GLUTAMINE PRODUCTION IN HIGH CONCENTRATIONS WITH ENERGY TRANSFER EMPLOYING GLUTAMINE SYNTHETASE FROM *MICROCOCCUS GLUTAMICUS*¹

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Glutamine was produced by coupling the glutamine synthetase from *Micrococcus glutamicus* with sugar fermentation of baker's yeast as an energy-generating system. With 429 units/ml of glutamine synthetase and 40 mg/ml of yeast cells, 120 mM of glutamate was converted completely to glutamine in 3–4 hr, and 160–170 mM of glutamine (approximately 23–25 g/l) was formed in 5 hr from 350 mM of the substrates glutamate and ammonium chloride with a yield of about 40%, based on the energy released by the yeast during sugar fermentation. The activity ratio of sugar fermentation and glutamine synthetase was again confirmed to be important for achievement of energy-coupling in systems with high concentrations of glutamate and ammonium chloride. Glutamine formation was partially stimulated by the addition of Mg^{2+} in concentrations at which yeast fermentation of sugar showed no response to the cation, but was greatly stimulated by the addition of a small amount of Co^{2+} . This same Co^{2+} effect was also observed in the reaction with cell-free extracts or toluol-treated cells of *M. glutamicus*.

We have established processes for the production of various substances, employing dried yeast cells as an enzyme source (1–9). The processes are driven by the energy released by the alcoholic fermentation of sugar by the yeast. We have also designed a new process in which the yeast fermentation of sugar is combined with an endergonic reaction catalyzed by an enzyme from a different microorganism (4, 10). The results suggest that the process offers the possibility of producing many compounds in high yield by using various biosynthetic reactions and high concentrations of substrates. We introduced the term “coupled fermentation

¹ Amino acids are L-isomer unless otherwise stated.

with energy transfer" for the process. The first step of the process is the accumulation of energy in the form of fructose 1,6-diphosphate (FDP) by yeast fermentation of sugar, based on the Harden-Young effect (11); the second is the coupling of the fermentation of FDP with endergonic reactions (energy-utilizing system) through an ATP-ADP system. The energy-utilizing system can involve the enzymes of yeast itself or those of other organisms.

In earlier papers (12, 13), which reported glutamine production employing a combination of baker's yeast cells and glutamine synthetase from *Gluconobacter suboxydans* as the first application of "coupled fermentation with energy transfer" for production of a nonphosphorylated compound, we pointed out some of the factors that influence glutamine formation, and which are thought to apply to the process generally. However, the glutamine synthetase of *G. suboxydans* was not suitable for practical production of glutamine on a large scale and in high concentration because the enzyme content of the organism is very low, making difficult its preparation and use of large amounts of the purified enzyme. On the other hand, we found that *Micrococcus glutamicus* ATCC 13032 forms a large amount of glutamine synthetase when grown on glutamate as a nitrogen source (14), and also established a simple, high-yield purification procedure for the enzyme (15). In the present paper, we report glutamine production in high yield by using the *M. glutamicus* enzyme and larger amounts of the substrates glutamate and ammonium chloride.

MATERIALS AND METHODS

Enzyme preparation. a) Dried baker's yeast: baker's yeast supplied by Oriental Co., Ltd. was dried as described previously (2). b) Glutamine synthetase: three preparations were used in this study. (1) Toluol-treated cells: *Micrococcus glutamicus* ATCC 13032 cells grown on glutamate as a nitrogen source (14, 15) were collected, washed and resuspended in 0.01 M potassium phosphate buffer (pH 7.0, 3 ml), then lysed with 0.3 ml toluol at 37° for 30 min with shaking. (2) Cell-free extracts: the cell suspension was treated by sonication for 10 min at 0–10°. After centrifugation at 14,000 × *g*, the supernatant was dialyzed against 0.01 M potassium phosphate buffer (pH 7.0). (3) Partially purified enzyme: the cell-free extracts were treated at 50° for 10 min, fractionated with ammonium sulfate, and purified by DEAE-cellulose column chromatography as described previously (15).

Reaction conditions. The standard reaction mixture for glutamine production contained 100–300 mM glucose, 100–300 mM potassium phosphate buffer (pH 7.0), 25–350 mM sodium glutamate, 25–350 mM ammonium chloride, 0–50 mM MgCl₂, 0–10 mM ATP, 0–429 units/ml glutamine synthetase preparation and 0–60 mg/ml dried yeast cells in a total volume of 2 ml. Reactions were carried out at 28° with shaking and terminated by immersing the reaction tubes in boiling

water for 3 min. One unit of glutamine synthetase was defined as the amount which forms 1 μ mol of γ -glutamylhydroxamate per min by the transferring reaction (14, 15).

Assays. Glutamine, glutamate, glucose, FDP and protein were determined as described previously (12, 13).

RESULTS AND DISCUSSION

Isolation and identification of glutamine

The mixture containing 200 mM glucose, 200 mM potassium phosphate buffer, 150 mM sodium glutamate, 150 mM ammonium chloride, 15 mM MgCl_2 , 2.5 mM ATP, 54 units/ml partially purified glutamine synthetase and 20 mg/ml dried yeast cells was incubated for 4 hr. After heating, the combined reaction mixture from 30 tubes each containing 2 ml was centrifuged. The precipitate was washed twice with water. The supernatants were combined, acidified with 1 N HCl to pH 2.0 and applied to a column of Dowex 50 $\times$ 8 (H^+ form, 2.4 $\times$ 15 cm). The column was washed with water, then with 2 N ammonium hydroxide. The ammonium hydroxide eluate was evaporated and the residue taken up in a small volume of water. The solution was adjusted to pH 9–10 with 1 N sodium hydroxide and loaded on a Dowex 1 $\times$ 2 column (acetate form, 2.4 $\times$ 15 cm). The column was washed with water and the unadsorbed fraction was concentrated to dryness. The residue was dissolved in a small volume of hot water. Hot ethanol was added to the solution until a faint turbidity formed, then the mixture was placed in a refrigerator. The white crystal deposited were recrystallized from water-ethanol. The specimen gave a single spot corresponding to authentic glutamine on paper chromatograms developed with various solvent systems. Elemental analysis gave the following result. Calculated for glutamine, $\text{C}_5\text{H}_{10}\text{O}_3\text{N}_2$: C, 41.09; H, 6.90; O, 32.84; N, 19.17. Found: C, 41.03; H, 6.94; O, 32.61; N, 19.26. The crystals decomposed at 184–185°, as did the authentic glutamine. The isolated and the authentic products were demonstrated to be identical in IR and PMR spectra (Figs. 1 and 2). The product could act as a substrate for the γ -glutamylhydroxamate forming reaction catalyzed by the glutamine synthetase of *M. glutamicus*. Glutamate was found in the acid-hydrolyzate of the isolate. From these results, the product was identified as L-glutamine.

Production of glutamine by use of partially purified glutamine synthetase

Figure 3 shows the time courses of glutamine formation with different amounts of glutamate and ammonium chloride as substrates. Glutamine formation was slow initially, then accelerated before the maximum accumulation of FDP, and proceeded rapidly in parallel with the degradation of FDP until it was consumed completely. This observation agreed well with the principle of “the coupled fermentation” described in the introductory paragraph. In addition, Fig. 3 indicates

Fig. 1. IR spectra of authentic L-glutamine [A] and isolated amino acid [B] (KBr tablet).

Fig. 2. PMR spectra of authentic L-glutamine [A] and isolated amino acid [B] in D₂O.

Fig. 3. Effect of concentration of glutamate and ammonium chloride on glutamine production.

The reaction mixture contained 200 mM glucose, 200 mM potassium phosphate buffer (pH 7.0), 15 mM $MgCl_2$, 2.5 mM ATP, 54 units/ml partially purified glutamine synthetase and 20 mg/ml dried yeast cells. The concentrations of both glutamate and ammonium chloride were (A) 25 mM, (B) 100 mM and (C) 150 mM. Symbols: ●, glutamine; ○, glucose; ▲, FDP.

that an increase in the amounts of substrates caused an increase in the final amounts of glutamine to some extent, but also retarded the glutamine synthesis. Analyses of glucose and FDP in the mixtures suggested that the retardation was the result of inhibition of sugar fermentation by the substrates. However, when the concentrations of glutamine synthetase and yeast cells were increased to 429 units/ml and 40 mg/ml, respectively, 120 mM glutamate was converted completely to glutamine in 3–4 hr incubation (Fig. 4-A). The maximum amount of glutamine formed (160–170 mM, approximately 23–25 g/l, Fig. 4-B) was 5–10 times greater than that with the *Gluconobacter* glutamine synthetase (12, 13). This indicates that the *Micrococcus* enzyme fixed about 40% of the energy (corresponding to 400 mM ATP) released from 200 mM glucose.

The influence of the concentrations of yeast cells and glutamine synthetase on glutamine formation with large amounts of substrates (Fig. 5) was almost the same as that with lower concentrations of substrates (12, 13); glutamine formation increased with the increase in glutamine synthetase but decreased with the increase in yeast cells above a certain level. In the mixtures containing larger amounts of yeast cells, glucose was consumed rapidly and most of the energy supplied by sugar fermentation was lost without being utilized by glutamine synthetase.

Fig. 4. Glutamine production in mixtures with larger amounts of glutamate and ammonium chloride.

The mixture contained 200 mM glucose, 200 mM potassium phosphate buffer (pH 7.0), 15 mM MgCl_2 , 429 units/ml partially purified glutamine synthetase and 40 mg/ml dried yeast cells. The concentrations of both glutamate and ammonium chloride were (A) 120 mM and (B) 350 mM. Symbols: ●, glutamine; ○, glutamate; ⊙, glucose; ▲, FDP.

Effect of metal ions and ATP on glutamine formation

Figure 6 shows the effect of Mg^{2+} and Co^{2+} addition on glutamine formation. Yeast fermentation of sugar was stimulated by less than 2 mM Mg^{2+} but further addition had little effect. On the other hand, the glutamine synthesizing reaction was not saturated even by 30–50 mM Mg^{2+} but was strikingly enhanced by the simultaneous addition of 5 mM Co^{2+} . The minimum effective amount of Co^{2+} was about 1 mM (Table 1).

As reported previously (16), the *Micrococcus* glutamine synthetase requires Mg^{2+} as a cofactor in the reaction. The Mg^{2+} saturation curves at fixed levels of ATP were sigmoidal and the maximum activities were seen at the Mg^{2+} -ATP ratio of 2: 1. Moreover, we found that addition of a small amount of Co^{2+} greatly enhanced the activity in the presence of insufficient Mg^{2+} to express the maximum activity. Stimulation of glutamine formation by Co^{2+} (Fig. 6) may be a reflection of the enzyme property mentioned above and may be practically meaningful for glutamine production. At the same time, however, the present result indicated that glutamine synthetase could not be saturated by Mg^{2+} , which completely activated yeast fermentation of sugar. This suggests the need for further investigation of the action of divalent cations on glutamine synthetase under the fermentative conditions.

Addition of ATP to the reaction mixture was less effective for glutamine production with a larger amount of yeast cells. Glutamine formation was delayed

Fig. 5. Effect of concentrations of glutamine synthetase and yeast cells on glutamine production.

The mixture contained 200 mM glucose, 200 mM potassium phosphate buffer (pH 7.0), 150 mM glutamate, 150 mM ammonium chloride, 15 mM MgCl_2 and 2.5 mM ATP. The amounts of glutamine synthetase and dried yeast cells were: (A) 54 units/ml and 20 mg/ml; (B) 215 units/ml and 20 mg/ml; (C) 429 units/ml and 20 mg/ml; (D) 54 units/ml and 40 mg/ml; (E) 215 units/ml and 40 mg/ml; (F) 429 units/ml and 40 mg/ml; (G) 54 units/ml and 60 mg/ml; (H) 215 units/ml and 60 mg/ml; (I) 429 units/ml and 60 mg/ml.

Fig. 6. Effect of Mg^{2+} and Co^{2+} on glutamine production.

The mixture contained 300 mM glucose, 300 mM potassium phosphate buffer (pH 7.0), 150 mM glutamate, 150 mM ammonium chloride, 25 units/ml partially purified glutamine synthetase and 40 mg/ml dried yeast cells. In Exp. A, the amounts of Mg^{2+} added were [A-1] 0 mM, [A-2] 2 mM, [A-3] 15 mM, [A-4] 30 mM and [A-5] 50 mM. In Exp. B, 5 mM Co^{2+} was added to each mixture of Exp. A. Symbols: ●, glutamine; ○, glucose.

Table 1. Stimulatory effect on glutamine production of various concentration of Co^{2+} .

Co^{2+} added (mM)	Glutamine formed (mM)	
	4 hr incubation	8 hr incubation
0	6.0	15.0
0.5	28.5	54.0
1	34.5	62.3
5	30.0	60.8
10	33.8	63.0

The mixture contained 300 mM glucose, 300 mM potassium phosphate buffer (pH 7.0), 150 mM glutamate, 150 mM ammonium chloride, 2 mM MgCl_2 , 25 units/ml partially purified glutamine synthetase and 40 mg/ml dried yeast cells. The concentration of CoCl_2 was varied as indicated.

Table 2. Utilization of cell-free extracts and toluol-treated cells of *M. glutamicus* as glutamine synthetase preparation.

Glutamine synthetase	Addition of Co^{2+}	Glutamine formed (mM)	
		4 hr incubation	8 hr incubation
Exp. A			
Cell-free ext., 1.4 mg/ml	—	2.3	6.7
	+	22.3	38.3
Exp. B			
Toluol-treated cells, 68 mg/ml (dry matter)	—	6.7	30.0
	+	75.0	98.3

The mixture contained 300 mM glucose, 300 mM potassium phosphate buffer (pH 7.0), 150 mM glutamate, 200 mM ammonium chloride, 5 mM MgCl_2 , and 40 and 50 mg/ml dried yeast cells in Exp. A and Exp. B, respectively. The amount of Co^{2+} added was 5 mM and that of glutamine synthetase preparation was as shown in the table.

due to inhibition of glutamine synthetase by AMP or ADP (14) formed in the mixtures containing more than 5 mM ATP (data not shown).

Glutamine production by use of cell-free extracts or toluol-treated cells of M. glutamicus as glutamine synthetase preparation

Table 2 indicates glutamine production with cell-free extracts or toluol-treated cells as the glutamine synthetase preparation. The stimulating effect of Co^{2+} on glutamine formation was again observed.

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