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Purification and properties of diaminopimelate decarboxylase of *Micrococcus glutamicus*

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Abstract. Diaminopimelate decarboxylase (EC 4.1.1.20) of *Micrococcus glutamicus* ATCC 13059 was purified to homogeneity. The enzyme had an apparent molecular weight of 191,000 as determined by gel filtration on Sephadex G-200. At protein concentrations of 20 and 10 μg per ml and in the absence of pyridoxal-5'-phosphate, it dissociated into a species of molecular weight 94,000. The polypeptide chain molecular weight as determined by sodium dodecyl sulphate Polyacrylamide gel electrophoresis was 100,000. The K_m for *meso* diaminopimelate was 0.5 mM and that for pyridoxal-5'-phosphate was 0.6 μM. Sulphydryl groups and pyridoxal-5'-phosphate were essential for activity and stability. The enzyme was inhibited significantly by L-lysine and DL-aspartic β-semialdehyde.

Keywords. *Micrococcus glutamicus;* diaminopimelate decarboxylase; pyridoxal phosphate ; *meso*-diaminopimelate.

Introduction

Diaminopimelate decarboxylase (*meso*-l,7-diaminopimelate carboxylase EC 4.1.1.20, abbreviated as diaminopimelate decarboxylase), the terminal enzyme of the lysine biosynthetic pathway in bacteria and in higher plants, has not been purified before from any source to homogeneity. The enzyme from *Escherichia coli* was purified 200-fold (White and Kelly, 1965). This enzyme preparation sedimented as a single peak but gave four bands on gel electrophoresis. The major band, representing 70% of the protein was assumed to be the decarboxylase. The enzymes has been purified 150 to 170-fold from *Lactobacillus arabinosus* (Norton and Chen, 1970) and from wheat germ (Mazelis and Creveling, 1978). The homogeneity of these preparations was not checked. The enzyme from *Micrococcus glutamicus* ATCC 13286 (a homoserine auxotroph) has been purified 350-fold (Vitale *et al.*, 1977). This preparation contained three other proteins as evidenced by its electrophoretic pattern on Polyacrylamide gels.

This paper describes the purification and properties of the diaminopimelate decarboxylase of *M. glutamicus* ATCC 13059, a wild-type strain. Several differences were observed between this enzyme and that reported from *M. glutamicus* ATCC 13286.

Abbreviations used: SDS, sodium dodecyl sulphate; buffer PM, 0.1M potassium phosphate buffer, pH 7.5, containing 20 mM 2-mercaptoethanol; pyridoxal—P, pyridoxal-5' phosphate.

Materials and methods

Chemicals. Most of the fine chemicals used were the products of Sigma Chemical Co. (St. Louis, Missouri, USA). (DL+*meso*)-2,6-diamino [1,7-^{14}C] pimelic acid was obtained from the Radiochemical Centre (Amersham, UK). All other chemicals were of analytical reagent grade. Commercial diamino [1,7-^{14}C] pimelic acid was diluted to the desired specific activity with *meso*-diaminopimelate. The DD- and LL-isomers together constituted less than 1% of the resulting diaminopimelate samples. *Meso*-diaminopimelate was isolated from commercial diaminopimelate by fractional crystallization by the method of Work (1963). It was chromatographically pure and was completely decarboxylated to lysine by purified diaminopimelate decarboxylase. LL-diaminöpimelate was isolated from the mother liquor obtained after the first crop of *meso*-diaminopimelate crystals were removed, by preparative paper chromatography (Work, 1962a). DL-aspartic-ß-semialdehyde was prepared by ozonolysis of DL-C-allylglycine by the method of Black and Wright (1955). Diaminopimelate-sepharose was prepared by coupling synthetic diaminopimelate containing 36% of the *meio*-isomer, to AH-Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden) using l-ethyl-3 (3-dimethyl-aminopropyl)-carbodiimide according to instructions provided by the manufacturer. It should contain at least 0.38 µmol of diaminopimelate per ml gel for satisfactory resolution.

Bacteria and growth

M. glutamicus ATCC 13059 was maintained on slants of peptone-yeast extract agar. Cells were grown in a medium containing glucose, 15%; NH_4C1, 1%; $MgSO_4.7H_2O$, 0.02%; urea, 0.25% K_2HPO_4, 0.01% (all constituents, w/v) and clarified corn steep liquor, 20% (v/v). Urea, K_2HPO_4 and corn steep liquor were autoclaved separately. pH was adjusted to 7.0 with ammonia just before inoculation. Corn steep liquor was clarified as follows: it was diluted with an equal weight of deionized water, neutralized to pH 7.0 with 10N NaOH, stirred continuously for 30 min and centrifuged at 3000 *g* for 10 min. The pellet was washed thrice with deionized water so that the volume of the pooled supernatant and washings was four times the weight of the corn steep liquor taken. The clarified corn steep liquor was autoclaved at 1.1 kg cm^{-2} for 20 min. A second precipitate formed. The supernatant was either collected by centrifugation under aseptic conditions or by decantation and used in the medium.

Cells were grown to late exponential phase (16 h) in 1 litre Erlenmeyer flasks on a rotary shaker at 30°C. They were harvested in cold, washed three times with large volumes of 0.03 M potassium phosphate buffer, pH 7.5, containing 0.02 M 2-mercaptoethanol and stored at —20°C until the preparation of cell-free extract.

Enzyme assay. Diaminopimelate decarboxylase was routinely assayed by measuring the rate of diaminopimelate disappearance as described by White (1971). The assay system of final volume 1 ml contained per ml, Tris.HCl buffer, pH 7.5, 100 µmol; *meso*-diaminopimeiate, 5 µmol; pyridoxal phosphate, 10 nmol and enzyme. A unit of activity is the amount of enzyme that catalyzes the disappearance of 1 µmol diaminopimelate per min at 37°C and specific activity is

the number of units per mg protein. The enzyme was also assayed by measuring $^{14}CO_2$ released from diamino 1[1,7^{-14}C] pimelate (Kobayashi, 1963). The assay system was the same as that described above, except that radioactive substrate was used. The reaction was terminated after 10 min by tipping in 0.2 ml of 6 N H_2SO_4. $^{14}CO_2$ was trapped in 10-20 µl hyamine hydroxide and counted in a Beckman model LS-100 liquid scintillation spectrometer.

Substrate specificity. Carbon dioxide released from compounds tested as substrates for diaminopimelate decarboxylase was measured manometrically (Work, 1962). The main compartment of the Warburg flask contained in 0.9 ml, imidazole buffer, pH 7.0, 100 µmol; pyridoxal phosphate, 10 nmol and enzyme, 3.3 µg. One side arm contained the test compound in 0.1 ml; the other contained 1 M citric acid, 0.1 ml. Total gas evolved in 1 h at 37°G was measured by tipping in citric acid. The values were expressed as those above a blank without substrate.

Determination of molecular weight. The molecular weight of the enzyme was determined by gel filtration at 4°C on a column (1.5X137 cm) of Sephadex G-200 by the method of Whitaker (1963). Bovine thyroglobulin (670,000), bovine serum albumin (67,000; 134,000) and ribonuclease from bovine pancreas (13,600) were used as markers.

Electrophoresis. Disc gel electrophoresis was carried out using formulations described by Davis (1964) and Gabriel (1971). Sodium dodecyl sulphate polyacrylamide gel electrophoresis was carried out by the method of Weber *et al.* (1972). Samples were heated in a boiling water bath in 0.01 M sodium phosphate buffer, pH 7.0, in the presence of 1% sodium dodecyl sulphate and 1% 2-mercaptoethanol for 2 min; cooled and dialyzed against running buffer before electrophoresis. Isoelectric focussing was carried out in 5% Polyacrylamide gels containing 0.8% Ampholine (Leaback and Rutter, 1968) in an LKB multiphor unit [LKB Produketer, Bromma, Sweden].

Gels were stained by the method of Fairbanks *et al.* (1971). They were scanned in a Joyce Loebl Chromoscan using a 620 nm filter. Enzyme was located in gels after electrophoresis by slicing them into pieces of 5.0 mm length, homogenizing with 0.5 ml of 0.1 M buffer PM, pH 7.5, left overnight at 5°C and assaying qualitatively and colorimetrically, the activity in an aliquot of the supernatant.

Miscellaneous. To remove bound pyridoxal phosphate, hydroxylamine was added to the enzyme solution to 0.5 mM. The enzyme (11 µg/ml) was then dialyzed against 100 volumes of 0.1M potassium phosphate buffer pH 7.5 containing 0.02M 2-mercaptoethanol and 0.5 mM hydroxylamine for 30-40 min. This was followed by dialysis against the same buffer without hydroxylamine (25 volumes) for at least 3 h with rapid stirring and change of buffer every 30 min.

Results

Enzyme purification

All purification steps were carried out at 4-8°C. Thawed cells were ruptured by grinding with three times their weight of alumina (Type 305, Sigma) and extracted with buffer PM (5 ml/g wet cells) with grinding (Gunsalus, 1955). The extract was

centrifuged at 16,000 *g* for 10 min and the residue re-extracted with buffer PM (3 ml/g wet cells). The two extracts were pooled and centrifuged once more at 16,000 *g* for 10 min to remove alumina that might have decanted over.

A 25% solution of streptomycin sulphate in buffer PM was added dropwise to the cell-free extract until a final streptomycin concentration of 2% (w/v) was reached. The solution was stirred well and kept overnight. Precipitated matter was removed by centrifugation at 16,000 *g* for 10 min.

The streptomycin sulphate supernatant was fractionated with ammonium sulphate at pH 7.5 (Green and Hughes, 1955). Proteins precipitating within 30 min between 45% and 60% saturation with ammonium sulphate were collected by centrifugation at 25,000 *g* for 20 min. They were taken up in buffer PM and dialysed exhaustively against the same buffer. The dialyzed enzyme solution was chromatographed on a column of DEAE-Sephadex (figure 1). Fractions containing the enzyme activity were pooled and concentrated by ultrafiltration through a PM 10 membrane (Amicon BV, Oosterhout NB, Holland).

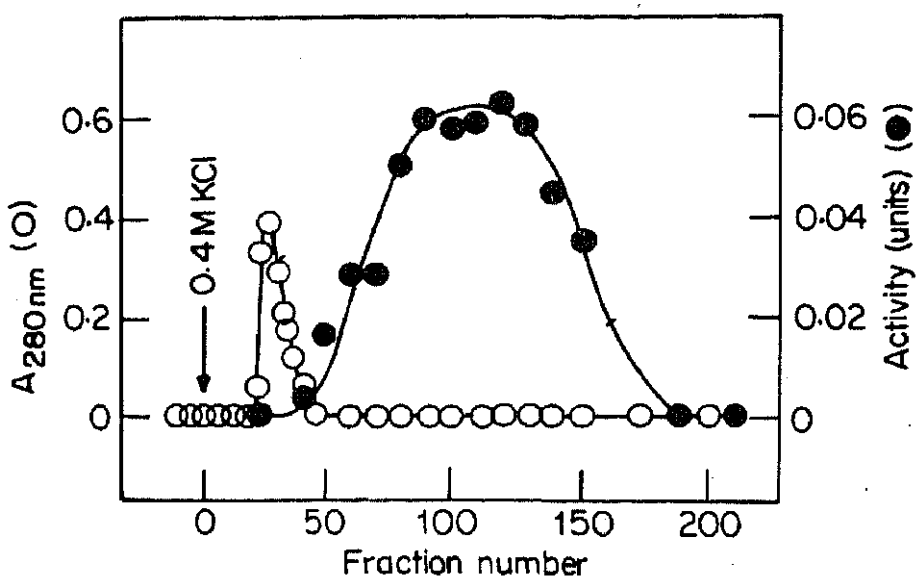

Figure 1. Chromatography of the 45-60% saturation ammonium sulphate fraction on DEAE-Sephadex. An aliquot containing 1,549 mg protein and 103 units of enzyme activity was loaded onto a column (3.3X20cm) of DEAE-SepJjadex A-50 equilibrated with buffer PM. The column was washed with buffer PM and then buffer PM containing 0.3 M KCl until the absorbance of the effluent at 280 nm was less than 0.05. The column was subsequently developed with buffer PM containing 0.4 M KCl. The flow rate was 71 ml per hour. Fractions 50-170 were pooled. Protein, O, activity, ●.

The DEAE-Sephadex eluate was adjusted to 0.2 M KCl by the addition of buffer PM and chromatographed on a column of diaminopimelate-Sepharose (figure 2A). Fractions containing enzymatic activity were pooled, dialyzed against buffer PM and rechromatographed on diaminopimelate Sepharose (figure 2B). Considerable loss of activity occurred on rechromatography. Further loss of activity could be prevented by the addition of 5 μM pyridoxal-*P* to the enzyme preparation. The eluate of the second diaminopimelate-Sepharose column was dialyzed against 0.01 M potassium phosphate buffer containing 7.5 mM 2-mercaptoethanol and 5 μM pyridoxal-*P* and concentrated with aquacide II.

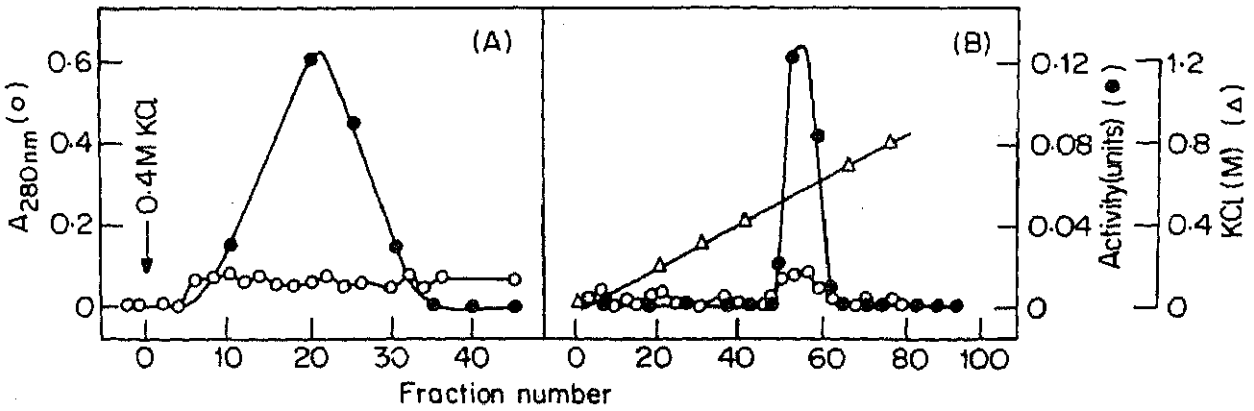

Figure 2. Chromatography of the DEAE-Sephadex eluate on diaminopimelate-Sepharose.

(A). 32.5 ml of concentrated DEAE-Sephadex eluate, containing 16.3 mg protein and 58.8 units of activity, was adjusted to 0.2 M KCl and loaded onto a column (1X23 cm) of diaminopimelate-Sepharose equilibrated with buffer PM containing 0.2 M KCl. The column was washed with the equilibration buffer and then, buffer PM containing 0.3 M KCl, until the absorbance at 280nm was negligible. The column was subsequently developed with buffer PM containing 0.4 M KCl. The flow rate was 36 ml per hour. Fractions 10-30 were pooled.

(B). Rechromatography on diaminopimelate-Sepharose. 129 ml eluate from the previous step (4.77 mg protein, 112 units activity) were loaded on a bed (1X23 cm) of diaminopimelate-Sepharose equlibrated with buffer PM. After washing the column, a linear gradient of KCl in buffer PM was applied. The two chambers of the gradient apparatus contained 100 ml of buffer PM and buffer PM containing 1 M KCl respectively. The flow rate was 24 ml per hour. Fractions 50-61 were pooled. Symbols: (O) protein (●) activity: (Δ) KCl gradient.

Aliquots (2.5 ml or less) of the concentrated enzyme were chromatographed on a 1.5X138 cm bed of Sephadex G-200 equilibrated with buffer PM containing 5 μM pyridoxal-*P*. Fractions with enzymatic activity were pooled. The results of the purification are shown in table 1.

Table 1. Purification of diaminopimelate decarboxylase of *M. glutamicus* ATCC 13059.

Fraction	Total activity[a] (units)	Total protein (mg)	Specific activity (units/mg)	Purification	Recovery (%)
Crude extract	316	5408	0.06	1	100
Streptomycin sulphate, 2%	266	4587	0.06	1	84
$(NH_4)_2SO_4$, 45-60%	205	3098	0.07	1	65
DEAE-Sephadex	127	35	3.62	61	40
Dia ninopimelate-sepharose	114	4.84	23.50	398	36
Diaminopimelate-Sepharose rechromatographed	42	2.07	20.50	347	13
Sephadex G-200	34	1.58	21.40	363	11

Wet-packed cells (145 g) was used to prepare the crude extract.

[a] The enzyme was assayed with (DL+*meso*)-diaminopimelate containing 53% of the *meso*-isomer. The specific activity of the purified preparation was 30.60 units/mg protein when *meso*-diaminopimelate was used.

Homogeneity

Polyacrylamide gel electrophoresis of fraction 7 at pH 7.0 in the presence or absence of 5 μM pyridoxal-*P* revealed four protein bands, all of which had the decarboxylase activity. At pH 9.0, five protein bands were observed. One prominent band (molecular weight 100,000) and three very minor bands (molecular weights 45,000; 50,000; 150,000) were observed on SDS-polyacrylamide gel electrophoresis (figure 3). A single protein band corresponding to pI 3.6 was observed on isoelectric focussing. These data indicate that the enzyme preparation was homogeneous.

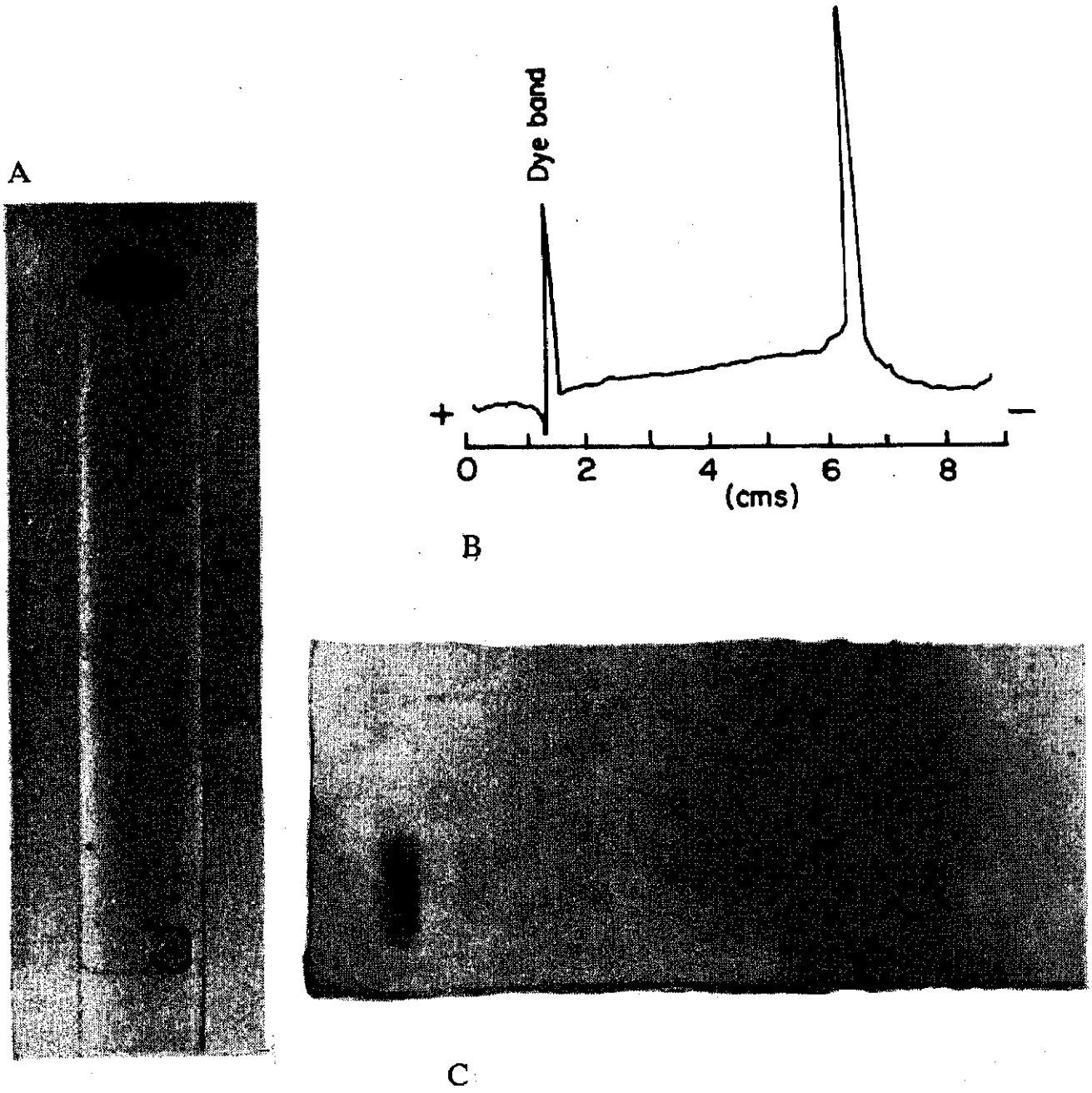

Figure 3. Electrophoresis of purified diaminopimelate decarboxylase. (A) SDS-polyacrylamide gel electrophoresis protein (50 μg) in a 10% gel. (B) A densitometer scan of the SDS-PAGE gel. (C) Isoelectric focussing of enzyme (75 μg) in the pH range 3-10. 1M NaOH and 1N H_3PO_4 were used as the cathode and anode wetting solutions. Electrophoresis was carried out at 8-9°C with a constant voltage of 400 V.

Conditions for optimal activity and stability

Diaminopimelate decarboxylase loses all activity upon freezing, in buffer PM containing 5 μM pyridoxal phosphate and 0.4 M KCl, the enzyme did not loose any activity in twelve weeks at 4°C. By themselves, pyridoxal phosphate and KCl were not equally effective in stabilizing the enzyme. Unless mentioned otherwise, the enzyme stored in the presence of pyridoxal phosphate and KCl was dialyzed against buffer PM when used for the following experiments. This preparation looses 25% activity in two weeks at 4°C.

The enzyme required sulphydryl groups for activity and stability. Added thiols were not essential for activity (table 2). However dithiothreitol, 2,3-dimercapto-propan-1-ol and reduced glutathione stimulated activity. Inhibition by sulphydryl blocking reagents such as *P*-hydroxymercuribenzoate, iodoacetamide and N-ethyl-maleimide was reserved by an excess of thiols (table 3). Heavy metal ions such as Hg^{2+} (1 mM) and Cu^{2+} (1 mM) and Zn^{2}+ inhibited the enzyme completely by (100%) by forming mercaptides. The inhibitions by Hg^{2+} and Cu^{2+} ions were reversed by 2-mercaptoethanol (2 mM) to the extent of 42% and 69% respectively. The inhibition by Zn^{2+} ion was not only reversed completely by dithiothreitol (5 mM) but the enzyme was also activated by 40%.

Table 2. The stability of the enzyme and the requirement for sulphydryl groups,

Time (days)	Specific activity (units/mg)			
	Experimental[a]		Control[b]	
	Without additions	+ Dithio-threitol (2mM)	Without additions	+ Dithio-threitol (2mM)
0	32.2	31.5	30.7	48.1
2	27.2	29.9	31.5	51.2
7	11.4	17.3	30.7	46.5
15	0.0	5.1	23.7	32.8

[a] The purified enzyme (33 μg) in buffer PM (1 ml) were loaded on a column (1 X23.5 cm) of Sephadex G-25 which had been equilibrated in 0.1 M potassium phosphate buffer, pH 7.5. The column was developed with the equilibration buffer. The material collected in the void volume was used as a source of enzyme. Pyridoxal-P was added only when the enzyme samples were assayed.

[b] The control was an aliquot of the enzyme sample loaded on the column.

The enzyme samples were stored at 4°C.

The enzyme had optimal activity at pH 7.4-7.6 in 0.1 M Tris-HCl buffer, at pH 7-7.1 in 0.1 M imidazole-HCl buffer, and at pH 7.5 in 0.1 M potassium phosphate buffer.

Table 3. Effect of thiols and sulphydryl-blocking reagents on enzyme activity.

Additions	Activity (%)
None	100
2-mercaptoethanol, 2 mM	99
Glutathione, 2 mM	126
2,3-dimercaptopropan-1-ol, 1 mM	130
Dithiothreitol (DTT) (1 to 10 mM)	135
p-hydroxymercuribenzoate (pHMB), 1 mM	100
pHMB, 1.7 mM	24
pHMB, 1.7 mM+DTT, 7 mM	137
pHMB, 5 mM	0
pHMB. 5 mM (IAM), 7 mM	0
Iodoacetamide ((AM), 1 mM	87
IAM, 2 mM	53
IAM, 2 mM+DTT, 5 mM	92
IAM, 5 mM	8
IAM, 5 mM+DTT, 5 mM	63
N-Ethylmaleimide (NEM), 1 mM	101
NEM, 5 mM	17
NEM, 5 mM+DTT, 5 mM	44

The assay system (final volume 1ml) containing Tris-HCl buffer pH 7.5, 100 μmol; pyridoxal phosphate, 10 nmol; enzyme 1 unit and the test compound was preincubated at 37°C for 5 min before starting the reaction with *meso*-diaminopimelate, 5 μmol.

For checking the reversal of inhibition, after the enzyme was allowed to react with test compound for 5 min, thiol was added. The reaction mixture was incubated further for atleast 5 min at 37°C, before starting the reaction with diaminopimelate.

Substrate specificity and concentration

The enzyme did not decarboxylate LL-diaminopimelate, L-lysine, D-lysine, LL-lysine, S-(2-aminoethyl)-L-cysteine or L-2-amino-pimelate (10 mM each), while *meso*-diaminopimelate (5mM) was 64% decarboxylated. From a double reciprocal plot of the substrate concentration and reaction rate (figure 4), the K_m for *meso*-diaminopimelate was found to be 0.5 mM. The enzyme preparation was free of the racemase (Work, 1962) activity.

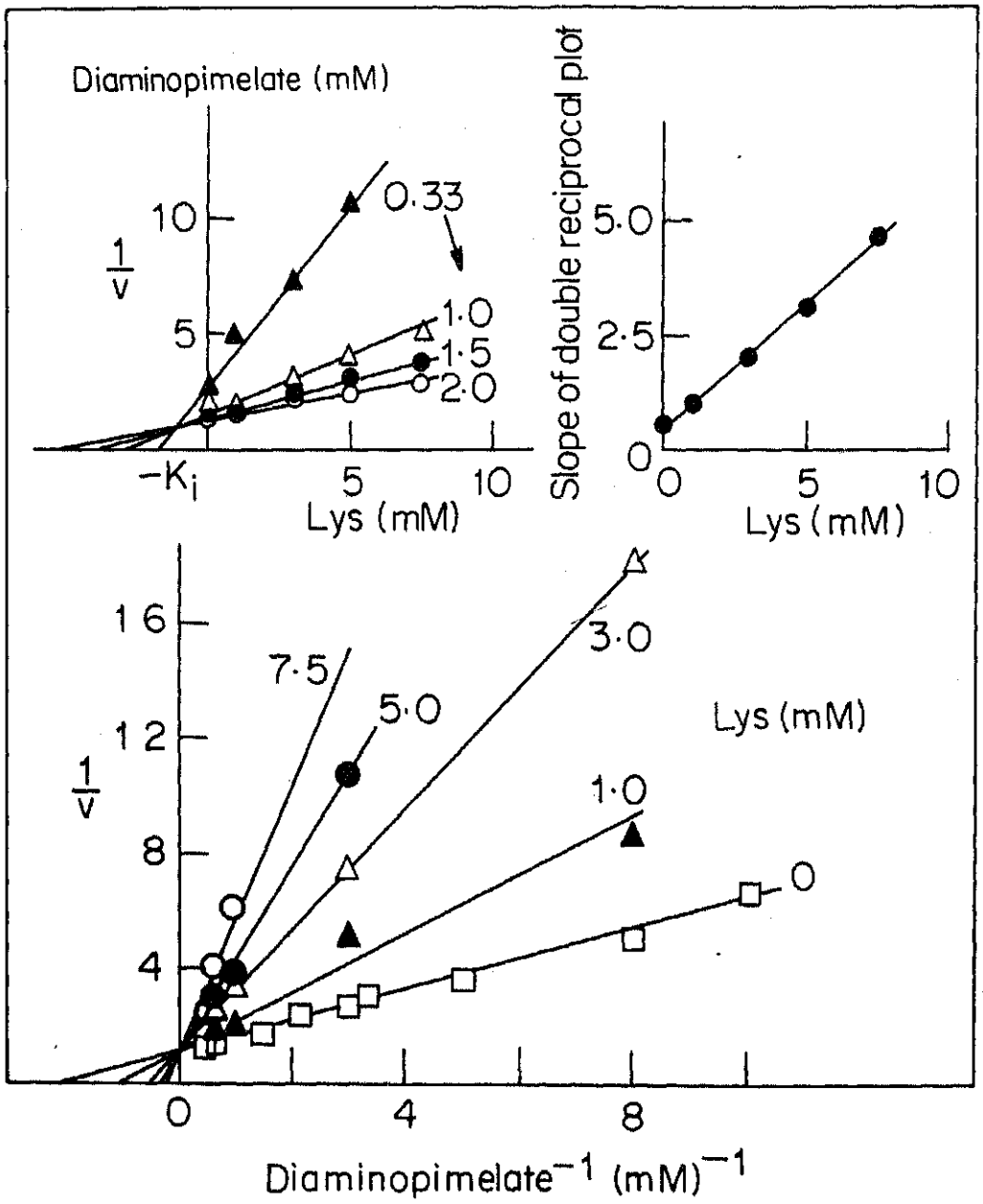

Figure 4. Inhibition of diaminopimelate decarboxylase by L-lysine with respect to diaminopimelate. Enzyme, (0.132 µg) was assayed with diamino [1,7-^{14}C] pimelic acid (A_2 pm) of specified activity 500 µCi per mol. Velocity of the reaction is expressed as the amount (in cpm) of $^{14}CO_2$ released in 10 min.

Cofactor requirement and concentration

When the DEAE-Sephadex eluate (Fraction 4, table 1) was assayed colorimetrically in the absence of added pyridoxal-*P*, no activity was detected. At this stage of purification, less than 1% of the maximal activity could be detected in the absence of added pyridoxal-*P* using the more sensitive radiochemical assay. When the purified enzyme had been stored in buffers containing pyridoxal-*P* it was not possible to remove bound pyridoxal-*P* merely by dialysis against buffer PM. In such cases, the enzyme was treated with hydroxylamine (Materials and Methods). The resultant enzyme preparation had about 3% of maximal activity in the absence of added pyridoxal-phosphate, when assayed radiochemically.

The K_m for pyridoxal phosphate of the 'resolved' enzyme as determined by a double reciprocal plot of pyridoxal-phosphate concentration against reaction rate was 0.6 µM (figure 5). The enzyme was inactive with pyridoxamine-5'-phosphate or pyridoxal. Pyridoxamine-5'-phosphate inhibited the activity and this was reversed by an excess of pyridoxal-*P*.

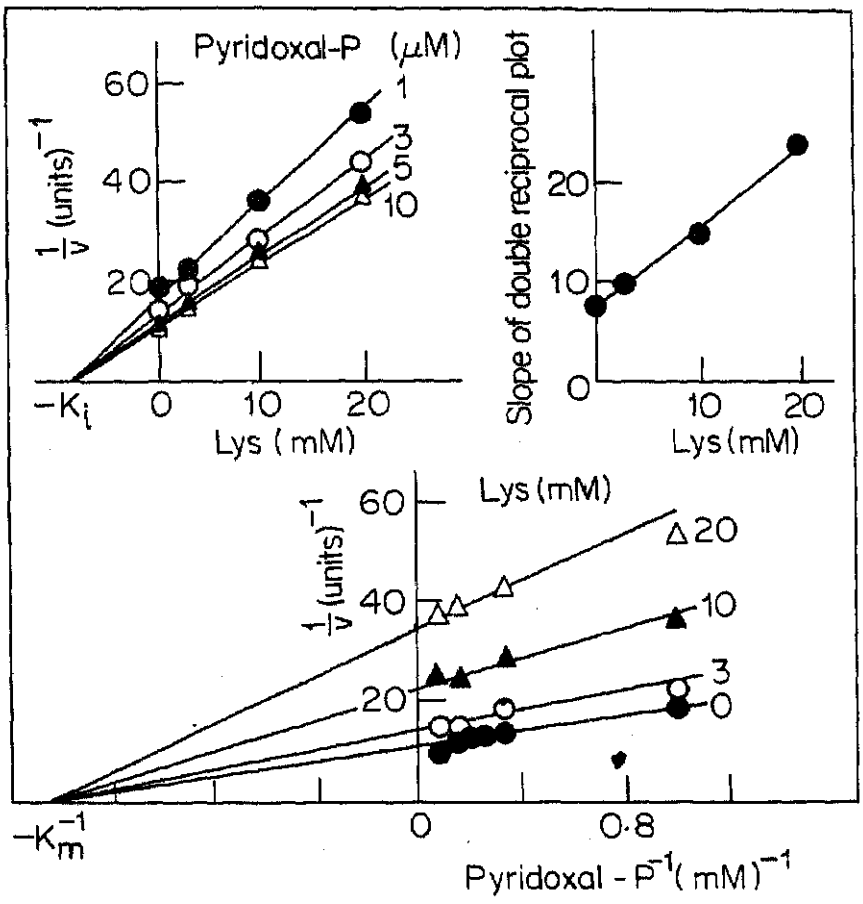

Figure 5. Inhibition of diaminopimelate decarboxylase by L-lysine with respect to pyridoxal-*P*. Three µg of 'resolved' enzyme was used for assay.

Effect of amino acids

Of the protein amino acids, only L-lysine inhibited the enzyme significantly and DL-aspartate-ß-semialdehyde did so to a lesser extent at saturating and lower concentrations of diaminopimelate (table 4). The other amino acids tested had no effect. Giutaraldehyde at the same concentrations did not inhibit the enzyme when the concentration of meso-diaminopimelate was 5 mM.

Inhibition by L-lysine with respect to *meso*-diaminopimelate fell into the class termed as 'single site pure-competitive' [(figure 4) (Segel, 1975)]. The K_i for lysine as determined by the Dixon plot was 1.2 mM. Inhibition by L-lysine was 'single site pure non-competitive' (Segel, 1975) with respect to pyridoxal phosphate the K_i for lysine was 9 mM (figure 5). Lysine inhibition could not be dissociated from activity by altering the pH of the assay by heating the enzyme at 50° C for 15 min or by assaying the presence of a partially (50%) inhibitory concentration of iodoaceta-

Table 4. Effect of amino acids on diaminopimelate decarboxylase activity.

Additions (10 mM)	Activity (%)	
	Diaminopimelate (0.6 mM)[a]	(5 mM)[b]
None	100	100
L-Pro	136	113[c]
L-Cys	81	86[c]
L-Arg	122	94
L-His	73	109
L-Lys	20	50
L-Thr	127	108
L-Met	65	108
L-Ile	109	102
L-Lys+L-Thr+L-Met+L-Ile	21	55
L-Leu+L-Val+L-Ile	81	97
L-Lys+L-Leu+L-Val+L-Ile	17	56
DL-aspartic-β-semialdehyde[d]	72	66

[a] Diamino [1,7-^{14}C] pimelate of specific activity 500 μCi per mol and 0.132 μg enzyme were used. The activity of the control sample was 5,500 cpm $^{14}CO_2$ released under Standard assay conditions.

[b] The enzyme was assayed colorimetrically. 3.3 μg enzyme was used per assay.

[c] Assayed radiochemically with diamino [1,7^{-14}C] pimelic acid of specific activity 0.067 μCi per mol. The activity of the control was 5,200 cpm $^{14}CO_2$ released. 1.32 μg protein was used per assay.

[d] The concentration of DL-aspartic-ß-semialdehyde was 1 and 2 mM respectively.

mide. These procedures were for dissociating activity from lysine inhibition— i.e. for desensitizing the enzyme if two sites are involved. Since lysine, a product of the reaction catalyzed by the decarboxylase, is a linear competitive inhibitor as evidenced by linear slopes replot (figure 5), it could be inferred that there are no additional sites for lysine binding other than the substrate binding site(s).

Inhibition by analogues of diaminopimelate

Structural analogues of diaminopimelate which were found to inhibit the decarboxylase are listed in table 5. D-Lysine, S-(2-aminoethyl)-L-cysteine, L-2, 4-diaminobutyrate, L-2-3-diaminopropionate, DL-2-aminoadipate, hexamethylenediamine, adipic acid, 6-aminohexanoic acid or glutaric acid (10 mM) had no effect at either concentration (0.6 mM or 5 mM) of diaminopimelate. It appears from these results that the best inhibitors are those that have two amino groups separated by five methylene groups. The efficacy of inhibition is increased by the presence of a carboxyl substituent in position 1 in the L-configuration.

Table 5. Inhibition of diaminopimelate decarboxylase activity analogues of diaminopimelate.

Additions (10 mM)	Activity (%)	
	Diaminopimelate[a] (0.6 mM)	Diaminopimelate[b] (5 mM)
None	100	100
(DL+*allo*)-Cystathionine	75	113
LL-Diaminopimelate	0.4	0.5[c]
LL-Cystine	58	83
L-Lysine methylester	31	81
Cadaverine	40	63
Putrescine	—	85
DL-2-Aminopimelic acid	49	105
Pimelic acid	42	97
L-Norleucine	45	76

[a, b, c] As in table 4.
— denotes 'not determined'.

Effect of other compounds

At a saturating concentration (5 mM) of *meso*-diaminopimelate, UDP-glucose (1 mM), UTP, UDP or UMP or a mixture of the three nucleotides (1 mM each), ATP, ADP, AMP or a mixture of the adenyl nucleotides (1 mM each) whether in the presence or absence of 1 mM $MgCl_2$ had no significant effect on enzyme activity. At a non-saturating concentration (0.6 mM) of *meso*-diaminopimelate, 47% activation was seen with a mixture of the adenyl nucleotides (1 mM each).

Molecular weight and subunit structure

The molecular weight of the enzyme was found to be concentration- and pyridoxal-*P* dependent (table 6). The molecular weight of the major polypeptide chain

Table 6. Molecular weight of diaminopimelate decarboxylase.

Conditions of chromatography		Approximate molecular weight
Sample loaded (μg)	Buffer used	
100	PM	191,000
20	PM	191,000; 94,000
10	PM	94,000
10	PM containing 5 μM pyridoxal-*P*	191,000
100	PM containing 5 μM pyridoxal-*P*	191,000

One ml enzyme in buffer PM (Fraction 5 table 1) which had no detectable activity (by chemical procedure) in the absence of added pyridoxal-*P*, was chromatatographed on a calibrated bed of Sephadex G-200 (Methods). Diaminopimelate decarboxylase activity was monitored in the eluate. The difference in elution volume between the two molecular weight species was 17 ml.

observed by sodium dodecyl sulphate Polyacrylamide gel electrophoresis was 100,000. It was inferred from these data that the decarboxylase was composed of two subunits of approximate molecular weight 100,000. Dimerization was induced by pyridoxal phosphate and higher protein concentrations. These data also suggest that the minor bands of molecular weight 45,000, 50,000 and 150,000 observed on electrophoresis may be fragments of the 94,000 and 191,000 molecular weight species which arose by incomplete reduction or dissociation or by proteolytic cleavage during electrophoresis.

Discussion

Diaminopimelate decarboxylase has been obtained from *M. glutamicus* ATCC 13059 in a homogeneous form. Other homogeneous enzymes are known to exhibit multiple enzymatically active bands on Polyacrylamide gel electrophoresis (due to association-dissociation) and move as a single band on SDS-polyacrylamide gels like the decarboxylase of *M. glutamicus* ATCC 13059 (Kleinshmidt and Kleiner, 1978; Feldberg and Datta, 1971). This is the first report of a homogeneous preparation of the enzyme. The enzyme from *M. glutamicus* ATCC 13286 showed four bands on Polyacrylamide gel electrophoresis at pH 7.0 of which only one band had enzyme activity (Vitale *et al.*, 1977). The specific activity of the enzyme purified from strain ATCC 13059 was about one-and-a-half times higher than that reported for the purified enzyme from strain ATCC 13286.

The procedure for the purification of the decarboxylase of *M. glutamicus* ATCC 13059 described in this work differs from those described earlier in the use of an affinity column. Immobilized pyridoxal-phosphate derivatives (Fukui *et al.*, 1975) were not useful, although the enzyme that was chromatographed had an absolute requirement of pyridoxal-*P* for activity. The decarboxylase from *M. glutamicus* ATCC 13059 resembles the enzyme from several other sources in its specificity for *meso*-diaminopimelate (White and Kelly, 1965; Dewey *et al.*, 1954; Shimura and Vogel, 1966; Vogel and Hirvonen, 1971) and its requirement of pyridoxal-phosphate (White and Kelly, 1975; Norton and Chen, 1970; White, 1971; Dewey *et al.*, 1954; Rosner, 1975; Grandgenett and Stahly, 1968)and of Sulphydryl groups (White and Kelly, 1965; Vitale *et al.*, 1977; Dewey *et al.*, 1954; Grandgenett and Stahly, 1968).

The enzyme required added sulphydryl groups for stability. The stimulation by certain dimercapto compounds probably indicates that some more free sulphydryls needed for activity are formed or these compounds are more efficient in protecting enzyme sulphydryl groups. The inhibition by heavy metal ions and p-hydroxymercuribenzoate and reversal by sulphydryl compounds are to be expected. At high concentrations of p-hydroxymercuribenzoate, the enzyme is perhaps denatured and no reactivation can occur. But the reversal of inhibition of N-ethylmaleimide and iodoacetamide by sulphydryl compounds is hard to explain. These reagents are presumed to combine irreversibly with enzyme-SH groups and treatment of these alkylated-S-enzymes is not expected to release the inhibition. Since the inhibition is released in this instance, it is possible that these reagents may bind other groups reversibly. There are other such examples in literature (Vijayalakshmi *et al.*, 1975). The K_m for *meso*-diaminopimelate of this enzyme is a

little lower than values reported for the enzyme from other bacterial sources (White and Kelly, 1965; Norton and Chen, 1970; Vitale *et al.,* 1977; Rosner, 1975; Grandgenett and Stahly, 1968; Kagan *et al.,* 1971) and a little higher than the values reported for the plant enzymes (Mazelis and Creveling, 1978; Shimura and Vogel, 1966; Vogel and Hirvonen, 1971; Mazelis *et al.,* 1976). The K_m for pyridoxal-*P* of the diaminopimelate decarboxylase of *M. glutamicus* ATCC 13059 was 0.6 μM and was lower than the values reported for other decarboxylases (Norton and Chen, 1970; Mazelis and Creveling, 1978; Rosner, 1975). The observations of Vitale *et al.,* (1977) led them to infer that inhibition of the decarboxylase by substrate analogues did not require both ends of the molecule to be polar; the contribution of size and pI appeared to be more important. The best inhibitor by far of the enzyme from *M. glutamicus* was LL-diaminopimelate. The others were not very effective; they were of a similar size as the substrate and with both ends polar excepting L-norleucine which inhibited equally well. The effects of diaminopimelate analogues on the enzyme from various sources (White and Kelly, 1965; Dewey *et al.,* 1954; Rosner, 19.75) are all slightly different from one another and from the effects on the enzyme from the two strains of *M. glutamicus.* These differences may be in part due to difference in assay conditions. The decarboxylases from most sources including the enzyme from *M. glutamicus* ATCC 13059 are inhibited by L-lysine (White and Kelly, 1965; Vitale *et al.,* 1977; Rosner, 1975; Grandgenett and Stahly, 1968); lysine inhibition is competitive with respect to *meso*-diaminopimelate. The enzyme from *M. glutamicus* ATCC 13059 was inhibited significantly by aspartic-ß-semialdehyde (at 2 mM) and much more strongly by LL-diaminopimelate (at 10 mM). Although several other amino acids inhibited or activated the enzyme (20-40%) at a non-saturating concentration of *meso*-diaminopimelate, these effects were not evident when higher concentrations of this compound were used. Since LL-diaminopimelate does not appear to be an obligatory/free intermediate in lysine biosynthesis in *M. glutamicus* (Leadlay, 1979), only the inhibitions by L-lysine and aspartic-ß-semialdehyde may be of physiological significance.

The molecular weight of the diaminopimelate decarboxylase from the two strains of *M. glutamicus* differ considerably. The enzyme from *M. glutamicus* ATCC 13286 had an approximate molecular weight of 53,000 (Vitale *et al.,* 1977) as determined by gel filtration. The enzyme from *M. glutamicus* ATCC 13059 consists of two polypeptide chains of approximate molecular weight 100,000. The enzyme from *Bacillus subtilis* (Grandgenett and Stahly, 1971) and *E. coli* (White and Kelly, 1965) have approximate molecular weights of 105,000 and 200,000 respectively. Although many amino acid decarboxylases have a subunit structure, this is the first report of the subunit structure of diaminopimelate decarboxylase. Association-dissociation occurring at low protein concentrations such as those used in these experiments, has been reported for the chorismate mutase-prephenate dehydratase of *Salmonella typhimurium* (Schmidt and Zalkin, 1971).

Thus, the diaminopimelate decarboxylase of *M. glutamicus* ATCC 13059 grossly resembles most other diaminopimelate decarboxylases, although it differs from the enzyme of *M. glutamicus* ATCC 13286 in several aspects.

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