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Assembly of the *Pseudomonas aeruginosa* Nonribosomal Peptide Siderophore Pyochelin: In Vitro Reconstitution of Aryl-4,2-bisthiazoline Synthetase Activity from PchD, PchE, and PchF[†]

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ABSTRACT: Three *Pseudomonas aeruginosa* proteins involved in biogenesis of the nonribosomal peptide siderophore pyochelin, PchD, PchE, and PchF, have been expressed in and purified from *Escherichia coli* and are found to produce the tricyclic acid hydroxyphenyl-thiazolyl-thiazolinyl-carboxylic acid (HPTT-COOH), an advanced intermediate containing the aryl-4,2-bis-heterocyclic skeleton of the bithiazoline class of siderophores. The three proteins contain three adenylation domains, one specific for salicylate activation and two specific for cysteine activation, and three carrier protein domains (two in PchE and one in PchF) that undergo posttranslational priming with phosphopantetheine to enable covalent tethering of salicyl and cysteinyl moieties as acyl-*S*-enzyme intermediates. Two cyclization domains (Cy1 in PchE and Cy2 in PchF) create the two amide linkages in the elongating chains and the cyclodehydrations of acylcysteine moieties into thiazolinyl rings. The ninth domain, the most downstream domain in PchF, is the chain-terminating, acyl-*S*-enzyme thioester hydrolase that releases the HPTT-*S*-enzyme intermediate to the observed tandem bis-heterocyclic acid product. A PchF-thioesterase domain active site double mutant fails to turn over, but a monocyclic hydroxyphenyl-thiazolinyl-cysteine (HPT-Cys) product continues to be released from PchE, allowing assignment of the cascade of acyl-*S*-enzyme intermediates involved in initiation, elongation, and termination steps.

When bacteria are in iron-limited environments, they often respond with the production of low-molecular weight iron chelators known as siderophores (Figure 1), with moderate [$K_d = 10^{-5}$ M for pyochelin (*1*−*3*)] to good [$K_d = 10^{-30}$ M for mycobactins (*4*)] to remarkable [$K_d = 10^{-52}$ M for enterobactin (*5*)] affinities for Fe(III). Siderophores fall into three general types, with (a) hydroxamates [mycobactin **3** (*6*) and exochelin (*7*)], (b) catechols [enterobactin **4** (*8*) and vibriobactin (*9*)], or (c) thiazolines [pyochelin **1** (*10*) and yersiniabactin **2** (*11*, *12*)] as iron-coordinating functional groups, but all three types are assembled by nonribosomal peptide synthetases (NRPS).[1] NRPS are a class of multimodular enzymes (*13*−*15*) also responsible for the generation of peptide-based secondary metabolites with antimicrobial [vancomycin (*16*) and penicillins (*17*)], immunosuppressive [cyclosporins (*18*)], anticancer [bleomycin (*19*)], or surfactant [surfactin (*20*)] activity. The opportunistic pathogen *Pseudomonas aeruginosa* makes both a high-affinity peptide hydroxamate, i.e., pyoverdin (*21*), and a low-affinity peptide bisthiazoline, i.e., pyochelin **1** (*10*), as siderophores that contribute to pathogenicity (*22*−*24*).

We are attempting to dissect the molecular organization and mechanism of the NRPS catalysts that make siderophores and antibiotics. In recent studies, we have reported the full reconstitution of enterobactin synthetase activity from the four *Escherichia coli* proteins EntB, EntD, EntE, and EntF in making the tris[*N*-(2,3-dihydroxybenzoyl)serine]trilactone **4** (*25*), and also the partial reconstitution (formation of the aryl-capped first thiazoline ring) of the yersiniabactin synthetase activity from the plague bacterium *Yersinia pestis* (*26*). Yersiniabactin **3** possesses two thiazolines and a thiazolidine, arising from enzymatic cyclization of cysteine residues during chain elongation on the carrier protein domains of the multidomain high-molecular weight protein 2 (HMWP2) and high-molecular weight protein 1 (HMWP1) of yersiniabactin synthetase. Pyochelin **2** clearly is structurally homologous to the left-hand portion of yersiniabactin **3**, and one anticipates its assembly from salicylate and two cysteine residues. Most intriguing is the sequential cyclization and dehydration in generating the tandem 4,2-linked thiazoline−thiazolidine pair in both yersiniabactin and pyochelin. Such tandem ring heterocycles, when fully aromatized

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[1] Abbreviations: PCP, peptidyl carrier protein; ArCP, aryl carrier protein; BSA, bovine serum albumin; CoA, coenzyme A; DTT, dithiothreitol; IPTG, isopropyl *β*-D-thiogalactopyranoside; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; P-pant, 4′-phosphopantetheine; PPTase, 4′-phosphopantetheinyl transferase; SOE, splicing by overlap extension; TCA, trichloroacetic acid; LSC, liquid scintillation counting; HPTT-COOH, hydroxyphenyl-thiazolyl-thiazolinyl-carboxylic acid; HPT-Cys, hydroxyphenyl-thiazolinyl-cysteine; NRPS, nonribosomal peptide synthetases; H6, hexahistidine; PP_i, pyrophosphate; HPLC, high-performance liquid chromatography.

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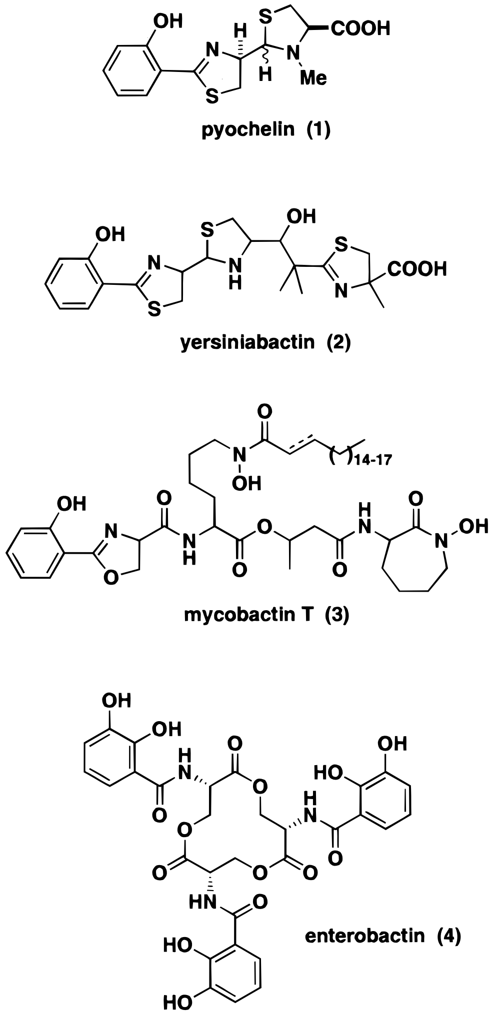

FIGURE 1: Structures of aryl-N-capped peptidic siderophores.

as the bithiazole, become the DNA-intercalating moieties in the anticancer drug bleomycin (*27*), but to date, their mechanism of formation from Cys-Cys dipeptide precursors has been obscure.

The *P. aeruginosa* genome sequencing project, as well as more directed genetic efforts on pyochelin assembly, have revealed two gene clusters, *pchEF* (*28*) and *pchDCBA* (*29*), required for pyochelin biosynthesis. PchA and PchB are involved in conversion of chorismate to salicylate, and PchC is homologous to thioesterases associated with NRPS. PchD (547 amino acids) is homologous to MbtA (*6*) and YbtE (*26*), the 2-hydroxybenzoyl-AMP ligases involved in the mycobactin and yersiniabactin biosynthetic pathways, respectively, making it likely that PchD fulfills the same role in pyochelin biosynthesis. As noted by Reimmann and colleagues, PchE (1438 amino acids) and PchF (1809 amino acids) have signature sequences for NRPS. By gene inactivation analysis, PchE has been shown to be required for production of dihydroaeruginoate (here termed hydroxyphenyl-thiazolinyl carboxylate or HPT-COOH), while PchF is required for production of the entire pyochelin molecule (*28*). The results of the gene inactivation analysis fit the predictions of domain and module organization by Reimmann and colleagues and the deductions we can make from the arrangement of four predicted domains in PchE and four in PchF. In particular, the first four domains of HMWP2 [residues 1−1491 (*26*)] presage the domain order of PchE, making it the likely starter subunit in pyochelin assembly. In this work, we report the overproduction in *E. coli* and purification of PchD, PchE, and PchF, and the posttranslational priming of the three carrier protein domains of PchE and PchF. We also demonstrate the competence of the reconstituted pyochelin synthetase in catalyzing the double cyclodehydration to yield the aryl-4,2-linked bis-heterocyclic skeleton of pyochelin, minus the reduction and N-methylation tailoring of the terminal thiazoline. This is the first report of an in vitro enzymatic biosynthesis of such tandem bis-heterocycles by full-length recombinant NRPS. Finally, the NRPS cosynthetic heterocyclization to yield 4,2-linked bis-heterocyclic rings reported here contrasts with the enzymes that posttranslationally heterocyclize serines and cysteines in the ribosomally synthesized pro-microcin to introduce the 4,2-linked tandem rings found in the mature antibiotic microcin B17 produced by *E. coli* (*30*). These two different strategies are the only enzymatic pathways known to date for formation of these bis-heterocyclic rings.

EXPERIMENTAL PROCEDURES

Materials and Recombinant DNA Techniques. Luria-Bertani (LB) medium was prepared and used for culturing *E. coli* strains and *P. aeruginosa* PA14 as previously described (*31*). Pyochelin production in *P. aeruginosa* PA14 in iron-limited medium (*32*) was confirmed by HPLC and thin-layer chromatography as reported previously (*28*, *33*), and by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF): m/z ($[M + H]^+$) calcd 325.1, found 325.2. Competent cells of *E. coli* strains DH5α and BL21(DE3) were purchased from GibcoBRL and Novagen, respectively. Plasmids pET28 and pET29 were obtained from Novagen. Restriction endonucleases, T4 DNA ligase, pLITMUS28, and pUC19 were obtained from New England Biolabs. Unlabeled coenzyme A (CoA) was purchased from Sigma. [^{3}H]CoA was prepared by DuPont New England Nuclear. [^{14}C]Salicylate, [^{35}S]-L-cysteine, and [^{32}P]PP$_i$ were purchased from DuPont New England Nuclear. Synthesis of the HPT-COOH standard has been reported previously (*26*). HPTT-COOH was synthesized and kindly provided by I. Mori. Sfp was purified as described previously (*34*). *P. aeruginosa* strain PA14 was cultured in LB medium, and its genomic DNA was isolated using QIAGEN-tip (Qiagen). Plasmid DNA preparation, gel extraction of DNA fragments, and purification of DNA amplified by polymerase chain reaction (PCR) were performed using QIAprep, QIAEX II, and QIAquick kits, respectively (Qiagen). PCRs were carried out using *Pfu* DNA polymerase as described by the enzyme supplier (Stratagene), except for the addition of DMSO (10%) and glycerol (3%) to the reaction mixtures. The fidelity of PCR-amplified DNA fragments was established by nucleotide sequencing after subcloning into the corresponding expression vector. The *pch* genes from *P. aeruginosa* PA14 exhibited a 1−1.5% variation when compared to those from *P. aeruginosa* PAO1, resulting in a ∼1% variation in amino acid sequence. The sequences from *P. aeruginosa* PA14 have been deposited in GenBank [accession numbers AF184620 (*pchD*), AF184621 (*pchE*), and AF184622 (*pchF*)]. DNA sequencing was performed on double-stranded template DNA by the Molecular Biology Core Facility of the Dana Farber Cancer Institute (Boston, MA). Oligonucleotide primers were obtained from Integrated

Table 1: Oligonucleotides Used for Amplification of Genes from *P. aeruginosa*

Gene	Primer name	Primer sequence [a]
pchE	E1	GAGAGCC**CATATG**GATCTGCCCCCCGATT
pchE	E2	GTTCGC**AAGCTT**TCATAGCACGCCCTCTTCCA
pchF	F1	AGGGCGT**CATATG**AGCCTCGGCGAACTGCTGGAAAC
pchF	F2	CGGACGT**AAGCTT**TCGGCTCGCCTCCTCTGGCA
pchD	D1	CGGAGAT**CATATG**ACTTCCTCGCCCGTCA
pchD	D2	CAGGCGG**AAGCTT**TGCGCGGGCCTCCAGCTCGC
pchC	C1	AGGCCCG**CATATG**AGCGCCGCCTGGGTCCGGCCGT
pchC	C2	GGAGTTT**AAGCTT**TGCAACCTCTCGGGAAAGGGCGT
pchF-Te	F3	CGCTACATGCTCCCGGCGCAGTT
pchF-Te	F4	TTCGACGCTGGCGGCGTCGGCACTA
pchF-Te	F5	CTGCTCGGCTACGCAGCCGGCGGGCTGGTC
pchF-Te	F6	ACCAGCCCGCCGGCTGCGTAGCCGAGCAGGT

[a] The bold sequences represent restriction sites used for cloning as specified in the text. Nucleotide changes introduced in *pchFTE* are underlined.

DNA Technologies Inc. The protein concentration was determined with Bio-Rad protein assay (Bio-Rad) or by using the protein's predicted molar extinction coefficient and absorpance at 280 nm.

Cloning pch Genes and Plasmid Constructions. The *pch* genes *pchE*, *pchF*, *pchC*, and *pchD* were amplified from *P. aeruginosa* strain PA14 genomic DNA using the primers shown in Table 1. The amplified *pchC*, *pchF*, and *pchD* fragments were digested with *Nde*I and *Hin*dIII and ligated to pET29b digested with the same enzymes to generate the pPch plasmids pPchC, pPchF, and pPchD, respectively. The amplified *pchE* fragment was digested with *Nde*I and *Hin*dIII and ligated to pET28b digested with the same enzymes to generate plasmid pPchE. Plasmids pPchC, pPchF, and pPchD express the translational fusions PchC-H6, PchF-H6, and PchD-H6, respectively, in which the hexahistidine (H6) tags encoded in the corresponding vectors are fused in-frame to the C-terminus of the Pch proteins. The plasmid pPchE expresses the translational fusion H6-PchE in which the H6 tag encoded in the vector is fused in-frame to the N-terminus of PchE. The pPch plasmids were introduced by transformation into *E. coli* strain DH5α, and then transferred to *E. coli* strain BL21(DE3) for expression and overproduction of the fusion proteins.

Construction of the PchF Thioesterase Mutant. The Cys1606Ala/Ser1607Ala double mutant of PchF was constructed via the splicing by overlap extension (SOE) method (*35*). To obtain useful cloning sites, the *Bsi*WI to *Hin*dIII fragment of pPchF was excised by restriction digestion and subcloned successively into pLITMUS28 and pUC19, yielding pLITMUS28-PchF(6458−10503) and pUC19-PchF(6458−10503), respectively. Using pPchF as a template, the 5′ and 3′ fragments of a mutant insert were amplified in the first round of PCR using primers F3 and F6 (5′ fragment) and F4 and F5 (3′ fragment). The fragments were gel-purified, combined, and used as a template for the second round of PCR, with F3 and F4 as primers. The resulting product was digested with *Bst*EII and *Not*I and ligated to the corresponding sites in pUC19-PchF(6458−10503), creating pUC19-PchFTE. This plasmid was then digested with *Bsi*WI and *Hin*dIII, and the insert was recovered and ligated to the corresponding sites in digested pPchF, creating pPchFTE. This plasmid was introduced into *E. coli* DH5α, and subsequently into *E. coli* BL21(DE3) for overproduction of PchF(Cys1606Ala/Ser1607Ala)-H6.

Overproduction and Purification of Pch Proteins in E. coli. For overproduction of H6-PchE, PchF-H6, PchFTE-H6, and PchD-H6, the *E. coli* BL21(DE3) strains containing the pPch plasmids were cultivated (1 L) with shaking (300 rpm) at 30 °C in LB broth containing 50 μg/mL kanamycin. Routinely, cultures were induced with 0.5 mM IPTG when they reached an OD$_{600}$ of 0.4. After induction, incubation was continued for a period of 4 h at 24 °C before the cells were harvested by centrifugation (10 min at 2000*g*) and resuspended in 30 mL of a solution of 0.2 M NaCl/25 mM Tris-HCl (pH 7.9) (buffer A). Following resuspension, cells were disrupted by two passages through a French pressure cell (18 000 psi). Cellular debris was removed from the lysate by centrifugation (30 min at 95000*g*). The fusion proteins were purified by nickel column chromatography using Ni-NTA Superflow resin according to the manufacturer's instructions (Qiagen). For purification, column loading was performed in buffer A, washing was done with 25 mM Tris-HCl (pH 7.9)/500 mM NaCl/5 mM imidazole (buffer B), and elution was accomplished with buffer B and an imidazole gradient increasing from 5 to 300 mM over 120 mL. Fractions of the eluant from this column were analyzed for the presence of the Pch proteins by SDS−PAGE. Fractions containing the Pch fusions were dialyzed against 25 mM Tris-HCl (pH 8.0)/2 mM DTT/10% glycerol (buffer C). After dialysis, the fractions were aliquotted, flash-frozen in liquid nitrogen, and stored at −80 °C.

Covalent Incorporation of [^{3}H]Phosphopantetheine into PchE and PchF. The incorporation of [^{3}H]phosphopantetheine was performed as described previously (*6*, *34*). Reaction mixtures (150 μL) contained 75 mM Tris-HCl (pH 7.5), 10 mM MgCl$_2$, 1 mM DTT, 25 μM [^{3}H]CoA (75 Ci/mol), 5 mM ATP, 5% glycerol, 500 nM Sfp, and 2.5 μM PchE or 2.5 μM PchF. After the reactions had been started with the addition of 10 μL of [^{3}H]CoA, samples (10 μL) were taken from the mixtures at specific times and the reactions quenched with 200 μL of 10% TCA and 50 μg of BSA. The precipitate was pelleted by centrifugation, washed two times with 10% TCA (1 mL), solubilized in 200 μL of 1 M Tris base and 0.5% SDS, and added to 3.5 mL of liquid scintillation fluid. The incorporated ^{3}H label was quantified by liquid scintillation counting (LSC).

Covalent Incorporation of [^{35}S]Cysteine into PchE and PchF. Aminoacylation was performed as described previously (*36*). The synthetases were aminoacylated in reaction mixtures (100 μL) containing 75 mM Tris-HCl (pH 7.5), 10 mM MgCl$_2$, 5 mM ATP, 0.1 mM CoA, 5% glycerol, 1 μM PchD, and 0.5 μM Sfp. PchE or PchF was included in the reaction mixtures at a concentration of 4 μM. The reaction mixtures were incubated at 30 °C for 1 h to allow phosphopantetheinylation prior to initiating the aminoacylation reaction with the addition of [^{35}S]Cys. After addition of 5 μL of [^{35}S]Cys (2 mM, 954 Ci/mol), 5 μL samples were taken at specific times and the reactions quenched with 200 μL of 10% TCA. The precipitate was pelleted by centrifugation, washed twice with 10% TCA (1 mL), solubilized in 200 μL of 1 M Tris base and 0.5% SDS, and added to 3.5 mL of liquid scintillation fluid before quantification of the incorporated ^{35}S label by LSC. Samples for autoradiography were quenched in 10 μL of 2× PAGE sample buffer without DTT. Samples were resolved using 7.5% gradient gels (Bio-Rad), stained with Coomassie Blue, soaked for 20 min in Amplify (Amersham), dried at 80 °C under vacuum, and exposed to X-ray film for 2 h at −70 °C.

Covalent Incorporation of [^{14}C]Salicylate and [^{14}C]-Salicylate Transfer from PchE to PchF. For assessing the incorporation of [^{14}C]salicylate into PchE, reaction mixtures (100 μL) contained 75 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 5 mM ATP, 5 mM Cys, 0.1 mM CoA, 5% glycerol, 1 μM PchD, 0.5 μM Sfp, and 2.5 μM PchE. Reaction mixtures were incubated at 30 °C for 30 min to allow phosphopantetheinylation prior to addition of 2.8 μL of [^{14}C]salicylate (1.8 mM, 55.5 Ci/mol). After addition of [^{14}C]salicylate, 5 μL samples were taken at specific times and the reactions quenched with 250 μL of 10% TCA. The precipitate was pelleted by centrifugation, washed two times with 10% TCA (1 mL), solubilized in 200 μL of 1 M Tris base and 0.5% SDS, and added to 3.5 mL of liquid scintillation fluid. The incorporated ^{14}C label was quantified by LSC. To test for transfer of [^{14}C]salicylate from PchE to PchF, and unless otherwise specified, reaction mixtures (100 μL) were as described above, but containing both PchE (7 μM) and PchF (5 μM). The reaction mixtures were incubated at 30 °C for 1 h to allow phosphopantetheinylation prior to addition of 5 μL of [^{14}C]salicylate (1.8 mM, 55.5 Ci/mol). After addition of [^{14}C]salicylate, 10 μL samples were taken at specific times and the reactions quenched with 10 μL of 2× PAGE sample buffer without DTT. The samples were resolved using 7.5% gels (Bio-Rad), stained with Coomassie Blue, soaked for 20 min in Amplify (Amersham), dried at 80 °C under vacuum, and exposed to X-ray film for 3 days at −70 °C.

ATP−[^{32}P]Pyrophosphate Exchange Reactions and K_m Determination. ATP−PP_i exchange was assayed as previously described (*6*, *36*). The reaction mixtures (100 μL) contained 75 mM Tris-HCl (pH 8.8), 10 mM NaCl, 5 mM ATP, 2 mM DTT, and 5% glycerol. For the K_m determination, the concentration of Cys or salicylate was varied. The enzymes PchF, PchE, and PchD were added to the reaction mixture to final concentrations of 40, 80, and 100 nM, respectively. The reactions were initiated by addition of 20 μL of sodium [^{32}P]pyrophosphate (5 mM, 2 μCi/μmol). After incubation at 30 °C for 10 min, the reactions were quenched by the addition of 0.5 mL of a charcoal suspension [1.6% (w/v) activated charcoal, 0.1 M tetrasodium pyrophosphate, and 0.35 M perchloric acid]. The charcoal was pelleted by centrifugation, washed three times with 1 mL of 0.1 M tetrasodium pyrophosphate and 0.35 M perchloric acid, resuspended in 0.5 mL of H_2O, and added to a scintillation vial containing 3.5 mL of scintillation fluid, and the bound radioactivity was determined by LSC.

Production and Detection of Salicylate-Containing Compounds. Unless otherwise specified, enzymatic reaction mixtures for production of salicylate-containing compounds (reconstitution reaction) contained 75 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 5 mM ATP, 0.1 mM CoA, 1 mM salicylate, 5 mM Cys, 5% glycerol, 2.5 μM PchE, 2.5 μM PchF, 1 μM PchD, and 0.5 μM Sfp. The reaction mixtures were incubated at 30 °C for 1 h to allow phosphopantetheinylation prior to initiating the reaction with the addition of ATP. After addition of ATP, incubation was continued and samples of 100 μL were taken at specific times, followed by acidification with 20 μL of 8.5% phosphoric acid. Salicylate-containing compounds were extracted with ethyl acetate (800 μL) as reported previously (*26*). The recovered organic fraction (700 μL) was dried under reduced pressure. The residue was dissolved in 300 μL of 30% acetonitrile/water and analyzed by HPLC using a Vydac C18 reverse-phase column (4.6 mm × 25 cm) on a Beckman Gold HPLC system with monitoring at 254 nm. Mobile phases were as follows: mobile phase A, 0.1 mL of formic acid and 0.2 mL of triethylamine in 1 L of water; and mobile phase B, acetonitrile and mobile phase A in a 4:1 mixture. Samples were eluted at a rate of 1 mL/min using a linear gradient of mobile phase B from 10 to 76% over the course of 17 min. Production of pyochelin in reconstitution reactions was monitored by HPLC using an extended gradient of mobile phase B from 10 to 100% over the course of 23 min (the pyochelin standard elutes at 22.2 min). The amount of HPTT-COOH and HPT-Cys present in the reconstitution reaction mixtures was calculated from the peak's area by comparison to reference curves (area vs nanomoles) prepared with the synthetic standards. The salicylate area was used to correct the area of the products to compensate for errors arising from sample processing.

Production and Isolation of Peaks I and II for Mass Spectrometry and ^{1}H NMR. A 10 mL reconstitution reaction mixture was prepared as indicated above. The reaction was quenched with 2 mL of 8.5% phosphoric acid, and the products were extracted with 50 mL of ethyl acetate. The organic fraction was dried under reduced pressure, and the residue was dissolved in 30 mL of 30% acetonitrile/water. The samples were resolved by HPLC using the procedures described above, and peaks I and II were collected from each injection. The purified peaks I and II were analyzed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF). ^{1}H NMR spectra of purified peaks I and II were acquired in CD_3OD with a Varian Unity 500 spectrometer and referenced to residual CHD_2OD. Coupling constants are given in hertz.

(1) Hydroxyphenyl-Thiazolyl-Thiazolinyl-Carboxylic Acid (HPTT-COOH). ^{1}H NMR: δ 8.25 (s, 1H), 7.96 (m, 1H), 7.35 (t, 1H, $J = 7.7$), 7.01 (d, 1H, $J = 8.0$), 6.96 (m, 1H), 5.31 (m, 1H), 3.76 (m, 1H). Chemical shifts were in good agreement with those obtained from a synthetic standard. MS (MALDI): m/z ($[M + H]^+$) calcd 307.02, found 307.71.

(2) Hydroxyphenyl-Thiazolinyl-Cysteine (HPT-Cys). Small amounts of material from the enzymatic reaction did not allow complete assignment of a ^{1}H spectrum. However, phenyl ring protons and the two α-peptidic protons were matched with those from a synthetic standard. MS (MALDI): m/z ($[M + H]^+$) calcd 327.05, found 327.73.

RESULTS

Purification of Recombinant PchE, PchF, PchD, and PchFTE. The postulated multidomain peptide synthetases PchF (1809 residues) and PchE (1438 residues) and the predicted salicyl-AMP ligase PchD (547 residues) from *P. aeruginosa* PA14 were overproduced in *E. coli* and purified. A double mutant PchF variant (PchFTE) with amino acid substitutions (Cys1606Ala and Ser1607Ala) in the thioesterase (TE) domain (Figure 2) was also constructed and purified. To facilitate purification, each enzyme was overproduced

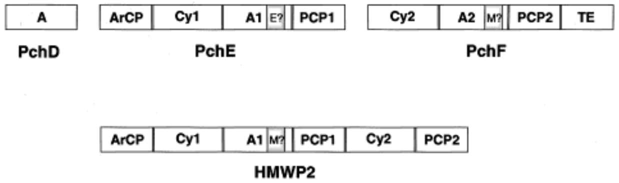

FIGURE 2: Domain organization of the PchDEF system and of HMWP2. The adenylation (A) domains, cyclization (Cy) domains, aryl and peptidyl carrier protein (ArCP and PCP) domains, and thioesterase (TE) domain are represented. The gray boxes represent inserts of ∼340 amino acid present in the adenylation domains. On the basis of a low level of homology and enzymatic activities required to produce the corresponding siderophores, the inserts have been suggested to have epimerase (E) or methyltransferase (M) activity. The prediction of domain organization for PchE and PchF has been reported by Reimmann (*28*), who proposed a reductase domain in place of the TE domain assigned here. The domain organization of HMWP2 has been reported previously (*26*).

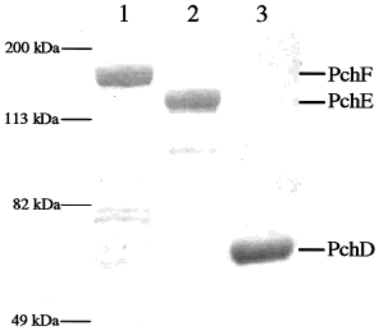

FIGURE 3: Purity of recombinant PchD-H6, H6-PchE, and PchF-H6 overproduced in and purified from *E. coli*. Protein samples were resolved by SDS−PAGE (10%) and stained with Coomassie blue. Lanes 1−3 were loaded with PchF-H6, H6-PchE, and PchD-H6, respectively, obtained after purification. The positions of molecular mass markers are denoted.

fused to a hexahistidine (H6) tag. The tags in PchE and PchF or PchFTE were placed at the N- and C-termini, respectively. The positions for the H6 tags were selected because they would leave the native N- and C-termini of PchF or PchFTE and PchE, respectively, free for potential protein−protein interactions. Interaction between these ends of the synthetases might be essential for transfer of acyl intermediates from PchE to PchF (see below).

The recombinant synthetases PchE, PchF, and PchFTE were insoluble when overproduced at 37 °C. When overproduced at this temperature, the recombinant proteins present in the cell lysates were pelleted by centrifugation, and no detectable synthetases remained in the supernatant as determined by SDS−PAGE. However, a fraction (∼20%) of the overproduced synthetases remained soluble when the proteins were overproduced at 24 °C. In contrast, the salicyl-AMP ligase PchD, bearing the H6 tag at its C-terminus, was substantially soluble when overproduced at 37 °C. The soluble enzymes were purified by nickel affinity chromatography. The final yields of PchE, PchF, and PchD were 8, 6, and 36 mg per liter of *E. coli* culture, respectively (Figure 3). The yield for the PchFTE mutant was 4 mg per liter, and the purity (not shown) was comparable to that of the wild-type PchF protein. Attempts to overproduce PchC, the postulated thioesterase (*29*) in the pyochelin biosynthesis pathway, fused to a C-terminal H6 tag or an N-terminal maltose binding protein tag were unsuccessful. Recombinant PchC could not be detected in *E. coli* upon induction as determined by SDS−PAGE.

Posttranslational Phosphopantetheinylation of the Apo Forms of PchE and PchF. In line with the multiple-carrier thiotemplate mechanism for acyl chain assembly of products by peptide synthetases (*37*), posttranslational modification of PchE and PchF by covalent attachment of the 4′-phosphopantetheine (P-pant) moiety of coenzyme A is essential for the biosynthetic competence of both synthetases (Figure 7, see below). On the basis of sequence analysis, there are two predicted target domains for phosphopantetheinylation in PchE, an N-terminal aryl carrier protein domain (ArCP) and a C-terminal peptidyl carrier protein domain (PCP1), and one internal peptidyl carrier protein domain (PCP2), in PchF (Figure 2). The ArCP, PCP1, and PCP2 domains contain conserved serine residues (Ser46 and Ser1385 in PchE and Ser1442 in PchF) whose side chains are the predicted sites of phosphopantetheinylation (*28*). Although posttranslational modification of PchE and PchF is not required in assessing amino acid activation (formation of cysteinyl-AMP intermediates; see below), phosphopantetheinylation is necessary in investigating the succeeding formation of the salicyl-*S*-ArCP and cysteinyl-*S*-PCP covalent enzyme intermediates and subsequent product formation by these synthetases. In the absence of an identifiable *P. aeruginosa* phosphopantetheine transferase (PPTase) to date, we used Sfp to posttranslationally modify PchE and PchF. Sfp is a robust PPTase from *Bacillus subtilis* responsible for the posttranslational modification of surfactin synthetase, but is able to modify a wide variety of heterologous carrier domains (*34*). The apo to holo conversion of the carrier domains in PchE (i.e., ArCP and PCP1) and PchF (i.e., PCP2) was investigated by monitoring the Sfp-dependent covalent incorporation of [^{3}H]phosphopantetheine into the synthetases (Figure 4A). For both synthetases, under the conditions of the assay, the Sfp-dependent phosphopantetheinylation exhibited well-behaved saturation kinetics and was completed after incubation for 30 min. The maximum percentage of phosphopantetheinylation measured for PchE was 100%, adjusted for the two potential modification sites (ArCP and PCP1). PchF, with a single carrier protein domain (PCP2), was phosphopantetheinylated with a stoichiometry of 67%. The stoichiometries of phosphopantetheinylation are in good agreement with the predicted number of target sites in the synthetases. We thus conclude that the PPTase Sfp from *B. subtilis* efficiently modifies these synthetases from *P. aeruginosa* and generates a high mole fraction of holo synthetases that can become covalently loaded during pyochelin assembly.

Formation of Acyl-AMP Intermediates: Activation of the Pyochelin Monomers. Prior to the formation of the salicyl-*S*-ArCP or cysteinyl-*S*-PCP covalent intermediates (Figure 7), salicylate and cysteine monomers have to be activated as adenylates. To determine whether the predicted cysteine adenylation domains (A1 and A2) of recombinant synthetases PchE and PchF and the predicted salicylate activating protein PchD would activate their respective substrates, the cysteine- and salicylate-dependent exchange of [^{32}P]PP$_i$ into ATP was analyzed. The exchange assay reflects the reversible formation of the tightly but not covalently bound acyl-AMP· enzyme intermediates. Both peptide synthetases were able to activate L-cysteine (Figure 4B,C). However, D-cysteine

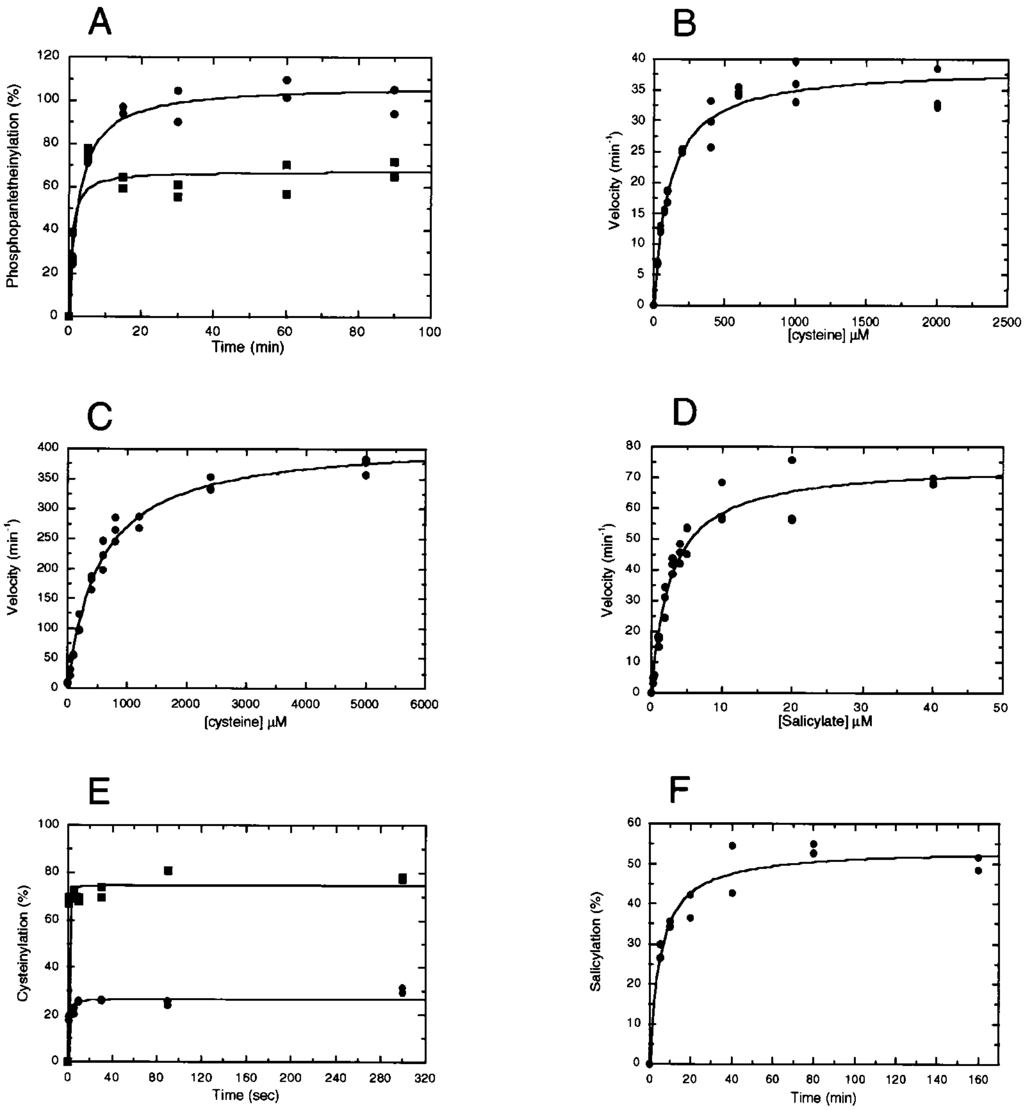

FIGURE 4: (A) Sfp-catalyzed phosphopantetheinyl group transfer from CoA to apo-PchE (●) and apo-PchF (■). PPTase activity was measured as the extent of incorporation of [^{3}H]P-pant into apo-proteins as monitored by radioassay. (B and C) Velocity of cysteine-dependent exchange of [^{32}P]PP$_i$ into ATP for PchE and PchF, respectively. (D) Velocity of salicylate-dependent exchange of [^{32}P]PP$_i$ into ATP for PchD. (E) In-cis aminoacylation of PchE (●) and PchF (■) with cysteine. (F) PchD-dependent in-trans aminoacylation of PchE with salicylate. The level of aminoacylation was measured as the extent of incorporation of [^{35}S]Cys (cysteinylation) or [^{14}C]salicylate (salicylation) into holo-proteins as monitored by radioassay. See Experimental Procedures for details.

was not a substrate (not shown) for either enzyme in this assay. PchF had a $k_{\rm cat}$ of 415 min^{-1} and a $K_{\rm m}$ for cysteine of 537 μM, while the $k_{\rm cat}$ and $K_{\rm m}$ for cysteine determined for PchE were 38 min^{-1} and 110 μM, respectively. Thus, both cysteine adenylation domains had comparable catalytic efficiencies: 0.4 μM^{-1} min^{-1} for A1 in PchE and 0.8 μM^{-1} min^{-1} for A2 in PchF. The catalytic competence of the adenylation domain of PchD, presumably responsible for the activation of salicylate, was also demonstrated (Figure 4D). Recombinant PchD had a $k_{\rm cat}$ of 74 min^{-1} and a $K_{\rm m}$ for salicylate of 2.8 μM, indicating a catalytic efficiency of 27 μM^{-1} min^{-1}. These results validate predicted monomer activation functions of PchE, PchF, and PchD, an essential prerequisite to pyochelin assembly.

Covalent Aminoacylation of PchF and PchE and Arylation of PchE: Loading of the Monomers. In accordance with the model for pyochelin assembly (Figure 7), the holo forms of PchE and PchF should carry the monomeric precursors (salicylate and cysteine) through a thioester linkage to the reactive thiol group of the posttranslationally introduced phosphopantetheine prosthetic group. The formation of salicyl-*S*-ArCP in PchE would require a second activity of the salicyl-AMP ligase PchD, i.e., salicyl transferase. The salicylation of the ArCP to form salicyl-*S*-PCP in PchE represents an acylation in-trans. In contrast, the formation of cysteinyl-*S*-PCP in PchE and PchF is an acylation in-cis, in which the cysteine transferase activity of each synthetase's adenylation domain loads cysteine on the PCP domain located within the same polypeptide. To determine whether the synthetases were capable of in-cis formation of cysteinyl-*S*-PCP, PchE and PchF were first phosphopantetheinylated using Sfp, and then the formation of the covalent acyl intermediates was monitored by measuring the extent of covalent incorporation of [^{35}S]cysteine into the holo synthetases by TCA precipitation and LSC. The results shown in Figure 4E demonstrate the competence of both holo synthetases for autoacylation. Under the conditions of the assay, the cysteinylation of PchE and PchF exhibited well-behaved saturation kinetics and was completed in less than 30 s. The stoichiometries of cysteine loading for holo-PchF

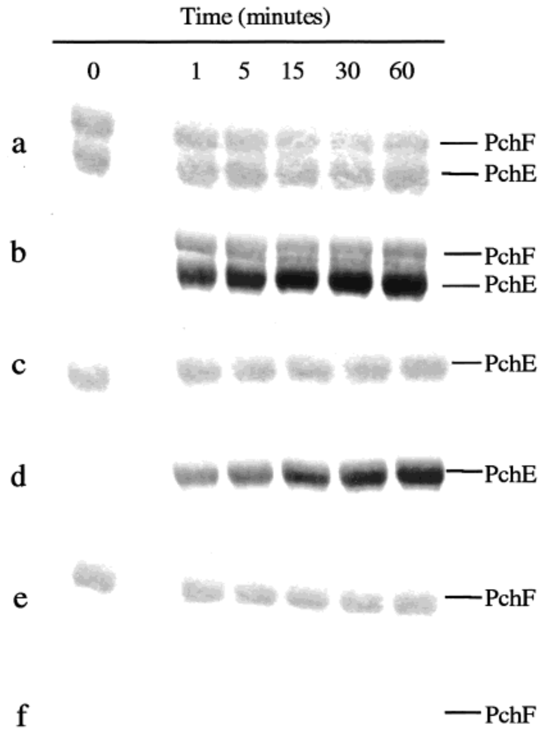

FIGURE 5: Transfer of a salicylate-containing intermediate from PchE to PchF. Panels a, c, and e show Coomassie blue-stained bands of the Pch proteins, and panels b, d, and f show autoradiographs of the corresponding proteins. Reaction mixtures contained 75 mM Tris-HCl (pH 7.5), 10.0 mM $MgCl_2$, 0.5 mM CoA, 5% glycerol, 5.0 mM cysteine, 5.0 mM ATP, 0.26 μM Sfp, 1.4 μM PchD, 7 μM PchE (panels a−d), and 5 μM PchF (panels a, b, e, and f). Reaction mixtures were incubated for 60 min at 30 °C prior to addition of 90 μM [^{14}C]salicylate (55 μCi/μmol). Samples (10 μL) were taken at the indicated times after addition of salicylate, the reactions quenched in SDS−PAGE loading buffer, and the mixtures resolved on a 7.5% SDS−PAGE. X-ray films were exposed to the gels for 3 days before being developed.

and holo-PchE were 75 and 27%, respectively. Incorporation of covalently bound ^{35}S-labeled cysteine into PchE and PchF was also validated and visualized by autoradiography (not shown) of samples resolved by SDS−PAGE as described in Experimental Procedures. The results proved the competence of the adenylation domains A1 and A2 to acylate their corresponding carrier protein domains, i.e., PCP1 and PCP2, respectively. The ability of PchD to transfer salicylate to the holo-ArCP of PchE was likewise monitored and confirmed. The results indicated a stoichiometry for salicylation of 54%, which was reached after incubation for 40−50 min (Figure 4F). Salicylation of holo-PchE was also visualized by SDS−PAGE and autoradiography (Figure 5d). Holo-PchF was in contrast not a substrate for salicylation by PchD (Figure 5f), consistent with regioselective recognition of PchE, and the predicted role of PchE as the first enzyme in pyochelin assembly.

Transfer of Covalently Bound Intermediates from PchE to PchF: Chain Elongation. The postulated mechanism for pyochelin biosynthesis outlined in Figure 7 predicts the first step in chain elongation is the formation of a salicyl-cysteine-*S*-enzyme intermediate. The intermediate is condensed by PchE and is covalently bound to PCP1 of the enzyme via a thioester linkage with the P-pant group. After presumed heterocyclization and dehydration of the salicyl-cysteinyl-*S*-enzyme to the hydroxyphenyl-thiazoline (HPT) species,

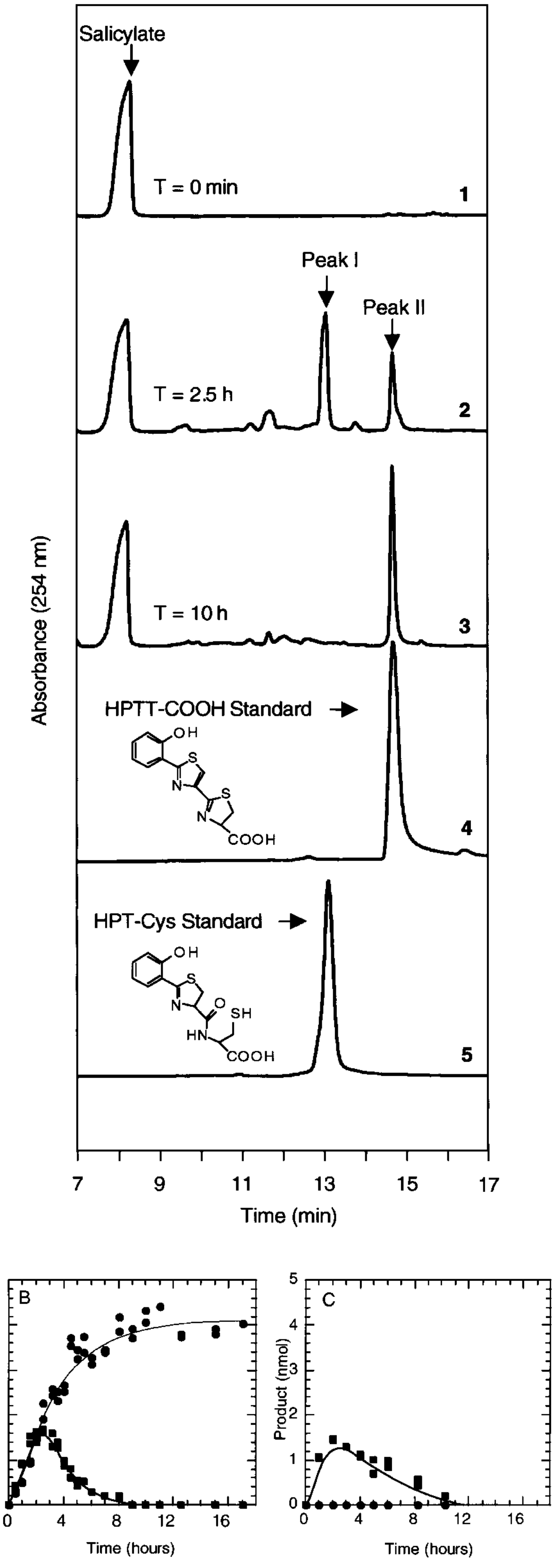

FIGURE 6: (A) HPLC trace showing the production of HPT-Cys (peak I) and HPTT-COOH (peak II) by the reconstituted PchDEF system. Traces 1−3 show the extracted products at 0, 2.5, and 10 h, respectively. Traces 4 and 5 show the elution profile of the synthetic standards HPTT-COOH and HPT-Cys, respectively. (B) Time-dependent accumulation of HPT-Cys (■) and HPTT-COOH (●) in reconstitution reaction mixtures containing PchD, PchE, and PchF. (C) Time-dependent accumulation of HPT-Cys (■) and HPTT-COOH (●) in reconstitution reaction mixtures where PchF was omitted. See Experimental Procedures for details.

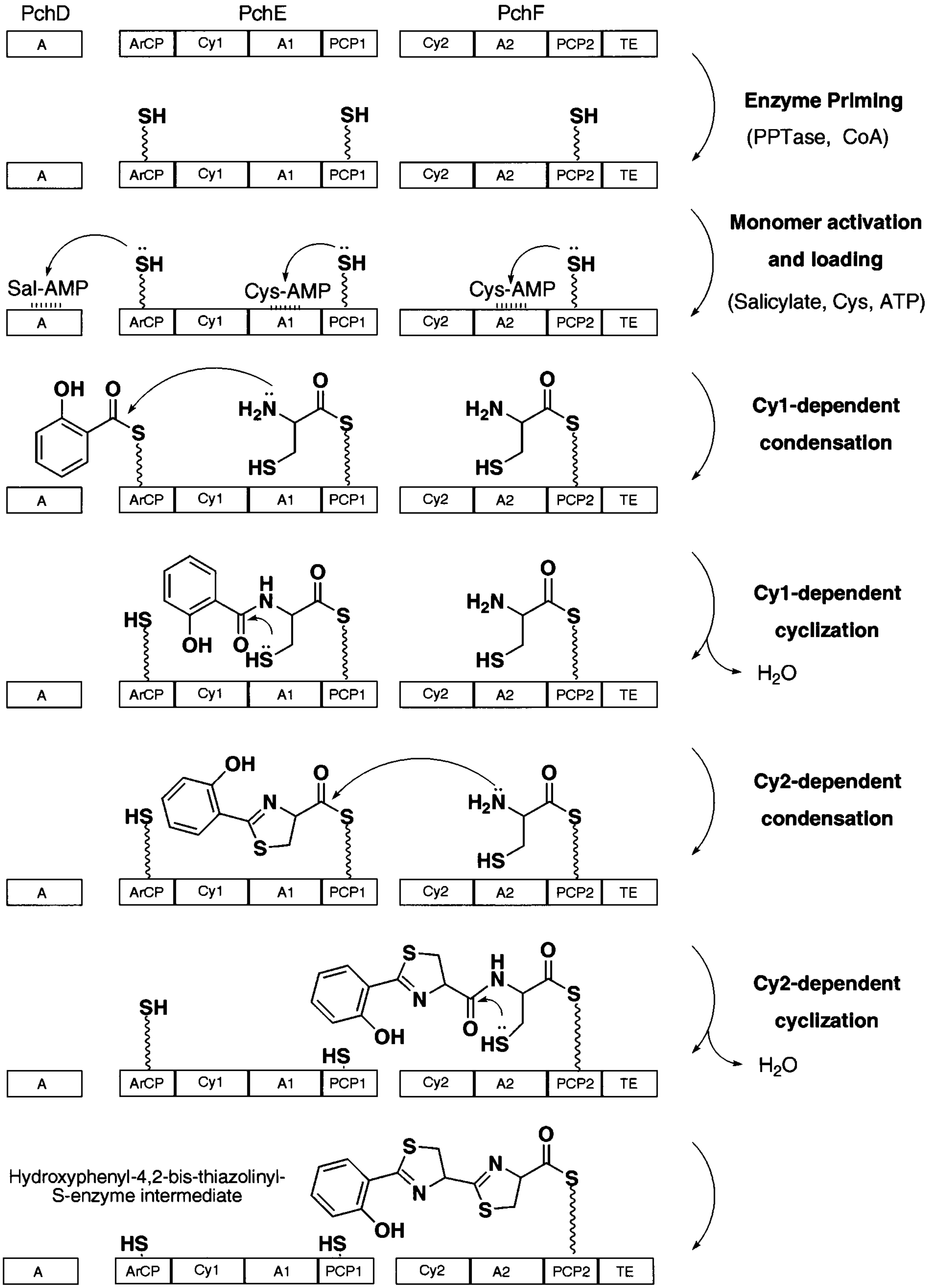

FIGURE 7: Model for PchDEF-dependent enzymatic synthesis of hydroxyphenyl-4,2-bisthiazoline carboxylate. The domain nomenclature is as indicated in the legend of Figure 2. The putative methyltransferase (M) and epimerase (E) domains shown in Figure 2 have been omitted here. The TE-mediated hydrolytic release and spontaneous oxidation steps of the hydroxyphenyl-4,2-bisthiazolinyl-*S*-enzyme intermediate are shown in Figures 8 and 9, respectively.

the HPT acyl group should be transferred to PchF for continuation of pyochelin assembly. To determine whether there was transfer of such an intermediate from PchE to PchF, we investigated the PchE-dependent incorporation of covalently bound ^{14}C-labeled salicylate into PchF. Figure 5b shows a time course for the incorporation of the covalently bound [^{14}C]salicylate-containing intermediate into PchF, assessed after SDS−PAGE separation of PchE and PchF and autoradiography. Incorporation of [^{14}C]salicylate into PchF was PchE-dependent. In the presence of PchE, the transfer of ^{14}C label into PchF was detected as early as 1 min after the reaction was initiated by the addition of [^{14}C]salicylate (Figure 5b). However, no labeling of PchF was detected when PchE was omitted from the reaction mixture (Figure 5f). The presence of cysteine was also necessary for labeling PchF (Figure 8C). The results demonstrate the predicted transfer of a salicylate-containing intermediate from PchE to PchF and together with the data depicted in Figure 4 establish the progression of covalent intermediates, first in PchE and then in PchF.

Assembly and Catalytic Release of Salicylate-Cysteine-Containing Products by PchE and PchF. In addition to monomer activation and acyl-*S*-enzyme intermediate formation, assembly of the pyochelin tricyclic (hydroxyphenyl-thiazolinyl-thiazolidinyl) backbone requires monomer condensation and cysteine cyclization activities (Figure 7). These activities are predicted to reside in the Cy1 and Cy2 domains of PchE and PchF, respectively (Figure 2). To assess Cy1 and Cy2 cyclization functions and assembly of bicyclic and tricyclic acyl-*S*-enzyme intermediates on PchE and PchF, respectively (Figure 9), the release of salicyl-N-capped cyclized products in reaction mixtures containing PchE, PchF, PchD (for salicylation of PchE), Sfp (for phosphopantetheinylation of the apo domains), and substrates CoA, ATP, Cys, and salicylate was investigated. These reconstitution reaction mixtures were incubated at 30 °C, and any released products were extracted and analyzed by HPLC as described in Experimental Procedures. Two prominent peaks whose areas increased over time were detected by HPLC (Figure 6A). The first of these, peak I, eluted with a retention time of 13.4 min. The other peak, peak II, eluted with a retention time of 15.1 min. The compounds in peaks I and II were identified as hydroxyphenyl-thiazolinyl-cysteine, HPT-Cys, and hydroxyphenyl-thiazolyl-thiazolinyl-carboxylic acid, HPTT-COOH, respectively. Peaks I and II had retention times that coincided with those of synthetic HPT-Cys and HPTT-COOH, respectively (Figure 6A). Furthermore, purified peak I and purified peak II coeluted when co-injected with synthetic HPT-Cys and HPTT-COOH, respectively (not shown). Purified peaks I and II were also submitted for mass spectrometric analysis. The masses determined for the products in peaks I (327.73 Da) and II (307.71 Da) were in good agreement with the masses determined for the synthetic standards of HPT-Cys and HPTT-COOH, respectively (Table 2). The ^{1}H NMR spectra of the two synthetic standards and of the purified products in peaks I and II were also recorded. The chemical shifts for peak II were in good agreement with those from synthetic HPTT-COOH. The amounts of purified peak I were insufficient for complete assignment of all protons, but several diagnostic chemical shifts were matched with those from a synthetic HPT-Cys standard. It is noteworthy that HPT-COOH (hydroxyphenyl-thiazolinyl-carboxylic acid or dihydroaeruginoic acid) has been isolated from *P. aeruginosa* (*29*). These results demonstrate unambiguously that the product contained in peak I is HPT-Cys and the product contained in peak II is HPTT-COOH, in which the first heterocycle has been oxidized from the expected thiazoline to the fully oxidized heteroaromatic thiazole (Figure 9). We believe this oxidation to be both nonenzymatic and facile, since attempts to prepare hydroxylphenyl-thiazolinyl-thiazolinyl-carboxylic acid synthetically resulted only in products with an aromatized middle ring.

The time-dependent accumulation of HPT-Cys and HPTT-COOH in the reconstitution reaction mixture is shown in Figure 6B. The product HPT-Cys (peak I) could be clearly detected after incubation for 30 min, accumulated for approximately 2 h, and then its concentration declined to undetectable levels. In contrast, the produced HPTT-COOH (peak II) accumulated during the first 8 h of incubation, after which its concentration remained constant. The apparent k_{cat} values observed over the linear range for the release of HPT-Cys and HPTT-COOH were 0.062 min^{-1} (for the first 1.5 h) and 0.056 min^{-1} (for the first 2.5 h), respectively. The maximum accumulation (1.6 nmol at 2.5 h) of HPT-Cys indicates that the reaction proceeded to render at least six catalytic turnovers before the concentration of the compound declined. The maximum level of accumulation of HPTT-COOH (4.0 nmol) corresponded to a total of 16 catalytic turnovers in the 150 min assay period.

HPTT-COOH was present only in reaction mixtures containing all the substrates and enzymes. However, HPT-Cys was also detected in reaction mixtures in which PchF was omitted (Figure 6C). The release of HPT-Cys by PchE in the absence of PchF indicated that the first heterocycle is assembled by this protein. Addition of PchF allowed the formation and subsequent hydrolytic release of the tricyclic HPTT-COOH (Figure 7). At this stage, the basic skeleton of the tricyclic pyochelin is completed, with an imine reduction and N-methylation remaining. Overall, the results demonstrate the catalytic competence of the reconstituted pyochelin synthetase to assemble the pyochelin backbone in vitro and the collinearity of the modular organization of the synthetases and the biosynthesis of the pyochelin aryl-4,2-linked bis-heterocyclic backbone.

In the absence of PchF, the concentration of PchE-produced HPT-Cys also declined over time (Figure 6C), as did the concentration of added synthetic HPT-Cys (∼2 nmol) in a reaction mixture in which either PchF or PchE was omitted (not shown). However, the added synthetic HPT-Cys did not disappear in reaction mixtures in which both PchE and PchF were omitted. Thus, the destruction of free HPT-Cys is PchE- and PchF-dependent, although by an as yet unidentified mechanism. Furthermore, to determine if free HPT-Cys could be used by PchF as a substrate for HPTT-COOH production, we assayed the formation of HPTT-COOH in a reconstitution reaction mixture from which salicylate was omitted, but synthetic HPT-Cys was added (∼100 nmol) at the time the reaction was started with ATP. This reconstitution reaction failed to produce HPTT-COOH, but the concentration of added HPT-Cys declined as previously observed (data not shown). These results indicated that free HPT-Cys cannot be utilized by PchF to form HPTT-COOH.

Release of HPT-Cys and HPTT-COOH and Function of the Thioesterase Domain of PchF: Chain Termination. The release of HPT-Cys from PchE is likely due to transthiolation of the HPT-*S*-PCP1 intermediate from PchE to adventitious

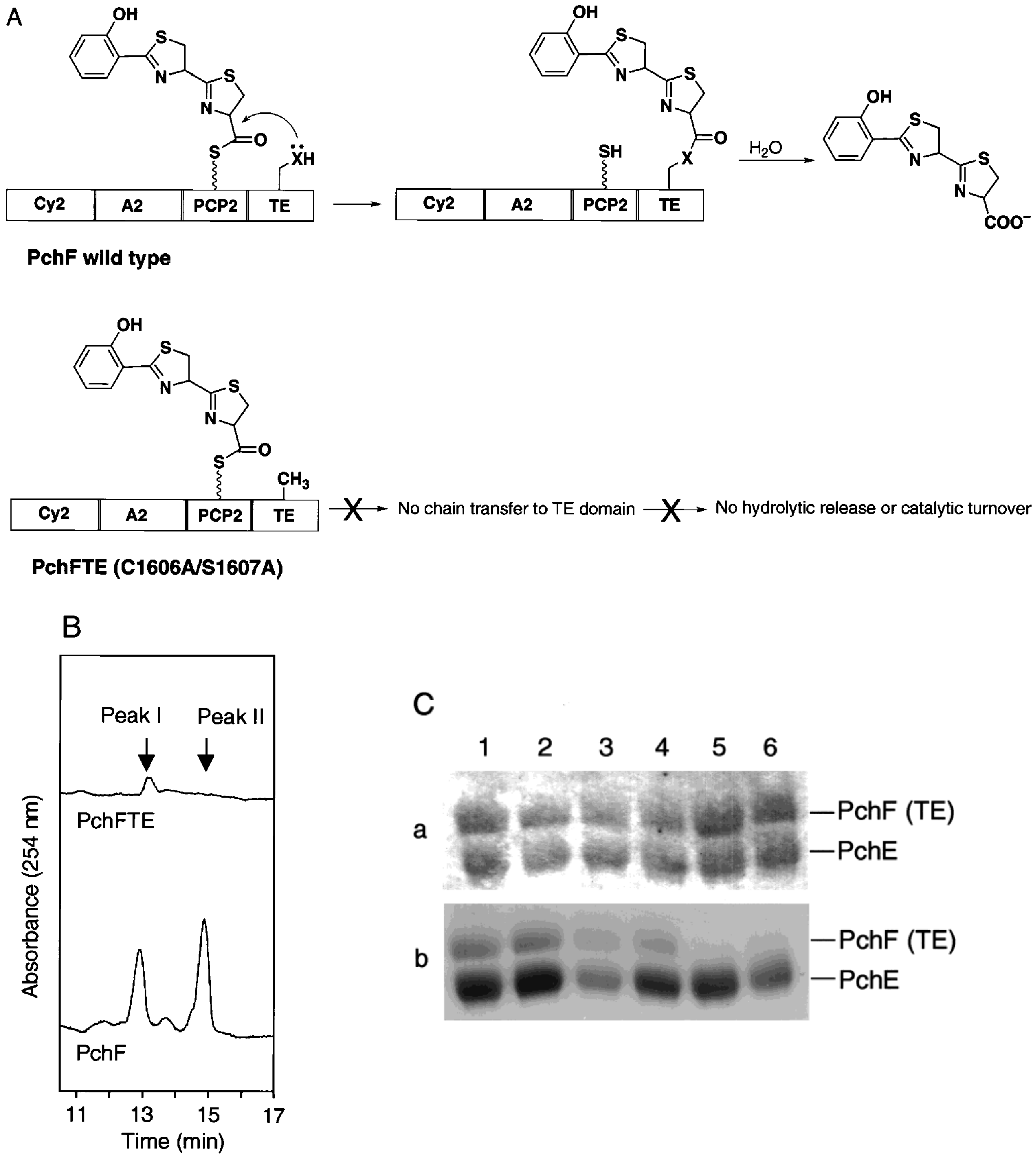

FIGURE 8: (A) Proposed TE-mediated hydrolytic release of HPTT-COOH from PchF. The side chain of the potential nucleophiles (Cys1606 and Ser1607) identified by sequence analysis of the TE domain are represented by XH (X being S or O). Acyl chain transfer and the subsequent hydrolytic release are abrogated in the double mutant PchFTE (Cys1606Ala and Ser1607Ala). (B) HPLC trace of HPT-Cys and HPTT-COOH products formed (after incubation for 7 h) in reconstitution reaction mixtures with double mutant PchFTE (top trace) or with the wild-type PchF (bottom trace). See Experimental Procedures for details. (C) Transfer of a salicylate-containing intermediate from PchE to PchF and PchFTE. Panel a shows the Coomassie blue-stained bands of Pch proteins, and panel b shows autoradiographs of the corresponding proteins. Reaction mixtures contained 75 mM Tris (pH 7.5), 10.0 mM $MgCl_2$, 0.5 mM CoA, 5.0 mM cysteine, 5.0 mM ATP, 5% glycerol, 0.26 μM Sfp, 1.4 μM PchD, 7 μM PchE, and 5 μM PchF (for lanes 1 and 2) or 5 μM PchFTE (for lanes 3 and 4). Lanes 5 and 6 were treated like lanes 2 and 4, respectively, except for the omission of cysteine from the reaction mixtures. Reaction mixtures were incubated for 60 min at 30 °C prior to addition of 90 μM [^{14}C]salicylate (55 μCi/μmol). Samples (10 μL) were taken 5 min after addition of salicylate (lanes 1 and 3) or 60 min after addition (lanes 2 and 4), the reactions quenched in SDS−PAGE loading buffer, and the mixtures resolved on a 7.5% SDS−PAGE. X-ray films were exposed to the gels for 2 days before being developed.

free cysteine in solution (Figure 9), as seen in the yersiniabactin system (*26*). On the other hand, the release of HPTT-COOH from PchF is presumed to be hydrolytic and mediated by the terminal thioesterase domain (Figures 8A and 9). In that case, such chain-terminating release from PchF should require a functional TE domain located at the C-terminus of the synthetase, so the predicted thioesterase active site was targeted by site-directed mutagenesis. In place of the expected

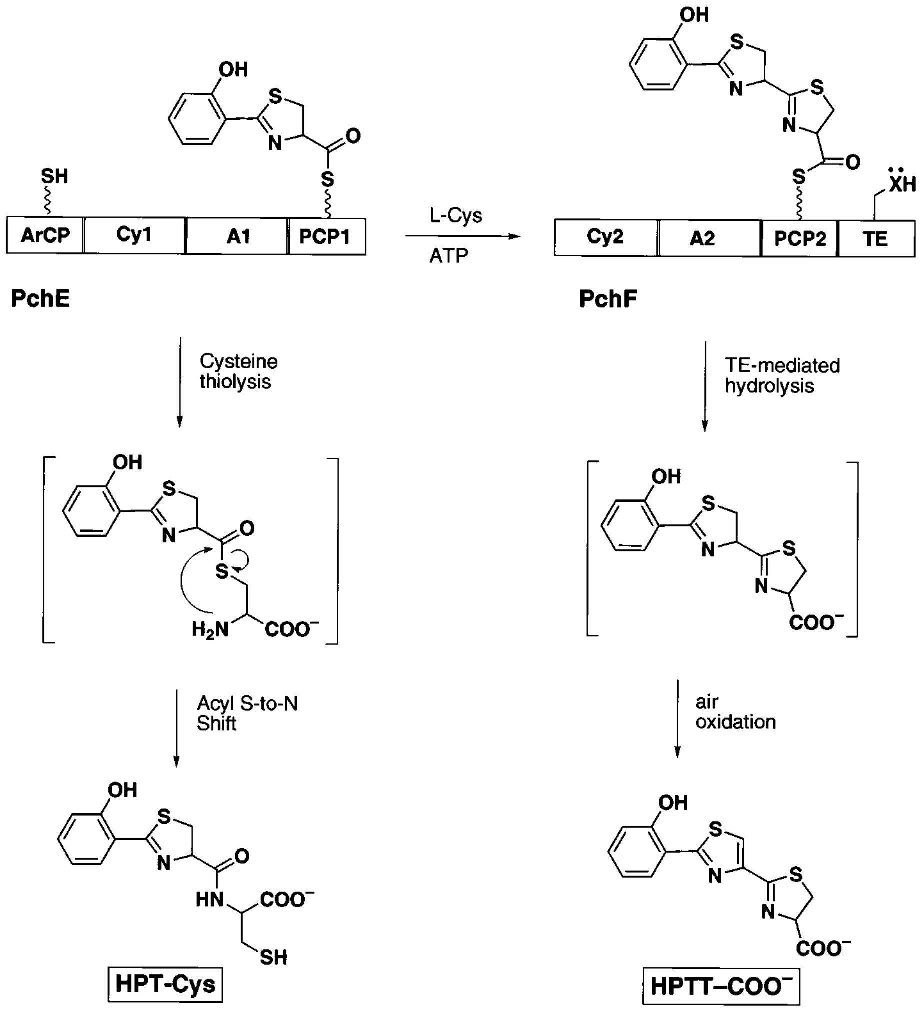

FIGURE 9: Elongation of acyl-*S*-enzyme intermediates on PchE and PchF, and thiolytic and hydrolytic release mechanisms.

Table 2: Mass Spectral Data for Standard Compounds and Purified Products

compound	calcd mass (Da)	mass determined for the synthetic standard[a] (Da)	mass determined for the purified product (Da)
HPTT-COOH[b]	306.01	307.27	307.71
HPT-Cys	326.04	327.68	327.73

[a] The masses ($[M + 1]^+$ values) were determined by MALDI-TOF mass spectrometry. [b] The mass corresponds to the compound with the spontaneously oxidized thiazole ring.

serine nucleophile in a conserved thioesterase signature motif, GxSxG (*38*–*40*), the active site motif of the thioesterase domain in PchF possesses cysteine in the GYCSG sequence, but notably next to a serine residue. Since either (Cys or Ser) residue could be the catalytic nucleophile in an HPTT-*S*-TE or HPTT-*O*-TE intermediate that is then subject to hydrolytic release of the full-length acyl chain, we decided to mutate both the Cys and Ser residues to address either eventuality. Thus, the constructed PchFTE mutant has both the Cys1606 and the Ser1607 residues substituted with Ala (Figure 8A). In the event, no HPTT-COOH could be detected in reconstitution reaction mixtures with the double mutant PchFTE (Figure 8B); however, HPT-Cys, whose formation is solely dependent on PchE, was produced as expected (confirming the mechanistic steps of Figure 9). If the HPTT acyl chain is indeed now stuck in place on the P-pant tether of the PCP2 domain of the PchFTE double mutant, then the mutant should still be able to incorporate a covalent salicyl moiety into such a tethered HPTT chain. As expected, the PchFTE mutant was loaded with [^{14}C]salicylate in a manner similar to that of the wild-type PchF (Figure 8C). Thus, the behavior of the PchFTE mutant validates the predicted requirement of the TE domain in the catalytic release of HPTT-COOH.

DISCUSSION

In this study, the three proteins (PchD, PchE, and PchF) from *P. aeruginosa* that comprise the core of the enzymatic assembly line for the aryl-4,2-linked bis-heterocyclic siderophore pyochelin have been overproduced in *E. coli* and purified. The recombinant peptide synthetases PchE and PchF were posttranslationally primed with phosphopantetheine at three carrier protein domains to create, together with the salicyl-AMP ligase PchD, a reconstitutable and active three-subunit (PchDEF) NRPS system in vitro. This work validates the predicted functions of the nine domains: three adenylation domains (two for cysteine and one for salicylate), three carrier protein domains (one aryl carrier protein domain and two peptidyl carrier proteins), two cyclization domains, and the chain-releasing thioesterase domain in the three-subunit system (PchDEF). By our analysis, PchF has a four-domain organization typical for a chain-elongating and -terminating

NRPS module: Cy2-A2-PCP2-TE. Interestingly, Cy2 is the first example of an N-terminal cyclization domain in an NRPS subunit. PchE has four clearly discernible NRPS-type domains: ArCP-Cy1-A1-PCP2 by our sequence-based predictions. The detection of intermediate transfer and thiolytic release of the HPT-*S*-enzyme intermediate as HPT-Cys and TE-mediated hydrolytic release of HPTT-*S*-enzyme as HPTT-COOH allowed assignment of the catalytic flux of covalent intermediates down the three-subunit (PchDEF), nine-domain assembly line and deconvolution of the chain initiation, elongation, and termination steps.

The collinearity between the modular organization of the synthetases and the biosynthesis of the pyochelin backbone has thus been demonstrated. Most notably, the in vitro tandem cyclization of adjacent cysteine residues to a 4,2-linked bisthiazoline unit is achieved cosynthetically with chain elongation. The sequential biosynthesis of heterocycles is directed by the two Cy domains. Cy1 (in PchE) is involved in the condensation reaction between the salicylate bound to the donor ArCP domain and the cysteine bound to the acceptor PCP1 domain. Cy1 is also responsible for the cyclization of the cysteine in the salicyl-cysteinyl-*S*-PCP1 intermediate in forming salicyl-thiazolinyl-*S*-PCP1. Analogously, Cy2 (in PchF) forms the peptide bond between the carbonyl carbon at the C-terminus of the salicyl-thiazolinyl intermediate bound to PCP1 (now acting as acyl donor) and the nitrogen of the amino group of the cysteine bound to the acceptor PCP2 to form the salicyl-thiazolinyl-cysteinyl-*S*-PCP2 intermediate. Cy2 subsequently cyclizes the C-terminal cysteine to form the second thiazoline heterocycle in the salicyl-thiazolinyl-thiazolinyl-*S*-PCP2 intermediate (Figure 7).

PchD is functionally and structurally cognate with the aryl N-capping initiator ligases MbtA and YbtE involved in the biosynthesis of the siderophores mycobactin (*6*) and yersiniabactin (*26*), respectively. In this work, we validated the anticipated salicyl-AMP ligase activity of PchD and also demonstrated its ability to catalyze salicyl transfer to the holo form of PchE but not to PchF. This regioselectivity of salicyl-*S*-PchE formation is consistent with specific protein−protein (e.g., PchD−PchE) interactions that determine the location of covalent monomer attachment. We have noted a similar regioselectivity in the mycobactin system in which MbtA transfers salicylate to the N-terminal holo-ArCP of the NRPS subunit MbtB of mycobactin synthetase, but not to an internal holo-PCP domain (*6*). Similar carrier protein domain selectivity has also been reported for YbtE (*26*). Thus, it is highly likely that the terminal thiol of the phosphopantetheinyl group of the holo-ArCP domain of PchE is the site of initial salicylation of PchE.

Both the Ser46 in the ArCP domain and Ser1385 in the PCP1 of PchE domain could be covalently primed with P-pant via action of the PPTase Sfp from *B. subtilis*. The doubly holo-PchE then gained the ability to be covalently thioesterified by two types of acyl substrates after their activation as adenylates: salicylate, by in-trans salicylation mediated by PchD on the N-terminal ArCP, and cysteine, by in-cis auto-aminoacylation mediated by the adjacent adenylation domain (A1) on the C-terminal PCP1. The fourth PchE domain, Cy1, is predicted to have peptide condensation and cyclization activity. Here we demonstrated the release of HPT-Cys by PchD and PchE in the absence of PchF, validating that prediction. The evidence presented in this work also shows that the apo-PCP2 domain in PchF can be modified by Sfp action and can be covalently loaded with the cysteine via cysteinyl-AMP generated by the adenylation domain (A2) in-cis. This species is the downstream nucleophile for chain growth via transfer of the HPT chain docked as the HPT-*S*-PCP1 electrophile at the C-terminus of PchE. Intersubunit (PchE to PchF) chain transfer was demonstrated by [^{14}C]salicyl transfer from PchE to PchF, validating the intersubunit directional flow. The initial transient product of peptide bond condensation in PchF would be HPT-cysteine-*S*-PCP2, and subsequent dehydrative cyclization produces HPTT-*S*-PCP2. The latter acyl-*S*-enzyme intermediate is transferred onto the chain-terminating C-terminal TE domain, followed by hydrolysis to release the HPTT-COOH product. The fact that the TE domain of PchF is participating in chain release and permitting multiple catalytic turnovers was assessed by determining the ability of a PchF double mutant (Cys1606Ala/Ser1607Ala) to release HPTT-COOH. The mutations eliminated the two potential catalytic nucleophiles identified by sequence analysis of the TE domain. Ether Cys1606 or Ser1607 could function as a nucleophile to form a HPTT-*S*-TE or HPTT-*O*-TE intermediate set up for hydrolytic release of the acyl chain. In the event, the double mutant failed to release HPTT-COOH, confirming that product release proceeds from the most downstream domain. We have therefore demonstrated the function and activity (directly and indirectly) for all nine domains of the PchD/PchE/PchF protein system.

The question arises as to why turnover to produce HPTT-COOH in the reconstituted PchDEF system is so slow ($k_{cat} \sim 0.05\ min^{-1}$). It is possible that some key component is still missing, such as the putative external thioesterase encoded by the *pchC* gene. For the in vivo biosynthesis of the lipopeptide surfactin produced by *B. subtilis*, two thioesterase activities are required. One of the activities resides in the C-terminal thioesterase domain of the NRPS subunit SrfA−C, the other in an independent thioesterase protein, SrfA-TE. Deletion of the gene fragment encoding the internal thioesterase domain or the gene for the external thioesterase leads to a 97 or 84% reduction in the level of surfactin production in vivo, respectively, while in the double mutant, surfactin production is completely abrogated (*41*). The number and type of thioesterases of the pyochelin system are analogous to that of the surfactin system (C-terminal TE domain of PchF and the external PchC). We attempted overproduction of PchC in *E. coli*, but the recombinant protein could not be detected in the surrogate host. Thus, the effect of PchC remains to be determined.

Although the k_{cat} value for HPTT-COOH release from the PchDEF-reconstituted system is very low, the transfer of salicyl-containing enzyme intermediates from PchE to PchF is quite rapid (10 s, not shown), suggesting that some step in chain elongation or termination in PchF is rate-limiting for turnover. There may be some editing or quality control check of the final acyl chain before it passes from the PCP2 to the TE or from the TE domain to solution. The HPTT acyl skeleton is two chemical steps away from pyochelin and requires imine reduction and N-methylation. It is possible that a reductase and a methyltransferase (see below) normally act in complex with PchD, PchE, PchF, and perhaps PchC, and exert control on the rate of acyl chain transfer to the TE domain. If this is true, then a fully reconstituted pyochelin synthetase complex may exhibit a higher k_{cat} value than the one measured here.

In their initial sequence analysis of PchF, Reimmann and colleagues (*28*) suggested the presence of a methyltransferase and a reductase domain upstream and downstream of the thiolation (PCP2) domain in PchF, respectively. In contrast, we believe that these activities are represented by as yet unidentified separate tailoring catalysts. We have recently found such a reductase domain fused to the NRPS catalyst Lys2 (*42*), and the conserved sequence hallmarks of such NAD(P)H-utilizing reductases are not in PchF (or PchE); instead, we have assigned and validated a functional thioesterase domain to the C-terminus of the PchF protein. It is thus likely that *P. aeruginosa* uses an external reductase, but the question of whether the thiazoline-to-thiazolidine reduction occurs cosynthetically or after hydrolytic release of the acyl chain from PchF remains to be answered. Also, as yet undetermined is the whereabouts of the *N*-methyltransferase required to N-methylate the thiazolidine and complete pyochelin synthesis. The N-methylation is likely to follow thiazoline reduction to thiazolidine since only in the tetrahydro ring is the nitrogen basic enough to be the nucleophile toward the methyl donor *S*-adenosylmethionine (SAM). While fungal NRPS such as cyclosporin synthetase have *N*-methyltransferase domains embedded in the synthetase (*18*), in the chloroeremomycin assembly the N-terminal leucine is probably methylated by an external *N*-methyltransferase (*16*). We have noted a 340-residue insert in the adenylation domain of the HMWP2 subunit of yersiniabactin synthetase that is somewhat homologous to methyltransferase domains. An equivalent insert is present in the A1 and A2 domains of PchE and PchF, but on addition of SAM or SAM and NADPH, the production of HPTT-COOH was actually inhibited and no peak corresponding to pyochelin detected by HPLC (not shown).

Reimmann and co-workers also proposed an epimerization domain located between the adenylation (A1) and thiolation (PCP1) domains of PchE, because earlier Rinehart and colleagues (*43*) had noted that pyochelin has the *R* configuration at C2 of the first thiazoline ring, suggesting its acyclic precursor is D-cysteine. Because the results presented here indicate that PchE (and PchF) does not activate D-cysteine, an epimerization function does indeed appear to be required. Where this function resides, as well as the timing of the stereochemical inversion of C2 of the L-cysteine activated by PchE, remains to be investigated.

Finally, we note that the isolated, bis-heterocyclic HPTT-COOH encompasses one thiazole and one thiazoline ring, both of which are oxidized relative to pyochelin. While we believe the second ring's final reduction is a matter of an unidentified enzymatic component, the oxidation of the first ring from a thiazoline to a thiazole is likely the result of facile air oxidation. Not only did we fail to identify any thiazoline−thiazoline enzymatic products, but all attempts to chemically synthesize hydroxyphenyl-thiazolinyl-thiazolinyl-COOH resulted in isolation of the thiazole-containing product described in detail here. It is possible that the extended conjugation gained by aromatization is the driving force for air oxidation, and that rapid reduction of the second ring to the thiazolidine is necessary for preventing this oxidation from occurring, either as the covalent HPTT-*S*-enzyme species or free in solution.

The ability to isolate, assay, and reconstitute the PchDEF assembly line to its Cys-Cys bis-heterocyclization activity sets the stage for further analysis and manipulation of multimodular siderophore synthetases. Of great importance is investigation into the unidentified activities noted above of reduction, N-methylation, and generation of the *R* configuration of the first thiazoline. However, the system will also facilitate analysis of the mechanism and timing of peptide bond formation and cyclodehydration chemistry. There are also issues such as transfer of intermediates and domain swapping between related NRPS subunits that will be investigated. For example, it is tempting to speculate that the four-domain PchE was an evolutionary precursor to the six-domain HMWP2 prior to its fusion of the Cy2-PCP2 domains of contemporary HMWP2. Given the very close correspondence of PchEF and HMWP2 systems, investigating the efficiency of intermediate transfer between the two systems and their domain portability and compatibility by constructing chimeric subunits will be instructive.

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