Purification and characterization of L-2,4-diaminobutyrate decarboxylase from *Acinetobacter calcoaceticus*

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Acinetobacter calcoaceticus **ATCC 23055 produces a large amount of 1,3-diaminopropane under normal growth conditions. The enzyme responsible, L-2,4-diaminobutyrate (DABA) decarboxylase (EC 4.1.1.–), was purified to electrophoretic homogeneity from this bacterium. The native enzyme had an M_r of approximately 108000, with a pI of 5·0, and was a dimer composed of identical or nearly identical subunits with apparent M_r 53000. The enzyme showed hyperbolic kinetics with a K_m of 1·59 mM for DABA and 14·6 μM for pyridoxal 5′-phosphate as a coenzyme. The pH optimum was in the range 8·5–8·75, and Ca^{2+} gave a much higher enzyme activity than Mg^{2+} as a cationic cofactor. *N*-γ-AcetylDABA, 2,3-diaminopropionic acid, ornithine and lysine were inert as substrates. The enzyme was different in subunit structure, N-terminal amino acid sequence and immunoreactivity from the DABA decarboxylase of *Vibrio alginolyticus* previously described.**

Introduction

Acinetobacters are aerobic Gram-negative coccobacilli highly prevalent in nature (Bouvet & Grimont, 1986), and the genus *Acinetobacter* has recently been moved to a new family *Moraxellaceae* together with the genera *Moraxella*, *Psychrobacter* and related organisms (Rossau *et al.*, 1991). Although acinetobacters are usually commensals, and not pathogenic for humans, they can cause nosocomial infections of clinical importance, including respiratory tract infections, bacteremia and meningitis (Beck-Sagué *et al.*, 1990, and references cited therein).

Some members of *Acinetobacter* have been reported to possess a characteristic polyamine distribution pattern with 1,3-diaminopropane (DAP) as a major component and very little putrescine, spermidine or spermine (Busse & Auling, 1988; Yamamoto *et al.*, 1991). It is generally accepted that DAP is a catabolic oxidation product of spermidine or spermine (Razin *et al.*, 1959; Morgan, 1980). However, in a previous paper (Yamamoto *et al.*, 1991), we showed that, in common with members of the genus *Vibrio* (Nakao *et al.*, 1989), all strains of *A. calcoaceticus* and *A. lwoffii* so far examined could decarboxylate L-2,4-diaminobutyric acid (DABA), producing DAP and CO_2.

In this paper, we describe the purification and characterization of DABA decarboxylase (EC 4.1.1.–) from *A. calcoaceticus* ATCC 23055, and compare its properties with those of the DABA decarboxylase from *Vibrio alginolyticus* previously reported (Nakao *et al.*, 1989).

Methods

Chemicals and materials. Dihydrochloride salts of DAP and DABA, sodium cyanoborohydride, carboxymethoxylamine and hydroxyapatite were purchased from Wako; DEAE-Sepharose CL-6B, Sephacryl S-200 HR, Mono Q HR and Ampholine were from Pharmacia LKB; pyridoxal 5′-phosphate (PLP) and standard protein kit for SDS-PAGE (SDS-6H) were from Sigma; L-2,3-diaminopropionic acid was from Fluka. Human γ-globulin (M_r 160000), BSA (66000) and ovalbumin (45000) from Sigma, and soybean lipoxygenase (102000) from Oriental Yeast (Japan) were used as standard proteins for analytical gel filtration. *N*-γ-AcetylDABA was synthesized as described by Benoiton & Leclerc (1965). All other chemicals were of reagent quality commercially available.

Growth conditions. The preculture of *A. calcoaceticus* ATCC 23055 grown overnight in nutrient broth was added as an inoculum (2%, v/v) to the same medium, which was vigorously shaken at 30 °C for 8 h. The cells were harvested by centrifugation at 5300 *g* at 4 °C and stored at −80 °C until enzyme purification.

Enzyme assay. The production of DAP was assayed in the standard mixture (1·0 ml), containing 100 μmol Tris/HCl, pH 8·5, 0·2 μmol PLP,

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

The amino acid sequence data reported in this paper have been submitted to PIR and have been assigned the accession numbers A41817 [DABA DC (A)] and B41817 [DABA DC (V)].

2·5 μmol $MgCl_2$, 15 μmol DABA and enzyme protein (≦100 μg), incubated at 37 °C for 15 min. DAP formed was determined by the method of Nakao *et al.* (1989) except for the use of a gas chromatograph with a hydrogen flame ionisation detector. The reproducibility of the assay method was assessed by calculating the recoveries of known amounts of DAP (10–150 nmol) added to assay mixtures denatured by the prior addition of $HClO_4$. The recovery for DAP was 95 ± 4% ($n = 10$). The formation of DAP was linear with respect to time (10–60 min) and the amount of enzyme protein present. One unit (U) of activity was defined as the amount catalysing the formation of 1 μmol DAP min^{-1} under standard assay conditions.

Enzyme purification. Unless otherwise noted, all operations were done at 0–5 °C and all dialyses were done against 100 vols of the specified buffers with two changes for 12–16 h. When the enzyme was eluted with buffers lacking PLP, an appropriate amount of 4 mM-PLP was added to each reservoir tube in advance to make a final concentration of 40 μM. Protein was determined by the Lowry or Bradford (1976) methods with BSA as a standard. For most column fractions, the protein elution pattern was obtained from A_{280}.

Frozen cells (190 g wet wt) were suspended in 950 ml 20 mM-Tris/HCl, pH 7·5, and 0·1 mM-EDTA (buffer A) containing 40 μM-PLP, and disrupted with an ultrasonic homogenizer. The suspension was centrifuged for 30 min at 40000 *g*, and a small portion of the supernatant was dialysed against the same buffer to determine the activity (crude extract). The supernatant was fractionated with ammonium sulphate, and the precipitate (50–70% saturation) was dissolved in a minimum volume of buffer A containing 40 μM-PLP and dialysed against the same buffer. The dialysed material was applied to a DEAE-Sepharose CL-6B column (3·7 × 27 cm) equilibrated with buffer A. After washing the column with one column vol. of the same buffer, the enzyme was eluted with a 1 litre linear gradient of 0–0·45 M-NaCl at a flow rate of 30 ml h^{-1}. The active fractions eluting at 0·35–0·39 M-NaCl were pooled and then dialysed for 4 h against 20 mM-potassium phosphate (buffer B), pH 6·8, containing 40 μM-PLP. The dialysed solution was rechromatographed on a DEAE-Sepharose CL-6B column (2·6 × 22 cm) equilibrated with buffer B. The enzyme was eluted with a 300 ml linear gradient of 0–0·45 M-NaCl in buffer B, and the active fractions eluting at 0·40–0·42 M-NaCl were collected. The enzyme solution was applied to a Sephacryl S-200 HR column (2·1 × 88 cm) equilibrated with buffer A containing 40 μM-PLP, 0·4 M-NaCl and 0·02% NaN_3, and the enzyme was eluted with the same buffer at a flow rate of 10 ml h^{-1}. The active fractions were combined and dialysed against 10 mM-potassium phosphate, pH 7·5, containing 40 μM-PLP. The dialysed enzyme solution was put on a hydroxyapatite column (1·65 × 10 cm) equilibrated with 10 mM-potassium phosphate, pH 7·5. After washing the column with 2 column vols of the same buffer, the enzyme was eluted with a 240 ml linear gradient of 10–200 mM-potassium phosphate at a flow rate of 10 ml h^{-1}. The active fractions eluting at 43–49 mM-potassium phosphate were subjected to HPLC (Waters 650) with a Mono Q HR 5/5 column equilibrated with buffer B, pH 7·5. After the column was washed with 10 ml of the same buffer, the enzyme was eluted with a 40 ml gradient of 0–1 M-NaCl at a flow rate of 0·5 ml h^{-1}. The active fractions eluting at around 0·62 M-NaCl were rechromatographed on the same column with a 20 ml gradient of 0–1 M-NaCl in buffer B, pH 6·8, and the enzyme was eluted at around 0·56 M-NaCl. The purified enzyme was dialysed against buffer A containing 40 μM-PLP and 0·02% NaN_3, and the dialysed solution was concentrated to about one-fifth of its original volume with an Amicon Centricon 10 and stored at 1 °C.

Electrophoretic analyses. PAGE of the native enzyme was done on a 7·5% (w/v) polyacrylamide slab gel as described by Williams & Reisfeld (1964). SDS-PAGE was done on a 10% (w/v) polyacrylamide slab gel as described by Laemmli (1970). Samples in 2% (w/v) SDS, 7% (v/v) glycerol and 5% (w/v) 2-mercaptoethanol were boiled at 100 °C for 3 min. Electrofocusing was done on a 5% (w/v) polyacrylamide gel plate containing 2·7% (w/v) Ampholine, pH 3·5–9·5, with solutions of 0·1 M-H_3PO_4 at the anode and 0·1 M-NaOH at the cathode (Wrigley, 1971) using a horizontal electrofocusing apparatus (Model AE-3230, Atto, Japan). Electrofocusing was continued with cooling for 4 h at 400 V. For pH determinations, the gel was sliced into 5 mm sections and each was suspended in 0·3 ml of water. Protein bands were visualized by staining with 0·25% Coomassie Brilliant Blue R-250 in 50% (v/v) methanol and 10% (v/v) acetic acid followed by destaining with 40% (v/v) ethanol and 10% (v/v) acetic acid. For monitoring the distribution of enzyme activity after native PAGE, the gels were sliced in 5 mm sections, and each was macerated overnight at 4 °C in buffer A containing 40 μM-PLP.

M_r determination. Analytical gel filtration of the native enzyme was done with a Sephacryl S-200 HR column (2·1 × 88 cm) calibrated with the standard proteins, as described by Andrews (1965). The M_r of the subunit was determined from relative mobility on SDS-PAGE compared with the standards.

Immunological methods. Antiserum against the *A. calcoaceticus* DABA decarboxylase was obtained from an adult male rabbit by subcutaneous injection of the purified enzyme (0·2 mg) in 1 ml of 10 mM-potassium phosphate, pH 7·5, containing 0·9% (w/v) NaCl, emulsified in an equal volume of Freund's complete adjuvant (Difco). After 2 weeks, the rabbit was given a booster shot with 0·1 mg of the enzyme emulsified in Freund's incomplete adjuvant (Difco) once a week for 2 weeks. Whole blood was drawn from a carotid after one week from the final booster injection. Blood was allowed to clot at 37 °C for 30 min and then kept at 4 °C overnight. The serum was separated from the clots by centrifugation, heat-inactivated at 56 °C for 30 min and stored at −80 °C. Antiserum against the *V. alginolyticus* DABA decarboxylase, which was purified from strain ATCC 17749 according to the procedures of Nakao *et al.* (1989), was prepared with the same method.

Ouchterlony double-diffusion analysis was done with 0·7% agar plates in 20 mM-Tris/HCl, pH 7·5, containing 0·9% NaCl and 0·04% NaN_3, for 24 h.

N-Terminal amino acid sequence analysis. The purified enzymes (about 1·5 nmol) from *A. calcoaceticus* and *V. alginolyticus*, which had been dialysed against 10 mM-potassium phosphate, pH 7·5, were used for automated Edman degradation with an Applied Biosystems 477A protein sequencer, equipped with an on-line HPLC apparatus Model 120A.

Results and Discussion

Purification of DABA decarboxylase

The highest total activity of DABA decarboxylase was obtained from cells growing in the late exponential phase (8 h after inoculation), and the enzyme activity was present exclusively in the soluble fraction. Addition of DABA at 5 mM to the medium brought about no significant increase in specific activity of the enzyme, but total DAP production (the sum of intra- and extracellular DAP) increased about five-fold compared to that without addition of DABA. This indicated that the enzyme intensively decarboxylated exogeneously added DABA to yield DAP.

Preliminary results with partially purified enzyme

Table 1. *Purification of DABA decarboxylase from A. calcoaceticus*

A typical purification from 190 g wet wt of cells is shown.

Step	(mg)	Activity (U)	Activity (%)	Specific activity (U mg^{-1})	Specific activity (-fold)
Crude extract	9718	80·4	100	0·008	1
50–70% Ammonium sulphate	4668	73·9	92	0·016	2
DEAE-Sepharose (pH 7·5)	284	80·4	100	0·283	35
DEAE-Sepharose (pH 6·8)	84	64·3	80	0·765	96
Sephacryl S-200 HR	29·8	54·6	68	1·83	228
Hydroxyapatite	2·9	15·2	18·9	5·24	655
Mono Q (0·25–1 M-NaCl)	0·61	6·71	8·3	11·0	1333
Mono Q (0–0·5 M-NaCl)	0·38	6·45	8·0	17·2	2150

indicated that PLP, but not dithiothreitol, enhanced stability of the enzyme, and that both PLP and Mg^{2+} were indispensable for full enzyme activity. The purification scheme is given in Table 1. The enzyme was purified 2150-fold, with an overall yield of 8%. The purified enzyme could be stored in buffer A containing 40 μM-PLP and 0·02% NaN_3 at 1 °C for at least two weeks without significant loss of activity. However, freezing and thawing the purified enzyme caused a severe loss of activity.

Properties of the purified enzyme

Purity, M_r and subunit structure. Native PAGE of the enzyme showed a single band, which coincided with the activity, indicating that the preparation was homogeneous. Electrofocusing gave a pI of 5·0. When subjected to Sephacryl S-200 gel filtration, the enzyme was eluted as a single protein peak with apparent M_r 108000 ± 1000 ($n = 3$). SDS-PAGE showed a single band with M_r 53000. These results indicate that the native enzyme is a dimer composed of identical or nearly identical subunits.

Effect of pH and temperature on the enzyme activity and stability. The enzyme exhibited maximum activity at pH 8·5–8·75 in 100 mM-Tris/HCl. More than 50% of maximum activity was observed at pHs 7·75 and 9·25. No loss of activity was found at pH 7·5–9·0 at 37 °C for 15 min. When the effect of temperature on the enzyme activity was examined at pH 8·5, the enzyme was most active at 45 °C, the specific activity being about 1·2-fold higher than that at 37 °C. When incubated at various temperatures for 15 min in 100 mM-Tris/HCl, pH 8·5, the enzyme was stable up to 40 °C.

Substrate specificity and kinetic parameters. The enzyme was specific for DABA; under the standard conditions, *N*-γ-acetylDABA, L-2,3-diaminopropionic acid, L-ornithine and L-lysine (each at 15 mM) were inert as substrates, as judged by the fact that the possible reaction products, monoacetylDAP, ethylenediamine, putrescine and cadaverine, were not detected by GLC analysis (Yamamoto *et al.*, 1982, 1984). Moreover, the enzyme was assumed to be inactive on D-BADA (this is commercially unavailable), since the K_m value for racemic DABA was about two-fold higher than that for L-DABA without any appreciable change in V. The enzyme exhibited typical Michaelis–Menten kinetics; the double reciprocal plot gave a K_m of 1·59 mM and a V of 20·0 μmol DAP min^{-1} (mg protein)$^{-1}$.

Requirement for PLP. Enzyme dialysed for 3 h against buffer A devoid of PLP showed an absolute dependence on PLP for its activity. However, such dialysis led to about 70% irreversible loss of activity. The approximate K_m value for PLP, determined from a double reciprocal plot of reaction rate versus PLP concentration using the dialysed preparation, was 14·6 μM. Treatment of the enzyme with sodium cyanoborohydride at 2 mM for 1 h at room temperature (Guirard & Snell, 1987) resulted in only 50% inactivation, indicating that the enzyme is relatively resistant to reduction with this agent. In contrast, the enzyme was inhibited by carboxymethoxylamine, an PLP-dependent enzyme inhibitor, 52% and 78% inhibition being observed at 0·5 mM and 1·0 mM, respectively.

Effect of divalent cations. When assayed without the addition of Mg^{2+}, the enzyme exhibited 14% of the activity in the presence of 2·5 mM-Mg^{2+} (Fig. 1), and was almost completely inhibited by the addition of 1 mM-EDTA. Ca^{2+} was more effective in activating the enzyme than Mg^{2+}; about 2·4-fold activation, in comparison to Mg^{2+}, at 2·5 mM-Ca^{2+} (Fig. 1). Sr^{2+} at 2·5 mM activated the enzyme to a degree similar to Mg^{2+}. However, Zn^{2+}, Mn^{2+}, Co^{2+} and Ni^{2+} were rather

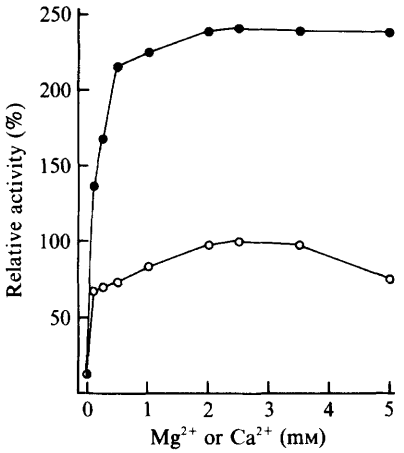

Fig. 1. Activation of DABA decarboxylase from *A. calcoaceticus* by Ca^{2+} and Mg^{2+}. The purified enzyme (0·3 μg) was incubated under standard assay conditions, except that $MgCl_2$ (○) or $CaCl_2$ (●) was added at the indicated concentrations. The relative activity is expressed as a percentage of the activity (75·2 nmol DAP formed in 15 min) observed at 2·5 mM-Mg^{2+}.

inhibitory at 2·5 mM. Na^+ and K^+ (up to 1 M) did not affect enzyme activity. The strong activation by Ca^{2+} distinctly sets the DABA decarboxylase of *A. calcoaceticus* apart, since, to our knowledge, this has never been reported for other amino acid decarboxylases.

N-Terminal amino acid sequences and immunological properties of the DABA decarboxylases from A. calcoaceticus and V. alginolyticus

The N-terminal amino acid sequences of the DABA decarboxylases from *A. calcoaceticus* and *V. alginolyticus* were determined and compared (Fig. 2). Neither enzyme had a methionine residue at the amino terminus and both gave unique N-terminal sequences with no evidence of homogeneity, agreeing with the inference drawn from SDS-PAGE that the DABA decarboxylase from *A. calcoaceticus* was very different in subunit M_r from that of *V. alginolyticus*, which was a tetramer composed of M_r 109000 subunits (Nakao *et al.*, 1989). Furthermore, the results of Ouchterlony double-diffusion analysis showed that the enzymes formed single well-defined precipitin bands with the respective antisera, but did not cross-react with the antiserum against the different source of enzyme (Fig. 3), indicating that the two enzymes have no detectable common antigenic determinants. Thus, very different proteins appear to have evolved independently to achieve what appears to be a common function.

It has been proposed that the levels of diamines such as DAP, putrescine and cadaverine are associated with

A. calcoaceticus

Val-Asp-Phe-Ala-Glu-His-Arg-Lys-Ala-Leu-
Leu-Trp-Asn-Asp-Ala-Glu-Ser-Ile-Ala

V. alginolyticus

Ser-Thr-Ala-Phe-Glu-Val-Asp-(Ser)-Asn-
Ile-Trp-Asn-Ile-Phe

Fig. 2. N-Terminal amino acid sequences of the DABA decarboxylases from *A. calcoaceticus* and *V. alginolyticus*.

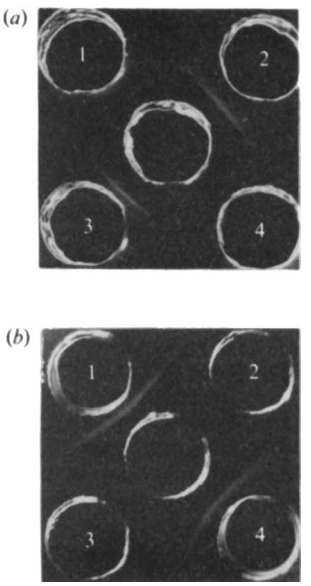

Fig. 3. Ouchterlony double-diffusion analysis of the purified DABA decarboxylases from *A. calcoaceticus* and *V. alginolyticus*. Addition to individual wells as follows: centre wells, pooled rabbit antisera (30 μl) against DABA decarboxylases from *A. calcoaceticus* (*a*) and *V. alginolyticus* (*b*); wells 1 and 4, DABA decarboxylase from *A. calcoaceticus* (5 μg); wells 2 and 3, DABA decarboxylase from *V. alginolyticus* (5 μg). There was no cross-reaction between preimmune serum and any of the proteins listed above.

environmental pH or osmotic pressure (Munro *et al.*, 1972; Morris & Fillingame, 1974; Poulin *et al.*, 1984). In addition, it has been reported that DAP can support the growth of an *Escherichia coli* mutant deficient in polyamine biosynthesis as effectively as putrescine (Morris & Jorstad, 1973). These data would mean that DAP production by acinetobacters may have physiological significance that is not yet recognized.

Enzymological studies with other *Acinetobacter* species are required to clarify whether the existence of this enzyme is unique to this genus. If so, the enzyme itself or the gene encoding it will be helpful as a biomarker for *Acinetobacter* upon identification and classification. Further efforts will be also directed towards elucidation of the biosynthetic pathway for DABA in this bacterium.

In this manner, the role and importance of the DABA decarboxylase and its product, DAP, might be further clarified.

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