Molecular Characterization of the Genes of Actinomycin Synthetase I and of a 4-Methyl-3-hydroxyanthranilic Acid Carrier Protein Involved in the Assembly of the Acylpeptide Chain of Actinomycin in Streptomyces*

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Actinomycin synthetase I (ACMS I) activates 4-methyl-3-hydroxyanthranilic acid, the precursor of the chromophoric moiety of the actinomycin, as adenylate. The gene acmA of ACMS I was identified upstream of the genes acmB and acmC encoding the two peptide synthetases ACMS II and ACMS III, respectively, which assemble the pentapeptide lactone rings of the antibiotic. Sequence analysis and expression of acmA in Streptomyces lividans as enzymatically active hexa-His-fusion confirmed the acmA gene product to be ACMS I. An open reading frame of 234 base pairs (acmD), which encodes a 78-amino acid protein with similarity to various acyl carrier proteins, is located downstream of acmA. The acmD gene was expressed in Escherichia coli as hexa-His-fusion protein (Acm acyl carrier protein (AcmACP)). ACMS I in the presence of ATP acylated the purified AcmACP with radioactive p-tolual acid, used as substrate in place of 4-MHA. Only 10% of the AcmACP from E. coli was acylated, suggesting insufficient modification with 4-phosphopantetheine cofactor. Incubation of this AcmACP with a holoe-ACP synthase and coenzyme A quantitatively established the holo-form of AcmACP. Enzyme assays in the presence of ACMS II shown that toluyl-AcmACP directly acylated the thioester-bound threonine on ACMS II. Thus, AcmACP is a 4-MHA carrier protein in the peptide chain initiation of actinomycin synthesis.

The actinomycins, a family of bicyclic chromopeptide lactones with strong antineoplastic activity (1), are produced by various streptomycete strains (2, 3). Common to all actinomycins is the chromophoric moiety actinocin, a unique phenoxazinone with strong antineoplastic activity (1), are produced by various streptomycete strains (2, 3). Common to all actinomycins is the chromophoric moiety actinocin, a unique phenoxazinone synthase (5) catalyzing the formation of actinocin (Fig. 1).

The assembly of the ACMSs into two and three modules, respectively, necessary for pentapeptide lactone assembly. Inspection of the protein sequence of ACMS II, however, showed that the conserved motif of the 4-phosphopantetheine cofactor attachment site occurred only twice, namely in the two amino acid acrylamide gel electrophoresis; 2,3-DHB, 2,3-dihydrobenzoic acid; PCR, polymerase chain reaction.

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‡ The abbreviations used are: 4-MHA, 4-methyl-3-hydroxyanthranilic acid; 4-MHB, 4-methyl-3-hydroxybenzoic acid; ACMS, actinomycin synthetase; ACP, acyl carrier protein; ORF, open reading frame; PMSF, phenylmethylsulfonyl fluoride; DTE, 1,4-dithiothreitol; PAGE, poly-
modules of this protein. Thus, the third 4'-phosphopantetheine cofactor, which was initially postulated to lie in front of the first (threonine) module (8), is not located on ACMS II. From the previous work on acmB and acmC in the acm gene cluster we got evidence that the 5'-end of the gene acmA of ACMS I is located upstream of the gene acmB of ACMS II and points in the opposite direction (13). Here we show the cloning and expression of acmA and the analysis of the gene product. More importantly, we found directly downstream of the gene of ACMS I a small open reading frame (ORF) encoding a small ACP (acmD), which is the protein harboring the missing third 4'-phosphopantetheine cofactor required for condensation of 4-MHA with threonine on ACMS II.

**EXPERIMENTAL PROCEDURES**

**Chemicals and Radiobiologicals—**4-MHA was synthesized as described (10). [3-14]C Toluic acid (8.3 Ci/mol) was from Sigma. Tetrasodium [32P] pyrophosphate (28.2 Ci/mmol) was from NEN Life Science Products, and [α-32P] ATP (400 Ci/mmol) was from Hartmann Analytik (Braunschweig, Germany). All other chemicals were of the highest grade commercially available.

**Strains and Cultures—**Streptomyces lividans 1326 was from the John Innes strain collection (Norwich, United Kingdom). It was grown according to standard protocols.

**Plasmids, DNA Manipulations, and Cloning and Sequencing Procedures—**Techniques for DNA isolation, manipulation, and transformation were as described by Sambrook et al. (17) and Hopwood et al. (14). The cosmid cosA1 carrying the ACMS gene cluster from S. chrysomallus ATCC 11523 was described previously (13). The plasmids used for subcloning fragments were pTZ18U (Amersham Pharmacia Biotech), pSP72 (Promega), pSL1180 (Amersham Pharmacia Biotech). Sequence determinations were performed with a Tag cycle sequencing kit (United States Biochemicals-Amersham Pharmacia Biotech sequencing kit US7100 or US7500) on plasmid DNA. Fragments were subcloned into pTZ18U. Sequence comparisons, multiple sequence alignments, and identity scores were computed with CLUSTAL W (18) or with the FASTA database search results (19). The plasmid for expression of acmA and acmB in S. lividans was pJL702 (20). For expression of acmA in E. coli, expression vector pQE30 (Qiagen) was used, and for expression of acmD in E. coli, pQE32 (Qiagen) was used. For PCRs, Vent DNA polymerase (BioLabs) was used according to the manufacturer's instructions.

**Heterologous Expression of acmA—**Expression of acmA in E. coli was as amino-terminal hexa-His-tagged fusion protein. The acmA was amplified with suitable restriction ends by PCR using cosmid cosA1 (13) as template. Forward and reverse primers were FACMA1 (5′-TAAGAG-GAAGCTGGATCCGCCGATAAATGGTG-3′) and RACMA2 (5′-TAG-GCTGGAATCCGGACCGAGGTGAA-3′), respectively. The resulting 1.6 kilobase pair PCR fragment was digested with BamHI and SalI and ligated into pQE30. In this construct the amino-terminal end of ACMS I would change from MADK- to MRGSHHHHHHGSADK-. Transformation into E. coli JM109 yielded plasmid pACMA1. After restriction analysis and control sequencing on both ends of the fragment, pACMA1 was transformed into E. coli strain M15. Cultures of M15/pACMA1 (1.6 liters of 2× YT medium, 100 μg/ml ampicillin, 25 μg/ml kanamycin) were grown at 30 °C to an A600 of 0.9 and then induced with 2 mM isopropylthiogalactoside. Cells were harvested after a further 14 h of incubation at 30 °C.

For the expression of acmA as carboxyl-terminal hexa-His-tagged fusion protein in S. lividans, acmA was placed as a translational fusion in frame into the ATG start codon of the melCI gene of Streptomyces plasmid pJL702 (20), which is under control of the mel promoter. The ATG start codon of melCI is contained in the unique SalI site of the plasmid. The acmA was amplified with synthetic primers to generate a matching 5'-SphI site in its start codon and to create a hexa-His encoding sequence between the last codon and the stop codon.

**Fig. 1.** Structure of actinomycin and 4-MHA pentapeptide lactone and scheme of their assembly by the actinomycin synthetases. Actinomycin is formed by oxidative condensation of two 4-MHA pentapeptide lactones. The 4-MHA pentapeptide lactone is assembled on actinomycin synthetases II and III, which activate the five amino acids of the peptide lactone ring in the indicated order. 4-MHA is adenylated by ACMS I. Peptide synthesis starts after acylation of threonine with activated 4-MHA on the surface of ACMS II. Sar, N-methylglycine; MeVal, N-methyl-l-valine; AdoMet, S-adenosyl-l-methionine.

![Actinomycin D](image_url)
fragment was isolated and ligated into SphI/BglII-cleaved pJLT02. Transformation into S. lividans resulted in plasmid pCAM11. The recombinant protein encoded by pCAM11 is changed at the amino terminus from MDAK- to MHHMKD- and at the carboxyl terminus from -AS to -ASHHHHHH. Microsequencing of the purified protein later confirmed the new amino-terminal sequence (not shown). For expression of acmA in S. lividans encoded by pCAM11 as hexa-His-tagged fusion protein, the transformation strain was grown at 30 °C (6 liters of YEME medium, 10 μg/ml thioestrepton) for 4 days and then harvested by suction filtration.

Heterologous Expression of acmB—The acmB was expressed in S. lividans from plasmid pACM5 as described previously (13).

Heterologous expression of acmD (AcmAACP)—The gene acmD encoding the 4-MHA carrier protein was expressed in E. coli as amino-terminal hexa-His-tagged fusion protein. The acmD gene was engineered by PCR with synthetic primers FACPI1 (5′-CCGAGATCTTC-GAAGGACGACATCAGGGCGAT-3′) and FACPI2 (5′-CGAGATCTGT-GCTGGGCCCCTGCGGCCGC-3′) using pA1sub27 as template. This generated a 276-base pair fragment with a 5′-Phosphopantetheine Transferase Gene (ORF C, 761 base pairs) flanking the nosiheptide resistance region upstream of the gene of ACMS II (12510 bp, 12510, 8.0), 300 mM KCl, 300 mM NaCl, 1 mM PMSF, 1 mM benzamidine (1 mM benzamidine) passed twice through a French press at 10,000 psi. After DNase I treatment (30 μg/ml) and with gentle stirring for 90 min, the supernatant (60 ml) was passed through a DEAE-cellulose column (8 × 3.5 cm) to which, at pH 7, ACMS I does not bind. Fractions with enzymatic activity were pooled (240 ml). Addition of 28 ml of 1 M Tris-HCl, pH 8.0, shifted pH from 7.0 to 8.0, and the solution was applied to a nickel-chelate resin column (2.8 × 1.6 cm). After washing the column with Buffer NW (see above), bound proteins were eluted with a 150-ml linear gradient of 0–250 mM imidazole in Buffer NW. Fractions of 3.5 ml were collected, and the fractions with enzymatic activity was pooled. This pool was diluted with 5 volumes of 15% glycerol (v/v), 4 mM DTE, 1 mM EDTA, 15% PMSF, 1 mM benzamidine and applied onto an α-aminohexyl-Sepharose 4B (Sigma) column (6.5 × 1 cm) for enzyme concentration. After washing with Buffer C (15% glycerol (v/v), 50 mM Tris-HCl (pH 8.0), 1 mM EDTA, 4 mM DTE, 1 mM PMSF, 1 mM benzamidine), the bound protein was eluted with a step of 0.3 M NaCl in Buffer C. The protein (total volume, 6 ml) was purified in 1.5-ml portions to homogeneity by gel filtration on an Superdex75 column (Amersham Pharmacia Biotech) previously equilibrated with Buffer B (15% glycerol (v/v), 100 mM Tris-HCl (pH 8.0), 1 mM EDTA, 4 mM DTE, 1 mM PMSF, 1 mM benzamidine). Protein with apparent homogeneity was in the rear half of the activity peak. The purified recombinant protein could be stored at −80 °C for at least 6 months without detectable loss of activity.

Purification of 4-MHA Carrier Protein (AcmAACP) Expressed in E. coli—Approximately 12 g of cell paste was suspended in 34 ml of Buffer NI (see above). After passage of the suspension through a French press at 10,000 psi, DNase I (25 μg/ml) was added, and the suspension was left on ice for 15 min with gentle stirring. After centrifugation (15 min at 20,000 × g), the supernatant was applied to a nickel-chelate column (3 × 1.6 cm) equilibrated with Buffer NI. The column was washed with 15 ml of the same buffer and 80 ml of Buffer NW until no more protein eluted from the column. A 100-ml linear gradient of 0–500 mM imidazole in Buffer NW afforded elution of hexa-His-tagged protein. The bulk of protein appeared at 100–200 mM imidazole, and the corresponding fractions were pooled. 1 mM DTE was added to give 4 mM final concentration, and 4.5-ml portions (3 mg protein) were gel filtrated on Ultrogel AcA34 (40 × 25 cm column dimensions) in Buffer B (see above). After this step, enzyme appeared to be pure as judged from SDS-PAGE. Typically, about 4 mg of total protein were obtained by this procedure. Protein could be stored at −80 °C for 4 weeks without loss of p-toluic binding activity in Buffer B.

Purification of Recombinant ACMS II Encoded by pACM5 from S. lividans—Partially purified recombinant ACMS II was prepared as described previously (13). Enzyme from the AcA34 gel filtration step was concentrated, and 4.5-ml portions (3 mg protein) were applied to a nickel-chelate column (3 × 1.6 cm) equilibrated with Buffer NI. The column was washed with 15 ml of the same buffer and 80 ml of Buffer NW until no more protein eluted from the column. A 100-ml linear gradient of 0–500 mM imidazole in Buffer NW afforded elution of hexa-His-tagged protein. The bulk of protein appeared at 100–200 mM imidazole, and the corresponding fractions were pooled. 1 mM DTE was added to give 4 mM final concentration, and 4.5-ml portions (3 mg protein) were gel filtrated on Ultrogel AcA34 (40 × 25 cm column dimensions) in Buffer B (see above). After this step, enzyme appeared to be pure as judged from SDS-PAGE. Typically, about 4 mg of total protein were obtained by this procedure. Protein could be stored at −80 °C for 4 weeks without loss of p-toluic binding activity in Buffer B.

Purification of Recombinant 4′-Phosphopantetheine Transferase (Holo-ACP Synthase) from S. actuosus Encoded by pPAN5—The resultant hexa-His-tagged fusion protein was purified analogous to AcmAACP as described above.

Analytical Methods—SDS-PAGE was performed according to Laemmli (22). Protein determinations were done according to Bradford

FIG. 2. Map of the gene acmA encoding ACMS I and its flanking regions on the chromosome of S. chrysomallus. Sequencing of the region upstream of the gene of ACMS II (acmD) led to identification of acmA as indicated by an arrow. Downstream of acmA, partially overlapping its 5′-end, follows acmD, which encodes the 4-MHA carrier protein AcmAACP. The SalI fragment in plasmid pA1sub27 was the template for PCR amplifications of acmA and acmD.
Antibodies against ACMS I from *S. chrysomallus* were raised in a rabbit by two administrations of 100-μg portions of the purified enzyme (at a 1-month interval) (courtesy of Prof. F. J. Fehrenbach, Robert-Koch-Institut, Berlin, Germany). The serum was used without further purification in dilutions of 1:1000 to 1:3000 for Western blot analysis by semidry blot standard techniques (provided by Biometra and Schleicher & Schuell). Anti-rabbit alkaline phosphatase conjugate (Sigma) served as second antibody for the purpose of nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate detection (both from Roche Molecular Biochemicals). Thin-layer chromatography of enzymatically formed acyl adenylates was performed as described (10).

**Enzyme Assays—** ATP-[32P]pyrophosphate exchange reactions and acyl adenylate formation tests with [α-32P]ATP were as described previously (11).

The recombinant 4-MHA carrier protein (AcmACP) was tested for thioester formation with p-[1-14C]toluic acid (used as 4-MHA substrate analogue) by measuring the formation of trichloroacetic acid-precipitable radioactivity. One unit of AcmACP was defined as the amount of enzyme that covalently binds 1 nmol of p-toluic acid in 30 min at 29 °C. Standard assay contained 14 mM p-[1-14C]toluic acid, 10 mM ATP, 45 mM MgCl₂, 280 mM purified recombinant ACMS I and 100 μl of AcmACP-containing protein fraction. Buffer was Buffer B (see above) and incubation was at 29 °C for 30 min. After addition of 6% trichloroacetic acid and leaving on ice for 15 min, precipitated proteins were collected by suction filtration on membrane filters ME 25 (Schleicher & Schuell). After washing and drying, filter bound radioactivity was counted in a Packard 1600CA scintillation counter. To visualize charged AcmACP as labeled band in SDS-polyacrylamide gels, precipitated proteins were resuspended in 70 μl of 15% (w/v) glycerol, 100 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1 mM PMSF, and 1% SDS. 2 μl of 45% sucrose-0.25% bromphenol blue was added. 5-μl aliquots were usually counted by liquid scintillation counting, and 10-μl portions were separated by SDS-PAGE (17.5% polyacrylamide). Gels were stained with Coomassie Brilliant Blue R250 (Serva) and, after drying, exposed to x-ray film (NIF100, Konica).

Measurement of activity of recombinant ACMS II was as described previously (13).

The assay to demonstrate the transfer of p-toluic acid from p-toluic acid-AcmACP-thioester to ACMS II-threonine thioester was the same as described above to measure charging of AcmACP but with the additional presence of ACMS II and its substrate threonine. Assays contained 200 nM ACMS II, 5.1 μM AcmACP (0.35 units), 0.046 units of partially purified ACMS II, 10 mM ATP, 50 mM MgCl₂, 14 mM p-[1-14C]toluic acid, 8 mM threonine and were incubated for 30 min at 29 °C. After addition of 5% trichloroacetic acid, protein was allowed to precipitate overnight on ice. Control reactions involved omission of one component from the reaction in each case. Precipitated proteins were collected, washed twice with 7% trichloroacetic acid and once with 6 ml of 3% trichloroacetic acid, and finally resuspended in a 350-μl solution of 15% (w/v) glycerol, 100 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1 mM PMSF, and 1% SDS. 9 μl of 45% sucrose-0.25% bromphenol blue was added. A 10-μl aliquot was analyzed by liquid scintillation counting, and 8 μl was applied onto a 35% polyacrylamide gel. Staining, drying, and autoradiography was as above.

Incubation of AcmACP with the holo-ACP synthase from *S. actosus* was performed in a total volume of 600 μl (Buffer B) at 30 °C for 45 min in the presence of 28 μM AcmACP, 1 μM holo-ACP synthase, 290 mM ACMS II, 10 mM p-[1-14C]toluic acid, 10 mM ATP, 20 mM MgCl₂ and 0.2 mM CoA. 80-μl aliquots were precipitated with 2 ml of 7% trichloroacetic acid and 50 μl of 3% bovine serum albumin at several time points.

**TABLE I**

| Enzyme | Amino acid | Organism/biosynthesis system | Position (amino acid) | Sequence |
|--------|------------|------------------------------|----------------------|----------|
| ACMS I | 472        | *S. chrysomallus*: Actinomycin | 136                  | VVLYVQFSGSTGSKPKVXGPAPA |
| SNBA²  | 582        | *S. pristinaespiralis*: Pristinamycin I | 191                  | RGVFLLSGTTAALPKLFRTH |
| ENTE   | 536        | *E. coli*: Enterobactin       | 184                  | VAYQFQSGTGTGPKLFRTH |
| YBTE   | 525        | *Yersinia enterocolitica*: Yersiniaibactin | 170                  | TALLLISGTTGPKLFRTH |
| LUC    | 550        | *Photinus pyralis*: Luciferin | 192                  | IALTIMSNGSGTGPKKWLP |
| RAPA   | 8563       | Streptomyces hygroscopicus: Ramapycin | 164                  | PAYLMWTSGGTGPKVLSTQ |
| ACMS II| 2611       | *S. chrysomallus*: Actinomycin | 611                  | VAYV1TSGGSGPKVNYTH |
| MTML   | 514        | Streptomyces argillaceus: Mythramycin | 174                  | VVCQQVTSGGTGPKVLRT |
| HBAÁB  | 539        | Rhodopseudomonas palustris: 4-hydroxy-benzoate-CoA-ligase | 166                  | IAHYQWSGGTGPKVMHH |
| 4CL2   | 542        | Nicotiana tabacum: 4-coumarate-CoA-ligase | 183                  | VVALPFSGTGPKVLRT |

⁰GenBank™ accession numbers: SNBA, X98515; ENTE, P10378; YBTE, Q56950; LUC, Q27758; RAPA, Q54297; ACMB, O68487; MTLM, E1311674; HBAÁ, Q55005; 4CL2, Q24146.
ligases and the activation domains of various peptide synthetases (Table I). Among these are the aromatic carboxylic acid-activating enzymes EntE and YbtE from *E. coli* and *Yersinia pestis*, respectively (24–26). Furthermore, the enzyme has sequence similarity (20% identity) to the hydroxypicolinic acid-adenylating enzyme SnbA from *Streptomyces pristinaespiralis* and to coumarate CoA ligase (24% identity) from *Petroselinum crispum*, a member of the acyl-CoA ligases (27, 28). The conservation in these sequences was always highest in the five so-called core regions characteristic for adenylating domains (29) (Table I), which leaves no doubt about the nature of the cloned gene. As predicted from the biochemical analyses of ACMS I obtained from *S. chrysomallus* (10, 11), no 4^-9^-phosphopantetheine attachment site was found in the enzyme sequence that distinguishes this enzyme from typical peptide synthetases, which bind their substrates as thioesters after activation as adenylate.

**TABLE II**

Purification of hexa-His-tagged ACMS I from *S. lividans*

| Purification step            | Volume | Protein | Activity | Specific activity | Purification | Yield |
|------------------------------|--------|---------|----------|------------------|--------------|-------|
| Crude extract                | 60     | 4800    |          |                  |              |       |
| DEAE-cellulose, pH 7.0       | 240    | 1104    | 240.7    | 0.22             | 1            | 100   |
| Nickel-chelate chromatography| 53.5   | 16.05   | 81.6     | 5.08             | 23.1         | 33.9  |
| Aminoethyl-Sepharose         | 6      | 4.5     | 81.6     | 13.7             | 62.5         | 25.6  |
| Superoxid™ 75 gel filtration | 19.5   | 1.55    | 30.5     | 19.6             | 89.1         | 12.7  |

**Expression of acmA—**To further characterize the *acmA* gene product, the gene was expressed in *E. coli* as an amino-terminal hexa-His-tagged fusion protein. Western blotting of protein extracts of *E. coli* M15 carrying the expression construct pACMA1 (see under “Experimental Procedures”) with antibodies directed against ACMS I from *S. chrysomallus* showed the presence of the expressed protein as a 45-kDa band in SDS-PAGE (not shown). Nickel-chelate chromatography of total extract from 6 M guanidine hydrochloride-treated cells and in parallel to the soluble fraction of non-denatured cells revealed that by far, most of the protein was in the insoluble fraction. Testing the soluble fraction revealed enzymatic activity as judged from the ATP-pyrophosphate exchange dependent on 4-MHA, 4-MHB, or *p*-toluic acid at an appreciable activity. The enzyme was purified with reference to the purification of the original protein from *S. chrysomallus* (see under “Experimental Procedures”). However, this resulted in very low enzyme yields (final total yield, 0.2% at a specific activity of 1.6 nanokatal/mg of protein), which made production of the enzyme in *E. coli* not amenable for our purposes.

To address the production of active enzyme in higher yields, *acmA* was engineered as a translational fusion into the ATG start codon of the melC gene on plasmid pIJ702, and the resultant plasmid, pACMA11, was transformed into *S. lividans*. In contrast to the construct used in *E. coli*, here the enzyme carried the hexa-His tail at its carboxyl-terminal end. Analyzing total and cytosolic protein extracts from the transformed strain and the control strain harboring pIJ702 by Western blotting revealed the presence of ACMS I after expression of *acmA* in *S. lividans* (Fig. 3). Moreover, the protein was exclusively in the soluble fraction (not shown). Total activity based on the protein present in the starting material was measured by the 4-MHB dependent ATP-pyrophosphate ex-
change and was found to be 3-fold higher than total enzyme activity in extracts of *S. chrysomallus* X2–18, an actinomycin-high producer (11). The purification is shown in Table II. Remarkably, about 50% of total activity did not bind to nickel-chelate matrix due to either masking of the hexa-His tail or its removal through proteolytic activities. Nevertheless, steps following such as adsorption to aminohexyl-Sepharose with subsequent elution with a salt step and gel filtration on Superdex™ 75 afforded purification of the enzyme to homogeneity with 12% yield and a specific activity of approximately 20 nanokatal/mg of protein. This specific activity is about 3-fold higher than estimated for the wild type enzyme, which apparently is due to the much shorter purification procedure of the recombinant protein (four versus eight steps). The recombinant enzyme was indistinguishable from the wild type enzyme with respect to acyl adenylate formation both from various benzene carboxylic acids and in the ATP-pyrophosphate exchange reaction dependent on these substrates (11). Likewise, the wild type enzyme, the recombinant ACMS I, did not catalyze the formation of a CoA thioester from any of the benzene carboxylic acids tested including 4-MHA.

Cloning and Sequencing of acmD, an ORF Transcriptionally Coupled to acmA—In the course of sequencing the ACMS I gene *acma*, immediately downstream of *acmA*, an ORF was identified that overlaps with its ATG start the stop codon of *acmA* indicating transcriptional and translational coupling between the two genes (Fig. 2). This suggested a close functional link between the gene products of *acma* and that ORF, which was named *acmD*. The *acmD* gene has a length of 236 base pairs, encoding a protein of 78 amino acids in length with a calculated molecular mass of 8691 Da and a pI of 3.83. Comparison of the deduced amino acid sequence of *acmD* with protein sequences in the data bank revealed similarity with various ACPs from bacteria involved in fatty acid and polyketide synthesis (Table III). These carry 4'-phosphopantetheine as prosthetic group attached to a conserved serine, which is also present in the *acmD* gene product (Table III). There was also similarity with DltC, the D-alanine carrier in lipoteichoic biosynthesis in *Lactobacillus casei* (30), the ACP domain of the enthB gene product of the enterobactin synthesis system in *E. coli* (31, 32), and the amino-terminal ACP extra-domain of the yersiniabactin synthetase HWMP2, a peptide synthetase involved in the biosynthesis of the acyltripeptide yersiniabactin in *Yersinia* strains (33, 34). The gene product of *acmD* appeared to be a suitable candidate for carrying the missing 4'-phosphopantetheine cofactor accepting 4-MHA in actinomycin biosynthesis and was therefore named AcmACP.

Expression of *acmD* as a Hexa-His-tagged Fusion Protein—For expression of *acmD* as hexa-His-tagged fusion protein the expression plasmid pACPI-Q1 (using vector pQE 32) was constructed. After transformation of *E. coli* strain M15 cultivation was performed at 30 °C and induction was with low concentrations of isopropylthiogalactoside to allow gradual expression of the gene. SDS-PAGE analysis of protein extracts of the *E. coli* strain transformed with plasmid pACPI-Q1 revealed an abundant protein of approximately 10 kDa, which was missing in the control strain carrying pQE32 (Fig. 4, lanes 1 and 2, respectively). The purification of the protein was by nickel-chelate chromatography and Ultrogel Ac A 54 gel filtration, which yielded an appreciable amount of pure protein (Fig. 4, lane 3). The total yield was 67%.

The ability of the protein to bind p-[^14]C]toluic acid in the presence of ACMS I and ATP was measured by the formation of trichloroacetic acid-stable radioactivity. Covalent substrate binding was also used to demonstrate the charged protein as a labeled band in SDS-PAGE as described under “Experimental Procedures.” Fig. 4, lanes 4 and 4’, shows that the protein was charged with p-[^14]C]toluic acid when ACMS I and ATP were present. No radiolabeling of the protein was seen when ATP (Fig. 4, lanes 5 and 5’) or ACMS I (Fig. 4, lanes 3 and 3’) were absent in the reaction mixture. These findings indicate that the AcmACP does not activate p-toluic acid per se and was charged only in case of prior activation of p-toluic acid by ACMS I. An estimate for the extent of 4'-phosphopantetheinylation was obtained by comparing the amount of radioactive substrate covalently bound to the protein and the total amount of AcmACP protein present in the assays. In two AcmACP preparations after gene expression in *E. coli*, only 10% of the AcmACP was found to bind ACMS I activated p-toluic acid. This apparently points to a low extent of phosphopantetheinylation of AcmACP in the foreign host *E. coli*. To increase the amount of phosphopantetheinylated AcmACP (holo-AcmACP) and also to demonstrate that the p-tolueic acid became bound to AcmACP via 4'-phosphopantetheine as prosthetic group, the acylation of AcmACP by ACMS I was performed in the additional presence of a holo-ACP synthase and CoA (35). We chose for these experiments the holo-ACP synthase from *S. actuosus* (21),
which was expressed from its gene as a hexa-His-tagged fusion protein in *E. coli* as described under “Experimental Procedures.” The holo-ACP synthase was added to the AcmACP/ACMS I incubations in 1 μM concentrations and incubated for different lengths of time with and without CoA. The data in Fig. 5 clearly show that with increasing length of incubation, the amount of AcmACP was reached. By contrast, in the absence of CoA the basal level of 10% remained constant during the whole 40 min incubation, which was also the case when no holo-ACP synthase was present (the latter not shown). These data illustrate the 1:1 stoichiometry between the enzyme, its cofactor, and the substrate p-toluic acid. Moreover, varying the ACMS I concentration (from 20 to 450 nM) in short (2 and 4 min) incubations with AcmACP revealed direct proportionality between the amount of ACMS I added and p-tolyl-AcmACP formed (not shown).

*p*-Tolyl-AcmACP Is Substrate of ACMS II—Once we had identified the *acmD* gene product as 4-MHA binding protein, we set out to elucidate the possible interaction between the AcmACP and ACMS II in the transfer of the aromatic carboxylic acid to the threonine moiety (i.e. the first amino acid in the pentapeptide lactone ring) activated by ACMS II. In order to exclude possible contamination of ACMS II with AcmACP when isolated from actinomycin-producing *S. chrysomallus*, we used the recombinant ACMS II expressed in *S. lividans* (13). This ensured that all components used for the enzyme reaction were derived from an actinomycin-free background. Data in Fig. 6 (lanes 4 and 4’) show that upon incubation of ACMS II with AcmACP, ACMS II and their substrates threonine, p-[^14]Ctoluic acid, and ATP, the bands of both ACMS II and AcmACP were significantly labeled. When the same experiment was performed without ATP (Fig. 6, lanes 3 and 3’) or without the AcmACP (lanes 5 and 5’), labeling of neither protein band was observed. This indicates that binding of p-toluic acid is ATP-dependent and that labeling of ACMS II only occurs from the charged AcmACP. In the absence of threonine, ACMS II became only faintly labeled, possibly due to the presence of residual trace amounts of threonine that had been loaded to the enzyme previously in *in vivo* conditions. (Fig. 6, lanes 2 and 2’) This findings clearly show that the p-toluic acid bound to AcmACP directly acylates the threonine covalently bound to ACMS II. Lanes 6 and 6’ in Fig. 6 show that when ACMS I is absent, neither band of AcmACP nor ACMS II are labeled, which clearly reveals that charging of both AcmACP and ACMS II is dependent on the prior activation of p-toluic acid as adenylate.

**DISCUSSION**

We have shown previously that the 4-MHA pentapeptide lactones, the penultimate precursors of the bicyclic actinomycins, are assembled by the two peptide synthetases, ACMS II and III. These enzymes from actinomycin-producing *S. chrysomallus* activate the five amino acids of the pentapeptide lactone ring as adenylate and thioesters, whereas 4-MHA is activated by a separate 4-MHA adenylylating enzyme, ACMS I. ACMS I does not bind its substrate as thioester as revealed by enzymatic testing and, as shown here, because of the absence of a 4’-phosphopantetheine attachment site in its sequence. Furthermore, ACMS I has no activity as an acyl-CoA ligase (11). It was shown previously that purified ACMS II binds threonine, the first amino acid of the pentapeptide chain, as a thioester, which can be acylated with nonnatural substrates such as p-tolyl-CoA or p-tolyl-phosphopantetheine thioesters, yielding p-tolyl-threonine (8). This suggested that 4-MHA (represented in these experiments by the structural analogue p-toluic acid) in natural conditions would be thioesterified to an unknown carrier, which most probably contained a 4’-phosphopantetheine cofac-

**FIG. 5.** Covalent binding of p-toluic acid to holo-AcmACP. AcmACP (28 μM) purified after expression in *E. coli* was incubated with p-[^14]Ctoluic acid (10 μM), ATP (10 μM), ACMS I (290 μM), and holo-ACP synthase (1 μM) in the presence (closed circles) or absence (open circles) of CoA (200 μM). The increase of the holo-form of the AcmACP from 10% basal level (mol % p-toluic acid per mol of AcmACP) was determined after trichloroacetic acid precipitation as described under “Experimental Procedures.”

**FIG. 6.** Formation of ACMS II bound p-[^14]Ctoluic acid from the 4-MHA carrier protein (AcmACP) to the threonine on ACMS II. The assay mixture to show the formation of p-[^14]Ctoluic acid on ACMS II (lane 4) contained 5.1 μM AcmACP, 14 μM p-[^14]Ctoluic acid, 10 mM ATP, 8 mM threonine, 200 μM ACMS I and ACMS II (0.016 units, expressed in and partially purified from *S. lividans*). Proteins were separated by SDS-PAGE (5%) (upper panel), and charged proteins were visualized by autoradiography for 8 weeks (lower panel). In the controls, ACMS II (lanes 1 and 1’), threonine (lanes 2 and 2’), ATP (lanes 3 and 3’), ACMS I (lanes 5 and 5’), and AcmACP (lanes 6 and 6’) were omitted.
Moreover, purified ACMS II from *S. chrysomallus* reacted, when charged with threonine, with chemically synthesized $p$-toluic acid-adenylate under formation of $p$-toluyl-threonine, suggesting that the $4\'\text{-phosphopantetheine}$ cofactor would be located on ACMS II. This view was supported by the fact that covalent binding of $p$-toluic acid to enzyme could be inhibited by sulphydryl blockers, such as dibromopropanone, $n$-ethylmaleimide, or iodoacetamide (8). Sequencing the ACMS II gene, however, revealed that ACMS II had only two $4\'\text{-phosphopantetheine}$ attachment sites, each located in the ACP domain of the threonine and valine modules (13).

The data presented here clearly show that the postulated additional $4\'\text{-phosphopantetheine}$ cofactor is located on a small 4-MHA carrier protein (AcmACP) encoded by *acmD* located downstream of the ACMS I gene *acmA* in the ACMS gene cluster (Fig. 2). The functional studies done with the purified protein expressed in the foreign host *E. coli* clearly reveal that AcmACP is specifically acylated by ACMS I with $p$-toluic acid (or 4-MHA).

**Fig. 7. Scheme of the events catalyzed by ACMS I, AcmACP, and ACMS II in formation of 4-MHA-threonine.** $p$-Toluic acid (substrate analogue of 4-MHA) was used as model substrate for ACMS I and AcmACP. Threonine, covalently bound on ACMS II, reacts with 4-MHA bound on AcmACP as thioester. Functional parts of ACMS II are schematically indicated: shaded boxes indicate the adenylation domains for threonine and valine, and E represents the epimerization domain. The black boxes indicate the peptidyl carrier domains responsible for binding of threonine and valine via thioesters. The HHXXXDG motifs in front of each activation domain signify the condensation domain. Numbers refer the amino acid residues of ACMS II sequence.
The acylated AcmACP then is used as substrate by ACMS II in the acylation of the covalently bound threonine. This is most probably with the assistance of the putative acyltransferase domain containing the HHIVMDAGF motif in the amino-terminal region of ACMS II found in front of the threonine module (Fig. 7). These results clearly assign the role as carrier in the transfer and thioesterification of 4-MHA to the acmD gene product and not to an ACP extradomain located on ACMS II.

The involvement of small ACPs and of ACP extradomains in peptide biosynthesis systems or related systems in bacteria has been realized only recently. A first example is the 8.9-kDa D-alanine carrier protein from Lactobacillus casei, which carries D-alanine through the cell membrane to membrane-associated lipoethioic acid and, in cooperation with an acceptor, forms a D-alanine ester with lipoethioic acid (30, 36). In the biosynthesis of the trilactone, enterobactin, which is a trimer of 2,3-dihydroxy-D-alanine through the cell membrane to membrane-associated alanine carrier protein from Salmonella typhimurium, a first example is the 8.9-kDa D-alanine carrier protein has been described. The role of the D-alanine carrier protein has been assigned to the transfer and thioesterification of the D-alanine ester.

These results clearly assign the role as carrier in the transfer and thioesterification of the D-alanine ester. This is most probably with the assistance of the putative acyltransferase domain containing the HHIVMDAGF motif in the amino-terminal region of ACMS II found in front of the threonine module (Fig. 7). These results clearly assign the role as carrier in the transfer and thioesterification of 4-MHA to the acmD gene product and not to an ACP extradomain located on ACMS II.

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