Purification and crystallization of benzoylformate decarboxylase

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(RECEIVED December 20, 1994; ACCEPTED February 27, 1995)

Abstract

A new large-scale purification method for benzoylformate decarboxylase from *Pseudomonas putida* has allowed us to undertake an X-ray crystallographic study of the enzyme. The previously observed instability of the enzyme was overcome by addition of 100 μ M thiamine pyrophosphate to buffers used in the purification. The final enzyme preparation was more than 97% pure, as determined by denaturing gel electrophoresis and densitometry. The mobility of the enzyme on a gel filtration column indicates that it is a tetramer of 57-kDa subunits. Large, single crystals of benzoylformate decarboxylase were grown from solutions of buffered polyethylene glycol 400, pH 8.5. The crystals diffract to beyond 1.6 Å resolution and are stable for days to X-ray radiation. Analysis of X-ray data from the crystals, along with the newly determined quaternary structure, identifies the space group as I222. The unit cell dimensions are $a = 82$ Å, $b = 97$ Å, $c = 138$ Å. An average V_m value for the crystals is consistent with one subunit per asymmetric unit. The subunits of the tetramer must be arranged with tetrahedral 222 symmetry.

Keywords: benzoylformate decarboxylase; crystallization; protein purification; thiamine pyrophosphate

Benzoylformate decarboxylase (EC 4.1.1.7) from *Pseudomonas putida* (ATCC 12633) is a thiamine pyrophosphate (TPP)-dependent enzyme that catalyzes the nonoxidative conversion of benzoylformate to benzaldehyde and carbon dioxide (Fig. 1). The enzyme is a component of the mandelate pathway, which allows bacteria to utilize (*R*)-mandelic acid as a sole carbon source by converting it to benzoic acid, which is then metabolized by the β -ketoadipate pathway and citric-acid cycle. The mandelate pathway, in addition to being one of the first pathways whose genes were recognized as being coordinately regulated (Stanier, 1947), has two special characteristics that have prompted its study. First, it has been suggested that the components of the pathway form a multienzyme complex in vivo (Halpin et al., 1981). Of the four components of the pathway, the structure of one (mandelate racemase) has been determined at high resolution (Neidhart et al., 1991), and the structure of another ((*S*)-mandelate dehydrogenase) has been modeled (Mittra et al., 1993). The determination of the structures of the remaining enzymes, benzoylformate decarboxylase and benzaldehyde

dehydrogenase, would allow for the first time the modeling of a complete multienzyme complex. Second, the mandelate pathway is present in only a few organisms, suggesting that the components of the pathway have only recently evolved to assume their present roles. The comparison of a “new” enzyme, one that has recently diverged from another with a different catalytic ability, to other, “older” enzymes that catalyze similar reactions, might reveal how proteins with related sequences evolve to differ drastically in catalytic activity and substrate specificity (Neidhart et al., 1990).

In addition to its implications for the study of the mandelate pathway in general, the determination of the structure of benzoylformate decarboxylase should fill some gaps in our understanding of TPP chemistry by enzymes and of the architecture of the active site in TPP-dependent decarboxylases. Recently, the structures of three TPP-requiring enzymes that are homologous to benzoylformate decarboxylase (Tsou et al., 1990; Robinson & Chun, 1993) have been solved: yeast pyruvate decarboxylase was determined to 2.4 Å resolution (Dyda et al., 1993), pyruvate oxidase was determined to 2.1 Å resolution (Muller & Schulz, 1993), and transketolase was determined to 2.0 Å resolution (Lindqvist et al., 1992; Nilsson et al., 1993; Nikkola et al., 1994). The conformation and the environment of

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Fig. 1. Conversion of benzoylformate to benzaldehyde and carbon dioxide.

the TPP in these three enzymes supports the proposal that the abstraction of a proton from C-2 of TPP, which initiates the enzyme-catalyzed reaction, is accomplished by a base contributed by the TPP itself, the 4'-amino group (Jordan & Mariam, 1978; Jordan et al., 1982). This mechanism requires activation by adjacent functional groups on the enzyme. A higher resolution view of enzyme-bound TPP would test the generality of this mechanism of TPP activation among different decarboxylases and elucidate the specifics of the mechanism in greater detail.

Although the residues that bind the TPP and the Mg^{2+} were identified in the previously determined structures, other residues involved in catalysis were not. The overall mechanism of TPP-dependent decarboxylation was initially proposed many years ago (Breslow, 1958). Studies of benzoylformate decarboxylase itself suggest the involvement of at least two active-site bases, one protonated and one unprotonated, in catalysis and substrate binding (Weiss et al., 1988). One base, presumably the 4'-amino group of TPP, abstracts the proton from C-2 of TPP. The resulting ylide attacks the carbonyl carbon of benzoylformate to form the first tetrahedral complex; decarboxylation results in a C2- α -carbanion, which abstracts a proton from a second protonated base. Dissociation of the second tetrahedral complex gives TPP, benzaldehyde, and carbon dioxide. The nature of the second base and the configuration of the two tetrahedral intermediates remains unknown, although inhibitor studies suggest that the absolute configuration of the first tetrahedral intermediate is *R* (Weiss et al., 1988). The high-resolution structure of benzoylformate decarboxylase alone and in complex with previously characterized inhibitors (Reynolds et al., 1988; Weiss et al., 1988) will address these questions.

This study describes the first large-scale purification of pure and stable benzoylformate decarboxylase. Crystals exceptionally well suited to X-ray analysis have been obtained. The crystal structure of benzoylformate decarboxylase will be the first of a prokaryotic nonoxidative decarboxylase to be reported, and

the first structure of a TPP-dependent enzyme to be determined to better than 2 Å resolution.

Results and discussion

Purification and characterization of benzoylformate decarboxylase

The purification procedure described here allows the isolation of a large quantity of pure benzoylformate decarboxylase in four steps, starting from the crude extract. Previous isolations of the enzyme from *P. putida* resulted in specific activities of 193 U/mg (Hegeman, 1970) and 16.8 U/mg (Wilcocks et al., 1992); benzoylformate decarboxylase, with a specific activity of 46 U/mg, has also been isolated from *Escherichia coli* expressing the *mdlC* gene (Tsou et al., 1990). Our purification is based on the latter method. However, in the present study, the cells were lysed by shearing with glass beads rather than with lysozyme treatment and sonication. Heat treatment and gel filtration were omitted because of the previously observed instability of the enzyme. A Fast Sepharose column was substituted for DEAE Sephacel and Mono Q columns to streamline the purification protocol. A phenyl Sepharose column was used as a final step to take advantage of a different principle of separation. Results of the new purification procedure are summarized in Table 1. The final benzoylformate decarboxylase preparation has a specific activity of 138 U/mg and is more than 97% pure, as determined by SDS-PAGE (Fig. 2) and densitometry.

In the previously reported purification, the specific activity of enzyme preparation decreased nearly fourfold in the last two

Fig. 2. Analysis of the purification of benzoylformate decarboxylase by SDS-PAGE. Aliquots of the enzyme preparation after each stage in the purification were separated by denaturing gel electrophoresis. The amount of each sample loaded was normalized for the amount of benzoylformate decarboxylase present in it. Lane 1, crude extract; lane 2, samples after ammonium sulfate fractionation; lane 3, Affi-Blue column; lane 4, Fast Sepharose column; lane 5, phenyl Sepharose column; and lane 6, molecular weight standards. Numbers at the right indicate the molecular mass of the markers in kilodaltons.

Table 1. Purification of benzoylformate decarboxylase

	Volume (mL)	Protein (mg)	Activity (units)	Specific activity (units/mg)
Crude extract	375	2,360	17,200	7.3
(NH ₄) ₂ SO ₄ fractionation	176	1,210	15,300	12.7
Affi-Blue	70	149	12,000	80.7
Fast Sepharose	31	37.5	6,200	165
Phenyl Sepharose	1.9	20.5	2,820	138

purification steps; enzyme instability seemed to correlate with increased dilution. We have found that the addition of 100 μ M TPP causes benzoylformate decarboxylase at any purification level to be stable at room temperature or at 4 °C for a week, even if it is diluted to a concentration of less than 0.1 mg/mL. During the last purification step, the phenyl Sepharose column, the enzyme showed decreased specific activity. This might be due to the absence of TPP; it was omitted from the buffers used in that step because TPP bound to the phenyl Sepharose column and its elution obscured the protein peak. However, the column was retained in the purification because by the criterion of gel electrophoresis (Fig. 2) it resulted in the removal of impurities from the enzyme preparation.

Glycerol, dithiothreitol, EDTA, and KCl had no apparent effect on the stability of the pure enzyme, though glycerol has a stabilizing effect on the crude benzoylformate decarboxylase. The isoelectric point of benzoylformate decarboxylase was determined to be 6.5. It is stable in the range of pH 5.5–7.5, but slowly loses activity above pH 8, a phenomenon also observed for pyruvate decarboxylase (Hübner et al., 1992).

The mobility of purified benzoylformate decarboxylase on a gel filtration column indicates that it is a tetramer with a molecular mass of 248 kDa. The observed molecular mass correlates well with that calculated for a tetramer of benzoylformate decarboxylase from its known sequence (Tsou et al., 1990): 230 kDa. Both the tetrameric quaternary structure and the molecular mass of benzoylformate decarboxylase are similar to those reported for other TPP-dependent decarboxylases, such as 2-oxoglutarate decarboxylase from *Euglena gracilis* (248 kDa; Shigeoka & Nakano, 1991), pyruvate decarboxylase from *Zymomonas mobilis* (240 kDa; Diefenbach & Duggleby, 1991), and benzoylformate decarboxylase (232 kDa) and phenylpyruvate decarboxylase (227 kDa) from *Acinetobacter calcoaceticus* (Barrowman & Fewson, 1985).

Production and characterization of benzoylformate decarboxylase crystals

The benzoylformate decarboxylase crystals we have obtained are large (up to $0.2 \times 0.5 \times 1.5$ mm) and diffract to at least 1.6 Å resolution. Analysis of diffraction patterns indicates clearly that the crystals exhibit the symmetry of space group I222, with unit cell dimensions $a = 82$ Å, $b = 97$ Å, $c = 138$ Å. It is impossible to distinguish between the space groups I222 and I2₁2₁2₁ through examination of general extinctions of any class of reflections because extinctions resulting from the screw axes are a subset of those resulting from body centering. However, in this case, the choice of a space group can be made on biochemical grounds. Given this unit cell, and assuming that a single subunit is present in an asymmetric unit, the calculated V_m value is 2.50, a typical value for protein crystals (Matthews, 1968). If, then, an asymmetric unit contains a single subunit, the four subunits in a tetramer must be related by crystallographic symmetry elements. This is possible only if those symmetry elements are pure rotation axes, and impossible if they are screw axes. Therefore, the space group of the benzoylformate decarboxylase crystals is I222, and the subunits of the tetramer must be arranged with tetrahedral 222 symmetry.

We have collected native data from benzoylformate decarboxylase crystals. The crystals are stable for days to X-rays, allowing a full data set (>90% complete to 1.8 Å) to be collected from

Fig. 3. Crystals of benzoylformate decarboxylase. The largest crystal pictured is approximately 0.3 mm in length.

a single one. When reduced in space group I222, the overall R -merge of the data was 5% on intensities. The crystals are often somewhat irregularly shaped (Fig. 3); however, they consistently diffract well and are well ordered (with a mosaicity value of 0.2–0.3, as refined by R-AXIS data reduction software). Thus, the macroscopic appearance of the crystals belies the underlying regularity of their crystal lattice.

We are now screening heavy-atom derivatives of benzoylformate decarboxylase crystals in order to determine the structure of the enzyme, to the limits of diffraction of the crystals, by the method of isomorphous replacement. The high-resolution structure of benzoylformate decarboxylase should contribute greatly to understanding the enzymatic utilization of the important co-factor TPP.

Materials and methods

Sepharose Fast Flow and phenyl Sepharose columns, and the molecular-weight standard kit for gel filtration and electrophoresis were purchased from Pharmacia. Affi-blue resin was obtained from Bio-Rad. EDTA, TPP, potassium phosphate, phenylmethylsulfonyl fluoride, bovine serum albumin, ammonium sulfate, benzoylformic acid, dithiothreitol, KCl, Tris, and glycerol were purchased from Sigma. Polyethylene glycol 400 was purchased from Serva Chemical. All other chemicals used were the highest available purity.

Analysis of enzyme purity and activity

Protein concentration was determined with the method of Bradford (1976; with a kit obtained from Bio-Rad) using bovine serum albumin as a standard. Enzyme activity was determined by monitoring the decrease of benzoylformate concentration versus time in a continuous assay (Hegeman, 1970). The enzyme preparation was separated by SDS-PAGE on a 12.5% gel using the Phastgel system from Pharmacia. The gel was stained with

Coomassie brilliant blue, and a densitometer was used to determine the purity of the enzyme.

Enzyme purification

Benzoylformate decarboxylase was expressed in *E. coli* containing the plasmid pBFDtrc as described previously (Tsou et al., 1990), except that the cells were induced with isopropyl- β -thiogalactopyranoside when they reached an OD of 1.2 (rather than 1.0), and the wet cells were stored at -80°C (rather than -20°C). Increasing the scale of the fermentation from 9 L to 60 L resulted in comparable yields.

All purification steps were performed at 4°C unless noted otherwise. The frozen cells (37.5 g) were thawed and suspended in a total volume of 200 mL of 50 mM potassium phosphate, pH 6.0, 0.1 mM TPP, 0.1 mM phenylmethylsulfonyl fluoride (buffer A). The cells were sheared with a bead beater. The ice-cold 400-mL bead-beating container was filled three-quarters full with cell suspension and subsequently filled to the top with beads, and was operated for four cycles of 1 min each. The lysate was removed and the beads were rinsed with additional buffer A to give a total volume of 400 mL, which was centrifuged at $100,000 \times g$ for 90 min.

Ammonium sulfate fractionation (50–75%)

The supernatant fraction (375 mL) was transferred to an 800-mL beaker and ammonium sulfate (110.6 g) was added slowly over the course of 10–15 min with stirring. The suspension was stirred for an additional 30 min and centrifuged for 20 min at $10,000 \times g$. The supernatant fraction (410 mL) was collected and additional ammonium sulfate (66 g) was added slowly over the course of 15–20 min with stirring. The suspension was stirred for 30 min and centrifuged at $10,000 \times g$ for 40 min. The pellet was suspended in 60 mL of 25 mM potassium phosphate, pH 6.0, 1 mM magnesium chloride, 0.1 mM TPP, 10% glycerol (buffer B) and dialyzed three times against 4 L of buffer B. A total volume of 76 mL of dialyzed sample was obtained.

Affi-Blue column

The dialyzed enzyme solution was concentrated to 34 mL in an Amicon ultrafiltration cell equipped with a 30,000-molecular-weight cut-off membrane, and loaded by gravity onto an Affi-Blue column (2.5×40 cm) that had been equilibrated with six bed volumes of buffer B. Protein was eluted from the column with buffer B at a flow rate of 16 mL/h, and 4.5-mL fractions were collected. Fractions containing the highest benzoylformate decarboxylase activity (25–41) were pooled.

Sepharose Fast Flow column

The sample was loaded by gravity onto a Sepharose Fast Flow column (2.5×40 cm) that had been equilibrated with 10 bed volumes of 50 mM potassium phosphate, pH 5.8, 0.1 mM TPP, 10% (v/v) glycerol (buffer C) at a flow rate of 250 mL/h. The column was washed with 170 mL of buffer C, followed by a 2-L gradient of buffer C with 0–1.0 M sodium chloride at a flow rate of 230 mL/h. Eluate was collected in 4-mL fractions; benzoylformate decarboxylase was eluted between 0.24 mM and 0.25 mM sodium chloride. Fractions 118–125 were pooled and

exchanged into 50 mM sodium phosphate, pH 6.0, 1 M ammonium sulfate, using an Amicon ultrafiltration cell, and concentrated to 4 mL.

Phenyl Sepharose column

The following step was performed at room temperature, using an FPLC system from Pharmacia. The column (1.0×60 cm) was equilibrated with 10 bed volumes of 50 mM sodium phosphate, pH 6.0, 1 M ammonium sulfate (buffer D) at a flow rate of 3 mL/min. The sample was loaded at 1 mL/min and the column washed with 35 mL of buffer D. The enzyme was eluted with a gradient of 1.0–0 M ammonium sulfate at a flow rate of 3 mL/min, and 8-mL fractions were collected. Fractions 46–50 were pooled, exchanged into 25 mM HEPES, pH 7.0, 0.2 mM TPP, 0.1 mM MgCl_2 , and concentrated to 1.9 mL.

Storage

The pure enzyme was concentrated to 10 mg/mL in 0.1 mM MgCl_2 , 0.2 mM TPP, 25 mM HEPES, pH 7.0, frozen in an acetone-dry ice bath, and stored at -80°C .

Determination of molecular weight and isoelectric point

The molecular weight of the pure enzyme was determined by gel filtration with a high-molecular-weight standard kit, following the instructions of the manufacturer. A Sepharose CL-6B column ($2.6 \text{ cm} \times 36 \text{ cm}$) was packed and equilibrated with 50 mM sodium phosphate, pH 6.0, 0.1 M sodium chloride at a flow rate of 6 mL/min. The calibration of the column with the protein standards was performed at 0.33 mL/min. The isoelectric point of the enzyme was determined with a broad range calibration kit (pI 5.2–7.35) using a Phastgel isoelectric focusing gel in the range of pH 5–8.

Crystallization

Crystals were grown at room temperature by hanging-drop vapor diffusion against a well solution of 22% polyethylene glycol 400, 0.15 M CaCl_2 , 0.1 M Tris, pH 8.5. Drops contained equal volumes (2 μL) of well solution and purified benzoylformate decarboxylase stored as described above. Under these conditions, large single crystals grow in 2–5 days, although they sometimes require seeding from a solution of crushed crystals of the same form diluted in 26% polyethylene glycol 400, 0.15 M CaCl_2 , 0.1 M Tris, pH 8.5. Crystals of similar morphology could also be obtained if the well solution contained 0.1 M HEPES, pH 7.5, rather than Tris. Crystals were mounted in quartz capillaries (Charles Supper Co.) for diffraction analysis.

Data collection

Crystals were kept at a temperature of 4°C during X-ray experiments with a stream of chilled air. Initial data were collected to 3 $\text{\AA}$ resolution on a Siemens X-100A multiwire proportional X-ray detector. X-rays were generated by an Eliot GX-6 rotating anode (30 kV $\times$ 30 mA) with a 0.5-mm focusing cup and a 0.3-mm collimator. The set of X-ray diffraction images was reduced to integrated indexed intensities and processed with XDS (Kabsch, 1988) to determine the unit cell dimensions, Laue sym-

metry, and space group. A second data set, to 1.6 Å resolution, was collected on an R-AXIS IIC image plate detector using 0.3-mm collimated monochromatized CuK α radiation from a Rigaku RU-200 rotating anode generator (50 kV $\times$ 150 mA). The diffraction images were reduced to integrated indexed intensities with the R-AXIS processing software PROCESS (T. Higashi, Rigaku Corporation, 1990) and MOSFLM (A.J. Wonocott, Imperial College, 1980).

Acknowledgments

This work was supported in part by NIH grants GM40570 (to G.L.K.) and GM26788 (to G.A.P. and D.R.), fellowship DRG-1194 of the Cancer Research Fund of the Damon Runyon-Walter Winchell Foundation (to M.S.H.), and in part by a grant from the Lucille P. Markey Charitable Trust. M.S.H. and A.M. contributed equally to this manuscript.

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