Xanthine oxidoreductase (XOR) is the prototypical member of the molybdenum hydroxylase family of proteins (1, 2). In humans, XOR catalyzes the hydroxylation of hypoxanthine to xanthine as well as xanthine to uric acid, and the mammalian enzyme exists in two alternative forms of the same gene product. Normally, the enzyme exists in a dehydrogenase form (xanthine oxidase) but can be readily converted to an oxidase that utilizes O2 exclusively (3). This conversion of xanthine dehydrogenase form utilizing NAD+ as the final electron acceptor from the enzyme’s flavin site but can exist as an oxidase that utilizes O2 for this purpose. Central to an understanding of the enzyme’s function is knowledge of purine substrate orientation in the enzyme’s molybdenum-containing active site. We report here the crystal structure of xanthine oxidase, trapped at the stage of a critical intermediate in the course of reaction with the slow substrate 2-hydroxy-6-methylpurine at 2.3 Å. This is the first crystal structure of a reaction intermediate with a purine substrate that is hydroxylated at its C8 position as is xanthine and confirms the structure predicted to occur in the course of the presently favored reaction mechanism. The structure also corroborates recent work suggesting that 2-hydroxy-6-methylpurine orients in the active site with its C2 carbonyl at the stage of a critical intermediate in the course of reaction with the enzyme’s flavin site (4). The specific sequence of these latter events varies from one substrate to another as well as with the reaction conditions (5). The catalytic sequence of XOR is thought to be initiated by abstraction of a proton from the Mo=O–OH group by a universally conserved active site glutamate residue (6), followed by nucleophilic attack of the resulting Mo=O– unit on the carbon center to be hydroxylated and concomitant hydride transfer to the FAD (via the [2Fe-2S] centers), and deprotonation of the Mo=O– group by a universally conserved active site glutamate residue (7). Oxidative hydroxylation of the purine substrate occurs at the molybdenum center, which results in the two-electron reduction of the enzyme. After internal electron transfer via the [2Fe-2S] centers to the FAD, reducing equivalents are passed to the final electron acceptor (O2 or NAD+). The crystal structure of the bovine enzyme has been determined (8), and it shows that the four redox-active centers of each monomer are found in separate, distinctly folding domains.

The catalytic cycle of XOR is thought to be initiated by abstraction of a proton from the Mo=O–OH group by a universally conserved active site glutamate residue (9), followed by nucleophilic attack of the resulting Mo=O– unit on the carbon center to be hydroxylated and concomitant hydride transfer to the FAD (via the [2Fe-2S] centers), and deprotonation of the Mo=O– group by a universally conserved active site glutamate residue (10). The specific sequence of these latter events varies from one substrate to another as well as with the reaction conditions, but the Mo=O– group is considered to be an obligate intermediate. When electron transfer proceeds product dissociation, a transient LMo=O(S)OR species is generated that gives rise to the well characterized “very rapid” EPR signal of the molybdenum center.

In addition to the glutamate residue thought to act as a general base in initiating the reaction, another highly conserved residue in the active site of the xanthine-utilizing enzymes (but not the closely related enzymes that oxidize aldehydes) is Arg-310 (numbering in xanthine dehydrogenase from Rhodobacter capsulatus, Arg-880 in the bovine enzyme) (11). As shown in Fig. 2, this residue lies some 10 Å from the molybdenum center, too far to participate directly in catalysis. In the structure of the
R. capsulatus enzyme with the mechanism-based inhibitor alloxanthine, however, Arg-310 is hydrogen-bonded to the pyrimidine subnucleus of the heterocycle via one of its carbonyl groups (10). Also, in a model of urate bound to reduced enzyme based on the crystal structure of the aldehyde oxidoreductase from Desulfovibrio gigas (9), the equivalent Arg is suggested to interact similarly with bound product.

We have recently demonstrated that Arg-310 of the R. capsulatus enzyme plays an important role in catalysis, contributing \( \frac{2}{11} \) to stabilization of the transition state and accounting for a 204-fold increase in rate acceleration with xanthine as substrate (11). Arg-310 (Arg-880 in the bovine enzyme) appears to lower the activation energy for the reaction by stabilizing negative charge accumulation on the heterocycle through an electrostatic interaction with the C6 carbonyl oxygen of substrate. This in turn implies that substrate binds in an orientation opposite to that seen in the structure of the (reduced) enzyme in complex with the mechanism-based inhibitor alloxanthine but like that seen in the model of urate bound to the D. gigas aldehyde oxidoreductase (9). This conclusion is supported by a kinetic study utilizing a homologous series of purine substrates, which fell into two groups (11). Those that are effective substrates of the wild-type enzyme, presumably binding in an orientation similar to xanthine and making use of the catalytic contribution of Arg-310 to rate acceleration, were strongly affected by mutation of Arg-310 to methionine. On the other hand, those that react slowly with wild-type enzyme (in particular 2,6-diaminopurine and 2-hydroxy-6-methylpurine) have functional groups at position 6 (amino and methyl, respectively) that prevented interaction with Arg-310. It was concluded that the poor substrates bound in an inverted orientation to that of xanthine, accounting for both their low reactivity with wild-type enzyme and relative insensitivity to loss of the active site arginine (11). This same principle of orientation is expected to hold true in the case of transition state stabilization by Arg-880 in the bovine enzyme.

To further examine the manner in which substrate orientation influences the manner in which the catalytic machinery of xanthine oxidoreductase is utilized, and to directly test our previous hypothesis regarding substrate orientation, we have crystallized bovine xanthine oxidase with the slow substrate 2-hydroxy-6-methylpurine (HMP) added to the mother liquor immediately prior to mounting and flash-freezing the crystals. We present here the structure at 2.3 Å resolution of the enzyme-substrate complex trapped at an intermediate stage in catalysis. The structure clearly shows substrate/product coordinated to the molybdenum center in an end-on fashion as previously proposed, and with the purine ring in an orientation fully consistent with our proposed mode of binding.

**EXPERIMENTAL PROCEDURES**

**Materials**—Magnetic bases for use with the automated express crystallography system at SGX-CAT were obtained from MAR-USA (Evanston, IL). Mounted cryoloops, magnetic cryovials, and crystal growth materials were obtained from Hampton Research (Aliso Viejo, CA). All chemicals and reagents were obtained at the highest quality/purity available from Sigma-Aldrich.

**Isolation of Xanthine Oxidase**—Xanthine oxidase was isolated from fresh, unpasteurized bovine milk (Waterman Farm, Ohio State University) according to published methods (12). The milk was obtained from a single animal, so as to minimize any heterogeneity in the qualities and characteristics of the isolated protein. Isolated enzyme was stored in \( \text{N}_2(\text{l}) \) and passed down a Sephadex G-25 column to remove salicylate prior to use.

**Crystallization, Data Acquisition, and Structure Determination**—Crystals were grown via the batch method, initially following previous conditions (13) and using microbridges to hold the batch solutions in the sealed wells of a 24-well tray. After a review of the literature (14), modifications were made in constructing the batch solutions. The most important modification involved premixing the enzyme and precipitant solution volumes together in an Eppendorf tube with very light agitation (no more then 5 s) prior to transfer into the concave indentation of the microbridge. The batch solutions contained 20 \( \text{µl} \) of enzyme solution mixed with 10 \( \text{µl} \) of the precipitant solution. We were also able to obtain similar crystals at half these volumes.

The final conditions that yielded the best crystals were a precipitant solution of 12–14% polyethylene glycol 8000, 0.1 M
potassium phosphate at initial pH 6.5, and 0.2 mM EDTA. The enzyme solution contained 10 mg/ml XO in 40 mM Tris-HCl initial pH 7.8, 20 mM pyrophosphate initial pH 8.5, 0.2 mM EDTA, and 5 mM dithiothreitol. The enzyme solution was allowed to sit on ice for 1 h following the addition of dithiothreitol, prior to beginning the construction of the well solutions. The final pH of the well solutions was 7.2 ± 0.1. Crystals grew after 2–3 days at 25 °C in darkness. Initially, we were only able to obtain large numbers of stellate clusters, owing to a high number of nucleation sites within the protein-precipitant batch solution. By lowering the precipitant concentration and substituting polyethylene glycol 8000 for polyethylene glycol 4000, we were able to obtain XO crystals in the form of rectangular plates over the same timeframe. These ranged from 0.1 to 1.0 mm in their longest dimension.

A 40–45% polyethylene glycol 200 solution at pH 9.0 containing all proportions of the enzyme and precipitant solutions was used as the cryoprotectant solution. This was introduced by buffer exchange using a micropipette. Following an exchange of greater than the original batch volume, the substrate (also in the same cryoprotectant solution) was introduced from a stock solution to give a 1 mM concentration in the new batch solution. Crystals were mounted and frozen after 1 min. Initial crystals were screened on the home rotating anode source at the Ohio State University. Final diffraction data were collected at Argonne National Laboratory on the SGX Pharmaceuticals, Inc. beamline using a wavelength of 0.9793 Å and an MARCCD 165 detector. Data sets were collected at 2.3-Å resolution for the XO/HMP complex.

Data were processed using the MOSFLM package of the CCP4 program suite (15, 16). The structure of the enzyme was determined by molecular replacement using the MOLREP package of CCP4, with protein data bank file 1FIQ as the search model (8). Following rigid body refinement in REFMAC of the CCP4 suite, the molybdopterin cofactor (MTE and MOS) was constructed, and the structure was refined using the restrained refinement protocol in REFMAC (17–21). Ini-

**FIGURE 2.** The active site molybdenum center of the bovine xanthine oxidase (PDB accession code 1FIQ) with corresponding residue numbers for the *Rhodobacter capsulatus* enzyme in parentheses (modified from (8)). Panel A shows the view from the perspective of an approaching substrate. The molybdenum itself is shown in teal blue, with apical and planar oxygen atoms (shown in red), a planar sulfur (shown in yellow), and the edenthiolate of a pterin ring in the metal's coordination sphere. Carbon atoms are in silver, oxygen atoms in red, sulfur atoms in yellow, and nitrogen atoms in blue. The two representations shown differ in being rotated 90° about the vertical axis, with panel B being 90° right of panel A. Substrate wedges between Phe-1009 and Phe-914 in binding at the active site. As shown, the positions of the Mo=O and Mo=S groups of the molybdenum coordination sphere have been reversed between apical and planar positions from those given in the Protein Data Bank file, given the evidence that it is the Mo=O rather than Mo=S that occupies the apical position of the square pyramidal coordination sphere of the molybdenum (as discussed in Ref. 25).

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TABLE 1

Statistics for data collection and refinement

| Statistic | XO with bound HMP |
|-----------|-------------------|
| PDB code  | 3B9J              |
| Space group | P2₁              |
| Resolution | 33.6-2.3 Å        |
| Wavelength (Å) | 0.9793 🔴      |
| Unique reflections (test set) | 114,520 (5,785) |
| Completeness % (highest resolution shell) | 96.3 (90.5) |
| I/σ(I) (highest resolution shell) | 13.5 (1.8) |
| Rmerge (highest resolution shell) | 19.4 (25.1) |
| Rfree (highest resolution shell) | 26.3 (32.5) |
| Ramachandran statistics (%) | 87.2, 11.0, 1.0, 0.7 |
| Mean coordinate error based on free R value (Å) | 0.260 |
| Mean coordinate error based on maximum likelihood (Å) | 0.196 |
| r.m.s. deviation bond length (Å) | 0.017 |
| r.m.s. deviation bond angles (°) | 1.8 |
| Average B value (Å²) | 22.7 |
| Number of non-hydrogen atoms in refinement | 19,852 |
| Number of waters | 861 |



- tially, non-crystallographic symmetry restraints (set at “medium” in the REFMAC interface) between analogous cofactors and domains of the two subunits of the XO dimer were used, but in subsequent rounds of refinement, removing the NCS restraints entirely resulted in the lowest free R-factor. The weighting term for geometric restraints was also adjusted in REFMAC to minimize R_free.

The 2-hydroxy-6-methylpurine molecule was constructed using the PRODRG2 server (22), and the resulting PDB file was built into the corresponding electron density observed in the XO active site using COOT and verified also in O (23, 24). Following the merging of the HMP with the refined XO structure, the resulting structure was refined again using restrained refinement in REFMAC. Water molecules were subsequently added to the XO electron density map after addition of the HMP molecules. The final R-factors were an R_cry (19.4%) and an R_free of 26.3% (Table 1). Images were rendered using PyMol.³

RESULTS

Overall Structure of XO with 2-Hydroxy-6-methylpurine—The crystals examined in the present study were monoclinic in space group P2₁, unlike previous structures of XOR but similar to recent work by Yamaguchi and coworkers (26). The overall dimensions of the unit cell were a = 133.2 Å, b = 73.8 Å, and c = 146.5 Å with angles of 90, 98.9, and 90° (Table 1). In this crystal form, specific residues that previously were disordered became more apparent in the electron density map. This was particularly true of residues 1316–1328 of one of the two monomers, which were added to fit ordered electron density, as was residue 1292 (glutamate), which was also absent in the previous model structure. The asymmetric unit possessed one molecule of the homodimeric enzyme.

As in previous structures of XOR, each monomer consisted of four domains; two N-terminal domains each with a [2Fe-2S] cluster, an FAD-binding domain, and a molybdenum portion of the protein with the molybdenum center sandwiched between two domains at the C-terminal end of the polypeptide (see Fig. 4). All cofactors were accounted for in the electron density map and were successfully fit into the crystal structure. The r.m.s. displacements for the α-carbons of each monomer in the final refined XO-HMP structure relative to that of 1FIQ were 0.345 and 0.340 Å, respectively. The relative orientations and distances between the redox-active centers were essentially identical to those reported previously (8).

As shown in Table 1, 0.7% of residues in the refined structure (15 residues) were in disallowed geometries. These residues are scattered at random throughout the 2442 amino acids of the structure. Twelve of these residues are fit within strongly observed electron density, and three of these residues are in regions of the structure for which somewhat weaker electron density was observed. These twelve amount to less than 1% of the amino acid residues in the refined structure. The latter three (Leu-1316 and Ala-1324 of one monomer and Glu-1292 of the other) were absent in the search model (1FIQ); the first two were added to fit newly ordered density beyond residue 1315 as mentioned above.

2-Hydroxy-6-methylpurine in the Active Site—In both subunits of the crystal structure, the heterocycle is docked with its C8 approaching the equatorial Mo–OH oxygen atom (O2) of the molybdenum center (Fig. 4). In doing so, it stacks on Phe-914 and has a side-on interaction with Phe-1009, consistent with previous work with the inhibitors alloxanthine and FYX-051 (5, 10). As shown in Fig. 4, the carbonyl oxygen at the C2 position of HMP interacts with Arg-880 in both active sites, while the methyl group at C6 is directed away from this residue, projecting into a hydrophobic pocket. This is the orientation expected on the basis of our previous work (11), in which it was suggested that this slow substrate binds in an orientation that precludes effective utilization of Arg-880 and presumably opposite that of the physiological substrate xanthine. The carbonyl at the C6 position of xanthine (Fig. 3) allows for effective use of Arg-880 in stabilizing negative charge accumulation at this position, but that at C2 is expected to be significantly less effective. With the C6 carbonyl replaced by a methyl group in HMP, the more favorable interaction is lost and the heterocycle

³ W. L. DeLano (2002) PyMOL, DeLano Scientific, San Carlos, CA.
binds in an inverted orientation that is less catalytically effective.

In the final structure, the C8 of HMP is positioned 2.2 Å from the equatorial oxygen of the molybdenum center in one active site of the dimer, and 1.6 Å in the other (as shown with omit density in Fig. 4). In this second site, there is obviously continuous electron density connecting C8 and the oxygen, indicating that catalysis has proceeded to the point of C–O bond formation (Fig. 4B). The distance here between Glu-1261 and N9 of HMP is 2.9 Å, suggesting a hydrogen bonding interaction. The C8 of substrate is 3.4 and 3.6 Å from the molybdenum in the two active sites of the dimer. The Mo–S distance in both sites is 2.0 Å with a mean coordinate error of 0.2 Å, most consistent with a Mo=S bond and either a MoV or MoV oxidation state for the metal, but not MoIV with a Mo–SH ligand. Notwithstanding the known difficulties in determining metal-ligand distances that are associated with Fourier truncation artifacts in the vicinity of a metal as heavy as molybdenum (27), particularly at the present resolution, we consider it likely that the Mo–S distance reflects a Mo=S, implying that the metal is in the MoV valence state (see below). Indeed, under conditions very similar to those explored here large amounts of the EPR active MoV state are known to accumulate (28, 29).

The two active sites appear to represent different points in the catalytic sequence. The first site (Fig. 4A), with the heterocycle positioned somewhat further from the molybdenum center, most likely represents the Michaelis complex for the reaction, with substrate positioned for hydroxylation but no C–O bond formation and the molybdenum center in the MoVI oxidation state prior to substrate coordination. In the second site (Fig. 4B), the reaction has obviously progressed to the point of C–O bond formation given the relatively shorter refined distance (with mean coordinate error considered) and evidence of $2F_o - F_c$ electron density between the HMP-C8 and planar molybdenum oxygen, presumably also then with reduction of the molybdenum to a +5 or +4 valence state. That the Mo–S distance suggests a Mo=S rather than Mo–SH in turn means that the molybdenum is at the MoV rather than MoIV state, an interpretation consistent with the known kinetic behavior of the enzyme with HMP in which high levels (>80%) of the species giving rise to the “very rapid” EPR signal accumulate in the steady state. The HMP molecule was not defined as being bonded to the planar molybdenum oxygen in either active site prior to refinement, and thus the distance given here is refined as separate MoOOS and HMP monomers in the electron density.

The orientation of bound product to the molybdenum in this species, coordinated to the molybdenum in a simple end-on fashion, is in fact that expected on the basis of ESEEM and ENDOR studies of this species, although the Mo–C distance of 3.4 Å seen here is somewhat longer than the 2.9 distance expected (30, 31). The orientation observed in the present crys-
tal structure has important mechanistic implications, because it is inconsistent with proposed mechanisms that lead to a side-on orientation of product in the molybdenum coordination sphere (32).

As indicated above, the orientation of the heterocycle with respect to Arg-880 is consistent with our predictions based on the kinetic behavior of wild-type enzyme and an R310M mutant of the xanthine dehydrogenase from *R. capsulatus* with a series of purine substrates. This orientation of HMP is thought to be an inferior one in which the catalytic power of Arg-880 is ineffectively utilized. Good substrates, including xanthine, are thought to bind in an orientation opposite to that of poor substrates such as the HMP utilized here. Given the significance of substrate orientation vis-à-vis the utilization of Arg-880 in catalysis, we positioned the substrate into the $2F_o - F_i$ density map following omission of the molecule from our structure with subsequent refinement (creating an “omit map”), as well as into the $F_o - F_i$ density map at $+2.5$ sigma. Fig. 4 illustrates the HMP molecule positioned into these unbiased electron densities. If the HMP molecule is positioned in the unbiased density opposite to our proposed orientation with the C6 position directed toward Arg-880 (but maintaining the HMP-C8 alignment with the molybdenum oxygen according to the proposed catalytic mechanism), both the C2 carbonyl and the C6 methyl groups project out of the density map. Despite this, we attempted to refine our structure with the heterocycle bound in the opposite orientation to that shown in Fig. 3, with the C6 methyl directed at Arg-880 and the C2 carbonyl directed into the hydrophobic pocket. In the subsequently refined structure and electron density map the orientation was not appropriate for catalysis. For each of the two active sites with this “inverted” orientation of HMP, the Mo–OH O2 appeared to hydrogen bond with N9 of the substrate at $2.3$ and $2.7$ Å, and C8 of the heterocycle was oriented toward Glu-1261 at $2.4$ and $3.3$ Å. The C6 methyl was $3.1$ and $2.9$ Å from Arg-880. Furthermore, several atoms of this refined structure, particularly C8 and N7 of the heterocycle, clearly lay outside the observed electron density. For these reasons this second orientation of substrate was deemed unsatisfactory.

**DISCUSSION**

In the present work, we observed the slow substrate 2-hydroxy-6-methylpurine both before and during catalysis in the active site of xanthine oxidase from bovine milk. The active site shown in Fig. 4A depicts the orientation of substrate relative to a fully oxidized molybdenum center (Mo(VI)) in what is presumably the catalytically relevant Michaelis complex. In the active site of Fig. 4B, the heterocycle has undergone oxidative hydroxylation at C8, and is now coordinated to the active site molybdenum via a Mo–O–C8 linkage. The molybdenum here appears to be in the Mo(V) state, as reflected by the short Mo–S bond distance of $2.0$ Å with a mean coordinate error of $0.2$ Å. The difficulties of interpreting the molybdenum oxidation state and bonding interactions with the sulfo- and oxo-ligands based on crystallographic refinement notwithstanding, the electron density between HMP/Mo–O$_2$ and previous data on catalytic intermediates with HMP led us to conclude that the molybdenum is indeed in the Mo(V) oxidation state. The orientation of substrate with respect to the molybdenum center seen here in Fig. 4B, with product coordinated to the metal via the catalytically introduced hydroxyl group in a simple end-on fashion, is consistent with that proposed previously on the basis of ESEEM and ENDOR studies of this EPR-active intermediate (although the Mo–C distance seen here is again somewhat longer than predicted) (30, 31). This orientation provides important support of our previously proposed reaction mechanism of xanthine oxidoreductase (33), involving base-assisted nucleophilic attack by the planar molybdenum oxygen on the C8 position of substrate, with concomitant hydride transfer to the planar sulfur.

In the structure determined here, HMP is oriented such that the C2=O oxygen interacts with Arg-880 and the C6 methyl group points away from it. This orientation is the more reasonable on chemical as well as structural grounds. In previous work we have shown that Arg-310 of *R. capsulatus* xanthine dehydrogenase (homologous to Arg-880 of the bovine enzyme examined here) plays an important role in catalysis (11). We have suggested that HMP binds in an inverted orientation to that seen with xanthine, and for this reason is unable to take effective advantage of transition state stabilization by Arg-880. This accounts for the low reactivity of substrates such as HMP and 2,6-diaminopurine with wild-type enzyme, and at the same time their relative insensitivity to mutation of the active site arginine to a methionine (11). This conclusion is supported by the work presented here, which clearly demonstrates HMP oriented as predicted, with its C6 position oriented away from Arg-880.

Finally, the observation of somewhat different structures in the two subunits of the dimeric enzyme deserves comment. With one subunit evidently having progressing part way through the catalytic sequence while the other is arrested at the point of substrate binding in the active site, it would appear that the enzyme, at least in the crystal, may be operating in a reciprocating fashion, with first one then the other subunit functioning catalytically. Such behavior has in fact previously been suggested for XOR (35). The manifestly different active site structures notwithstanding, however, it is not evident what structural constraints at the subunit interface (which is in the molybdenum-binding portion of the enzyme) might give rise to such reciprocating behavior. Specifically, the two subunits have essentially identical conformations, and there is no evident structural mechanism by which the structural and functional status of the active site of one subunit might be conveyed to the subunit interface and transmitted to the other subunit. The α-carbon r.m.s. deviations between the two subunits in the structure reported here are 0.289 Å for the xyz displacement and 2.74 Å$^2$ for the B-factor displacement. The only obvious structural difference between the two subunits is the ordered residues near the end of the two main chains, nearer the flavin-binding domains of each subunit rather than the subunit interface region. The newly resolved residues in the one subunit of the present structure actually increase the structural match between the two subunits (one subunit continues to reside 1320 at the C terminus, and the second increased from residue 1315 to 1328 in the present structure). Given difficulties in fitting these newly resolved residues in our structure into the relatively weak electron density in certain places, such
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differences in conformation as are observed in this region should be taken with caution. Nevertheless, we clearly observe only one crystallographically defined subunit progressing partially through the catalytic sequence and cannot exclude some specific mechanism of site-site interaction that is not evident at the present 2.3-Å resolution. Work to address this specific issue is presently under way.

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