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PN 10028 **Crystalline L-aspartate 4-carboxy-lyase**

L-aspartate 4-carboxy-lyase (E.C. 4.1.1.12), which catalyses the formation of α-alanine and CO_2 by the β-decarboxylation of L-aspartate, has been previously demonstrated to be present in a number of micro-organisms, including *Desulfovibrio desulfuricans*[1], *Nocardia globerula*[2] and *Clostridium perfringens*[3,4]. The enzyme has been partially purified from *Cl. perfringens* by NISHIMURA, MANNING AND MEISTER[4] and has been shown to contain firmly-bound pyridoxal 5'-phosphate; aspartate decarboxylation was stimulated by the further addition of pyridoxal 5'-phosphate and α-keto acids[1–4]. This communication describes the crystallization, and some of the properties, of L-aspartate 4-carboxy-lyase from *Achromobacter* sp.

Cultures of *Achromobacter* d-15 (ref. 5) were grown at 30° in 80 l of basal salts medium containing 25 mM-ammonium *d*(+)tartrate as sole source of carbon and nitrogen and harvested several hours after cessation of logarithmic growth. *Step I. Cell extract.* Cells (35 g dry wt.) were suspended in water to 580 ml and were disrupted by passage through a French Pressure Cell at 12 000 lb/in², followed by treatment for 5 min in an "M.S.E." ultrasonic disintegrator operating at 1.4 A. The extract thus obtained was centrifuged at 25 000 × *g* for 30 min, the precipitated material was discarded and the supernatant solution was diluted to 16 mg of protein/ml with potassium maleate (pH 5.0), L-aspartate and pyridoxal 5'-phosphate to final concentrations, respectively, of 50 mM, 10 mM and 0.1 mM; the pH was adjusted to 5.0 with 1 N acetic acid. *Step II. Heat treatment.* The solution was kept at 50° for 1 h, cooled, mixed with protamine sulphate ("ex-herring"; L. Light & Co.; 1 g/20 g of protein) and ammonium sulphate (to 30% saturation), and was centrifuged at 77 000 × *g* until the supernatant solution was clear (3–12 h). The solution was adjusted to pH 7.0 with 15 N NH_4OH. *Step III. Ammonium sulphate fractionation.* Ammonium sulphate was added to 50% saturation; the resultant precipitate, collected by centrifugation for 30 min at 15 000 × *g*, was discarded. Further addition of ammonium sulphate, to 68% saturation, yielded a precipitate which was collected by centrifugation and dissolved in 0.1 M sodium acetate (pH 5.0). *Step IV. pH Fractionation.* The dissolved precipitate was dialysed against 0.1 M sodium acetate (pH 7.0). Material precipitated in the dialysis tube was collected by centri-

TABLE I

SUMMARY OF PURIFICATION PROCEDURE

L-aspartate 4-carboxy-lyase was assayed manometrically at 30°. Evolution of carbon dioxide was measured over 30 min after addition of L-aspartate. The main compartments of Warburg flasks contained, in a final volume of 1.8 ml, 200 μmoles of sodium acetate (pH 5.0), 0.5 μmole of sodium pyruvate and enzyme (20–2000 μg of protein). L-aspartic acid (20 μmoles) was added from the side-arm after 15 min equilibration. 1 Unit of enzyme is defined as that quantity which catalyses the evolution of 1 μmole of carbon dioxide/min under these conditions.

Step	*Material*	*Protein concentration (mg/ml)*	*Total volume (ml)*	*Total protein (mg)*	*Specific activity (units/mg of protein)*	*Total units*	*Yield (% of initial enzyme content)*
I	Cell extract (pH 5.0)	16	1340	21 400	0.44	9500	100
II	Heat-treated extract (pH 7.0)	1.8	1000	1 800	2.78	5010	53
III	Ammonium sulphate, 50–68% fraction	17.4	27	470	7.92	3720	39
IV	Sodium acetate extract (pH 5.0)	12.8	10	128	26.8	3430	36
V	CM-cellulose supernatant	5	10	50	48.3	2415	25
VI	Crystals	6.6	5	33	73.0	2410	25
	Recrystallized	—	—	—	73.4	—	—

fugation and extracted with 0.1 M sodium acetate (pH 5.0). *Step V. CM-cellulose treatment.* The extract was stirred for 15 min with CM-cellulose powder (4 mg/mg of protein). The suspension was centrifuged and the supernatant solution was dialysed overnight against 0.1 M sodium acetate (pH 7.0). *Step VI. Crystallization.* Enzymically active protein precipitated on dialysis but readily dissolved in 0.5 M sodium acetate (pH 7.0). Drop-wise addition of water, to reduce the buffer concentration to approx. 0.2 M, produced a faint turbidity; on standing overnight, this increased in intensity and yielded crystalline enzyme. This procedure resulted in a 160-fold purification with an overall yield of 25% (Table I).

The crystalline enzyme catalysed the β-decarboxylation of 73 μmoles* of L-aspartate/min/mg of protein at 30°; this is more than ten times the activity of the best preparation previously reported[4], even though that was assayed at 37°. The activity of the crystalline enzyme, expressed as μmoles of L-aspartate decarboxylated/min/μmole of bound pyridoxal 5'-phosphate (see below) was 3860 at 30°, which is of the same order as the value of 4750 at 37°, calculated from the work of NISHIMURA *et al.*[4] for the enzyme from *Cl. perfringens*.

The stimulation of enzymic activity by pyridoxal 5'-phosphate and pyruvate observed with the L-aspartate 4-carboxy-lyase from *Cl. perfringens*[4] was observed also with the crystalline enzyme from *Achromobacter* (Table II). The low activity of the enzyme in the absence of activators was increased 2-fold by the addition of pyridoxal 5'-phosphate and more than 6-fold by the addition of pyruvate.

The spectrum of the crystalline enzyme at pH 7.0 exhibited two peaks at 280 mμ

* Specific activities of up to 135 were obtained in subsequent preparations, when the crystallized enzyme was diluted with 0.5% bovine serum albumen before assay. The amount of crystalline protein binding 1 mole of pyridoxal 5'-phosphate varied from 53 000 × *g* to 112 000 × *g*; the most active enzyme had an activity of 10 000 μmoles of L-aspartate decarboxylated/min/μmole of bound pyridoxal 5'-phosphate at 30°.

TABLE II

EFFECT OF PYRIDOXAL 5'-PHOSPHATE AND PYRUVATE ON ENZYMIC ACTIVITY

The complete system for assay of L-aspartate 4-carboxy-lyase was that described under Table I but also contained 0.5 μmole of pyridoxal 5'-phosphate in the main compartment of Warburg flasks.

Assay system	Specific activity of enzyme (μmoles of CO_2 evolved/min/mg of protein)
Complete	60.5
Pyridoxal 5'-phosphate omitted	33.0
Pyruvate omitted	16.6
Pyridoxal 5'-phosphate and pyruvate omitted	8.3

and 360 mμ with an absorbancy ratio of 4.2 : 1 (Fig. 1,A). The enzyme at Step V had a small peak at 410 mμ which disappeared on further purification. Acidification of the enzyme with HCl to pH < 3 resulted in the disappearance of the peak at 360 mμ (Fig. 1,A); subsequent treatment of this material with phenylhydrazine, by

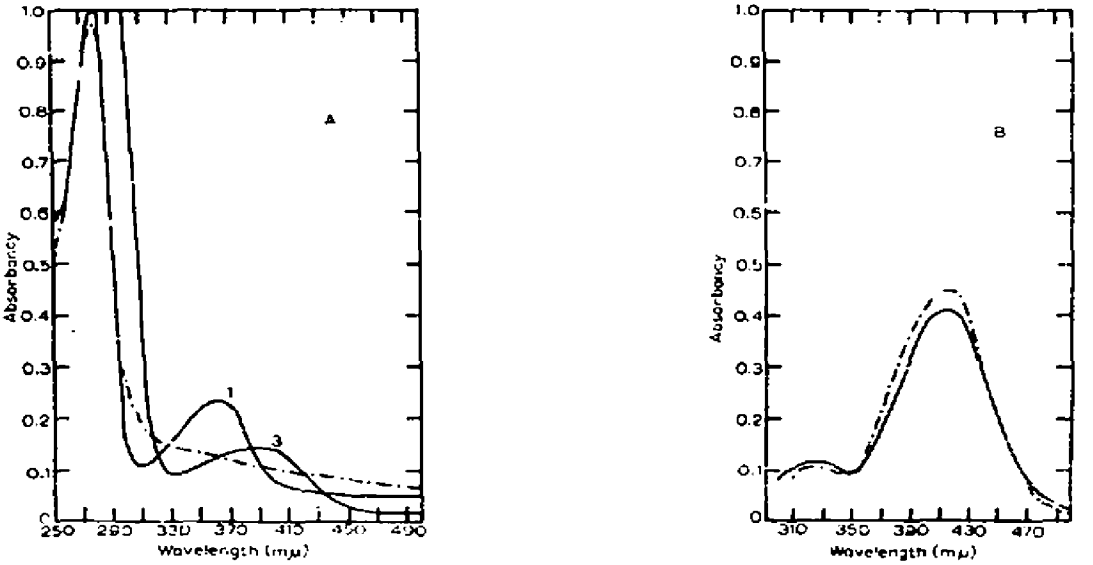

Fig. 1A. Absorption spectra of L-aspartate 4-carboxy-lyase. Curve 1, 0.965 mg protein in 1.0 ml 0.25 M sodium acetate (pH 7.0); Curve 2, Enzyme (as in Curve 1) 30 min after the addition of 0.1 ml 2 N HCl to bring the pH to 2.7; Curve 3. Enzyme (as in Curve 1) after the addition of 0.1 ml 1 N NaOH to bring the pH to 9.0.

Fig. 1B. Absorption spectra of the phenylhydrazones of pyridoxal 5'-phosphate and the chromophore of L-aspartate 4-carboxy-lyase. (———) To the enzyme (as in Curve 2, Fig. 1A) was added 0.05 ml phenylhydrazine reagent (2 g phenylhydrazine hydrochloride in 100 ml of 10 N H_2SO_4). The precipitated protein was removed by centrifugation and the spectrum was recorded 15 min after the addition of the reagent. (—·—) 0.019 μmole of pyridoxal 5'-phosphate in 0.95 ml of water was treated with 0.05 ml phenylhydrazine reagent and the spectrum was recorded after 10 min. All spectra were recorded with an Optica recording spectrophotometer and were corrected for dilution effects.

the method of WADA AND SNELL[6], resulted in the rapid formation of a compound with a peak at 410 mμ. The spectrum is closely similar to that of the phenylhydrazone of authentic pyridoxal 5'-phosphate (Fig. 1,B). 1 mole of pyridoxal 5'-phosphate phenylhydrazone was found per 53 000 g of protein in this preparation; this was in good agreement with the results of microbiological assay for vitamin B_6 in the acid-hydrolyzed enzyme, by the method of MORRIS *et al.*[7], which showed 1 mole of pyridoxal 5'-phosphate to be present per 52 500 g of protein (± 5%).

The spectrum of the enzyme in 0.1 N NaOH had a peak at 390 mμ (Fig. 1,A) indicative of free pyridoxal 5'-phosphate. The enzyme lost its activity under these conditions and could not be reactivated by added pyridoxal 5'-phosphate.

The spectral properties of L-aspartate 4-carboxy-lyase are of considerable interest since this enzyme appears to be unique in its requirement for an activator in addition to the bound pyridoxal 5'-phosphate. The peak at 360 mμ has not previously been found in pyridoxal 5'-phosphate enzymes in the pH range of 4–7 which usually have a peak at 415–430 mμ due to Schiff base formation (see ref. 8). Therefore the peak at 360 mμ may be due to the binding of pyridoxal 5'-phosphate in an unusual form. The nature of this binding is currently under investigation.

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Department of Biochemistry,
University of Leicester,
Leicester (Great Britain)

EDITH M. WILSON

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