The State of Reduction of Molybdopterin in Xanthine Oxidase and Sulfite Oxidase*

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Methods have been devised to examine the spectral properties and state of reduction of the pterin ring of molybdopterin (MPT) in milk xanthine oxidase and the Mo-containing domain of rat liver sulfite oxidase. The absorption spectrum of the native pterin was visualized by difference spectroscopy of each protein, denatured anaerobically in 6 M guanidine hydrochloride (GdnHCl), versus a sample containing the respective apoprotein and other necessary components. The state of reduction of MPT was also probed using 2,6-dichlorobenzenoneindophenol (DCIP) to measure reducing equivalents/MPT, after anaerobic denaturation of the protein in GdnHCl in the presence or absence of Hg²⁺. In the case of xanthine oxidase the data indicate that the terminal sulfide ligand of Mo causes the reduction of a native dihydride form of MPT to the tetrahydride level. This reduction does not occur if Hg²⁺ is added prior to denaturation of the protein. Based on its observed behavior, the native MPT in the Mo cofactor of xanthine oxidase is postulated to exist as a quinonoid dihydroypterin.

Quantitation of DCIP reduction by MPT of Mo fragment of sulfite oxidase showed a two-electron oxidation of MPT, even when the Mo fragment was denatured in the presence of Hg²⁺ to prevent internal reduction reactions due to sulfhydryls or sulfide. Difference spectra of DCIP-treated versus untreated Mo fragment showed that MPT had been fully oxidized. These data indicate that the native MPT in sulfite oxidase must be a dihydro isomer different from that in xanthine oxidase.

Many of the structural aspects of the Mo cofactor (1, 2) have only recently been revealed through studies of its biological derivative, urothione (3), two oxidized degradation products, form A and form B (4, 5), and a carboxamidomethyl derivative (6). One feature which has still not been investigated is the native state of reduction of the MPT ring, which was presumed to exist as a tetrahydride form primarily on the basis of the extreme air lability of the Mo cofactor upon its release from protein (3). Since the Mo cofactor appears to be a universal element of molybdoenzymes (with the exception of nitrogenase), it is of importance to determine if MPT exists in the same state of reduction in every molybdoenzyme. The oxidation-reduction potentials of the Mo centers in molybdoenzymes span a range of approximately 700 mV (7), but the factors responsible for such wide variation are not known. Since MPT, like any other pterin, has several available states of reduction with limited air stability, such as tetrahydro, 5,6-dihydro, 6,7-dihydro, 5,8-dihydro, and quinonoid dihydro, the existence of different states in molybdoenzymes could be postulated to explain these differences. This possibility would derive credence if differences in the oxidation state or form of MPT in any two molybdoenzymes with differing potentials can be demonstrated.

Rat liver sulfite oxidase and milk xanthine oxidase were selected for the studies reported here because the oxidation-reduction potentials of the Mo centers in the two enzymes differ by nearly 400 and 200 mV for the MoVI → MoV and MoV → MoIV transitions, respectively (7). Therefore, if no difference in the state of reduction of MPT is found between these enzymes, then a role for MPT in poising Mo center oxidation-reduction potentials could be discounted.

Another reason for studying the state of reduction of MPT is to address the possible role of MPT in the catalytic activity of molybdoenzymes. Some nonfunctional forms of molybdoenzymes, including those which occur naturally and those which can be produced chemically, may be inactive as a result of alterations in the state of reduction of MPT. Xanthine oxidase is one enzyme which exists, as purified, in active and inactive forms. Determining the state of reduction of MPT in these forms is clearly an important first step in understanding the possible role of MPT in the catalytic activity.

The extreme instability of the Mo cofactor has made it impossible to isolate the native molecule from protein for direct structural studies. Therefore, it became necessary to design less direct methods for determining the state of reduction of MPT. Obtaining the absorption spectrum of MPT could reveal its state of reduction, since pterins in various states of reduction do exhibit distinct spectra. However, spectral studies of MPT in an intact molybdoenzyme are obviated by the presence of one or more additional strong chromophores, such as flavin, heme, and Fe/S centers, which totally mask the comparatively low absorbance of MPT. Even the weak chromophore formed by Mo and its thiol ligands supersedes the absorption spectrum of MPT (8). The absorption spectrum of MPT cannot be revealed by subtraction of such chromophores from the spectrum of a native molybdoenzyme because each interacts with the protein in ways which influence the overall spectrum. Thus, the summation of the absorbance due to all chromophores is not equivalent to the intact spectrum.

To circumvent these problems, we have devised a technique...
for examining the absorption spectrum of MPT anaerobically released but not isolated from the enzyme components. The technique involves denaturation of the enzyme so that the interfering chromophores are destroyed or can be subtracted by difference spectroscopy. We have also developed a method for detecting reduced forms of MPT under similar denaturing conditions by using 2,6-dichlorobenzeneindophenol (DCIP), which is known to oxidize tetrahydropterins (9). In spite of the presence of other prosthetic groups, this method can accurately quantitate the amount of DCIP-oxidizable pterin under a variety of experimental conditions. The results obtained with xanthine oxidase and sulfite oxidase are presented here.

**EXPERIMENTAL PROCEDURES**

**Enzymes**

Xanthine oxidase was purified from bovine milk by the procedure of Wood et al. (10). The enzyme was separated into fully active, MPT-free, and low activity fractions using the Sepharose 4B/folate affinity chromatography procedure detailed elsewhere (11). Apoprotein of xanthine oxidase for difference spectroscopy was prepared by denaturation of MPT-free xanthine oxidase in 6 M guanidine hydrochloride (GdnHCl), followed by gel filtration on a PD-10 column (Bio-Rad) equilibrated with 6 M GdnHCl, pH 7.0, in order to separate flavin and other small molecules from the apoprotein. Xanthine/O2 activity was assayed spectrophotometrically by monitoring the increase in absorption at 295 nm due to uric acid production. Reaction buffer contained 0.1 mM xanthine, 0.1 mM pyrophosphate, and 0.2 mM EDTA at pH 8.3, 25 °C. Specific activity is expressed as AFR (activity-to-flavin ratio), which is defined as the absorbance change/min at 295 nm divided by the enzyme absorbance at 450 nm.

Sulfite oxidase was purified from rat livers and chicken livers as described previously (12, 13). Further purification was achieved using a Sepharose S-300 column or a Pharmacia PPLC Superose-12 column. Mo fragment of rat liver sulfite oxidase was isolated after cleavage of the 10,000 M, heme-containing domain with trypsin, as previously detailed (9). The Mo fragment was purified by FPLC on a Superose-12 column in 50 mM potassium phosphate, pH 7.8. The Mo fragment concentration was determined by organic phosphate analysis following the procedure of Ames (14), assuming that each Mo fragment dimer contains two phosphorylated Mo cofactors. The Mo fragment was dialyzed in 50 mM Tris buffer, pH 7.5, prior to phosphate analysis. Apoprotein of Mo fragment for use in difference spectroscopy was isolated by anaerobic denaturation of Mo fragment in 6 M GdnHCl containing 10 mM dithiothreitol (DTT). The denatured protein was then gel-filtered on a PD-10 column in the same buffer. In order to effect complete removal of molybdopterin and interfering chromophores to the reference cuvette was not needed in this case.

**Difference Spectroscopy**

The difference spectrum of MPT in xanthine oxidase was obtained using a solution of apoprotein of xanthine oxidase in 6 M GdnHCl, pH 7.0, 1 mM EDTA, and an appropriate amount of FAD as a reference against an anaerobic solution of active xanthine oxidase denatured in 6 M GdnHCl and 1 mM EDTA, pH 7.0. The GdnHCl solution was made anaerobic utilizing a cuvette with a sidearm and an adjustable stopcock designed so that N2 could be bubbled through the solution for 30 min before sealing the cuvette and mixing the enzyme in the cuvette sidearm with the solution. Prior to placement in the sidearm, the enzyme was made anaerobic in a rubber-stoppered vial by repeated evacuation and flushing with N2. The prepurified N2 was deoxygenated by passage through a column of heated dispersed copper pellets (Catalyst R3-11, Chemalog). Absorption and difference spectra were obtained using a Shimadzu UV240 or UV260 spectrophotometer.

The difference spectra of denatured Mo fragment of sulfite oxidase versus its apoprotein were obtained similarly, except that additions of other chromophores to the reference cuvette was not needed in this case.

**DCIP Reduction Experiments**

The amount of DCIP reduced in any experiment was calculated from the decrease in absorbance at 600 nm using a molar extinction of 20,000, which was determined by measuring the decrease in absorbance of 600 nm due to addition of DCIP to an anaerobic solution of 50 mM potassium phosphate, pH 7.0, containing 32 mM xanthine oxidase and 20 μM DCIP. DCIP reduction experiments were designed as follows.

1) Xanthine Oxidase Model System—The model system designed to mimic xanthine oxidase contained 0.9 ml of 7.2 M GdnHCl at pH 7.0 and 45 μl of 1.2 M potassium phosphate at pH 9.28 in a stoppered cuvette which was made anaerobic by repeated evacuation and flushing with N2 for 20 min. To the cuvette, 50 μl of 25 mM mercuric chloride and 11 μl each of 1 mM FAD, 1 mM sodium molybdate, 1 mM DTT, and 0.5 mM spinach ferredoxin were added from anaerobic stock solution vials using gas tight Hamilton syringes. Following the addition of 25 μl of 1.87 mM DCIP, the OD600 was measured. Next, tetrahydrobiopterin (H4B) or 7,8-dihydrobiopterin (H5B) was added to a final concentration of 10 μM. The change in absorbance in 15 min was recorded.

2) Xanthine Oxidase System—DCIP reduction by MPT released from xanthine oxidase was measured in a stopped cuvette containing an anaerobic solution of 6 M GdnHCl and either 50 mM potassium phosphate or 100 mM Tris-HCl, pH 7.0. Xanthine oxidase (160-200 μg) was added anaerobically by syringe to yield a final enzyme concentration of 3.5-5.0 μg/ml. Allowing 5 min for equilibration, 1.28 μM H2O2 was added to the GdnHCl reaction mixture (described for the xanthine oxidase system) after denaturation of the enzyme. After 2 min, 25 μl of 1.87 mM DCIP was added, yielding a typical final volume of 1.175 ml. The OD600 was recorded after 15 min. The initial OD600 control value was determined in experiments where xanthine oxidase was replaced with buffer in the reaction cuvette. The control absorbance minus the absorbance resulting after reduction of DCIP by MPT was used to calculate the % of expected DCIP reduction, which is the ratio of the change in OD600 seen versus the change expected from a two-electron oxidation of the known MPT concentration. Enzyme and pterin concentrations in these experiments were selected to give a change in OD600 of 0.10-0.20.

3) Sulfite Oxidase System—The DCIP reduction by MPT released from rat and chicken liver sulfite oxidase or from Mo fragment was measured in the same manner as the procedure described for xanthine oxidase. A model system for the denaturation of rat and chicken liver sulfite oxidase contained 3.7 μM hemoglobin and 10 μM H4B or H5B in place of enzyme.

4) Other Systems—The sulfide addition experiments were conducted by adding the conditions described for the xanthine oxidase system by adding 50 mM Tris-HCl, pH 7.5, to the reaction mixture. After 5 min, 25 μl of 1.87 mM DCIP was added to oxidize the MPT, followed by 5 min by 10 μl of 10 mM DTT to react with the excess DCIP and to reduce the oxidized MPT. After 5 min, excess mercuric chloride (25-50 μl) was added, to bind any unreacted DTT. Finally, a second addition of DCIP was made anaerobic utilizing a cuvette with a sidearm and an adjustable stopcock designed so that N2 could be bubbled through the solution for 20 min before sealing the cuvette and mixing the enzyme in the cuvette sidearm with the solution. Prior to placement in the sidearm, the enzyme was made anaerobic in a rubber-stoppered vial by repeated evacuation and flushing with N2. The prepurified N2 was deoxygenated by passage through a column of heated dispersed copper pellets (Catalyst R3-11, Chemalog). Absorption and difference spectra were obtained using a Shimadzu UV240 or UV260 spectrophotometer.

**Cyanide Treatment**

Xanthine oxidase was incubated aerobically with 10 mM KCN in 50 mM potassium phosphate, pH 7.5, for 1 or 4 h at room temperature in greater than 95% loss of xanthine oxidase activity. The enzyme was then passed through a PD-10 gel filtration column to remove the cyanide prior to measuring the extent of DCIP reduction using the standard conditions outlined for the xanthine oxidase system.
Molybdopterin in Xanthine Oxidase and Sulfite Oxidase

Xanthine oxidase was denatured anaerobically in a solution of 6 M GdnHCl and 2 mM EDTA, pH 7.0, in two rubber-septum-stoppered cuvettes for which a spectral base line had been recorded. After denaturation of the enzyme in each cuvette, potassium phosphate buffer was added to give a final concentration of 50 mM, followed by mercaptoethanol to 0.5 mM. To the sample cuvette, 40 μM DCIP was added, followed in 5 min by DTT to 0.4 mM in order to bleach excess DCIP. To the reference cuvette, a premixed solution of DCIP and DTT was added. Although both cuvettes contained equal concentrations of these reagents, the MPT in the reference cuvette was never exposed to oxidized DCIP. The difference spectrum was then recorded. An identical procedure was used for the Mo fragment of rat sulfite oxidase except that the concentration of mercaptoethanol was 0.1 mM.

Materials

Ultrapure guanidine hydrochloride was obtained from Schwarz/Mann. Sodium 2,6-dichlorobenzenoindophenol was from Fisher. Tetrahydrobiopterin, 7,8-dihydrobiopterin, and biopterin were purchased from Dr. B. Schircks Laboratories, Jona, Switzerland. Pterin concentrations were calculated based on an εmax of 8710 M⁻¹ cm⁻¹ for tetrahydrobiopterin (15), an εmax of 6200 M⁻¹ cm⁻¹ for 7,8-dihydrobiopterin (16), and a measured εmax of 6480 M⁻¹ cm⁻¹ for biopterin. Spinach ferredoxin was a gift from Dr. Henry Kamin (Duke University). Trypsin and hemoglobin (bovine blood) were purchased from Sigma.

RESULTS

Difference Spectroscopy

Fig. 1 shows the absorption spectrum of active xanthine oxidase (A) and the changes which occur upon anaerobic denaturation in 6 M GdnHCl. This treatment destroys the Fe/S centers and the Mo ligand field, leaving visible only the absorption bands of FAD and protein (B). Addition of stoichiometric FAD to the reference cuvette produced a difference spectrum dominated by the large absorbance due to protein but with virtually no absorbance beyond 340 nm (C). EDTA was included to abolish charge transfer complexes which arise in such concentrated solutions. Even at this stage, it was clear that no oxidized pterin was present, since a typical oxidized pterin; therefore, such apoprotein could not be used for difference spectroscopy. The top panel of Fig. 2 shows the typical difference spectrum obtained under these conditions. Comparison of the spectrum to spectra of biopterin in various states of reduction (middle panel) indicated that MPT in this system was neither in an oxidized nor in the stable 7,8-dihydro form. In control experiments with 7,8-H₂B and apoprotein, no spectral distortion of H₂B due to high protein concentration was seen, and no reduction of H₂B to H₂ was ever detected.

The spectral study of MPT in rat liver sulfite oxidase was simplified by removing the interfering chromophore, heme, by tryptic cleavage of the enzyme to yield Mo fragment which is easily separated from the heme-containing domain. The only chromophores other than protein in the Mo fragment are MPT and the chromophore created by Mo ligation. Thus, the subtractive process in difference spectroscopy of Mo fragment was much less complicated than that required for xanthine oxidase. The absorption difference spectrum of Mo fragment anaerobically denatured in 6 M GdnHCl versus absorption band, apoprotein which had been isolated from MPT-free xanthine oxidase by removal of the FAD and other small molecules using gel filtration in 6 M GdnHCl was also included in the reference cuvette. Similar attempts to isolate apoprotein from active xanthine oxidase always yielded apoprotein contaminated by tightly bound or covalently attached oxidized pterin; therefore, such apoprotein could not be used for difference spectroscopy. The top panel of Fig. 2 shows the absorption difference spectrum of MPT in the reference cuvette. The A₅₀ of spectrum A is approximately 0.22.

Fig. 1. Difference spectroscopy procedure for examining MPT in denatured xanthine oxidase. Shown are the absorption spectra of 2.96 μM xanthine oxidase with AFR = 136 in A, 50 mM potassium phosphate buffer and 0.2 mM EDTA at pH 7.0; B, anaerobic 6 M GdnHCl and 1 mM EDTA at pH 7.0, and C, as in B, but with the calculated amount of FAD (5.2 μM) added to the GdnHCl blank. Spectrum A is truncated in order to enhance the features of the denatured enzyme in curves B and C. The A₅₀ of spectrum A is approximately 0.22.

Fig. 2. Top panel, the absorption difference spectrum of MPT in xanthine oxidase, obtained by recording the difference spectrum of an anaerobic solution of 2.33 μM xanthine oxidase (AFR = 184) in 6 M GdnHCl and 1 mM EDTA, pH 7.0, versus a reference containing apoprotein of xanthine oxidase and 4.66 μM FAD in 6 M GdnHCl and 1 mM EDTA, pH 7.0. Middle panel, absorption spectra of A, 12.3 μM biopterin; B, 10.1 μM 7,8-dihydrobiopterin; and C, 10.6 μM tetrahydrobiopterin, in 6 M GdnHCl at pH 7.0. Spectrum C was obtained under anaerobic conditions. Bottom panel, the absorption difference spectrum of MPT in Mo fragment of rat liver sulfite oxidase, obtained by recording the difference spectrum of an anaerobic solution of 15 μM Mo fragment in 6 M guanidine hydrochloride and 1 mM EDTA, pH 7.0, versus a reference containing apoprotein of Mo fragment in the same buffer.
denatured apoprotein demonstrates that native MPT in sul-
fite oxidase is also not in the fully oxidized or 7,8-dihydro-
state (Fig. 2, bottom panel). Although a shoulder in the spec-
trum is consistently found in the 350-390 nm region, the
absorbance of this feature is not sufficient to account for the
presence of an oxidized pterin, which would show an absorb-
ance of 0.2-0.3 at this pterin concentration. The significance
of the shoulder in the difference spectrum is unclear, but it
was reproducibly observed. Thus, it might be a real spectral
feature of the MPT form in sulfite oxidase.

The main feature of both difference spectra is an absorp-
tion band near 300 nm, indicating that MPT may be in a tetra-
hydro state, or in an unstable dihydro form with spectral
properties similar to a tetrahydropterin. Documented exam-

DCIP Reduction

**Xanthine Oxidase**—Since it was possible that internal redox
reactions in the denatured enzyme solution might alter the
native state of MPT, a method was devised in which DCIP
was used to quantitate the amount of tetrahydropterin present
under conditions where such reduction reactions could be
eliminated. As a control, a system modeling the environment of
denatured xanthine oxidase in 0 M GdnHCl was generated
by adding FAD, Fe/S centers (in ferredoxin), DTT, and H2B
to an anaerobic 6 M GdnHCl solution. The concentrations of
FAD, H2B, and ferredoxin were equal to the concentrations
of FAD, MPT, and Fe/S centers present in a xanthine oxid-
ase/GdnHCl solution. Reaction of free sulfides with DCIP was
completely abolished by Hg2+ addition. The amount of DCIP reduced by a known pterin concentra-
tion was measured. As expected, H2B showed nearly stoichi-
ometric 2e- reduction of DCIP (90% of expected), whereas
H2B was unreactive (3%). Thus, the method was able to
accurately detect H2B despite the complexity of the reaction
mixture.

For MPT in xanthine oxidase, DCIP reduction was first
used to confirm the tetrahydro state of reduction seen by
difference spectroscopy when internal reduction reactions
were not prevented. Hg2+ was added to the GdnHCl/enzyme
solution after denaturation had occurred. The extents of
DCIP reduction obtained in this manner using xanthine ox-
idase of different AFR values are shown in Fig. 3. The data
reveal a linear relationship between the AFR of the xanthine
oxidase, which is a measure of the fraction of active molecules,
and the % of DCIP reduction seen. A control experiment was
performed where enzyme was denatured aerobically overnight
to permit full air oxidation of MPT, then DCIP reduction was
measured following Hg2+ addition. No DCIP reduction was
detectable, indicating not only that the pterin was responsible
for the DCIP reduction anaerobically, but also that Hg2+ was
able to fully block all protein sulfhydryls and sulfide. If Hg2+
was omitted, over 1900% of the expected DCIP reduction was
described as stoichiometric 2e- reduction of DCIP (90% of expected). An explanation for the higher than expected values became evi-
dent when DCIP reduction by MPT-free xanthine oxidase
was measured. Since this form has been shown to lack Mo
cofactor (11), no reduction was expected, yet 36% reduction
was seen, very likely representing some reducing species, such as
Fe(II), which exists transiently in the complex-denatured
solution. DCIP reduction by MPT-free xanthine oxidase
which had been denatured aerobically overnight was minimal,
supporting the existence of a transient species. It is very likely
that solutions of active enzyme contain the species as well.
Subtracting 36% from the reduction value obtained for active
enzyme gives 92% of the DCIP reduction expected for a 2e-
oxidation of MPT, suggesting that MPT might be a tetrahy-
dropterin under these conditions.

An explanation for the linear relationship of AFR to % of
expected DCIP reduction is that the terminal cyanosulfur on the Mo in the Mo cofactor is responsible for the reduction of a dihydro form of MPT to the tetrahydro state
upon denaturation. The AFR of xanthine oxidase does de-
crease as the amounts of inactive desulfo and demolybdo
forms increase; thus, most of the MPT released from enzyme
with low AFR would remain in the native dihydro state of
oxidation in the absence of the concomitant release of the
terminal sulfide.

To eliminate sulfhydryls or free sulfide as possible internal
sources of MPT reduction, the experiment was modified so
that Hg2+ was present during, rather than being added after,
denaturation of xanthine oxidase of varying AFR, prior to
assaying DCIP reduction. As shown in Fig. 3, any reduction
above the basal 36% is virtually totally abolished, indicating
that Hg2+ had prevented the adventitious reduction of MPT
from its native state. Therefore, these data suggest that the
native state of reduction of MPT in xanthine oxidase must
be a relatively unstable dihydro species, different from stable
7,8-dihydropterins such as H2B which are not reducible by
sulphydryl compounds. The presence of Hg2+ during denat-
uration of MPT-free xanthine oxidase did not result in abolition
of DCIP reduction, indicating that the transient reducing
species was unaffected.

Several other important features of the data in Fig. 3 must
be emphasized and explained. Extrapolating to AFR = 0, the
amount of DCIP reduction (52%) indicates that even when
fully desulfo and/or demolybdo enzyme is used, a small
amount (16%) of DCIP reduction over the basal level (36%)
would be seen. This small amount may reflect a limited ability
of other reductive agents in the denatured solution, such as
protein sulphydryls or sulfide from Fe/S centers, to reduce
MPT. This ability would be totally abolished when Hg2+
was included, as indicated by the y axis intercept value (34%)
obtained by extrapolation of the data points obtained in the
presence of Hg2+. The small positive slope of the line suggests
that Hg2+ does not totally abolish reduction by the terminal
sulfur ligand of fully active enzyme but does show an increasing
ability to abolish all reduction as enzyme AFR decreases. This
effect may be due to the increasing ability of Hg2+ to

![Fig. 3. DCIP reduction by MPT of xanthine oxidase prepara-
tions with varying AFR. The amount of reduction is expressed
as the % of DCIP reduction expected for the MPT concentration
present, assuming a 2 e- reduction of one DCIP/MPT. Experimental
conditions are detailed under "Experimental Procedures." The DCIP
reduction by MPT was measured A (○), following anaerobic
denaturation of xanthine oxidase for 5 min prior to Hg2+ addition; and B (●),
following anaerobic denaturation of xanthine oxidase in the presence
of Hg2+ for 5 min.](image-url)
more efficiently bind all terminal sulfide present as the ratio of free sulfide to Hg²⁺ decreases (with decreasing AFR).

To provide corroboration for the purported role of the terminal S in MPT reduction, DCIP reduction experiments were conducted in which exogenous Na₂S was included during denaturation. The concentration of sulfide included was six times the concentration arising from the released terminal sulfurs in a denatured solution of active enzyme, since higher levels would likely be required to compensate for the fact that terminal sulfur is normally released in the immediate vicinity of released MPT. As shown in Fig. 4, when sulfide was included in an experiment using low activity xanthine oxidase (AFR = 45), the amount of DCIP reduction seen increased to 130% of expected, which is the normal value obtained for fully active enzyme. Sulfide addition did not alter the level of DCIP reduction seen using highly active xanthine oxidase, indicating that the Hg²⁺ levels which were increased to block the excess sulfide from reacting with DCIP were adequate.

7,8-H₂S which was incubated with sulfide under the same conditions was unable to reduce DCIP. A control in which enzyme was incubated aerobically in GdnHCl prior to sulfide addition and quantitation of DCIP reduction showed no DCIP reduction, again indicating that excess sulfide did not interfere with the assay, and that the observations made using low AFR enzyme were not artifactual. These results indicate that the inactive desulfo and demolybdo forms of xanthine oxidase contain MPT in the same native state of reduction as fully active xanthine oxidase but that under normal denaturation conditions MPT in the inactive forms does not get reduced to the tetrahydro state in the absence of label sulfide. The native state of reduction of MPT in xanthine oxidase most consistent with the data presented is a dihydro form other than a stable 7,8-dihydro. One possibility is the quinonoid dihydro form, shown in Fig. 5A.

Since naturally occurring low AFR xanthine oxidase is isolated as a mixture of active, desulfo and demolybdo forms, cyanide treatment of xanthine oxidase was used to make inactive desulfo preparations (19) with an AFR near 0 in an attempt to further examine the role of the terminal sulfur in MPT reduction. As shown in Table I, when preparations of xanthine oxidase, regardless of the initial AFR, were chemically desulfurated and then used in DCIP reduction experiments, instead of seeing the low levels of DCIP reduction expected (<16%) for enzyme with AFR = 0, an average of 52% (when basal level of 36% is subtracted) was seen. Increasing the length of enzyme exposure to CN⁻ or waiting 24 h before assaying DCIP reduction by a treated sample did not alter the results.

There are several possible explanations for these aberrant results. First, the MPT in the desulfo form occurring as a result of CN⁻ treatment may differ from that which is found naturally, although this seems unlikely since CN⁻ treatment of enzyme with low AFR would yield a sample containing both types of desulfo molecules, yet such a sample showed results identical to those for CN⁻-treated fully active enzyme. Another possibility is that CN⁻ not only desulfurates the molecules but also tightly binds to some part of the enzyme or pterin, and interferes with the measurement of DCIP reduction. Finally, the chemical process of desulfuration may actually reduce MPT from its native state to tetrahydro to some extent, which could account for the average 52% DCIP reduction seen. CN⁻-treated MPT-free xanthine oxidase did yield somewhat higher (18%) levels of DCIP reduction than that for the untreated enzyme, but provided no conclusive evidence.

Inclusion of Hg²⁺ during denaturation of CN⁻-treated xanthine oxidase lowered the extent of DCIP reduction by only one-third, yielding the same level regardless of the initial AFR of the enzyme. These data suggest that most of the reduction seen was due to a moiety (possibly MPT) already in a reduced state prior to denaturation.

**Sulfite Oxidase**—To quantitate the reducing equivalents of MPT in the Mo fragment of rat liver sulfite oxidase, its ability to reduce DCIP was examined. Since sulfite oxidase lacks the terminal sulfur on the Mo, having instead two oxo ligands (20), the purified enzyme does not contain inactive forms and

![Fig. 4. Sulfide addition experiments. DCIP reduction by MPT was measured following denaturation of xanthine oxidase samples for 5 min in the presence (□) and in the absence (■) of added Na₂S, prior to Hg²⁺ addition. Details of the reaction conditions are given under "Experimental Procedures." Xanthine oxidase with AFR = 45 was denatured aerobically overnight prior to Na₂S and Hg²⁺ additions.](image)

**TABLE I**

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<th>Sample</th>
<th>Treatment</th>
<th>Initial/ Final</th>
<th>Initial/ Final</th>
<th>% of expected</th>
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<td>AFR</td>
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*The 36% basal level of reduction has been subtracted from these values.*
does not pose the complications encountered when studying the state of reduction of MPT in xanthine oxidase. The studies of MPT in the Mo fragment showed that, irrespective of whether the Mo fragment was denatured in the presence or absence of mercurials, the amount of DCIP reduction corresponded to a two-electron oxidation of MPT (Table II). The experiments with enzyme denaturation in the presence of mercurials were included as a means for detecting any internal redox reactions occurring in the solution which might alter the native reduction state of MPT. Since no difference was seen in the ability of MPT to reduce DCIP in the two experiments, it is believed that no adventitious reduction of MPT had occurred. In the experiments in which enzyme was denatured in the absence of mercurials, it should be noted that mercurials were included following enzyme denaturation in order to prevent the reduction of DCIP by protein sulfhydryls. When Mo fragment was denatured in the presence of sodium sulfide prior to the measurement of DCIP reduction, the level of DCIP reduction achieved was nearly unchanged.

A control experiment was performed in which Mo fragment was denatured aerobically overnight to permit full air oxidation of MPT, then DCIP reduction was measured. As shown in Table II, the ability of the sample to reduce DCIP was abolished, as expected. Another control demonstrated that the system was able to accurately quantitate the two-electron oxidation of H$_2$B to H$_2$B, as 96% of the expected DCIP reduction was seen. H$_2$B was unable to reduce DCIP.

Intact rat and chicken liver sulfite oxidase yielded significantly different results in the DCIP reduction assay system than those obtained with rat liver Mo fragment. As shown in Table II, both enzymes demonstrated approximately a 50% decrease in ability to reduce DCIP. One possible cause of this discrepancy was the presence of hem on the system which might in some way interfere with DCIP reduction. To test this possibility, a model system was designed which included hemoglobin as a source of heme and protein in the assay solution and reduced forms of biopterin as a pterin source. Inclusion of hemoglobin alone caused no DCIP reduction. The ability of H$_2$B to reduce DCIP was decreased 50% in the presence of hemoglobin, indicating that heme does interfere in the system.

State of MPT after Oxidation by DCIP

Xanthine Oxidase—Since DCIP reduction experiments in dicated that only 2e$^{-}$/MPT were transferred to DCIP, the

<table>
<thead>
<tr>
<th>Sample</th>
<th>Pterin conc.</th>
<th>% of expected DCIP reduction after denaturation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mo fragment</td>
<td>8.6 µM</td>
<td>92%</td>
</tr>
<tr>
<td>Mo fragment + sulfide</td>
<td>8.6 µM</td>
<td>98%</td>
</tr>
<tr>
<td>Tetrahydrobiopterin (H$_2$B)</td>
<td>11 µM</td>
<td>96%</td>
</tr>
<tr>
<td>Dihydrobiopterin (H$_2$B)</td>
<td>10 µM</td>
<td>3%</td>
</tr>
<tr>
<td>Holoenzyme model system</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hemoglobin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hemoglobin + H$_2$B</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rat liver sulfite oxidase</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Amount of reduction is expressed as the % of expected change in OD$_{300}$ due to the reduction of DCIP, assuming a 2e$^{-}$ reduction of one DCIP/MPT. Experimental conditions are detailed under “Experimental Procedures.”

Sulfite Oxidase—To determine if the two-electron oxidation/MPT of denatured Mo fragment produced a dihydro or fully oxidized pterin, the difference spectrum of DCIP-treated versus unoxidized denatured samples of Mo fragment was obtained, as shown in Fig. 6 (bottom panel). For the concen-
Reversible oxidation/reduction of MPT in xanthine oxidase

<table>
<thead>
<tr>
<th>Sample</th>
<th>% of expected DCIP reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xanthine oxidase</td>
<td></td>
</tr>
<tr>
<td>with AFR = 50</td>
<td>56</td>
</tr>
<tr>
<td>with AFR = 77</td>
<td>30</td>
</tr>
<tr>
<td>with AFR = 87</td>
<td>81</td>
</tr>
<tr>
<td>with AFR = 169</td>
<td>95</td>
</tr>
<tr>
<td>Tetrahydrobiopterin</td>
<td>2</td>
</tr>
<tr>
<td>Xanthine oxidase with AFR = 77, denatured aerobically and assayed after 16 h</td>
<td>0</td>
</tr>
</tbody>
</table>

*The experiments were conducted as detailed under "Experimental Procedures." Tris buffered GdnHCl denaturation mixtures were used.

Reversible Oxidation/Reduction of MPT

**Xanthine Oxidase**—To determine the dihydro form to which MPT is oxidized by DCIP, DTG was added in 20-fold molar excess of the pterin concentration after initial reaction of MPT with DCIP, followed by excess Hg**+**+, then more DCIP in order to determine whether tetrahydro-MPT had been formed. It is reported that quinonoid dihydropterins are reducible by thiol reagents (17, 18, 22), and we have demonstrated that dihydro- H*B cannot be reduced using the conditions just described. H*B did not show any reversibility in either Tris or potassium phosphate buffer systems. This result was to be expected since the quinonoid form of biopterin rapidly rearranges (within 1 min) to the 7,8-dihydro form (17). Reversibility was seen, however, using xanthine oxidase, and the extent of reduction appeared to be unrelated to the AFR of the enzyme used (Table III). These results also indicate that DTG cannot reduce the putative quinonoid-MPT as effectively as sulfide, supporting the proposed idea that the terminal sulfide is responsible for the reduction of MPT which occurs upon enzyme denaturation in GdnHCl. A control in which enzyme was denatured aerobically overnight before the experiment showed no DCIP reduction, as expected, since the fully oxidized pterin formed should not be reducible with DTT.

To test the stability of the putative quinonoid dihydro form of MPT, a series of experiments were conducted in which the length of time between the initial oxidation of MPT by DCIP and the addition of DTG was increased by increments of 15 min. The ability of DTT to reduce DCIP was nearly abolished in 60 min.

**Sulfite Oxidase**—In contrast to MPT of xanthine oxidase which appeared to undergo reversible oxidation/reduction, the DCIP-oxidized MPT of Mo fragment showed limited ability (15%) to be reduced.

**DISCUSSION**

The results of the difference spectroscopy experiments clearly show that the native state of reduction of MPT in xanthine oxidase is neither the fully oxidized nor the stable 7,8-dihydro form. The fact that MPT is reducible by sulfide or to some extent by DTT also indicates that a stable 7,8-dihydro is not a possibility, as evidenced by the fact that 7,8-dihydrobipterin was not reducible under these conditions. The DCIP reduction studies and the sulfide addition experiments provide sufficient evidence that the native state is not the fully reduced tetrahydro form. Therefore, it seems reasonable to conclude that the native state of reduction of MPT in xanthine oxidase is the readily reducible quinonoid dihydro form.

The formation of quinonoid dihydrobipterin in the aromatic amino acid hydroxylase reactions has been documented (17, 23); thus, it is of interest that another biochemically significant pterin, MPT, appears to exist in this quinonoid dihydro form, at least in xanthine oxidase. Although quinonoid dihydropterins are generally highly unstable transient species (such as quinonoid-H*B), several have been shown to be relatively stable. Quinonoid-6-methylbihydropterin, quinonoid-6,6-dimethylbihydropterin, and quinonoid-6,7-dimethylbihydropterin have reported half-lives, respectively, of 56 min in 0.2 M Tris-HCl, pH 7.8 (24), 4 h in 0.1 M Tris-HCl, pH 7.4 (18), and 25 min in Hepes, pH 8.0 (25). It seems likely that the tight noncovalent binding of MPT to the enzyme contributes to the stabilization of the quinonoid state, even in the presence of 6 M GdnHCl. The 4-carbon side chain at C-6, with its two sulfurs and phosphate, may also play a significant role.

In xanthine oxidase, no correlation has been found between changes in the state of MPT reduction and enzyme inactivation. The sulfide addition experiments presented here strongly indicate that the naturally occurring desulfo and demolybdo inactive forms of xanthine oxidase contain MPT in the same native quinonoid dihydro state as fully active enzyme. Difference spectra of MPT, which were obtained using either fully active enzyme or enzyme with a low AFR, are all similar to that of tetrahydropterins. This result appears at first to be contradictory, since the DCIP reduction experiments showed that MPT of enzyme with low AFR is a mixture of fully reduced and quinonoid forms due to the deficiency of terminal sulfide. However, quinonoid dihydropterins have been shown to be very similar spectrally to tetrahydropterins (17, 21, 18, 24); thus, the spectral results are consistent with the conclusions presented here. More support for the quinonoid dihydro-MPT designation for both active and inactive xanthine oxidase is derived from the difference spectra obtained after DCIP treatment, which indicate that neither enzyme form yields significant oxidized pterin.

The experiments presented here also demonstrate that MPT in denatured Mo fragment of sulfite oxidase is oxidized by DCIP by two electrons, even when the possibility of internal redox reactions in the denatured enzyme solution is eliminated. This finding is in agreement with the fact that sulfite oxidase does not have the terminal sulfur which is found in xanthine oxidase. The MPT in sulfite oxidase was found to be in the fully oxidized state after oxidation by DCIP, both by difference spectroscopy and by the inability of DTT to reduce the DCIP-treated MPT. Taken in sum, the results indicate that the native state of reduction of MPT in sulfite oxidase is at the dihydro level.

The ability of MPT in sulfite oxidase to react with DCIP and the spectral properties of MPT demonstrate that it cannot be the stable 7,8-dihydropterin. The absorption band around 300 nm in the difference spectrum of MPT supports the existence of a less stable dihydro structure. Although the spectrum does resemble the documented spectra of several unstable quinonoid dihydropterins (17, 18), the dihydro form in sulfite oxidase is not necessarily quinonoid. Other unstable dihydro forms which have not been characterized, such as 5,6-dihydro, 6,7-dihydro, and 5,8-dihydro forms, may also have absorption spectra which resemble those of quinonoid forms.

In fact, it is quite clear that MPT in sulfite oxidase cannot
be in a quinonoid dihydro form, by comparison of its behavior to that of MPT in xanthine oxidase. In xanthine oxidase, MPT in its native quinonoid dihydro state cannot be oxidized by DCIP, whereas in sulfite oxidase MPT in its native state is oxidized by DCIP. By ruling out the quinonoid dihydro and 7,8-dihydro pterin forms, other structures for the dihydro-MPT in sulfite oxidase must be considered. Several possibilities are shown in Fig. 5(B–D). Little information exists concerning some of the dihydro pterin isomers due to their instability. In the case of sulfite oxidase, one such unstable form may well be stabilized by interaction with protein. That we are able to detect different dihydro isomers in denatured enzyme solutions suggests that the pterins are not fully dissociated from protein under the conditions used. If such unstable forms were truly free of protein-stabilizing effects, it might be expected that these would isomerize to a stable form such as 7,8-dihydro.

An interesting finding in these studies was that heme appeared to interfere with the DCIP reduction assay in some way, consistently causing a 50% decrease in the expected extent of reduction, which suggests that only one electron/MPT was transferred to DCIP. A possible explanation for this result is that the free heme in the assay solution oxidizes the pterin (MPT or H$_2$B) by one electron, yielding a one- or three-electron intermediate which can then react with only half as much DCIP. Precedence for such a mechanism is found in the rapid oxidation of tetrahydropterin by ferricytochrome $c$ to yield a three-electron pterin intermediate which can then donate one electron to O$_2$ or to another ferricytochrome $c$ (26).

The experimental approach presented in this paper for probing the electronic state of the pterin ring was necessitated by the extreme lability of MPT once it is extracted from the protein environment. A degree of stability, as determined from the retention of biological activity, can be invested on protein-stabilizing effects, such as ascorbates or dithionite are present (27). However, such conditions could well alter the native state of MPT released from a molybdoenzyme and are unlikely to be useful for defining the chemical nature of the enzyme-bound molecule.

In summary, the data presented here provide presumptive evidence for the existence of dihydro forms of MPT in xanthine oxidase and sulfite oxidase. While the evidence also suggests that the two enzymes contain different forms of dihydropterin, the possibility that both enzymes contain the same form and that, under the conditions used in these studies, the pterin in one of the enzymes had isomerized to a different form cannot be excluded. These findings do not yet substantiate a role for the state of reduction of MPT in influencing the oxidation/reduction potentials of the Mo in molybdoenzymes, but do strengthen the likelihood of MPT involvement in enzyme function, given the reactivity of the dihydropterin forms found in both of the molybdoenzymes that were examined. The techniques and results described herein represent the basis for future studies aimed at exploring any possible role of the pterin ring in the catalytic activities of molybdoenzymes.

REFERENCES