The Enzymes of Proline Biosynthesis in *Escherichia coli*

THEIR MOLECULAR WEIGHTS AND THE PROBLEM OF ENZYME AGGREGATION

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1. By using Bio-Gel A1.5M and Sephadex G-150 columns, crude cell-free extracts of *Escherichia coli* were fractionated to demonstrate the existence of a proline-biosynthetic aggregate. 2. Sephadex G-150 resolves two glutamyl kinases that are inhibited by proline, with mol.wts. of 125000 and 38000, the reactions of which are Mg^{2+} -dependent. The heavier species is more sensitive to inhibition by proline. 3. γ -Glutamyl phosphate reductase and 1-pyrroline-5-carboxylate reductase (EC 1.5.1.2) have mol.wts. of approx. 125000 and 190000 respectively, the specific activity of the latter being 5×10^3 -fold greater than either of the other two biosynthetic enzymes or of the total pathway *in vivo*. 4. Bio-Gel A1.5M chromatography gave a single glutamyl kinase of mol.wt. 250000, and the possibility of this being a constituent of an enzyme complex is discussed.

Proline-auxotrophic mutants of *Escherichia coli* may be divided into two distinct groups, strains of one group excreting a compound capable of supporting the growth of strains in the other, but not vice versa (Vogel & Davies, 1952; Strecker, 1956; Baich & Pierson, 1965). The metabolite concerned, 1-pyrroline-5-carboxylate, is in equilibrium with glutamic γ -semialdehyde (Rodwell, 1969). A very active NADPH-dependent pyrroline-5-carboxylate reductase, reducing pyrroline-5-carboxylate to proline, has been identified in extracts of *E. coli* (Baich & Pierson, 1965) and shown to be insensitive to inhibition by proline. A defect in this enzyme leads to pyrroline-5-carboxylate excretion. A pyrroline-5-carboxylate reductase, inhibited by proline and NADPH, and with mol.wt. 300000, has been reported (Rossi *et al.*, 1977).

The mechanism of semialdehyde formation from glutamate is subject to some uncertainty, although most evidence now points to two enzymes being involved. Two contiguous gene loci are responsible for the conversion (Bachman *et al.*, 1976; Condamine, 1971). Baich (1969*a*) has partially purified an enzyme that, in the presence of glutamate, ATP, Mg^{2+} and hydroxylamine, gives γ -glutamyl hydroxamate, indicating the prior synthesis of an activated glutamyl moiety. The enzyme also catalysed P_i release from ATP when hydroxylamine or imidazole and glutamate were present. Both reactions could be inhibited by proline. It was accordingly suggested that the enzyme, a kinase, produced γ -glutamyl phosphate, a hypothesis supported by the evidence of other

carboxyl-activated amino acids in the reactions of glutamine synthetase (EC 6.3.1.2) (Krishnaswamy *et al.*, 1962), aspartate kinase (EC 2.7.2.4) (Black & Wright, 1953, 1954), glutathione synthesis (Orlowski & Meister, 1971) and that of glutamyl-tRNA^{Glu} synthesis in *Bacillus subtilis* (Wilcox & Nirenberg, 1968). A similar kinase, not sensitive to proline inhibition, has been identified from *Brevibacterium flavum* (Yoshinaga *et al.*, 1975).

The second of the two enzymes participating in semialdehyde synthesis has been described by Baich (1971). In the presence of pyrroline-5-carboxylate (itself in equilibrium with glutamic γ -semialdehyde) it catalyses a P_i -dependent reduction of NADP⁺, suggesting that γ -glutamyl phosphate is reduced to glutamic γ -semialdehyde during proline biosynthesis.

The existence of the intermediate, γ -glutamyl phosphate, is still conjecture. Although its chemical synthesis was reported by Katchalsky & Paecht (1954), it is an extremely labile compound, susceptible to hydrolysis and nucleophilic attack on the activated carboxyl group, which prevents its detection under physiological conditions and its use as an experimental substrate. The need to protect this hypothesized intermediate from contact with the numerous nucleophiles and water of the cytosolic environment during its transfer from the kinase to the phosphate reductase led to the suggestion that the two enzymes are physically associated, allowing passage of the glutamyl phosphate directly from one enzyme to the other (Baich, 1969*a*).

The present paper considers the isolation and partial purification of a proline-biosynthetic aggregate by gel chromatography. Although the existence of an enzyme complex has not been demonstrated

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unequivocally, the data remain consistent with a complex of the first two pathway enzymes being necessary for the synthesis of pyrroline-5-carboxylate from glutamate. The molecular weights of the three biosynthetic enzymes have been established, together with the discovery that glutamyl kinase is itself a multimeric protein with catalytically active subunits. The different molecular-weight species also differ in some of their kinetic properties.

Experimental

Organisms and culture media

The *E. coli* strains used in this study were as shown in Table 1.

The culture media and growth procedures were similar to those used previously (Hayzer & Moses, 1978).

The *proC* genotype was confirmed by incubating cells grown overnight at 37°C in medium 63 containing D-glucose (0.2%, w/v), threonine and leucine (200 µg of each/ml) and L-glutamate (50 mM). After 30 min a 1.0 ml sample was withdrawn, the cells were killed with trichloroacetic acid [0.5 ml; aq. 10% (w/v) solution] and the debris was removed by centrifugation (10 min at 10000g). A sample (0.25 ml) was assayed for pyrroline-5-carboxylate content by using an equal volume of *o*-aminobenzaldehyde (0.125%, w/v, in ethanol). Control flasks contained the same cell culture but included L-proline (5.0 mg/ml). Strains with the *proC* locus demonstrated pyrroline-5-carboxylate excretion that could be inhibited by proline.

Set-3 strains were checked for proline excretion by being streaked on medium 63/agar plates (containing glucose, threonine and leucine) at right-angles to, but not touching, streaks of strain X278 (*proB*). After

incubation for 48 h at 37°C cross-feeding of the proline auxotroph indicated excretion. None of these strains could excrete pyrroline-5-carboxylate; thus cross-feeding was not due to a *proC* lesion.

Preparation of cell-free extract and gel chromatography

These have been described by Hayzer & Moses (1978).

Enzyme assays

(a) *Glutamyl kinase*. The procedure was based on that of Baich (1969a), making use of the reaction:

In a final volume of 0.5 ml were: ATP (disodium salt), 10 mM; L-glutamate, 100 mM; MgCl₂, 20 mM; NH₂OH·HCl, 100 mM; enzyme sample, 0.25 ml. The solvent was 0.1 M-Tris/HCl buffer, pH 7.4, containing 6 mM-2-mercaptoethanol. NH₂OH·HCl was freshly prepared as a 4 M aq. solution and neutralized with NaOH.

All components were mixed to give a stock solution with concentrations twice the final values, and portions (0.25 ml) dispensed into assay tubes. The reactions were started by adding the enzyme samples, mixed and incubated at 37°C for up to 3 h. To terminate the reaction and measure the γ -glutamyl hydroxamate, 0.75 ml of a mixture of Fe(NO₃)₃·9H₂O (10%, w/v), trichloroacetic acid (5%, w/v) and 10 M-HCl (6.67%, v/v) was added, the tubes were centrifuged at 5000g for 3–4 min to remove denatured protein and the A₅₃₅ was recorded against an appropriate blank. The concentration of the γ -glutamylhydroxamate formed was calculated from the molar absorption coefficient of the Fe-hydroxamate complex of 761 M⁻¹·cm⁻¹ determined with an authentic sample of hydroxamate. To identify the glutamyl kinase of the proline-biosynthetic pathway a parallel assay was used which included L-proline (100 mM) as a feedback inhibitor.

(b) *Glutamyltransferase*. The reaction is:

In a final volume of 0.5 ml were: ADP (sodium salt), 0.36 mM; glutamine, 45.5 mM; MgCl₂, 18 mM; NH₂OH·HCl, 91 mM; Na₃AsO₄, 36.4 mM; enzyme sample, 0.25 ml. The procedure was identical with that for glutamyl kinase, except that the control assays used a mixture of glycine, serine, alanine, histidine and tryptophan (all 100 mM, except tryptophan, 50 mM), which are known to be inhibitors of

Table 1. *Strains of Escherichia coli* used in this study

Designation	Genotype	Source
Set 1		
X210	F ⁻ , <i>thi</i> , <i>str</i> , <i>leu</i> , <i>thr</i> , <i>T</i> 1,5,6, <i>lac</i> γ , <i>pro</i> ⁺	R. Curtiss, III
X680	as for X210, but <i>proA</i>	R. Curtiss, III
X278	as for X210, but <i>proB</i>	R. Curtiss, III
X277	as for X210, but <i>proC</i>	R. Curtiss, III
Set 2		
X454	F ⁻ , <i>thi</i> , <i>str</i> , <i>leu</i> , <i>lys</i> , <i>trp</i> , <i>met</i> , <i>purE</i> , <i>pro</i>	R. Curtiss, III
X462	as for X454, but <i>proA</i>	R. Curtiss, III
X474	as for X454, but <i>proB</i>	R. Curtiss, III
X478	as for X454, but <i>proC</i>	R. Curtiss, III
Set 3		
XA21-4)	F ⁻ , <i>thi</i> , <i>str</i> , <i>lac</i> <i>i</i> ₃₀ ⁺ <i>z</i> _{M15} ^{del} <i>y</i> ⁺ <i>ac</i> ⁺	H. Tristram
XA21-6)		

glutamine synthetase (Woolfolk & Stadtman, 1967). In assays with Mn^{2+} as the bivalent cation, precipitation of some $Mn_3(AsO_4)_2$ was unavoidable, and this was removed by centrifugation for 10 min at 5000g before stock solutions were used.

(c) *γ -Glutamyl phosphate reductase*. The assay system contained, in a total volume of 1.0 ml: $NADP^+$, 0.25 mM; KH_2PO_4 (adjusted to pH 7.4 with KOH), 100 mM; $MgCl_2$, 20 mM; pyrroline-5-carboxylate, 1.25 mM; Tris/HCl, pH 7.4, 250 mM; enzyme sample, 0.25 ml. A stock solution with all components except the enzyme sample was prepared and used within 12 h. The assay was started by adding the enzyme and the increase in A_{340} at 31°C was recorded with an SP.1800 u.v. spectrophotometer (Pye-Unicam, Cambridge, U.K.) against a water blank. Controls had either pyrroline-5-carboxylate or P_i omitted. The activity of the glutamyl phosphate reductase was recorded as the difference between that with the 'complete' system and the appropriate control, and catalysed the reaction:

The instability of γ -glutamyl phosphate in aqueous solution requires that this enzyme be assayed in the reverse direction to that of biosynthesis.

(d) *1-Pyrroline-5-carboxylate reductase*. The assay system contained, in a total volume of 1.0 ml: $NADPH$, 0.25 mM; $MgCl_2$, 20 mM; pyrroline-5-carboxylate, 1.25 mM; Tris/HCl, pH 7.4, 250 mM (containing 6 mM-2-mercaptoethanol); enzyme sample, 0.25 ml (or less if required, the volume being adjusted with 0.1 M-Tris/HCl buffer, pH 7.4). The assay was started by adding the enzyme and the decrease in A_{340} at 31°C was recorded against a water blank. Controls lacking pyrroline-5-carboxylate were always used to correct for any $NADPH$ oxidase activity.

Synthesis and assay of 1-pyrroline-5-carboxylate

Details were given by Hayzer & Moses (1978).

Protein determinations

Protein was measured by the procedure of Lowry *et al.* (1951), with crystalline bovine serum albumin (fraction V) as the standard.

Sources of reagents

Amino acids, trichloroacetic acid and inorganic compounds were supplied by BDH, Poole, Dorset, U.K. γ -Glutamyl hydroxamate, all cofactors and protein standards were from Sigma (London) Chemical Co., London S.W.6, U.K., except for aldolase, which was purchased from Boehringer Corp. (London) Ltd., London W.5, U.K.

Results

Glutamyl kinase activity of crude cell-free extracts

Previous attempts (H. Gamper & V. Mořes, unpublished work) to demonstrate a glutamyl kinase inhibited by proline in crude extracts of *E. coli* have failed; even though there was extensive formation of the expected hydroxamate, proline had no effect on its production.

To lessen the effect of any masking by the more active glutamine synthetase, cells were grown on glucose and NH_4^+ as the carbon and nitrogen sources respectively, as opposed to the glycerol and glutamate of the earlier work. The result was to yield a higher adenylation state of glutamine synthetase, i.e. with an AMP moiety bound to each of the subunits of the enzyme, thus giving a decrease in the Mg^{2+} -dependent activity (Kingdon & Stadtman, 1967). Although the change in the growth medium decreased the rate of hydroxamate formation in the presence of Mg^{2+} by 82%, proline was still unable to inhibit the reaction, suggesting that during the incubation period proteinases in the extract released sufficient proline to inhibit the glutamyl kinase, so that glutamine synthetase alone was being detected.

Glutamyl kinase activity after fractionation of crude extract on Bio-Gel A1.5 M

Both glutamyl kinase and glutamyltransferase were measured, the latter because it is a known activity of glutamine synthetase (Woolfolk *et al.*, 1966; Tate & Meister, 1973) and could therefore be used to distinguish that system from the glutamyl kinase susceptible to inhibition by proline.

The state of adenylation of glutamine synthetase also modifies certain properties of this reaction: in particular, Mn^{2+} activates the transferase activity of both the adenylated and the non-adenylated species, giving a greater specific activity with the latter, whereas Mg^{2+} gives a reaction only with the adenylated form (Stadtman *et al.*, 1970). Consequently the greater the degree of adenylation (ranging from 1 to 12 AMP molecules/molecule of glutamine synthetase), the less the Mg^{2+} -dependent activity and the greater the Mn^{2+} -dependent activity. The conditions of growth adopted in the present work were designed to give the adenylated species in anticipation of avoiding the masking of the proline-inhibited glutamyl kinase.

With extract from strain X210 (*pro*⁺), the glutamyl kinase assay gave two peaks of activity on Bio-Gel chromatography. The mixture of five amino acids (as described in the Experimental section for the transferase assay) failed to affect either of the two reactions, whereas proline decreased or eliminated the second peak only (Fig. 1a). The proline-inhibited activity was eluted in fractions 25–26, corresponding to a

Fig. 1. Glutamyl kinase activity in fractions eluted from Bio-Gel A1.5M after application of crude extract of strain X210 (wild-type)

(a) Mg²⁺-dependent activity. ▲, Control assay; ○, +proline (100mM); □, +amino acid mixture; ●, proline-inhibitable kinase (by difference). (b) Mn²⁺-dependent activity. ▲, Control assay; ○, +proline (100mM); □, +amino acid mixture; ●, activity inhibited by the amino acid mixture (by difference).

mol.wt. approx. 250000 as estimated from the calibration curve reported by Hayzer & Moses (1978, Fig. 8). The enzyme responsible for peak I had a mol.wt. of about 550000, compared with the known mol.wt. of glutamine synthetase, 600000 (Shapiro & Ginsberg, 1968; Valentine *et al.*, 1968).

The identity of peak-I enzyme was confirmed as glutamine synthetase by replacement of Mg²⁺ by Mn²⁺, resulting in a dramatic increase in this activity, which could be inhibited by the amino acid mixture, but not by proline (Fig. 1b). Furthermore the glutamyltransferase assay showed a single peak in the same position as peak I, which was inhibited by the five amino acids and activated by Mn²⁺ (Fig. 2).

Synthesis of hydroxamate was totally dependent on the presence of glutamate or glutamine (in the kinase and transferase assays respectively), but a broad peak of hydroxamate formation independent of cofactor, metal and anion was established, centring on fractions 22–23. This residual activity was not normally corrected for and it would account for the extensive trailing of peak I in the transferase assay, arguing against the proline-inhibited enzyme being capable of a similar reaction.

Cell extracts from strains X278 (*proB*) and X277 (*proC*), defective in the second and third enzymes of the proline pathway respectively, gave profiles of

enzyme activity identical with those of the *pro*⁺ strain. In no case was any capacity for hydroxamate synthesis eluted after fraction 30.

Crude cell extract of strain X680 (*proA*), defective in the gene for the glutamyl kinase, was completely different. Glutamine synthetase was again located, mainly as the adenylated form, as witnessed by its greater activity with Mn²⁺ ions (Fig. 3a). No glutamyl kinase sensitive to proline was observed. The fractions with the high Mg²⁺-dependent kinase activity were eluted earlier than expected for a proline-inhibited glutamyl kinase (fractions 22–23 as opposed to 25–26). Further, the peak-II activity from strain X680 was independent of ATP and Mg²⁺ and appeared to be similar to the cofactor-independent activity of the other three strains. The glutamyltransferase assay (Fig. 3b) gave a prominent peak in the same position, in addition to that corresponding to glutamine synthetase, again requiring only glutamine.

Fractionation of crude cell extract on Sephadex G-150

Glutamyl kinase and glutamyltransferase activities. To improve the resolution of the two enzymes glutamine synthetase and the proline-inhibited glutamyl kinase, a column of Sephadex

Fig. 2. Glutamyltransferase activity in fractions eluted from Bio-Gel A1.5 M after application of crude extract of strain X210

Assay system with Mg^{2+} (■) and Mn^{2+} (□); assay system + amino acid mixture + Mg^{2+} (●); assay system + proline (100 mM) + Mg^{2+} (○).

G-150 was used, which had a smaller fractionation range than Bio-Gel A1.5M. The results of the glutamyl kinase assay, after fractionation of extracts of strains X210 and X680, are shown in Figs. 4(a) and 4(b). With the extract from the *pro*⁺ strain, two proline-sensitive glutamyl kinase activities were located in the fractions, with mol.wts. of 125 000 (peak B) and 38 000 (peak C) (Fig. 5). Peak A represents glutamine synthetase, as shown by the effects of changes in the bivalent cations on the transferase assay (Fig. 6). Extracts of strain X680 gave only the lighter species of the kinase (peak C) and, unlike that with Bio-Gel, proline was inhibitory (Fig. 4b). Strains X278 and X277 gave activity profiles similar to those of strains X680 and X210 respectively (Fig. 7).

As noted with the Bio-Gel column, extract of strain X680 passed through Sephadex G-150 gave a very prominent cofactor-independent 'glutamyltransferase' activity that was eluted in approximately the same position as the peak-B kinase. With this assay all other extracts showed glutamine synthetase only. The possibility was considered that this peak of cofactor-independent activity from strain X680 was due to a glutaminase, or possibly an asparaginase with the ability to hydrolyse glutamine (Campbell & Mashburn, 1969). Indeed, fractions eluted after application of strain-X210 extract contained a very active asparaginase (Fig. 8) together with a glutaminase, although the profiles of the two peaks suggests that they were not the same enzyme. Both activities gave a peak in fractions 23–25, close to the strain-

Fig. 3. Glutamyl kinase and glutamyltransferase activities in fractions eluted from Bio-Gel A1.5 M after application of crude extract of strain X680 (*proA*)

(a) Glutamyl kinase: assay system with Mg^{2+} (▲) and Mn^{2+} (□); Mg^{2+} + proline (100 mM) (○); increase in activity after substitution of Mn^{2+} for Mg^{2+} (by difference) (△). (b) Glutamyltransferase: assay system with Mg^{2+} (▲); without cofactors (●).

X680 transferase; the latter may perhaps have been due to one or other of these two enzymes.

The pattern of two proline-inhibited glutamyl kinases being associated with the *pro*⁺ and *proC* strains, whereas only one species was found with the *proA* and *proB* strains, was not observed with the other set of *E. coli pro*[−] mutants carrying similar lesions, nor was the profile of transferase activity the same (Table 2). *E. coli* XA21 derivative strains, which are efficient proline excretors and hence presumably defective in the feedback regulation of the proline-biosynthetic pathway, also displayed two kinases inhibited by proline.

Neither kinase species was active with Mn^{2+} as the bivalent cation (Fig. 4a), but they did differ in their sensitivities to proline, that in peak B being totally inhibited by 25 mM-proline whereas that in peak C

Fig. 4. Glutamyl kinase activity in fractions eluted from Sephadex G-150 after application of crude extracts from strains X210 (wild-type; a) and X680 (*proA*; b)

$\blacktriangle$, Assay with Mg^{2+} ; $\circ$, with Mg^{2+} + 100mM-proline; $\square$, assay with Mn^{2+} ; $\bullet$, proline-inhibited kinase activity (by difference).

required 100–200mM-proline for maximum inhibition (Fig. 9).

The two enzymes were not interconvertible: after chromatography of strain-X210 extract, fractions 22–27 containing kinase B were pooled, concentrated by ultrafiltration and re-applied to the Sephadex column. Fractions 29–34 containing kinase C were similarly treated. In neither case did one kinase give rise to the other. Fractions 22–27 contained a residual amount of glutamine synthetase activity (Fig. 9).

A most significant feature of the results with Sephadex G-150 is that in no instance was a proline-inhibited glutamyl kinase found with a mol.wt. of

250000, i.e. one that would correspond to the activity seen with the Bio-Gel A1.5M separation.

Glutamyl phosphate reductase and pyrroline-5-carboxylate reductase activities The glutamyl phosphate reductase was assayed in the reverse direction to that for proline biosynthesis (see the Experimental section). Strain-X210 (*pro*⁺) extract gave no fractions capable of reducing NADP⁺. The probable explanation is a coupling between glutamyl phosphate reductase and that of pyrroline-5-carboxylate reductase, so that any NADPH formed was immediately re-oxidized.

This was overcome by the use of strain-X277 (*proC*) extract, lacking an active pyrroline-5-carboxyl-

Fig. 5. Calibration curve for the Sephadex G-150 column. The positions of the two species of the glutamyl kinase are indicated. (1), Dextran blue; (2), thyroglobulin; (3), fibrinogen; (4), pyruvate kinase; (5), aldolase; (6), bovine serum albumin; (7), ovalbumin; (8), carbonic anhydrase; (9), cytochrome c.

ate reductase. With such a preparation, fractions from Sephadex G-150 were identified that contained a pyrroline-5-carboxylate and P_i -dependent $NADP^+$ reductase activity (Fig. 7a), the enzyme having a mol.wt. of 125000. No other reductase peak was found with this assay and the strain-X277 extract was devoid of any $NADPH$ -dependent pyrroline-5-carboxylate reductase. Extracts from strains X210, X278 and X680 all contained a highly active pyrroline-5-carboxylate reductase (Fig. 7b) with a mol.wt. of 180000–200000. The specific activity of this enzyme was approx. 100 times that of either the kinase or phosphate reductase. Because of the presence of this enzyme it was not possible to assay for glutamyl phosphate reductase activity of strain-X278 and thus to demonstrate its absence. It should be noted that the assays were all carried out on fractions from the Sephadex columns, and although this resolved the two reductases the separation was not absolute, the residual pyrroline-5-carboxylate reductase being sufficient to couple with the very much less active glutamyl phosphate reductase.

Discussion

It has been demonstrated in the preceding paper (Hayzer & Moses, 1978) that passage of wild-type extract through Bio-Gel A1.5M gave fractions that, after concentration by ultrafiltration, were capable of synthesizing proline (or pyrroline-5-carboxylate) from glutamate; the molecular weight of this activity was in the range 2.9×10^5 – 5×10^5 , tending to the lower rather than the upper limit. The identification now of a single proline-inhibited glutamyl kinase of

Fig. 6. Glutamyltransferase activity in fractions eluted from Sephadex G-150 after application of crude extracts from strains X210 (wild-type; a) and X680 (*proA*; b) ▲, Assay with Mg^{2+} ; ○, with amino acid mixture; ●, without ADP.

mol.wt. 250000 suggests either that this is the size of the enzyme aggregate or that, on concentration of the fractions, an enzyme of this size combined with a glutamyl phosphate reductase to give a complex approaching 500000 mol.wt.

Information from fractionation on Sephadex G-150 supports the conclusion that what was observed on the Bio-Gel A1.5M column was an aggregate of both the glutamyl kinase and the glutamyl phosphate reductase, each of mol.wt. 125000. This supposes the aggregate of 250000 mol.wt. to have been stable to passage through Bio-Gel A1.5M, though dissociable on Sephadex G-150 into equal-sized subunits. Furthermore the glutamyl kinase is either a tetramer or a trimer. The possibility that the kinase observed with Bio-Gel was confused with the peak-B species from Sephadex G-150 is

Fig. 7. Enzyme activities of the proline-biosynthetic pathway of two strains after passage of crude extracts through Sephadex G-150

(a) Strain X277 (*proC*); (b) strain X278 (*proB*). ▲, Total kinase activity; ○, with proline (100 mM); ●, proline-inhibitable kinase activity (by difference); ■, glutamyl phosphate reductase; □, pyrroline-5-carboxylate reductase. Extracts of strains X210 and X680 gave enzyme activity patterns similar to that of strain X278 (b).

inconsistent with the reproducibility of the results and the calibration of the columns.

Contrary to the concept of an enzyme aggregate stable on only one type of gel is the observation that, with Sephadex G-150, extracts from strains X680 and X278 failed to give the higher-molecular-weight species of proline-inhibited kinase (peak B).

Fig. 8. Asparaginase and glutaminase activity in fractions eluted from Sephadex G-150 after application of crude extract of strain X210 (wild-type)

All assay tubes contained: $\text{NH}_2\text{OH}\cdot\text{HCl}$, 100mM; glutamine or asparagine, 50mM; enzyme sample, 0.125ml; all dissolved in Tris/HCl buffer, pH7.4, containing 6mM-2-mercaptoethanol, in a final volume of 0.25ml. After a 3h incubation period at 37°C, the Fe^{3+} -hydroxamate assay solution was added (0.375ml) and A_{535} recorded after removal of the denatured protein. ○, Asparaginase; ●, glutaminase.

Since both strains had genetic lesions believed to affect the enzymes that should make up the aggregates, peak B may be said to represent an enzyme complexed with the glutamyl phosphate reductase. Peak C would then contain the dissociated protein. These conclusions make the results with Bio-Gel more difficult to explain (unless they indicate the presence of a self-aggregated kinase or complex), and difficulties of interpretation remain.

Set-2 strains did not give patterns explicable in terms of an aggregate of mol.wt. 125000, i.e. with a heavy kinase being lost in *proA* and *proB* extracts. The observed pattern of data is further complicated by the possibility that in the intact complex hydroxylamine is prevented from reacting with the glutamyl phosphate intermediate: the need to protect γ -glutamyl phosphate was, after all, the reason for postulating in the first place an aggregate of the proline-biosynthetic enzymes. So the assay procedure for γ -glutamyl kinase, which depends on the use of hydroxylamine, may determine only that part of the enzyme activity not associated with a complex.

The differences in sensitivity of the kinase to proline inhibition indicate that the enzyme may undergo a conformational change when it is aggregated that affects its kinetic properties. A similar explanation may hold for the fact that Baich (1969a) found the kinase to be 80% inhibited by 1.0mM-proline, whereas in the present work (peak C, the lighter kinase) 200mM-proline was required before inhibition was total, although it should also be noted that Baich (1969a) measured P_i release, whereas we have determined hydroxamate formation.

Failure to detect a proline-resistant glutamyl kinase from proline-excreting strains might have resulted from: (i) the strains not being resistant to

Table 2. Summary of the glutamyl kinase and glutamyltransferase activity profiles of nine strains of *E. coli* after fractionation of the crude extracts on Sephadex G-150

Abbreviation: N.T., not tested.

Strain	Proline genotype	Kinase activity			Transferase activity	
		Peak A (glutamine synthetase)	Peak B	Peak C	Peak I (glutamine synthetase)	Peak in fractions 21-26*
X210	<i>pro</i> ⁺	+	+	+	+	—
X680	<i>proA</i>	+	—	+	+	+*
X278	<i>proB</i>	+	—	+	+	—
X277	<i>proC</i>	+	+	+	+	—
X454	<i>pro</i> ⁺	+	+	+	+	+†
X462	<i>proA</i>	+	+	+	+	‡
X474	<i>proB</i>	+	—	+	+	‡
X478	<i>proC</i>	+	—	+	+	‡
XA21 (4)	<i>pro</i> ⁺	+	+	+	N.T.	N.T.

* This activity did not require ADP, AsO_4^{3-} or bivalent cations.

† Prominent peak in fractions 27-32 only, which did not require cofactors.

‡ The peak was not a constant feature with this strain.

Fig. 9. Glutamyl kinase activity eluted from Sephadex G-150 after re-passage of fractions 22-27 (a) and fractions 29-34 (b), previously eluted from the same column after application of crude extract from strain X210 (wild-type) ▲, Glutamyl kinase activity; ○, with 25 mM-proline; ●, with 100 mM-proline; □, Mg^{2+} replaced by Mn^{2+} .

feedback control by proline, proline excretion resulting from some other mechanism, perhaps a pathway by-passing the kinase in the synthesis of glutamic γ -semialdehyde; (ii) the glutamyl kinase being modified when aggregated so that it lost its sensitivity to proline, whereas the dissociated enzyme, seen in the column effluent, was inhibitable; (iii) the assay conditions of 10 mM-ATP and 100 mM-glutamate being such as to over-ride the resistance and to permit the normal proline inhibition; (iv) the proline concentration of 100 mM *in vitro* was sufficient to inhibit the reaction, whereas the intracellular concentrations themselves had no effect.

The experiments described in this paper were designed to investigate the possible existence of an enzyme aggregate associated with the proline-biosynthetic pathway. The evidence obtained is of such a nature that one is unable to say for certain that the aggregate exists. The appropriate molecular weights of the glutamyl phosphate reductase and pyrroline-5-carboxylate reductase have been established as 125 000 and 190 000 respectively. γ -Glutamyl kinase, the first enzyme in the sequence, is probably a multimeric protein of mol.wt. 125 000, consisting of four subunits each of mol.wt. 25 000-40 000. If there is a protein aggregate, its molecular weight is likely to be either 125 000 or 250 000. Baich (1969b) has suggested that the enzymes of proline biosynthesis are mem-

brane-bound. If disruption of the cells in the French press detaches them from this membrane-bound location, the aggregate that seems to form in solution at high concentrations of protein might represent just that part of the total multimeric structure that can form an association in the absence of the membrane. To that extent the aggregate in solution may be an artifact, although yet representing an echo of the real state of affairs in the living cell.

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