The steady state and rapid reaction kinetics of an E232A coli XDH shows a preference for NAD (XO) by oxidation of sulfhydryl residues or by proteolysis (1, 2). cell, although it can readily be converted to an oxidase form dehydrogenase form, XDH, and exists mostly as such in the tally different from that of all other monooxygenase/hydroxylative titration and steady state kinetics on recombinant and is highly homologous to homodimeric eukaryotic the proposed role of this residue as an active site base indicating at least a 10^7 decrease in catalytic effectiveness for this variant. This result is fully consistent with the proposed role of this residue as an active site base that initiates catalysis.

Xanthine dehydrogenase (XDH, EC 1.1.1.204) catalyzes the hydroxylation of xanthine to uric acid with NAD^+ as the electron acceptor. R. capsulatus XDH forms an (αβ)₂ heterotetramer and is highly homologous to homodimeric euarkytic xanthine oxidoreductases. Here we first describe reductive titration and steady state kinetics on recombinant wild-type R. capsulatus XDH purified from Escherichia coli, and we then proceed to evaluate the catalytic importance of the active site residues Glu-232 and Glu-730. The steady state and rapid reaction kinetics of an E232A variant exhibited a significant decrease in both k_{cat} and k_{val} as well as increased K_a and K_v values as compared with the wild-type protein. No activity was determined for the E730A, E730Q, E730R, and E730D variants in either the steady state or rapid reaction experiments, indicating at least a 10^2 decrease in catalytic effectiveness for this variant. This result is fully consistent with the proposed role of this residue as an active site base that initiates catalysis.

Xanthine dehydrogenase (XDH, EC 1.1.1.204) catalyzes the hydroxylation of hypoxanthine to xanthine and xanthine to uric acid, the last two steps in the formation of urate. A water molecule rather than O₂ is the ultimate source of oxygen incorporated into the product, and the reaction is thus fundamentally different from that of all other monooxygenase/hydroxylase systems. The mammalian enzyme is synthesized as the dehydrogenase form, XDH, and exists mostly as such in the cell, although it can readily be converted to an oxidase form (XO) by oxidation of sulfhydryl residues or by proteolysis (1, 2). XDH shows a preference for NAD^+ reduction at the FAD site but is also able to react with molecular oxygen, whereas XO fails to react with NAD^+ and exclusively uses dioxygen as its substrate. The conversion of XDH to XO is of considerable medical interest, because the enzyme is implicated in hyperuricemia, and its activity is proposed to play a role in post-ischemic reperfusion injury (3). The mammalian enzyme is a homodimer with a molecular mass of 290 kDa, with each monomer acting independently in catalysis. Each subunit contains one molybdenum cofactor (MocO, consisting of a MoOS(OH) core coordinated to 1 eq of molybdopterin), two [2Fe-2S] clusters that are distinguishable by EPR, and 1 eq of FAD (4). The oxidation of xanthine takes place at the MocO, and the electrons thus introduced are transferred via the two FeS centers to FAD by intramolecular electron transfer. Rhodobacter capsulatus XDH is a cytoplasmic enzyme with an (αβ)₂ heterodimeric structure, with the two subunits encoded by the xdhA and xdhB genes, respectively (5). The redox-active cofactors are bound to two different subunits; the FAD and [2Fe2S] centers are found in the XDH subunits, and the MocO is found in the XDHB subunits. This complement of redox-active centers provides the enzyme with the capacity to hold a maximum of 6 reducing eq per functioning αβ unit (corresponding to one intact subunit in the case of the vertebrate enzymes) upon reoxidation; under anaerobic conditions each can react with 3 eq of xanthine. Unlike the mammalian oxidoreductases, but like avian XDH, R. capsulatus XDH is isolated with high reactivity toward NAD^+ and does not undergo the conversion to the oxidase form (6). In addition to xanthine, XDH is reducible by NADH (7, 8). The recently solved crystal structures of the mammalian xanthine oxidoreductase (XOR) from bovine milk (9) and the homologous bacterial XDH from R. capsulatus (10) have paved the way for a detailed structural and functional analysis of these enzymes. In previous work (6) we have reported a system for the heterologous expression of R. capsulatus in Escherichia coli. The bacterial XDH thus represents an ideal system for the generation of site-directed mutants by which the catalytic roles of various active site residues at the molybdenum center can be evaluated.

Here we describe a detailed kinetic characterization of wild-type XDH by steady state and rapid reaction kinetics, and we then compare and contrast this with the behavior of site-specific mutations of two glutamate residues at the active site of the enzyme. The two active site glutamate residues that have been targeted, Glu-232 and Glu-730, have been proposed to be involved in either substrate binding or in rate acceleration of substrate oxidation by base catalysis. Our kinetic results with Glu-232 and Glu-730 variants clearly identify these residues as important for catalysis, and we show that residue Glu-730 is essential for catalysis by acting as an active site base that initiates substrate oxidation. Our data provide the first mutagenic study of amino acid residues at the active site of a xanthine oxidoreductase.

**EXPERIMENTAL PROCEDURES**

**Protein Purification—**R. capsulatus XDH was purified using the procedure described by Leimkühler et al. (6) with some modifications. Instead of chromatography on phenyl-Sepharose, the protein was puri-
Catalytic Mechanism of *R. capsulatus* **XDH**

The active form of XDH was eluted from the affinity column with 20 mM Tris, 0.2 mM EDTA, pH 7.8, containing 15% pyrophosphate and 20 mM salicylate. Both fractions were separately concentrated using ultrafiltration, gel filtered using a PD-10 gel filtration column (Amersham Biosciences), and finally stored in 20 mM Tris, 0.2 mM EDTA, 2.5 mM dithiothreitol, pH 7.8. By using PCR mutagenesis amino acid exchanges E232A, E730A, E730Q, E730D, and E730R were introduced into *R. capsulatus* XDH. The generated XDH variants were purified by nickel-nitrilotriacetic acid and Q-Sepharose chromatography, and XDH variant E232A was additionally purified on Sepharose 4B/folate gel.

**Enzyme Assays**—Enzyme assays were carried out at 25 °C in 20 mM Tris, 0.2 mM EDTA, pH 7.8, in a final volume of 1 ml. Routine assay mixtures contained 1 mM xanthine. XDH activities with various electron acceptors were determined by monitoring the absorbance changes as follows: NADH (1 mM; 340 nm), O2 (air-saturated buffer; 295 nm), or cytochrome c (16.7 μM; 550 nm). XDH concentration was determined from the absorbance at 465 nm using the extinction coefficient of 31.6 mm−1 cm−1 for the native enzyme. The extinction coefficient was determined on the basis of FAD content after acid precipitation. The activity to flavin ratio (AFR) was obtained by dividing the change in absorbance/min at 295 nm in the presence of NADH by the absorbance at 465 nm of the enzyme used in the assay at 25 °C.

**Reducive Titration with Sodium Dithionite**—Reducive titration of XDH was carried out in 1 ml of 20 mM Tris, 0.2 mM EDTA, pH 7.8, at 20 °C. Anaerobic enzyme was prepared in an all-glass apparatus (12) by using oxygen-free argon obtained by passing the argon gas through a column of copper with calcium oxide pellets (Osaka Sanso). Sodium dithionite was dissolved in the same buffer and made anaerobic by bubbling with oxygen-free argon for more than 10 min and put in a gas-tight glass syringe. Concentrations of sodium dithionite were determined by titration with a solution of FAD of known concentration. Absorption spectra were recorded with a Hitachi U-3200 spectrophotometer in a double-beam mode.

**Absorbance Spectra during Anaerobic Reduction with Xanthine and NADH**—XDH in 1 ml of 20 mM Tris, 0.2 mM EDTA, pH 7.8, was put in the main compartment of an anaerobic cuvette, and 50 μl of 10 mM xanthine or NADH were put in the side arm. The solutions in the main compartment and side arm were made anaerobic by repeat evacuation and flushing with argon. The reaction was started by mixing the two solutions and was allowed to proceed at 20 °C. Recording of the absorbance spectrum was repeated over the range of 250 to 800 nm with a Beckman DU-7400 diode array spectrophotometer.

**Rapid Reaction Kinetics of Recombinant Protein**—Rapid half-reaction experiments were carried out under anaerobic conditions in 20 mM Tris, 0.2 mM EDTA, pH 7.8, at 4 °C with an Applied Photophysics SX-18MV kinetic spectrophotometer with a 1-cm observation path length. For recombinant wild-type XDH, enzyme concentrations of 4 μM were mixed in equal volumes of various concentrations of xanthine and NADH. Absorbance changes were monitored at 460 and 620 nm in time periods ranging from 0.05 to 1 s, and the observed rate constants 

\[ k_{\text{obs}} \]

were then plotted against substrate concentration to obtain the reduction constant (\( k_{\text{red}} \)) and the dissociation constant (\( K_a \)). For the E232A variant, the enzyme concentrations of 30–40 μM were mixed in equal volumes with various concentrations of xanthine (24–520 μM). Absorbance changes were monitored at 460 nm in time periods ranging from 10 to 200 s. The E730A, E730Q, E730D, and E730R variants were too slow to be observed on the stopped flow and were therefore reacted under anaerobic conditions between 5 and 24 h while periodically measuring an absorbance spectrum.

**Quantitative Analysis of the Amount of Cyanosoluble Sulfur Present in *R. capsulatus* XDH**—In order to assess quantitatively the degree of incorporation of both molybdenum and the acid-labile sulfur (present as Mo=S) in the molybdopterin coordination sphere, the amount of Mo=S present in *R. capsulatus* XDH was determined by the cyanolysis method of Westley (13), which quantitates the amount of labile sulfur released as thiocyanate upon reaction with cyanide. 2 mg of XDH in 50 mM HEPES, pH 7.2, were incubated with 100 mM KCN overnight at 4 °C. Subsequently, the protein fraction was separated from the soluble fraction by ultrafiltration. Released SCN was determined by the addition of 500 μl of ferric nitrate reagent (100 g of Fe(NO3)3·9H2O and 200 ml of 65% HNO3 per 1500 ml) to 500 μl of the protein-free soluble fraction. Thiocyanate (complexed with iron) was quantitated by \( A_{520} \) using an SCN standard curve.

**RESULTS**

**Purification of Active *R. capsulatus* XDH by Folate Affinity Chromatography**—*R. capsulatus* XDH is a member of the MoOS family of molybdenum enzymes, containing Mo=O and Mo=S groups in the molybdenum coordination sphere (10). It has been shown that fully active XDH can be separated from inactive species (e.g. desulfo or demolybdy enzyme) by using affinity chromatography on Sepharose 4B/folate gel (11). To obtain molybdenum-saturated *R. capsulatus* XDH, enzyme purified by nickel-nitrilotriacetic acid and Q-Sepharose chromatography was applied to a folate affinity column. Inactive *R. capsulatus* XDH, lacking molybdenum or both molybdenum and the catalytically essential sulfur, was eluted from the column with buffer containing 10% pyrophosphate, whereas active enzyme possessing the fully assembled molybdenum center, including the catalytically essential Mo=S group, was eluted with buffer containing 15% pyrophosphate and 20 mM salicylate. Visible absorption spectra of recombinant XDH purified after folate affinity chromatography are shown in Fig. 1. The active portion of the enzyme showed an absorbance ratio of \( A_{440}/A_{550} \) of 3.12 and an \( A_{490}/A_{550} \) ratio of 4.9, reflecting a high purity of the enzyme, as well as a FAD to iron content of 1:4 (Table I). On the basis of FAD content, the molar extinction coefficient for *R. capsulatus* XDH was calculated as 31.6 mM−1 cm−1. In contrast, the less tightly bound enzyme was mainly inactive and was determined to lack substantial amounts of the Mo=S group (Table I, data not shown). After separation of the inactive species, the active portion of *R. capsulatus* XDH showed an AFR (as defined under “Experimental Procedures”) of 1750. As shown in Table II, analysis of the amount of cyanosoluble sulfur present in *R. capsulatus* XDH gave a value of 80% of Mo=S present in the enzyme. This shows that in contrast to the bovine enzyme, *R. capsulatus* XDH is purified in a form possessing an almost full complement of the Mo=S ligand required for activity (14).

**Reduction of *R. capsulatus* XDH by Xanthine, NADH, and Sodium Dithionite**—Before characterizing variants of *R. capsulatus* xanthine dehydrogenase generated by site-directed mutagenesis, it was first necessary to establish the behavior of the wild-type enzyme. The reduction of wild-type enzyme by xanthine and NADH was thus examined under anaerobic conditions. Fig. 2A shows the spectra of 13.3 μM oxidized XDH and reduced enzyme 15 s and 135 min after mixing with 0.5 mM xanthine. The reduction level reached around 80% of the initial absorbance at 465 nm immediately after mixing, and the enzyme was completely reduced after an incubation time of 135...
**Table I**

| XDH protein after | Q-Sepharose | Sepharose 4B/folate gel |
|---|---|---|
| Total yield of protein (mg) | 82.27 | 10.5 |
| % PP, 10% PP, 15% PP/20 mm salicylate | 10.5 | 46.68 |
| $A_{466}/A_{350}$ | 7.25 | 7.8 |
| $A_{466}/A_{530}$ | 3.16 | 3.19 |
| Xanthine::NAD activity$^a$ | 23.84 | 3.69 |
| $\text{AFR}^b$ | 1173 | 213 |

$^a$ Xanthine::NAD activity was recorded at 340 nm.

$^b$ AFR was obtained by dividing the change in absorbance/min at 295 nm in the presence of NAD by the absorbance at 465 nm of the enzyme used in the assay.

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**Table II**

| XDH variant | Wild-type | E22A/7E30A | E730D | E730Q | E730R |
|---|---|---|---|---|---|
| % Moco content$^a$ | 100 | 108 | 33 | 92 | 109 |
| Mol of S per subunit$^a$ | 0.76 | 0.83 | 0.22 | 0.32 | 0.48 |

$^a$ Moco was quantitated as described previously (26). Moco content of wild-type *R. capsulatus* XDH was set to 100%, and the amount of Moco determined in the active site variants was compared to that value.

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**Table III**

| XDH variant | Wild-type | E22A | 7E30A | E730D | E730Q | E730R |
|---|---|---|---|---|---|---|
| % Moco content | 100 | 108 | 33 | 92 | 109 | 81 |
| Mol of S per subunit | 0.76 | 0.83 | 0.22 | 0.32 | 0.48 | 0.62 |

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Absorbance at 530 nm, consistent with the formation of the flavin semiquinone radical with simultaneous reduction of the FeS centers to an appreciable degree. Addition of the next 2 eq resulted in little increase in absorbance at 630 nm with continued decrease in absorbance at 466 and 530 nm. This suggests that the flavin semiquinone level remained unchanged, whereas the FeS centers became reduced to a greater degree. The last 2 reducing eq caused a continuous and uniform loss of the remaining absorbance at all wavelengths, indicating reduction of flavin semiquinone to the hydroquinone. These data are consistent with the reductive titration of chicken liver XDH (17), pointing to a similar order of oxidation-reduction potentials for both enzymes.

**Steady State Kinetics and Superoxide Generation of Wild-type Enzyme—Steady state kinetics of wild-type *R. capsulatus* XDH were performed by varying the concentrations of xanthine and NAD (see “Experimental Procedures”). From the data, $k_{\text{cat}}$/xanthine$^1$ is calculated as $108 \pm 1.5 \text{ s}^{-1}$, $K_m$/xanthine, as 64.5 ± 0.9 μM (Table III), and $K_m$/NAD, as 103 ± 2.5 μM. Because the enzyme used in the assay was only 80% active, the corrected turnover number for the fully functional enzyme is 135 s$^{-1}$. This value is six times higher than that reported for chicken liver XDH of 22.5 s$^{-1}$ (16) and is similar to that of *Comamonas acidovorans* XDH (18). The bacterial enzymes thus react much faster with xanthine as substrate, which explains the higher activities (expressed as AFR) of the enzyme in standard assays as compared with bovine milk XO that have been described previously (6). Substrate inhibition at xanthine concentrations higher than 200 μM was not observed for *R. capsulatus* XDH, a phenomenon described for bovine XO that is associated with substrate binding to reduced rather than oxidized forms of the enzyme generated in steady state (19, 20).

*R. capsulatus* XDH, like avian XDH, is isolated with high reactivity toward NAD$^+$ and low reactivity toward oxygen as electron acceptor and does not convert to an oxidase form (6). The absorption spectrum observed when enzyme is reacted with xanthine under aerobic conditions indicates that *R. capsulatus* XDH is largely reduced in the steady state, consistent with a lower overall reactivity toward O$_2$ as compared with NAD$^+$ as oxidizing substrate (Fig. 4). The predominant flavin species is the semiquinone form, as evidenced by the increase in absorption at 620 nm relative to the oxidized enzyme (Fig. 4) which is typical for the dehydrogenase form of the XORs at pH 7.8. It is known, however, that the dehydrogenase forms of the vertebrate enzymes produce a larger amount of O$_2$ than do the oxidase forms (16, 21). The production of O$_2$ during turnover is conveniently monitored by including cytochrome c into the reaction mixture, the reduction of cytochrome c by O$_2$ being considerably faster than the spontaneous dismutation of O$_2$. The amount of O$_2$ produced in the reaction mixture is obtained by comparison against a blank reaction run under the same conditions in the presence of saturating superoxide dismutase (16). By using this method we observe that the reduction of cytochrome c is the same with or without the addition of superoxide dismutase, with 1.9 mol of cytochrome c reduced per min/mol of enzyme. In comparison, 2.5 mol of urate/min/mol of enzyme were produced using O$_2$ as electron acceptor, so that during turnover with xanthine and O$_2$, *R. capsulatus* XDH produces 38% of O$_2$ radicals. This is comparable with results with chicken liver XDH, which produces 40–44% O$_2$ radicals, and rat XDH, which produces 34% O$_2$ radicals, during turnover with xanthine and O$_2$ (16).

**Kinetics of Reduction of Wild-type *R. capsulatus* XDH by Xanthine and NADH**—Finally, with regard to the wild-type enzyme, we have examined the kinetics of reduction by both xanthine and NADH, using a stopped-flow spectrophotometer.
As shown in Fig. 5A, the reaction as followed at several different wavelengths was biphasic. The first phase of the reaction has a spectral change reflecting substantial reduction of the flavin with significant accumulation of both semiquinone and hydroquinone, as well as FeS reduction. This phase is dependent on the concentration of xanthine with values of \( k_{\text{red(xanthine)}} \) and \( K_d(\text{xanthine}) \) (Fig. 5B). For both bovine XO and chicken liver XDH, the overall rate-limiting step is thought to be dissociation of urate from the reduced molybdenum center (for chicken XDH, \( k_{\text{lim}} \) at 4 °C) (17). Given that turnover is substantially faster in the bacterial enzymes as compared with enzyme from vertebrate sources, product release must be considerably faster than is seen with the vertebrate enzymes. The second phase of the reaction with xanthine has a similar rate following the reaction at either 460 or 620 nm (about 3 s\(^{-1}\)) and is independent of the xanthine concentration. The second phase resulted in the disappearance of the flavin semiquinone radical (no change in absorbance at 620 nm) and a further decrease of the absorbance at 460 nm, reflecting reduction of the flavin semiquinone onto the hydroquinone, with additional reduction of the FeS centers.

We have also analyzed the kinetics of reduction of XDH\textsuperscript{ox} by NADH. Although NADH is the product of the physiological reaction, when present in excess it can efficiently reduce XDH\textsuperscript{ox} to the four-electron level, effectively backing up the reaction (7). As with xanthine, reaction of XDH\textsuperscript{ox} with NADH gave a biphasic reaction as shown in Fig. 6A. The first phase of the reaction is very fast, and the rate constant \( k_{\text{red(NADH)}} \) has been estimated as 1093 s\(^{-1}\) (Fig. 6B). As seen with xanthine, the spectral change associated with the first phase showed FeS reduction and flavin semiquinone accumulation. Unlike the reaction with xanthine, however, the rate constant for the second phase exhibits hyperbolic dependence on the NADH concentration and \( k_{\text{red(NADH)}} \) and \( k_{\text{d(NADH)}} \) values were determined to be 98.4 \pm 1.0 s\(^{-1}\) and 26.8 \pm 0.9 \mu M, respectively (Fig. 6B). A possible explanation for this observation is that the second NADH molecule reacts with two-electron reduced XDH with the following equilibrium (Scheme 1).

\[
\begin{align*}
\text{FeSI}_{\text{red}} & \rightleftharpoons \text{FeSI}_{\text{ox}} \\
\text{Mo}^{V} & \rightleftharpoons \text{Mo}^{IV} \\
\text{FADH}^{+} & \rightarrow \text{FAD} + \text{NADH} \\
\text{FeSI}_{\text{ox}} & \rightleftharpoons \text{FeSI}_{\text{red}} + K_{d(\text{NADH})} \\
\text{FADH}_{2} & \rightarrow \text{FAD} + \text{NADH} \\
\text{FeSI}_{\text{ox}} & \rightleftharpoons \text{FeSI}_{\text{red}} + K_{d(\text{NADH})}
\end{align*}
\]

Scheme 1

Steady State and Rapid Reaction Kinetics of XDH Variants E232A, E730A, E730D, E730Q, and E730R—The crystal structure of R. capsulatus XDH with alloxanthine bound at the active site has shown that the six-membered ring of the inhibitor interacts with Glu-232, Arg-310, and Glu-730 (Fig. 7A) suggesting that these amino acids are either involved in substrate binding or in the rate acceleration of substrate oxidation.

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\( ^3 \) The prevailing model for interpreting the spectral changes associated with the observed kinetic phases of the molybdenum hydroxylases is the rapid equilibrium model as originally proposed by Olson et al. (8). According to this model, upon dissociation of product urate from the reduced molybdenum center, reducing equivalents equilibrate between the several redox-active centers of the enzyme according to their relative reduction potentials, and do so rapidly relative to the rate of product dissociation. It is for this reason that the kinetics of the reaction at the Moco, which is essentially colorless, can be monitored by the accumulation of reducing equivalents in the FAD and FeS centers of the enzyme.
The two active site glutamate residues are highly conserved to those identified in the active site of Desulfovibrio gigas aldehyde oxidoreductase, and on the basis of this structure a mechanism for hydroxylation has been proposed involving base-assisted proton abstraction from the attacking Mo—OH group by the glutamate residue corresponding to Glu-730 in R. capsulatus XDH (22). With the kinetic and spectroscopic results for the wild-type enzyme in hand, it is now possible to analyze the importance of these two glutamate residues by site-directed mutagenesis. Thus, the variants E232A, and E730A, E730Q, E730D, and E730R of R. capsulatus XDH have been generated as described under “Experimental Procedures.” Whereas the E232A variant as purified by folate affinity chromatography yields an enzyme with a comparable Mo=S content as found with the wild-type protein (Table II), amino acid exchanges of Glu-730 resulted in an altered affinity to Sepharose 4B/folate gel so that no separation of nonfunctional forms of the protein was possible. Thus, the Mo=S content of these variants was lower in comparison to wild-type XDH (Table II), and it was necessary to correct for this lower content in the steady state kinetic analysis. Analysis of the cyanolysable sulfur showed that wild-type XDH and the E232A variant were purified with a Mo=S content of about 80%, whereas a lower amount of the catalytically competent portions of the Glu-730 variants, the E730A in particular, contained the terminal sulfur ligand (Table II). In contrast, the purified XDH variants contained a full complement of [FeS] centers and FAD as the wild-type protein.

The steady state kinetics of the recombinant proteins with varying concentrations of xanthine and 1 mM NAD+ were obtained by monitoring absorbance increases at 340 nm for NADH production. The kinetic parameters $k_{cat/}\text{xanthine}$; $K_M(\text{xanthine})$ and $k_{cat/}\text{Km}$ at pH 7.8 are given in Table III. The $k_{cat/}\text{xanthine}$ and $K_M(\text{xanthine})$ for the E232A mutant protein are found to be $4.4 \pm 0.54 \text{ s}^{-1}$ and $163 \pm 35 \mu\text{M}$, respectively. In contrast, repeated experiments with the Glu-730 variants showed no activity, and therefore the $k_{cat/}\text{xanthine}$ and $K_M(\text{xanthine})$ could not be determined. It is clear that these variants are profoundly compromised in catalytic power, even after taking into account the lower level of Mo=S content of the E730A relative to other recombinant forms of the enzyme.

The rapid reaction kinetic parameters $k_{red(\text{xanthine})}$ and $K_{d(\text{xanthine})}$ at 4 °C were determined for the E232A and Glu-730 variants by using stopped-flow spectrophotometry, following reaction by the absorbance decrease at 460 nm associated with reduction of the enzyme as done above for the wild-type enzyme (Table III). Because enzyme reduction is followed directly in these experiments, the lower molybdenuim content of the Glu-730 variants did not complicate quantitation; to the extent that the nonfunctional forms of the enzyme are present, there will be a larger background absorbance, but the spectral change associated with reduction of that portion of the enzyme population possessing the Mo=S group can still be detected and the kinetics accurately determined. The rate of reduction slowed by a factor of 12 from $67.3 \pm 1.3 \text{ s}^{-1}$ for the wild-type enzyme to $5.5 \pm 0.52 \text{ s}^{-1}$ for the E232A mutant. The $K_{d(xanthine)}$ for the E232A mutant is 409 ± 51 µM, which is an increase from the wild-type enzyme by a factor 12 (and generally consistent with the steady state results). On the other hand, repeated attempts to determine the $k_{red}$ for the Glu-730 variants were unable to detect enzyme reduction by xanthine even after extended overnight incubations, using concentrations of the functional form of the mutant that should have given absorbance changes on the order of 0.3. Cyanolysis controls, as described under “Experimental Procedures,” confirmed that the enzyme sample indeed had the levels of Mo=S incorporation that were expected, and the absence of observable enzyme reduction could not be attributed to a low Mo=S content (from Table II). We conservatively estimate $t_{1/2}$ for reduction of the Glu-730 variants to be no shorter than 8 h, indicating that the rate of reaction at high xanthine concentrations is at least $\sim 10^4$-fold slower than seen with wild-type enzyme. In contrast to the impaired reduction by xanthine, all XDH variants were reduced by NADH to the same extent as the wild-type enzyme. In contrast to the impaired reduction by xanthine, all XDH variants were reduced by NADH to the same extent as the wild-type enzyme.
almost full complement of the Mo=S ligand required for functionality. *R. capsulatus* XDH, like avian XDH, was isolated here with high reactivity toward NAD and low reactivity toward oxygen as electron acceptor and did not convert to an oxidase form (6). However, *R. capsulatus* XDH produced about 38% superoxide radicals because of the accumulation of the flavin semiquinone during reduction which produces O2 after reacting with O2, whereas enzyme forms containing FADH2 react with O2 forming H2O2 (21, 23). This result is consistent with the superoxide stoichiometries for chicken liver XDH (40–44% O2) and rat XDH (34% O2) and is consistent with *R. capsulatus* XDH being a true dehydrogenase. This underscores the fact that the dehydrogenase form of the enzyme generates substantially higher amounts of O2 during catalysis than determined for bovine XO (30%).

The turnover number for *R. capsulatus* XDH with xanthine was found to be about six times higher than that for the chicken liver enzyme, illustrating modest differences in the catalytic activities of the two enzymes. Because the rate-limiting step is thought to be a release of urate from reduced enzyme, this indicates minimally that product release must be considerably faster in the bacterial enzyme than is the case with the vertebrate enzymes. We find that reduction of *R. capsulatus* XDH with xanthine or NADH proceeds biphasically. In contrast, the reduction of chicken liver XDH was determined to occur in three phases, with the middle phase being dependent on product release (17).

By way of addressing the catalytic importance of specific amino acid residues known to be present in the active site, we have investigated the kinetic behavior of two site-directed mutants of the *R. capsulatus* XDH. Our kinetic results clearly establish that Glu-232 is important in both substrate binding (as reflected in *Km*) and transition state stabilization (as reflected in *kcat*). Both the steady state and rapid kinetics indicate considerably slower rates for the E232A mutant. The *kcat* decreases by a factor of 16 from wild type, and *kred* from the rapid half-reaction exhibits a 12-fold decrease from wild type. The E232A variant also has a 100-fold larger dissociation constant for substrate binding than does the wild-type enzyme, indicating that this residue also plays an important role in substrate binding at the active site. From Arrhenius theory, a 1 order of magnitude change in *kcat* indicates that Glu-232 contributes 1.4 kcal/mol in transition stabilization to the overall reaction, and the 100-fold change in *Kd* indicates from

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**Table III**

| Kinetic parameters for *R. capsulatus* XDH and variants E232A, E730A, E730D, E730Q, and E730R with xanthine |
|---------------------------------------------------------------|
|                  | Steady state | Reductive half-reaction |
|                  | *kcat* | *Km* | *kcat*/*Km* | *kred* | *Kd* | *kred*/*Kd* |
| Wild-type XDH    | 108 ± 1.5 | 64.5 ± 0.9 | 167 ± 0.9 | 67.3 ± 1.3 | 33.6 ± 2.3 | 203 ± 0.14 |
| E232A            | 4.4 ± 0.54 | 163 ± 35 | 2.7 ± 0.58 | 5.5 ± 0.52 | 409 ± 57 | 1.3 ± 0.18 |
| E730A            | ND | ND | ND | ND | ND | ND |
| E730D            | ND | ND | ND | ND | ND | ND |
| E730Q            | ND | ND | ND | ND | ND | ND |
| E730R            | ND | ND | ND | ND | ND | ND |

* Kinetic data were recorded in 20 mM Tris, 0.2 mM EDTA, pH 7.8.
* No activity detectable.
XDH and NADH (final concentrations of 5.5 and 4.68 mM) were mixed anaerobically as described in Fig. 5. This amounted to a minimum of 10 kcal/mol toward stabilization of the ES complex.

Our kinetic work with the E730A, E730Q, E730D, and E730R variants indicated that Glu-730 is catalytically essential; the rapid half-reaction kinetic results of all variants did not indicate any significant reduction, even after prolonged incubation with excess substrate. Based on our results we conclude that mutation of Glu-730 in general decreases it functioning as an active site base that abstracts protons from the Mo-OH group (Fig. 7B). This amounts to a minimum of 10 kcal/mol in transition state stabilization from Arrhenius theory. Because all amino acid exchanges of Glu-730 gave the same carbon atom of the substrate. In addition, the equatorial Mo=S group of the oxidized enzyme has become protonated to afford Mo-SH on reduction of the molybdenum center (25). Glu-1261 was proposed to act as a general base by abstracting the proton from Mo-OH, and the new protonated Glu-1261 is stabilized by forming a hydrogen bond to the N-1—nitrogen of the substrate (25). In light of the mechanistic and structural similarities among bovine XOR and R. capsulatus XDH, our results with the Glu-730 variants of R. capsulatus XDH fully support an essential catalytic role for this residue and are consistent with it functioning as an active site base that abstracts protons from the Mo-OH group (Fig. 7B), which then undertakes a nucleophilic attack on the C-S position of substrate. Concomitant hydride transfer to the Mo=S group yields an intermediate with the product directly coordinated to the MoV center (Fig. 7B), which was shown in the crystal structure of the bovine XDH FYX-051 complex.

Acknowledgment—We thank Annika Krause (Bruinschweig, Germany) for technical assistance.

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FIG. 6. A, time courses of absorption at 460 and 620 nm of R. capsulatus XDH after mixing with 45.1 μM NADH using a stopped-flow apparatus. B, dependence on NADH concentration of observed rate constants (k_{obs}) for first (filled square) and second (open circle) phases. XDH and NADH (final concentrations of 5.5 and 4.68–225 μM, respectively) were mixed anaerobically as described in Fig. 5.

\[ \Delta G^\circ = -RT \ln \left( \frac{k_{\text{obs}}}{k_{\text{mutant}}} \right) \]

that it contributes 2.8 kcal/mol toward stabilization of the ES complex.

The pH dependence studies of bovine XO with substrate xanthine have yielded bell-shaped curves with pK_a values of 7.4, attributable to substrate, and 6.6, assigned to an active site base (24) leading to a mechanism for bovine XO, in which Glu-1261 (corresponding to Glu-730 in the R. capsulatus XDH) acts as an active site base in initiating catalysis (4). Recently, the crystal structure of bovine XDH with an intermediate in the hydroxylation reaction of the slow substrate FYX-051 has been solved (25). The crystal structure showed a carbon-oxygen bond of the product that is complexed to the molybdenum atom of the cofactor, and the complex indicates that the catalytically labile Mo-OH oxygen has formed a bond with a carbon atom of the substrate. In addition, the equatorial Mo=S group of the oxidized enzyme has become protonated to afford Mo-SH on reduction of the molybdenum center (25). Glu-1261 was proposed to act as a general base by abstracting the proton from Mo-OH, and the new protonated Glu-1261 is stabilized by forming a hydrogen bond to the N-1—nitrogen of the substrate (25). In light of the mechanistic and structural similarities among bovine XOR and R. capsulatus XDH, our results with the Glu-730 variants of R. capsulatus XDH fully support an essential catalytic role for this residue and are consistent with it functioning as an active site base that abstracts protons from the Mo-OH group (Fig. 7B), which then undertakes a nucleophilic attack on the C-S position of substrate. Concomitant hydride transfer to the Mo=S group yields an intermediate with the product directly coordinated to the MoV center (Fig. 7B), which was shown in the crystal structure of the bovine XDH FYX-051 complex.

Acknowledgment—We thank Annika Krause (Bruinschweig, Germany) for technical assistance.
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*J. Biol. Chem.* 2004, 279:40437-40444. doi: 10.1074/jbc.M405778200 originally published online July 20, 2004

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