Mutational Analysis of the Epimerization Domain in the Initiation Module PheATE of Gramicidin S Synthetase†

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ABSTRACT: The epimerase (E) domain of the three-domain (ATE) initiation module of *Bacillus brevis* gramicidin S synthetase equilibrates the Cα configuration of the phenylalanyl moiety presented as Phe-S-4′-phosphopantetheine-modified (Ppant) acyl enzyme. Mutants at 22 residues of this E domain that are conserved across the approximately 450 residue E domains of nonribosomal peptide synthetases were constructed, and the PheATE* derivatives expressed in *Escherichia coli* as C-terminal His tag fusions and then purified and assayed for three activities: (1) the L-Phe Cα-[^{3}H] exchange to solvent, (2) the rate of approach to D-Phe/L-Phe-S-Ppant acyl enzyme equilibrium from either L- or D-Phe, and (3) the rate of Phe-Pro dipeptidyl-S-Ppant enzyme formation with the downstream ProCAT module. We found that for wild-type PheATE epimerization is much faster than subsequent condensation, leading to a 1.9:1 ratio of D-Phe-S-Ppant/L-Phe-S-Ppant acyl enzyme. Only D-Phe is then transferred to yield D-Phe-L-Pro-S-Ppant ProCAT acyl enzyme. Among the mutants generated, three PheATE* constructs, H753A, D757S, and Y976A, showed no detectable Cα-^{3}H washout, while E892A and R896A were among a larger set partially impaired. All these mutants were dramatically impaired in approach to D-Phe/L-Phe-S-Ppant equilibrium from either D- or L-Phe, while another construct, D767S, was asymmetrically impaired only for D-to-L-Phe direction. In the D-Phe-L-Pro dipeptidyl-S-Ppant condensation assay, the H753A and E892A forms of PheATE* were only slightly active from L-Phe but unimpaired from D-Phe; N975A epimerizes faster than Y976A from L-Phe. When the chirality of the Phe-Pro-diketopiperazine released product was analyzed the D,L/L,L ratio from wild-type PheATE and ProCAT was 98:2. From E892A and N975A it was comparably 95:5 and 92:8, but H753A and Y976A yielded 56% of the L,L-product, reflecting a gain of function to transfer L-Phe. The 98:2 preference of wild-type PheATE for D-Phe transfer reflects the kinetically controlled stereopreference of the condensation (C) domain of ProCAT for the D-Phe-S-Ppant donor substrate. It may be that other NRPS C domains immediately downstream of E domains will likewise be D-selective.

One hallmark of nonribosomal peptide antibiotics is the presence of D-amino acids, often in substantial abundance (*1*, *2*). For example, the tripeptide precursor of penicillins and cephalosporins is L-δ-aminoadipyl-L-cysteinyl-D-valine, while four of seven constituents in the glycosylated backbone of vancomycin group heptapeptide antibiotics are D-amino acids. The D-configuration of such residues may be in part utilized to slow proteolytic degradation of peptide antibiotics, given that most proteases exhibit selectivity for L-amino acid residues. On the other hand, they may also play a functional role, e.g., by setting conformers and side chains for subsequent processing steps, such as the oxidative cyclizations in penicillin and vancomycin biosyntheses.

The D-amino acids found in nonribosomal peptide synthetase-, (NRPS-) generated antibiotics or siderophores can arise from two routes: (1) the direct activation and incorporation of D-amino acids by the gate-keeping adenylation (A) domains or (2) in situ epimerization of an L-aminoacyl Cα center during the peptide chain elongation process. While the first route is known for the initiation module of cyclosporin A synthetase (D-Ala-specific) (*3*), the second mechanism seems to represent the more general solution by NRPSs (*1*, *2*). The reaction is effected by distinct catalytic epimerization (E) domains, ca. 450 aa in size, embedded within each module that converts an L-amino acid to the corresponding D-isomer.

Generally, NRPSs display a multimodular organization of semi-autonomous domains, and core domains include the A domain for ATP-dependent activation of the cognate amino acid as aminoacyl-O-AMP, and its paired peptidyl carrier protein [PCP; also known as thiolation (T) domain] (*1*, *2*, *4*). Each carrier protein domain bears a posttranslationally

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[1] Abbreviations: aa-S-Ppant T, aminoacylated thioester form of cofactor 4′-phosphopantetheine-modified holo thiolation domain; A, adenylation domain; ATP, adenosine 5′-triphosphate; BSA, bovine serum albumin; C, condensation domain; CoASH, coenzyme A; DKP, diketopiperazine; DTT, D,L-dithiothreitol; E, epimerization domain; EDTA, ethylenediaminetetraacetic acid; HPLC; high-performance liquid chromatography; LSC, liquid scintillation counting; MES, 2-(*N*-morpholino)ethanesulfonic acid; NRPS; nonribosomal peptide synthetase; PCR, polymerase chain reaction; Ppant, 4′-phosphopantetheine; PP_i, inorganic pyrophosphate; T, thiolation domain; TCA, trichloroacetic acid; TLC, thin-layer chromatography; Tris; tris(hydroxymethyl)-aminomethane.

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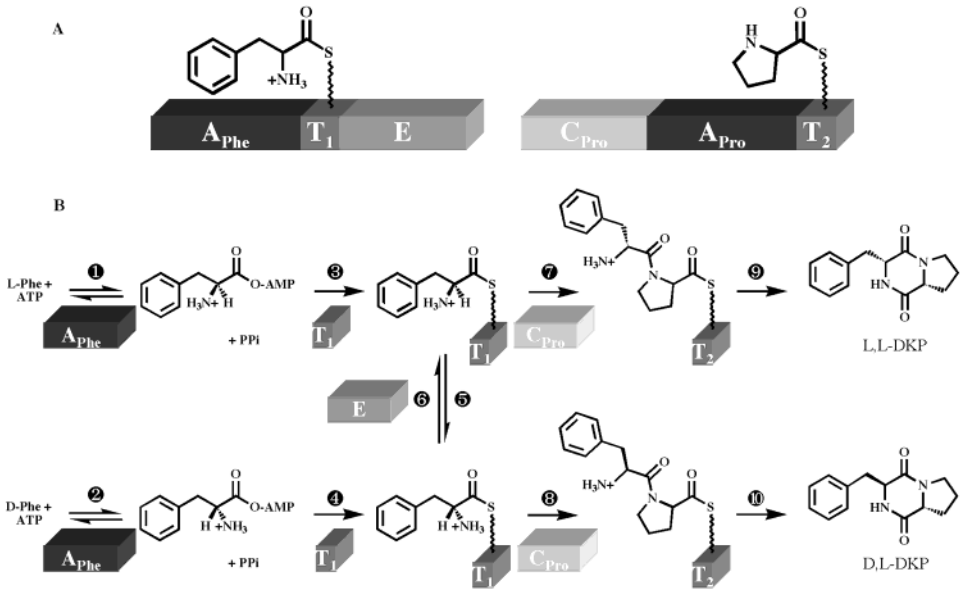

FIGURE 1: Bis-modular NRPS system under investigation. (A) The domain organization of the initiation module PheATE and the elongation module ProCAT is shown with their subdomains highlighted: A domains (A_{Xaa}, black), holo thiolation domain (T, dark gray), peptide bond-forming C domain (C_{Pro}, light gray), and epimerase domain (E, medium gray). (B) Stereoselective formation of D-Phe-L-Pro and L-Phe-L-Pro DKP, catalyzed by the bimodular system, has been dissected into a ten-step reaction sequence: steps 1 + 2, ATP-dependent formation of L/D-Phe-O-AMP (catalyst A_{Phe}); steps 3 + 4, transfer to the paired T domain and formation of L/D-Phe-S-Ppant (requires holo T_1); steps 5 + 6, interconversion (epimerization) of L/D-Phe-S-Ppant (catalyst E); steps 7 + 8, translocation of L/D-Phe to ProCAT and formation of L/D-Phe-L-Pro-S-Ppant dipeptidyl enzyme (requires L-Pro-S-Ppant T_2, catalyst C); and steps 9 + 10, autocatalytic release of L,L- or D,L-DKP.

introduced phosphopantetheinyl (Ppant) thiol to which the aminoacyl moiety of the aminoacyl adenylate is transferred to yield aminoacyl-S-Ppant T domain (*5*, *6*). Peptide bond formation between two pairs of AT domains requires a third type of core domain, the condensation (C) domain, responsible for the peptide bond-forming step (*7*). Thus each NRPS elongation module has a CAT triadic core, and in an AT_n-CAT_{n+1} double module competent for chain elongation, the growing aminoacyl (or peptidyl) chain is in thioester attachment to the upstream T_n domain and is transferred to the attacking aminoacyl monomer in thioester linkage to the downstream T_{n+1} domain.

E domains are auxiliary domains that can be accommodated by modules where the amino acid to be activated is L-configured but is incorporated into the growing peptidyl chain as the D-isomer. They can be embedded either in initiation modules (ATE) as in gramicidin S synthetase (*8*), where the first residue to be incorporated is D-Phe, or in internal elongation modules (CATE) as in the third module of AcvA synthetase (*9*−*11*), to provide a D-Val residue in penicillin precursor biosynthesis. Since the PheATE module of gramicidin S synthetase showed only low levels of racemase activity with the free amino acid (*12*), the Phe-S-Ppant thioester is the most probable candidate for a thermodynamically activated substrate as pointed out also by Stein et al. (*13*). Thus an ATE initiation module would be able to epimerize the L-aminoacyl-S-Ppant enzyme into the D-aminoacyl-S-Ppant species (*14*), while the E domain in CATE elongation modules are thought to epimerize the corresponding peptidyl-S-Ppant acyl enzyme intermediates (*15*, *16*).

The initiation module PheATE (Figure 1A) is a good starting point for analysis of E-domain mechanism and specificity because of its simplicity, but also in part because the A domain of this ATE module will activate both L- and D-Phe (*13*, *14*). Thus, both L-Phe-S-Ppant and the D-Phe-S-Ppant T domain are accessible in cis as substrates for the E domain. Another advantage of the PheATE/ProCAT system is that purified PheATE of the gramicidin S synthetase (GrsA) will interact with the next module ProCAT to produce the Phe-Pro-S-Ppant dipeptidyl enzyme (Figure 1) and that the Phe-Pro dipeptide is spontaneously released and assayable as the Phe-Pro diketopiperazine (DKP) (*7*). Since in the wild-type system only D-Phe is transferred to ProCAT, this opens up the question how L-Phe transfer is suppressed and whether discrimination in favor of D-Phe is achieved by the C or E domain. Apparently the E domain is somehow required for the intermodular aminoacyl (D-Phe) transfer, since the corresponding PheAT double domain is incompetent for both D- and L-Phe transfer (*7*). Further efforts to express the E domain separately in functional form have failed so far, although a PheTE construct is functional for epimerization when loaded with Phe in trans by the action of PheA (this study; data not shown).

Among the questions about E domain function are (1) how rapidly the internal L/D-Phe-S-Ppant thioester equilibrium is established, both on an absolute scale and relative to the rate of the subsequent condensation reaction, and (2) what residues of E domains are important for Cα-H cleavage and reprotonation starting from either D- or L-Phe-S-Ppant substrates. To address these questions, mutants in residues conserved across 45 E domains (Figure 2) have been constructed in the E domain of the tridomain GrsA (here PheATE) and assayed to monitor the following three stages of E domain function: (1) L-Phe-Cα-H abstraction and exchange into solvent; (2) the rate and extent of epimerization of Phe-S-Ppant enzyme by measuring the ratio of D-Phe-S-Ppant/L-Phe-S-Ppant, approaching from either the L- or D-Phe direction; and (3) the catalytic competence for PheATE mutants to form dipeptide with ProCAT.

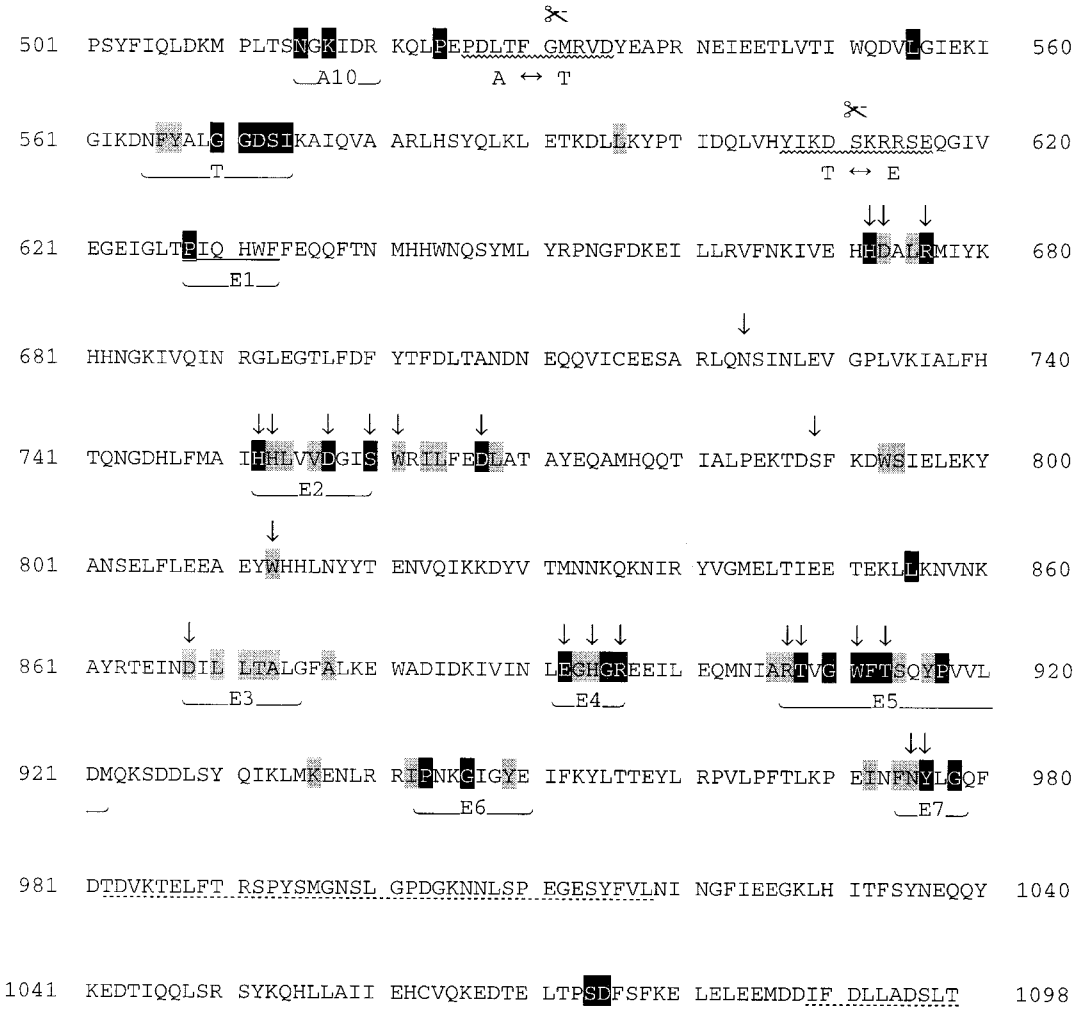

FIGURE 2: Primary sequence of the C-terminus of PheATE shown as an archetype of 45 E domains identified in NRPS. Sequence alignments of E domains revealed 23 absolutely invariant residues (black boxes) and 30 highly conserved residues (gray boxes). Core motifs are highlighted in accordance with ref *1*, and targets for site-directed mutagenesis (this work) are indicated by arrows. Wavy lines mark the junctures between PheA and PheTE, as well as between PheAT and PheE. Dotted lines indicate regions of low overall similarity between internal and C-terminal E domains.

EXPERIMENTAL PROCEDURES

Cloning and Expression of PheATE Mutants. All PheATE E-domain mutants were constructed by site-directed mutagenesis of pPheATE (*7*) with the QuikChange site-directed mutagenesis system from Stratagene. Design of the primers, PCR amplification of the entire plasmid, and cloning of the amplified DNA was performed in accordance with the manufacturer's protocol. Standard procedures were applied for DNA manipulations and the preparation of the recombinant plasmids in *Escherichia coli* strain XL1-Blue. The integrity of all constructs was confirmed by DNA sequencing at the Molecular Biology Core Facilities of the Dana-Farber Cancer Institute (Boston, MA).

Expression of the mutant genes and purification of the His_6-tagged proteins was accomplished as described previously (*7*). All constructs are appended by a C-terminal GSRSHHHHHH tag, and as judged by SDS−PAGE, most proteins could be purified to apparent homogeneity by single-step Ni^{2+}-affinity chromatography (compare Table 1). Fractions containing the recombinant proteins were pooled and dialyzed against assay buffer (20 mM MES, pH 8.0, 100 mM sodium chloride, 10 mM magnesium chloride, and 1 mM EDTA). The proteins could be stored at −80 °C over several months with no observable loss of activity, and protein concentrations were determined by use of the calculated extinction coefficients for their absorbance at 280 nm (A_{280nm}): 138 690 M^{-1} cm^{-1} for PheATE and all PheATE* mutants except W761A and W911A (A_{280nm} = 133 000 M^{-1} cm^{-1}), as well as Y976A (A_{280nm} = 137 410 M^{-1} cm^{-1}). Preparation of holo ProCAT was performed as described previously (*7*).

Priming of heterologously expressed proteins was achieved by incubation with CoASH and *Bacillus subtilis* Ppant transferase Sfp as described previously (*17*, *18*). Reaction mixtures in assay buffer contained 2.5 μM apoenzyme, 100 μM CoASH, and 25 nM Sfp and were incubated for 30 min at 37 °C.

ATP−Pyrophosphate Exchange Assay. The ATP−pyrophosphate exchange reactions were performed to examine activity, specificity, and correct A-domain folding of all recombinant proteins purified. Reaction mixtures in assay buffer (final volume 100 μL) contained 0 or 2 mM D-Phe and 250 nM enzyme. The reaction was initiated by the addition of 2 mM ATP, 0.2 mM tetrasodium pyrophosphate, and 0.15 μCi (16.06 Ci/mmol) of tetrasodium [^{32}P]pyro-

Table 1: List of Generated PheATE* Mutants and Their Catalytic Properties (i.e., Rates)

PheATE* mutant	solubility[a]	folding[b]	Cα-proton washout (min^{-1})	epimerization: L-to-D direction (step 5)[c]		epimerization: D-to-L direction (step 6)[c]		DKP formation: with L-Phe		DKP formation: with D-Phe	
				min^{-1}	% D-Phe	min^{-1}	% D-Phe	min^{-1}	% L,L	min^{-1}	% L,L
wt	+	+	>100	>2	66	>2	66	0.1	2	0.07	0
H672A	O	−	12	>2	70	0.3	76	0.07	4	0.03	nd[d]
D673A	−	−	nd	nd	nd	nd	nd	nd	nd	nd	nd
D673S	+	O	7	1	40	<0.01	99	<0.001	0	nd	nd
R676A	O	−	nd	nd	nd	nd	nd	<0.001	0	nd	nd
N724A	+	+	>100	>2	70	>2	63	0.08	3	0.07	nd
H752A	+	+	0.8	>2	58	>2	66	0.08	5	0.06	nd
H753A	+	+	<0.01	<0.01	2	<0.01	95	<0.001	40	0.08	0
D757A	−	−	nd	nd	nd	nd	nd	nd	nd	nd	nd
D757S	O	O	<0.01	<0.01	0	<0.01	99	<0.001	2	nd	0
S760A	+	+	>100	>2	65	>2	65	0.07	5	0.07	nd
W761A	+	+	3	>2	53	>2	70	0.08	3	0.06	nd
D767S	+	+	>100	>2	63	<0.01	97	0.1	2	nd	0
S789A	+	−	0.5	0.06	38	nd	nd	nd	nd	nd	nd
W813A	O	−	>100	2	64	nd	nd	nd	nd	nd	nd
D868S	+	+	>100	>2	63	0.9	71	0.09	1	0.08	nd
E892A	+	+	14	<0.01	22	<0.01	100	<0.001	5	0.03	0
H894A	+	+	0.5	>2	54	>2	70	0.11	5	0.12	nd
R896A	O	O	1	<0.01	0	<0.01	100	0.01	4	0.04	0
R907A	O	−	15	>2	42	nd	nd	nd	nd	nd	nd
T908A	+	−	>100	>2	51	nd	nd	nd	nd	nd	nd
W911A	+	+	0.2	2	47	1.1	60	0.05	5	0.07	nd
T913A	+	+	>100	>2	71	>2	69	0.09	2	0.08	nd
N975A	+	+	0.03	0.8	59	0.4	64	0.03	8	0.09	0
Y976A	+	+	<0.01	0.9	54	1.3	70	0.003	56	0.1	0
H753A/N975A	+	+	<0.01	<0.01	0	<0.01	98	<0.001	36	0.08	nd

[a] As indicated by the SDS−PAGE analysis of supernatant and pellet after cell lysis. [b] As indicated by the results of the ATP−PP_i exchange assay. [c] As shown in Figure 1B. [d] nd, not determined.

phosphate (NEN) and incubated at 37 °C for 10 min. Reactions were quenched by adding 0.5 mL of a stop mix containing 1.2% (w/v) activated charcoal, 0.1 M tetrasodium pyrophosphate, and 0.35 M perchloric acid. Subsequently, the charcoal was pelleted by centrifugation, washed once with 1 mL of water, and resuspended in 0.5 mL of water. After addition of 3.5 mL of liquid scintillation fluid (Ultima Gold; Packard), the charcoal-bound radioactivity was determined by liquid scintillation counting (LSC) in a Beckman LS1801 scintillation counter.

Radioassay for the Detection of Cα-H Washout. The proton-transfer reaction between enzyme and C2-proton of the substrate was investigated by loading two different species of Phe. Ring-labeled L-[4-^{3}H]Phe was used to internally calibrate the system for loading with 1 equiv of label. In contrast, loading with L-[2,3-$^3H_2$]Phe can give 1 or 2 equiv of label, depending on whether the Cα-proton is progressively washed out. Reaction mixtures in assay buffer (final volume 400 μL) contained 2 mM ATP and 500 nM enzyme. The reaction was initiated by the addition of 1.48 μM [^{3}H]Phe (ring or chain labeled; 30 or 15 Ci/mmol, respectively) and incubated at 37 °C. At various time points, 50 μL samples were taken and immediately quenched by the addition of 0.8 mL of 10% TCA (w/v) and 20 μL of BSA solution [2% (w/v)]. The TCA precipitate was washed once with 0.5 mL of 10% TCA (w/v) and solubilized in 150 μL of 1 M Tris, and the acid-stable label was quantified by LSC as described above.

Radio-TLC Assay for Detection of Phe-S-Ppant Epimerization. To investigate the conversion of L-Phe-S-Ppant enzyme to the D-Phe-S-Ppant species (and vice versa), PheATE and mutants were allowed to activate and covalently load [^{14}C]Phe of high specific activity. Reaction mixtures in assay buffer (final volume 200 μL) containing 8.9 μM L-[^{14}C]Phe (450 mCi/mmol) or 71.4 μM D-[^{14}C]Phe (56 mCi/mmol) as well as 500 nM enzyme were incubated for 3 min at 37 °C. After quenching with 0.8 mL of 10% TCA (w/v), the precipitates were washed in succession with 1 mL of ether/ethanol (1:3 v/v) and 1 mL of ether. The [^{14}C]Phe-S-Ppant/enzyme complexes were hydrolyzed by incubation with 100 μL of 0.1 N potassium hydroxide for 30 min at 75 °C, extracted with 1 mL of methanol, and centrifuged at 4 °C and 13 000 rpm for 30 min. Subsequently, supernatants containing the released [^{14}C]Phe were transferred to fresh tubes. After removal of the solvent under vacuum, the residues could be dissolved in 10 μL of 50% methanol (v/v) and applied (2 μL) to chiral TLC plates (Aldrich) in order to separate D- and L-isomers. The TLC was developed in solvent A (acetonitrile/water/acetic acid, 4:1:1 v/v/v). Autoradiographies of TLC plates were obtained with BAS−IIIS imager plates after 3−24 h of exposure on a Bio-Imaging Analyzer BAS1000 (Fuji), and analyzed densitometrically with NIH Image 1.6.1 software.

TLC separation and subsequent autoradiography was also utilized to confirm the integrity of both [^{14}C]Phe substrates. These studies revealed an optical purity of >99% for L-[^{14}C]-Phe and 95% for D-[^{14}C]Phe (data not shown).

Radioassay for the Detection of Phe-Pro Diketopiperazine Formation. To assay for dipeptidyl-S-Ppant product formation, holo-ProCAT was loaded with L-[^{3}H]Pro and mixed with purified holo PheATE (wild type or mutant) that had been loaded beforehand with either L- or D-Phe. Reaction mixtures in assay buffer (final volume 200 μL) contained 2 mM ATP and 1 μM enzyme, as well as the cognate amino

acid (1.33 μM L-[5-^{3}H]Pro (30 mCi/mmol) or 4 mM L- or D-Phe). Simultaneously, 200 μL of each solution was preincubated at 37 °C to ensure a complete aminoacylation of both modules. After 3 min, the condensation reaction was initiated by combining equal volumes of PheATE (wild type or mutants) and ProCAT, and at various time points 50 μL samples were taken and immediately quenched by the addition of 0.5 mL of 10% TCA (w/v). The reaction mixtures were extracted with 0.5 mL of butanol/chloroform (4:1 v/v), the organic layers were washed once with 0.5 mL of 0.1 M sodium chloride, and the amount of extractable label (DKP) was quantified by LSC.

Analysis of the Stereochemistry of Diketopiperazines. The identity of released DKP diastereoisomers was established by HPLC with coinjection with authentic standards, whose stereochemistry had been verified by ^{1}H NMR spectroscopy. Reaction mixtures in assay buffer (final volume 200 μL) containing 2 mM ATP, 1 μM PheATE (wild type or mutant), 1 μM ProCAT, 1.48 μM L-[4-^{3}H]Phe (27 mCi/mmol), and 2 mM L-Pro were incubated overnight at 37 °C. After extraction with 0.5 mL of butanol/chloroform (4:1 v/v), the organic layers were washed once with 0.5 mL of 0.1 M sodium chloride and transferred to fresh tubes. Subsequently, the organic solvent was removed under vacuum. The remainders of each reaction were dissolved in 800 μL of 30% methanol, blended with authentic L,L and D,L-DKP standards and applied to a reverse-phase HPLC column (Vydac, C18, 4.6 × 250 mm, 5 mμ, 300 Å) equilibrated with the same buffer. D,L and L,L isoforms of Phe-Pro DKP could be separated by applying a linear gradient from 30% to 60% methanol (flow rate 1 mL/min) over 15 min. In the relevant time frame (between 8.5 and 13 min), samples were taken (6 s/fraction) and examined for their absorbance at 220 nm, as well as the amount of tritium label (by LSC).

For preparation and characterization of the standards, 2 mL reactions were performed under essentially the same conditions except both substrates, L-Phe and L-Pro, were used at 4 mM. The DKPs were prepared and purified as described above. The two products, D-Phe-L-Pro DKP and L-Phe-L-Pro DKP, were collected separately, dried, dissolved in $CDCl_3$, and analyzed on a Bruker AMX 500 spectrometer. Characteristic chemical shifts in the proton nuclear magnetic resonance spectra of both compounds were in agreement with published data (*19*), and resonance peaks could be assigned to the following protons: m at 1.56−2.20 ppm (for D,L-DKP) or 1.80−2.35 ppm (for L,L-DKP) to Pro Cδ-H and Pro Cγ-H, m at 2.96 ppm (D,L) or 4.05 ppm (L,L) to Pro Cα-H, dd at 3.08−3.16 ppm (D,L) or 2.80 + 3.60 ppm (L,L) to Phe Cβ-H, m at 3.52 ppm (D,L) or 3.64 ppm (L,L) to Pro Cδ-H, dd at 4.24 ppm (D,L) or 4.32 ppm (L,L) to Phe Cα-H, br s at 6.19 ppm (D,L) or 5.67 ppm (L,L) to N−H, and m at 7.25 ppm (D,L) or 7.27 ppm (L,L) to aromatic Phe C-H.

RESULTS

Homology Searches To Select Sites for E-Domain Mutations. E domains of NRPSs lack significant homology with known amino acid and carboxylic acid racemases (e.g., mandelate racemases) (*1*). They share, however, a signature sequence motif HHxxxDxxSW (the so-called His motif) with a superfamily of acyl transferases (*20*). It has been noted that this motif is also conserved within C domains of NRPSs (*20*) and that the second His and the Asp residues are indispensable for C-domain activity (*7*, *21*). To identify possible catalytic key residues of NRPS epimerases, we started our study with alignments of the primary sequences of E domains. A scan of 45 E domains revealed an overall similarity ranking from 16% (between PksF and Hts1) to 99% (between LchAA and LicA), with an average percentage of similarity of 36% (data not shown). Among all E domains investigated, we found 23 absolutely invariant residues, of which 9 have aliphatic and 14 functionalized side chains (Figure 2). This alignment also revealed 30 highly conserved (but not invariant) residues, of which 15 have functionalized side chains.

Generation and Characterization of E-Domain Mutants of PheATE. In this study, 12 invariant and 10 conserved residues have been mutated and one double mutant (H753A/N975A) was constructed (Figure 2 and Table 1). The first round of mutations was an alanine scan and included the conserved loci HDxxR (positions 672−676) and HHxxxDxx-SW (His motif or E2; positions 752−761), as well as the motifs E4 (ExHxR; positions 892−896), E5 (RTxxWxT; positions 907−913) and E7 (NY; positions 975 and 976). While the first two motifs are conserved between E and C domains, the latter are characteristic only for E domains (*1*). The mutant subunits (PheATE*) were individually expressed with C-terminal His_6-tags in *E. coli* and purified by Ni^{2+}-affinity chromatography. Of these 21 PheATE derivatives expressed, 14 could be purified to homogeneity in equivalent quantities, whereas 7 mutants revealed a decreased solubility that allowed us to do only a basic characterization of these constructs (Table 1). In this connection, the largest influence was observed for the substitution of two Asp residues, D673 and D757, indicating that these residues may (also) play a structural role. To solve this problem, we applied the suggestions of Bordo and Argos for safe substitutions in site-directed mutagenesis (*22*). They observed conserved topological structures in various enzyme families that allowed the structural equivalency of residues in homologous structures, predicting that the preferred substitute for an Asp residue, especially when topologically exposed, is a Ser. Thus, a second round of mutagenesis of two invariant (D757 and D767) and two conserved (D673 and D868) Asp moieties was performed toward serine. These Asp-to-Ser mutants did in fact display an improved solubility and, with the exception of mutant D757S, all constructs could be also purified to homogeneity in equivalent amounts.

To test for correct folding, all constructs were subjected to a D-Phe-dependent ATP−PP_i exchange assay, which assesses the activity (and selectivity) of an unmutated portion (A domain) of the tridomain PheATE derivatives. The assay revealed that for 18 (of 25) mutants the adenylation activity could be maintained, indicating that their structures cannot have changed much if at all. The remaining seven constructs sustained a drop in adenylation activity and therefore are likely to be affected in folding (Table 1).

Assays of Epimerase Domain Functions in NRPSs. The natural substrate of PheATE E domain is the adjacent Phe-S-Ppant T domain, formed by the tandem action of coupled A and T core domains. To form this covalent acyl-S-Ppant T domain intermediate, each T domain must have a specific serine side chain (Figure 2; S573) converted from inactive apo to active HS-Ppant holo form (*5*, *13*). This posttransla-

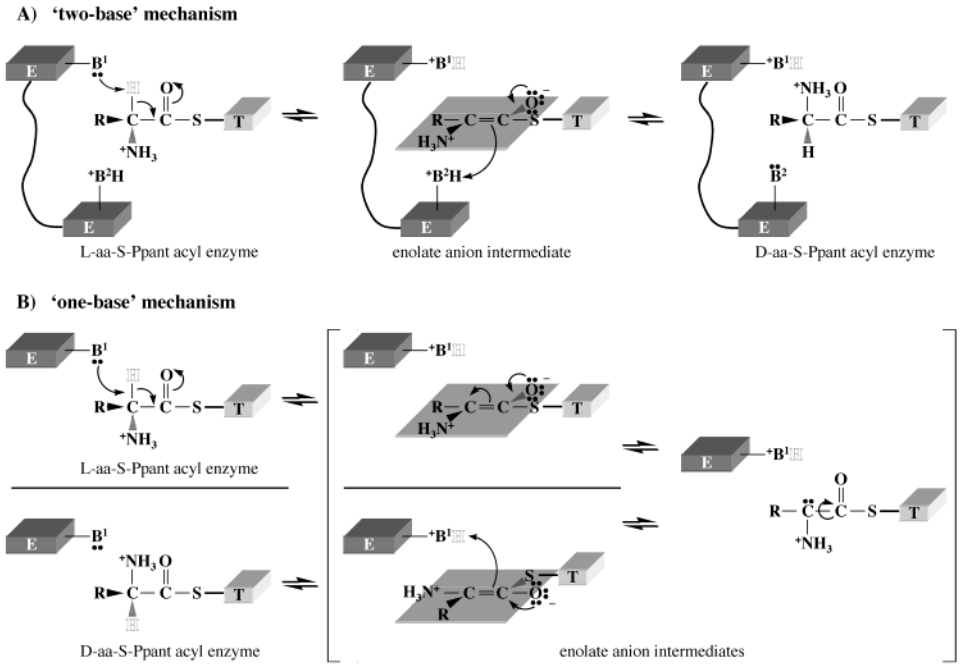

FIGURE 3: Alternative mechanisms to describe Cα-proton transfer during aminoacyl-S-Ppant epimerization. In the two-base mechanism (A), an enzymic base deprotonates the α-carbon, which is then reprotonated by the conjugate acid of a second base. In contrast, in a one-base mechanism (B), a single enzymic base serves to both de- and reprotonate the aminoacyl-S-Ppant. Experimental basis for the differentiation between both mechanisms is the exchange between substrate and solvent of the abstracted Cα-proton (outlined).

tional modification requires the catalytic action of a dedicated Ppant transferase (*6*), which, however, does not exist in the heterologous *E. coli* expression host used. Consequently, all PheATE* mutants were purified as apoproteins and converted in vitro into the active holo form by incubation with the *Bacillus subtilis* Ppant transferase Sfp before assays were run that assessed thiolation and/or epimerization activity (*17*, *18*).

The E domain works in cis on the upstream aminoacyl-S-Ppant T domain, so the covalent tethering of the two domains means on epimerization of L-Phe-S-Ppant T domain to D-Phe-S-Ppant (and vice versa) that the epimerized product stays covalently attached to PheATE. Consequently, the PheATE initiation module is not freed up for another catalytic turnover until transfer of the D-Phe moiety to the downstream ProCAT module in dipeptide bond formation (Figure 1). So PheATE on its own is in this sense stoichiometric in its epimerization capacity, and assays of the E-domain catalysis must deal with both the sensitivity issue and how to detect product that remains covalently bound. On the other hand, although substrate and product of the epimerization reaction are covalently held on the T domain, the adjacent E domain could be reversibly removing the Cα-H and adding it back until the L/D ratio has equilibrated in the E active-site microenvironment. This circumstance forms the basis of the first assay explored.

Enzymatic Cleavage of the Cα−H Bond of L-Phe by PheATE and PheATE Mutants.* During the epimerization of L- to D-Phe by PheATE, one expects at some stage cleavage of the Cα−H bond to yield the Cα-carbanion as a planar-stabilized transition state or intermediate that could be reprotonated back either to the L-Phe or D-Phe species. Two alternative mechanisms have been described to categorize enzymes that catalyze such proton transfers (Figure 3) (*23*). In the two-base mechanism, one enzymic base removes the Cα proton from the substrate, and the conjugate acid of a second enzymic base delivers a proton back to the opposite face. In contrast, in a one-base mechanism, a single enzymic base serves to both deprotonate and reprotonate the aminoacyl-S-Ppant. Consequently, for a two base mechanism and starting with L-[2-^{3}H]-Phe, there would be no label retained after epimerization to the D-Phe-S-Ppant-acyl enzyme product, while under the same conditions in a one-base mechanism there could be fractional to complete internal return of the radiolabel to substrate carbanion intermediate (Figure 3), based on the competition between proton transfer to solvent or to carbanionic-S-Ppant T domain.

We investigated the proton transfer reaction of PheATE and PheATE* mutants by allowing the enzymes to load with two different forms of regioselectively tritiated Phe, ring-labeled L-[4-^{3}H]Phe and chain-labeled L-[2,3-^{3}H$_2$]Phe. The former should give no washout to solvent and therefore can serve to calibrate stoichiometry of Phe-S-Ppant formation. In contrast, the tritium label at the Cα locus of chain-labeled Phe may progressively get lost to solvent. At a given time point and under the same conditions, ring-labeled Phe calibrates the system for the loading of 1 equiv of label, while the 2,3-chain-labeled Phe can give 1 to 2 equiv, depending on whether the Cα-proton is washed out or not.

Using this assay, we found that wild-type Phe-S-Ppant PheATE enzyme lost 1 equiv of label, presumably the Cα-proton, completely at the earliest time point. Determination of the exact rate constant for the abstraction will require rapid quench reaction kinetics, but the k_{cat} may be at least several per second (>100 min^{-1}), since the washout was complete within 5 s (shortest time measurable; data not shown). Notably, this limit matches the rate for the acylation of holo-PheATE but is substantially faster than subsequent translocation/condensation (D-Phe-L-Pro-S-Ppant formation) between PheATE and ProCAT (about 1.8 min^{-1}) or noncatalytic

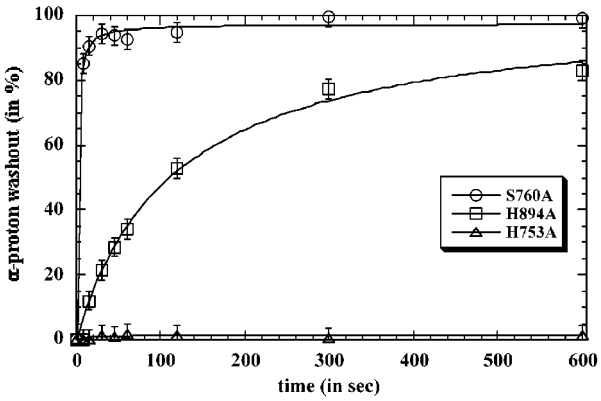

FIGURE 4: Exchange of Cα-proton between L-[2,3-$^3H_2$]Phe substrate and solvent. The proton-transfer reaction was investigated by loading two different species of Phe onto PheATE* mutants: ring-labeled L-[4-^{3}H]Phe to internally calibrate the system for loading and L-[2,3-$^3H_2$]Phe that may progressively wash out to solvent. Samples were taken at various time points and immediately quenched with 10% TCA (w/v), and the acid-stable label was quantified by LSC. On the basis of these studies, PheATE* mutants can be categorized in three groups: (1) those that apparently approach wild-type rates (e.g., S760A; ○) (2) those that are at least somewhat impaired in Cα-[^{3}H] exchange (e.g., H894A; □), and (3) those mutants whose Cα-[^{3}H] washout is essentially at background level (e.g., H753A; △). Rates for all mutants are given in Table 1.

release of the cyclic D-Phe-L-Pro DKP (about 0.5 min^{-1}) (*7*, *18*). Thus, epimerization of L-Phe is definitely not the rate-limiting step in the biosynthesis of cyclic decapeptide antibiotic gramicidin S. Control experiments confirmed that the observed washout depends on ATP, active holo Ppant enzyme, and presence of the E domain (data not shown). This is consistent with the mechanistic formulation that the Cα-H bond is not cleaved at stage of the Phe-O-AMP intermediate sequestered in the A domain but only after formation of L-Phe-S-Ppant (*13*). We separately showed that the PheAT double domain fragment of GrsA could covalently load with tritiated L-Phe but did not wash out the Cα-[^{3}H] radioactivity (data not shown), thereby substantiating that the E domain is the catalyst for the proton-transfer reaction observed and that L-Phe-S-Ppant is the species undergoing washout by the E domain of PheATE.

With both the assay and the role of the E domain established in wild-type PheATE, we proceeded to investigate the ability of the PheATE* mutants to abstract and wash out the Cα-proton. These studies, whose results are summarized in Table 1 and Figure 4, revealed that the E-domain mutants can be categorized into three groups:

The first group is formed by those mutants that apparently approach wild-type rates for the washout of tritium label. Among others, it comprises four constructs with mutations in invariant residues (S760A, D767S, T908A, and T913A), and in the measurable time frame their rates cannot be distinguished from that of wild-type PheATE.

The majority of the PheATE* E domain mutants revealed at least some impairment in their ability to wash out the Cα-proton from L-Phe. The group comprises six mutations in invariant positions (H672A, R676A, H752A, E892A, R896A, and W911A), and the loss of catalytic activity ranges from ≥6-fold (E892A; 15 min^{-1}) to ≥1500-fold (N975A; 0.03 min^{-1}).

The studies identified three PheATE* mutants (H753A, D757S, and Y976A) whose ability to wash out the Cα-proton is essentially at background level. At an estimated limit of signal-to-noise ratio of 2%, there was no measurable wash out of tritium label over a period of 1 h.

These results suggest that the second His (H753), but not the first His (H752), of the His motif (Figure 2; E2), as well as the residues D757 and Y976 may be essential for the deprotonation of L-Phe-S-Ppant. The data allow no statement about whether these residues will be also required for the abstraction and washout of the Cα-proton from D-Phe-S-Ppant, since the corresponding D-[2-^{3}H]Phe substrate was not available.

Interconversion of L-Phe-S-Ppant and D-Phe-S-Ppant Acyl Enzymes (Rates and Equilibrium Position). It has been shown that PheATE activates in an ATP-dependent manner equally well the L- and D-enantiomers of Phe and racemizes them (*13*, *14*). However, it is not known whether the E domain fully equilibrates the L- and D-configurations of Phe-S-Ppant substrates before translocation and chain elongation of the D-Phe moiety only to the ProCAT. This stereoselectivity could be a property of either E or C domain, and thus the question arises whether the internal L-Phe-S-Ppant/D-Phe-S-Ppant equilibrium on PheATE is held close to unity or if one isomer predominates and then selectively reacts with the downstream C domain.

To address this issue and any effect mutations in the E domain might have on this phase of E-domain action, the configuration of the Phe-S-Ppant thioester in the aminoacyl-S-Ppant/enzyme covalent intermediate was analyzed. For this purpose, holo-PheATE enzymes (wild type and mutants) were allowed to activate and covalently load L-[^{14}C]Phe of high specific activity (450 mCi/mmol). The [^{14}C]Phe-S-Ppant/enzyme thioester complexes were separated from excess substrate, washed, and hydrolyzed with 0.1 N potassium hydroxide. The released [^{14}C]Phe could then be applied to chiral TLC plates to determine the stereochemistry of the released amino acids and thereby of the Phe-S-Ppant enzymes.

As shown in Figure 5, wild-type holo-PheATE produces radiolabeled D- and L-Phe in spots of about comparable intensity after 0.5 min of incubation. Efforts to determine a time course for the conversion of L-Phe-S-Ppant enzyme to D-Phe-S-Ppant form failed at 37 °C because the reaction was too rapid. Even when the temperature was progressively lowered to 5 °C and the reaction was quenched within 10 s after initiation, the wild-type enzyme still had attained its final D-Phe-S-Ppant/L-Phe-S-Ppant ratio. As in the assay of Cα-proton abstraction, rapid quench kinetics will be required to determine reaction rates. However, the temperature and time dependence studies gave 72 separate data points, allowing at least a good statistical evaluation of the D-Phe-S-Ppant /L-Phe-S-Ppant ratio of 1.9:1 (66% D-Phe-S-Ppant acyl enzyme), as well as the assessment of the accuracy (error limits) of the TLC densitometrical assay at about ±3%.

To evaluate whether the 1.9:1 ratio of D-Phe-S-Ppant to L-Phe-S-Ppant enzyme obtained starting from L-Phe was in fact the equilibrium position for PheATE, the studies were repeated starting from D-[^{14}C]Phe. As shown in Figure 5, the same ratio could be obtained when coming from this opposite direction. Thus, the final ratio between D-Phe- and L-Phe-S-Ppant stereoforms of this E domain deviates from

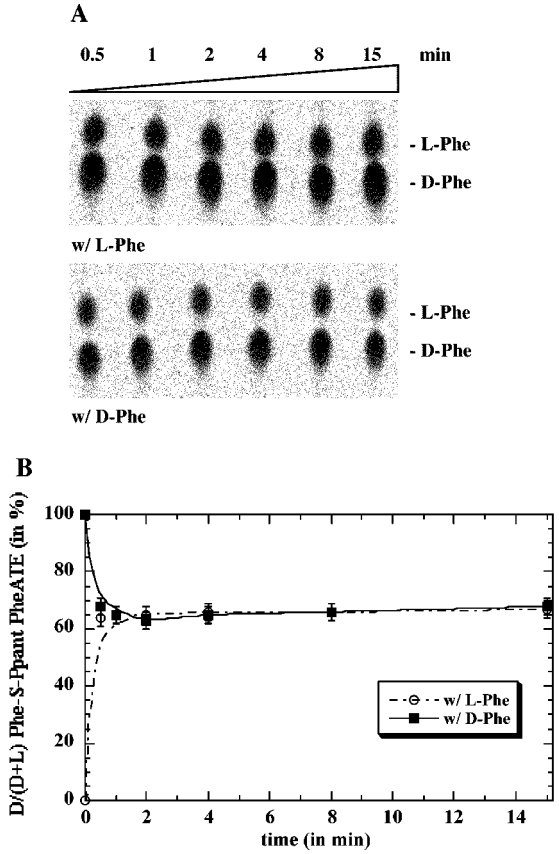

FIGURE 5: L-to-D and D-to-L conversion in the wild-type Phe-S-Ppant/PheATE thioester complex. Holo-PheATE was allowed to load D- and L-[^{14}C]Phe of high specific activity. Reaction mixtures were incubated at 37 °C and quenched with 10% TCA (w/v) at various time points. Subsequently, the [^{14}C]Phe-S-Ppant/enzyme complexes were hydrolyzed, and released [^{14}C]Phe was applied to chiral TLC plates to separate D- and L-isomers. (A) TLC plates were developed in acetonitrile/water/acetic acid (4:1:1 (v/v/v) and the radiolabel was visualized by autoradiography. Examples are shown for time courses coming from either L-Phe (Figure 1B; steps 1 → 3 → 5 ↔ 6) or D-Phe (steps 2 → 4 → 6 ↔ 5). (B) Autoradiographies were further analyzed densitometrically with NIH Image. Each individual point combines the data from 12 (L-Phe; ○) or 4 (D-Phe; □) independent experiments, respectively.

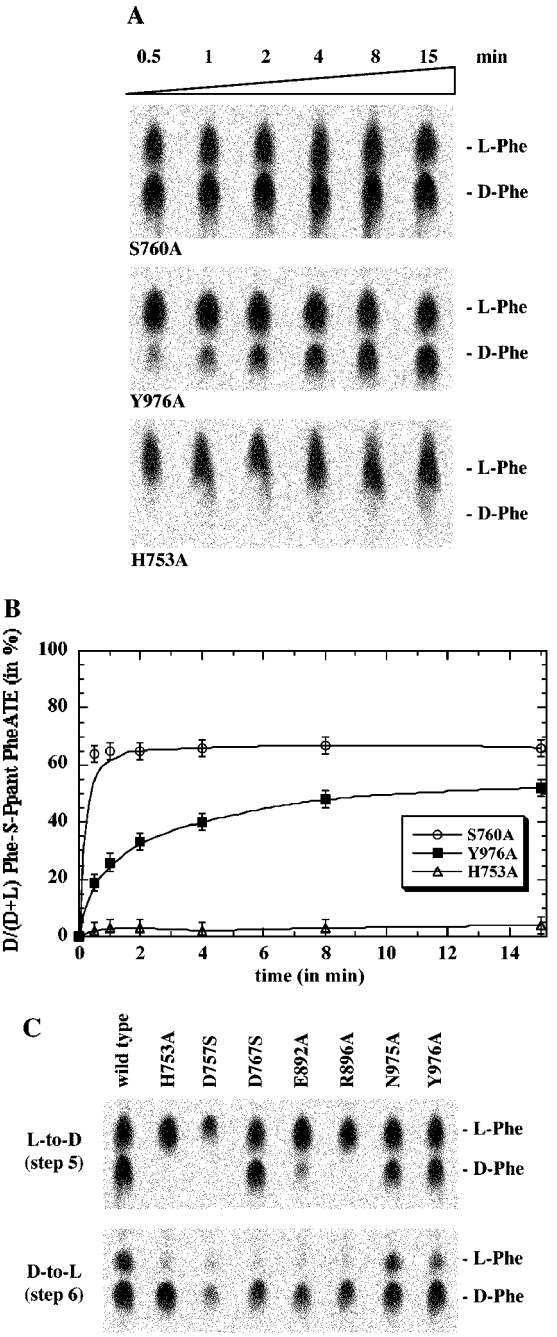

FIGURE 6: Phe-S-Ppant interconversion in PheATE* E-domain mutants. The mutants can be categorized in three groups: (1) those that apparently approach wild-type rates (e.g., S760A; ○), (2) those that are at least somewhat impaired in L- to D-Phe-S-Ppant conversion (e.g., Y976A; □), and (3) those mutants that are essentially at background level for step 5 (Figure 1B; e.g., H753A; △). Shown are (A) the autoradiography and (B) the densitometric analysis for one member of each group. Rates for all mutants are given in Table 1. (C) For the most dramatically affected PheATE* mutants, samples from a single time point (4 min) were applied to TLC. Autoradiography is shown for reactions coming from either L-Phe (blocked in step 5) or D-Phe (blocked in step 6).

the 1:1 ratio expected from a true racemase, imply that the asymmetric microenvironment at the T−E bidomain interface may stabilize one isomer marginally but measurably over the other. To determine how mutations in the E domain will affect this microenvironment and alter the kinetics and equilibrium for the conversion of L-Phe-Ppant and D-Phe-S-Ppant, we next investigated the PheATE* mutants, with representative results in Figure 6.

Some mutants (e.g., N724A, S760A, W761A, and T913A) gave L-Phe-S-Ppant/D-Phe-S-Ppant ratios and conversion rates equivalent to the wild type and so (by this assay) were not impaired in E-domain function (Table 1 and Figure 6a,b). Two mutants (H753A and D757S; E2) that do not wash out any Cα-H are also dramatically impaired in epimerization starting from either L- or D-Phe-S-Ppant enzyme, consistent with an essential role in steps of epimerization common to both Phe-S-Ppant isomers (Table 1 and Figure 6). Interestingly, we could also identify two groups of PheATE* E-domain mutants that appear to be affected differentially in one or more steps shown in Figures 3: deprotonation (as assessed by Cα-H washout) and reprotonation (as assessed by conversion of L- to D-Phe-S-Ppant or D- to L-Phe-S-Ppant). For example the PheATE* double mutant N975/Y976 (E domain motif E7; Figure 2), is dramatically impaired in Cα-H washout (>1000-fold) but 1 order of magnitude less disabled (about >90-fold) in epimerization rates coming from either L- or D-Phe-S-Ppant enzyme (Table 1 and Figure 6A,B). Most pronounced representatives of the second group

are also found clustered within the primary structure of the E domain (ExHxR; E4). Mutants E892A and R896A are only moderately impaired in Cα-H washout (≥6-fold and ≥90-fold) but are substantially slowed in epimerization of both substrates, L- and D-Phe-S-Ppant (Figure 6C). They are presumably not the catalytic base(s) but could be crucial in some other role, e.g., stabilization of the enolate anion intermediate (Figure 3).

Notably, all four of the dead PheATE* derivatives (H753A, D757A, E892A, and R896A) bear mutations in invariant positions, and with a limiting signal-to-noise ratio of 5%, neither revealed a detectable conversion of either D- or L-Phe-S-Ppant acyl enzyme isomer within a period of 1 h. Moreover, neither construct shows an asymmetry in steps 5 and 6 (Figure 1B) and epimerization of L- and D-Phe-S-Ppant are equally impaired (Figure 6C).

In contrast, the PheATE* mutant D767S is asymmetrically inhibited in the approach to equilibrium position of D-Phe-S-Ppant/L-Phe-S-Ppant. Conversion of D-Phe-S-Ppant to the corresponding L-isomer is at least 200-fold reduced (<0.01 min^{-1}) compared the rate in the opposite L- to D-Phe-S-Ppant direction (Figure 6C and Table 1; >2 min^{-1}, equivalent to wild-type PheATE). Since this PheATE* mutant folds properly (as judged by ATP−PP_i exchange) and washes out Cα-H from L-Phe-S-Ppant (Table 1) at rapid rates, this result could indicate that D767 might be specifically involved in the D- to L-Phe-S-Ppant half reaction (Figure 3).

Dipeptidyl-S-Ppant and DKP Formation: Translocation of D- or L-Phe from PheATE to ProCAT.* Prior studies revealed that only D-Phe is transferred from donor Phe-S-Ppant PheATE to acceptor L-Pro-S-Ppant ProCAT in order to form the corresponding D,L-dipeptidyl-S-Ppant enzyme (*7*, *24*). Thus, the results just noted about equilibration to a ratio of 2:1 D-Phe-S-Ppant/L-Phe-S-Ppant in the PheATE acyl donor raises the questions of how L-Phe translocation to ProCAT is suppressed and how efficiently the C domain of ProCAT can or will transfer the L-Phe moiety from Phe-S-Ppant PheATE* mutants.

To assay for the formation of dipeptidyl-S-Ppant ProCAT nascent product, tritiated L-Pro was used and was allowed to load onto holo-ProCAT in the presence of ATP. Subsequently, the L-Pro-S-Ppant enzyme was mixed with purified holo-PheATE (wild type or E-domain mutant) that had been loaded in a preincubation with ATP and either L-Phe or D-Phe. Once translocation of Phe from PheATE to ProCAT occurs by C-domain catalysis, the radiolabeled Phe-Pro dipeptidyl moiety is autoreleased by cyclization to the diketopiperazine (DKP; Figure 1B). This product is readily extractable into organic solvent and can be analyzed by LSC and HPLC. Wild-type PheATE as aminoacyl donor yields D-Phe-L-Pro-DKP/L-Phe-L-Pro-DKP in a ratio of 98:2 for about a 50:1 stereopreference for D-Phe over L-Phe transfer (Table 2 and Figure 7).

On the basis of the results obtained by LSC (data not shown), the PheATE* mutants can be categorized in three groups. The majority of PheATE* mutants, especially constructs that were already little or not affected in Cα-H washout and L to D conversion (e.g., H752A, S760A, and W761A), revealed no effect on DKP formation when assayed with either D- or L-Phe (Table 1). An HPLC analysis of the stereochemistry of the Phe-Pro DKPs generated with these mutants revealed that only the D,L diastereomer is formed in measurable quantities (data not shown). A second group is impaired in any kind of DKP formation (with D- and L-Phe) but all its members are without exception impaired in folding (Table 1), which makes an assessment of this effect uninterpretable, and they are not considered further.

Table 2: HPLC Analysis of DKP Stereochemistry

enzyme	quantity in	total DKP	D,L-DKP	L,L-DKP
wild type	μM	621	609	12
	%	100	98	2
H753A	μM	296	179	117
	%	100	60	40
E892A	μM	584	552	32
	%	100	95	5
N975A	μM	718	663	55
	%	100	92	8
Y976A	μM	425	188	238
	%	100	44	56
H753A/	μM	95	61	34
N975A	%	100	64	36

The most interesting group consists of four PheATE* E mutants (H753A, D757S, E892A, and Y976A) that are significantly impaired in rates of DKP formation when loaded with L-Phe. In line with previous results for Cα-H washout and L- to D-Phe-S-Ppant acyl PheATE conversion, it is no surprise to find the H753A mutant among this group, since this construct is affected in all stages of Phe-S-Ppant epimerization. This mutant sustained a ≥500-fold loss in catalytic activity (<0.001 min^{-1} for H753A versus 0.5 min^{-1} for the wild type). However, its catalytic activity was not completely destroyed, since HPLC analysis of DKP stereochemistry (overnight reaction; see Experimental Procedures) revealed the production of small amounts of both D,L- and L,L-(Phe-Pro)DKP with a ratio of about 3:2 (Table 2 and Figure 7). The identity of both diastereomers was confirmed by ^1H NMR spectroscopy (data not shown) (*19*), manifesting a substantial increase in the fraction of L-Phe transferred by the C domain of ProCAT. This relative accumulation of L,L-DKP cannot be simply due to a failure to accumulate D-Phe-S-Ppant, since other mutants do not reveal this capability. For example, PheATE* mutant E892A not only was impaired in L- to D- and D- to L-Phe-S-Ppant conversion but also sustained a significant 240-fold drop in catalytic efficiency in the DKP formation (Table 1). The D,L-DKP/L,L DKP ratio is on a wild-type level of 95:5 (Table 2 and Figure 7). Likewise, N975A also yields a ratio of 92:8, but the adjacent PheATE* mutant Y976A, like H753A, shows an extraordinarily high percentage of 56% L,L-DKP of the total DKP released (Table 2 and Figure 7). A stalling of L-Phe-S-Ppant on PheATE (as provided by a slow conversion to the corresponding D-isomer) is not by itself sufficient to obtain a translocation of L-Phe from PheATE* mutants E892A and N975A to L-Pro-S-Ppant ProCAT but is manifested in H753A and Y976A.

No PheATE construct (wild type or mutant) led to the formation of L,L-DKP when provided with D-Phe (data not shown), but they all were competent for the formation of natural D,L-DKP. This result offers the first evidence that nonfunctional E domains, e.g., in PheATE* mutants H753A or E892 can fulfill an architectural bridging role during the transfer of D-Phe to ProCAT, because PheAT, with the E domain deleted, is incompetent even when provided with D-Phe (*7*).

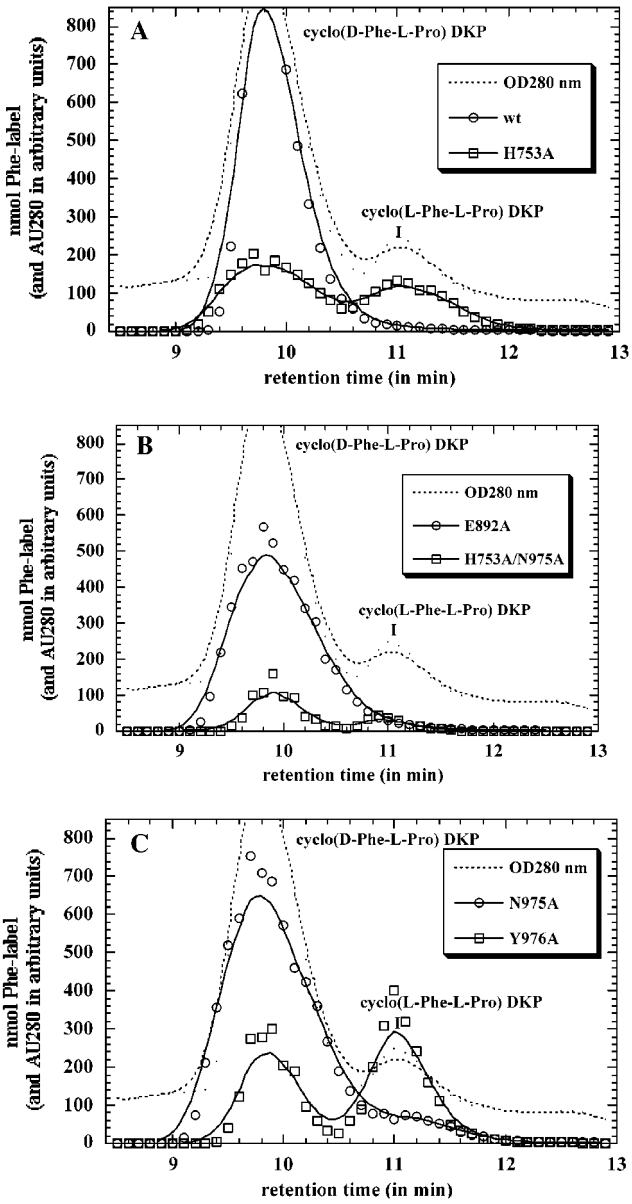

FIGURE 7: Analysis of the stereochemistry of Phe-Pro DKPs. To assay for dipeptidyl-S-Ppant product formation and stereochemistry, holo-PheATE (wild type and mutant) and holo-ProCAT were incubated together overnight with L-[^{3}H]Phe, L-Pro, and ATP. After extraction with butanol/chloroform (4:1 v/v), the organic layers were washed with 0.1 M sodium chloride and dried under vacuum. The residue was dissolved in 30% methanol, blended with L,L- and D,L-DKP standards and applied to a reverse-phase HPLC column. Both isoforms of Phe-Pro DKP can be separated by applying a linear gradient from 30% to 60% methanol (flow rate 1 mL/min) over 15 min. In the relevant time frame (between 8.5 and 13 min), samples were taken (6 s/fraction) and examined for their absorbance at 220 nm, as well as the amount of tritium label (by LSC). Shown are the results for samples obtained with (A) wild-type Phe (○) and H753A (□), (B) E892A (○) and H753A/N975A (□), as well as (C) N975A (○) and Y976A (□). Results of peak integration are summarized in Table 2.

DISCUSSION

The E domain of the initiation module PheATE of gramicidin S synthetase is studied here as a prototype of 45 E domains identified to date in NRPS catalysts. E domains are found in two modular locales in NRPS assembly lines: (1) in N-terminal, chain-initiating modules (ATE) and (2) in internal elongation modules (CATE) (*1*, *2*). In both cases, the E domain is inserted between the T domain that serves as growing acyl chain donor and the immediately downstream C domain in the next module that catalyzes the peptide bond-forming step. For example, the two-module assembly line AT_1-CAT_2 will make an L,L-dipeptidyl-S-Ppant T_2 acyl enzyme intermediate to start the NRPS template, while insertion of an E domain in the first module yields the AT_1E-CAT_2 bis-module that will make the D,L-dipeptidyl-S-Ppant intermediate. The latter case has been established, e.g., in gramicidin S and tyrocidin biosynthesis with initiation module PheATE and elongation module ProCAT located on separate polypeptides (Figure 1A). When D-amino acid residues appear in internal sites in nonribosomal peptides, the E domain is found in CAT_nE-CAT_{n+1} bis-elongation modules and specifies epimerization of the Cα-center of the S1 residue in the nascent peptidyl-S-Ppant chain docked at T_n.

As a first step in defining structure/function attributes of the PheATE E domain, mutation of 12 conserved residues to alanine (or serine) were affected, along with 10 highly conserved residues also clustered in six signature sequence motifs (Figure 2). Most of the PheATE* mutants could be heterologously expressed and purified as His_6-tagged forms from *E. coli* and subjected to functional analysis. Three assays were developed to probe one or more steps of E-domain function: (1) cleavage of the Cα-[^{3}H] bond of L-Phe, (2) approach to equilibrium of the D/L-Phe-S-Ppant acyl enzyme stereoisomers, and (3) rates of transfer of the D-Phe and/or L-Phe moieties to the Pro-S-Ppant acyl enzyme on the ProCAT module, catalyzed by the C domain in the peptide bond-forming step (Figure 1B; step 7 + 8). These studies have resulted in five mutants, H753A, D757S, D767S, E892A, and Y976A, with substantially altered kinetics in one or more steps of E-domain function. The described assays may be applicable to any E domain in any NRPS module.

Previously, wild-type PheATE (GrsA) has been studied by Kanda et al. (*12*) as a racemase for free Phe, by assaying tritium washout to solvent from L-[2-^{3}H]Phe or "wash-in" from ^3H_2O to D- or L-Phe. However, the observed rates were exceedingly slow (0.01−0.05 min^{-1}): 2 orders of magnitude below the condensation rate of ProCAT for D-Phe transfer [Figure 1B, step 8; 1.8 min^{-1} (*7*, *18*)) and some 4 orders of magnitude slower than the tritium washout reported here for L-[2-^{3}H]Phe-S-Ppant from PheATE (>100 min^{-1}). It is now clear, especially in the light of more recent work from Stein et al. (*5*, *13*), which established that every NRPS T domain does contain a Ppant prosthetic group, that the E domain of PheATE is not a phenylalanine racemase but a Phe-S-Ppant epimerase. This is generalizable to all the other NRPS E domains (although the substrate may be either an aminoacyl- or peptidyl-S-Ppant) (*12*, *14*, *16*, *25*). By this criterion, E domains are immediately distinct from amino acid racemases that are cofactor-independent, e.g., for proline (*23*) or glutamate (*26*−*28*), and from PLP-dependent amino acid racemases, e.g., for alanine (*29*−*31*) or serine (*32*). On the other hand there is some mechanistic analogy between the two cofactor-dependent PLP- and Ppant-linked catalysts (Figure 8A). Each of them covalently tethers the aminoacyl moiety to a coenzyme, the PLP via the substrate amino group in imine linkage and the Ppant E domains via the substrate carboxyl group in thioester linkage. In each case, the covalent

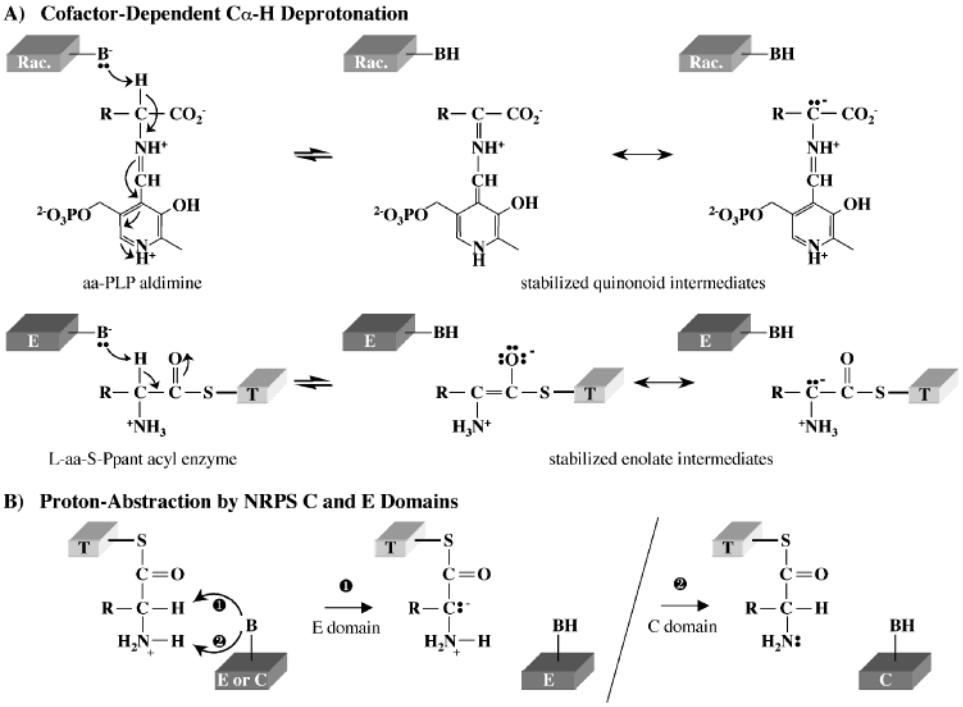

FIGURE 8: Mechanistic parallels between E domains and PLP-dependent racemases, as well as NRPS E and C domains. In panel A is shown how two cofactors, PLP and HS-Ppant, can be used to labilize the Cα-proton in amino acid racemization and aminoacyl-S-Ppant epimerization by Cα-carbanion formation. Panel B shows the target of the primary base of NRPS C and E domains, as well as the corresponding acyl-S-Ppant intermediate formed

tethering activates the Cα-H of the aminoacyl moiety for cleavage, since the Cα-carbanionic transition state is now stabilized by electron delocalization into the cofactor (Figure 8A), lowering energy barriers and accelerating catalytic rates.

The tritium washout from L-[2-^{3}H]Phe to solvent requires the PheATE module to realize steps 1, 3, and 5 of Figure 1B. Since we separately showed that L-Phe-O-AMP formation in step 1 and L-Phe-S-Ppant thioester formation in step 3 resulted in no tritium release to water, the Cα-[^{3}H] cleavage step is indeed localized to step 5. This E-domain-mediated detritiation process is so fast that it is completed in less than 5 s at ice-bath temperature. Therefore, determination of the rate constant for step 5 will require stopped-flow quench studies with substrate quantities of wild-type PheATE. Some of the PheATE* mutants were substantially impaired in detritiation but were unaffected in steps 1 and 3, localizing the mutational deficit to step 5, the L to D direction of E-domain catalysis. These constructs, H753A, D757S, N975A, and Y976A, are initial candidates for the catalytic base required for Cα-H cleavage of L-Phe-S-Ppant acyl enzyme (Figure 3). To obtain the corresponding rates for step 6, the Cα-H cleavage of the D-Phe-S-Ppant acyl enzyme, will require custom synthesis of D-[2-^{3}H]Phe of equivalently high specific radioactivity.

Two conclusions are already clear from these washout experiments with L-Phe as starting substrate. First, the rate of epimerization, which could be underestimated by both a tritium kinetic isotope effect and only fractional loss to solvent in any turnover event, is much faster than step 8, the C-domain-mediated peptide bond-forming catalytic chain transfer step that in turn is much faster than step 10 (discussed below). Second, the loss of the Cα-H argues for a Cα-carbanionic thioester intermediate in epimerase catalysis (Figure 3) and that the enzyme-B^1[^{3}H]$^+$ species exchanges a proton with solvent. The current tritium washout data from only L-Phe are insufficient to determine whether tritium washout rates are asymmetric and whether a one-base or two-base epimerase mechanism is thereby favored, although the complete and instantaneous Cα-[^{3}H] washout of wild-type PheATE indicates the two-base variant (*23*).

The high rate of net Cα-[^{3}H] washout from L-Phe (>100 min^{-1}) compared to the rate of D-Phe transfer (1.8 min^{-1}; step 8) to yield D-Phe-L-Pro-DKP (0.5 min^{-1}; step 10) suggests that the D- and L-Phe-S-Ppant isomers on the T domain of PheATE should come to configurational equilibrium before aminoacyl transfer to ProCAT. This prediction was validated explicitly by the second assay for E-domain function, starting with either L-[^{14}C]Phe or D-[^{14}C]Phe and monitoring the ratio of D-Phe-S-Ppant/L-Phe-S-Ppant acyl enzyme intermediate. This ratio was obtained by quenching of the PheATE protein into trichloroacetic acid at different time points to isolate the acid-stable Phe-S-Ppant enzyme, followed by base hydrolysis to release the free [^{14}C]Phe and D/L-isomer ratio determination by chiral TLC. This ratio of D/L-Phe was equated to the ratio of D-Phe-S-Ppant/L-Phe-S-Ppant T domain. Again, wild-type PheATE had reached its configurational equilibrium for the D-Phe-S-Ppant/L-Phe-S-Ppant ratio by the first time point, as expected from data of detritiation assay. But that equilibrium position was not 1:1 but rather 1.9:1 in favor of the D-Phe-S-Ppant over the L-Phe-S-Ppant species. While not structurally interpretable at this point, it does validate that the chiral microenvironment of the E-domain active site can selectively stabilize the D-Phe-S-Ppant acyl enzyme.

Two additional kinds of evidence were provided by analysis of the rate of approach to this configurational equilibrium of D/L-Phe-S-Ppant acyl enzyme by PheATE* mutants. There were subtle, but detectable shifts in the D/L-

Phe-S-Ppant equilibrium ratio as readout of some energetic change in the E-domain active site (Table 1). More dramatic were the rate effects in slowing down steps 5 (L-Phe-S-Ppant going to D-Phe-S-Ppant enzyme) and step 6 (D-Phe-S-Ppant going to L-Phe-S-Ppant enzyme) for some of the E*-domain mutants. The predictive correlation that mutants impaired in L-Phe Cα detritiation should be slowed in step 5 held true for H753A and D757S. Also inactive in this assay were E892A and R896A, while N975A and Y976A were only slowed but still measurably active. Most intriguingly, these latter examples imply that epimerization of L-Phe-S-Ppant (step 5) can also be achieved without exchange of Cα-[^{3}H] with solvent. This observation may be only explainable by means of protein crystallography, since it conflicts with most of our results that clearly suggest a two-base rather than a one-base mechanism for proton abstraction. Evidence for this latter mechanism is, i.e., the fast and complete loss of the Cα-[^{3}H] into solvent for wild type and most mutants, as well as the selective stabilization of D-Phe-S-Ppant enzyme.

The third assay utilized both wild type PheATE and PheATE* mutants to assess the impairment of mutated E* domains for Phe transfer to ProCAT in the dipeptide bond-forming step. Since PheATE will activate both L- and D-Phe in the presence of ATP but only D-Phe is found in the antibiotic, one could assay steps 1−3−5−8−10 (see Figure 1B) from L-Phe and steps 2−4−8−10 from D-Phe. As anticipated, given the block in step 5, the H753A, D757S, E892A, and R896A PheATE E-domain mutants were essentially inactive in D-Phe-L-Pro-DKP formation when starting from L-Phe. The flux from D-Phe was normal or only slightly impaired (E892A). Consistent with the earlier two assays, two additional PheATE* mutants, N975A and Y976A, were slowed by about 25-fold in generating D,L-DKP from L-Phe, but not from D-Phe. At this juncture H753 and D757 are leading candidates for part of the catalytic apparatus involved in the deprotonation of L-Phe-S-Ppant acyl enzyme to yield the carbanionic thioester species (Figure 3). In this connection, given the observed consequences of H753A mutation, it is even more surprising that some few E domains in the databases lack this residue, PksF (H′Q) (*33*) and PpsE (H′A) (*34*), although it has not yet been tested whether those E domains are still functional. The residue(s) that may be involved in reprotonation to or deprotonation of D-Phe-S-Ppant enzyme are not clear, but considering the selective block in step 6, D767 may be part of the catalytic apparatus responsible for cleaving the Cα-H of D-Phe-S-Ppant acyl enzyme.

Given that the wild-type PheATE activates L-Phe and catalyzes a preequilibrium mixture of 1.9:1 D-Phe/L-Phe-S-Ppant enzyme before the transfer to Pro-S-Ppant ProCAT, the conclusion arises that the C domain of ProCAT must be stereoselective, rejecting the L-Phe-S-Ppant conformer and selecting the D-conformer for acyl transfer. By extension it may be that other NRPS C domains downstream of E domains are D-selective for the aminoacyl-S-Ppant donor or the S1 residue in a peptidyl-S-Ppant donor. Validation of this hypothesis would be important not only for knowledge about the iterative catalytic domains of NRPSs but also for choosing C domains in combinatorial biosyntheses where D-residues are desired. That the ProCAT C domain is D-stereoselective and not absolutely D-stereospecific is indicated by the PheATE* mutants H753A and Y976A. Although in wild-type PheATE the D/L-Phe transfer ratio is 98:2 as concluded from chiral HPLC of the D,L and L,L-DKP products, when H753 and Y976 are mutated and flux drops from L-Phe through step 5, the Phe-Pro-DKP formed is now 40−60% L,L. When no D-Phe-S-Ppant acyl enzyme is formed because of the epimerase block and L-Phe-S-Ppant accumulates, the ability of the C domain of ProCAT to slowly transfer L-Phe is revealed. However, a simple stalling of L-Phe-S-Ppant on PheATE* cannot explain all observations, since the formation of D-Phe-S-Ppant in Y976A mutant is still faster than steps 8 and 10. Moreover, some PheATE* mutants such as E896A almost exclusively transfer D-Phe, although its formation from L-Phe-S-Ppant enzyme is dramatically impaired. Thus, the basis of D-Phe selectivity by ProCAT C domain may be a mixture of architectural recognition and kinetic removal of D-Phe-S-Ppant from the equilibrium mix at the PheATE T domain.

The E domain of the initiation module PheATE not only is important for producing and selectively translocating the sterechemically correct D-Phe but also makes some crucial architectural contribution to catalytic efficiency of ProCAT as a peptide bond-forming catalyst. The E-domain deletion protein PheAT is completely inactive in the condensation assay, even when loaded with D-Phe in thioester linkage to the Ppant prosthetic group (*7*), while nonfunctional E domains in PheATE* mutants can fulfill an architectural bridging role. It will be interesting to know if this is a generalizable attribute of E domains in other NRPS assembly lines. Given (1) the apposition of E and C domains in NRPS catalysts (*1*, *2*), (2) the suggestion that E domains may have arisen from C domain duplication (based on structural and functional homology; *1*, *20*), (3) the requirement for the conserved His and Asp (His motif; H753 and D757 PheATE E domain) in both C-domain (*7*, *21*) and now E-domain function, and (4) the placement of that His in the active site of the acyl transferase superfamily member chloramphenicol acetyltransferase (*20*, *35*, *36*), there may be both commonality of mechanism in proton abstraction (Figure 8b) and a structural basis for E/C domain interactions.

At internal E domain sites in NRPS assembly lines, the T_n-E pair $(CAT)_{n-1}-(CATE)_n-(CAT)_{n+1}$ modules could act to epimerize L-aminoacyl-S-Ppant T_n domain before condensation with the growing peptide chain docked at T_{n-1} catalyzed by C_n, or after chain transfer (*15*, *16*) when the peptidyl-S-Ppant T_n would be the substrate for the adjacent E domain before C_{n+1} action. The timing of epimerization on aminoacyl- or peptidyl-S-Ppant T_n may depend both on the relative rates of E_n versus C_n and C_{n+1} as well as the D versus L chirality preference of the C domain that use the aminoacyl-S-Ppant T_n as acceptor (here C_n) and the peptidyl-S-Ppant T_n as chain donor (here C_{n+1}). In either kinetic variant the internal E domains must equilibrate L/D chirality much faster than C-domain peptide bond-forming steps in order not to leave up to half the chains in a nonelongatable stereoisomer.

With regard to E-domain versus C-domain mechanism, one can actually say little about C-domain mechanism of peptide bond formation, other than the expectation that the downstream aminoacyl-S-Ppant substrate will need to be deprotonated at the NH_{3+} group to create the nucleophilic amino nitrogen ($-NH_2$) required for condensation. In some analogy (Figure 8B) the aminoacyl-S-Ppant substrate must

be deprotonated on Cα. Both mechanisms require a catalytic base, in the C and E domains, in similar spatial relationship to the aminoacyl-S-Ppant T domain and the H753 and conserved His equivalent in C domains could be part of that catalytic apparatus (*20*).

With regard to architecture and interaction and how E domains can be required for the action of the paired downstream C domain and even modulate its D- versus L-stereoselectivity in chain transfer, perhaps the C/E domains interact to form a transient heterodimer. This could put constraints on approach and orientation of the partner T domains as substrates and could control presentation of a D-Phe-, versus L-Phe-S-Ppant T domain to a C domain. The oligomerization state of modules in NRPS assembly lines is unknown, while modular fatty acid synthases and modular polyketide synthases are evidently dimeric with communication in trans (*37*−*39*). One practical consequence could be that pairs of E and downstream C domains may need to be spliced together in combinatorial manipulations, e.g., to alter use of an L-amino acid to a D-amino acid at a particular way station in the assembly line for a peptide antibiotic.

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