STUDIES ON LYSINE FERMENTATION III. α , ϵ -DIAMINOPIMELIC ACID ACCUMULA- TION AND DIAMINOPIMELIC ACID DECARBOXYLASE

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Received for publication, December 9, 1960

L-Lysine accumulation by various auxotrophs of microorganism was described in previous papers (1, 2). Detailed studies were carried out on high lysine producer, a homoserine-requiring mutant of *Micrococcus glutamicus* (3, 4). And it became clear that both homoserine and threonine inhibit enzyme activity of lysine producing system from glucose and ammonium salt. Furthermore, the presence of α , ϵ -diaminopimelic acid (DAPA) and DAPA decarboxylase in lysine producing strain was demonstrated (5).

This paper deals with the results of studies on DAPA-decarboxylase of various strains including lysine requiring mutants and on DAPA accumulation by lysine requiring strains.

A pathway for lysine biosynthesis is also discussed in this paper.

METHODS

The mutants of *Micrococcus glutamicus* (3, 4) were used in all experiments. Induction of these mutants were described in separate papers (6). Genealogy of *M. glutamicus* was shown in Fig. 1.

Large test tubes, each of which contains 10 ml of a fermentation medium were used in culture experiments of DAPA accumulation. A test tube shaker was used in these experiments. Other conditions were the same as described in previously (1, 2). Growth experiments were conducted with the same method as previous papers (7, 8).

The same method as that of previous paper was used in lysine analysis. DAPA in culture broth was estimated by the method modified by GILVARG's (9) as the final volume to become to 20 ml. In the experiment using cell suspensions, in order to eliminate the disturbance of lysine on assay, paper-chromatographic separation was used as previous treatment. RHULAND *et al*'s (10) solvent system was used in this paperchromatography. After the spot of DAPA was cut finely, the method described above was employed.

RESULTS

1. *Growth Response of Lysine Requiring Strains*

Growth response of M. 615-338-433-1 to lysine was shown in Fig. 3. M. 534-Co 146-5 responded slowly to lysine and turbidity was low. Some experiments were attempted to clarify the cause of this slow and low response of M. 534-Co 146-5 but further studies are still necessary. As

Fig. 1. Genealogy of used mutants of *Micrococcus glutamicus*.

shown in Table 1, M. 615-338-433-1 responded slowly to DAPA. Both M. 534-Co 146-5 and M. 7032-2-10 did not respond to DAPA.

2. *Isolation and Identification of DAPA*

It was found that M. 534-Co 146-5 and M. 7032-2-10 accumulate DAPA in culture broths. Accumulation of DAPA was apparent by examination using RHULAND *et al*'s (10) paperchromatographic technique which was designed for detection of DAPA. But to confirm its accumulation, isolation and identification of DAPA from culture broth was attempted.

After precipitation of cells and calcium carbonate by centrifuge, the supernatant obtained was adjusted to pH 2 with hydrochloric acid. Then this acidified broth was applied to the column (3×31 cm) of Dowex 50X-2 and 0.3% ammonia water was used for elution. Each fraction of eluted liquid was examined by paperchromatography. Glutamic acid was eluted first, then glutamic acid plus alanine, alanine plus DAPA plus trace amounts of valine, finally followed by DAPA in this order. The last coming fractions of DAPA were collected and concentrated under reduced pressure. After decolorization of the concentrate with active carbon, alcohol was added to the concentrate and precipitated DAPA was isolated by filtration. This

Table 1. Growth response of lysine requiring strains to DAPA. (optical density)

Added substance		Lysine·HCl		Lysine·HCl 50 μg/ml + nicotinic acid 5 μg/ml	DAPA		DAPA 50 μg/ml + nicotinic acid 50 μg/ml			DAPA 50 μg/ml + L-methionine 50 μg/ml	DAPA 200 μg/ml + L-methionine 50 μg/ml	
		50 μg/ml	10 μg/ml		100 μg/ml	20 μg/ml						
Strain		23 hr.		23 hr.	72 hr.		23 hr.	48 hr.	72 hr.	72 hr.	72 hr.	
615-338-433-1		0.137	0.069	0.795	0	0	0	±	+	(1.16)	0	
Monocolony isolate of 615-338-433-1	No. 1			0.242	0	0	0	±	+	(1.14)	0	
	No. 2			0.142	0	0	0	±	+	(1.08)	0	
534-Co 146-5										0	0	0

Table 2. DAPA decarboxylase in lysine requiring strains and parent strains

Strain	Nutritional requirement	CO ₂ evolved (µl)		DAPA change*	Lysine produced	Con- clusion**
		30 min.	60 min.			
M. 534	Wild	27	37	Disappear partly	+ (0.27 mg/m)	+
M. 534-Co 146	L-methionine	19	38	Disappear partly	±	+
M. 534-Co 146-5	L-methionine + L-lysine	0	0	Did not disappear	—	—
M. 615-338-433	Nicotinic acid			Disappear partly	±	+
M. 615-338-433-1	Nicotinic acid + L-lysine			Disappear partly	+	+
M. 7032-2	Wild			Disappear partly	+ (0.24)	+
M. 7032-2-10	Lysine			Did not disappear	—	—

* By paper chromatography.

** + = presence of DAPA decarboxylase, — = absence of DAPA decarboxylase.

isolated DAPA was analyzed without further recrystallization. Analytical data and theoretical value is as follows:

Analytical: C 42.9%, H 7.72%, N 14.40%

Theoretical: C 44.2%, H 7.42%, N 14.73%

Infrared spectrum of sample was the same with that of DAPA obtained from fermentation broth in Chas. Pfizer & Co., Inc. (U.S.A.). DAPA from Chas. Pfizer Co. and our sample are both mixture of meso and LL-isomers of DAPA.

3. *DAPA Decarboxylase in Lysine Requiring Strain*

Presence of DAPA decarboxylase in lysine requiring mutants and their parent strains was examined. Summarized data of several repeated experiments were presented in Table 2. In some strains, decarboxylation of DAPA was not noticeable by the manometric method using acetone dried celles as an enzyme preparation, but the presence of DAPA decarboxylase was estimated by the disappearance of DAPA and appearance of lysine. The absence of DAPA decarboxylase in both lysine requiring mutants, M. 534-Co 146-5 and 7032-2-10 was concluded in every methods. DAPA disappeared partly by M. 615-338-433-1 that responded to DAPA slowly (Table 1). And it may be estimated that this strain has DAPA decarboxylase and

Fig. 2. Standard curve for colorimetric assay of DAPA using paperchromatographic technique.

blocked before DAPA in lysine biosynthesis. But activity of the DAPA decarboxylase of this strain may have become weak since trace amount of DAPA was accumulated in a medium (screening medium (6)). Slow response of this strain to DAPA may be due to a permeability factor as described in previous paper.

About normal amount of meso-DAPA was found in cells of M. 615-338-433-1 cultured on lysine by the examination of the hydrolysate of the cells. How this DAPA was synthesized? Possibility of back mutation is low in view of the results of the experiment shown in Table 1. Therefore, the cause of this DAPA synthesis seems to be interesting.

4. DAPA Accumulation by Lysine Requiring Mutants

Influence of the concentration of biotin and lysine those are added in media as growth supporting substances was investigated. The results with M. 534-Co 146-5 are shown in Table 3. In this case L-methionine which is required by this strain was added in concentration of 50 $\mu\text{g}/\text{ml}$. In the absence of biotin (though trace amounts of biotin was brought from the seed medium), growth was poor and tendency of glutamic acid accumulation in place of DAPA accumulation was recognized. With limited concentrations of lysine, DAPA was accumulated and the accumulation was maximum in the case where L-lysine·HCl was added in concentrations of 62.5 $\mu\text{g}/\text{ml}$ and

Fig. 3. Growth response of M. 615-338-433-1 to lysine (nicotinic acid 5 $\mu\text{g}/\text{ml}$).

biotin in 30 $\mu\text{g/L}$. Accumulated DAPA was found to be a mixture of meso and LL-isomers by the examination with RHULAND *et al*'s (10) paperchromatographic technique.

The similar results were obtained with another strain M. 7032-2-10 which is derived from different parent strain (Fig. 1). With this strain 2.0 mg of DAPA per ml. of the medium was accumulated in the medium containing 250 μg L-lysine·HCl per ml. Similar experiment was also undertaken with *Escherichia coli* 26-26 (obtained from Dr. B. D. DAVIS) using the media supplemented some trace elements to those of Table 3. In this case too,

Table 3. Influence of biotin and lysine concentrations on DAPA accumulation

Biotin	Lysine·HCl	Cell	DAPA	Alanine	Valine	Glutamic acid
30 $\mu\text{g/l}$	1000 $\mu\text{g/ml}$	10.0 mg/ml	trace	⦿	+	⦿
	500	9.4	"	⦿	+ ~ ⦿	⦿
	250	7.4	"	⦿	±	+
	125	8.6	1.0	⦿	+	+
	62.5	8.0	2.5	+	±	+
	31.5	5.3	2.4	+	±	+
	15.6	5.3	1.7	—	—	—
	7.8	5.3	1.3	±	±	+
	0	5.5	1.3	+	+	+
0	1000	3.8	trace	⦿	—	⦿
	500	3.1	"	⦿	—	⦿
	250	3.6	"	+	—	⦿
	125	3.3	"	+	—	⦿
	62.5	3.1	0.5	+	+	⦿
	31.2	2.9	0.8	±	+	⦿
	15.6	6.1	0.4	±	—	+
	7.8	3.3	0.9	±	—	+
	0	3.2	0.9	±	—	+

Basal medium: cerelese 7.5%, $(\text{NH}_4)_2\text{SO}_4$ 1.5%, K_2HPO_4 0.5%, KH_2PO_4 0.05%, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.025%, CaCO_3 1.0%, L-methionine 50 $\mu\text{g/ml}$.

Incubation time: 96 hr.

limited addition of lysine was necessary for accumulation of DAPA. Two milligram of DAPA per ml. of the medium was accumulated in the medium containing L-lysine·HCl 62.5 μg per ml but almost no DAPA was accumulated with high concentrations of lysine (500-1000 μg L-lysine·HCl/ml).

5. Influence of Amino Acids on DAPA Accumulation

Enzyme activity of lysine accumulating system in a lysine producing strain was inhibited specifically by homoserine or threonine as shown in previous paper (2). Therefore, inhibition of DAPA accumulation or DAPA

decarboxylation will be expected if lysine is synthesized via DAPA. From this standpoint, the influence of homoserine and threonine on DAPA accumulation was studied. Aspartic acid was also tested since it seems to be a precursor of DAPA in *E. coli* (9). Since arginine was reported to compete with lysine (8), it was tested in expectation of the increasing effect of the DAPA accumulation as in the valine accumulation by an isoleucine-requiring mutant where leucine and valine increased the valine accumulation (11). Glutamic acid

Table 4. Influence of amino acids on DAPA accumulation

Added amino acid	Diaminopimelic acid (mg/ml)		
	$\mu\text{g/ml}$	Test tube (120 hr.)	250 ml Erlenmeyer flask (96 hr.)
DL-threonine	1000	0.73	0.50
	500	0.68	0.58
	250	0.63	0.56
	125	.500	0.56
DL-homoserine	1000	0.75	0.56
	500	0.73	0.54
	250	0.67	0.50
	125	0.60	0.75
L-glutamic acid	1000	0.60	
	500	0.45	
	250	0.47	
	125	0.32	
DL-alanine	1000	0.52	
	500	0.63	
	250	0.60	
	125	0.57	
L-arginine	1000	1.05	0.92
	500	0.85	0.54
	250	0.72	0.58
	125	0.80	0.40
L-aspartic acid	1000	0.52	
	500	0.54	
	250	0.63	
	125	0.55	
None	0	0.67	0.57

Basal medium: glucose 7.5%, $(\text{NH}_4)_2\text{SO}_4$ 1.5%, K_2HPO_4 0.05%, KH_2PO_4 0.05%, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.025%, CaCO_3 1.0%, biotin 30 $\mu\text{g/l}$, L-methionine 50 $\mu\text{g/ml}$, L-lysine $\cdot \text{HCl}$ 50 $\mu\text{g/ml}$.

and alanine were also included in the test. The results of the experiment were shown in Table 4. No significant influence was recognized with any amino acid tested, although arginine showed a slight increase of DAPA accumulation.

The similar result was obtained in the similar experiment including asparagine with another lysine requiring strain M. 7032-2-10 derived from a different parent strain. With *E. coli* 26-26 too, homoserine and threonine showed no significant inhibition on DAPA accumulation in the concentration range of the amino acids (0-1000 $\mu\text{g/ml}$) except a slight inhibition was observed with 1000 μg of DL-homoserine per ml of media.

6. Inhibition of DAPA Synthesis with Washed Cell Suspension by Lysine

Influence of amino acids on DAPA synthesis from glucose and ammonium salt by washed cell suspension was examined. The cell suspension used in this experiment was obtained with the following procedure. The seed culture medium of M. 534-Co146-5 was inoculated to a 2 L flask which contained 400 ml of the fermentation medium. The composition of the fermentation medium was as follows: Cerelose 8.0%, $(\text{NH}_4)_2\text{SO}_4$ 1.5%, K_2HPO_4 0.05%, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.025%, biotin 30 $\mu\text{g/L}$, CaCO_3 1.0%, L-methionine 50 $\mu\text{g/ml}$, L-lysine-HCl 5 $\mu\text{g/ml}$. After culturing for 2 days, cell was harvested by centrifuge with previous elimination of CaCO_3 . After washing 2 times

Fig. 4. Infra-red spectrum of DAPA (upper : sample, bottom; DAPA of Pfizer Co.).

with saline water, the cell was finally suspended in M/15 phosphate buffer (pH 7.5). Reaction mixture in large test tube was cultured on test tube shaker at 28°C.

The mixture of meso and LL- isomer of DAPA was synthesized by cell suspension as in fermentation broth. Lysine inhibited DAPA synthesis significantly as shown in Table 5. But threonine and homoserine showed almost no inhibition on DAPA synthesis.

Table 5. Influence of amino acids on DAPA formation by washed cell suspension

Added amino acid	DAPA synthesized
None	364 μ g/ml
None, no glucose	100
DL-threonine 10^{-2} M	252
DL-homoserine 10^{-2} M	270
L-lysine 10^{-2} M	120
L-glutamic acid 10^{-2} M	314
L-alanine 10^{-2} M	242

Reaction mixture: M/15 M phosphate buffer (pH 7.5) 1 ml, $MgCl_2$ (10μ moles) 1 ml, NH_4Cl (200μ moles) 1 ml, glucose (100μ moles) 0.5 ml, cell (in 15/M phosphate buffer, 100 mg) 1 ml, amino acid (final 10^{-2} M) 0.5 ml.

7. Influence of Amino Acids on DAPA Decarboxylation

As described above, homoserine and threonine showed no clear inhibition on DAPA synthesis, while lysine inhibited significantly DAPA synthesis. Therefore, the experiment to test the influence of these amino acid on DAPA decarboxylation was attempted. Acetone dried cell of M. 901, the lysine producer, was used as enzyme preparation. Synthetic DAPA was used in the experiment shown in Table 6 and DAPA isolated from fermentation broth was used in the experiment shown in Table 7. In each flask 1.9 mg of

Table 6. Influence of amino acids on DAPA decarboxylation (–1).

Added amino acids		CO ₂ evolved (μ l)			
	M	10 min.	20 min.	30 min.	60 min.
None	1.6×10^{-2}	13	24	29	31
L-lysine	"	0	0	0	1
L-methionine	"	4	10	17	26
L-glutamic acid	"	12	26	26	16
L-leucine	"	12	12	21	24

Synthetic DAPA 1.9 mg was used as substrate.

Table 7. Influence of amino acids on DAPA decarboxylation (-2).

Added amino acid	M	experimented no.	CO ₂ evolved (μ l)			
			10 min.	20 min.	30 min.	60 min.
None	1.6×10^{-2}	3	12	21	30	46
DL-valine	"	2	12	22	29	50
DL-threonine	"	2	16	24	34	54
DL-homoserine	"	3	20	31	49	57
L-serine	"	1	20	33	43	62
Glycine	"	1	24	30	47	67
L-phenylalanine	"	1	24	39	52	56
L-lysine	"	2	0	0	0	1
	1×10^{-2}	1			13	22
	1×10^{-3}	1			33	23
L-arginine	1.6×10^{-2}	2	7	12	20	29
	1×10^{-2}	1			30	56
	1×10^{-3}	1			48	71
L-histidine	1.6×10^{-2}	2	4	6	8	12
	1×10^{-2}	1			11	32
	1×10^{-3}	1			28	52

DAPA from fermentation broth 1.9 mg was used as substrate.

DAPA was added as substrate. Amino acids were added in the concentration shown in the tables. Other conditions were the same as that described previously (5). Homoserine and threonine showed no inhibition even in the concentration of 1.6×10^{-2} M, while lysine, arginine and histidine in high concentration inhibited the DAPA decarboxylation. Some inhibitory effect was recognized with glutamic acid.

DISCUSSION

Lysine biosynthesis via DAPA was already discussed in previous paper (5). In the present studies, DAPA accumulation by lysine requiring strains which lost DAPA decarboxylase was demonstrated. Biosynthetic pathway of lysine via DAPA was firstly suggested by DAVIS (12) by the same phenomenon in *E. coli*. Upon this fact one can not help taking DAPA as "obligatory intermediate" in lysine biosynthesis.

On the other hand, as previously reported (2) in the experiment using the washed cell suspension, lysine synthesis from glucose and ammonium salt was inhibited specifically by homoserine and threonine. Furthermore, it was found that these amino acids inhibit the enzyme activity rather than enzyme synthesis of lysine synthesizing system. Therefore, inhibition of DAPA accumulation or DAPA decarboxylation will be expected if lysine is synthesized via DAPA. But as described above, both homoserine and threonine showed no inhibitory effect either on DAPA synthesis or DAPA decarboxylation while lysine inhibited DAPA synthesis specifically. These

facts can not be explained but to presume the difference of the so called "control mechanism" by strain or biosynthetic pathway of lysine excluding DAPA or 2 biosynthetic pathways of DAPA.

Biosynthetic pathway excluding DAPA was already suggested by ROBERT's *et al* (13) from their studies on amino acid biosynthesis using C^{14} with a strain of *E. coli* different from the strain of B. D. DAVIS. Recently, MEADOW and WORK studied the DAPA synthesis of the lysine requiring strain of *E. coli* by the same technique as that of ROBERTS *et al.* and suggested that DAPA excreted in media is synthesized via a different pathway with that of cellular DAPA. This suggestion is based on the fact that with non-labeled glucose and labeled aspartate or pyruvate, radioactivity was incorporated into cellular DAPA but not into exogenous DAPA, while with labeled glucose or acetate radioactivity was incorporated into both DAPAs. Thus, in *E. coli* many data on lysine biosynthesis including other findings are not necessarily explained rationally. The complexity of lysine biosynthesis in *M. glutamicus* may be suggested by the findings described in this paper.

SUMMARY

α , ϵ -Diaminopimelic acid (DAPA) was accumulated by two lysine requiring strains of *Micrococcus glutamicus* in limited lysine concentration in media. These strains showed neither DAPA decarboxylase nor growth response to DAPA.

A slow growth response to DAPA and DAPA decarboxylase was found in another lysine requiring strain. DAPA accumulation was not recognized by this strain, although trace amounts of DAPA were accumulated in some medium.

Enzyme activity of lysine accumulating system in a lysine-producing strain was inhibited specifically by homoserine or threonine as shown in previous paper. Therefore, inhibition of DAPA accumulation or DAPA decarboxylation will be expected if lysine is synthesized via DAPA. But both homoserine and threonine showed no inhibitory effect either on DAPA synthesis or DAPA decarboxylation while lysine inhibited DAPA synthesis specifically.

From these results, biosynthetic pathway of lysine via DAPA and different pathways of lysine and DAPA were discussed.

ACKNOWLEDGMENT

The synthetic diaminopimelic acid used in this and previous works that are commercially unavailable was supplied to us by Professor M. MATSUI, Tokyo University to whom we take this opportunity of expressing our thanks.

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