands occur between 400–500 nm, and overlap the corresponding spectra of both iron-sulfur centers and the flavin and thus can only be observed after these latter spectral components are

zine to dithionite-reduced enzyme (Fig. 10), also arise from a charge-transfer species, the Mo(IV)-lumazine complex in this instance. Enzyme reduced with xanthine has a CD spectrum identical to that observed with dithionite (19).

The Effect of Substitution in the Pteridine Ring on the Formation of the Long Wavelength Band—The following pteridines were added to dithionite-reduced xanthine oxidase at about 50 μM: 2,4-dihydroxy-7-methyl-pteridine, 2-amino-4-hydroxypteridine (pterin), 2-amino-4,6-dihydroxypteridine (xanthopterin), 2-amino-4,7-dihydroxypteridine (isoxanthopterin), and 2-amino-4,6,7-trihydroxypteridine (leucopterin). In no case was a significant stable increase in absorbance observed between 600–700 nm. However a long wavelength species can be observed when pterin and xanthopterin are reacted anaerobically with xanthine oxidase in the stopped flow apparatus (Table I). The time course of the resultant spectrophotometric changes are qualitatively similar to those observed with lumazine (Fig. 1). In particular, there is a rapid increase in absorbance at 650 nm with an associated extinction change of 3700 M⁻¹ cm⁻¹. Similar data (not shown) have been obtained with deflavo enzyme.

**DISCUSSION**

The characterization of the molybdenum center of xanthine oxidase is routinely achieved using electron paramagnetic resonance spectroscopy and a wealth of valuable spectral and kinetic data has been obtained on a variety of species using this approach (3). The principal limitations of this technique are that it is restricted to Mo(V) (Mo(III) is observable by EPR but it is questioned whether this lower valence is acces-

Fig. 9. Anaerobic titration of dithionite-reduced xanthine oxidase with lumazine. 23 μM total xanthine oxidase (activity to flavin ratio = 117) was reduced with small increments of dithionite solution until the absorbance changes at 454 nm were complete. Small portions of 2.4 mM lumazine up to a maximum of 1.4 mol of lumazine/mol of flavin were then added. The inset shows the difference spectrum obtained by manually subtracting the spectrum of fully reduced enzyme with that obtained after the addition of 1.4 molar equivalents of lumazine, expressing the change in molar absorbance/mole of active enzyme. All of the absorbance at 454 nm was restored on reoxidation with air.

Fig. 10. Circular dichroism spectra of the charge-transfer species. Top, absolute CD spectra of resting xanthine oxidase, enzyme reduced by dithionite and reduced enzyme to which 74 μM violapterin has been added. The inset shows resting enzyme on a compressed scale. The experimental conditions are identical to those of Fig. 6. Bottom, difference CD spectra of dithionite-reduced enzyme-violapterin complex minus dithionite-reduced enzyme, and dithionite-reduced enzyme lumazine complex minus dithionite-reduced enzyme. The lumazine data was obtained as part of the experiment described in Fig. 9. This correspondence in wavelength maximum in the near infrared is strong evidence for assigning this CD band to the molybdenum-violapterin complex and implies that the newly developed CD bands present at other wavelengths also originate from this charge transfer species. By extension we conclude that the CD changes observed on addition of lumazine to dithionite-reduced enzyme (Fig. 10), also arise from a charge-transfer species, the Mo(IV)-lumazine complex in this instance. Enzyme reduced with xanthine has a CD spectrum identical to that observed with dithionite (19).

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The characterization of the molybdenum center of xanthine oxidase is routinely achieved using electron paramagnetic resonance spectroscopy and a wealth of valuable spectral and kinetic data has been obtained on a variety of species using this approach (3). The principal limitations of this technique are that it is restricted to Mo(V) (Mo(III) is observable by EPR but it is questioned whether this lower valence is accessible in this enzyme (3)) and the measurements are most commonly made at very low temperatures in the frozen state. This has several inherent disadvantages. 1) The possibility of pH changes during the freezing process with consequent modification in the EPR characteristics (20); 2) possible variations of redox potentials with temperature leading to changes in electron distribution patterns (21); and 3) the requirement of rapid freezing techniques for kinetic studies; these are both more cumbersome and less precise than, say, conventional stopped flow spectroscopy.

The only well documented optical species previously assigned to molybdenum is observed in the interaction of Mo(IV) with alloxanthine (4). The ensuing absorbance bands occur between 400–500 nm, and overlap the corresponding spectra of both iron-sulfur centers and the flavin and thus can only be observed after these latter spectral components are
accounted for. It is possible that this Mo(IV)-allopurinol spectral species does arise during catalytically relevant times, but is masked by the other large spectral changes occurring simultaneously, and consequently is not readily exploited as a tool in elucidating the mechanism of this enzyme. Thus, the discovery of a charge-transfer complex between molybdenum and certain pteridine substrates, notably lumazine, which lead to quite intense absorption bands at long wavelengths where xanthine oxidase exhibits only a weak intrinsic absorption, should substantially facilitate the study of the interaction of this metal with its substrates.

The evidence that this stable charge-transfer complex is associated with the molybdenum is 2-fold. First, inactivation of this center by either cyanide or allopurinol renders the enzyme incapable of forming this complex. Second, removal of the flavin from xanthine oxidase has essentially no influence on either the rate of formation or spectral characteristics of the charge-transfer species. Since there is no reason to believe that the iron-sulfur centers will form a complex with substrate or product, we conclude that the long wavelength charge-transfer absorbance is due to an interaction between molybdenum and pteridine, analogous to the previously reported complex of xanthine oxidase with its substrates. The isothionic bond of the molybdenum redox system should substantially facilitate the study of the interaction of this metal with its substrates.

There are several similarities between the charge-transfer complexes formed with violapterin and alloxanthine. In both cases the mode of reduction appears unimportant and the appropriate charge-transfer complex can be formed on mixing either parent substrate with resting enzyme or their respective products with dithionite-reduced enzyme.

Both complexes are formed with active enzyme. However, the Mo=O coordination which appears to be the site of cyanide is necessary for binding of violapterin and a modification of the molybdenum center such as Mo=S to Mo=O (22) appears to render the enzyme incapable of reacting with this compound. However alloxanthine does bind to cyanide-inactivated xanthine oxidase (23).

On the other hand, there is at least one significant difference between these two types of complexes. The reaction of the reduced enzyme-allocanthine species with O$_2$ is extremely slow ($t_{1/2} = 5.5$ h). In this reaction, the flavin and iron-sulfur centers are reoxidized rapidly but the oxidation of the Mo(IV) allocanthine complex is slow although both ferricyanide and phenazine methosulfate facilitate this process (3). By contrast, all of the functional groups are readily reoxidized when the reduced xanthine oxidase-violapterin complex is reacted with oxygen. Evidently, allocanthine but not violapterin, substantially raises the potential of the molybdenum redox system such that in enzyme containing 1-2 electrons the fraction of fully reduced flavin is extremely small (1). Because oxygen reacts predominantly if not exclusively with reduced flavin, the removal of the final two electrons occurs very slowly in the presence of alloxanthine.

Complete characterization of the kinetics and equilibrium of formation of stable charge-transfer complex has proved to be difficult. Although the equilibrium process appears to saturate, it cannot be described by a unique dissociation constant and the kinetics of binding appear to require a minimum of two steps to explain the absorbance and fluorescence changes that are observed. This complexity can have several explanations.

First, the reduction of xanthine oxidase by dithionite is not well understood. Eight electrons are consumed in this reaction while only six are readily accountable by the four prosthetic groups. The fate of these additional two electrons is controversial. Massey (25) has postulated the conversion of Mo(VI) to Mo(III) while Bray (24) has invoked the reduction of a disulfide: the addition of these last two electrons is associated with the development of the "slow" molybdenum EPR signal although this is probably the result of reduction of molybdenum in nonfunctional enzyme. Thus, reduction by dithionite is complex and yields a heterogeneous population of reduced enzyme within times that are acceptable for experimental manipulation (e.g. 24 h). Second, the kinetic complexity might represent alternative modes of binding of the ligand to the molybdenum. The long known observation that xanthine oxidase can oxidize purine→hypoxanthine→uric acid in discrete steps (25) implies several acceptable orientations of the purine with respect to the molybdenum. This interpretation is supported by the demonstration by EPR of two discrete complexes of xanthine with partially reduced enzyme (3). Third, it is possible that on prolonged incubation, lumazine reacts with the slight excess of dithionite present in the reaction mixture to yield tetrahydro-derivatives. Such reactions have been obtained on boiling lumazine for 2 min in 0.1 M NaOH and 0.25 M dithionite (26).

Although the reaction of both violapterin and lumazine with xanthine oxidase is complicated, it is clear that each heterocycle reacts directly with molybdenum producing a high affinity charge-transfer complex. The formation of this complex leads to intense absorbance and circular dichroism bands between 550-700 nm. In addition, both lumazine and violapterin are fluorescent, and this fluorescence is partially quenched on reaction with the enzyme. Thus, we have a system of considerable potential for the investigation of the reaction of the hydroxylolation process catalyzed by the molybdenum center of xanthine oxidase since many of the enzyme species involved in the reductive half-reaction can be visualized spectrophotically.

Note Added in Proof—R. Hille and V. Massey (1982) J. Biol. Chem. 257, 8898-8901 have recently provided direct evidence that dithionite reduces a disulfide bond in xanthine oxidase.

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