FK506 Maturation Involves a Cytochrome P450 Protein-Catalyzed Four-Electron C-9 Oxidation in Parallel with a C-31 O-Methylation

Dandan Chen,^{a,b} Lihan Zhang,^c Bo Pang,^a Jing Chen,^{a,c} Zhinan Xu,^b Ikuro Abe,^c Wen Liu^a

State Key Laboratory of Bioorganic and Natural Products Chemistry, Shanghai Institute of Organic Chemistry, Chinese Academy of Sciences, Shanghai, China^a; Department of Chemical and Biological Engineering, Zhejiang University, Hangzhou, China^b; Graduate School of Pharmaceutical Sciences, The University of Tokyo, Tokyo, Japan^c

FK506, structurally similar to FK520 and rapamycin, is an α -keto amide bonding-containing, macrolide natural product that exhibits potent immunosuppressive activity and moderate antifungal activity. FK506 biosynthesis requires a hybrid polyketide synthase (PKS)-nonribosomal peptide synthetase (NRPS) system to construct the skeleton of the macrolide. The mechanism for tailoring this macrolide to furnish FK506 remains poorly understood. In this study, we report a maturation paradigm common for FK506, FK520, and rapamycin, by characterizing two conserved regiospecific, post-PKS-NRPS modifications in an FK506-producing *Streptomyces tsukubaensis* strain. A cytochrome P450 protein, FkbD, catalyzes a less common, four-electron oxidation at C-9 to give a rarely found α -keto amide group, whereas a methyltransferase, FkbM, is responsible for O-methylation at C-31 to afford a methoxy group. Both FkbD and FkbM are highly tolerant in their substrate choice; therefore, the order of FkbD- and FkbM-catalyzed reactions is interchangeable in the FK506 biosynthetic pathway. Inactivation of *fkbD* produced a new intermediate, 9-deoxo-FK506, which displayed antifungal activity lower than that of FK506. Taking previously reported bioassay results regarding the intermediates 9-deoxo-31-O-demethyl-FK506 and 31-O-demethyl-FK506 into account, it is clear that the modifications catalyzed by FkbD and FkbM are of importance to reach the full biological activity of FK506 by forming a key structure motif that is necessary for interaction of the molecule with the receptor and, subsequently, the downstream intracellular responses.

FK506, originally isolated from *Streptomyces tsukubaensis* in 1984 (1), is an immunosuppressive drug that is widely used in clinics to reduce a patient's immune activity against allogeneic organ transplant. It interacts with a receptor, known as FK506 binding protein 12 (FKBP12), to form an FK506-FKBP12 complex. The complex then targets calcineurin and inhibits its calmodulin-dependent Ser/Thr phosphatase activity (2), leading to the arrest of T cell proliferation at the G₀-G₁ stage in mammal (3). This calcineurin-based action also has an adverse effect on the growth of various fungi, therefore endowing FK506 with moderate antifungal activity (4, 5). FK506 is an α -keto amide bond-containing, macrolide natural product structurally similar to FK520 (coproduct of FK506 in *S. tsukubaensis*) and rapamycin but distinct in the ring size and appended functionalities (Fig. 1A). The entire biosynthetic gene cluster of FK506 was recently cloned and characterized (6, 7), allowing for comparative analysis with those of FK520 (8) and rapamycin (9) to account for their generality and specificity in biosynthesis.

FK506 biosynthesis involves a hybrid polyketide synthase (PKS)-nonribosomal peptide synthetase (NRPS) system to construct the 23-membered macrolide (10). FkbABC, which are type I PKSs, assemble the polyketide backbone by using 4,5-dihydroxycyclohex-1-enecarboxylic acid, a chorismate derivative, as a starter unit (11). The NRPS FkbP is responsible for extending the resulting linear polyketide intermediate by an unnatural amino acid residue, L-pipecolate, and yields 9-deoxo-31-O-demethyl-FK506 (compound 1) via intramolecular lactonization for further tailoring (see Fig. 5A) (12, 13). During the macrolide-forming process, the PKSs for FK506 incorporate two unusual extender units, methoxymalonyl (MOM) (also common for FK520) and allylmalonyl (AM) (unique for FK506), to afford two methoxy groups at C-13 and C-15 and one allyl side chain at C-21, respec-

tively (7, 14–16). Genetic enhancement of the *in vivo* supply of MOM and AM improved FK506 production (17), therefore providing a strategy potentially helpful for increasing the yields of other secondary metabolites that contain pathway-specific building blocks.

The biosynthetic gene clusters of FK506, FK520, and rapamycin share two closely linked and highly conserved tailoring genes (Fig. 1B). Their encoded products include a cytochrome P450 oxidoreductase-like protein, FkbD, and an S-adenosylmethionine (SAM)-dependent methyltransferase, FkbM. *In vivo* investigations into *fkbM* and *fkbD* were previously carried out by Shafiee and coworkers (18–20) and showed that they both are essential for FK506 maturation: (i) inactivation of *fkbM* accumulated 31-O-demethyl-FK506 (compound 2), (ii) compound 2 can be converted into FK506 by the cell extract of a recombinant *Streptomyces lividans* strain in which *fkbM* was expressed in *trans*, and (iii) inactivation of *fkbD*, which was speculated to have a polar effect on the expression of the downstream gene *fkbM*, produced 9-deoxo-31-O-demethyl-FK506 (compound 1). Thus, FkbM is known to be a regiospecific O-methyltransferase responsible for methylating the *meta*-hydroxyl group of 4,5-dihydroxycyclohex-1-enecarboxylic acid at C-31 in FK506. On the other hand, the func-

Received 9 January 2013 Accepted 15 February 2013

Published ahead of print 22 February 2013

Address correspondence to Wen Liu, wliu@mail.sioc.ac.cn.

Supplemental material for this article may be found at <http://dx.doi.org/10.1128/JB.00033-13>.

Copyright © 2013, American Society for Microbiology. All Rights Reserved.

doi:10.1128/JB.00033-13

FIG 1 Structures and partial gene clusters of FK506, FK520, and rapamycin. (A) Structures of FK506 and its analogues FK520 and rapamycin. The macrocyclic scaffolds derived from the same precursors are shown in dotted frames. (B) Organization and sequence comparison of the homologous genes encoding post-PKS-NRPS modifications for FK520, FK506, and rapamycin. ID, sequence identity.

tion of FkbD remains poorly understood. The unsolved questions include (i) whether or not FkbD alone can catalyze a four-electron oxidation at C-9 to afford a 9-oxo group of FK506, given the fact that two-electron oxidation (e.g., hydroxylation and dehydrogenation) is most common for P450 enzyme-catalyzed reactions, and (ii) how and when the FkbD-catalyzed reaction takes place, the characterization of which may provide insights into the formation of a pharmaceutically important structure motif that features a rarely found, α -keto amide group common for FK506, FK520, and rapamycin (Fig. 1A). In this study, we answer these questions on the basis of *in vivo* and *in vitro* characterization of FkbD and FkbM in FK506 biosynthesis. Identification of a new intermediate, 9-deoxo-FK506 (compound 3), also facilitates evaluation of the effect of the key structure motif variation on the biological activity of FK506 by combining data from previously reported bioassay studies.

MATERIALS AND METHODS

General materials and methods. Bacterial strains and plasmids used in this study are summarized in Table S1 in the supplemental material. Primer sequences are listed in Table S2 in the supplemental material. Chemicals and biochemical materials were purchased from standard commercial sources unless otherwise stated.

DNA isolation and manipulation in *Escherichia coli* and *S. tsukubaensis* were performed according to standard protocols (21). Primer synthesis and DNA sequencing were performed at Shanghai GeneCore Biotechnology Inc.

High-performance liquid chromatography (HPLC) analysis was carried out with an Agilent 1200 HPLC system (Agilent, USA) and a Diamonsil C₁₈ 5- μ m reverse-phase column (250 by 4.6 mm, catalog no. 99603; Dikma, USA). The analytical method was developed with a flow rate of 1 ml/min, column temperature at 60°C, and UV detection at 215 nm. For fermentation product analysis, an isocratic program (40% solvent A–60% solvent B) lasting 35 min was applied; for *in vitro* enzymatic reaction analysis, the column was eluted over an 80-min gradient as follows: constant 95% solvent A–5% solvent B at 0 to 10 min, a linear gradient to 60%

solvent A–40% solvent B at 10 to 20 min, a linear gradient to 45% solvent A–55% solvent B at 20 to 22 min, and constant 45% solvent A–55% solvent B at 22 to 80 min (solvent A, H₂O; solvent B, CH₃CN). HPLC-mass spectrometry (HPLC-MS) analysis was performed on a Shimadzu 2010 liquid chromatograph-mass spectrometer (Shimadzu, Japan) under the same conditions. High-resolution electrospray ionization MS (HR-ESI-MS) analysis was carried out on an IonSpec 4.7 T Fourier transform mass spectrometer (IonSpec, USA). Nuclear magnetic resonance (NMR) data were recorded on a Bruker DRX-400 NMR spectrometer (Bruker, Germany). Measurement of UV-visible (UV-vis) absorbance was performed on a Jasco V-530 UV-vis spectrophotometer (Jasco, Japan). Kinetic parameters of FkbD and FkbM were analyzed by using Origin 8.0 software (OriginLab, USA).

Inactivation of *fkbD*, *fkbM*, and *fkbDM*. The genomic DNA of *S. tsukubaensis* ZJU01 served as the template for PCR amplification.

To inactivate *fkbD*, a 2.04-kb fragment obtained by using primers pDLf and pDLr and a 2.04-kb fragment obtained by using primers pDRf and pDRr were initially cloned into the pMD19-T vector, giving pFL2011 and pFL2012, respectively. After sequencing to confirm fidelity, the 2.04-kb EcoRI/XbaI and 2.04-kb XbaI/HindIII fragments were recovered and then coligated into the EcoRI/HindIII site of pKC1139, yielding the recombinant plasmid pFL201, in which a 501-bp in-frame coding region (corresponding to amino acids [aa] 112 to 278 of the deduced product FkbD) of *fkbD* was deleted.

To inactivate *fkbM*, a 2.11-kb fragment obtained by using primers pMLf and pMLr and a 2.03-kb fragment obtained by using primers pMRf and pMRr were initially cloned into the pMD19-T vector, giving pFL2021 and pFL2022, respectively. After sequencing to confirm fidelity, a recombinant plasmid, pFL202, was constructed according to the same strategy, in which a 354-bp in-frame coding region (corresponding to aa 74 to 191 of the deduced product FkbM) of *fkbM* was deleted.

To inactivate *fkbD* and *fkbM* simultaneously, the 2.04-kb EcoRI/XbaI insert of pFL2011 and the 2.03-kb XbaI/HindIII insert of pFL2022 were coligated into the EcoRI/HindIII site of pKC1139 to generate pFL203, in which a 1,403-bp coding region (corresponding to aa 112 to 388 of the deduced product FkbD and aa 1 to 191 of the deduced product FkbM) of the contiguous genes *fkbD* and *fkbM* was deleted.

The constructs pFL201, pFL202, and pFL203 were introduced individually into *S. tsukubaensis* ZJU01 by intergeneric conjugation from *E. coli* ET12567/pUZ8002 (21). According to a procedure described previously (22), the exconjugants were subjected to a double-crossover recombination event, leading to the generation of the recombinant strains FL201 ($\Delta fkbD$), FL202 ($\Delta fkbM$), and FL203 ($\Delta fkbDM$), respectively. The genotype for each mutation was validated by PCR amplification and sequencing (see Fig. S1 in the supplemental material).

Structural elucidation of 9-deoxo-FK506. Strain cultivation and compound extraction were carried out according to procedures described previously (17). HR-ESI-MS $[M + Na]^+$ $m/z = 812.4917 \pm 0.002$, (calculated for $C_{44}H_{71}NO_{11}Na$, 812.4919); for 1H and ^{13}C NMR (400 and 100 MHz, respectively; $CDCl_3$), see Fig. S2 and S3 in the supplemental material; UV-vis λ_{max} , 192 nm.

Expression and purification of FkbD and FkbM. To express *fkbD*, a 1.17-kb *fkbD*-containing fragment was obtained by PCR amplification with primers pDf and pDr and then cloned into the pMD19-T vector to yield pFL2041. After sequencing to confirm fidelity, the 1.17-kb insert was recovered from pFL2041 and ligated into the NdeI/EcoRI site of pET28a, yielding pFL204 for producing FkbD in an N-terminally 6×His-tagged form. Similarly, a 0.78-kb *fkbM*-containing fragment was amplified with primers pMf and pMr and then subcloned to give pFL2051. After sequencing to confirm fidelity, a recombinant construct, pFL205, was constructed for producing FkbM in an N-terminally 6×His-tagged form.

The constructs pFL204 and pFL205 were introduced individually into *E. coli* BL21(DE3) by transformation, yielding two recombinant strains, FL204 and FL205, respectively. Each strain was cultured in Luria-Bertani (LB) medium supplemented with 50 μ g/ml kanamycin at 37°C and 250 rpm until the optical density at 600 nm (OD_{600}) reached 0.6 to 0.8. To produce FkbM, 0.1 mM isopropyl- β -D-thiogalactopyranoside (IPTG) was added to the culture before further incubation at 22°C at 250 rpm for 20 h. To produce FkbD, 0.1 mM IPTG, 0.1 mM $Fe(NH_4)_2(SO_4)_2$, and 0.5 mM 5-aminolevulinic acid (ALA) were added to the culture before further incubation at 18°C at 250 rpm for 28 h. Purification of the proteins and determination of their concentrations were carried out according to methods described previously (23) (see Fig. S4 in the supplemental material).

Spectrophotometric characterization of FkbD. To characterize FkbD as a cytochrome P450 protein, a CO binding difference spectrum was operated as previously described (24, 25). A spectrum of FkbD within the range of 350 to 600 nm was recorded after 30 s of bubbling (see Fig. 3A).

The substrate binding assay of FkbD was performed as described previously (24). The spectra of the FkbD-containing solution, recorded from 350 to 600 nm, were obtained following the addition of substrates dissolved in dimethyl sulfoxide (DMSO) (see Fig. S5 in the supplemental material). With a spectrum of a blank control serving as the baseline, difference spectra were obtained by successive addition of different concentrations of substrate ($[S]$) (compound 3 or 1) (see Fig. 3B). The spectrophotometric experiments were carried out in triplicate. The values of delta absorbance units (ΔAU) were determined by subtracting the trough absorbance at 419 nm from the peak absorbance at 392 nm, which were then fitted by nonlinear least-square regression to the equation $\Delta AU = \Delta AU_{max}[S]/([S] + K_D)$ to determine K_D (equilibrium dissociation constant) values (see Fig. S6 in the supplemental material).

Characterization of FkbD-catalyzed C-9 oxidation in vitro. A 50- μ l reaction mixture in 10% (vol/vol) DMSO containing 50 μ M substrate (compound 3 or 1), 1 mM NADPH, 0.1 U/ml ferredoxin-NADP⁺ reductase, 50 μ g/ml ferredoxin, 10 mM glucose-6-phosphate (glucose-6-P), 1 U/ml glucose-6-P dehydrogenase, and 2 μ M FkbD in 50 mM Tris-HCl buffer (pH 7.5) was incubated at 30°C for 2 h. For a negative control, FkbD was inactivated by heating at 100°C for 15 min. Reactions were initiated by addition of FkbD and terminated by addition of an equal volume of methanol to precipitate the enzyme. After removal of the precipitate by centrif-

ugation, the supernatant was subjected to HPLC and HPLC-MS analyses (see Fig. 3D).

Determination of pH dependence of the FkbD-catalyzed reaction.

The 50- μ l reaction mixtures containing 1 μ M FkbD and 50 μ M compound 1 (other components were identical to those described above) in 50 mM piperazine-*N,N'*-bis(2-ethanesulfonic acid) (PIPES) (pH 6.0 to 7.0) or Tris-HCl (pH 7.5 to 8.5) buffer were incubated at 30°C for 2 h and were then subjected to HPLC analysis to determine the pH dependence of FkbD-catalyzed oxidation (see Fig. S7 in the supplemental material). All the tests were repeated in duplicate.

Determination of apparent kinetic parameters of the FkbD-catalyzed reaction. Determination of apparent K_m' and k_{cat}' values of FkbD-catalyzed hydroxylation was performed by a continuous assay measuring NADPH consumption at 340 nm. Referring to an established method (24), 1 μ M FkbD, 0.1 U/ml ferredoxin-NADP⁺ reductase, 50 μ g/ml ferredoxin, and various concentrations of substrate (compound 3 or 1) in PIPES buffer (pH 7.0) were mixed in a microquartz cuvette (Starna, United Kingdom) mounted in a constant-temperature cell holder equilibrated to 30°C. Spectral acquisition at 340 nm was initiated by addition of 250 μ M NADPH, and the data were acquired for a 300-s run time. All the tests were performed on three parallel samples. A standard curve of NADPH concentrations was made from 50 μ M to 250 μ M. The absorbance of a blank control without substrate addition served as the baseline. The average initial substrate hydroxylation velocity was fitted by nonlinear least-square regression to a substrate inhibition equation, $v = V_{max}/(1 + K_m'/[S] + [S]/K_i)$, to determine the kinetic parameters (see Fig. S8 in the supplemental material).

Characterization of FkbM-catalyzed C-31 O-methylation in vitro. A 50- μ l reaction mixture in 10% (vol/vol) DMSO containing 50 μ M substrate (compound 1 or 2), 0.5 mM SAM, 4 mM $MgSO_4$, and 2 μ M FkbM in 50 mM Tris-HCl buffer (pH 7.5) was assayed. For a negative control, FkbM was inactivated by heating at 100°C for 15 min. Reactions were initiated by addition of FkbM, and the mixtures were then incubated at 30°C for 2 h. To terminate the reactions, an equal volume of methanol was added to the mixtures to precipitate the enzyme. After removal of the precipitate by centrifugation, the supernatant was subjected to HPLC and HPLC-MS analyses (see Fig. 4B).

Determination of pH dependence of the FkbM-catalyzed reaction.

The 50- μ l reaction mixtures containing 1 μ M FkbM and 50 μ M compound 1 (other components were identical to those described above) in 50 mM PIPES (pH 6.0 to 7.0) or Tris-HCl (pH 7.5 to 8.5) buffer were incubated at 30°C for 1 h and were then subjected to HPLC analysis to determine the pH dependence of FkbM-catalyzed, SAM-dependent O-methylation (see Fig. S9 in the supplemental material). All the tests were repeated in duplicate.

Determination of kinetic parameters of the FkbM-catalyzed reaction. A time course for the FkbM-catalyzed reaction was monitored to determine the initial rate conditions. The 50- μ l reaction mixtures containing 1 μ M FkbM and 50 μ M substrate (compound 1 or 2) (other components were identical to those described above) in 50 mM Tris-HCl buffer (pH 7.5) were incubated at 30°C for 1, 3, 5, 7, 10, 15, 20, 25, 30, 45, and 60 min (see Fig. S10 in the supplemental material). Consumption of compounds 1 and 2 was fitted to a linear equation to obtain the initial velocity.

To determine the kinetic parameters, the 50- μ l reaction mixtures contained 1 μ M FkbM and various concentrations of substrate (compound 1 or 2) (other components were identical to those described above) in 50 mM Tris-HCl buffer (pH 7.5). Each assay mixture was incubated at 30°C for 5 min, and each assay was repeated in triplicate. The average initial velocities were fitted by nonlinear least-square regression to the Michaelis-Menten equation, $v = V_{max}/(1 + K_m'/[S])$, to determine the kinetic parameters (see Fig. S11 in the supplemental material).

Bioassay of 9-deoxo-FK506. For evaluating antifungal activity, each compound was diluted in DMSO and tested as described previously (1). The test strains of *Aspergillus terreus*, *Fusarium solani*, and *Candida albi-*

FIG 2 HPLC analysis of fermentation products from *S. tsukubaensis* strains. The products were characterized to be FK506 in ZJU01 (wild type), 9-deoxy-FK506 (compound 3) in FL201 (*fkbD* deletion strain), 31-*O*-demethyl-FK506 (compound 2) in FL202 (*fkbM* deletion strain), and 9-deoxy-31-*O*-demethyl-FK506 (compound 1) in FL203 (*fkbDM* deletion strain). mAU, milli-absorbance units.

cans were precultivated in a modified Sabouraud agar culture (4% glucose, 1% polypeptone, 1% agar) at 30°C for 48 h. An agar piece of approximately 1 by 1 cm² was inoculated onto another Sabouraud agar culture containing gradient concentrations of the test compounds. After incubation for 4 days at 30°C, zones of inhibition were checked to determine MIC values (see Fig. S12 in the supplemental material).

RESULTS

Production of 9-deoxy-FK506 (compound 3) by in-frame deletion of *fkbD*. Considering that *fkbD* and *fkbM* reside in a single operon for coupled transcription (20), we chose an in-frame deletion of *fkbD* in an FK506-producing, wild-type *S. tsukubaensis* strain to avoid any potential polar effects on the expression of the downstream gene *fkbM*. This led to the generation of mutant strain FL201 for product examination (Fig. 2). FL201 lost the ability to produce FK506 but apparently accumulated a new product, compound 3, with a UV absorption pattern similar to that of FK506. Compound 3 was then isolated and purified from the culture broth of FL201 for structural elucidation. HR-ESI-MS analysis showed its positive $[M + Na]^+$ ion at m/z 812.4917, establishing the molecular formula as $C_{44}H_{71}NO_{11}$ (m/z calculated 812.4919). Furthermore, the ¹H and ¹³C NMR spectra of compound 3 supported its identity to 9-deoxy-FK506 (see Fig. S2 and S3 in the supplemental material). This compound is distinct from FK506 by possessing a saturated C-9 atom, indicating that FkbD participates in regiospecific oxidation at C-9.

In vitro assay for the C-9 oxidation activity of FkbD. We expressed *fkbD* in *E. coli* BL21(DE3) by supplementing Fe²⁺ and ALA to facilitate ferriprotophyrin complex formation for constructing the prosthetic group of cytochrome (25). Recombinant FkbD

in an N-terminally 6×His-tagged form was purified to homogeneity for characterization of the protein. Under reductive conditions, the addition of CO to the FkbD-containing solution resulted in a shift of the maximum absorption from 419 nm to 446 nm, characteristic of all cytochrome P450 enzymes (24). Compound 3 was then incubated with FkbD for *in vitro* assays of enzymatic activity, and the reaction mixtures were subjected to time course analysis by HPLC-MS and HR-ESI-MS. In the presence of FkbD, compound 3 was consumed accordingly, with the generation of a new product, product 4, and after a longer incubation, the end product FK506 appeared (Fig. 3D). The pH dependence of the reaction was also investigated, showing the highest activity of FkbD at pH 7.0 (see Fig. S7 in the supplemental material). This finding suggested that compound 4 can be an intermediate during the FkbD-catalyzed conversion of compound 3 into FK506. Consequently, compound 4 was fractionated and incubated with FkbD, which indeed showed that this compound can be converted into FK506 under the same reaction conditions (Fig. 3D). HR-ESI-MS analysis eventually established the molecular formula of compound 4 as $C_{44}H_{71}NO_{12}$ ($[M + Na]^+$ m/z calculated 828.4869, found 828.4883), supporting its identity to 9-hydroxyl-FK506, which differs from FK506 by the introduction of a hydroxyl group at C-9. Therefore, FkbD acts on C-9 as a regiospecific cytochrome P450 oxidase capable of catalyzing a successive two-step, four-electron oxidation to convert substrate 3 into FK506 via the intermediate compound 4.

Production of 31-*O*-demethyl-FK506 (compound 2) by deletion of *fkbM* and production of 9-deoxy-31-*O*-demethyl-FK506 (compound 1) by deletion of *fkbDM*. For the timing of FkbD-catalyzed oxidation and FkbM-catalyzed *O*-methylation in the FK506 maturation process, we prepared their potential substrates, compounds 1 and 2, from the mutant *S. tsukubaensis* strains. Consistent with previous findings (20), inactivation of *fkbM* led to the generation of strain FL202, which was capable of producing compound 2, whereas inactivation of both *fkbD* and *fkbM* resulted in the generation of strain FL203, which produced compound 1 (Fig. 2). Further HR-ESI-MS analyses confirmed their identities as 31-*O*-demethyl-FK506 ($[M + Na]^+$ m/z calculated 812.4556 for $C_{43}H_{67}NO_{12}$, found 812.4545) for compound 2 and 9-deoxy-31-*O*-demethyl-FK506 ($[M + Na]^+$ calculated m/z , 798.4763 for $C_{43}H_{69}NO_{11}$; found 798.4766) for compound 1, respectively.

Kinetic analysis of FkbD-catalyzed oxidation. We first evaluated whether or not FkbD had the ability to convert compound 1 into compound 2 under the same reaction conditions as those described above. In the presence of FkbD, compound 1 was consumed accordingly with the generation of a new product, compound 5, and after longer incubation, the end product, compound 2, indeed appeared (Fig. 3D). Compound 5 was fractionated and then subjected to HR-ESI-MS analysis, which established the molecular formula as $C_{43}H_{69}NO_{12}$ ($[M + Na]^+$ m/z calculated 814.4712, found 814.4739). Thus, compound 5 could be a hydroxylated product, 9-hydroxyl-31-*O*-demethyl-FK506, generated by one-step, two-electron oxidation at C-9. These findings indicated that FkbD can tolerate compound 1 as the substrate, to afford the 9-oxo group of compound 2 via the intermediate compound 5.

To determine the substrate specificity, we subsequently analyzed the apparent kinetic parameters of FkbD in the presence of compounds 3 and 1, respectively, by examining the consumption of NADPH, a cosubstrate in FkbD-catalyzed oxidation, as an elec-

FIG 3 FkbD activity assays *in vitro*. (A) UV-vis absorbance of FkbD under reducing conditions with or without CO. A Soret peak at 446 nm appeared after binding of CO to 10 μ M FkbD. Abs, absorbance. (B) Substrate binding spectra for 9-deoxo-FK506 (compound 3) and 9-deoxo-31-*O*-demethyl-FK506 (compound 1) bound to FkbD. Various concentrations of compound 3 (i) or compound 1 (ii) (2, 5, 10, 15, 20, 30, 40, 50, 75, and 100 μ M) were mixed with 10 μ M FkbD in 50 mM PIPES buffer (pH 7.0). The mixtures were maintained at 30°C for 20 min to achieve steady-state binding. Difference spectra were obtained with a UV-vis absorption curve of a blank control serving as the baseline. (C) FkbD-catalyzed regiospecific oxidation of compound 3 (or compound 1) to afford FK506 (or compound 2), likely via the intermediate compound 4 (or compound 5). (D) *In vitro* conversion of compound 3 to FK506 and compound 4 in the absence (i) or presence (ii) of active FkbD, with authentic compound 3 (iii) and FK506 (iv) as the standards; of compound 1 to compounds 2 and 5 in the absence (v) or presence (vi) of active FkbD, with authentic compounds 1 (vii) and 2 (viii) as the standards; of compound 4 to FK506 in the absence (ix) or presence (x) of active FkbD, with compound 4 (xi) from the semipreparation as the standard; and compound 5 to compound 2 in the absence (xii) or presence (xiii) of active FkbD, with compound 5 (xiv) from the semipreparation as the standard. Compound 1, 9-deoxo-31-*O*-demethyl-FK506; compound 2, 31-*O*-demethyl-FK506; compound 3, 9-deoxo-FK506; compound 4, 9-hydroxyl-FK506; compound 5, 9-hydroxyl-31-*O*-demethyl-FK506.

TABLE 1 Apparent kinetic parameters of FkbD-catalyzed oxidation

Substrate	Mean K'_m (μM) $\pm$ SD	Mean k'_{cat} (min^{-1}) $\pm$ SD	Mean K'_i (μM) $\pm$ SD	Mean k'_{cat}/K'_m ($\text{mM}^{-1} \text{min}^{-1}$) $\pm$ SD
Compound 3	5.90 $\pm$ 0.40	1.04 $\pm$ 0.03	107.22 $\pm$ 8.39	175.46 $\pm$ 12.69
Compound 1	11.78 $\pm$ 3.84	1.14 $\pm$ 0.19	85.87 $\pm$ 36.45	97.13 $\pm$ 35.57

tron donor. This examination was performed by monitoring the first-step reaction for C-9 hydroxylation, which proceeds much faster than the second-step reaction for 9-oxo generation, and by fitting of the observed data into a substrate inhibition equation, $v = V_{\max}/(1 + K'_m/[S] + [S]/K'_i)$. With the ferredoxin and ferredoxin-NADP⁺ reductase from spinach as a heterologous electron transfer partner system, the conversion of compound 3 to compound 4 gave kinetic values comparable to those for the transformation of compounds 1 to 5 (Table 1; see also Fig. S8 in the supplemental material). Furthermore, we measured the substrate binding efficiency of FkbD according to a characteristic manner of cytochrome P450 proteins, to which substrate binding results in a typical blue shift from 419 nm to 392 nm. This allowed the determination of K_D values of $4.31 \pm 1.34 \mu\text{M}$ for compound 3 and $8.20 \pm 0.91 \mu\text{M}$ for compound 1 (see Fig. S6 in the supplemental material). Thus, FkbD has no apparent preference for compounds 3 and 1, both of which can be oxidized by FkbD with a similar efficiency.

In vitro assay of FkbM-catalyzed O-methylation and its kinetic analysis. We expressed FkbM in *E. coli* and purified it in an N-terminally 6 $\times$ His-tagged form. Substrate 1 or 2 was then incubated with FkbM for *in vitro* assays of methyltransferase activity. In the presence of SAM, FkbM efficiently catalyzed 31-O-methylation by completely converting compound 1 to compound 3 and converting compound 2 to FK506 (Fig. 4B), validating that this enzyme is a SAM-dependent, regiospecific O-methyltransferase. The pH dependence of the reaction was also investigated, showing the highest activity of FkbM at pH 7.5 (see Fig. S9 in the supplemental material). All subsequent bioactivity assays were performed at this pH to obtain the steady-state kinetic parameters.

The time course of FkbM-catalyzed O-methylation showed an increase in the amount of the product (compound 3 or FK506) in parallel with a decrease in the amount of the substrate (compound 1 or 2). The reaction was linear with respect to time for approximately 10 min (see Fig. S10 in the supplemental material). The kinetic parameters were measured by providing saturated SAM (see Fig. S11 in the supplemental material), showing that the conversion of compound 1 into compound 3 was comparable to the biotransformation of compound 2 into FK506 in efficiency (Table 2). According to these results, FkbM also has no apparent preference in substrate specificity and catalyzes the 31-O-methylation of compounds 1 and 2 with a similar efficiency.

Bioassay of new intermediate compound 3. The new intermediate compound 3, 9-deoxy-FK506, was subjected to a bioassay against various fungi along with the parent compound FK506 as the control. Both compound 3 and FK506 had no activity against an FK506-resistant strain of *Candida albicans*. For FK506-sensitive strains of *Aspergillus terreus* and *Fusarium solani*, the activity of compound 3 dramatically decreased, showing a MIC of 1.00 $\mu\text{g/ml}$ against *A. terreus*, which is 25-fold higher than that of FK506 (0.04 $\mu\text{g/ml}$), and a MIC of 10 $\mu\text{g/ml}$ against *F. solani*, which is 5-fold higher than that of FK506 (2.0 $\mu\text{g/ml}$) (see Fig. S12

FIG 4 FkbM activity assays *in vitro*. (A) FkbM-catalyzed regiospecific O-methylation of compound 1 (or compound 2) to afford compound 3 (or FK506). (B) *In vitro* conversion of compound 1 to compound 3 in the absence (i) or presence (ii) of active FkbM, with authentic compounds 1 (iii) and 3 (iv) as the standards, and of compound 2 to FK506 in the absence (v) or presence (vi) of active FkbM, with authentic compound 2 (vii) and FK506 (viii) as the standards.

in the supplemental material). This finding indicated that the saturation of the C-9 group of FK506 has a negative effect on anti-fungal activity.

DISCUSSION

Natural products with an origin of polyketide, peptide, or the polyketide-peptide hybrid have long been appreciated for their

TABLE 2 Kinetic parameters of FkbM-catalyzed O-methylation

Substrate	Mean K_m (μM) $\pm$ SD	Mean k_{cat} (min^{-1}) $\pm$ SD	Mean k_{cat}/K_m ($\text{mM}^{-1} \text{min}^{-1}$) $\pm$ SD
Compound 1	8.09 $\pm$ 0.41	1.60 $\pm$ 0.02	197.27 $\pm$ 10.16
Compound 2	5.16 $\pm$ 0.31	2.07 $\pm$ 0.03	402.18 $\pm$ 24.29

FIG 5 FkbD-catalyzed four-electron oxidation in FK506 biosynthesis. (A) Hybrid PKS-NRPS system-programmed formation of the macrolide skeleton, which can undergo two parallel postmodifications to furnish the mature product FK506. KS, ketosynthase; AT, acyltransferase; ACP, acyl carrier protein; A, adenylation; C, condensation; PCP, peptidyl carrier protein. (B) Proposed mechanism for the FkbD-catalyzed reaction. The first two-electron oxidation results in the formation of a hydroxyl group at C-9, while the second two-electron oxidation eventually affords the 9-oxo group, which could proceed either by direct dehydrogenation or by dehydration via a diol intermediate.

remarkable pharmaceutical importance (26). To reach full biological activity, biosynthesis often requires variable post-PKS, post-NRPS, or post-PKS-NRPS modifications to further functionalize the main skeleton (27, 28), which may improve the binding affinity of the molecule for the target(s) in cells. This rule is applicable to FK506. On the basis of characterization of the cytochrome P450 oxidase FkbD and the *O*-methyltransferase FkbM, we now establish that FK506 maturation involves the activities of these two proteins to decorate the polyketide-peptide hybrid macrolide, by affording the 9-oxo and 31-*O*-methyl groups, respectively. Miller and coworkers showed previously that the 9-deoxo-31-*O*-demethyl (compound 1) and 31-*O*-demethyl (compound 2) FK506 derivatives are much less potent in biological activity than the parent compound FK506 (19). Together with the bioassay in this study regarding 9-deoxo-FK506 (compound 3), it can be con-

cluded that lack of any functional group, 9-oxo, 31-*O*-methyl, or both, would result in a decrease in biological activity. Previous crystallography studies revealed that these two groups are of importance for the binding affinity of FK506 for FKBP12 (29, 30); thus, the less functionalized FK506 structure may likely weaken FK506-FKBP12 complex formation and consequently affect the downstream intracellular responses.

Interestingly, FkbD-catalyzed C-9 oxidation appears to be independent of FkbM-catalyzed 31-*O*-methylation according to the comparisons of relevant kinetic parameters and substrate binding constants, suggesting that these two regiospecific modifications are competing in the FK506 maturation pathway. The intermediate compound 1 can go through the interchangeable reaction in order to reach the mature product FK506, either via the 31-*O*-demethyl derivative compound 2 or via the 9-deoxo derivative

compound 3 as the intermediate (Fig. 5A). Both FkbD and FkbM are highly tolerable in their substrate choice, consistent with the fact that FK520, the coproduct in *S. tsukubaensis* distinct from FK506 in the substitution at C-21 of an ethyl instead of an allyl (17), utilizes the same biosynthetic pathway to furnish the 9-oxo and 31-O-methyl groups. Rapamycin maturation also shares these two modifications (31); however, whether or not they proceed in an interchangeable way remains to be determined.

Single cytochrome P450 protein-catalyzed four-electron oxidation is less common. All known examples, to our knowledge, have recently been found in the biosynthesis of terpenoids, including pentalen-13-al (32), solavetivone (33), and albaflavenone (34). Characterization of FkbD-mediated 9-oxo formation in FK506 biosynthesis now expands this reaction paradigm to the field of polyketide-peptide hybrid natural products. These characterized conversions share an alcohol as the intermediate (often as the major product *in vitro*), indicating that the process can be separated into two steps, as proposed previously by Zhao and coworkers (34). For FkbD, introduction of a hydroxyl takes place for the first two-electron oxidation at C-9, and the resulting alcohol intermediate may be released from the enzyme *in vitro* (given the relatively rapid generation of this intermediate as the major product in the reaction process). Followed by rebinding to the active site of FkbD, the alcohol intermediate can then undergo the second two-electron oxidation to eventually afford the rare α -keto amide group. Mechanistically, the second oxidation step could proceed either by direct dehydrogenation or by dehydration via a diol intermediate (Fig. 5B) (35). It should be noted that we have not detected any alcohol intermediate in the FK506-producing *S. tsukubaensis* strain, suggesting that the turnover rate of the oxidation of the alcohol intermediate *in vivo* is much higher than that *in vitro*.

In conclusion, we have uncovered the maturation process of FK506 by characterizing the regiospecific post-PKS-NRPS modifications in *S. tsukubaensis*, which is also common for the naturally occurring analogues FK520 and rapamycin. The reactions include a cytochrome P450 protein-catalyzed two-step, four-electron oxidation and an O-methylation that can take place in an interchangeable order. Taking previous investigations regarding the structure-bioactivity relationship into account, it is clear that these two modifications are of importance for furnishing the characteristic key structure motif to fully exhibit the biological activity of FK506.

ACKNOWLEDGMENTS

This work was supported in part by grants from the NNSF (20832009, 21176214, and 91213303), the 973 Program (2010CB833200 and 2012CB721100), and the CAS (KJCX2-YW-H201) of China.

REFERENCES

- Kino T, Hatanaka H, Hashimoto M, Nishiyama M, Goto T, Okuhara M, Kohsaka M, Aoki H, Imanaka H. 1987. FK-506, a novel immunosuppressant isolated from a *Streptomyces*. 1. Fermentation, isolation, and physicochemical and biological characteristics. *J. Antibiot.* 40:1249–1255.
- Liu J, Farmer JD, Lane WS, Friedman J, Weissman I, Schreiber SL. 1991. Calcineurin is a common target of cyclophilin-cyclosporine-A and FKBP-FK506 complexes. *Cell* 66:807–815.
- Tocci MJ, Matkovich DA, Collier KA, Kwok P, Dumont F, Lin S, Degudicibus S, Siekierka JJ, Chin J, Hutchinson NI. 1989. The immunosuppressant FK506 selectively inhibits expression of early T-cell activation genes. *J. Immunol.* 143:718–726.
- Foor F, Parent SA, Morin N, Dahl AM, Ramadan N, Chrebet G, Bostian KA, Nielsen JB. 1992. Calcineurin mediates inhibition by FK506 and cyclosporine of recovery from alpha-factor arrest in yeast. *Nature* 360:682–684.
- Kunz J, Hall MN. 1993. Cyclosporine-A, FK506 and rapamycin—more than just immunosuppression. *Trends Biochem. Sci.* 18:334–338.
- Motamedi H, Shafiee A. 1998. The biosynthetic gene cluster for the macrolactone ring of the immunosuppressant FK506. *Eur. J. Biochem.* 256:528–534.
- Mo S, Kim DH, Lee JH, Park JW, Basnet DB, Ban YH, Yoo YJ, Chen SW, Park SR, Choi EA, Kim E, Jin YY, Lee SK, Park JY, Liu YA, Lee MO, Lee KS, Kim SJ, Kim D, Park BC, Lee SG, Kwon HJ, Suh JW, Moore BS, Lim SK, Yoon YJ. 2011. Biosynthesis of the allylmalonyl-CoA extender unit for the FK506 polyketide synthase proceeds through a dedicated polyketide synthase and facilitates the mutasynthesis of analogues. *J. Am. Chem. Soc.* 133:976–985.
- Wu K, Chung L, Revill WP, Katz L, Reeves CD. 2000. The FK520 gene cluster of *Streptomyces hygroscopicus* var. *ascomyceticus* (ATCC 14891) contains genes for biosynthesis of unusual polyketide extender units. *Gene* 251:81–90.
- Schwecke T, Aparicio JF, Molnar I, Konig A, Khaw LE, Haydock SF, Oliynyk M, Caffrey P, Cortes J, Lester JB, Bohm GA, Staunton J, Leadlay PF. 1995. The biosynthetic gene-cluster for the polyketide immunosuppressant rapamycin. *Proc. Natl. Acad. Sci. U. S. A.* 92:7839–7843.
- Motamedi H, Cai SJ, Shafiee A, Elliston KO. 1997. Structural organization of a multifunctional polyketide synthase involved in the biosynthesis of the macrolide immunosuppressant FK506. *Eur. J. Biochem.* 244:74–80.
- Andexer JN, Kendrew SG, Nur-e-Alam M, Lazos O, Foster TA, Zimmermann AS, Warneck TD, Suthar D, Coates NJ, Koehn FE, Skotnicki JS, Carter GT, Gregory MA, Martin CJ, Moss SJ, Leadlay PF, Wilkinson B. 2011. Biosynthesis of the immunosuppressants FK506, FK520, and rapamycin involves a previously undescribed family of enzymes acting on chorismate. *Proc. Natl. Acad. Sci. U. S. A.* 108:4776–4781.
- Gatto GJ, Boyne MT, Kelleher NL, Walsh CT. 2006. Biosynthesis of pipecolic acid by RapL, a lysine cyclodeaminase encoded in the rapamycin gene cluster. *J. Am. Chem. Soc.* 128:3838–3847.
- Gatto GJ, McLoughlin SM, Kelleher NL, Walsh CT. 2005. Elucidating the substrate specificity and condensation domain activity of FkbP, the FK520 pipecolate-incorporating enzyme. *Biochemistry* 44:5993–6002.
- Carroll BJ, Moss SJ, Bai LQ, Kato Y, Toelzer S, Yu TW, Floss HG. 2002. Identification of a set of genes involved in the formation of the substrate for the incorporation of the unusual “glycolate” chain extension unit in ansamitocin biosynthesis. *J. Am. Chem. Soc.* 124:4176–4177.
- Kato Y, Bai LQ, Xue Q, Revill WP, Yu TW, Floss HG. 2002. Functional expression of genes involved in the biosynthesis of the novel polyketide chain extension unit, methoxymalonyl-acyl carrier protein, and engineered biosynthesis of 2-desmethyl-2-methoxy-6-deoxyerythronolide B. *J. Am. Chem. Soc.* 124:5268–5269.
- Goranovic D, Kosec G, Mrak P, Fujs S, Horvat J, Kuscer E, Kopitar G, Petkovic H. 2010. Origin of the allyl group in FK506 biosynthesis. *J. Biol. Chem.* 285:14292–14300.
- Chen DD, Zhang Q, Zhang QL, Cen PL, Xu ZN, Liu W. 2012. Improvement of FK506 production in *Streptomyces tsukubaensis* by genetic enhancement of the supply of unusual polyketide extender units via utilization of two distinct site-specific recombination systems. *Appl. Environ. Microbiol.* 78:5093–5103.
- Shafiee A, Motamedi H, Chen T. 1994. Enzymology of FK-506 biosynthesis—purification and characterization of 31-O-desmethylFK-506 O-methyltransferase from *Streptomyces* sp. MA6858. *Eur. J. Biochem.* 225:755–764.
- Shafiee A, Motamedi H, Dumont FJ, Arison BH, Miller RR. 1997. Chemical and biological characterization of two FK506 analogs produced by targeted gene disruption in *Streptomyces* sp. MA6548. *J. Antibiot.* 50:418–423.
- Motamedi H, Shafiee A, Cai SJ, Streicher SL, Arison BH, Miller RR. 1996. Characterization of methyltransferase and hydroxylase genes involved in the biosynthesis of the immunosuppressants FK506 and FK520. *J. Bacteriol.* 178:5243–5248.
- Kieser T, Bibb MJ, Buttner MJ, Chater KF, Hopwood DA. 2000. Practical *Streptomyces* genetics. John Innes Foundation, Norwich, United Kingdom.
- Liao RJ, Liu W. 2011. Thiostrepton maturation involving a deesterifica-

- tion-amidation way to process the C-terminally methylated peptide backbone. *J. Am. Chem. Soc.* 133:2852–2855.
23. Zhang Q, Chen DD, Lin J, Liao RJ, Tong W, Xu ZN, Liu W. 2011. Characterization of NocL involved in thiopeptide nocathiacin I biosynthesis: a [4Fe-4S] cluster and the catalysis of a radical S-adenosylmethionine enzyme. *J. Biol. Chem.* 286:21287–21294.
 24. Lambalot RH, Cane DE, Aparicio JJ, Katz L. 1995. Overproduction and characterization of the erythromycin C-12 hydroxylase, EryK. *Biochemistry* 34:1858–1866.
 25. Ding W, Deng W, Tang MC, Zhang Q, Tang GL, Bi YR, Liu W. 2010. Biosynthesis of 3-methoxy-5-methyl naphthoic acid and its incorporation into the antitumor antibiotic azinomycin B. *Mol. Biosyst.* 6:1071–1081.
 26. Walsh CT. 2004. Polyketide and nonribosomal peptide antibiotics: modularity and versatility. *Science* 303:1805–1810.
 27. Fischbach MA, Walsh CT. 2006. Assembly-line enzymology for polyketide and nonribosomal peptide antibiotics: logic, machinery, and mechanisms. *Chem. Rev.* 106:3468–3496.
 28. Rix U, Fischer C, Remsing LL, Rohr J. 2002. Modification of post-PKS tailoring steps through combinatorial biosynthesis. *Nat. Prod. Rep.* 19: 542–580.
 29. Van Duyne GD, Standaert RF, Karplus PA, Schreiber SL, Clardy J. 1991. Atomic-structure of FKBP-FK506, an immunophilin-immunosuppressant complex. *Science* 252:839–842.
 30. Becker JW, Rotonda J, McKeever BM, Chan HK, Marcy AI, Wiederrecht G, Hermes JD, Springer JP. 1993. FK-506-binding protein—3-dimensional structure of the complex with the antagonist L-685,818. *J. Biol. Chem.* 268:11335–11339.
 31. Gregory MA, Hong H, Lill RE, Gaisser S, Petkovic H, Low L, Sheehan LS, Carletti I, Ready SJ, Ward MJ, Kaja AL, Weston AJ, Challis IR, Leadlay PF, Martin CJ, Wilkinson B, Sheridan RM. 2006. Rapamycin biosynthesis: elucidation of gene product function. *Org. Biomol. Chem.* 4:3565–3568.
 32. Quaderer R, Omura S, Ikeda H, Cane DE. 2006. Pentalenolactone biosynthesis. Molecular cloning and assignment of biochemical function to PtII, a cytochrome p450 of *Streptomyces avermitilis*. *J. Am. Chem. Soc.* 128:13036–13037.
 33. Takahashi S, Yeo YS, Zhao YX, O'Maille PE, Greenhagen BT, Noel JP, Coates RM, Chappell J. 2007. Functional characterization of premnaspirodien oxygenase, a cytochrome P450 catalyzing regio- and stereospecific hydroxylations of diverse sesquiterpene substrates. *J. Biol. Chem.* 282:31744–31754.
 34. Zhao B, Lin X, Lei L, Lamb DC, Kelly SL, Waterman MR, Cane DE. 2008. Biosynthesis of the sesquiterpene antibiotic albaflavone in *Streptomyces coelicolor* A3(2). *J. Biol. Chem.* 283:8183–8189.
 35. Wood AW, Swinney DC, Thomas PE, Ryan DE, Hall PF, Levin W, Garland WA. 1988. Mechanism of androstenedione formation from testosterone and epitestosterone catalyzed by purified cytochrome-P-450b. *J. Biol. Chem.* 263:17322–17332.