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 (β/α)\(_8\)-barrel domain of the bidomain proteins. The assigned physiological functions in the muconate factorizing 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/NAAR; 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 (β/α)\(_8\)-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-dl-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-acrylate. Curiously, the value of $k_{cat}/K_m = 370 \text{M}^{-1} \text{s}^{-1}$ 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).

When the NAAAR was assayed for the dehydration reaction catalyzed by OSBS, the value of $k_{cat}/K_m$ was measured as $2.5 \times 10^5 \text{M}^{-1} \text{s}^{-1}$. Although this value is less than that for diffusion-controlled reactions, it is similar to that measured for the homologous OSBS from B. subtilis, $7.5 \times 10^5 \text{M}^{-1} \text{s}^{-1}$. 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 $1.7 \times 10^4$ and $2.0 \times 10^5 \text{M}^{-1} \text{s}^{-1}$, 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 (β/α)_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 ≥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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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 a 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).

H NMR (500 MHz, DMSO): 12.22 ppm (2H, s, COOH), 8.13 ppm (1H, t, J = 7.0 Hz, CONH), 4.19–4.13 ppm (2H, m, CONHCH), 2.43–2.30 ppm (4H, m, HOC6H4CH2CH2CO), 1.22 ppm (3H, d, J = 7.5 Hz, CH2CH3).

Mass spectrometry (ESI negative mode): observed, 188.05 (M − H); expected for C14H11NO5, 189.06 (M).

Cloning, Expression, and Protein Purification of the Member of the GNAT Superfamily. The gene encoding the
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 MgSO₄, 0.4 mM of each dNTP, and 40 pmol of both a forward primer (AseI recognition site; 5’-CCGCAGGGGAGAACATTAAATGCTCCCGTAGC- AATC-3’) and a reverse primer (BamHI recognition site; 5’-CCTTCTCCTTTCTGGAGATCTTTC-CTCACC-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 LB broth (LB supplemented with 100 µg/mL ampicillin) for 48 h and harvested by centrifugation. No isopropyl-β-D-thiogalactopyranoside (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 resin (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 (NH₄)₂SO₄ for further purification. The protein was eluted with a linear gradient of 0.4–0 M (NH₄)₂SO₄, 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 MgCl₂. 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 (NdeI recognition site; 5’-CAGGGG-GG- GAGGCCCATATGAAAGAAATTGTTCAGCAGATG-3’) and a reverse primer (BamHI recognition site; 5’-CTTTC- AACTTCTGGAGATCTTTC-CTCACC-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 OD₆₀₀ 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. Phenylmethanesulfonylfluoride (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.

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 (acyetyl- 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 (NH₄)₂SO₄, and 2 mM 4,4′-dipyridyl disulfide. The rate of formation of 4-thiopyridine 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 MnCl₂. 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 CdCl₂ 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 MnCl₂ by quantitating the decrease in absorbance of SHCHC (ε = 2400 M⁻¹cm⁻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. azerea*, *T. thermophilus* (both strains HB8 and HB27), and *G. kaustophilus* HTA426 (Figure 1). We could not obtain the strain of *A. azerea* 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-acetyltransferase 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-acetyltransferase. 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-acetyltransferase is an efficient catalyst for the N-succinylation of hydrophobic and basic d-amino acids.

**Table 1: Activity Screen of the N-Acyltransferase with Succinyl-CoA, Acetyl-CoA, and Both d- and l-Amido Acids**

<table>
<thead>
<tr>
<th>Compound</th>
<th>d-amino acid</th>
<th>l-amino acid</th>
<th>d-amino acid</th>
<th>l-amino acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ala</td>
<td>1.2</td>
<td>0</td>
<td>0.070</td>
<td>0</td>
</tr>
<tr>
<td>Val</td>
<td>5.6</td>
<td>0</td>
<td>5.3</td>
<td>0</td>
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<tr>
<td>Leu</td>
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<td>0.088</td>
<td>3.5</td>
<td>0.0051</td>
</tr>
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<td>Ile</td>
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<td>0</td>
<td>2.2</td>
<td>0.0013</td>
</tr>
<tr>
<td>Met</td>
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<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Phe</td>
<td>3.0</td>
<td>0.0072</td>
<td>0.0026</td>
<td>0.0026</td>
</tr>
<tr>
<td>Tyr</td>
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<td>0.95</td>
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</tr>
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<td>Trp</td>
<td>1.9</td>
<td>0.0040</td>
<td>0.41</td>
<td>0</td>
</tr>
<tr>
<td>Pro</td>
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<td>0</td>
<td>0.0015</td>
<td>0.0014</td>
</tr>
<tr>
<td>Gly</td>
<td></td>
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<td>0.018</td>
</tr>
<tr>
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<td>0.27</td>
<td>0.0083</td>
<td>0.023</td>
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<td>Cys</td>
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<td>nd</td>
</tr>
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<td>Asn</td>
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<td></td>
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<td>Gln</td>
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<td></td>
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<tr>
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<td>Arg</td>
<td>9.8</td>
<td>0.013</td>
<td>3.3</td>
<td>0.0018</td>
</tr>
</tbody>
</table>

* 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.
of the amino acid product. We attempted to measure the

\[ R \]

ninhydrin to quantitate the formation of the

\[ \text{shown}. \]

Inspection of the data in the Table reveals that the

\[ \text{member of the Zn}^{2+} \text{dithiopyridine reagent}. \]

measure kinetic constants for the L-enantiomers of some

\[ \text{N}\text{-succinyl-L-amino acids. Enzymatic activity only could be} \]

detected for the hydrolysis of some N-succinyl-l-amino acids.

Less than 0.1% of this activity could be detected for

\[ \text{N}\text{-formyl-}, \text{N}\text{-formimino-}, \text{N}\text{-acyl-}, \text{and} \]

N-succinylaminolaccases. Enzymatic activity only could be
detected for the hydrolysis of some 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-succinyltransferase of unknown function, succinyl-CoA: D-amino acid N-succinylationtransferase; (2) homologue of OSBS/NAAAR, N-succinylaminol acid racemase (NSAR); and (3) hydrolyase, 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 may 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. 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.

\[ ^{\text{a}} \text{Not determined because the amino acid reacts with the 4,4'-dithiopyridine reagent}. \]

\[ ^{\text{b}} \text{Not determined due to low solubility in the assay}. \]

\[ ^{\text{c}} \text{Rate measured at 50 mM; the low rate prevented kinetic analysis}. \]

\[ ^{\text{d}} \text{Saturation could not be achieved, so only the value of} k_{\text{cat}}/K_m \text{could be determined}. \]

N-Succinyl-L-His 1.5 ± 0.1 130 ± 20 (1.4 ± 0.3) \times 10^1

\[ \text{N-Succinyl-L-Arg} 0.9 ± 0.1 310 ± 65 (2.9 ± 0.7) \times 10^1 \]

\[ \text{N-Succinyl-L-Thr} \quad 0.71 \pm 0.03 83 ± 13 (8.6 ± 1.3) \times 10^1 \]

\[ \text{N-Succinyl-L-Gln} 0.65 ± 0.04 89 ± 18 (7.3 ± 1.5) \times 10^1 \]

\[ \text{N-Succinyl-L-Glu} 0.073 \pm 0.003 \quad - \quad - \quad - \]

\[ \text{N-Succinyl-L-Asn} 0.87 \pm 0.03 22 ± 2 41 ± 3 \]

\[ \text{N-Succinyl-L-Gly} 5.7 ± 0.4 170 ± 30 (3.4 ± 0.6) \times 10^1 \]

\[ \text{N-Succinyl-L-Asp} 0.0004^\text{c} \quad - \quad - \]

\[ \text{N-Succinyl-L-Arg} 1.0 ± 0.1 310 ± 65 (2.9 ± 0.7) \times 10^1 \]

\[ \text{N-Succinyl-L-Thr} 0.71 ± 0.03 83 ± 13 (8.6 ± 1.3) \times 10^1 \]

\[ \text{N-Succinyl-L-Arg} 0.9 ± 0.1 310 ± 65 (2.9 ± 0.7) \times 10^1 \]

\[ \text{N-Succinyl-L-His} 1.5 ± 0.1 130 ± 20 (1.4 ± 0.3) \times 10^1 \]

\[ \text{N-Succinyl-L-Fum} 0.0001^\text{c} \quad - \quad - \quad - \]

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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)₆-barrel domain. Previously, we had recognized the MLE, OSBS, and L-Ala-t/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-t/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-t/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.

**REFERENCES**