Esters of Mandelic Acid as Substrates for (*S*)-Mandelate Dehydrogenase from *Pseudomonas putida*: Implications for the Reaction Mechanism†

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ABSTRACT: (*S*)-Mandelate dehydrogenase (MDH) from *Pseudomonas putida* is a flavin mononucleotide (FMN)-dependent enzyme that oxidizes (*S*)-mandelate to benzoylformate. In this work, we show that the ethyl and methyl esters of (*S*)-mandelic acid are substrates for MDH. Although the binding affinity of the neutral esters is 25−50-fold lower relative to the negatively charged (*S*)-mandelate, they are oxidized with comparable k_{cat}s. Substrate analogues in which the carbonyl group on the C-1 carbon is replaced by other electron-withdrawing groups were not substrates. The requirement of a carbonyl group on the C-1 carbon in a substrate suggests that the negative charge developed during the reaction is stabilized by delocalization to the carbonyl oxygen. Arg277, a residue that is important in both binding and transition state stabilization for the activity with (*S*)-mandelate, is also critical for transition state stabilization for the esters, but not for their binding affinity. We previously showed that the substrate oxidation half-reaction with (*S*)-mandelate has two rate-limiting steps of similar activation energies and proceeds through the formation of a charge-transfer complex of an electron-rich donor and oxidized FMN [Dewanti, A. R., and Mitra, B. (2003) *Biochemistry 42*, 12893−12901]. This charge-transfer intermediate was observed with the neutral esters as well. The observation of this electron-rich intermediate for the oxidation of an uncharged substrate to an uncharged product, as well as the critical role of Arg277 in the reaction with the esters, provides further evidence that the MDH reaction mechanism is not a concerted transfer of a hydride ion from the substrate to the FMN, but involves the transient formation of a carbanion/ene(di)olate intermediate.

Flavin mononucleotide (FMN)-dependent enzymes that oxidize (*S*)-α-hydroxyacids to α-ketoacids constitute a large family found in both bacteria and eukarya, including plants and mammals. The mechanism of the first half-reaction involving substrate oxidation and FMN reduction is expected to be similar for all the enzymes, given the extensive sequence and structural similarity in this enzyme family, including highly conserved active site residues (*1*−*6*). The second half-reaction involving reoxidation of the reduced flavin is different and depends on the type of electron acceptor used by the individual enzymes. These enzymes use substrates with a wide range of side-chain sizes, from the small two-carbon glycolic acid, to long-chain aliphatic hydroxyacids, or the aromatic mandelic acid for (*S*)-mandelate dehydrogenase (MDH)[1] from *Pseudomonas putida* (*7*−*10*). However, to date, they have been known to primarily oxidize hydroxyacid anions to ketoacid anions. The only exceptions so far are the long-chain hydroxyacid oxidase from rat kidney, which can oxidize α-(*S*)-amino acids as well as an ionized form of 2-hydroxyphenylacetohydroxamic acid, and (L)-pantoyllactone dehydrogenase from *Nocardia asteroides*, which is able to oxidize a hydroxylactone to a ketolactone (*8*, *11*, *12*). In the present study, we identify methyl and ethyl esters of (*S*)-mandelic acid as substrates for MDH. Although the affinity of MDH for the neutral esters is substantially lower than that for (*S*)-mandelate, the k_{cat} values are quite comparable.

The mechanism of the substrate oxidation half reaction has been extensively studied, particularly for flavocytochrome b_2 from *Saccharomyces cerevisae*, lactate monooxygenase from *Mycobacterium smegmatis*, lactate oxidase from *Aerococcus viridans*, and MDH (*13*−*16*). The majority of the evidence supports a mechanism in which the oxidation of the α-hydroxyacid anion proceeds stepwise through a carbanion intermediate (*16*−*18*). Structural analyses of glycolate oxidase, flavocytochrome b_2, and MDH are compatible with this mechanism (*2*, *5*, *6*). The carbanion is stabilized by two conserved arginine residues (Arg165 and Arg277 in MDH) that interact with the substrate carboxylate group (*19*−*21*). However, an alternative mechanism in which a hydride ion is transferred from the α-carbon of the substrate to the FMN in a concerted step has also been proposed (*22*). In this case, the role of Arg277 would be to neutralize the negative charge on the carboxylate group as the geometry of the α-carbon changes from tetrahedral to planar, parallel to hydride transfer.

We have recently shown that the reaction of MDH with (*S*)-mandelate involves formation of a distinct, transient intermediate at low temperature (*23*). The intermediate is a charge-transfer complex of oxidized FMN and an electron-

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[1] Abbreviations: MDH, (*S*)-mandelate dehydrogenase.

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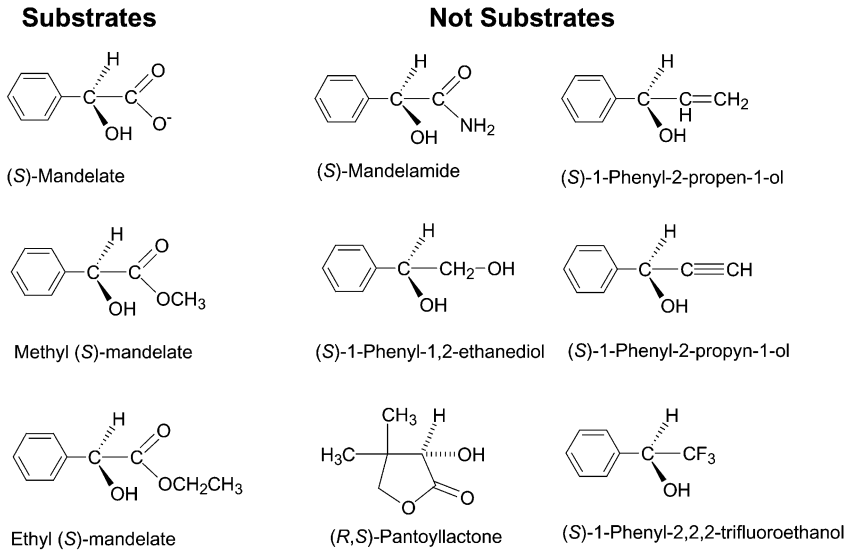


FIGURE 1: Structures of the various compounds used in this study.

rich donor. We presented evidence that supported the assignment of this electron-rich donor to be a carbanion/enediolate species. In this study, we show that the oxidation of the hydroxyesters involves a similar intermediate, further supporting our claim that it is not a Michaelis complex of oxidized FMN and an anionic substrate, but a distinct catalytic intermediate. Thus, the oxidation of both (*S*)-mandelate and the esters go through a similar electron-rich carbanion/ene(di)olate intermediate.

The kinetic parameters of ester oxidation by the R277K and R277G mutants of MDH show that Arg277 has no influence on the binding affinity of the esters. However, similar to our previous observation for the oxidation of (*S*)-mandelate, Arg277 plays a critical role in the oxidation of the esters as well (*19*). This demonstrates that the transition state for ester oxidation must be negatively charged. Interestingly, substrate analogues that did not possess a carbonyl group on the C-1 carbon were not substrates. This further supports the critical role of Arg277 in stabilizing the negative charge generated during the reaction with both (*S*)-mandelate and the esters, through interaction with the oxyanion of the enolate species.

EXPERIMENTAL PROCEDURES

Materials. (*S*)-Mandelamide was a gift from Dr. Michael J. McLeish. All chemicals, including the hydroxyester substrates, were purchased from Fluka, Lancaster, Sigma-Aldrich or TCI and were of the highest possible analytical grade. All the compounds shown in Figure 1, with the exception of (*S*)-mandelate and (*S*)-1-phenyl-1,2-ethanediol, were not highly soluble in water; therefore, concentrated stocks were made in dimethylformamide. When additions were made from these stock solutions, the dimethylformamide concentration was kept below 3% in both steady-state and stopped-flow assays. Control experiments showed that 3% dimethylformamide in the assay buffer does not affect the activity of MDH.

Methods. Determination of the Extent of Hydrolysis of Ethyl and Methyl Mandelates. It was confirmed that the esters of mandelic acid were not appreciably hydrolyzed to the acid prior to or during the enzymatic assay by thin-layer chromatography (TLC) on silica gel G plates. In one experiment, the esters were incubated in the assay buffer at pH 7.5 for 10 min, followed by TLC in an ethyl acetate/hexane (30:70) solvent system. In another experiment, an enzymatic assay was carried out, followed by lyophilizing the entire reaction. The remaining solid was extracted in 10% dimethylformamide and subjected to TLC in the same solvent system as above together with (*S*)-mandelate, benzoylformate, and the benzoylformate esters. In both cases, single clear spots corresponding to the esters were observed. Proton NMR experiments also confirmed these results. When the enzymatic reaction was subjected to proton NMR analysis, a clear peak for the methoxy protons was observed in the NMR spectrum (3.6 ppm using TMS as an internal standard); the aromatic protons and the methoxy protons were present in the expected ratio of 5:3. These observations confirmed that the activity measured was due to the esters and not their hydrolysis products.

*Enzyme Purification. wt*MDH and the mutant enzymes were solubilized with detergent and purified using established protocols as described earlier (*19*). Protein concentrations were estimated by measuring the free FMN released upon boiling the protein solutions for 5 min.

Steady-State and Pre-Steady-State Kinetics. Steady-state activities were measured at 4 or 20 °C, in 0.1 M potassium phosphate, pH 7.5, containing 1 mg/mL bovine serum albumin, 1 mM phenazine methosulfate, and 100−150 μM dichloroindophenol, as described previously (*16*).

The low activity of R277G toward hydroxyesters could be enhanced by the addition of exogenous 1-methylguanidine, in a manner similar to that reported previously (*19*). Since R277G was unstable at 1-methylguanidine concentrations >100 mM, rescued activities were measured at 1-methylguanidine concentrations below 80 mM. The apparent K_ms for the ester substrates for R277G were determined in the presence of 80 mM 1-methylguanidine at pH 7.5.

Stopped-flow data were collected at different temperatures in 0.1 M potassium phosphate, pH 7.5, under anaerobic conditions. Solutions were made anaerobic by saturating them with argon. Time-dependent spectra of the appearance

Table 1: Steady-State and Pre-Steady-State Kinetic Parameters for *wt*MDH and the Arg277 Mutant Enzymes[a]

	*wt*MDH	R277K	R277G
(*S*)-Mandelate[b]			
k_{cat} (s^{-1})	360 ± 8	66 ± 2	0.23 ± 0.07
K_m (mM)	0.12 ± 0.01	5.6 ± 0.4	17 ± 5
k_{red} (s^{-1})	402 ± 16	67 ± 4	0.27 ± 0.02
K_d (mM)	0.19 ± 0.03	5.2 ± 0.8	17.5 ± 3.5
Methyl (*S*)-mandelate			
k_{cat} (s^{-1})	151 ± 8	2.3 ± 0.1	2.5 ± 0.3
K_m (mM)	11.3 ± 1.4	10.3 ± 1.8	7.4 ± 1.4
k_{red} (s^{-1})	177 ± 15	3.0 ± 0.1	3.4 ± 0.1
K_d (mM)	11.0 ± 2	9.3 ± 1.0	10.5 ± 0.8
Ethyl (*S*)-mandelate			
k_{cat} (s^{-1})	204 ± 7	2.6 ± 0.1	0.3 ± 0.0
K_m (mM)	2.9 ± 0.3	2.7 ± 0.3	3.7 ± 0.0
k_{red} (s^{-1})	223 ± 12	3.4 ± 0.1	0.22 ± 0.02
K_d (mM)	5.1 ± 0.6	4.0 ± 1.4	3.0 ± 0.6

[a] Pre-steady-state assays were performed at 20 °C in 0.1 M potassium phosphate, pH 7.5. For steady-state assays, 100−150 μM dichloroindophenol, 1 mg/mL bovine serum albumin, and 1 mM phenazine methosulfate were also included. k_{red} is the rate of FMN reduction, obtained from measuring the decrease in absorbance at 460 nm. [b] Values are from ref *20*.

and decay of the intermediate between 350 and 750 nm were recorded at different temperatures using a photodiode array accessory connected to the stopped-flow spectrophotometer. In routine measurements, overall FMN reduction at 4 or 20 °C was monitored by measuring the decrease in absorbance at 460 nm. A transient intermediate could be observed with the ester substrates at 4 °C, just as was previously described for (*S*)-mandelate (*23*). The rates of the appearance and breakdown of this intermediate were measured at 560 nm. The data were fitted to double exponential equations, unless noted otherwise, using the Pro/K software supplied by Applied Photophysics.

Instrumentation. Steady-state kinetics were measured with a thermostated Varian (Cary 1E) spectrophotometer. Stopped-flow data were collected using a thermostated Applied Photophysics System (SX 18-MV) spectrophotometer equipped with a photodiode array accessory.

RESULTS

Ethyl (S)-Mandelate and Methyl (S)-Mandelate Are Substrates for MDH. The carboxylate esters of (*S*)-mandelic acid, ethyl and methyl (*S*)-mandelates (Figure 1) were both rapidly oxidized by *wt*MDH (Table 1). TLC as well as proton NMR experiments, together with other indirect evidence elaborated in Discussion, confirmed that the observed activity was due to the esters and not their hydrolysis product, the acid anion. MDH is very efficient toward its natural substrate, (*S*)-mandelate, with a k_{cat} of 360 s^{-1} and a K_m of 0.1 mM measured at 20 °C (Table 1). Comparable k_{cat}s were obtained with methyl and ethyl (*S*)-mandelate, 151 and 204 s^{-1}, respectively, although the K_ms were much higher, 11 and 3 mM.

The kinetics of the reductive half-reaction, that is, flavin reduction upon addition of substrate, was followed in stopped-flow experiments at 20 °C, by measuring the decrease in absorbance at 460 nm. For (*S*)-mandelate, the k_{cat} and k_{red} are similar, indicating that the chemical step involving the reductive half-reaction is rate limiting at 20 °C (*20*). This was also found to be true for both ester substrates. As shown in Table 1, the rate of flavin reduction, k_{red}, for the esters is 40−55% of the value for (*S*)-mandelate, confirming that the hydroxyesters can be efficiently oxidized to the ketoesters. However, the K_d data obtained for the esters show that the affinity of *wt*MDH for the esters is 25−50-fold lower than that for the hydroxyacid anion (Table 1).

Surprisingly, the amide derivative of mandelic acid, (*S*)-mandelamide (Figure 1), was neither a substrate for MDH nor a competitive inhibitor of the (*S*)-mandelate activity. The only homologue of MDH that has been shown to use a neutral substrate thus far is L-pantoyllactone dehydrogenase from *N. asteroides*, which can use a lactone as substrate (*12*). (D,L)-Pantoyllactone was tested as a substrate for *wt*MDH; no activity was observed. The constrained structure of this lactone may prevent it from optimally binding at the active site of MDH; also, the lack of β-unsaturation may prevent it from being utilized as a substrate (Figure 1). We have shown earlier that hydroxyacids that are saturated at the β-carbon are rather poor substrates for MDH (*16*).

Effect of Replacing the Carboxylate Group of Mandelic Acid with Unsaturated or Electron-Withdrawing Groups. Since our results indicated that hydroxyesters were substrates for MDH, we tested other neutral compounds as substrates in which the carboxylate group of mandelic acid is replaced by unsaturated or electron-withdrawing groups. Three such compounds, (*S*)-1-phenyl-2-propyn-1-ol, (*S*)-1-phenyl-2-propen-1-ol, and (*S*)-1-phenyl-2,2,2-trifluoroethanol (Figure 1), showed no detectable activity with MDH, although they were all inhibitors of the (*S*)-mandelate activity. Inhibition by (*S*)-1-phenyl-2,2,2-trifluoroethanol was characterized further. It was observed to be a competitive inhibitor of (*S*)-mandelate with a K_i of 2.6 ± 0.6 mM.

Earlier we had shown that another neutral compound, (*S*)-1-phenyl-1,2-ethanediol, which lacks unsaturation at the C-1 carbon, is a weak inhibitor of the (*S*)-mandelate activity with *wt*MDH, with a K_i of 53.8 ± 0.1 mM (*16*).

The Activity of the Arg277 Mutants toward the Ester Substrates. Arg277 is a strictly conserved residue in the active site of MDH. We have previously reported that replacing Arg277 by the charge-conserved lysine residue results in a ∼6-fold lower k_{cat} and k_{red} with (*S*)-mandelate compared to *wt*MDH, but a ∼25-fold lower affinity (*19*, *20*). The R277G mutant is an extremely poor enzyme, with a k_{cat} and a k_{red} that are ∼1500-fold lower, and a ∼100-fold lower affinity for (*S*)-mandelate compared to *wt*MDH (*19*, *20*). In this study, we measured both the steady state and pre-steady-state kinetic parameters of the oxidation of the ester substrates by R277K and R277G (Table 1). Similar to the case reported earlier with (*S*)-mandelate, the steady-state and stopped-flow kinetic parameters for the esters are comparable for both Arg277 mutants, indicating that the reductive half-reaction is rate-limiting for the Arg277 mutants, as observed for *wt*MDH (*20*). R277K has surprisingly low k_{cat}s and k_{red}s for both esters, ∼2.5−3.5 s^{-1}, in contrast to (*S*)-mandelate. Another notable difference is that the affinities (K_ds) for the ester substrates for *wt*MDH and both Arg277 mutants are similar, unlike for (*S*)-mandelate. Interestingly, although R277K is a much better enzyme than R277G with (*S*)-mandelate as substrate, this is no longer the case with the esters, especially methyl (*S*)-mandelate (Table 1). Thus, Arg277 appears to play a vital role in the catalysis of not just (*S*)-mandelate, but the neutral ester substrates as well.

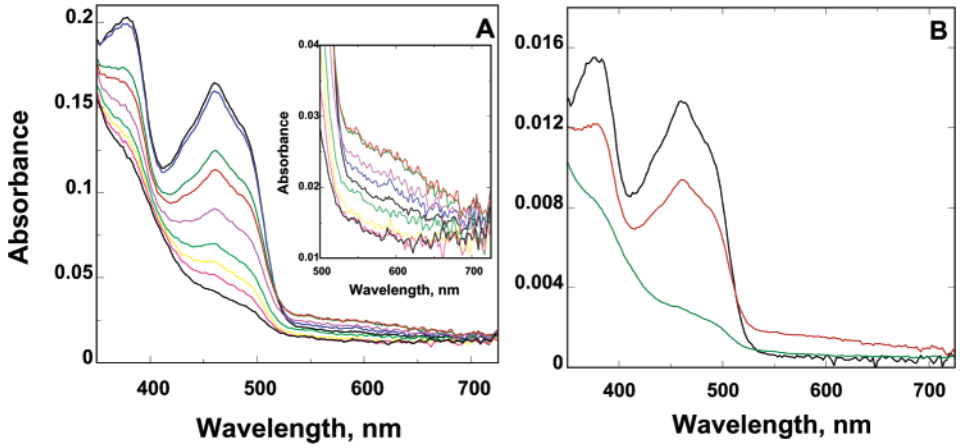


FIGURE 2: (A) Absorbance spectra recorded for 30 μM *wt*MDH, in 0.1 M potassium phosphate, pH 7.5 under anaerobic conditions at 4 °C. Spectra, in descending order, were recorded in a stopped-flow spectrophotometer equipped with a diode-array accessory, 1.2, 3.6, 6, 8.4, 13, 23, 35, 56, and 239 ms after the addition of 20 mM ethyl (*S*)-mandelate. The inset shows an expanded view of the absorbance changes from 500 to 725 nm. (B) Comparison of the spectrum of oxidized *wt*MDH (black), reduced with ethyl (*S*)-mandelate (green) and the simulated spectrum of the charge-transfer complex obtained with the ethyl ester (red). The simulated spectrum was obtained from real-time spectra using the Pro/K software.

The Activity of R277G toward Ester Substrates Could Be Enhanced by Exogenous Chemicals. As we previously reported, the low activity of R277G toward (*S*)-mandelate can be increased ~300-fold using 1-methylguanidine in solution (*19*). R277G has a k_{cat} of 2.5 s^{-1} and a K_m of ~7 mM toward methyl (*S*)-mandelate; the corresponding values for ethyl (*S*)-mandelate are 0.3 s^{-1} and ~4 mM. The k_{cat}s for both esters could be increased by the addition of 1-methylguanidine to the assay medium. Other compounds tested as potential rescue agents include guanidine, 1-ethylguanidine, imidazole, 1-methylimidazole, ethylamine, and propylamine. Unlike rescue of the activity with (*S*)-mandelate, none of these compounds showed an appreciable increase in the activities with the esters, with the exception of 1-ethylguanidine, which showed a slight increase.

When the activity of R277G was measured in the presence of both 1-methylguanidine and the esters, the increased activity showed saturation behavior with respect to both the rescue agent and the substrate, implying that the rescue agent, substrate, and enzyme form a ternary complex before catalysis, as had also been observed for (*S*)-mandelate (*19*). In the presence of 80 mM 1-methylguanidine, the k_{cat} of R277G toward methyl (*S*)-mandelate could be increased 3-fold to ~8 s^{-1}, whereas the k_{cat} of R277G for ethyl (*S*)-mandelate could be increased from 0.3 to ~10 s^{-1}. K_r values, defined as the concentration of the rescue agent at which half the maximal increase in activity is observed, were measured by varying the concentration of the rescue agent at a fixed ester concentration (*19*). The K_r value obtained was ~50 mM for rescue of the activity with both esters, in contrast to the value of 9.5 mM obtained for the rescue of the activity with (*S*)-mandelate for R277G by 1-methylguanidine (*19*). Using a fixed concentration of 1-methylguanidine (80 mM), the apparent K_ms for methyl and ethyl (*S*)-mandelate were 5.5 ± 0.6 and 7.0 ± 1.1 mM, respectively. These values are of the same order of magnitude as those obtained in the absence of a rescue agent (Table 1).

The Appearance of an Intermediate during the Course of the Reaction. We have recently reported that during the reaction of *wt*MDH with (*S*)-mandelate, a transient intermediate is observed prior to the reduction of the enzyme-bound FMN at low temperature (*23*). The spectrum of this intermediate indicated that it is a charge-transfer complex of oxidized FMN with an electron-rich species, most likely the carbanion/enolate generated in the first step of the catalytic reaction. We tested whether this transient intermediate was observed with the neutral esters as substrates. Figure 2A shows the changes in absorbance spectra in the 350−725 nm range at 4 °C for *wt*MDH following the addition of ethyl (*S*)-mandelate. The spectra were recorded in the stopped-flow spectrophotometer equipped with a diode-array accessory. Just as with (*S*)-mandelate, a distinct intermediate is observed prior to the full reduction of FMN. The intermediate has absorbance between 550 and 750 nm, a region in which neither oxidized nor reduced FMN absorbs (inset to Figure 2A). Figure 2B shows a simulated spectrum of the charge-transfer intermediate, obtained from the time-dependent spectra in Figure 2A, using the model

$$E_{ox} \rightarrow I \rightarrow E_{red}$$

and the Pro/K software supplied by Applied Photophysics. The intermediate retains ~70% of the absorbance of oxidized FMN at 460 nm, and also has long-wavelength absorbance with a low extinction coefficient above 550 nm. This spectrum is very similar to the one obtained with (*S*)-mandelate (*23*). A similar transient intermediate was also observed with methyl (*S*)-mandelate, although the amplitude at 560 nm was slightly lower than that with the ethyl ester.

Figure 3 shows the absorbance changes at 460 and 560 nm following addition of 20 mM ethyl (*S*)-mandelate to *wt*MDH at 4 °C. Similar results were obtained for methyl (*S*)-mandelate. The data could be fitted to two exponential equations with the two rate constants describing the process:

$$E{\cdot}FMN_{ox} + S \xrightarrow{k_1} I \xrightarrow{k_2} E{\cdot}FMN_{red}$$

where $E{\cdot}FMN_{ox}$ is oxidized MDH, I is the intermediate, and $E{\cdot}FMN_{red}$ is fully reduced MDH.

Dependence of k_1 and k_2 on Substrate Concentration. The dependence of the rates of formation and disappearance of the intermediate, k_1 and k_2, on the substrate concentration

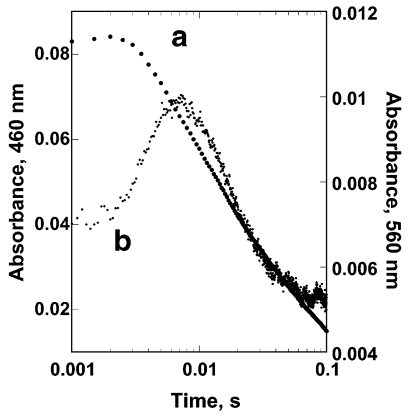


FIGURE 3: Changes in absorbance at (a) 460 and (b) 560 nm obtained for 10 μM *wt*MDH following the addition of 10 mM ethyl (*S*)-mandelate (final concentration) in 0.1 M potassium phosphate, pH 7.5 under anaerobic conditions at 4 °C.

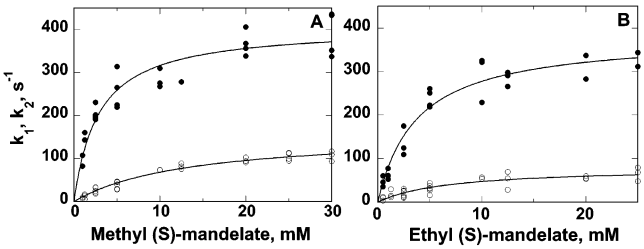


FIGURE 4: Dependence of the rate of formation, k_1 (●) and the rate of disappearance, k_2 (○) of the charge-transfer intermediate on the concentration of (A) methyl (*S*)-mandelate and (B) ethyl (*S*)-mandelate in 0.1 M potassium phosphate, pH 7.5 under anaerobic conditions at 4 °C. 25 μM *wt*MDH was used in the experiments. The data were fitted to a hyperbolic equation.

Table 2: Steady-State and Pre-Steady-State Kinetic Parameters for *wt*MDH with (*S*)-Mandelate and the Ester Analogues at 4 °C[a]

	(*S*)-mandelate[b]	methyl (*S*)-mandelate	ethyl (*S*)-mandelate
k_{cat} (s^{-1})	134 ± 3	55.9 ± 3	78 ± 3
K_m (mM)	0.11 ± 0.00	7.7 ± 1.3	2.4 ± 0.2
k_1 (s^{-1})	398 ± 26	407 ± 18	382 ± 21
$K_{1(half-saturation)}$ (mM)	0.19 ± 0.04	2.9 ± 0.5	3.8 ± 0.7
k_2 (s^{-1})	121 ± 10	160 ± 9.5	80 ± 9.5
$K_{2(half-saturation)}$ (mM)	0.36 ± 0.08	13.4 ± 1.8	6.9 ± 2.0

[a] Assay conditions were as described in Table 1. Since there are two components to k_{red} (the rate of FMN reduction) at 4 °C, k_1 and k_2 (measured by monitoring absorbance changes at 560 nm) are reported instead of k_{red}. [b] Values are from ref *23*.

was determined from absorbance changes at 560 nm at 4 °C, for different methyl and ethyl (*S*)-mandelate concentrations (Figure 4). The data were fitted to a two-exponential equation to generate k_1 and k_2. The values for k_1 and k_2 at saturating methyl (*S*)-mandelate concentrations were 407 ± 18 and 160 ± 10 s^{-1}, respectively, while k_1 and k_2 at saturating ethyl (*S*)-mandelate concentrations were 382 ± 21 and 80 ± 10 s^{-1}, respectively (Table 2). Substrate concentrations at half-saturation values of k_1 and k_2 were 2.9 ± 0.5 and 13 ± 2 mM for methyl (*S*)-mandelate, and 3.8 ± 0.7 and 6.9 ± 2 mM for ethyl (*S*)-mandelate, respectively. The corresponding k_{cat} and K_m values for the steady-state reaction at 4 °C are also shown in Table 2.

Temperature Dependence of the Reaction with Esters. Just as the case with (*S*)-mandelate, the intermediate was not observed when the reaction with the esters was monitored at room temperature (*23*). For (*S*)-mandelate, k_1 and k_2 both increase with temperature, such that k_1 is too fast to measure at room temperature. We investigated the temperature dependence of k_1 and k_2 for the esters, by monitoring the changes in absorbance at 560 nm at different temperatures. As shown in Figure 5A,B, k_1 increases rapidly with temperature for both esters, such that at temperatures ≥ 10 °C, the intermediate is formed within the dead time of the instrument. For the ethyl ester, k_2 also increases with temperature; however, the dependence of k_2 on temperature for methyl (*S*)-mandelate is much less pronounced than for (*S*)-mandelate or ethyl (*S*)-mandelate (Figure 5A,B; *23*).

The data in Figure 5A,B were replotted as ln(k/T) versus 1/T (Figure 5C,D) and fitted to a linear form of the Eyring equation:

$$k = (k_B/h)T \exp(\Delta S^{\neq}/R) \exp(-\Delta H^{\neq}/RT)$$

$\Delta S^{\neq}$ and $\Delta H^{\neq}$ were calculated from the intercept and slope of the plot, respectively. The activation energy, $\Delta G^{\neq}$, was calculated at 25 °C from the relationship

$$\Delta G^{\neq} = \Delta H^{\neq} - T\Delta S^{\neq}$$

At 25 °C, $\Delta H^{\neq}$ and $\Delta S^{\neq}$ for k_1 were 88 kJ/mol and 121 J (K mol)$^{-1}$, respectively, for methyl (*S*)-mandelate as substrate and 57 kJ/mol, and 9 J (K mol)$^{-1}$ respectively, for ethyl (*S*)-mandelate as substrate (Table 3). The corresponding $\Delta H^{\neq}$ and $\Delta S^{\neq}$ for k_2 were 62 kJ/mol and 15 J (K mol)$^{-1}$, respectively, for methyl (*S*)-mandelate and 79 kJ/mol and 78 J (K mol)$^{-1}$ for ethyl (*S*)-mandelate as substrate. From these values, we obtain $\Delta G^{\neq}$ values of 52 and 58 kJ/mol for k_1 and k_2, respectively, for methyl (*S*)-mandelate and 54 and 56 kJ/mol respectively, for ethyl (*S*)-mandelate at 25 °C. The corresponding values for (*S*)-mandelate are also included in Table 3.

DISCUSSION

Many members of the FMN-dependent (*S*)-α-hydroxyacid oxidizing enzyme family have been extensively studied; however, to date, their physiological substrates were reported to be the carboxylate anions of hydroxyacids or amino acids. The only exception was (L)-pantoyllactone dehydrogenase from *N. asteroides*, which can utilize a cyclic hydroxyester as a substrate but not a hydroxyacid (*12*). In another study, long-chain hydroxyacid oxidase was shown to use 2-hydroxyphenylacetohydroxamate, with 10−100-fold less efficiency relative to the best substrate, mandelate; the authors concluded that an ionized form of this hydroxamic acid was the actual substrate (*11*).

In the present study, we show that neutral α-hydroxyesters are substrates for MDH. The binding affinity of the esters to MDH is much lower compared to the acid anion. In contrast, the esters are oxidized with k_{cat}s similar to (*S*)-mandelate. Thus, while a negative charge on the substrate is clearly important for binding, it is not necessary for catalysis. As expected, the active site of MDH has evolved to bind the acid anion efficiently, since the physiological substrate for MDH is (*S*)-mandelate and not its esters (*24*). However, the fact that MDH, a member of the α-hydroxyacid oxidizing enzyme family, is able to utilize hydroxyesters suggests that neutral derivatives could be the true substrates

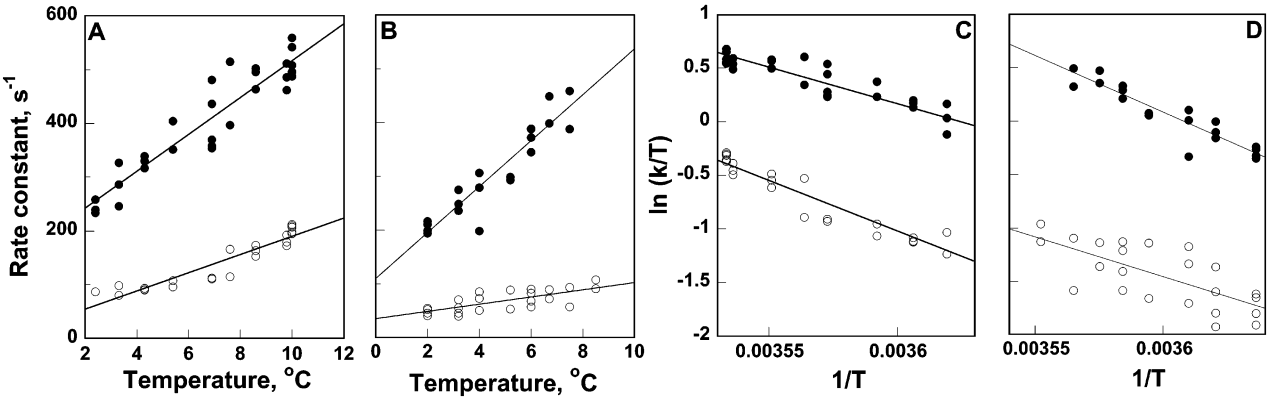


FIGURE 5: Temperature dependence of the rate of formation, k_1 (●) and rate of disappearance, k_2 (○) of the intermediate for ethyl (*S*)-mandelate (A and C) and methyl (*S*)-mandelate (B and D). Reactions were in 0.1 M potassium phosphate, pH 7.5, using 25 μM *wt*MDH and 20 and 50 mM ethyl and methyl (*S*)-mandelate, respectively, under anaerobic conditions.

Table 3: Activation Energy Parameters for the Intermediate[a]

	$\Delta H^{\neq}$ (kJ mol^{-1})	$\Delta S^{\neq}$ (J mol^{-1} K^{-1})	$\Delta G^{\neq}$ (kJ mol^{-1})
(*S*)-Mandelate[b]			
k_1	74.9 ± 6.7	76.5 ± 6.7	52
k_2	63.5 ± 7.2	25.2 ± 2.9	56
Methyl (*S*)-mandelate			
k_1	88.1 ± 7.9	120.8 ± 10.7	52
k_2	62.4 ± 13.5	15.3 ± 3.5	58
Ethyl (*S*)-mandelate			
k_1	57.0 ± 4.4	9.0 ± 0.7	54
k_2	78.9 ± 5.4	78.1 ± 5.4	56

[a] The values of $\Delta H^{\neq}$ and $\Delta S^{\neq}$ were calculated using data from Figure 5C,D. $\Delta G^{\neq}$ was calculated at 25 °C. [b] Values are from ref *23*.

for other, as yet uncharacterized, members of this large enzyme family. It is to be noted that the k_{cat}/K_m parameter for ethyl (*S*)-mandelate is 5-fold higher than for methyl (*S*)-mandelate. This, together with the ~4-fold stronger binding affinity of the ethyl ester relative to the methyl ester, suggests that in the absence of a negative charge on the substrate, the larger and more hydrophobic ethyl group participates in a favorable hydrophobic interaction with the active site. It is possible that the active sites of hitherto uncharacterized members of this protein family may have evolved to increase affinity for neutral substrates by exploiting such hydrophobic interactions. Using the K_d values of *wt*MDH for the hydroxy-esters and (*S*)-mandelate from Table 1, we can calculate the contribution of the negative charge of (*S*)-mandelate toward binding affinity to be at least 1.9−2.4 kcal/mol at 25 °C.

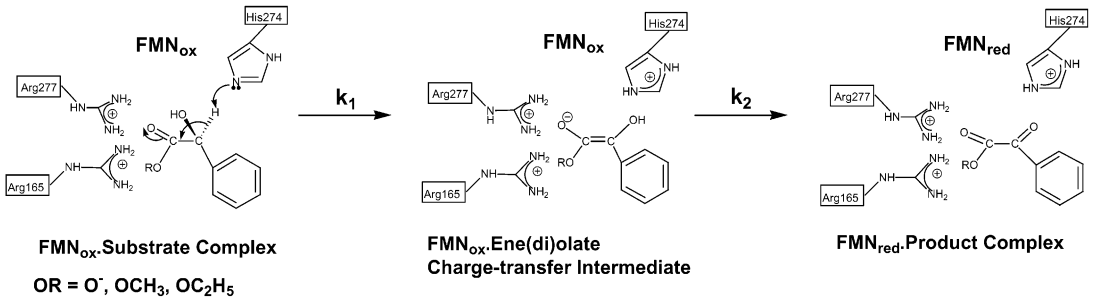
Other neutral compounds that have different functionalities on the C-1 carbon, (*S*)-1-phenyl-2,2,2,-trifluoroethanol, (*S*)-1-phenyl-2-propen-1-ol and (*S*)-1-phenyl-2-propyn-1-ol, were not substrates for MDH but inhibitors, as is (*S*)-1-phenyl-1,2-ethanediol (*16*). These results imply that the presence of a carbonyl group at the C-1 carbon atom is essential for catalysis; the inductive electron-withdrawing effect provided by the unsaturated ethylenic and acetylenic groups, or the trifluoromethyl group, which would all be expected to lower the pK_a of the α-proton, is not sufficient. Mechanistic evidence that we have presented to date overwhelmingly support the formation of a carbanion intermediate following abstraction of the α-proton in the MDH reaction mechanism. The requirement of a carbonyl group on the C-1 carbon implies that the transition state is stabilized by delocalizing the negative charge of the carbanion on the carbonyl oxygen to form the enolate or enediolate intermediate (Scheme 1). The predominantly enolate/enediolate form of the transition state is supported by other data as discussed later. Other flavoenzymes that involve proton abstraction from the α-carbon in the first step of the reaction mechanism include dihydroorotate dehydrogenase and acylCoA dehydrogenase (*25*, *26*). The former utilizes a negatively charged substrate, whereas the latter oxidizes a neutral thioester. However, in both cases, the substrate α-carbon is adjacent to a carbonyl group.

Surprisingly, (*S*)-mandelamide was neither a substrate nor a competitive inhibitor for MDH. Thus, it does not appear to bind at the active site of MDH at all. The most likely explanation is that the amide hydrogens and the guanidinium groups of the two arginines at the active site have a strong unfavorable interaction.

The Role of Arg277 in Ester Oxidation. Arg277 plays a critical role for the reaction with (*S*)-mandelate; it binds the substrate primarily through a hydrogen bond between the guanidinium side-chain and the carboxylate group of the substrate, and it stabilizes the transition state through charge interactions (*19*). Results obtained for the Arg277 mutants with the esters show that unlike (*S*)-mandelate, neither the positive charge nor the guanidinium side-chain of this residue is essential for binding the neutral esters. However, it is extremely important for the transition state stabilization of the reaction with the esters. Thus, the transition state for the neutral ester substrates must also be negatively charged, in agreement with a carbanion/enolate intermediate for the mechanism (Scheme 1). In the case of the alternative mechanism suggested for this enzyme family, that of a direct hydride transfer from the substrate α-carbon to the FMN, it could be argued that the positive charge of Arg277 is important because it neutralizes the negative charge on the substrate as the geometry of the α-carbon changes from tetrahedral to planar, parallel to hydride transfer for (*S*)-mandelate (*22*). However, Arg277 would not be expected to affect the transition state for the reaction with the neutral esters. As our observations show, this is clearly not the case.

Interestingly, the charge-conservative mutation, R277K, is not significantly better at oxidation of the esters compared to R277G, especially the methyl ester, in contrast to (*S*)-mandelate. It is possible that in the absence of a charge on the ester substrate, the lysine at residue 277 is displaced from its optimal position and cannot stabilize the transition state.

Scheme 1



In contrast, the diffused charge of the guanidinium side-chain of Arg277 and its capacity to form multiple hydrogen bonds probably holds it in optimal orientation in the active site, irrespective of whether the substrate itself is charged or not. Another interesting point of note is that the rather poor R277G mutant is a better enzyme with the esters than with (*S*)-mandelate. This is probably due to a more favorable interaction of the ethyl and methyl groups in the empty pocket created by the R277G mutation, compared to the charged carboxylate anion of (*S*)-mandelate, such that the negatively charged transition state can be stabilized by the second arginine in the active site, Arg165 (Scheme 1). We have shown earlier that the conserved Arg165 also plays a role in the catalysis of (*S*)-mandelate (*20*).

The low activity of R277G toward esters can be rescued by exogenous 1-methylguanidine, although to a lesser extent and with a weaker binding affinity relative to (*S*)-mandelate; additionally, other guanidines and imidazoles were ineffective (*19*). These results also suggest that exogenous guanidines and imidazoles are able to bind at the active site of R277G and form a ternary complex with the substrate with higher affinity and with more optimal orientation when the substrate is negatively charged. At the same time, these data clearly show that a guanidinium side-chain is also important for the oxidation of the neutral esters.

An Intermediate in the Reaction Pathway for the Esters at Low Temperature. The MDH reaction mechanism goes through the formation of a distinct and extremely short-lived intermediate at low temperature (*23*). The intermediate, only observed with fast substrates that have high k_{cat}s, is a charge-transfer complex of oxidized FMN with an electron-rich species. Evidence pointed to the carbanion/enediolate intermediate as the charge-transfer donor. The oxidation of the ester substrates proceeds through a similar charge-transfer complex at low temperatures. In fact, the observation that this intermediate is formed with the neutral esters, which have comparable k_{cat}s but rather weak affinities relative to the hydroxyacid anion, further reinforces our earlier claim that the intermediate arises from the negatively charged carbanion/ene(di)olate ion, and not the substrate or product of the reaction. Similar to our earlier observation for (*S*)-mandelate, the FMN reductive reaction for the esters has two steps of similar activation energies, defined by the rate constants, k_1 and k_2, the rates of formation and breakdown of the charge-transfer intermediate, with k_1 being 2.5−5-fold faster than k_2 (Scheme 1). Just as we observed with (*S*)-mandelate, both k_1 and k_2 have a dependence on substrate concentration. This is likely due to the values of k_1 and k_2 being very similar to each other so that they are difficult to separate. If the intermediate was a long-lived species, we would expect it to have no dependence on substrate concentration at all.

A comparison of the kinetic parameters obtained at 4 and 20 °C for (*S*)-mandelate and the two esters reveals that for (*S*)-mandelate and ethyl (*S*)-mandelate, the rate-limiting step occurs in the first, FMN reduction, half-reaction at both temperatures (Tables 1 and 2). In contrast, for methyl (*S*)-mandelate, there is a change in the rate-limiting step with temperature. At 20 °C, the rate-limiting step is clearly FMN reduction and substrate oxidation (Table 1). However, at 4 °C, k_1 and k_2 are much faster than the k_{cat}, implying that a step beyond FMN reduction is rate-limiting (Table 2). A logical explanation is that for methyl (*S*)-mandelate at 4 °C, product release is partly rate-limiting. Therefore, while the ethyl ester is a better overall substrate at both 4 and 20 °C, both k_1 and k_2 are higher for the methyl ester compared to the ethyl ester at 4 °C.

Thermodynamic Parameters for the Intermediate. Free energies of activation for the steps described by the rate constants k_1 and k_2 are quite similar for (*S*)-mandelate and the esters, ∼52−58 kJ/mol, although the $\Delta G^{\neq}$ for the breakdown of the intermediate is slightly higher for all three substrates, as expected from a slightly lower value for k_2. Interestingly, the change in entropy is much lower for the disappearance of the intermediate relative to its formation for (*S*)-mandelate and methyl (*S*)-mandelate, suggesting that the intermediate resembles the product more than the substrate. In the conversion from substrate to product, the α-carbon must change geometry from tetrahedral to planar—this entails a change in the position of the large phenyl ring. The lower change in entropy for the second rate-limiting step indicates that the intermediate has a structure in which the α-carbon is already planar; it is more likely to be an ene(di)olate species than a carbanion (Scheme 1).

However, for ethyl (*S*)-mandelate, changes in both the enthalpy and entropy of activation are higher for the step describing breakdown of the intermediate (Table 3); also, the change in entropy for the formation of the intermediate is quite small. A likely explanation is that the presence of the larger, hydrophobic ethyl group prevents the intermediate from existing fully as the enolate ion; as a result, the conversion from a carbanion to product involves a greater rearrangement at the active site.

The Esters Are the Substrates and not the Hydrolysis Product, the Carboxylate Acid Anion. A concern in the studies reported in this work is that the activity observed with methyl and ethyl (*S*)-mandelates is due to a small amount of the hydrolyzed acid, (*S*)-mandelate. As stated earlier, TLC and NMR data were used to confirm that there was no hydrolyzed acid present. The evidence from the

Arg277 studies supports this conclusion. The behavior of both R277K and R277G are sufficiently different for (*S*)-mandelate and the esters for us to be certain that the activity measured was not due to (*S*)-mandelate itself. The effect of rescue agents on the activities of R277G toward (*S*)-mandelate and the esters are also quite different. Additionally, whereas (*S*)-mandelate has the same rate-limiting steps at 4 and 20 °C, the situation is clearly different for methyl (*S*)-mandelate. These observations rule out the possibility that the observed activity of the esters is due to the presence of the hydroxyacid.

Conclusions. Although MDH is more efficient toward its physiological substrate, (*S*)-mandelate, it is able to oxidize α-hydroxyesters with k_{cat}s approaching that of the α-hydroxyacid anion. The presence of a carbonyl group on the C-1 carbon appears to be an essential requirement in a substrate, suggesting that the extra negative charge developed during the reaction is stabilized by delocalization onto the oxyanion. Arg277, formerly shown to be critical for both substrate binding and transition state stabilization for the (*S*)-mandelate activity, is also critical for transition state stabilization for the hydroxyesters, but not for their binding affinity. Similar to our earlier observation with (*S*)-mandelate, the substrate oxidation/FMN reduction half-reaction in *wt*MDH has two rate-limiting steps of similar activation energies and proceeds through the formation of a distinct transient intermediate for the neutral esters as well. The intermediate is a charge-transfer complex of an electron-rich donor and oxidized FMN. We have previously shown that the electron-rich donor is a carbanion/enolate species for the reaction with (*S*)-mandelate. The observation of this intermediate for the oxidation of an uncharged substrate to an uncharged product, as well as the critical role of Arg277 in the reaction with the esters, provides further evidence that the MDH reaction mechanism is not a concerted transfer of a hydride ion from the substrate to the FMN but involves the transient formation of a carbanion/ene(di)olate intermediate.

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