Elucidating the Substrate Specificity and Condensation Domain Activity of FkbP, the FK520 Pipecolate-Incorporating Enzyme†

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ABSTRACT: Rapamycin, FK506, and FK520 are potent immunosuppressant natural product macrocycles generated by hybrid polyketide synthase (PKS)/nonribosomal peptide synthetase (NRPS) systems in streptomycetes. An important functional element within these molecules is an L-pipecolate moiety that is incorporated into the completed polyketide chain by the action of RapP/FkbP, a four-domain NRPS that also putatively serves to cyclize the chain after amino acid insertion. Here we report the expression and purification of recombinant FkbP from the FK520 biosynthetic pathway. Using a combination of radioassays and Fourier transform mass spectrometry, we demonstrate that once FkbP has been phosphopantetheinylated in vitro, its peptidyl carrier protein domain can be successfully loaded with L-pipecolic acid and, to a lesser extent, L-proline. The first condensation domain of FkbP is shown to be active through the successful acetylation of aminoacyl-*S*-FkbP using the appropriately loaded terminal acyl carrier protein from the PKS array, FkbA, as the chain donor. Site-directed mutagenesis confirmed that the N-terminal condensation domain catalyzes the transfer reaction. Acetylation of prolyl-*S*-FkbP was more rapid and occurred to a greater extent than that of pipecolyl-*S*-FkbP, a trend which was also observed with alternative acyl chain donors. These observations suggest that the adenylation domain of FkbP serves as the primary selectivity filter for pipecolate incorporation.

The biosynthesis of many clinically important natural products is achieved through the action of two related enzyme families: nonribosomal peptide synthetases (NRPSs)[1] and polyketide synthases (PKSs) (*1*, *2*). These large, multimodular proteins are subdivided into discrete domains, each possessing a distinct catalytic function that contributes to the biosynthetic process. Adenylation (A) and acyl transferase (AT) domains, found within NRPSs and PKSs, respectively, are responsible for activating and capturing amino acid and acyl monomer units. These substrates are covalently loaded onto the phosphopantetheinyl moieties of acyl (ACP) and peptidyl (PCP) carrier protein domains, and then joined together in an assembly line manner by intervening condensation (C) and ketosynthase (KS) domains. Chain release is typically achieved by nucleophilic attack at the terminal thio- or oxoester. This nucleophile can be a heteroatom from within the chain itself, resulting in macrocyclization of the product that is concomitant with its release. Formation of the macrocycle is often critical for biological activity, and is observed in a number of compounds currently used or under investigation as therapeutics, including erythromycin, daptomycin, nystatin, and epothilone (*2*).

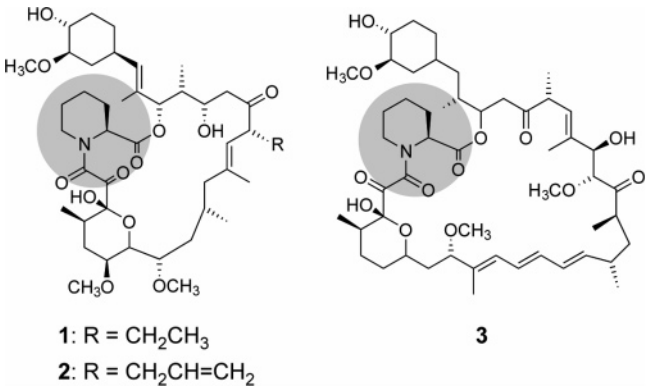

FIGURE 1: FK520 (**1**), FK506 (**2**), and rapamycin (**3**). The pipecolate moieties are shaded.

The macrolide immunosuppressants (Figure 1), including FK520 (**1**), FK506 (**2**), and rapamycin (**3**), represent an important subset of macrocycles generated by tandem arrays of PKS and NRPS biosynthetic machinery (*3*−*5*). Isolated from a collection of *Streptomyces* species (*6*−*8*), these compounds potently suppress the immune system and have been utilized as candidate therapies for the prevention of

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[1] Abbreviations: NRPS, nonribosomal peptide synthetase; PKS, polyketide synthase; A domain, adenylation domain; AT domain, acyltransferase domain; ACP, acyl carrier protein; PCP, peptidyl carrier protein; C domain, condensation domain; KS domain, ketosynthase domain; FKBP, FK506-binding protein; PPIase, peptidyl-prolyl *cis*−*trans*-isomerase; PIE, pipecolate-incorporating enzyme; FTMS, Fourier transform mass spectrometry; CoA, coenzyme A; IPTG, isopropyl β-D-thiogalactopyranoside; EDTA, ethylenediaminetetraacetic acid; TCEP, tris(2-carboxyethyl)phosphine hydrochloride; TE domain, thioesterase domain; PPTase, phosphopantetheinyl transferase.

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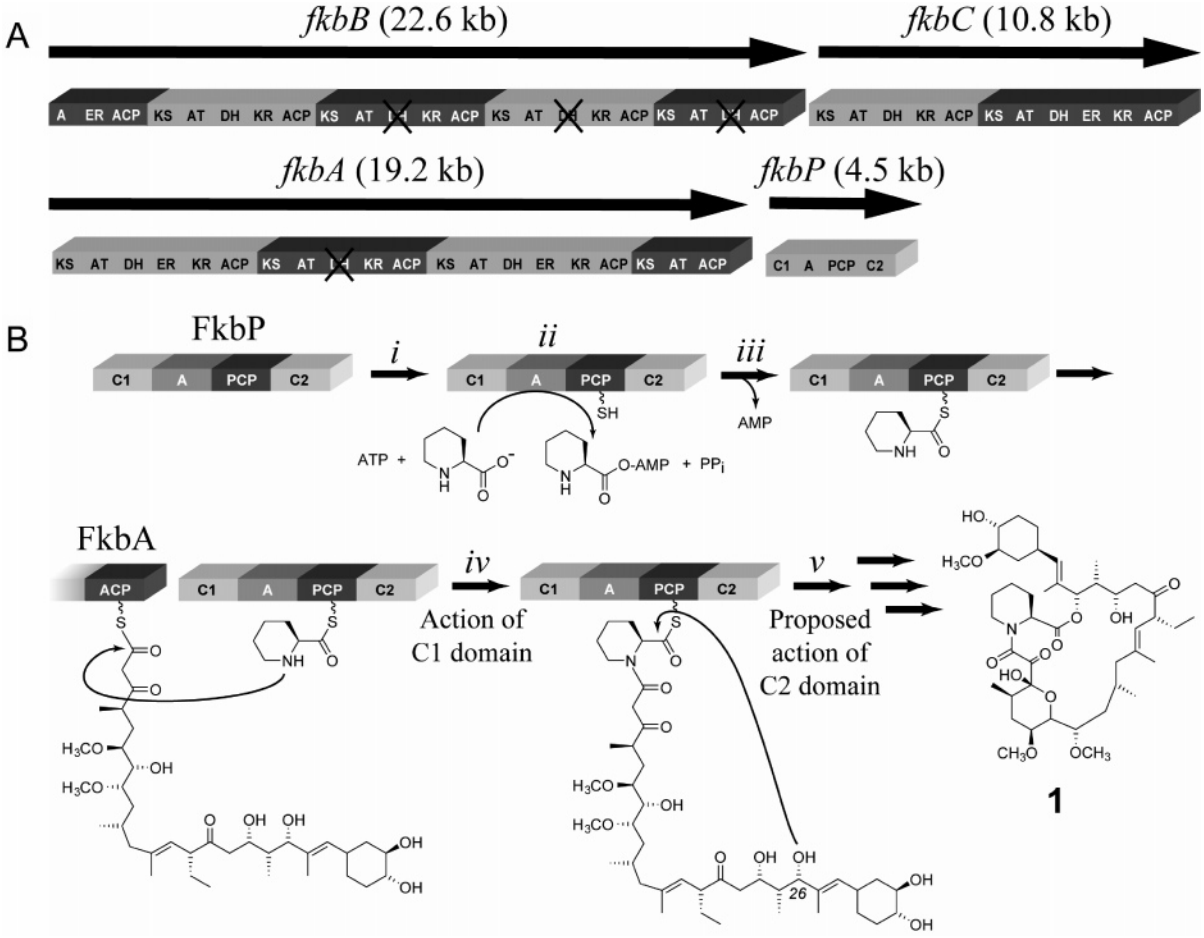

FIGURE 2: (A) Biosynthetic gene cluster for the PK/NRP portion of FK520. (B) Presumed mechanism of FkbP. (*i*) Apo-FkbP is phosphopantetheinylated to its corresponding holo form. (*ii*) The A domain activates L-pipecolate to the corresponding adenylate, which is then (*iii*) tethered to the cofactor thiol on the PCP domain. (*iv*) One of the C domains catalyzes the transfer of the completed polyketide chain to the pipecolate amine. (*v*) The remaining C domain performs the macrocyclization with concomitant release of the chain. Additional tailoring steps yield the final product **1**.

organ transplant rejection (*9*, *10*), the treatment of inflammatory dermatologic disorders (*11*), and the inhibition of coronary artery restenosis after stent placement (*12*). While acting by different mechanisms (*13*), each of these macrolides initially interacts with a class of proteins known as FKBPs (FK506-binding proteins), which belong to the enzyme family of peptidyl-prolyl *cis*−*trans*-isomerases (PPIases) (*14*).

Critical to the interaction between the macrolide and the FKBP is a pipecolate moiety, which extends into the PPIase active site (*15*−*18*). This group is derived from L-pipecolic acid, a nonproteinogenic, six-membered proline analogue. This amino acid is inserted into the growing polyketide chain by the action of a pipecolate-incorporating enzyme (PIE) (*19*). In the FK520 producer strain, *Streptomyces hygroscopicus* ssp. *ascomyceticus*, this PIE is a 161 kDa, four-domain NRPS known as FkbP (Figure 2A). After activation of L-pipecolate to the corresponding adenylate by the A domain and covalent attachment to the adjacent PCP domain, two critical bond-forming reactions are catalyzed by the remaining two C domains (Figure 2B). One of these C domains is presumed to mediate the attack of the pipecolyl secondary amine onto the thioester of the terminal ACP domain within the PKS enzyme FkbA, yielding the acyl−pipecolyl intermediate. The remaining C domain putatively releases the chain from the protein by mediating intramolecular nucleophilic attack of the C-26 hydroxyl onto the pipecolyl thioester.

These two C domains are of significant interest as their respective activities have not yet been assigned and they integrate two key functional components into the final product: the pipecolate moiety and the macrolactone. Additionally, the interaction between FkbP and FkbA involves the convergence of a PKS and an NRPS array in generating a hybrid PK/NRP natural product. Interactions of this type are particularly important as they represent an opportunity to greatly expand the diversity of bioengineered natural products produced by these systems (*20*, *21*). Previously, Nielsen et al. (*22*) reported A domain activity of FkbP using an ATP−PP_i exchange assay, and demonstrated putative pipecolyl thioester formation between radiolabeled L-pipecolic acid and the enzyme purified from harvested mycelia of *S. hygroscopicus*. Additionally, investigation of the recombinant PIE from the rapamycin biosynthetic pathway (RapP) purified from *Streptomyces coelicolor* revealed formation of a pipecolyl−RapP intermediate (*19*).

In this study, we use recombinant FkbP from the FK520 biosynthetic pathway, expressed from *Escherichia coli* and phosphopantetheinylated in vitro to characterize the kinetics and specificity of the adenylation and aminoacylation reactions. Through the use of both radioassays and Fourier transform mass spectrometry (FTMS) (*23*−*25*), we demon-

strate transfer of acyl substrates from the terminal ACP of FkbA to aminoacyl-*S*-FkbP. Our results suggest that the A domain dictates the degree of amino acid selectivity in the incorporation reaction, in contrast to the observed gatekeeper function imposed by the C and cyclization (Cy) domains of other NRPS systems (*26*, *27*). Moreover, through the use of site-directed mutagenesis, we are able to assign the acyl transfer function to the amino-terminal C domain of FkbP.

EXPERIMENTAL PROCEDURES

Materials. Chemically competent TOP10 and BL21(DE3) *E. coli*, the pBAD-TOPO TA expression kit, and NuPAGE Novex 4−12% Bis-Tris gels were obtained from Invitrogen. The pET22b and pET28a plasmids were purchased from Novagen. All restriction endonucleases, T4 DNA ligase, and calf intestinal phosphatase were obtained from New England Biolabs. Synthetic DNA oligonucleotides were purchased from Integrated DNA Technologies and used without further purification. PCRs were run with either Pfu Turbo DNA polymerase or Herculase Enhanced DNA polymerase from Stratagene. Ni−NTA chromatography resin was purchased from Qiagen. [1-^{14}C]Acetyl-CoA and Amplify were obtained from Amersham Biosciences. Sodium [^{32}P]pyrophosphate was purchased from Perkin-Elmer. BAS-IIIs phosphorimager plates were obtained from Fuji. DNA sequencing and quantitative amino acid analysis were performed at the Molecular Biology Core Facilities at the Dana-Farber Cancer Institute (Boston, MA). Sequencing-grade modified trypsin was purchased from Promega. Nanospray emitter nozzles and tips were purchased from Advion Biosciences. All other materials were purchased from Sigma-Aldrich.

Isolation of Genomic DNA. Streptomyces hygroscopicus ssp. *ascomyceticus* was obtained from ATCC (strain 55087), inoculated in seed medium supplemented with 0.5% glycine (*22*), and grown at 27 °C for 9 days. Genomic DNA was isolated using the Bactozol kit (Molecular Research Center).

Cloning of fkbP and fkbA-ACP Genes. The *fkbP* open reading frame was amplified from genomic DNA with Herculase Enhanced DNA polymerase using primers PF1 (5′-GGA ATT CCA TAT GAC AAT ACC CGG TAA AAG GCC CGC GAC-3′) and PR1 (5′-GAT ATT CTC GAG CTC GCT TTC CAC GTA ACC GGT GAC GC-3′). *Nde*I and *Xho*I restriction sites are underlined. The resulting 4.5 kb PCR product was purified by agarose gel electrophoresis and ligated into pBAD-TOPO to generate the pBAD-FkbP construct. This plasmid was digested with *Nde*I and *Xho*I, and the purified ORF insert was ligated into similarly digested pET22b, yielding a construct, pET22b-FkbP, with a C-terminal hexahistidine tag with a LEHHHHHH-COO$^-$ sequence. Missense mutations at nucleotide positions 112 and 238 were repaired by amplifying a fragment of the *fkbP* ORF encompassing the region from the start codon to a unique *Msc*I site at nucleotide position 772 using Pfu Turbo and primers PF1 (vide supra) and PR2 (5′-GGA AGG CCG CCA GCA GGG TCA TGA A-3′). Subsequent digestion with *Nde*I and *Msc*I, followed by ligation into the aforementioned pET22b-FkbP construct, digested with *Nde*I and *Msc*I, yielded the repaired expression plasmid pET22b-FkbP-(rep1).

A DNA fragment encoding the C-terminal ACP domain of FkbA was amplified from genomic DNA using Pfu Turbo and primers AF1 (5′-GGA ATT CCA TAT GGC GGA GTC CGA GCC GGA GGA C-3′) and AR1 (5′-GGA ATT CCT CGA GTC ATC GGT AGT CCT TCC AGG TAT GGG-3′). *Nde*I and *Xho*I sites are underlined. When translated, this fragment extends from residue 6255 to the C-terminus of FkbA. The resulting PCR product was digested with *Nde*I and *Xho*I, purified by agarose gel electrophoresis, and ligated into similarly digested pET28a to generate the *fkbA(ACP)* expression construct with an N-terminal hexahistidine tag with a $^+$H$_3$N-MGSSHHHHHHSSGLVPRGSH sequence.

Introduction of the D162N Mutation into fkbP. Mutagenesis was performed by sequence overlap extension (*28*). In the first round, two separate PCRs were run on the pET22b-FkbP(rep1) plasmid template. The two sets of primers were PF1 and PmutR1 (5′-CGA CCA GCC GTT GCC GGC GAC ATG-3′) and PmutF1 (5′-CAT GTC GCC GGC AAC GGC TGG TCG-3′) and PR2. The mutagenic site is underlined. In the second round, the products of the first round were used as the template for amplification with the outer primers (PF1 and PR2). The product of this reaction was digested with *Nde*I and *Msc*I, purified by agarose gel electrophoresis, and ligated into similarly digested pET22b-FkbP(rep1) to yield mutated expression construct pET22b-FkbP(D162N).

Protein Overexpression and Purification. Expression plasmids were transformed into *E. coli* strain BL21(DE3) and grown in LB medium supplemented with either 100 μg/mL ampicillin for FkbP and FkbP(D162N) or 30 μg/mL kanamycin for FkbA(ACP). Each liter of culture was inoculated with a 10 mL overnight starter culture. Cultures were grown at 25 °C to an OD_{600} of 0.6−0.9, induced with 60 μM IPTG, and then incubated at 16 °C for an additional 16 h. Cells were harvested by centrifugation (20 min at 6000*g*), resuspended in lysis buffer [400 mM NaCl, 25 mM Tris (pH 8.0), and 10% glycerol], and lysed by two passages through a French press at 12000−16000 psi. Ni−NTA resin (1 mL per 3 L culture) and 2 mM imidazole were added to the clarified lysate and allowed to batch bind for 2 h at 4 °C. The resin was washed with 5 column volumes of 2 mM imidazole in lysis buffer. The protein was eluted with a step gradient of increasing imidazole concentrations in lysis buffer (5, 20, 40, 60, 200, and 500 mM). FkbP and FkbA(ACP) each typically eluted in the 40, 60, and 200 mM fractions, which were pooled and dialyzed at 4 °C in two steps: 4 h in 100 mM NaCl, 50 mM Tris (pH 8.0), 1 mM EDTA, and 10% glycerol, followed by 4 h in 100 mM NaCl, 50 mM Tris (pH 8.0), 1 mM TCEP, and 10% glycerol. Concentrated protein solutions were stored at −80 °C.

Protein concentrations were determined by quantitative amino acid analysis. Results from this analysis were correlated with concentrations determined by UV absorption at 280 nm for FkbP or by the Bradford assay for FkbA(ACP).

ATP−PP$_i$ Exchange Assay. Reactions were carried out at room temperature in a total volume of 200 μL containing 50 mM Tris (pH 7.5), 10 mM $MgCl_2$, 3 mM TCEP, 1 mM ATP, 1 mM sodium [^{32}P]pyrophosphate (2 Ci/mol), 230 nM FkbP, and various concentrations of amino acids. Reactions were initiated by the addition of protein. At 5 and 10 min, 50 μL of this reaction was quenched into 500 μL of 1.6% (w/v) activated charcoal, 4.5% (w/v) tetrasodium pyrophosphate, and 3.5% (v/v) perchloric acid. The charcoal was collected by centrifugation, washed twice with 500 μL of 4.5% (w/v) tetrasodium pyrophosphate and 3.5% (v/v)

perchloric acid, then resuspended in 500 μL of wash solution, added to scintillation fluid, and counted. The amount of bound radioactivity was converted to reaction velocity using the specific activity of [^{32}P]pyrophosphate. Velocity was plotted against amino acid concentration and fitted to the Michaelis−Menten equation in Mathematica (Wolfram Research). Each reaction was carried out in duplicate.

Acyl Transfer from FkbA(ACP) to FkbP Assessed by Radioassay. FkbP (2.5 μM) was combined with Sfp (1.4 μM) and coenzyme A (CoA, 150 μM) in the stock reaction buffer [50 mM Tris (pH 7.5), 5 mM $MgCl_2$, and 5 mM TCEP] and incubated at room temperature for 1 h to allow for complete phosphopantetheinylation. Amino acid (5 mM) and ATP (5 mM) were then added, and the reaction mixture was incubated at room temperature for an additional 2 h. In parallel, FkbA(ACP) (11 μM) was combined with Sfp (2.7 μM) and [1-^{14}C]acetyl-CoA (180 μM, 56 Ci/mol) in the stock reaction buffer, and incubated at room temperature for 1 h. To initiate transfer, the FkbA(ACP) reaction mixture was combined with the FkbP reaction mixture at a 2:1 FkbA(ACP):FkbP molar ratio. At specific time points, aliquots of the transfer reaction were removed and quenched in an equal volume of 2× nonreducing SDS−PAGE sample loading buffer. Samples were run on a NuPage Novex 4−12% Bis-Tris gel, Coomassie-stained and destained, soaked in Amplify for 30 min, dried, and exposed to a BAS-IIIs phosphorimager plate for 8−16 h at room temperature.

Covalent Modifications of FkbP Measured by FTMS. Aminoacylation and acyl transfer reactions were set up in an identical manner as described above, with the exception that cold acyl-CoAs were used in place of the ^{14}C-labeled acetyl-CoA. Two hours after initiation of the transfer by the combination of the FkbP and FkbA(ACP) reactions, samples were treated with trypsin in a 1:2.5 (w/w) ratio of protease to enzyme. After proteolysis at 30 °C for 5 min, the digestions were quenched in 30% formic acid and lyophilized. The addition of acid did not precipitate the enzymes. The lyophilized pellets were resuspended in 100 mM NH_4-OAc (pH 4), 6 M urea, 5 mM TCEP, and 10% CH_3CN for 1 h at room temperature prior to injection onto a Jupiter C4 reversed phase column (4.6 mm × 150 mm). The digestions were fractionated in a linear gradient of 30 to 70% CH_3CN in 0.1% TFA over 40 min at 1 mL/min. One minute fractions were collected, lyophilized, and stored at −20 °C. Prior to MS analysis, the lyophilized fractions were resuspended in 20−40 μL of electrospray solvent (49% CH_3CN, 50% H_2O, and 1% formic acid).

Samples were directly infused into a custom-built quadrupole−FTMS hybrid instrument using a Nanomate 100 nanoelectrospray robot (Advion Biosciences, Ithaca, NY). The resulting spray was passed through a resistively heated metal capillary and skimmer before external accumulation for a total of 0.5 s in an octupole (*29*). After accumulation, the ions were shuttled to the ICR cell through a quadrupole that can function either as a simple ion guide or as a selective filter for defined *m*/*z* windows. The values reported in this study are monoisotopic molecular masses (M_r) unless otherwise indicated. External calibration of the spectra was based on bovine ubiquitin (8559.64 Da). Increased mass accuracy was achieved for selected spectra using two to three peaks of known identity as internal calibrants.

The isotopic distributions of the active site peptides were fit using a least-squares algorithm provided by the FTMS data analysis program THRASH (*30*). By comparison of the least-squares fit to a theoretical fit of the distribution, the *m*/*z* values of both the monoisotopic and most abundant isotopic peaks of the peptides were determined. The percent occupancy of any active site peptide modification form (i.e., holo, loaded, or condensed species) was determined by comparing the integrated intensity of the form's three most abundant isotopes (including all adducts such as sodium, potassium, and formylation) to the total abundance of all the differentially modified states of the peptide. The percent occupancies of all charge states were then averaged to provide the total percent occupancy of the peptide form. The accuracies of percent occupancy values between spectra of active sites bearing different intermediates are semiquantitative due to possible differences in the ionization efficiencies of the differentially modified peptide ions, but have been shown to be precise to ∼5% (*23*).

RESULTS

Cloning of FkbP and FkbA(ACP). The open reading frame of *fkbP* was amplified by PCR from *S. hygroscopicus* ssp. *ascomyceticus* genomic DNA in a single 4.5 kb product. However, sequencing of this construct revealed three discrepancies with the published nucleotide sequence (*3*): C112 → T, C238 → T, and A1739 → G. We considered these mutations a likely result of errors introduced by the DNA polymerase used in the amplification reaction. The first two of these result in highly nonconservative amino acid changes, R38W and R80C; they were successfully repaired by amplification of a portion of the ORF spanning these mutations, followed by replacement of this fragment in the expression construct. The mutation at nucleotide position 1739 introduces a Q580R substitution in a region of the A domain that is not part of a consensus motif (*31*). Moreover, the location of this residue is predicted to be solvent-exposed and distant from the enzyme active site on the basis of sequence comparisons to the phenylalanine-activating A domain from gramicidin S synthetase 1, for which a three-dimensional structure is known (*32*). As the A domain of FkbP proved to be highly active with appropriate selectivity (vide infra), this mutation was not repaired.

Detection of FkbP Active Site Peptides Using FTMS. To directly interrogate the PCP domain of FkbP using FTMS, the peptide containing the active site serine must first be identified from a proteolytic mixture. Trypsin digestion followed by reversed phase fractionation resulted in the identification of 59 peptides, providing 64% coverage of the primary sequence of FkbP within 20 ppm. A molecular ion, observed in fraction 20, putatively matched the peptide harboring the active site serine within 15 ppm (1991.01 Da, Figure 3A). The identity of this peptide was confirmed using tandem mass spectrometry (Figure 1 of the Supporting Information). Further investigation of the proteolytic mixture yielded only one other active site peptide harboring a missed cleavage site at Arg^{1014} and Val^{1015}, present at a lower abundance than the fully digested peptide. As this peptide represents a minor proteolytic component, it will not be discussed further in this study. Incubation of FkbP with Sfp and CoA resulted in a 100% shift in the mass of the exhaustive peptide to 2331.24 Da, indicating complete

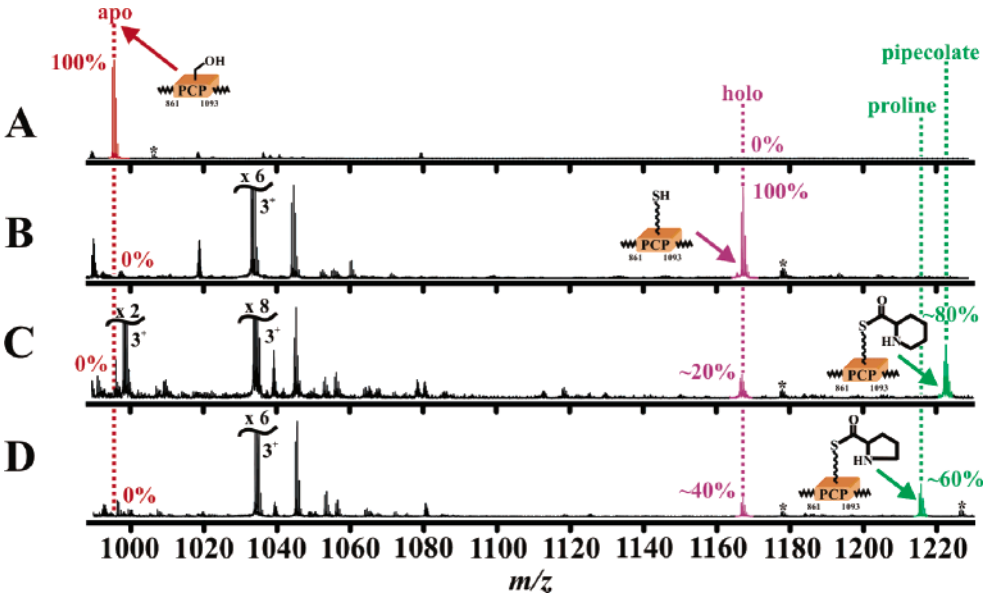

FIGURE 3: Differential modifications of the active site peptide correlating to the PCP domain of FkbP. The asterisks denote adducts, and unrelated peaks have been truncated for spectral clarity (i.e., ×6): (A) apo form (2^+, 1991.01 Da), (B) holo form (2^+, 2331.24 Da), (C) pipecolyl-*S*-FkbP (2^+, 2442.33 Da), and (D) prolyl-*S*-FkbP (2^+, 2428.17 Da).

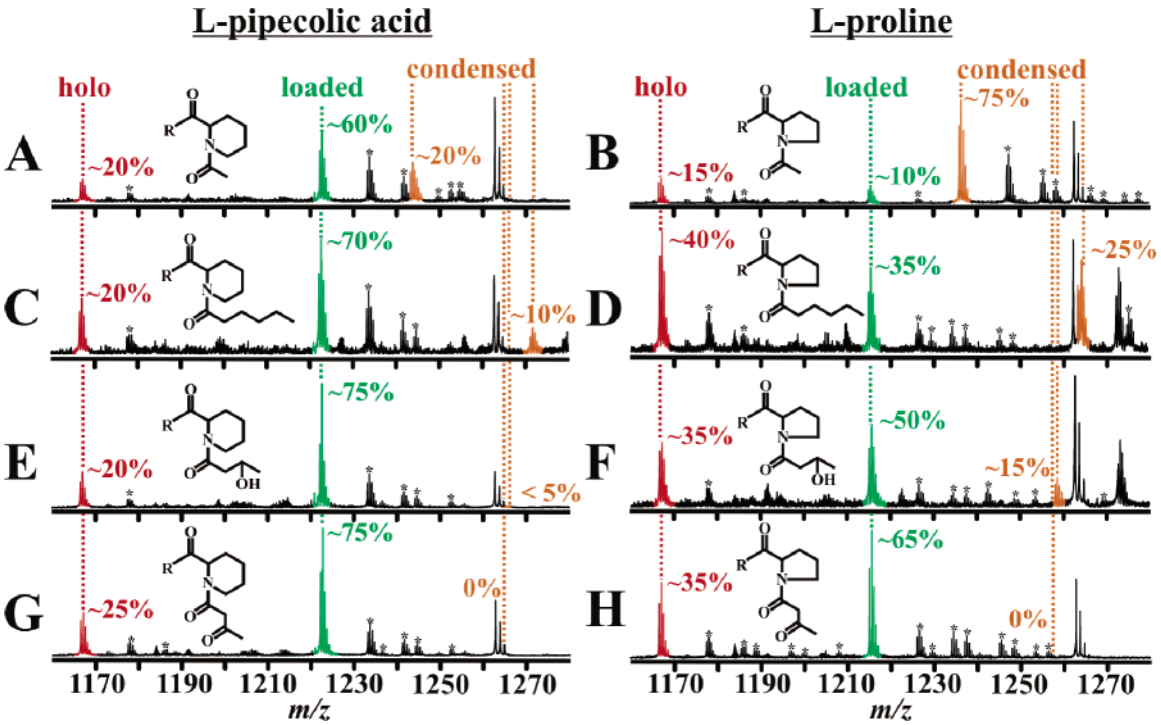

FIGURE 4: Condensation of acyl substrates from FkbA onto pipecolyl-*S*-FkbP and prolyl-*S*-FkbP: (A) acetyl-pipecolyl-*S*-FkbP (2^+, 2484.33 Da), (B) acetyl-prolyl-*S*-FkbP (2^+, 2470.18 Da), (C) *n*-hexanoyl-pipecolyl-*S*-FkbP (2^+, 2540.42 Da), (D) *n*-hexanoyl-prolyl-*S*-FkbP (2^+, 2526.40 Da), (E) *β*-hydroxybutyryl-pipecolyl-*S*-FkbP (2^+, 2528.42 Da), (F) *β*-hydroxybutyryl-prolyl-*S*-FkbP (2^+, 2514.33 Da), (G) acetoacetyl-pipecolyl-*S*-FkbP (no condensation product), and (H) acetoacetyl-prolyl-*S*-FkbP (no condensation product). The asterisks denote adducts.

phosphopantetheinylation of the active site (Figure 3B). Addition of ATP and L-pipecolic acid further shifted the mass of the peptide by 111.09 Da over that of the holo form to 2442.33 Da, demonstrating successful loading of the active site with the thioester-bound intermediate at ~80% occupancy (Figure 3C).

Measurement of A Domain Activity by ATP−PP$_i$ Exchange. The activity and substrate specificity of the A domain of FkbP were assayed by detection of amino acid-stimulated exchange of the radiolabel from [^{32}P]pyrophosphate to ATP. Michaelis−Menten kinetic parameters for exchange were obtained for each of the following amino acids: L-pipecolic acid, D-pipecolic acid, and L-proline (Table 1). No exchange was detected in the presence of L-alanine. These data clearly indicate that the preferred substrate for the A domain is L-pipecolic acid, with a catalytic efficiency (k_{cat}/K_m) comparable to that observed for other A domains in NRPS systems (*33*, *34*). This process is highly stereoselective, as indicated by the strong preference for the L-isomer over the D-isomer. The reduced activity of the A domain in

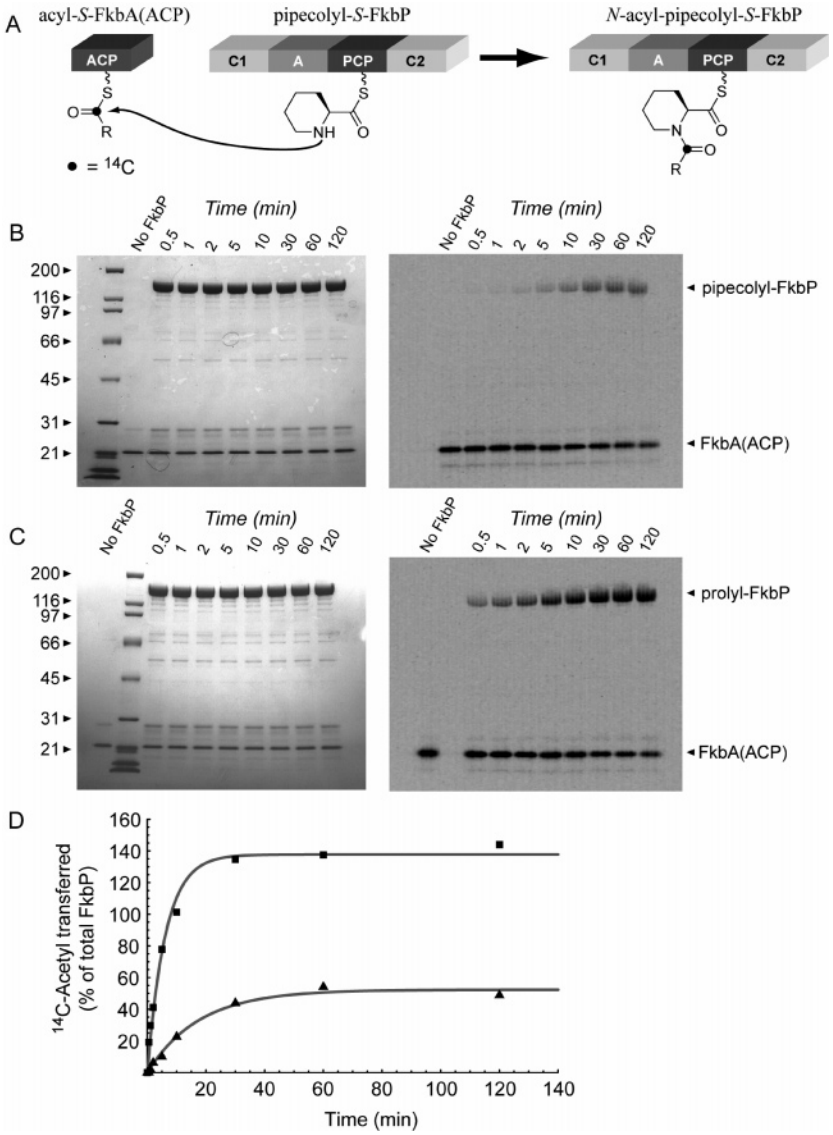

FIGURE 5: (A) Schematic of the radioassay for C1 domain function. (B) SDS−PAGE analysis of the time-dependent transfer of [1-^{14}C]-acetyl from FkbA(ACP) to pipecolyl-*S*-FkbP. The Coomassie-stained gel is on the left, and the autoradiogram is on the right. (C) Transfer experiment using prolyl-*S*-FkbP as the acyl chain acceptor. (D) Quantitation of the autoradiograms presented in panels B (▲) and C (■). The best fit to a first-order reaction rate equation is shown for each. The plateau in the prolyl curve above 100% is considered to be a result of errors in gel quantitation and protein concentration.

Table 1: Michaelis−Menten Kinetic Constants for A Domain Activity of FkbP, Measured by Amino Acid-Stimulated ATP−PP_i Exchange

substrate	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m (mM^{-1} min^{-1})
L-pipecolic acid	1.1 ± 0.1	74 ± 22	67
L-proline	33 ± 2	38 ± 8	1.2
D-pipecolic acid	60 ± 2	47 ± 2	0.78
L-alanine	−	−	−

Table 2: Selection of Noncognate Substrates for PCP Loading by FkbP

substrate	% occupancy[a]
L-pipecolic acid	~80
L-proline	~60
cis-4-hydroxy-L-proline	~20
trans-4-hydroxy-L-proline	~20
L-thioproline	~25
L-alanine	<5
L-azetidinecarboxylic acid	not incorporated
N-methyl-L-proline	not incorporated
D-proline	not incorporated
D-pipecolic acid	not incorporated

[a] Determined by the FTMS assay after a 2 h incubation.

the presence of L-proline (k_{cat}/K_m reduced by 50-fold) when compared with that in the presence of L-pipecolic acid corresponds well to the previously published characterization of FkbP isolated from harvested *Streptomyces* mycelia (*22*).

Specificity of PCP Aminoacylation Measured by FTMS. While activity in the ATP−PP_i assay provides an indication of A domain substrate specificity, it is not sufficient to demonstrate successful aminoacylation of the PCP domain (*22*). To this end, covalent intermediates were detected directly on the active site peptide of FkbP using FTMS. Incubation of holo-FkbP with L-pipecolic acid and L-proline in the presence of ATP revealed a preference for L-pipecolic acid, successfully incorporating 20% more substrate than L-proline within 2 h (panel C vs panel D of Figure 3). As shown in Table 2, several other proline and pipecolate analogues were incorporated into the active site, although

to a lesser extent than the aforementioned substrates. Generally, the enzyme appears to be tolerant toward substitution of the proline ring but is intolerant to D-isomers and methylation of the amine. L-Alanine was observed to aminoacylate at <5% occupancy, and D-pipecolic acid was not incorporated at all, in contrast to the ATP−PP_i exchange assay results.

Acetate Transfer from FkbA(ACP) to Aminoacyl-FkbP. Two complementary methods were utilized to observe the activity of the first condensation function of FkbP: transfer of an acyl chain from the upstream ACP of FkbA to the pipecolyl-*S*-PCP. The first assay entailed visualization of the active site peptide using FTMS for the formation of the *N*-acetyl-pipecolyl-*S*-FkbP and *N*-acetyl-prolyl-*S*-FkbP intermediates. Incubation of acetyl-*S*-FkbA(ACP) with pipecolyl-*S*-FkbP or prolyl-*S*-FkbP resulted in the formation of the condensed intermediates, as depicted in panels A and B of Figure 4. The calculated percent occupancies of condensed intermediates after a 2 h reaction suggest that the formation of the *N*-acetyl-prolyl-*S*-FkbP product is more efficient than the formation of the *N*-acetyl-pipecolyl-*S*-FkbP intermediate, despite the fact that L-pipecolic acid is the native substrate and serves as a more suitable substrate for both adenylation and aminoacylation (vide supra).

The second assay assessed the transfer of a radiolabeled acetyl group from FkbA(ACP) to aminoacyl-*S*-FkbP using protein gel electrophoresis. FkbA(ACP) was loaded with [1-^{14}C]acetyl-CoA using Sfp and then incubated with FkbP that had been phosphopantetheinylated with Sfp and CoA and aminoacylated in the presence of ATP and either L-pipecolic acid or L-proline (Figure 5A). At various time points, the reaction was quenched in nonreducing loading buffer and separated by SDS−PAGE. The transfer of the acetyl group was observed by detection of radioactivity in the higher-molecular mass band. Over the course of 2 h, the signal is readily apparent in the pipecolyl-*S*-FkbP (Figure 5B) and prolyl-*S*-FkbP bands (Figure 5C). Consistent with the results observed by FTMS, acetyl transfer to prolyl-*S*-PCP occurred to a greater extent than transfer to pipecolyl-*S*-PCP (Figure 5D). Moreover, these data indicate that acetyl transfer to prolyl-*S*-FkbP is more rapid than that to pipecolyl-*S*-FkbP by approximately 3-fold ($t_{1/2}^{pro}$ = 4 min vs $t_{1/2}^{pip}$ = 12 min).

Transfer of Alternate Acyl Substrates. To test the substrate tolerance of the condensation reaction, several CoA analogues were examined for their condensation efficiency using FTMS. Of particular interest are acyl chains that mimic the structure of the native upstream acyl intermediate. Incubation of pipecolyl- and prolyl-*S*-FkbP with either *n*-hexanoyl- or *β*-hydroxybutyryl-*S*-FkbA(ACP) yielded the expected condensation products (panel C vs panel D of Figure 4 and panel E vs panel F of Figure 4). As with the acetyl substrate, condensation onto prolyl-*S*-FkbP appeared to proceed with a greater efficiency than with pipecolyl-*S*-FkbP. However, when pipecolyl- and prolyl-*S*-FkbP were incubated with acetoacetyl-*S*-FkbA(ACP), no condensed intermediates were observed in either case (panels G and H of Figure 4).

Effect of N-Terminal C Domain Knockout Mutation on Acyl Transfer. Site-directed mutagenesis was utilized to probe the role of the N-terminal C domain on chain transfer. C domains contain a characteristic HHX_3DG motif that is critical for catalytic activity (*35*). Mutation of the conserved aspartate to asparagine has been shown to severely impair condensation function (*36*). The corresponding mutation in the N-terminal C domain of FkbP, D162N, was introduced into the FkbP expression construct using sequence overlap extension. The corresponding protein product, FkbP(D162N), was expressed and purified in a manner identical to that of its wild-type counterpart. FkbP(D162N) was as soluble as FkbP, and the activity of its A domain, as measured by the ATP−PP_i exchange assay, was comparable to that of wild-type FkbP (data not shown), suggesting that the mutation did not affect protein stability or folding. Assessment of acyl transfer from acyl-*S*-FkbA(ACP) to aminoacyl-*S*-FkbP(D162N) was performed using both the radioassay and the FTMS method as previously described (Figure 6). Formation of the condensed species was not detected by either method, regardless of the amino acid substrate (L-pipecolic acid or L-proline).

DISCUSSION

The immunosuppressant natural products rapamycin, FK506, and FK520 possess macrocyclic lactone scaffolds that provide conformational constraints required for recognition by their binding proteins (the FKBPs) (*15*−*18*) and interaction of the drug−FKBP complexes with downstream target proteins such as calcineurin (*37*) and FRAP (*38*). While the FK506/520 and rapamycin scaffolds possess differing polyketide portions, the common core motif contains the sole amino acid moiety, the six-membered L-pipecolate (shaded portions of Figure 1). This nonproteinogenic amino acid is derived from L-lysine by the presumed action of the cyclodeaminase RapL/FkbL (*39*), also encoded in the FK506/520 and rapamycin biosynthetic gene clusters.

As pipecolate is an amino acid, it is anticipated that a NRPS module would be responsible for its activation and subsequent installation into the natural product. Indeed, the *fkbP* and *rapP* genes (from the FK506/520 and rapamycin biosynthetic gene clusters, respectively) encode the single NRPS modules in these hybrid NRPS/PKS assembly lines. In the case of FK520 biosynthesis, the linear gene order suggests the 11 modules of the PKS, distributed over three subunits, yield the full-length acyclic polyketidyl chain tethered in a thioester linkage to the most downstream ACP domain of FkbA (Figure 2B). It is presumed that the lone NRPS module, FkbP, acts at the end of the assembly line process, forming both the N7−C8 amide bond and the C1−C26 lactone C−O bond (*19*).

Unlike most PKS, NRPS, and NRPS/PKS hybrid assembly lines, FkbP lacks a C-terminal thioesterase (TE) domain (*40*). Inspection of the FkbP sequence reveals the canonical C−A−PCP domain order, typical of NRPSs, followed by a second C domain. This C1−A−PCP−C2 arrangement suggests that FkbP performs two condensation steps. Herein, we report successful reconstitution of FkbP as well as validation of its adenylation and aminoacylation activities. Further, we demonstrate successful abolition of condensation activity through the use of site-directed mutagenesis within the C1 domain. From these data, we propose that the C1 domain performs the N7−C8 acylation of the pipecolyl-*S*-PCP amino group while the C2 domain catalyzes macrolactonization to create the C1−C26 bond, with subsequent release of preFK520.

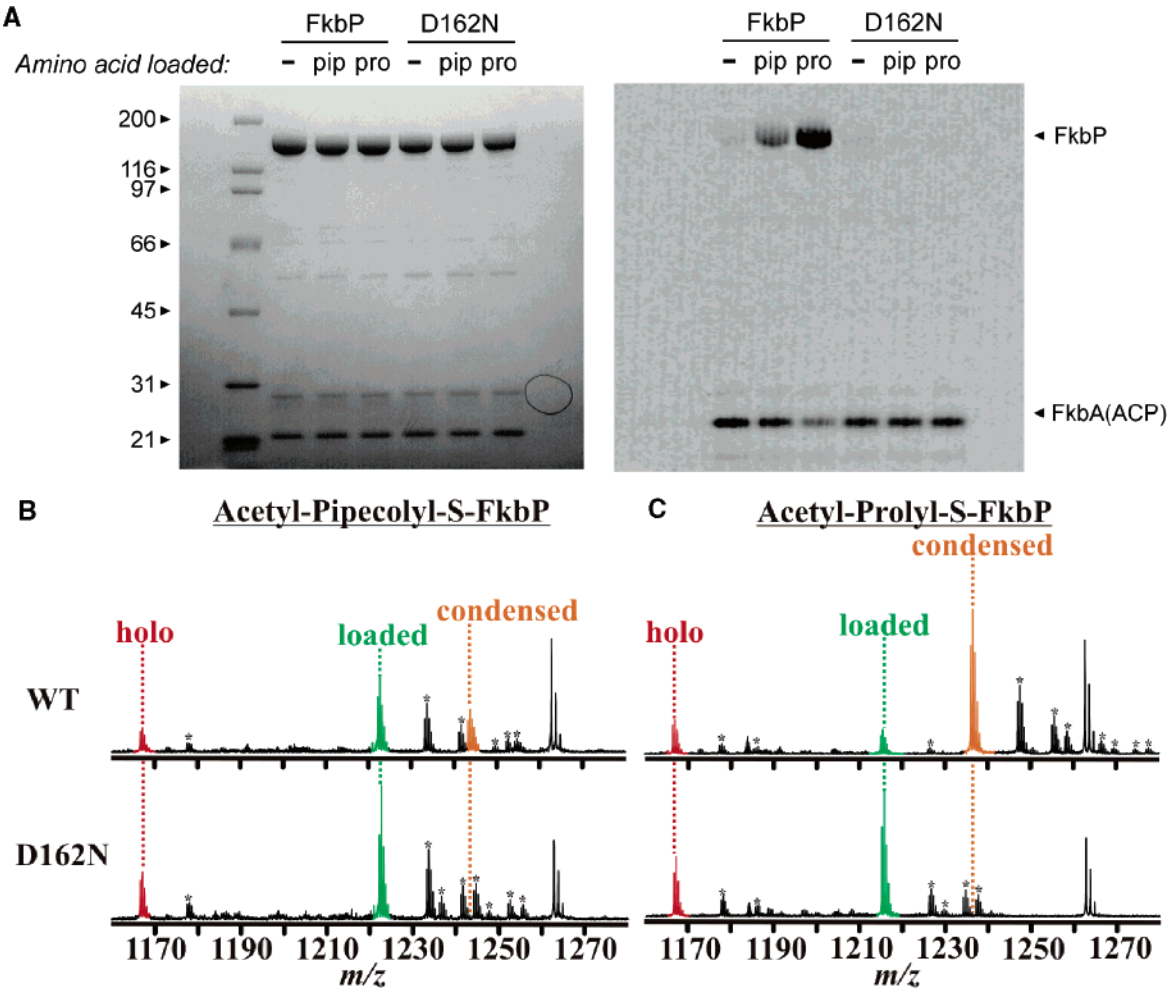

FIGURE 6: Effect of the amino-terminal C domain active site mutation (D162N) on acyl transfer. (A) Radioassay of the transfer reaction. [1-^{14}C]Acetyl-*S*-FkbA(ACP) and either holo-FkbP with no loaded amino acid (−), pipecolyl-*S*-FkbP (pip), or prolyl-*S*-FkbP (pro)—with or without the D162N mutation—were incubated for 60 min before SDS−PAGE analysis. The Coomassie-stained gel is on the left, and the autoradiogram is on the right. (B) Demonstration of activity loss with acetyl-pipecolyl-*S*-FkbP. (C) Demonstration of activity loss with acetyl-prolyl-*S*-FkbP.

After the 160 kDa FkbP had been obtained in soluble form, A domain activity was validated through the use of an ATP−PP_i exchange assay. Incubation of FkbP with either L-pipecolic acid or L-proline demonstrated that the A domain was functional for both substrates. The ability to activate both the cyclic six- and five-membered amino acids was expected, given that a small amount of natural product with the prolyl moiety in place of the pipecolyl group can be isolated from fermentations of the FK506 and rapamycin producer strains (*41*, *42*).

To assay the subsequent transfer of the activated substrates to the terminal thiol of the phosphopantetheinylated PCP domain, the active site serine was directly interrogated using high-performance mass spectrometry. Trypsin digestion of the intact FkbP resulted in the observation of the apo form active site peptide, with no evidence of modification by native *E. coli* phosphopantetheinyl transferases (PPTases) (*43*), consistent with previous observations of RapP expressed and purified from *E. coli* (*19*). Since the PCP domain was not primed in vivo, the *Bacillus subtilis* PPTase, Sfp, was used to generate the holo form of FkbP in vitro (*44*). Subsequent incubation with ATP and either L-pipecolic acid or L-proline permitted the first direct evidence of a thioester-bound intermediate at the PCP domain within the NRPS modules of the macrolide immunosuppressant biosynthetic gene products.

To evaluate the proposed C1 domain function, an assay for acylation of the aminoacyl-*S*-FkbP intermediate was required. To this end, we cloned and expressed in soluble form the isolated C-terminal ACP domain of FkbA as an 18 kDa protein fragment. As the full-length linear acyl chain precursor is not readily available, we chose an acetyl group as the simple surrogate acyl donor. Using either unlabeled acetyl-CoA or [1-^{14}C]acetyl-CoA and Sfp, the apo-ACP was converted to the necessary acetyl-*S*-pantetheinyl-ACP intermediate (Figure 5; Figure 2 of the Supporting Information). Initiation of acetyl transfer was achieved by mixing the two acyl-*S*-pantetheinyl proteins, and the anticipated *N*-acetyl-pipecolyl/prolyl-*S*-FkbP products were detected using either SDS−PAGE or FTMS.

Surprisingly, when prolyl-*S*-FkbP was used as the acetyl acceptor, the formation of the *N*-acetyl condensed product was more robust than when using pipecolyl-*S*-FkbP. Elucidation of the time dependence of transfer revealed that formation of the *N*-acetylated aminoacyl species was ~3-fold faster and occurred to a greater extent by approximately 2.5-fold with prolyl-*S*-FkbP than with pipecolyl-*S*-FkbP. Given that the pipecolate-incorporated natural product is the

predominant form isolated, these data may implicate the A domain as the primary gatekeeper for discrimination between L-pipecolic acid and L-proline. However, in the case of the natural acyl chain donor, the kinetics of transfer by the C1 domain may or may not favor the six-membered ring over the five-membered ring analogue. Nevertheless, these results indicate that proline can maintain the biosynthetic flux through the PKS/NRPS pathway, consistent with the published report that inhibition of pipecolate biosynthesis and addition of excess proline lead to the preferential production of prolylrapamycin in the rapamycin producer strain (*42*).

Since the simple two-carbon acetyl donor was a suitable substrate for transfer, we further evaluated *n*-hexanoyl-*S*-ACP, β-hydroxybutyryl-*S*-ACP, and 3-ketobutyryl-*S*-ACP as acyl donors. As shown in Figure 4, *n*-hexanoyl and β-hydroxybutyryl condensation onto both prolyl and pipecolyl moieties were detected, but no transfer was observed with the 3-ketobutyryl chain. One might have anticipated the 3-ketobutyryl donor to be the best mimic of the distal portion of the native full-length acyl donor; however, the effects of enolization of this substrate on transfer are unclear. Moreover, formation of the pyranose ring may precede transfer to FkbP, resulting in the conversion of the β-keto group at this position to the hemiacetal. Studies on the synthesis and assay of longer acyl chain mimics of the linear biosynthetic acyl chain are currently underway. It will be of particular interest to determine if greater yields of acyl transfer to prolyl-*S*-FkbP than to pipecolyl-*S*-FkbP for these substrates continue the trend seen with the short acyl chain transfers.

In addition, we used site-directed mutagenesis to validate the proposal that acyl transfer to pipecolyl-*S*-FkbP or prolyl-*S*-FkbP is mediated by C1, anticipated by the function of such C domains in canonical C−A−PCP NRPS modules. The Asp^{162} residue in the conserved HHX_3DG motif within the N-terminal C domain was mutated to Asn, a mutation previously shown to severely impair the function of the C domain in other NRPS systems. Using both the [^{14}C]acetyl transfer assay and FTMS analysis, no transfer was observed for any acyl substrates to either pipecolyl-*S*-FkbP(D162N) or prolyl-*S*-FkbP(D162N). These data confirm the role of the C1 domain of FkbP as the catalyst for peptide bond formation between the completed polyketide chain and the pipecolyl secondary amine.

This study establishes that three of the four domains in the recombinant C1−A−PCP−C2 NRPS module FkbP are functional and sets the stage for assay of C2. The expectation is that C2 will catalyze intramolecular lactone formation from an appropriately situated hydroxyl substituent in the acyl chain. In vivo, the FK520 PKS/NRPS hybrid assembly line synthesizes a 23-membered macrolactone. We will now proceed to evaluate placement of OH groups at shorter distances in the acyl chains transferred by C1 to evaluate the ability of C2 to catalyze cyclorelease. Such future studies should provide information about what variant size macrolactones can be generated by the lone NRPS module of the FK506/520 and rapamycin assembly lines. In turn, this would validate the idea that the single nonproteinogenic amino acid pipecolate is embedded in the otherwise polyketide scaffold by the double C domains of the NRPS module and might suggest such four-domain catalysts could be useful as insertases of amino acids into other polyketide backbones.

SUPPORTING INFORMATION AVAILABLE

Dissociation of the apo peptide harboring the active site serine (Figure 1) and time course of FkbA (ACP) loading with acetyl-CoA (Figure 2). This material is available free of charge via the Internet at http://pubs.acs.org.

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