The Phosphonopyruvate Decarboxylase from *Bacteroides fragilis*

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The *Bacteroides fragilis* capsular polysaccharide complex is the major virulence factor for abscess formation in human hosts. Polysaccharide B of this complex contains a 2-aminoethylphosphonate functional group. This functional group is synthesized in three steps, one of which is catalyzed by phosphonopyruvate decarboxylase. In this paper, we report the cloning and overexpression of the *B. fragilis* phosphonopyruvate decarboxylase gene (aepY), purification of the phosphonopyruvate decarboxylase recombinant protein, and the extensive characterization of the reaction that it catalyzes. The homotrimeric (41,184-Da subunit) phosphonopyruvate decarboxylase catalyzes

\[ k_{\text{cat}} = 10.2 \pm 0.3 \text{ s}^{-1} \]

the decarboxylation of phosphonopyruvate \( (K_m = 3.2 \pm 0.2 \mu M) \) to phosphonoacetaldehyde \( (K_i = 15 \pm 2 \mu M) \) and carbon dioxide at an optimal pH range of 7.0–7.5. Thiamine pyrophosphate \( (K_m = 13 \pm 2 \mu M) \) and certain divalent metal ions \( (\text{Mg(II) } K_m = 82 \pm 8 \mu M; \text{Mn(II) } K_m = 13 \pm 1 \mu M; \text{Ca(II) } K_m = 78 \pm 6 \mu M) \) serve as cofactors. Phosphonopyruvate decarboxylase is a member of the α-ketodecarboxylase family that includes sulfopropyruvate decarboxylase, acetohydroxy acid synthase/acetolactate synthase, benzoylformate decarboxylase, glyoxylate carboligase, indole pyruvate decarboxylase, pyruvate decarboxylase, the acetyl phosphate-producing pyruvate oxidase, and the acetate-producing pyruvate oxidase. The Mg(II) binding residue Asp-260, which is located within the thiamine pyrophosphate binding motif of the α-ketodecarboxylase family, was shown by site-directed mutagenesis to play an important role in catalysis. Pyruvate \( (k_{\text{cat}} = 0.05 \text{ s}^{-1}, K_m = 25 \text{ mM}) \) and sulfopropyruvate \( (k_{\text{cat}} = 0.05 \text{ s}^{-1}; K_i = 200 \pm 20 \mu M) \) are slow substrates for the phosphonopyruvate decarboxylase, indicating that this enzyme is promiscuous.

*Bacteroides fragilis* is a human pathogen that causes intra-abdominal abscess formation in its host (1, 2). The bacterial capsular polysaccharide complex is the major virulence factor for abscess formation. The capsular polysaccharide complex is composed of three distinct polysaccharides, polysaccharides A, B, and C (3–6). These polysaccharides consist of repeating units that contain a zwitterionic motif of negative and positive charged groups. The zwitterionic charge motif plays an essential role in the induction of the host defense response, which leads to abscess formation. The 2-aminoethylphosphonate (AEP) \(^1\) unit of polysaccharide B contributes the positive and negative charges that form the zwitterionic motif (see Fig. 1). AEP is the phosphonate counterpart to phoshethanolamine, a common lipid polar head-group. The P-C bond of AEP is resistant to both chemical and enzymatic hydrolysis. The AEP unit is found in proteins (7), lipids (8, 9–12), and polysaccharides (4) located at the cell surfaces in certain parasitic organisms. These AEP conjugates either participate in host infection, as in the case of the *B. fragilis* polysaccharide B, or they are responsible for the persistence of the parasite within the host.

The presence of AEP in the *B. fragilis* polysaccharide B was first demonstrated by NMR structural analysis (3). More recently, the polysaccharide B biosynthetic pathway gene locus was sequenced (13). Three genes, *aepX*, *aepY*, and *aepZ*, which encode proteins that share significant sequence identity with the three enzymes of the AEP biosynthetic pathway, are included within this locus (Fig. 2). To our knowledge, this is the first known example of the AEP biosynthetic pathway gene cluster in a bacterium. Moreover, the opportunity now exists for the isolation of the three pathway enzymes for mechanistic study and inhibitor design. Because the AEP pathway enzymes are not present in humans, they are excellent candidates for drug targeting.

What is presently known about the AEP biosynthetic pathway and the three enzymes that catalyze it has resulted from a “patch-work” effort. The AEP biosynthetic pathway was first discovered in *Tetrahymena pyriformis* (14–17). In this organism, AEP is incorporated into phospholipids, which form the plasma membrane. The AEP pathway was shown to consist of the three steps depicted in Fig. 3. In the first step of the reaction, P-enolpyruvate is converted to phosphonopyruvate (Ppyr). P-enolpyruvate mutase, the enzyme that catalyzes this step, has been isolated from several different organisms and characterized (18–20). The conversion of P-enolpyruvate to Ppyr is thermodynamically unfavorable \( (K_m \sim 1 \times 10^{-3}) \), and thus, the ensuing decarboxylation step catalyzed by Ppyr decarboxylase is required to drive the Ppyr-forming reaction forward. The *T. pyriformis* Ppyr decarboxylase is membrane-bound and difficult to isolate for characterization (21). What we do know about this enzyme derives from the study of the bacterial enzyme which functions in biosynthetic pathways leading to bialaphos, fosfomycin, and phosphinothricin tripeptide in *Streptomyces hygroscopicus* (22), *Streptomyces woundrensis* (23, 24), and *Streptomyces viridogenanes* (25), respectively. In these pathways, P-enolpyruvate mutase and Ppyr decarboxylase collaborate to form the common precursor phosphonoacetaldehyde (Pald). The *S. hygroscopicus* Ppyr decarboxylase has been isolated and its native size and its cofactor requirement (viz. thiamine pyrophosphate and Mg(II)) have been defined (26).

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\(^{1}\) P-enolpyruvate, phosphoenolpyruvate; Ppyr, phosphonopyruvate; TPP, thiamine pyrophosphate; TAPS, N-tris(hydroxymethyl)methyl-3-amino- propanesulfonic acid; Pald, phosphonoacetaldehyde; MES, 4-morpholineethanesulfonic acid.

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\(^{1}\) The abbreviations used are: AEP, 2-aminoethylphosphonate;
As with the Ppyr decarboxylase of *Tetrahymena pyriformis*, the third enzyme of the pathway, Pald transaminase proved to be membrane-bound and difficult to isolate. Using partially purified enzyme, Kim (21) was able to demonstrate catalysis of pyridoxal phosphate-dependent transamination of Pald with L-alanine functioning as the ammonium group donor. A related transaminase can be found in bacteria adapted for the use of L-glutamate (32). Nevertheless, the bacterial AEP transaminase (27–31). It, too, is dependent on pyridoxal phosphate; however, the ammonium group acceptor is not the physiological reaction is catalyzed in the direction of Pald formation, this enzyme has become known as AEP transaminase (27–31). It, too, is dependent on pyridoxal phosphate, and its transamination direction and study of the three pathway enzymes. In this paper, we report the cloning and overexpression of the *B. fragilis* Ppyr decarboxylase gene (*aepY*), purification of the protein, and for the first time, an in-depth study of the Ppyr decarboxylase.

**EXPERIMENTAL PROCEDURES**

**Materials**—Thiamine pyrophosphate chloride (TPP), dihydrol-β-nicotinamide adenine dinucleotide (β-NADH), yeast alcohol dehydrogenase, and the buffers used in protein purification and kinetic assays were purchased from Sigma and used without further purification. Phosphonopyruvate and sulfopyruvate were synthesized according to the published methods (33, 34). Recombinant *Bacillus cereus* phosphonoacetaldehyde hydratase was purified as described previously (35). *B. fragilis* genomic DNA was purchased from American Type Culture Collection (ATCC 25285D). The primers used in PCR-based DNA amplification were custom synthesized at Invitrogen. For the cloning of the phosphonopyruvate decarboxylase gene, the sequence of the 5’ to 3’ primer was ATTCAGACGCATATGGTAAGTGTA and that of the 3’ to 5’ primer was TCTTTCATCTGACATCTGATCCTGCT, with the introduced restriction sites underlined. The enzymes used in DNA manipulation were purchased from Invitrogen and used with the buffers provided.

**PCR-based Cloning of Phosphonopyruvate Decarboxylase Gene**—The genomic DNA template was denatured (30 min at 94 °C) before adding *Phu* DNA polymerase. The target gene was amplified by 20 cycles of 94 °C denaturation for 1 min, 55 °C annealing for 50 s, and 73 °C elongation for 3.5 min. The PCR product was purified by electrophoresis and digested using *Nde*I and *Bam*HI restriction enzymes. The digest was ligated to an *Nde*I-*Bam*HI-cleaved fragment of *P. aeruginosa* phosphonopyruvate decarboxylase gene, the sequence of the 5’ to 3’ primer was ATTCAGACGCATATGGTAAGTGTA and that of the 3’ to 5’ primer was TCTTTCATCTGACATCTGATCCTGCT, with the introduced restriction sites underlined. The enzymes used in DNA sequencing carried out at the Center for Genetics in Medicine, University of New Mexico School of Medicine, Albuquerque, NM.

**Protein Purification**—*E. coli* BL21(DE3) cells, transformed with the named *P. aeruginosa* phosphonopyruvate decarboxylase gene, were grown in 1.2 liters × 4-LB media containing 100 µg/ml ampicillin. Following an 8-h induction period with 0.2 mM isopropyl-β-D-thiogalactopyranoside, the cells were harvested by centrifugation at 6500 rpm, 4 °C (all purification steps were carried out at 4 °C except where noted). The cell pellet was suspended in 100 ml of 50 mM potassium phosphate containing 5 mM MgCl₂ and 1 mM diithiothreitol, pH 7.5 (referred to as buffer A, hereafter). Cells were lysed at 1000 p.s.i. in a French pressure cell and then centrifuged at 20,000 rpm for 30 min. The supernatant was subjected to ammonium sulfate precipitation. The 40–85% fraction was collected by centrifugation and dissolved in buffer A for overnight dialysis against buffer A. The dialysate was chromatographed on a DEAE-Sepharose column (3.0 × 60 cm) (equilibrated with buffer A) using a 1.4-liter linear gradient of KCl (0.15–0.60 M) in buffer A as eluant. The Ppyr decarboxylase-containing fractions (eluted at ~0.35–0.40 M KCl) were identified using the spectrophotometric activity assay (described in the following section) and analyzed by SDS-PAGE. The desired Ppyr decarboxylase-containing fractions were combined and chromatographed on a hydroxyapatite column (3.0 × 40 cm) equilibrated with buffer A, using a 1.4-liter linear gradient of phosphate (0–0.25 M) in buffer A as eluant. The fractions containing the Ppyr decarboxylase were concentrated in 50 mM K⁺ HEPES buffer containing 5 mM MgCl₂, 1 mM MnCl₂, and 1 mM diithiothreitol, pH 7.5 (buffer B) for storage at ~80 °C. Yield: 3.7 mg wet cell (or 22 mg of cell culture/liter).

**Site-directed Mutants**—The site-directed mutants E213A, D258A, and D260A were prepared by PCR and commercial primers using the clone *P. aeruginosa* phosphonopyruvate decarboxylase pET 3a as a template. The PCR product was purified by electrophoresis and digested using *Nde*I and *Bam*HI restriction enzymes. The digest was ligated to an *Nde*I- and *Bam*HI-cleaved fragment of *P. aeruginosa* phosphonopyruvate decarboxylase gene and then used to transform competent *E. coli* BL21(DE3) cells. The gene sequence was verified by DNA sequencing carried out at the

**Phosphonopyruvate Decarboxylase**

![Fig. 1. The structure of the *B. fragilis* bacterial capsular polysaccharide, polysaccharide B (4).](image)

![Fig. 2. The *B. fragilis* polysaccharide B biosynthetic pathway gene locus (13). The three AEP biosynthetic pathway genes are: aepX (P-β-enolpyruvate mutase), aepY (Ppyr decarboxylase), and aepZ (Pald transaminase). The arrows designate the transcription direction for each open reading frame.](image)
FIG. 3. The AEP biosynthetic pathway in *T. pyriformis*.

FIG. 4. Coomassie Blue-stained SDS-PAGE of the *B. fragilis* Ppyr decarboxylase isolated at each stage of the purification procedure. Lane 1, protein standards; lane 2, total soluble protein; lane 3, the protein fraction precipitated between 40–85% ammonium sulfate at 0 °C; lane 4, combined Ppyr decarboxylase fraction after DEAE-Sepharose column; lane 5, combined Ppyr decarboxylase fraction after hydroxylapatite column.

\[ \log Y = \log(C/(1 + [H]/K_h + K_h/[H])) \]  

where \( Y \) is \( k_{cat}/K_m \), \( C \) is the hydrogen ion concentration, \( C \) is the acid dissociation constant, and \( K_h \) is the base dissociation constant.

**Screening of Alternative Substrates and Inhibitors—Substrate activity was observed with pyruvate and sulfopyruvate. The pyruvate reaction was monitored at 340 nm (\( \Delta \varepsilon = 6,200 \text{ M}^{-1} \text{ cm}^{-1} \)) by using the alcohol dehydrogenase/NADH coupling system. The 1 ml of reaction mixture contained 50 mM K HEPES (pH 7.3, 25 °C), 5 mM MgCl₂, 1 mM MnCl₂, 1 mM wild-type phosphonoacetaldehyde dehydrogenase, 10 units of alcohol dehydrogenase, 0.02 mM metal-free Ppyr decarboxylase, and various concentrations of metal ions. The reactions were monitored at 340 nm (\( \Delta \varepsilon = 6,200 \text{ M}^{-1} \text{ cm}^{-1} \)). The initial velocity data were analyzed using Equation 1. The sulfopyruvate reaction was monitored by \(^1\text{H} \) NMR. Sulfopyruvate (1 mM) was incubated for 1.5 h in 1 ml of D₂O containing 0.5 mM Ppyr decarboxylase, 1 mM MgCl₂, 0.4 mM TPP, and 10 mM K HEPES (pH 7.6, 25 °C) before recording the NMR spectrum. \(^1\text{H} \) NMR spectra were measured with a Bruker Advance 500-MHz NMR spectrometer using D₂O as the solvent and at a probe temperature of 24–26 °C. The chemical shift data are reported with respect to the external reference (trimethylsilyl)-propanesulfonic acid for \(^1\text{H} \) NMR. Control reactions were carried out under the same conditions, except that Ppyr decarboxylase was omitted.

**Pald Inhibition of Ppyr Decarboxylase Catalyzed Decarboxylation of Pyruvate—The 1-ml reaction mixtures contained 50 mM K HEPES, 5 mM MgCl₂, 1 mM MnCl₂, 10 mM pyruvate, 10 units of alcohol dehydrogenase, 0.15 mM NADH, 1.5 mM TPP, 1.8 mM Ppyr decarboxylase, and various concentrations of Pald (0–300 \( \mu \text{M} \)). Initial velocities were measured at 340 nm and analyzed using Dixon Equation 3 for a competitive inhibition pattern.

\[ \frac{1}{v} = \frac{K_m}{V_{max} [S]} + \frac{1}{V_{max} [I]} + \frac{1}{K_h \cdot [S]} \]  

where \( v \) is the initial velocity, \( V_{max} \) is the maximum velocity, \( [S] \) is the substrate concentration (here it is 10 mM pyruvate), \( K_m \) is the Michaelis-Menten constant of pyruvate, \( [I] \) is the inhibitor concentration and \( K_h \) is the inhibition constant.
The theoretical molecular weight of the phosphonopyruvate decarboxylase calculated from the amino acid sequence was 41,184, which agrees with the experimental molecular weight of 41,199, measured by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. The subunit size estimated by SDS-PAGE analysis was 49 kDa compared with the native protein size of 120 kDa determined by gel filtration chromatography. Thus, the quaternary structure of Ppyr decarboxylase appears to be homotrimeric. (The *S. hygroscopicus* native Ppyr decarboxylase molecular size was reported as 135 kDa (26)).

**Sequence Homologs**—At the time of this writing, there are a total of seven Ppyr decarboxylase sequences listed in the Protein Data Bank. The Ppyr decarboxylase-encoding genes in *B. fragilis* (NCBI protein data bank ID code AAG26466) (378 amino acids), *Bacteroides thetaiotaomicron* (NCBI protein data bank ID code NP_810632) (374 amino acids), *Amycolatopsis orientalis* (NCBI protein data bank ID code CAB45023) (371 amino acids), and *Clostridium tetani E88* (NCBI protein data bank ID code NP_782297) (376 amino acids) are positioned between the genes encoding homologs of P-enolpyruvate mutase and AEP transaminase. Pairwise sequence alignments made with the *B. fragilis* Ppyr decarboxylase (which activity has been demonstrated in this work) demonstrated 76, 35, and 43% sequence identity, respectively. The other three Ppyr decarboxylases (401, 384, and 397 amino acids long, respectively) function in the bialaphos, fosfomycin, and phosphinothricin carboxylases (401, 384, and 397 amino acids long, respectively) and therefore do not have sequence homology with the bialaphos enzymes. Three additional Ppyr decarboxylases listed in the protein data bank (384, 334, and 397 amino acids) are positioned within the genes encoding homologs of P-enolpyruvate mutase and AEP transaminase. The other three Ppyr decarboxylases (401, 384, and 397 amino acids long, respectively) function in the bialaphos, fosfomycin, and phosphinothricin carboxylases (401, 384, and 397 amino acids long, respectively) and therefore do not have sequence homology with the bialaphos enzymes. Three additional Ppyr decarboxylases listed in the protein data bank (384, 334, and 397 amino acids) are positioned within the genes encoding homologs of P-enolpyruvate mutase and AEP transaminase.

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**Results and Discussion**

**Protein Purification and Size Determination**—The DNA sequence of the cloned gene agreed with the published sequence (GenBank™ accession number AF285774_6). The recombinant Ppyr decarboxylase was purified to homogeneity (see Fig. 4) by using the 4-step protocol summarized in Table I in an overall yield of 3.7 mg/g wet cells. The steady-state kinetic constants for catalyzed decarboxylation of phosphonopyruvate are $K_{cat} = 10.2 \pm 0.3 \text{ s}^{-1}$; $K_m = 3.2 \pm 0.2 \text{ mM}$; and $k_{cat}/K_m = 3.2 \times 10^6 \text{ s}^{-1} \text{ M}^{-1}$, as determined at 25 °C under optimal reaction conditions (viz. pH 7.3, 5 mM MgCl$_2$, 1 mM MnCl$_2$, 200 μM TPP).

The metal ion dependence of the steady-state kinetic constants for Ppyr decarboxylase catalysis was measured in the presence of saturating phosphonopyruvate (50 μM) and TPP (1.5 mM) at pH 7.3 and 25 °C. Co(II), Ni(II), and Zn(II) were not activators.

| Purification steps                  | Total protein | Total activity | Specific activity | Activity recovery | Purification |
|------------------------------------|---------------|----------------|------------------|------------------|--------------|
| Extract from 30 g of wet cells     | 10            | 6200           | 0.62             | 100              | 3            |
| Ammonium sulfate (40-85%)          | 2             | 3500           | 1.8              | 60               | 3            |
| DEAE-Sepharose                     | 0.20          | 2200           | 10.5             | 35               | 17           |
| Hydroxyapatite                     | 0.11          | 1344           | 12.1             | 20               | 20           |

$^*$ One activity unit is defined as the amount of enzyme required to produce 1 μmol of Pald/min in 50 mM HEPES, pH 7.3, 5 mM MgCl$_2$ and 1.5 mM TPP at 25 °C.

**TABLE II**

| Metal activator | Activator $K_m$ μM | $k_{cat}$ s$^{-1}$ | $k_{cat}/K_m$ | % activity-fold |
|-----------------|--------------------|-------------------|---------------|----------------|
| Mg(II)          | 82 ± 8             | 8.7 ± 0.2         |               | 1              |
| Mn(II)          | 13 ± 1             | 10.2 ± 0.3        |               | 1              |
| Ca(II)          | 78 ± 6             | 9.3 ± 0.2         |               | 1              |

**FIG. 5.** The decarboxylation reactions of the α-keto carboxylate substrates for the Ppyr decarboxylase-containing enzyme family.

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Sulfopyruvate decarboxylase (39) and Ppyr decarboxylase are more distant members of a family of TPP- and Mg(II)-dependent decarboxylases that includes acetohydroxy acid synthase/acetolactate synthase (40), benzoylformate decar-
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Boxylase (41), glyoxylate carboligase, indole pyruvate decarboxylase, pyruvate decarboxylase, the acetyl phosphate-producing pyruvate oxidase, and the acetate-producing pyruvate oxidase (42) (sequence identities between \textit{B. fragilis} Ppyr decarboxylase and these proteins with a sequence range of 23–29\%). The chemical reactions catalyzed by these enzymes are shown in Fig. 5. The common chemistry catalyzed by the family of enzymes is the decarboxylation of an $\alpha$-keto carboxylate using the TPP cofactor as an “electron sink.”

**Metal Ion and TPP Activation**—Metal-free Ppyr decarboxylase, prepared by exhaustive dialysis, is inactive. The metal ions Mg(II), Mn(II), Ca(II), Co(II), Ni(II), and Zn(II) were tested (at a concentration of 1 or 5 mM) as activators of the metal-free enzyme in the presence of 1.5 mM TPP and saturating Ppyr (50 \textmu M). Only Mg(II), Mn(II), and Ca(II) were activators. The $k_{\text{cat}}$ and $K_m$ values (corresponding to the $K_d$ for metal ion dissociation from the enzyme-TPP-Ppyr-M(II) complex) are listed in Table II. Whereas the $k_{\text{cat}}$ values of the Mg(II)-, Mn(II)-, and Ca(II)-activated decarboxylase are similar ($\sim 10$ s$^{-1}$), the $K_m$ for Mn(II) (13 \textmu M) is significantly smaller than the $K_m$ values of Mg(II) and Ca(II) (80 \textmu M).

**TPP-free Ppyr decarboxylase**, also prepared by exhaustive dialysis, is inactive. The $K_m$ for TPP activation was determined by measuring the initial velocity of the Mg(II) (5 mM)/Mn(II) (1 mM)-activated Ppyr decarboxylase-catalyzed decarboxylation of Ppyr (saturating at 50 \textmu M). The initial velocity data defined the $k_{\text{cat}}$ and $TPP K_m$ profile.

![Fig. 6. The ClustalW sequence alignment representing the Ppyr decarboxylase-containing enzyme family. The individual sequences shown were picked as representatives of the subfamilies. The stringently conserved residues across all the subfamilies are highlighted in yellow, and the TPP signature motif is boxed. The polar residues interacting directly with Mg(II) or TPP are indicated by *, and the polar residues conserved across Ppyr decarboxylases and sulfooxyurate decarboxylases are highlighted in gray and marked by \#. The residues are numbered for the \textit{B. fragilis} Ppyr decarboxylase. PPyr\_Bfra, \textit{B. fragilis} Ppyr decarboxylase (NCBI protein data bank ID code AAG26468); SPyr\_Meth, sulfooxyurate decarboxylase from \textit{Methanosarcina acetivorans} str. C2a (NCBI protein data bank ID code NP_618188); Asyn\_Ecol, the acetolactate synthase isozyme I large subunit from \textit{E. coli} CFT073 (NCBI protein data bank ID code NP_756456); POxi\_Ecol, pyruvate oxidase from \textit{E. coli} O157 (NCBI protein data bank ID code NP_296643); BFdc\_Pseu, benzoylformate decarboxylase from \textit{Pseudomonas putida} (NCBI protein data bank ID code P20906).](image)

![Fig. 7. pH rate profiles of Ppyr decarboxylase catalysis. See “Experimental Procedures” for details. The apparent $pK_a$ of 6.1 ± 0.1 and $pK_a$ of 8.5 ± 0.1 were obtained by computer fitting the log $k_{\text{cat}}$ profile to Equation 2, and $pK_a$ of 6.7 ± 0.2 and $pK_a$ of 7.7 ± 0.2 were obtained by computer fitting the log ($k_{\text{cat}}/K_m$) profile.](image)

**TABLE III**

| Enzyme          | $k_{\text{cat}}$ \textmu M | Ppyr $K_m$ \textmu M | $k_{\text{cat}}/K_m$ s$^{-1}$ \textmu M$^{-1}$ |
|-----------------|-----------------------------|----------------------|-----------------------------------------------|
| Wild type       | 10.2 ± 0.3                  | 3.2 ± 0.2            | 3.2 × 10$^4$                                  |
| E213A           | 0.48 ± 0.01                 | 2.6 ± 0.3            | 1.8 × 10$^3$                                  |
| D258A           | 0.96 ± 0.01                 | 2100 ± 50           | 4.6 × 10$^2$                                  |
| D260A           | 0.010 ± 0.001               | 17.1 ± 0.8           | 5.8 × 10$^2$                                  |
Mg(II) and TPP-binding Residues—The results described above show that Ppyr decarboxylase, like the other α-keto decarboxylases of this enzyme family, requires TPP and Mg(II) as cofactors. Based on the known three-dimensional structures of several of these family members (41, 45–50), it has been shown that the Mg(II) cofactor functions to anchor the TPP by coordinating to two of the phosphate oxygen atoms and to the side chains of an active site Asp and Asn. The Asp and Asn residues are located at opposite ends of the TPP-binding sequence motif (51) illustrated in Fig. 6. In Ppyr decarboxylase, the Asp residue is Asp-260. The site-directed mutant D260A was prepared to test the contribution of Asp-260 to catalysis. The steady-state kinetic constants of this mutant were determined to be $k_{cat} = 0.01 \, s^{-1}$, $K_m = 17 \, \mu M$, and $k_{cat}/K_m = 6 \times 10^5 \, s^{-1} \, M^{-1}$. Whereas the $K_m$ is increased only 5-fold, the catalytic turnover rate of the mutant enzyme is 1000-fold lower than that of the wild-type enzyme (Table II). Thus, Asp-260 is an important contributor to catalysis. This result is consistent with the assignment of the TPP-binding sequence motif in Ppyr decarboxylase as indicated in Fig. 6.

The N1(H) of the pyridinium ring is bound through a H-bond to a Glu side chain located near the N terminus. The Ppyr decarboxylase and sulfopyruvate decarboxylase (<400 amino acids) are both considerably smaller than the TPP-binding subunits of acetolactate synthase, benzoylformate decarboxylase, and pyruvate oxidase/dehydrogenase (~600 amino acids). Nevertheless, the ClustalW sequence alignment (see Fig. 6) identifies this Glu as stringently conserved within the enzyme superfamily, including the Ppyr decarboxylase and sulfopyruvate decarboxylase subfamilies. In the B. fragilis Ppyr decarboxylase this TPP-binding residue is Glu-49.

Mutagenesis Probe of Carboxylate Residues Unique to the Sulfopyruvate and Ppyr Decarboxylases—Aside from total charge (−1 and −2, respectively) of the anionic C (3) substituent, the structures of sulfopyruvate and phosphonopyruvate are quite similar. In this study, sulfopyruvate was shown to be a competitive inhibitor of Ppyr decarboxylase (at pH 7.25°C) with a $K_i = 200 \pm 20 \, \mu M$. Furthermore, 1H NMR analysis of a reaction mixture initially containing 0.5 μM Ppyr decarboxylase, 1 mM sulfopyruvate, 1 mM MgCl2, and 0.4 mM TPP in 10 mM KHEPES (pH 7.6, 25°C) revealed that 15% of the sulfopyruvate had been converted to sulfoacetaldehyde (identified by the aldehyde proton signal at 9.3 parts/million) within the 1.5-h incubation period. Because sulfopyruvate is a competitive inhibitor of this TPP-binding sequence motif in Ppyr decarboxylase as indicated in Fig. 6, for which the E213A mutant was prepared. The kinetic constants obtained for the E213A mutant ($k_{cat} = 0.5 \, s^{-1}$, $K_m = 2.6 \, \mu M$, and $k_{cat}/K_m = 2 \times 10^6 \, s^{-1} \, M^{-1}$) indicated only a 20-fold reduction in activity. In contrast to Asp-258 and Asp-260, Glu-213 does not appear to play an important role in Ppyr decarboxylase catalysis.

The Importance of the Phosphonopyruvate “Phosphono” Group in Ppyr Decarboxylase Substrate Recognition—Phosphonopyruvate was shown to be a slow substrate with the kinetic constants $k_{cat} = 0.05 \, s^{-1}$, $K_m = 25 \, \mu M$, and $k_{cat}/K_m = 2 \times 10^3 \, s^{-1} \, M^{-1}$. A comparison of these values with those obtained with phosphonopyruvate (Table III) reveals the importance of the pyruvate C(3)-PO3−2 group in substrate activity. Using pyruvate as the substrate for the Ppyr decarboxylase, Pald was shown to be a competitive inhibitor with a $K_i = 15 \pm 2 \, \mu M$. The large difference in the values of the phosphonopyruvate and pyruvate $K_i$ constants (3 μM versus 25 μM) and in values of the Pald (−2 charge on the phosphono group) and sulfopyruvate (−1 charge on the sulfo group) $K_i$ constants (15 versus 200 μM), shows that the phosphonopyruvate “phosphono” group, and not the carboxyl group, is the major source of enzyme-substrate binding energy.

pH Dependence of Catalysis—The pH rate profile analysis defined pH 7.0–7.5 as the pH range for optimal Ppyr decarboxylase catalysis. Both the log $k_{cat}$ and log ($k_{cat}/K_m$) pH profiles were bell-shaped (Fig. 7). Computer fitting of the log $k_{cat}$ profile gave an apparent $pK_a$ of 6.1 ± 0.1 for the essential base within the enzyme-substrate complex, and an apparent $pK_a$ of 8.2 ± 0.1 for the essential acid within the enzyme-substrate complex. Computer fitting of the log ($k_{cat}/K_m$) profile gave an apparent $pK_a$ of 6.7 ± 0.2 for the essential base within the uncomplexed substrate or enzyme and an apparent $pK_a$ of 7.7 ± 0.2 for the essential acid within the uncomplexed substrate or enzyme.

Conclusion—The identity of the resultant product of the aepY gene in the B. fragilis genome has been shown to be Ppyr decarboxylase. This enzyme is a trimer of a 41,184-Da subunit, which shares sequence homology (global and cofactor-binding sequence motifs) with members of a superfamily of TPP- and Mg(II)-dependent α-keto decarboxylases that includes sulfopyruvate decarboxylase, pyruvate decarboxylase and oxidase, acetolactate synthase, and benzoylformate decarboxylase. The phosphonopyruvate C(3)-PO3−2 group plays an important role in Ppyr decarboxylase binding. Nevertheless, sulfopyruvate and pyruvate are slow substrates for the Ppyr decarboxylase, indicative of low substrate specificity.
16. Warren, W. A. (1968) Biochim. Biophys. Acta 156, 340–346
17. Deleted in proof
18. Kim, A., Kim, J., Martin, B. M., and Dunaway-Mariano, D. (1998) J. Biol. Chem. 273, 4443–4448
19. Jia, Y., Lu, Z., Huang, K., Herzberg, O., and Dunaway-Mariano, D. (1999) Biochemistry 38, 14165–14173
20. Liu, S., Lu, Z., Jia, Y., Dunaway-Mariano, D., and Herzberg, O. (2002) Biochemistry 41, 10270–10276
21. Kim, J. B. (1994) Investigations of 2-Aminoethylphosphonate Biosynthetic Enzymes in Tetrahymena Pyriformis. Ph.D. thesis, University of Maryland, College Park, MD
22. Nakashita, H., Kozuka, K., Hidaka, T., Hara, O., and Seto, H. (2000) Biochim. Biophys. Acta 1490, 159–162
23. Thompson, C. J., and Seto, H. (1995) Biotechnology 28, 197–222
24. Seto, H., and Kuzuyama, T. (1999) Nat. Prod. Rep. 16, 589–596
25. Schwartz, D., Recktenwald, J., Pelzer, S., and Wolfe, R. S. (1999) FEMS Microbiol. Lett. 163, 149–157
26. Nakashita, H., Watanabe, K., Hara, O., Hidaka, T., and Seto, H. (1997) J. Antibiot. (Tokyo) 50, 212–219
27. Cassaigne, A., Lacoste, A. M., and Neuzil, E. (1976) C. R. Hebd. Seances Acad. Sci. Ser. D Sci. Nat. 282, 1637–1639
28. Jiang, W., Metcalf, W. W., Lee, K. S., and Wanner, B. L. (1995) J. Bacteriol. 177, 6411–6421
29. Parker, G. F., Higgins, T. P., Hawkes, T., and Robson, R. L. (1999) J. Bacteriol. 181, 389–395
30. Ternan, N. G., and Quinn, J. P. (1998) Syst. Appl. Microbiol. 21, 346–352
31. La Nauze, J. M., Rosenberg, H., and Shaw, D. C. (1970) Biochim. Biophys. Acta 128, 332–350
32. Kim, A. D., Baker, A. S., Dunaway-Mariano, D., Metcalf, W. W., Wanner, B. L., and Martin, B. M. (2002) J. Bacteriol. 184, 4134–4140
33. Anderson, V. E., Weiss, P. M., and Cleland, W. W. (1984) Biochemistry 23, 2779–2786
34. White, R. H. (1986) Biochemistry 25, 5304–5308
35. Baker, A. S., Ciocci, M. J., Metcalf, W. W., Kim, J., Babbitt, P. C., Wanner, B. L., Martin, B. M., and Dunaway-Mariano, D. (1998) Biochemistry 37, 9305–9315
36. Apel, R. D., Bairch, A., and Hochstrasser, D. F. (1994) Trends Biochem. Sci. 19, 258–260
37. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
38. DiMarco, A. A., Bobik, T. A., and Wolfe, R. S. (1990) Annu. Rev. Biochem. 59, 355–394
39. Groen, M., Xu, H., and White, R. H. (2000) J. Bacteriol. 182, 4862–4867
40. Chipman, D., Barak, Z., and Schloss, J. V. (1998) Biochim. Biophys. Acta 1385, 401–419
41. Hasson, M. S., Muscate, A., McLeish, M. J., Polovnikova, L. S., Gerlt, J. A., Kenyon, G. L., Petsko, G. A., and Ringe, D. (1998) Biochemistry 37, 9918–9930
42. Schellenberger, A. (1998) Biochim. Biophys. Acta 1385, 177–186
43. Tate, J. R., and Nixon, P. F. (1987) Anal. Biochem. 160, 78–87
44. Jung, E. H., Takeuchi, T., Nishino, K., and Belew, Y. (1988) Int. J. Biochem. 20, 1255–1259
45. Muller, Y. A., Lindqvist, Y., Furey, W., Schulz, G. E., Jordan, F., and Schnei- der, G. (1993) Structure 1, 95–103
46. Muller, Y. A., and Schulz, G. E. (1993) Science 259, 965–967
47. Dyda, F., Furey, W., Swaminathan, S., Sax, M., Farrenkopf, B., and Jordan, F. (1993) Biochemistry 32, 6165–6170
48. Arjunan, P., Umland, T., Dyda, F., Swaminathan, S., Furey, W., Sax, M., Farrenkopf, B., Gao, Y., Zhang, D., and Jordan, F. (1996) J. Mol. Biol. 256, 590–600
49. Dobritzsch, D., Konig, S., Schneider, G., and Lu, G. (1998) J. Biol. Chem. 273, 20196–20204
50. Pang, S. S., Duggleby, R. G., and Guddat, L. W. (2002) J. Mol. Biol. 317, 249–262
51. Hawkins, C. F., Borges, A., and Perham, R. N. (1989) FEBS Lett. 255, 77–82