Prephenate decarboxylases: a new prephenate-utilizing enzyme family that performs non-aromatizing decarboxylation en route to diverse secondary metablolites

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Abstract

Prephenate is the direct precursor of phenylpyruvate and 4-hydroxyphenylpyruvate in the biogenesis of phenylalanine and tyrosine by action of the decarboxylative, aromatizing enzymes prephenate dehydratase and dehydrogenase, respectively. The recent characterization of BacA in bacilysin biosynthesis as a non-aromatizing decarboxylase reveals a new route from prephenate in the biosynthesis of nonproteinogenic amino acids. This study describes two additional enzymes, AerD from *Planktothrix agardhii* and SalX from *Salinispora tropica*, that utilize the central building block prephenate for flux down distinct pathways to amino acid products, representing a new metabolic fate for prephenate and establishing a new family of non-aromatizing prephenate decarboxylases.

Prephenate has been known for decades as the product of the celebrated 3,3-sigmatropic rearrangement of chorismate, a reaction that is classically followed by formation of the aromatic amino acids phenylalanine and tyrosine (1). Prephenate dehydratase and prephenate dehydrogenase are the enzymes that direct prephenate to Phe and Tyr by decarboxylation and aromatization of the cyclohexenyl ring with concomitant loss of the C-7 hydroxide or hydride, respectively. Recently we characterized the *Bacillus subtilis* prephenate dehydratase homolog BacA, which is involved in the biosynthesis of bacilysin (**1**,Figure 1), and catalyzes the first step of a 4-enzyme pathway that transforms prephenate to a distinct non-aromatic amino acid product (2). Like prephenate dehydratase, BacA decarboxylates prephenate at C-4 yet uniquely protonates C-6 or C-6' to yield the endocyclic diene dihydro-4-hydroxyphenylpyruvate (H$_2$HPP, **5**,Figure 2A) (2). This diene is then subject to nonenzymatic isomerization to the exocyclic diene in a reaction that can be dramatically accelerated by BacB to yield the indicated H$_2$HPP regioisomer **6**. Here we identify two homologs to BacA from distinct microbial secondary metabolite pathways that catalyze the same non-aromatizing decarboxylation of prephenate, thus establishing a new family of enzymes. The first of these is AerD from the cyanobacterium *Planktothrix agardhii,* which is involved in the production of the nonribosomal glycopeptides

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aeruginoside 126A and B (**3**,Figure 1) (3). These natural products contain the unusual amino acid residue 2-carboxy-6-hydroxy-octahydroindole (Choi) (**7**) (4,5). The second enzyme is SalX in the marine actinomycete *Salinispora tropica* biosynthetic pathway to the proteasome inhibitor salinosporamide A (**2**) (6–8). Specifically, SalX is relevant to the production of the nonproteinogenic amino acid building block cyclohexenylalanine (**9**; H$_4$Phe) (9).

In a study of nonribosomal peptide synthetase gene clusters in the cyanobacterium *P. agardhii* CYA126/8, gene insertions led to the discovery and identification of novel aeruginoside glycopeptides (3). Aeruginosin natural products, which are potent inhibitors of serine proteases, typically contain the unusual amino acid residue Choi (4). Three of the genes in the aeruginosin cluster, *aerDEF*, were found to be relevant to Choi production (3). Ishida et al. noted the similarity of *aerDEF* to the *bacABC* genes in the bacilysin gene cluster in *B. subtilis*. However, at the time of the aeruginosin gene cluster discovery, the specific roles of the BacABC proteins were unknown, so it was unclear what catalysts the congeneric *aer* genes were encoding. For example, it was hypothesized that arogenate, not prephenate, might be the entry point onto the Choi pathway (3). As noted by Ishida et al., AerD is 33.8% identical to BacA, so we sought to determine whether AerD was in fact a catalytic homolog of BacA. Because the aeruginoside producer *P. agardhii* CYA126/8 and its DNA were not readily available, we chose to use a synthetic gene for AerD (DNA 2.0), which was optimized for expression in *Escherichia coli* (Figure S2). This gene was cloned into a pET-28b vector equipped with an N-terminal His$_6$ tag. As shown in Figure S3, excellent overproduction of soluble protein ensued, allowing purification of 14 mg/liter of culture. We assayed AerD with prephenate as a substrate utilizing UV scanning kinetics and readily observed the conversion to an A$_{258}$ nm species, indicative of endocyclic H$_2$HPP diene **5** (Figure 2,S4a). As we had found in studies with BacA from *B. subtilis*, H$_2$HPP **5** non-enzymatically isomerized to the thermodynamically favored conjugated exocyclic diene **6** (2). ^{1}H NMR analysis validated that AerD ultimately generates H$_2$HPP **6** (Figure S4b, Table S1). The k_{cat} (245 ± 1 min^{-1}) and K_m (170 ± 3 μM) values for prephenate conversion to initial endocyclic diene **5** for AerD compare favorably with those for BacA. Thus it is very likely prephenate is the physiological substrate for AerD in the initial step in construction of the bicyclic amino acid Choi.

Salinosporamide A (**2**) from *S. tropica* CNB-440 has a high density of functionality built into its scaffold. The bicyclic β-lactone-γ-lactam framework is a latent warhead and the C-2 chloroethyl side chain acts as a covalent trap for irreversible capture of the proteasome (10). The third element of the salinosporamide scaffold is the nonproteinogenic 3-hydroxyl-L-cyclohexenylalanyl residue (referred to here as a β-hydroxylated H$_4$Phe (**9**)), which governs the binding specificity of the natural product to the proteasome (11). Examination of the salinosporamide biosynthetic gene cluster has indicated a set of *sal* genes that could be involved in H$_4$Phe formation, among them SalX (8). Although our initial proposal suggested SalX might function similar to a prephenate dehydratase for a putative dihydroprephenate substrate (12), we decided to test it for conversion of prephenate to **5** and then **6**, given 35% sequence identity to BacA. Toward this end, the *salX* gene was amplified from *S. tropica* genomic DNA and ligated into an N-His$_8$ vector for expression in *E. coli*. As shown in Figure S3, moderate overproduction of soluble enzyme was obtained at 5 mg/liter of culture (Figure S3). UV assays revealed that SalX also converts prephenate to endocyclic diene H$_2$HPP **5** as the initial product before spontaneous isomerization to exocyclic diene **6** (Figure 2,S4a), which was further confirmed by ^{1}H NMR (Figure S4b, Table S2). While SalX had a 16–22 fold lower k_{cat} than AerD and BacA, it also had a lower K_m for prephenate. The SalX k_{cat}/K_m catalytic efficiency ratio of 0.73 min^{-1} μM^{-1} is only 2–4 fold lower than BacA and AerD, making it likely that prephenate is a physiologic substrate for SalX. To further probe whether prephenate is indeed the natural substrate for SalX and if

H$_2$HPP **6** is an intermediate in H$_4$Phe production, feeding studies were preformed with a previously described *S. tropica salX$^−$* disruption mutant that lacks salinosporamide production (9). H$_2$HPP **6** was generated and purified from an *in vitro* transformation of prephenate by SalX and then added aseptically to a 50 mL culture of the Δ*salX S. tropica* mutant. After 5 days the culture was extracted and HPLC analysis indicated restoration of salinosporamide production (Figure S5), which was confirmed by LCMS analysis with authentic standards. This chemical complementation confirmed that **6** is a pathway intermediate of **2** biosynthesis in *S. tropica*.

We establish here a new family of prephenate decarboxylases that produces a non-aromatized H$_2$HPP product. The three new members of this family, BacA, AerD and SalX, are capable of initiating divergent pathways that transform the primary metabolite prephenate into distinct non-proteinogenic amino acids used in natural product production. The intriguing aspect of these enzymes is that while decarboxylation occurs similar to prephenate dehydratases and dehydrogenases, these enzymes avoid aromatization by delivery of a proton to C-6 or C-6' to yield a diene product. The net result is conversion of the 5,8-cyclohexadiene-7-ol in prephenate to a 4,8-cyclohexadiene-7-ol in the H$_2$HPP product **5**. Isotopic labeling studies in *S. tropica* demonstrated that both halves of the cyclohexenyl ring of prephenate are distinguished during the biosynthetic transformation, suggesting a regioselectivity on the part of SalX (12). A similar biosynthetic observation was established in studies of the conversion of prephenate to the antibiotic 2,5-dihydrophenylalanine (13). However, additional detailed stereochemical analyses will be necessary to determine if all three enzymes share the same regio- and stereoselectivity in regards to protonation. Two possible mechanistic scenarios arise, one in which the reaction could involve a concerted pericyclic reaction where the C-4 carboxylate becomes protonated and in turn donates a proton to C-6 (or C-6') as CO$_2$ is evolved, or a second mechanism in which a BH$^+$ conjugate acid in the enzymatic active site acts as the proton source. We are presently pursuing crystallization trials with BacA, AerD, and SalX to shed light on the mechanism and determine how these enzymes avoid the aromatization of prephenate that is typical of prephenate dehydratase and prephenate dehydrogenase-catalyzed transformations.

An initial BLAST search finds many other examples of proteins homologous to these enzymes, several of which are located near potential biosynthetic enzymes in host genomes such as *Nodularia spumigena, Clostridium celluloytican, Photorhabdus luminescens,* and *Erwinia amylovora*, the latter of which is known to produce 2,5-dihydrophenylalanine (DHPA, (14). DHPA is also produced in a variety of *Streptomyces* species, with recent data indicating production in *P. luminescens* (15) as well. Isotopic feeding studies in *S. arenae* demonstrated that DHPA is derived from prephenate (13), which suggests this enzyme family may be diverting prephenate into unique small molecule products across a broad array of microorganisms.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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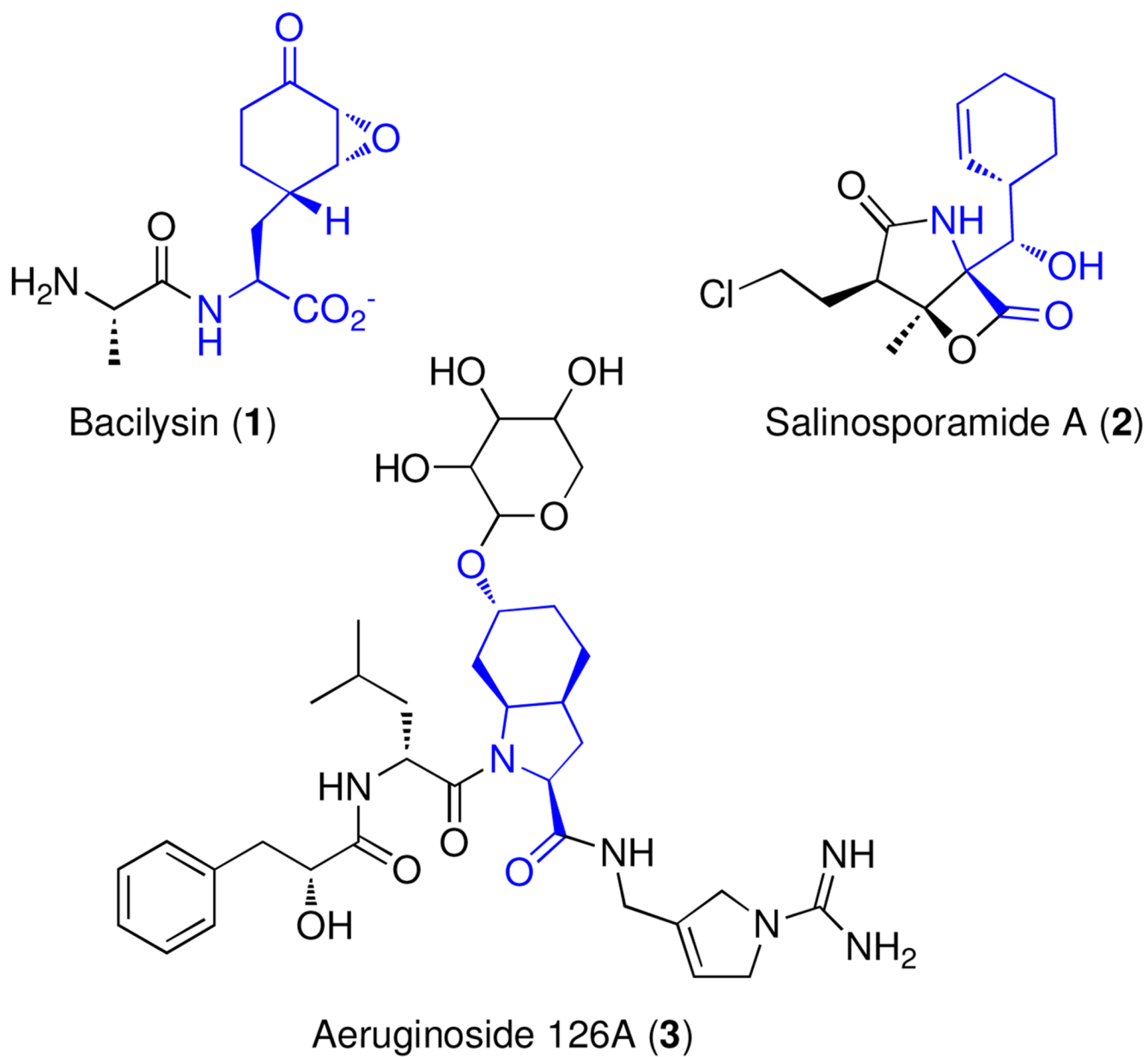
Figure 1.
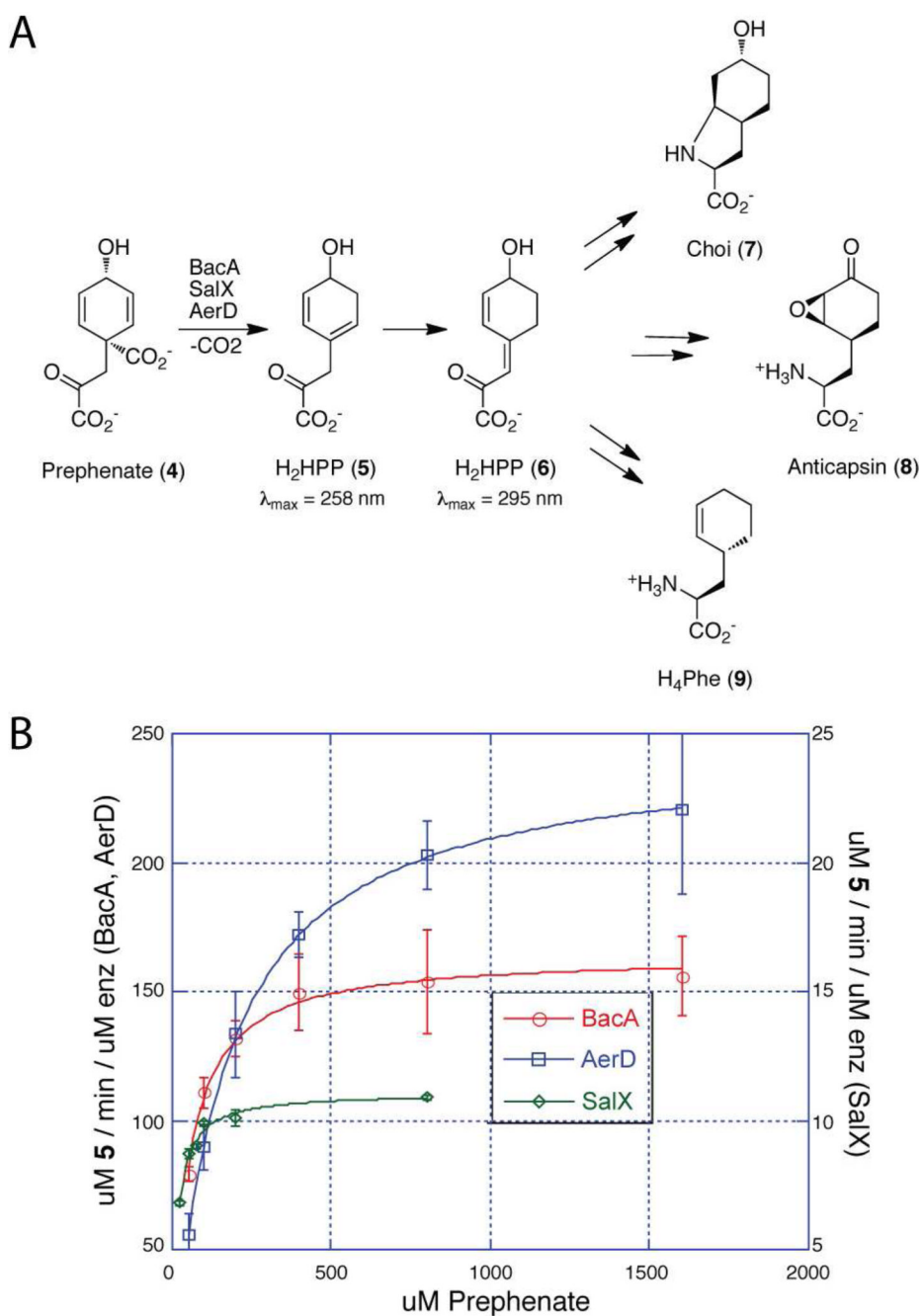Natural products containing amino acids (blue) derived from prephenate.

Figure 2.
A) Transformation of prephenate first to H_2HPP **6**, which is eventually incorporated into various amino acids. B) Kinetics of BacA, AerD and SalX.