The Role of Arginine 310 in Catalysis and Substrate Specificity in Xanthine Dehydrogenase from Rhodobacter capsulatus*

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The rapid reaction kinetics of wild-type xanthine dehydrogenase from Rhodobacter capsulatus and variants at Arg-310 in the active site have been characterized for a variety of purine substrates. With xanthine as substrate, $k_{\text{red}}$ (the limiting rate of enzyme reduction by substrate at high $[S]$) decreased ~20-fold in an R310K variant and 2 × 10$^4$-fold in an R310M variant. Although Arg-310 lies on the opposite end of the substrate from the C-8 position that becomes hydroxylated, its interaction with substrate still contributed ~4.5 kcal/mol toward transition state stabilization. The other purines examined fell into two distinct groups: members of the first were much less effectively hydroxylated by the wild-type enzyme but were strongly affected by the exchange of Arg-310 to methionine (with a reduction in $k_{\text{red}}$ greater than 10$^3$), whereas members of the second were much less effectively hydroxylated by wild-type enzyme but also much less significantly affected by the amino acid exchanges (with a reduction in $k_{\text{red}}$ less than 50-fold). The effect was such that the 4000-fold range in $k_{\text{red}}$ seen with wild-type enzyme was reduced to a mere 4-fold in the R310M variant. The data are consistent with a model in which “good” substrates are bound “correctly” in the active site in an orientation that allows Arg-310 to stabilize the transition state for the first step of the overall reaction via an electrostatic interaction at the C-6 position, thereby accelerating the reaction rate. On the other hand, “poor” substrates bound upside down relative to this “correct” orientation. In so doing, they are unable to avail themselves of the additional catalytic power provided by Arg-310 in wild-type enzyme but, for this reason, are significantly less affected by mutations at this position. The kinetic data thus provide a picture of the specific manner in which the physiological substrate xanthine is oriented in the active site relative to Arg-310 and how this residue is used catalytically to accelerate the reaction rate (rather than simply bind substrate) despite being remote from the position that is hydroxylated.

The molybdenum-containing hydroxylases represent a unique solution to the hydroxylation of carbon centers. Other monooxygenases introduce an oxygen atom derived from O$_2$ and consume two reducing equivalents (along with the two removed from the substrate to be hydroxylated) in reducing O$_2$ to water. The molybdenum enzymes, on the other hand, utilize water itself as the ultimate source of the oxygen atom incorporated into product. In the case of enzymes such as xanthine dehydrogenase, not only are molybdenum enzyme reducing equivalents not consumed in carrying out the catalyzed reaction, but in fact physiologically useful reducing equivalents in the form of NADH are generated. As such, these enzymes represent a unique solution to the chemistry of hydroxylation, and the requisite cleavage of a carbon-hydrogen bond that accompanies it (1).

The xanthine dehydrogenase from Rhodobacter capsulatus is an ($\alpha\beta$)$_2$ heterotetramer comprising two copies each of the XdhA and XdhB gene products (2). XdhA possesses two different [2Fe-2S] iron-sulfur clusters of the spinach ferredoxin variety as well as FAD, with each redox-active center located in a separately folded and contiguous domain of the polypeptide. XdhB possesses a molybdenum center with a square pyramidal LMo$^{5+}$OS(OH) coordination sphere, with L representing a unique pyranopterin cofactor coordinated to the metal via an enedithiolate sidechain. This organic cofactor is common to all molybdenum- and tungsten-containing enzymes, with the sole exception of the multinuclear molybdenum- and iron-containing active site of nitrogenase (1). The structure of the R. capsulatus enzyme is known (3), and it bears strong structural as well as sequence homology to other members of the molybdenum hydroxylase family of enzymes (including bovine xanthine oxidoreductase (4, 5) and quinoline 2-oxidoreductase from Pseudomonas putida (6)). The structure of the active site of the R. capsulatus xanthine dehydrogenase is shown in Fig. 1.

The molybdenum hydroxylases, which also include aldehyde oxidases from vertebrate, plant, and bacterial sources, are thought to share a common reaction mechanism, which has been best worked out in the case of the bovine enzyme (7–9). Catalysis is initiated by abstraction of a proton from the Mo-OH group by a universally conserved active site glutamate residue (7) followed by nucleophilic attack on the carbon center to be hydroxylated and concomitant hydride transfer to the Mo=S of the molybdenum center. This reaction yields an LMo$^{5+}$(SH)(OR) intermediate, with OR representing the now hydroxylated product coordinated to the molybdenum via the newly introduced hydroxyl group. The catalytic sequence is completed by displacement of the bound product from the molybdenum coordination sphere by hydroxide from solvent.
electron transfer out of the molybdenum center to the FAD (via the [2Fe-2S] centers), and deprotonation of the Mo-SH to give the original, oxidized LMoOS(OH) form of the center. The specific sequence of these latter events varies with the substrate used and the reaction conditions, but the LMo IV(SH)(OR) species is considered an obligatory intermediate.

In addition to the glutamate residue thought to act as a general base, another highly conserved residue in the active site of the xanthine-utilizing enzymes (but not the aldehyde-utilizing ones) is Arg-310 (Arg-880 in the bovine enzyme) (Fig. 1). This residue lies some 10 Å from the molybdenum center, too far to participate directly in catalysis. In the structure of the enzyme with the mechanism-based inhibitor alloxaanthine, however, Arg-310 is hydrogen-bonded to the back side of the heterocycle via one of the carbonyl groups of the latter (3). Also, in a model of urate binding to reduced enzyme based on the crystal structure of the aldehyde oxidoreductase from Desulfovibrio gigas (7), the equivalent Arg is suggested to interact similarly with bound product. In the present work, we examined the catalytic role of Arg-310 and found, surprisingly, that its exchange to methionine results in a 20000-fold decrease in its interaction with substrate is used to accelerate reaction rate of enzyme reduction upon mixing with substrate). Thus, approximately 80% active.

**EXPERIMENTAL PROCEDURES**

**Protein Purification**—Recombinant *R. capsulatus* xanthine dehydrogenase was purified using the procedure described by Leimkuhler et al. (11), with affinity chromatography on a Sepharose 4B/folate gel as the final step. By using PCR mutagenesis, amino acid exchanges R310M and R310K were introduced into *R. capsulatus* xanthine dehydrogenase. The generated variants were expressed under the same conditions as the wild-type enzyme and purified by nickel-nitrilotriacetate chromatography, Q-Sepharose, and size exclusion chromatography. The purified enzymes were concentrated by ultrafiltration, gel-filtered using a PD-10 gel filtration column (GE Healthcare), equilibrated with 50 mM Tris, 1 mM EDTA, 2.5 mM DTT, pH 7.5, and stored at −70 °C until used. The iron content of wild-type xanthine dehydrogenase and the R310M and R310K variants was determined to be in a range of 93 to 95%, whereas the molybdenum content varied for the proteins: 99% for wild-type xanthine dehydrogenase, 67% for the R310M variant, and 50% for the R310K variant, as analyzed by ICP-OES (Perkin Elmer Optima, DV2100). The levels of molybdenum saturation correspond well with the amount of the pterin cofactor present in the enzymes as determined by conversion to Form A described previously (11). The wild-type enzyme was determined to be approximately 80% active.

**Enzyme Assays and Rapid Reaction Kinetics**—Routine enzyme assays were carried out as described previously (10) at 25 °C in 20 mM Tris, 0.2 mM EDTA, pH 7.8, monitoring the absorbance change at 340 nm due to reduction of NAD (+) to NADH. The enzyme concentration was determined from the absorbance at 465 nm using an extinction coefficient of 31.6 mm M −1 cm −1 (11). Reductive half-reaction experiments were performed under anaerobic conditions using an Applied Photophysics SX-18MV kinetic spectrophotometer with a 1-cm observation path length. Standard reaction conditions were 20 mM Tris, 0.2 mM EDTA, pH 7.8, at 4 °C. In a typical experiment, enzyme at a concentration of 12–14 μM was mixed with an equal volume of substrate solution, the latter at concentrations ranging from 20 μM to 2.0 mM. The reaction was monitored at 460 and 620 nm over an appropriate time scale, and the observed kinetic transients fit to exponentials to obtain *k* obs.

For those substrates for which *k* obs varied with substrate concentration, *k* obs was then plotted against substrate concentration to obtain *k* red, the limiting rate of reduction at high [S], and the dissociation constant *K* d. For those substrates (e.g. 2-hydroxy-6-methylpurine, as seen previously with the bovine enzyme (12)) for which *k* obs did not vary, the average of the observed values was taken as *k* red.

Tris hydrochloride was from Fisher Scientific. Xanthine, 1-methylxanthine, 2-thioxanthine, and 6-thioxanthine were from Sigma, 2,6-diaminopurine from Aldrich, and 2-hydroxy-6-methylpurine from the Sigma-Aldrich Library of Rare Chemicals. Other reagents were of the highest purity available commercially and were used without further purification.

**RESULTS**

**Site-directed Mutagenesis of Arg-310 of *R. capsulatus* Xanthine Dehydrogenase**—Both active site residues Glu-232 and Glu-730 in the *R. capsulatus* enzyme have been shown to be
Arg-310 in Xanthine Dehydrogenase from R. capsulatus

The Reaction of Wild-type R. capsulatus Xanthine Dehydrogenase and R310K/R310M Variants with Xanthine—The reductive half-reaction of wild-type R. capsulatus xanthine dehydrogenase exhibits a hyperbolic dependence of the observed rate constant on substrate concentration, yielding at 4 °C values for \( k_{\text{red}} \) and \( K_d \) of 29 s\(^{-1}\) and 25 \( \mu \)M, respectively, with a value for \( k_{\text{red}}/K_d \) of \( 1.2 \times 10^6 \text{M}^{-1}\text{s}^{-1} \), in reasonable agreement with previous work (10). Upon substitution of Arg-310 with lysine, the corresponding values are 1.6 s\(^{-1}\), 45 \( \mu \)M and \( 3.7 \times 10^4 \text{M}^{-1}\text{s}^{-1} \), indicating that this conservative mutation significantly compromises but does not abolish the catalytic power of the enzyme. Mutation to methionine, on the other hand, reduces \( k_{\text{red}} \) to 0.0017 s\(^{-1}\), a reduction of more than \( 10^5 \) from the value seen with the wild-type enzyme. In the case of the R310M variant, no substrate concentration dependence of the observed rate constant was seen over the range of 20 \( \mu \)M to 2.0 mM, as has been observed previously with the slow substrate 2-hydroxy-6-methylpurine with bovine xanthine oxidase (12).

This contrasting behavior of the wild-type enzyme and R310M variant notwithstanding, it is evident from a comparison of \( k_{\text{red}} \) values alone that Arg-310 contributes \( \sim 4.5 \) kcal/mol toward transition state stabilization in the wild-type enzyme. As indicated in Table 1, the R310K variant was generally more active than the R310M variant for a variety of purine substrates, indicating that retaining the positive charge at position 310 gave rise to considerably less dramatic consequences on the kinetic behavior of the enzyme. It is thus evident that a positively charged residue at position 310 leads to significant transition state stabilization in the enzyme-catalyzed reaction.

The very great effect on \( k_{\text{red}} \) upon mutating Arg-310 to methionine is surprising given that Arg-310 lies \( \sim 10 \) Å from the active site molybdenum and on the opposite end of the substrate from the site that is hydroxylated (implying that a significant amount of the free energy available to the system from the favorable interaction between substrate and Arg-310 is used to stabilize the transition state rather than simply bind to substrate). As seen in Fig. 3, however, a positive charge in the vicinity of the C-6 position is expected to resonance-stabilize the negative charge accumulating on the ring as a result of nucleophilic
Arg-310 in Xanthine Dehydrogenase from R. capsulatus

**TABLE 1**

Rapid reaction kinetic parameters for wild-type *R. capsulatus* xanthine dehydrogenase and R310K and R310M variants

| Substrate          | Wild type | R310K | R310M |
|--------------------|-----------|-------|-------|
|                    | $k_{\text{red}}$ | $K_d$ | $k_{\text{red}}/K_d$ | $k_{\text{red}}$ | $K_d$ | $k_{\text{red}}/K_d$ | $k_{\text{red}}$ | $K_d$ | $k_{\text{red}}/K_d$ |
| Xanthine           | 67.3 ± 3.10 | 33.6 ± 0.07 | 2.00 ± 0.00 | 1.63 ± 0.05 | 44.7 ± 6.00 | 0.0365 | 0.00219 ± 0.0005 | ND | ND |
| 2-Thioxanthine     | 29.2 ± 1.00 | 24.9 ± 0.40 | 1.16 ± 0.06 | 1.037 ± 0.006 | 46.33 ± 40.00 | 0.000799 | 0.000167 ± 0.0005 | ND | ND |
| 6-Thioxanthine     | 14.49 ± 0.80 | 40.89 ± 10.00 | 0.354 ± 0.01 | 1.26 ± 0.02 | 9.43 ± 1.00 | 0.134 | 0.00540 ± 0.0002 | 15.05 ± 3.00 | 0.000359 |
| 1-Methylxanthine   | 22.36 ± 0.70 | 24.10 ± 4.00 | 0.928 ± 0.01 | 0.0135 ± 0.005 | ND | ND | 0.00547 ± 0.0002 | ND | ND |
| 2,6-Diaminopurine  | 1.68 ± 0.007 | 0.094 ± 0.01 | 0.0213 ± 0.005 | ND | ND | 0.0837 ± 0.004 | 10.66 ± 5.00 | 0.0785 | |
| 2-Hydroxy-6-methylpurine | 0.168 ± 0.003 | 9.52 ± 1.00 | 0.0176 | 0.01775 ± 0.0006 | ND | ND | 0.00175 ± 0.0006 | ND | ND |

*From Ref. 10.
Present work.
ND, not determined.
Global fit of diode array detector data set using two kinetic steps.
Global fit of diode array detector data set using one kinetic step.

attack at C-8. As discussed further below, the fact that this stabilization occurs at C-6 rather than C-2 has implications regarding the orientation of substrate in the active site for productive catalysis.

The Reaction of Additional Purine Substrates with Wild-type Xanthine Dehydrogenase—In addition to xanthine, we also examined the reaction of *R. capsulatus* xanthine dehydrogenase with 1-methylxanthine, 2-thioxanthine, 6-thioxanthine, 2,6-diaminopurine, and 2-hydroxy-6-methylpurine, compounds for which the structures are shown in Fig. 4. These purines were selected taking into consideration that xanthine dehydrogenase is also able to hydroxylate at C-2 and C-6 as well as C-8, when these positions are available; each member of the present set is substituted in such a way that it can only be hydroxylated at the C-8 position (as is xanthine), and as such they constitute a proper homologous series in which to examine substrate specificity. The results of this kinetic study are tabulated in Table 1.

Both 2- and 6-thioxanthine were found to be effective substrates for the *R. capsulatus* xanthine dehydrogenase, with $k_{\text{red}}$ and $K_d$ values comparable with those seen with xanthine. These two thio derivatives have also previously been found to be effective substrates for bovine xanthine oxidase (13). By contrast, 1-methylxanthine, a good substrate for the bovine enzyme, exhibits a 10-fold reduced $k_{\text{red}}$ and 2-fold increased $K_d$ with the *R. capsulatus* enzyme. Although it is among the best substrates for the bovine enzyme (13), it is rather less effective than xanthine as a substrate for the *R. capsulatus* enzyme. On the other hand, 2-hydroxy-6-methylpurine and especially 2,6-diaminopurine are manifestly poorer substrates than xanthine (or either of the thio derivatives) for the wild-type *R. capsulatus* enzyme, with $k_{\text{red}}$ values of 0.17 s$^{-1}$ and 0.007 s$^{-1}$, respectively.

The Reaction of Additional Purine Substrates with the R310K and R310M Variants of *R. capsulatus* Xanthine Dehydrogenase—The trend seen with xanthine as substrate was generally observed for the other purines used in this study, with reactivity moderately reduced in the R310K variant and significantly reduced in the R310M variant. The exception was seen with 2-hydroxy-6-methylpurine, where the R310K variant was actually modestly more active than the wild-type enzyme, by a factor of ~4. Interestingly, there was a very small range of values for $k_{\text{red}}$ for the R310M variant with the substrates used, a factor of only 4 as compared with 4000 for wild-type enzyme (or 13,000 for the wild-type bovine enzyme (13)).

![FIGURE 3. Proposed stabilization of negative charge accumulation in the transition state by Arg-310.](image)

![FIGURE 4. The purine substrates used in the present study. Shown are xanthine, 2-thioxanthine, 6-thioxanthine, 1-methylxanthine, 2-hydroxy-6-methylpurine, 2,6-diaminopurine. The structure for alloxanthine is shown for comparison.](image)
**DISCUSSION**

On the basis of the experiments described above, it is evident that the substrates investigated herein fall broadly into two groups: members of the first are effective substrates of the wild-type enzyme and are hydroxylated significantly less effectively by the R310M variant, whereas members of the second are poor substrates of the wild-type enzyme but are much less affected by mutation of Arg-310 to methionine. Included in the first group is the physiological substrate xanthine, in which hydroxylation proceeds more than 104-fold more slowly in the variant than in the wild-type enzyme. Given that Arg-310 is quite removed from the site where hydroxylation occurs, it is interesting that \( k_{\text{red}} \) is affected to the extent that it is upon mutation of Arg-310 to methionine. It is evident that a considerable amount of the free energy available to the enzyme from the favorable interaction of this residue with substrate (some 4.5 kcal/mol) is used not to stabilize the E-S complex but instead to stabilize the transition state, thereby accelerating the reaction. How this likely occurs is illustrated in Fig. 3, where it can be seen that interaction of Arg-310 with the C-6 carbonyl group of substrate stabilizes negative charge accumulation on the heterocycle that accompanies nucleophilic attack at C-8.

Effective neutralization of negative charge accumulation on substrate in the course of the reaction is best achieved if substrate binds in the active site with the orientation shown in Fig. 3, with the \( C_6 = O \) carbonyl in a position to interact with Arg-310. In the crystal structure of the reduced *R. capsulatus* enzyme with the tight-binding inhibitor alloxanthine, however, it is the carbonyl equivalent to \( C_2 = O \) of xanthine (somewhat confusingly, the numbering convention of heterocycles is such that the equivalent carbonyl of alloxanthine is designated \( C_6 = O \) (14)) that interacts with Arg-310 (3). It is important to recognize, however, that in the complex with reduced enzyme, alloxanthine sits ~2 Å closer to the molybdenum than does nascent product in the course of the reaction, because alloxanthine coordinates directly to the molybdenum via N-8 of its pyrazole ring, whereas the C-8 position of nascent product is bridged to the metal via the hydroxyl oxygen that is introduced catalytically (Fig. 5). Being somewhat further removed from the molybdenum, it is possible that the catalytically preferred orientation of substrate is in fact inverted relative to the orientation seen crystallographically in the alloxanthine complex. Indeed, a model for urate bound to xanthine oxidoreductase based on the structure of the *D. gigas* aldehyde oxidoreductase (which, unusually among the aldehyde-utilizing enzymes, possesses Arg at position 504, equivalent to Arg-310 in the *R. capsulatus* enzyme) has Arg-504 interacting with the \( C_6 = O \) rather than \( C_2 = O \) carbonyl group of the docked product (7).

FIGURE 5. Schematics of the orientations of alloxanthine and urate (the product of enzyme action on xanthine) coordinated to the reduced molybdenum center of xanthine dehydrogenase. The orientation shown for alloxanthine is that seen in the crystal structure of the reduced *R. capsulatus* enzyme in complex with the inhibitor (3). That shown for bound product is as described under ”Discussion” and in Ref. 7.

*In generating this model, it was necessary to exchange Phe-425 in the structural model of the *D. gigas* enzyme to a Glu and to assume that the hydroxyl groups of Tyr-535 and Tyr-622, which are both phenylalanine in the xanthine-utilizing enzymes, do not alter the mode of substrate binding. It is further to be noted that Tyr-622 of the *D. gigas* enzyme, which was not shown in the original model (7), occupies a significantly different orientation relative to the molybdenum center than does the corresponding Phe-459 (Phe-1009 in bovine) in the subsequently determined *R. capsulatus* and bovine structures.*
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(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 (7)). This conclusion is supported by the results of a kinetic study utilizing a homologous series of purines; 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, are strongly affected by the exchange of Arg-310 to methionine, whereas those that react most slowly with enzyme (2,6-diaminopurine and 2-hydroxy-6-methylpurine) have functional groups at position 6 (amino and methyl, respectively) that prevent interaction with Arg-310. We suggest that these substrates bind in an inverted orientation to that seen with xanthine, accounting for their low reactivity. Because these purines are not able to take advantage of transition state stabilization by Arg-310 in wild-type enzyme, they are expected to be less sensitive to its mutation to methionine, as was in fact observed. The disparity in reaction rate among the purine substrates used here with the R310M variant is 1000-fold less extensive than with the wild-type enzyme, indicating that Arg-310 plays a significant role in determining the substrate specificity of xanthine dehydrogenase. Finally, it is worth noting that the catalytic role for Arg-310 proposed here also provides an explanation as to why the arginine is not generally conserved in the aldehyde oxidases, because with these enzymes negative charge accumulation is expected to be restricted to the carbonyl oxygen of substrate, much nearer the molybdenum center.

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Addendum—Since our manuscript was submitted, Nishino and co-workers (16) have reported the properties of several mutants of human xanthine oxidoreductase, including an R881M mutant analogous to the R310M mutant considered here with the R. capsulatus xanthine dehydrogenase. By steady-state kinetic analysis, these authors find no detectable activity in the mutant, consistent with our observation here that $k_{\text{cat}}$ is reduced by a factor of 20,000. Interestingly, and in contrast with the bacterial enzyme, these workers find that activity toward benzaldehyde as substrate is only moderately affected by the mutation in the human enzyme.

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