Purification and Characterization of Phosphopantetheine Adenylyltransferase from *Escherichia coli*  

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Phosphopantetheine adenylyltransferase (PPAT) catalyzes the penultimate step in coenzyme A (CoA) biosynthesis: the reversible adenylation of 4'-phosphopantetheine yielding 3'-dephospho-CoA and pyrophosphate. Wild-type PPAT from *Escherichia coli* was purified to homogeneity. N-terminal sequence analysis revealed that the enzyme is encoded by a gene designated *kdtB*, purported to encode a protein involved in lipopolysaccharide core biosynthesis. The gene, here renamed *coaD*, is found in a wide range of microorganisms, indicating that it plays a key role in the synthesis of 3'-dephospho-CoA. Overexpression of *coaD* yielded highly purified recombinant PPAT, which is a homohexamer of 108 kDa. Not less than 50% of the purified enzyme was found to be associated with CoA, and a method was developed for its removal. A steady state kinetic analysis of the reverse reaction revealed that the mechanism of PPAT involves a ternary complex of enzyme and substrates. Since purified PPAT lacks dephospho-CoA kinase activity, the two final steps of CoA biosynthesis in *E. coli* must be catalyzed by separate enzymes.

Coenzyme A (CoA) is an essential cofactor in numerous biosynthetic, degradative, and energy-yielding metabolic pathways and is involved in the control of several key reactions in intermediary metabolism (1). CoA also donates the 4'-phosphopantetheine cofactor to the acyl carrier protein of the fatty acid synthase complex (2).

The synthesis of CoA occurs in five steps which, utilize pantetheinyl (vitamin B₅), cysteine, and ATP (for review, see Ref. 3). In all systems studied, the rate of CoA biosynthesis appears to be regulated by feedback inhibition of the first enzyme of the pathway, pantetheinyl kinase (4–7). *In vitro* studies of pantetheinyl kinase from *Escherichia coli* showed that (a) CoA and, to a lesser extent, its acyl thioesters are competitive inhibitors with respect to ATP and (b) the *Kₗ* values are within the physiological range of intracellular CoA concentrations (6). Studies of the intermediates in CoA biosynthesis have shown that both pantetheinyl and 4'-phosphopantetheine can accumulate in the cell (8). Hence, in addition to control of CoA synthesis on the level of pantetheinyl kinase, further modulation of flux through the pathway could occur at phosphopantetheine adenylyltransferase (PPAT), which catalyzes the penultimate step in the pathway (Fig. 1), the reversible adenylation of 4'-phosphopantetheine to form 3'-dephospho-CoA (dPCoA) and pyrophosphate (PP_i). Regulation of this step may control the reverse reaction of 4'-phosphopantetheine arising either from the turnover of the 4'-phosphopantetheinyl cofactor of the acyl carrier protein (8) or the cleavage of CoA by a phosphodiesterase (9).

Despite the above arguments for a role in the regulation of CoA biosynthesis, PPAT has not been the subject of a detailed study. Enzymes with PPAT activity have been purified from a number of different organisms. In mammals PPAT has been shown to be part of a complex that also includes dPCoA kinase, the effector of the final step in the biosynthetic pathway, which catalyzes the phosphorylation of the 3'-hydroxyl group of the ribose ring of dPCoA. The bi-functional complex (“CoA synthase”) was purified from pig liver (10) and shown to exist in solution as a homodimer with subunits of 57 kDa. Limited proteolysis of the latter revealed that the subunits are identical and that each subunit contains both PPAT and dPCoA kinase (11). Although similar bi-functional complexes have been partially purified from other mammalian sources (12, 13), the PPAT of baker's yeast (14) was identified as part of a much larger (375–400 kDa) CoA-synthesizing protein complex, which contained six different enzyme activities involved in the synthesis and metabolism of CoA. The complex could be separated into two components, the smallest of which (10–15 kDa) contained both PPAT and dPCoA kinase activities. Prior to the present study, the only bacterial PPAT studied in detail is that from *Brevibacterium ammoniagenes* (15), which, in contrast to the mammalian and yeast enzymes, is not part of a (multienzyme complex and behaves as a trimeric protein of 108 kDa with subunits of 35.4 kDa).

Thus far none of the above proteins shown to possess PPAT activity has been extensively characterized. Here we report the purification of the wild-type enzyme from *E. coli*, and the identification of its gene sequence, previously attributed to *kdtB* and now designated *coaD*. The overexpression of *coaD* yielded sufficient protein for a characterization of PPAT, which included kinetic studies and determination of the molecular mass (and subunit structure) of the protein. PPAT appears to catalyze the reverse reaction (the formation of 4'-phosphopantetheine and ATP) by a sequential (ternary complex) mechanism, in which the order of addition of substrates is not yet known. Hydrodynamic and cross-linking studies suggest that PPAT is hexameric, behaving as a dimer of trimers.

**EXPERIMENTAL PROCEDURES**

*Materials*—dPCoA, p-pantetheine, PP_i, reagent, and DEAE-Pheoncle were purchased from Sigma. Dilithium CoA, Sephadex G-25, Sepharose 4B, and the following chromatography columns: PD-10, Resource Q (1 ml), HiPrep Sephacryl S-100 High Resolution (2.6 × 60 cm),

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The abbreviations used are: CoA, coenzyme A; DTT, dithiothreitol; dPCoA, 3'-dephospho-coenzyme A; PP_i, pyrophosphate; PPAT, phosphopantetheine adenylyltransferase; FPLC, fast protein liquid chromatography; PAGE, polyacrylamide gel electrophoresis.
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and HiLoad Q-Sepharose (2.6 × 10 cm), were obtained from Amersham Pharmacia Biotech. Trisodium ATP, disodium CTP, disodium NADP, disodium NADH, monosodium phosphoenuolyvurate, recombinant hexokinase, and glucose-6-phosphate dehydrogenase from yeast, as well as pyruvate kinase and lactate dehydrogenase from rabbit muscle, were purchased from Roche Molecular Biochemicals. 3-Deoxy-D-xylulose-5-phosphate synthase and 1-deoxy-D-xylulose-5-phosphate reductoisomerase from Toronto Research Chemicals. 4B. The resulting resin (Red-Sepharose) contained 631 nmol of bound dye/g of moist gel (16).

Microorganisms and Growth Conditions—E. coli JM101 was grown in 0.5-liter cultures of 2× YT medium (16 g of tryptone, 10 g of yeast extract, and 5 g of NaCl per liter) in 2-liter flasks at 37 °C and 250 rpm. Cells were harvested in the late exponential growth phase (after 8–9 h) and stored at −20 °C before use.

Enzyme Assays—PPAT activity was assayed in the reverse direction using hexokinase and glucose-6-phosphate dehydrogenase to couple ATP production to NADPH reduction (17). The 1-ml assay mixture consisted of 0.1 mM dPCoA, 2 mM PP, 2 mM MgCl2, 1 mM NADPH, 5 mM glucose, 4 units of hexokinase, and 1 unit of glucose-6-phosphate dehydrogenase in 50 mM Tris-HCl buffer, pH 8.0, containing 1 mM DTT (2× TD buffer). Steady state kinetic measurements were carried out with 40 mM PPAT in the assay mixture. Kinetic parameters were determined by fitting the obtained experimental data simultaneously to the equation for a sequential reaction mechanism using Igor for Apple Macintosh (WaveMetrics, Lake Oswego, OR).

dPCoA kinase assays followed the production of ADP from dPCoA and ATP using pyruvate kinase and lactate dehydrogenase as coupling enzymes to monitor NADH oxidation (18). The 1-ml assay mixture consisted of 0.1 mM dPCoA, 2 mM PP, 2 mM MgCl2, 2.5 mM phosphoenolpyruvate, 0.16 mM NADH, 5 units of pyruvate kinase, and 5 units of lactate dehydrogenase in 2× TD buffer.

Nucleotidyltransferase activity was assayed by monitoring the production of PP by the method of O’Brien (19) using a commercially available coupled enzyme system (PP, reagent). The 1-ml assay mixture contained 1 or 5 mM 3-deoxy-D-xylulose-5-phosphate acid, 2 mM CTP or ATP, 4 mM MgCl2, and 250 μl of PP, reagent in 2× TD buffer.

All enzyme assays were carried out at 25 °C, and the change in absorbance at 340 nm was monitored. One unit of activity corresponds to the formation of 1 μmol of product/min using an extinction coefficient of NADP/PH of 6,220 M−1 cm−1. Rates were corrected for nonspecific oxidation of NADH or reduction of NADPH.

Pyrophosphorylase of CoA and dPCoA by PPAT—CoA or dPCoA (1 mM) was mixed with 20 mM PP, and 10 μM PPAT in 2× TD buffer. Each reaction mixture was incubated for 60 min or 48 h (30 min for dPCoA) at room temperature, diluted with water to 20 ml and applied to a DEAE-Sephasel column (1.0 × 9.5 cm), equilibrated with 20 ml of 0.3 m HCl, 30 ml of water, and 10 ml of water containing 0.5 mM DTT respectively. The column was washed with water and the bound compounds were eluted with a gradient from 0 to 0.5 m NaCl in 60 ml and from 0.2 to 0.6 m NaCl (30 ml) at 0.5 ml/min. The amounts of substrates remaining and products formed were determined as follows.

PP was determined by the method of O’Brien (19) using PP, reagent.

4′-Phosphopantetheine was determined by monitoring the production of PP, in the forward reaction of PPAT by the method described above.

The 1-ml assay mixture consisted of a 4′-phosphopantetheine-containing sample, 2 mM ATP, 4 mM MgCl2, 40 μM PPAT, and 250 μl of PP, reagent in 2× TD buffer. ATP was determined by the method of Lamprecht and Trautschold (17). CoA was determined by a method adapted from Barnes and Weitzman (20).

Protein Determination—Protein determinations were carried out by the method of Bradford (21) using bovine serum albumin as standard. In the last steps of the purification of wild-type PPAT, the amount of protein was estimated from the chromatography profiles using a specific absorption coefficient at 280 nm (ε280 nm) of 0.523 (this paper).

Purification of Native PPAT—All purification steps were carried out at 4 °C except for the FPLC steps, which were carried out at room temperature. Wet cell paste (69.5 g) of E. coli JM101 was resuspended in 70 ml of 50 mM Tris-HCl buffer, pH 8.0, containing 5 mM DTT, 0.2 mM phenylmethanesulfonyl fluoride, and 1 mg of DNase. Cells cooled on ice were disrupted by sonication (10 bursts of 60 s). Cellular debris was removed by centrifugation (30,000 × g for 20 min).

The supernatant was divided into two equal fractions, which were separately applied to a DEAE-Sephasel column (2.2 × 20.5 cm), equilibrated with 25 mM Tris-HCl buffer, pH 8.0, containing 0.5 mM DTT (TD buffer). The column was washed with TD buffer, and the proteins were eluted with a 400-ml linear gradient from 0 to 0.5 m NaCl in TD buffer at 1 ml/min. Active fractions were pooled and concentrated to 25 ml by ultrafiltration (10-kDa cut-off membrane). The concentrate was dialyzed overnight against 1.5 liters of 10 mM HEPES-NaOH buffer, pH 8.0, containing 10 mM MgCl2 and 0.5 mM DTT (HMD buffer) and applied to a Red-Sepharose column (1.6 × 20 cm), equilibrated with HMD buffer. Proteins were allowed to bind to the resin for 10 min before the column was washed with HMD buffer at 1 ml/min. Most of the PPAT activity did not bind to the resin. The active fractions of both runs were pooled and concentrated to 26.5 ml and applied to a Sephadex G-25 column (2.6 × 32 cm), equilibrated with HMD buffer. Proteins were eluted with HMD buffer at 2 ml/min. The fractions containing the bulk of the proteins were separated from fractions containing material with a lower molecular weight (monitored by the absorbance at 280 nm), and then pooled and concentrated to 25 ml. This step was repeated, and the eluate was concentrated to 24.1 ml and reapplied to a fresh Red-Sepharose column (1.6 × 20 cm), equilibrated with HMD buffer. Most of the PPAT activity now bound to the resin, and proteins were eluted with a 400-ml linear gradient from 0 to 1.0 m NaCl in HMD buffer at 1 ml/min. Active fractions were pooled, concentrated to 12.5 ml, and dialyzed overnight against 1.5 liters of 10 mM HEPES-NaOH buffer, pH 8.0, containing 0.5 mM DTT (HD buffer).

The dialysate was applied to a Resource Q FPLC column (1 ml), equilibrated with HD buffer. The column was washed with HD buffer and eluted with a 20-ml linear gradient from 0.2 to 0.4 m NaCl in HD buffer. Active fractions were pooled (2 ml), desalted by passage through a PD-10 gel filtration column, and reapplied to the Resource Q column. PPAT was eluted using the same gradient.

The active fractions were pooled (2.4 ml) and applied to a HiPrep Sephacryl S-100 High Resolution FPLC column (2.6 × 60 cm), equilibrated with 10 mM HEPES-NaOH buffer, pH 8.0, containing 0.15 m NaCl and 0.5 mM DTT (HSD buffer). Proteins were eluted with HSD buffer at 1 ml/min. The active fractions were combined, concentrated to 0.5 ml, and stored at −80 °C. The Sephacryl S-100 column was calibrated using ovo transferrin (77.0 kDa), ovalbumin (42.7 kDa), carbonic anhydrase (30.0 kDa), myoglobin (17.2 kDa), and cytochrome c (12.3 kDa) as molecular size standards.

Cloning and Overexpression of the coaD Gene—The coaD gene was amplified using E. coli JM101 genomic DNA as a template in the polymerase chain reaction. The forward primer incorporated a XbaI

FIG. 1. Reaction catalyzed by PPAT.
restoration process and a ribosome binding site in front of the start codon: 5'-GCTCTAAGCTATGAGGAAGATATCATATGCAACGGGGC-GAT-3'. The reverse primer incorporated a BamHI restriction site preceding the stop codon: 5'-GGGGTACCGTCGACGCTAACC-3'. The amplification cycle was as follows: denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min, and extension at 72 °C for 2 min. Amplification was performed for 25 cycles, followed by a final extension step at 72 °C for 10 min. The resulting polymerase chain reaction product was subcloned into XbaI/BamHI cleaved pUC19. The resulting plasmid was designated pUC/coaD. E. coli JM101 was transformed with pUC/coaD to yield an overexpressing strain. All steps were carried out using standard molecular biology procedures (22).

**Purification of Recombinant PPAT—** E. coli JM101-pUC/coaD was cultured on 2× YT medium supplemented with 100 μg/ml ampicillin and harvested in the stationary growth phase (after 16–24 h). Cells (35.3 g) were disrupted to give a cell-free extract, and ion-exchange chromatography on DEAE-Sephacel was performed as described above. Active fractions were pooled, concentrated to 30 ml, and dialyzed overnight against 1.5 liters of HMD buffer. The dialysate was applied to a Red-Sepharose column, but most of the PPAT activity did not bind to the resin and was collected in the flow-through. This was directly applied to a HiLoad Q Sepharose FPLC column (2.6 × 10 cm), equilibrated with HD buffer. The column was washed with HD buffer and proteins eluted with a 250-mM linear gradient from 0.25 to 0.45 M NaCl in HD buffer at 5 ml/min. Active fractions were pooled, concentrated to 8.1 ml, and applied to a Sephacryl S-100 column. Proteins were eluted with HSD buffer at 1 ml/min. The active fractions were pooled, concentrated to 13 ml (about 20 mg/ml), and stored at −80 °C.

**Removal of PPAT-bound 260-nm Absorbing Chromophores—** SDS-PAGE was carried out in 15% polyacrylamide gels using the Tris-glycine buffer system of Laemmli (23).

**Protein and DNA Sequencing—** SDS-PAGE was carried out with 7 μg purified wild-type PPAT and the protein was electroblotted onto polyvinylidene difluoride as described by Towbin et al. (24). The transfer buffer used contained 48 mM Tris, 39 mM glycine, 10% methanol, and 0.03% SDS. The membrane was stained with 0.1% Coomassie Brilliant Blue R-250 in 50% methanol and submitted for commercial N-terminal sequencing.

The subcloned coaD gene was sequenced using the T7 sequencing kit (Amersham Pharmacia Biotech). The double-stranded DNA template was denatured and annealed to the forward and reverse M13 primers as described by Martsen et al. (25).

**Cross-linking with Glutaraldehyde—** Cross-linking experiments were carried out following the procedure of Seizen et al. (26). PPAT was exchanged into 0.2 M triethanolamine buffer, pH 8.0, using a PD-10 gel filtration column. Glutaraldehyde was dissolved in this buffer and dialyzed overnight against 800 ml of CD buffer. Precipitated protein was removed by centrifugation at 48,000 × g for 5 min and the supernatant applied to a Red-Sepharose II column. PPAT was eluted with CSD buffer containing 0.15M NaCl (CSD buffer). PPAT was eluted with CSD buffer at 1 ml/min. Fractions with a ΔA260/A280 ratio higher than 1.0 were pooled, concentrated to 7.0 ml, and reapplied to the Sephacryl S-100 column. Further elution was performed using the Tris-EDTA buffer system of Laemmli (23).

**Removal of PPAT-bound 260-nm Absorbing Chromophores—** Purification of PPAT from E. coli JM101

### RESULTS

**Purification of PPAT—** In order to purify PPAT from E. coli, the procedure published for the B. ammoniagenes enzyme (15) was followed initially. This comprises cell breakage, polymerase chain reaction, followed by ion-exchange chromatography (PPAT eluting at approximated 0.34 M NaCl). However, PPAT involves elution by a salt gradient from a Red-Sepharose column. However, PPAT failed to bind to this resin unless it had been subjected to gel filtration to remove low molecular weight (less than 1000) compounds. Nevertheless, both Red-Sepharose steps (before and after gel filtration) remained part of the procedure since the combination of these three steps improved the purification greatly (Table I). The last step in the published procedure involves affinity chromatography on Red-Sepharose. However, PPAT failed to elute from the column in buffers containing up to 0.5 mM dPCoA. Finally, PPAT was purified to homogeneity using high resolution anion exchange and gel filtration chromatography (Table I).

**Cloning, Overexpression, and Purification of Recombinant PPAT—** Purification of PPAT from E. coli JM101

### Table I

| Purification step | Protein | Activity | Specific activity | Yield | Purification |
|-------------------|---------|----------|------------------|-------|--------------|
| Cell-free extract | 7134    | (4.38)   | 0.00061          | 100   | 1            |
| DEAE-Sephalocel | 579     | 4.38     | 0.0076e          | 100   | 13           |
| Red-Sepharose I | 160     | 3.98     | 0.0253           | 91    | 41           |
| Sephadex G-25  | 149     | 3.59     | 0.0243           | 82    | 39           |
| Red-Sepharose II| 1.88    | 1.72     | 0.0760           | 29    | 1500         |
| Resource Q      | 0.0694  | 1.41     | 20.3             | 32    | 33,300       |
| Sephacryl S-100| 0.0205  | 0.457    | 22.3             | 23    | 36,600       |

* The amount of protein was estimated using a molecular extinction coefficient for PPAT at 280 nm of 9330 M⁻¹ cm⁻¹ (this paper).

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**FIG. 2. SDS-PAGE analysis of the purification of wild-type PPAT.** Samples of each step in the purification of wild-type PPAT were subjected to SDS-PAGE in a 15% polyacrylamide gel and stained by silver staining. Lane M, molecular weight markers; lane 1, cell-free extract of E. coli JM101; lane 2, DEAE-Sephalocel eluate; lane 3, Red-Sepharose I eluate; lane 4, Red-Sepharose II eluate; lane 5, Resource Q eluate; lane 6, Sephacryl S-100 eluate.
the cell-free extract showed that the protein was soluble and had a greatly increased specific activity for PPAT (from 0.00061 to 1.0 units/mg). The DNA sequence of the cloned gene was determined and was identical to the published sequence (28). Therefore, the kdb gene was renamed coaD.

For the purification of the recombinant PPAT from *E. coli* JM101-pUC/coaD, the same approach was followed as described above (Table II). After the initial union exchange chromatography step, the fractions containing PPAT activity were applied to Red-Sepharose. Almost all of the activity did not bind to the resin, but, unlike the wild-type enzyme, the bound compound(s) could not be removed by gel filtration on Sephadex G-25 (see below). The enzyme was further purified to homogeneity using high resolution anion exchange and gel filtration chromatography (Fig. 4). The final preparation was very stable on storage and on freezing and thawing; no loss in activity was found when the protein was stored at 80 °C for a period of 5 months. After storage for more than 6 months at 20 °C, 89% of the original activity remained.

The specific activity of recombinant PPAT was 9.1 units/mg. This is less than half the specific activity determined for the purified wild-type enzyme. The difference is most probably due to errors in the estimation of the amount of protein in the latter steps of the purification of the wild-type enzyme. The specific absorption coefficient at 280 nm (A280) was determined using the method of van Iersel et al. (43). The average value of four different recombinant PPAT preparations was 0.523 ± 0.007. Using the calculated molecular weight of 17,837, this gives a molecular extinction coefficient of 9,330 M⁻¹ cm⁻¹.

### Identification and Removal of the Bound Compound(s) from the Recombinant PPAT

The absorption spectrum of the purified PPAT is shown in Fig. 5. The spectrum shows a broad absorption maximum between 250 and 280 nm, indicating that one or more compounds with absorption maxima in this region (most likely nucleotides) are bound to the protein. A solution of protein in citrate buffer, pH 5.0, and subsequent application to a Sephacryl S-100 column in the same buffer (see "Experimental Procedures") gave a molecular extinction coefficient of 9,330 M⁻¹ cm⁻¹.

The data were obtained with 35.3 g of wet cell paste. For experimental details, see "Experimental Procedures."

## Table II

| Purification step | Protein | Activity | Specific activity | Yield | Purification |
|-------------------|---------|----------|-------------------|-------|--------------|
| Cell-free extract | 3287    | 3307     | 1.0               | 100%  | 1.0          |
| DEAE-Sepharose    | 995     | 3366     | 3.4               | 102%  | 3.4          |
| Red-Sepharose     | 524     | 2488     | 4.8               | 75%   | 4.8          |
| HiLoad Q          | 357     | 2502     | 7.0               | 76%   | 7.0          |
| Sephacryl S-100   | 248     | 2249     | 9.1               | 68%   | 9.1          |

### Fig. 4. SDS-PAGE analysis of the purification of recombinant PPAT

Samples of each step in the purification of recombinant PPAT were subjected to SDS-PAGE in a 15% polyacrylamide gel and stained with Coomassie Brilliant Blue R-250. Lane M, molecular weight markers; lane 1, cell-free extract of *E. coli* JM101-pUC/coaD; lane 2, DEAE-Sepharose eluate; lane 3, Red-Sepharose run-through; lane 4, HiLoad Q eluate; lane 5, Sephacryl S-100 eluate.
were carried out in which CoA (1 mM) was incubated at room temperature to determine whether CoA is a substrate for PPAT, two experiments were performed at different protein concentrations (approximately 0.4, 0.8, and 1.2 mg/ml) were obtained by sedimentation equilibrium experiments in an analytical ultracentrifuge. Three experiments at different protein concentrations (approximately 0.4, 0.8, and 1.2 mg/ml) were carried out, and an average value of 108 ± 2 kDa was obtained, indicating that all protein-bound CoA had been removed.

**Determination of the Native Molecular Mass**—During purification, the enzyme eluted from the calibrated Sephacryl S-100 column with an elution volume equivalent to a mass of 71.2 ± 1.1 kDa, which suggested that in solution the enzyme exists as a homotetramer. A different result, however, was obtained by sedimentation equilibrium experiments in an analytical ultracentrifuge. Three experiments at different protein concentrations (approximately 0.4, 0.8, and 1.2 mg/ml) were carried out, and an average value of 108 ± 2 kDa was obtained, indicating that native PPAT is a homohexamer.

Further experiments, using gluteraldehyde as a cross-linking reagent, were carried out at three different molecular ratios of enzyme to cross-linking reagent. At the low ratio of PPAT to gluteraldehyde (1:1.8), the PPAT monomer (17.8 kDa) was observed together with one other product with a mass of 37.3 kDa, which is likely to be the PPAT dimer (Fig. 6). At the intermediate ratio (1:18), two additional products are present with masses of 39.4 and 53.7 kDa, the latter of which is clearly the PPAT trimer. The other product is probably also a dimer, which is formed at a later stage by a more complex cross-linking and therefore runs at a slightly higher mass than the initially formed dimer. In addition, some high molecular mass products are formed, which are very likely multi-protein complexes. At the high ratio (1:180), initially all four products (monomer, two forms of dimer, and trimer) were present in the reaction mixture (results not shown), but after incubation times longer than 30 min only multi-protein complexes are observed. No product with a mass equal to that of the PPAT tetramer (71.2 kDa) was observed under any of the experimental conditions tested.

**Pyrophosphorylation of CoA and dPCoA by PPAT**—To determine whether CoA or dPCoA is a substrate for PPAT, two experiments were carried out in which CoA (1 mM) was incubated at room temperature with 20 mM PP, and 10 μM PPAT. After either 60 min or 48 h, the reactions were stopped and the products were analyzed. No decrease in the CoA concentration (1.07 and 1.02 mM, respectively) and only a slight decrease in the PP concentration (18.3 and 18.7 mM, respectively) were observed. Furthermore, no formation of 4′-phosphopantetheine was observed even after 48 h. A similar conversion reaction was carried out with 1 mM dPCoA instead of CoA. The mixture was incubated for 30 min at room temperature, and analysis of the products showed the formation of 0.73 mM 4′-phosphopantetheine and 0.77 mM ATP. Therefore, it is clear that CoA is not a substrate for PPAT.

**Steady State Kinetic Analysis of PPAT**—The steady state kinetics of PPAT were investigated by measuring the initial rate of the reverse reaction at varying concentrations of one substrate at fixed concentrations of the second substrate (Fig. 7, A and B). The intersecting lines obtained in both graphs are consistent with a mechanism in which a ternary complex between the enzyme and both substrates is formed before catalysis. The data were fitted to this model, and the following steady state parameters were calculated: $k_{cat} = 3.3 ± 0.1$ s$^{-1}$ (equivalent to 11.5 ± 0.5 units/mg), $K_m$ (dPCoA) = 7.0 ± 1.4 μM, and $K_m$ (PP) = 0.22 ± 0.04 mM. No substrate inhibition was observed for dPCoA at concentrations up to 200 μM. The possibility of substrate inhibition by PP could not be determined since it inhibits the coupling enzymes at concentrations higher than 2 mM.

**Effect of pH on the PPAT Activity**—PPAT had a narrow pH optimum with a maximum at 6.9. Although the activity at pH 8.0 is 68% of the maximum, the assays were carried out routinely at pH 8 because of the preference of hexokinase, one of the coupling enzymes, for pH values above 8 (44).

**Is PPAT a Cytidlyltransferase?**—It has been suggested (45) that the protein encoded by the *kdtB* gene is 3-deoxy-d-manno-2-octulosonic acid cytidlyltransferase. To eliminate this possibility, the preparation of PPAT purified after overexpression of *kdtB* was assayed in the forward direction with 3-deoxy-d-manno-2-octulosonic acid as a substrate in the presence of 2 mM ATP or CTP. No activity was found at concentrations of 1 and 5 mM 3-deoxy-d-manno-2-octulosonic acid with either nucleotide.

**DISCUSSION**

A procedure has been developed for the purification of PPAT from *E. coli*, which yields homogenous protein after a 36,600-fold purification. Although the procedure yielded only small amounts of protein (20.5 μg from 69.5 g of wet cell paste), it was...
sufficient for N-terminal amino acid sequence analysis. A data base search using the N-terminal sequence suggested that PPAT of E. coli was the protein encoded by kdtB (28); the 10 residues that were determined for PPAT are identical to its predicted N-terminal sequence (Fig. 3), and the molecular mass of the deduced kdtB protein (17.8 kDa) approximates that of purified PPAT (18.4 ± 0.3 kDa). No function for the kdtB protein has been demonstrated, but since it is located next to a group of CoA and PPAT. Therefore, it is reasonable to assume that some of the fully conserved residues (Fig. 3), namely His-18, Lys-42, Arg-51, Arg-91, Asp-95, and Glu-99, could play a role in the binding of CoA. The enzyme proved to be quite stable under these conditions since the yield was more than 80%.

Purified PPAT failed to show any activity in an assay for dPCoA kinase. Therefore, as for B. ammoniagenes (15), the last two steps of CoA biosynthesis in E. coli are catalyzed by separate enzymes. Since PPAT is part of a bifunctional enzyme complex (with dPCoA kinase) in mammalian systems (10) and of a multi-enzyme complex (with among others dPCoA kinase) in bakers' yeast (14), there seems to be a clear difference in the organization of the enzymes of CoA biosynthesis in prokaryotic and eukaryotic organisms.

Results from gel filtration chromatography suggested that PPAT is a homotetramer of 71.2 kDa. However, this method is dependent on the protein shape requiring appropriate standards for calibration (48). Further analysis by sedimentation equilibrium centrifugation, a shape-independent method, was carried out, the results indicating that PPAT behaves as a homohexamer of 108 kDa. This result was supported by cross-linking experiments in which the enzyme was incubated with glutaraldehyde (Fig. 6). Therefore, we propose that in solution PPAT is a homohexamer and that the presence of glutaraldehyde it is cross-linked into two identical trimers. The only other purified bacterial PPAT, the enzyme from B. ammoniagenes, is reported to be a trimeric protein of 108 kDa with subunits of 35.4 kDa (15). This is about twice the molecular mass of all other PPAT monomers found to date. Unfortunately, there are no protein or DNA sequence data available on PPAT from B. ammoniagenes, and the nature of this difference remains unclear.

The kinetic parameters determined for the reverse reaction \( \kappa_{cat} = 3.3 \text{ s}^{-1}, K_{cat}dPCoA = 7 \mu M, \text{ and } K_{cat}(PP) = 0.22 \text{ mm} \) are very similar to those reported (49) for the PPAT activity of the bi-functional pig liver enzyme (7.7 s\(^{-1}\), 11 \mu M, and 0.19 mm, respectively). No comparison could be made with the PPAT of B. ammoniagenes since kinetic parameters for the reverse assay have not been reported.

Steady state kinetic studies demonstrated that the PPAT
reaction proceeds by a ternary complex mechanism, although
the order of substrate binding has yet to be determined. Similar
mechanisms have been reported for other members of the
nucleotideyltransferase superfamily, such as glycerol-3-phos-
phate cytidylyltransferase (50) and class I aminoacyl-tRNA
synthetases (51). In all the latter case, activation of amino acids
occurs via an in-line displacement mechanism following nucleo-
philic attack by the carboxyl group of the amino acid on the
\( \alpha \)-phosphate of ATP, and structure-based reaction mechanisms
for this activation have been described (52–54). In all the pro-
posed mechanisms for class I aminoacyl tRNA synthetases, a
highly conserved HXGH sequence is envisaged to play an im-
portant role in ATP binding and especially in the stabilization
of the pentacoordinate transition state since site-directed mu-
tagenesis studies have shown that neither of the histidines of
this conserved sequence participates in catalysis as a proton-
donating residue (55, 56). The HXGH motif is highly conserved
in the nucleotideyltransferase superfamily (45) and the histi-
dines in this motif have been reported to have a similar func-
tion in glycerol-3-phosphate cytidylyltransferase (50) and phos-
pholipase cytidylyltransferase (57). In all but one of the PPAT
sequences, the first histidine residue has been replaced by a the
nine forming a TXGH motif, which is found near the N
terminus of the protein (Fig. 3). A similar change has been
found in the motif of tryptophanyl-tRNA synthetase without
affecting its catalytic function (58). Therefore, it is reasonable
to propose that this sequence plays a similar role in the cata-
lytic mechanism of PPAT by stabilizing the pentacoordinate
transition state formed by the nucleophilic attack of 4′-phos-
phopantetheine on the \( \alpha \)-phosphate of ATP.

Site-directed mutagenesis can be employed to determine if
the role of the TXGH motif in the catalytic mechanism of PPAT
is the same as that of the HXGH motif of class I aminoacyl
tRNA synthetases. However, a full description of the catalytic
mechanism will be greatly aided by the availability of a high
resolution three-dimensional structure of PPAT, determined by
x-ray diffraction (59).

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