

Evolution of Enzymatic Activity in the Enolase Superfamily: Functional Studies of the Promiscuous *o*-Succinylbenzoate Synthase from *Amycolatopsis*[†]

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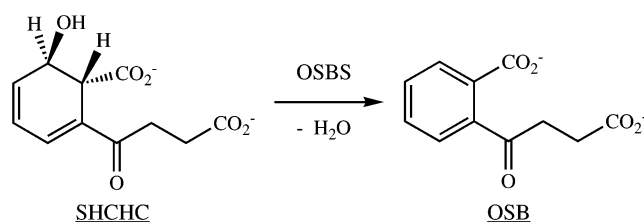
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ABSTRACT: *o*-Succinylbenzoate synthase (OSBS) from *Amycolatopsis*, a member of the enolase superfamily, catalyzes the Mn²⁺-dependent exergonic dehydration of 2-succinyl-6*R*-hydroxy-2,4-cyclohexadiene-1*R*-carboxylate (SHCHC) to 4-(2'-carboxyphenyl)-4-oxobutyrates (*o*-succinylbenzoate or OSB) in the menaquinone biosynthetic pathway. This enzyme first was identified as an *N*-acylamino acid racemase (NAAAR), with the optimal substrates being the enantiomers of *N*-acetyl methionine. This laboratory subsequently discovered that this protein is a much better catalyst of the OSBS reaction, with the value of $k_{\text{cat}}/K_{\text{M}}$, for dehydration, $2.5 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$, greatly exceeding that for 1,1-proton transfer using the enantiomers of *N*-acetylmethionine as substrate, $3.1 \times 10^2 \text{ M}^{-1} \text{ s}^{-1}$ [Palmer, D. R., Garrett, J. B., Sharma, V., Meganathan, R., Babbitt, P. C., and Gerlt, J. A. (1999) *Biochemistry* 38, 4252–8]. The efficiency of the promiscuous NAAAR reaction is enhanced with alternate substrates whose structures mimic that of the SHCHC substrate for the OSBS reaction, for example, the value of $k_{\text{cat}}/K_{\text{M}}$ for the enantiomers of *N*-succinyl phenylglycine, $2.0 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$, is comparable to that for the OSBS reaction. The mechanisms of the NAAAR and OSBS reactions have been explored using mutants of Lys 163 and Lys 263 (K163A/R/S and K263A/R/S), the putative acid/base catalysts identified by sequence alignments with other OSBSs, including the structurally characterized OSBS from *Escherichia coli*. Although none of the mutants display detectable OSBS or NAAAR activities, K163R and K163S catalyze stereospecific exchange of the α -hydrogen of *N*-succinyl-(*S*)-phenylglycine with solvent hydrogen, and K263R and K263 catalyze the stereospecific exchange the α -hydrogen of *N*-succinyl-(*R*)-phenylglycine, consistent with formation of a Mn²⁺-stabilized enolate anion intermediate. The rates of the exchange reactions catalyzed by the wild-type enzyme exceed those for racemization. That this enzyme can catalyze two different reactions, each involving a stabilized enediolate anion intermediate, supports the hypothesis that evolution of function in the enolase superfamily proceeds by pathways involving functional promiscuity.

The reactions catalyzed by members of the mechanistically diverse enolase superfamily are initiated by abstraction of the α -proton of a carboxylate anion substrate to yield an enediolate intermediate stabilized by coordination to a divalent cation (usually Mg²⁺); this intermediate is directed to product by an active site acid (1, 2). The muconate lactonizing enzyme (MLE)¹ subgroup of the superfamily is characterized by a highly conserved Lys-X-Lys motif at the end of the second β -strand and a Lys at the end of the sixth β -strand in the (β/α)₈-barrel domain of the bidomain

Scheme 1



structure. These Lys residues are located on opposite faces of the active site, thereby allowing proton-transfer reactions to/from either face of the substrate/enediolate anion intermediate. To date, three different reactions are known to be catalyzed by members of the MLE subgroup: an intramolecular cycloisomerization reaction catalyzed by MLE, a 1,1-proton-transfer reaction catalyzed by L-Ala-D/L-Glu epimerase (3), and a dehydration reaction catalyzed by *o*-succinylbenzoate synthase (OSBS). OSBS catalyzes the very exergonic syn dehydration of 2-succinyl-6*R*-hydroxy-2,4-cyclohexadiene-1*R*-carboxylate (SHCHC) to yield 4-(2'-carboxyphenyl)-4-oxobutyrates (*o*-succinylbenzoate or OSB) (Scheme 1).

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¹ Abbreviations: MLE, muconate lactonizing enzyme; MR, mandelate racemase; NAAAR, *N*-acylamino acid racemase; NARM, *N*-acetyl-(*R*)-methionine; NASM, *N*-acetyl-(*S*)-methionine; NSRM, *N*-succinyl-(*R*)-methionine; NSSM, *N*-succinyl-(*S*)-methionine; NSRPG, *N*-succinyl-(*R*)-phenylglycine; NSSPG, *N*-succinyl-(*S*)-phenylglycine; OSB, 4-(2'-carboxyphenyl)-4-oxobutyrates (*o*-succinylbenzoate); OSBS, *o*-succinylbenzoate synthase; SHCHC, 2-succinyl-6*R*-hydroxy-2,4-cyclohexadiene-1*R*-carboxylate.

The OSBS-catalyzed reaction occurs in the biosynthesis of menaquinone, which is required for anaerobic growth by many eubacteria and some archaea (4). As a result, genes encoding OSBSs have been identified in the genomes of more than 70 microorganisms (5). Curiously, the pairwise sequence identities of the encoded OSBSs are often <15%, perhaps as a result of the highly exergonic nature of the reaction (6). The rate acceleration for the OSBS-catalyzed reaction is only 1.8×10^{11} (7) versus values of 1.1×10^{17} for the fumarase-catalyzed reactions and 1.7×10^{15} for mandelate racemase-catalyzed reactions (8, 9), two other reactions initiated by abstraction of an α -proton of a carboxylate anion substrate. The unusual divergence of sequence among isofunctional homologues may reflect the requirement for only a modest rate acceleration, which may relax geometric requirements for the placement of the active site acid/base catalyst(s) vis a vis the substrate and enediolate intermediate.

That the OSBS reaction is efficiently catalyzed by active sites in highly divergent sequence contexts suggests that some OSBSs may be functionally promiscuous, that is, they catalyze another reaction as well as the OSBS reaction (10). Perhaps the extreme sequence divergence results from convergent evolution from multiple progenitors within the enolase superfamily, so the potential functional promiscuity may provide a clue as to the identities of the various progenitors. In this context, the discovery that the OSBS from *Amycolatopsis* also catalyzes a 1,1-proton transfer reaction is not surprising. This enzyme first was identified by its ability to catalyze the racemization of a variety of *N*-acylamino acids, so it was designated *N*-acylamino acid racemase (NAAAR) (11); the enantiomers of *N*-acetyl methionine were reported as the best substrates (12). Subsequently, this laboratory discovered that NAAAR is a much better catalyst of the OSBS reaction: the value of k_{cat}/K_M for the OSBS-catalyzed dehydration of SHCHC, $2.5 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$, greatly exceeds that for racemization of *N*-acetyl methionine, $3.1 \times 10^2 \text{ M}^{-1} \text{ s}^{-1}$ (6). Accordingly, this OSBS is an example of a naturally promiscuous enzyme.

Several structures are available for the OSBS from *Escherichia coli*, including those determined for the wild-type enzyme both in the presence and in the absence of the OSB product (13). These structures confirm the identities of the potential acid/base Lys residues, Lys 133 and Lys 235, that had been identified in sequence alignments; these are located on opposite faces of the active site at the ends of the second and sixth β -strands in the $(\beta/\alpha)_8$ -barrel domain. Subsequent structural studies of the catalytically inactive K133R mutant (14) revealed the absolute configuration of the SHCHC substrate and lead to the conclusion that the dehydration reaction to form the OSB product necessarily proceeds via a syn elimination reaction in which Lys 133 at the end of the second β -strand is the only acid/base catalyst, first abstracting the α -proton and then, as the conjugate acid, facilitating departure of the hydroxide leaving group. That Lys 235 at the end of the sixth β -strand is also essential suggests that it may stabilize the enolate intermediate via π -cation electrostatic interactions.

Although the OSBS from *E. coli* and the promiscuous OSBS from *Amycolatopsis* share only 19% sequence identity, alignment of their sequences, along with those of the other OSBSs that can be identified in the databases (5), allows

the prediction that Lys 163 in the promiscuous OSBS is the acid/base catalyst that mediates the syn dehydration reaction, and Lys 263 stabilizes the enolate intermediate via a π -cation interaction.

In this paper, we describe characterization of the mechanisms of the NAAAR and OSBS reactions catalyzed by the protein from *Amycolatopsis* and report that the promiscuous NAAAR reaction can be enhanced by changing the structure of the *N*-acylamino acid substrate so that it more closely resembles that of the SHCHC substrate for the OSBS reaction. Furthermore, although the syn dehydration catalyzed by this OSBS evidently utilizes Lys 163 at the end of the second β -strand as the sole acid/base catalyst, the mechanism of the NAAAR (1,1-proton transfer) reaction also requires Lys 263 at the end of the sixth β -strand, with Lys 163 functioning as the (*R*)-specific base and Lys 263 functioning as the (*S*)-specific base. These studies provide additional support for our earlier suggestion that the identities of the acid/base reaction catalyzed by the members of the enolase superfamily are determined by the residues that direct substrate specificity (15).

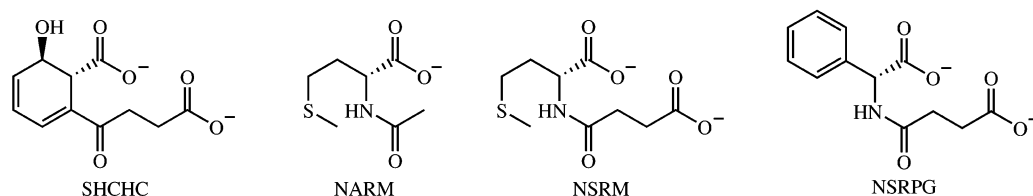
MATERIALS AND METHODS

E. coli strains XL1-Blue and BL-21 (DE3) were obtained from Stratagene. *Taq* DNA polymerase and restriction enzymes were purchased from Invitrogen. Oligonucleotide primers were synthesized by Operon (Alameda, CA) or by Biosynthesis (Lewisville, TX). Deuterated reagents were obtained from Cambridge Isotopes Ltd. All other reagents were of the highest quality grade commercially available.

NMR spectra were recorded using Varian Unity Inova 500 and 600 MHz NMR spectrometers. Spectrophotometric assays for OSBS activity were performed with a Perkin-Elmer Lambda-14 spectrophotometer. Assays for racemization activity were performed with a Jasco DIP-370 digital polarimeter. PCR reactions were performed using a MJ Research PTC-200 Peltier Thermal Cycler.

Synthesis of *N*-Succinylamino Acids. *N*-Succinylamino acids were synthesized by combining the enantiopure amino acid (66 mmol) with succinic anhydride (73 mmol, 1.1 equiv) in 50 mL of glacial acetic acid and heating the solution to 110 °C with stirring for 10 min. The solution was cooled, and the solvent was evaporated to give a viscous syrup. The syrup was dissolved in 50 mL of H₂O, and the pH was adjusted to 7. The solution was filtered through an Amicon 10 000 MWCO PES membrane and purified by anion-exchange chromatography on a column of Dowex AG-1 \times 8-400, eluted with a gradient of 0–0.5 M LiCl; the bislithium salt of the *N*-succinylated amino acid was detected by optical rotation and precipitated by the addition of 10:1 acetone/methanol. *N*-succinyl-(*R*)-methionine: $[\alpha]_{\text{Na589}}^{20} = -1.69^\circ$; ¹H NMR (500 MHz, ²H₂O) δ 1.84 (m, 2H), δ 1.94 (s, 3H), δ 2.34 (m, 6H), δ 4.12 (dd, *J* = 4.4, 4.5 Hz). *N*-succinyl-(*S*)-methionine: $[\alpha]_{\text{Na589}}^{20} = +1.21^\circ$; ¹H NMR (500 MHz, ²H₂O) δ 1.84 (m, 2H), δ 1.94 (s, 3H), δ 2.34 (m, 6H), δ 4.12 (dd, *J* = 4.4, 4.5 Hz). *N*-succinyl-(*R*)-phenylglycine: $[\alpha]_{\text{Na589}}^{20} = -77.6^\circ$; ¹H NMR (500 MHz, ²H₂O) δ 2.28 (m, 2H), δ 2.38 (m, 2H), δ 5.02 (s, 1H), δ 7.23 (m, 5H). *N*-succinyl-(*S*)-phenylglycine: $[\alpha]_{\text{Na589}}^{20} = +49.0^\circ$; ¹H NMR (500 MHz, ²H₂O) δ 2.28 (m, 2H), δ 2.38 (m, 2H), δ 5.02 (s, 1H), δ 7.23 (m, 5H).

Scheme 2



Site-Directed Mutagenesis. The template for mutagenesis was the 1.11 kb gene encoding NAAAR from *Amycolatopsis* sp. T-1–60 chromosomal DNA subcloned into the pET17b vector. The QuikChange site-directed mutagenesis method was utilized to introduce the desired mutations. The mutations were confirmed by sequencing performed at the W. M. Keck Biotechnology Center (Urbana, IL).

Protein Expression and Purification. The extinction coefficients and predicted masses of proteins were calculated based on the amino acid sequence (<http://expasy.cbr.nrc.ca/tools/protparam.html>). Mutant OSBSs were expressed in *E. coli* strain BL-21 (DE3) at 30 °C without induction by IPTG. The cells were lysed by sonication, and the mutant proteins were purified to electrophoretic homogeneity by consecutive chromatographies on DEAE-Sephacel and Resource-Q columns (6). Fractions containing protein were identified by OD₂₈₀ and SDS–PAGE. Electrospray ionization mass spectrometry (ESI-MS) was used to confirm the expected masses (within a 0.1% error).

Circular Dichroism. Wild-type and the mutant versions of OSBS *Amycolatopsis* were subjected to circular dichroism (CD) studies at the Laboratory of Fluorescence Dynamics at the University of Illinois. The various proteins were dialyzed against 50 mM Tris-HCl, pH 8.0, containing 0.1 mM MnCl₂; the samples were adjusted to protein concentrations of ~0.3 mg/mL. The CD spectra were obtained with a Jasco 720 spectropolarimeter using 0.1 cm quartz (QS) cuvettes. The samples were scanned five times at room temperature over the 190–350 nm wavelength range (0.5 nm increments), and the scans were averaged. The CD spectra were recorded in the absence of any ligands.

Spectrophotometric Assay for OSBS Activity. OSBS activity was measured at 25 °C using varying concentrations of SHCHC, 50 mM Tris-HCl, pH 8.0, and 0.1 mM MnCl₂ by quantitating the decrease in absorbance at 310 nm ($\Delta\epsilon = -2400 \text{ M}^{-1} \text{ cm}^{-1}$). The kinetic isotope effect for the OSBS reaction was measured using the same assay, substituting α -²H-SHCHC and comparing the resulting rates.

Polarimetric Assay for NAAAR Activity. NAAAR activity was measured at 25 °C using varying concentrations of substrate (0.25–100 mM), 50 mM Tris-HCl, pH 8.0, 0.1 mM MnCl₂, and 0.1 mg of wild-type or 1.0 mg of mutant enzyme by quantitating the decrease in optical rotation at 405 nm, with a 10 s integration time and a 10 cm cell length.

Kinetic Overshoot Experiments. Racemization in either ¹H₂O or ²H₂O was monitored by the polarimetric assay using 20 mM (*R*)- or (*S*)-*N*-succinylphenylglycine, 50 mM Tris-[*d*₁₁]-Tris-²HCl, pD 8.0, 0.1 mM MnCl₂, and 1.0 mg of wild-type enzyme.

Kinetic Studies Using ¹H NMR Spectroscopy. Wild-type and mutant proteins were exchanged into ²H₂O using an Amicon stirred cell. A 10 mL aliquot of protein was concentrated to 2 mL; 8 mL of ²H₂O was added, and the

protein solution was again concentrated to 2 mL. This process was repeated three times. Samples for ¹H NMR analysis (800 μ L) contained 20 mM (*R*)- or (*S*)-*N*-succinylphenylglycine, 0.5 mg of protein, 50 mM [²H₁₁]-Tris-²HCl, pD 8.0, and 0.1 mM MnCl₂ in ²H₂O. Samples were monitored for incorporation of ²H into the substrate at the α -hydrogen position after overnight incubation at 25 °C. In subsequent experiments, the rates of exchange, k_{exch} , were quantitated using saturating concentrations of the enantiomers of *N*-succinylphenylglycine (20 mM) by monitoring the change in intensity of the α -proton as a function of time.

RESULTS AND DISCUSSION

These experiments described in this manuscript were performed to elucidate the mechanisms of the OSBS and NAAAR reactions catalyzed by the promiscuous OSBS from *Amycolatopsis*. In particular, we sought to elucidate the roles of the putative acid/base catalysts, Lys 163 and Lys 263, in the observed functional promiscuity. These catalysts are predicted to be located on opposite faces of the active site at the ends of the second and sixth β -strands in the (β/α)₈-barrel domain. Additionally, enhancement of the NAAAR reaction by altering the structure of the substrate to mimic that of the SHCHC substrate for the OSBS reaction might allow insights into how accidental functional promiscuity can be exploited by nature to evolve new natural functions.

Enhancing the Promiscuous NAAAR Reaction. When an enolase superfamily member from *Amycolatopsis* was identified as NAAAR, the best substrates for the racemization reaction were reported to be the enantiomers of *N*-acetyl- and *N*-propylmethionine (11, 12). With the discovery that the physiological function of this protein is catalysis of the OSBS reaction (6), we thought that the unnatural NAAAR reaction might be enhanced by altering the structure of the *N*-acylamino acid substrates to more closely resemble SHCHC, the substrate for the OSBS reaction (Scheme 2).

Converting the acetyl group of *N*-acetyl-(*R*)-methionine (NARM), and its enantiomer *N*-acetyl-(*S*)-methionine (NASM), to a succinyl group in *N*-succinyl-(*R*)-methionine (NSRM), and its enantiomer *N*-succinyl-(*S*)-methionine (NSSM), to mimic the succinyl side chain of SHCHC increases the value of $k_{\text{cat}}/K_{\text{M}}$ of a factor of ~300 (Table 1). Replacing methionine with the more hydrophobic phenylglycine in *N*-succinyl-(*R*)-phenylglycine (NSRPG), and its enantiomer *N*-succinyl-(*S*)-phenylglycine (NSSPG), increases the value of $k_{\text{cat}}/K_{\text{M}}$ by another factor of 10, resulting in a 3000-fold increase in efficiency for the NAAAR reaction so that it approaches that of the OSBS reaction.

That the efficiency of the nonphysiological 1,1-proton transfer reaction can approach that of the OSBS reaction is striking. The OSBS reaction is a syn dehydration involving the direct participation of only a single acid/base catalyst

Table 1: Values of the Kinetic Constants for the NAAAR and OSBS Reactions^a

substrate	k_{cat} (s ⁻¹)	K_{m} (M)	$k_{\text{cat}}/K_{\text{m}}$ (M ⁻¹ s ⁻¹)
NARM	4.8	9.8×10^{-3}	4.9×10^2
NASM	6.4	1.1×10^{-2}	5.9×10^2
NSRM	110	6.5×10^{-3}	1.7×10^4
NSSM	110	5.9×10^{-3}	1.8×10^4
NSRPG	190	9.4×10^{-4}	2.0×10^5
NSSPG	190	9.5×10^{-4}	1.9×10^5
SHCHC	120	4.8×10^{-4}	2.5×10^5

^a Assay conditions are described in the text.

(initially as a base and then as an acid; (14)) whereas the NAAAR reaction, a 1,1-transfer reaction, is necessarily catalyzed by acid/base catalysts on opposite faces of the substrate (vide supra).

The quantitative ability of this protein to catalyze promiscuous racemization reactions is perhaps even more impressive when the rate accelerations for the various reactions are compared. The rate constant, k_{non} , for the nonenzymatic OSBS reaction, 1.6×10^{-10} s⁻¹ (7), exceeds those for the nonenzymatic dehydration reaction catalyzed by fumarase, 1.1×10^{-14} s⁻¹ (9), and the nonenzymatic racemization reaction catalyzed by mandelate racemase, 3×10^{-13} s⁻¹ (8). In the following, we assume that the values of k_{non} for the fumarase and mandelate racemase reactions are reliable estimates for the values of k_{non} for racemization of *N*-acylalkylamino acids and *N*-acylaryl amino acids, respectively. Using these values of k_{non} , the rate acceleration ($k_{\text{cat}}/k_{\text{non}}$) for the OSBS reaction is 7.5×10^{11} , and those for the racemization reactions are 4.4×10^{14} (NARM), 1.1×10^{16} (NSRM), and 6.3×10^{14} (NSRPG). Thus, because the values of k_{non} for the promiscuous racemization reactions are significantly less than that for the OSBS reaction, the rate accelerations for the promiscuous reactions significantly exceed that for the natural reaction.

We recently reported the introduction of functional promiscuity in single mutants of both an L-Ala-D/L-Glu epimerase and a muconate lactonizing enzyme II, with the promiscuity being the ability to catalyze the OSBS reaction (15). Taken together, our previous observations and those reported in this manuscript support the conclusion that the active sites in the enolase superfamily are predisposed for acid/base catalysis, with the identity of the reaction determined by the structure of the ligand that is able to bind in the active site.

Kinetic Characterization of Mutant Enzymes. On the basis of sequence alignments with other OSBSs, including the structurally characterized protein from *E. coli*, Lys 163 and Lys 263 located on opposite sides of the active site at the ends of the second and sixth β -strands in the (β/α)₈-barrel domain were considered to be appropriately located to function as acid/base catalysts and were targeted for mutagenesis. Six mutants were constructed and purified to homogeneity: K163A, K163R, K163S, K263R, K263S, and K163R/K263R (the K263A mutant was constructed but was insoluble). No OSBS or NAAAR activity could be detected for any of the mutants using spectrophotometric and polarimeter assays, respectively (Table 2). In our previously reported studies of the monofunctional OSBS from *E. coli*, the analogous substitutions for the homologous Lys 133 and

Table 2: Values of the Rates of the OSBS, NAAAR, and Substrate Exchange Reactions for Wild-Type and Mutant Enzymes^a

	OSBS activity	NAAAR activity	exchange activity	
	k_{cat} (s ⁻¹)	k_{cat} (s ⁻¹)	NSSPG k_{cat} (s ⁻¹)	NSRPG k_{cat} (s ⁻¹)
WT	120	178	490	380
K163A	$<3 \times 10^{-5}$	$<2 \times 10^{-4}$		
K163R	$<3 \times 10^{-5}$	$<2 \times 10^{-4}$	430	
K163S	$<3 \times 10^{-5}$	$<2 \times 10^{-4}$	0.29	
K263R	$<3 \times 10^{-5}$	$<2 \times 10^{-4}$		190
K263S	$<3 \times 10^{-5}$	$<2 \times 10^{-4}$		100
K163R/K263R	$<3 \times 10^{-5}$	$<2 \times 10^{-4}$		

^a Assay conditions are described in the text.

Lys 235 residues also yielded catalytically inactive proteins (14).

Exchange of Substrate Hydrogen with Solvent Hydrogen in the NAAAR Reaction. Mandelate racemase (MR), a well-characterized member of the enolase superfamily, was the model for exploring the effects of the mutants on the NAAAR reaction. Although the H297N mutant of the (*R*)-specific acid/base catalyst in MR has no detectable racemase activity, exchange of the α -protium of (*S*)- but not (*R*)-mandelate with solvent deuterium is efficiently catalyzed (16). In an analogous set of experiments, we investigated the ability of mutants of the OSBS from *Amycolatopsis* to catalyze α -proton exchange in the promiscuous NAAAR reaction.

Each mutant was incubated overnight in ²H₂O with either NSRPG or NSSPG; exchange was monitored by ¹H NMR, and the reactive substrate-mutant pairs were identified. In subsequent experiments, the values of the rate constants for exchange, k_{exch} , were quantitated using a saturating concentration of substrate (20 mM), thereby allowing comparison with k_{cat} for the racemization reaction (Table 2). When either Lys 163 or Lys 263 was replaced with Arg or Ser, solvent-derived deuterium was incorporated into one of the enantiomeric substrates: mutants of Lys 163 catalyzed exchange of NSSPG, and mutants of Lys 263 catalyzed exchange of NSRPG. Thus, we conclude that Lys 163 is the (*S*)-specific and Lys 263 is the (*R*)-specific acid/base catalyst.

Although we did not prepare α -deuterated samples of either NSRPG or NSSPG, we observed a significant solvent deuterium isotope effect on k_{cat} for the racemization of each protiated enantiomer (NSRPG, $k_{\text{H}}/k_{\text{D}} = 2.6$; NSSPG, $k_{\text{H}}/k_{\text{D}} = 2.2$). If the reaction coordinate is symmetrical, comparable substrate deuterium isotope effects are expected.

The values of k_{exch} for the wild-type enzyme using both NSRPG and NSSPG exceed those for k_{cat} for racemization. We performed kinetic overshoot experiments in which the progress of racemization of protiated samples of both NSRPG and NSSPG were compared in both H₂O and ²H₂O. Assuming no exchange of the substrate enantiomer with solvent deuterium during the reaction, a transient excess of the product enantiomer is expected when the reaction is performed in ²H₂O (17, 18). However, in our experiments, no overshoot was observed with either enantiomeric substrate in either H₂O or ²H₂O (Figure 1), although the initial rates of racemization were diminished in ²H₂O, as expected. We attribute this observation to faster exchange of the substrate enantiomer with solvent than racemization. Thus, the absence

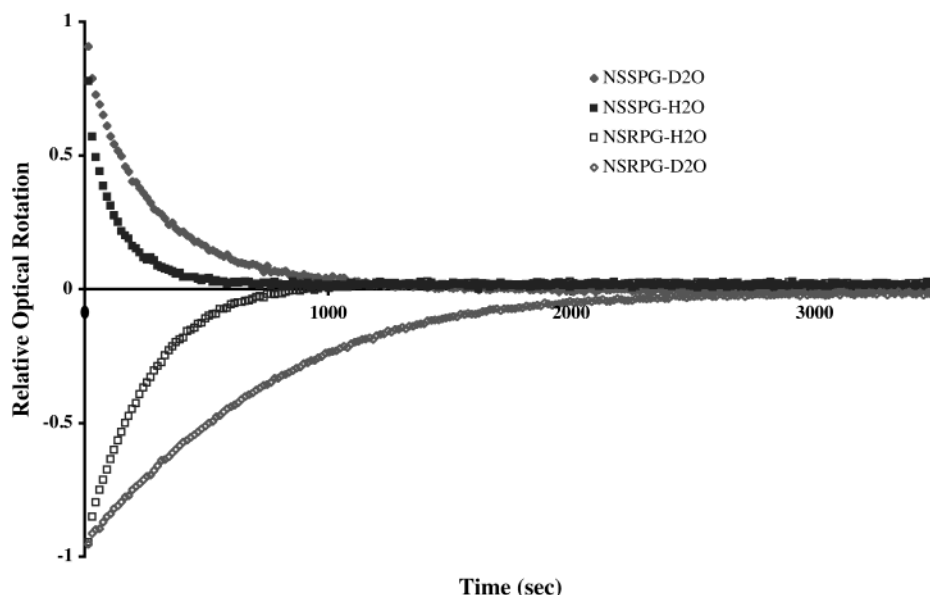


FIGURE 1: Kinetic overshoot experiments for NSRPG and NSSPG in both $^1\text{H}_2\text{O}$ and $^2\text{H}_2\text{O}$. The time-dependence of the optical rotation, expressed relative to the initial value, is displayed. The experimental conditions are described in the text.

of overshoot is in agreement with our demonstration that k_{exch} exceeds k_{cat} for both enantiomers (Table 2).

Mechanisms of the OSBS- and NAAAR-Catalyzed Reactions. Our data allow consistent mechanisms for both reactions. The mechanism of the OSBS reaction is based on the trans configuration of the SHCHC substrate that requires that the dehydration is necessarily a syn β -elimination (14). Accordingly, in analogy with the mechanism of the OSBS from *E. coli* (14), we propose that this reaction proceeds via an E1cb mechanism in which α -proton abstraction of SHCHC by Lys 163 forms a Mn^{2+} -stabilized enediolate anion intermediate that collapses to OSB with the assistance of the conjugate acid of Lys 163 (Figure 2A). Lys 263 likely forms a π -cation interaction with the SHCHC substrate, with this interaction enhanced as negative charge develops as the intermediate is formed by abstraction of the α -proton. The phenotypes of our mutants are consistent with Lys 163 and Lys 263 both being required for catalysis, as outlined.

The promiscuous NAAAR reaction necessarily involves a two-base 1,1-proton transfer mechanism. Racemization occurs via base-catalyzed abstraction of the α -proton to form a Mn^{2+} -stabilized enediolate anion intermediate (Figure 2B). The existence of this intermediate is supported by (1) our observation that the values of k_{exch} exceed those for k_{cat} for racemization and (2) some mutants catalyze exchange in the absence of racemization. The intermediate is diverted to product with the assistance of an active site acid. Our studies allow Lys 163 to be assigned as the (*R*)-specific base and Lys 263 as the (*S*)-specific base (Figure 2B).

The mechanisms of the two reactions are similar but distinct. Both mechanisms require the participation of Lys 163 and Lys 263. The OSBS reaction utilizes Lys 163 as the single acid/base catalyst, whereas the NAAAR reaction uses both Lys residues as acid/base catalysts. The conscription of Lys 263 from stabilization of the enediolate intermediate in the OSBS reaction to direct participation in the 1,1-proton transfer reactions in the NAAAR reaction is a striking example of functional divergence induced by the altered structures and reactivities of the substrates for the natural and unnatural reactions, respectively.

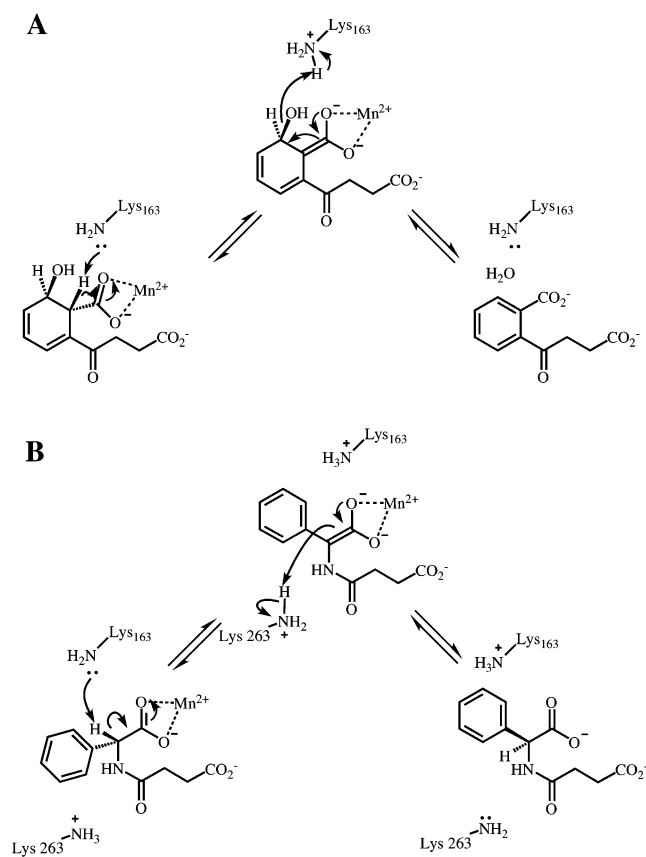


FIGURE 2: Panel A, the mechanism of the OSBS reaction. Panel B, the mechanism of the NAAAR reaction. Although the normal divalent metal ion utilized by members of the enolase superfamily is Mg^{2+} (1), the assays performed in this study utilized Mn^{2+} because it provides a ~ 3 -fold enhancement in the rate of the NAAAR reaction relative to Mg^{2+} .

Conclusions. The OSBS from *Amycolatopsis*, originally identified because of its ability to catalyze the NAAAR reaction (12), has been characterized for both reactions. Altering the structure of the substrate for the NAAAR reaction to mimic that of the substrate for the OSBS reaction increases the efficiency of the promiscuous reaction by a

factor of 10^3 . The phenotypes of mutants of both Lys 163 and Lys 263 are consistent with the results of previous studies of the reactions catalyzed by the OSBS from *E. coli* (a syn dehydration that uses a single acid/base catalyst; (14)) and MR (a 1,1-proton transfer that uses two acid/base catalysts; (19)). That the enzyme from *Amycolatopsis* can catalyze two different reactions, each involving a stabilized enediolate anion intermediate, supports the hypothesis that evolution of function in the enolase superfamily proceeds by pathways involving functional promiscuity. Our conclusion that this enzyme catalyzes both a one-base syn dehydration reaction (OSBS) and a two-base 1,1-proton transfer reaction (NAAAR) supports our earlier suggestion that active sites in the enolase superfamily are hard-wired for acid/base chemistry, with the identity of the reaction determined by the residues that determine substrate specificity, and therefore, allow the substrates for alternate reactions to fortuitously bind in the active site (15).

The structures of complexes of the promiscuous OSBS from *Amycolatopsis* with the OSB product as well as with the various *N*-acylamino acid substrates described in this paper are in progress and will be reported in due course (20).

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