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Altered Molecular Form of Acyl Carrier Protein Associated with β-Ketoacyl-Acyl Carrier Protein Synthase II (*fabF*) Mutants

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Acyl carrier protein (ACP) is a required cofactor for fatty acid synthesis in *Escherichia coli*. Mutants lacking β-ketoacyl-ACP synthase II activity (*fabF1* or *fabF3*) possessed a different molecular species of ACP (F-ACP) that was separated from the normal form of the protein by conformationally sensitive gel electrophoresis. Synthase I mutants contained the normal protein. Complementation of *fabF1* mutants with an F′ factor harboring the wild-type synthase II allele resulted in the appearance of normal ACP, whereas complementation with an F′ possessing the *fabF2* allele (a mutation that produces a synthase II enzyme with altered catalytic activity) resulted in the production of both forms of ACP. The structural difference between F-ACP and ACP persisted after the removal of the 4′-phosphopantetheine prosthetic group, and both forms of the protein had identical properties in an in vitro fatty acid synthase assay. Both ACP and F-ACP were purified to homogeneity, and their primary amino acid sequences were determined. The two ACP species were identical but differed from the sequence reported for *E. coli* E-15 ACP in that an Asn instead of an Asp was at position 24 and an Ile instead of a Val was at position 43. Therefore, F-ACP appears to be a modification of ACP that is detected when β-ketoacyl-ACP synthase II activity is impaired.

Acyl carrier protein (ACP) is one of the most abundant proteins in *Escherichia coli* and functions as an acyl-group carrier in bacterial fatty acid biosynthesis and as an acyl donor in membrane phospholipid biogenesis (for reviews, see references 19 and 24). The complete primary sequence of *E. coli* ACP is known (29). The protein contains a preponderance of acidic residues throughout the sequence and relatively few positively charged residues, which are clustered at the amino terminus. ACP has a molecular weight of 8,847, and the acyl moiety is bound as a thioester to the sulfhydryl group located at the terminus of the 4′-phosphopantetheine prosthetic group, which is attached to Ser-36 of the protein via a phosphodiester linkage. The ACP prosthetic group turnover cycle is the only known posttranslational modification of ACP. [ACP]synthase catalyzes the transfer of 4′-phosphopantetheine from coenzyme A (CoA) to apo-ACP (7), and [ACP]phosphodiesterase (28) cleaves the prosthetic group from ACP. The intracellular concentration of apo-ACP is very low (11), and the rate of prosthetic group turnover appears to be related to the cellular CoA content (12).

E. coli possess two forms of β-ketoacyl-ACP synthase that catalyze the condensation of malonyl-ACP with the growing fatty acid chain (4, 9). Synthases I and II differ in their elutions from hydroxylapatite, their pH optima, their heat stabilities, and their substrate specificities and are encoded by distinct structural genes (for reviews, see references 5 and 23). Synthase I mutants (*fabB*) are unable to synthesize unsaturated fatty acids and require an exogenous unsaturated fatty acid supplement for growth (26). Strains that lack β-ketoacyl-ACP synthase II activity (*fabF1* or *fabF3*) are unable to elongate palmitoleic acid to *cis*-vaccenic acid but do not exhibit a growth defect (5, 8–10). Strains that do not properly regulate fatty acid composition in response to temperature harbor a mutation (*fabF2*) that is allelic with *fabF1*, providing compelling evidence that synthase II is responsible for the compositional changes that occur in the membrane following a temperature shift (6). Other than these alterations in fatty acid composition, defects in β-ketoacyl-ACP synthase activity have not been correlated with other cellular processes. In this paper, we report a modification of ACP structure in strains harboring mutations in β-ketoacyl-ACP synthase II activity.

MATERIALS AND METHODS

Materials. Sources of supplies were as follows: New England Nuclear Corp., β-[3-^{3}H] alanine (specific activity, 40 Ci/mol) and [2-^{14}C]malonyl-CoA (specific activity, 50 mCi/mol); Schwarz/Mann, [4,5-^{3}H]leucine (specific activity, 55 Ci/mol); ICN Radiochemicals, ^{14}C-labeled amino acid mixture (specific activity, 50 mCi/mol); Amersham Corp., ACS scintillation cocktail; Whatman, Inc., DEAE (DE-52)-cellulose; Bio-Rad Laboratories, electrophoresis supplies; Sigma Chemical Co., β-alanine, bovine serum albumin, amino acids; and Analtech, Silica Gel H thin-layer chromatography plates. All other materials were reagent grade or better.

Bacterial strains and growth conditions. The bacterial strains used in this study were derivatives of *E. coli* K-12 and are listed in Table 1. Strains were grown in minimal medium E (30) supplemented with thiamine (0.0001%), glucose (0.4%), and required amino acids (0.1%). The *panD* mutants were grown in the presence of 4 μM β-alanine. Strain SJ51 was constructed from strain SJ16 by the selection method of Maloy and Nunn (14). Strain SJ86 (*panD fabF1*) was constructed by transduction of strain SJ51 (*panD*) with P1 bacteriophage grown on strain CY288 (*fabF1 zfc*::Tn*10*). The tetracycline-resistant recombinants were screened for the presence of *cis*-vaccenate by gas-liquid chromatography and for the normal β-ketoacyl-ACP synthase II protein by using 10% polyacrylamide gels to fractionate cells labeled with β-[3-^{3}H]alanine (20).

Analytical techniques. Cell extracts were prepared by a freeze-thaw lysis procedure that was detailed elsewhere (13). Conformationally sensitive gel electrophoresis and fluorography were performed as described previously (11). The

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TABLE 1. Bacterial strains

Strain	Relevant genotype	Source
DM47	*fabF1 recA*	27
DM83	*fabF3 fabB20*	27
CY288	*fabF1 zcf*::Tn*10*	9
CY320	*pyrC*	27
CY321	*fabF1*(Cvc[a])	27
CY322	*fabF2*(Vtr[a])	27
CY328	*fabB15 panD*	27
SJ16	*panD2 zad-220*::Tn*10*	12
SJ51	Tet[s] of strain SJ16	This study
SJ86	*fabF1 zcf*::Tn*10 panD2*	This study
UB1005	*metB1* (parent of strain SJ16)	2

[a] Phenotype designation.

14-cm separating gel was 20% polyacrylamide, and electrophoresis was performed at 37°C. Purified ACP and F-ACP had a tendency to resolve into two bands in this gel electrophoresis system, but the molecular basis for this phenomenon is unknown. Proteins cross-linked with either ACP or CoA were detected with 10% polyacrylamide gels (20). Preparation of affinity-purified ACP antibodies, immunoaffinity chromatography, the partial purification of [ACP] phosphodiesterase, and the reaction conditions for removal of the ACP prosthetic group were the same as described previously (11). Cell extracts used for the fatty acid synthase assays were prepared as described previously (18, 31), and [1-^{14}C]malonyl-CoA incorporation was used as a monitor of fatty acid formation. After 20 min at 37°C, the incubations were terminated by the addition of 0.1 M KOH and heated for 1 h at 100°C in a sealed tube to release bound acyl moieties. The mixture was acidified with 1 N HCl and extracted with diethyl ether, the ether phase was dried, and the extract was reacted overnight with methanol containing 3% HCl. The distribution of label among the fatty acid methyl ester subclasses was determined by argentation thin-layer chromatography (3), and fatty acid compositions were determined by gas-liquid chromatography (13).

Purification and sequencing of ACP and F-ACP. ACP was purified from *E. coli* K-12 strain UB1005, a derivative of strain W1655F$^-$ (1), and F-ACP was purified from strain SJ86. Approximately 250 g of either strain was lysed in a French pressure cell, and the unbroken cells and debris were removed by centrifugation. Solid ammonium sulfate was added to the supernatant to achieve 80% saturation, and the precipitate was removed by centrifugation. The ammonium sulfate supernatant was adjusted to pH 4.1 with acetic acid, and the precipitate was allowed to flocculate for 12 h at 4°C and was then collected by centrifugation. The pellet was dissolved by adjusting the pH to 8.4, the insoluble material was removed by centrifugation, and the supernatant was dialyzed against 10 mM Tris hydrochloride (pH 8.5)–0.1 mM EDTA. The sample was lyophilized and loaded onto a Sephacryl S-200 column (1 by 140 cm) which was developed at a flow rate of 6 ml/h with 50 mM Tris hydrochloride (pH 7.0)–50 mM KCl–5 mM β-mercaptoethanol. The ACP peak was located by using conformationally sensitive gel electrophoresis (11), pooled, and applied to a DEAE-cellulose column (DE-52) equilibrated with 10 mM bis-Tris hydrochloride (pH 6.0)–5 mM β-mercaptoethanol. The 10-ml column was eluted with a 500-ml gradient from 0.1 to 0.5 M NaCl in the same buffer. ACP eluted at 0.37 M NaCl, and the peak fractions were pooled, dialyzed, and lyophilized. The ACP preparations were homogeneous as judged by uv spectroscopy (21), nuclear magnetic resonance spectroscopy (15, 16, 25), and conformationally sensitive gel electrophoresis (11).

The primary amino acid sequences of ACP and F-ACP were determined by using an Applied Biosystems gas-phase sequenator. The native proteins were used to obtain the sequence of the first 50 residues. The carboxy-terminal 27 residues were sequenced as follows. First, the ACP species was reacted with acetic anhydride to acetylate all free amino groups (25), and this reaction was judged to be complete by conformationally sensitive gel electrophoresis (25). The samples were then desalted and lyophilized. The acetylated ACP (2 mg; 0.23 μmol) was dissolved in 1 ml of 70% formic acid containing 10 μmol of CNBr and incubated for 24 h at 25°C. The new amino terminus generated by this method was used to initiate the sequencing of residues 45 to 77.

RESULTS

An altered form of ACP (F-ACP) in *fabF* mutants. Strains SJ51 (*panD*) and SJ86 (*panD fabF1*) were labeled with β-[3-^{3}H]alanine, and the extracts were analyzed by native gel electrophoresis. β-[3-^{3}H]alanine is the precursor to pantothenate and specifically labels CoA and the prosthetic group of ACP. Five labeled protein bands were detected in strain SJ16 (Fig. 1A). The P1 band was the [^{3}H]CoA-binding protein described by Rock (20), and the band marked ? was also reported previously (20, 27), but the identity of the tritiated ligand has not been determined. As expected, strain SJ86 exhibited a defect in [*Pan*-^{3}H]ACP binding to the band identified as β-ketoacyl-ACP synthase II (27) and also appeared to have a different molecular form of ACP (Fig. 1A). This observation was confirmed by labeling these two strains

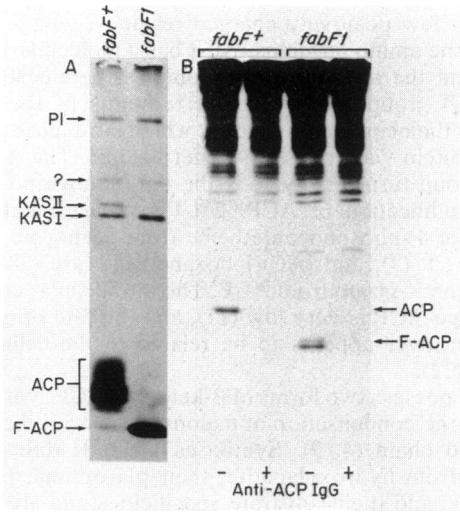

FIG. 1. A different ACP species (F-ACP) in a *fabF1* mutant. (A) strain SJ51 (*panD*), left lane; strain SJ86 (*panD fabF1*), right lane. Both strains were grown in the presence of β-[3-^{3}H]alanine (4 μM), Triton X-100 lysates were prepared, and the extracts were fractionated by electrophoresis with 10% polyacrylamide gels as described in Materials and Methods. The band labeled P1 was the CoA-binding protein (20), and the band labeled ? was observed in earlier work but has not been characterized (20, 27). KASI and KASII indicate the position of the two β-ketoacyl-ACP synthases cross-linked to ACP (20, 27). (B) Strains SJ51 (*panD*) and SJ86 (*panD fabF1*) were grown to stationary phase in the presence of [4,5-^{3}H]leucine (40 μCi/ml), and the radioactive proteins were fractionated with 20% polyacrylamide gels as described in Materials and Methods. In the indicated lanes, the cell extract was adsorbed with affinity-purified ACP-specific antibodies prior to electrophoresis.

with [4,5-^{3}H]leucine (Fig. 1B). Normal ACP was not detected in strain SJ86 (*fabF1*), but instead, there was a faster-migrating band (F-ACP) that was related to ACP as shown by its specific interaction with affinity-purified ACP antibodies. A panel of strains containing different *fabF* alleles was screened to determine whether the *fabF* mutation correlated with the occurrence of F-ACP (Fig. 2). The *fabF1* isolate (strain CY320) possessed F-ACP, as did the independent *fabF3* isolate (strain DM83). Normal ACP was detected in strains harboring the *fabF2*(Vtr) defect. In this lesion, β-ketoacyl-ACP synthase II activity was maintained, although the presence of this enzyme results in defective temperature control over the product distribution of fatty acid biosynthesis and the overproduction of *cis*-vaccenate (6). Strain CY328 was defective in β-ketoacyl-ACP synthase I activity (*fabB15*) and contained the normal ACP form. Tetracycline-resistant isolates from the transduction of strain SJ51 with P1 phage grown on strain CY288 (*fabF1 zcf*::Tn*10*) were analyzed for the *fabF1* defect by gas chromatography of the fatty acid methyl esters prepared from lipid extracts and for the F-ACP defect by gel electrophoresis of extracts from cells labeled with β-[3-^{3}H]alanine. The appearance of F-ACP coincided with a deficiency in *cis*-vaccenic acid content in all cases, illustrating that these two processes are genetically linked. Supplementing strain SJ86 (*fabF1*) with exogenous *cis*-vaccenate to supply the missing product of β-ketoacyl-ACP synthase II activity did not change the F-ACP phenotype. These data show that the appearance of F-ACP correlates with a reduction in β-ketoacyl-ACP synthase II activity.

Complementation analysis. Complementation of the *fabF1* mutation in strain DM47 with an F′ factor harboring the wild-type *fabF*$^+$ gene resulted in the appearance of the normal form of ACP (Fig. 3). This result indicates that a diffusible factor produced by the F′ plasmid corrects the F-ACP defect. Complementation with an F′ factor that carried the *fabF2* allele (a mutation that produced a synthase II with altered catalytic activity [6]) resulted in a mixture of the two ACP forms in vivo (Fig. 3). The phenomenon of intra-allelic complementation explains this interesting result. β-Ketoacyl-ACP synthase II is a dimer of identical subunits (9), and *fabF1* mutants produce defective enzyme subunits in vivo (27). Therefore, the combination of defective synthase II subunits derived from the *fabF1* allele with the altered polypeptides produced by the *fabF2* allele has the net effect of lowering the overall β-ketoacyl-ACP synthase II activity. This conclusion is supported by the observation that the fatty acid composition of the *fabF1*/F′ *fabF2* strain is intermediate between the compositions of strains containing either the *fabF1* or *fabF2* mutation (27). These data support the hypothesis that the appearance of F-ACP is due to an alteration in β-ketoacyl-ACP synthase II enzymatic activity.

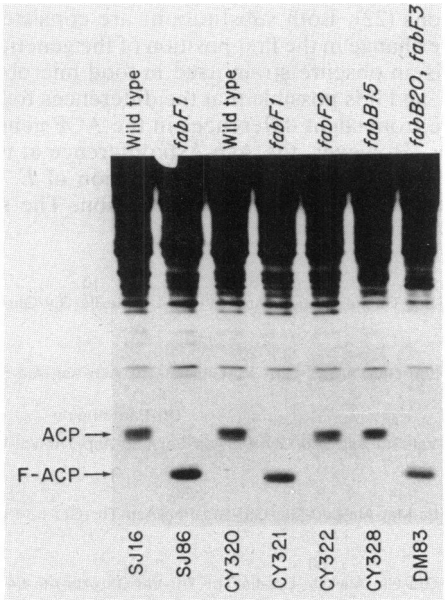

FIG. 2. Occurrence of F-ACP in strains defective in β-ketoacyl-ACP synthase activity. Strains were labeled with [4,5-^{3}H]leucine (40 μCi/ml) during the logarithmic phase of growth and lysed by the freeze-thaw procedure, and the extracts were fractionated with 20% polyacrylamide gels as described in Materials and Methods. The strain designations are given along the bottom of the figure, and the relevant genotypes are given along the top.

FIG. 3. Complementation of the *fabF1* mutation with F′ factors harboring either the *fabF*$^+$ or *fabF2* alleles. The strains were labeled during logarithmic growth with [4,5-^{3}H]leucine (40 μCi/ml), lysed by the freeze-thaw procedure, and analyzed with 20% polyacrylamide gels as described in Materials and Methods.

Nature of the F-ACP modification. The electrophoretic mobility of F-ACP is consistent with the molecule's being an acyl-ACP. However, treatment of F-ACP with either 1 M hydroxylamine or 1 M dithiothreitol at 37°C did not change the electrophoretic mobility of F-ACP, although these two agents released [1-^{14}C]palmitate from [1-^{14}C]palmitoyl-ACP. To rule out a modification of the ACP prosthetic group, the 4′-phosphopantetheine was removed from F-ACP by using [ACP]phosphodiesterase (Fig. 4). Incubation of either [*Pan*-^{3}H]F-ACP or [*Pan*-^{3}H]ACP with [ACP]phosphodiesterase resulted in the complete removal of 4′-phospho[^{3}H]pantetheine. In a parallel experiment, ACP labeled in the amino acid backbone was quantitatively converted to apo-ACP that was clearly resolved from ACP by gel electrophoresis (Fig. 4). In contrast, incubation of amino acid-labeled F-ACP with [ACP]phosphodiesterase did not change the electrophoretic mobility of F-ACP (Fig. 4). These data suggest that the modification in F-ACP occurs on the amino acid backbone and not on the prosthetic group of the protein.

Purification and primary sequence of F-ACP. To confirm that an alteration in the ACP gene was not responsible for

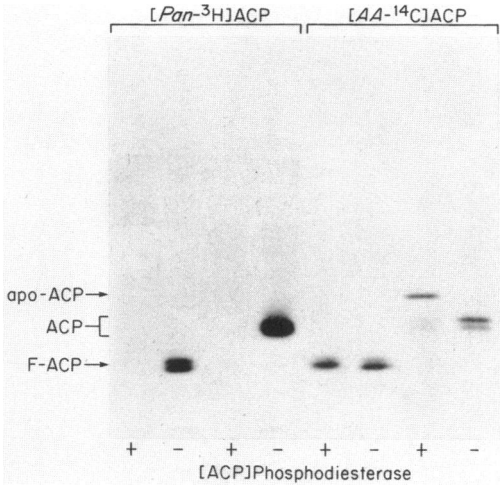

FIG. 4. Removal of the 4'-phosphopantetheine prosthetic group from ACP or F-ACP with [ACP]phosphodiesterase. Strains SJ16 (*panD*) and SJ86 (*panD fabF1*) were labeled during logarithmic growth with a mixture of ^{14}C-amino acids (100 μCi/ml), and the respective ^{14}C-labeled ACP species ([*AA*-^{14}C]ACP) were purified by immunoaffinity chromatography (11). [*Pan*-^{3}H]ACP and [*Pan*-^{3}H]F-ACP were prepared from the same two strains labeled with β-[3-^{3}H]alanine (4 μM) and purified by ion-exchange chromatography (11). The products of the [ACP]phosphodiesterase digestion (11) of these four ACP species were analyzed with 20% polyacrylamide gels as described in Materials and Methods.

the occurrence of F-ACP in *fabF* mutants and to search for possible modifications of the amino acid backbone, ACP from strains UB1005 and SJ86 (*fabF1*) was purified to homogeneity and the primary amino acid sequence was determined (Fig. 5). There was no difference between the primary sequence determined for ACP and that determined for F-ACP. However, there were two differences between the ACP sequences we determined (Fig. 5) and the previously published sequence derived from *E. coli* E-15 ACP (29). We detected an Asn instead of an Asp at position 24 and an Ile instead of a Val at position 43. Thus, F-ACP appears to be a modification of ACP that cannot be detected by the sequencing procedure.

Function of F-ACP in fatty acid synthesis. The ability of F-ACP to support the reactions of fatty acid biosynthesis was tested in a cell-free system. F-ACP was as effective as ACP in supporting in vitro incorporation of [1-^{14}C]malonyl-CoA into fatty acids. Likewise, the product distribution ($C_{16:0}$ plus $C_{18:0}$, 55%; $C_{16:1}$, 20%, and $C_{18:1}$, 25%) was not affected by the type of ACP used in the assay, indicating that F-ACP is used as an acyl carrier in the synthesis of *cis*-vaccenate. [*Pan*-^{3}H]ACP was not converted to [*Pan*-^{3}H]F-ACP by extracts prepared from strain SJ86 (*fabF1*). Extracts from this strain were defective in the production of *cis*-vaccenate regardless of the type of ACP added to the assay mixture. There was no indication that F-ACP has different biochemical properties from those of ACP in fatty acid biosynthesis.

DISCUSSION

ACP structure was altered in strains that have impaired β-ketoacyl-ACP synthase II activity. Strains harboring mutations that abolish synthase II activity (*fabF1* and *fabF3*) invariably possessed F-ACP (Fig. 2). The alteration in ACP structure was specific for defects in synthase II activity, since mutants lacking synthase I activity possessed normal ACP (Fig. 2). An active, but altered form of β-ketoacyl-ACP synthase II was produced in *fabF2* mutants, and these strains had the normal form of ACP (Fig. 2). However, reduction in the activity of these mutant polypeptides by intra-allelic complementation with a *fabF1* allele (Fig. 3) reduces the synthesis of *cis*-vaccenate (27) and resulted in the appearance of a mixture of F-ACP and ACP in vivo (Fig. 3). The exact modification of the ACP structure engendered by the lack of β-ketoacyl-ACP synthase II activity remains to be established. The modification did not involve the 4'-phosphopantetheine prosthetic group (Fig. 4), nor was it removed by hydroxylamine. F-ACP was not due to a mutation in the ACP gene. Complementation analysis indicated that there is a diffusible product that cures the F-ACP defect (Fig. 3). Amino acid sequencing of the two ACP forms (Fig. 5) did not reveal differences in the primary structure of the two proteins. The ACP modification may be related to an unknown function or to regulatory control of β-ketoacyl-ACP synthase II activity. The product distribution is normal when F-ACP is used as the cofactor in the fatty acid synthase system prepared from wild-type cells; however, this type of assay system is only an approximation of the intracellular conditions and may not reveal subtle differences in structure-reactivity relationships that are important in vivo. The physiological significance of this alteration in ACP structure must await more detailed examination of the interaction of F-ACP thioester derivatives with the enzymes of fatty acid and phospholipid biosynthesis.

We found two differences between the sequence determined in 1968 from *E. coli* E-15 (29) and the sequence obtained in the present study from *E. coli* K-12. At position 24, Asn was found instead of Asp, and at position 43, Ile was present instead of Val. Both of these changes are conservative amino acid substitutions that would not be expected to influence the predicted helical secondary structure of ACP in these regions (22). Both substitutions are consistent with a single-base change in the first position of the genetic code. *E. coli* E-15 is an obscure strain used in food microbiology by Werkman, and it is possible that the differences found in our study arise from slight differences in the ACP gene in these two strains. However, the Asp-Asn difference at residue 24 could also have resulted from deamidation of *E. coli* E-15 ACP during protein or peptide purification. The sequence-

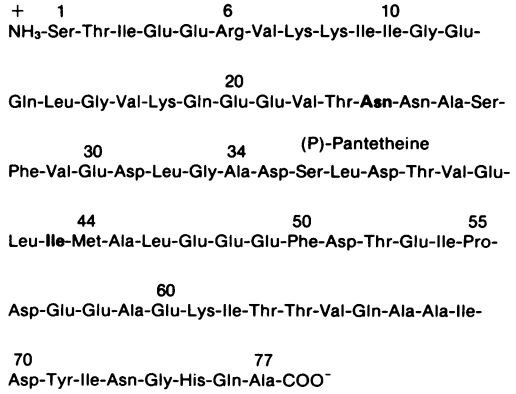

FIG. 5. The complete amino acid sequence of ACP from *E. coli* K-12. The two differences between the published *E. coli* E-15 sequence (29) and the *E. coli* K-12 sequence at positions 24 and 43 are indicated in boldface type.

specific assignments of the proton nuclear magnetic resonance signals of ACP are also consistent with Asn at position 24 (T. A. Holak and J. H. Prestegard, personal communication). These data give the exact primary amino acid sequence and molecular weight (8,860) of *E. coli* K-12 ACP and will permit the precise interpretation of high-resolution nuclear magnetic resonance spectra (15, 16, 25) and X-ray crystallography patterns (17).

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