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Coenzyme A Biosynthesis: Steric Course of 4′-Phosphopantothenoyl-L-cysteine Decarboxylase†

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ABSTRACT: 4′-Phosphopantothenoyl-L-cysteine decarboxylase (*P*PC decarboxylase) was partially purified from rat liver. 4′-Phosphopantothenoyl[2-$^2H_1$]-L-cysteine was synthesized and converted by *P*PC decarboxylase to 4′-phospho[1-$^2H_1$]pantetheine. The product was degraded by reduction with Raney nickel followed by acidic hydrolysis to [1-$^2H_1$]ethylamine. The latter was converted to the (−)-camphanamide derivative, NMR studies of which revealed that the deuterium was located in the *pro*-1*S* position. Also, unlabeled 4′-phosphopantothenoyl-L-cysteine was incubated with *P*PC decarboxylase in D_2O, giving, after degradation, the (−)-camphanamide of (1*R*)-[1-$^2H_1$]ethylamine. The results show that the decarboxylation takes place with retention of configuration. These results are discussed in terms of possible mechanisms for the decarboxylation.

The decarboxylation of 4′-phosphopantothenoyl-L-cysteine (*P*PC)[1] (**1**) (see Chart I) to 4′-phosphopantetheine (*P*P) (**2**) by 4′-phosphopantothenoyl-L-cysteine decarboxylase [4′-phospho-*N*-(D-pantothenoyl)-L-cysteine carboxy-lyase, EC 4.1.1.36] (*P*PC decarboxylase) constitutes a step in the biosynthesis of the ubiquitous acyl group carrier coenzyme A (Abiko, 1975). Two purifications of this enzyme have been reported, one using rat liver (Abiko, 1970, 1967) and the other horse liver (Scandurra et al., 1974) as source. The decarboxylases obtained from the two sources appeared to be very similar. The enzyme has an absolute requirement for the 4′-phosphate of the substrate and does not decarboxylate pantothenoylcysteine. We now report the results of our studies on the steric course of the decarboxylation of *P*PC, which show that the decarboxylation proceeds in a *retention* mode.

Materials and Methods

^1H NMR spectra were run on a Varian EM-360 and on a Bruker WM-250 instrument. ^2H NMR spectra were run on

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[1] Abbreviations: *P*PC decarboxylase, 4′-phosphopantothenoyl-L-cysteine decarboxylase; *P*PC, 4′-phosphopantothenoyl-L-cysteine; *P*P, 4′-phosphopantetheine; Tris, tris(hydroxymethyl)aminomethane; SW, spectral width; PW, pulse width; RD, relaxation delay; LB, line broadening.

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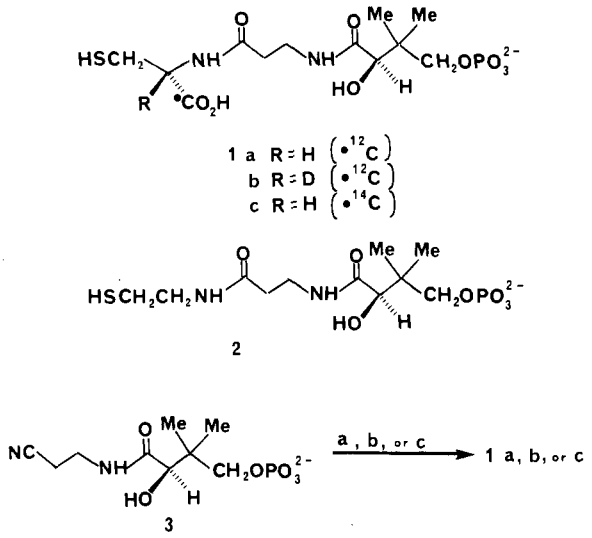
Chart I[a]



[a] Reagents indicated on arrow are as follows: a, L-cysteine; b, [2-$^2H_1$]-L-cysteine; c, [1-^{14}C]-DL-cysteine (product 1c is *RS* at C-2).

a Bruker WM-500 instrument. Gas chromatograms were run on a Varian 920 instrument. Liquid scintillation counting was performed on a Nuclear Chicago Mark III instrument. Samples were dissolved in 10 mL of New England Nuclear Aquasol. Radiochromatograms were scanned by using a Nuclear Chicago Actigraph III instrument. Thin-layer chromatography (TLC) was performed by using Analtech cellulose MN300 plates. Frozen rat livers were obtained from Pel-Freeze Biologicals, Rogers, AR. [1-^{14}C]-DL-Cysteine was obtained from RPI Corp. High-performance liquid chromatography (HPLC) was performed by using a Waters instrument equipped with an M-6000A pump, a U6K injector, and a Model 450 variable wavelength detector set at 200 nm. The column used was a Waters Associates μBondapak C_{18} 3.7 mm × 30 cm column. The solvent was 0.020 M potassium phosphate, pH 3.5, at 1.0 mL/min. Postcolumn detection of thiols was performed by directing the outlet from the column through two Kratos Model URS 050 postcolumn reaction systems connected in series. Into the first reaction system was pumped 0.025 M *o*-phthalaldehyde in 50% MeOH–H_2O at 0.75 mL/min. Into the second reaction system was pumped 0.010 M taurine in 0.40 M sodium borate buffer, pH 9.4, at 0.75 mL/min. Each reactor was equipped with a 1.0-mL capacity reaction chamber. Each reagent was filtered through a 0.45-μm filter and degassed by pumping before use. The outlet from the second reactor was directed into a Kratos Model FS950 fluorometer equipped with a 365-nm excitation filter and a 418-nm emission filter.

Synthesis of 4′-Phosphopantothenoyl[2-$^2H_1$]-L-*cysteine* (**1b**) *and 4′-Phosphopantothenoyl*[1-^{14}C]-DL-*cysteine* (**1c**). *S*-Benzyl-L-cysteine (10.5 g) was treated with D_2O (20 mL), and the mixture was freeze-dried. The deuterium-exchanged product was then treated with acetic anhydride (100 mL) and D_2O (13 mL) at reflux for 2 min. The solvents were removed under reduced pressure, and the acetic anhydride–D_2O treatment was repeated twice. The product was dissolved in hot acetone, and the solution was treated with charcoal, filtered, and concentrated. After standing at 25 °C, and then at 0 °C, *N*-acetyl-*S*-benzyl[2-$^2H_1$]-DL-cysteine (8.1 g) was recovered. The ^1H NMR (D_2O–NaOD) showed no signal for the α-proton. A portion of the product was then resolved by using acylase according to a literature procedure (Greenstein & Winitz, 1961). The resultant *S*-benzyl[2-$^2H_1$]-L-cysteine was further converted to [2-$^2H_1$]-L-cysteine hydrochloride by published methods (Greenstein & Winitz, 1961). This product (940 mg) was then converted by the method of Nagase (1967) to 4′-phosphopantothenoyl[2-$^2H_1$]-L-cysteine, (**1b**), 1.6 g: ^2H NMR (^2H-depleted H_2O) δ 2.23 (integral 26.0), with a minor impurity absorbing at δ 1.27 (integral 4.3).

[1,1′-$^{14}C_2$]-DL-Cysteine (0.68 mg, 100 μCi) was mixed with DL-cysteine (1.21 g, 10 mmol) and converted, as described by Scandurra et al. (1974), to 4′-phosphopantothenoyl[1-^{14}C]-DL-cysteine (**1c**), 2.5 g (10 μCi/mmol). Radiochromatography [solvent, *n*-BuOH–HOAc–H_2O (5/2/3)] showed one main radioactive peak (ca. 85%, R_f 0.29) and a minor radioactive impurity (ca. 15%, R_f 0.44; probably pantothenoyl-DL-cysteine).

Purification and Assay of PPC Decarboxylase. Frozen rat liver (70 g, kept at −70 °C prior to use) was stirred for 2 min in 140 mL of cold (4 °C) potassium phosphate buffer (0.015 M, pH 7.6) and then homogenized in a Waring blender at top speed for 2 min. The homogenate was adjusted to pH 5.7 with 10% H_3PO_4, and then to pH 5.5 with 1 M KH_2PO_4. The mixture was kept at 0 °C for 30 min and then by stirring in an 80 °C water bath raised to 57 °C over 20 min and kept at this temperature for 5 min. The mixture was then cooled in ice–H_2O and centrifuged at 40000*g* for 30 min. The supernatant (160 mL) was treated with $(NH_4)_2SO_4$ (28 g, added continuously over 28 min), while it was stirred at 0 °C. After 20 min of additional stirring, the mixture was centrifuged at 40000*g* for 30 min. The precipitated protein was redissolved in 5 mL of 0.015 M potassium phosphate buffer, pH 7.6, and dialyzed against 800 mL of this same buffer for 20 h at 4 °C. The resultant solution was used for conversions of ^{14}C- or ^2H-labeled *P*PC to *P*P. Typically, the dialysate contained ca. 14 units/mL *P*PC decarboxylase with a specific activity of ca. 2 units/mg of protein.

*P*PC assays were performed as described (Scandurra et al., 1974), but with 4′-phosphopantothenoyl[1-^{14}C]-DL-cysteine as substrate. Protein assays were performed by using the Bio-Rad protein assay kit, with bovine serum albumin as standard.

Conversion of 4′-Phosphopantothenoyl[2-^2H]-L-*cysteine to 4′-Phospho*[1-$^2H_1$]*pantetheine; Degradation of 4′-Phospho*-[1-$^2H_1$]*pantetheine to the* [1-$^2H_1$]*Ethylamine* (−)-*Camphanamide Derivative.*[2] [2-^2H]*P*PC (**1b**) (180 mg, 0.33 mmol) was dissolved in a solution (60 mL) containing Tris base (0.605 g, 5 mmol) and L-cysteine (1.36 g, 11 mmol), pH 8.0, and treated with 5 mL of the *P*PC decarboxylase solution prepared as described above, under N_2 at 37 °C with stirring for 20 h. The conversion of *P*PC to *P*P was followed by HPLC and essentially stopped after 4–5 h with ca. 20–30% conversion. The mixture was then passed through a 2 cm × 10 cm column of Dowex 50W-X8, 50–100 mesh, H^+ form. HPLC analysis indicated the absence of cysteine in the eluate, which was then freeze-dried. The resultant product (0.25 g) was dissolved in EtOH–H_2O (1/1, 20 mL) and treated with freshly prepared W-2 Raney nickel (Mozingo, 1955) (200 mg) at reflux for 5 h under argon. The mixture was cooled and filtered, and the filtrate was evaporated under reduced pressure. The residue (0.12 g) was treated with 2 N HCl (3.5 mL) at reflux

[2] The conditions for the reduction–hydrolysis sequence were established by using commercially available pantethine as a model. By use of deuterated Raney nickel prepared from Raney nickel alloy in NaOD–D_2O, it was established that no significant hydrogen migration occurs in the reduction. The isolated ethylamine hydrochloride had ca. 88% D in the methyl group and only ca. 12% D in the methylene, as shown by deuterium NMR.

for 4 h. The solution was then made alkaline with excess concentrated NaOH and placed in a distillation apparatus having an argon inlet in place of a thermometer. The solution was warmed at 60 °C while the volatile materials were passed directly into a solution of concentrated HCl (1 mL) in MeOH (9 mL) over $2^1/_2$ h. The acidic solution was then evaporated under reduced pressure to yield crude [1-$^2H_1$]ethylamine hydrochloride, ca. 3 mg. This was treated with (−)-camphanyl chloride (50 mg) in dry pyridine (0.2 mL) at 20 °C for 18 h. Ether (2 mL) was added, and the extract was washed (2 N HCl, H_2O, 10% $NaHCO_3$), dried (Na_2SO_4), and evaporated to yield ca. 6 mg of crude product. The pure [1-$^2H_1$]ethylamine (−)-camphanamide was isolated by preparative gas chromatography ($^1/_4$ in. × 6 ft column containing 15% SE-30 on 80/100-mesh Supelcoport at 178 °C; retention time, 7 min): yield, ca. 1 mg; NMR, see legend to Figure 2.

Conversion of Unlabeled 4′-Phosphopantothenoyl-L-cysteine to 4′-Phospho[1-$^2H_1$]pantetheine; Degradation of 4′-Phospho[1-$^2H_1$]pantetheine to the [1-$^2H_1$]Ethylamine (−)-Camphanamide Derivative.[2] Unlabeled *P*PC (180 mg) was converted to [1-$^2H_1$]*P*P exactly as described in the preceding section, except that the Tris–cysteine buffer was prepared by using D_2O (60 mL) in place of H_2O (60 mL). The decarboxylation product was converted to the [1-$^2H_1$]ethylamine (−)-camphanamide derivative as in the preceding section: yield, ca. 2 mg; ^2H NMR, see legend to Figure 3.

Synthesis of (1RS)-[1-$^2H_1$]Ethylamine. Acetaldoxime (0.50 g, 8.5 mmol) was dissolved in absolute EtOH (75 mL) and $CHCl_3$ (2 mL) and was reduced with deuterium in the presence of PtO_2 (150 mg) at 200 psi in a Parr 450-mL pressure reactor for 90 min (Secrist & Logne, 1972). The solution was filtered and the solvent evaporated under reduced pressure to yield [1-$^2H_1$]ethylamine hydrochloride as a semicrystalline solid, 0.4 g: NMR (D_2O) δ 1.40 (d, 3 H, J = 7 Hz) and 3.30 (q, 1 H, J = 7 Hz). A portion of the product was converted, as described above, to the (−)-camphanamide, a portion of which was purified by preparative gas chromatography (GC) as described above: ^2H NMR, see text.

RESULTS

Our approach to the determination of the stereochemistry of this reaction was to incubate 4′-phosphopantothenoyl[2-$^2H_1$]-L-cysteine (**1b**) with the decarboxylase, and then to analyze the chirality in the [1-$^2H_1$]-β-mercaptoethylamide unit of the resultant *P*P by reductive degradation to [1-^2H]-ethylamine. The complementary experiment, in which unlabeled *P*PC was subjected to decarboxylation in a D_2O-containing medium, was also performed, leading (after degradation) to an enantiomeric sample of [1-^2H]ethylamine. These samples of (presumably) stereospecifically deuterated ethylamine were then subjected to chirality analysis by a recently published method (Parker, 1983).

Since neither *P*PC nor *P*P are commercially available, we first undertook the syntheses of these compounds. *P*P (**2**) was synthesized by a published method (Moffatt & Khorana, 1961) from commercially available pantethine. *P*PC (**1a**) was synthesized by the method of Nagase (1967) from pantothenonitrile 4′-phosphate (**3**). The products, isolated as noncrystalline barium salts, proved rather difficult to analyze for chemical purity by the published method [TLC or paper chromatography, with detection by the Hanes–Isherwood spray (Hanes & Isherwood, 1949)]. In our hands, these compounds tended to run as elongated streaks, and the detectability was poor.

As an alternative method of analysis, we reexamined a published HPLC method for the separation for all of the intermediates in the biosynthesis of coenzyme A, including *P*PC and *P*P (Halvorsen & Skrede, 1980). Unfortunately, the detection method for *P*PC and PP was rather awkward, involving the collection of fractions that were assayed for radioactivity content. Thus, nonradioactive *P*PC or *P*P could not be detected by this method. We found, however, that these compounds could be detected by UV at 200 nm, albeit with low sensitivity and with a very noisy background having many extraneous peaks due to (trace) impurities. The method was greatly improved by addition of a postcolumn reaction detector system recently reported for use with thiols (Nakamura & Tamura, 1981). Thus *P*PC and *P*P could be readily separated and detected with high sensitivity with only a moderate amount of unidentified background absorption (Figure 1). The detection sensitivities for *P*PC and *P*P were nonequal, that for *P*P being ca. 3-fold greater than that of *P*PC. This HPLC method proved to be very useful in following the enzymatic conversions of unlabeled or deuterium-labeled *P*PC to their respective decarboxylation products.

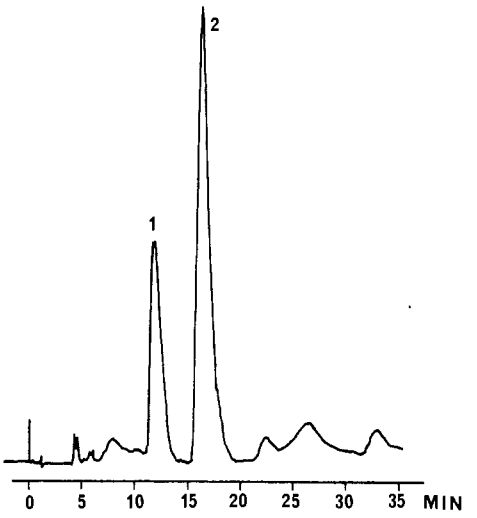


FIGURE 1: HPLC chromatogram of equimolar mixture of synthetic *P*PC and *P*P: peak 1, *P*PC; peak 2, *P*P; other peaks, unidentified impurities. For conditions, see Materials and Methods.

We next undertook the purification of the required *P*PC decarboxylase. Fresh rat liver was initially used as enzyme source, since this was reported to have the highest enzymatic activity of any animal source (Scandurra et al., 1974) (the relative activity of various nonanimal sources does not appear to have been reported). However, we later found that frozen rat livers, obtained from a commercial source, had the same activity as fresh livers, and therefore these were used for most of our work. The method of enzyme purification used (see Materials and Methods) was, however, based mainly on that reported for the horse liver enzyme, as this method appeared to be much less complicated. For preparative conversions of *P*PC, the enzyme was purified only through the ammonium sulfate precipitation stage and had adequate activity for our purposes at that stage. Enzyme assays were carried out essentially as described by Scandurra et al. (1974), except that, instead of *P*PC synthesized from [U-^{14}C]-L-cysteine, we used as substrate *P*PC synthesized from [1-^{14}C]-DL-cysteine.

The required deuterated *P*PC (**1b**) was then synthesized from **3**. This was incubated with partially purified *P*PC decarboxylase, and the reaction was followed by HPLC until no further change occurred. The reaction could never be carried to completion. In fact, typically, conversions of less than 30% were usually observed. This may, in part, be due to loss of the essential 4′-phosphate by residual phosphatase activity.

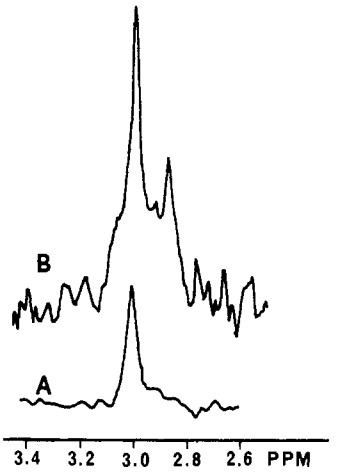
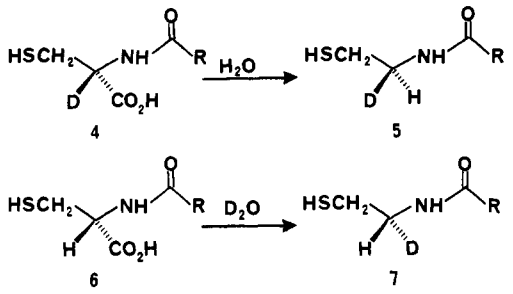


FIGURE 2: ^{1}H-decoupled ^{2}H NMR spectra, run at 76.77 MHz in 3.30 mm o.d. (2.34 mm i.d.) Wilmad coaxial cells contained in a 5 mm o.d. outer tube, in C_6H_6 solutions. (A) Product obtained after incubation of [2-$^2H_1$]*P*PC with *P*PC decarboxylase, followed by Raney nickel reduction, acidic hydrolysis, and derivatization of the resultant [1-$^2H_1$]ethylamine with (−)-camphanyl chloride; SW, 1000 Hz, 0.977 Hz/point; PW, 30 μs; RD, zero; LB, 2 Hz; 22956 scans; repetition rate, 1.024 s. (B) Sample A, admixed with an equal amount of (1*RS*)-[1-$^2H_1$]ethylamine (−)-camphanamide: SW, 800 Hz, 0.781 Hz/point; PW, 30 μs; RD, zero; LB, 1 Hz; 17044 scans; repetition rate, 1.28 s.

Scheme I



Also, the decarboxylase from rat liver (Abiko, 1967) [but not that from horse liver (Scandurra et al., 1974)] was found to be inhibited by the decarboxylation product, *P*P. After incubation, the excess cysteine (used in the buffer) and proteins were removed by filtering the mixture through a cation-exchange resin. The crude product was then reduced with Raney nickel. Following hydrolysis with 2 N HCl, [1-$^2H_1$]ethylamine hydrochloride was isolated and was converted (in very low overall yield) to the (−)-camphanamide derivative. The ^{2}H NMR spectrum of this product showed a single peak at δ 2.98 (Figure 2A). After addition of an equal amount of the (1*RS*)-[1-$^2H_1$]ethylamine (−)-camphanamide derivative, two peaks were visible, at δ 2.87 and 3.00, the downfield peak being ca. 3-fold greater in intensity (Figure 2B). Thus, it is clear that the deuterium resonance of the biosynthesis product is appearing in the *downfield* position, which Parker (1983) has shown to be due to the *pro*-1*S* position of ethylamine. It follows that the biosynthesis product is labeled as shown in **5** and that therefore the eliminated carboxyl was replaced by hydrogen with *retention* of configuration, **4** → **5** (Scheme I).

In the complementary experiment, unlabeled *P*PC (**1a**) was incubated with the decarboxylase in a buffer prepared by using D_2O in place of H_2O. The crude product was converted, as before, to [1-$^2H_1$]ethylamine hydrochloride, which was converted to the (−)-camphanamide derivative. Again, a single deuterium resonance, at δ 2.87, was observed (Figure 3A). After adding a small amount of the (1*RS*)-[1-$^2H_1$]ethylamine (−)-camphanamide derivative, a ^{2}H NMR spectrum (Figure 3B) similar to that shown in Figure 2B, but with reversed peak intensities, was obtained. Thus, the steric course of the decarboxylation, **6** → **7**, was confirmed by this experiment.

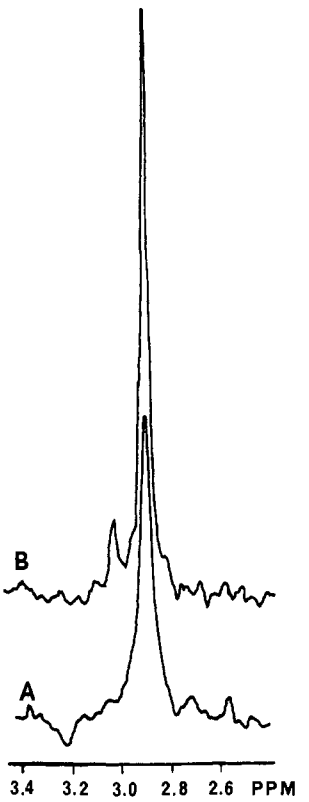
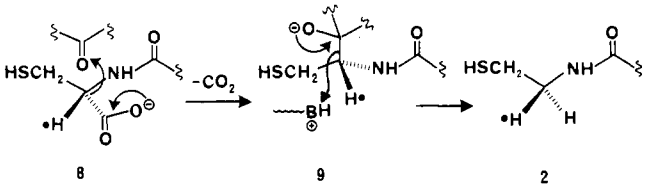


FIGURE 3: ^{1}H-decoupled ^{2}H NMR spectra, run at 76.77 MHz in 3.30 mm o.d. (2.34 mm i.d.) Wilmad coaxial cells contained in a 5 mm o.d. outer tube, in C_6H_6 solutions. (A) Product obtained after incubation of unlabeled *P*PC in a D_2O-containing buffer with *P*PC decarboxylase, followed by Raney nickel reduction, acidic hydrolysis, and derivatization of the resultant [1-$^2H_1$]ethylamine with (−)-camphanyl chloride: SW, 1000 Hz, 0.488 Hz/point; PW, 40 μs; RD, zero; LB, 2 Hz; 421 scans; repetition rate, 2.048 s. (B) Sample A, admixed with a small amount of (1*RS*)-[1-$^2H_1$]ethylamine (−)-camphanamide: SW, 1000 Hz, 0.488 Hz/point; PW, 40 μs; RD, zero; LB, 1 Hz; 1049 scans; repetition rate 2.048 s.

Discussion

The steric courses of a large number of decarboxylases have been determined (Walsh, 1979). The results have shed considerable light on the mechanisms of such reactions. The enzyme investigated in the present study was of particular interest to us because its mechanism must differ substantially from the mechanisms of other decarboxylases. There appears no obvious activation pathway (e.g., oxidation of a β-hydroxyl group or formation of a Schiff base type intermediate). In fact, the formation of a Schiff base intermediate is impossible, and the enzyme is actually inhibited by pyridoxal phosphate. Evidence has been reported which suggests that the enzyme may have a carbonyl group (of unknown nature) at the active site (Scandurra et al., 1974). Thus *P*PC decarboxylase (from horse liver) was strongly inhibited by hydroxylamine, phenylhydrazine, or sodium borohydride. Decarboxylases containing an active site pyruvoyl group are well-known (Recsei & Snell, 1970), but clearly the carbonyl group of *P*PC decarboxylase cannot be involved in Schiff base formation with the substrate, as shown for the pyruvoyl group of histidine decarboxylase (Recsei & Snell, 1970). Possibly, the carbonyl group may serve as an anchor for the essential sulfhydryl group of *P*PC, with the formation of a thiohemiacetal or thiohemiketal intermediate. This carbonyl group (which would

Scheme II: Hypothetical Reaction Mechanism for *P*PC Decarboxylase



then have to be highly conjugated) may be responsible for the reported (Scandurra et al., 1974) UV absorption of the purified enzyme, which shows a shoulder at 370–440 nm, decreased by addition of mercaptoethanol.

Alternatively, the carbonyl may act as a temporary acceptor of the presumed carbanion resulting from CO_2 elimination, with the formation of an intermediate such as **9** (Scheme II). Subsequent fragmentation of **9**, possibly with proton delivery by an active site lysine residue (whose presence is suggested on the basis of the observed inhibition by pyridoxal phosphate), would then yield the product **2**. The decarboxylation and protonation steps (Scheme II) might reasonably be formulated as proceeding with inversion of configuration, resulting in overall retention as observed. Obviously, further studies on this interesting enzyme will be required to confirm the presence and exact nature of the various reactive groupings in the active site.

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L-Alanosine: A Noncooperative Substrate for *Escherichia coli* Aspartate Transcarbamylase†

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ABSTRACT: L-Alanosine, an antibiotic produced by *Streptomyces alanosinicus*, can be used by *Escherichia coli* aspartate transcarbamylase as a substrate instead of L-aspartate. The Michaelis constant of the catalytic subunit for this analogue is about 10 times higher than that for the physiological substrate, and the catalytic constant is about 30 times lower. The saturation curve of the native enzyme for L-alanosine indicates the lack of homotropic cooperative interactions between the catalytic sites for the utilization of this compound. It appears therefore that L-alanosine is unable to promote the allosteric transition. However, *N*-(phosphonoacetyl)-L-aspartate, a "bisubstrate analogue" of the physiological substrates, stimulates the reaction. This phenomenon is very similar to that reported by Foote and Lipscomb [Foote, J., & Lipscomb, W. N. (1981) *J. Biol. Chem. 256*, 11428–11433] concerning the reverse reaction using carbamylaspartate. The reaction is normally sensitive to the physiological effectors ATP and CTP. The significance of these results for the mechanism of the allosteric regulation is discussed.

Escherichia coli aspartate transcarbamylase (EC 2.1.3.2) catalyzes the first reaction of the pyrimidine pathway, that is, the carbamylation of the amino group of aspartate by carbamyl phosphate. This reaction is feedback-inhibited by CTP and stimulated by ATP. *E. coli* aspartate transcarbamylase (ATCase)[1] is made by the association of two

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[1] Abbreviations: ATCase, aspartate transcarbamylase; PALA, *N*-(phosphonoacetyl)-L-aspartate; T and R forms, tight and relaxed forms of the enzyme having low and high affinity, respectively, for the substrate aspartate; Tris, tris(hydroxymethyl)aminomethane; PEI–cellulose, poly(ethylenimine)–cellulose.

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