GLUTAMATE KINASE ACTIVITY IN BREVIBACTERIUM FLAVUM : RELATIONSHIP BETWEEN L-PROLINE AND L-GLUTAMINE BIOSYNTHESIS

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In the preceding paper (Yoshinaga et al., 1966a), an isoleucine auxotrophic mutant, strain No.14-5 (ATCC No.15940), derived from Brevibacterium flavum 2247 (ATCC No.14067), a glutamic acid-producing bacterium, was found to produce a very large amount of L-proline directly from glucose and inorganic nitrogen in the medium, and cultural conditions favorable for L-proline production by this mutant were demonstrated (Yoshinaga et al., 1966b). The present paper deals with the relationship between L-proline and L-glutamine biosynthesis in this isoleucine auxotrophic mutant, especially with the participation of glutamate kinase reaction in the synthesis of both amino acids in this microorganism.

L-Proline is synthesized in Escherichia coli from L-glutamic acid by the following reactions (Vogel and Davis, 1952).

L-glutamic acid $\longrightarrow$ L-glutamic γ-semialdehyde (1)

L-glutamic γ-semialdehyde $\rightleftharpoons$ L-Δ'-pyrroline-5-carboxylic acid $\longrightarrow$ L-proline (2)

This pathway was also found in Neurospora crassa (Vogel and Bonner, 1954) and Torula utilis (Abelson and Vogel, 1955), and an in vitro enzymatic conversion of glutamic γ-semialdehyde to L-proline (reaction 2) was studied in detail (Strecker, 1957 ;

Yura and Vogel, 1959). The reaction of a carboxyl group to an aldehyde group (reaction 1), however, has not yet been elucidated. This step appears to be analogous to the reduction of L-aspartic acid to aspartic β-semialdehyde by way of the phosphorylated intermediate, β-aspartyl phosphate (Black and Gray, 1953 ; Black and Wright, 1955), through the participation of ATP, as suggested by Strecker (1957) and Smith (1957). Similarly, the reduction of N-acetyl glutamic acid to N-acetyl glutamic γ-semialdehyde in the synthesis of L-ornithine requires an intermediate phosphorylation (Udaka and Kinoshita, 1958 ; Baich and Vogel, 1962).

Enzyme extracts were prepared, at 4°, from cells of strain No.14-5 grown for 24 hr in the following production medium containing, per liter, glucose (as starch hydrolyzate) 100g, $(NH_4)_2SO_4$ 60g, KH_2PO_4 1.0g, $MgSO_4 \cdot 7H_2O$ 8.0g, $FeSO_4 \cdot 7H_2O$ 0.01g, $MnSO_4$ 0.01g, L-isoleucine 150mg, "Ajieki" (solution of amino acids mixture, Ajinomoto Co., Inc., Tokyo) 1.0ml, D-tartaric acid 1.0g, biotin 450μg, thiamine hydrochloride 100μg and $CaCO_3$ 50g. Fermentation was carried out as previously described (Yoshinaga et al., 1966b). Cells were collected by centrifugation after separating $CaCO_3$ and washed twice with 0.75% K_2SO_4 solution, then were usually disrupted in 0.1M Tris-H_2SO_4 buffer, pH 7.6, by sonic oscillator for 40 min (10KC), but were ground with alumina equivalent to the wet cells' weight for the assay of L-proline from L-glutamic acid. In the latter case, supernatant obtained by elimination of alumina by centrifugation at 900×G was used as a cell homogenate, while a transparent cell-free extract was prepared after separation of cell debris from this cell homogenate or from sonicate by centrifugation at 10,000×G. Protein was estimated by the method of Lowry (1951) or by the

Table 1 L-Proline Formation from L-Glutamate

Additions	L-Proline formed		Hydroxamate formed**
	With L-glutamate	Without L-glutamate*	
	(μmoles)	(μmoles)	
None	0.8	0.0	0.000
$MgSO_4 \cdot 7H_2O$	2.9	0.5	0.000
$MgSO_4 \cdot 7H_2O$+ATP	8.9	1.3	0.000
$MgSO_4 \cdot 7H_2O$+ATP+NH_2OH	0.0	0.0	0.194

* Endogenous L-glutamate was 12.7 μmoles.

**ΔOD at 540 mμ : Blank obtained in the presence of endogenous L-glutamate was substracted from the values obtained.

The following reaction mixture was incubated at 31° for 5 hr in a final volume of 3.0 ml : 100 μmoles of L-glutamate (adjusted to pH 7.6 with KOH), 100 μmoles of Tris-H_2SO_4 pH 7.6 and 1.0 ml of cell homogenates (84.9 mg as protein). The concentration of additions was as follows : 60 μmoles of $MgSO_4 \cdot 7H_2O$, 60 μmoles of ATP and 400 μmoles of hydroxylamine hydrochloride (adjusted to pH 7.6 with KOH). L-Proline formed was determined by paperchromatography using n-BuOH-CH_3COOH-H_2O (4:1:2 by volume) system, and measured by microbiological assay (Leuconostoc citrovorum ATCC 8081). Hydroxamate formed was determined spectrophotometrically, according to modified method of Lipmann and Tuttle (1945). The reaction mixture was treated with 0.75 ml of 10%$FeCl_3$-6.6%trichloroacetic acid-0.7N HCl. After centrifugation, an optical density of the supernatant was measured at 540 mμ as a relative amount of hydroxamate formed.

Table 2 Essential Components for Glutamate Kinase Reaction Mixture

Conditions	Hydroxamate formed*		
	0	30 min	3 hr
Complete system	0.053	0.114	0.299
- ATP	0.063	0.053	0.078
- L-Glutamate	0.062	0.085	0.115
- $MgSO_4 \cdot 7H_2O$	0.065	0.084	0.096
- Enzyme preparation	0.000	0.000	0.000

*Optical density at 540 mμ.

The complete reaction mixture was incubated at 31° in a volume of 0.5 ml : 50 μmoles of L-glutamate (adjusted to pH 7.6 with KOH). 10 μmoles of ATP, 10 μmoles of $MgSO_4 \cdot 7H_2O$, 200 μmoles of hydroxylamine hydrochloride (adjusted to pH 7.6 with KOH), 15 μmoles Tris-H_2SO_4 pH 7.6 and 0.25 ml (0.25 mg as protein) of cell-free extracts.

method of Warburg and Christian as modified by Layne (1957). All spectrophotometric measurements were perfomed with a Hitachi Model 139 UV-VIS Spectrophotometer.

As shown in Table 1, evidence that the conversion of L-glutamic acid to L-proline was increased with high energy phosphate and Mg^{++}, was obtained by using both cell homogenates and cell-free extracts of strain No.14-5 mentioned above. In addition, when hydroxylamine, a trapping agent of acyl compounds (Lipmann and Tuttle, 1945), was added into the above-mentioned reaction mixtures, none of L-proline was formed from L-glutamic acid. This finding suggests the presence of enzymes which catalyze the phosphorylation of L-glutamic acid (glutamate kinase reaction) and catalyze the reduction of its phosphorylated intermediate to glutamic γ-semialdehyde as demonstrated in the case of the conversion of L-aspartic acid to aspartic β-semialdehyde.

Glutamate kinase activity was found to occur in the cell-free extracts of this microorganism, while this glutamate kinase reaction appeares to be the reaction 3.

$$\text{L-glutamic acid} + \text{ATP} \xrightarrow{Mg^{++}} [\gamma\text{-glutamyl phosphate}] + \text{ADP} \quad (3)$$

In this reaction, glutamyl phosphate formed is considered to be γ-glutamyl phosphate (Elliott, 1951). The enzymatic activity determined by the conventional hydroxamic method is shown in Table 2. Since the absence of L-glutamic acid, ATP, or Mg^{++} in the reaction mixtures resulted in a marked decrease in hydroxamate formation, the reaction most likely proceeds according to the reaction 3.

Although no conclusive demonstration of glutamyl phosphate reductase was shown, glutamyl phosphate formed seems to be converted to L-proline by way of glutamic γ-semialdehyde through

Table 3 Relation between L-Proline, L-Glutamine and Hydroxamate Formation

Conditions	L-Proline formed		L-Glutamine formed		Hydroxamate formed**
	With L-glutamate	Without L-glutamate*	With L-glutamate	Without L-glutamate	
	(μmoles)		(μmoles)		
Complete system	4.70	0.52	0.00	0.00	0.000
+ $(NH_4)_2SO_4$	0.00	0.00	9.59	1.44	0.000
+ NH_2OH	0.00	0.00	0.00	0.00	0.212

* Endogenous L-glutamate was 4.69 μmoles.
** ΔOD at 540 mμ.
The complete reaction mixture was incubated at 31° for 5 hr in a volume of 3.0 ml : 100 μmoles of L-glutamate (adjusted to pH 7.6 with KOH), 60 μmoles of $MgSO_4 \cdot 7H_2O$, 60 μmoles of ATP, 100 μmoles of Tris-H_2SO_4 pH 7.6 and 1.5 ml of cell-free extracts (18.0 mg as protein). The concentration of additions as follows : 100 μmoles of $(NH_4)_2SO_4$ and 400 μmoles of hydroxylamine hydrochroride (adjusted to pH 7.6 with KOH). L-Glutamine formed was determined by paperchromatography using phenol saturated with water system, and measured by Warburg manometric method (Tsunoda et al., 1961).

the participation of another enzyme ; because glutamic γ-semialdehyde in the reaction mixtures was detected by paper chromatograohic methods (Smith, 1957). Glutamyl phosphate, therefore, is concluded to be an intermediate in the biosynthesis of L-proline.

The above-mentioned assay method of glutamate kinase activity, however, is the same as that of glutamine synthetase activity using the conventional hydroxamic method (Elliott, 1948). Brevibacterium flavum 2247 was able to produce a large amount of L-glutamine (Tsunoda et al., 1961), and strain No.14-5 has also such ability under some condition, especially in the presence of 4 μg/liter of biotin. Table 3 shows that cell-free extracts prepared from strain No.14-5 which was cultured under the condition where L-proline was accumulated but not L-glutamine, were able to convert L-glutamic acid to L-glutamine. The conclusion drawn from Table 3 is that glutamyl phosphate is a common precursor in the biosynthesis of both L-proline and L-glutamine. Accordingly, the conventional hydroxamic method, which measures glutamyl phosphate formed, should be applied to the assay of glutamate kinase activity, and it would be correct to measure L-glutamine formed itself, in the presence of L-glutamic acid, ATP, Mg^{++} and ammonium ion, as the assay method of so called glutamine synthetase activity.

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