Note

Promotion of Reductive Amination of α -Ketoglutarate to L-Glutamate in *Brevibacterium flavum* No. 2247 under Electro-energizing Conditions*

Masayoshi IWAHARA and Motoyoshi HONGO

Department of Applied Microbial Technology,
The Kumamoto Institute of Technology,
Ikeda 4-22-1, Kumamoto 860, Japan

Received April 14, 1981

Two pathways have been postulated for the L-Glu production in L-Glu fermentation bacteria.¹⁾ One is the reductive amination²⁾ and the other is the transamination.³⁾ In these bacteria, activities of transaminases were detected but the activity of aspartase is weak. So it was shown that glutamate is mainly synthesized through the reductive amination of α -KG.²⁾ In the strain used in the present experiments we detected only slight aspartase activity. As shown in Table I, abundant L-glutamate dehydrogenase (GDH) and isocitrate dehydrogenase (ICDH) activity occurs. The activity of GDH is always greater than that of ICDH at any period of the growth phase of this bacterium, suggesting that the amount of NADP compared with NADPH is a rate determining factor. Shingu *et al.*⁶⁾ reported that the higher specific activity of GDH as compared with that of ICDH and other enzymes is the characteristic of L-Glu fermentation bacteria. This strain is also considered to have the same characteristic.

As reported previously,⁷⁾ L-Glu production occurred when E-E fermentation was conducted in the cathode compartment with redox dyes having a low E_0' such as NR added. This is considered to be due to the promotion of the reductive amination in the bacterial cell. As shown previously,⁷⁾ when a redox dye with an E_0' similar to that of NADP was added to the broth or reaction mixture, the dye received the electron from the cathode which resulted in a promotion of the reductive amination. To confirm this the following experiments were conducted with the resting cells and the cell free extract.

Experiment I. Resting cells were used:

It is known that L-Glu fermentation bacteria grown

* Electrochemical Studies on Fermentation. Part III. for Parts I and II, see ref. 7.

Abbreviations: E-E, electro-energizing; E-E I, electro-energizing incubation; α -KG, α -ketoglutarate; JG, janus green; NR, neutral red; BV, benzyl viologen; MV, methyl viologen.

TABLE I. CHANGES OF ICDH AND GDH ACTIVITY IN THE COURSE OF CULTURE

Brev. flavum No. 2247 was grown in 40 ml of medium F⁷⁾ at 30°C for 20 hr on a reciprocal shaker (120 strokes/min). The cells were harvested by centrifugation at 9000 $\times g$ for 10 min at 2°C and washed 2 times with 0.05 M phosphate buffer at pH 7.5. The cell pellet was suspended in 40 ml of 0.05 M phosphate buffer at pH 7.5, and aerated for 2 hr (starved culture). The cells were harvested by centrifugation at 9000 $\times g$ for 10 min at 2°C and washed 2 times with sterilized tap water (washed cells). The cell pellet was suspended in a three fold volume of 0.05 M phosphate buffer at pH 7.5, and the suspension was treated with a sonicator, Kubota 200M (Kubota Commercial Co., Ltd.), at 9 kHz for 30 min at 0°C. The broken cell suspension was centrifuged at 9000 $\times g$ for 20 min at 2°C, and a cell free extract was obtained.

Culture time (hr)	Enzyme activity		
	ICDH*	GDH*	GDH ICDH
10	0.65	3.00	4.6
20	1.00	2.80	2.8
30	0.45	1.50	3.3

* ICDH and GDH activities were determined by the same methods as Kornberg⁴⁾ and Stricker,⁵⁾ respectively. The reaction mixture contained 200 μ mol Tris-HCl buffer (pH 7.5), 20 μ mol $MgCl_2 \cdot 6H_2O$, 200 μ mol DL-isocitrate, 0.4 μ mol NADP and cell free extract (5~7 mg protein) in a final volume of 3 ml.

under biotin sufficient conditions do not incorporate α -KG, but those grown under biotin deficient condition get to incorporate α -KG.⁶⁾ In this experiment, the reaction mixture was placed in the cathode compartment of the apparatus shown in Fig. 1 and E-E I was carried out. The results are shown in Table II, L-Glu production increased by E-E. When JG or NR was added before passing a direct current, L-Glu production became twice that of no E-E I control. Thus, about 15% of α -KG added as a substrate changed to L-Glu showing the promotion of reductive amination by E-E.

Experiment II. Cell free extract was used:

The effect of E-E on L-Glu production from citrate using cell free extract was investigated. The results are shown in Table III. The effect of redox dyes was not observed in the case of no E-E I. However, in the case of E-E I with NR, the reaction rate increased and L-Glu production was about 25% higher than that of no E-E I control, and 13% higher than that of E-E I without redox

FIG. 1. Apparatus for Electro-energizing Incubation.

A, cathode compartment (reaction vessel); B, cathode (mercury); C, reaction mixture; D, spin bar; E, magnetic stirrer; F, sampling port; G, silicone rubber stopper; H, gas outlet; I, agar bridge; J, anode compartment; K, anode (platinum); L, anolyte (10% KCl); M, direct current power supply; N, variable resistor; O, voltmeter; P, ammeter.

dyes. In this case reactions were carried out in micro-aerobic conditions. Under such conditions, the NADPH produced may be easily re-oxidized enzymatically, but the reoxidized NADP may be re-reduced directly or indirectly by E-E, minimize the loss of NADPH by oxidation resulting in the promotion of L-Glu production. This assumption is supported by the report of Kinoshita *et al.*^{2,9)} In the results of Experiments I and II, the promotion of reductive amination by E-E I were observed more effectively in the case of intact cells than in that of cell free extract. It can be suggested that E-E may have an effect upon cellular permeability for α -KG and L-Glu. Further details will be reported in a following paper.

REFERENCES

- 1) K. Yamada, S. Kinoshita, T. Tsunoda and K. Aida, "Aminosan Hakko," Kyoritsu Shuppan, Tokyo, 1972.
- 2) S. Kinoshita, K. Tanaka and S. Akita, *Nippon Nôgeikagaku Kaishi*, **34**, 589 (1960).
- 3) I. Shiio, S. Otsuka and T. Tsunoda, *J. Biochem.*, **48**, 110 (1960).
- 4) A. Kornberg, "Methods in Enzymology," Vol. I, ed.

TABLE II. PROMOTION OF REDUCTIVE AMINATION OF α -KG TO L-GLU IN RESTING CELLS OF *Brev. flavum* No. 2247

The reaction mixture contained 400 μ mol of sodium α -ketoglutarate, 600 μ mol of NH_4Cl , 0.8 μ mol of redox dyes and 100 mg of dry cells in a final volume of 8 ml of 0.1 M phosphate buffer (pH 7.5). After incubation at 30°C for 6 hr, the reaction was stopped and the L-Glu produced was determined according to the method of Watanabe *et al.*¹⁰⁾

Redox dyes added (0.1 mM)	L-Glu produced (mg/ml)	
	No E-E I (Control)	E-E I*
No addition	15	55
JG	15	105
NR	18	100
BV	20	80
MV	20	85

* Direct current of 1.0 mA/cm² at 1.5 V was applied.

TABLE III. EFFECTS OF E-E I ON L-GLU PRODUCTION FROM CITRATE USING CELL FREE EXTRACT OF *Brev. flavum* No. 2247.

The reaction mixture contained 400 μ mol of sodium citrate, 600 μ mol of NH_4Cl , 0.2 mg of NADP, 0.8 μ mol of redox dyes and 1 ml of cell free extract (6~7 mg protein) in a final volume of 8 ml of 0.1 M phosphate buffer (pH 7.5). After incubation at 30°C for 3 or 6 hr, the reaction was stopped and the L-Glu produced was determined.

Redox dyes added (0.1 mM)	L-Glu produced (mg/ml)			
	Incubation time (hr)			
	3		6	
	No E-E I (Control)	E-E I*	No E-E I (Control)	E-E I*
No addition	288	310	450	495
JG	288	333	459	530
NR	297	351	464	560
BV	285	330	440	510
MV	302	362	453	554

* Direct current of 1.5 mA/cm² at 3 V was applied.

by S. P. Colowick and N. O. Kaplan, Academic Press Inc., New York, N. Y., 1955, p. 685.

- 5) H. J. Stricker, "Methods in Enzymology," Vol. II, ed. by S. P. Colowick and N. O. Kaplan, Academic Press Inc., New York, N. Y., 1955, p. 211.
- 6) H. Shingu, K. Haneda, T. Sano and G. Terui, *J.*

- Ferment. Technol.*, **49**, 668 (1971).
- 7) M. Hongo, and M. Iwahara, *Agric. Biol. Chem.*, **43**, 2075, 2083 (1979).
- 8) I. Shiio, S. Otsuka and M. Takahashi, *J. Biochem.*, **51**, 56 (1962).
- 9) K. Tanaka, S. Akita, K. Kimura and S. Kinoshita, *Amino Acids*, **1**, 62 (1959).
- 10) Y. Watanabe, K. Watanabe, F. Koide, T. Saito and K. Shimura, *Nippon Nôgeikagaku Kaishi*, **34**, 620 (1960).
-