THE SIGNIFICANCE OF GLUTAMIC DEHYDROGENASE IN GLUTAMIC ACID FERMENTATION

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Received for Publication, August 10, 1962

In the previous papers (1–3), it was reported that TPN-linked glutamic dehydrogenase plays a principal role in the biosynthesis of L-glutamic acid in *Micrococcus glutamicus*.

It has long been known that the pathway of ammonia uptake in microorganisms is either through TPN- or DPN-linked glutamic dehydrogenase (4, 5) or through aspartase (6). Recently, the occurrences of L-alanine dehydrogenase (7, 8) and L-leucine dehydrogenase (9) were reported, suggesting alternative mechanisms of ammonia uptake. HONG *et al.* (10) showed that *Bacillus* having glutamic dehydrogenase lacked alanine dehydrogenase and *vice versa*. HALPERN *et al.* (11) reported that the ratio of the activity of glutamic dehydrogenase to that of aspartase in *Escherichia coli* varied depending upon the compositions of a medium such as nitrogen sources. In order to get further insight into the cause of above-mentioned diversity in the mechanism of ammonia uptake, the activities of various enzymes involved in ammonia uptake were examined with *M. glutamicus*.

The present paper concerns with the recognition of glutamic dehydrogenase as the primary enzyme acting in the process of ammonia uptake *M. glutamicus* irrespective of the cultural conditions so far tested.

EXPERIMENTALS

Used Strains and Cultivation—*Micrococcus glutamicus* No. 541, No. 560 (both biotin requiring strains) and several mutants (12) of this species were used. *Escherichia coli* No. 2 obtained by KITAHARA *et al.* (13) and *Bacillus megaterium* B-5-5 obtained by KINOSHITA *et al.* (14) were also used. These two strains were known to possess a strong activity of aspartase and excrete a considerable amount of L-aspartic acid in the medium containing fumaric acid. One loopful of the cells grown on a bouillon agar slant for one day at 30° was transferred to 20 ml of the seed medium in 250 ml flask consisting of 2% glucose, 1% peptone, 0.5% meat extract and 0.25% NaCl. In the case of the mutants, 0.01% nutrient required was also included in the medium. The cultivation was conducted with rotary shaking (200 r.p.m.) for 24 hours at 30°. The seed was then transferred, in 10% inoculum size, to a final medium and it was grown under similar conditions. As the final media were used both the media having the same composition

as the seed media and the chemically defined media (15) consisting of 10% glucose, 0.05% KH_2PO_4 , 0.05% K_2HPO_4 , 0.05% $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$, 0.002% $\text{MnSO}_4 \cdot 4 \text{H}_2\text{O}$, 0.002% $\text{FeSO}_4 \cdot 7 \text{H}_2\text{O}$, 0.2% $(\text{NH}_4)_2\text{SO}_4$, 0.5% urea, 0.5 to 20 $\mu\text{g/L}$ of biotin and 0.001% phenol red. In the chemically defined media, pH was controlled during fermentation to 7.0 to 8.0 by the addition of urea.

Preparation of Cell-free Extracts—After 24 hours' cultivation, the cells were harvested by centrifugation at $1000 \times g$ for 15 minutes and washed twice with 0.85% NaCl solution. The cells were then suspended in deionized water in the concentration of 100 mg cells per milliliter and disrupted by 10 KC sonic oscillation for 30 minutes at 0° . The supernatant obtained after the removal of cell-debris by centrifugation at $9000 \times g$ for 15 minutes was used as the enzyme preparation.

Assay Procedure for Enzyme Activity—The following assay systems were used for the determination of the activities of enzymes in the extracts. In each case, the activity was expressed by the difference between the values obtained for the complete system and the system without the respective substrate.

Aspartase: The complete assay system contained 50 μ moles of L-aspartate, 1 μ mole of 5'-adenylic acid, 100 μ moles of phosphate buffer, and 5 mg of cell-free extract as protein in a final volume of 2.5 ml (pH 8.0). The activity was expressed by μ moles of ammonia released during 2 hours of stationary incubation at 30° .

Glutamic-aspartic transaminase: The complete assay system contained 50 μ moles of L-aspartate, 100 μ moles of α -ketoglutarate, 20 μg of pyridoxal phosphate, 200 μ moles of phosphate buffer, and 4 mg of cell-free extract as protein in a final volume of 2.0 ml (pH 8.0). The activity was expressed by μ moles of L-glutamate formed during 1 hour of stationary incubation at 30° .

L-Alanine dehydrogenase: The complete assay system (8) contained 10 μ moles of L-alanine, 1 μ mole of TPN or DPN, 125 μ moles of 2-amino-2-methyl-1,3 propanediol-HCl buffer, and 1 mg of cell-free extract as protein in a final volume of 3.0 ml (pH 9.8). The activity was expressed by the increase in absorbancy at $340 \text{ m}\mu$ during 5 minutes (the increment in optical density at $340 \text{ m}\mu$ between the readings at 15 and 75 seconds after making the last addition was multiplied by 5) per g protein. In the cell-free extracts used a comparatively strong activity of DPNH oxidase was observed. This activity, however, was inhibited 20% to 40% by 10^{-2} M cyanide or azide and almost completely by anaerobic condition. Consequently, for the check of DPN-linked dehydrogenases, the reactions were carried out in a THUNBERG tube type cell under anaerobic conditions.

Glutamic-alanine transaminase: Both the assay system and the determination of the enzyme activity were the same as described in the case of glutamic-aspartic transaminase except that L-aspartate was replaced by L-alanine.

L-Leucine dehydrogenase: The complete assay system (9) contained 40 μ moles of L-leucine, 1 μ mole of TPN or DPN, 150 μ moles of glycine buffer, and 1 mg of cell-free extract as protein in a final volume of 3.0 ml (pH 11.0).

The reactions were carried out at 30° in the same way as described in the case of L-alanine dehydrogenase and the activity was expressed similarly.

L-Glutamic dehydrogenase: The complete assay system (3) contained 50 μ moles of L-glutamate, 1 μ mole of TPN or DPN, 100 μ moles of phosphate buffer, and 1 mg of cell-free extract as protein in a final volume of 3.0 ml (pH 7.8). The reactions were carried out at 30° and the activity was expressed in the same way as used for L-alanine dehydrogenase.

Analysis —

L-Aspartic acid: The enzymic reaction was stopped by heating the reaction mixture in boiling water for 5 minutes. After the removal of coagulated protein by centrifugation, L-aspartic acid in the supernatant was determined by the microbioassay using *Leuconostoc mesenteroides* p 60 (16).

L-Glutamic acid: After the same deproteinization as described above, L-glutamic acid was determined manometrically with the use of L-glutamic decarboxylase of *Escherichia coli* (17).

Ammonia: The reactions were stopped by the addition of equal volumes of 10% trichloroacetic acid. After deproteinization by centrifugation, ammonia in the supernatant was determined by the combination of the micro-diffusion analysis (18) and the colorimetric assay (19).

Protein: The protein in the extracts was precipitated by the addition of 10% trichloroacetic acid and redissolved in 1 N NaOH. The protein contents of the solutions were measured by the FOLIN-CIOCALTEU reagent (20) and expressed as the equivalent amounts of egg-white albumin.

Assay of Dehydrogenase Activities: Assays were carried out with Beckman DU spectrophotometer.

RESULTS

Synthesis of Aspartate from Fumarate and Synthesis of Glutamate from Citrate by the Extracts of Various Species of Bacteria.

To compare the activities of two of the most common systems of the ammonia uptake, one involving aspartase, the other involving glutamic dehydrogenase, the activity to synthesize aspartate from fumarate and the activity to synthesize glutamate from citrate were examined with the extracts of various species of bacteria grown on the glucose bouillon media for 24 hours (in the case of the auxotrophs, with the addition of 0.01% nutrient required). The results are shown in Table 1. Both *E. coli* No. 2 and *Bacillus megaterium* B-5-5 were the bacteria selected specifically for the production of aspartate from fumarate and they were known to possess the strong activity of aspartase (13, 14).

Table 1 shows that *M. glutamicus* and its mutants were much weaker in the synthesis of aspartate from fumarate, compared with the other bacteria mentioned above. Therefore, it can be safely said that aspartase does not play any substantial role in the ammonia uptake in *M. glutamicus*. On the contrary, all strains of *M. glutamicus* synthesized glutamate quantitatively

Table 1. Synthesis of Aspartate from Fumarate and Synthesis of Glutamate from Citrate

Enzyme Source	(1) L-aspartate produced from fumarate	(2) L-glutamate produced from citrate
	μ moles	μ moles
<i>Escherichia coli</i> No. 2	9.1	10.8
<i>Bacillus megaterium</i> B-5-5	9.1	0.4
<i>M. glutamicus</i> 560	0.0	46.5
<i>M. glutamicus</i> 541	0.0	46.4
<i>M. glutamicus</i> 901 (hom ⁻)	0.8	46.8
<i>M. glutamicus</i> 534-Co 147 (thr ⁻)	0.9	48.7
<i>M. glutamicus</i> 615-305 (ura ⁻)	1.0	40.0

Reaction mixture:

- (1) Fumarate 50 μ moles, NH₄Cl 100 μ moles, phosphate 100 μ moles, Mg⁺⁺ 2 μ moles, cell-free extract 10 mg as protein, pH 7.2, final volume 2.5 ml. Stationary, for 3.5 hrs., at 30°C.
- (2) Citrate 50 μ moles, NH₄Cl 100 μ moles, phosphate 100 μ moles, Mg⁺⁺ 2 μ moles, cell-free extract 10 mg as protein, pH 7.8, final volume 2.5 ml. Stationary, for 3.5 hrs., at 30°C.

from citrate. The mechanism concerned was previously shown to be the coupling reaction between TPN-linked isocitric dehydrogenase and TPN-linked glutamic dehydrogenase (1). From the comparative studies with the other species of bacteria as shown in Table 1, it became more clear that the ammonia uptake in *M. glutamicus* is carried out by glutamic dehydrogenase and practically not by aspartase.

The results about the auxotrophs shown in Table 1 deserve particular attention. In the order given in the table, these auxotrophs are lysine (21), homoserine (22), and orotic acid (23) producing strains. All of these products are known to be synthesized from aspartic acid (24, 25). The fact that aspartate was synthesized from fumarate only in trace amount while glutamate was produced almost quantitatively from citrate by the extracts of these strains indicates that aspartate required for the synthesis of lysine, homoserine, and orotic acid could be supplied by the action of glutamic dehydrogenase and glutamic-aspartic transaminase much faster than by the action of aspartase. Indeed, in all of these extracts, the activity of glutamic-aspartic transaminase was demonstrated to be as strong as that in *M. glutamicus* No. 541 shown in Table 2.

Table 2. Activities of aspartase, glutamic-aspartic transaminase, L-alanine dehydrogenase, glutamic-alanine transaminase, L-leucine dehydrogenase and L-glutamic dehydrogenase of *M. glutamicus* grown on different levels of biotin

Biotin conc. for growth $\mu\text{g/L}$	Aspartase		Glutamic-aspartic transaminase		L-alanine dehydrogenase		Glutamic-alanine transaminase		L-leucine dehydrogenase		L-glutamic dehydrogenase	
	+ AMP	- AMP	+ PP	- PP	TPN	DPN	+ PP	- PP	TPN	DPN	TPN	DPN
0.5	0.4	0.4	17.3	16.9			3.9	3.6			105	
2.5	1.1	0.0	19.0	19.0	0.0		0.0	0.0			160	0.0
5	0.7	0.0	18.8	19.5	0.0	0.0	0.0	0.0			180	
10	1.4	0.0	16.0	14.9	0.0	0.0	0.5	2.0	0.0	0.0	123	
20	0.6	0.8	6.7	4.8	0.0	0.0	2.9	2.6			70	

PP: Pyridoxal phosphate AMP: 5'-Adenylic acid

Used Strain: *M. glutamicus* 541

Activities of Enzymes Involved in Ammonia Uptake in the Extracts of M. glutamicus Grown on Different Levels of Biotin.

The mechanism of the ammonia uptake in *M. glutamicus* can be characterized as described above. In the following, the effects of biotin levels in the growth medium upon the mechanism of ammonia uptake were studied in detail. Biotin is an essential growth factor for this microorganism (15) and is shown to exert profound effects upon the metabolic activities of this microorganism (26, 27). *M. glutamicus* No. 541 was grown in the chemically defined media having different levels of biotin and the activities of the enzymes were assayed with the cell-free extracts prepared from the cells grown in these different cultural conditions. The assay systems and the methods of determination of the enzyme activities are described in Experimentals. The results are shown in Table 2.

The activities of aspartase were shown to be extremely low in all preparations. Only about 1μ mole of ammonia was released in 2 hours from 50μ moles of aspartate by the extract having 5 mg protein. Difference in the biotin concentration in the growth media and the addition of the presumed cofactor of aspartase (28, 29), 5'-adenylic acid to the reaction mixture gave only minor changes to the enzyme activities of the cell-free extracts.

The considerably strong activities of glutamic-aspartic transaminase were detected in all preparations. About 20μ moles of glutamate were produced in 1 hour from 50μ moles of aspartate by the extract having 4 mg protein. However, difference in the biotin concentration in the growth media gave practically no effect upon the activities of the enzyme. No effect of the addition of pyridoxal phosphate to the reaction mixture was observed, indicating that biotin deficiency did not lower the intracellular

contents of pyridoxal phosphate to affect the activity of the transaminase.

No activity of alanine dehydrogenase was detected with the addition of either TPN or DPN.

The activity of glutamic-alanine transaminase was detected definitely, although it was weaker than that of glutamic-aspartic transaminase.

No activity of leucine dehydrogenase was detected with the addition of either TPN or DPN.

As previously reported (3), strong activities of TPN-linked glutamic dehydrogenase were observed in all preparations, but the activity of DPN-linked glutamic dehydrogenase was undetectable. Difference in the biotin concentration in the growth media brought about no significant change in the activity of TPN-linked glutamic dehydrogenase.

From the results described above, all of the following three possibilities of the ammonia uptake and consequently of the route of glutamic acid synthesis were practically excluded, i.e. (1) the combination of aspartase and glutamic-aspartic transaminase, (2) the combination of alanine dehydrogenase and glutamic-alanine transaminase, and (3) the combination of leucine dehydrogenase and transaminase.

Therefore, the mechanism of ammonia uptake in *M. glutamicus* can be summarized as follows: TPN-linked glutamic dehydrogenase is the primary enzyme for the uptake of ammonia and L-glutamic acid is produced by the action of this dehydrogenase without preceding formation of other amino acids. The characteristic feature of this mechanism of ammonia uptake does not change with the concentration of biotin in the growth media.

DISCUSSIONS

The mechanism of ammonia uptake in microorganisms has now been understood to be rather diverse, since the discovery of the dehydrogenases causing reductive amination (7-9) besides DPN- or TPN-linked glutamic dehydrogenase. Furthermore, it was thought to vary not only from species to species (10) but also according to cultural conditions (11).

In view of the recent findings of diversity in the mechanism of ammonia uptake in microorganisms, the extensive re-examination of the activities of enzymes involved in the ammonia uptake was conducted with *M. glutamicus*. The result was the recognition of TPN-linked glutamic dehydrogenase as the principal enzyme for the ammonia uptake in this microorganism. Although the various metabolic activities of *M. glutamicus* were shown to be regulated by the biotin concentration in the growth medium (26, 27), no appreciable change was observed in the activities of enzymes involved in the ammonia uptake. Only minor changes were noticed in the activity of aspartase, the general picture of ammonia uptake remaining, however, the same as described above.

The finding that the relative predominance of glutamic dehydrogenase over aspartase remains unchanged in many auxotrophs of *M. glutamicus*

which produce amino acids or a pyrimidine base of aspartic acid family indicates that this property is inherent to this species of bacteria and quite resistant to mutagenic treatment.

On the basis of the results obtained in the present work, it can be assumed that the alpha-amino groups of aspartic acid and alanine are supplied from that of glutamic acid and, in its consequence, the amino acids belonging to glutamic acid family and aspartic acid family (30) are synthesized directly or indirectly via the synthesis of glutamic acid in *M. glutamicus*.

SUMMARY

1. The activity to synthesize aspartic acid from fumaric acid and ammonia and the activity to synthesize glutamic acid from citric acid and ammonia were examined with the cell-free extracts obtained from several strains of *M. glutamicus* and their mutants grown on glucose bouillon media. All of them were found to possess a strong activity of the latter but practically no activity of the former.

2. The activities of aspartase, glutamic dehydrogenase, alanine dehydrogenase, leucine dehydrogenase, glutamic-aspartic transaminase, and glutamic-alanine transaminase were examined with the cell-free extracts obtained from the cells of *M. glutamicus* (parent strain) grown on different levels of biotin. Only glutamic dehydrogenase and transaminases were detected in ample amounts. No significant difference was found in the activities of enzymes involved in the ammonia uptake in the extracts of the cells grown on the different levels of biotin.

3. From the results 1 and 2, it is concluded that in *M. glutamicus* the principal mechanism of ammonia uptake is effected by TPN-linked glutamic dehydrogenase irrespective of the biotin level in the growth medium, and consequently, the biosynthesis of the amino acids belonging to glutamic acid family and aspartic acid family proceeds directly or indirectly via the synthesis of glutamic acid.

ACKNOWLEDGEMENT

The author wishes to thank Dr. Shukuo KINOSHITA and Dr. Katsunobu TANAKA for their valuable advices throughout this study.

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