Purification and Properties of *Escherichia coli* 4′-Phosphopantothenoylcysteine Decarboxylase: Presence of Covalently Bound Pyruvate†

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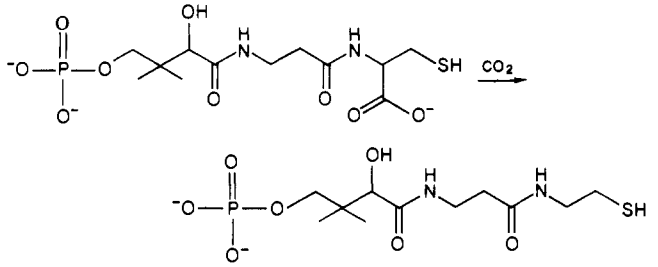
ABSTRACT: 4′-Phosphopantothenoylcysteine decarboxylase was purified 900-fold from *Escherichia coli* B with an overall yield of 6%. The enzyme migrates as a single band with a molecular weight of 35 000 ± 3000 in 10% polyacrylamide gel electrophoresis under denaturing conditions. The native enzyme has an apparent molecular weight of 146 000 ± 9000 as determined by a gel exclusion column. At pH 7.6 and 25 °C, K_m = 0.9 mM and V_{max} = 600 nmol/(min·mg of protein). The pH optimum for V_{max} is between 7.5 and 7.7. Hydroxylamine, phenylhydrazine, potassium cyanide, and sodium borohydride as well as pyridoxal phosphate and pyridoxal inactivated the enzyme. The enzyme contains covalently bound pyruvate as suggested by the isolation of [^{3}H]lactate and pyruvate from [^{3}H]$NaBH_4$-reduced enzyme and native enzyme, respectively. One mole of [^{3}H]lactate was isolated per 39 000 g of [^{3}H]$NaBH_4$-reduced and completely inactivated enzyme, and 1 mol of pyruvate was isolated per 31 000 ± 4000 g of native enzyme. Mild base treatment released lactate and pyruvate from the reduced and the native enzymes, respectively, suggesting the pyruvate is attached to the enzyme by an ester bond. These findings are in accord with similar results obtained with the horse liver enzyme (R. Scandurra, personal communication). The presence of covalently bound pyruvate in the bacterial and mammalian enzymes suggests that pyruvate plays a major role in the mechanism of action.

4′-Phosphopantothenoylcysteine decarboxylase [4′-phospho-*N*-(D-pantothenoyl)-L-cysteine carboxy-lyase, EC 4.1.1.36] (PPC-DC)[1] is a key enzyme in the biosynthesis of coenzyme A and catalyzes the decarboxylation of 4′-phosphopantothenoylcysteine to 4′-phosphopantetheine.



Unlike most other biological decarboxylations which occur β to a carbonyl group or some other structure that can act as an electron sink, no apparent electron sink is available for the decarboxylation of 4′-phosphopantothenoylcysteine. The reaction catalyzed by PPC-DC is thus different from most enzymic decarboxylations and therefore is of interest. Partial purification of PPC-DC from rat liver (Abiko, 1967) and horse liver (Scandurra et al., 1974) was reported. Evidence was obtained which suggested that the horse liver enzyme contained covalently bound pyruvate (Scandurra et al., 1979). The rat liver enzyme was shown to catalyze the decarboxylation with the retention of configuration (Aberhart et al., 1985).

We have purified PPC-DC 900-fold from *Escherichia coli* with 6% overall yield. The purified enzyme migrates as a single sharp band in polyacrylamide gel electrophoresis under denaturing conditions. The *E. coli* enzyme was found to have approximately one functional pyruvate group per subunit. Furthermore, the pyruvoyl group can be released by mild base hydrolysis, suggesting an ester-type linkage is involved instead of an amide bond as found in other pyruvoyl decarboxylases.

Experimental Procedures

Materials. All reagents were from commercial sources and used without further purification unless otherwise noted. Pantothenonitrile 4′-phosphate (I) and pantetheine 4′-phosphate (II) were synthesized according to Nagase (1967). L-[^{14}C(U)]Cysteine (50 μL, 306 mCi/mmol) from New England Nuclear was diluted 1000-fold with carrier L-cysteine hydrochloride. L-[^{35}S]Cysteine hydrochloride (0.5 mCi, 47 mCi/mmol) from Amersham was 100-fold diluted with carrier L-cysteine hydrochloride. ^{14}C- and ^{35}S-labeled 4′-phosphopantothenoylcysteine, respectively, were synthesized from these compounds according to Nagase (1967) and Scandurra et al. (1974). Sodium [^{3}H]borohydride (528 mCi/mmol) was obtained from New England Nuclear and diluted with 100 mg of cold carrier in 5 mL of 0.01 N NaOH. Frozen *E. coli* B cell paste grown in the minimal medium and harvested at $^3/_4$ log was obtained from Grain Processing Co. (Muscatine, IA).

NMR Spectra. All NMR spectra were recorded on a Varian XL-300 spectrometer with tetramethylsilane as an external reference.

Enzyme Assay. For detection of the activity in the minimal incubation volume, a modified Warburg vessel was made. Two transverse holes were made in a 1.5-mL microcentrifuge tube with a 25-G needle, and the holes were sealed with small pieces of labeling tape. Inside this tube were placed the enzyme

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[1] Abbreviations: DTT, dithiothreitol; EDTA, ethylenediaminetetraacetate; *E. coli*, *Escherichia coli*; FPLC, fast-protein liquid chromatography; HPLC, high-pressure liquid chromatography; P_i, inorganic phosphate; POPOP, 1,4-bis(5-phenyloxazol-2-yl)benzene; PPO, 2,5-diphenyloxazole; PPC, 4′-phosphopantothenoylcysteine; PPC-DC, 4′-phosphopantothenoylcysteine decarboxylase; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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solution and 0.1 M Tris-HCl buffer, pH 7.6, in a total volume of 40 μL, and 10 μL of 0.2 M DTT solution in water was added to the mixture. On top of the solution, in a microcentrifuge tube, was put a 250-μL pipet tip whose point was cut and sealed off with flame. This inner pipet container, serving as the central well of the conventional Warburg vessel, contained 50 μL of 20% KOH. The tube was then closed. Through one of the holes in the tube, 10 μL of 17 mM solution of ^{14}C-labeled 4′-phosphopantothenoylcysteine in water was injected to initiate the reaction. The hole was immediately resealed with a piece of tape. The tube was gently vortexed and incubated for 10 min at 25 °C. At the end of the incubation, 50 μL of 5 N H_2SO_4 was injected through the other hole. The tube was then resealed, vortexed, and further incubated for 30 min at 25 °C. After this second incubation the tube was opened and the KOH solution in the inner pipet tip was retrieved. The KOH solutions was mixed with 2 mL of absolute EtOH and 10 mL of scintillation cocktail in a 25-mL counting vial. The scintillation cocktail was prepared by mixing 4 g of PPO and 50 mg of POPOP in 1 L of toluene. After the solution stood for 3 h at room temperature, stable radioactivity was measured in a Beckman LS 1800 liquid scintillation counter. No radioactivity was detected in control experiments with or without boiled enzyme.

One enzyme unit is defined as the amount of protein that releases 1 nmol of CO_2 per minute at 25 °C. The protein concentration was measured by the method of Lowry et al. (1951) using bovine serum albumin (Sigma) as a standard.

Purification of PPC-DC from E. coli. All procedures were carried out at temperatures between 0 and 4 °C. All buffers used for the purification of the enzyme contained 1 mM EDTA.

Frozen *E. coli* cell paste (350 g) was blended in 250 mL of 10 mM KP_i, pH 7.0, in a Waring blender. The homogenized cell solution was then sonicated by using a Branson sonicator at the maximal power setting. The sonication was continued for 45 s with continuous stirring and with 30-s intervals between sonication. This was repeated an additional 9 times. The homogenate was then centrifuged for 45 min at 14000*g*, and the pellet was discarded.

To the supernatant was added dropwise 25° (v/v) of a 2% solution of salmine protamine sulfate (Calbiochem; A grade) prepared in water and pH adjusted to 7.0 with KOH. The resulting cloudy solution was centrifuged for 30 min at 14000*g*, and the pellet was discarded. This supernatant was directly applied on a DEAE column (4.5 × 50 cm; Whatman DE 23) preequilibrated with 10 mM KP_i, pH 7.0. The column was washed with 1.2 L of the same buffer solution at a flow rate of 1.2 mL/min. The same flow rate was maintained during the linear gradient of 0–350 mM KCl in 10 mM KP_i, pH 7.0, buffer (total gradient volume 4 L). Fractions (22 mL) were collected and assayed for protein (absorbance at 280 nm) and enzyme activity. The third protein peak (fractions 75–90) after the start of the gradient contained the enzyme activity. These fractions were pooled and concentrated in a Diaflo ultrafiltration cell with a PM-10 membrane (Amicon). At the same time the solution was desalted by repeated dilution of the concentrated solution with 10 mM KP_i, pH 7.0, buffer.

The concentrated and desalted protein solution was then applied onto a hydroxyapatite column (Bio-Rad) prepared in a 50-mL disposable syringe (3 × 8 cm) and preequilibrated with 10 mM KP_i, pH 7.0, buffer. After the protein solution was absorbed, the column was washed with 100 mL of the same buffer. PPC-DC was then eluted from the column by a linear gradient of 10–400 mM KP_i, pH 7.0, buffer (total gradient) volume 400 mL). Fractions (8 mL) were collected and assayed for protein (A_{280} reading) and PPC-DC activity. The protein peak fractions containing PPC-DC (fractions 22–36) were pooled. The protein solution was again concentrated and desalted by using a Diaflo ultrafiltration cell as described above. The final concentrated solution was in 10 mM KP_i, pH 7.0.

An aliquot of this protein solution (7 mg, 1.1 mL) was injected on a mono Q column (anion exchanger) connected to a Pharmacia FPLC system and eluted with a KP_i gradient. Fractions (1 mL) were collected and assayed for the enzyme activity. PPC-DC was eluted as a broad peak over the KP_i gradient of 0.09–0.11 M KP_i, pH 7.0. After the above FPLC step was repeated an additional 5 times, the PPC-DC peaks were pooled and concentrated in a Diaflo ultrafiltration cell with a PM-10 membrane. The protein solution was further concentrated to 250 μL by using a Centricon-10 (Amicon).

The enzyme was further purified in an HPLC size exclusion column. Aliquots of the protein solution (20 μL) were injected on a Spherogel–TSK 3000SW column (7.5 × 300 mm; Altex) connected to a Beckman 112 HPLC system. The enzyme was eluted with 0.3 M KP_i, pH 7.0, buffer, at a 0.4 mL/min flow rate. Two protein peaks were detected. The first peak was the major peak and contained more than 80% of the applied activity. The enzyme peaks were pooled and concentrated by using a Centricon-10.

Molecular Weight Estimation. The HPLC gel filtration column described above was calibrated with the following standard protein samples (Sigma); sweet potato β-amylase (M_r 200 000), yeast alcohol dehydrogenase (M_r 150 000), bovine serum albumin (M_r 66 000), and bovine erythrocyte carbonic anhydrase (M_r 29 000). The void volume of the column was measured with blue dextran (M_r 2 000 000). The molecular weight of native PPC-DC was then determined on the basis of this calibration.

SDS–Polyacrylamide Gel Electrophoresis. SDS–polyacrylamide gel electrophoresis was carried out in a vertical slab gel as initially described by Laemmli (1970). Proteins were separated in a 10% polyacrylamide gel with a 5% polyacrylamide stack. For the estimation of the subunit molecular weight, a mixture of the standard molecular weight markers was used (Sigma). The standards were bovine serum albumin (M_r 66 000), egg albumin (M_r 45 000), rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (M_r 36 000), bovine erythrocyte carbonic anhydrase (M_r 29 000), and PMSF-treated trypsinogen (M_r 24 000).

Inactivation of PPC-DC. The reaction mixture contained 5 μL of PPC-DC solution (3.4 μg/μL in 10 mM KP_i, pH 7.0), 10 μL of DTT (0.2 M in H_2O), inhibitor (1.0 M NH_2OH in 1 M KP_i, pH 7.5; 0.5 M phenylhydrazine in 0.1 M KP_i, pH 7.5; 1.0 M KCN in 0.1 M KP_i, pH 7.5; 0.5 M $NaBH_4$ in 10 mM NaOH; 0.2 M pyridoxal in H_2O; 0.2 M pyridoxal phosphate in H_2O), and 0.1 M Tris-HCl, pH 7.6, to a total volume of 50 μL. After incubation at 25 °C for 10 min, the remaining activity of the enzyme was measured by injection of 10 μL of 17 mM ^{14}C-labeled substrate as described. The control contained everything except inhibitors.

Substrate Protection against Inactivation by $NaBH_4$. The enzyme was preincubated for 1 min with various concentrations of unlabeled substrate before the addition of $NaBH_4$. For each set of experiments, the following incubation mixture was prepared: 25 μL of enzyme (3.4 μg/μL in 10 mM KP_i, pH 7.0), 50 μL of DTT (0.2 M in H_2O), unlabeled substrate (17 mM in H_2O), and 0.1 M Tris-HCl, pH 7.6, to a total volume of 250 μL. After a 1-min incubation on ice, 10 μL of 0.5 M

$NaBH_4$ in 10 mM NaOH was added. The reaction mixture was vortexed and left at 25 °C. At time intervals 50-μL aliquots were withdrawn and assayed for the remaining activity of the enzyme.

Reduction of PPC-DC with [^{3}H]NaBH$_4$. PPC-DC (0.5 mg, 1.7 mg/mL in 80 mM KP_i, pH 7.0) was reduced with 5–25 μL of [^{3}H]$NaBH_4$ stock solution (0.536 M in 10 mM NaOH). After incubation at 25 °C for 10 min, 40 μL of acetone were added. The pH of the reaction mixture was about 8.5 (paper). The solution was further incubated for 30 min at 25 °C in an open flask before being exhaustively dialyzed against 10 mM KP_i, pH 7.0, at 4 °C. The protein concentration (Lowry et al., 1951) of the dialyzed solution and remaining activity were measured. A parallel control was done with nonreduced enzyme. An aliquot of dialyzed solution was withdrawn and measured for determination of radioactivity. The protein was lyophilized with the addition of 2 μL of ethyl-L-lactic acid (Aldrich). The lyophilized protein and ethyl-L-lactic acid were then dissolved in 150 μL of 0.1 N NaOH. After refluxing at 100 °C for 15–30 min, the solution was neutralized with 1.0 N HCl and passed through a Dowex 50WX8 column (H^+ form; prepared in a disposable Pasteur pipet; total volume 0.5 mL). The column was washed and eluted with H_2O until no more radioactivity was detected in the eluent (about 2 mL). The eluent was neutralized with 1.0 N NaOH and concentrated by rotary evaporation under reduced pressure. Half of the concentrated solution was injected on an HPLC column for organic acid analysis (HPX 87H; 300 × 7.8 mm; Bio-Rad) coupled to a differential refractometer (Waters Associates). The column was eluted with 5 mM H_2SO_4 at a flow rate of 0.4 mL/min. Fractions (0.4 mL) were collected and measured for ^{3}H radioactivity in 4 mL of ACS scintillation cocktail (Amersham). A control was run by treating an equivalent amount of bovine serum albumin with [^{3}H]$NaBH_4$ under the conditions described above.

Paper Chromatography of the Base Hydrolysate of Reduced PPC-DC. The other half of the above base hydrolysate that had been passed through the Dowex 50 column and neutralized was lyophilized and dissolved in 50 μL of H_2O. The solution was streaked on a Whatman 3MM paper and chromatographed in EtOH–NH_4OH–H_2O (32:1:7). On one part of the paper was spotted DL-lactate as the standard. After 10 cm development, the portion of the paper with the standard lactate was cut and sprayed with 0.1% (EtOH) 2,6-dichlorophenolindophenol. The lactate was visualized as a pink spot on the light blue background (R_f = 0.51). The remaining part of the paper was then cut into 0.5 cm wide pieces parallel to the development line and put into vials with 1 mL of H_2O. After a gentle shaking, 12 mL of ACS cocktail (Amersham) was added, and the vials were counted for radioactivity.

Reduction of Ethyl Pyruvate with [^{3}H]NaBH$_4$. In a parallel experiment with the reduction of PPC-DC with sodium [^{3}H]borohydride, 20 μL of ethyl pyruvate (Aldrich) in 280 μL of 80 mM KP_i, pH 7.0, was reduced with 25 μL of [^{3}H]$NaBH_4$ stock solution (0.536 M in 10 mM NaOH). After a 10-min incubation at 25 °C, the reaction was stopped by addition of 40 μL of acetone. The solution was lyophilized and base hydrolyzed as described above for the reduction of enzyme. The reaction mixture was then neutralized and concentrated by rotary evaporation under reduced pressure. The concentrated solution was then applied on the organic acid analysis column and eluted with 5 mM H_2SO_4 as above. The lactate peak was pooled.

Lactate Assay. The amount of lactate formed by reduction of ethyl pyruvate was measured by the L-lactate dehydrogenase assay coupled to glutamate–pyruvate transaminase (Noll, 1966), assuming the racemic mixture of DL-lactate was formed. The standard curve was generated by assaying known amounts of L-lactic acid. From this quantitation of lactate, the specific radioactivity of [^{3}H]lactic acid from the reduction of ethyl pyruvate was determined.

Release of Pyruvate from PPC-DC by a Mild Base Treatment. PPC-DC (0.5 mg, 1.7 mg/mL in 80 mM KP_i, pH 7.0) was lyophilized and dissolved in 0.2 mL of 0.1 N NaOH. After the solution was stirred at 25 °C for 1–2 h, it was neutralized with 1 N HCl. The resulting cloudy solution was centrifuged, and the supernatant was assayed for pyruvate by L-lactate dehydrogenase (Kubowitz & Ott, 1943). From the standard curve prepared by assaying known amounts of sodium pyruvate, the amount of pyruvate released from the enzyme was measured.

N-D-Pantothenoyl-L-alanine 4′-Phosphate (III). Pantothenic acid 4′-bis(2,2,2-trichloroethyl)phosphate (82 mg, 0.15 mmol) (Ciardelli et al., 1981) was dissolved in 3 mL of dry tetrahydrofuran, and the solution was cooled to −15 °C in a dry ice–acetone bath. To this solution were added 16 μL of *N*-methylmorpholine (0.15 mmol) and 19 μL of isobutyl chloroformate (0.15 mmol). After the solution was stirred at −15 °C for 5 min, 37 mg of L-alanine benzyl ester hydrochloride (0.17 mmol) and 19 μL of *N*-methylmorpholine (0.17 mmol) in 3 mL of tetrahydrofuran were added. After the reaction mixture was stirred for 15 min in the cold bath, it was concentrated to dryness by rotary evaporation under reduced pressure. The residue was dissolved in 10 mL of ethyl acetate and successively washed with 5 mL each of H_2O, 5% citric acid, 10% $NaHCO_3$, and H_2O. The organic layer was dried over $MgSO_4$ and concentrated to a yellow oil. Without further purification, thus formed 4′-bis(2,2,2-trichloroethyl)-phosphopantothenoylalanine benzyl ester was first reduced with zinc in tetrahydrofuran to remove trichloroethyl groups (Carson, 1980). The benzyl group was subsequently removed by catalytic hydrogenation over palladium in methanol–1 N acetic acid (1:1). The solution was concentrated and passed through a Dowex 50WX8 (H^+) column (0.7 × 10 cm) and washed with 5 mL of H_2O. The combined eluates were concentrated to dryness by rotary evaporation in vacuo, and the residue was dissolved in 10 mL of H_2O. The aqueous solution was adjusted to pH 7.2 with 0.1 N $Ba(OH)_2$. The solution was then lyophilized to 32.5 mg of a fluffy white powder of *N*-D-pantothenoyl-L-alanine 4′-phosphate barium salt (0.057 mmol; overall yield 38%): ^{1}H NMR (D_2O) δ 0.80 (s, 3 H), 0.92 (s, 3 H), 2.52 (t, 2 H), 2.61 (q, 1 H), 2.93 (d, 3 H), 3.38 (dd, 1 H), 3.50 (m, 2 H), 3.66 (dd, 1 H), 4.08 (s, 1 H).

D-Pantothenic Acid Methyl Ester 4′-Phosphate (IV). Pantothenic acid 4′-bis(2,2,2-trichloroethyl)phosphate (100 mg, 0.18 mmol) in 10 mL of diethyl ether was methylated with diazomethane. After the solution was concentrated to dryness, the residue was taken up in 5 mL of tetrahydrofuran and reduced with zinc dust and acetic acid (Carson, 1980). The remaining zinc was filtered, and the filtrate was concentrated to about 1 mL. The solution was then passed through a Dowex 50 (H^+) column (0.7 × 10 cm) and worked up as described above. The white powder of D-pantothenic acid methyl ester 4′-phosphate barium salt was obtained with 26% overall yield (21 mg): ^{1}H NMR (D_2O) δ 0.82 (s, 3 H), 0.96 (s, 3 H), 2.56 (t, 2 H), 3.41 (dd, 1 H), 3.51 (m, 2 H), 3.69 (dd, 1 H), 3.70 (s, 3 H), 4.09 (s, 1 H).

N-D-Pantothenoyl-L-cysteine Methyl Ester 4′-Phosphate (V). This compound was prepared from D-pantothenonitrile

4′-phosphate and L-cysteine methyl ester by following the general procedure for the synthesis of *N*-D-pantothenoyl-L-cysteine 4′-phosphate by Nagase (1967). The crude product was tested without further purification as an inhibitor of the PPC-DC.

O-D-*Pantothenoyl-depsi-ambo-cysteine 4′-Phosphate (VI).* 2-Diazo-3-(benzylthio)propionic acid benzyl ester was synthesized from *S*-benzyl-L-cysteine benzyl ester *p*-tosylate (Chemical Dynamics Co.) by following the general procedure of Takamura et al. (1975). The product was purified on a silica gel (60–200 mesh) by eluting with chloroform: overall yield 36%; ^{1}H NMR ($CDCl_3$) δ 3.42 (s, 2 H), 3.86 (s, 2 H), 5.22 (s, 2 H), 7.36 (m, 10 H). Pantothenic acid 4′-bis-(2,2,2-trichloroethyl)phosphate (0.84 g, 1.50 mmol) in 15 mL of chloroform was added dropwise to a stirred solution of the 2-diazo-3-(benzylthio)propionic acid benzyl ester (0.47 g, 1.50 mmol) in 10 mL of chloroform at 25 °C. The reaction mixture was refluxed for 1 h and then cooled to room temperature. The coupled product, 4′-bis(2,2,2-trichloroethyl)phosphopantothenoyl-depsi-*S*-benzyl-ambo-cysteine benzyl ester, was isolated on silica gel GF plates eluting with ethyl acetate; R_f = 0.55. Successive removal of benzyl groups by hydrogenation over palladium and trichloroethyl groups by zinc reduction yielded the crude product of *O*-D-pantothenoyl-depsi-ambo-cysteine 4′-phosphate zinc salt. This crude product was tested as an inhibitor of PPC-DC.

Test of Various Substrate Analogues as Inhibitors of PPC-DC. The incubation mixture contained 15 μL of PPC-DC (2.5 μg/μL in 10 mM KP_i, pH 7.0), 10 μL of 0.2 M Tris-HCl, pH 8.0, 10 μL of 0.2 M DTT, and the aqueous solution of substrate analogue to a total volume of 60 μL. To this solution in the assay vial 10 μL of [^{14}C]PPC was injected either immediately or after 30 min of incubation at 25 °C. The reaction was stopped as described in the assay procedure, and the amount of $^{14}CO_2$ was measured accordingly. Controls contained substrate only.

Reduction of PPC-DC in the Presence of PPC. To test if the enzymatic mechanism of decarboxylation involves an imine intermediate, with the carbonyl functional group of the enzyme, the following experiments were carried out to trap such an intermediate. Tris-HCl buffer, pH 8.0, (5 μL, 0.2 M), 20 μL of PPC-DC (5.0 μg/μL in 30 mM KP_i, pH 7.0), 10 μL of 0.2 M DTT, and 20 μL of [^{35}S]PPC (18.0 mM in H_2O, 2.98 × 10^5 cpm/μmol) were mixed together. After 1 min of standing at 25 °C, 100 μL of 2 M sodium cyanoborohydride was added. After 2 min, 200 μL of 1 M HCl was injected into the solution to stop the reaction. The reaction mixture was incubated at 25 °C for 30 min with frequent shaking. Finally, 1.1 mL of 15% trichloroacetic acid was added to precipitate the protein. After centrifugation, the pellet was dissolved in 50 μL of 6 M urea and this solution was applied on an HPLC sizing column (Spherogel–TSK 3000SW; 7.5 × 300 mm; Altex) connected to a Beckman 112 HPLC system. The column had been equilibrated with 6 M urea in 0.1 M KP_i, pH 7.0, buffer. The column was eluted with the same buffer at 0.5 mL/min flow rate. Fractions (0.5 mL) were collected and measured for protein (Bradford, 1976) and also for radioactivity. The latter was measured by taking a 0.4-mL aliquot of the fraction into 1.0 mL of H_2O and 4.0 mL of ACS scintillation cocktail (Amersham). Parallel control experiments were run in the absence of sodium cyanoborohydride.

RESULTS

Enzyme Purification. Table I summarizes the purification of *E. coli* PPC-DC. Upon gel exclusion chromatography two peaks (a and b) showed the enzyme activity, although peak b had a one-fifth of the specific activity of peak a. The elution volume of peak a corresponded to a protein with M_r 146 000 ± 9000. Peak b corresponded to a molecular weight of approximately 30 000. When the protein was incubated overnight at 30 °C prior to application on the column, peak b increased at the expense of peak a. When analyzed by SDS–polyacrylamide electrophoresis, peak a migrated as a single sharp band corresponding to a molecular weight of 35 000 ± 3,000. Peak b showed additional protein bands as well as the 35 000 band. It is presumed that peak b contains monomeric PPC-DC along with other proteins. Peak a represents native PPC-DC.

Table I: Purification of *E. coli* PPC-DC

	protein (mg)	vol (mL)	concn (mg/mL)	total units	units/mg of protein
sonication	30740	580	53	21000	0.69
25% protamine sulfate	9040	1000	8.8	14600	1.6
DEAE-52	320	450	0.71	5000	15.4
hydroxyapatite	41	6.6	6.3	3800	93
FPLC (mono Q)	3.9	0.25	16	1600	400
HPLC (gel permeation)	2.0	1.1	1.8	1200	600

Table II: Inactivation of *E. coli* PPC-DC by Various Carbonyl Reagents and Pyridoxal[a]

	concn (mM)	remaining act. (%)
none		100
hydroxylamine	100	22
	20	48
phenylhydrazine	100	0
	20	21
KCN	100	0
	20	32
$NaBH_4$	50	0
	10	25
pyridoxal	50	0
pyridoxal 5′-phosphate	50	0

[a] See text for experimental details.

V_{max} and K_m of E. coli PPC-DC. At pH 7.6 and 25 °C V_{max} was 600 nmol/(min·mg of protein). The K_m at pH 7.6 and 25 °C was 0.9 mM.

Inactivation of PPC-DC by Carbonyl Reagents and Pyridoxal. As shown in Table II, carbonyl reagents as well as pyridoxal and pyridoxal phosphate inactivate the PPC-DC. Inactivation of horse liver PPC-DC by NH_2OH, phenylhydrazine, and sodium borohydride was reported (Scandurra et al., 1974). Pyridoxal phosphate also inactivates the horse liver enzyme (Scandurra et al., 1974). The degrees of inactivation under similar reaction conditions are comparable for the two enzymes. Sodium borohydride irreversibly inactivates the enzyme with no reactivation after an exhaustive dialysis. The inactivation rate by $NaBH_4$ decreases in the presence of substrate, and the protective effect by substrate displays saturation behavior. Half-maximal protection is observed at a substrate concentration close to K_m. At infinite substrate concentration the rate of inactivation approaches zero. These results suggest that $NaBH_4$ reduces a structure at the active site.

Reduction of PPC-DC with [^{3}H]$NaBH_4$. Enzyme was reduced with several concentrations of [^{3}H]$NaBH_4$, and the amount of radioactivity incorporated into the protein was determined as well as the remaining catalytic activity (Table III). The data indicate that a considerable amount of nonspecific labeling occurred. The reduced, inactive protein was then treated with base (see Experimental Procedures), and the amount of material that was not retained by Dowex 50, H^+,

Table III: ^3H Analysis of [^3H]$NaBH_4$-Reduced *E. coli* PPC-DC[a]

[^3H]$NaBH_4$ (mM)	cpm/mg of protein	inactivation[b] (%)	cpm/mg of protein eluted from Dowex 50
0	0	0	0
10	250000	76	41000
50	356000	100	59000
100	440000	100	56000
50[c]	320000		0

[a] For experimental details, see text. [b] Remaining activity was measured after dialysis. [c] Bovine serum albumin was used as a control, in place of PPC-DC.

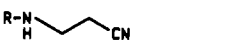
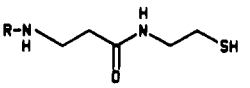
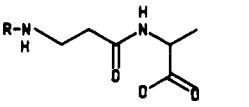
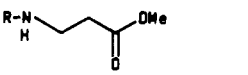
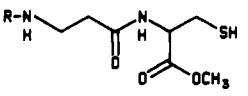
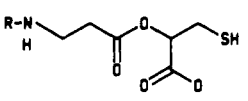
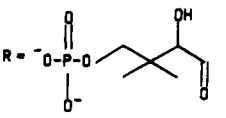
i.e., uncharged or negatively charged molecules, was determined. As the data in Table III show, this material is a fraction of the total radioactivity incorporated into the protein. Furthermore, increasing the amount of the reducing agent, the reduction time, or the base hydrolysis time did not increase the amount of radioactivity, which did not bind to the cation exchanger. No radioactive material is isolated when serum albumin is substituted for PPC-DC.

Identification of Dowex 50 Effluent. In order to identify the radioactive substance that did not bind to the Dowex 50 column, the material was examined with several chromatographic systems. One system, the HPLC organic acid column, affords good separations between many organic acids and alcohols including lactate, acetate, pyruvate, glycerate, 2-hydroxybutyrate, 3-hydroxybutyrate, 1,2-propanediol, 1,3-butanediol, and 2,3-butanediol, among others. More than 90% of the radioactivity found in the Dowex 50 effluent, when applied on the organic acid column, comigrated with the carrier DL-lactic acid. It was also found to comigrate as a single sharp peak when injected together with DL-[^3H]lactic acid prepared from ethyl pyruvate as described. On ascending paper chromatography on Whatman 3MM paper in EtOH–NH_4OH–H_2O (32:1:7), DL-lactate migrates as a single spot with R_f = 0.51. Again, more than 90% of the applied radioactivity comigrated with the authentic DL-lactate. These data suggested that the enzyme contains pyruvate bound as ester at the active site, in agreement with the data of Scandurra (1979).

To further characterize this component of the active site, the enzyme was subjected to base hydrolysis and the reaction mixture was assayed for pyruvate with lactic dehydrogenase. One mole of pyruvate was released from PPC-DC per 31000 ± 4,000 g of enzyme. Increasing the exposure to base from 1 to 2 h did not increase the amount of pyruvate. Control experiments with ethyl pyruvate established that after 60 min the amount of pyruvate detectable by this assay procedure reaches a maximum (89% of the theoretical amount) and does not change upon longer exposure. No pyruvate was detected when L-lactate dehydrogenase was added to 0.5 mg of native PPC-DC without base treatment.

We carried out an experiment to quantitatively determine the amount of [^3H]lactate released upon [^3H]$NaBH_4$ reduction and base treatment. It is difficult to assay the specific activity of [^3H]$NaBH_4$. We, therefore, reduced ethyl pyruvate with the same solution of [^3H]$NaBH_4$ that was used to inactivate the enzyme. After reduction, the reaction mixture was exposed to the same basic conditions used with the enzyme. The reduction and subsequent base hydrolysis of ethyl pyruvate with [^3H]$NaBH_4$ produced two major products. They were identified as lactate and 1,2-propanediol with the HPLC organic acid column. The ratio of lactate to propanediol was 93:7, based on peak areas. The lactate peak was assayed for the amount of lactate as described under Experimental Procedures, and thus the specific radioactivity of [^3H]lactate was determined as 2340 cpm/nmol of DL-lactate, assuming the racemic mixture was formed. From this specific radioactivity of [^3H]lactate and the total radioactivity isolated as DL-lactate from reduced PPC-DC, 1 mol of DL-lactate was estimated per 39000 g of reduced enzyme.

Table IV: Various Substrate Analogues as Inhibitors of *E. coli* PPC-DC

COMPOUND		HIGHEST CONCENTRATION TESTED (mM)	% INHIBITION
CYSTEINE		25	4
CYSTEAMINE		17	<1
	(I)	34	22
	(II)	6	<5
	(III)	10	<2
	(IV)	22	<10
	(V)[a]	~15	
	(VI)[a]	~10	
			

[a] Crude compounds were tested. Since no inhibition was detected at concentrations indicated, no further purification and analysis were attempted.

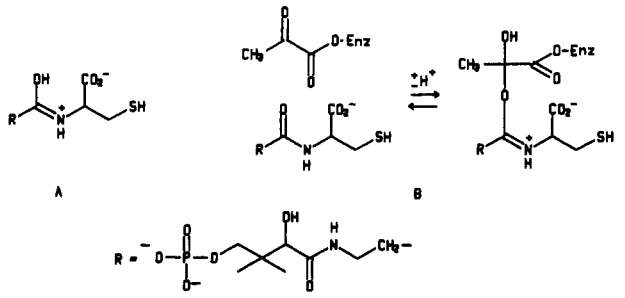
Various Substrate Analogues as Inhibitors of E. coli PPC-DC. Table IV summarizes the results of testing various substrate analogues as possible inhibitors of PPC-DC. None of the tested compounds was effective as an inhibitor even at high concentration.

Attempts To Trap Reaction Intermediate(s) of the Enzymatic Decarboxylation of PPC. Attempts were made to trap enzyme–substrate adducts by acid precipitation of the protein that had been exposed to labeled substrate and $NaCNBH_3$. However, no labeled protein was isolated. Negative results are not informative. Failure to trap an intermediate with $NaCNBH_3$ could be due to inaccessibility of the intermediate to $NaCNBH_3$ or due to the fact that the Schiff base intermediate is not involved.

Discussion

PPC-DC contains covalently bound pyruvate. This conclusion is based upon the isolation of pyruvate upon mild base hydrolysis and on the release of [^3H]lactate after reduction with [^3H]$NaBH_4$ and subsequent base hydrolysis. One mole of pyruvate was released per 31000 ± 4000 g of enzyme, and

Scheme I: Possible Participation of an Ylide in the Decarboxylation of 4′-Phosphopantothenoylcysteine



1 mol of DL-[^{3}H]lactate was released per 39 000 g of enzyme. Therefore, 1 mol of pyruvate is present per enzyme subunit.

The release of lactate and pyruvate by a mild base treatment of reduced and unreduced *E. coli* PPC-DC, respectively, indicates the pyruvate is linked to the enzyme as an ester rather than as an amide. In cases where pyruvate is bound to a protein connected by amide bonds, lactate was not released even after 30-min incubations of $NaBH_4$-reduced enzymes at 100 °C in 0.1 N NaOH (Hodgins & Abeles, 1969; Riley & Snell, 1968). Moreover, a pyruvoyl amide, pyruvoylglycine, was found to undergo irreversible transformation to an uncharacterized structure at pH 12 at 25 °C within 2.5 h, and isolation of pyruvate from this structure failed (Errera & Greenstein, 1947). Fu and Greenstein also observed similar reactions with pyruvoylalanine and pyruvoylphenylalanine [1951; also Greenstein and Winitz (1961)].

None of the substrate analogues with various degrees of structural departure from the substrate showed any significant inhibition of the decarboxylase, indicating the tight substrate specificity of the enzyme. Both the horse liver enzyme and the rat liver enzyme have been reported to be highly substrate specific (Abiko, 1967; Scandurra et al., 1974, 1979). The rat liver enzyme was reported to be inhibited by the product pantetheine 4′-phosphate (K_i = 0.43 mM; Abiko, 1967), whereas no significant product inhibition was observed for the horse liver enzyme (Scandurra et al., 1974) and the *E. coli* enzyme.

The presence of covalently bound pyruvate in both *E. coli* and horse liver enzymes (Scandurra et al., 1979) indicates the general mechanistic role of pyruvate. The decarboxylase differs from histidine decarboxylase in that the pyruvyl moiety is bound in an ester linkage rather than an amide linkage (Recesei & Snell, 1984). The residue must therefore be introduced by a different mechanism, possibly a posttranslational modification.

A simple mechanism for the enzymatic decarboxylation could involve decarboxylation of the ylide represented by structure A (Scheme I). A similar nitrogen ylide mechanism has been proposed for orotidine-5′-phosphate decarboxylase, which decarboxylates orotidine 5′-phosphate to uridine 5′-phosphate (Beak & Siegel, 1976). The covalently bound pyruvate may participate in the reaction by forming an adduct with the enol oxygen of the amide (structure B, Scheme I) and thereby increasing the positive charge on the amide nitrogen, which would facilitate the decarboxylation.

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