Biosynthesis of Proline in *Pseudomonas aeruginosa*

PROPERTIES OF γ -GLUTAMYL PHOSPHATE REDUCTASE AND 1-PYRROLINE-5-CARBOXYLATE REDUCTASE

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(Received 1 December 1978)

γ -Glutamyl phosphate reductase, the second enzyme of proline biosynthesis, catalyses the formation of L-glutamic acid 5-semialdehyde from γ -glutamyl phosphate with NAD(P)H as cofactor. It was purified 150-fold from crude extracts of *Pseudomonas aeruginosa* PAO 1 by DEAE-cellulose chromatography and hydroxyapatite adsorption chromatography. The partially purified preparation, when assayed in the reverse of the biosynthetic direction, utilized L-1-pyrroline-5-carboxylic acid as substrate and reduced NAD(P)⁺. The apparent K_m values were: NAD⁺, 0.36 mM; NADP⁺, 0.31 mM; L-1-pyrroline-5-carboxylic acid, 4 mM with NADP⁺ and 8 mM with NAD⁺; P_i, 28 mM. 3-(Phosphonoacetyl-amido)-L-alanine, a structural analogue of γ -glutamyl phosphate, inhibited this enzyme competitively ($K_i = 7$ mM). 1-Pyrroline-5-carboxylate reductase (EC 1.5.1.2), the third enzyme of proline biosynthesis, was purified 56-fold by (NH₄)₂SO₄ fractionation, Sephadex G-150 gel filtration and DEAE-cellulose chromatography. It reduced L-1-pyrroline-5-carboxylate with NAD(P)H as a cofactor to L-proline. NADH ($K_m = 0.05$ mM) was a better substrate than NADPH ($K_m = 0.02$ mM). The apparent K_m values for L-1-pyrroline-5-carboxylate were 0.12 mM with NADPH and 0.09 mM with NADH. The 3-acetylpyridine analogue of NAD⁺ at 2 mM caused 95% inhibition of the enzyme, which was also inhibited by thio-NAD(P)⁺, heavy-metal ions and thiol-blocking reagents. In cells of strain PAO 1 grown on a proline-medium the activity of γ -glutamyl kinase and γ -glutamyl phosphate reductase was about 40% lower than in cells grown on a glutamate medium. No repressive effect of proline on 1-pyrroline-5-carboxylate reductase was observed.

1-Pyrroline-5-carboxylate is a key intermediate in the metabolism of proline. In *Pseudomonas aeruginosa* it could arise by an ornithine 5-aminotransferase-catalysed reaction from L-ornithine and 2-oxoglutarate (Voellmy & Leisinger, 1975), by the oxidation of L-proline by proline oxidase (Clarke & Ornston, 1975), or from L-glutamate via γ -glutamyl phosphate by γ -glutamyl phosphate reductase, the second enzyme of proline biosynthesis. γ -Glutamyl phosphate formed by γ -glutamyl kinase from L-glutamate and ATP, presumably while in the enzyme-bound form, is converted into glutamic acid 5-semialdehyde by the mediation of NAD(P)H- γ -glutamyl phosphate reductase (Baich, 1969, 1971). This reaction is analogous to the syntheses of N-acetylglutamic acid 5-semialdehyde in arginine biosynthesis (Vogel & McLellan, 1970) and of aspartic acid 4-semialdehyde and 6-aminoadipic acid 2-semialdehyde (Δ^1 -piperidine-6-carboxylic acid) in the biosynthesis of lysine (Black & Wright, 1955; Larson *et al.*, 1963).

In the third step of proline biosynthesis glutamic acid 5-semialdehyde, which exists in equilibrium

with 1-pyrroline-5-carboxylic acid is reduced by the NAD(P)H-1-pyrroline-5-carboxylate reductase (EC 1.5.1.2) to proline. Although this reaction is irreversible in *Escherichia coli* (Rossi *et al.*, 1977), it is probably reversible in *Clostridium sporogenes* (Costilow & Cooper, 1978).

Part of our efforts to elucidate the enzymology, the genetic organization and the regulation of the interconversions of glutamic acid, proline and ornithine in *P. aeruginosa* resulted in the partial purifications and characterizations of γ -glutamyl phosphate reductase and 1-pyrroline-5-carboxylate reductase, which are described in the present paper.

Materials and Methods

Micro-organisms and growth conditions

Strains of *P. aeruginosa* PAO, media, conditions of growth, and preparation of cell extracts have been described in the preceding paper (Krishna & Leisinger, 1979). Crude extracts for the assay or the purification of γ -glutamyl phosphate reductase were prepared

in buffer P (25 mM-potassium phosphate, pH 7.0, containing 0.1 mM-dithiothreitol).

Chemicals

DL-1-Pyrroline-5-carboxylic acid was prepared from 5-hydroxylysine by KIO_4 oxidation (Williams & Frank, 1975). L-1-Pyrroline-5-carboxylic acid and N^2 -acetyl-L-glutamic acid 5-semialdehyde were enzymically made from L-ornithine and N^2 -acetyl-L-ornithine respectively, by using partially purified N^2 -acetyl-L-ornithine 5-aminotransferase from *P. aeruginosa* (Hayzer *et al.*, 1979; Voellmy & Leisinger, 1975). L-Aspartic acid 3-semialdehyde and L- Δ^1 -piperidine-6-carboxylic acid were prepared by using crude extracts of brewer's yeast (Larson *et al.*, 1963). 3-(Phosphonoacetyl-amido)-L-alanine, a structural analogue of γ -glutamyl phosphate, was a generous gift from Dr. B. R. Horn, Department of Chemistry, Rensselaer Polytechnic Institute, Troy, NY, U.S.A. 5-Oxo-L-proline was obtained from Fluka A.G., Buchs, Switzerland.

Enzyme assays

γ -Glutamyl phosphate reductase. This was assayed in the reverse of the biosynthetic direction by measuring the formation of NAD(P)H from NAD-(P)⁺ and 1-pyrroline-5-carboxylic acid. The reaction mixtures comprised, in a total volume of 1 ml: potassium phosphate buffer, pH 6.5, 50 mM; DL-1-pyrroline-5-carboxylic acid, 4–5 mM or L-1-pyrroline-5-carboxylic acid, 2–3 mM; NAD(P)⁺, 1 mM; and crude extract (0.6–1.2 mg of protein). In the reference cell potassium phosphate buffer was replaced by imidazole/HCl, pH 6.5, 50 mM, to correct for the phosphate-independent oxidation of 1-pyrroline-5-carboxylate by 1-pyrroline-5-carboxylate dehydrogenase (EC 1.5.1.12). For assaying γ -glutamyl phosphate reductase in crude extracts the 3-acetylpyridine analogue of NAD⁺ (2 mM) was included in the reaction mixture. This analogue inhibited 1-pyrroline-5-carboxylate reductase, which strongly interfered with the γ -glutamyl phosphate reductase assay. Small portions from the purification steps were dialysed against 5 mM-imidazole buffer, pH 6.5, before assays to remove phosphate. A_{340} measurements were made on a double-beam recording spectrophotometer during incubations of 5 to 10 min at 30°C. One unit of enzyme activity catalyses the reduction of 1 μ mol of NADP⁺/min under these conditions.

1-Pyrroline-5-carboxylate reductase (EC 1.5.1.2). This was determined by measuring the oxidation of NADH in a 1 ml reaction mixture containing 50 mM-imidazole/HCl, pH 6.5, 0.2 mM-NADH, 0.5 mM-DL-1-pyrroline-5-carboxylate and an appropriate amount of crude extract (20–50 μ g of protein) or partially purified enzyme to yield linear rates over a 5 min period at ambient temperature (22°C). When crude extracts were used appropriate corrections were

made by using blanks where 1-pyrroline-5-carboxylate was omitted. One unit is defined as the amount of enzyme that oxidizes 1 μ mol of NADH/min under standard assay conditions.

L-Glutamate dehydrogenase (EC 1.4.1.2). This was estimated by the method of Adachi & Suzuki (1977).

Proline oxidase. This was assayed colorimetrically with *o*-aminobenzaldehyde, as described by Williams & Frank (1975).

Sucrose-density-gradient centrifugation

Sucrose-density-gradient centrifugations were carried out in a Beckman Spinco ultracentrifuge (model L2-65B) at 2°C for 12 h at 40000 rev./min with an SW 50.1 rotor (149000g, r_{av} 8.35 cm). Samples (0.2 ml) containing the markers yeast alcohol dehydrogenase, cytochrome *c*, and human haemoglobin along with the preparation to be analysed were layered on 4.6 ml of a 5–20% (w/v) sucrose gradient. $s_{20,w}$ values were calculated as described by Martin & Ames (1961). Marker enzymes were assayed by standard methods (Bergmeyer, 1974).

Results

Partially purified enzyme preparations

Cells of the wild-type strain *P. aeruginosa* PAO 1 grown on nutrient yeast broth served as a source of enzyme for the partial purification of γ -glutamyl phosphate reductase (Table 1) and of 1-pyrroline-5-carboxylate reductase (Table 2). γ -Glutamyl phosphate reductase was also purified from cells of strain PAO 853, which lacked 1-pyrroline-5-carboxylate reductase. The yield and the purification factor were similar to a purification with strain PAO 1 as an enzyme source.

Figs. 1 and 2 show the final purification steps applied to γ -glutamyl phosphate reductase and to 1-pyrroline-5-carboxylate reductase, hydroxyapatite chromatography and DEAE-cellulose chromatography respectively. The partially purified preparations of the two reductases that eluted from these columns revealed five to six bands on polyacrylamide-gel electrophoresis. Both preparations were free of proline oxidase, 1-pyrroline-5-carboxylate dehydrogenase, and glutamate dehydrogenase. All the experiments on the properties of γ -glutamyl phosphate reductase and 1-pyrroline-5-carboxylate reductase were performed with partially purified preparations. Both of these enzymes exhibited pH optima of 6.2–6.5. The two reductases of proline biosynthesis together with a partially purified preparation of γ -glutamyl kinase (Krishna & Leisinger, 1979) were subjected to sedimentation in 5–20% sucrose gradients as described in the Materials and Methods section. The $s_{20,w}$ values for γ -glutamyl kinase, γ -glutamyl phosphate reductase and 1-pyrroline-5-carboxylate reductase were 5.6, 6.2, and

Table 1. *Partial purification of γ -glutamyl phosphate reductase*

The steps in the purification procedure were as follows. Step 1: cells (20g wet wt.) of strain PAO 1, suspended in 45 ml of buffer P (25 mM-potassium phosphate, pH 7.0, containing 0.1 mM-dithiothreitol) were broken by passage through the French press (7000 Lbf/in², 48400 kPa, and centrifuged at 30000g for 60 min. Step 2: streptomycin sulphate (2%, w/v, final concentration) was added to the supernatant and the pellets obtained on centrifugation were discarded. The supernatant was dialysed against 2 litres of buffer P. Step 3: non-diffusible material was applied to a Whatman DE-52 DEAE-cellulose column (2.5cm $\times$ 49cm) equilibrated with buffer P and eluted with a 0–.5M-KCl gradient in the same buffer at 60ml/h. Fractions containing γ -glutamyl phosphate reductase were combined, concentrated and dialysed against buffer P. Step 4: the dialysis-residue was applied to a column (0.5cm $\times$ 12cm) of hydroxyapatite. Protein was eluted stepwise with 50mM- and 125mM-potassium phosphate, pH 7.0. γ -Glutamyl phosphate reductase activity was eluted at the latter phosphate concentration (Fig. 1). Fractions containing enzyme activity were pooled, dialysed against buffer P and stored in smaller portions at –40°C.

 γ -Glutamyl phosphate reductase activity

Step	Fraction	Protein (mg)	Specific activity (units/mg)	Activity	Yield (%)	Fold purification
				(units)		
1	Crude extract	1600.0	0.0016	2.56	100	1.0
2	Streptomycin sulphate	1429.0	0.0020	2.86	111	1.2
3	DEAE-cellulose	17.0	0.0575	0.98	38	36.0
4	Hydroxyapatite	5.7	0.2180	1.24	48	136.0

Table 2. *Partial purification of 1-pyrroline-5-carboxylate reductase*

Steps 1–5: 50g (wet wt.) of cells from strain PAO 1 was broken and processed through streptomycin precipitation, (NH₄)₂SO₄ precipitation, gel filtration on Sephadex G-150, and DEAE-cellulose chromatography as described in the preceding paper (Krishna & Leisinger, 1979). The 1-pyrroline-5-carboxylate reductase activity eluted between 0.2 and 0.25M-KCl in the salt gradient applied to the final DEAE-cellulose column (Fig. 2).

1-Pyrroline-5-carboxylate reductase activity

Step	Fraction	Protein (mg)	Specific activity (units/mg)	Activity	Yield (%)	Fold purification
				(units)		
1	Crude extract	4325	0.059	255	100	1.0
2	100000g supernatant	3168	0.063	200	78	1.1
3	(NH ₄) ₂ SO ₄ (40–80%)	2746	0.090	247	97	1.5
4	Sephadex G-150	403	0.625	252	98	10.6
5	DEAE-cellulose	42	3.317	139	55	56.0

Fig. 1. *Hydroxyapatite chromatography of γ -glutamyl phosphate reductase*

The concentrated fraction from step 3 (Table 1) was applied to a hydroxyapatite column (0.5cm $\times$ 12cm) and subjected to stepwise elution with 50 and 125mM-potassium phosphate buffer, pH 6.5. The flow rate was 5ml/h and 0.25ml fractions were collected. Symbols: $\circ$, A_{280} ; $\bullet$, enzymic activity.

Fig. 2. *Elution profile of 1-pyrroline-5-carboxylate reductase from DEAE-cellulose*

Partially purified enzyme from the gel-filtration step (Sephadex G-150, step 4; Krishna & Leisinger, 1979) was loaded on a DEAE-cellulose column (1.6cm $\times$ 28cm) and eluted with a 0.15 to 0.35M-KCl gradient. The start of the gradient is indicated by an arrow. The flow rate was 60ml/h and the fraction volume was 1.7ml. Symbols: $\circ$, protein; $\bullet$, enzymic activity.

5.6S (Fig. 3), which correspond to molecular weights of 95000, 110000, and 95000 respectively. When the molecular weights were estimated by gel filtration on Sephadex G-150 values of 84000, 100000, and 94000 were obtained (results not shown).

Substrate specificities of γ -glutamyl phosphate reductase and L-pyrroline-5-carboxylate reductase

Compounds tested as potential substrates or co-substrates were included at the same concentrations as the normal substrates in the standard assay mixtures. The compounds tested with both enzymes were: DL-1-pyrroline-5-carboxylate, L-1-pyrroline-5-carboxylate, L- Δ^1 -piperidine-6-carboxylate, *N*²-acetyl-L-glutamic acid 5-semialdehyde, L-aspartic acid 4-semialdehyde, NAD⁺, NADH, NADP⁺, and NADPH.

For γ -glutamyl phosphate reductase, assayed in the reverse direction of the biosynthetic reaction, L-1-pyrroline-5-carboxylate was found to be the best substrate. It reduced NAD⁺ and NADP⁺ in the presence of P_i or arsenate. When 50mM-potassium

arsenate, pH6.5, was used in place of potassium phosphate, the γ -glutamyl phosphate reductase was about 50% as active. L- Δ^1 -Piperidine-6-carboxylate was about 10% as active as L-1-pyrroline-5-carboxylate in the standard assay. The apparent K_m was 0.36mM for NAD⁺ and 0.31mM for NADP⁺. For L-1-pyrroline-5-carboxylate the apparent K_m was 4mM with NADP⁺ (Fig. 4a) and 8mM with NAD⁺.

Fig. 3. *Sucrose-density-gradient-centrifugation profiles* Samples of 0.2ml consisting of the appropriate markers (1mg of each) and the partially purified enzymes of proline biosynthesis (1.1 units of the first, 0.02 unit of the second and 0.1 unit of the third enzyme), were layered on 5–20% sucrose gradients in 50mM-imidazole buffer, pH6.5, and centrifuged at 149000g (SW 50.1 rotor, r_{av} 8.35) for 12h, fractionated and analysed. Recovery of the activities of the proline-biosynthetic enzymes after centrifugation was about 70%. Symbols: □, γ -glutamyl kinase (E1); △, γ -glutamyl phosphate reductase (E2); ●, L-pyrroline-5-carboxylate reductase (E3); ○, alcohol dehydrogenase; ▲, haemoglobin; ■, cytochrome c.

Fig. 4. *Substrate affinities of 1-pyrroline-5-carboxylate reductase and γ -glutamyl phosphate reductase* (a) Double-reciprocal plots of velocity of γ -glutamyl phosphate reductase against L-pyrroline-5-carboxylate and P_i (fixed, 50mM), at NADP⁺ concentrations of 0.2mM (○), 0.6mM (△), and 1.0mM (□). (b) Double-reciprocal plots of velocity of 1-pyrroline-5-carboxylate reductase against L-1-pyrroline-5-carboxylate, at NADPH concentrations of 0.043mM (△), 0.06mM (□) and 0.1mM (●).

1-Pyrroline-5-carboxylate reductase utilized only the L-isomer of DL-1-pyrroline-5-carboxylic acid. Fig. 4(b) shows that the saturation curve for L-1-pyrroline-5-carboxylate was hyperbolic at different concentrations of NADPH. The apparent K_m for L-1-pyrroline-5-carboxylate was 0.09 mM with NADH and 0.12 mM with NADPH. L- Δ^1 -Piperidine-6-carboxylate was about 10% as active as L-1-pyrroline-5-carboxylate, whereas N^2 -acetyl-L-glutamic acid 5-semialdehyde and L-aspartic acid 4-semialdehyde were not substrates. NADH with an apparent K_m of 0.05 mM was a better co-substrate than NADPH (apparent K_m of 0.20 mM). The reaction catalysed by 1-pyrroline-5-carboxylate reductase was irreversible.

Inhibitors of γ -glutamyl phosphate reductase and 1-pyrroline-5-carboxylate reductase

Various thiol-blocking reagents, proline analogues (5-oxo-L-proline, DL-3,4-didehydropyrroline, L-thio-proline, and L-2-azetidine-4-carboxylic acid), nucleotide analogues (thio-NAD⁺, thio-NADP⁺ and the 3-acetylpyridine analogue of NAD⁺), and heavy-metal ions were tested as inhibitors of the two reductases of proline biosynthesis under standard assay conditions. In general these compounds had little effect on the activity of γ -glutamyl phosphate reductase, whereas they inhibited 1-pyrroline-5-carboxylate reductase to different degrees.

Buffers free of thiol compounds were used when thiol-blocking reagents were tested. *p*-Chloromercuribenzoate and iodoacetamide at 1 mM completely inactivated 1-pyrroline-5-carboxylate reductase. *N*-Ethylmaleimide (1 mM) inhibited the enzyme by 60%. Preincubation of the enzyme for 5 min at 20°C with 2 mM-dithiothreitol prevented this inhibition.

L-Proline at 50 mM inhibited the γ -glutamyl phosphate reductase by 25% and 1-pyrroline-5-carboxylate reductase by 75%. Among proline analogues, 2 mM-L-2-azetidine-4-carboxylic acid caused 50% inhibition of the 1-pyrroline-5-carboxylate reductase; others had no significant effect.

Derivatives of NAD⁺ inhibited the 1-pyrroline-5-carboxylate reductase; the 3-acetylpyridine analogue of NAD⁺ (2 mM) brought about 95% inhibition. Thio-NAD⁺ and thio-NADP⁺ inhibited this enzyme 65% at 0.2 mM. However, in concentrated solutions these two compounds turned yellow. Hence thio-NAD⁺ and thio-NADP⁺ were of limited use in inhibiting 1-pyrroline-5-carboxylate reductase to assay γ -glutamyl phosphate reductase in crude extracts. Heavy-metal ions inactivated 1-pyrroline-5-carboxylate reductase (Ag⁺ at 0.1 mM almost completely and Hg²⁺ at 1.0 mM by 75%).

Inhibition of γ -glutamyl phosphate reductase by 3-(phosphonoacetyl-amido)-L-alanine

3-(Phosphonoacetyl-amido)-L-alanine, a non-reactive structural analogue of γ -glutamyl phosphate, has been shown to bind to glutamine synthetases from *Escherichia coli* and from pea seeds. It inhibited *E. coli* glutamine synthetase competitively with L-glutamate (Wedler & Horn, 1976). γ -Glutamyl phosphate is postulated as an intermediate in proline biosynthesis and thus, in the assay system used in the present paper, is the assumed product formed by γ -glutamyl phosphate reductase from 1-pyrroline-5-carboxylic acid and P_i. We have therefore tested whether γ -glutamyl phosphate reductase from *P. aeruginosa* is inhibited by 3-(phosphonoacetyl-amido)-L-alanine. The analogue is competitive against 1-pyrroline-5-carboxylic acid, thus suggesting the formation of a γ -glutamyl phosphate-enzyme complex in the reaction catalysed by γ -glutamyl phosphate reductase. With NADP⁺ as a co-substrate the K_i value of 3-(phosphonoacetyl-amido)-L-alanine was 7 mM (Fig. 5), with NAD⁺ a K_i of 4.6 mM was obtained.

Regulation of enzyme synthesis in the proline-biosynthetic pathway

Regulation of enzyme synthesis in amino acid-biosynthetic pathways seems to be rare in pseudo-

Fig. 5. Inhibition of γ -glutamyl phosphate reductase by 3-(phosphonoacetyl-amido)-L-alanine

Lineweaver-Burk plots of velocity against L-1-pyrroline-5-carboxylate concentrations with P_i fixed at 50 mM and NADP⁺ at 1 mM. Symbols: Δ , 2 mM-3-(phosphonoacetyl-amido)-L-alanine; $\circ$, 6 mM-3-(phosphonoacetyl-amido)-L-alanine; $\square$, no inhibitor.

monads (Clarke & Ornston, 1975). To test whether this generalization applied also to the pathway of proline synthesis we have compared the specific activities of the three proline biosynthetic enzymes in cells grown on L-glutamate as the only carbon and nitrogen source [growth rate constant (μ) = 0.65 h⁻¹] with the specific activities measured in extracts from cells grown on L-proline as the only source of carbon and nitrogen (μ = 0.55 h⁻¹). Table 3 shows that the first and the second enzyme of the pathway were repressed by about 40% during growth on proline, whereas the specific activity of 1-pyrroline-5-carboxylate reductase was not affected by the growth conditions. It thus appears that there is little regulation of the synthesis of γ -glutamyl kinase and γ -glutamyl phosphate reductase by proline.

Gene-enzyme relationships

In the preceding paper (Krishna & Leisinger, 1979) the mutation *pro-73* was assigned on the basis of enzyme measurements to *pro A*, the structural gene for γ -glutamyl kinase. Crude extracts of three proline-auxotrophic mutants representing the three genetic classes of proline auxotrophs were also assayed for γ -glutamyl phosphate reductase and 1-pyrroline-5-carboxylate reductase (Table 4). As expected from feeding experiments (Voellmy & Leisinger, 1975), the extract from strain PAO 853 (*pro-70*) was devoid of 1-pyrroline-5-carboxylate

reductase, whereas in the extract from strain PAO 831 (*pro-71*) there was 10 times less γ -glutamyl phosphate reductase activity than in the wild type. The results of the enzymic analysis of the three proline auxotrophs are thus in accord with the genetic data (Pemberton & Holloway, 1972) and show that the *pro-70* mutation corresponds to *pro C*, the structural gene for 1-pyrroline-5-carboxylate reductase, whereas *pro-71* is a mutation in *pro B*, the structural gene for γ -glutamyl phosphate reductase.

Discussion

Three enzymes specifically implicated in the biosynthesis of proline by *P. aeruginosa* were partially characterized in the present and preceding papers. The properties of the enzyme activities described are consistent with the pathway of proline synthesis proposed in *E. coli* (Vogel & Davis, 1952; Baich, 1969) and leading from glutamate via a γ -carboxy-activated intermediate and glutamic acid 5-semialdehyde (1-pyrroline-5-carboxylate) to proline. In *Pseudomonas* γ -glutamyl phosphate is suggested as the first intermediate in this sequence by the properties of a proline inhibitable γ -glutamyl kinase (Krishna & Leisinger, 1979) and of a γ -glutamyl phosphate reductase. The latter enzyme, assayed in the reverse of the biosynthetic direction, required 1-pyrroline-5-carboxylate, NAD⁺ or NADP⁺, and P_i as substrates. P_i was replaceable by arsenate. 3-(Phos-

Table 3. Regulation of enzyme synthesis in the proline-biosynthetic pathway of *P. aeruginosa*

The wild-type strain PAO 1 was grown on medium P containing either 20 mM-L-glutamate or 20 mM-L-proline as the only carbon and nitrogen source. Cell extracts were prepared and enzyme activities were determined as described in the Materials and Methods section and by Krishna & Leisinger (1979). Specific activities reported for the different enzymes represent the arithmetic mean computed from three independent experiments $\pm$ s.d.

Growth	Specific activity (unit/mg of protein)			
	Glutamine synthetase	γ -Glutamyl kinase	γ -Glutamyl phosphate reductase	1-Pyrroline-5-carboxylate reductase
L-Glutamate	0.236 $\pm$ 0.017	0.165 $\pm$ 0.011	0.0082 $\pm$ 0.0006	0.102 $\pm$ 0.016
L-Proline	0.195 $\pm$ 0.010	0.088 $\pm$ 0.015	0.0051 $\pm$ 0.0002	0.103 $\pm$ 0.006

Table 4. Gene-enzyme relationships in the proline-biosynthetic pathway

The wild-type strain and three proline-auxotrophic mutants were grown overnight in Fernbach flasks on nutrient yeast broth (Krishna & Leisinger, 1979). γ -Glutamyl phosphate reductase and 1-pyrroline-5-carboxylate reductase were determined in crude extracts, dialysed against 5 mM-imidazole buffer, pH 6.5. The results for γ -glutamyl kinase are from Krishna & Leisinger (1979).

Strain	Specific activity (unit/mg of protein)		
	γ -Glutamyl kinase	γ -Glutamyl phosphate reductase	1-Pyrroline-5-carboxylate reductase
PAO 1	0.066	0.0051	0.077
PAO 879	0.004	0.0041	0.069
PAO 831	0.053	0.0004	0.085
PAO 853	0.071	0.0036	0.002

phonoacetyl-amido)-L-alanine, a structural analogue of γ -glutamyl phosphate, inhibited the enzyme competitively with 1-pyrroline-5-carboxylate. 1-Pyrroline-5-carboxylate reductase from *P. aeruginosa*, the third enzyme of proline biosynthesis, exhibited similar specificity towards NADH and NADPH as the enzymes isolated from several rat tissues (Meister *et al.*, 1957; Smith & Greenberg, 1957; Adams & Goldstone, 1960; Peisach & Strecker, 1962; Herzfeld *et al.*, 1977) and from soya-bean leaves (Miller & Stewart, 1976). It differed from the *Neurospora crassa* and *E. coli* 1-pyrroline-5-carboxylate reductases in that NADPH was the preferred substrate in these two cases (Yura & Vogel, 1959; Rossi *et al.*, 1977). The 3-acetylpyridine analogue of NAD⁺ (2 mM) inhibited the 1-pyrroline-5-carboxylate reductase from *P. aeruginosa* over 95%. It had no effect on γ -glutamyl phosphate reductase, a property that permitted measurement of this enzyme in crude extracts containing 1-pyrroline-5-carboxylate reductase. The molecular weight of *Pseudomonas* 1-pyrroline-5-carboxylate reductase was estimated as 95 000, a value considerably lower than those determined for the corresponding enzymes from *E. coli* (320 000, Rossi *et al.*, 1977) and *Clostridium sporogenes* (200 000; Costilow & Cooper, 1978).

Further evidence for the involvement in proline biosynthesis of the enzyme reactions reported in the present paper was obtained by the enzymic analysis of proline-auxotrophic mutants of *P. aeruginosa*. In each of three extracts prepared from representatives of the three genetic classes of proline auxotrophs a different proline-biosynthetic enzyme was lacking. The availability of specific enzyme assays applicable to crude extracts thus allowed a preliminary exploration of two questions concerning the organization of proline biosynthesis: the importance of the formation of a complex between the first and the second enzyme of the pathway for the channeling of the labile intermediate γ -glutamyl phosphate and the existence of regulatory mechanisms preventing futile cycles between glutamate and glutamic acid 5-semialdehyde and also between 1-pyrroline-5-carboxylate and proline.

The formation of an aggregate between γ -glutamyl kinase and γ -glutamyl phosphate reductase from *E. coli* has been suggested by Baich (1969). Gamper & Moses (1974) observed that the formation *in vitro* of proline from glutamate by crude French-press extracts from *E. coli* was sensitive to dilution and postulated that dissociation of an enzyme complex between the first and the second enzyme was responsible for this phenomenon. Recently, in crude extracts from the same organism, a proline-inhibitable γ -glutamyl kinase of mol.wt. 250 000 was detected, which may be part of a complex of proline-biosynthetic enzymes of this size (Hayzer & Moses, 1978). In our experiments with *P. aeruginosa* γ -

glutamyl kinase and γ -glutamyl phosphate reductase from crude extracts or from partially purified enzyme preparations (Fig. 3) sedimented separately in sucrose gradients and gave distinct activity peaks on gel filtration on Sephadex G-100 or G-150. Also, when mutant extracts were analysed, the sedimentation profile of γ -glutamyl kinase or γ -glutamyl phosphate reductase in sucrose gradients was not affected by the absence of either enzyme activity (results not shown). Whereas these observations do not exclude the existence of an enzyme complex *in vivo*, they indicate that such an aggregate would be labile and might dissociate during the preparation of cell extracts by the method we have used.

1-Pyrroline-5-carboxylate occupies a central position in the biosynthesis and the degradation of proline as well as in the degradation of arginine and ornithine by *P. aeruginosa* (Voellmy & Leisinger, 1975). Its formation, as well as its utilization by the reduction to proline, the oxidation to glutamate, or the transamination to ornithine, are expected to be regulated at the level of enzyme synthesis and/or activity. In the present study we have tested the regulation of the biosynthetic reactions involved in the metabolism of 1-pyrroline-5-carboxylate. Although enzyme repression seems to be of minor importance (Table 3), the synthesis of 1-pyrroline-5-carboxylate is likely to be regulated at the level of enzyme activity by proline that feedback inhibits γ -glutamyl kinase (Krishna & Leisinger, 1979). 1-Pyrroline-5-carboxylate reductase from *E. coli* is reported to be inhibited by proline and by NADP⁺ (Rossi *et al.*, 1977). The *Pseudomonas* enzyme was inhibited by proline, but not by high concentrations of NADP⁺ and NAD⁺. It seems, however, unlikely that the inhibition of this enzyme by high concentrations of proline (50 mM) is of physiological significance in regulating the conversion of 1-pyrroline-5-carboxylate to proline. Induction of the catabolic enzyme 1-pyrroline-5-carboxylate dehydrogenase may be more important in directing the utilization of pyrroline-5-carboxylate by either the biosynthetic or the degradative pathway.

We thank Dr. Bradley Horn and Dr. David Hayzer, Mikrobiologisches Institut, ETH, Zürich, Switzerland, for generously supplying 3-(phosphonoacetyl-amido)-L-alanine and L-1-pyrroline-5-carboxylic acid respectively. This study was supported by the Swiss National Foundation for Scientific Research (project no. 3.5090.75).

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