Acyl Carrier Protein Is a Cellular Target for the Antibacterial Action of the Pantothenamide Class of Pantothenate Antimetabolites*

Yong-Mei Zhang‡§, Matthew W. Frank‡, Kristopher G. Virga¶, Richard E. Lee‡, Charles O. Rock‡, and Suzanne Jackowski‡

From the ‡Protein Science Division, Department of Infectious Diseases, St. Jude Children’s Research Hospital, Memphis, Tennessee 38105 and the ¶Department of Pharmaceutical Sciences, University of Tennessee Health Science Center, Memphis, Tennessee 38163

Pantothenate is the precursor of the essential cofactor coenzyme A (CoA). Pantothenate kinase (CoaA) catalyzes the first and regulatory step in the CoA biosynthetic pathway. The pantothenate analogs N-pentylpantothenamide and N-heptylpantothenamide possess antibacterial activity against Escherichia coli. Both compounds are substrates for E. coli CoaA and competitively inhibit the phosphorylation of pantothenate. The phosphorylated pantothenamides are further converted to CoA analogs, which were previously predicted to act as inhibitors of CoA-dependent enzymes. Here we show that the mechanism for the toxicity of the pantothenamides is due to the inhibition of fatty acid biosynthesis through the formation and accumulation of the inactive acyl carrier protein (ACP), which was easily observed as a faster migrating protein using conformationally sensitive gel electrophoresis. E. coli treated with the pantothenamides lost the ability to incorporate [1-14C]acetate to its membrane lipids, indicative of the inhibition of fatty acid synthesis. Cellular CoA was maintained at the level sufficient for bacterial protein synthesis. Electrospray ionization time-of-flight mass spectrometry confirmed that the inactive ACP was the product of the transfer of the inactive phosphopantothenamide moiety of the CoA analog to apo-ACP, forming the ACP analog that lacks the sulfhydryl group for the attachment of acyl chains for fatty acid synthesis. Inactive ACP accumulated in pantothenamide-treated cells because of the active hydrolysis of regular ACP and the slow turnover of the inactive prosthetic group. Thus, the pantothenamides are pro-antibiotics that inhibit fatty acid synthesis and bacterial growth because of the covalent modification of ACP.

CoA is the major acyl group carrier in living systems and is synthesized by a universal series of reactions beginning with the vitamin (B₅) pantothenate (1). All of the genes and enzymes involved in the CoA biosynthetic pathway have been identified in Escherichia coli (Fig. 1). The first step in the pathway is catalyzed by the key rate-controlling pantothenate kinase (CoaA) (ATP:pantothenate 4'-phosphotransferase; EC 2.7.1.33). Cysteine is next added to the phosphopantothenate by 4'-phosphopantothenoyl cysteine synthase and rapidly decarboxylated to phosphopantetheine. These two steps are carried out by a bifunctional polypeptide, phosphopantothenoylcysteine synthetase decarboxylase (denoted CoaBC, formally Dfp) (2). The last two steps are carried out by two separate enzymes, namely phosphopantetheine adenylyltransferase (CoaD) (3, 4) followed by the addition of the 3'-ribose phosphate by dephospho-CoA kinase (CoaE) (5). E. coli is capable of de novo pantothenate biosynthesis (1) or can import pantothenate from the medium via a sodium-dependent active transport process (6–8). CoA is also required for the synthesis of ACP, the acyl group carrier in bacterial fatty acid synthesis. The phosphopantetheine moiety of CoA is transferred to serine 36 of apo-ACP by [ACP]synthase (AcpS) (9, 10), and the ACP prosthetic group is removed from the protein by [ACP]hydrolyase. The initial report identifying the [ACP]hydrolyase gene is incorrect (11, 12); therefore, the gene responsible for [ACP]hydrolyase activity remains to be isolated. Nonetheless, ACP prosthetic group turnover is very rapid in vivo, with turnover proceeding at a rate 4 times faster than the rate of new ACP synthesis (13, 14).

The pantothenamides are a class of pantothenate analogs, exemplified by the N-pentylpantothenamide (N5-Pan) and N-heptylpantothenamide (N7-Pan) (Fig. 1), that inhibit E. coli growth (15). These pantothenamides are substrates for CoaA, and the phosphorylated derivatives are used as substrates by CoaD and CoaE to produce the CoA analogs ethylidethia-CoA and butyldethia-CoA (16). The rate of conversion of N5-Pan to the CoA analog is more rapid than the conversion of pantothenate to CoA. This discovery led to the conclusion that the mechanism for the toxicity of N5-Pan is due to its biosynthetic conversion to the CoA analog ethylidethia-CoA, which acts as an inhibitor of CoA-utilizing and acetyl-CoA-utilizing enzymes (16). We have examined the metabolism of pantothenamide antimetabolites, specifically N5-Pan and N7-Pan, in further detail and conclude that their biological effects are exerted through the transfer of the inactive 4'-phosphopantothenamide moiety from the CoA analogs to ACP, resulting in the accumu-

* This work was supported by National Institutes of Health Grants GM 62896 and GM 34496, Cancer Center (CORE) Support Grant CA 21765, and the American Lebanese Syrian Associated Charities. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ To whom correspondence should be addressed: Protein Science Division, Dept. of Infectious Diseases, St. Jude Children’s Research Hospital, 332 N. Lauderdale, Memphis, Tennessee 38105-2794. Tel.: 901-495-5624; Fax: 901-495-3099; E-mail: yongmei.zhang@stjude.org.

¶ Department of Pharmaceutical Sciences, University of Tennessee Health Science Center, Memphis, Tennessee 38163

1 The abbreviations used are: CoaA, pantothenate kinase; ACP, acyl carrier protein; AcpH, [ACP]hydrolyase; AcpS, [ACP]synthase; CoaD, 4'-phosphopantetheine adenylyltransferase; CoaE, dephospho-CoA kinase; ESI, electrospray ionization; MIC, minimal inhibitory concentration; MS, mass spectrometry; N5-Pan, N-pentylpantothenamide; N7-Pan, N-heptylpantothenamide; PanF, pantothenate permease; TOF, time-of-flight.
The elution solvent was then removed under high heat and vacuum on a rotary evaporator to complete dryness. The resulting free acid obtained in this procedure (4 g, 18.2 mmol) was a viscous, straw-colored oil, which was then redissolved in 10 ml of dry dimethylformamide. The 10-ml solution was split into two 5-ml fractions and placed into 100-ml reaction vessels. To each solution an additional 10 ml of dry dimethylformamide was added. For the synthesis of N5-Pan, amylamine (1.16 ml, 10 mmol) and diphenylphosphoryl azide (2.24 ml, 10 mmol) were added to the first pantothenate solution. For the synthesis of N7-Pan, heptylamine (1.48 ml, 10 mmol) and diphenylphosphoryl azide (2.24 ml, 10 mmol) were added to the second pantothenate solution. The reaction mixtures were then cooled to 0 °C, and triethylamine (1.39 ml, 10 mmol) was added to each. The reactions were stirred at 0 °C for 2 h, followed by stirring at room temperature overnight. The reaction volumes were reduced by rotary evaporation under high vacuum to remove dimethylformamide. Each of the products was then purified by flash column chromatography on a silica gel first with 100% ethyl acetate followed by a linear gradient of 100.0 to 95.5 chloroform/methanol. The fractions containing each of the products were collected and concentrated in vacuo, yielding both products as white powders (1.6 g with 60% yield and 2.1 g with 71% yield, respectively).

**Determination of Minimal Inhibitory Concentrations**—The MICs of the pantothenamides against the *E. coli* strains were determined by a broth microdilution method. Bacterial culture was grown to mid-log phase in 1% tryptone broth before being diluted 30,000-fold in the same medium. A 0.1-ml aliquot of the diluted cell suspension (3,000–5,000 colony forming units) was used to inoculate each well of a 96-well plate (U-bottom with low evaporation lid) containing 100 μl of tryptone broth with the indicated concentration of inhibitors. To study the effect of pantothenate on the MICs, the tryptone broth was supplemented with 100 μM pantothenate. The plate was incubated at 37 °C for 20 h before being read by a Fusion™ universal microplate analyzer (Packard Instrument Co.) at 600 nm. The growth of cells treated with an equal volume of the solvent vehicle was considered as 100%.

**1-14C-Acetate Labeling of Membrane Lipids**—Strain ANS1 was grown to early log phase (20 Klett units) in M9 minimal medium supplemented with 0.1% casamino acids, 0.4% glycerol, and 0.0005% thiamine. The culture was harvested by centrifugation. The cell pellets were washed with phosphate-buffered saline and resuspended in 100 μl of M9. The total cellular lipids were extracted with chloroform-methanol (19), and the incorporated 14C-isotope in lipids in the chloroform phase was quantitated by scintillation counting. To test whether N5-Pan interferes with the protein synthesis in *E. coli*, we labeled strain ANS1 cells with 1 μl of the L-[^3-H]alanine mixture (specific activity, 54 μCi/μmol). Aliquots of 1-ml cell suspension containing 100 μM N5-Pan for 0.5, 1, 2, and 3 h (labeled with the L-[^3-H]alanine acid mixture for 15 min) were collected on Millipore HA 0.45 μm filters. The filters were washed with 5 ml of phosphate-buffered saline and transferred to scintillation vials with 3 ml of scintillation fluid to count the incorporated 3H-isotope. Tetracycline (200 μg), a known protein synthesis inhibitor, and Me2SO were included as controls.

**β-[^3-H]Alanine Labeling of CoA in N5-Pan-treated Cells**—Strain SJ16 was grown on M9 minimal medium supplemented with 0.5 μM β-[^3-H]Alanine, 0.1% casamino acids, 0.4% glycerol, and 0.0005% thiamine to early log phase (20 Klett units). N5-Pan was added to the growing cells to a final concentration of 10 μM. One-milliliter aliquots of cells were removed at 0.5, 1, 2, and 4 h after the addition of N5-Pan. Cells were harvested with centrifugation and lysed with lysozyme and Triton X-100 (100 μl). CoA and ACP in 10 μl of the cell lysate were separated on a pre-activated silica gel H thin layer plate (Analtech), which was developed in butanol/acetic acid/H2O at 5:2:4 (v/v/v). The 3H-labeled CoA and pathway intermediates were detected using an AR-2000 TLC Imaging Scanner (BIOSCAN).

**Effects of Exogenous Fatty Acids on the MIC of N5-Pan for Streptococcus pneumoniae R6**—To test whether inhibition of N5-Pan for *S. pneumoniae* strain R6 was determined using the microdilution method described above, and a defined semisynthetic medium (C+Y medium) (20) was used to replace the tryptone broth. To test whether fatty acid can reverse the inhibition of N5-Pan, the C+Y growth medium was supplemented with 0.1% oleate, and 10 mg/ml bovine serum albumin was added as a carrier of the fatty acid. The same amount of bovine serum albumin was added to the controls lacking fatty acids.

**Pantothenamides Target ACP**

**Fig. 1. Metabolism of pantothenate and pantothenamides in *E. coli***. Pantothenate and pantothenamides are phosphorylated by pantothenate kinase CoaA. Next, cysteine is added to the phosphopantothenate (P-Pan), which is decarboxylated to phosphopantothenine (P-PanSH) by phosphopantothenate-cysteine lyase and the 4’-phosphopantothenoylcysteine synthetase/decarboxylase CoaBC enzyme complex. P-N5-Pan is not a substrate for CoaBC, because it lacks the carboxyl group. Phosphopantothenine adenyltransferase, denoted CoaD, catalyzes the transfer of an adenylyl group to P-PanSH and forms dephospho-CoA and dephospho-N5-CoA, which, in turn, are phosphorylated to form CoA and N5-CoA by phospho-CoA kinase, denoted CoaE. The phosphopantothenoyl moiety of Coa is transferred by (ACP)synthase, denoted AcpS, to apo-ACP to form ACP for fatty acid biosynthesis. N5-CoA can be used by AcpS to produce N5-ACP, an inactive analog of ACP that lacks the sulphydryl group for acyl chain attachment. The prosthetic group of ACP is hydrolyzed by [ACP]hydrolase, denoted AcpH, whereas N5-ACP is not a good substrate for AcpH.
Conformationally Sensitive Gel Electrophoresis and Western Blot of ACP—Strain ANS1 was grown on M9 minimal medium supplemented with 0.1% casamino acids, 0.4% glycerol, and 0.0005% thiamin to early log phase (20 Klett units). N5-Pan or N7-Pan was added to the growing cells to a final concentration of 4× their MIC concentrations (100 μM for N5-Pan; 50 μM for N7-Pan), and cells were harvested by centrifugation at the end of 3 h after the addition of pantothenamide. Control cells were treated with an equal volume of the solvent vehicle Me2SO. Cells were then lysed with lysozyme and Triton X-100, and the lysates were centrifuged to get rid of cellular debris. The cell-free lysates of the N5-Pan treated cells, the N7-Pan treated cells, and the control cells were loaded onto a 13% acrylamide gel containing 0.5 M urea, and gel electrophoresis was performed under non-denaturing conditions. The separated proteins were electroblotted onto a polyvinylidene difluoride membrane, and the ACP was detected by immunoblotting using the ECF detection kit (Amersham Biosciences). The ACP-specific antibody (21) was used at 1:500 dilution as the primary antibody, and the anti-rabbit IgG conjugated with alkaline phosphatase was used as the secondary antibody (1:2500 dilution). The blot was developed using the ECF detection reagents according to the manufacturer’s instructions, and the fluorescent signal was detected using a Typhoon 9200.

Purification of ACP—Strain ANS1 was grown on M9 minimal medium supplemented with 0.1% casamino acids, 0.4% glycerol, and 0.0005% thiamin. N5-Pan was added to the early log phase culture to 100 μM, and the cells were incubated for 3 h in the presence of N5-Pan. Control cells were given the same amount of Me2SO solvent vehicle. Cells were harvested by centrifugation at the end of the incubation and lysed with a French press at 20,000 p.s.i. The cell-free extract was obtained by ultra-centrifugation of 200,000 g at 4 °C for 1 h. ACP was purified from the cell-free extract with an anion exchange DE52 column followed by gel filtration. Briefly, the cell-free extract was loaded on a DE52 column equilibrated in 10 mM Bis-Tris (pH 6.5). After the column was washed with 10 column volumes of 10 mM Bis-Tris (pH 6.5) an 10 column volumes of the same buffer containing 0.15 M LiCl, ACP was eluted with 10 mM Bis-Tris (pH 6.5) containing 0.45 M LiCl. The fractions with the ACP were pooled and concentrated before being loaded onto a Superdex 75 gel filtration column that was equilibrated in 20 mM Tris-HCl (pH 7.4). The fractions with the purified ACP were pooled and concentrated.

Mass Spectrometry of ACP—The intact protein mass determination of ACP purified from N5-Pan-treated and control cells was performed by the Hartwell Center for Bioinformatics and Biotechnology at St. Jude Children’s Research Hospital. Approximately 200 pmol of the purified ACP was diluted to 50 μl with 10 mM Tris-HCl at pH 8. The protein was loaded on a weak anion exchange nano-extraction cartridge (Western Analytical, Murrieta, CA) and washed extensively with water at pH 8 (pH was adjusted with 12.5% ammonium hydroxide) to remove the buffer salts. The protein was eluted from the column with 0.5 M acetic acid to a final acetonitrile concentration of 50%. Mass measurements were performed using a Micromass LCT ESI-TOF spectrometer (Micromass Inc., Beverly, MA) equipped with a Z-spray electrospray interface (Micromass Inc.). A flow rate of 200 nℓ/min was maintained using a VLP200 syringe pump (Harvard Apparatus, Holliston, MA), and the other instrument settings were those typically used for protein measurements on this instrument. Deconvolution of the protein spectrum was accomplished using the maximum entropy algorithm of the MassLynx software (Micromass Inc.) (22).

ACP Turnover in N5-Pan-treated Cells—Strain ANS1 was grown on M9 minimal medium supplemented with 0.1% casamino acids, 0.4% glycerol, and 0.0005% thiamin to early log phase (20 Klett units). N5-Pan was added to the growing cells to a final concentration of 100 μM. After 2, 5, 10, 15, 30, 45, and 60 min of N5-Pan addition, 5-ml aliquots of the cell suspension were removed and harvested by centrifugation. The remaining cells were collected on a Millipore HA 0.45 μM filter on a vacuum manifold and washed with pre-warmed M9 medium to remove the N5-Pan. The cells were then resuspended and diluted to 20 μl of 20 Klett units pre-warmed M9 medium followed by 4 h of incubation at 37 °C. During the 4-h incubation, 5-ml aliquots of cell suspension were removed and harvested by centrifugation at the following time points: 30, 60, 90, 120, 150, 180, and 240 min. The cell pellets were lysed with lysozyme and Triton X-100 (200 μl). Proteins in 10 μl of cell free lysates were separated by electrophoresis on a 13% polyacrylamide gel containing 0.5 M urea under non-denaturing conditions (23). The ACP was detected by immunoblotting as described above.

RESULTS

Antibacterial Activity of the Pantothenamides—The antimicrobial activity of the pantothenamides was tested against E. coli strain ANS1, which is defective in TolC-dependent efflux. N5-Pan and N7-Pan inhibited the growth of strain ANS1 with MIC values of 25 and 12 μM, respectively (Fig. 2, A and B). We and others have shown previously that N5-Pan and N7-Pan are both substrates and inhibitors of the coaA gene product (16, 24). Thus, we added pantothenate to the growth medium to test whether it would attenuate the inhibitory effect of the compounds on bacterial growth. Supplemen
growth medium with 100 μM pantothenate increased the MIC values only by 2-fold for both compounds (Fig. 2, A and B). Furthermore, overexpression of the E. coli coaA gene on a multi-copy vector failed to rescue the cells from the inhibitory effects of N5-Pan and N7-Pan (Fig. 2, A and B), indicating that cell growth inhibition was not due to the competitive inhibition of the phosphorylation of pantothenate and that CoaA was not the target of the antimicrobial action of the pantothenate antimetabolites.

Transport of the Pantothenamides—TolC is an outer membrane protein that is involved in the type I secretion of proteins and efflux of small molecules and toxic compounds (17). N5-Pan inhibited UB1005, the tolC" wild-type parent of E. coli strain ANS1, with an MIC of 25 μM, which is similar to that of the TolC-defective strain ANS1 (Fig. 2, A and C), suggesting that the cell permeability of N5-Pan is independent of TolC. For this reason, the later metabolic experiments were performed using N5-Pan. In contrast, wild-type strain UB1005 was much more resistant to N7-Pan than strain ANS1. The MIC of N7-Pan against strain UB1005 was > 200 μM, much higher than that of strain ANS1 (Fig. 2, B and C), demonstrating that N7-Pan is a substrate for a TolC-dependent efflux pump(s). The pantothenate permease (PanF) of E. coli catalyzes the concentrative uptake of pantothenate by a sodium ion cotransport mechanism (6–8). Strain DV1 is defective in PanF and has lost the ability to utilize extracellular pantothenate for growth (6). To determine whether the uptake of the pantothenamides was through the PanF permease, the MIC values of the compounds for strain DV1 were obtained and compared with the panF" wild-type strain UB1005. The MIC values of both compounds for strain DV1 were the same as those for strain UB1005 (Fig. 2C), indicating that the uptake of the pantothenamides was independent of the PanF transport system.

Formation of Faster Migrating ACPs in Cells Treated with the Pantothenamides—Strauss and Begley showed that N5-Pan was converted to a CoA analog by the enzymes in the CoA biosynthetic pathway and that the analog intermediates were better substrates for CoaD and CoaE in vitro (16). They concluded that the favorable conversion of N5-Pan to the CoA analog could account for its antibacterial activity by competing with Coa and acetyl-CoA for enzymes that utilize these cofactors. To test whether the Coa analogs were used by AcpS to modify ACP, we studied the effects of the pantothenamides on the ACP. Strain ANS1 was grown to early log phase, and either 100 μM N5-Pan, 50 μM N7-Pan, or Me2SO was added to the separate cultures (Fig. 3A). The growth curves showed that there was no difference in the replication rate among the three conditions during the first two doubling periods after the addition of the inhibitors (Fig. 3A). Only after 1.5 h of incubation in the presence of the inhibitors did cell replication start to slow down in the cultures with the pantothenamides, and the N7-Pan-treated cells stopped growing altogether at the end of the 3 h of incubation. The delayed toxicity of the pantothenamides suggested that the mechanism of the antibacterial activity required their incorporation into a cellular component rather than the direct inhibition of an essential enzyme for bacterial growth.

ACP and its thioesters can be separated at alkaline pH on a polyacrylamide gel containing a low concentration of urea (25). Acyl-ACPs migrate faster than free ACP, and the longer the chain length, the faster the migration. Cell-free extracts of strain ANS1 treated with 100 μM N5-Pan, 50 μM N7-Pan, or Me2SO from the above experiments were separated on a 13% polyacrylamide gel containing 0.5 M urea and electroblotted onto a polyvinylidene difluoride membrane. Immunoblotting with the anti-ACP primary antibody showed that cells treated with the inhibitors accumulated ACPs that migrated faster than regular ACP, with the ACP from the N7-Pan-treated cells being the fastest migrating protein (Fig. 3A, inset). Only trace amounts of normal ACP could be detected in N5-Pan- and N7-Pan-treated cells (Fig. 3A, inset). The faster migrating protein bands were also confirmed to be ACP by matrix-assisted laser desorption/ionization-TOF MS on the basis of both MS and MS/MS spectra.

Intact Mass Determination of ACP—The 4′-phosphopantetheine prosthetic group of ACP is post-translationally transferred from CoA to apo-ACP by AcpS (Fig. 1). N5-Pan is converted to a CoA antimetabolite, ethyldethia-CoA, in which a pentyl
group replaced the \( \beta \)-mercaptoethylamine of CoA (16). To confirm that the CoA analogs were used by AcpS to produce the faster migrating ACPs, ACP was purified by anion exchange and gel filtration chromatography for ESI-TOF MS to determine the intact mass of the protein from cells treated with N5-Pan or control cells treated with Me\(_2\)SO. The ACP purified from the control cells exhibited a mass of 8848.94 Da (Fig. 3B), consistent with the expected mass of ACP (8848.70 Da). The calculated mass of the ACP with the modification by ethylene-thia-CoA was 8858.70 Da. The experimental mass of the ACP purified from cells treated with N5-Pan (renamed N5-ACP hereafter) was 8858.91 Da (Fig. 3C), demonstrating that the ethylene-thia-CoA was used as a substrate by AcpS to modify apo-ACP. Because the prosthetic group of N5-ACP is longer than that of the regular ACP by two carbons, N5-ACP resembled acetyl-ACP on the conformationally sensitive gel and migrated faster than the normal ACP (Fig. 3A, inset). Similarly, ACP from cells treated with N7-Pan (renamed N7-ACP hereafter) was equivalent to butyryl-ACP and migrated even faster than N5-ACP on the gel (Fig. 3A, inset).

**Inhibition of Fatty Acid Synthesis by the Pantothenamides**—ACP is an essential component for the bacterial fatty acid synthase system (type II) as a carrier of the growing acyl chain to the pathway enzymes. The acyl group is covalently attached to ACP by forming a thioester linkage with the sulphydryl group of the 4\(^-\)phosphopantetheine moiety. Because N5-ACP and N7-ACP lack the sulphydryl group for the attachment of the acyl chain, these two proteins are inactive for fatty acid synthesis. Acetate is a precursor for bacterial fatty acid synthesis, and fatty acid production can be monitored by measuring [1-\(^{14}\)C]acetate incorporation into the membrane lipids. To determine the effect of N5-Pan on bacterial fatty acid synthesis, N5-Pan was added to an early log phase culture of strain ANS1 to 100 \( \mu \)M followed by the addition of [1-\(^{14}\)C]acetate. As expected, the incorporation of \(^{14}\)C-isotope into the membrane lipids was significantly reduced within 1 h of treatment (Fig. 4A) in comparison to control cells treated with an equal volume of the solvent Me\(_2\)SO, even though the N5-Pan treated cells were still replicating (Fig. 3A). Fatty acid synthesis was completely inhibited by N5-Pan within 3 h of incubation, although the inhibitory effect of N5-Pan was not as potent as that of the established fatty acid synthesis inhibitor cerulenin (Fig. 4A). N5-Pan did not interfere with the protein synthesis, as shown by the labeling experiments with the L-[\(^{3}\)H]amino acid mixture (Fig. 4B). To test whether the CoA levels were affected by N5-Pan, we labeled the \( \beta \)-alanine auxotrophic strain S/J16 with \( \beta \)-[3-\(^{3}\)H]alanine. The \(^{3}\)H-labeled CoA, ACP, and the CoA synthetic pathway intermediates were separated on a thin layer chromatography plate. The results showed that the CoA level in N5-Pan-treated cells remained unchanged within 2 h of treatment, whereas the normal ACP level dropped to below 10% of the control. The unchanged CoA level was consistent with the continued protein synthesis (Fig. 4B), because the primary physiological effect of reduced CoA is the inhibition of protein synthesis (26).

To verify that fatty acid synthesis is the target of the antibacterial activity of N5-Pan, we supplemented the growth medium with fatty acids to rescue cells from the inhibitory effect of N5-Pan. The addition of oleate to the growth medium failed to reverse growth inhibition by N5-Pan in strain ANS1. This result was anticipated, because the essential \( \beta \)-hydroxymyristic acid for lipid A synthesis in *E. coli* can not be provided from the medium. Thus, we selected the Gram-positive bacterium *S. pneumoniae* strain R6 for the phenotype complementation experiment. N5-Pan exhibited similar antibacterial activity against *S. pneumoniae* strain R6 with an MIC value of 25 \( \mu \)M.

The addition of 0.1% oleate to the C+Y medium increased the MIC value by 4-fold to 100 \( \mu \)M (Table I). Similarly, the MIC value of cerulenin, an established fatty acid synthesis inhibitor, was increased by supplementation with oleate (Table I). These data, together with results from the above labeling experiments, point to the inhibition of fatty acid synthesis through the formation of inactive ACPs as the primary target for the pantothenate antituberculoses.

**Accumulation of Inactive ACP**—The 4\(^-\)phosphopantetheine prosthetic group of ACP is metabolically active in *E. coli* (14). The rapid turnover rate of the prosthetic group reaches 4% of the ACP pool per minute during pantothenate starvation. The active turnover of the prosthetic group is achieved through the coordinated action of two enzymes, AcpS and the uncharacter-
The ACP prosthetic group is incorporated following de novo synthesis of the protein and also by turnover of the prosthetic group on the protein during growth (13, 14). Rapid turnover accounts for the speedy incorporation of the analogs into ACP (Fig. 5). The fact that the ACP analog does not disappear with the same kinetics suggests that the ACP analog may not be a good substrate for AcpH (Fig. 5). The protein that catalyzes ACP hydrolysis to apo-ACP has not been identified, so we were unable to directly test this idea using an in vitro assay with purified enzyme. Rapid ACP prosthetic group turnover coupled with the resistance of N5-ACP to hydrolysis ensures that the inactivation of ACP is rapid and persistent. Thus, fatty acid synthesis halts when the level of active ACP falls below the amount needed to support the pathway.

The effects of the accumulation of the N5-ACP and N7-ACP are reminiscent of the observed toxicity of apo-ACP accumulation (29). This work shows that even in the presence of sufficient active ACP to support fatty acid synthesis, the expression of the inactive apo-ACP is toxic because of its ability to non-productively interact with and inhibit the essential enzymes that utilize acyl-ACP substrates (29). When cells were treated with a pantothenamide, not only is inactive ACP generated by the replacement of the prosthetic group with the pantothenamide intermediates, but the amount of intracellular normal ACP is reduced.

The understanding of the mechanism of action of the pantothenamides opens the potential for the development of new antimetabolites that target ACP and fatty acid biosynthesis. One challenge in the development of such compounds is the requirement that they be metabolized by CoaA, CoaD, CoaE, and AcpS. The AcpS step is likely to accept a broad range of Coa analogs, because a range of Coa thioesters are known to be substrates for the transference (30), illustrating that the AcpS active site accommodates a range of modifications at the terminal sulfhydryl. Similarly, the CoaA-ADP-pantothenate ternary complex structure (24) shows how the pantothenamides are able to dock into the pantothenate kinase active site and suggest that there are a number of potential moieties that could be attached to the pantothenamide scaffold. Less is known about the substrate specificity of the CoaD and CoaE enzymes.

Whole cell screening of pantothenamide analogs will be required not only to determine whether they are efficiently utilized by the pathway enzymes but also to determine whether they can effectively penetrate the cell membrane permeability and efflux pump barriers. Our data rule out the pantothenate

| Compound   | MIC (μM) | Fatty acid | +0.1% Oleate |
|------------|----------|------------|-------------|
| N5-Pan     | 25       | 100        | >500        |
| Cerulenin  | 100      |            |             |

Notes:

- Bovine serum albumin (10 mg/ml) was added to the growth medium as a carrier for the fatty acid. The same amount of bovine serum albumin was included in the control without fatty acid (−Fatty acid).
- No inhibition was observed at the highest concentration tested.

**DISCUSSION**

We have shown that the pantothenamides inhibit bacterial growth through their incorporation into and inactivation of the ACP component of the type II fatty acid synthase system. The initial report of pantothenamide antibacterial activity suggested that this class of compounds interfered with pantothenate metabolism (15), and our data support this idea to some extent because the addition of pantothenate to the medium does render the cells somewhat more resistant to the pantothenamides (Fig. 2). However, extracellular pantothenate cannot prevent the growth inhibition, suggesting that the presence of the pantothenamide itself is toxic. Subsequently, Strauss and Begley (16) demonstrated that the pantothenamides were converted to CoA analogs by the biosynthetic pathway (Fig. 1) and suggested that these inactive CoA analogs interfered with intermediary metabolism. However, the requirement for CoA in rich medium is very low. The primary physiological effect of reduced CoA levels is the inhibition of protein biosynthesis (26), which is primarily due to the inability to produce glutamate when cells are grown in minimal medium (27). This limitation occurs when the CoA drops to 5% of the normal level in cells grown in minimal medium, and the CoA requirement is even lower in rich broth (28). Our data show that the CoA levels in cells treated with N5-Pan were relatively unchanged and that protein synthesis was unaffected (Fig. 4B), suggesting that reduced CoA is not the underlying cause of pantothenamide toxicity. This led us to examine and demonstrate that the CoA analogs were incorporated into ACP by AcpS, thereby inactivating fatty acid synthesis (Figs. 3 and 4). The supplementation of exogenous fatty acid to the growth medium conferred upon S. pneumoniae strain R6 more resistance to N5-

**FIG. 5.** Accumulation of N5-ACP in E. coli. Regular ACP and N5-ACP were separated on a 13% polyacrylamide gel containing 0.5 M urea and detected by immunoblotting with the anti-ACP antibody. The far left lane is 100 ng of pure ACP as a control. Aliquots of cells were removed at different time points after the addition and removal of N5-Pan for immunoblotting as described under "Experimental Procedures." The arrows indicate when N5-Pan was added and removed. The numbers above each lane represent minutes and indicate the time when samples were removed for analysis.
transporter as a mechanism for the uptake of the pantothena-
mines and show that the TolC-dependent efflux pumps render
Gram-negative cells more resistant to the N5-Pan while having
no effect on the N5-Pan (Fig. 2). These data suggest that the
more hydrophobic pantothename structures will be less ef-
effective because of their affinity for this class of efflux systems.
Another complication in designing antimetabolites is the diver-
sity of pantothenate kinase isozymes in bacteria. Whereas the
coaA gene characterized in E. coli is widespread, Staphyloco-
coccus aureus possesses a pantothenate kinase whose primary
sequence is distinct from its E. coli counterpart (12.7% identi-
cal). Nonetheless, this version of pantothenate kinase is also
inhibited by N5-Pan and N7-Pan, and the analogs are toxic to
S. aureus (31). Other bacteria such as Pseudomonas aeruginosa
and Helicobacter pylori do not have a recognizable pantothena-
tate kinase gene in their chromosomes, although they clearly
have the other four genes that comprise the CoA biosynthetic
pathway (32, 33). In these organisms, the gene for pantotha-
ate kinase needs to be identified, and its ability to use the
pantothename substrates should be examined. Finally,
there is no information on the metabolism or action of panto-
thenate on mammalian cells, clearly a prerequisite for de-
termining the potential for this class of compounds being de-
veloped as antimicrobials.

Acknowledgments—We thank Kiran Kodali and Dr. Clive Slaughter
at the Hartwell Center of St. Jude Children’s Research Hospital for the
mass spectrometry experiments.

REFERENCES
1. Jackowski, S. (1996) in Escherichia coli and Salmonella typhimurium: Cellu-
lar and Molecular Biology (Neidhardt, F. C., Curtiss, R., Gross, C. A.,
Ingraham, J. L., Lin, E. C. C., Low, K. B., Magasanik, B., Reznikoff, W.,
Riley, M., Schaechter, M., and Umbarger, H. E., eds) Vol. 1, pp. 687–694,
American Society for Microbiology Press, Washington, D. C.
2. Strauss, E., Kinsland, C., Ge, Y., McLafferty, F. W., and Begley, T. P. (2001)
J. Biol. Chem. 276, 13513–13516
3. Geerlof, A., Lewendon, A., and Shaw, W. V. (1999) J. Biol. Chem. 274,
27105–27111
4. Izard, T., and Geerlof, A. (1999) EMBO J. 18, 2021–2030
5. Mishra, P., Park, P. R., and Drueckhammer, D. G. (2001) J. Bacteriol. 183,
2774–2778
6. Vallari, D. S., and Rock, C. O. (1985) J. Bacteriol. 164, 136–142
7. Vallari, D. S., and Rock, C. O. (1985) J. Bacteriol. 162, 1156–1161
8. Jackowski, S., and Alix, J.-H. (1990) J. Bacteriol. 172, 3842–3848
9. Polacco, M. L., and Cronan, J. E., Jr. (1981) J. Biol. Chem. 256, 5750–5754
10. Flugel, R. S., Hwangbo, Y., Lambalot, R. H., Cronan, J. E., Jr., and Walsh,
C. T. (2000) J. Biol. Chem. 275, 959–968
11. Fischl, A. S., and Kennedy, E. P. (1990) J. Bacteriol. 172, 5445–5449
12. Nakanishi, M., Yatome, C., Ishida, N., and Kitade, Y. (2001) J. Biol. Chem.
276, 46394–46399
13. Powell, G. L., Elevson, J., and Vagelos, P. R. (1969) J. Biol. Chem. 211,
5610–5624
14. Jackowski, S., and Rock, C. O. (1984) J. Biol. Chem. 259, 1891–1895
15. Clifton, G., Bryant, S. R., and Skinner, C. G. (1970) J. Bacteriol. 102,
137, 1156–1161
16. Jackowski, S., and Rock, C. O. (1983) J. Biol. Chem. 258, 15186–15191
17. Jackowski, S., and Rock, C. O. (1985) J. Biol. Chem. 260, 926–932
18. Bligh, E. G., and Dyer, W. J. (1959) Can. J. Biochem. Physiol. 37, 911–917
19. Jackowski, S., and Rock, C. O. (1984) J. Biol. Chem. 259, 1891–1895
20. Ivey, R. A., Zhang, Y.-M., Virga, K. G., Hevener, K., Lee, R. E., Rock, C. O.,
Jackowski, S., and Park, H.-W. (2004) J. Biol. Chem. 279, 35622–35629
21. Heath, R. J., and Rock, C. O. (1995) J. Biol. Chem. 270, 26538–26542
22. Jackowski, S., and Park, H.-W. (2002) Trends Biochem. Sci. 27, 3–6
23. Jackowski, S., and Park, H.-W. (2004) J. Biol. Chem. 279, 35622–35629
24. Ivey, R. A., Zhang, Y.-M., Virga, K. G., Hevener, K., Lee, R. E., Rock, C. O.,
Jackowski, S., and Park, H.-W. (2004) J. Biol. Chem. 279, 35622–35629
25. Heath, R. J., and Rock, C. O. (1995) J. Biol. Chem. 270, 26538–26542
26. Jackowski, S., and Rock, C. O. (1985) J. Biol. Chem. 260, 866–871
27. Kestin, D. H., Zhang, Y., and Cronan, J. E., Jr. (1996) J. Bacteriol. 178,
2662–2667
28. Vallari, D. S., and Jackowski, S. (1988) J. Bacteriol. 170, 3961–3968
29. Kestin, D. H., Carey, M. R., and Cronan, J. E., Jr. (1995) J. Bacteriol. 177,
22229–22235
30. Gehring, A. M., Lambalot, R. H., Cronan, J. E., Jr., and Walsh, C. T. (1997)
Chem. Biol. 4, 17–24
31. Choudhry, A. E., Mandichak, T. L., Broskey, J. P., Egolf, R. W., Kinsland, C.,
Begley, T. P., Seefeld, M. A., Ku, T. W., Brown, J. R., Zalacain, M., and
Ratnam, K. (2003) Antimicrob. Agents Chemother. 47, 2051–2055
32. Osterman, A. L. (2002) J. Bacteriol. 184, 4555–4572
33. Osterman, A., and Overbeek, R. (2003) Curr. Opin. Chem. Biol. 7, 238–251