Arginine 165/Arginine 277 Pair in (*S*)-Mandelate Dehydrogenase from *Pseudomonas putida*: Role in Catalysis and Substrate Binding†

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ABSTRACT: (*S*)-Mandelate dehydrogenase from *Pseudomonas putida* belongs to a FMN-dependent enzyme family that oxidizes (*S*)-α-hydroxyacids. Active site structures of three homologous enzymes, including MDH, show the presence of two conserved arginine residues in close juxtaposition (Arg165 and Arg277 in MDH). Arg277 has an important catalytic role; it stabilizes both the ground and transition states through its positive charge as well as a hydrogen bond [Lehoux, I. E., and Mitra, B. (2000) *Biochemistry 39*, 10055−10065]. In this study, we examined the role of Arg165 and the overall importance of the Arg165/Arg277 pair. Single mutants at Arg165 as well as double mutants at Arg165 and Arg277 were characterized. Our results show that Arg165 has a role similar to, but less critical than, that of Arg277. It stabilizes the transition state through its positive charge and the ground state through a charge-independent interaction, most likely, a hydrogen bond. Though the k_{cat}s for the charge-conserved mutants, R165K and R277K, were only 3−5-fold lower than those of wild-type MDH (*wt*MDH), the k_{cat} for R165K/R277K was ∼350-fold lower. Thus, at least one arginine residue is required for the optimal substrate orientation and catalysis. Stopped-flow studies show that the FMN reduction step is completely rate-limiting for both *wt*MDH and the arginine mutants, with the possible exception of R165E. Substrate isotope effects indicate that the carbon−hydrogen bond-breaking step is only partially rate-limiting for *wt*MDH but fully rate-limiting for the mutants. pH profiles of R165M conclusively show that the pK_a of 9.3 in free *wt*MDH does not belong to Arg165.

(*S*)-Mandelate dehydrogenase (MDH),[1] an enzyme in the mandelate metabolic pathway in *Pseudomonas putida*, oxidizes (*S*)-mandelate to benzoyl formate (*1*). It belongs to an FMN-dependent enzyme family that specifically oxidizes the (*S*)-enantiomer of α-hydroxyacids to α-ketoacids. The members of this family share extensive sequence similarity (*2*, *3*). Structural analyses of three enzymes, glycolate oxidase from spinach, flavocytochrome b_2 (L-lactate dehydrogenase) from *Saccharomyces cerevisiae*, and a soluble form of the membrane-bound MDH, show that they have highly homologous structures and identical active site architectures (*4*−*6*). In particular, two conserved arginine residues that are close together, Arg165 and Arg277 (MDH numbering), are part of the active site (Figure 1). The mechanism of the substrate oxidation and flavin reduction half-reaction can involve either the formation of a carbanion intermediate that subsequently transfers an electron pair to FMN or the transfer of the substrate α-hydrogen as a hydride ion to FMN. The majority of experimental evidence gathered to date for a number of enzymes in this family, including MDH, supports the former mechanism (*7*−*9*). It is likely that two arginine residues are required to stabilize the carbanion intermediate generated from the carboxylate anion substrate in the absence of metal ion cofactors.

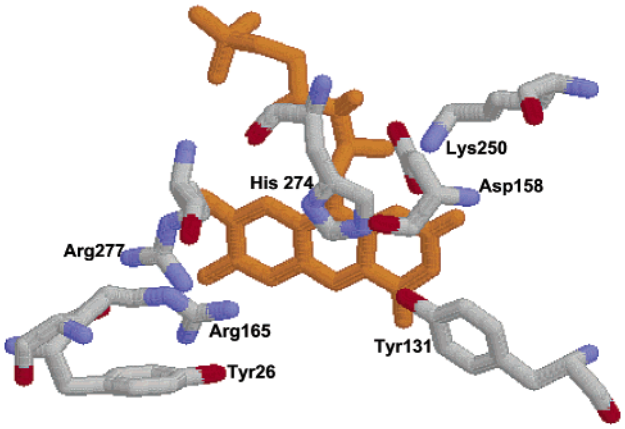

FIGURE 1: Active site geometry of MDH-GOX2, a soluble mutant of MDH, showing Arg165 and Arg277.

In the structure of the product-bound form of flavocytochrome b_2, the homologue of Arg277 interacts with the carboxylate group of the ketoacid product (*5*). Site-specific mutagenesis of this arginine residue in lactate monooxygenase and lactate oxidase resulted in inactive enzymes (*10*, *11*). However, using both charge-conserved and neutral substitutions in MDH, we showed that the positive charge of Arg277 plays critical roles in both binding and oxidation

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[1] Abbreviations: BSA, bovine serum albumin; DCPIP, dichloroindophenol, sodium salt; $^{D}k_{cat}$, primary substrate kinetic isotope effect on k_{cat}; MDH, (*S*)-mandelate dehydrogenase.

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of the hydroxyacid anion substrate (*12*). The residue corresponding to Arg165 occupies two different conformations in the various available structures. In the free enzyme structure of glycolate oxidase, the side chain of this residue is stacked against that of the second arginine residue (*13*). However, in the structures of the sulfite-bound form of flavocytochrome b_2 and the negatively charged inhibitor-bound form of glycolate oxidase as well as in the MDH structure in which a sulfate ion is bound at the active site, the side chain of Arg165 and its homologues points toward the active site (*6*, *14*, *15*). Together with two other strictly conserved arginine residues, Arg277 and Arg165 also form part of a channel that may guide the anionic substrate into the active site (*16*). Thus, Arg165 may have multiple functions.

To probe the importance of the positive charge at residue 165 and that of the guanidinium side chain in particular, we have characterized the site-specific mutant enzymes R165K, R165G, R165M, and R165E. Additionally, to understand the interesting juxtaposition of two positively charged arginine residues in the active site of this enzyme family, we have also characterized the double mutants R165K/R277K, R165K/R277G, R165G/R277K, and R165G/R277G. We report pre-steady-state kinetic data for *wt*MDH and the mutants; our data clearly show that the steps involving FMN reduction are rate-limiting for all the enzymes. We also report the pH dependence of the R165M mutant enzyme. Previously, we had proposed that the residue with a pK_a of 9.7 in free *wt*MDH that is critical for catalysis is Arg277, though other residues could not be ruled out as candidates (*9*). Our data conclusively show that this pK_a does not belong to Arg165 and suggest that it does belong to Arg277.

EXPERIMENTAL PROCEDURES

Materials

Oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA). Reagents were purchased from commercial sources and were of the highest possible analytical grade. For the determination of the primary substrate kinetic isotope effect, [α-^{2}H]-(*S*)-mandelic acid was enzymatically prepared as described previously (*17*).

Methods

Genetic Engineering Methods. The mutations at residue 165 in *wt*MDH were generated by polymerase chain reaction methods. The oligonucleotides used for constructing the mutants R165K, R165G, R165M, and R165E had the sequences 5′-CGCGCTCCTTATAGCCGTT-3′, 5′-CGCGCTCTCCATAGCCGTT-3′, 5′-CGCGCTCCATATAGCCGTT-3′, and 5′-AACGGCTATGAAGAGCGCG-3′, respectively. The mutants R277K and R277G have been described previously (*12*). The double mutants R165K/R277K, R165G/R277K, R165K/R277G, and R165G/R277G were generated as follows. The plasmids containing R165G, R165K, R277G, and R277K were individually digested with the restriction enzyme *Sph*I to generate two fragments for each. The bases encoding residue 165 were located on the small fragment, while the bases encoding residue 277 were located on the large fragment. The fragments were purified separately. Appropriate small and large fragments were then ligated together to generate the double mutants. The orientation of ligation was checked by appropriate digestions with restriction enzymes. The entire sequences of all eight mutant genes were verified by automated DNA sequencing (DNA Sequencing Core Facility, Wayne State University).

Enzyme Purification. All the mutants were generated with carboxyl-terminal histidyl tags in the same expression vector as *wt*MDH (*9*). Growth of cells and purification of the mutant proteins were carried out using protocols described previously (*9*, *12*). Typically, cells were grown at 37 °C until midlog phase, followed by overnight induction with isopropyl β-thiogalactopyranoside at room temperature. The proteins were extracted from the membranes with 0.06% Triton X-100 and purified with buffers containing 0.1% Tween 80; the critical micellar concentrations of Triton X-100 and Tween 80 under these conditions are ∼0.01−0.06 and 0.0015%, respectively. Protein concentrations were estimated by measuring the amount of free FMN released upon boiling the protein solutions for 5 min. The extinction coefficient of MDH-bound FMN was not used to directly estimate protein concentrations because of errors introduced in the measured values due to light scattering caused by the protein/detergent solution.

Steady-State and Pre-Steady-State Kinetics. Unless noted otherwise, steady-state activities were measured in 0.1 M potassium phosphate buffer (pH 7.5) containing 1 mg/mL bovine serum albumin (BSA), 1 mM phenazine methosulfate, and 100−150 μM dichloroindophenol (DCPIP), at 20 °C, as described previously for *wt*MDH (*9*). Substrate kinetic isotope effects were measured using [α-^{2}H]-(*S*)-mandelate. The pH dependencies of the kinetic parameters for R165M and *wt*MDH were measured in a buffer containing 0.052 M ethanolamine, 0.052 M Tris, 0.1 M MES, 1 mg/mL BSA, and 200−230 μM DCPIP; the pH was adjusted using concentrated HCl or KOH. The decrease in absorbance of DCPIP was measured at 522 nm. Equations to fit the data have been described previously (*9*). Stopped-flow data were collected at 20 °C in 0.1 M potassium phosphate (pH 7.5) under anaerobic conditions. Solutions were made anaerobic by saturating them with argon. FMN reduction was monitored by following the decrease in absorbance at 460 nm. The data were adequately described by a single-exponential fit, unless noted otherwise.

Binding of Inhibitors. The binding of all the inhibitors was assessed at 20 °C with the exception of sulfite, which was assessed at 4 °C. The dissociation constants for sulfite, (*R*)-mandelate, and 1-phenylacetate were obtained from spectral titration of the oxidized mutant enzymes as described previously (*12*). The inhibition constant for phenylethanediol was obtained from steady-state measurements (*9*).

Chemical Rescue of the Activity of Arg165 Single and Arg165/Arg277 Double Mutants. We have previously shown that the low k_{cat} for R277G was increased to near-*wt*MDH levels by the addition of imidazoles or guanidines to the assay buffer (*12*). The ability of these chemicals to "rescue" the activity of the Arg165 mutants were also tested in procedures similar to those previously described (*12*). Typically, the potential rescue agent (100 mM) was added to the assay mixture containing 50 mM potassium phosphate (pH 7.5), 1 mg/mL BSA, 1 mM phenazine methosulfate, 120 μM DCPIP, 0.02−100 μM mutant enzyme, and 40−60 mM (*S*)-mandelate. The highest concentration of rescue agent that

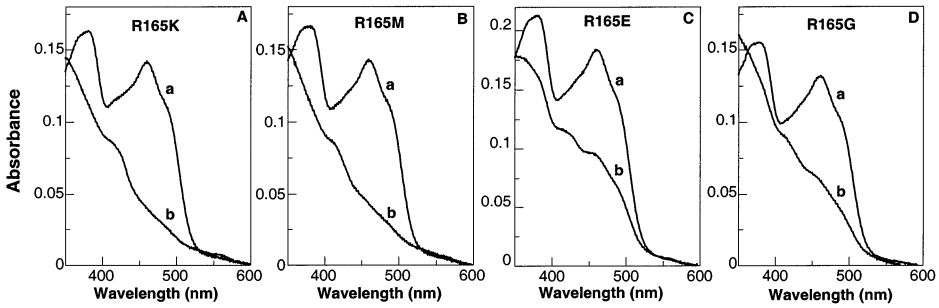

FIGURE 2: Absorbance spectra of the Arg165 single mutants in 20 mM potassium phosphate (pH 7.5) with 5% glycerol and 0.1% Tween 80: (A) 10 μM R165K, (B) 10 μM R165M, (C) 14 μM R165E, and (D) 10 μM R165G. For each panel, spectrum a is that of the oxidized protein and spectrum b was recorded after 20 mM (*S*)-mandelate was added under anaerobic conditions.

was used was 100 mM, since our previous experience has shown that some rescue agents at concentrations higher than 100 mM are deleterious for the activity of mutant proteins, but not *wt*MDH.

Instrumentation. UV−visible spectra were recorded with a Varian (Cary 1E) spectrophotometer. Stopped-flow data were collected using an Applied Photophysics System SX 18.MV spectrophotometer.

RESULTS

Purification and Properties of the R165 Single and Double Mutants. The levels of expression of R165K, R165M, and R165K/R277K were comparable to that of *wt*MDH. However, the level of expression of the single mutants decreased in the following order: R165K ∼ R165M > R165E > R165G. The double mutants (R165K/R277G, R165G/R277K, and R165G/R277G) were expressed at lower levels than *wt*MDH; in particular, R165K/R277G and R165G/R277G were poorly expressed. During extraction of the mutant enzymes from the membrane, it was critical to maintain the detergent concentration at or slightly above its critical micellar concentration. Use of higher detergent concentrations resulted in two enzyme populations, both with normal FMN spectra, but one active and the other inactive. These observations are similar to those made earlier for the Arg277 mutants (*12*). As a consequence of using low concentrations of detergents for protein extraction, the yields for purified proteins were typically low, and in some instances, they could not be completely separated from a small amount of a heme-containing impurity protein. Also, we observed that in general, the purified Arg165 mutants were less stable than *wt*MDH and the Arg277 mutants.

Figure 2 shows the absorbance spectra of the Arg165 single mutant. The spectra are similar to that of *wt*MDH, suggesting that the substitutions do not significantly affect the spectral properties of FMN. The R165K and R165M mutants were fully reduced on addition of (*S*)-mandelate; in contrast, R165E and R165G could be only partially reduced. This was consistently observed for a number of different protein preparations. The absorbance spectra of the Arg165/Arg277 double mutants show that the FMN spectral properties are also unaltered in these mutants (Figure 3). All four double mutants were less stable than the single Arg165 mutants in terms of long-term storage. For example, *wt*MDH retains activity for more than 1 year upon storage at −70 °C. However, the single Arg165 mutants lost activity after 2−3 months at −70 °C, while the double mutants were inactivated after 2−3 weeks in storage.

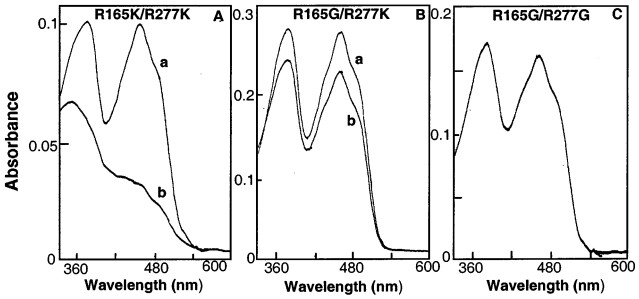

FIGURE 3: Absorbance spectra of the Arg165/Arg277 double mutants in 20 mM potassium phosphate (pH 7.5) with 5% glycerol and 0.1% Tween 80: (A) 8.4 μM R165K/R277K, (B) 22 μM R165G/R277K, and (C) 14.5 μM R165G/R277G. In panels A and B, spectrum a is that of the oxidized protein and spectrum b was recorded after 20 mM (*S*)-mandelate was added under aerobic conditions. Panel C shows the spectra of oxidized R165G/R277G and R165G/R277G after 20 mM (*S*)-mandelate was added.

Table 1: Steady-State and Pre-Steady-State Kinetic Parameters for the Arg165 and Arg277 Mutants Using (*S*)-Mandelate as a Substrate[a]

	k_{cat} (s^{-1})	K_m (mM)	k_{red} (s^{-1})	K_d (mM)	$^Dk_{cat}$
*wt*MDH	360 ± 8	0.12 ± 0.01	402 ± 16	0.19 ± 0.03	2.1
R165K	120 ± 1.8	1.5 ± 0.1	122 ± 3	2.2 ± 0.2	4.8
R165G	13.2 ± 0.6	5.8 ± 0.7	11.1 ± 0.8	6.5 ± 1.5	5.9
R165E	4.4 ± 0.2	12.0 ± 1.4	13.5 ± 0.6	15.7 ± 1.9	4.8
R165M	18.4 ± 0.25	4.4 ± 0.9	14.7 ± 0.8	9.8 ± 1.4	4.9
R277K	66 ± 2	5.6 ± 0.4	67 ± 4	5.2 ± 0.8	4.6
R277G	0.23 ± 0.07	17 ± 5	0.27 ± 0.02	17.5 ± 3.5	ND
R165K/R277K	1.1 ± 0.02	15.2 ± 0.8	1.1 ± 0.05	15.5 ± 1.9	6.4
R165G/R277K	0.04 ± 0.002	31.4 ± 3.4	ND	ND	ND
R165K/R277G	NM				
R165G/R277G	NM				

[a] Steady-state assays were performed in 0.1 M potassium phosphate (pH 7.5) containing 100−150 μM DCPIP, 1 mg/mL BSA, and 1 mM phenazine methosulfate at 20 °C. Pre-steady-state measurements were performed at 20 °C in 0.1 M potassium phosphate (pH 7.5). $^Dk_{cat}$ is the primary substrate kinetic isotope effect on k_{cat}. ND, not determined. NM, not measurable.

Kinetic Parameters. The kinetic parameters of *wt*MDH and the Arg165/Arg277 mutants are summarized in Table 1. R165K, in which the positive charge is conserved, was a fairly competent enzyme, with a k_{cat} that was only 3-fold lower than that of *wt*MDH and a K_m that was 12-fold higher. When Arg165 was replaced with neutral residues, as in R165G and R165M, the k_{cat} was decreased by 20−30-fold while the K_m was increased 35−50-fold compared to that of *wt*MDH. R165E, in which the charge at residue 165 has been completely reversed, still retained activity and had a k_{cat}/K_m that was only 10−20-fold lower than those of R165G and R165M. We have previously reported the steady-state kinetic

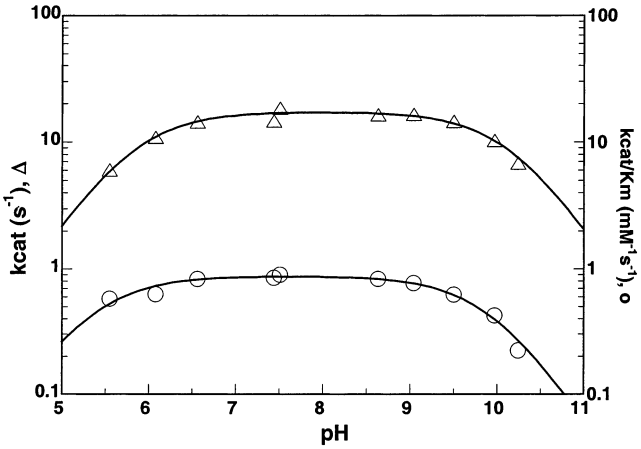

FIGURE 4: pH dependence of the k_{cat} (△) and k_{cat}/K_m (○) values for R165M at 20 °C using (*S*)-mandelate as a substrate. The lines are fits to the equation $y = Y(1 + 10^{pK_1-pH} + 10^{pH-pK_2})^{-1}$. The buffer used over the entire pH range was made up of 0.052 M ethanolamine, 0.052 M Tris, and 0.1 M MES. The pK_a values that were obtained were 5.8 ± 0.1 and 10.1 ± 0.1 for the k_{cat} pH profile and 5.4 ± 0.1 and 9.9 ± 0.1 for the k_{cat}/K_m pH profile.

parameters for the Arg277 mutants (Table 1; *12*). R277K has a k_{cat} only 5-fold lower than that of *wt*MDH, but a 50-fold higher K_m. Thus, both charge-conserved single mutants, R165K and R277K, have relatively high k_{cat}s. However, surprisingly, the charge-conserved double mutant, R165K/R277K, has a k_{cat} that is 330-fold lower than that of *wt*MDH. R165G/R277K was even less active, while R165K/R277G and R165G/R277G had no measurable activity.

The kinetics of the reductive half-reaction, that is, flavin reduction upon addition of (*S*)-mandelate, were followed in stopped-flow experiments for *wt*MDH and the Arg165/Arg277 mutants (Table 1). The rate of flavin reduction, k_{red}, and the K_d data obtained for *wt*MDH are similar to the steady-state k_{cat} and K_m values, indicating that the steps involving the FMN reductive half-reaction are rate-limiting for *wt*MDH. The k_{red} and the K_d data obtained for all the Arg165 and Arg277 single mutants as well as for R165K/R277K also show a similar pattern, with the possible exception of R165E. Thus, the decrease in overall catalytic rates for these mutants compared to that of *wt*MDH results from a decrease in the FMN reduction rate. R165E has a k_{cat} 3-fold lower than k_{red}, suggesting that a step after the FMN reduction step, possibly product release, may also contribute partially to the overall catalytic rate.

The primary substrate kinetic isotope effects on k_{cat} ($^Dk_{cat}$) are shown in Table 1. *wt*MDH has a $^Dk_{cat}$ of 2.1, implying that the substrate carbon−hydrogen bond-breaking step is only partially rate-limiting for this enzyme. $^Dk_{cat}$ increased from 2.1 for *wt*MDH to 4.8−5.9 for the Arg165 and Arg277 single mutants as well as for R165K/R277K at pH 7.5. Thus, the carbon−hydrogen bond-breaking step is fully rate-limiting for all the mutants, including R165K, which has a k_{red} only ~3-fold lower than that of *wt*MDH.

The pH dependencies of the k_{cat} and k_{cat}/K_m parameters were determined for R165M (Figure 4). The shapes of the pH profiles for R165M are identical to those of *wt*MDH (*12*). The pK_a values calculated from the k_{cat} pH profile were 5.8 ± 0.1 and 10.1 ± 0.1, while those calculated from the k_{cat}/K_m pH profile were 5.4 ± 0.1 and 9.9 ± 0.1. The corresponding pK_as obtained for *wt*MDH under similar conditions were 4.7 ± 0.1 and 10.1 ± 0.1 for the k_{cat} pH profile and 5.4 ± 0.2 and 9.3 ± 0.1 for the k_{cat}/K_m pH profile. These values are similar to those previously reported for *wt*MDH (*9*, *12*).

Table 2: Dissociation Constants of Negatively Charged Ligands with *wt*MDH and the Arg165/Arg277 Mutants[a]

	sulfite (μM)	1-phenylacetate (mM)	(*R*)-mandelate (mM)	(*S*)-mandelate (mM)
*wt*MDH	22 ± 0.3	12 ± 0.8	2.9 ± 0.2	0.19 ± 0.03
R165K	132 ± 3.5	11 ± 0.9	3.1 ± 0.2	2.2 ± 0.2
R165G	67 ± 10	67 ± 4	73 ± 2	6.5 ± 1.5
R277K	15.7 ± 1.7[b]	12 ± 3.1[b]	7.2 ± 1.0[b]	5.2 ± 0.8
R277G	500 ± 65[b]	22.5 ± 3.1[b]	19.5 ± 1.6[b]	17.5 ± 3.5
R165K/R277K	43 ± 1.8	11.0 ± 0.7	ND	15.5 ± 1.9
R165G/R277K	52 ± 3.1	20.3 ± 2.1	700 ± 60	ND
R165G/R277G	1133 ± 17	ND	ND	NM

[a] K_d values were measured in 20 mM potassium phosphate (pH 7.5) at 20 °C with the exception of sulfite, the value of which was measured at 4 °C. The K_d values for (*S*)-mandelate are also included. ND, not determined. NM, not measurable. [b] From ref *12*.

Chemical Rescue of the Activity of the Arg165 Mutants. The ability of chemical "rescue" agents such as guanidine, imidazole, and amine derivatives to increase the activity of R165G and R165M was tested. Unlike our previous observations for the R277G mutant, none of these reagents was effective in increasing the activity of the Arg165 mutants. Of the double mutants, only R165K/R277G displayed any rescued activity. This double mutant normally has no measurable activity toward (*S*)-mandelate. However, activity could be measured in the presence of 1-methylguanidine hydrochloride in the buffer (data not shown). The rescued activity showed saturation behavior with respect to both the rescue agent and the substrate. In the presence of a saturating concentration (80 mM) of 1-methylguanidine hydrochloride, R165K/R277G displayed a k_{cat} of 0.53 s^{-1} and an apparent K_m of 17.6 mM for (*S*)-mandelate. The concentration of 1-methylguanidine hydrochloride required to reach half of the maximum rescued activity was 18.6 mM. In contrast to this low level of rescue, the low intrinsic activity of R277G (0.23 s^{-1}) could be increased up to 200 s^{-1} in the presence of 1-methylguanidine hydrochloride (*12*).

Binding of Negatively Charged Ligands to the Arg165 Mutants. The contribution of Arg165 to the binding of negatively charged ligands was investigated by measuring the dissociation constants of sulfite, a ligand that forms a covalent adduct with FMN, as well as two competitive inhibitors, 1-phenylacetate and (*R*)-mandelate (Table 2). For comparison, the K_d values for the substrate, (*S*)-mandelate, are also included in Table 2. The reversible adduct of sulfite with FMN in *wt*MDH has a K_d of 22 μM at 4 °C and pH 7.5 (*9*). The affinity for sulfite was not dramatically affected in the R165K or R165G single mutants, suggesting that a positive charge at Arg165 is not essential for stabilizing the FMN−sulfite adduct. In contrast, removal of a positive charge at Arg277 results in a 25-fold lowering of binding affinity for sulfite in the R277G mutant (Table 2; *12*). Similarly, the R165G/R277G double mutant also showed an ~50-fold lower sulfite binding affinity, while the binding affinity of the R165K/R277K and R165G/R277K double mutants remained relatively unchanged.

1-Phenylacetate and (*R*)-mandelate are negatively charged competitive inhibitors of MDH; binding of these ligands

produces small changes in the oxidized FMN spectrum of the mutant enzymes, similar to those observed for *wt*MDH (data not shown) (*12*). K_ds were calculated by measuring the absorbance differences at 500−510 nm as a function of inhibitor concentration. A comparison of the K_d values for *wt*MDH, and the Arg165 and Arg277 mutants, shows that binding is unaffected as long as positive charges are present at Arg165 and Arg277. Thus, R165K and R277K have K_ds for these two ligands similar to that of *wt*MDH. However, when either positive charge is removed, as in R165G and R277G, the K_ds increase 2−6-fold relative to that of *wt*MDH. Interestingly, the affinity for (*R*)-mandelate is much more affected in R165G as well as R165G/R277K than that for 1-phenylacetate. Thus, it appears that the higher affinity for (*R*)-mandelate in *wt*MDH compared to that for 1-phenylacetate is at least partly due to the presence of a positive charge at Arg165.

We also measured the dissociation constant for the binding of the neutral inhibitor, phenylethanediol, to R165K and R165G. Phenylethanediol is a poor competitive inhibitor of *wt*MDH with a K_d of 61 ± 5 mM (*9*). The K_d values of phenylethanediol for R165K and R165G were lower than that for *wt*MDH (17 ± 2 and 24 ± 1 mM, respectively). The Arg165 mutations may result in a lower energetic cost for binding this neutral inhibitor than *wt*MDH.

DISCUSSION

Structural studies of the FMN-dependent α-hydroxyacid-oxidizing enzymes have shown that two arginine residues in MDH, Arg165 and Arg277, are strictly conserved in the active site. We have previously shown that Arg277 plays a critical role in substrate binding affinity and in transition-state stabilization (*12*). It may also play an important role in overall protein stability, since mutagenesis of the corresponding arginine in homologous enzymes led to unstable proteins or proteins with dramatically lower activities (*10*, *11*). The role of the second conserved arginine residue, Arg165 in MDH, was investigated in this study, as well as the necessity of conserving two *arginine* residues in the active site, as opposed to lysine residues or one of each.

Role of Arg165 in Substrate Binding and Catalysis. Our previous work has clearly established the importance of an arginine residue at position 277; the positive charge is required for efficient catalysis, and a hydrogen bond is required for binding both the substrate and the transition state (*12*). The kinetic parameters for the single Arg165 mutants show that Arg165 plays a similar though less important role (Table 1). Removal of the positive charge at Arg165 decreases the FMN reduction rate by ∼40-fold, whereas removal of the positive charge from Arg277 resulted in an ∼1600-fold lower FMN reduction rate. Surprisingly, activity is retained even in the R165E mutant. Substrate binding affinities, reflected by K_d values, show that replacing Arg165 with a lysine residue results in an ∼10-fold lower affinity; removal of the positive charge results in a further ∼3-fold loss in binding affinity. Thus, Arg165 appears to contribute to catalysis through its positive charge and to substrate binding through a possible hydrogen bond with the guanidinium group. The $^{D}k_{cat}$ values in Table 1 are significantly higher for the Arg165 mutants than for *wt*MDH; this suggests that Arg165 specifically plays a role in the α-carbon−hydrogen bond-breaking step, possibly by stabilizing the negatively charged carbanion intermediate. These results are consistent with similar mutations in the homologous enzymes, flavocytochrome b_2 and lactate oxidase from *Aerococcus viridans* (*11*, *20*). The arginine to lysine substitution was characterized in detail in flavocytochrome b_2; it was concluded that this arginine residue is indeed catalytically important and interacts with the substrate in both the ground and transition states. A comparison of the structures of flavocytochrome b_2 in the presence and absence of sulfite suggests that this arginine is stacked against the second arginine in the free enzyme and forms an electrostatic interaction with a conserved aspartate (*13*). However, it adopts a different conformation and faces toward the active site when negatively charged ligands are bound (*14*). In lactate oxidase, the homologous arginine was altered to lysine and methionine (*11*). The results obtained with these substitutions are similar to our results with MDH, leading to a similar conclusion that this arginine residue plays a role in catalysis, though it is not as important as the second arginine residue. In contrast, the homologous arginine to lysine substitution in lactate monooxygenase from *Mycobacterium smegmatis* resulted in an extremely poor enzyme, with very low affinity for substrate (*21*). It is possible that the arginine residue in lactate monooxygenase has a different role than the homologous arginines in this enzyme family.

All four single Arg165 mutants as well as the four Arg165/Arg277 double mutants displayed normal FMN spectra and were able to form an adduct with sulfite. This also included R165E, in which the positive charge at residue 165 is replaced with a negative charge. The K_d for the FMN−sulfite adduct depends on the redox potential of FMN, steric factors, and stabilizing interactions at the active site (*18*, *19*). The data in Table 2 show that even though the positive charge at Arg277 greatly influences the strength of the adduct, the one at Arg165 does not play an important role in stabilizing the FMN−sulfite adduct.

However, the positive charge at Arg165 does influence the binding of the competitive inhibitors 1-phenylacetate and (*R*)-mandelate, as does the positive charge at Arg277 (Table 1). While the affinity for these negatively charged inhibitors was only slightly affected in R165K, R165G exhibited a 6-fold weaker affinity for 1-phenylacetate and an ∼33-fold lower affinity for (*R*)-mandelate. This is an interesting contrast to the affinity for the substrate, (*S*)-mandelate, which is affected much more by the removal of arginine specifically than by the removal of the positive charge at residue 165.

Specific Role of the Arg165/Arg277 Pair. The charge-conserved single mutants, R165K and R277K, were able to oxidize (*S*)-mandelate with fairly high k_{cat}s, though substrate affinity was rather diminished in both mutants. However, much to our surprise, the R165K/R277K mutant had an ∼330-fold lower k_{cat} and an ∼130-fold lower substrate affinity. Additionally, the double mutants R165K/R277G, R165G/R277K, and R165G/R277G, in which arginine residues were completely absent from the active site, had extremely poor or no measurable activities. It is thus clear that it is not sufficient to simply retain positive charges at residues 165 and 277; arginine residues are specifically required. Optimal substrate binding requires hydrogen bonds from the guanidinium groups of Arg277 and also possibly Arg165 at the active site (*12*). When only one guanidinium

Table 3: Differences in Binding Energies of the Enzyme−Substrate Complex ($\Delta\Delta G_S$) and in Activation Energies of k_{cat} ($\Delta\Delta G^{\ddagger}$) and k_{cat}/K_m ($\Delta\Delta G_T^{\ddagger}$) between *wt*MDH and the Arg165/Arg277 Mutants at 20 °C[a]

	$\Delta\Delta G_S$ (kcal/mol)	$\Delta\Delta G^{\ddagger}$ (kcal/mol)	$\Delta\Delta G_T^{\ddagger}$ (kcal/mol)
R165K	−1.5	0.6	2.1
R165G	−2.3	1.9	4.2
R165E	−2.7	2.6	5.3
R165M	−2.1	1.8	3.9
R277K	−2.3	1.0	3.3
R277G	−2.9	4.3	7.2
R165K/R277K	−2.8	3.4	6.2
R165G/R277K	−3.3	5.3	8.6

[a] The energy differences were calculated using data from Table 1.

group is present, as in R165K or in R277K, the single hydrogen bond with the substrate carboxylate group is able to orient the substrate such that the reaction can proceed, though the affinity is much weaker. However, when both guanidinium groups are absent, the substrate affinity is not only extremely weak, but it is also likely that the substrate is not optimally oriented for catalysis. Another reason for the invariance of these two arginine residues is that it may be difficult for two lysine residues, with their localized positive charges, to remain in proximity in the free enzyme; as a result, they are displaced from their original positions and cannot stabilize the negatively charged transition state. Studies with neutral substrates support this hypothesis (A. R. Dewanti, Y. Xu, and B. Mitra, manuscript in preparation). A comparison of the catalytic abilities of R277K and R165K/R277K also suggests that the role of Arg165 in substrate binding and catalysis becomes much more important when the second arginine at position 277 is removed.

There is a possibility that the low activity of the R165K/R277K mutant is not due to the removal of both arginines but rather due to a conformational change, since the double mutants are less stable than the single mutants. However, it is to be noted that the double mutants display normal FMN spectra and have similar affinities for sulfite and 1-phenylacetate. Moreover, R165K/R277K is stable during purification and can be stored for a few weeks without a loss of activity. Therefore, it is unlikely that the large reduction in activity can be attributed merely to a conformational change. Structural studies may help to resolve this issue in the future.

Binding and Activation Energy Provided by Arg165 and Arg277. Using the kinetic parameters in Table 1, we calculated the differences in the binding energies of the enzyme−substrate (ES) complex, $\Delta\Delta G_S$, as well as the activation energies for k_{cat}, $\Delta\Delta G^{\ddagger}$, and k_{cat}/K_m, $\Delta\Delta G_T^{\ddagger}$, between *wt*MDH and the Arg165/Arg277 mutants (Table 3). These free energy differences were calculated using eqs 1−3, respectively (*22*).

$$\Delta\Delta G_S = RT \ln[K_{m(WT)}/K_{m(mutant)}] \quad (1)$$

$$\Delta\Delta G_T^{\ddagger} = RT \ln[(k_{cat}/K_m)_{(WT)}/(k_{cat}/K_m)_{(mutant)}] \quad (2)$$

$$\Delta\Delta G^{\ddagger} = \Delta\Delta G_T^{\ddagger} + \Delta\Delta G_S = RT \ln[(k_{cat})_{(WT)}/(k_{cat})_{(mutant)}] \quad (3)$$

The data in Table 3 confirm our conclusion that the positive charge at Arg165 contributes to transition-state stabilization, whereas a charge-independent interaction of ∼1.5 kcal/mol contributes to ground-state stabilization of the substrate. This is also true of Arg277. Previously, using chemical rescue agents, we were able to confirm that this charge-independent interaction with Arg277 is a hydrogen bond from the guanidinum side chain to the substrate carboxylate group. This is likely to be true for Arg165 also, though we cannot confirm it since we were unable to rescue the loss in activity for R165G or R165M. Table 3 shows that there are losses of ∼1.5 and ∼2.3 kcal/mol in binding energies with the charge-conserved mutations, R165K and R277K, respectively, compared to that of *wt*MDH. The loss in binding energy with R165K/R277K is 2.8 kcal/mol, less than the sum of the individual $\Delta\Delta G_S$ values. In contrast, there are destabilizations of the transition state of ∼0.6 and ∼1.0 kcal/mol with R165K and R277K, respectively. However, in R165K/R277K, the transition state is destabilized by 3.4 kcal/mol, twice the sum of the individual $\Delta\Delta G^{\ddagger}$ values. Thus, the two arginine residues in the active site do not act independently; there appears to be a synergistic effect between the two that is lost when both are replaced with lysine residues.

In the crystal structure of glycolate oxidase in the absence of substrate, product, or any inhibitor, the guanidinium groups of the corresponding arginines, Arg164 and Arg257, are 4 Å from each other (*4*). Arg164 interacts with the conserved Asp167 and is 2.7 Å from its carboxylate group. Upon binding a negatively charged ligand, Arg164 moves away from Asp167 (the distance becomes 5 Å) and instead faces the inhibitor. In the structures of both flavocytochrome b_2 and MDH-GOX2, when a negative ion is bound at the active site, the corresponding arginine is distant from the conserved aspartate and faces inward into the active site. In this conformation, both arginines are able to interact with the oxygen atoms of the negatively charged ligand. This arginine−oxyanion−arginine interaction has been observed in other protein structures (*23*). It is likely that when either or both arginines are replaced with lysines, thereby replacing the diffused positive charges of the guanidinium groups with the localized positive charges of amino groups, a favorable interaction with the negatively charged substrate and more negatively charged carbanion intermediate is lost.

pH Dependence of the Kinetic Parameters for R165M. The pH profiles for *wt*MDH had shown that a residue with an important role in catalysis has an apparent pK_a of 9.3 in the free enzyme that is shifted to 10.1 on binding (*S*)-mandelate (*9*, *12*). We previously suggested that the most likely residue with this pK_a is Arg277, though other residues in the active site could not be ruled out as candidates. The pH profiles of R165M, in which there is no positive charge at residue 165, reveal that the alkaline pK_a of the free enzyme is shifted to 9.9 in this mutant. Since R165M displays an alkaline pK_a, our data conclusively prove that this pK_a does not belong to Arg165. Additionally, this pK_a does not belong to the two conserved tyrosine residues in the active site (Y. Xu and B. Mitra, unpublished observations) or to the FMN (pK_a > 10). Thus, the likelihood that it belongs to Arg277 still exists, though it cannot be conclusively proven. We had earlier suggested that the lower than normal pK_a for Arg277 could be due to the proximity of a second positive charge at Arg165. Therefore, we expected that if the positive charge were removed from residue 165, the pK_a would be restored

to a more "normal" value for an arginine residue (typically >12). For example, removal of one of two proximal lysine residues (Lys116) in acetoacetate decarboxylase increases the pK_a of the catalytically important Lys115 from 6.0 to >9.2 (*24*−*26*). Instead in MDH, there is a modest increase in the pK_a from 9.3 in free *wt*MDH to 9.9 in R165M. If the pK_a of 9.3 in free *wt*MDH does belong to Arg277, then other factors, such as its proximity to the electropositive FMN, are expected to lower its pK_a, in addition to the positive charge at Arg165. In any case, as discussed above, structural studies have shown that in the free enzymes, the two arginines are stacked against each other and >4 Å apart; they would not be expected to influence each other greatly (*4*, *13*).

Chemical Rescue of Activity. In a previous study, we showed that the low k_{cat} of R277G could be increased to near-*wt*MDH levels with exogenous guanidines or imidazoles (*12*). This chemical rescue effect enabled us to delineate the importance of the hydrogen bond with Arg277 in substrate binding. In this study, we tested for chemical rescue of activity for the Arg165 single mutants. Surprisingly, a variety of exogenous guanidines or imidazoles had no effect on activity. This may be due to the relative inaccessibility of residue 165 to rescue agents in solution or, more possibly, due to the absence of a tight binding site for the rescue agent. The activity of the R165K/R277G double mutant could be enhanced by rescue agents, similar to the rescue observed for R277G. Normally, the activity of R165K/R277G was below the level of detection; however, in the presence of 1-methylguanidine and saturating (*S*)-mandelate concentrations, a k_{cat} of 0.5 s^{-1} and a K_m of 17 mM were measured. This activity is most probably due to the ability of 1-methylguanidine to bind in the space normally occupied by Arg277 and carry out its function to a limited extent.

CONCLUSIONS

In this study, we have used site-specific mutagenesis to examine the role of the conserved Arg165 in MDH, as well as the overall importance of the Arg165/Arg277 pair. Arg165 was replaced with lysine, methionine, glutamate, and glycine. Kinetic characterization of the four mutants clearly shows that Arg165 plays a role in substrate binding and catalysis, though its role is not as important as that of Arg277. The positive charge at Arg165 stabilizes the transition state, but contributes only weakly to substrate binding. A charge-independent interaction, possibly a hydrogen bond, between Arg165 and the substrate contributes to ground-state stabilization and increased binding affinity. The Arg165/Arg277 double mutants show that lysines cannot replace arginine residues at the active site; R165K/R277K is an extremely poor enzyme. pH studies conclusively show that a pK_a of 9.3 in free *wt*MDH does not belong to Arg165, and suggest that it belongs to Arg277. Stopped-flow kinetic analysis shows that the FMN reduction step is completely rate-limiting for *wt*MDH, though the carbon−hydrogen bond-breaking step is only partially rate-limiting, as shown by substrate isotope effects. For the Arg165 and Arg277 mutants, the FMN reduction step is fully rate-limiting also, with the possible exception of R165E. Additionally, the carbon−hydrogen bond-breaking step is fully rate-limiting for all the mutants.

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