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Purification and Characterization of the Acyl Carrier Protein of the *Streptomyces glaucescens* Tetracenomycin C Polyketide Synthase

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The acyl carrier protein (ACP) of the tetracenomycin C polyketide synthase, encoded by the *tcmM* gene, has been expressed in both *Streptomyces glaucescens* and *Escherichia coli* and purified to homogeneity. Expression of the *tcmM* gene in *E. coli* results mainly in the TcmM apo-ACP, whereas expression in *S. glaucescens* yields solely the holo-ACP. The purified holo-TcmM is active in a malonyl coenzyme A:ACP transacylase assay and is labeled by radioactive β-alanine, confirming that it carries a 4′-phosphopantetheine prosthetic group.

Polyketide synthases (PKSs) are key enzymes for the biosynthesis of polyketides, one of the largest groups of secondary metabolites, and have been classified into two categories. Type I PKSs are large multifunctional proteins, such as 6-methylsalicylic acid synthase from *Penicillium patulum* (1) and the recently reported *eryA* PKS for erythromycin biosynthesis in *Saccharopolyspora erythraea* (4, 6); type II PKSs, in contrast, are multiprotein complexes consisting of several individual enzymes. Molecular genetic analyses of several streptomycetes have led to the characterization of the genes encoding a number of different type II PKSs (11). These studies have revealed a highly conserved gene organization and a high degree of amino acid sequence similarity. Knowledge of PKS enzymology has languished, however, because of the apparent intractability of in vitro assay and purification of the constituent enzymes.

We are studying the tetracenomycin (TCM) C PKS of *Streptomyces glaucescens* (2) as a model of the type II enzymes to provide answers to some of the intriguing questions concerning the enzymology of such proteins. In this article, we describe the expression, in both *S. glaucescens* and *Escherichia coli*, of the *tcmM* acyl carrier protein (ACP) gene and the subsequent purification of this ACP in its holoenzyme form. With the availability of the TcmM protein in large quantity, we can study its structure and explore its interaction with other components of the TCM PKS.

Malonyl-CoA:ACP transacylase assay. The TcmM ACP was assayed for its ability to serve as an acceptor of malonate from malonyl coenzyme A (malonyl-CoA) catalyzed by a crude malonyl-CoA transacylase of *S. glaucescens*. Cells in the mid-exponential phase of growth were suspended in 100 mM sodium phosphate (pH 7.2)–2 mM dithiothreitol (DTT)–0.1 mM phenylmethylsulfonyl fluoride–1 mM EDTA–10% glycerol (10 ml/g of cells). Lysozyme (2 mg/ml) was added, and the mixture was allowed to incubate at room temperature for 2 h. To this viscous slurry, solid $MgCl_2$ was added to a final concentration of 25 mM, followed by about 0.5 mg of DNase. The resulting slurry was incubated on ice for 1 h and then centrifuged (13,600 × *g*, 20 min, 4°C) to yield a cell extract. The extract was brought to 0.3 M NaCl and 0.1% polyethylenimine by adding solid NaCl and a neutral 10% (vol/vol) solution of polyethylenimine, and the mixture was centrifuged as described before to give a clear supernatant. Solid ammonium sulfate (302 g/liter) was added to the supernatant with stirring, and the precipitate resulting after centrifugation (25,420 × *g*, 20 min, 4°C) was discarded. More ammonium sulfate (208 g/liter) was added to the supernatant, and the preparation was again centrifuged as described before to collect the pellet that contained the malonyl-CoA transacylase. This pellet was taken up in 20 mM sodium phosphate (pH 7.2)–2 mM DTT–20% glycerol (buffer I) to a final protein concentration of 5 mg/ml and was used as the malonyl-CoA transacylase in the ACP assay described below.

A typical TcmM assay solution contained 20 mM sodium phosphate (pH 7.2), 2 mM DTT, 10 μl of the malonyl-CoA transacylase, 10 μl of [2-^{14}C]malonyl-CoA in 10 mM phosphoric acid (pH 3.5) (8,000 cpm), and the ACP sample (10 to 50 μl) in a final volume of 100 μl. The complete assay solution without malonyl-CoA was preincubated at room temperature for 10 min, the reaction was then started by the addition of malonyl-CoA, and the assay was terminated after 1.5 min by the addition of 100 μl of a bovine serum albumin solution (10 mg/ml) and 400 μl of a 20% (vol/vol) trichloroacetic acid solution. Precipitated proteins were collected on a GF/B filter (Whatman, Maidstone, England) and washed with a 10% (vol/vol) trichloroacetic acid solution. The precipitated [2-^{14}C]malonyl-CoA–ACP retained on the filter was counted to determine the TcmM activity after subtraction of the activity observed in appropriate blank controls.

Expression of the *tcmM* gene in *S. glaucescens* yields solely the TcmM holo-ACP. To express the *tcmM* gene, pWHM701 was constructed as follows. A 1.1-kb *Sph*I-*Sst*I fragment that carries the *tcmM* gene was transferred from pWHM114 (2) into replicative form M13mp18 (22), similarly digested. An *Sph*I site was then introduced at the beginning of the *tcmM* gene by using the Amersham oligonucleotide-directed in vitro mutagenesis system (Amersham Corp., Arlington Heights, Ill.) and the synthetic oligodeoxynucleotide 5′-GGAGGTTCAGCATGCCCCAG-3′. Creation of the *Sph*I site required two nucleotide changes (underlined); the G-to-A transition replaces the wild-type GTG translation initiation codon of *tcmM* with an ATG codon. The entire *tcmM* gene was sequenced and transferred as a 314-bp *Sph*I-*Sst*I fragment into pIJ702 (13), analogously digested. This places the *tcmM* gene downstream of the putative

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TABLE 1. A summary of the steps for the purification of the TcmM ACP produced in *S. glaucescens* and *E. coli*

Steps[a]	Protein (mg) in:		
	S. glaucescens cells[b]	Exponential-phase *E. coli* cells[c]	Stationary-phase *E. coli* cells[d]
Crude cell extract	4,500	754.4	517.7
PEI[e] precipitation (supernatant)	4,100	731.4	485.8
$(NH_4)_2SO_4$ and glacial acetic acid precipitation (pellet)	982	112.0	210.0
Q Sepharose	81.5		
Sephacryl S-200		22.2	17.7
Mono Q	28.5	1.26	1.12
Phenyl Superose	0.855	0.775	0.828 (apoenyzme form) 0.053 (holoenzyme form)
Superose 6	0.44		

[a] As described in the text.
[b] From 70 g of cells.
[c] From 5.5 g of cells.
[d] From 3.3 g of cells.
[e] PEI, polyethylenimine.

ribosome binding site of the *mel* operon copper transport protein under the control of the *melC1* promoter in pIJ702.

S. glaucescens WMH1077 (formerly GLA.5-1) transformed with pWHM701 was grown in R2YENG medium (15) containing thiostrepton (20 μg/ml) at 30°C and 300 rpm for 28 h. Cells (25 g/liter) were harvested and washed, a cell extract was prepared as described earlier, and polyethylenimine (0.1%) and ammonium sulfate (302 g/liter) precipitation followed. After discarding this precipitate, more ammonium sulfate (208 g/liter) was added to the supernatant and the precipitate was collected by centrifugation (25,420 × *g*, 20 min, 4°C). The remaining supernatant was adjusted to pH 3.9 with glacial acetic acid and stirred overnight at 4°C; centrifugation followed as described before. The combined ammonium sulfate and acid pellets were dialyzed against 25 mM Tris-HCl (pH 8.0)–1 mM DTT (buffer II) and applied to a Q Sepharose fast-flow (Pharmacia, Piscataway, N.J.) column (2.6 by 18 cm) at room temperature. The column was washed with buffer II and developed with a linear 240-ml gradient from 0 to 0.5 M NaCl in buffer II at a flow rate of 6 ml/min. Fractions from this and succeeding purification steps were assayed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) performed on a PhastSystem (Pharmacia) and by the malonyl-CoA–ACP transacylase assay to determine the presence of TcmM. The fractions were then pooled and dialyzed against 25 mM Tris-HCl (pH 8.0)–1 mM DTT-0.2 M NaCl (buffer III) and applied to a Mono Q HR 10/10 column (Pharmacia) at room temperature. The column was washed with buffer III and developed with a linear 120-ml gradient from 0.2 to 0.5 M NaCl in buffer III at a flow rate of 4 ml/min. The pooled fractions containing the ACP were brought to 1.8 M ammonium sulfate by adding solid ammonium sulfate and applied to a Phenyl Superose HR 5/5 column (Pharmacia) at room temperature. The column was washed with 50 mM sodium phosphate (pH 7.2)–1 mM DTT (buffer IV) containing 1.8 M ammonium sulfate and then developed with a linear 15-ml gradient from 1.8 to 0 M ammonium sulfate in buffer IV at a flow rate of 0.5 ml/min. The pooled fractions containing the ACP from the Phenyl Superose column were concentrated in Centricon 10 filtration units (Amicon, Danvers, Mass.) and applied to a Superose 6 HR 10/30 column (Pharmacia) at room temperature. The protein was eluted with 20 mM sodium phosphate (pH 7.2)–2 mM DTT–150 mM NaCl (buffer V) at a flow rate of 0.5 ml/min. The final fractions exhibited a single band by SDS-PAGE.

The results of purification of the TcmM ACP produced in *S. glaucescens* are summarized in Table 1. Like various fatty acid synthase (FAS) ACPs (17, 18), the TCM PKS ACP behaves abnormally on a gel filtration column with an apparent M_r of 21,000 and in SDS-PAGE with an apparent M_r of 5,500 (8). (The expected M_r is 9,292 [2].) Purified TcmM is active in the malonyl-CoA–ACP transacylase assay, suggesting that the apo-ACP has been posttranslationally modified by the attachment of the 4′-phosphopantetheine prosthetic group. This was confirmed by in vivo labeling with [3-^{3}H]β-alanine, which specifically labels the 4′-phosphopantetheine group (9, 12, 21): [^{3}H]TcmM was recovered from *S. glaucescens* WMH1077(pWHM701) that had been grown in 500 ml of R2YENG medium containing thiostrepton (20 μg/ml) and [3-^{3}H]β-alanine (10 μCi).

Expression of the *tcmM* gene in *E. coli* mainly results in the TcmM apo-ACP. *E. coli* K38 transformed with plasmids pGP1-2 and pIJ2238 (8) was used as described by Gramajo et al. (8). Cells were grown at 30°C in 2× YT medium (8) containing kanamycin and ampicillin (50 μg/ml each). When the culture reached an A_{600} of 1.5 to 2.0 (exponential phase of growth), the temperature was shifted to 42°C and growth was continued for 2 h. Cells (5.5 g/liter) were then harvested and washed sequentially with 0.5 M NaCl and 0.1 M sodium phosphate (pH 7.2). TcmM was purified to homogeneity by the procedure described above with the following exceptions. The combined ammonium sulfate and glacial acetic acid pellets were dissolved in a minimum volume of buffer I and applied to a Sephacryl S-200 HR (Pharmacia) column (2.6 by 60 cm). Fractions were collected by elution with buffer V at room temperature at a flow rate of 2.0 ml/min. After gel filtration, the ACP was further purified on Mono Q HR 10/10 and Phenyl Superose HR 5/5 columns as described earlier (Table 1). After desalting by passage through a Vydac (Hesperia, Calif.) protein C_4 reverse-phase high-pressure liquid chromatography column developed with a linear 50-ml gradient from 5 to 95% acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 1.0 ml/min, the amino-terminal sequence of the purified TcmM was determined by the automated Edman degradation method. The first 10 amino acid residues (the V shown in Fig. 2 was not present) were identical to those predicted from the sequence of the *tcmM* gene (2).

Interestingly, the TcmM ACP purified from *E. coli* was inactive in the malonyl-CoA–ACP transacylase assay. To determine if this ACP was modified by attachment of the

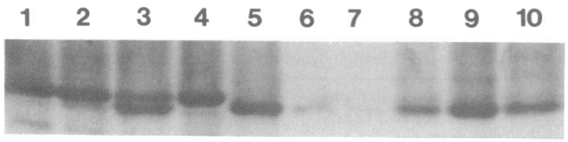

FIG. 1. Immunoblot of the conformationally sensitive PAGE of the TcmM ACP produced in *E. coli* and *S. glaucescens*. Lanes: 1, *E. coli* K38(pGP1-2/pIJ2238) culture induced in the exponential phase; 2, *E. coli* K38(pGP1-2/pIJ2238) culture induced in the stationary phase; 3, mixture of the TcmM apo- and holo-ACPs; 4, TcmM apo-ACP; 5, TcmM holo-ACP; 6, 12-h-old *S. glaucescens* culture; 7, 18-h-old *S. glaucescens* culture; 8, 28-h-old *S. glaucescens* culture; 9, 28-h-old *S. glaucescens* WMH1077(pWHM701) culture; 10, TcmM ACP purified from *S. glaucescens* WMH1077(pWHM701). Proteins were transferred to polyvinylidene difluoride membranes (Immobilon P; Millipore, Bedford, Mass.), and goat anti-rabbit immunoglobulin G conjugated with alkaline phosphatase (Sigma Chemical Co., St. Louis, Mo.) was employed as the second antibody.

4′-phosphopantetheine prosthetic group, a sample of the purified ACP was analyzed by electrospray mass spectrometry (3, 17). The major peak had a molecular weight of 8,963.33 ± 6.76, which is in reasonable agreement with the molecular weight of the apoenzyme (8,951.88) predicted from the sequence of the *tcmM* gene. The same sample was also analyzed by ^{31}P nuclear magnetic resonance (19), and no ^{31}P resonance was observed, confirming the absence of the 4′-phosphopantetheine group. Finally, when *E. coli* K38(pGP1-2/pIJ2238) was grown in 500 ml of 2× YT medium containing kanamycin and ampicillin (50 μg/ml each) and [3-^{3}H]β-alanine (10 μCi), the purified TcmM ACP was not ^{3}H labeled in vivo, although the *E. coli* FAS ACP was labeled in this experiment (data not shown).

In most cases, ACP genes from diverse species are expressed in *E. coli* exclusively (7, 9) or predominantly in their holoenzyme form (17). Since this was not our experience, the conditions for producing the TcmM holo-ACP in *E. coli* were explored. We observed that the ratio of apo- to holo-ACP varied as a function of the growth conditions of *E. coli* K38(pGP1-2/pIJ2238). To increase the amount of the holo-ACP, *E. coli* K38(pGP1-2/pIJ2238) was grown at 30°C in 2× YT medium containing kanamycin and ampicillin (50 μg/ml each) to an A_{600} of approximately 10 (stationary phase of growth). The temperature was raised to 42°C for 30 min and then lowered to 30°C for an additional 2 h. The cells (4.4 g/liter) were harvested and washed, and TcmM was purified as described earlier, during which we found that the TcmM apo- and holo-ACPs could be resolved on Mono Q or Phenyl Superose columns. As summarized in Table 1, 5 to 10% of the total TcmM isolated from the stationary-phase cells was in the holoenzyme form and was active in the malonyl-CoA–ACP transacylase assay.

It is interesting that induction of expression of the *tcmM* gene in *E. coli* stationary-phase cells results in the production of the TcmM holo-ACP to a small extent. It may be that the concentration of the *E. coli* ACP synthetase that transfers the 4′-phosphopantetheine group from CoA to a serine residue of the ACP (16) (see Fig. 2) is higher in the stationary phase of growth than in the exponential phase of growth or that the concentration of the competing FAS apo-ACP is lower. The shorter duration of the heat induction with the stationary-phase cells may also have reduced the risk of inactivation of the *E. coli* ACP synthetase (3). Alternatively, the apo-TcmM might have resulted from rapid degradation of the holo-TcmM by the *E. coli* ACP phosphodiesterase (16).

Western blot (immunoblot) analysis of TcmM apo- and holo-ACP produced in *E. coli* and *S. glaucescens*. ACPs tend to undergo conformational changes in alkaline pH, and different forms can be resolved by conformationally sensitive PAGE analysis (19). With the purified TcmM apo- and holo-ACPs as standards, conditions for conformationally sensitive PAGE were developed to resolve the TcmM apo- and holo-ACPs on 20% polyacrylamide gels under reducing conditions (19) (Fig. 1, lanes 3 to 5).

Cross-reactivity among ACPs from diverse species is known, leading to the conclusion that such proteins have similar three-dimensional conformations or, at least, similar epitopes (9, 14). This led us to examine whether anti-TcmM ACP antibodies cross-react with other ACPs. Anti-TcmM antibodies raised against denatured TcmM apo-ACP in rabbits by standard methods (10) reacted specifically with the TcmM apo- and holo-ACPs (Fig. 1, lanes 3 to 5 and 10). Cross-reactivity between the anti-TcmM and the *E. coli* or *S. glaucescens* FAS ACPs was not observed, however, nor was there cross-reactivity between anti-*E. coli* FAS ACP (12) and the TcmM apo- and holo-ACPs (data not shown). Thus, there could be a significant overall conformational difference between the *E. coli* FAS ACP and the TCM PKS ACP, even though the region surrounding prosthetic group attachment site serine is highly conserved among TcmM and other ACPs (11) (Fig. 2). These results suggest that the poor yield of holo-TcmM from *E. coli* may result from poor recognition of the TcmM substrate by *E. coli* ACP synthetase, even though this enzyme has been reported to have relatively broad substrate specificity (7, 9, 17).

Finally, we determined the extent to which the TcmM apo-ACP is modified in *S. glaucescens* by attachment of the prosthetic group, since in *E. coli* it appears that all of the FAS ACP is maintained in the holoenzyme form (12). Cultures of wild-type *S. glaucescens* were grown in

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PKS                      1                                                               50                                                     99
S. coelicolor ACP       matlLTtDDL rralVEcAGE tDGtDLsGdf LDlrF.EDiG YDSLALmEta arLEsrYGVS IPDDvagrVd TpRelLDLIN GalAEAA*.. .........
S. violaceoruber ACP    .marLTLDgL rtilVacAGE dDGVDLsGdi LDitF.EELG YDSLALmEsa sriErELGVa laDgDinEel TpRvlLDLVN GaQAEAA*.. .........
TcmM ACP                .vpqigLprL vEiirEcAGD pDerDLDGdi LDvtY.qDLG YDSiALlEis akLEqDLGVS IPgE...Elk TpRhtLhLVN tetAgeva*. .........

FAS
E. coli ACP             .......sti eErvkkiiGE QlGVkqEevt dnasFvEDLG aDSLdtvElV mALEEEFdte IPDEEaEkIt TvqaaiDyIN GhQA*..... .........
Sac. erythraea ACP      ....drkEiF eErieqvlaE QlGIpaEqit eEadLrEDLG mDSLdLvElV sALEDEvGmr VeqsqlEgIe TvghvMELtl dlvArlAtas aadkpeaas*
S. glaucescens ACP      ..maaTqEEi vaglaDivnE iaGIpvEdvq LDksFtDDLd vDSLsMvEvV vAaEErFdVk IPDEDvknlk TvgdatEyIl khQA*..... .........
Consensus               ----LTL--L -E--VE-AGE QDG-DLEG-- LD--F-EDLG YDSLAL-E-V -ALEEE-GVS IPDED-EEI- T-R--LDLIN G-QAEAA--- ---------
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FIG. 2. Alignment of the deduced amino acid sequence of the TCM PKS ACP (2) with *E. coli* FAS ACP (11), *S. erythraea* FAS ACP (17), *S. glaucescens* FAS ACP (5), *Streptomyces coelicolor* PKS ACP (11), and *Streptomyces violaceoruber* PKS ACP (20). The serine of the prosthetic group attachment site is shown in boldface type. Capital letters indicate identity of two or more amino acids in a vertical column. Stars indicate translation stops.

R2YENG medium at 30°C and 300 rpm for 12, 18, and 28 h, and the TcmM ACP produced was compared with that from exponential- and stationary-phase cultures of *E. coli* K38(pGP1-2/pIJ2238). Cells were harvested and washed, and cell extracts were prepared as described earlier. As expected, the TcmM holo-ACP was not observed in the cell extract prepared from *E. coli* cells induced in the exponential phase of growth (Fig. 1, lane 1), while the holo-ACP was readily detected in the extract from *E. coli* cells induced in the stationary phase of growth, although the apo-ACP predominated (Fig. 1, lane 2). In contrast, only the holo-ACP was produced in *S. glaucescens* over the 28-h period (Fig. 1, lanes 6 to 9), implying the presence of a very active ACP synthetase. This finding indicates that the ratio of apoenzyme to holoenzyme forms of TcmM is unlikely to play a regulatory role in polyketide biosynthesis. This is one reason that we have been able to increase the production of TCM C biosynthetic intermediates by introducing extra copies of the *tcmM* gene into *S. glaucescens*, which ultimately increases the production of the TcmM holo-ACP (5). As expected from the timing of TCM C production (5), expression of the *tcmM* gene was not detected in *S. glaucescens* until 18 h (Fig. 1, lanes 6 to 8). This coincides with the time of expression of *tcmK* gene (8).

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