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Characterization of a γ-Glutamyl Kinase from *Escherichia coli* That Confers Proline Overproduction and Osmotic Tolerance

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Mutation(s) in the *proBA* operon of *Escherichia coli* confers proline overproduction and enhanced osmotic tolerance in enteric bacteria (L. N. Csonka, Mol. Gen. Genet. 182:82–86, 1981; M. J. Mahan and L. N. Csonka, J. Bacteriol. 156:1249–1262, 1983). A glutamate-dependent ATPase assay was developed and used to determine *proB*-encoded γ-glutamyl kinase activity in the absence of glutamate-γ-semialdehyde dehydrogenase. This assay indicated that the feedback insensitivity of mutant γ-glutamyl kinase was independent of glutamate-γ-semialdehyde dehydrogenase. However, the capacity of glutamate-γ-semialdehyde dehydrogenase from the osmotolerant mutant to interact with the kinase was altered in thermal stability, suggesting that mutations in both *proB* and *proA* may be required for osmotolerance.

In *Escherichia coli*, proline is synthesized from glutamate by the following series of reactions.

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

$$\gamma\text{-glutamyl phosphate} + \text{NADPH} \rightarrow \text{glutamate-}\gamma\text{-semialdehyde} + \text{NADP}^+ + P_i \quad (2)$$

$$\text{glutamate-}\gamma\text{-semialdehyde} \xrightarrow{\text{spontaneous}} \text{1-pyrroline-5-carboxylate}$$

$$\text{1-pyrroline-5-carboxylate} + \text{NADPH} \rightarrow \text{proline} + \text{NADP}^+ \quad (3)$$

γ-Glutamyl kinase, the focus of this communication, catalyzes reaction 1, the activation of glutamate to γ-glutamyl phosphate (3). The only known regulatory mechanism of the proline biosynthetic pathway is the feedback inhibition of this enzyme by proline (3). In reaction 2, γ-glutamyl phosphate is reduced to glutamate-γ-semialdehyde by the action of glutamate-γ-semialdehyde dehydrogenase (4, 10). Evidence has been presented that γ-glutamyl kinase and glutamate-γ-semialdehyde dehydrogenase occur as a complex (11, 21) and that γ-glutamyl kinase activity is undetectable in the absence of the dehydrogenase (21). The product of reactions 1 and 2, glutamate-γ-semialdehyde, cyclizes spontaneously to form an internal Schiff base, 1-pyrroline-5-carboxylate, which in turn is reduced to proline by pyrroline-5-carboxylate reductase (20). The genes coding for these enzymes are *proB*, *proA*, and *proC* for γ-glutamyl kinase, glutamate-γ-semialdehyde dehydrogenase, and pyrroline-5-carboxylate reductase, respectively. *proC* is located at about 9 min on the *E. coli* chromosome map (2), while both *proA* and *proB* map at about 6 min on the *E. coli* chromosome (2). *proA* and *proB* have been sequenced and shown to form an operon with the direction of transcription from *proB* to *proA* (8).

In addition to its roles in protein synthesis and as carbon and nitrogen sources, proline can act as a protectant against osmotic stress. In this mechanism, proline (or other osmoprotectants) protects the cell against osmotic stress by accumulating in the cytosol to restore the optimum osmotic differential between the cell and the environment (13, 23). The ability of proline to enhance the growth rate of *Salmonella oranienburg* grown at inhibitory levels of osmotic strength was reported by Christian (5, 6). That observation led to the isolation of a rare mutant strain of *Salmonella typhimurium* (TL126) that carries a mutation which confers both the overproduction of proline, via its usual biosynthetic pathway, and osmotolerance (7).

The mutation *pro-74* arose in *E. coli* episome F'_{126} which was carried by strain TL126. Recently, a 10.4-kilobase fragment of the F'_{126} (*pro-74*) episome was cloned into pBR322 and found to contain *proBA* (16). The mutation conferring proline overproduction was localized in *proB* (16). The authors suggested two possible changes in *proB* to account for the proline overproduction phenotype. Either the γ-glutamyl kinase (*proB*) specific activity is increased compared with that of wild type or the feedback sensitivity is lost allowing the kinase to produce an excess of the intermediate, γ-glutamyl phosphate.

Even though the mutation for proline overproduction is in *proB* (γ-glutamyl kinase), it is possible that a double mutation, one in *proB* and the other in *proA* (glutamate-γ-semialdehyde dehydrogenase), is necessary for the osmotolerance phenotype. The fact that the osmotolerance phenotype is very rare (7) is consistent with this possibility.

In this report, a kinetic analysis of γ-glutamyl kinase and glutamate-γ-semialdehyde dehydrogenase is carried out to identify the biochemical nature of the *pro-74* mutation. Preliminary results have appeared elsewhere (L. T. Smith, Fed. Proc. 43:1691, 1984).

MATERIALS AND METHODS

Materials. All chemicals were reagent grade and were used without further purification. Pyrroline-5-carboxylate was regenerated from its 2,4-dinitrophenylhydrazone derivative with acetophenone as described previously (17).

Bacterial strains and culture conditions. The bacterial strains and plasmids used in this work are given in Table 1. The complex medium used was LB (18) and the minimal medium was M63 (18) supplemented with 20 mg of thiamine per liter. Solid media contained, in addition, 15 g of agar per liter. Antibiotic supplements used, when indicated, were 25 mg of ampicillin per liter and 12.5 mg of tetracycline per liter.

Growth measurements. Bacterial cultures were grown in LB plus tetracycline for about 5 h, subcultured into M63 plus tetracycline at a 1:50 dilution, and grown to saturation overnight. The cells were subcultured into minimal medium plus tetracycline, allowed to double two times, and used to

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Reference or source
E. coli		
CSH26	Δ(*lac-proBA*) *thi ara*	18
CM26	CSH26 containing pLA1	16
CM126	CSH26 containing pLA101	16
CM126V	CSH26 containing pLA101V	This study
Plasmids		
pLA1	pBR322 with 10.4-kilobase insert containing wild-type *proBA*	16
pLA101	pBR322 with 10.4-kilobase insert containing mutant *proBA* (*pro-74*)	16
pLA101V	Spontaneous variant of pLA101 conferring less proline overproduction and osmotolerance than pLA101	This study

inoculate (1%) the final minimal medium containing tetracycline with or without 0.5 M NaCl. Cells were grown aerobically at 37°C, and growth was monitored with a Klett-Summerson colorimeter. To ensure that the plasmid had remained intact in the bacterial host at the end of each experiment, a sample of cells was diluted and plated on M63 supplemented with 0.23 g of proline per liter or M63 supplemented with tetracycline. For CM126 and CM126V, the cells were also tested for proline overproduction by stabbing colonies from the M63-tetracycline plates into M63 containing a lawn of CSH26 (Pro^-). A halo of growth of CSH26 was observed within 12 h around the stabs of the proline overproducers.

Measurement of intracellular proline concentration. Cells were grown to the late-log phase as described above for growth measurements. From 1 to 5 ml of culture was rapidly filtered, and the proline was extracted from the filtered cells with 5% perchloric acid. A sample of the centrifuged extract was lyophilized, and the proline was quantitated by amino acid analysis on a Beckman 6300 amino acid analyzer.

Enzyme preparation. Starter cultures were grown in LB medium plus tetracycline overnight and used to inoculate 1 liter of the same medium. Cultures were grown aerobically at 37°C for about 16 h. All further operations were carried out at 0 to 4°C except where noted. The cells were harvested by centrifugation at 5,000 × *g* for 10 min, and the cell paste was suspended in 8 ml of 50 mM Tris hydrochloride–1 mM dithiothreitol, pH 7.0 (Tris-dithiothreitol). Cells were disrupted by two passages through a French press at 4,000 lb/in^2 at room temperature, and the debris was removed by centrifugation at 31,000 × *g* for 35 min. The resulting crude extract was either dialyzed against two changes of Tris-dithiothreitol and then frozen at −70°C or was frozen undialyzed and thawed when needed for the following steps.

To separate γ-glutamyl kinase from glutamate-γ-semialdehyde dehydrogenase the following procedures were carried out. A 0.75-ml portion of 10% streptomycin sulfate was added, with stirring, to 5 ml of undialyzed crude extract over a 10-min period. The mixture was allowed to stir for 5 min further and to stand on ice for 20 min before being centrifuged at 27,000 × *g* for 15 min. The supernatant solution was brought to 35% saturation of ammonium sulfate, slowly with stirring, with solid ammonium sulfate. The mixture was stirred for 5 min, kept on ice for 15 min, and then centrifuged at 27,000 × *g* for 15 min. The precipitate was saved, and the supernatant was brought to 40% saturation of ammonium sulfate and centrifuged as outlined above, and the precipitate was discarded. A fraction precipitating between 40 and 75% saturation of ammonium sulfate was similarly made. The 0 to 35% fraction containing γ-glutamyl kinase and the 40 to 75% fraction containing glutamate-γ-semialdehyde dehydrogenase were each dissolved in 2 ml of Tris-dithiothreitol, dialyzed against two changes of buffer, and stored at −70°C.

Heat treatment. To test for the temperature sensitivity of γ-glutamyl kinase the following methods were used. Enzyme (100 μl) was incubated at 45°C in a water bath for the indicated times and then plunged into ice to stop the heat denaturation. Two minutes after the last tube was cooled, the tubes containing enzyme were quickly warmed to room temperature and immediately assayed for activity. The assay was initiated by the addition of the complete assay mixture into the tube containing the heat-treated enzyme. The effect of proline and other compounds on the heat stability of γ-glutamyl kinase was tested in the following way. Enzyme (150 μl) was diluted 10% with the compound to be tested. The mixture was heated and cooled as above, and the compound removed from the treated enzyme by centrifugal gel filtration (19) before assaying for activity. The values obtained were normalized to an untreated, diluted sample that was subjected to centrifugal gel filtration.

Enzyme and protein assays. Except where otherwise indicated, γ-glutamyl kinase activity was assayed by the method of Hayzer and Leisinger (9). The production of γ-glutamyl hydroxamate from glutamate, ATP, and NH_2OH was measured after a 5-min incubation at 37°C. Where indicated in the results section, the appearance of inorganic phosphate was used as a method to monitor γ-glutamyl kinase activity (ATPase assay) from ammonium sulfate-fractionated crude extracts. This assay utilizes the fact that in the absence of glutamate-γ-semialdehyde dehydrogenase, γ-glutamyl phosphate is rapidly hydrolyzed to glutamate and inorganic phosphate (12). The same conditions were used as for the hydroxamate assay, except that the hydroxylamine was omitted. The reaction was stopped with 0.75 ml of 6.5% trichloroacetic acid, the mixture was centrifuged, and 50 μl was removed to assay for the presence of inorganic phosphate (1). A correction was made for glutamate-independent ATPase. Under these assay conditions, nonenzymatic ATP hydrolysis was undetectable, and the assay was linear with time (up to 7 min) with up to 50 μl of enzyme. Glutamate-γ-semialdehyde dehydrogenase activity was assayed by measuring pyrroline-5-carboxylate-dependent $NADP^+$ reduction at room temperature by the method of Hayzer and Leisinger (10), except that 4 mM $NADP^+$ was used. Protein concentration of cell extracts and bacterial cultures was determined by the method of Lowry et al. (15) using bovine serum albumin as the standard.

RESULTS

Growth rates and intracellular proline requirement for osmotolerance. In view of the fact that three recombinant strains are now available, it was of interest to determine the relative osmotolerance and intracellular proline concentration of these strains and to compare these parameters with the enzymatic properties of the γ-glutamyl kinases. The generation times of all three strains were about the same when grown in minimal medium (Table 2). However, when

TABLE 2. Growth rates and intracellular proline accumulation of *E. coli* strains carrying wild-type or mutant γ-glutamyl kinase[a]

Strain	Growth rate (generations/h)		Intracellular proline concn (nmol of proline/mg of protein)	
	No NaCl	0.5 M NaCl	No NaCl	0.5 M NaCl
CM26	1.0	0.07	ND[b]	ND
CM126V	0.89	0.09	23 ± 11	40 ± 11
CM126	0.95	0.19	40 ± 5	690 ± 20

[a] Cultures were grown in M63 with or without 0.5 M NaCl as indicated.
[b] ND, Not detectable (<1.9 nmol of proline/mg of protein).

the medium was supplemented with 0.5 M NaCl, the mutant, CM126, grew at more than twice the rate of the wild type, CM26. The variant grew only about 30% better than the wild type.

Table 2 also lists the intracellular proline concentrations of CM26, CM126, and CM126V cultures grown in minimal medium with or without added NaCl. In the absence of salt, proline was undetectable in the wild-type strain, CM26, while the proline levels in CM126V and CM126 were at least 10-fold and 20-fold higher, respectively, than in the wild type. When the cultures were grown with 0.5 M NaCl, the intracellular concentration of proline increased to 40 and 690 nmol/mg of protein for CM126V and CM126, respectively, while in strain CM26, proline was undetectable. These results demonstrate that the variant, which shows an intermediate level of osmotolerance, also contains an intermediate level of proline, relative to CM26 and CM126.

Sensitivity of γ-glutamyl kinase to feedback inhibition by proline. Mahan and Csonka (16) proposed that γ-glutamyl kinase from the proline-overproducing mutant has either a higher specific activity in the cell or is less sensitive to feedback inhibition by proline compared with the wild-type enzyme, thus allowing an increased production of the intermediate, γ-glutamyl phosphate. To determine which of these possibilities is correct, the activity of γ-glutamyl kinase from crude extracts of CM26, CM126, and CM126V was measured as a function of proline concentration in the assay mixture. The mutant enzyme was less feedback inhibited by proline (Fig. 1): the concentration of proline required to decrease the velocity 50% (apparent K_i) was 0.089 mM for the wild type and 32 mM for the mutant, while the specific activity in the crude extract of both was approximately 35 nmol/min per mg. The residual level of activity remaining at high concentrations of proline is a contaminating activity because *E. coli* CSH26 (Δ*proBA*) bearing pBR322 gave a similar low level of activity (7 to 8 nmol/min per mg of protein). The apparent K_i for proline of γ-glutamyl kinase from CM126V was 1.4 mM (Fig. 1), an intermediate value between those of the kinases from CM26 and CM126, while the specific activity was nearly the same as that for the other two kinases. The addition of 0.5 M NaCl to the assay mixture did not alter the apparent K_i for proline in the wild type or mutant. Nor did salt in the growth medium significantly affect the specific activity of the resulting kinase. CM126 grown in M63 or M63 plus 0.5 M NaCl contained about the same amount of γ-glutamyl kinase (41 to 45 nmol/min per mg).

Together, Fig. 1 and Table 2 demonstrate the inverse relationship of feedback inhibition by proline to osmotic tolerance and proline overproduction in three recombinant strains.

Separation of γ-glutamyl kinase and glutamate-γ-semialdehyde dehydrogenase. To investigate the biochemical nature of the mutation in γ-glutamyl kinase in more detail and to assess the possibility that glutamate-γ-semialdehyde dehydrogenase also carries a mutation, the kinase was separated from the dehydrogenase by ammonium sulfate precipitation. Because γ-glutamyl kinase activity is undetectable in the absence of glutamate-γ-semialdehyde dehydrogenase in the hydroxamate assay (21), a new method was developed to measure enzyme activity that relies on the observation that γ-glutamyl phosphate is readily hydrolyzed to glutamate and P_i in solution (12). The scheme for this reaction is glutamate + ATP → γ-glutamyl phosphate + ADP → glutamate + P_i. The net reaction is the breakdown of ATP to ADP and P_i, a glutamate-dependent ATPase in which the appearance of P_i is measured. Columns 3 and 5 of Table 3 show that about 90% of the γ-glutamyl kinase ATPase activity is in the 0 to 35% ammonium sulfate fraction, while all of the glutamate-γ-semialdehyde dehydrogenase activity is in the 40 to 75% ammonium sulfate fraction. By the hydroxamate assay, the activity of both the 0 to 35% and 40 to 75% fractions was low. When these fractions were combined, however, the activity was more than additive (Table 3, columns 1 and 2). These results demonstrate that the two enzymes can be separated by ammonium sulfate precipitation.

Feedback inhibition of γ-glutamyl kinase assayed with glutamate-γ-semialdehyde dehydrogenase from CM26 or CM126. With the kinase and dehydrogenase separated, changes in the sensitivity to feedback inhibition can be determined with respect to the glutamate-γ-semialdehyde dehydrogenase; i.e., does the source of dehydrogenase (wild type or mutant) affect the feedback inhibition properties of the kinase, in view of the fact that these enzymes probably exist as a complex in the cell (11, 21)? The sensitivity of wild-type γ-glutamyl kinase to feedback inhibition was the same regardless of the source of glutamate-γ-semialdehyde dehydrogenase (data not shown). Similar results were obtained with the mutant γ-glutamyl kinase assayed with either

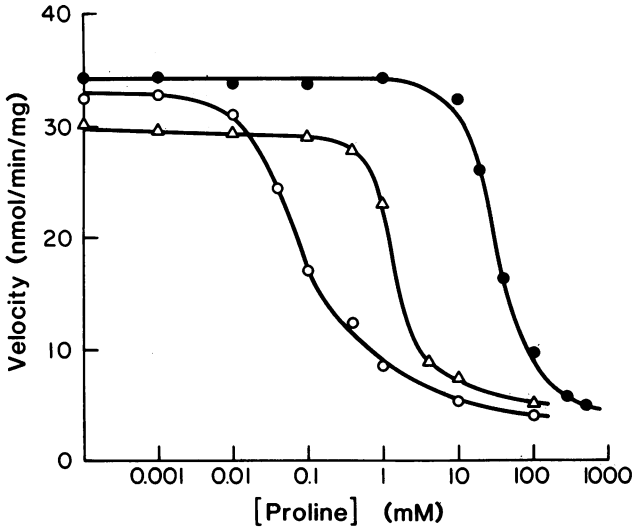

FIG. 1. Feedback inhibition of γ-glutamyl kinase by proline. γ-Glutamyl kinase activity from dialyzed crude extracts was measured as a function of proline concentration in the assay mixture. The hydroxamate assay procedure was used, and the sources of extract were wild-type strain CM26 (○), proline-overproducing strain CM126 (●), and the variant CM126V (△).

TABLE 3. γ-Glutamyl kinase and glutamate-γ-semialdehyde dehydrogenase activities after ammonium sulfate fractionation[a]

Strain and fraction	γ-Glutamyl kinase				Glutamate-γ-semialdehyde dehydrogenase (nmol/min·assay)
	Hydroxamate assay		ATPase assay		
	nmol/min·assay	$\frac{C}{A+B}$[b]	nmol/min·assay	$\frac{C}{A+B}$	
CM26 (wild type)					
A. 0–35%	16		94		ND[c]
B. 40–75%	18		10		105
C. 0–35% + 40–75%	75	2.2	110	1.1	73
CM126 (mutant)					
A. 0–35%	12		96		ND
B. 40–75%	13		16		189
C. 0–35% + 40–75%	90	3.6	102	0.9	120

[a] From crude extracts from strains CM26 and CM126, γ-glutamyl kinase and glutamate-γ-semialdehyde dehydrogenase were separated by ammonium sulfate fractionation. The 0 to 35% fraction and 40 to 75% fraction were tested independently for γ-glutamyl kinase activity, using either the hydroxamate or ATPase assay procedure. 50 μl of enzyme was used per assay. Also, 50 μl of the 0 to 35% fraction was combined with 50 μl of the 40 to 75% fraction and assayed together. The activity of glutamate-γ-semialdehyde dehydrogenase in the ammonium sulfate fractions was determined by using 10 μl of enzyme per assay. The values reported here, however, have been multiplied by 5 so a direct comparison of the activities between the two enzymes can be made.

[b] The value obtained for kinase activities with the 0 to 35% and 40 to 75% fractions recombined was divided by the sum of the values obtained for the activity in each fraction.

[c] ND, Not detectable (<0.4 nmol/min·50 μl of enzymes).

mutant or wild-type dehydrogenase and kinase from the variant assayed with dehydrogenase from the wild type or variant (data not shown). Thus, the mutation for the loss of feedback sensitivity lies solely in the *proB* gene.

Temperature sensitivity of γ-glutamyl kinase. To determine if the mutation in γ-glutamyl kinase results in a less stable protein, crude extracts of CM26 and CM126 were incubated at 45°C for various lengths of time before assaying for activity. The mutant kinase was much less heat stable than the wild type (Fig. 2). The initial rate of loss of activity of the mutant enzyme was too rapid to quantitate, but appeared to be severalfold greater than that of the wild type, which initially decreased in activity at a rate of about 6% per min. The control extract, CSH26 carrying pBR322, contained 8 to 9 nmol/min per mg of protein of activity, all of which was insensitive to heat treatment. This result may account for the activity of γ-glutamyl kinase from CM26 and CM126 remaining at 10 min in Fig. 2.

The temperature sensitivity of γ-glutamyl kinase did not translate into a temperature-sensitive mutation; that is, cultures of CM126 grown at 30°C did not show more osmotolerance than those grown at 37°C, relative to a wild-type culture (data not shown).

The possibility that substrates can protect the kinase against denaturation was investigated. Glutamate and ATP had no effect; however, both wild-type and mutant γ-glutamyl kinases were protected against denaturation when extracts were heated in the presence of proline (Fig. 3). The amount of proline needed for maximal protection of wild-type and mutant kinases was determined. Each enzyme was incubated at 45°C for 7 min in the presence of various

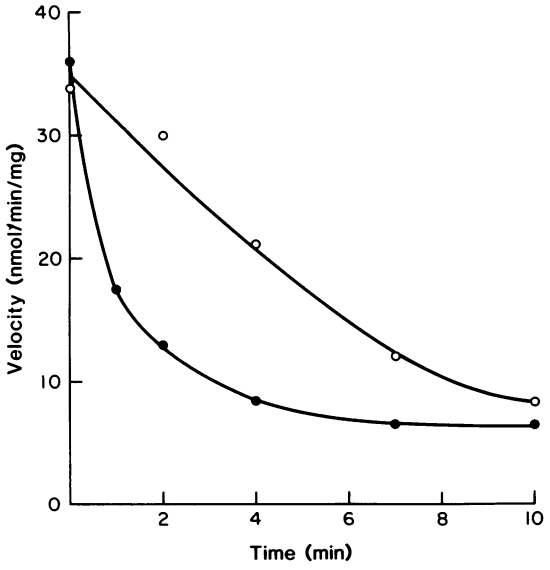

FIG. 2. Sensitivity of wild-type and mutant γ-glutamyl kinases to heat treatment. Dialyzed crude extracts containing γ-glutamyl kinase from strains CM26 (○) and CM126 (●) were incubated at 45°C for the indicated times and then assayed for activity by the hydroxamate procedure.

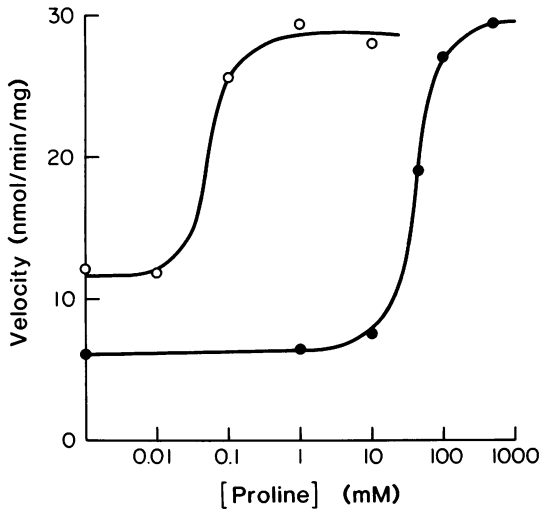

FIG. 3. Proline-mediated protection against heat denaturation. Dialyzed crude extracts from strains CM26 (○) and CM126 (●) were incubated for 7 min at 45°C with proline at the concentrations indicated. After the incubation period, the proline was removed from the extract, and the extract was assayed for γ-glutamyl kinase activity by the hydroxamate assay.

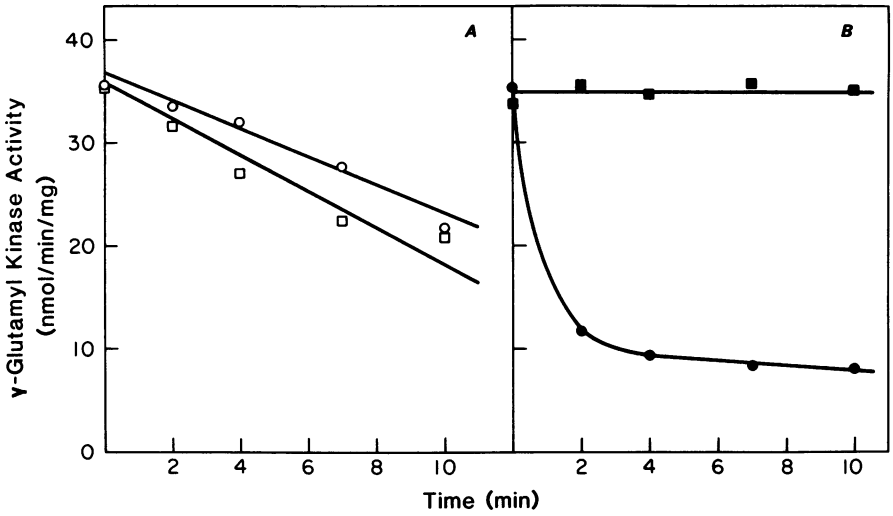

FIG. 4. Stability toward heat treatment of the γ-glutamyl kinase and the glutamate-γ-semialdehyde dehydrogenase components of the kinase assay. γ-Glutamyl kinase was assayed by the hydroxamate assay which requires the presence of both γ-glutamyl kinase and glutamate-γ-semialdehyde dehydrogenase to observe activity. In panel A, an ammonium sulfate fraction from wild-type CM26 extract was used, and either γ-glutamyl kinase (○) or glutamate-γ-semialdehyde dehydrogenase (□) was incubated at 45°C for the times indicated. After the incubation, the treated enzyme was recombined with the complementary enzyme that was untreated. In panel B, a similar experiment was carried out with the kinase (●) or dehydrogenase (■) from the mutant (CM126) incubated at 45°C.

concentrations of proline, and then the proline was removed from the crude extract by centrifugal gel filtration before assaying for kinase activity. The amount of proline needed to retain nearly all of the enzyme activity correlated with the amount of proline needed to saturate the allosteric site (see Fig. 1); i.e., about 1 mM for the wild type and 300 mM for the mutant enzyme (Fig. 3). This result implies that the mutation(s) causing the instability toward heat treatment also causes the decreased binding ability of proline (loss of feedback inhibition).

Temperature sensitivity of glutamate-γ-semialdehyde dehydrogenase in the kinase assay. The possibility that both γ-glutamyl kinase and glutamate-γ-semialdehyde dehydrogenase contributed to the differences in the temperature sensitivity between the wild-type and mutated enzymes was assessed. Ammonium sulfate-fractionated γ-glutamyl kinase was incubated at 45°C and recombined with untreated dehydrogenase for the hydroxamate assay of γ-glutamyl kinase activity (Fig. 4). The results obtained were similar to those shown in Fig. 2.

The converse experiment was also carried out; i.e., the dehydrogenase was heat treated and then recombined with untreated kinase. After a 10-min incubation, the wild type lost almost half of the kinase activity, while the mutant remained completely stable (Fig. 4). These results suggest that *proA* (glutamate-γ-semialdehyde dehydrogenase) may also carry a mutation.

These changes in kinase activity seen with treated glutamate-γ-semialdehyde dehydrogenase are not reflected in dehydrogenase activity itself. When the dehydrogenase was heat treated and assayed for its own activity in the absence of the kinase, no decrease in activity was observed when either mutant or wild-type extracts were heated at 45°C for 10 min (data not shown). Hence, the changes occurring in the dehydrogenase are too subtle to affect gross changes in its activity, but these changes are substantial enough to alter the protein-to-protein interactions within the kinase-dehydrogenase complex.

Biochemical analysis of γ-glutamyl kinase by the ATPase assay. The results of the experiments with γ-glutamyl kinase presented in Fig. 1 to 4 were obtained by the hydroxamate assay, which requires the presence of glutamate-γ-semialdehyde dehydrogenase as well as the kinase. The ATPase assay, however, does not require the enzyme complex. Therefore, it was of interest to compare the proline sensitivity and heat stability of γ-glutamyl kinase by the ATPase assay to the results obtained with the hydroxamate assay. Using the ATPase assay, γ-glutamyl kinase from ammonium sulfate-fractionated crude extracts of CM26 and CM126 was undetectable (<5% of the maximum) at 1 and 485 mM proline, respectively, and a 4-min incubation at 45°C resulted in a loss of 35% of the activity of the wild-type kinase (CM26) and a loss of 78% of the mutant activity (CM126). These results confirm the conclusions drawn above, that is, glutamate-γ-semialdehyde dehydrogenase is not involved in the change in feedback inhibition by proline of γ-glutamyl kinase and is only subtly involved in the changes in heat stability of the mutant kinase-dehydrogenase complex in vitro.

DISCUSSION

The results given in this report correlate the sensitivity to proline feedback inhibition of γ-glutamyl kinase to two physiological parameters: proline overproduction and osmotic stress tolerance (Fig. 1 and Table 2). This correlation was made with three strains: the mutant, the wild type, and a spontaneous variant with properties intermediate between the wild type and the mutant. The feedback inhibition patterns of the kinases were independent of the presence of glutamate-γ-semialdehyde dehydrogenase. This result was obtained by using the hydroxamate assay with dehydrogenase from either CM26 or CM126, and by using the ATPase assay, which is carried out in the absence of dehydrogenase.

While the values for the K_i of proline differed dramatically, the specific activity of γ-glutamyl kinase from crude extracts of all three strains was about the same. Interestingly, Lodato et al. (14) isolated two proline-overproducing mutant fibroblast lines that show alterations in pyrroline-5-

carboxylate synthase (enzyme analogous to the γ-glutamyl kinase–glutamate-γ-semialdehyde dehydrogenase complex). One mutant contained a synthase that was less sensitive to feedback inhibition by proline and ornithine, a result similar to that described in this report. The other mutant, however, contained a pyrroline-5-carboxylate synthase of unusually high specific activity.

Smith et al. (21) characterized a proline-overproducing mutant from *E. coli*. They found that the γ-glutamyl kinase from the mutant was about 100-fold less sensitive to feedback inhibition than that from the wild type. This value is intermediate between CM126V and CM126. One would speculate that the mutation Smith et al. isolated would confer a level of osmotic stress tolerance intermediate between CM126V and CM126.

One consequence of the mutation in γ-glutamyl kinase is the decreased stability toward heat treatment compared with the wild type. Proline protects γ-glutamyl kinase against heat denaturation, and the proline concentration needed for maximum protection is the same as that needed to saturate the allosteric site. The significance of these results is that the altered allosteric site which allows a decrease in sensitivity to feedback inhibition seems to also confer a decreased stability in the structure of the protein.

The results given in this report support the view that the feedback-insensitive γ-glutamyl kinase is required for osmotolerance. However, the fact that the proline levels increased even more when cells were grown with 0.5 M NaCl (Table 2) indicates that other factors are involved in the osmotic tolerance phenotype. For example, the rate at which proline is degraded by or excreted from the cell may decrease in cultures grown at high osmotic strength, thus allowing a higher level of proline to accumulate. Also, the concentration of glutamate, the substrate of γ-glutamyl kinase, in cells grown at low osmotic strength may be limiting for maximal kinase activity. Hence, an increase in the biosynthesis of glutamate in cells grown at inhibitory osmotic strength would be important. Indeed, the glutamate pool size increases in several bacterial species when they are grown under high osmotic strength (22, 24) and may increase in *E. coli* as well. Finally, Fig. 4 demonstrates that the *proA* enzyme (glutamate-γ-semialdehyde dehydrogenase) from CM126 may be more heat stable than the wild type. This result raises the possibility that there is a mutation in *proA* which, although not involved in the change in feedback inhibition of γ-glutamyl kinase, may be involved in the osmotolerance phenotype. For example, it may be responsible for the increased intracellular proline concentration in cells grown at elevated salt concentrations.

The possibility of a double mutation could also account for the extreme rarity of the osmotolerance phenotype. Of the 107 azetidine-2-carboxylate-resistant mutants isolated, only one, TL126, also conferred markedly enhanced osmotolerance (7). Hence, the possibility of a double mutation, one in *proB* which codes for a proline-insensitive γ-glutamyl kinase and one in *proA* which codes for an altered dehydrogenase, may be necessary for the osmotolerance phenotype. Presently, *proB* and *proA* are being sequenced to determine what base changes have occurred.

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