Aminoacyl-CoAs as Probes of Condensation Domain Selectivity in Nonribosomal Peptide Synthesis

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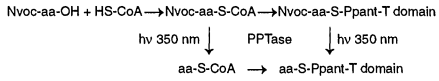
In nonribosomal biosynthesis of peptide antibiotics by multimodular synthetases, amino acid monomers are activated by the adenylation domains of the synthetase and loaded onto the adjacent carrier protein domains as thioesters, then the formation of peptide bonds and translocation of the growing chain are effected by the synthetase's condensation domains. Whether the condensation domains have any editing function has been unknown. Synthesis of aminoacyl–coenzyme A (CoA) molecules and direct enzymatic transfer of aminoacyl-phosphopantetheine to the carrier domains allow the adenylation domain editing function to be bypassed. This method was used to demonstrate that the first condensation domain of tyrocidine synthetase shows low selectivity at the donor residue (D-phenylalanine) and higher selectivity at the acceptor residue (L-proline) in the formation of the chain-initiating D-Phe-L-Pro dipeptidyl-enzyme intermediate.

Peptide antibiotics such as penicillins and cephalosporins, vancomycin, bacitracin, actinomycin D, the antitumor peptide bleomycin, as well as the immunosuppressant cyclosporin A are all assembled on nonribosomal peptide synthetases (NRPSs). The identity and sequence of amino acid residues in these nonribosomal peptides is dictated by the organization of sets of iterated modules in the megasynthetases (*1, 2*). Each module activates a specific amino acid by means of a pair of closely coupled domains: an adenylation (A) domain produces an aminoacyl–*O*-adenosine monophosphate (aa-O-AMP) that is then covalently tethered in thioester linkage to the phosphopantetheinyl (Ppant) prosthetic group (*3*) of the adjacent thiolation (T) domain (also known as a peptidyl carrier protein). The peptidyl chain grows directionally in incremental steps of elongating acyl-S-enzyme intermediates. Peptide bond formation and chain translocation occur each time an upstream donor peptidyl-S-Ppant is attacked by a downstream acceptor aminoacyl-S-Ppant nucleophile, a process under the catalytic control of condensation (C) domains (Fig. 1). For an NRPS to be functional, each T domain must have a specific serine side chain converted from an inactive apo to a Ppant-holo form by dedicated phosphopantetheinyl transferases (PPTases).

Previous work to increase the diversity of peptide antibiotics has indicated that the A domains are the main fidelity-conferring units for monomer selection, activation, and transfer to the paired T domains, on the basis of substitutions of different A domains in surfactin synthetase (*4*). With the report of the x-ray structure of the phenylalanine-activating A domain (*5*) of the gramicidin S-synthetase complex (*6*), structure-function mutagenesis studies will help define rules for amino acid recognition by A domains. In contrast, little is known about the tolerance of the peptide bond–forming C domains to elongate alternate acyl donors from the upstream T domain or to add alternate aminoacyl monomers at the downstream acceptor T domain. In some cases, A domains have activated structural homologs of their substrate amino acid, and the C domains have been shown to accept these miscognate substrates (*7*). For any approaches, however, to reprogram NRPS biosynthetic machineries, further knowledge concerning the selectivity of C domains would be crucial.

The approach we have taken to directly examine the selectivity at both the donor and acceptor sites of C domains required three steps: (i) synthesis of aminoacyl-S-coenzyme A molecules (aa-S-CoAs) to serve as potential substrates to bypass the A domains, (ii) enzymatic loading of aminoacyl-S-Ppant moieties onto the apo forms of upstream and downstream T domains, and (iii) evaluation of C domain–mediated peptide bond formation to yield dipeptidyl-S-enzyme product.

The synthesis of aa-S-CoAs was undertaken (Eq. 1) with unblocked CoASH and 6-nitroveratryloxycarbonyl (Nvoc)–protected amino acids, with PyBOP (*8*) as a condensation reagent to yield Nvoc-aa-S-CoAs (*9*). The amino acids required N-protection to avoid cross condensation reactions between the amines and the product thioesters. The Nvoc group was removed by photodeprotection in aqueous solutions (15 min, 350 nm, 4°C) without damage to any proteins that were present, yielding either the free aa-S-CoA or an aa-S-enzyme immediately before their desired use.



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The stability of free aa-S-CoAs to hydrolysis at physiological pH and temperature was critical for them to be used as substrates. High-performance liquid chromatography (HPLC) analysis showed that L-Phe-S-CoA had a half-life of 6 hours at pH 7 (37°C) and of 3 hours at pH 8, long enough to be used in the enzymatic experiments reported here. As anticipated, Nvoc-L-Phe-S-CoA had a substantially longer half-life (>24 hours, pH 7). Nuclear magnetic resonance analysis in D_2O of the C_2–H on L-Phe-S-CoA also established that nonenzymatic racemization was negligible in these time frames, thus chirality was maintained. The Nvoc group is chromophoric [Nvoc-L-Ala-OH: molar extinction coefficient ε (348 nm) $\approx$ 6400 cm^{-1} M^{-1}] and proved useful as an ultraviolet marker in quantitation and enzymatic transfer studies before photodeprotection.

We next addressed whether Nvoc-aa-S-CoAs would serve as substrates for PPTases that normally transfer the free thiol-containing Ppant moiety of CoASH onto the side chain of a specific serine residue in apo T domains. Kinetic efficiency comparisons were conducted with *Bacillus* PPTase, Sfp (*10*), and the 11-kD apo T domain, PheT, of the first module of the gramicidin S-synthetase complex as well as the larger substrates PheAT and PheATE (*11, 12*). Transfer of the aa-S-Ppant moieties to the apo T domains was monitored by two kinds of assays: a native–polyacrylamide gel electrophoresis (PAGE) gel shift assay that resolves apo and holo forms of the T domain, and an assay that quantifies the incorporation of the Nvoc chromophore on the protein by gel filtration and measurement of the absorbance at 350 nm. Using both methods we found that Nvoc-protected and free aa-S-CoAs were substrates with catalytic efficiencies (catalytic rate constant k_{cat} = 15 to 22 min^{-1}, Michaelis constant K_m = 9 to 27 μM, k_{cat}/K_m = 0.7 to 1.9 min^{-1} μM^{-1}) approaching that of the physiological substrate CoASH (k_{cat} = 14 min^{-1}, K_m = 14 μM, k_{cat}/K_m = 1.0 min^{-1} μM^{-1}) (*13*).

Mass spectrometric characterization of the aa-CoAs (D- and L- isomers of Phe-S-CoA, Ala-S-CoA, and L-Leu-S-CoA) and their respective products, after transfer of the aa-S-

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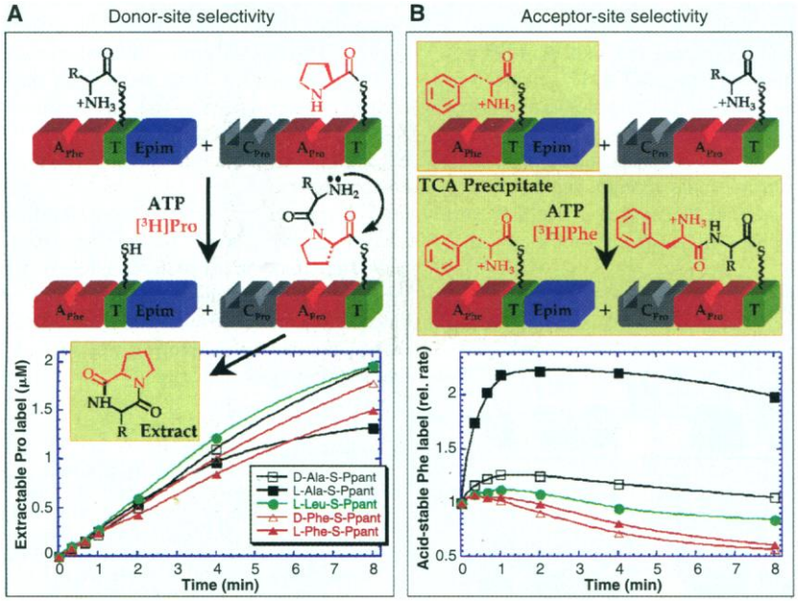
Ppant moieties to PheT by Sfp, showed that the experimental results are in agreement with the anticipated mass increases (*14*). Thus Sfp, the *Bacillus* PPTase dedicated to priming the apo T domains of the seven modules of the surfactin synthetase complex (*10*), did not distinguish between CoASH and aminoacylated derivatives, including those with the large Nvoc substituent.

To address whether a peptide bond–forming C domain would tolerate the mischarged donor and acceptor aa-S-Ppant T domains and make novel combinations of peptides, we turned to a system that uses PheATE and ProCAT (*6, 15*) (Fig. 1). This system has the advantage that the donor and acceptor T domains are on separate enzymes, allowing each to be loaded independently. Additionally, peptide bond formation can be detected by substrate translocation between separate protein modules. There is an epimerase (E) domain in the donor module PheATE, allowing analysis of D- and L-isomer utilization. The preference for proline in the acceptor module ProCAT leads to spontaneous diketopiperazine (DKP) formation and release of product from the enzyme, facilitating detection of radiolabeled products.

To assess the donor-site selectivity, apo PheATE (*11*) was loaded with Nvoc-aa-S-CoAs by means of Sfp action and photodeprotected to give the aa-S-PheATE intermediates (*16*). Holo ProCAT was loaded with [^{3}H]Pro and ATP, giving [^{3}H]prolyl-S-ProCAT. Figure 1A shows the rate of [^{3}H]DKP formation for each of the aa-S-PheATE intermediates upon incubation of the loaded enzymes. Indeed, the rates of release of [^{3}H]DKP differed little among the products derived from loading the mis-cognate substrates (L-Phe, D-Ala, L-Ala, and L-Leu) compared with the natural intermediate (D-Phe) in the donor position (*17*). On the other hand, when the selectivity of this C domain for the downstream nucleophilic aminoacyl group on the acceptor T domain was examined, greater selectivity was evident. Only L-Ala-S-ProCAT (of the five acceptors tested) was competent in this system (Fig. 1B) (*16*), yielding D-Phe-L-Ala-S-ProCAT at rates (k_{cat} = 1.4 min^{-1}) comparable to D-Phe-L-Pro-S-ProCAT formation (k_{cat} = 1.8 min^{-1}).

To further validate these results, two controls were carried out. It was established that apo ProCAT could be covalently modified with Nvoc-D-Ala-S-CoA and Nvoc-L-Ala-CoA by incorporation of the Nvoc chromophore into the protein (*14*). Subsequently, we demonstrated by transfer of [^{3}H]Phe to ProCAT that a dipeptidyl-S-enzyme is selectively formed between D-Phe and L-Ala (but not D-Ala) (Fig. 2A) (*12, 16*). The D-Ala-S-



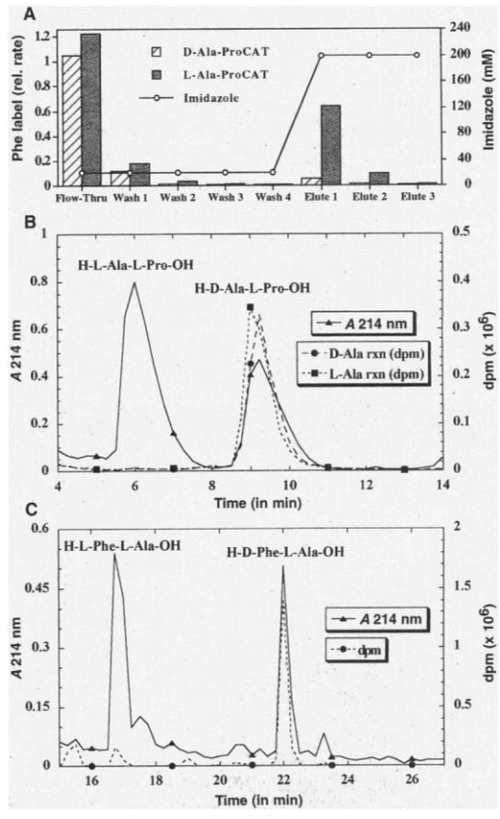


Fig. 1. (Left) Investigation of C domain selectivity for donor and acceptor aminoacyl-S-Ppant substrates. PheATE and ProCAT (*11*) are shown with their subdomains highlighted: A domains (A_{xxx}, red), carrier T domains (green), peptide bond–forming C domains (C_{xxx}, gray), and epimerase domain (Epim, blue). (**A**) To investigate the donor-site selectivity of C domains, we loaded ProCAT with [^{3}H]Pro and loaded PheATE with five different aminoacyl groups: D- and L-Phe, D- and L-Ala, and L-Leu (*16*). Upon mixing the loaded modules, the respective aminoacyl moieties should be transferred to ProCAT, yielding deacylated holo-PheATE and dipeptidyl-ProCAT. The proline-containing dipeptide-thioesters autocyclize, liberating radiolabeled diketopiperazines (DKPs), which are extracted and quantified by liquid scintillation counting (LSC) (yellow boxed item). The rates for DKP formation for all tested substrates were in the same range ($0.4 \leq k_{cat} \leq 0.5$ min^{-1}), indicating a low selectivity toward the aminoacyl donor (*17*). (**B**) To test acceptor-site selectivity of the C domain, we loaded ProCAT with five aa-S-CoAs (*16*) and loaded PheATE with [^{3}H]Phe. Peptide bond formation occurs with the transfer of radiolabel from [^{3}H]Phe-S-PheATE onto the aa-S-ProCAT. This translocation will liberate the T domain of the initiation module, which is rapidly reacylated by [^{3}H]Phe-adenylate. The molar quantity of precipitable radiolabel (yellow boxed items) should increase from 1 to 2 if [^{3}H]Phe is transferred from PheATE to ProCAT. **Fig. 2. (Right)** Characterization of the dipeptide products. (**A**) Transfer of [^{3}H]Phe from PheATE to ProCAT loaded with either L-Ala-S-CoA (solid bars) or D-Ala-S-CoA (striped bars). Condensation reactions were initiated by the addition of [^{3}H]Phe-S-PheATE. His-tagged ProCAT (*15*) was isolated on Ni^{2+}-nitrilotriacetic acid columns after 1 min. Columns were washed (MES buffer pH 8.0, 20 mM imidazole) and subsequently eluted (MES buffer pH 8.0, 200 mM imidazole). In each fraction, total protein was precipitated [10% TCA (w/v) and 2% BSA (w/v)], and the acid-stable label was quantified by LSC. (**B**) HPLC analysis of Ala-Pro dipeptide chirality by coinjection of radiolabeled enzyme products with D-Ala-L-Pro and L-Ala-L-Pro standards. Apo PheATE was loaded with D- and L-Ala-S-CoA and incubated with [^{3}H]Pro-S-ProCAT (*15*). After 4 min, the reactions were quenched [10% TCA (w/v)] and dipeptides were released with performic acid (*22*) and analyzed by HPLC (C18, 4.6×250 mm, 5 μm, 0 to 30% acetonitrile/0.1% trifluoroacetic acid over 30 min). Fractions were collected and analyzed for absorbance (*A*) at 214 nm (solid line) and by LSC (broken lines). (**C**) HPLC analysis of Phe-Ala dipeptide chirality. ProCAT was loaded with L-Ala-S-CoA and incubated with [^{3}H]L-Phe-S-PheATE. Dipeptides were analyzed by coinjection with L-Phe-L-Ala and D-Phe-L-Ala standards. Dpm, disintegrations per minute.

ProCAT partner is presumably incompetent because of incorrect chirality. The failure of D-Phe-S-ProCAT could be explained similarly, but L-Phe-S-ProCAT and L-Leu-S-ProCAT may fail as substrates if the C domain also discriminates for steric size.

Given that this C domain would not use D-Ala or D-Pro as acceptor, we analyzed the preference for D- or L- at the donor site for the formation of Ala-L-Pro and Phe-L-Ala. Both D-Ala-S-CoA and L-Ala-S-CoA yielded only D-Ala-L-[^{3}H]Pro when dipeptide was released from ProCAT before DKP formation (Fig. 2B). This proves that the E domain can accept substrates other than phenylalanine and that epimerization of L-Ala-S-PheATE is faster than peptide bond formation. When the released Phe-Ala dipeptide product was analyzed (Fig. 2C), a 9:1 mixture of D-Phe-L-Ala:L-Phe-L-Ala was obtained, indicating that this C domain can transfer L-Phe at a slow competitive rate. Future studies with inactivating point mutations in the E domain will be required to decipher the donor stereopreference of this C domain.

In general, one would expect the C domains at internal elongation sites in NRPS complexes to be relatively nonselective for the upstream electrophilic acyl partners in condensation reactions, because growing chains of diverse length and steric bulk are translocated. On the other hand, the downstream acceptor T domain always bears a monomeric aminoacyl thioester as the attacking nucleophile. The C domain presumably deprotonates the NH_3^+ of these monomers to generate the NH_2 group by means of a histidine base in a highly conserved HHxxxDG motif (H, His; D, Asp; G, Gly) (*15, 18*) and may register size, shape, and α-carbon chirality toward that nucleophilic partner. The interaction of C domains and downstream T domains must also occur to control the timing of peptidyl chain growth, and there have been speculations about a waiting position (*19*) and kinetic trapping of the aa-S-Ppants to explain how the timing of acyl chain growth is controlled.

The evidence presented here predicts the existence of a binding pocket that could also explain the accurate initiation of the nonribosomal biosynthetic process. Figure 3 specifies three subsites (*20*) for the interaction of the Ppant tether of a T domain with nearby A and C domains: loading site, acceptor site, and donor site. At the loading site, the Ppant-SH terminus must find the aa-O-AMP generated in the paired A domain and capture the aminoacyl moiety as the aa-S-Ppant (for example, Pro-S-T domain). The Pro-S-Ppant$_2$ tether must then reach upstream to the acceptor site of the C_{Pro} domain and find the upstream Phe-S-Ppant$_1$, docked perhaps in a donor site on C_{Pro}. Finally, the D-Phe-L-Pro-S-Ppant$_2$ must reach downstream to the C_{Val} condensation site for chain elongation to the tripeptide–T domain stage. Our results provide evidence for an acceptor-binding, but not a donor-binding, site on C_{Pro}. The ability to bypass adenylation domain specificity represents a general approach to interrogate the mechanism and specificity of various catalytic domains within megasynthetases.

In the functionally and architecturally related polyketide synthetases, monomer acyl groups are presented as activated, diffusable acyl-S-CoAs (*1*). Our results show that N-deblocked aa-S-CoAs have sufficient hydrolytic stability to qualify as candidates for diffusable intermediates in amino acid metabolism, yet aa-S-CoAs are unknown in contemporary organisms. Instead, cells use oxoesters to the 2′ or 3′ termini of the tRNAs that are kinetically and thermodynamically more stable than thioesters. There is evidence that some aa-tRNA synthetases will make small amounts of aa-S-CoAs from CoASH, albeit with low catalytic efficiency. (*21*). This raises the possibility of an evolution of the aa-tRNA synthetases from the A domains of the NRPSs. The unsuitability of aa-S-CoAs may not be due to hydrolytic lability but rather to rapid transthiolation by high glutathione concentrations (1 to 10 mM) in cells, randomizing the activated aminoacyl groups away from the CoASH recognition determinant. This susceptibility to thiol capture may be the reason aminoacyl and peptidyl thioesters are kinetically sequestered away from bulk solution in the T domain (carrier protein) way stations of the peptide megasynthetases.

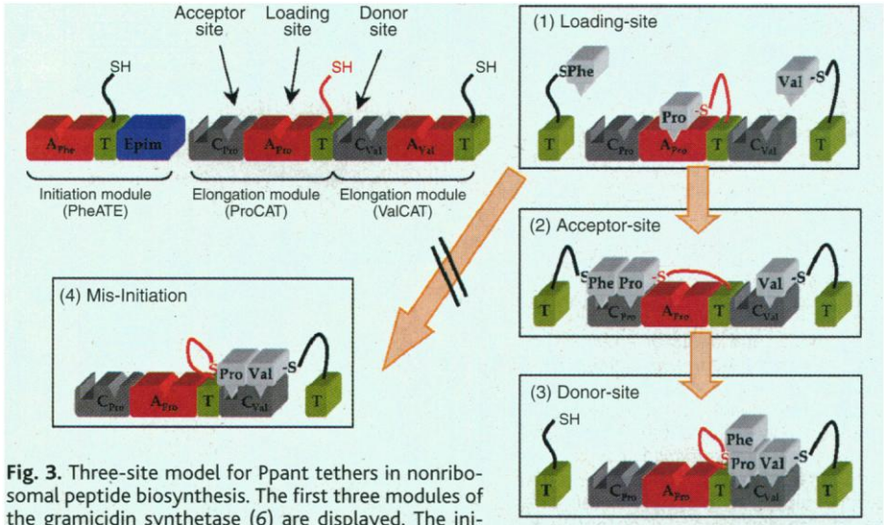


Fig. 3. Three-site model for Ppant tethers in nonribosomal peptide biosynthesis. The first three modules of the gramicidin synthetase (*6*) are displayed. The initiation module PheATE activates and epimerizes the first amino acid (Phe) as a separate subunit from the modules that activate the second (ProCAT) and third (ValCAT) amino acids. To account for the multiple tasks of acceptor recognition, donor recognition, and chain translocation, the Ppant arm of a T domain is postulated to adopt three distinct configurations (*20*), detailed here for the T domain of ProCAT. In the loading site (panel 1), the free thiol of Ppant is acylated by Pro-O-AMP that was formed by the adjacent A_{Pro} domain. The Pro-S-Ppant arm must reach upstream to the acceptor site (panel 2) on the C_{Pro} domain and wait for the donor Ppant from the upstream T domain (here Phe-S-Ppant-T) to fill the donor site. At this juncture the C domain catalyzes peptide bond formation and the dipeptidyl (D-Phe-L-Pro)-S-Ppant arm can exit the C_{Pro} domain and swing downstream to the donor site (panel 3) in the C_{Val} domain. For an orderly progression, to avoid incomplete chains and chain misinitiation at internal sites (panel 4), there must be carefully orchestrated kinetic control of occupancy of the C domain donor and acceptor sites for the Ppant arms of every T domain. The PheATE initiation module has no C domain to trap its aminoacylated T domain and presumably can only occupy the donor site on the C_{Pro} domain to initiate chain growth.

References and Notes

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10 to 70% methanol in 50 mM KH_2PO_4 over 25 min, 10 ml/min, monitor at 380 nm). Next, the HPLC-purified mixture was desalted on the same column (methanol was removed on a rotary evaporator, and the sample loaded in H_2O and eluted with 90% methanol) and lyophilized, yielding the purified Nvoc-aa-S-CoA (40 to 80% yield) as a yellow solid. For preparation of the deprotected aa-S-CoAs, the solution of Nvoc-aa-S-CoA collected from the HPLC purification was directly photolysed (4°C, 350 nm, 1 hour), purified by HPLC again (four injections, TSK gel C18 5×20 cm, 5 μm, 120 Å, 0 to 40% methanol in 50 mM KH_2PO_4 over 25 min, 10 ml/min, monitor at 280 nm), and desalted. After removal of methanol on a rotary evaporator, the sample was lyophilized to give the aa-S-CoA (50 to 80% yield) as a white solid.

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11. PheATE, PheAT, PheTE, and PheT are deletion mutants or derivatives of the phenylalanine (Phe)–activating enzyme GrsA, containing combinations of the A domain, the T domain, and the epimerization (E) domain. ProCAT is the recombinant first module of TycB, containing a proline (Pro)–activating A domain, a T domain, and the upstream C domain.
12. The construction of the expression plasmids pPheATE and pProCAT was described previously (*15*). We amplified the insert of the His-tagged T domain clone pPheT (PheATE residues 523 to 613) from chromosomal DNA of *Bacillus brevis* (American Type Culture Collection 9999) with Turbo-Pfu polymerase (Stratagene) using the manufacturer's protocol and the following 5′-modified oligonucleotides: 5′-Nco I, 5′-ATACCATGGCGGAACCTGATTTAACTTTCGG-3′, and 3′-Bgl II, 5′-ATAAGA TCTTTTACTATCTTTTATATA-ATGAACG-3′. The amplified DNA was purified, digested with Nco I and Bgl II, and ligated into the expression vector pQE60. DNA sequencing of pPheT and mass spectrometric analysis of the purified gene product revealed two modifications: a $His^{84} \rightarrow Gln$ substitution caused by a CAT-to-CAA transversion, and posttranslational removal of the NH_2-terminal methionine. Neither modification interfered with the apo-to-holo conversion of this T domain. The enzymatic integrity of all proteins was confirmed in the adenosine triphosphate (ATP)–pyrophosphate exchange and thioester formation assays (*15*). Apo-to-holo conversion of the T domain was verified by phosphopantetheinylation with [^{3}H]CoASH and Sfp (*10*). A derivative of pPheATE, featuring a cleavable His tag, was obtained by recloning the 3.3-kb *grsA* fragment from pPheATE into pET28b. After expression and affinity purification with Ni^{2+}–nitrilotriacetic acid resin, the pure protein was dialyzed against MES buffer, pH 7.0, and cleaved with thrombin. The protein solution was gel-filtered (P-30 Micro Bio-Spin columns; Bio-Rad) and applied to a Ni^{2+}–nitrilotriacetic acid column, providing PheATE in the flow-through, which was then used for the described [^{3}H]Phe-transfer assay. His-tagged and untagged PheATE are enzymatically identical.
13. Phosphopantetheinylations were performed in MES buffer pH 7.0 (50 mM MES, 100 mM NaCl, 10 mM $MgCl_2$, and 1 mM EDTA) containing 25 μM PheT and 2 to 100 μM CoASH (or derivative). Reactions were initiated by the addition of 25 nM Sfp. At various time points, 100-μl samples were taken and quenched with 10 μl of EDTA (0.5 M). After addition of 25 μl of sample buffer [40% glycerol (v/v), 20% β-mercaptoethanol (v/v) and bromophenol blue (5 mg/l)], 25-μl samples were applied to native tris-tricine gels [H. Schagger and G. von Jagow, *Anal. Biochem.* **166**, 368 (1987)]. The β-mercaptoethanol hydrolyzed the aa-S-Ppant-T thioesters, resulting in conversion to holo T domains, thus eliminating errors in quantitation due to partial hydrolysis during separation. Coomassie-stained gels were analyzed densitometrically with NIH Image 1.6.1 software. For larger peptide synthetase fragments, a spectrophotometric assay was used. Single reactions (100 μl) containing 25 μM T domain (15 μM PheAT or PheTE) and 2 to 100 μM Nvoc-D-Phe-S-CoA were run with or without Sfp for 45 min, quenched by addition of 10 μl of EDTA (0.5 M), gel-filtered (P-6 Micro Bio-Spin Columns; Bio-Rad), and analyzed for absorbance at 350 nm. The values reported were normalized for protein content at 220 nm, and the reliability of the method was confirmed by comparison with identical samples in the PAGE assay. Kinetic constants are as follows (P, PAGE assay; A, absorbance assay). For PheT acceptor: CoASH (P, k_{cat} = 14 min^{-1}:K_m = 14 μM: k_{cat}/K_m = 1 μM^{-1} min^{-1}), Nvoc-D-Ala-S-CoA (P, 20:27:0.7), Nvoc-L-Ala-S-CoA (P, 18:20:0.9), Nvoc-D-Phe-S-CoA (P, 18:13:1.4) and (A, 16:9:1.8), D-Phe-S-CoA (P, 15:8:1.9), Nvoc-L-Phe-S-CoA (P, 17:17:1), L-Phe-S-CoA (P, 22:19:1.2). For PheAT acceptor: Nvoc-D-Phe-S-CoA (A, 17:16:1.1). For PheTE acceptor: Nvoc-D-Phe-S-CoA (A, 15:16:0.9).
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16. Introduction of aa-S-Ppant from Nvoc-aa-S-CoAs into PheATE and ProCAT was accomplished as follows: 2.5 μM apo enzymes were incubated for 30 min at 37°C with 25 μM Nvoc-aa-S-CoA, 50 nM Sfp (*10*), and 5% dimethyl sulfoxide (v/v) in MES buffer pH 7.0. The reaction mixtures were gel-filtered (P-6 Micro Bio-Spin Columns; Bio-Rad) to remove the excess of CoA derivatives and to exchange the MES buffer pH 8.0. Deprotection of the Nvoc group was performed in an ultraviolet reactor (maximum wavelength λ_{max} = 350 nm, distance = 50 to 100 mm; Rayonet Photochemical Reactor, Branford, CT) for 15 min on ice, and the aminoacylated proteins were instantly used in condensation reactions. To ensure a complete aminoacylation of the second module, we incubated the respective holo enzyme with its cognate, radiolabeled substrate: 1 μM holo enzyme was incubated at 37°C in MES buffer, pH 8.0, containing 2 mM ATP and 4.4 μM L-[4-^{3}H]Phe (27.0 Ci/mmol) or 4.8 μM L-[5-^{3}H]Pro (24.7 Ci/mmol). Simultaneously, a 1 μM solution of the other aminoacylated module in MES buffer, pH 8.0, was also incubated at 37°C. After 3 min, the condensation reaction was initiated by combining equal volumes of both solutions. At various time points, 100-μl samples were taken and immediately quenched by the addition of 0.8 ml of 10% trichloroacetic acid (TCA) (w/v) and 20 μl of bovine serum albumin (BSA) solution [2% (w/v)]. The TCA precipitate was washed once with 0.5 ml of 10% TCA (w/v), and the acid-stable label was quantified by LSC. The supernatants 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 NaCl, and the amount of extractable label (DKP) was quantified by LSC (*15*).
17. The rate of formation of the dipeptidyl-S-T domain intermediate is about three to four times as fast as the subsequent release of the corresponding DKP product. For the wild-type system, the following rates were determined: 1.8 min^{-1} for the formation of D-Phe-L-Pro-S-Ppant and 0.5 min^{-1} for DKP release. The donor-site assay only assesses the slower DKP formation, so no argument can be made about possible rate differences for the formation of various aa-L-Pro dipeptide intermediates, except that they are no slower than 0.4 to 0.5 min^{-1}.
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Functional Arteries Grown in Vitro

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A tissue engineering approach was developed to produce arbitrary lengths of vascular graft material from smooth muscle and endothelial cells that were derived from a biopsy of vascular tissue. Bovine vessels cultured under pulsatile conditions had rupture strengths greater than 2000 millimeters of mercury, suture retention strengths of up to 90 grams, and collagen contents of up to 50 percent. Cultured vessels also showed contractile responses to pharmacological agents and contained smooth muscle cells that displayed markers of differentiation such as calponin and myosin heavy chains. Tissue-engineered arteries were implanted in miniature swine, with patency documented up to 24 days by digital angiography.

Atherosclerotic vascular disease, in the form of coronary artery and peripheral vascular disease, is the largest cause of mortality in the United States (*1*). Surgical mainstays of therapy for affected vessels less than 6 mm in diameter include bypass grafting with autologous veins or arteries (*2*); however, adequate tissue for bypass conduits is lacking in many patients. Artificial materials, when used to bypass arteries that are less than 6 mm in diameter, have thrombosis rates greater than 40% after 6 months (*3*). Although novel approaches for producing small-caliber arterial grafts have been developed, problems with mechanical properties (*4*) or the utilization of neonatal cells (*5*) have heretofore prevented clinical implementation.

We report here the development of tech-

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