Solid Phase 4′-Phosphopantetheinylation: Fungal Thiolation Domains are Targets for Chemoenzymatic Modification

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No data exist on the ability of thiolation domains from fungal non-ribosomal peptide synthetases to undergo 4′-phosphopantetheinylation, using either biotinylated or fluorescently labeled coenzyme A analogues, mediated by 4′-phosphopantetheinyll transferases (PPTase). Yet, this is a key requirement to confirm the amino acid recognition function, and coding potential, of either non-ribosomal peptide synthetases or recombinantly expressed regions of these enzymes (e.g., didomains or modules). Moreover, determination of 4′-phosphopantetheinylation activity remains cumbersome. Here, we demonstrate that a recombinant fungal PPTase catalyzes the solution-phase transfer of either biotin- or fluorescein-labeled 4′-phosphopantetheine region of coenzyme A to a fungal thiolation domain, which is either part of a non-ribosomal peptide synthetase didomain (72 kDa), derived from Aspergillus fumigatus, or fused to a non-native protein (glutathione s-transferase). Significantly, we demonstrate that this reaction can unexpectedly occur when the target protein (4.4 pmol) is immobilized on a solid surface. These findings (i) confirm that thiolation domains of fungal origin, in native or non-native configuration, can accept modified 4′-phosphopantetheine residues via PPTase-mediated labeling and (ii) illustrate a novel, high-throughput method to determine PPTase activity.

INTRODUCTION

Phosphopantetheinyl transferases (PPTase) catalyze the transfer of 4′-phosphopantetheine from coenzyme A (CoA) to the carrier domain of a range of enzymes involved in non-ribosomal peptide, polyketide, and fatty acid biosynthesis (1). Non-ribosomal peptide (NRP) synthetases are produced in the active (apo-) form and are post-translationally modified by PPTase to the active (holo-) form by the covalent attachment of CoA-derived 4′-phosphopantetheine to a reactive serine residue in thiolation (T) domains (also referred to as peptidyl carrier protein (PCP)) within the NRP synthetase (2). Initial strategies for assaying 4′-phosphopantetheinylation involved the use of [3H]-pantetheine CoA and liquid scintillation counting (3), HPLC separation, and analysis of purified proteins (4). More recently, the application of protein mass spectrometry for the determination of apoenzyme 4′-phosphopantetheinylation has been reported (2, 5). These conventional methods for assaying PPTase activity, however, can be costly and cumbersome and significantly limit high-throughput operations (6).

An elegant method of covalently labeling T/PCP domains of NRP synthetases, using biotinylated or fluorescently labeled CoA, and a recombinant Bacillus subtilis encoded PPTase, Sfp, in PPTase-mediated reactions has been described (6). These authors used Sfp to catalyze modification of carrier proteins with a single fluorescent or affinity reporter, providing a sensitive means of protein visualization, Western blot identification, and affinity purification. This labeling technique also has utility in the identification of carrier protein domains from lysates of native cultures, as a proportion of carrier proteins in the lysate may be present in an unmodified inactive state. Application of the technique to the 6-deoxyerythronolide B synthase (DEBS) pathway from Saccharopolyspora erythraea resulted in the identification of a 150 kDa protein indicative of native DEBS (6).

The ability of PPTases to post-translationally modify carrier proteins using low molecular mass chemical labels represents a novel and efficient tool for protein labeling and has significant implications in biomolecular research. This approach has been adapted to fluorescence imaging of cellular events involving transferrin-mediated iron uptake (7) where the transferrin receptor 1 (TfR1) was fused to a PCP, and the resultant TfR1-PCP fusion specifically labeled with a fluorophore via the action of Sfp. In an independent study, an 11-residue peptide, DSLE-FLASKL, predicted to be a suitable target for modification by the B. subtilis Sfp was fused to the C- and N-termini of target protein and subsequently labeled with a biotin tag by the PPTase (8). The majority of reporter labeling of carrier proteins by promiscuous PPTases have been limited to in vitro and cell surface protein labeling (9–11). A chemoenzymatic route to protein modification in vivo using a fluorescent marker has also been reported (12). This mechanism centered on the ability of native enzymes present in E. coli, CoAA, and CoAD and CoAE to sequentially modify a fluorescent pantetheine analogue to result in a fluorescently labeled CoA analogue. Coexpression of the carrier protein VibB and the PPTase Sfp in the presence of the fluorescent pantetheine analogue resulted in the fluorescent modification of the VibB carrier protein in vivo.

The PPTase-mediated modification of bacterial pyochelin NRP synthetase carrier proteins PchE and PchF using fluorescein (Fl)-CoA analogues has also been reported (13). These in vitro 4′-phosphopantetheinylation assays used 2 µM B. subtilis Sfp, 300 µM Fl-CoA, and PchE/ PchF carrier proteins at concentrations of 10 and 21 µM, respectively. The detection of the modified carrier proteins in this study was enabled by Fourier transform mass spectrometry whereby mass shifts were observed for reporter labeled peptides.

In vitro 4′-phosphopantetheinylaion assays have been used to investigate the effect of inhibitors on PPTase activity (14).
Phosphopantetheinylation are cumbersome and time-consuming. Characterization, and the assay systems for detection of amounts of the secreted PPTase has hindered complete enzyme date, the compatibility of this fungal PPTase has not been

\[ \mu \]

PCR reactions also included 10 \( \mu \)g of genomic DNA per PCR reaction. In a total volume of 20 \( \mu \)L. PCR reactions comprised an

Aspergillus fumigatus strain ATCC 26933 was grown (250 mL cultures) at 37 °C in minimal essential medium (MEM) (5% (v/v) containing fetal calf serum (FCS)) for 2 days. Genomic DNA was isolated by crushing fungal mycelia in liquid N\(_2\) followed by phenol/chloroform extraction, washing with 70% (v/v) ethanol and final DNA resuspension in 10 mM Tris-HCl, 1 mM EDTA pH 8.0 (200 μL). PCR was carried out using AccuTaq LA polymerase (Sigma) using a total of 5 ng of genomic DNA per PCR reaction. PCR reactions also included 10× AccuTaq reaction buffer, 0.25 μM of each dNTP, 1.0 μM forward and reverse primer (Table 1) in a total volume of 20 μL. PCR reactions comprised an initial step of 60 s denaturation at 95 °C, followed by 30 cycles consisting of 60 s at 95 °C, 30 s at 55 °C, 90 s at 72 °C, and finally 360 s at 72 °C. Amplicons were analyzed by agarose gel electrophoresis and visualized using an Eagle-Eye II digital still video system (Stratagene, CA, USA). DNA sequence analysis was carried out on a commercial basis by MWG Biotech (Germany).

Cloning and Expression of the Nonribosomal Peptide Synthetase Gene Fragment, pes3\(_{AT}\). The pes3\(_{AT}\) amplicon, which is derived from the 5′ end of the entire pes3 gene (Figure 1), was initially cloned into the TOPO cloning vector and subsequently cloned into pProEx expression vectors (Invitrogen) according to the manufacturer’s instructions. pProEx-pes3\(_{AT}\), the expression vector containing the region encoding the initial AT domain of pes3 (A. fumigatus pes3\(_{AT}\)) (Figure 1), was transformed into E. coli according to the manufacturer’s guidelines (Invitrogen). Protein expression was induced by the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG; 0.6 mM final concentration) and monitored by SDS-PAGE, Western blot analysis using monoclonal antibody reactivity against a His\(_6\) fusion peptide, and MALDI-ToF mass spectrometry (15).

Recombinant pes3\(_{AT}\) Purification and Refolding. Recombinant pes3\(_{AT}\) was purified under denaturing conditions using Ni-NTA metal-chelate affinity chromatography in the presence of 6 M guanidine—HCl. Purified recombinant Pes3\(_{AT}\) (250 μg/mL) in 50 mM sodium carbonate pH 9.4 containing 6 M guanidine—HCl was serially dialyzed into 50 mM sodium carbonate pH 9.4 containing 8 M urea, then 4 M urea, followed by dialysis into 50 mM sodium carbonate buffer pH 9.4 to facilitate solubilization.

Generation of Recombinant Fusion Protein (T-GstB) Containing the Pes3\(_{AT}\) Derived Thiolation Domain. The region encoding the thiolation (PCP) domain from pes3\(_{AT}\) was fused to A. fumigatus glutathione s-transferase B (gstB (16)) in order to assess the utility of the domain for non-native protein labeling (Figure 1). PCR cloning primers incorporated EcoRI and HindIII restriction sites to facilitate PCR amplification and directional subcloning of the T region (Table 1). This amplicon (196 bp) was subsequently digested with EcoRI and HindIII and ligated to the pProEx-gstB expression vector (16), which had also been digested with EcoRI and HindIII. The expression vector containing T-GstB was then transformed into E. coli DH5α according to refs 16 and 17. Protein expression was induced by the addition of IPTG (0.6 mM final concentration) and monitored by SDS-PAGE and Western blot analysis. Purification of the T-GstB fusion protein was carried out as previously described (2).

**Table 1. Oligonucleotide Primers Designed for Directional Cloning of pes3\(_{AT}\) and T/PCP Region Sequences into the pProEx-GstB Expression Vector**

<table>
<thead>
<tr>
<th>gene</th>
<th>primer sequences (5′-3′)</th>
<th>amplicon (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pes3(_{AT})</td>
<td>F GAGACCTGAGACTACGCGGAATGGAA</td>
<td>1800</td>
</tr>
<tr>
<td>pes3(_{AT})</td>
<td>R GAGAAAGGATCCATGGAACGCCGGTCCGAG</td>
<td>196</td>
</tr>
<tr>
<td>T/PCP</td>
<td>F GAGAGATCGTGGAGCCGGTCCGAG</td>
<td>196</td>
</tr>
<tr>
<td>T/PCP</td>
<td>R GAGAGATCTGGACGGTCTCTGTCCGAG</td>
<td>196</td>
</tr>
</tbody>
</table>

**Bioinformatic Analysis.** Preliminary sequence data were obtained from The Institute for Genomic Research Website at http://www.tigr.org. The unannotated genome of Aspergillus fumigatus was initially investigated to identify open reading frames corresponding to novel NRP synthetases. One open reading frame, which encoded a putative NRP, was identified and was subsequently termed pes3 (Figure 1).

**Fungal DNA Isolation and PCR Amplification.** A. fumigatus strain ATCC 26933 was grown (250 mL cultures) at 37 °C in minimal essential medium (MEM) (5% (v/v) containing fetal calf serum (FCS)) for 2 days. Genomic DNA was isolated by crushing fungal mycelia in liquid N\(_2\) followed by phenol/chloroform extraction, washing with 70% (v/v) ethanol and final DNA resuspension in 10 mM Tris-HCl, 1 mM EDTA pH 8.0 (200 μL). PCR was carried out using AccuTaq LA polymerase (Sigma) using a total of 5 ng of genomic DNA per PCR reaction. PCR reactions also included 10× AccuTaq reaction buffer, 0.25 μM of each dNTP, 1.0 μM forward and reverse primer (Table 1) in a total volume of 20 μL. PCR reactions comprised an

**EXPERIMENTAL METHODS**

Figure 1. Diagrammatic representation of (A) Pes3 domain architecture, and (B) thiolation (T)-GstB fusion protein. The first adenylation domain and thiolation domain from module 1 of Pes3 (Pes3\(_{AT}\)) were selected for recombinant protein production. Wavy lines shown in A indicate covalently tethered phosphopantetheine moieties to T domains (shaded). The T-GstB fusion protein is also illustrated showing the Pes3 thiolation domain (T) fused to the GstB protein. The N- and C-termini of both proteins are indicated.

Here, the efficacy of novel anthranilic acid inhibitors of the B. subtilis acyl carrier protein synthase (AcpS), which catalyzes the transfer of 4′-phosphopantetheinyl group from CoA onto a conserved serine residue present on an E. coli acyl carrier protein (ACP), was assessed and found to inhibit IC\(_50\) (μM) values of 0.8–32.3. The effect of these inhibitors was determined in a solution phase assay format, which involved incubation of the AcpS (a PPTase) in the presence of a GST-linked ACP carrier protein and a biotin-CoA reporter analogue at room temperature. Detection of the biotin-labeled ACP was achieved by an overnight incubation with streptavidin—aliphophycocyanin conjugate followed by time-resolved fluorescence detection.

A PPTase from Aspergillus fumigatus has recently been expressed in, and purified from, recombinant baculovirus infected Spodoptera frugiperda (Sf\(_6\)) insect cell supernatants. Subsequent mass spectrometric detection of a 4′-phosphopantetheinylated peptide confirmed that this secreted PPTase post-translationally modified a recombinant NRP synthetase T domain from A. fumigatus Pes1 in the presence of CoA (2). To date, the compatibility of this fungal PPTase has not been demonstrated with modified CoA derivatives; moreover, limited amounts of the secreted PPTase has hindered complete enzyme characterization, and the assay systems for detection of 4′-phosphopantetheinylation are cumbersome and time-consuming.

![Diagram](https://via.placeholder.com/150)
Bradford analysis and the supernatants divided into 0.5 mL aliquots and stored at −20 °C for further use.

For purification of intracellular PPTase, infected Sf6 insect cells (5 × 10⁶) were resuspended in lysis buffer (5 mL; 50 mM Na₂HPO₄, 300 mM NaCl, 10 mM imidazole pH 8.0) containing 0.05% (w/v) sodium deoxycholate and sonicated (Bandelin Sonopuls, Progen Scientific Ltd., UK) for 3 × 5 s at a maximum power for 5 min with 30 s intervals on ice and the resultant lysates centrifuged at 10 000g for 10 min at 4 °C. Purification proceeded under native conditions using Ni-NTA metal-chelate affinity chromatography. Optimal protein yields were obtained using an optimized batch purification approach which entailed the incubation of the PPTase lysate to the Ni-NTA resin for 2 h with gentle inversion at 4 °C. Subsequently, the protein/resin complex was packed into a column to facilitate washing and elution steps. Purified PPTase was eluted with 250 mM imidazole and subsequently dialyzed into Ppant buffer.

**Coenzyme A Analogue Synthesis and Solution Phase PPTase Activity Analysis.** Coenzyme A (CoA) was covalently labeled through an available sulhydryl group by reaction with either maleimido-dPEG₄-biotin (MB) (Quanta BioDesign Ltd., Ohio, USA) or 5'-iodoacetamidofluorescein (Sigma-Aldrich) according to ref 6 with modifications (Supporting Information). All reactions were allowed to proceed on ice for 30 min, followed by 10 min incubation at room temperature, and terminated by addition of 1 M DTT (1 µL). 4'-Phosphopantetheinylation reactions using PPTase and recombinant Pes3ₐt were carried out as follows: Reaction mixtures for the in vitro 4'-phosphopantetheinylation assay contained Pes3ₐt (4.4 µM), PPTase (0.08 µM), biotin-CoA (8.4 µM) in 75 mM Tris-HCl, 5 mM DTT, and 10 mM MgCl₂, pH 8.0 (final volume: 100 µL). Reaction mixtures were incubated at 37 °C for 1 h. After incubation, reactions were terminated by the addition of 10% (w/v) trichloroacetic acid (900 µL) and centrifuged at 10 000g for 10 min. Pellets were resuspended in 1 M Tris-HCl pH 8.0 and analyzed by SDS-PAGE and Western blot analysis, using direct probing with streptavidin–horse radish peroxidase (HRP) (1/500) and ECL chemiluminescent substrate (Pierce Biotech), to visualize biotinylated Pes3ₐt. Fluorescence detection was also undertaken by directly scanning SDS-PAGE gels, when fluorescence-Coomassie (Fl-CoA) (6) was employed instead of biotin-CoA (6), using a Typhoon variable mode imager (GE Healthcare) at excitation and emission wavelength at 488 and 520 nm, respectively. Scanned gels were subsequently stained with Coomassie Brilliant Blue R. Alternatively, reaction mixtures were directly added to Nunc Maxisorb microwells (1 h), washed using phosphate buffered saline 0.05% (w/v) Tween-20 (PBST), and fluorescence detected using a plate reader (Bio-Tech, Synergy HT) with excitation and emission values of 490 and 520 nm, respectively.

**Microwell Coating with Recombinant Pes3ₐt, T-GstB, or GstB for Use in Solid Phase 4'-Phosphopantetheinylation Assays.** Microtiter plates were coated with purified recombinant Pes3ₐt (2 µg/mL) diluted in 50 mM sodium carbonate buffer pH 9.6 containing 0.01% (w/v) sodium dodecyl sulfate (100 µL/well). Plates were incubated at 37 °C for 2 h and washed twice with PBST (200 µL). Blocking solution (1% (w/v) bovine serum albumin and 10% (w/v) sucrose in 50 mM sodium carbonate buffer pH 9.6; 200 µL/well) was applied to the wells and stored at 4 °C overnight. Blocking solution was decanted from plates, and any residual solution was removed by tapping the plate upside down onto absorbent paper and transferring the plate to a 37 °C incubator for 1 h prior to use. Microwells were also individually coated with the T-GstB and GstB, respectively (5 µg/mL), for subsequent use in the solid phase 4'-phosphopantetheinylation assays. Microwell coating of T-GstB and GstB and subsequent 4'-phosphopantetheinylation assays were carried out as described for Pes3ₐt.***

**Solid Phase 4'-Phosphopantetheinylation Using Biotin-CoA.** Reagents were prepared in triplicate in 1.5 mL microfuge tubes as outlined in Table 2. Aliquots (100 µL) of each sample were placed in wells of 96-well plates (Nunc Maxisorb) which had been previously coated with Pes3ₐt (2 µg/mL). Plates were incubated for 1 h at room temperature to facilitate enzymatic reaction. Plates were then washed twice with PBST. Streptavidin–HRP (1/2000 dilution; 100 µL/well) was added and plates incubated at 37 °C for 1 h. Plates were washed 4 times with PBST. Excess liquid was removed from the wells by tapping the plate out on absorbent paper. Substrate (3,3',5,5'-tetramethylbenzidine; TMB; 100 µL/well) was applied to the wells and the absorbance values read at 450/630 nm using a plate reader (Bio-Tech, Synergy HT).

**Solid Phase 4'-Phosphopantetheinylation Using Fl-CoA.** Reagents were prepared in triplicate in 1.5 mL microfuge tubes as outlined in Table 3. Aliquots (100 µL) of each sample were placed in wells of black 96-well plates (Nunc Maxisorb) which had been previously coated with Pes3ₐt (2 µg/mL). Plates were incubated for 1 h at room temperature to facilitate enzymatic reaction. Plates were then washed twice with PBST and excess liquid removed by tapping the plates on absorbent paper. Fluorescence was detected using a plate reader (Bio-Tech, Synergy HT) with excitation and emission values of 490 and 520 nm, respectively.

**PPTase Characterization Using Solid Phase 4'-Phosphopantetheinylation Assay.** Temperature and pH optima, along with PPTase reaction time, were determined. In addition, the effect of protease inhibitors (phenylmethylsulfonyl fluoride; 1516 Bioconjugate Chem., Vol. 20, No. 8, 2009 Stack et al.
PMSF inclusion on PPTase cell lysate stability was investigated. Student’s t test was used to assess the statistical significance of relevant results. Absorbance values for control lysate (lacking PPTase) were subtracted from PPTase lysates for calculation of relative PPTase activity (%). The mean absorbance value for purified PPTase was set at 100% with all other values (PPTase lysate and biotin-CoA controls) expressed as a percentage of this.

RESULTS

Recombinant Protein Expression, Purification, and Solubilization. Pes3AT was found to be highly insoluble under nondenaturing conditions (data not shown) and was solubilized by addition of 6 M guanidine·HCl containing 1 mM DTT to a final concentration of 30 mg/mL or 5 mg/g cells. SDS-PAGE analysis and Western blot analysis confirmed the required purity of Pes3AT (Figure 2). The 72 kDa Pes3AT band observed on the Coomassie stained gel was excised and subjected to MALDI-ToF mass spectrometry. Resultant tryptic digest peptide monoiso-

Table 4. Peptides Identified Following Trypsin Digestion of Recombinant Pes3AT and MALDI-ToF Mass Spectrometry Analysis

<table>
<thead>
<tr>
<th>m/z (Da)</th>
<th>identified peptides</th>
</tr>
</thead>
<tbody>
<tr>
<td>900.39</td>
<td>RYADWMAKYLVMKR</td>
</tr>
<tr>
<td>1656.85</td>
<td>KKSVWTMVAMLAIMK</td>
</tr>
<tr>
<td>1590.88</td>
<td>KRILDTTEAPLIVHR</td>
</tr>
<tr>
<td>1523.84</td>
<td>KGVPPHSIATSIR</td>
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<tr>
<td>1930.95</td>
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<td>1288.737</td>
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<tr>
<td>1273.61</td>
<td>HREDGDVLYHLLGR</td>
</tr>
<tr>
<td>1531.66</td>
<td>KVAEHSTDTSADNER</td>
</tr>
</tbody>
</table>

*The peptides identified represent 20% coverage of the Pes3AT and confirm the identity of the protein.

topic m/z values (Table 4), compared to those of an in silico digest of Pes3AT, represented 20% sequence coverage thereby providing conclusive confirmation of Pes3AT identity. Pes3AT was solubilized under nondenaturing conditions by dilution into Ppant buffer containing 8 M urea (4 mL) at 0.5 mg/mL Pes3AT followed by serial dialysis into Ppant diluent (final concentration: 0.41 mg/mL (5.75 µM)). This solubilized Pes3AT preparation was aliquoted and stored at −20 °C for subsequent use. The 36 kDa T-GstB fusion protein was purified from E. coli cell lysates resulting in a T-GstB yield of 13 mg/mL or 5 mg/g cells (Figure 3).

Following infection with recombinant baculovirus encoding A. fumigatus PPTase, Spodoptera frugiperda insect cell lysates were prepared by sonication in the presence of 0.05% (w/v) sodium deoxycholate in Ppant diluent. Purified PPTase was obtained by incubation with Ni-NTA affinity resin for 2 h followed by elution with 250 mM imidazole and dialysis into Ppant diluent (yield: 0.33 mg/10⁸ cells) (Figure 3).

Labeled CoA Conjugate Synthesis. Biotin-CoA conjugates were prepared by reacting MB with CoA to form a stable thioether-mediated conjugate for subsequent assay development. Prior to addition of free DTT to scavange any unreacted MB, a new method was deployed to determine the efficiency of biotin–CoA conjugate formation, which involved determination of residual CoA using aldrithiol-4 (4,4′-dithiodipyridine) reagent (Supplementary information). This analysis confirmed ef-
fectively 90%+ efficiency of biotin-CoA conjugate synthesis and extends the utility of aldrithiol-4 and other reagents used for thiol group determination (18). Previously, identical biotin-CoA analogues were synthesized, the unincorporated biotin reporter extracted via the use of scavenger resins, and the resultant biotin-CoA reporter subsequently used for the high-sensitivity detection (100 pg) of the biotin labeled recombinant VibB carrier protein domain (6). However, we observed minimal background protein modification once DTT was added to biotin-CoA conjugates, which implies that removal of unreacted MB may not always be necessary. A fluorescently linked CoA analogue was also synthesized using 5′-iodoacetamidofluorescein (5′-IAF), which alkylates the sulfhydryl group of CoA. Fluorescent detection offers the potential advantage of direct analysis of labeled proteins without the need for enzyme conjugates and chemiluminescent substrates (19). The production of fluorescein-CoA was confirmed by TLC analysis; however, evidence of unreacted 5′-IAF in the fluorescein-CoA sample was also observed (data not shown). As with biotin-CoA conjugates, it was found that subsequent addition of DTT eliminated nonspecific fluorescent labeling of target proteins. In addition, RP-HPLC analysis was also used to detect the production of fluorescein-CoA conjugates with the appearance of a new peak with a lower retention time of 17.0 min for the fluorescein-CoA test specimens which is likely due to the higher polarity of Fl-CoA molecule compared to 5′-IAF (data not shown).

4′-Phosphopantetheinylation of Pes3_AT with Biotin- and Fluorescein-CoA is Mediated by PPTase. Pes3_AT was subject to specific 4′-phosphopantetheinylation by PPTase present in insect cell lysates (Figure 4a). Here, modification of the Pes3_AT (4.4 µM) was clearly detectable using PPTase-containing insect cell lysates (17–1700 µg/mL total protein), with a weak signal, corresponding to specific 4′-phosphopantetheinylation, at 3 µg/mL total cell protein. No 4′-phosphopantetheinylation was detectable in the presence of control insect cell lysate, thereby providing confirmation that modification of Pes3_AT with biotin-CoA was mediated specifically by PPTase and not as a result of direct nonspecific labeling of Pes3_AT with residual MB. Omission of Pes3_AT from the reaction likewise produced a negative result, which confirmed that proteins in the insect cell lysate were not labeled nonspecifically with biotin-CoA (Figure 4a). Replacement of biotin-CoA with fluorescein-CoA as the PPTase substrate also facilitated post-translational modification of Pes3_AT (Figure 4b). Here, Pes3_AT (4.44 µM) was labeled using both purified PPTase (0.08 µM) and PPTase present in insect cell lysate (34 µg/mL total cell protein). No 4′-phosphopantetheinylation was detectable in the absence of either PPTase or Pes3_AT. Overall, these observations confirm that the Pes3_AT thiolation domain can be post-translationally modified by PPTase with either biotinylated or fluorescently labeled CoA.

High-Throughput Detection of 4′-Phosphopantetheinylation. Reaction mixtures containing combinations of purified PPTase, Pes3_AT, and fluorescein-CoA were incubated in solution for 1 h and subsequently transferred to black microtiter plates,
incubated for a further 1 h, followed by washing with PBS (2×) to remove unbound fluorescein-CoA. Fluorescent analysis at 490/520 nm (exc/em) (Figure 5) indicated that fluorescently labeled Pes3AT had bound to the plate and was detectable by fluorescence analysis. Fluorescence labeling was predominantly mediated by PPTase modification; however, nonspecific labeling was also evident (up to 25%) in the absence of PPTase. No fluorescence was detectable in the absence of Pes3AT.

For technical reasons, direct detection of biotinylated Pes3AT on microwells was not possible as described for fluorescent Pes3AT above (data not shown). However, 4'-phosphopantetheinylation was possible using microwells precoated with Pes3AT (4.4 pmol), and stabilized by BSA addition (Figure 6a). To ensure that biotin labeling of the bound Pes3AT protein was the result of PPTase activity and not due to the presence of insect cell proteins present in the crude cell lysate, control reactions were included whereby uninfected insect cell lysates (at identical total protein concentration) were incubated in the Pes3AT coated microwells in the presence of biotin-CoA, and resultant absorbance values compared to those obtained from incubation with PPTase insect cell lysate. Background activity was apparent in uninfected cell lysate controls compared to the biotin-CoA and PPTase cell lysate specimens to PPTase absent from reactions. Ppant buffer control: Ppant buffer only. Ppant buffer and fluorescein-CoA (Fl-CoA) only: Pes3AT and PPTase absent from reactions.

DISCUSSION

Despite recent advances in PPTase-mediated labeling of T/PCP or ACP domains of prokaryotic origin, via biotinylated or fluorescently modified CoA analogues, minimal information on this approach for eukaryotic (fungal) T domain modification is available. Moreover, facile determination of PPTase activity has remained a challenge. Here, data are presented which confirm that T domains of fungal origin can be post-translationally modified, both in solution and when immobilized on a solid surface, using a combination of modified CoA analogues and a functional PPTase. We also demonstrate that T domains can be transferred to a non-native protein (GstB) and that this fusion protein (T-GstB) is also a substrate for PPTase-mediated modification. Finally, it appears that the solid phase assay format presents a new strategy for determination of PPTase activity which is faster than current methods and is compatible with high-throughput functionality.

The gene encoding Pes3 represents the largest NRPS open reading frame in the A. fumigatus genome. Here, we have expressed the first AT didomain of Pes3 and a T-GstB fusion protein, in E. coli at high yields (5 mg/g cells). We have previously cloned and expressed recombinant PPTase in the baculovirus expression system, purified from PBS washes of intact cells by subsequent Ni-NTA chromatography, and shown it to be capable of transferring the Ppant group from CoA to the T domain derived from a distinct A. fumigatus NRPS (pes1) (2). However, in the present work we utilize PPTase, of intracellular origin, which was extracted in high yields (3.33 mg/10⁶ cells) and purified by Ni-NTA affinity chromatography. Interestingly, PPTase activity was detectable in crude cell lysates, indicating that enzyme purification is not always essential.

**Figure 5.** Solid phase 4'-phosphopantetheinylation assay using Fl-CoA. After solution phase incubation, 4'-phosphopantetheinylation reactions (and appropriate controls) were coated on black microtiter plates and subsequently analyzed by fluorescence with excitation and emission at 490 and 520 nm, respectively. Results given represent the mean (±SE) of the experiment performed in triplicate. All reaction constituents: Pes3AT; PPTase; Fl-CoA. PPTase negative control: PPTase absent from reactions. Ppant buffer control: Ppant buffer only. Ppant buffer and fluorescein-CoA (Fl-CoA) only: Pes3AT and PPTase absent from reactions.

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**Figure 5.** Solid phase 4'-phosphopantetheinylation assay using Fl-CoA. After solution phase incubation, 4'-phosphopantetheinylation reactions (and appropriate controls) were coated on black microtiter plates and subsequently analyzed by fluorescence with excitation and emission at 490 and 520 nm, respectively. Results given represent the mean (±SE) of the experiment performed in triplicate. All reaction constituents: Pes3AT; PPTase; Fl-CoA. PPTase negative control: PPTase absent from reactions. Ppant buffer control: Ppant buffer only. Ppant buffer and fluorescein-CoA (Fl-CoA) only: Pes3AT and PPTase absent from reactions.

**Figure 6c.** Here, nonspecific modification (approximately 25% of the PPTase reaction) was also evident.

### DISCUSSION

Despite recent advances in PPTase-mediated labeling of T/PCP or ACP domains of prokaryotic origin, via biotinylated or fluorescently modified CoA analogues, minimal information on this approach for eukaryotic (fungal) T domain modification is available. Moreover, facile determination of PPTase activity has remained a challenge. Here, data are presented which confirm that T domains of fungal origin can be post-translationally modified, both in solution and when immobilized on a solid surface, using a combination of modified CoA analogues and a functional PPTase. We also demonstrate that T domains can be transferred to a non-native protein (GstB) and that this fusion protein (T-GstB) is also a substrate for PPTase-mediated modification. Finally, it appears that the solid phase assay format presents a new strategy for determination of PPTase activity which is faster than current methods and is compatible with high-throughput functionality.

The gene encoding Pes3 represents the largest NRPS open reading frame in the A. fumigatus genome. Here, we have expressed the first AT didomain of Pes3 and a T-GstB fusion protein, in E. coli at high yields (5 mg/g cells). We have previously cloned and expressed recombinant PPTase in the baculovirus expression system, purified from PBS washes of intact cells by subsequent Ni-NTA chromatography, and shown it to be capable of transferring the Ppant group from CoA to the T domain derived from a distinct A. fumigatus NRPS (pes1) (2). However, in the present work we utilize PPTase, of intracellular origin, which was extracted in high yields (3.33 mg/10⁶ cells) and purified by Ni-NTA affinity chromatography. Interestingly, PPTase activity was detectable in crude cell lysates, indicating that enzyme purification is not always essential.
Figure 6. (A) Diagrammatic representation of solid phase 4′-phosphopantetheinylation. Immobilized thiolation domains (e.g., in Pes3\textsubscript{AT}) (green circles) are stabilized by the addition of BSA (dashed line). The reactive serine residue (−OH) on the thiolation domain can then be modified with biotin-CoA in the presence of PPTase. PPTase activity is then indirectly detected by addition of streptavidin−HRP conjugate and TMB substrate. Fluorescein-CoA (Fl-CoA) can be substituted for an analogous, that crude PPTase preparations can be used to effect in vitro 4′-phosphopantetheinylation, and also that the enzyme produced within recombinant baculovirus-infected insect cells is active. Detection of fluorescein-labeled Pes3\textsubscript{AT} (4.4 μM) was achieved using 7.3 μM fluorescein-CoA and using either 0.08 μM purified PPTase and crude PPTase cell lysate at a concentration of 0.034 mg/mL (estimated to contain 0.92 μM PPTase). Thus, the molar ratios of purified PPTase, carrier protein, and fluorescein-CoA used were approximately 1:55:90. The PPTase-mediated modification of the Pyochelin NRP synthetase carrier proteins PchE and PchF using similar fluorescein-CoA analogues has been reported (13). The in vitro 4′-phosphopantetheinylation assays used in that study, however, used 2 μM B. subtilis Sfp, 300 μM fluorescein-CoA, and PchE/PchF carrier proteins at concentrations of 10 and 21 μM, respectively. The molar ratios of PPTase, carrier protein, and fluorescein-CoA in the present study were 1:5−10:150, respectively, similar to fluorescein-CoA−PPTase assay described herein; however, the assay of ref 13 required approximately 5-fold more carrier protein and 25 times more PPTase (Sfp), respectively.

In order to eliminate the time-consuming electrophoretic step associated with labeled carrier protein detection and expedite analysis, we attempted direct immobilization of reaction mixtures to microwells, subsequent to in vitro 4′-phosphopantetheinylation followed by visualization and absorbance detection. This approach was unsuitable for use with biotin-CoA reporter analogues as the biotin moiety exhibited high nonspecific binding to microwells thereby masking any possibility of detecting PPTase mediated Pes3\textsubscript{AT} labeling (data not shown). However, the approach was successfully applied to in vitro 4′-phosphopantetheinylation assays using a fluorescein-labeled CoA analogue whereby 3-fold greater fluorescence was detectable in specimens containing PPTase, Fl-CoA and Pes3\textsubscript{AT} compared to specimens containing Fl-CoA and Pes3\textsubscript{AT} alone (Figure 5). Although extensive validation of this approach has
mediated protein modification is up to 75% complete by 1 h.
PPTase activity. Optimal reaction time is 2 h; however, PPTase-
test was used to calculate
represent the mean (\( \pm \) SE) of three individual experiments. Student’s \( t \) test was
used to calculate \( P \)-values for the difference between the control (24
\( ^\circ \)C + protease inhibitors) and the following conditions: 4 \( ^\circ \)C (+/−
protease inhibitors \( P = 0.003; P = 0.0009 \)), 24 \( ^\circ \)C (- protease inhibitors \( P = 0.033 \)), 37 \( ^\circ \)C (+/−
protease inhibitors \( P = 0.005; P = 0.0002 \)), and 50 \( ^\circ \)C (+ protease inhibitors \( P = 0.00001; P = 0.00002 \)).

(A) The effect of temperature on PPTase activity in the
presence of protease inhibitors. Results given represent
the mean (\( \pm \) SE) of three individual experiments. Student’s \( t \) test was
used to calculate \( P \)-values for the difference between the control (24
\( ^\circ \)C + protease inhibitors) and the following conditions: 4 \( ^\circ \)C (+/−
protease inhibitors \( P = 0.003; P = 0.0009 \)), 24 \( ^\circ \)C (- protease inhibitors \( P = 0.033 \)), 37 \( ^\circ \)C (+/−
protease inhibitors \( P = 0.005; P = 0.0002 \)), and 50 \( ^\circ \)C (+ protease inhibitors \( P = 0.00001; P = 0.00002 \)).

(B) The effect of protease inhibitors on PPTase activity. Results
are expressed as percent (% relative to the highest level, i.e., pH 8.0,
and represent the mean (\( \pm \)SE) of three individual experiments. Student’s \( t \) test was
used to calculate \( P \)-values for the difference between the control (pH 8.0)
and the following pH conditions: 4.0 \( (P = 0.01) \), 6.5 \( (P = 0.008) \),
and 10.0 \( (P = 0.0003) \). (C) The effect of the reaction time on
PPTase activity. Optimal reaction time is 2 h; however, PPTase-
mediated protein modification is up to 75% complete by 1 h.

A number of groups have confirmed that proteins fused to
either T domains of bacterial origin, or peptides derived from
phage display, can be covalently tethered to immobilized CoA,
and this observation has been proposed as a new strategy for
protein array preparation (9, 20). However, to date, labeled
pantetheine transfer to immobilized T domains, in either native
or non-native configuration, has not been demonstrated. The
75 amino acid T domain used in our study is equivalent in size
to other fungal and bacterial T domains but considerably longer
than ybbR-like tags introduced by other groups (8). The use of
the T domain of fungal (A. fumigatus) origin as a target for
C-terminal labeling of Pes3AT and N-terminal labeling of
T-GstB, respectively, suggests that domain acceptor function
is independent of position in the substrate protein. We have
observed that purified Pes3AT or T-GstB can be stably and
irreversibly attached to microwells, blocked to minimize
nonspecific binding, and followed by PPTase-mediated bioti-
nylation or fluorescent labeling using either biotin-CoA or
fluorescein-CoA. This finding represents a new strategy for
assessment of both T domain functionality and PPTase activity
determination and potentially for the assessment of PPTase/AcpS/Sfp inhibitors, which have been proposed as potential drug
targets (14).

Our data indicate that 0.33 \( \mu g \) (4.4 pmol) Pes3AT can be
detected using 29 \( \mu g \) (0.7 nmol) of recombinant PPTase (in either
purified or crude form) in the presence of biotin-CoA via solid phase phosphopantetheinylation. It also suggests that
any conformational restriction due to Pes3AT immobilization
does not significantly impede phosphopantetheinylation. More-
over, chemoenzymatic detection is statistically significant \( (P < 0.05) \) over protein labeling in the absence of recombinant
PPTase. Importantly, we also demonstrate that the T domain
derived from Pes3AT can be fused to an unrelated protein and
also be subjected to solid phase phosphopantetheinylation. This
confirms the discrete functionality of the T domain and strongly
suggests that they can be used as a fusion partners for unrelated
proteins to facilitate targeted labeling procedures.

In order to optimise the \( \text{in vitro} \) 4'-phosphopantetheinylation
assay and to further characterize PPTase activity, assays were
performed at different reaction temperatures. Initial \( \text{in vitro} \) 4'-
phosphopantetheinylation assays using the A. fumigatus encoded
recombinant PPTase (2) were carried out at a reaction temper-
ature of 37 \( ^\circ \)C; however, optimal PPTase activity occurs at 25
\( ^\circ \)C with 100% increase in the extent of Pes3AT modification
observed compared to that at 37 \( ^\circ \)C. \( \text{In vitro} \) 4'-phosphopan-
tetheinylation assays employing the use of the B. subtilis PPTase
Sfp, were carried out at room temperature and 30 \( ^\circ \)C,
respectively (6, 13). Furthermore, the \( \text{in vitro} \) phosphopantethe-
inlation assays used to assess the affect of PPTase (AcpS)
specific inhibitors on the ability of the B. subtilis AcpS to post-
translationally modify the ACP carrier protein were also
performed at 25 \( ^\circ \)C (14).

Subsequent to determination of optimal PPTase reaction
temperature, the effect of pH on PPTase activity was also
investigated and revealed that optimal PPTase activity was
observed at pH 8.0 (100%), with a significant decrease in
PPTase activity to 45%, 65%, and 35% for pH 4.0, pH 6.5,
and pH 10.0, respectively. These observations are in accord-
ance with those for the \( \text{in vitro} \) phosphopantetheinylation assays used
for B. subtilis AcpS type PPTase, which were also carried out
at pH 8.0 (14). However, a clear pH optimum at pH 6.0 for the
B. subtilis Sfp type PPTase, with activity reducing to less than
20% of the optimum pH for pH 5.0 and pH 7.0, respectively,
has been reported (3). Thus, it is clear that optimal reaction
conditions should be established for each specific PPTase or
equivalent AcpS/Sfp enzyme.
In summary, we have demonstrated that solid phase in vitro 4'-phosphopantetheinylation, using either biotin- or fluorescent-coenzyme A substrate analogues, of fungal thiolation domains in native or non-native configuration is possible. Moreover, we have conclusively shown that the solid phase assay format presents a new strategy for determination of PPTase activity, which is superior to current approaches and highly likely to be compatible with high-throughput screening systems for PPTase inhibitor identification. We also suggest that solid phase 4'-phosphopantetheinylation may be compatible with the ongoing search for peptide mimotopes, in part via phage display, of T domain functionality (21, 22).

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Supporting Information Available: Experimental procedures and results relating to the synthesis efficiency of biotin-coA. This material is available free of charge via the Internet at http://pubs.acs.org.

LITERATURE CITED


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