Biosynthesis of Proline in *Pseudomonas aeruginosa*

PARTIAL PURIFICATION AND CHARACTERIZATION OF γ -GLUTAMYL KINASE

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A γ -glutamyl kinase (ATP-L-glutamate 5-phosphotransferase) was purified about 85-fold from crude extracts of *Pseudomonas aeruginosa* strain PAO 1 by $(\text{NH}_4)_2\text{SO}_4$ precipitation, molecular-sieving by Sephadex G-150 and DEAE-cellulose chromatography. The molecular weight of this enzyme was 84000. The preparation catalysed formation of γ -glutamyl hydroxamate from L-glutamate, ATP and Mg^{2+} or Mn^{2+} with concomitant hydrolysis of ATP to ADP+P_i. L-Proline inhibited the γ -glutamyl kinase activity by 50% at 5 mM and almost completely at 30 mM. The inhibition by L-proline was non-competitive, whereas L-methionine-DL-sulphoximine inhibited the enzyme competitively. Proline was found to inhibit the γ -glutamyl kinase activity of the wild-type strain and of representatives of two of the three transductional classes of proline-auxotrophic mutants. Strain PAO 879, a mutant representing the third transductional class of proline auxotrophs, lacked proline-inhibitable γ -glutamyl kinase. Thiol-blocking reagents inhibited the γ -glutamyl kinase and this effect was prevented by dithiothreitol.

The biosynthesis of proline has been envisaged to occur in a sequence of three enzymic reactions. The first step, the activation of the γ -carboxy group of L-glutamic acid, requires MgATP^{2-} and is catalysed by a γ -glutamyl kinase (ATP-L-glutamate 5-phosphotransferase, EC 2.7.2.-). It is inhibited by L-proline in *Escherichia coli* (Baich, 1969). However, γ -glutamyl phosphate, the postulated product of the first reaction and the substrate of the second, has never been isolated owing to its instability. The second enzyme, γ -glutamyl phosphate reductase, at the expense of a molecule of NADPH, converts γ -glutamyl phosphate into L-glutamic acid 5-semialdehyde (Baich, 1971). It was proposed that in *E. coli* the first two enzymes might exist in a complex, converting L-glutamate, ATP and NADPH into L-glutamic acid 5-semialdehyde (Baich, 1971; Gamper & Moses, 1974; Hayzer & Moses, 1978). The latter compound cyclizes spontaneously to 1-pyrroline-5-carboxylic acid, which is reduced in an irreversible reaction to L-proline by the third enzyme 1-pyrroline-5-carboxylate reductase (EC 1.5.1.2) and NAD(P)H (Meister *et al.*, 1957; Smith & Greenberg, 1957; Adams & Goldstone, 1960; Strecker, 1971; Miller & Stewart, 1976; Rossi *et al.*, 1977; Herzfeld *et al.*, 1977).

The properties of the proline biosynthetic enzymes in micro-organisms are largely unknown. The difficulties associated with the identification of the γ -glutamyl kinase and γ -glutamyl phosphate reduc-

tase among the interfering activities present in crude extracts and the relative instability of these two enzymes may be partly the reason for this. It would thus be useful to obtain information on these enzymes and on their intermolecular relationships and to compare them with enzymes of similar function such as glutamine synthetase and enzymes of arginine biosynthesis.

Our attempts to delineate the biosynthetic pathway of proline in *Pseudomonas aeruginosa* in terms of the enzymic steps resulted in the partial purification and characterization of the enzymes involved: the γ -glutamyl kinase, the γ -glutamyl phosphate reductase and the 1-pyrroline-5-carboxylate reductase. The properties of the first enzyme, γ -glutamyl kinase, are reported in the present paper. Comparison of the properties of the second and third enzymes are reported in the following paper (Krishna *et al.*, 1979).

Materials and Methods

Micro-organism and growth conditions

P. aeruginosa PAO 1, a wild-type strain (A.T.C.C. 15692), and the three proline-auxotrophic mutants PAO 831 (*ade-66, his-151, ura-21, thi-1, pro-71, ese-14, FP*⁻), PAO 853 (*ade-66, his-151, ura-21, thi-1, pro-70, ese-14, FP*⁻) and PAO 879 (*ade-136, leu-8, pro-73, ese-20, chl-2, FP*⁻) were obtained from Professor B.

Holloway, Department of Genetics, Monash University, Clayton, Vic., Australia. For the purification of γ -glutamyl kinase, strain PAO 1 was grown at 37°C, as described by Haas & Leisinger (1975a) in a F-0300 fermenter (Chemap A.G., Männedorf, Switzerland) on 250 litres of double-strength medium P (Leisinger *et al.*, 1972) with 40mM-L-glutamate as the sole source of carbon and nitrogen. The cell paste obtained was stored at -20°C for several months without decrease in specific activity of γ -glutamyl kinase. Alternatively strain PAO 1 and the mutant strains were grown in Fernbach flasks on minimal medium P containing either 20mM-L-glutamate or 20mM-L-proline as the only carbon and nitrogen source or on nutrient yeast broth (0.8% nutrient broth, 0.5% yeast extract, 0.8% NaCl) at 37°C on a rotary shaker. Cells for enzyme assays were harvested in the late exponential phase at a cell density of approx. 10^9 cells/ml, washed with 0.9% NaCl and stored at -20°C.

Chemicals

All the chemicals and reagents were of analytical grade, or of the highest purity commercially available. Amino acids, nucleotides, coenzymes for the dehydrogenases, inorganic pyrophosphatase, phosphoenolpyruvate, pyruvate kinase, lactate dehydrogenase and all the markers used for molecular-weight estimations were products of Boehringer, Mannheim, Germany, or of Sigma Chemical Co., St. Louis, MO, U.S.A. DE-52 DEAE-cellulose was from Whatman, Maidstone, Kent, U.K. Nutrient broth and yeast extract were from Difco Laboratories, Detroit, MI, U.S.A.

Buffers

Buffer 1 consisted of 50mM-imidazole/HCl, pH 6.9, 0.5mM-dithiothreitol, 1mM-phenylmethanesulphonyl fluoride and 10% (w/v) glycerol. It was used for the preparation of crude extracts. Buffer 2 was 2mM and buffer 3 was 10mM with respect to imidazole/HCl, but both were otherwise similar to buffer 1. For studying the effect of thiol-blocking reagents buffer 4 was employed. It did not contain dithiothreitol, but was otherwise similar to buffer 1. All steps pertaining to preparation and fractionation of cell extracts were done at 0-4°C.

Crude extracts

Crude extracts for enzyme assays were prepared by passing 5 ml of a 20% (w/v) cell suspension in buffer 1 through a French pressure cell at a pressure of 7000 lbf/in² (48 000 kPa). Cell debris was sedimented by centrifugation at 30 000g for 40 min. The resulting crude extract was dialysed for 3 h against three changes of 300 ml each of buffer 2.

Enzyme assays

γ -Glutamyl kinase activity was assayed in the forward direction essentially as described by Rowe *et al.* (1970) for glutamine synthetase. The reaction mixture contained, in a total volume of 0.25 ml, 50mM-imidazole/HCl, pH 6.5, 10mM-ATP (adjusted to pH 6.5 with NaOH), 20mM-MgCl₂, 4mM-dithiothreitol, 50mM-L-glutamate (sodium salt), 100mM-hydroxylamine (adjusted to pH 6.5 just before use) and up to 0.03 unit of γ -glutamyl kinase to start the reaction. When the activity was determined in crude extracts 2mM-NaF was added. Reaction mixtures from which ATP was omitted provided blanks for an ATP-independent γ -glutamyl hydroxamate-forming activity present in these extracts. All these incubations were done in the presence and in the absence of 5mM-L-proline. After incubation at 37°C for 15 min the reaction was terminated by the addition of 1.0 ml of a solution containing 2.5% (w/v) FeCl₃ and 6% (w/v) trichloroacetic acid in 2.5M-HCl. The absorbance of the hydroxamate-Fe³⁺ complex was measured at 535 nm. The molar absorption coefficient of this complex under the conditions described was 340 M⁻¹·cm⁻¹ as determined by a standard curve prepared with γ -glutamyl hydroxamate. Incubation mixtures containing 5mM-L-proline were used for blank values also during enzyme purification. When the partially purified γ -glutamyl kinase was assayed, blank values were obtained by measuring product formation in the presence of 40mM-L-proline. Non-specific reactions like that of glutamine synthetase activity were thus eliminated. One unit of γ -glutamyl kinase is defined as the amount of enzyme catalysing the proline-inhibitable formation of 1 μ mol of γ -glutamyl hydroxamate per min under assay conditions. γ -Glutamyl kinase was also assayed in 50mM-imidazole/HCl, pH 6.5, by the L-glutamate-dependent release of P_i from ATP by the procedure of Shapiro & Stadtman (1970) for the assay of glutamine synthetase of *E. coli*. In both assay methods product formation was a linear function of time (up to 30 min) and protein concentration provided that not more than 0.06 unit of enzyme per ml of reaction mixture was used.

Glutamine synthetase was estimated as γ -glutamyl-transferase activity. γ -Glutamyl phosphate reductase was assayed by measuring the increase in A_{340} with 1-pyrroline-5-carboxylate, P_i and NADP⁺ as substrates (Baich, 1971; Krishna *et al.*, 1979) and L-glutaminase as described by Borghild *et al.* (1970). Inorganic pyrophosphatase was measured by the method of Heppel (1955), and ATPase as described by Lowry *et al.* (1954). ADP determinations were performed by a coupled-assay procedure (Storer & Cornish-Bowden, 1976a). P_i was determined by the micro-method of Taussky & Shorr (1953).

Enzyme purification

Frozen cells (50 g) were thawed and suspended in 100 ml of buffer 1. The pH of this suspension was adjusted to 6.9 with a few drops of 0.1 M-NaOH. The cells were broken by passage through a French press at about 7000 lbf/in² (48 000 kPa). The viscous exudates from the pressure cell were combined and centrifuged at 30 000 g for 40 min to obtain a crude extract. Further clarification of the crude extract was brought about by centrifugation at 100 000 g for 90 min. A streptomycin sulphate solution (20 g/litre) was added to the S-100 supernatant to a final concentration of 0.2% (w/v). The suspension was allowed to stand for 30 min and was then centrifuged and the sediment was discarded. The supernatant was brought to 40% (w/v) saturation with solid (NH₄)₂SO₄, centrifuged after 15 min and the pellet was discarded. The (NH₄)₂SO₄ concentration was raised to 60% saturation and the suspension was centrifuged; the precipitate, which contained about 60% of the total γ -glutamyl kinase activity, was saved. The supernatant was treated with more (NH₄)₂SO₄ to obtain 80% saturation and centrifuged. The sediment contained about 20% of the γ -glutamyl kinase activity. The 40–60% and the 60–80% saturation (NH₄)₂SO₄ fractions were combined and will be referred to as the 40–80% (NH₄)₂SO₄ fraction.

One half portion of the 40–80% (NH₄)₂SO₄ fraction was dissolved in buffer 1 (20 ml) and dialysed for 2 h against 2 litres of buffer 2. It was next applied by upward flow to a Sephadex G-150 column (5 cm $\times$ 87 cm) equilibrated with buffer 2 and eluted with the same buffer. The flow rate was 60 ml/h and fractions of 5.5 ml were collected. Fractions containing γ -glutamyl kinase were concentrated by ultrafiltration (Amicon PM-30 membrane) and applied to a DEAE-cellulose column (1.6 cm $\times$ 28 cm) equilibrated with buffer 3. Protein was eluted with a linear gradient of 0.15 M to 0.35 M-KCl in buffer 3. The flow rate was 60 ml/h and 1.7 ml fractions were collected. The fractions (52–65) containing high specific activity of γ -glutamyl kinase were pooled and dialysed against two changes of 100 ml each of buffer 2. The final concentration of glycerol in the partially purified enzyme was increased to 25% and portions of 0.4 ml were stored at -40°C .

Molecular-weight estimation

Gel filtration on a Sephadex G-100 column (1.6 cm $\times$ 61 cm) was used for estimating the molecular weight of γ -glutamyl kinase. The molecular weights of the markers used were: *Pseudomonas* sp. galactose dehydrogenase, 102 000; horse liver alcohol dehydrogenase, 80 000; ovalbumin, 45 000; and horseradish peroxidase, 40 000. Ovalbumin was monitored by measuring the A_{280} and the marker enzymes were

measured by standard methods (Bergmeyer, 1974). The column was equilibrated with buffer 2 and was eluted with the same buffer at a flow rate of 2.5 ml/h. The void volume (V_0) and the total bed volume (V_t) were measured by using Blue Dextran 2000 and Bromophenol Blue. Sucrose-density-gradient centrifugations were done by the method of Martin & Ames (1961).

Protein determination

Protein was estimated by the colorimetric method described by Lowry *et al.* (1951) and by spectrophotometric methods (Waddell, 1956; Layne, 1957).

Gel disc electrophoresis

Polyacrylamide-gel disc electrophoresis was performed by using 75 μg of the partially purified enzyme by the method described by Davis (1964); 7.5% gels were used and Coomassie Brilliant Blue R-250 was employed for staining.

Results

Partial purification of γ -glutamyl kinase

The γ -glutamyl kinase was purified about 85-fold from glutamate-grown cells of *P. aeruginosa* PAO 1 as described in the Materials and Methods section. The results of a typical purification are presented in Table 1. The determination of γ -glutamyl kinase in crude extracts was complicated by the high background of glutamate-activating enzymes forming glutamyl hydroxamate under assay conditions, but not inhibited by proline. (NH₄)₂SO₄ precipitation and gel filtration on Sephadex G-150 removed most of these contaminating activities. Chromatography on DEAE-cellulose (Fig. 1) eliminated the remaining interfering activities and yielded an enzyme preparation that had a stringent substrate specificity and was 95% inhibited by 40 mM-L-proline. It showed three strong discrete bands and three minor bands when subjected to analytical gel electrophoresis and was found to contain very small amounts of enzyme activities related to proline biosynthesis and of glutaminase: γ -glutamyl phosphate reductase was enriched 2-fold. 1-Pyrroline-5-carboxylate reductase and L-glutaminase were present at 30 and 6% of the activities found in crude extract.

Further purification of γ -glutamyl kinase has presented difficulties in that the enzyme was unstable in subsequent purification steps. Thus all the experiments reported in the present paper were performed with the partially purified enzyme. During storage over 2 months at -40°C it lost about 40% activity, but then remained stable.

Table 1. *Partial purification of γ -glutamyl kinase*

Crude extracts were prepared from 50g wet wt. of frozen cells (strain PAO 1) and fractionated as described in the Materials and Methods section.

Step	Stage	Protein (mg)	Volume (ml)	γ -Glutamyl kinase activity			
				Specific activity (units/mg)	Activity (units)	Yield (%)	Fold purification
1	Crude extract	3838	190	0.15	576	100	1.0
2	100 000g supernatant	3142	168	0.16	503	87	1.1
3	40–80% saturation $(\text{NH}_4)_2\text{SO}_4$	1435	40	0.30	431	75	2.0
4	Sephadex G-150 (pooled + concentrated)	158	9	1.39	219	38	9.5
5	DEAE-cellulose (pooled + concentrated)	14	5	12.68	178	31	86.8

Fig. 1. DEAE-cellulose chromatography of γ -glutamyl kinase

Experimental details are described in the Materials and Methods section. Symbols: ○, protein; Δ, γ -glutamyl kinase activity not inhibited by 5mM-L-proline; ▲, γ -glutamyl kinase activity.

Properties of γ -glutamyl kinase

The elution volume of the enzyme and the marker proteins on Sephadex G-100 were determined. An apparent mol.wt. of 84000 for γ -glutamyl kinase was estimated graphically from a plot of the ratio of the elution volume to the void volume (V_e/V_0) against the logarithms of the molecular weights of the marker proteins (Andrews, 1970). By sucrose-density-gradient centrifugation the mol.wt. was 94000 (Krishna *et al.*, 1979).

A pH against activity profile of γ -glutamyl kinase is shown in Fig. 2. Maximal activity was observed at pH 6.0–6.3, which was also the range for maximal inhibition by L-proline. Several analogues of L-glutamate and ATP were tested as substrates for γ -glutamyl kinase by both methods of assay, hydroxamate formation and release of P_i . N-Acetyl-L-glutamate, 5-oxo-L-proline, D-glutamate, L-aspartate,

Fig. 2. pH-dependence of γ -glutamyl kinase activity and inhibition by proline

Assays were performed under standard conditions except for variations of the buffers: sodium acetate (50mM)/acetic acid (○, ●); 50mM-imidazole/HCl (Δ, ▲); 50mM-Tris/HCl (□, ■); 50mM-glycine/NaOH (▽, ▼). Open symbols denote activity in the absence of L-proline and filled symbols activity in the presence of 10mM-L-proline.

L-methionine-DL-sulphoximine, L-asparagine, 2-amino-L-adipate, 2-amino-L-n-valerate, CTP, ITP, ADP, GDP, IDP, and acetyl phosphate were ineffective. L-Glutamine, 5-ethyl-L-glutamate, and 5-methyl-L-glutamate were respectively 10, 5 and 6% as active as L-glutamate in the γ -glutamyl kinase reaction. GTP could serve as a phosphate donor with about 10% of the efficiency of ATP. L-Methionine-DL-sulphoximine inhibited the enzyme in the presence of ATP and Mg^{2+} . This inhibition was competitive with L-glutamate. Among different cations tested as substitutes for Mg^{2+} , only Mn^{2+} was effective. These ions were inhibitory for γ -glutamyl

Table 2. *Products of γ -glutamyl kinase reaction*

The standard assay conditions were: 0.005 unit of the partially purified γ -glutamyl kinase and 10 units of inorganic pyrophosphatase per 0.25 ml of the incubation mixture. One unit of pyrophosphatase releases 1 μ mol of P_i from PP_i /min per mg of protein at 25°C.

System	Time of incubation (min)	Products formed (μ mol)			
		Hydroxamate assay		P_i -release assay	
		γ -Glutamyl hydroxamate	P_i	ADP	P_i
Complete	5	0.162	0.168	0.172	0.180
	15	0.445	0.475	0.456	0.500
	30	0.855	0.895	0.868	0.930
Complete (+ pyrophosphatase)	5	0.176	0.184	0.165	0.195
	15	0.462	0.496	0.470	0.500
	30	0.860	0.915	0.900	1.055
Incomplete (L-glutamate omitted)	5	0.025	0.025	0.017	0.028
	15	0.025	0.046	0.017	0.058
	30	0.025	0.070	0.017	0.088

Fig. 3. *Effect of L-proline concentration on saturation of γ -glutamyl kinase by L-glutamate and ATP*

By using the hydroxamate assay in (a) L-glutamate and L-proline concentrations were varied from 2.5 to 50mM and from 0 to 30mM respectively. In (b) concentrations of ATP and L-proline were varied from 2.5 to 50mM and from 0 to 30mM. The concentration of Mg^{2+} was maintained at twice that of ATP.

kinase activity at concentrations higher than 20mM. Cd^{2+} and Hg^{2+} inhibited the reaction completely at 0.1mM.

p-Chloromercuribenzoate and *N*-ethylmaleimide at 0.125mM caused complete inhibition of the enzyme. Iodoacetamide at 0.125mM brought about 60% inhibition. Preincubation of the enzyme with 0.25mM-dithiothreitol for 5min partially protected the enzyme against attack of these thiol-blocking reagents. L-Glutamate and ATP separately or together did not protect.

Products of the γ -glutamyl kinase reaction

Table 2 shows that under the conditions of the hydroxamate assay γ -glutamyl hydroxamate and P_i are formed in stoichiometric amounts. ADP was not measurable when hydroxylamine was a component of the incubation mixture, as the latter inhibited the phosphoenolpyruvate kinase system used in the ADP assay. Inclusion of inorganic pyrophosphatase in the incubation mixture was without significant effect on the amounts of P_i released, thus suggestive of hydrolysis of ATP to ADP and P_i in the presence of L-glutamate. When the formation of ADP and P_i was followed under the conditions of the P_i release assay, slightly higher amounts of P_i than ADP were observed.

Effect of substrate saturation on the γ -glutamyl kinase reaction

The apparent K_m value for L-glutamate was determined from experiments wherein the concentrations of L-glutamate and ATP were varied (2.5 to 50mM). The Mg^{2+} concentrations were maintained

at twice that of ATP providing for the MgATP^{2-} complex (Storer & Cornish-Bowden, 1976b). From the double-reciprocal plots an apparent K_m of 12mM for L-glutamate was derived. When ATP was the variable substrate and L-glutamate the fixed substrate a series of sigmoid double-reciprocal plots was obtained.

In the presence of L-proline the saturation function for L-glutamate remained hyperbolic up to 10mM-L-proline, but it was sigmoidal at 20 and 30mM-L-proline (Fig. 3a). However, plots of the enzyme activity as a function of ATP concentration were non-hyperbolic. The sigmoidicity increased with increasing proline concentration (Fig. 3b).

Product inhibition

γ -Glutamyl kinase was inhibited by ADP and P_i . At 5mM these reaction products led to a decrease in enzyme activity of 7–12% (Fig. 4). Under standard assay conditions a final concentration of 2mM with respect to the products of the reaction was reached. The inhibition caused by ADP and P_i was not cumulative. L-Proline had no synergistic influence.

Inhibition by proline and proline analogues

L-Proline, DL-3,4-didehydroproline, DL-1-pyrroline-5-carboxylic acid, L-thiopropine (L-thiazoli-

Fig. 4. Inhibition of enzyme activity by added ADP, P_i and L-proline

ADP and P_i were added to the reaction mixtures separately and together (equimolar) at concentrations of 2.5 to 40mM. Partially purified enzyme (0.034 unit per tube) was used. The hydroxamate assay was used. Symbols: $\circ$, P_i ; $\bullet$, ADP; $\triangle$, ADP and P_i ; $\blacktriangle$, ADP and P_i in the presence of 5mM-L-proline.

dine-4-carboxylic acid), L-azetidine-2-carboxylic acid, L-4-hydroxyproline, L-proline amide, L-prolylglycine, glycyl-L-proline, D-proline, L-arginine, L-ornithine, and L-5-oxopyrrolidine-2-carboxylic acid (5-oxo-L-proline) were tested at 2–40mM concentrations as inhibitors of γ -glutamyl kinase. Inhibition of the enzyme by 50% was caused by 3mM-L-azetidine-2-carboxylic acid, 5mM-L-proline, 9mM-DL-3,4-didehydroproline, and 12mM-L-thiopropine (Fig. 5). L-Ornithine and 5-oxo-L-proline inhibited about 10% at 10mM. All other compounds were ineffective.

γ -Glutamyl kinase activity in proline-auxotrophic mutants

Genetic markers of proline auxotrophy have been mapped in three different loci on the chromosome of *P. aeruginosa* PAO (Pemberton & Holloway, 1972). Three proline-auxotrophic mutants, each representing one of the three genetic classes of proline auxotrophs, were grown on nutrient yeast broth as described in the Materials and Methods section. The amount of proline-inhibitable γ -glutamyl kinase activity in cell extracts of the proline auxotrophs was compared with the proline-inhibitable γ -glutamyl kinase activity in extracts of the wild-type strains. As shown in Table 3 strain PAO 879 (*pro-73*) was devoid of proline-inhibitable γ -glutamyl kinase, whereas strains PAO 831 (*pro-71*) and PAO 853 (*pro-70*) showed wild-type amounts of this activity. One thus concludes that *pro-73*, which has been

Fig. 5. Effect of proline analogues on γ -glutamyl kinase activity

L-Azetidine-2-carboxylic acid ($\circ$), L-proline ($\triangle$), 3,4-didehydro-DL-proline ($\blacktriangle$) and L-thiopropine ($\bullet$) were tested by the hydroxamate assay.

Table 3. Inhibition of γ -glutamyl kinase by L-proline in wild-type and in proline-auxotrophic mutants of *P. aeruginosa*. Crude extracts were prepared from the strains PAO 1, PAO 831, PAO 853 and PAO 879 grown on nutrient yeast broth and assayed for γ -glutamyl kinase activity $\pm$ 10mM-L-proline.

Strain	γ -Glutamyl hydroxamate formed (μ mol/min per mg of protein)		Proline-inhibitable γ -glutamyl kinase activity (units/mg of protein) $\pm$ M.D.*
	No proline	10mM-Proline	
PAO 1	0.146	0.080	0.066 $\pm$ 0.007
PAO 831	0.115	0.062	0.053 $\pm$ 0.015
PAO 853	0.122	0.051	0.071 $\pm$ 0.008
PAO 879	0.094	0.098	0.004 $\pm$ 0.010

* M.D. is the mean deviation computed from four determinations.

mapped at 40min on the *Pseudomonas* chromosome, represents a lesion in *pro A*, the structural gene of γ -glutamyl kinase.

Discussion

γ -Glutamyl kinase, the first enzyme of proline biosynthesis, became detectable in crude extracts when the French press was used for cell breakage. In extracts prepared by sonication, γ -glutamyl kinase activity was not detectable. Several properties of γ -glutamyl kinase support the notion that the enzyme is specifically involved in proline biosynthesis. Thus the enzyme was inhibited by proline and by proline analogues. Extracts prepared from a strain belonging to one of the three transductional groups of proline-auxotrophic mutants (Pemberton & Holloway, 1972) were devoid of γ -glutamyl kinase activity (Table 3). Also, the specific substrate requirement of the purified γ -glutamyl kinase and the formation of almost equal amounts of γ -glutamyl hydroxamate, ADP and P_i (Table 2) are consistent with the involvement of an enzyme-bound γ -glutamyl intermediate similar to that of glutamine synthetase or γ -glutamylcysteine synthetase (Krishnaswamy *et al.*, 1962; Todhunter & Purich, 1975; Meister & Tate, 1976; Wedler & Horn, 1976). This view is strengthened by the fact that γ -glutamyl kinase of *P. aeruginosa* is also competitively inhibited by L-methionine-DL-sulphoximine (Ronzio *et al.*, 1969). Furthermore, the second enzyme of the pathway, for which γ -glutamyl phosphate probably is the substrate, was inhibited competitively by 3-(phosphonoacetyl-amido)-L-alanine, a structural analogue of the postulated substrate (Krishna *et al.*, 1979).

Relatively high concentrations of L-proline were needed to inhibit γ -glutamyl kinase from *P. aeruginosa* in crude extracts as well as in partially purified preparations. The enzyme from *E. coli* has been reported to be 50% inhibited by 0.05mM-L-proline (Baich, 1969), whereas 5mM-L-proline was necessary to cause the same degree of inhibition of the *Pseudomonas* enzyme. Although higher concentrations of

proline effected almost complete inhibition of γ -glutamyl kinase, 5mM- or 10mM-proline was used as the inhibitory concentration for assaying the enzyme in crude extracts. This was necessitated by the observation that some of the hydroxamate-forming activities contained in crude extracts of *P. aeruginosa* were stimulated by higher concentrations of proline. The units of γ -glutamyl kinase as defined in the Materials and Methods section were thus arbitrary. For crude extracts we cannot exclude the possibility that hydroxamate-forming activities (e.g. glutamine synthetase) other than γ -glutamyl kinase were also inhibited by 5–10mM-proline. The fact that extracts of the proline-auxotrophic mutant PAO 879 contained the same amount of γ -glutamylhydroxamate-forming activity in the presence and in the absence of 10mM-L-proline (Table 3) makes this possibility unlikely. Although this is strong evidence against inhibition of glutamine synthetase by proline, we have not studied the effect of proline on purified glutamine synthetase from *P. aeruginosa*.

It is noteworthy that the γ -glutamyl kinase of proline biosynthesis in *Brevibacterium flavum* was unaffected by proline (Yoshinaga *et al.*, 1975). In *P. aeruginosa* the saturation curves for L-glutamate were hyperbolic at lower concentrations of proline and exhibited sigmoidicity at higher concentrations, possibly due to substrate exclusion (Segel, 1975). With ATP, however, a non-hyperbolic velocity response was obtained in the absence of proline. It became sigmoidal when the proline concentration was increased. The catalytic activity of γ -glutamyl kinase of *P. aeruginosa* thus appears to be regulated by proline and ATP. In contrast with this, it has been shown for two related enzymes, namely for γ -glutamyl kinase from *E. coli* (Baich, 1969) and for N-acetylglutamic acid 5-phosphotransferase from *P. aeruginosa* (Haas & Leisinger, 1975b) that the target substrate (Sanwal, 1970) is not ATP, but glutamate or N-acetylglutamate respectively. The unusual regulatory properties of γ -glutamyl kinase from *P. aeruginosa*, namely its relative insensitivity to L-proline and its co-operativity with respect to ATP,

may become meaningful when the enzyme functions in its proper environment *in vivo*, possibly as a complex with γ -glutamyl phosphate reductase.

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