Evolution of Enzymatic Activities in the Enolase Superfamily: *N*-Succinylamino Acid Racemase and a New Pathway for the Irreversible Conversion of D- to L-Amino Acids[†]

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ABSTRACT: Members of the mechanistically diverse enolase superfamily catalyze reactions that are initiated by abstraction of the α-proton of a carboxylate anion to generate an enolate anion intermediate that is stabilized by coordination to a Mg^{2+} ion. The catalytic groups, ligands for an essential Mg^{2+} and acid/base catalysts, are located in the $(\beta/\alpha)_8$-barrel domain of the bidomain proteins. The assigned physiological functions in the muconate lactonizing enzyme (MLE) subgroup (Lys acid/base catalysts at the ends of the second and sixth β-strands in the barrel domain) are cycloisomerization (MLE), dehydration (*o*-succinylbenzoate synthase; OSBS), and epimerization (L-Ala-D/L-Glu epimerase). We previously studied a putatively promiscuous member of the MLE subgroup with uncertain physiological function from *Amycolatopsis* that was discovered based on its ability to catalyze the racemization of *N*-acylamino acids (*N*-acylamino acid racemase; NAAAR) but also catalyzes the OSBS reaction [OSBS/NAAAR; Palmer, D. R., Garrett, J. B., Sharma, V., Meganathan, R., Babbitt, P. C., and Gerlt, J. A. (1999) *Biochemistry 38*, 4252−4258]. In this manuscript, we report functional characterization of a homologue of this protein encoded by the genome of *Geobacillus kaustophilus* as well as two other proteins that are encoded by the same operon, a divergent member of the Gcn5-related *N*-acetyltransferase (GNAT) superfamily of enzymes whose members catalyze the transfer an acyl group from an acyl-CoA donor to an amine acceptor, and a member of the M20 peptidase/carboxypeptidase G2 family. We determined that the member of the GNAT superfamily is succinyl-CoA:D-amino acid *N*-succinyltransferase, the member of the enolase superfamily is *N*-succinylamino acid racemase (NSAR), and the member of the M20 peptidase/carboxypeptidase G2 family is *N*-succinyl-L-amino acid hydrolase. We conclude that (1) these enzymes constitute a novel, irreversible pathway for the conversion of D- to L-amino acids and (2) the NSAR reaction is a new physiological function in the MLE subgroup. The NSAR is also functionally promiscuous and catalyzes an efficient OSBS reaction; intriguingly, the operon for menaquinone biosynthesis in *G. kaustophilus* does not encode an OSBS, raising the possibility that the NSAR is a bifunctional enzyme rather than an accidentally promiscuous enzyme.

The assignment of physiological function to proteins discovered in genome projects is a major challenge in genomic biology. In favorable cases, function can be assigned by homology; for example, proteins that catalyze the same reaction on the same substrate (members of families) often, but not always, share ≥40% sequence identity. Proteins that share lower levels of similarity often can be assigned to either specificity-diverse or functionally diverse superfamilies. In the former case, for example, the serine protease superfamily, the chemical reactions catalyzed by the members are the same, but the identity of the substrate differs (*1*). In the latter case, for example, members of the enolase and amidohydrolase superfamilies, neither the chemical reaction nor the substrate is the same (*2*). In either case, the function of the protein cannot be assigned without further experimentation.

The members of the mechanistically diverse enolase superfamily catalyze reactions that are initiated by abstraction of the α-proton of a carboxylate anion to generate an enolate anion intermediate that is stabilized by coordination to a Mg^{2+} ion (*3*, *4*). The sequences of the currently recognized members of the superfamily can be grouped into three distinct active site contexts based on the identities and positions of shared general acid/base catalysts in the $(\beta/\alpha)_8$-barrel domain of the bidomain structure: the enolase subgroup in which a Lys is located at the C-terminal end of the sixth β-strand; the mandelate racemase (MR[1]) subgroup in which a His−

Asp dyad is located at the C-terminal ends of the seventh and sixth β-strands, respectively; and the muconate lactonizing enzyme (MLE) subgroup in which Lys residues are located at the C-terminal ends of both the second and sixth β-strands.

To date, all of the reactions that have been assigned to the enolase superfamily are initiated by enolization of a carboxylate anion substrate. However, this partial reaction occurs in a number of overall reaction contexts, including dehydration (β-elimination), racemization/epimerization (1,1-proton transfer), and cycloisomerization (intramolecular β-elimination). Further complicating the problem of functional assignment of homologues discovered in genome projects, the same chemical reaction (but with different substrate specificities) can be catalyzed by members of different subgroups. Dehydration reactions can be catalyzed by members of all three subgroups: the dehydration of 2-phosphoglycerate catalyzed by enolase, the dehydration of D-glucarate catalyzed by D-glucarate dehydratase (and other acid sugar dehydratases; MR subgroup), and the dehydration of 2-succinyl-6-hydroxy-2,4-cyclohexadienyl-1-carboxylate (SHCHC) catalyzed by o-succinylbenzoate synthase (OSBS; MLE subgroup). 1,1-Proton-transfer reactions can be catalyzed by members of two subgroups: the reaction catalyzed by mandelate racemase (MR subgroup) and the reaction catalyzed by L-Ala-D/L-Glu epimerase (MLE subgroup).

Furthermore, we have discovered that members of the enolase superfamily may be functionally promiscuous, providing yet another ambiguity in unequivocal functional assignment. An example pertinent to the present manuscript is a protein from a species of *Amycolatopsis* that was first described based on its ability to catalyze the racemization of N-acylamino acids and is a member of the MLE subgroup of the enolase superfamily (5). This N-acylamino acid racemase (NAAAR; gi:975627) was identified in a screen of 30 000 microorganisms so that a catalyst for racemization of N-acylamino acids could be obtained for use in a commercially viable, coupled-enzyme process for the conversion of a racemic mixture of an N-acylamino acids to enantiomerically pure amino acids using either a D- or L-acylase. Curiously, the value of $k_{cat}/K_m = 370$ M^{-1} s^{-1} for racemization of N-acetylmethionine, the best substrate then described, is considerably less than the diffusion-controlled limit that characterizes an optimally evolved catalyst (6). One of our laboratories then published an explanation for this low value for k_{cat}/K_m following recognition that the sequence of the NAAAR shares 43% sequence identity with the OSBS from *Bacillus subtilis* (gi:16080130) which is encoded by a gene located in an operon for menaquinone biosynthesis (7).
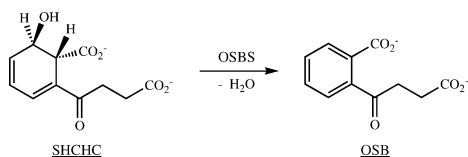
SHCHC → OSB (OSBS, – H$_2$O)

When the NAAAR was assayed for the dehydration reaction catalyzed by OSBS, the value of k_{cat}/K_m was measured as 2.5×10^5 M^{-1} s^{-1}. Although this value is less than that for diffusion-controlled reactions, it is similar to that measured for the homologous OSBS from *B. subtilis*, 7.5×10^5 M^{-1} s^{-1}. On this basis, we "assigned" the OSBS function to the protein from *Amycolatopsis* and considered the less efficient NAAAR reaction to be an example of catalytic promiscuity (OSBS/NAAAR).

In later studies of the OSBS/NAAAR, we synthesized N-succinyl methionine and N-succinyl phenylglycine as structural analogues of the SHCHC substrate for the OSBS reaction and discovered that these were significantly better substrates for the NAAAR reaction than N-acetyl methionine; the values for k_{cat}/K_m were 1.7×10^4 and 2.0×10^5 M^{-1} s^{-1}, respectively (8). We interpreted the increases in kinetic constants associated with modification of the structure of the substrate for the NAAAR reaction as enhancement of an accidental catalytic promiscuity. Structural studies of the OSBS/NAAAR in the presence of the OSB product of the OSBS reaction and the N-succinyl methionine and N-succinyl phenylglycine substrates for the NAAAR reaction provided a structure-based explanation for the promiscuity (9). The distal portions of the OSB product for the OSBS reaction and the substrates for the NAAAR reaction were bound in a hydrophobic pocket, formed from residues contributed by the 20s and 50s loops in the capping domain. Regarding the placement of the catalytic residues with respect to the substrate/product, Lys 163 located at the C-terminal end of the second β-strand in the $(\beta/\alpha)_8$-barrel domain is appropriately positioned to catalyze the syn-elimination of water from SHCHC in the OSBS reaction (10). Lys 163 and Lys 263 located at the C-terminal end of the sixth β-strand are appropriately positioned to catalyze the 1,1-proton-transfer reaction that equilibrates the enantiomers of the N-succinyl-amino acids in the NAAAR reaction.

As the sequence databases have continued to expand, ~40 proteins from Gram-positive bacteria and archaea now can be identified that share $\geq 35\%$ sequence identity with the OSBS/NAAAR from *Amycolatopsis* (J. A. Gerlt, unpublished observations). Whereas the deposited DNA sequence that encodes the protein from *Amycolatopsis* provided only partial sequence for the flanking genes, the deposited DNA sequences for many of the homologues provide complete genomic context because these were obtained in genome projects. Some of the homologues from Gram-positive bacteria are encoded by genes located in menaquinone biosynthesis operons, allowing the OSBS function to be unequivocally assigned to these proteins (e.g., *Bacillus anthracis*, *B. subtilis*, and *Enterococcus faecalis*).

However, some of the homologues are located in a distinct, but shared, operon context. This includes a protein from *Amycolatopsis azurea* (gi:18461712) that shares 89% sequence identity with the OSBS/NAAAR from the *Amycolatopsis* species identified in the screen for the racemization catalyst (5); comparison of the DNA sequences encoding OSBS/NAAAR and the homologue from *A. azurea* reveals that the sequences flanking the genes are highly homologous, suggesting an identical operon context for both proteins. However, the functions of many of the homologues of the OSBS/NAAAR from *Amycolatopsis* are uncertain, because either the genomes do not contain recognizable genes

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[1] Abbreviations: GNAT, Gcn5-related N-acyltransferase; IPTG, isopropyl-β-D-thiogalacto-pyranoside; LB, Luria broth; MLE, muconate lactonizing enzyme; MR, mandelate racemase; NAAAR, N-acylamino acid racemase; NSAR, N-succinylamino acid racemase; OSBS, o-succinylbenzoate synthase; SHCHC, 2-succinyl-6-hydroxy-2,4-cyclohexadienyl-1-carboxylate.
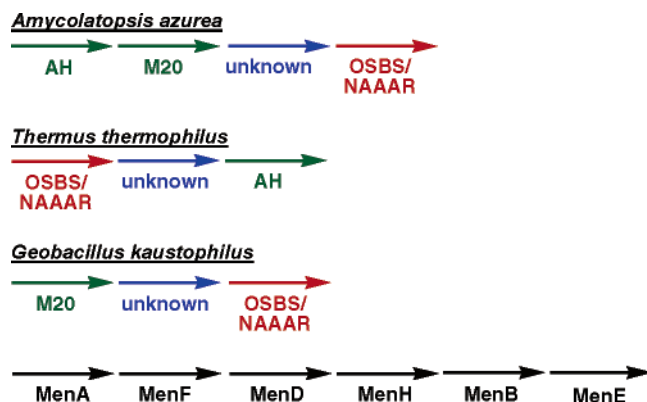
FIGURE 1: Operon context for homologues of the OSBS/NAAAR from *Amycolatopsis*. The genes colored in red encode homologues of the OSBS/NAAAR; the genes colored in blue encoded a protein of "unknown" function that is a member of the GNAT superfamily, and the genes colored in green encoded hydrolases (M20, M20 peptidase/carboxypeptidase G2 family; AH, amidohydrolase/urease/phosphotriesterase superfamily). For *G. kaustophilus*, the operon encoding the enzymes in the menaquinone biosynthetic pathway is also shown (Men A, 1,4-dihydroxy-2-naphthoate octaprenyltransferase; MenB, 1,4-dihydroxynaphthoyl-CoA synthase; MenD, 2-succinyl-6-hydroxy-2,4-cyclohexadienyl-1-carboxylate synthase; MenE, *o*-succinylbenzoate:CoA ligase; MenF, menaquinone-specific isochorismate synthase; and MenH, 1,4-dihydroxynaphthoyl-CoA hydrolase.

encoding 1,4-dinaphthoyl-CoA synthase, a highly conserved enzyme in the menaquinone biosynthetic pathway, or the gene encoding another member of the MLE subgroup is proximal to genes encoding enzymes in the menaquinone pathway.

The genome context of the OSBS/NAAAR encoded by *A. azurea* is shown in Figure 1. The gene upstream of the OSBS/NAAAR is annotated as "unknown;" it is preceded by two genes encoding hydrolases, one (labeled M20 in the figure) encoding a protein annotated as a member of the M20 peptidase/carboxypeptidase G2 family (*11*) and the second (labeled AH in the figure) annotated as encoding a D-amino acid hydrolase in the amidohydrolase/urease/phosphotriesterase superfamily (*12*). Importantly, homologues that share >30% sequence identity with the "unknown" protein often are encoded by genes in these menaquinone pathway-distinct operons that encode homologues of OSBS/NAAAR.[2] In addition, several, but not all, of these operons encode hydrolases from the M20 peptidase/carboxypeptidase G2 family and/or amidohydrolase/urease/phosphotriesterase superfamily. The genome contexts of the OSBS/NAAAR homologues in *Thermus thermophilus* and *Geobacillus kaustophilus* (the latter the subject of this manuscript) are displayed in Figure 1. The conserved occurrence of genes encoding a homologue of the OSBS/NAAAR, the "unknown" protein, and at least one hydrolase suggests that these operons encode the same metabolic pathways in different organisms. If the identity of this pathway could be determined, the physiological function of the OSBS/NAAAR from *Amycolatopsis* and its presumed isofunctional homologues encoded by genes in similar operon contexts in other organisms could be assigned.

Although the "unknown" is alternatively annotated as "chorismate synthase" in several organisms, BLAST and PSI-BLAST searches of the databases using the sequences of the various "unknowns" reveal that these are divergent members of the GNAT superfamily of enzymes that transfer an acyl group from an acyl-CoA donor to an amine acceptor (*13*). Because we previously discovered that the putative racemase promiscuity of the OSBS/NAAAR from *Amycolatopsis* is significantly enhanced by the *N*-succinyl modification (*8*), we hypothesized that the operons encode a pathway in which an amino acid is N-succinylated using succinyl-CoA to provide the physiological substrate for the OSBS/NAAAR; continuing with this hypothesis, the product of the racemization reaction would be the substrate for the hydrolase. With this sequence of reactions, the pathway would accomplish the irreversible conversion of a D-amino acid to an L-amino acid, or vice versa. In this manuscript, we provide evidence supporting this hypothesis obtained by studies of the substrate specificities of the proteins encoded by the three-gene operon in *G. kaustophilus* (Figure 1). As a result, we conclude that (1) the encoded pathway accomplishes the irreversible conversion of D-amino acids to L-amino acids and (2) *N*-succinylamino acid racemase (NSAR) activity is a physiological function of the OSBS/NAAAR from *Amycolatopsis* and its homologues in organisms whose genomes contain operons that also encode the succinyl-CoA:D-amino acid *N*-acyltransferase (formerly the "unknown" protein).

MATERIALS AND METHODS

Libraries of N-Acylated Amino Acids. Most of the *N*-acetylamino acids were purchased from either Sigma Chemical Company or Novabiochem. The acetylated derivatives of D-serine, D-threonine, D-glutamate, D-glutamine, and D-histidine were prepared by a modification of the procedure described in the next section for *N*-succinyl-L-alanine except that acetic anhydride was substituted for succinic anhydride. The *N*-formimino- and *N*-formylamino acids were prepared as previously described (*14*).

Preparation of N-Succinylamino Acids. With few exceptions (lysine and arginine) *N*-succinylamino acids were prepared by the addition of succinic anhydride to the amino acid in a solvent of acetic acid. The preparation of *N*-succinyl-L-alanine is described here as a general procedure along with the associated physical constants. The descriptions of the syntheses for the remaining *N*-succinylamino acids can be found in the Supporting Information. In a 100 mL flask were added L-alanine (3.6 g, 40 mmol), succinic anhydride (4.0 g, 40 mmol), and acetic acid (40 mL). The mixture was heated to 50−60 °C for 5 h. After removal of the solvent, the solid residue was recrystallized from ethyl acetate and methanol to obtain a white solid in a yield of 61% (4.6 g).

^{1}H NMR (500 MHz, DMSO): 12.22 ppm (2H, s, COO$\underline{H}$), 8.13 ppm (1H, t, J = 7.0 Hz, CON$\underline{H}$), 4.19−4.13 ppm (1$\underline{H}$, m, CONH$\underline{H}$), 2.43−2.30 ppm (4$\underline{H}$, m, HO$_2$CC$\underline{H}_2$C$\underline{H}_2$CO), 1.22 ppm (3$\underline{H}$, d, J = 7.5 Hz, CHC$\underline{H}_3$).

Mass spectrometry (ESI negative mode): observed, 188.05 (M − H); expected for $C_7H_{11}NO_5$, 189.06 (M).

Cloning, Expression, and Protein Purification of the Member of the GNAT Superfamily. The gene encoding the

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[2] The remainder of the "unknown" proteins are encoded by genomes that also encode homologues of the OSBS/NAAAR, but the genes encoding the "unknown" protein and the OSBS/NAAAR are not colocalized in operons.

member of the GNAT superfamily (gi:56419460) was PCR-amplified from *G. kaustophilus* HTA426 genomic DNA kindly provided by Professor Takami, Japan Agency for Marine-Earth Science and Technology. The reaction (100 μL) contained 1 ng of DNA, 10 μL of 10× Pfx Amplification Buffer, 1 mM MgSO$_4$, 0.4 mM of each dNTP, and 40 pmol of both a forward primer (*Ase*I recognition site; 5′-CCG-CAGGGGGGAGAACATTAATATGCCTCTGTCGCTGCA-AATC-3′) and a reverse primer (*Bam*HI recognition site; 5′-CCTCTTCCCCTTTCTGGGATCCTCATTCGTTTC-TC-CTCACCC-3′). The amplification was performed using a PTC-200 Gradient Thermal Cycler (MJ Research) with the following parameters: 94 °C for 3 min followed by 40 cycles at 94 °C for 1 min, a gradient temperature range of 45−65 °C for 1 min and 15 s, and 68 °C for 3 min followed by a final extension at 68 °C for 10 min. The amplified gene was cloned into a modified pET15b (Novagen) vector in which the N-terminal His-tag contains 10 His residues. The protein was expressed in *Escherichia coli* strain BL21 (DE3). Transformed cells were grown at 37 °C in Luria broth (LB; supplemented with 100 μg/mL ampicillin) for 48 h and harvested by centrifugation. No isopropyl-β−D-thiogalacto-pyranoside (IPTG) was added to induce protein expression. The cells were resuspended in binding buffer (5 mM imidazole, 0.5 M NaCl, and 20 mM Tris-HCl, pH 7.9) and lysed by sonication (Fischer Scientific 550 Sonic Dismembrator). The lysate was cleared by centrifugation, and the His-tagged protein was purified using a column of chelating Sepharose Fast Flow (Amersham Biosciences) charged with Ni^{2+}. Cell lysate was applied to the column in binding buffer, washed with 15% elute buffer (1 M imidazole, 0.5 M NaCl, and 20 mM Tris-HCl, pH 7.9)−85% wash buffer (60 mM imidazole, 0.5 M NaCl, and 20 mM Tris-HCl, pH 7.9), and eluted with 50% binding buffer−50% strip buffer (100 mM EDTA, 0.5 M NaCl, and 20 mM Tris-HCl, pH 7.9). The purified protein was subjected to SDS gel electrophoresis, and the protein was >95% pure as assessed by SDS gel electrophoresis. The N-terminal His-tag was removed with thrombin (Amersham Biosciences) according to the manufacturer's instructions. The His-tag-cleaved protein was dialyzed against 20 mM Tris, pH 8.0. An absorption coefficient at 280 nm of 52 750 M^{-1} cm^{-1} was used for determination of protein concentration.

Cloning, Expression, and Protein Purification of the Homologue of OSBS/NAAAR. The gene encoding the homologue of the OSBS/NAAAR from *Amycolatopsis* (gi: 56419461) was PCR-amplified from genomic DNA isolated from *G. kaustophilus* HTA426 as described in the previous section using a forward primer (*Ase*I recognition site; 5′-GCAGAAAGGGGAAGATTAATGGCGATCAACATCG-AGTACG-3′) and a reverse primer (*Bam*HI recognition site; 5′-CTTTTATCATCCTGGATCCTTATGCCG-TCGCCG-TACGATG-3′). The amplified gene was cloned into a modified pKK223-3 (Amersham) vector and expressed in an OSBS-deficient strain of *E. coli* (*15*). Transformed cells were grown at 37 °C in LB broth (supplemented with 100 μg/mL ampicillin and 50 μg/mL kanamycin) for 48 h after 0.5 mM IPTG induction at a cell density corresponding to an OD$_{600}$ of ~0.6. The cells were resuspended in low salt buffer (20 mM Tris, pH 8.0, and 5 mM MgCl$_2$) and lysed by sonication. The lysate was cleared by centrifugation and applied to a DEAE Sepharose Fast Flow column (26 mm ×

70 cm) in low salt buffer. The column was washed with 800 mL of low salt buffer, and the protein was eluted with a linear gradient of 0−50% NaCl (1 M) over 1600 mL in wash buffer. Fractions containing the protein were identified by SDS−PAGE analysis and applied to a Phenyl Sepharose 6 Fast Flow (Amersham Biosciences) column (16 mm × 20 cm) in 0.4 M (NH$_4$)$_2$SO$_4$ for further purification. The protein was eluted with a linear gradient of 0.4−0 M (NH$_4$)$_2$SO$_4$, and the fractions showing >95% purity by SDS gel electrophoresis were pooled and concentrated. The purified protein was dialyzed against 20 mM Tris, pH 8.0, and 5 mM MgCl$_2$. An absorption coefficient at 280 nm of 58 680 M^{-1} cm^{-1} was used for determination of protein concentration.

Cloning, Expression, and Protein Purification of the M20 Peptidase. The gene encoding the member of the M20 peptidase/carboxypeptidase G2 family (gi:56419459) was PCR-amplified from genomic DNA from *G. kaustophilus* HTA426 following the procedure described previously using a forward primer (*Nde*I recognition site; 5′-CAGCGG-GG-GAGAGCCATATGAAAGAAATTGTTCAGCAGATG-3′ and a reverse primer (*Bam*HI recognition site; 5′-CTTTC-AACTGTTCGGGATCCTCAATGATTTGCAGCGACAG-3′). The amplified gene was cloned into the pET17b (Novagen) vector and expressed in *E. coli* strain BL21 (DE3). Cultures were grown at 14−16 °C in LB containing 100 μg/mL ampicillin. Protein expression was initiated by adding IPTG to a final concentration of 0.2 mM at a cell density corresponding to an OD$_{600}$ of ~0.6, and the cells were allowed to grow overnight. The cells were harvested by centrifugation at 11 000 rpm for 15 min and resuspended in 50 mM sodium HEPES buffer, pH 7.5. Phenylmethanesul-fonylfluoride (1.0 mM) was added to the cell suspension. The cells were disrupted by sonication at 0 °C for 30 min, and the soluble protein was recovered from the lysed cells by centrifugation at 11 000 rpm for 15 min. The supernatant was incubated with DNase I (100 μg/mL) for 1 h at 0 °C. The protein solution was filtered with a 0.2 μm filter (CORNING) and loaded onto a Superdex-200 gel filtration column (Amersham Pharmacia) equilibrated with 50 mM sodium HEPES, pH 7.5. The pooled fractions containing the desired protein were rapidly frozen and stored at −78 °C. The purified protein was subjected to SDS gel electrophoresis, and the major protein band on the gel exhibited the expected molecular weight (43 kDa). The purity of the isolated protein was >50% as assessed by SDS gel electrophoresis. An absorption coefficient at 280 nm of 37 650 M^{-1} cm^{-1} was used for the determination of protein concentration. Edman sequencing of the N-terminal five amino acids of the purified enzyme by the Protein Chemistry Laboratory of Texas A&M University gave Met-Lys-Glu-Ile-Val. The sequence is consistent with the deposited protein sequence.

Spectrophotometric Assay for N-Acyltransferase Activity. A modification of a continuous spectrophotometric assay (*16*) was used to screen potential substrates for *N*-acyltransferase activity. The transferase activity was quantitated at 25 °C using 5 mM acyl-CoA (acetyl- or succinyl-CoA) and 5 mM amino acid (D- or L-amino acid) in 50 mM Tris, pH 7.9, containing 0.15 mM EDTA, 0.1 mM (NH$_4$)$_2$SO$_4$, and 2 mM 4,4′-dipyridyl disulfide. The rate of formation of 4-thiopyr-idine was quantitated by measuring the increase in absorbance at 324 nm (ϵ = 19 800 M^{-1} cm^{-1}).

After the initial screen revealed that the transferase was specific for succinyl-CoA and D-amino acids, the values of kinetic constants were measured using varying concentrations of D-amino acids at a fixed 5 mM concentration of succinyl-CoA. The kinetic constants for methionine and cysteine could not be measured because of their reactivity with the 4,4′-dipyridyl disulfide reagent.

Polarimetric Assay for N-Acylamino Acid Racemization. The racemization of N-acylamino acids was measured at 25 °C using varying concentrations of substrates in 50 mM HEPES, pH 7.9, and 0.1 mM MnCl$_2$. The change in optical rotation was quantitated at Hg 405 nm using a JASCO P-1010 polarimeter, with a 10 s integration time and a 10 cm path length cuvette (*5*). The low values for the specific rotations of N-succinyl-L-Lys, -Asn, -Gln, and -Glu prevented measurement of k_{cat} and K_m, so only the observed rate using 50 mM substrate is reported. The negligible specific rotation of the N-succinyl-L-threonine precluded its use in these assays.

Assay for the Hydrolysis of N-Acylamino Acids. A modification of a colorimetric ninhydrin-based assay (*17*) was adopted to screen for substrates for N-acylamino acid hydrolysis. The Cd-ninhydrin reagent solution was prepared by dissolving 0.8 g of ninhydrin in 80 mL of 99.5% ethanol and 10 mL of acetic acid, followed by the addition of 1 g of CdCl$_2$ dissolved in 1 mL of water. Routinely, 100 μL aliquots of sample solution and 330 μL of the Cd-ninhydrin reagent were mixed and heated in a 96-well block for 10 min in an 84 °C water bath. After the block was cooled to room temperature, 250 μL of the reaction solution from each well was transferred to a 96-well UV–visible plate, and the absorbance at 507 nm was read with a SPECTRAmax plate reader from Molecular Devices. The initial screen for potential substrates was conducted at 25 °C with libraries of the following set of N-acylamino acid derivatives at a concentration of 5 mM using 50 mM sodium HEPES, pH 7.4, as buffer: N-succinyl-L-amino acids (plus phenylglycine), N-succinyl-D-amino acids (except lysine, arginine, and histidine), N-acetyl-L-amino acids (except proline and lysine), N-acetyl-D-amino acids (except proline, lysine, arginine, cysteine, and methionine), N-formimino-D-glutamate, and N-formimino-L-amino acids (including alanine, glutamate, methionine, glycine isoleucine, valine, tryptophan, aspartate, leucine, and phenylalanine). The kinetic constants for the N-succinyl-L-amino acid derivatives were determined using the same reaction conditions by the removal of aliquots at various times and measuring the concentration of the free amino acid.

Spectrophotometric Assay for OSBS Activity. OSBS activity was measured at 25 °C using 2-hydroxy-6-succinyl-2,4-cyclohexadiene carboxylate (SHCHC), 50 mM HEPES, pH 7.9, and 0.1 mM MnCl$_2$ by quantitating the decrease in absorbance of SHCHC (ϵ = 2400 M^{-1}cm^{-1} at 310 nm). SHCHC was prepared from chorismate as previously described (*7*).

RESULTS AND DISCUSSION

As noted in the Introduction, the operons encoding a homologue of OSBS/NAAAR, the "unknown", and at least one hydrolase are found in *A. azurea*, *T. thermophilus* (both strains HB8 and HB27), and *G. kaustophilus* HTA426

Table 1: Activity Screen of the N-Acyltransferase with Succinyl-CoA, Acetyl-CoA, and Both D- and L-Amino Acids

compound	succinyl-CoA		acetyl-CoA	
	D-amino acid	L-amino acid	D-amino acid	L-amino acid
Ala	1.2[a]	0	0.070	0
Val	5.6	0	5.3	0
Leu	1.9	0.088	3.5	0.0051
Ile	2.1	0	2.2	0.0013
Met	nd[b]	nd[b]	nd[b]	nd[b]
Phe	3.0	0.0072	0.0026	0.0026
Tyr	3.0	0	0.95	0.0015
Trp	1.9	0.0040	0.41	0
Pro	0	0	0.0015	0.0014
Gly	-	0.35	-	0.018
Ser	0.27	0.0083	0.023	0.0014
Thr	1.0	0	0.062	0.0028
Cys	nd[b]	nd[b]	nd[b]	nd[b]
Asn	0.46	0	0.015	0.0022
Gln	0.099	0	0.068	0.0023
Asp	0.0021	0	0	0.0020
Glu	0.014	0	0	0.00019
Lys	12	0	3.6	0.011
Arg	9.8	0.013	3.3	0.0018

[a] The observed rates (in s^{-1}) using 5 mM concentrations of the CoA esters and 5 mM concentrations of the amino acids. [b] Not determined because the amino acid reacts with the 4,4′-dithiopyridine reagent.

(Figure 1). We could not obtain the strain of *A. azurea* for which partial genome sequence data is available, and we have not yet been able to express and purify all three proteins from *T. thermophilus*. However, we have been able to express and purify the three proteins from *G. kaustophilus*. Our strategy was to discover the substrate specificities of all three enzymes, with the expectation that when integrated these would reveal the identity of the metabolic pathway as well as the function of each enzyme in that pathway.

Substrate Specificity of the N-Acyltransferase of Unknown Function. The "unknown", assumed to catalyze an N-acyltransferase reaction, was purified and screened for activity using the assay described in Materials and Methods. Both the D- and L-enantiomers of 17 of the 20 common amino acids (excluding the achiral Gly and both Met and Cys which interfere with the spectrophotometric assay that measures the formation of a reactive thiol) were used as acceptor substrates. Acetyl- and succinyl-CoA were used as the donor substrates, because the known biosynthetic pathways for (basic) L-amino acids utilize N-acetyl- and N-succinylamino acids as intermediate metabolites. We used single, fixed concentrations of the amino acids (5 mM) and of the acyl-CoA donors (5 mM) to screen the substrate specificity of the N-acyltransferase. The rates observed in these assays are summarized in Table 1.

We then determined the values of the kinetic constants (k_{cat}, K_m, and k_{cat}/K_m) for various D-amino acids using a fixed concentration (5 mM) of succinyl-CoA; these are displayed in Table 2. Inspection of these data reveals that the N-acyltransferase is an efficient catalyst for the N-succinylation of hydrophobic and basic D-amino acids.

Substrate Specificity of the Homologue of OSBS/NAAAR. The homologue of OSBS/NAAAR was purified and assayed as described in Materials and Methods. A polarimetric assay is the most convenient for racemization of N-acylamino acids. However, this assay is limited by the specific rotations of the substrates, as noted in Materials and Methods. The kinetic

Table 2: Kinetic Constants for *N*-Succinyl-CoA:D-Amino Acid *N*-Succinyltransferase

compound	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (M^{-1} s^{-1})
D-Ala	1.6 ± 0.2	12 ± 3	130 ± 1
D-Val	2.3 ± 0.1	0.46 ± 0.1	(5.0 ± 0.9) × 10^3
D-Leu	3.2 ± 0.2	0.50 ± 0.2	(6.4 ± 0.7) × 10^3
D-Ile	1.6 ± 0.1	0.39 ± 0.2	(4.1 ± 0.4) × 10^3
D-Met	nd^a	nd^a	nd^a
D-Phe	2.4 ± 0.2	0.094 ± 0.04	(2.6 ± 0.1) × 10^4
D-Tyr	nd^b	nd^b	nd^b
D-Trp	1.4 ± 0.1	0.25 ± 0.05	(5.6 ± 0.1) × 10^3
D-Pro	0.0043^c	-	-
Gly	-	-	22 ± 2^d
D-Ser	-		24 ± 1
D-Thr	2.6 ± 0.2	21 ± 4	130 ± 1
D-Cys	nd^a	nd^a	nd^a
D-Asn	0.87 ± 0.03	22 ± 2	41 ± 3
D-Gln	2.3 ± 0.1	18 ± 1	130 ± 2
D-Asp	-	-	2.0 ± 0.2
D-Glu	-	-	1.2 ± 0.1
D-Lys	12 ± 0.7	2.7 ± 0.7	(4.5 ± 0.3) × 10^3
D-Arg	11 ± 1	0.89 ± 0.3	(1.2 ± 0.1) × 10^4

^{a} Not determined because the amino acid reacts with the 4,4′-dithiopyridine reagent. ^{b} Not determined due to low solubility in the assay. ^{c} Rate measured at 50 mM; the low rate prevented kinetic analysis. ^{d} Saturation could not be achieved, so only the value of k_{cat}/K_m could be determined.

Table 3: Kinetic Constants for the Racemization of *N*-Succinyl-L-Amino Acids Catalyzed by *N*-Succinylamino Acid Racemase

compound	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (M^{-1} s^{-1})
N-Succinyl-L-Ala	89 ± 9	2.6 ± 1.0	(3.4 ± 0.3) × 10^4
N-Succinyl-L-Val	92 ± 7	2.5 ± 0.7	(3.7 ± 0.4) × 10^4
N-Succinyl-L-Leu	17 ± 1	1.3 ± 0.2	(1.3 ± 0.1) × 10^4
N-Succinyl-L-Ile	35 ± 2	3.7 ± 0.8	(9.4 ± 0.8) × 10^3
N-Succinyl-L-Met	53 ± 1	3.6 ± 0.4	(1.4 ± 0.1) × 10^4
N-Succinyl-L-Phe	19 ± 1	0.8 ± 0.2	(2.3 ± 0.2) × 10^4
N-Succinyl-L-Tyr	52 ± 4	2.6 ± 0.7	(2.0 ± 0.2) × 10^4
N-Succinyl-L-Trp	7.9 ± 0.5	7.1 ± 1.6	(1.1 ± 0.2) × 10^3
N-Succinyl-L-Pro	0		0
N-Succinyl-L-Ser	45 ± 7	3.0 ± 1.3	(1.5 ± 0.2) × 10^4
N-Succinyl-L-Thr	-^{a}	-^{a}	-^{a}
N-Succinyl-L-Cys	-^{b}	-^{b}	7.1 ± 0.3^b
N-Succinyl-L-Asn	2.5 ± 0.2^c	-^{c}	-^{c}
N-Succinyl-L-Gln	9.0 ± 0.1^c	-^{c}	-^{c}
N-Succinyl-L-Asp	0.071 ± 0.001^c	-^{c}	-^{c}
N-Succinyl-L-Glu	0.073 ± 0.03^c	-^{c}	-^{c}
N-Succinyl-L-Lys	2.1 ± 0.1^c	-^{c}	-^{c}
N-Succinyl-L-Arg	0.014 ± 0.001^c	-^{c}	-^{c}
N-Succinyl-L-His	2.0 ± 0.2	3.8 ± 1.3	530 ± 40

^{a} Not determined because of low specific rotation. ^{b} Saturation could not be achieved, so only the value of k_{cat}/K_m could be determined. ^{c} Rate measured at 50 mM; the low specific rotation prevented kinetic analysis.

constants are displayed in Table 3. We also attempted to measure kinetic constants for the L-enantiomers of some N-acetylated amino acids; however, these were inferior substrates with values of $k_{cat}/K_m \leq 50$ M^{-1} s^{-1} (data not shown). Inspection of the data in the Table reveals that the homologue of OSBS/NAAAR is an efficient catalyst for the racemization of hydrophobic, polar, and some basic *N*-succinyl-D/L-amino acids.

Substrate Specificity of the Hydrolase. The hydrolase, a member of the Zn^{2+}-dependent M20/carboxypeptidase G2 family, was partially purified and assayed as described in Materials and Methods.[3] The most convenient assay uses ninhydrin to quantitate the formation of the α-amino group of the amino acid product. We attempted to measure the

Table 4: Kinetic Constants for the Hydrolysis of *N*-Succinyl-L-Amino Acids Catalyzed by *N*-Succinyl-L-Amino Acid Hydrolase^a

compound	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (M^{-1} s^{-1})
N-Succinyl-L-Ala	4.0 ± 0.1	25 ± 5	(1.6 ± 0.3) × 10^5
N-Succinyl-L-Val	2.2 ± 0.1	50 ± 11	(4.4 ± 1.0) × 10^4
N-Succinyl-L-Leu	3.6 ± 0.3	130 ± 20	(2.9 ± 0.6) × 10^4
N-Succinyl-L-Ile	0.5 ± 0.04	90 ± 14	(5.6 ± 1.0) × 10^3
N-Succinyl-L-Met	3.8 ± 0.2	180 ± 20	(2.1 ± 0.3) × 10^4
N-Succinyl-L-Phe	0.97 ± 0.04	34 ± 8	(2.8 ± 0.6) × 10^4
N-Succinyl-L-Trp	0.34 ± 0.02	32 ± 6	(1.1 ± 0.2) × 10^3
N-Succinyl-Gly	5.7 ± 0.4	170 ± 30	(3.4 ± 0.6) × 10^4
N-Succinyl-L-Ser	2.8 ± 0.2	50 ± 10	(5.9 ± 1.2) × 10^4
N-Succinyl-L-Thr	0.71 ± 0.03	83 ± 13	(8.6 ± 1.3) × 10^3
N-Succinyl-L-Gln	0.65 ± 0.04	89 ± 18	(7.3 ± 1.5) × 10^3
N-Succinyl-L-Arg	0.9 ± 0.1	310 ± 65	(2.9 ± 0.7) × 10^3
N-Succinyl-L-His	1.5 ± 0.1	130 ± 20	(1.4 ± 0.3) × 10^4

^{a} The rates of hydrolysis for the *N*-succinyl derivatives of L-glutamate, L-tyrosine, L-aspartate, L-asparagine, L-lysine, and L-cysteine at concentrations of 1.0 mM were <5% the rate for the hydrolysis of 1.0 mM *N*-succinyl-L-alanine; these were not subjected to detailed kinetic analyses.

kinetic constants for the hydrolysis of the D- and L-enantiomers of *N*-formyl-, *N*-formimino-, *N*-acetyl-, and *N*-succinylamino acids. Enzymatic activity only could be detected for the hydrolysis of some *N*-succinyl-L-amino acids. Less than 0.1% of this activity could be detected for *N*-formyl-, *N*-formimino-, and *N*-acetyl-D/L-amino acids. The kinetic constants for the hydrolyses of *N*-succinyl-L-amino acids are displayed in Table 4. Inspection of the data in Table 4 reveals that the hydrolase is an efficient catalyst for the hydrolysis of hydrophobic, small, and some basic *N*-succinyl-L-amino acids.

Assignment of Functions and Identification of the Pathway. On the basis of the experimentally determined substrate specificities and kinetic constants for all three enzymes, we assign the following functions: (1) *N*-acyltransferase of unknown function, succinyl-CoA:D-amino acid *N*-succinyltransferase; (2) homologue of OSBS/NAAAR, *N*-succinyl-amino acid racemase (NSAR); and (3) hydrolase, *N*-succinyl-L-amino acid hydrolase. Taken together, the assigned functions permit identification of this novel metabolic pathway as the irreversible conversion of D-amino acids to their L-enantiomers (Figure 2).

Given the considerable energetic cost of this transformation that involves stoichiometric utilization of succinyl-CoA, this pathway must serve an important role in the physiology of the encoding organisms. We speculate that the pathway may be involved in the detoxication of one or more D-amino acids: a reversible, one-step pathway using a "conventional" amino acid racemase likely would not allow as efficient scavenging and removal of the toxic substance from the environment. Alternatively, the pathway simply could provide a strategy to utilize D-amino acids as carbon source, although other bacteria have devised simpler and energetically less costly solutions to this problem, including both PLP-dependent and -independent amino acid racemases.

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[3] Although we could not obtain the hydrolase as a homogeneous preparation, in control experiments, we demonstrated that extracts of *E. coli* strain BL21 (DE3) transformed with the "empty" vector did not catalyze significant hydrolyses of *N*-acylated amino acids, whereas extracts of this strain (from which the partially purified preparation was obtained) catalyzed only the hydrolyses of *N*-succinyl-L-amino acids.
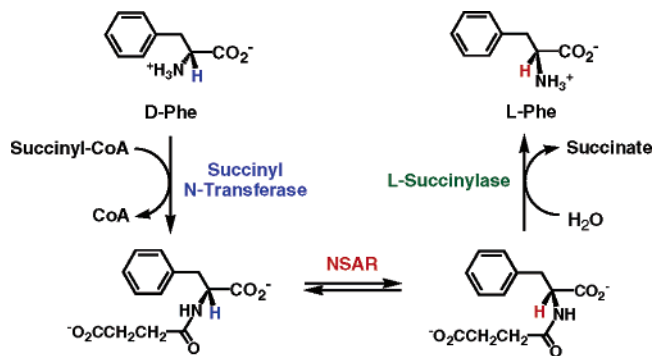
FIGURE 2: Irreversible pathway for the conversion of D- to L-amino acids, with the enantiomers of phenylalanine given as an example, showing the reactions catalyzed by the enzymes functionally assigned in this study.

With this functional assignment for the member of the enolase superfamily that catalyzes the NSAR reaction, we note a functional parallel to the purpose for which "original" NAAAR was sought and discovered in *Amycolatopsis* (*7*): in that case, the equilibration of the enantiomers of *N*-acetylamino acids so that an achiral mixture could be irreversibly and quantitatively converted to enantiomerically pure amino acids in a commercially viable process. Now, we recognize a biological role of the enzyme is to irreversibly transform enantiomerically pure D-amino acids to enantiomerically pure L-amino acids, thereby facilitating the survival and/or growth of the organism.

Functional Promiscuity of the NSAR. Because we discovered that the "original" NAAAR from *Amycolatopsis* was an effective catalyst of the OSBS reaction (*7*), we also assessed whether the NSAR from *G. kaustophilus* catalyzes the OSBS reaction. Indeed, it is an efficient catalyst of the OSBS reaction: the values of k_{cat} and k_{cat}/K_m are 180 s^{-1} and 1.9×10^6 M^{-1} s^{-1}, respectively.

Intriguingly, the genome of *G. kaustophilus* contains an operon for menaquinone biosynthesis (Figure 1, last panel) that includes the genes that encode all of the enzymes in the expected pathway *except OSBS* (MenC). In addition to NSAR that is a member of the MLE subgroup, the only other member of the enolase superfamily encoded by the *G. kaustophilus* genome belongs to the MR subgroup and can be assigned the D-galactonate dehydratase function based on sequence homology [61% sequence identity with the functionally and structurally characterized enzyme from *E. coli* K-12 (*18, 19*)]. Because all known OSBSs belong to the MLE subgroup of the enolase superfamily (M. E. Glasner, J. A. Gerlt, and P. C. Babbitt, unpublished observations), this raises the possibility that the NSAR encoded by the operon for irreversible conversion of D-amino acids to L-amino acids is actually a bifunctional [or "moonlighting" (*20, 21*)] protein that also functions in the menaquinone biosynthetic pathway as the required OSBS. An analogous situation may apply to the presumed isofunctional homologues of NSAR in other organisms, including the completely sequenced *Deinococcus radiodurans* and *T. thermophilus* that are taxonomically classified based on the structures of their menaquinones, although genes encoding other enzymes in the menaquinone biosynthetic pathway cannot be identified in the genomes of these organisms. *A. azurea* also contains menaquinone; however, a complete genome sequence is not available for this organism. So, the "original" OSBS/NAAAR

might be a bifunctional, rather than a promiscuous, protein, with its initial discovery based on screening for a racemization catalyst for physiologically "incorrect" *N*-acetylamino acids rather than the "correct" *N*-succinylamino acids (*5*). We currently are testing this possibility by disrupting the gene encoding NSAR in *G. kaustophilus*; because menaquinone is required for anaerobic growth, this lesion would prevent anaerobic growth if the protein functions in both pathways.

Evolution of Function in the MLE Subgroup. The functional assignment of *N*-succinylamino acid racemase (NSAR) establishes the fourth physiological reaction catalyzed by the MLE subgroup in which conserved acid/base Lys residues are located at the C-terminal ends of both the second and sixth β-strands of the (β/α)₈-barrel domain. Previously, we had recognized the MLE, OSBS, and L-Ala-D/L-Glu epimerase reactions. Despite the conserved Lys acid/base functional groups, the active site motifs that support these reactions are functionally and phylogenetically distinct: the MLE reaction is associated with a Glu located at the C-terminal end of the eighth β-strand; the L-Ala-D/L-Glu epimerase reaction is associated with a DxD motif located at the end of the eighth β-strand, and the OSBS reaction is associated with the absence of these motifs, and usually a Gly in their place, at the end of the eighth β-strand. The proteins that catalyze the NSAR reactions are members of the latter group, so the ability to catalyze a 1,1-proton-transfer reaction (L-Ala-D/L-Glu epimerase and NSAR) evolved more than once in the MLE subgroup.

We assume that the homologues of NSAR encoded by operons that also encode isofunctional homologues of the succinyl-CoA:D-amino acid *N*-succinyltransferase will share the ability to catalyze racemization of *N*-succinylamino acids. However, based on comparisons of the specificity-determining residues predicted to be located in their active sites [based on sequence alignments and the structure of the protein from *Amycolatopsis* (*9*)], we expect that some of these will have substrate specificities that differ from that we measured for the NSAR from *G. kaustophilus*. We are investigating the substrate specificities of these homologues so that the structural bases for specificity-driven evolution of function in the MLE subgroup can be better understood (*15, 22*).

SUPPORTING INFORMATION AVAILABLE

Description of the syntheses of the *N*-succinylamino acids used. This material is available free of charge via the Internet at http://pubs.acs.org.

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