Biochemical and Molecular Analyses of the *Streptococcus pneumoniae* Acyl Carrier Protein Synthase, an Enzyme Essential for Fatty Acid Biosynthesis*

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Acyl carrier protein synthase (AcpS) is an essential enzyme in the biosynthesis of fatty acids in all bacteria. AcpS catalyzes the transfer of 4'-phosphopantetheine from coenzyme A (CoA) to apo-ACP, thus converting apo-ACP to holo-ACP that serves as an acyl carrier for the biosynthesis of fatty acids and lipids. To further understand the physiological role of AcpS, we identified, cloned, and expressed the *acpS* and *acpP* genes of *Streptococcus pneumoniae* and purified both products to homogeneity. Both *acpS* and *acpP* form operons with the genes whose functions are required for other cellular metabolism. The *acpS* gene complements an *Escherichia coli* mutant defective in the production of AcpS and appears to be essential for the growth of *S. pneumoniae*. Gel filtration and cross-linking analyses establish that purified AcpS exists as a homotrimer. AcpS activity was significantly stimulated by apo-ACP at concentrations over 10 μM and slightly inhibited at concentrations of 5–10 μM. Double reciprocal analysis of initial velocities of AcpS at various concentrations of CoA or apo-ACP indicated a random or compulsory ordered bi bi type of reaction mechanism. Further analysis of the inhibition kinetics of the product (3',5'-ADP) suggested that it is competitive with respect to CoA but mixed (competitive and noncompetitive) with respect to apo-ACP. Finally, apo-ACP bound tightly to AcpS in the absence of CoA, but CoA failed to do so in the absence of apo-ACP. Together, these results suggest that AcpS may be allosterically regulated by apo-ACP and probably proceeds by an ordered reaction mechanism with the first formation of the AcpS-apo-ACP complex and the subsequent transfer of 4'-phosphopantetheine to the apo-ACP of the complex.

The biosynthesis of fatty acids is known to be required for the growth of bacteria as fatty acids are essential components of bacterial membrane lipids and lipopolysaccharides (1, 2). The fatty acid biosynthetic pathway in bacteria is well characterized (1, 2). Bacteria utilize the type II or dissociated, fatty acid synthase system for fatty acid synthesis (1, 2, 4–7). The holo-acyl carrier protein (holo-ACP)1 plays an essential role as an acyl carrier for fatty acid precursors, growing acyl intermediates, and nascent fatty acid products (1–5).

ACP is a small acidic protein in bacteria (1, 2) or a small domain of the type I fatty acid synthase in eukaryotes (3). ACP in *Escherichia coli* is encoded by the *acpP* gene (1, 2). The newly synthesized ACP, or apo-ACP, is not functional in fatty acid synthesis. The conversion of apo-ACP to holo-ACP by ACP synthase (AcpS) is required for its functionality (1, 2, 4, 5). AcpS catalyzes the transfer of the 4'-phosphopantetheine moiety from coenzyme A (CoA) onto a serine residue of apo-ACP, thereby converting apo-ACP to holo-ACP (1, 2, 4–7). The holo-ACP formed then mediates the transfer of acyl intermediates by the covalent attachment of all acyl intermediates via their carboxyl group to the thiol group of the 4'-phosphopantetheine group of holo-ACP (1–8). Thus, AcpS also plays an essential role in fatty acid biosynthesis.

Homologues of AcpS and ACP have been identified in many bacterial genomes sequenced to date (9–14). *E. coli* AcpS has been well studied (4–8, 15). The *acpS* gene from *E. coli* forms an operon with the upstream gene, *pdxJ*, whose function is required for vitamin B₂₅ biosynthesis (16, 17). The *acpS* gene was originally identified as *dpj* (downstream of *pdxJ*) whose function, although unknown, was required for the growth of *E. coli* (16, 17). Later, the landmark biochemical study by Lamblot and Walsh (5) led to the identification of Dpj as AcpS. *E. coli* AcpS is a small, highly basic protein of about 14 kDa (5). The *E. coli* enzyme has been purified and characterized (5). The purified AcpS appears to be a homodimer (5). The enzyme exhibits a broad substrate specificity and can utilize a variety of ACPs that are required for many diverse aspects of cellular metabolism (5–15, 18–22). These results indicate that AcpS may be able to participate in other metabolism besides fatty acid biosynthesis in the cell. Purified AcpS also exhibits activity with a variety of CoA derivatives (15). Finally, AcpS is a very low abundance protein in *E. coli* (4, 5). In contrast, ACP is a very abundant protein that was estimated to be present at 25,000–60,000 molecules/cell (1, 2, 17). The majority of ACPs present in the cell are found to be holo-ACP (1, 2, 25, 26).

Although *E. coli* AcpS is well studied, the reaction mecha-

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1 The abbreviations used are: ACP, acyl carrier protein; AcpS, acyl carrier protein synthase; CoA, coenzyme A; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; PCR, polymerase chain reaction; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; sulfo-EGS, ethylene glycolbis(succinimidyl)succinate; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]-glycine.

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nism of AcpS remains unknown. In addition, only the AcpS from *E. coli*, a rod-shaped, Gram-negative bacterium, has been thoroughly characterized to date. It still remains to be determined whether AcpS from Gram-positive bacteria would play the same physiological role. Finally, AcpS appears to possess all of the features necessary for a good antibacterial target, such as its essential nature, widespread existence in bacteria, and unique catalytic position in a pathway (fatty acid biosynthesis). Thus, AcpS might be a valuable antibacterial target for identifying novel antimicrobial agents. To better understand the function of AcpS in *Streptococcus pneumoniae*, a sphere-shaped, Gram-positive bacterium and also a major human pathogen of the upper respiratory tract, and to explore AcpS as an antibacterial target, we first cloned and expressed the *acpS* and *acpP* genes of *S. pneumoniae* and characterized both gene products. The results of this study show that *S. pneumoniae* AcpS shares many biochemical properties with *E. coli* AcpS but also exhibits major differences with respect to their native structures and substrate regulations. In addition, the results of this study suggest that AcpS proceeds by an ordered reaction mechanism with the first formation of the enzyme-apo-AcpP intermediate from apo-Acp followed by the transfer of 4'-phosphopantetheine from CoA to the apo-Acp of the complex. Finally, both *acpP* and *acpS* form complex operons with the genes whose functions are not required for fatty acid biosynthesis.

**EXPERIMENTAL PROCEDURES**

**Materials**—Unless specified otherwise, all fine chemicals were purchased from Sigma. All fast protein liquid chromatography resins and columns used for protein purification and strains and reagents for construction, expression, and purification of GST-fused proteins were obtained from Amersham Pharmacia Biotech. Luzia Bertani (LB) broth medium was purchased from Bio 101, Inc. (Vista, CA). All polycrylamide gels and reagents were purchased from Novex (San Diego, CA). SYPRO Orange and Bradford protein assay reagents were purchased from Bio-Rad, and ethylene glycolbis(succinimidylsuccinate) (sulfo-

**Construction of the acpP and acpS Genes of S. pneumoniae (hex) R6**—The *acpP* and *acpS* genes were cloned from *S. pneumoniae* by PCR as described before (27). All of the reagents, plasmids, and cell lines used for cloning and expression were the same as those described before (27). Based on the initial sequence of *acpS*, it was thought that the *acpP* open reading frame started at the second Met codon (see GenBank accession number AF278617). Thus, the primers responding to this sequence were designed and used for cloning the *S. pneumoniae* *acpS* gene (see below). However, we have recently realized that the open reading frame of the *acpP* gene probably starts at the first Met codon rather than the second codon (GenBank accession number AF278617). Thus, the *acpS* gene cloned and expressed might lack the first two codons encoding the amino acid residues of Met-Arg. To clone the *acpS* gene, the following PCR primers were designed and used to amplify the *acpS* gene for cloning into *E. coli* expression systems. The 5' PCR primer (5'-CGCGGATCCTATTTTGCGACGCGATTTG-3') was designed at the ATG start codon of *acpS* and contains BamHI and NdeI sites for cloning purposes. The 3' PCR primer (5'-CGCGGATCCTAGTTCGACGCGATTTG-3') was designed at the stop codon of *acpP* and contains a BamHI site after the stop codon. Using these primers, *acpS* was PCR-amplified from *S. pneumoniae* for 25 cycles under the conditions as described before (27). Five PCR products were combined, and a portion of the pooled PCR products was digested with BamHI. The BamHI-digested PCR fragment was cloned into pCZA342, a low copy number plasmid (28) that had been digested with BamHI and dephosphorylated with calf intestinal alkaline phosphatase. *acpS* from several pCZA342 clones was sequenced, and a clone containing the consensus *acpS* gene sequence was used for constructing expression systems. This pCZA342 clone was digested with NdeI and BamHI. The NdeI–BamHI DNA fragment containing *acpS* was subcloned into pET-11a (Novagen). The resulting construct was designated as pRBP-19. The pCZA342 clone was also digested with BamHI, and the BamHI fragment of *acpS* was subcloned into pGEX-2T, resulting in pRBP-20.

To clone the *acpP* gene, the following PCR primers were used for amplification: the 5' PCR primer (5'-CCGCGATCCTATTTTGCGACGCGATTTG-3') and the 3' PCR primer (5'-CCGCGATCCTATTTTGCGACGCGATTTG-3'). Using these primers, *acpP* was PCR-amplified from *S. pneumoniae* as above. The PCR products were digested above. The PCR products were digested above. The PCR product was digested with BamHI. The BamHI-digested PCR fragment was cloned into pCZA342. The pCZA342 clone was digested with NdeI and BamHI. The NdeI–BamHI DNA fragment containing *acpP* was subcloned into pET-11a (Novagen), resulting in pRBP-16.

**Construction of AcpS and AcpP from S. pneumoniae**—LY128 (E. coli BL21 [pRBP-16]) was grown at 35°C in LB broth medium supplemented with 100 μg/ml ampicillin. The overnight culture (40 ml) was then inoculated into 1000 ml of LB medium supplemented with ampicillin and grown with shaking at 250 rpm until an *A*<sub>600</sub> of 0.5–0.6 was reached. The culture was induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside for 3 h. Cells were harvested by centrifugation at 4500 × g for 4°C for 8 min, washed twice in phosphate-buffered saline (PBS), resuspended in 50 mM citrate phosphate, pH 6.0, and disrupted by passing twice through a French pressure cell. The resulting cell extract was centrifuged at 160,000 × g for 40 min at 4°C. The supernatant fraction was collected and applied to a 15S Source Q column (2.5 × 8 cm) that had been equilibrated with 50 mM citrate phosphate, pH 6.0 (buffer A). The column was washed with buffer A and eluted with 0–1 M KCl in buffer A. Fractions (7 ml each) were collected. The presence of AcpS in the fractions was detected by SDS-PAGE analysis (16% Tricine gels) (29). The fractions containing AcpS were pooled and applied to a S-100 Sepharose preparative gel filtration fast protein liquid chromatography column (5.0 × 60 cm) equilibrated with 50 mM Tris-HCl, pH 7.0, 100 mM KCl. The fractions containing AcpS were collected, adjusted with glyc erol to a final concentration of 15% (v/v), and stored in small aliquots at −70°C. Protein concentration was determined using a protein assay kit (Bio-Rad) with bovine serum albumin as a standard (30).

LY135 (E. coli XL1 Blue (mRF’/pRBP-20)) was grown, harvested, disrupted, and centrifuged as described above. The supernatant fraction was applied to a glutathione-Sepharose 4B column (10 ml) that had been equilibrated with 100 ml of PBS. The column was washed with 100 ml of PBS, and the GST-AcpS fusion protein was eluted with 10 mM glutathione in PBS. Fractions were analyzed by SDS-PAGE (12% gly cine), and those fractions containing GST-AcpS were pooled, dialyzed against 50 mM Tris-HCl, pH 7.0 (4 liter), adjusted with glycerol to a final concentration of 15% (v/v), and stored at −70°C as described above.

LY140 (E. coli BL21 (pLySuV/pRBP-16) was grown, induced, harvested, and disrupted as described above. The resulting cell extract was centrifuged as described above. The supernatant fraction was collected and applied to a 15S Source Q column (2.5 × 8 cm) that had been equilibrated with 50 mM Tris-HCl, pH 8.0, 100 mM KCl (buffer C). The column was washed with 100 ml of buffer C and eluted with a linear gradient of 0.1–1.0 M KCl in buffer C. Fractions (7 ml each) were collected, and the presence of AcpP in the fractions containing AcpP was detected by SDS-PAGE as described above (16% tricine gels). The fractions containing apo-AcpP were pooled and applied to a S-100 Sepharose gel filtration column (5.0 × 60 cm) equilibrated with 50 mM Tris-HCl, pH 7.0, 100 mM KCl. The column was eluted with the same buffer. Fractions (10 ml each) containing apo-AcpP were collected, analyzed by electrospray mass spectrometry, and stored at −70°C as described above.

**Analysis of AcpS by Gel Filtration Column Chromatography**—To determine the native structure of AcpS, a purified AcpS preparation (375 μg) was applied to a S-75 Superdex gel filtration column (HR 1.0 × 30 cm), equilibrated with 50 mM Tris-HCl, pH 7.0, 50 mM KCl, 10 mg/ml of MgCl<sub>2</sub>. The column was calibrated with the protein molecular weight standards (Sigma). The effect of detergent or salt on the native structure of AcpS was analyzed by treating AcpS with 6 M CHAPS or 50–500 mM KCl before and during column chromatography.

**Sedimentation centrifugation analysis of AcpS** was carried out using an XLA ultracentrifuge (Beckman Instruments, Fullerton, CA). A purified AcpS preparation (adjusted to 0.2 and 0.4 mg/ml) was centrifuged at 16,000 rpm for 24 h at 22°C. The absorbance at 280 nm as a function of the radius of gyration for the system reaches equilibrium was analyzed using XL-AWX-1.1, a non-data least-squares fit data analysis program. Partial specific volume of AcpS was calculated to be 0.721 ml/g based on its amino acid sequence. The molecular weight of AcpS was determined using a global fit of the two data sets collected with 0.2 and 0.4 mg/ml samples.

Cross-linking experiments were performed as follows. Purified AcpS and apo-AcpP preparations (1 ml each) were dialyzed against 2 liters of
ACP Synthase of S. pneumoniae

20 mM potassium phosphate buffer, pH 7.0, at 4°C for 18 h. The dialyzed AcpS (163 μM) and apo-ACP (94 μM) preparations were mixed without or with 19.5 and 9.4 mM sulfo-EGS, respectively, and the mixtures were incubated at room temperature for 30 min. The reactions were stopped by the addition of 50 mM Tris-base followed by incubation at room temperature for 30 min. The resulting AcpS and apo-ACP preparations treated without or with the cross-linker (10 μl) were mixed with an equal volume of Tricine sample buffer and analyzed by SDS-PAGE (16% Tricine gels).

To determine whether AcpS binds directly to apo-ACP or CoA in the absence of the other substrate, a purified AcpS preparation (27 μM) was first mixed with 10 mM MgCl₂ and then either 100 μM apo-ACP or 50 μM CoA. The mixture was incubated at room temperature for 30 min and subjected to gel filtration column chromatography (S-75 Superdex) under the conditions described above. The fractions containing the AcpS-apo-ACP complex and unbound apo-ACP were analyzed by SDS-PAGE (16% Tricine gels), SYPRO Orange staining, and mass spectrometry.

Enzyme Assay and Kinetics—Unless otherwise indicated, reaction mixtures contained 50 mM Tris-HCl, pH 7.0, 10 mM MgCl₂, 2.5–50 μM CoA, 0.25–6.0 μM purified apo-ACP of S. pneumoniae, and 3.7 nM purified AcpS of S. pneumoniae and were incubated at 37 °C for 9 min. Reactions were stopped by the addition of 50 mM EDTA. The formation of holo-ACP was measured by the HPLC method or the trichloroacetic acid precipitation method. To determine Kₘ values, we cloned and expressed the acpS gene that encodes AcpS of S. pneumoniae—like other ACPs (1, 2, 20, 34–36), this suggests that a genetic reorganization event consisting of the complex operons that currently exist in the bacteria is also different from those of the acpP genes in E. coli, and other organisms (20, 34–36). This suggests that a genetic reorganization event might have occurred during evolution, which resulted in the formation of the complex operons that currently exist in the organisms such as S. pneumoniae.

The subunits of S. pneumoniae AcpS and apo-ACP exhibit molecular weights virtually identical to those of E. coli AcpS and apo-ACP, respectively. Both proteins also share 38% identities with their counterparts in E. coli. The pl value of S. pneumoniae AcpS was estimated to be 6.5, which is much lower than 9.98, the pl value of E. coli AcpS (16, 17). Therefore, S. pneumoniae AcpS is significantly less basic than E. coli AcpS. Like other ACPs (1, 2, 20, 34–36), S. pneumoniae apo-ACP is very acidic, with a pl value of only 3.4.

Finally, we tested whether the S. pneumoniae acpS gene complements E. coli mutant strain, HT253, defective in the production of AcpS (17). HT253 contains a mini-Tn10 insertion in the pdxJ gene, which is upstream of and forms an operon with a product that encodes glycerol 3-phosphate acyltransferase, z is involved in different aspects of cellular metabolism such as histidine biosynthesis (histC encoding histidinol phosphate aminotransferase), lipid biosynthesis (pdxX, required for the pl values of 6.5 and 3.4—16, 17). Therefore, S. pneumoniae AcpS is significantly less basic than E. coli AcpS. Like other ACPs (1, 2, 20, 34–36), S. pneumoniae apo-ACP is very acidic, with a pl value of only 3.4.

RESULTS

Identification and Organization of the acpS and acpP Genes of S. pneumoniae—To understand the function of AcpS in the biosynthesis of fatty acids in S. pneumoniae, we cloned and expressed the acpS gene as well as the acpP gene that encodes a substrate of AcpS. Both genes were identified from our S. pneumoniae data base (32) using the E. coli acpS and acpP gene sequences as queries in the BLAST program (33). The acpS gene, 369 base pairs long, encodes a protein consisting of 122 amino acid residues with a predicted molecular mass of 13.7 kDa (GenBank™ accession number AF276617). The acpS gene appears to be organized into an operon with the genes in the order argF-aroF-acpS-alr-recG, since there are long non-coding regions located upstream of argF and downstream of recG. Thus, the acpS operon appears to consist of the genes that are required in aromatic amino acid biosynthesis (aroF and aroG encoding 3-deoxy-D-arabino-heptulosonate 7-phosphate synthases), cell wall biosynthesis (alr encoding N-alanine racemase), and DNA recombination (recG). In this regard, the genomic organization of acpS in S. pneumoniae is quite different from that of acpS in E. coli, since acpS in E. coli consists of an operon with its upstream pdxJ gene that is required for vitamin B₉ biosynthesis (16, 17).

The acpP gene, 234 base pairs long, encodes a protein consisting of 77 amino acid residues with a predicted molecular mass of 8.7 kDa (GenBank™ accession number AF276618). The acpP gene appears to consist of an operon with the genes in the order hisC-unknown-plsX-acp. There are very long non-coding regions located in the upstream of hisC and downstream of acpP. Like the acpS operon, the genes in the acpP operon are also involved in different aspects of cellular metabolism such as histidine biosynthesis (hisC encoding histidinol phosphate aminotransferase), lipid biosynthesis (pdxX, required for the pl values of 6.5 and 3.4—16, 17). Therefore, S. pneumoniae AcpS is significantly less basic than E. coli AcpS. Like other ACPs (1, 2, 20, 34–36), S. pneumoniae apo-ACP is very acidic, with a pl value of only 3.4.

Finally, we tested whether the S. pneumoniae acpS gene complements E. coli mutant strain, HT253, defective in the production of AcpS (17). HT253 contains a mini-Tn10 insertion in the pdxJ gene, which is upstream of and forms an operon with acpS (16, 17). The mini-Tn10 carries two divergent tetra-cycline-inducible promoters (17). In the absence of tetracycline, HT253 could not grow on LB plates because the mini-Tn10 insertion in pdxJ blocks the transcription of the acpS gene. Thus, the growth of HT253 is tetracycline-dependent. When the acpS gene (pRBP123, acpS carried on pGEX-2T) was introduced into HT253, this mutant strain was able to grow on LB medium without the supplementation of tetracycline and isopropyl-1-thio-β-D-galactopyranoside. Apparently, the basal level expression of acpS without isopropyl-1-thio-β-D-galacto-
pyranoside induction was sufficient for the complementation of HT253. This result clearly shows that the \textit{S. pneumoniae} \textit{acpS} gene complements the \textit{E. coli} mutant deficient in the production of AcpS. Attempts to inactivate the \textit{acpS} gene of \textit{S. pneumoniae} through genetic insertional mutagenesis failed (28), indicating that \textit{acpS} is essential for the growth of \textit{S. pneumoniae}.

\textbf{Expression, Purification, and Identification of the AcpS and ACP of \textit{S. pneumoniae}—}The \textit{acpS} and \textit{acpP} genes identified were cloned into expression vectors and expressed in \textit{E. coli} (see “Experimental Procedures”). Both AcpS and apo-ACP were highly expressed in \textit{E. coli} and exhibited the molecular weights predicted (Fig. 1). The overexpressed AcpS was purified to apparent homogeneity in two steps (Fig. 1A) using Source S-cation exchange and gel filtration column chromatography. The overexpressed \textit{S. pneumoniae} apo-ACP was also purified to apparent homogeneity (Fig. 1B) in two steps using Source Q-anion exchange and gel filtration column chromatography.

To confirm the purified proteins as AcpS and ACP, we performed N-terminal sequencing and mass spectrometric analyses. The first 9 amino acid residues of purified AcpS were determined to be MIVGHGIDI, a sequence that is identical to the predicted amino acid sequence for the protein encoded by the cloned \textit{acpS} gene. This encoded protein is predicted to have a molecular weight of 13,388. Consistent with this predicted value, mass spectrometric analysis showed that purified AcpS had a molecular weight of 13,390. Thus, the purified protein is \textit{S. pneumoniae} AcpS.

N-terminal sequencing analysis also showed that purified apo-ACP exhibited the predicted amino acid sequence (data not shown). When subjected to mass spectrometric analysis, purified apo-ACP was found to exhibit two peaks, the major peak with a molecular mass of 8834 Da (about 80% of the total protein) and the minor peak with a molecular mass of 8861 Da (20%) that is 26 Da larger than that of the major species. The predicted molecular weight for \textit{S. pneumoniae} apo-ACP is 8706, thus in agreement with the results of mass spectrometric analysis. Mass spectrometric analysis further showed that both apo-ACPs were converted to holo-ACP upon their reaction with AcpS, since the molecular weights of both ACPs were increased by 341 Da, corresponding to the molecular weight of the 4'-phosphopantetheine group (data not shown). Finally, mass spectrometric analysis showed that the presence of holo-ACP was not detectable in the apo-ACP preparations (data not shown).

The mobility of apo-ACP and holo-ACP was examined by native gel electrophoresis followed by staining with SYPRO Orange (see “Experimental Procedures”). Holo-ACP was found to migrate more slowly than apo-ACP (data not shown). The complete conversion of apo-ACP to holo-ACP was confirmed as evidenced by the fact that the molecular weight of ACP was increased from 8,834 Da (apo-ACP) to 9,174 Da (holo-ACP) upon the treatment of apo-ACP with AcpS, CoA, and Mg\(^2+\). Thus, unlike \textit{E. coli} holo-ACP (5), \textit{S. pneumoniae} holo-ACP migrates more slowly than apo-ACP.

\textbf{Determination of the Native Structures of \textit{S. pneumoniae} AcpS and ACP—}To determine the molecular weight of native AcpS, we subjected a purified AcpS preparation to gel filtration column chromatography analysis. AcpS was eluted in the fractions corresponding to a molecular mass of 38 kDa (Fig. 2A, peak B). This result suggests that AcpS is a homotrimer with a predicted molecular mass of 41 kDa (GenBank\textsuperscript{TM} accession number AF276617). To confirm this further, a purified AcpS preparation was subjected to sedimentation analysis. This analysis showed that purified AcpS had a molecular mass of 39

\footnote{P. Treadway, unpublished results.}
kDa, which is consistent with that of gel filtration analysis. Finally, when a purified AcpS preparation was subjected to cross-linking (see “Experimental Procedures”) followed by SDS-PAGE analysis, two protein bands were observed (Fig. 3). The two bands had molecular masses of 10.4 and 28.2 kDa, respectively, thus corresponding to the monomeric and trimeric forms of AcpS (Fig. 3, lane 2). Taken together, these results show that the AcpS of *S. pneumoniae* is a trimeric enzyme. The trimeric structure of AcpS appears to be stable, since AcpS still retained its native structure in the presence of 6 mM CHAPS or 50–500 mM KCl during gel filtration column chromatography (data not shown).

When apo-ACP was also subjected to gel filtration column chromatography, it was eluted in the fractions corresponding to a molecular mass of 17 kDa, thus indicating that apo-ACP may exist as a dimer (Fig. 2A). Apo-ACP has been shown to behave abnormally on gel filtration columns due to its molecular asymmetry in shape (39, 40). To examine further whether apo-ACP is a dimer, purified apo-ACP was subjected to cross-linking followed by SDS-PAGE analysis. Only one protein band was observed, which had a molecular mass of 5.6 kDa (Fig. 3B). Thus, this result shows that apo-ACP is a monomeric protein. The result of the gel filtration column analysis was consistent with the previously reported anomalous behavior of apo-ACP on gel filtration columns (39, 40).

**Kinetic Characterization of *S. pneumoniae* AcpS**—To elucidate the reaction mechanism of AcpS, we examined its substrate specificity and kinetics. The purified AcpS of *S. pneumoniae*, when assayed by the HPLC method, exhibited an optimal activity at 45–50 °C and pH 6.5 and was stable at 22–65 °C. AcpS was able to utilize a number of CoA derivatives as substrates and exhibited the following relative activities: 100 (CoA), 91 (acetyl-CoA), 76 (desulfo-CoA), 65 (acetoacetyl-CoA), 12 (malonyl-CoA), and 0 (dephospho-CoA). Thus, like *E. coli* AcpS and *B. subtilis* Sfp protein, *S. pneumoniae* AcpS utilizes different CoA derivatives as substrates (15, 41).

*S. pneumoniae* AcpS appears to exhibit Michaelis-Menten kinetics when assayed at various CoA concentrations and apo-ACP concentrations lower than 10 μM (Fig. 4A). AcpS activity increased in a dose-dependent manner at the apo-ACP concentrations of 0.5–5 μM (Fig. 4A). Then, when the concentration of apo-ACP approached 10 μM, AcpS activity decreased (Fig. 4A). This result is consistent with the observation that apo-ACP is inhibitory to AcpS at higher concentrations (4–6, 15). However, a further increase of apo-ACP concentrations (>10 μM) was accompanied with a significant increase in AcpS activity (Fig. 4A). As a result, two separate substrate saturation curves were obtained at low and high concentrations of apo-ACP (Fig. 4, B and C). The double reciprocal plot analyses indicated that AcpS had *Km* (for apo-ACP) values of 0.5 ± 0.08 and 109 ± 6.8 μM and *Vmax* values of 2439 ± 243 (kcat = 1.7 ± 0.17 s⁻¹) and 13,659 ± 1290 (kcat = 9.3 ± 0.9 s⁻¹) nmol/min/mg at the low and high concentrations of apo-ACP, respectively. Thus, at higher apo-ACP concentrations, the affinity of AcpS for apo-ACP was significantly decreased (approximately 200-fold), but its catalytic activity was significantly increased (5-fold). Together, these results indicate that the *S. pneumoniae* AcpS may be allosterically regulated by its substrate, apo-ACP.

When a fixed apo-ACP concentration and various CoA concentrations were used, a hyperbolic substrate saturation curve was obtained for AcpS (Fig. 5A). The apparent *Km* and *Vmax* values of AcpS were determined to be 11.5 ± 0.9 μM (for CoA) and 3976 ± 73 nmol/min/mg (kcat = 2.7 ± 0.05 s⁻¹), respectively (Fig. 5B). The kcat values determined for AcpS at low apo-ACP concentrations were in good agreement (1.7 versus 2.7 s⁻¹).

Since the trichloroacetic acid precipitation method has often been used for the assay of AcpS activity (4–6), we also characterized the kinetic properties of the enzyme using this assay method. This assay utilizes [³H]CoA as a substrate for AcpS. The apparent *Km* values of the enzyme for apo-ACP and CoA were determined to be 1.3 ± 0.7 and 7.1 ± 0.4 μM, respectively. The *Vmax* (kcat) values determined were 4179 ± 182 (2.8 ± 0.04 s⁻¹) nmol/min/mg. Thus, the kinetic parameters determined by the trichloroacetic acid precipitation method are in general agreement with those obtained by the HPLC method. However, we did notice that the trichloroacetic acid precipitation method tended to generate variations significantly higher than those of the HPLC method, especially when apo-ACP was below 1 μM.

Although *E. coli* AcpS has been extensively studied (4–7), the kinetic mechanism of the enzyme is unknown. To further elucidate the kinetic mechanism of *S. pneumoniae* AcpS, we analyzed the double reciprocal plots of the initial velocities of the enzyme at fixed concentrations of one substrate versus various concentrations of the other substrate (42). This analysis yielded *Km* and *Vmax* values that are similar to those deter-
mined before (data not shown). As shown in Fig. 6A, the double reciprocal plots of the initial velocities of AcpS obtained at the various CoA and fixed apo-ACP concentrations yielded an intersecting pattern. The same pattern was obtained when various concentrations of apo-ACP and fixed concentrations of CoA were used (Fig. 6B). Together, these results suggest that AcpS proceeds by a random or compulsory bi bi type but not a ping-pong (double displacement) type of reaction mechanism (42).

To differentiate these two possible reaction mechanisms, we analyzed the kinetics of product inhibition. AcpS activity was examined in the presence of 3',5'-ADP. As shown in Fig. 7A,
when various CoA concentrations were used, the double reciprocal plots yielded a simple competitive pattern with a $K_i$ of 6.0 mM (Fig. 7C). However, when various apo-ACP concentrations were used, the double reciprocal plots yielded a linear mixed pattern with a $K_i$ of 2.5 mM (Fig. 7, A and D). Since the patterns of inhibition with respect to CoA and apo-ACP are competitive and mixed, respectively, these results suggest that apo-ACP is probably the first substrate to bind to the enzyme, which is followed by CoA (Ref. 42; see "Discussion").

The Binding of apo-ACP and CoA to AcpS—To determine the order of substrate binding to AcpS, we analyzed the binding of CoA and apo-ACP to purified AcpS by gel filtration column chromatography, mass spectrometry, or filter binding assays. We reasoned that if CoA binds to AcpS first and forms an enzyme-substrate complex that is required for the next reaction with apo-ACP, then a stable enzyme-substrate complex should be detectable. When a mixture of CoA and purified AcpS that had been incubated at room temperature for 30 min (see "Experimental Procedures") was subjected to gel filtration column chromatographic, mass spectrometry, or filter binding assays, the presence of CoA was not detectable in the fractions containing purified AcpS (data not shown). Thus, CoA did not appear to bind to AcpS in the absence of apo-ACP. To further examine the binding of CoA to AcpS, a mixture of purified AcpS and $[^3H]$CoA that had been incubated under the same conditions was subjected to a filter binding assay (see "Experimental Procedures"). There was no evidence that $[^3H]$CoA was bound to AcpS, since the radioactivity of $[^3H]$CoA was not detectable after washing (data not shown). Thus, CoA does not appear to bind to AcpS in the absence of apo-ACP.

To examine whether apo-ACP binds to AcpS in the absence of CoA, we subjected a mixture of AcpS and apo-ACP (apo-ACP/AcpS = 5:1) to gel filtration column chromatography and analyzed the column fractions by SDS-PAGE (see "Experimental Procedures"). Two protein peaks were observed (Fig. 2A). The leading peak (peak A) had a molecular mass of approximately 53 kDa as judged by gel filtration analysis (Fig. 2A). Consistent with the formation of the AcpS-apo-ACP complex, the presence of apo-ACP was also detected in the fractions containing purified AcpS (Fig. 2B). Together, these results show that apo-ACP can bind to AcpS in the absence of CoA.

**DISCUSSION**

In this study, we have identified the acpS and acpP genes from *S. pneumoniae* and purified and characterized their gene products. Sequencing analysis shows that both acpS and acpP form complex operons with the genes whose functions are required for other cellular metabolism. We have established, by using a variety of biochemical approaches, that purified AcpS and apo-ACP existed as a homotrimer and monomer, respectively, and that apo-ACP bound tightly to AcpS, but CoA failed to do so. Kinetic analysis suggests that AcpS proceeds by an ordered reaction mechanism with the first formation of the enzyme-apo-ACP intermediate from apo-ACP and the subsequent transfer of the 4'-phosphopantetheine group from CoA to apo-ACP.

The biochemical identification of *dpj* as *acpS* (5) is significant. This work has led to the subsequent identification of a number of AcpS-like enzymes in different bacterial species that are required for the biosynthesis of polyketides, enterobactin siderophore, and others (7) and has established the cross-functionality of ACP and AcpS in different biosynthetic systems (6, 7, 15, 18–22, 41). The AcpS from *E. coli*, a rod-shaped, Gram-
negative bacterium, is the best characterized enzyme among the AcpS-like enzymes identified. It is a small, basic protein with a molecular weight of 13,922 that can utilize a variety of CoA derivatives as substrates and is inhibited by apo-ACP at higher concentrations (>10 μM) (4, 6, 15). This apparent inhibition results from the electrostatic interaction between the basic AcpS enzyme and the acidic apo-ACP (6). Likewise, the AcpS from *S. pneumoniae*, a sphere-shaped, Gram-positive bacterium, is also a small protein with a virtually identical size, can use different CoA derivatives, and is slightly inhibited by apo-ACP at similar concentrations although stimulated at higher concentrations.

Both *E. coli* and *S. pneumoniae* AcpS enzymes also appear to exhibit similar kinetic properties. Purified *E. coli* AcpS had *Km* values of 1.5 and 50 μM for apo-ACP and CoA, respectively, and a *kcat* value of 1–2 s⁻¹ (5, 6). At low apo-ACP concentrations, purified *S. pneumoniae* AcpS had *Km* values of 0.5 and 11.3 μM for apo-ACP and CoA, respectively, and a *kcat* value of 1.8–2.5 s⁻¹. Clearly, both enzymes have significantly higher (20–30-fold) affinities for apo-ACP than for CoA. It is known that the intracellular CoA and ACP concentrations vary depending on the stage of growth and carbon sources (43–45). The CoA and total ACP pools were estimated to be 20–90 and 4–10 pmol/10⁸ cells, respectively (23, 24, 26, 43–45). Thus, the CoA and total ACP concentrations in the cell are approximately 400–1800 and 90–200 μM, respectively, if the cell volume is assumed to be 0.5 μM³ (46). Interestingly, the relative affinities of both AcpS enzymes determined for their substrates apparently correlate with the estimated concentrations of CoA and ACP. These kinetic properties and the in vivo concentrations suggest that under physiological conditions, the concentrations of both CoA and ACP are probably over the *Km* values of AcpS even if apo-ACP is a small fraction of the total ACP pool. Therefore, both substrates might not be a rate-limiting factor in the conversion of apo-ACP to holo-ACP in the cell. This may explain why ACP exclusively exists as holo-form (1).

One of the major differences between *E. coli* and *S. pneumoniae* AcpS enzymes appears to be their native structures. *E. coli* AcpS was reported to be a homodimer with a molecular mass of 28 kDa (5). *S. pneumoniae* AcpS is shown, in this study, as a homotrimer. Several lines of evidence support the conclusion that *S. pneumoniae* is a trimeric enzyme. The gel filtration analysis showed that purified AcpS had a molecular mass of 38 kDa. Since AcpS has a molecular mass of 13.7 kDa based on its DNA sequence, a homotrimer of AcpS is predicted to have a molecular mass of 41 kDa, which is consistent with the result of gel filtration analysis. In addition, the sedimentation analysis showed that purified AcpS had a molecular mass of 39 kDa. Finally, the results of our cross-linking experiments clearly demonstrated the presence of two predominant protein species with molecular masses of 10.4 and 28.2 kDa, respectively, as resolved by SDS-PAGE. Taken together, these results establish that *S. pneumoniae* AcpS does exist as a homotrimer. In light of this finding, it is possible that AcpS enzymes from Gram-positive bacteria may differ from those of Gram-negative bacteria with respect to their native structures. It also remains possible that *E. coli* AcpS might exist as a homotrimer. It should be noted that *E. coli* AcpS is significantly more positively charged than *S. pneumoniae* AcpS. It is possible that *E. coli* AcpS might migrate faster than *S. pneumoniae* AcpS during gel filtration column chromatography. As a result, *E. coli* AcpS appears to behave as a dimer as judged by gel filtration analysis (5).

The other difference between *E. coli* and *S. pneumoniae* AcpS enzymes appears to lie in their regulation by the substrate, apo-ACP. Both enzymes are inhibited by apo-ACP at similar concentrations (6). However, the activity of *S. pneumoniae* AcpS, but not that of *E. coli* AcpS, is significantly stimulated by apo-ACP at even higher concentrations (Fig. A4). The stimulation by apo-ACP (Fig. A4) suggests that *S. pneumoniae* AcpS may be allosterically regulated by apo-ACP at higher concentrations. As a result, at higher apo-ACP concentrations (>15 μM), the affinity of *S. pneumoniae* AcpS for apo-ACP is significantly decreased (200-fold), and its catalytic activity is also increased (5-fold) (Fig. 4, B and C). These changes in the affinity and activity of the enzyme at high apo-ACP concentrations might have relevant physiological significance. Under the conditions where apo-ACP is overproduced in the cell, an AcpS with increased *Km* for apo-ACP and *Vmax* clearly could be significantly more efficient in the conversion of apo-ACP to holo-ACP, thereby preventing the accumulation of high levels of apo-ACP in the cell that is known to be toxic (47).

Among all AcpS-like enzymes, *E. coli* AcpS enzyme has been the most extensively studied biochemically. Studies have been primarily focused on the substrate specificity and cross-functionality of the enzyme in other analogous systems (4–7, 15, 18–22, 41). Despite the extensive characterization of the *E. coli* AcpS, the reaction mechanism of the enzyme has not been determined. In this study, we attempted to elucidate the reaction mechanism of the enzyme. The analysis of the initial velocities of AcpS obtained at fixed concentrations of one substrate and various concentrations of another reveals that AcpS probably proceeds by a random or ordered compulsory bi bi reaction mechanism, because an intersecting pattern was obtained regardless of which substrate (CoA or apo-ACP) was the fixed one or the varied one (42) (Fig. 6). To further investigate the possible mechanism, the inhibition kinetics of 3′,5′-ADP, one of the reaction products, was examined. This analysis indicates that AcpS appears to proceed by an ordered reaction mechanism with the first formation of the AcpS-apo-ACP intermediate and the subsequent transfer of 4′-phosphopantetheine from CoA onto apo-ACP. The mode of inhibition by 3′,5′-ADP with respect to CoA is competitive when apo-ACP is the fixed substrate and CoA is the varied substrate (Fig. 7). The competitive inhibition with respect to CoA indicates that CoA only binds to the enzyme-apo-ACP intermediate. The mode of inhibition by 3′,5′-ADP with respect to apo-ACP is mixed, i.e., a combination of competitive and noncompetitive inhibition when apo-ACP is the varied substrate and CoA is the fixed substrate (Fig. 7B). The mixed type of inhibition by 3′,5′-ADP with respect to apo-ACP suggests that 3′,5′-ADP binds to the free enzyme and the enzyme-apo-ACP intermediate. Thus, inhibition was competitive with respect to apo-ACP when 3′,5′-ADP binds to the free enzyme and noncompetitive with respect to apo-ACP when 3′,5′-ADP binds to the enzyme-apo-ACP intermediate. This proposed reaction mechanism for AcpS is consistent with the results of the substrate-binding experiments. Under the conditions tested, apo-ACP bound tightly to AcpS in the absence of CoA, but CoA failed to do so in the absence of apo-ACP. Taken together, these results suggest that the reaction mechanism of AcpS is ordered rather than random and that the formation of the enzyme-apo-ACP intermediate occurs first before the transfer of 4′-phosphopantetheine from CoA onto apo-ACP.

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