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Purification and Characteristics of a γ-Glutamyl Kinase Involved in *Escherichia coli* Proline Biosynthesis

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γ-Glutamyl kinase, the first enzyme of the proline biosynthetic pathway, was purified to homogeneity from an *Escherichia coli* strain resistant to the proline analog 3,4-dehydroproline. The enzyme had a native molecular weight of 236,000 and was apparently comprised of six identical 40,000-dalton subunits. Enzymatic activity of the protein was detectable only in assays containing highly purified γ-glutamyl phosphate reductase, the second enzyme of the proline pathway. Plots of γ-glutamyl kinase activity as a function of glutamate concentration were sigmoidal, with a half-saturation value for glutamate of 33 mM, whereas plots of enzyme activity as a function of ATP concentration displayed typical Michaelis-Menten kinetics with a K_m for ATP of 4×10^{-4} M. Enzyme activity was insensitive to the glutamate analog L-methionine-DL-sulfoximine, but ADP was a potent competitive inhibitor. Characteristics of the enzyme were compared with those of a γ-glutamyl kinase partially purified from a 3,4-dehydroproline-sensitive *E. coli*. These results indicated that the only major difference was that the enzyme from the 3,4-dehydroproline-resistant strain was 100-fold less sensitive to feedback inhibition by proline.

Proline biosynthesis in *Escherichia coli* occurs through a series of three enzymatic reactions catalyzed by the gene products of the *proB*, *proA*, and *proC* loci:

$$\text{L-glutamate} + \text{ATP} \xrightarrow[proB]{Mg^{2+}} \gamma\text{-glutamyl phosphate} + \text{ADP} \quad (1)$$

$$\gamma\text{-glutamyl phosphate} + \text{NADPH} \xleftrightarrow{proA} \text{L-glutamic acid 5-semialdehyde} + \text{NADP}^+ + \text{P}_i \quad (2)$$

$$\text{L-glutamic acid 5-semialdehyde} \xleftrightarrow{\text{spontaneous}} \Delta^1\text{-pyrroline-5-carboxylate}$$

$$\Delta^1\text{-pyrroline-5-carboxylate} + \text{NADPH} \xrightarrow{proC} \text{L-proline} + \text{NADP}^+ \quad (3)$$

Reaction 1 is catalyzed by γ-glutamyl kinase (ATP:L-glutamate 5-phosphotransferase, EC 2.7.2.11) and most likely results in the production of an extremely labile intermediate, γ-glutamyl phosphate (1, 8). Due to the lability of this compound, it has been proposed that γ-glutamyl kinase functions in a complex with the second enzyme of the pathway, γ-glutamyl phosphate reductase (1) (GP-reductase; L-glutamate 5-semialdehyde:NADP$^+$ oxidoreductase [phosphorylating], EC 1.2.1.41). The product of reaction 2, L-glutamic acid 5-semialdehyde, undergoes a spontaneous cyclization to Δ^1-pyrroline-5-carboxylate, which is converted to proline through the action Δ^1-pyrroline-5-carboxylate reductase (L-proline:NAD[P]$^+$ 5-oxidoreductase, EC 1.5.1.2), the third enzyme in the pathway (1, 22).

The second and third enzymes of proline biosynthesis have been purified and characterized (5, 9, 19); however, the first enzyme, γ-glutamyl kinase, has only been studied in cell-free extracts or crude preparations (1, 2). Regulation of proline biosynthesis is thought to be exerted primarily through feedback inhibition of γ-glutamyl kinase by proline (1, 3). *E. coli* mutants which excrete proline can be obtained by selection for resistance to the toxic proline analog 3,4-dehydroproline (3, 21). Synthesis of L-glutamic acid 5-semialdehyde in these strains is less sensitive to feedback inhibition by proline, but the enzymatic basis for these mutants has not been documented.

The lack of pure γ-glutamyl kinase has hampered studies of regulation in the proline pathway and prevented analysis of the proposed proline biosynthetic complex (1, 6, 10). To more fully understand the enzyme organization and regulation of proline biosynthesis we initiated a biochemical analysis of the *proBA* gene products. In this report we describe the purification of a γ-glutamyl kinase (*proB*) and a GP-reductase (*proA*) and present evidence that an interaction between these two enzymes is necessary for γ-glutamyl kinase activity. In addition, it was demonstrated that mutation to 3,4-dehydroproline resistance (hence proline excretion) was directly attributable to a γ-glutamyl kinase enzyme with decreased sensitivity to proline feedback inhibition.

MATERIALS AND METHODS

Bacterial strains and growth. Strain BRL1945(pAD-13) is a *recA* derivative of the *E. coli* K-12 strain K802 (*hsdR2 supE lacY galK metA*) containing the plasmid pAD-13. Plasmid pAD-13 contains the *proBA* genes from a 3,4-dehydroproline-resistant *E. coli* strain cloned into the expression vector pGW7. The genes have been cloned into this vector in such a manner as to be under the control of a temperature-inducible λ P_L promoter (5; A. H. Deutch, K. E. Rushlow, and C. J. Smith, manuscript in preparation). Strain BRL806 is a

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χ278 (*proAB*, obtained from R. Curtiss III) derivative containing the plasmid pGE-1. This plasmid is a 15.8-kilobase derivative of plasmid pLC-7-19 (4), which contains the wild-type *proBA* genes from *E. coli*.

Bacterial cultures were grown in a complex medium (YET broth; 10 g of Bacto-Tryptone, 5 g of yeast extract, 5 g of NaCl per liter) for all experiments. For enzyme purification from strain BRL1945(pAD-13), 1-liter cultures of YET broth containing 100 μg of ampicillin per ml were grown at 30°C with shaking to mid-log phase (absorbance at 660 nm [A_{660}] of 1.0). The culture temperature was then increased to 42°C and incubation was continued for 2 h. Cultures were harvested by centrifugation, washed in TD buffer (50 mM Tris, 1 mM dithiothreitol, pH 7.2), and stored as cell pellets overnight at 4°C. Strain BRL806 was grown in YET broth at 37°C with shaking until mid-log phase and then harvested as described above.

Enzyme assay. Two assays for γ-glutamyl kinase activity were utilized. The hydroxamate assay was essentially that described by Hayzer and Leisinger (7), and the reaction mixture contained the following in a final 0.25-ml volume at pH 7.0: 50 mM L-glutamate, 10 mM ATP, 20 mM $MgCl_2$, 100 mM hydroxylamine · HCl, 50 mM Tris base, and enzyme plus water. The reaction was carried out at 37°C for 15 to 30 min and then terminated by the addition of 1 ml of stop mix (55 g of $FeCl_3 \cdot 6H_2O$, 20 g of trichloroacetic acid, 21 ml of HCl per liter). The amount of γ-glutamyl hydroxamate was determined from the A_{535} by comparison to a standard curve prepared with authentic γ-glutamyl hydroxamate (Sigma Chemical Co.). One unit of activity was defined as the amount of enzyme required to produce 1 μmol of γ-glutamyl hydroxamate per min.

The second assay for γ-glutamyl kinase was a coupled assay based on the oxidation of NADPH by GP-reductase. The reaction contained the following in a final 1-ml volume at pH 7.0: 50 mM L-glutamate, 5 mM ATP, 20 mM $MgCl_2$, 0.15 mM NADPH, 50 mM Tris base, 0.5 U of GP-reductase, and enzyme plus water. GP-reductase was the highly purified protein prepared as described below. The reaction was initiated by the addition of NADPH, and the decrease in A_{340} at room temperature was recorded. One unit of γ-glutamyl kinase was the amount of enzyme necessary to oxidize 1 μmol of NADPH per min. In crude cell-free extracts, 1 U of γ-glutamyl kinase activity by the hydroxamate assay was equal to about 11 U of activity as measured by the coupled assay.

GP-reductase activity was measured by using the assay of Hayzer and Leisinger (7) except that the reaction mixture contained in a final 1-ml volume at pH 7.0: 2.5 mM DL-Δ^1-pyrroline-5-carboxylate, 1 mM NADP, 100 mM KH_2PO_4, 50 mM imidazole base, and enzyme plus water. The increase in A_{340} was recorded at room temperature, and 1 U of GP-reductase was defined as the amount of enzyme necessary to produce 1 μmol of NADPH per min.

Analytical methods. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was performed as described previously (15). Proteins were electrophoresed on 10% polyacrylamide slab gels (150 by 170 by 1.5 mm) containing 0.1% SDS, and protein bands were visualized by staining with Coomassie brilliant blue R-250.

Bio-Gel A-1.5 chromatography was performed with a column (95.5 by 1.5 cm) equilbrated with TD buffer. Protein standards were eluted at a flow rate of 8.1 ml h^{-1} and detected by A_{280}. The elution volumes of γ-glutamyl kinase and GP-reductase were determined by measurement of enzymatic activity.

DL-Δ^1-Pyrroline-5-carboxylate was synthesized by the procedure of Williams and Frank (23). Protein concentration was estimated by the method of Lowry et al. (16) as modified (18).

Enzyme purification. Cell-free extracts were prepared by resuspending cell pellets (0.5 g [wet weight] per ml) in TD buffer followed by sonication as described previously (5). Cellular debris was removed by centrifugation at 25,000 × *g* for 30 min at 4°C. All subsequent purification procedures were conducted at 4°C.

(i) γ-Glutamyl kinase. Cell-free extract (53 ml) was applied to a DEAE-cellulose column (2.5 by 40 cm) equilibrated with TD buffer. Protein was eluted from the column with a 0 to 0.5 M linear gradient of NaCl in 1.2 liters of TD buffer. Fractions (8.2 ml) containing γ-glutamyl kinase activity were pooled and applied directly to a column (2.5 by 15 cm) of Procion Red Agarose (Bethesda Research Laboratories) previously equilibrated with TD buffer. The column was washed with two volumes of TD buffer, and the protein was eluted with a 0.5 to 1.5 M linear gradient of KCl in 500 ml of TD buffer. Fractions containing γ-glutamyl kinase activity were pooled and dialyzed overnight against 100 volumes of TD buffer. This material was applied to a column (1.5 by 10 cm) of Cibacron Blue Agarose (Bethesda Research Laboratories), washed with 2 volumes TD buffer, and eluted with a linear gradient of ATP (0 to 200 mM) plus $MgCl_2$ (0 to 100 mM) in 150 ml of TD buffer adjusted to pH 7.0. The fractions containing γ-glutamyl kinase activity were pooled and dialyzed against 200 volumes of phosphate buffer (5 mM KH_2PO_4, 1 mM dithiothreitol, pH 7.0). This material was then applied to a column (1.5 by 27 cm) of hydroxylapatite, and the column was developed by running a linear gradient from 5 to 300 mM KH_2PO_4 (plus 1 mM dithiothreitol, pH 7.0). Fractions containing active enzyme were dialyzed against TD buffer and frozen at −70°C until needed.

(ii) GP-reductase. Cell-free extract (38 ml) was applied to a DEAE-cellulose column (2.5 by 40 cm) equilibrated with TD buffer. The enzyme was eluted with a linear gradient of NaCl (0 to 0.5 M) in 1.2 liters of TD buffer. The fractions containing GP-reductase activity were pooled and dialyzed two times against 20 volumes of phosphate buffer (10 mM KH_2PO_4, 1 mM dithiothreitol, pH 7.0). This material was then applied to a column (1.5 by 27 cm) of hydroxylapatite equilibrated with the same buffer. Active enzyme was eluted by washing the column with an additional 150 ml of the phosphate buffer. Eluant containing GP-reductase was applied directly to a second DEAE-cellulose column (2.5 by 15 cm), and active enzyme was eluted with a linear gradient of NaCl (0 to 0.5 M) in 500 ml of TD buffer. The most active fractions were pooled, dialyzed against TD buffer, and frozen at −70°C until needed.

RESULTS

Purification of γ-glutamyl kinase and GP-reductase. The use of GP-reductase as a reagent in the coupled assay required large amounts of the enzyme; thus, a simplified purification procedure based on the work of Hayzer and Leisinger (9) was developed. The γ-glutamyl kinase/GP-reductase-overproducing strain BRL1945(pAD13) was used as a source of the enzyme, and the purification scheme summarized in Table 1 consisted of only three steps. The final yield of GP-reductase activity was nearly 50% and represented a 46-fold purification over the crude extract. The purified enzyme was essentially free of contaminating protein and appeared as a single band after SDS-polyacrylamide gel electrophoresis (Fig. 1). The estimated molecular

TABLE 1. Purification of GP-reductase[a]

Enzyme fraction	Volume (ml)	Total protein (mg)	Total activity (U)	Recovery (%)	Sp act (U mg^{-1})	Fold purification
Crude extract	38	1,307	810	100	0.6	1
DEAE-cellulose	240	142	525	65	3.7	5.9
Hydroxylapatite	370	20.5	480	59	23.4	38
DEAE-cellulose	24.6	13.2	381	47	28.8	46

[a] Starting material was four 1-liter cultures of strain BRL1945(pAD13) grown as described in the text.

weight was about 42,000. This purification scheme consistently yielded between 12 and 20 mg of pure GP-reductase, which remained enzymatically stable for several months when stored at −70°C.

Initial attempts to purify γ-glutamyl kinase by using the hydroxamate assay to follow activity were for the most part unsuccessful. The enzyme could be fractionated by ammonium sulfate precipitation (45 to 65% saturation) and then recovered after Sephadex G-200 column chromatography. γ-Glutamyl kinase prepared in this manner appeared to be very stable, but all subsequent purification procedures resulted in 100% loss of enzyme activity. These results suggested the possibility that failure to purify the enzyme further was due to an inability to detect the enzyme activity rather than instability of the protein itself. Therefore, a coupled assay (see methods) employing the second enzyme of the proline biosynthetic pathway, GP-reductase, was developed to more accurately reflect the in vivo activity of γ-glutamyl kinase.

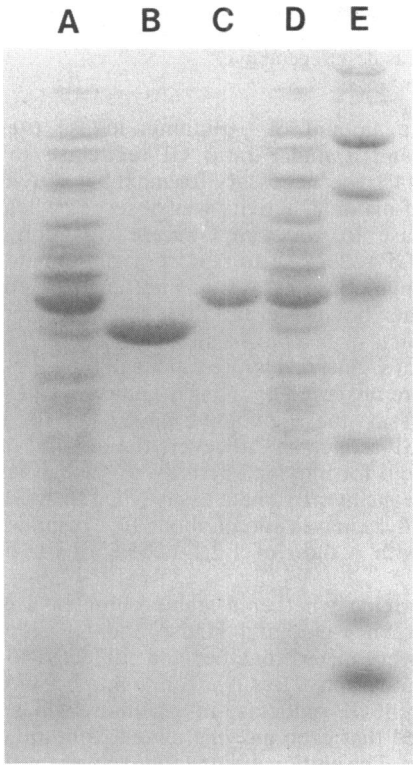

FIG. 1. SDS-polyacrylamide gel electrophoresis of γ-glutamyl kinase (DHP^r) and GP-reductase. Polyacrylamide gels (10%), prepared as described in the text, were used to analyze the purity of both enzymes. Lane A, 70 μg of protein of crude extract from strain BRL1945(pAD13); lane B, 5 μg of protein of purified γ-glutamyl kinase (DHP^r); lane C, 3 μg of protein of purified GP-reductase; lane D, 70 μg of protein of crude extract from strain BRL1945(pAD13). Lane E, high-molecular-weight standards (Bethesda Research Laboratories, Inc.): cytochrome *c*, 12,300; β-lactoglobulin, 18,400; α-chymotrypsinogen, 25,700; ovalbumin, 43,000; bovine serum albumin, 68,000; phosphorylase b, 92,500; myosin (H chain), 200,000.

Use of the coupled assay for detection of γ-glutamyl kinase activity facilitated its purification as summarized in Table 2. The source of enzyme was the 3,4-dehydroproline-resistant, γ-glutamyl kinase/GP-reductase-overproducing strain BRL1945(pAD13). DEAE-cellulose chromatography of crude extracts followed by chromatography on the affinity matrix Procion Red Agarose resulted in a 20-fold purification of the enzyme. Total γ-glutamyl kinase activity recovered from the Procion Red Agarose column was greater than the amount applied; this stimulation cannot be explained, but a similar observation was made when the matrix was used for the purification of Δ^1-pyrroline-5-carboxylate reductase (5). Chromatography on a second affinity matrix, Cibacron Blue Agarose, resulted in an apparent decrease in total enzyme activity. However, analysis by SDS-polyacrylamide gel electrophoresis indicated that this step was necessary for the selective removal of two contaminating protein bands. The final step, hydroxylapatite chromatography, resulted in the recovery of 5 mg of γ-glutamyl kinase, representing a yield of about 20%. The enzyme was free of contaminating proteins and contained no GP-reductase activity. Analysis on SDS-polyacrylamide gels (Fig. 1) showed a single protein band with a molecular weight of 40,000. Enzyme prepared in this manner and stored at −70°C showed no appreciable loss of activity over 3 months of storage. γ-Glutamyl kinase purified from strain BRL1945(pAD13) will be designated as γ-glutamyl kinase (DHP^r) for the remainder of this paper.

γ-Glutamyl kinase was also partially purified from strain BRL806, which contains the wild-type *proBA* genes on a plasmid and is sensitive to the proline analog 3,4-dehydroproline. Crude extracts containing 0.05 U per mg of protein were subjected to DEAE-cellulose chromatography followed by Procion Red Agarose chromatography as described above. Active enzyme eluted from Procion Red Agarose was purified 14-fold (0.71 U per mg of protein) with a yield of 20%; no detectable GP-reductase activity was observed in this material. Enzyme obtained from BRL806 will be designated as γ-glutamyl kinase (w^+) for the remainder of this paper.

Properties of γ-glutamyl kinase. Activity of γ-glutamyl kinase (DHP^r or w^+) displayed a linear response with respect to the amount of protein added when measured by the coupled assay. For γ-glutamyl kinase (DHP^r), this response range was between 0.15 and 0.8 μg of protein per assay. The reaction was also linear with respect to time for at least 10 min. In addition, preincubation of the complete assay mixture minus NADPH for up to 10 min had no effect on the enzyme stability or activity or on the rate or extent of the reaction.

The product formed during the coupled assay was apparently L-glutamic acid 5-semialdehyde (Δ^1-pyrroline-5-carboxylate). This was determined by addition of 0.5 U of homogeneous Δ^1-pyrroline-5-carboxylate reductase (*proC*) (5) to the coupled assay. Addition of this enzyme resulted in an increase in the rate of NADPH oxidation to exactly twice that of an assay mixture without Δ^1-pyrroline-5-carboxylate reductase (data not shown). This is the precise result expected since an additional molecule of NADPH would be utilized for every L-glutamatic acid 5-semialdehyde (Δ^1-pyrroline-5-carboxylate) molecule produced. Addition of DL-Δ^1-pyrroline-5-carboxylate to the standard coupled assay in the absence of Δ^1-pyrroline-5-carboxylate reductase had no effect on enzyme activity.

The pH versus activity profile of γ-glutamyl kinase (DHP^r and w$^+$) with the coupled reaction was determined in Tris buffer. Maximal activity was observed over a broad range between pH 6.5 and 7.0, and then activity declined sharply, with only 50% of the maximal activity observed at pH 6.0 or 7.5. The pH optimum for GP-reductase alone is 7.0, with 50% maximal activity at pH 6.5 and 7.8 (9). The molecular weight of native γ-glutamyl kinase (DHP^r) was estimated to be 236,000 (three determinations) as determined by elution from a Bio-Gel A-1.5 column calibrated with known molecular weight markers (Fig. 2). The molecular weight of GP-reductase was also determined and found to be approximately 268,000 (two determinations).

Requirement for GP-reductase. γ-Glutamyl kinase (DHP^r and w$^+$) purified by the coupled assay showed absolutely no activity when measured by the commonly used hydroxamate assay. Production of γ-glutamyl hydroxamate could be restored by the addition of highly purified GP-reductase as shown for γ-glutamyl kinase (DHP^r) in Fig. 3. The amount of GP-reductase required to restore maximal activity far exceeded the amount of γ-glutamyl kinase present in the reaction, and a molar ratio GP-reductase to γ-glutamyl kinase of 44:1 was necessary for maximal activity. Approximately half-maximal activity was observed when the ratio of GP-reductase to γ-glutamyl kinase was only 1.6:1. The ability of GP-reductase to restore hydroxamate production in γ-glutamyl kinase assays was not due to a protein effect, since neither bovine serum albumin (50 μg per assay) nor crude extracts from a *proBA* strain (χ278) could stimulate the synthesis of γ-glutamyl hydroxamate in these assays. Similar results were observed by using γ-glutamyl kinase (w$^+$). The coupled assay for γ-glutamyl kinase by its very nature required GP-reductase; however, the amount of GP-reductase required for maximal activity was much less than that required in the hydroxamate assay (Fig. 3). A GP-reductase-to-γ-glutamyl kinase ratio of about 10:1 resulted in maximal activity, with a ratio of 1.2:1 necessary for half-maximal activity.

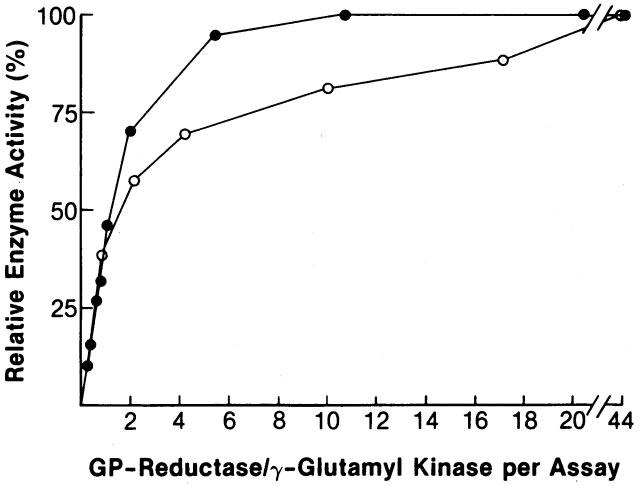

FIG. 3. Effect of GP-reductase on the activity of γ-glutamyl kinase (DHP^r). γ-Glutamyl kinase activity as determined by the hydroxamate assay (○) was measured as a function of the amount of GP-reductase added to each assay. The standard hydroxamate assay contained 0.55 μg of γ-glutamyl kinase and from 0 to 27 μg of GP-reductase. The ratio of GP-reductase to γ-glutamyl kinase was calculated by using molecular weights of 236,000 for γ-glutamyl kinase and 268,000 for GP-reductase. γ-Glutamyl kinase activity determined by the coupled assay (●) was measured as a function of GP-reductase added. The coupled assay contained 0.22 γg of γ-glutamyl kinase and 0 to 10.9 μg of GP-reductase. Ratios were determined as described above.

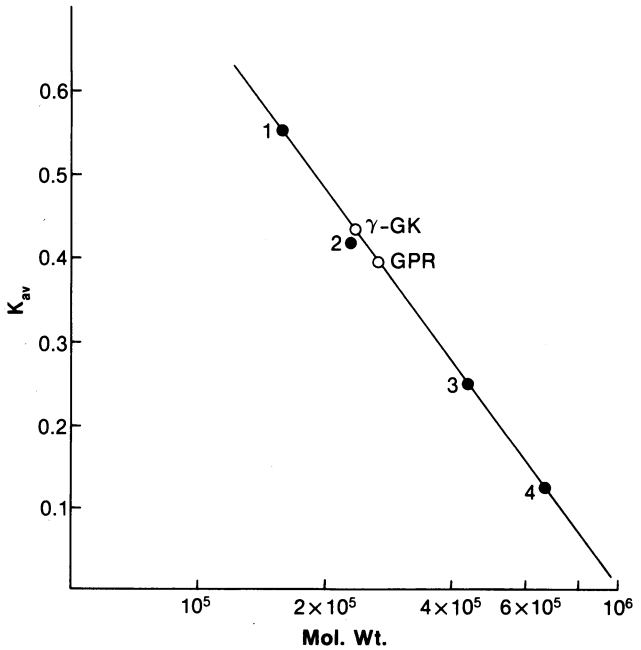

FIG. 2. Molecular weight estimation of γ-glutamyl kinase (DHP^r) and GP-reductase by BioGel A-1.5 chromatography. Standard proteins and their molecular weights were as follows: 1, aldolase, 158,000; 2, catalase, 232,000; 3, ferritin, 440,000; 4, thyroglobulin, 669,000. Abbreviations: γ-GK, γ-Glutamyl kinase (DHP^r); GPR, GP-reductase.

To determine whether a stable complex was formed in vitro between γ-glutamyl kinase and GP-reductase, both enzymes were mixed together and subjected to Bio Gel A-1.5 chromatography. Results from a number of experiments with different GP-reductase-to-γ-glutamyl kinase (DHP^r) ratios showed that each enzyme eluted from the column as a single peak. The elution volume for each was nearly identical to that observed when the enzymes were chromatographed separately (Fig. 2 and data not shown).

Kinetic studies with γ-glutamyl kinase. Plots of enzyme activity versus glutamate concentration for both γ-glutamyl kinase (DHP^r and w$^+$) were nonhyperbolic (Fig. 4). The concentrations of glutamate which yielded half-maximal activity were 33 and 37 mM for the DHP^r and w$^+$ enzymes, respectively. The glutamate analog L-methionine-DL-sulfoximine had no effect on either of the γ-glutamyl kinases when tested at concentrations as high as 50 mM. Addition of

TABLE 2. Purification of γ-glutamyl kinase (DHP^r)[a]

Enzyme fraction	Volume (ml)	Total protein (mg)	Total activity (U)	Recovery (%)	Sp act (U mg^{-1})	Fold purification
Crude extract[b]	53	1,823	1,065	100	0.58	1
DEAE-cellulose	160	864	814	76	0.94	1.6
Procion Red Agarose	54	83	980	92	11.8	20.2
Cibicron Blue Agrose	41	32.8	375	35	11.5	19.6
Hydroxylapatite	29.7	5.02	206	19.5	41	70.7

[a] The starting material was six 1-liter cultures of strain BRL1945(pAD13) grown as described in the text. γ-Glutamyl kinase activity was measured by the standard coupled assay.

[b] γ-Glutamyl kinase activity in crude extracts was calculated by subtracting the activity observed in the absence of ATP from that observed in the complete assay mixture and then dividing by 2. This calculation corrected for background activity and accounted for the oxidation of NADPH by Δ^1-pyrroline-5-carboxylate reductase, which was present in crude extracts.

proline to reaction mixtures of either γ-glutamyl kinase (DHP^r or w$^+$) increased the sigmoidicity of the glutamate saturation curves and increased the concentration of glutamate necessary for half-maximal activity (Fig. 4). However, the DHP^r γ-glutamyl kinase was about 100-fold less sensitive to proline inhibition than the w$^+$ enzyme (Fig. 4 and 5). Fifty percent inhibition of the w$^+$ enzyme was observed in the presence of 7×10^{-6} M proline, whereas 7×10^{-4} M proline was required to inhibit 50% of the DHP^r enzyme activity. In addition, both γ-glutamyl kinases were sensitive to inhibition by proline analog 3,4-dehydroproline, but the DHP^r enzyme was about 250-fold less sensitive to this inhibition. The DHP^r and w$^+$ enzymes were inhibited 50% by 5×10^{-3} and 2×10^{-5} M 3,4-dehydroproline, respectively (data not shown).

In contrast to glutamate, plots of enzyme activity as a function of ATP concentration displayed typical Michaelis-Menten kinetics. Double-reciprocal plots for both γ-glutamyl kinases were used to estimate the K_m values for ATP, and the values obtained were 4×10^{-4} and 5×10^{-4} M for the DHP^r and w$^+$ (data not shown) enzymes, respectively. ADP was found to strongly inhibit the γ-glutamyl kinase reaction. Double-reciprocal plots of enzyme activity versus ATP concentration at different ADP concentrations suggest this was a competitive inhibition, and the estimated K_i for ADP was 6×10^{-5} M (data not shown). Nucleotide monophosphates such as AMP, TMP, GMP, and CMP had no effect on γ-glutamyl kinase (DHP^r and w$^+$) activity.

DISCUSSION

The purification procedures described for γ-glutamyl kinase (DHP^r) and GP-reductase yielded pure, apparently homogeneous proteins. In wild-type *E. coli*, both of these enzymes exhibit very low specific activities, making their measurement difficult. One important factor contributing to the success of these purification procedures was the use of strain BRL1945(pAD13) containing the *proBA* DHP^r genes cloned in an expression vector (Deutch et al., in preparation). A second factor important to purification was use of the coupled assay for detection of γ-glutamyl kinase activity. Previous attempts to purify the enzyme by using the hydroxamate assay have failed (1, 10, 14; D. J. Hayzer, personal communication) because of the apparent requirement for GP-reductase. Studies with *Pseudomonas aeruginosa* showed that a partially purified preparation of the enzyme was enriched twofold for GP-reductase activity, and the γ-glutamyl kinase activity was unstable to all subsequent purification steps (14). These results suggest a similar requirement for GP-reductase by *P. aeruginosa* γ-glutamyl kinase.

The molecular weight of the γ-glutamyl kinase (DHP^r) subunit was 40,000 (Fig. 1), and this agrees closely with the smallest form (38,000) of a proline-inhibitable kinase observed upon fractionation of *E. coli* crude extracts (10). These results are also consistent with analysis of the *proB* DNA sequence, which predicts a single polypeptide of 38,952 molecular weight (Deutch et al., in preparation). The native form of γ-glutamyl kinase (DHP^r) was estimated to have a molecular weight of 236,000, suggesting a composi-

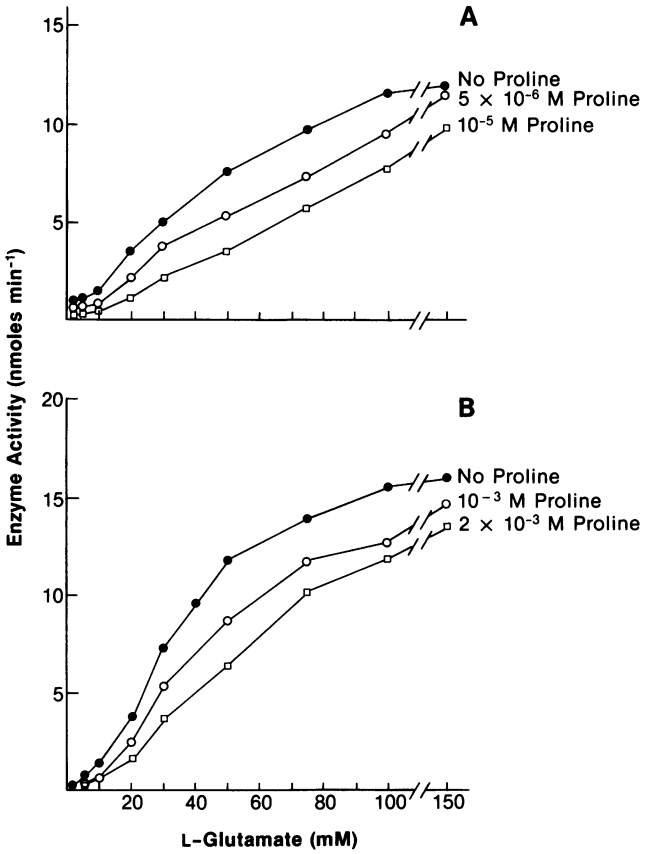

FIG. 4. Effect of L-glutamate concentration on the activity of γ-glutamyl kinase in the presence or absence of L-proline. The standard coupled assay was used to measure activity except that the concentrations of L-glutamate and L-proline were altered as indicated. Results were the average of three independent experiments. (A) γ-Glutamyl kinase (w$^+$). Each assay contained 10.4 μg of protein. (B) γ-Glutamyl kinase (DHP^r). Each assay contained 0.275 μg of protein.

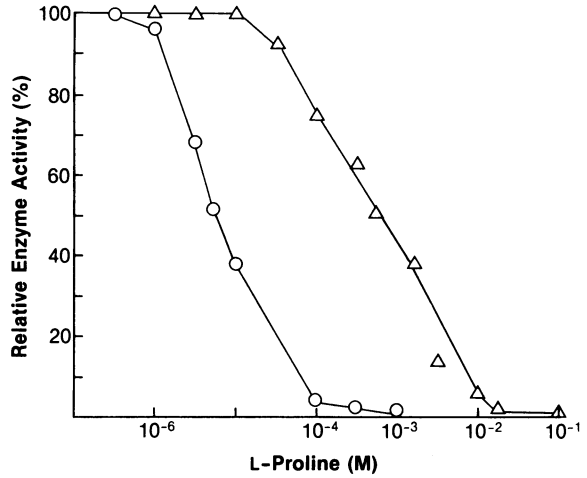

FIG. 5. Effect of L-proline on γ-glutamyl kinase activity. The standard coupled assay containing 50 mM glutamate was used to measure activity in the presence of proline as indicated. Symbols: ○, γ-glutamyl kinase (w^+); △, γ-glutamyl kinase (DHP^r).

tion of six identical subunits (Fig. 2). GP-reductase was also composed of a single subunit species with an estimated molecular weight of 42,000 (Fig. 1). This size estimate is reasonably close to that of a previous report of 47,000 (9) and is in close agreement with the 43,503 molecular weight deduced from the DNA sequence of the *proA* gene (Deutch et al., in preparation). Results of BioGel A-1.5 chromatography of native GP-reductase suggested that the enzyme had a molecular weight of 268,000 (Fig. 2). This is in contrast to the previously published molecular weight of 189,000 (9) but may possibly be accounted for by differences in purification or extraction procedures or both. Based on the data presented here, GP-reductase is probably composed of six identical subunits.

The most noteworthy observation of the present communication was a lack of detectable γ-glutamyl kinase activity in hydroxamate assays without GP-reductase added (Fig. 3). These results together with previously published reports (1, 8, 10) strongly suggest that a γ-glutamyl kinase-GP-reductase enzyme complex is required for proline biosynthesis in *E. coli*. In addition, two lines of evidence suggest that GP-reductase need not be enzymatically active to restore γ-glutamyl kinase activity. First, γ-glutamyl kinase activity was observed in the hydroxamate assays which contained GP-reductase but lacked NADPH, a cofactor required for GP-reductase activity. Second, a number of γ-glutamyl kinase-positive GP-reductase-negative mutants have been previously identified by the hydroxamate enzyme assay (7). The most likely explanation for the ability to detect these strains is a mutation in the *proA* gene which results in an enzymatically inactive protein still capable of interacting with γ-glutamyl kinase.

Enzyme complexes are not uncommon in amino acid biosynthesis, and as with the γ-glutamyl kinase-GP-reductase system, the *E. coli* anthranilate synthetase complex catalyzes sequential reactions in an enzymatic pathway. Activity of anthranilate synthetase component I (*trpE*) with glutamine as substrate requires the presence of phosphoribosyl anthranilate transferase (component II, *trpD*) or enzymatically inactive, immunologically cross-reacting material (13). Component I, however, does retain activity with ammonia as substrate in the absence of component II, and it has been shown that component II contains the glutamine binding site (13, 17). We believe it is unlikely that GP-reductase contains any of the substrate binding sites for γ-glutamyl kinase; rather, the results support the idea that the labile *proB* end product, γ-glutamyl phosphate, exists in an enzyme-bound state (1) and that GP-reductase interacts with γ-glutamyl kinase, effecting the release of γ-glutamyl phosphate which can then be measured as the hydroxamate derivative. Enzyme complexes may also have an affect on the regulation of its components as shown by the altered sensitivity to tryptophan of anthranilate synthetase components in the complexed state (13). There is currently no evidence for this type of interaction between GP-reductase and γ-glutamyl kinase, but it is clear the GP-reductase from a 3,4-dehydroproline-resistant mutant can function normally with the wild-type γ-glutamyl kinase and apparently does not alter the sensitivity to proline inhibition.

The enzyme data described above indicate that some type of enzyme complex is required for γ-glutamyl kinase activity; however, our initial experiments with gel filtration chromatography failed to detect the proposed complex. The combined molecular weight of the enzyme complex would be on the order of 500,000, but no γ-glutamyl kinase or GP-reductase activity was seen in the corresponding BioGel A-1.5 fractions. It is possible that chromatographic conditions were not adequate for complex formation. Although the *E. coli* anthranilate complex does not appear to have any special requirements for complex formation (12, 13), *Bacillus subtilis* anthranilate synthase and its glutamine-binding component only form a complex in the presence of the substrate glutamine (11). Future studies should be directed toward defining the parameters necessary for the GP-reductase-γ-glutamyl kinase interaction.

The substrate saturation kinetics of γ-glutamyl kinase (DHP^r and w^+) activity were nonhyperbolic with respect to glutamate concentration (Fig. 4). Generally, the enzyme was insensitive to small changes in glutamate concentration, and high levels of this substrate were required for saturation. A similar response for γ-glutamyl kinase in crude, GP-reductase-contaminated enzyme preparations has been noted previously (1, 3). The concentration of glutamate necessary for half-maximal activity was in the range of 30 to 40 mM for both the DHP^r and w^+ enzymes. This concentration is about 10-fold greater than the glutamate levels normally seen in the cell (20), and the reasons for such a high value are not currently known. One possibility is that a positive effector metabolite which modulates γ-glutamyl kinase activity has not yet been identified.

Proline (or 3,4-dehydroproline) was found to inhibit both the DHP^r and w^+ γ-glutamyl kinase enzymes, but a 100-fold greater concentration of proline was required to inhibit the DHP^r enzyme than was required to inhibit the w^+ enzyme (Fig. 5). The effect of proline was to decrease the affinity of the enzyme for glutamate at low or intermediate concentrations of this substrate, but the inhibition could be partially overcome at very high glutamate concentrations (Fig. 4). There was no measureable effect of proline on GP-reductase activity of the purified preparations used in this study. In that the same GP-reductase enzyme was used for the determination of both DHP^r and w^+ γ-glutamyl kinase activity, it appears that mutation to 3,4-dehydroproline resistance is due to an altered γ-glutamyl kinase enzyme.

The substrate saturation kinetics when ATP was the variable substrate displayed the typical Michaelis-Menten relationship. The apparent K_m values obtained for the DHP^r and w^+ enzymes were very close, 4×10^{-4} and 5×10^{-4} M,

respectively. Strong competitive inhibition was observed when ADP, but not AMP, was added to reaction mixtures. The fact that both the DHP^r and w$^+$ enzymes were competitively inhibited to the same extent by ADP (data not shown) is a clear indication that modulation by ADP and proline are independent and that ADP probably binds at the ATP site.

From the results discussed above, it would appear that γ-glutamyl kinase activity is regulated in at least three ways. First, the enzyme is insensitive to small changes in glutamate concentration. This apparent negative cooperativity would insulate proline biosynthesis from rapid changes in glutamate pool levels, thus allowing the cell to maintain a relatively constant level of proline production under a variety of growth conditions. A second mode of control involves inhibition by ADP. Regulation at this level would make proline biosynthesis responsive to cellular energy levels and ensure there was no drain on ATP pools. Finally, γ-glutamyl kinase activity is controlled by the end product of the pathway, proline. This point of control is by far the most important, since removal of this control by mutation (e.g., 3,4-dehydroproline resistance) leads to excretion of proline. This idea is supported by evidence that the only difference between DHP^r γ-glutamyl kinase and the w$^+$ enzyme is that the former shows decreased sensitivity to proline inhibition. All other characteristics of these enzymes appear to be nearly identical.

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