Recognition of Hybrid Peptidyl Carrier Proteins/Acyl Carrier Proteins in Nonribosomal Peptide Synthetase Modules by the 4′-Phosphopantetheinyl Transferases AcpS and Sfp*

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The acyl carrier proteins (ACPs) of fatty acid synthase and polyketide synthase as well as peptidyl carrier proteins (PCPs) of nonribosomal peptide synthetases are modified by 4′-phosphopantetheinyl transferases from inactive apo-enzymes to their active holo forms by transferring the 4′-phosphopantetheiny moiety of coenzyme A to a conserved serine residue of the carrier protein. 4′-Phosphopantetheinyl transferases have been classified into two types; the AcpS type accepts ACPs of fatty acid synthase and some ACPs of type II polyketide synthase as substrates, whereas the Sfp type exhibits an extraordinarily broad substrate specificity. Based on the previously published co-crystal structure of Bacillus subtilis AcpS and ACP that provided detailed information about the interacting residues of the two proteins, we designed a novel hybrid PCP by replacing the Bacillus brevis TycC3-PCP helix 2 with the corresponding helix of B. subtilis ACP that contains the interacting residues. This was performed for the PCP domain as a single protein as well as for the TycA-ACP domain within the nonribosomal peptide synthetase module TycA from B. brevis. Both resulting proteins, designated hybrid PCP (hPCP) and hybrid TycA (hTycA), were modified in vitro during heterologous expression in Escherichia coli (hPCP, 51%; hTycA, 75%) and in vitro with AcpS as well as Sfp to 100%. The designated hTycA module contains two other domains: an adenylation domain (activating phenylalanine to Phe-AMP and afterward transferring the Phe to the PCP domain) and an epimerization domain (converting the PCP-bound l-Phe to D-Phe). We show here that the modified PCP domain of hTycA communicates with the adenylation domain and that the co-factor of holo-hPCP is loaded with Phe. However, communication between the hybrid PCP and the epimerization domain seems to be disabled. Nevertheless, hTycA is recognized by the next proline-activating elongation module TycB1 in vitro, and the dipeptide is formed and released as diketopiperazine.

A large number of natural bioactive peptides (e.g. vancomycin, cyclosporine, tyrocidine, and surfactin) are produced by bacteria and fungi via a template-directed, nucleic acid-independent mechanism carried out by multienzyme complexes called nonribosomal peptide synthetases (NRPSs)* (1–3). The biosynthetic mechanism of NRPS resembles that of fatty acid synthases (FASs) and polyketide synthases (PKSs), which catalyze fatty acids synthesis and production of polyketides like erythromycin and tetracycin (4, 5).

NRPSs have a modular architecture; each module is responsible for the incorporation of one building block into the nascent peptide chain. A module can be subdivided into domains, each representing the single enzymatic activity necessary for synthesis and modification of the peptide chain. The adenylation (A) domain is responsible for substrate recognition and activation as aminoacyladenylate at the expense of ATP (6, 7). The downstream PCP domains are the sites of intermediate binding and serve as carrier units (8). The peptide bond formation between covalently bound intermediates on two consecutive modules is catalyzed by a condensation (C) domain (9, 10) (Fig. 1). During this peptide assembly, optional modifications of the substrates can occur, depending on the presence of modifying domains within the corresponding module. For example, epimerization (E) domains catalyze the conversion of incorporated L-amino acids to their D-isomers (2).

NRPSs, FASs, and PKSs contain one or more small PCP or ACP domains that are 80–100 amino acids in length that carry the building blocks and elongated intermediates to the different catalytic centers. Depending on the architecture of the multienzyme complex, these carrier proteins appear as integrated domains or as individual proteins. The PCP domains in NRPSs and the acyl carrier proteins (ACPs) in FASs and PKSs share a conserved sequence motif (GX/D/H/S/L/I/D/K) containing an invariant serine residue (11). During posttranslational modification the 4′-phosphopantetheine (Ppant) co-factor is transferred from coenzyme A and attached to this serine residue converting the carrier proteins from the inactive apo form to the active holo form (4) (Fig. 2). Dedicated 4′-phosphopantetheinyl transferases (PPTases) catalyze the nucleophilic attack of the hydroxyl side chain of the conserved carrier protein serine residue onto the 5′-β-pyrophosphate linkage of coenzyme A (CoASH) in a magnesium ion dependent reaction (4). During peptide or polyketide synthesis, the activated substrates and elongation intermediates are covalently linked as thioesters to the thiol moiety of the 4′-Ppant.

PPTases have been classified into two types based on sub-

* This work was supported by the Deutsche Forschungsgemeinschaft and Fonds der Chemischen Industrie. The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
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1 The abbreviations used are: NRPS, nonribosomal peptide synthetase; ACP, acyl carrier protein; FAS, fatty acid synthase; PCP, peptidyl carrier protein; PKS, polyketide synthase; Ppant, 4′-phosphopantetheine; PPTase, 4′-phosphopantetheine transferase; A, adenylation; C, condensation; E, epimerization; HPLC, high pressure liquid chromatography; DKP, diketopiperazine; CoASH, coenzyme A (where SH represents the free thiol group present in this co-factor).
strate specificity; the AcpS type of primary metabolism and the Sfp type of secondary metabolism (4, 12). PPTases of the AcpS type are about 120 amino acids in length and have a pI value of about 9.6; they are found in almost every known microorganism and are responsible for the modification of fatty acid ACPs. Biochemical studies demonstrated that ACPs of type II PKS ism and are responsible for the modification of fatty acid ACPs. The well characterized Sfp exhibits an extraordinarily broad substrate specificity and is able to modify all carrier proteins so far tested including PCPs of NRPSs and ACPs of both FASs (14), too.

**FIG. 1. Peptide bond formation by NRPS (C-A-PCP) modules.**

**Reaction 1**, the A domain recognizes and activates a specific amino acid as aminoacyladenylate (AMP-a1) at the expense of ATP. **Reaction 2**, the aminoacyladenylate (aa1) is then transferred to the 4’-Ppant of the PCP domain. **Reaction 3**, the C domain catalyzes the peptide bond formation between amino acids attached to PCPs of adjacent modules.

**EXPERIMENTAL PROCEDURES**

**General Techniques**

_E. coli_ M15, which carries the plasmid pREP4 providing the _lacI_ gene for efficient repression of transcription, was transformed with the described plasmids. The proteins were overproduced as previously described (10). Expression was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (final concentration) at an _A_260 of 0.6, and the cells were allowed to grow for an additional 2 h before being harvested by centrifugation at 4,500 × _g_ at 4 °C. The cell pellet was resuspended in buffer A (50 mM Hepes, 150 mM NaCl, pH 7.8) and disrupted by three passages through a cooled French pressure cell. The resulting cell extract was centrifuged at 36,000 × _g_ and 4 °C for 30 min. Protein purification using Ni²⁺-nitriotriacetic acid affinity chromatography was carried out as previously described. Purified protein was dialyzed against the respective assay buffer, brought to 10% glycerol (v/v), and stored at –80 °C. Tyc3-PCP (hereafter referred to as PCP), TycA, TycB1, AcpS form _B. subtilis_ and Sfp-His6 were produced and purified as previously described (10). Protein concentrations were determined based on the calculated extinction coefficient at 280 nm: PCP, 9,530 M⁻¹ cm⁻¹; hPCP, 9,530 M⁻¹ cm⁻¹; hTycA-His6, 133,580 M⁻¹ cm⁻¹; hTycB1, AcpS form _B. subtilis_, and Sfp-His6 produced and purified as previously described (10). Protein concentrations were determined based on the calculated extinction coefficient at 280 nm: PCP, 9,530 M⁻¹ cm⁻¹; hPCP, 9,530 M⁻¹ cm⁻¹; hTycA-His6, 133,580 M⁻¹ cm⁻¹.

**Posttranslational Modification of the Enzymes by Ppant Transferase and CoASH in Vivo**

Priming of heterologously expressed proteins was achieved by incubation of 1 μM apo-enzyme with 100 μM CoASH and 25 mM recombinant _B. subtilis_ Ppant transferase Sfp or AcpS for 30 min at 37 °C (16, 21) prior to the addition of any amino acid.

**Radioassay for the Detection of Posttranslational Modification of the Enzymes**

AcpS and Sfp activity was assayed by using a radioactive assay method essentially as described previously (22). This method measures the incorporation of the 3H-labeled 4′-phosphopantetheine group from [3H]CoA into apo-enzymes. Reaction mixtures containing 75 mM MES/HCl, pH 6.0 (in the case of AcpS 50 mM Tris/HCl, pH 8.8), 10 mM MgCl₂, 1 μM of the respective protein, 20 μM CoA, 200 nM [3H]CoA (specific activity, 40 Ci/mmol, 0.85 mCi/ml), and 2.2–5.6 nM AcpS of _B. subtilis_ or 25 μM Sfp were incubated at 37 °C for 30 min. The reactions were stopped by the addition of 0.8 ml of ice-cold 10% trichloroacetic acid (w/v) and 15 μl of bovine serum albumin (25 mg/ml). Precipitated protein was collected by centrifugation at 13,000 rpm and 4 °C for 15 min using a microcentrifuge. The pellet was washed twice with 0.8 ml of ice-cold trichloroacetic acid (w/v) and resuspended in 180 μl of formic acid. The resulting suspension was mixed with 3.5 ml of Rotiszint Eco Plus scintillation fluid (Roth, Karlsruhe, Germany) and counted using a
Kinetic and HPLC Analysis of hPCP and PCP

For kinetic studies, the amount of holo-carrier protein formed was determined by an HPLC method. 800-μl reaction mixtures containing, 1–125 μM apo-PCP or 5–135 μM apo-hPCP, 50 mM Tris/HCl, pH 8.8 (75 mM MES/HCl, pH 6.0, in the case of Sfp) 12.5 mM MgCl₂, 2 mM DTT, 1 mM CoA and 11.2 mM AcpS of B. subtilis or 10 mM Sfp were incubated at 37 °C for 10 min. The reaction was stopped, and the protein was precipitated by the addition of 10% trichloroacetic acid. The reaction mixtures were centrifuged for 30 min at 13,000 rpm and 4 °C in a microcentrifuge. The pellet was subsequently resuspended in 120 μl of 50 mM Tris/HCl, pH 8.8. A 100-μl sample of this solution was injected onto a reversed phase HPLC column (Nucleosil C18, 250 mm, 5 μm, 300 A, Macherey and Nagel) equilibrated with 60% solvent A (H₂O with 0.1% trifluoroacetic acid) in the case of PCP.

Kinetic Incorporation of [3H]Phosphopantetheine into TycA and hTycA

The incorporation of [3H]phosphopantetheine was performed as described previously (22). The reaction mixtures (100 μl) contained 50 mM Tris/HCl, pH 8.8 (in the case of Sfp 75 mM MES/HCl, pH 6.0), 10 mM MgCl₂, 20 μM CoA, 200 nM [3H]CoA (specific activity, 40 Ci/mmol and 0.95 Ci/mmol), 25 mM ACPS or Sfp, and 1 μM apo-TycA or -hTycA. The reactions were started by the addition of the PPTase and were incubated at 37 °C for 30 min. The reactions were quenched with 800 μl of 10% trichloroacetic acid (w/v) and 15 μl of 25 mg/ml bovine serum albumin. The mixture was incubated on ice for 15 min. The precipitate was pelleted by centrifugation (13,000 rpm for 30 min in a microcentrifuge), washed twice with 1 ml of 10% trichloroacetic acid (w/v), and subsequently solubilized in 180 μl of concentrated formic acid. The redissolved protein was mixed with 3.5 ml of Rotiszint Eco Plus scintillation fluid (Roth), and the amount of radioactivity incorporated was quantified using a 1900CA Tri-Carb liquid scintillation (Packard).

Covalent Incorporation of [14C]Phenylalanine into TycA and hTycA

To investigate the interaction between the A domain and the PCP of TycA and hTycA, both modules were covalently loaded with L- or D-[14C]Phe. Aminoacylation was performed as described previously (10, 23). The synthetases were aminoacylated in reaction mixtures (100 μl) containing assay buffer (75 mM Tris-HCl, pH 8.5, 10 mM MgCl₂, 5 mM ATP), 1 μM holo-TycA or holo-hTycA, and 2 μM L- or D-[14C]Phe (500 mCi/mmol) or 2 μM L- or D-[14C]Phe (56 mCi/mmol) for specific times, and samples were taken and quenched with 0.8 ml of ice-cold 10% trichloroacetic acid (w/v) and 15 μl of 25 mg/ml bovine serum albumin. The mixture was incubated on ice for 15 min. The precipitate was pelleted by centrifugation (13,000 rpm for 30 min in a microcentrifuge), washed twice with 1 ml of 10% trichloroacetic acid (w/v), and solubilized in 180 μl of concentrated formic acid. The redissolved protein was mixed with 3.5 ml of liquid scintillation fluid, and the amount of incorporated radioactivity was quantified by liquid scintillation analysis (Packard).

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), TycA[PheATE] and hTycA[PheATE]...
been loaded with L-Pro beforehand to assay for dipeptidyl-S-Ppant released [14C]Phe were transferred to fresh tubes, the solvent was removed under vacuum, and the pellet was dissolved in 10 mM potassium hydroxide at 75 °C to ensure a complete aminoacylation of both apo-ACP by AcpS and Sfp. Reaction mixtures containing the acyl carrier protein (1 mM), [3H]CoA, AcpS of B. subtilis (5.6 nM), or Sfp (25 nM) were incubated for 30 min at 37 °C. Shown is the modification of the respective acyl carrier protein in percent by AcpS (black columns) and Sfp (gray columns). This qualitative assay showed that AcpS and Sfp both recognize the designed hPCP. In addition Sfp also recognizes all other carrier proteins tested, whereas AcpS modifies ACP but not the TycC3-PCP that was used for the creation of the hPCP.

were allowed to activate and covalently load l- or d-[14C]Phe (24). Reaction mixtures in assay buffer containing 1 mM holo-enzyme as well as 8.9 μM l-[14C]Phe (450 mCi/mmol) or 71.4 μM d-[14C]Phe (56 mCi/mmol) were incubated for 3 min at 37 °C (final volume, 200 μl). At defined time points, the samples were taken and quenched with 0.8 ml of ice-cold 10% trichloracetic acid (w/v). After 15 min of incubation on ice the precipitate was centrifuged (4 °C, 13,000 rpm, 20 min in a microcentrifuge), and the pellet was washed in succession twice with 1 ml of ice-cold 10% trichloracetic acid, 1 ml of ether/ethanol (3:1 v/v), and 1 ml of ether. The pellet was subsequently dried at 37 °C.

The acid-stable thioester [14C]Phe-S-Ppant was hydrolyzed by incubation with 100 μl of 100 mM potassium hydroxide at 75 °C for 15 min. The extraction of the cleaved amino acid was carried out by the addition of 1 ml of methanol and centrifugation at 4 °C at 13,000 rpm for 30 min in a microcentrifuge. Subsequently, the supernatants containing the released [14C]Phe were transferred to fresh tubes, the solvent was removed under vacuum, and the pellet was dissolved in 10 μl of 50% ethanol (v/v) and applied to chiral TLC plates. The plates were developed in acetonitrile/water/methanol (4:1:1 v/v/v) as solvent. The radioactivity was quantified using the supplied Radioisotope Thin Layer Analyzer software. The autoradiography was utilized to confirm the integrity of both [14C]Phe substrates. These studies revealed an optical purity of >99% for l-[14C]Phe and 95% for d-[14C]Phe.

**HPLC Analysis for the Detection of Phe-Pro Diketopiperazine (DKP) Formation**

The identity of released DKP diastereoisomers was established by HPLC analysis using internal standards. Purified holo-TycA or hTycA was loaded with either l- or d-Phe and mixed with holo-TycB1 that had been loaded with l-Pro beforehand to assay for dideptidyl-S-Ppant product formation (25). 200-μl reaction mixtures in assay buffer contained 1 μM enzyme as well as the cognate amino acid (2.5 mM l-Pro and 2.5 mM l- or d-Phe). Simultaneously, 200 μl of each solution were preincubated at 37 °C to ensure a complete aminocyclization of both modules. After 5 min, the condensation reaction was initiated by combining equal volumes of TycA or TycA and TycB1, and at various time points 50-μl samples were taken and immediately quenched by the addition of 0.45 ml of 10% trichloracetic acid (w/v). The reaction mixtures were extracted with 0.5 ml of butanol/chloroform (4:1 v/v). The organic solvent was transferred to fresh tubes and subsequently removed under vacuum. The pellet was dissolved in 50 μl of methanol and applied to a reversed-phase HPLC column (GROM-SIL120-ODS-4 HE, C18, 60 × 3 mm inner diameter, 5 μm, from GROM, Herrenberg, Germany) equilibrated with 22.5% methanol, n.t. and L.l. isofomers of Phe-Pro DKP could be separated by isocratic elution using 25% methanol as solvent (temperature, 23 °C; flow rate, 1 ml/min) over 10 min. In the relevant time frame (5.5 and 6.5 min, respectively), the samples were examined for their absorbance at 214 nm. This qualitative assay showed that AcpS and Sfp both recognize the designed hPCP. In addition Sfp also recognizes all other carrier proteins tested, whereas AcpS modifies ACP but not the TycC3-PCP that was used for the creation of the hPCP.

**Circular Dichroism of PCP, hPCP, and ACP**

CD spectra were collected with a Jasco J-810 spectropolarimeter in a 0.1-cm water jacketed cell at 25 °C. The samples were dialyzed against 10 mM sodium phosphate, pH 7. The spectra of the protein (final concentration, 10 μM) were recorded from 260 to 190 nm in continuous mode with a scanning speed of 10 nm/min, a time constant of 2 s, and a bandwidth of 1 nm (26). In some experiments the samples were measured and 0.01 volumes of 1 M MgCl₂ were added directly, and the CD spectrum was remeasured after 30 min of incubation time for the analysis of the effect of Mg²⁺ ion on the secondary structure of carrier proteins. Five spectra of each protein were measured, and after spectral subtraction of a blank sample, quantitative deconvolution of CD spectra

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**Fig. 4. Sequence alignment of the acyl carrier protein superfamily.** Comparison of sequence alignments between ACPs of FAS, PCP, and PCP’s in NRPS. Invariant residues as well as highly conserved residues are shaded in black. The four bars at the bottom of the figure represent the helical regions of TycC3-PCP. The white bar symbolizes helix 2 of TycC3-PCP that was later replaced by the corresponding region of ACP. The solid arrows indicate the serine residue to which the co-factor 4’-phosphopantetheine is attached. The dashed arrow points to amino acid 58, a position that is not present in PCP’s.

**Fig. 5. Posttranslational modification of apo-PCP, apo-hPCP, apo-LPCP by AcpS and Sfp.** Reaction mixtures containing the acyl carrier protein (1 mM), [3H]CoA, AcpS of B. subtilis (5.6 nM), or Sfp (25 nM) were incubated for 30 min at 37 °C. Shown is the modification of the respective acyl carrier protein in percent by AcpS (black columns) and Sfp (gray columns). This qualitative assay showed that AcpS and Sfp both recognize the designed hPCP. In addition Sfp also recognizes all other carrier proteins tested, whereas AcpS modifies ACP but not the TycC3-PCP that was used for the creation of the hPCP.
**RESULTS**

**Construction of hPCP and hTycA as a New Class of Peptidyl Carrier Proteins**—The motivation behind this study is the construction of a hybrid acyl carrier protein that can be modified in vitro and in vivo by AcpS and Sfp. We have constructed a distinct hPCP through replacement of the region coding for helix 2 in the TycC3-PCP with the corresponding region of ACP (14 amino acids) from *B. subtilis* as a single domain (Fig. 3). Using the same strategy, we subsequently constructed a hTycA through the replacement of the corresponding helix in the PCP of TycA, the initiation module (comprising three domains: A, PCP, and E) of the tyrocidine synthetase. Both hybrid proteins were constructed on the DNA level using oligonucleotides carrying the genetic information encoding helix 2 of ACP of *B. subtilis*.

The structure of AcpS from *B. subtilis* co-crystallized with CoA and ACP was described previously by Parris et al. (17). It revealed detailed insight into the protein-protein interaction between AcpS and ACP. It was possible to identify those residues of ACP that are mainly responsible for this interaction. Most of them are located in helix 2 of ACP (numbering according to Xu et al. (28)). ACPs and PCPs have a similar function and secondary structure. The sequence similarity between ACPs and PCPs, however, is very low; it is concentrated in the immediate neighborhood of the invariant serine residue (2, 18), whereas the sequence similarity between ACPs of FAS and PKS is high. The similarity of the loop between helices 1 and 2 and of the orientation of helix 2 in ACPs is high, whereas sequence similarity between PCPs can only be found in helix 1 and in the loop between helix 1 and 2. PCPs were subdivided according to their location in NRPS modules into two groups: PCP<sub>PKS</sub> upstream of E domains and PCP<sub>ACS</sub> upstream of C domains (25). The major differences between PCPs of the two groups are located in the loop between helix 1 and 2 and in helix 2: 1) the sequence in the neighborhood of the invariant serine residue of PCP<sub>PKS</sub> is GGS/K/L/I/QXXXRL, whereas for PCP<sub>ACS</sub>s it is GGS/LK; 2) helix 2 of PCP<sub>PKS</sub>s contains three positively charged amino acids, whereas PCP<sub>ACS</sub> contain only two, and the amino acid (Gln) at position 50 in PCP<sub>ACS</sub>s is highly conserved; and 3) PCP<sub>PKS</sub> have a deletion of one amino acid at position 58 (numbering according to the NMR structure of TycC3-PCP. This amino acid is localized at the end of helix 2 (Fig. 4).

**Overproduction and Purification of hPCP, PCP, and hTycA**—Recombinant hPCP, PCP, and hTycA were obtained by heterologous overexpression of the hPCP, PCP, and hTycA genes in *E. coli* M15 as C-terminal His<sub>6</sub> tag fusion proteins and subsequent purification by metal affinity chromatography and gel filtration. SDS-PAGE analysis (data not shown) revealed two bands in the case of hPCP, indicating a significant apo to holo conversion by *E. coli* AcpS during heterologous expression, just as it was reported for the ACP from *B. subtilis* and *E. coli* (21, 29). In the case of PCP and hTycA only one band could be obtained (data not shown). Per liter of cell culture, 20 mg of hPCP (99% purity), 22 mg of PCP (98% purity), and 15 mg of hTycA (85% purity) were obtained. Additionally, in this study we were able to show that Sfp is able to recognize and modify the hybrid FAS/NRPS carrier protein hPCP.

The conversion of hPCP and PCP from the apo to the holo form by AcpS and Sfp was determined by a radio assay. In this assay, the transfer of the [3H]Ppant moiety of CoA to the strictly conserved serine residue of hPCP and PCP, which is catalyzed by a PPTase, was detected (Fig. 2). AcpS (25 nM) and Sfp (25 nM) were incubated with 1 μM apo-hPCP or apo-PCP for 30 min at 37 °C. The amount of holo-protein formed from the various ACP and PCP proteins by each PPTase was investigated using a Liquid Scintillation Counter assay. apo-hPCP was 100% phosphopantetheinylated by both PPTases, whereas apo-PCP was converted to 100% holo-PCP only by Sfp. By contrast, AcpS was unable to phosphopantetheinylate native PCP (Fig. 5). These results were verified by HPLC.

**Biochemical Characterization of hPCP and PCP**—An HPLC method was used to determine the catalytic activity of *B. subtilis* AcpS and Sfp with both hPCP and TycC3-PCP. The apo to holo ratios of the purified recombinant proteins were determined to be 49% to 51% for hPCP and 93% to 7% for PCP, indicating that PCP was much less phosphopantetheinylated during expression in *E. coli* by either endogenous EntD or Yhu than was hPCP (4, 8, 30). Reaction mixtures containing 11.2 nM AcpS of *B. subtilis* or 10 nM Sfp were incubated at 37 °C for 10 min. The kinetic constants were determined through a Michaelis-Menten fit of the data sets, which yielded a *K<sub>m</sub>* of 21.6 ± 0.5 μM and a *k<sub>cat</sub>* of 14.35 ± 0.5 min<sup>-1</sup>. The *K<sub>m</sub>* of Sfp for hPCP was determined to be 26 ± 6 μM, and the *k<sub>cat</sub>* was determined to be 96 ± 4 min<sup>-1</sup>. The *K<sub>m</sub>* of Sfp for PCP was 4.45 ± 1 μM, and the *k<sub>cat</sub>* was 96 ± 4 min<sup>-1</sup> (Table 1). In contrast to the observations made by Mootz et al. (21), who determined different kinetic constants for AcpS at low and high apo-ACP concentration, only one *K<sub>m</sub>* and *k<sub>cat</sub>* value could be determined in the case of hPCP.

| Substrate | AcpS | Sfp |
|-----------|------|-----|
| apo-PCP (2–140 μM) | 21.6 ± 3 | 14.35 ± 0.5 |
| apo-hPCP (2–180 μM) | 0.2 ± 10.3 | 22 ± 2 |
| apo-ACP (2–8 μM) | 68 ± 11 | 125 ± 9 |
| apo-ACP (20–200 μM)<sup>a</sup> | 1.4 ± 0.3 | 1.7 ± 0.1 |

<sup>a</sup> ND, not determined.

<sup>b</sup> Mootz et al. (21).
Posttranslational Modification of hTycA with AcpS and Sfp of B. subtilis and Communication of the PCP with the A Domain in hTycA—A radio assay was applied to determine the catalytic activity of B. subtilis AcpS and Sfp with the parent TycA and hTycA. Reaction mixtures contained 25 nM AcpS or Sfp, and 1 μM of parent TycA or hTycA (apo-holo mixtures as obtained after heterologous expression) were incubated at 37 °C for 30 min. Incubation of parent TycA with either AcpS or Sfp showed that only Sfp was able to modify its PCP domain (Table II; this value was arbitrarily set to 100%), whereas AcpS did not recognize the protein. Purified hTycA (apo-holo mixture) was modified by AcpS and Sfp to 100% holo form with [3H]CoA in vitro. hTycA had been modified to a great extent during heterologous expression; thus only the remaining apo-protein (25%) was radio-labeled during the in vitro experiment (Table II), which is equivalent to a complete conversion to holo-hTycA. To determine the apo to holo ratio of the heterologously expressed hTycA (1 μM, apo-holo mixture), purified hTycA was incubated in the presence of [14C]Phe for 3 min at 37 °C. At defined time points samples were taken, and the bound amino acid was cleaved off by alkaline hydrolysis and applied to chiral TLC plates. The plates were developed, and the proportion of L- to D-Phe was determined using a two-dimensional radio scanner. In the case of hTycA with ATP in the absence of a PPTase, 75% of the protein became aminocarboxylated with [14C]Phe. Additionally, preincubation of hTycA with AcpS or Sfp in vitro increased the aminocarboxylation efficiency with [14C]Phe to 100% (Table III). In a control experiment, 5% of the parent TycA (1 μM, apo-holo mixture) with ATP and without PPTase became aminocarboxylated with [14C]Phe, indicating that the parent TycA was much less phosphopantetheinylated during heterologous expression by either EntD or Yhhu than was hTycA (4, 8, 30). After additional preincubation in vitro with Sfp, the aminocarboxylation activity increased to 100%. In contrast, AcpS has had no effect in vitro (Table III).

To resolve the communication between the A and hPCP domains in hTycA, we applied a radio assay. The hTycA and parent TycA were each allowed to covalently bind either L- or D-[14C]Phe. We found that both parent TycA and hTycA were aminocarboxylated to 100% as Phe-S-Ppant-Enzymes at the first order of magnitude of the reaction kinetics (31).

Communication of hPCP Domain with the Epimerization Domain in hTycA—The natural substrate of the E domain of TycA is the adjacent Phe-S-Ppant-PCP domain (24). To study the communication between the PCP and the E domains in hTycA, the extent of conversion of D- and L-[14C]Phe (and vice versa) catalyzed by the E domain was investigated. Although the DNA sequence of hTycA was correct, this protein was not able to convert D- or L-[14C]Phe, indicating a disturbed PCP-E communication. As a control experiment, parent TycA was utilized. As described previously, the equilibrium position of ~70% D-Phe-S-Ppant was reached within 30s (25).

Communication of hTycA with TycB1 and DKP Formation Assay—TycB1, the first module of the tyrocidine synthetase B, being the natural substrate of TycA (23), was used to explore the intermodule communication of hTycA with the C domain of another NRPS module. Prior studies reported that only D-Phe is transferred from TycA to TycB1, forming the D-Phe-L-Pro (10). HPLC was used for the identification of noncatalytically released DKP. The holo-hTycA was incubated with D-Phe or L-Phe, holo-TycB1, L-Pro, and ATP. No product could be detected in the case of holo-hTycA with L-Phe as substrate, whereas parent TycA, under the same conditions, was able to transfer D-Phe to TycB1, resulting in DKP formation. However, when holo-hTycA was incubated with D-Phe instead of L-Phe, DKP could be detected (25% of parent TycA). This result supports the finding that hTycA cannot convert L-Phe to D-Phe, which would be required for DKP formation. Additionally, utilizing an S562A mutant of TycA in the same assay did not result in the formation of any DKP with either L- or D-Phe. The rate of DKP formation under the reaction condition of parent TycA with L-Phe was 45 ± 1 min⁻¹ and that with D-Phe was 56 ± 1.3 min⁻¹, whereas in the case of hTycA it was 14 ± 0.7 min⁻¹ with D-Phe as substrate (Table IV). The decreased rate of the latter indicates an impaired communication of hTycA with the C domain of TycB1.

**DISCUSSION**

For the NRPSs, PKS, and FAS multienzyme complexes to become functionally active, the embedded PCP and ACPs need to be primed with the L-Ppant co-factor moiety of CoA by the dedicated PPTase. The PPTases of the AcpS type have, in contrast to the Sfp type PPTases, a narrow substrate specificity and are only able to modify ACPs of FAS and type II PKS, whereas they show absolutely no activity toward PCPs of NRPSs. We present in this work the construction of the first hybrid PCP/ACP protein that can be modified by AcpS and Sfp in vitro and in vivo.

From the co-crystal structure of B. subtilis AcpS with its natural substrate ACP, detailed insight into the amino acid residues involved in the interaction of the two proteins was obtained (17). The interaction at the interface between the basic AcpS and the acidic ACP is primarily hydrophilic in nature. This interface consists mostly of helix α1 of AcpS and helix α3 of ACP. Zhang et al. (32) have recently identified the
ACP docking site on β-ketoacyl-ACP synthase III (FabH) (both E. coli proteins) using computational analysis that was later verified by site-directed mutagenesis. Again, most residues of ACP involved in the interaction can be found in helix α2. This knowledge raised the question of whether the residues in helix 2 of acyl and peptidyl carrier proteins may represent the determinants that other proteins such as AcpS and Sfp use to distinguish them, prompting us to carry out a careful comparison of the different acyl carrier protein species.

The NMR solution structures of E. coli and B. subtilis FAS-ACP, Streptomyces coelicolor actinorhodin PKS-ACP, and B. brevis TycC3-PCP show no significant differences in the overall structure, a four-helix bundle. Members of this superfamily are characterized primarily by two structural motifs; helix 1 is in anti-parallel orientation and helix 4 is in parallel orientation to helix 2, whereas the orientation and length of helices 1, 3, and 4 vary. The most obvious difference between ACPs and PCPs is the electrostatic surface potential, especially around the invariant serine residue and adjacent helix (2). The second noticeable difference is the overall pI value, B. subtilis FAS-ACP, TycC3-PCP, and the hPCP designed in this study have pI values of 3.8, 7.6, and 4.9, respectively (Fig. 7). The corresponding PPTases, AcpS of primary metabolism and Sfp of secondary metabolism, have pI values of 9.6 and of 5.6, respectively. How is the interaction of the carrier protein with the corresponding PPTase mediated? The co-crystal structure has shown that the carrier protein part of the interface consists mostly of helix 2 of ACPs (numbering according to Xu et al. (28)) or, if this can be generalized, of the corresponding region (helix 2) of PCPs (numbering according to the NMR structure of TycC3-PCP (18)). In addition, sequence alignments (Fig. 4) have shown that this very helix is a consistent structural element within the different subfamilies (ACP or PCP’s or PCPβα) but differs significantly between species. Hence, the choice of one carrier protein over another as substrate by a certain PPTase is probably determined by differences in charge and hydrophobic nature of these helices. Additionally, the overall charge of the whole carrier protein as well as steric reasons may also play a role in protein-protein recognition. The replacement of the amino acid making up helix 2 in the TycC3-PCP with the corresponding region of ACP from B. subtilis for the single PCP domain as well as for the PCP domain of the initiation module TycA demonstrates that the exchange of 14 amino acids in PCP and 12 amino acids in TycA allows these proteins still to be recognized by Sfp and, for the first time, by AcpS as well. The kinetic studies with Sfp indicated that the $k_{\text{cat}}/K_m$ for hPCP lies between that for PCP and ACP, corresponding to a 6-fold drop compared with PCP and a 12-fold increase in catalytic efficiency compared with ACP. The $k_{\text{cat}}$ values of Sfp for hPCP and PCP are the same, whereas the $k_{\text{cat}}$ value for ACP is about eight times lower compared with that for hPCP. Based on these findings, we propose that the hPCP is more closely related to PCP than to ACP, as expected, because about 84% of its amino acids derive from PCP. In addition, hPCP seems to be a more suitable substrate for Sfp than is ACP. In agreement with this hypothesis, we found only one $K_m$ and $k_{\text{cat}}$ value for the substrate hPCP, whereas Mootz et al. (21) determined two $K_m$ and $k_{\text{cat}}$ values of Sfp as well as AcpS for the substrate apo-ACP at low (2–8 μM) and higher (20–200 μM) concentrations. Interestingly, the $K_m$ of AcpS for the modification of hPCP is three times lower than the $K_m$ for ACP. The $k_{\text{cat}}$ for hPCP, on the other hand, is about 10 times lower than that for ACP. Thus, the catalytic efficiency of AcpS with hPCP is diminished by a factor of three compared with the natural substrate ACP. The simple interpretation of this data suggests that although AcpS is now capable of recognizing the new protein, the catalytic efficiency is low so that the hPCP would not be a good replacement of a PCP or an ACP. Despite the fact that Mootz et al. (21) determined the catalytic efficiency of Sfp with ACP to be even lower (Table I), they were able to construct B. subtilis HM0489 that contains Sfp as the sole PPTase. The phenotype of this strain is that of the wild type, so Sfp seems to be competent enough to serve both primary (FAS) and secondary (NRPS) metabolism. In light of these results, we propose that hPCP is close to a universal carrier protein as far as PPTases are concerned.

The CD spectra of PCP, ACP, and hPCP at pH 7 showed that all three proteins have a similar secondary structure. Interestingly, the α-helix content of the proteins is concentration-dependent. The acyl carrier proteins probably undergo a conformational change as their concentration increases, which may partially explain why two $K_m$ and $k_{\text{cat}}$ values could be determined for the ACPs of B. subtilis and Streptococcus pneumoniae at high and low concentrations (21, 33).

The crystal structures revealed that AcpS functions as a trimer (17, 33), whereas Sfp acts as a monomer (12, 16). Comparison of the two protein structures has shown that the active site of Sfp is shallower and wider than that of AcpS. It was argued that this is in accord with the size of their natural substrates, because ACP is a small distinct acidic protein in contrast to the large NRPS modules with neutral PCPs. The PCPs of NRPS modules seem to fit nicely into the shallow Sfp active site but not into the narrow AcpS active site. In this work, we were able to show that both hPCP and hTycA were modified in vivo by E. coli AcpS during heterologous expression (hPCP, 51%; hTycA, 70%) and in vitro by AcpS of B. subtilis as well as Sfp to 100%, whereas the corresponding parent proteins TycC3PCP and TycA were modified to 7 and 6%, respectively. The percentage of modification was increased at prolonged induction times; when hTycA was expressed for 3 h instead of 2 h, more than 85% of the protein present was halo-hTycA. These observations illustrate nicely that the polarity of the active site is more important for the determination of substrate specificity than structural considerations.

### Table IV

|                  | Parent TycA | hTycA |
|------------------|-------------|-------|
| $k_{\text{cat}}$ | 45 ± 1.1 | ND* |
| $k_{\text{cat}}$ | 56 ± 1.3 | 14 ± 0.7 |

* ND, not determined.
Recently, Linne et al. (25) reported the biochemical investigation of a set of hybrid proteins with domain fusions between A and PCP domains as well as PCP and E domains. They described that epimerization activity was only observed in the case of fusion proteins where the PCP domain originated from modules containing an E domain (PCPE). Moreover, analysis of sequence alignments revealed that the differences between PCPCs compared with PCPEs are localized within helices 2 and 3 of PCPs. They suggested that this region is important for a proper interaction of PCP and E domains. Our data strongly support this proposal. We were able to investigate the communication between the PCP and two other domains of hTycA, the A and E domains. Kinetic studies of the aminoacylation reaction of hTycA and parent TycA showed that the A domain does not differentiate very strictly between PCP substrates. The PCP domain has a much greater influence in the case of the epimerization reaction of hTycA, however, because the E domain was not capable of converting covalently bound Phe from the L- to D-isomer. The communication between the hPCP and E domains is disabled because of the swap of 12 amino acids in the PCP domain of hTycA.

TycB1-CAT, the first module of TycB and natural substrate of TycA, was used to explore the intermodule communication of hTycA with another NRPS module. The aminoacylated d-Phe-hTycA was able to transfer its amino acid onto aminoacylated L-Pro-TycB1-CAT, and the product D-Phe-L-Pro-DKP was formed but with a significant lower rate than with the parent TycA. Prior studies reported that the incubation of an epimerization domain deletion mutant of GrsA (PheAT), the first module of the gramicidin synthetase from B. brevis, with TycB1-CAT did not result in formation of DKP (10). In contrast, the inactivation of the E domain by point mutations resulted in product formation activity only if D-Phe was used as substrate. These results suggested that the E domain is necessary for the recognition by the C domain of TycB1-CAT and that the C domain is specific for D-Phe. In the case of hTycA, however, the intact epimerization domain still exists, but communication with TycB1-CAT was nevertheless impaired. In the light of these results it seems plausible that the intermodule communication is established by a combination of PCP and E domain rather than by the E domain alone.

The main goal of this study was to determine the region of ACPs or PCPs that is recognized by PPTases of the AcpS and Sfp types. Additionally, our data present the first recombinant NRPS system containing a designed PCP domain that does not require a dedicated NRPS-PPTase but is efficiently primed by the E. coli PPTase AcpS. It has frequently been necessary to co-express Sfp when expressing NRPSs heterologously in E. coli.
coli} to ensure complete apo to holo conversion. Using this new hybrid approach, it would now be possible (in some cases) to obtain fully phosphopantetheinylated NRPSs independent of the host used for expression.

Acknowledgments—We thank Thomas Weber for providing plasmid pQE70-TycC3-PCP and Dirk Schwarzer and Uwe Linne for discussions and critical comments on the manuscript. We thank Henning Mootz for providing plasmid pTycA and Antje Schäfer for excellent technical assistance.

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