Role of Arginine 277 in (*S*)-Mandelate Dehydrogenase from *Pseudomonas putida* in Substrate Binding and Transition State Stabilization†

Isabelle E. Lehoux and Bharati Mitra*

Department of Biochemistry and Molecular Biology, School of Medicine, Wayne State University, Detroit, Michigan 48201

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ABSTRACT: (*S*)-Mandelate dehydrogenase from *Pseudomonas putida* is an FMN-dependent α-hydroxy acid dehydrogenase. Structural studies of two homologous enzymes, glycolate oxidase and flavocytochrome b_2, indicated that a conserved arginine residue (R277 in MDH) interacts with the product carboxylate group [Lindqvist, Y., Branden, C.-I., Mathews, F. S., and Lederer, F. (1991) *J. Biol. Chem. 266*, 3198−3207]. The catalytic role of R277 was investigated by site-specific mutagenesis together with chemical rescue experiments. The R277K, R277G, R277H, and R277L proteins were generated and purified in active forms. The k_{cat} for the charge-conserved mutation, R277K, was only 4-fold lower than wt-MDH, but its K_m value was 40-fold lower; in contrast, k_{cat}s for R277G, R277H, and R277L were 400−1000-fold lower than for wt-MDH and K_m values were 5−15-fold lower compared to R277K. The K_ds for negatively charged competitive inhibitors were relatively unaffected in all four R277 mutants. The k_{cat} for R277G could be enhanced by the addition of exogenous guanidines or imidazoles; the maximum rescued k_{cat} was ∼70% of the wt-MDH value. Only reagents that were positively charged and could function as hydrogen bond donors were effective rescue agents. Our results indicate that R277 plays a major role in transition state stabilization through its positive charge—consistent with a mechanism involving a carbanion intermediate. The positive charge has a relatively small contribution toward substrate binding. R277 also forms a specific hydrogen bond with both the substrate and the transition state; this interaction contributes significantly to the low K_m for (*S*)-mandelate.

(*S*)-Mandelate dehydrogenase (MDH)[1] from *Pseudomonas putida*, catalyzes the oxidation of (*S*)-mandelate to benzoylformate. MDH belongs to a family of FMN-dependent (*S*)-α-hydroxy-acid oxidases and dehydrogenases that includes glycolate oxidase from spinach, flavocytochrome b_2s [L-lactate or (*S*)-mandelate dehydrogenase] from yeast, lactate monooxygenase from *Mycobacterium smegmatis*, lactate oxidase from *Aerococcus viridans*, and the long chain α-hydroxy acid oxidase from mammals (*1*, *2*). On the basis of structural as well as sequence similarities, the reductive half reaction, involving oxidation of the α-hydroxy acid to α-keto acid with the concomitant reduction of FMN, is likely to proceed through the same mechanism for all these enzymes (*3*−*6*). The second half-reaction in which FMN is reoxidized is different for the various enzymes. Unlike the oxidases, the dehydrogenases do not react with molecular oxygen. Instead, they react with an intramolecular heme in the case of the flavocytochrome b_2s, or a component of the electron transport chain in the case of the bacterial membrane-bound dehydrogenases, including MDH.

Biochemical evidence for a number of enzymes in this family suggests that the oxidation of the α-hydroxy acid proceeds through a carbanion intermediate (*7*−*9*). Three-dimensional structural analyses of glycolate oxidase and flavocytochrome b_2 are compatible with this mechanism (*6*). A conserved histidine residue (H274 in MDH) may initiate the reaction by abstracting the α-proton from the substrate (Figure 1). Two conserved arginine residues (R165 and R277 in MDH) that interact with the substrate carboxylate group may play critical roles in substrate binding as well as in stabilization of the postulated highly negatively charged carbanion intermediate. The structural data are also compatible with an alternative mechanism in which H274 abstracts the hydroxyl proton from the substrate and promotes the transfer of a hydride ion from the α-carbon to the FMN cofactor (*10*). Thus, the exact mechanism of α-hydroxy acid oxidation by these FMN-dependent enzymes is still equivocal.

One of our goals is to understand the function of highly conserved residues at the active site of MDH in binding and catalysis. Using mutagenesis and chemical rescue experiments, we recently demonstrated that H274 is the critical base that initiates the reaction and also plays a role in specifically binding the (*S*)-enantiomeric substrate (*11*). In the present study, we have investigated the function of R277. To date, charge-conserved mutations of the equivalent arginine in homologous enzymes (R376K in flavocytochrome b_2, R293K in lactate monooxygenase and R268K in lactate oxidase) have resulted in enzymes with extremely low activities, consistent with a critical role of this residue in

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* Address correspondence to this author. E-mail: bmitra@med.wayne.edu. Phone: (313) 577-0040. Fax: (313) 577−2765.

[1] Abbreviations: BSA, bovine serum albumin; DCPIP, dichloroindophenol, sodium salt; $^{D}k_{cat}$ and $^{D}k_{cat}/K_m$, substrate kinetic isotope effects on k_{cat} and k_{cat}/K_m, respectively; LB medium, Luria-Bertani medium; MDH, (*S*)-mandelate dehydrogenase; PMS, phenazine methosulfate.

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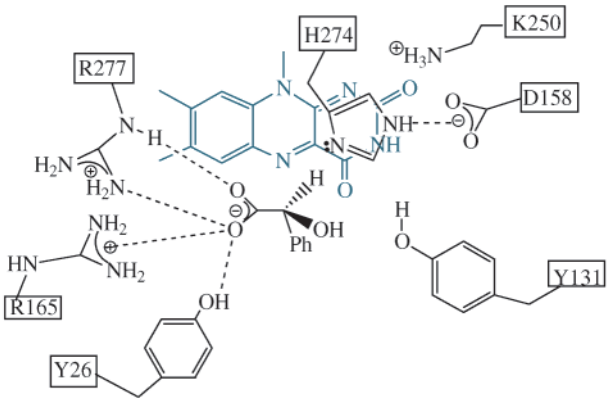


FIGURE 1: Active site of MDH with the substrate modeled in one possible orientation (adapted from ref *6*).

catalysis (*6*, *12*−*14*). In preliminary reports, we described the effects of mutating R277 in MDH to lysine, glycine, and leucine (*15*, *16*). The conservative mutation R277K was active, but R277G and R277L had no activity. The R277 mutants, especially the ones with neutral side chains, became inactivated to various extents during purification of the proteins. Thus, due to the low activities and the instability of the mutants of this arginine residue, the nature of its contributions to the reaction mechanism has remained unknown to date. R277 is one of four conserved arginines in this enzyme family. R277 is located at the active site and has been postulated to play an important catalytic role. The other three arginines, including R165, form a positively charged channel leading to the active site, presumably to attract the anionic substrate (*17*). R277 may also participate in an energetically favorable R165−R277 short-range interaction in the free enzyme (*18*). As such, it may play a critical role in maintaining the correct active-site structure and allowing substrate to bind with high affinity. Therefore, it is not surprising that mutations of R277 lead to unstable proteins.

The mutants, R277K, R277G, R277L, and R277H were generated in stable and active forms using a modified purification protocol. Kinetic characterization of these enzymes, including chemical rescue experiments, suggest that the positive charge at R277 plays a relatively minor role in substrate binding, but plays a major role in transition state stabilization. R277 also forms a specific hydrogen bond with both the substrate and the transition state but not with lower-affinity competitive inhibitors; this hydrogen bond contributes significantly to the substrate binding energy.

EXPERIMENTAL PROCEDURES

Materials. Oligonucleotides were purchased from Integrated DNA Technologies, Coralville, IA. Ni(II)-resin was either from Invitrogen or Qiagen. Imidazole, guanidine salts, and their derivatives and all other chemicals 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 (*19*).

Methods. Genetic Engineering Methods. The mutations at residue 277 in wt-MDH were generated by polymerase chain reaction methods. The oligonucleotides used for constructing the mutants R277L, R277K, R277G, and R277H had the sequences 5′-CACGGCGGTCTGCAACTCG-3′, 5′-CACGGCGGTAAGCAACTCG-3′, 5′-CACGGCGGTGGACAACTCG-3′, and 5′-CACGGCGGTCATCAACTCG-3′, respectively. The entire sequences of all four mutant genes were determined by DNA sequence analysis. The altered proteins were generated with carboxy-terminal histidyl tags in the same expression vector as wt-MDH. We have previously shown that the histidyl tag does not affect the properties of wt-MDH (*9*). The amounts of the R277 mutant proteins produced upon induction were similar to that of wt-MDH.

Enzyme Purification. The mutant enzymes R277G, R277H, and R277L were observed to be unstable and rapidly inactivated when purified according to the protocol previously used for wt-MDH (*9*). Therefore, the protocol was modified as follows. Typically, cells were grown at 37 °C till mid-log phase, followed by overnight induction with isopropyl β-thiogalactopyranoside. Membranes were prepared as described previously (*9*). They were then suspended in 100 mL of 20 mM potassium phosphate, pH 7.5, containing 5% glycerol and 1 mM phenylmethanesulfonylfluoride. The proteins were solubilized from the membranes with 0.04−0.06% Triton X-100. The solubilized proteins were purified on a Ni(II)-affinity column as described earlier, except that Triton X-100 was replaced by 0.1% Tween 80 in all the buffers. R277G, R277L, and R277H were active and stable when purified by this modified protocol. Both wt-MDH and R277K were active when purified in the presence of Triton X-100; however, the stability of both proteins increased significantly when they were purified using Tween 80. Additionally, the k_{cat} increased and the K_m decreased for R277K. Using the modified protocol, typical yields were low for both wt-MDH and the mutant proteins (∼0.5 mg of protein/g of starting cell paste). All the enzymes were >95% pure as seen on sodium dodecyl sulfate-polyacrylamide gels (data not shown). Protein concentrations were estimated by measuring the free FMN released upon boiling the protein solutions for 5 min as well as by the bicinchoninic acid reagent using bovine serum albumin as standard (Sigma). The two methods yielded similar results for all the enzymes.

Activity Assays. Activities were routinely assayed in 0.1 M potassium phosphate buffer, pH 7.5, containing 1 mg/mL BSA, 1 mM phenazine methosulfate (PMS), and 100−150 μM dichloroindophenol (DCPIP), at 20 °C, as described previously for wt-MDH, except as noted (*9*). The formation of H_2O_2 was measured in a coupled-enzyme assay using horseradish peroxidase and *o*-dianisidine. In this case, PMS and DCPIP were omitted from the assay mixture; 0.01 mg/mL horseradish peroxidase and 0.5 mM *o*-dianisidine were included. Formation of the *o*-dianisidine radical cation, which reflects the oxidase activity of the enzyme, was monitored at 460 nm and at 25 °C (ϵ = 11.3 mM^{-1} cm^{-1} at 460 nm). The pH dependence of the kinetic parameters of R277K as well as of wt-MDH purified according to the modified protocol were measured in a buffer containing 0.052 M ethanolamine, 0.052 M Tris and 0.1 M Mes, using the same equations to fit the data as those described earlier (*9*).

Binding of Negatively Charged Ligands. The binding of all the inhibitors was measured at 20 °C with the exception of sulfite, which was measured at 4 °C. The dissociation constants for sulfite, (*R*)-mandelate, and 1-phenylacetate were

obtained from spectral titration of the oxidized R277 mutant enzymes as described previously for wt-MDH (*11*). Binding constants for (*R*)-mandelate and 1-phenylacetate were also measured kinetically in competitive inhibition experiments (K_is) for R277G (in the presence of 1-methylguanidine), WT, and R277K. Binding of 1-phenylacetate to R277G (in the absence of 1-methylguanidine), R277H, and R277L was only measured through spectral titrations due to the low k_{cat}s and high K_ms of these mutant enzymes.

Chemical Rescue of the Activity of R277G. The low k_{cat} for R277G could be enhanced by the addition of imidazoles or guanidines to the assay buffer, but not amines. Typically, the ability of a particular chemical reagent to rescue activity was tested in an assay mixture containing 50 mM potassium phosphate, pH 7.5, 1 mg/mL BSA, 1 mM PMS, 120 μM DCPIP, 0.02−100 μM of the R277G protein, 40−60 mM (*S*)-mandelate, and high concentrations of the potential rescue agent. The magnitude of the rescued k_{cat} was unaffected by the order of addition of the assay components and did not increase following preincubation of the enzyme with the rescue agent prior to the assay. High concentrations of guanidine hydrochloride, up to 0.4 M, did not affect the activity of wt-MDH or R277K. However, guanidine hydrochloride at concentrations >0.1 M was deleterious for R277G, presumably due to denaturation of the protein.

To study the influence of the size of the rescue agent on the efficiency of rescue, we determined the k_{cat} and K_r (concentration of rescue agent which gives half of the maximal rescued k_{cat}) with guanidine hydrochloride, 1-methylguanidine hydrochloride, and 1-ethylguanidine sulfate. These reagents have similar pK_a values but increasing sizes of the substituent on the guanidinium group. The buffer used was 50 mM potassium phosphate, pH 7.5, containing 1 mg/mL BSA, 1 mM PMS, and 120 μM DCPIP. The ionic strength was maintained at 0.2 with potassium chloride. The (*S*)-mandelate concentration was kept fixed at 60 mM for these assays. Data were fitted to eq 1.

$$V = \frac{k_{cat}[\text{rescue agent}]}{K_r + [\text{rescue agent}]} \quad (1)$$

Similarly, the effect of the charge carried by the rescue agent on the efficiency of rescue was determined by assaying R277G with 4-methylimidazole and 1-methylguanidine hydrochloride at pHs 7.0 and 8.5. In each case, the buffer contained 25 mM Tris chloride, 1 mg/mL BSA, 1 mM PMS, and 120 μM DCPIP; the (*S*)-mandelate concentration was kept constant at 60 mM and the ionic strength was maintained at 0.1. The rescue agent was added from a 2 M stock adjusted to pH 7.0 or 8.5. The data were fitted to eq 1.

The effect of the rescue agent on the apparent K_m for (*S*)-mandelate for R277G was examined by measuring the K_m in the presence of 10 and 80 mM 1-methylguanidine hydrochloride in the standard assay buffer at pH 7.5.

Free Energy Calculations. Differences in the binding energies of the enzyme−substrate (ES) complex and the activation energies for k_{cat}/K_m and k_{cat} for wt-MDH and the R277 mutant enzymes were calculated using eqs 2, 3, and 4, respectively (*20*). The differences in binding energy of the competitive inhibitor, 1-phenylacetate, were calculated using eq 5.

$$\Delta\Delta G_S = RT \ln [K_{m(WT)}/K_{m(mutant)}] \quad (2)$$

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

$$\Delta\Delta G^{\ddagger} = \Delta\Delta G_T^{\ddagger} + \Delta\Delta G_S = RT \ln [(k_{cat})_{(WT)}/(k_{cat})_{(mutant)}] \quad (4)$$

$$\Delta\Delta G_I = RT \ln [K_{d(WT)}/K_{d(mutant)}] \quad (5)$$

Instrumentation and Data Analysis. UV−vis spectra were recorded with a Varian (Cary 1E) spectrophotometer. Data were analyzed with Kaleidagraph for the Macintosh (Synergy Software).

RESULTS

Purification and Spectral Properties of the R277 Mutants. In a preliminary study, we reported that the R277K enzyme, when purified in the presence of the detergent Triton X-100, was active; however, its k_{cat}/K_m was 1000-fold lower than the wt-MDH value (*16*). An earlier study of the R277K mutant, using nonhistidyl tagged protein and a longer purification protocol, yielded an enzyme with even lower k_{cat} and k_{cat}/K_m values (*15*). In this work, using a modified purification scheme, we obtained a highly stable and active R277K mutant enzyme with a k_{cat}/K_m only 135-fold lower than that of wt-MDH purified using the same protocol (Table 1). wt-MDH, purified by this modified protocol, was significantly more stable, especially at extremes of pH.

R277L, R277G, and R277H were rather unstable and were obtained as 1:1 mixtures of apo- and holoproteins when purified in the presence of Triton X-100. They were completely inactive and did not appear to bind either the substrate or negatively charged competitive inhibitors (*16*). Since R277G is highly active in the presence of rescue agents, particularly 1-methylguanidine, the loss in activity could be followed during the purification procedure. We observed that solubilization of the enzyme from membranes using 1% Triton X-100 yielded a protein that showed no activity with (*S*)-mandelate in the presence or absence of any rescue agent. The mutant enzymes could be purified and maintained in an active state only when the proteins were solubilized from the membranes with concentrations of Triton X-100 close to its CMC (critical micellar concentration), typically 0.04−0.06%. The amount of protein solubilized increased with longer times of incubation with the detergent, up to 12 h. Unlike Triton X-100, protein solubilized with Tween 80 was active; however, the yields were much lower than with Triton X-100. Therefore, the optimized purification protocol involved extraction of the protein from the membrane with low concentrations of Triton X-100, followed by purification on a Ni(II)-affinity column in the presence of Tween 80. The mutant enzymes purified by this method were stable and active; however, the yields were typically 10% of those obtained previously (*9*).

The spectra of the R277 mutant enzymes as well as that of wt-MDH are shown in Figure 2. wt-MDH, a dehydrogenase, is completely reduced by the substrate, (*S*)-mandelate, under aerobic conditions, since it reacts slowly with oxygen,

Table 1: (A) Steady-State Kinetic Parameters of wt-MDH and the R277 Mutant Enzymes Using (*S*)-Mandelate as Substrate and (B) Comparison of the Kinetic Parameters for wt-MDH and R277G in the Presence of 80 mM 1-Methylguanidine[a]

A

	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (mM^{-1} s^{-1})	$^Dk_{cat}$	$^Dk_{cat}/K_m$
WT	270 ± 3.6	0.12 ± 0.02	2250	2.3 ± 0.1	3.0 ± 0.3
R277K	73 ± 0.36	4.4 ± 0.09	16.6	4.6 ± 0.3	5.1 ± 0.4
R277H[b]	1.52 ± 0.06	19.2 ± 1.7	0.079	6.5 ± 0.7	nd
R277L	0.27 ± 0.02	73 ± 11	0.0037	nd	nd
R277G	0.64 ± 0.04	47 ± 7	0.014	nd	nd

B

	wt-MDH				R277G + 80 mM 1-methylguanidine			
	k_{cat} (s^{-1})	K_m (mM)	rel[c] k_{cat}/K_m	$^Dk_{cat}$	k_{cat} (s^{-1})	K_m (mM)	rel[c] k_{cat}/K_m	$^Dk_{cat}$
(*S*)-mandelate	270 ± 3.6	0.12 ± 0.02	1	2.3	201 ± 4	3.6 ± 0.3	1	2.2
(*S*)-3-phenyllactate	0.90 ± 0.02	4.3 ± 0.02	9.3×10^{-5}	5.9	0.074 ± 0.004	40.3 ± 5.7	3.3×10^{-5}	nd
(*R,S*)-2-hydroxy-octanoate[d]	0.5 ± 0.1	0.75 ± 0.1	3.0×10^{-4}	4.8	0.14 ± 0.01	10.3 ± 1.3	2.4×10^{-4}	nd

[a] Assays were performed in 0.1 M potassium phosphate, pH 7.5, containing 100−150 μM DCPIP, 1 mg/mL BSA, and 1 mM PMS. $^Dk_{cat}$, primary substrate kinetic isotope effect on k_{cat}. $^Dk_{cat}/K_m$, primary substrate kinetic isotope effect on k_{cat}/K_m. nd, not determined. [b] Assays for R277H were performed at pH 6.5. The k_{cat} for R277H at pH 7.5 was 0.3 s^{-1}. [c] Relative to the value for (*S*)-mandelate. [d] Values for wt-MDH are from ref *9*.

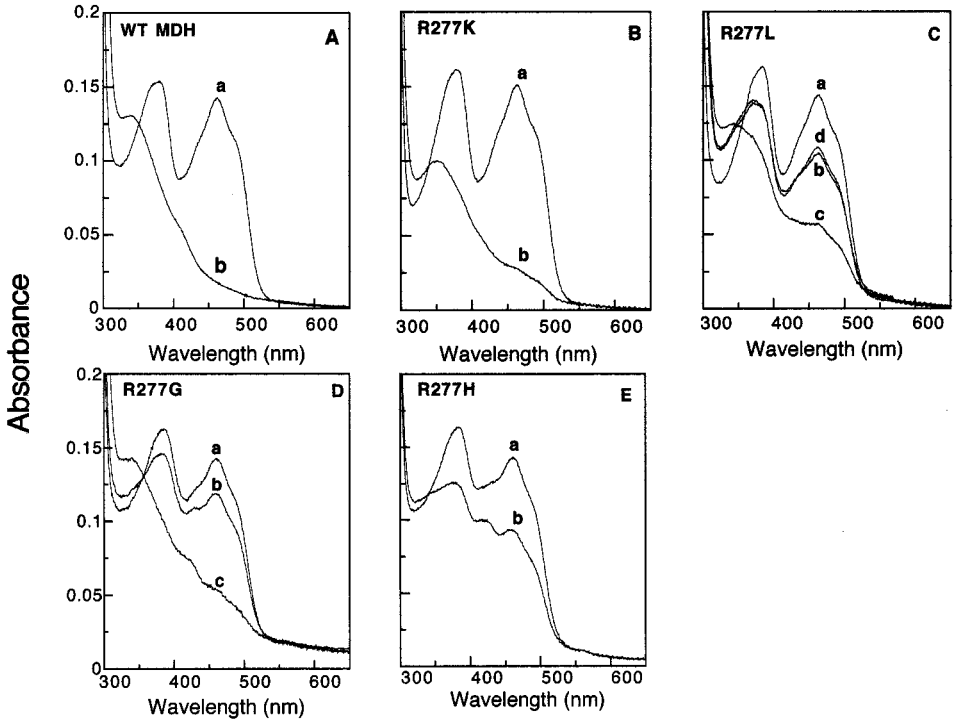


FIGURE 2: Absorbance spectra of wt-MDH and the R277 mutants in 20 mM potassium phosphate, pH 7.5, with 5% glycerol and 0.1% Tween 80 under aerobic conditions. (A) wt-MDH (15 μM), (B) R277K (15 μM), (C) R277L (15 μM), (D) R277G (10 μM), and (E) R277H (11 μM). For each panel, spectrum a is that of the oxidized protein; spectrum b was recorded after 20 mM (*S*)-mandelate was added under aerobic conditions. In panel C, spectrum c was recorded after the enzyme in spectrum b was incubated in partially anaerobic conditions (anaerobic conditions were created by flushing N_2 in the enzyme sample for 1 min). Spectrum d was recorded after the sample in spectrum c was reexposed to aerobic conditions. In panel D, spectrum c was recorded after the addition of 20 mM 1-methylguanidine to spectrum b.

with a rate of 0.1 s^{-1} (*21*). The rate of reactivity toward oxygen was unaffected in all four R277 mutant proteins as measured by hydrogen peroxide formation in aerobic buffers. R277K was completely reduced by (*S*)-mandelate in aerobic buffers. R277G, R277L, and R277H could only be partly reduced under aerobic conditions; further reduction was achieved when the buffer was made partially anaerobic. It is to be noted that the buffers could not be made completely anaerobic due to the leakiness caused by the presence of detergent. In the presence of 20 mM 1-methylguanidine hydrochloride, a fully reduced spectrum was obtained for R277G even under aerobic conditions. 1-Methylguanidine hydrochloride did not affect the spectra of either R277H or R277L (data not shown).

Steady-State Kinetics. All four mutants, R277K, R277H, R277G, and R277L, were active with (*S*)-mandelate as substrate. The kinetic parameters, including the primary substrate kinetic isotope effects, are summarized in Table 1A together with those for wt-MDH. The conservative mutant, R277K, was a fairly competent enzyme, with a k_{cat} that was only 4-fold lower compared to wt-MDH; however, surprisingly, its K_m was 40-fold higher. $^Dk_{cat}$ increased from

Table 2: Comparison of Dissociation Constants of Negatively Charged Ligands to wt-MDH and the R277 Mutants[a]

	1-phenylacetate (mM)	(*R*)-mandelate (mM)	sulfite (μM)	(*S*)-mandelate (mM)
WT	5.6 ± 1.0^b 5.6 ± 0.1^c	3.8 ± 0.2^b	30.4 ± 0.3^d	0.12 ± 0.02
R277K	12.3 ± 3.1^b 18.6 ± 1.0^c	7.2 ± 1.0^b	15.7 ± 1.7	4.4 ± 0.1
R277H	56 ± 17^b	nd	nd	19.2 ± 1.7^e
R277L	60 ± 4^b	nd	nd	73 ± 11
R277G	22.5 ± 3.1^b	19.5 ± 1.6^b	500 ± 65	47 ± 7
R277G + 80 mM 1-methylguanidine	9.3 ± 0.6^c	nd	nd	3.6 ± 0.3

[a] K_is were measured in 0.1 M potassium phosphate, pH 7.5, containing 100−150 μM DCPIP, 1 mg/mL BSA, and 1 mM PMS; K_ds were measured in 20 mM potassium phosphate, pH 7.5, containing 5% glycerol. The K_m values for the substrate, (*S*)-mandelate, are also included. nd, not determined. [b] Dissociation constants obtained from direct spectral titrations of oxidized FMN (K_d). [c] Dissociation constants obtained from competitive inhibition experiments (K_i). [d] From ref *9*. [e] The K_m for R277H was measured at pH 6.5.

2.3 ± 0.1 for wt-MDH to 4.6 ± 0.3 for the R277K mutant at pH 7.5, $^D k_{cat}/K_m$ also increased from 3.0 ± 0.3 for wt-MDH to 5.1 ± 0.4 for R277K. wt-MDH can utilize 2-hydroxy-3-butynoate, vinylglycolate, 3-phenyllactate, or 2-hydroxyoctanoate as substrates but with significantly higher K_ms compared to (*S*)-mandelate (*9*). No activity was detected with these hydroxy acids for R277K, probably due to very high K_ms. Activities obtained with the substrates, (*R,S*)-4-chloromandelate, (*R,S*)-4-bromomandelate, and (*R,S*)-4-methoxymandelate, were comparable to the values obtained with (*S*)-mandelate (data not shown).

When the positively charged residue at position 277 was replaced by a neutral residue (glycine, histidine, or leucine), the k_{cat} decreased 400−1000-fold compared to wt-MDH (at neutral and alkaline pHs for R277H, where it is likely to be unprotonated) (Table 1). The K_m increased 5−15-fold compared to the value for the R277K mutation. The activity obtained with R277H was higher at lower pH (<pH 7.0); the k_{cat} and K_m for this mutant reported in Table 1A were measured at pH 6.5.

The pH dependence of the k_{cat} and k_{cat}/K_m parameters were determined for R277K but not for R277G, R277L, and R277H, due to the high K_ms of these latter mutants for the substrate. Figure 3 shows the pH profiles for R277K together with those for wt-MDH prepared according to the modified protocol. The shapes of the pH profiles for the two enzymes are similar. The pK_a values calculated from the k_{cat} pH profile were 5.1 ± 0.1 and 9.9 ± 0.1 whereas the pK_a values calculated from the k_{cat}/K_m pH profile were 4.9 ± 0.1 and 9.4 ± 0.1 for the R277K mutant. The corresponding pK_as obtained for wt-MDH were 4.8 ± 0.1 and 10.3 ± 0.1 for the k_{cat} pH profile and 5.1 ± 0.1 and 9.7 ± 0.1 for the k_{cat}/K_m pH profile.

Binding of Negatively Charged Ligands to the R277 Mutants. To investigate the role of arginine 277 in binding negatively charged ligands, K_ds were measured for the binding of (*R*)-mandelate and 1-phenylacetate, two competitive inhibitors of MDH, and sulfite, a ligand that forms a reversible, covalent adduct with FMN. The results are summarized in Table 2, together with the K_m values obtained for the substrate, (*S*)-mandelate. Binding of both (*R*)-mandelate and 1-phenylacetate produce changes in the oxidized FMN spectrum. However, the difference spectra produced by 1-phenylacetate were different for the R277 mutants compared to wt-MDH (Figure 4). K_ds were calculated from reciprocal plots of the absorbance changes at 502−507 nm versus the concentration of inhibitor (Figure 4 and Table 2). The dissociation constant for 1-phenylacetate binding was also determined by kinetic methods in competitive inhibition experiments. The K_d and the K_i values obtained by the two methods were in reasonably good agreement for both wt-MDH and R277K (Table 2).

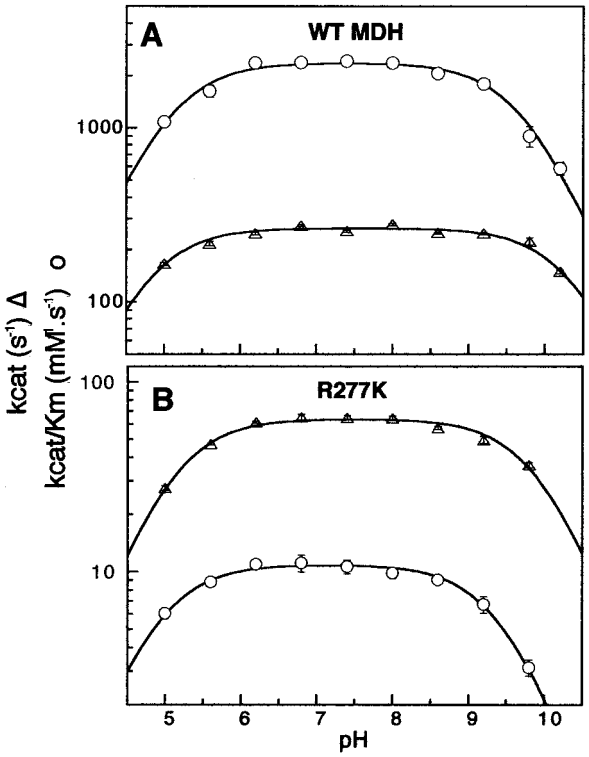


FIGURE 3: pH dependence of the k_{cat} (△) and k_{cat}/K_m (○) values for (A) wt-MDH and (B) R277K at 20 °C using (*S*)-mandelate as substrate. The lines are fits to the equation $y = Y[1 + 10^{(pK_1 - pH)} + 10^{(pH - pK_2)}]^{-1}$. The buffer used over the entire pH range was made up of 0.052 M ethanolamine, 0.052 M Tris and 0.1 M Mes. The pH was adjusted using concentrated HCl or KOH. Assays were performed by measuring the decrease in absorbance of 200−230 μM DCPIP at 522 nm in the presence of 1 mg/mL of BSA. The pK_a values obtained for R277 were 5.1 ± 0.1 and 9.9 ± 0.1 for the k_{cat} pH profile and 4.9 ± 0.1 and 9.4 ± 0.1 for the k_{cat}/K_m pH profile. The pK_a values obtained for wt-MDH were 4.8 ± 0.1 and 10.3 ± 0.1 for the k_{cat} pH profile and 5.1 ± 0.1 and 9.7 ± 0.1 for the k_{cat}/K_m pH profile.

Sulfite forms a covalent adduct with the oxidized FMN in the α-hydroxy acid oxidases/dehydrogenases. The dissociation constant of the adduct of sulfite with wt-MDH is 30 μM (*9*). The strength of the FMN-sulfite adduct was

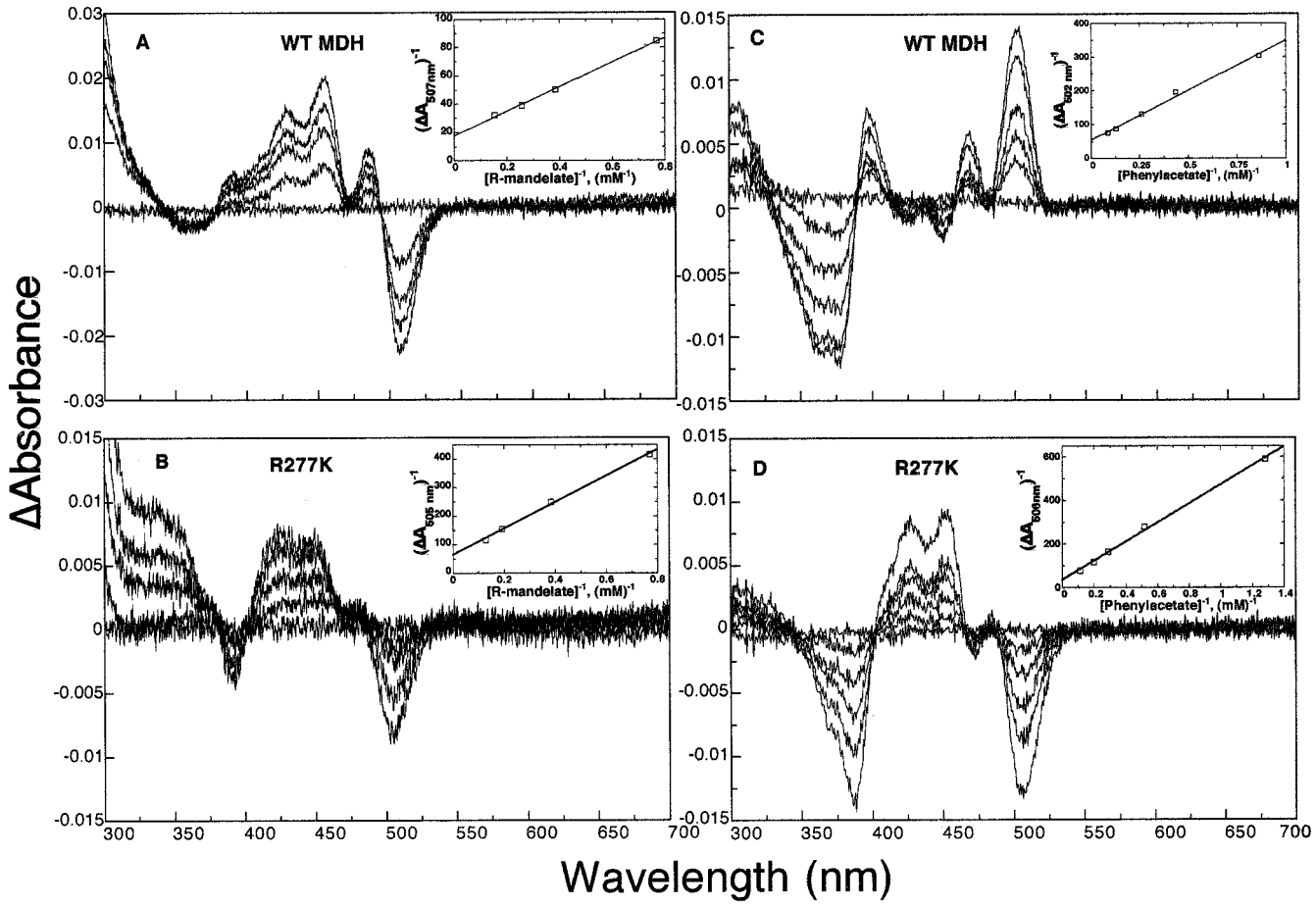


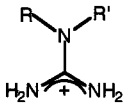
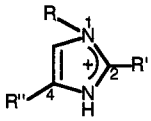
FIGURE 4: Difference spectra obtained for wt-MDH and R277K upon binding of (*R*)-mandelate and 1-phenylacetate at 20 °C in 20 mM phosphate, pH 7.5, containing 0.1% Tween 80 and 5% glycerol. (A) Difference spectra were obtained following the addition of 0, 1.3, 2.6, 3.9, and 6.5 mM (*R*)-mandelate to 30 μM wt-MDH. (B) Difference spectra were recorded following the addition of 0, 1.3, 2.6, 5.2, and 7.8 mM (*R*)-mandelate to 15 μM R277K. (C) Difference spectra were recorded after the addition of 0, 1.2, 2.3, 3.9, 7.7, and 11.6 mM 1-phenylacetate to 30 μM wt-MDH. (D) Difference spectra were recorded following the addition of 0, 0.8, 1.9, 3.5, 5.0, and 8.9 mM 1-phenylacetate to 50 μM R277K. K_ds were calculated from the double-reciprocal plots shown in the insets for each panel.

measured for R277K and R277G to investigate whether the environment around the FMN was significantly altered by the R277 mutations. The proteins were titrated with increasing concentrations of sulfite (data not shown). The K_ds obtained for R277K and R277G were 15 and 500 μM, respectively (Table 2).

Chemical Rescue of the Activity of R277G. The R277G mutant enzyme had low but measurable activity with (*S*)-mandelate (Table 1A). This low k_{cat} could be significantly enhanced by the addition of guanidine to the assay buffer (Tables 1B and 3). No guanidine-dependent increase in k_{cat} was detected with R277L or R277H. A variety of chemicals were tested for their ability to rescue the activity of R277G; the results are summarized in Table 3. Imidazoles and guanidines were found to be effective rescue agents. In addition to guanidine, 1-methyl, 1-ethyl, and 1-aminoguanidines were effective at rescue, with 1-methylguanidine displaying the maximal stimulation of k_{cat} of all the rescue agents tested. The disubstituted 1,1-dimethylguanidine as well as 1-nitroguanidine were unable to increase the k_{cat} of R277G. Imidazole, 2-methyl, and 4-methylimidazoles were able to stimulate the k_{cat} of R277G to different extents. No enhancement was detected with 1-methylimidazole. None of the amines tested acted as rescue agents, an unexpected finding since R277K was highly active.

The rescued activity showed saturation behavior with respect to both the rescue agent and the substrate, implying that the enzyme, the substrate and the rescue agent form a ternary complex prior to catalysis (Figures 5 and 6). Figure 5 shows the activity obtained with R277G at increasing concentrations of (*S*)-mandelate in the presence of saturating concentrations (80 mM) of 1-methylguanidine hydrochloride. Under these conditions, the k_{cat} obtained for R277G was 201 $\pm$ 4 s^{-1} compared to $\sim$0.6 s^{-1} in the absence of any rescue agent; this reflects an enhancement of $\sim$300-fold. Moreover, the rescued k_{cat} compares well with the wt-MDH k_{cat} value of $\sim$270 s^{-1}. Remarkably, $^{D}k_{cat}$ for the rescued activity of R277G at saturating (*S*)-mandelate and 1-methylguanidine concentrations was 2.2, similar to the wt-MDH value and lower than that observed with the conservative mutation, R277K (Table 1A). The apparent K_m for (*S*)-mandelate for the rescued activity of R277G was 3.6 $\pm$ 0.3 mM at saturating 1-methylguanidine concentrations (Figure 5 and Table 1B), in contrast to the K_m of 47 $\pm$ 7 mM obtained in the absence of 1-methylguanidine (Table 1A). In the presence of 10 mM 1-methylguanidine, which is the concentration at which half the maximal rate enhancement was observed (K_r) (Table 3), the apparent K_m for (*S*)-mandelate had an intermediate value of 16 $\pm$ 0.7 mM (data not shown).

Table 3: Compounds Tested as Potential Rescue Agents of R277G[b]

Chemicals tested for rescue of k_{cat}		Rate enhancement	K_r or highest concentration tested*, mM
Guanidine derivatives:			
	Guanidine (R, R'=H)	58	35 ± 5
	1-Methylguanidine (R=H; R'=CH_3)	314	9.5 ± 1
	1-Ethylguanidine (R= H; R' =CH_2CH_3)	260	52 ± 7
	1,1-Dimethylguanidine (R, R'= CH_3)	not detected	(60*)
	1-Aminoguanidine (R=H; R'=NH_2)	175	26 ± 2
	1-Nitroguanidine (R=H; R'=NO_2)	not detected	(20*)
Imidazole derivatives:			
	Imidazole (R, R', R'''= H)	63	51 ± 2
	4-methylimidazole (R, R'= H; R''= CH_3)	28[a]	(50*)
	2-methylimidazole (R, R''= H; R'= CH_3)	9[a]	(50*)
	1-methylimidazole (R', R''= H; R= CH_3)	not detected	(50*)
Primary Amines:			
$R-NH_3^+$	(R = H, CH_3, CH_2CH_3, $CH_2CH_2CH_3$, $CH_2CH_2CH_2CH_3$, $CH_2CH_2NHCH_3$)	not detected	(30*)

[a] Measured at the highest concentration tested. [b] k_{cat}s were measured in 50 mM potassium phosphate, pH 7.5, containing 1 mg/mL BSA, 1 mM PMS, 120 μM DCPIP, 0.02-100 μM of the R277G protein and 40−60 mM (*S*)-mandelate. K_r is the concentration of the rescue agent at which half of the maximal k_{cat} was observed. The rate enhancement is the ratio of the k_{cat}s obtained for R277G in the presence and absence of the rescue agents at either saturating concentration or the highest concentration tested [indicated by an asterisk (*)].

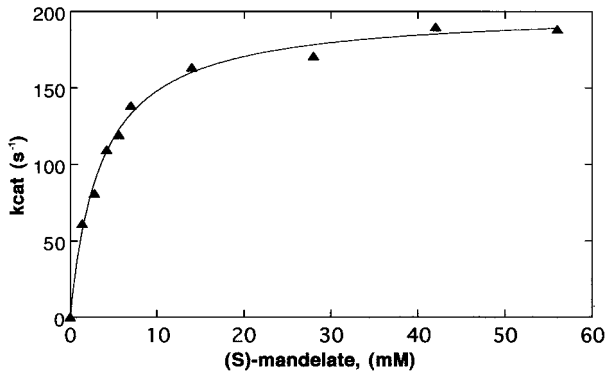


FIGURE 5: Determination of the apparent K_m for (*S*)-mandelate for R277G in the presence of 80 mM 1-methylguanidine hydrochloride in 100 mM potassium phosphate, pH 7.5, 1 mM PMS, 1 mg/mL BSA, and 120 μM DCPIP. The line is a fit to the Michaelis−Menten equation.

The kinetic parameters for two slow substrates, (*R*,*S*)-2-hydroxyoctanoate and (*S*)-3-phenyllactate, were also measured for R277G in the absence and presence of 1-methylguanidine hydrochloride. No activity was detected with these substrates in the absence of any rescue agent. However, in the presence of the rescue agent (80 mM), R277G was able to oxidize these substrates (Table 1B).

To study the effect of the charge of the rescue agent on the efficiency of rescue, we measured the activity of R277G at two pHs, 7.0 and 8.5, with two different rescue agents, 4-methylimidazole and 1-methylguanidine hydrochloride (Figure 6). Comparable k_{cat}s were obtained with 1-methylguanidine at both pHs. However, the degree of rescue by 4-methylimidazole was different at the two pHs, with almost no rescue observed at pH 8.5, in contrast to pH 7.0. 1-Methylguanidine has a pK_a of ∼13.5 and is therefore fully protonated at both pHs 7 and 8.5. 4-Methylimidazole has a

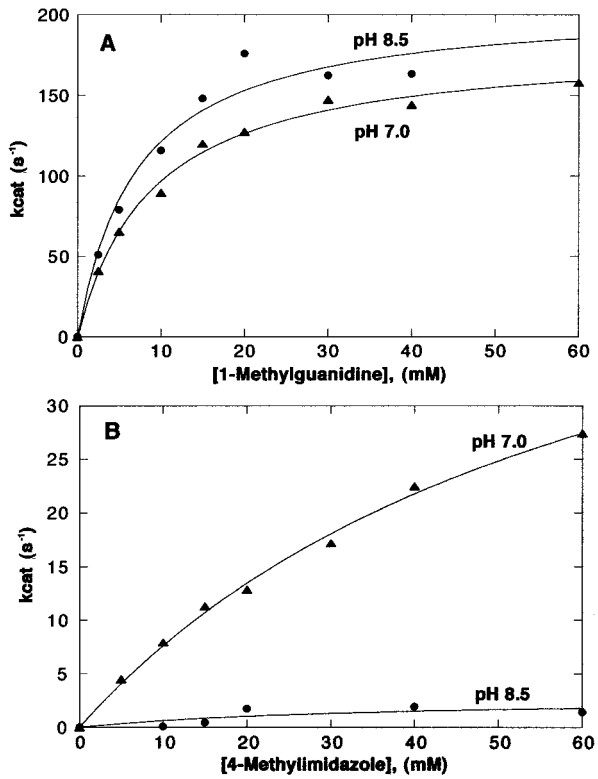


FIGURE 6: k_{cat} for R277G as a function of (A) total 1-methylguanidine hydrochloride and (B) total 4-methylimidazole concentrations (protonated and unprotonated forms) at pH 8.5 (●) and 7.0 (▲) in the presence of 60 mM (*S*)-mandelate. Activity was measured in 25 mM Tris chloride with 1 mM PMS, 1 mg/mL BSA, and 120 μM DCPIP. The lines are fits to the Michaelis−Menten equation. The k_{cat} and apparent K_m obtained were (A) 207 ± 16 s^{-1} and 7.1 ± 1.7 mM at pH 8.5 and 183 ± 5 s^{-1} and 8.9 ± 0.9 mM at pH 7.0 for 1-methylguanidine, and (B) 57 ± 5 s^{-1} and 65 ± 8 mM at pH 7.0 for 4-methylimidazole.

pK_a of 7.6 (ACD Labs), and hence, it is ~90% unprotonated at pH 8.5. Thus, a positively charged agent is required for effective rescue of the activity of R277G.

The influence of the size of the rescue agent on the rescue efficiency was tested by comparing the ratio, k_{cat}/K_r, obtained with guanidine hydrochloride, 1-methylguanidine hydrochloride and 1-ethylguanidine sulfate at 60 mM (*S*)-mandelate at pH 7.5 (Table 3). These three agents have comparable pK_as (~13.5) and are completely positively charged at this pH. 1-Methylguanidine showed the highest rescued k_{cat}, 200 s^{-1}, and the lowest observed K_r of all the rescue agents tested, 9.5 mM. The maximal k_{cat} measured with 1-ethylguanidine was similar to that with 1-methylguanidine, but its K_r was ~5-fold higher (52 ± 7 mM). Guanidine was the least efficient rescue agent of the guanidine derivatives tested, with a 6-fold lower maximal rescued k_{cat} and a relatively high K_r (35 ± 5.4 mM). Thus, there appeared to be no correlation between the size of the rescue agent and the efficiency of catalysis. This precluded the use of a Bronsted analysis of the reaction using guanidine derivatives with different pK_as.

DISCUSSION

MDH, in common with other members of the FMN-dependent-α-hydroxy-acid oxidase and dehydrogenase family, has four conserved arginines in its primary sequence. Structural information indicated that one of these, R277, binds the carboxylate end of the substrate and product and may play an important mechanistic role. Earlier studies of the arginine to lysine substitution in homologous enzymes reported low activities or inactivation during purification (*6*, *12*−*14*). In preliminary studies with MDH, we also observed that mutants at residue R277 became inactivated to various extent during purification. In the present study, we were able to generate active and stable mutant enzymes using a modified purification protocol. The modified protocol also resulted in significantly more stable preparations of wt-MDH and R277K. Characterization of altered enzymes containing both positively charged as well as neutral substitutions at R277, together with chemical rescue experiments, enabled us to clearly separate the different roles of R277 in binding and catalysis.

Purification and Spectral Characterization of the Mutants. When the R277 mutant enzymes were purified using the detergent Triton X-100, only R277K had detectable activity; the neutral substitutions were inactive. Interestingly, the inactive proteins all showed normal FMN spectra and were able to form adducts with sulfite, an observation similar to that made for lactate monooxygenase (*12*). We determined that the loss of activity resulted from the use of Triton X-100. Other detergents, for example, dodecylmaltoside, that were highly effective at solubilizing the proteins, also had the same deleterious effect on activity. Tween 80, a detergent that was *not* efficient at extracting the proteins from the membranes, also did not inactivate the proteins. Our modified purification protocol involved solubilization of the protein with Triton X-100, followed by further purification in the presence of Tween 80. Addition of Triton X-100 at later stages of the purification process or to the purified enzymes resulted in immediate inactivation of the enzymes. Our observations suggest that when the R277 mutants are removed from the membranes with low concentrations of detergents and purified, they remain tightly associated with certain phospholipids that help maintain the correct active-site structure. Detergents such as Triton X-100 and dodecylmaltoside disrupt these stabilizing interactions. Inactivation could be the result of an active site that is unable to bind and orient the substrate properly but can incorporate the FMN cofactor. It is to be noted that the membrane-bound MDH is the only enzyme in its family for which the lysine substitution of this conserved arginine is highly active. Its association with phospholipids may be advantageous in providing additional stability to the structure, particularly for the mutant enzymes, so long as these stabilizing factors remain associated with the enzyme after extraction from membranes. In support of this conclusion, it was observed that L-lactate dehydrogenase from *Escherichia coli*, another membrane-bound member of this enzyme family, gained further activity when the solubilized enzyme was reconstituted into phospholiposomes (*22*).

All four R277 mutants were stable and active; the high k_{cat} obtained with R277K is in contrast to the reports for lactate oxidase, lactate monoxygenase and flavocytochrome b_2 (*6*, *12*, *13*). It is possible that the very low k_{cat}s reported for the arginine to lysine substitution in the homologous enzymes were a result of either inactivation of the proteins during purification, or their inability to bind the substrate in the correct orientation. The FMN spectra of the R277 mutant enzymes were similar to that of wt-MDH. In each case, the enzyme-bound FMN formed an adduct with sulfite; however, the dissociation constant of the adduct varied depending on the nature of the substituent at R277. The K_d for the FMN-sulfite complex is a function of the redox potential of FMN, steric factors as well as stabilizing interactions at the active site (*23*, *24*). The strength of the adduct for the R277K mutant was 2-fold greater than for wt-MDH. For R277G, the dissociation constant was ~15-fold higher. Thus, not surprisingly, a charged residue at position 277 is necessary for a strong adduct of FMN with sulfite, though it cannot be ruled out that the higher K_d for the R277G mutant is primarily due to a change in the redox potential of FMN in R277G.

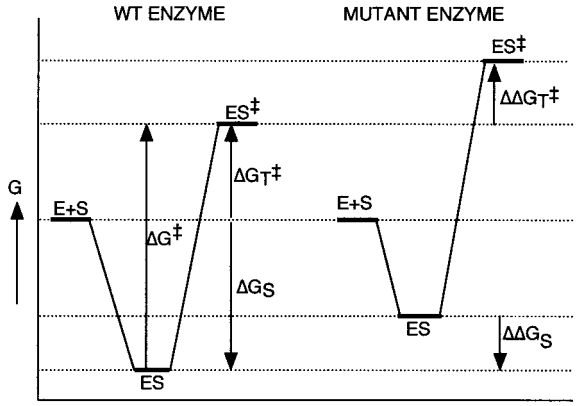
Specific Role of R277 in Substrate Binding and Catalysis. The steady-state kinetic parameters for the R277 mutants reveal that R277 contributes substantially to catalysis (Table 1). The conservative replacement of the arginine residue by lysine in R277K resulted in only a 4-fold lower k_{cat} compared to wt-MDH, whereas mutations in which the positive charge at residue 277 was completely deleted—R277L, R277G, and R277H—had dramatically decreased turnover rates. For wt-MDH, the rate-limiting step in the reaction mechanism is the oxidation of (*S*)-mandelate, since the steady-state k_{cat} has the same value as the rate of the reductive half-reaction, k_{red}; $^Dk_{cat}$ is also identical to $^Dk_{red}$ (B. Mitra, unpublished observations). The results with the R277 mutants clearly establish the importance of a positive charge at residue 277 for efficient catalysis. The lower k_{cat} for R277K compared to wt-MDH appears to be due to a decrease in the rate of the α-carbon−hydrogen bond-breaking step, since both $^Dk_{cat}$ and $^Dk_{cat}/K_m$ are significantly higher for R277K. It should be noted that the k_{cat} for R277G can be enhanced significantly by not only exogenous guanidines but also imidazoles. Also, the FMN spectra of the R277 mutants are similar to that of wt-MDH. Thus, the low k_{cat}s of the neutral substitutions at R277 are not the result of major structural perturbations of

Table 4: Differences in Binding Energies of the Enzyme−Substrate Complex ($\Delta\Delta G_S$), the Enzyme−Inhibitor (1-phenylacetate) Complex ($\Delta\Delta G_I$), 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 R277 Mutants at 25 °C[a]

	$\Delta\Delta G_S$ (kcal/mol)	$\Delta\Delta G^{\ddagger}$ (kcal/mol)	$\Delta\Delta G_T^{\ddagger}$ (kcal/mol)	$\Delta\Delta G_I$[b] (kcal/mol)
R277K	−2.1	0.8	2.9	−0.4
R277H	−3.0	3.1	6.1	−1.2
R277L	−3.8	4.1	7.9	−1.3
R277G	−3.5	3.6	7.1	−0.7
R277G + 80 mM 1-methylguanidine	−2.0	0.2	2.2	−0.2[c]

[a] The energy differences were calculated as described in Materials and Methods using data from Tables 1 and 2. [b] K_d values (Table 2) were used to calculate $\Delta\Delta G_I$. [c] The apparent K_i (Table 2) was used.

Scheme 1



the active site, but are likely to be the result of the missing side-chain of R277.

In contrast to the k_{cat}, the K_m for (*S*)-mandelate decreased 40-fold for the charge-conserved mutation, R277K, compared to wt-MDH. However, the K_m was only ∼5−15-fold lower for R277G, R277H, and R277L compared to R277K. It should be noted that for wt-MDH, the K_m and K_d for (*S*)-mandelate were observed to be similar when the parameters for the reductive reaction were measured (B. Mitra, unpublished observations). Binding of the competitive inhibitors 1-phenylacetate and (*R*)-mandelate were only slightly affected in the R277 mutants, including R277K, compared to wt-MDH. Taken together, these results imply that R277 plays an important role in substrate binding but a lesser role in binding competitive inhibitors.

Using the steady-state kinetic parameters, we calculated differences in binding energies of the enzyme−substrate complex as well as the activation energies for k_{cat} and k_{cat}/K_m for wt-MDH and the R277 mutants (Table 4, Scheme 1) (*20*). $\Delta\Delta G_S$ values, representing the difference in binding energies of the ES complex, show that even with the charge-conserved mutation, R277K, there is a loss of ∼2.1 kcal/mol in binding energy. In contrast, the positive charge at 277 contributes only about 1.5 kcal/mol to the binding energy since replacing the lysine by a neutral residue decreases ΔG_S by 3.8 and 3.5 kcal/mol in R277L and R277G, respectively, compared to wt-MDH. This points to a specific interaction of the arginine residue with the substrate, worth 2.1 kcal/mol, that is charge independent. As discussed below, chemical rescue experiments suggest that that this interaction is a hydrogen bond between the arginine ϵN and a carboxylate oxygen of the substrate. This is also supported by structural analysis of flavocytochrome b_2 where a hydrogen bond was observed between the corresponding arginine, R376, and the carboxylate oxygen of the product, pyruvate (*4*−*6*). The hydrogen-bond donor capability from the ϵN position is missing in the R277 mutants, including R277K. Interestingly, the difference in binding energies between the substrate, (*S*)-mandelate and the inhibitor, 1-phenylacetate, is 2.3 kcal/mol for wt-MDH. The similarity of this value and that calculated above for the charge-independent interaction of R277 with the substrate implies that this interaction occurs only when the substrate binds and is absent when negatively charged inhibitors bind to wt-MDH. On the other hand, the binding energy difference between the neutral inhibitor, (*S*)-phenylethanediol, and 1-phenylacetate is ∼1.4 kcal/mol for wt-MDH, suggesting that the weaker charge interaction of R277 with the carboxylate group is present when both the substrate and negatively charged inhibitors bind to the active site (*9*). These conclusions are supported by the free energy differences of binding of 1-phenylacetate to wt-MDH and the R277 mutants (Table 4). Therefore, it is likely that the substrate, (*S*)-mandelate, and the negatively charged inhibitors, 1-phenylacetate and (*R*)-mandelate, bind with somewhat different geometries.

The differences in activation energies for k_{cat} ($\Delta\Delta G^{\ddagger}$) between wt-MDH and the R277 mutants, summarized in Table 4, suggest that the transition state is stabilized relative to the enzyme−substrate complex by both a positive charge at R277 as well as a hydrogen bonding interaction. The differences in activation energies for k_{cat}/K_m ($\Delta\Delta G_T^{\ddagger}$) for wt-MDH and the R277 mutants indicate that the major, *new* contribution of R277 to transition state ($ES^{\ddagger}$) stabilization comes from the electrostatic interaction with the positive charge at R277. This is because the $\Delta\Delta G_T^{\ddagger}$ between wt-MDH and R277K is only <1 kcal/mol but 3.5−4 kcal/mol between wt-MDH and R277L/G. Therefore, we can conclude that a positive charge at position 277 in MDH contributes substantially toward transition state stabilization and only weakly to substrate binding. On the other hand, a hydrogen bond plays an important role in binding both the substrate and the transition state. These conclusions may have important mechanistic implications: if the mechanism involves the formation of a carbanion intermediate, a positively charged arginine could be envisioned to stabilize it. This conserved R277 appears to be oriented in the active site such that its positive charge does not interact optimally with the substrate but interacts with the highly negatively charged, planar carbanion/enediolate intermediate. In fact, this may be one of the strategies employed by the FMN-dependent α-hydroxy-acid oxidases and dehydrogenases to facilitate carbanion formation from anionic substrates.

Interpretation of the data obtained with the R277H mutant is complicated by the uncertainty associated with the protonation state of H277 at pH 6.5 (Table 1). The pK_a of H277 is expected to be <6, due to the proximity of other positively charged residues at the active site (for example, R165). As histidine 277 becomes protonated, the k_{cat} for the mutant should increase. However, it is not possible to measure the maximal k_{cat} associated with the fully protonated H277 since at low pH, the active-site base, H274, is likely to be protonated and cause complete inactivation of MDH (*11*). We observed that for R277H, the k_{cat} increased with decreasing pH in the range pH 8.5 to 6.0, consistent with the requirement of a positive charge for catalysis. However, the k_{cat} at pH 6.0 was much lower than for R277K, suggesting that either H277 is not fully protonated at this pH or that it is not optimally positioned for catalysis.

pH Dependence of the Kinetic Parameters for R277K. Though the pH profiles for wt-MDH prepared by the modified protocol are quite similar to those reported in a previous study, the pK_as obtained in the alkaline range are slightly higher (*9*). The alkaline pK_as obtained in this study are 10.3 and 9.4 for the k_{cat} and k_{cat}/K_m pH profiles, respectively, as opposed to 9.6 and 8.9, reported previously (*9*). wt-MDH prepared by the new protocol is significantly more stable at high pH. We believe that the lower pK_as in the alkaline range reported earlier are due to decreased stability of the enzyme. However, despite these differences, the overall conclusions from the pH profile for wt-MDH remains the same. A residue, important for activity, has an apparent pK_a of 9.7 in the free enzyme that is shifted to 10.3 on binding (*S*)-mandelate. This pK_a was assigned to either R277 or the second conserved arginine in the active site, R165. The pH dependence of the kinetic parameters of R277K reveals that the overall pH profiles of k_{cat} and k_{cat}/K_m for R277K and wt-MDH are very similar. The R277K enzyme displayed an alkaline pK_a of 9.4 in the free enzyme that is shifted to 9.9 upon binding the substrate. This lowering of the pK_a from 9.7 in free wt-MDH to 9.4 in R277K would seem to imply that this pK_a does belong to R277, though a more significant change would have been expected upon replacing an arginine residue by a lysine. However, this result should be interpreted with caution since R277K is less stable compared to wt-MDH at high pH. We cannot rule out the possibility that the slightly lower pK_as obtained for R277K relative to wt-MDH are artifacts due to the decreased stability of R277. Thus, we have yet to unequivocally determine the identity of the catalytically important residue with the pK_a of 9.7 in the active site of wt-MDH. It is not R165, the second conserved arginine in MDH (Y. Xu and B. Mitra, manuscript in preparation).

Chemical Rescue of R277G. The low k_{cat} of R277G could be rescued with exogenous guanidines or imidazoles. Therefore, we decided to characterize this chemically rescued activity in order to verify if indeed the charge-independent interaction of R277 with both the substrate and the transition state was a hydrogen bond. Guanidines and imidazoles were able to markedly enhance the k_{cat} of R277G for both (*S*)-mandelate as well as the poor substrates, 3-phenyllactate and 2-hydroxyoctanoate. The extent of rescue at two pHs with 4-methylimidazole and 1-methylguanidine hydrochloride reinforces our previous conclusion that a positive charge at residue 277 is necessary for high k_{cat} levels. Among the guanidine derivatives we tested, 1-nitro and 1,1-dimethylguanidine were unable to rescue the k_{cat} of R277G. 1-Nitroguanidine may not bind to the active site due to the presence of the nitro group. It is also possible that it binds in an orientation in which it cannot interact productively with the substrate. 1,1-Dimethylguanidine has the same pK_a as monosubstituted guanidines but is missing a hydrogen atom on its N-1 position and thus lacks a hydrogen bond donor capability at N-1. All the imidazoles tested enhanced the k_{cat} of R277G except 1-methylimidazole. Again, this substituted imidazole cannot act as a hydrogen-bond donor from its N-1 position, even though its pK_a is similar to that of the other imidazoles. We did not detect any rescued activity with a series of amines. This may result from weak binding of amines at the active site. The structures of flavocytochrome b_2 and glycolate oxidase show that in the absence of substrate/product, the ϵN of R277 interacts with H274 through a water molecule; this water molecule is displaced upon substrate binding (*5*). This hydrogen bond interaction with the water molecule may be important for the binding of the rescue agent to R277G. Therefore, those reagents that can participate in a hydrogen bond with the water molecule, may be able to bind to the active site and effectively rescue k_{cat}. Thus, to efficiently substitute for the function of R277 at the active site, the rescue agent clearly needs to be positively charged as well as be a potential hydrogen-bond donor.

Conclusions. In this study we have used site-specific mutagenesis together with chemical rescue of catalytic activity to examine the role of the conserved arginine277 in MDH. This arginine was replaced by lysine, histidine, leucine, and glycine. The R277H, R277L, and R277G mutants were rapidly inactivated when purified using the detergent, Triton X-100, suggesting that R277 plays an important role in maintaining the integrity of the active site and allowing substrate to bind with high affinity. A modified purification protocol yielded stable and active proteins. Kinetic characterization of the four mutants clearly shows that a positive charge at residue 277 stabilizes the transition state, but contributes only weakly to substrate binding. This requirement of a positive charge to stabilize the transition state suggests that the reaction proceeds through a highly negatively charged intermediate and is consistent with a mechanism involving a carbanion intermediate. Arginine277 also has a specific interaction with both the substrate and the transition state that is essential in binding (*S*)-mandelate with high affinity. The nature of this interaction was examined by chemical rescue experiments. The low k_{cat} of the R277G mutant could be rescued up to 300-fold with exogenous guanidines and imidazoles. Only chemical agents that possessed both a diffused positive charge as well as a hydrogen bond donor capability were effective rescue agents. This supports our conclusion that the specific hydrogen bond between R277 and a carboxylate oxygen of (*S*)-mandelate, suggested by structural and modeling data, is important for substrate binding.

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