Purification and Some Properties of a Novel L-2,4-Diaminobutyric Acid Decarboxylase from Vibrio alginolyticus

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Previous investigations have shown that members of the genus Vibrio possess a novel enzyme activity decarboxylating L-2,4-diaminobutyric acid (DABA) to 1,3-diaminopropane (DAP). In this paper we describe the purification, by about 3600-fold, of the enzyme from V. alginolyticus. The purified enzyme was apparently homogeneous, and had a specific activity of 4200 nmol DAP min⁻¹ (mg protein)⁻¹. The enzyme protein has an $M_r$ of 450000 ± 20000 and is apparently comprised of four identical subunits ($M_r$, 109000 ± 1000). Neither 2,3-diaminopropionic acid, ornithine, lysine nor arginine served as substrates. Some properties of the enzyme were determined. Cultivation of this bacterium in the presence of added DABA brought about increased production of norspermidine (NSPD), characteristically present in this species as well as DAP, suggesting that the enzyme may be functionally implicated in the formation of DAP, a biosynthetic precursor of NSPD.

INTRODUCTION

Norspermidine (NSPD), a C₁-lacking analogue of spermidine, is a general constituent of members of the genus Vibrio (Yamamoto et al., 1983) and is biosynthesized from a Schiff base formed between 1,3-diaminopropane (DAP) and L-aspartic β-semialdehyde by two enzymic reactions (Yamamoto et al., 1986a). Furthermore, vibrios synthesize NSPD in much larger amounts than spermidine (Yamamoto et al., 1986c), which is generally assumed to be the only known precursor of DAP (De Rosa et al., 1978; Aleksijevic et al., 1979; Paulin et al., 1983; Tabor & Tabor, 1985). This finding prompted us to examine the possibility of another mode of DAP biosynthesis. Recently, we found a novel enzyme, designated L-2,4-diaminobutyric acid (DABA) decarboxylase, that showed a strong preference for DABA as the substrate and yielded DAP and CO₂ as the products (Yamamoto et al., 1986b). Such an enzyme activity had previously not been reported in any organism.

In this paper, we present details on purifying to apparent homogeneity DABA decarboxylase from V. alginolyticus. Some basic properties of the purified enzyme are described. Further, since our attempts to demonstrate the natural occurrence of DABA in vibrios have hitherto been unsuccessful, the bacterium was grown in the presence of added DABA in order to evaluate the possible involvement of DABA decarboxylase in producing DAP for NSPD biosynthesis.

METHODS

Bacterial strain and growth conditions. The strain used was V. alginolyticus ATCC 17749. It was precultured at 37 °C for 12 h in medium A [0.45% yeast extract (Difco); 0.1% Bacto-casitone (Difco); basal inorganic salts (Yamamoto et al., 1983); 0.5% NaCl (unless otherwise noted); final pH 7.5]. Several 2 litre Erlenmeyer flasks, each containing 1 litre of the same medium, were inoculated with 20 ml of the preculture and mounted on a rotary shaker at 37 °C. After 6–8 h growth, the cells were harvested by centrifugation for 10 min at 5300 g at 4 °C, and

Abbreviations: DAP, 1,3-diaminopropane; NSPD, norspermidine; DABA, L-2,4-diaminobutyric acid; PLP, pyridoxal 5'-phosphate.

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washed once with ice-cold 0-5% NaCl with 10 mM-MgCl2. Cell yield was 5-6 g wet weight l-1. Cell pellets were often stored at -30 °C before enzyme purification. No change in enzyme activity was noted for at least 2 months.

Time-course study of enzyme activity during growth at different NaCl concentrations. Inocula (20 ml) prepared by growth in medium A containing 0-5, 2 and 5% (w/v) NaCl were each added to 1 litre of medium A containing NaCl at the corresponding concentrations. After 2, 6 and 10 h incubation, samples (200-400 ml) were aseptically withdrawn from the growing cultures. The cells were harvested as described above except that washing was done with a solution containing 10 mM-MgCl2 and NaCl at the same concentration as that used for cultivation. Cells were immediately suspended in 0.1 mM-PLP, and disrupted in a salt-ice bath with an ultrasonic homogenizer (Nissei US-300). The sonicate was centrifuged at 40000 g for 30 min and the supernatant was dialysed for 12 h against 500 vols buffer A, changing the buffer twice. The dialysed cell extract was immediately assayed for enzyme activity.

Enzyme assay. The assay mixture (total volume 1 ml) contained 100 mM-Tris/HCl, pH 8.25, 7.5 mM-DABA, 0-1 mM-PLP, 0-4 mg BSA and the enzyme protein (<100 mg). After incubation at 37 °C for 30 min, the reaction was stopped by the addition of 0.4 ml 20% (w/v) HClO4. DAP formed was determined by a highly sensitive GLC method (Yamamoto et al., 1986b). One unit of activity was defined as the amount of enzyme that catalysed the formation of 1 nmol DAP min-1 under the standard conditions. Protein was determined by the method of Lowry with BSA as the standard.

Purification of DABA decarboxylase. Unless otherwise noted all operations, except for FPLC which was done at room temperature, were done at 4°C or in an ice bath and all dialyses were done against 100 vols of the specified buffers for 12 h. The final procedures adopted for the purification of DABA decarboxylase are shown in Table 1. The frozen cells (240 g) were thawed and suspended in 1 litre of buffer A. Samples consisting of 250 ml of cell suspension were placed in 500 ml beakers kept in a salt-ice bath and sonicated with an ultrasonic homogenizer. Cell debris was removed by centrifugation. A small portion of the combined supernatant was dialysed against buffer A to assay the activity (crude extract). The supernatant was fractionated with solid (NH4)2SO4. The protein precipitated between 35 and 50% saturation was collected by centrifugation at 20000 g for 20 min, dissolved in buffer A and dialysed against two changes of the same buffer. After dialysis the (NH4)2SO4 fraction (60 ml) was freed from insoluble material by centrifugation; the supernatant was divided into three 20 ml portions, each of which was loaded onto a Sephacryl S-300 column (2-1 x 91 cm) equilibrated with buffer A containing 0.4 M-NaCl and 0.02% NaN3. The column was eluted with the same buffer at a flow rate of 10 ml h-1; 3 ml fractions were collected. The active fractions (tube nos 43-50) were pooled (24 ml), dialysed against buffer A and then applied onto a DEAE-Sephacore CL-6B column (2-2 x 25 cm) equilibrated with 20 mM-Tris/HCl, pH 7.5. After washing the column with at least 200 ml of the same buffer, a linear gradient of NaCl, 0-1 M in 300 ml 20 mM-Tris/HCl, pH 7.5, was applied at a flow rate of 15 ml h-1. Fractions (5 ml) were collected in tubes containing 50 μl 4 mM-PLP. The active fractions, eluting between 0.57 and 0.64 M-NaCl, were pooled (25 ml) and dialysed for 4 h against buffer A. This preparation was rechromatographed in the same way. The active fractions were pooled (25 ml), dialysed against 20 mM-potassium phosphate buffer, pH 7.5, containing 0.04 mM-PLP and applied onto a hydroxyapatite column (2-65 x 23 cm) equilibrated with the same buffer. After washing the column with 3 column vols of the same buffer, the enzyme was eluted with a 560 ml linear gradient of 20-200 mM-potassium phosphate buffer, pH 7.5, at a flow rate of 10 ml h-1. Fractions (8 ml) were collected in tubes containing 80 μl 4 mM-PLP. The active fractions eluting between 112-124 mM-potassium phosphate were pooled (24 ml) and dialysed against buffer A. This fraction was divided into 8 ml portions, each of which was injected onto a Pharmacia FPLC system equipped with a Mono Q column (HR; 5 x 0.5 cm), washed with 10 ml 20 mM-Tris/HCl, pH 7.5, containing 0.25 M-NaCl, and then eluted with a 60 ml linear gradient of 0.25-1 M-NaCl in 20 mM-Tris/HCl, pH 7.5, at a flow rate of 0.5 ml h-1. Fractions (1 ml) were collected in ice-cold tubes containing 8 μl 4 mM-PLP. The active fractions, eluting at around 0.6 M-NaCl, were pooled (18 ml) and dialysed for 4 h against buffer A. The whole was injected again onto the FPLC system, and eluted as just described. The peak fraction (1 ml) was dialysed for 4 h against buffer A containing 0.02% NaN3 and stored at 4°C after concentration to about 0.5 ml by using a Centricon 30 (Amicon).

Electrophoretic analyses. Isoelectric focusing was done on a 4% (w/v) polyacrylamide gel plate (0.8 mm thick; 4.5 x 11 cm) containing 2% (w/v) Ampholine pH 3.5-9.5 using a Bio-Rad horizontal isoelectric focusing apparatus (model 1415). The samples were run at an initial setting of 5 W for 5 h and then at 1500 V for 5 h. For monitoring the distribution of enzyme activity after electrophoresis, the gel was sliced horizontally into 5 mm sections, each of which was macerated in buffer A overnight at 4°C to elute the enzyme. SDS-PAGE was done by using a 7.5% (w/v) polyacrylamide slab gel according to the method of Laemmli (1970). Before loading, the enzyme sample was heated for 3 min with 0.1% SDS and 10% (w/v) glycerol in a boiling-water bath. The gel was stained for protein with Coomassie brilliant blue R-250. The relative mobility was determined for M, markers as well as for the enzyme.

Gel filtration. The M, of the native enzyme was determined by the method of Andrews (1965). A Sephacryl S-300 column (2-1 x 91 cm), equilibrated with buffer A containing 0.4 M-NaCl and 0.02% NaN3, and calibrated with known M, standard proteins, was used.
Table 1. Purification of DABA decarboxylase from V. alginolyticus

A typical purification from 240 g wet wt of cells is shown.

<table>
<thead>
<tr>
<th>Step</th>
<th>Total protein (mg)</th>
<th>Total activity (units)</th>
<th>Specific activity [units (mg protein)^(-1)]</th>
<th>Purification (fold)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>6279</td>
<td>7211</td>
<td>1.15</td>
<td></td>
<td>100</td>
</tr>
<tr>
<td>Crude extract 1. Ammonium sulphate (35-50%) precipitation</td>
<td>2006</td>
<td>9328</td>
<td>4.65</td>
<td>4.0</td>
<td>129</td>
</tr>
<tr>
<td>2. Sephacryl S-300</td>
<td>617</td>
<td>6096</td>
<td>9.88</td>
<td>8.6</td>
<td>84</td>
</tr>
<tr>
<td>3. 1st DEAE-Sepharose CL-6B</td>
<td>279</td>
<td>4576</td>
<td>16.4</td>
<td>14.3</td>
<td>63</td>
</tr>
<tr>
<td>2nd DEAE-Sepharose CL-6B</td>
<td>123</td>
<td>3752</td>
<td>30.5</td>
<td>26.5</td>
<td>52</td>
</tr>
<tr>
<td>4. Hydroxyapatite</td>
<td>23-6</td>
<td>3184</td>
<td>134.9</td>
<td>117</td>
<td>44</td>
</tr>
<tr>
<td>5. 1st FPLC</td>
<td>1-43</td>
<td>1659</td>
<td>1160</td>
<td>1009</td>
<td>23</td>
</tr>
<tr>
<td>2nd FPLC</td>
<td>0.256</td>
<td>1078</td>
<td>4210</td>
<td>3661</td>
<td>15</td>
</tr>
</tbody>
</table>

Cultivation in the presence of added DABA and polyamine determination. An inoculum (4 ml), prepared as described above, was added to 200 ml of a synthetic medium (Yamamoto et al., 1983) containing 2% (w/v) NaCl and DABA at the indicated concentrations, and then cultivated for 6 h. After centrifugation, DAP and NSPD both in cells and in growth medium (supernatant) were determined by the GLC method (Yamamoto et al., 1982). Cell protein was determined by a modified Lowry method (Clark, 1984) with BSA as the standard.

Chemicals. DABA and M, markers for SDS-PAGE were obtained from Sigma; DEAE-Sepharose CL-6B, Sephacryl S-300 and M, markers for gel filtration were from Pharmacia; Ampholine was from LKB; \( \Delta^L \)-[\( \Delta^L \)-arginine \( [270 \text{ mCi mmol}^{-1} (10 \text{ GBq})] \) was from ICN. All other chemicals were of analytical or reagent grade.

RESULTS

Time-course of DABA decarboxylase activity

*V. alginolyticus*, a weak halophile, can grow over a wide range of NaCl concentrations (0.5-7%, w/v). Thus, in order to define a good enzyme source for purification, DABA decarboxylase activity was determined in cells during growth at 0.5, 2 and 5% (w/v) NaCl. In all cases, a peak in specific activity was observed after 2 h growth, corresponding to the early exponential phase; thereafter specific activities declined differently. The burst of enzyme activity after 2 h in the respective cultures corresponded with a rapid increase in cellular NSPD content (Yamamoto et al., 1986c). A small amount of DAP [1-2 nmol (mg cell protein)^\(-1\)] was detected in cells around 2 h. The total DABA decarboxylase activity in cells grown at 0.5% NaCl reached its highest level after 6 h, and was much greater than in cells grown at 2 or 5% NaCl. Further, after sonic disruption, almost all the enzyme activity of cells grown at 0.5% NaCl was in the soluble fraction, which incidentally also had the maximum specific activity. Therefore, the soluble fraction of cells grown at 0.5% NaCl for 6-8 h was used for enzyme purification, according to the scheme given in Table 1.

Properties of the enzyme

Stability. The final preparation could be stored at 4 °C and pH 7.5 in the presence of PLP and NaN\(_3\) for 3 weeks without significant loss of activity. The addition of PLP was indispensable for storage of the enzyme. Freezing and thawing the purified enzyme caused almost complete loss of activity.

Purity and isoelectric point. The purified enzyme migrated as a single protein band in both isoelectric focusing and SDS-PAGE (Fig. 1). The protein band detected after isoelectric focusing coincided with enzyme activity and had a pl of 6.8. SDS-PAGE on a 10% (w/v) gel also gave a single protein band. Unfortunately, PAGE under non-denaturing conditions, as described by Davis (1964), on 4, 5 or 6% (w/v) separating gels and a 4% (w/v) stacking gel at 15 mA, failed to give a single band at a suitable position on the gels: 5 and 6% gels gave a severely tailed protein band along which enzyme activity was dispersed; and on a 4% gel a single band was observed comigrating with bromophenol blue.
Fig. 1. SDS-PAGE of the purified DABA decarboxylase. Lane A, purified enzyme (8 µg); lane B, marker proteins (from the top: myosin; β-galactosidase; phosphorylase b; BSA; egg albumin).

*M* of enzyme and subunit. The *M* of the purified enzyme, as determined with a calibrated column of Sephacryl S-300, was 450000 ± 20000. The *M* of the protein band upon SDS-PAGE was estimated to be 109000 ± 1000 (Fig. 1) and was unaltered when the protein was reduced with 1% (v/v) 2-mercaptoethanol before electrophoresis. Thus the native enzyme appears to be a tetramer of subunits of identical size which are not linked by disulphide bonds.

Substrate specificity and kinetic parameters. L-2,3-Diaminopropionic acid, L-ornithine, L-lysine and L-arginine were tested as potential substrates under the standard assay conditions. The decarboxylation activity of the purified enzyme towards the first three amino acids was evaluated by the GLC method (Yamamoto et al., 1986b); 14CO2 evolution from carboxyl-labelled arginine was monitored by the procedure of Tyagi et al. (1983). None of these four amino acids showed detectable substrate activity at 7.5 mM, indicating that the enzyme is highly specific for DABA. In addition, 10 mM concentrations of these amino acids did not inhibit decarboxylation of DABA (5 mM). When the purified enzyme was incubated with increasing concentrations of racemic DABA, *V*max did not change appreciably, whereas the *K* of the racemate was 171 µM, about 2-fold that for the L-isomer (see below). This indicates that the enzyme is neither active on nor inhibited by the D-isomer (this is commercially unavailable). Moreover, carboxynorspermidine[NH2(CH2)3NH(CH2)3CH2(NH2)CO2H], which is structurally similar to DABA and is decarboxylated by an enzyme present in vibrios to yield NSPD (Yamamoto et al., 1986a), did not serve as a substrate for DABA decarboxylase.

The enzyme exhibited typical Michaelis–Menten kinetics; a Lineweaver–Burk plot gave a *K* of 81 µM for DABA and a *V*max of 4300 nmol DAP min–1 (mg protein)–1.

*pH* optimum. The effect of pH on enzyme activity was examined in the range 5.8–10.0. The enzyme showed a broad pH optimum (7.5–8.75), more than half the maximal activity being observed at pH 6.5 and 9.3.

PLP requirement and its *K* value. Overnight dialysis of the purified enzyme against buffer devoid of PLP led to >25% irreversible loss of activity. Thus PLP was added to all buffers used for extraction and dialysis and to tubes collecting fractions. If PLP was omitted from the assay mixture, the activity of such a dialysed preparation decreased markedly, but 19% of the control
Vibrio diaminobutyric acid decarboxylase

Table 2. Enhanced production of DAP and NSPD in V. alginolyticus grown in the presence of DABA

The bacterium was grown at 37 °C for 6 h in 200 ml of the synthetic medium containing 2% (w/v) NaCl and DABA at the indicated concentrations. After harvesting the cells, DAP and NSPD were determined in both cells and supernatant (in each case the total protein was about 20 mg).

<table>
<thead>
<tr>
<th>DABA concn (mM)</th>
<th>Cells</th>
<th>Supernatant</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DAP [nmol (mg cell protein)^(-1)]</td>
<td>NSPD</td>
</tr>
<tr>
<td>0</td>
<td>ND</td>
<td>5-4</td>
</tr>
<tr>
<td>1</td>
<td>0-21</td>
<td>7-9</td>
</tr>
<tr>
<td>5</td>
<td>0-24</td>
<td>9-3</td>
</tr>
<tr>
<td>10</td>
<td>0-21</td>
<td>10-5</td>
</tr>
</tbody>
</table>

ND, Not detectable; -, not determined.

activity was still observed. In order to demonstrate this requirement for PLP more clearly, the enzyme was dialysed in the presence of L-cysteine to remove bound PLP (Wu & Morris, 1973). No activity was observed when PLP was omitted from the assay mixture, whereas addition of 0-1 mM-PLP reactivated the enzyme to 56% of the control activity, obtained with the enzyme dialysed in the absence of L-cysteine. The \( K_m \) value for PLP was determined using this preparation. Hyperbolic saturation kinetics were observed and a \( K_m \) of 5-6 \( \mu \)M was obtained.

In accordance with the above observations, the enzyme was inhibited by incubating with inhibitors of PLP-dependent enzymes, such as isonicotinic acid hydrazide, carboxymethoxylamine and pyridoxal. The most pronounced inhibition was observed with carboxymethoxylamine. Inhibition by 25 mM-pyridoxal could be reversed completely by addition of 1 mM-PLP.

Other properties. In assays to determine the effects of various metal ions, BSA was omitted from the assay mixture. Although, as reported earlier (Yamamoto et al., 1986b), 20 mM-Mg\(^{2+}\) stimulated the activity of the crude enzyme by about 30%, it had a slight inhibitory effect (about 5%) on the purified enzyme at the same concentration. The chloride or sulphate salts of other metal ions were tested with the purified enzyme. None were activators; on the contrary, Fe\(^{2+}\), Zn\(^{2+}\), Hg\(^{2+}\) and Ag\(^{+}\) (all 1 mM) severely inhibited the enzyme (>90%); Ca\(^{2+}\) (1 mM) had a slight inhibitory effect (10%). NaCl and KCl (up to 700 mM) and EDTA did not affect enzyme activity. Dithiothreitol (1-5 mM) neither stabilized nor stimulated the enzyme. Activity was unaffected by GTP and ATP, both of which have been known to stimulate bacterial ornithine decarboxylases (Hölttä et al., 1972; Paulin & Pösö, 1983).

Enhanced production of DAP and NSPD in cells grown in the presence of DABA

In spite of our efforts, we have hitherto been unable to demonstrate the natural occurrence of DABA in *Vibrio*. However, if DABA decarboxylase is indeed implicated in NSPD biosynthesis, one would expect that exogenous supply of DABA to the growth medium would prompt DAP production and eventually lead to increased accumulation of NSPD. Experiments, therefore, were planned to test this. As shown in Table 2, the content of NSPD was actually increased by increasing the concentration of DABA. DAP, which had not been detected in cells grown for 6 h in the absence of DABA, was also found but the amount remained constant regardless of the concentration of added DABA. In contrast, a large amount of DAP was detected in the medium, implying that overproduced DAP was excreted. A comparative experiment was done with *Escherichia coli* K12, but there was no increase in DAP and no appearance of NSPD. Moreover, when *V. alginolyticus* was grown for 6 h in the presence of 5 mM-DAP, the NSPD content increased about 1-5-fold, whereas the specific activity of DABA decarboxylase fell from 78 (control) to 37 units (mg protein)^(-1). Neither DABA nor DAP had any effect on the growth rate at the concentrations used. These results suggest that DABA decarboxylase may be involved in the formation of DAP as a biosynthetic precursor of NSPD.
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Discussion

It is well established that ornithine and/or arginine decarboxylases and lysine decarboxylase respectively, are responsible for the formation of the naturally occurring diamines putrescine and cadaverine (Boeker & Snell, 1972; Morris & Fillingame, 1974; Pegg & Williams-Ashman, 1981). However, an enzyme capable of catalysing the decarboxylation of DABA, a lower analogue of these amino acids, has so far not been described for any organism. Thus it is generally accepted that the source of DAP (which itself is widely distributed in nature) is spermidine and/or spermine, because both are ubiquitous constituents of living cells and oxidation activities towards these polyamines, leading to DAP production, can be detected in micro-organisms and plants (Morgan, 1980; Smith, 1985). However, an oxidation activity towards spermidine, leading to DAP production, could not be detected in vibrios, whereas a new activity, catalysing the decarboxylation of DABA to yield DAP, was observed in some Vibrio species (Yamamoto et al., 1986b).

In this study, DABA decarboxylase was isolated as an apparently homogeneous protein from V. alginolyticus. It has an $M_r$ of 450 000 as determined by gel filtration, while SDS-PAGE gives an $M_r$ of 109 000: thus the native enzyme is most likely a tetramer. The purified enzyme showed a strict specificity for DABA. As with most decarboxylases (Boeker & Snell, 1972), it required PLP for activity and stability.

In addition, we have presented experimental results suggesting that DABA decarboxylase may function in vivo to produce DAP as the precursor for NSPD synthesis. Thus: (a) the presence of enzyme activity closely corresponded in time to NSPD accumulation; (b) an appreciably enhanced production of both DAP and NSPD occurred when V. alginolyticus was grown in the presence of DABA. It is therefore unlikely that the catalysis of DAP formation observed represents a non-specific or artefactual activity of an enzyme that has some other role in this species.

DABA has been detected as one of the components of the peptide antibiotics polymyxins, produced by Bacillus species (Paulus et al., 1964), in mucoprotein of Corynebacteria (Perkins & Cummins, 1964), and in the free form in plants (Nigam & Ressler, 1966) and in animals (Ackermann & Menssine, 1960; Nakajima et al., 1967). Furthermore, an enzyme activity capable of synthesizing DABA has been reported in a Xanthomonas species (Rao et al., 1969) and in Bacillus colistinus (Ito et al., 1969). Based on such reports, it is not unlikely that vibrios also have the ability to produce DABA. Our failure to demonstrate the occurrence of DABA in vibrios could be explained by assuming that once DABA has been produced, it is immediately converted to DAP. Preparation of a mutant deficient in DABA decarboxylase or elucidation of the biosynthetic pathway for DABA would help to resolve this problem. These lines of studies are in progress.

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References


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