Mechanism of L-Threonine and L-Lysine Production by Analog-resistant Mutants of *Corynebacterium glutamicum*[†]

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Homoserine dehydrogenases and aspartokinases in L-threonine- or L-threonine and L-lysine-producing mutants derived from *Corynebacterium glutamicum* KY 9159 (Met⁻) were studied with respect to the sensitivity to the inhibition by end products, L-threonine and L-lysine. The activities of homoserine dehydrogenases in the mutants which produced L-threonine or L-threonine and L-lysine were slightly less susceptible to the inhibition by L-threonine than the activity in the parent strain, KY 9159. The aspartokinases in the threonine-producing mutants, KY 10484 and KY 10230, which were resistant to α -amino- β -hydroxyvaleric acid (AHV, a threonine analog) and more sensitive to thialysine (a lysine analog) than the parent, were sensitive to the concerted feedback inhibition by L-lysine and L-threonine by about the same degree as KY 9159. The aspartokinase in an AHV- and thialysine-resistant mutant, KY 10440, which was derived from KY 10484 and produced about 14 mg/ml of L-threonine in a medium containing 10% glucose was less susceptible to the concerted feedback inhibition than KY 10484 or KY 9159, although the activity was still under the feedback control. In the parent strain, L-threonine activated aspartokinase activity in the absence of ammonium sulfate, an activator of the enzyme, but partially inhibited the activity in the presence of the salt. On the other hand, the enzyme of KY 10440 was activated by L-threonine either in the presence or in the absence of the salt. In another AHV- and thialysine-resistant mutant, KY 10251, which was derived from KY 10230 and produced both 9 mg/ml of L-threonine and 5.5 mg/ml of L-lysine, L-threonine and L-lysine simultaneously added hardly inhibited the activity of aspartokinase.

Implications of these results are discussed in relation to L-threonine or L-lysine production, AHV or thialysine resistance and regulation of L-threonine biosynthesis in these mutants.

In a previous paper,²⁾ it was reported that an α -amino- β -hydroxyvaleric acid (AHV, a threonine analog)- and thialysine (a lysine analog)-resistant mutant, KY 10440, derived from *Corynebacterium glutamicum* KY 9159 (Met⁻) produced about 14 mg/ml of L-threonine with a medium containing 10% glucose. Another AHV- and thialysine-resistant mutant, KY 10251, which was also derived from KY 9159, produced both 9 mg/ml of L-threonine and 5.5 mg/ml of L-lysine. In the course of derivation of these mutants, authors found that AHV-resistant mutants, KY 10484 and KY 10230, the parent of KY 10440 and KY 10251 respectively, were much more suscep-

ible to the growth inhibition by thialysine than KY 9159. In *C. glutamicum* L-threonine biosynthesis was proved to be controlled by the end products, L-threonine, L-lysine and L-methionine,^{9,10)} as shown in Fig. 1. Aspartokinase was subject to the concerted feedback inhibition by L-lysine and L-threonine, and homoserine dehydrogenase was controlled by feedback inhibition by L-threonine and repression by L-methionine.

It was suggested that L-threonine or L-lysine production and AHV or thialysine resistance (or sensitivity) in the mutants might be due to the regulatory abnormality of the enzymes in L-threonine and L-lysine biosynthesis.

Therefore, in the present study, homoserine dehydrogenases and aspartokinases in the

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FIG. 1. Regulation of Threonine Biosynthesis in *C. glutamicum*.

mutants and the parent strain were examined with regard to the regulatory properties. Relations among L-threonine or L-lysine production, AHV or thialysine resistance (or sensitivity) and enzymic abnormality in the mutants are discussed.

MATERIALS AND METHODS

Organisms. The genealogy of the strains used is presented in Fig. 2. The derivations and characterizations of the mutants were described previously.²⁾

Cultures. Ten milliliters of a seed medium in a large test tube (2.5×19 cm) were inoculated with one loopful of an organism grown on a bouillon agar slant, and incubated on a reciprocal shaker. After incubation for 24 hr at 30°C, 2 ml of this seed culture were transferred into a 250 ml Erlenmeyer flask with a baffling plate containing 20 ml of the medium A supplemented with 100 µg/ml of L-methionine. The

flask was incubated on a rotary shaker (220 rpm, 5 cm dia.) for 24 hr at 30°C. The compositions of the seed medium and medium A were described previously.²⁾

Preparation of enzyme solution. The culture broth was first centrifuged at low speed to eliminate calcium carbonate from the broth. The broth was again centrifuged to collect the cells. After washing twice with 0.9% NaCl solution, the cells were suspended in 0.1 M Tris-HCl buffer (pH 8.0) containing 0.02 M of β-mercaptoethanol. The cell suspension containing 50~60 mg dry wt. of organisms/ml was treated with ultrasonic oscillator (10 KC) for 30 min under cooling. The cell debris was removed by centrifugation at 15,000×g for 30 min. Five volume of saturated ammonium sulfate solution was added slowly to the supernatant fluid. The precipitates formed were collected by centrifugation and dissolved in the same buffer. The resulting solution was used for enzyme assays.

Enzyme assays. Aspartokinase [ATP: L-Aspartate

FIG. 2. Genealogy of Mutants of *Corynebacterium glutamicum* Producing L-Threonine or L-Threonine and L-Lysine.

^{a)} The value expressed the amount of L-threonine (solid underline) or L-lysine (broken underline) produced in medium A supplemented with 100 µg/ml of L-methionine.

^{b)} AHV; α-amino-β-hydroxyvaleric acid, S-Lys; thialysine.

4-phosphotransferase (2.7.2.4.)] activity was determined as described by Black and Wright,³¹ by measuring the aspartate hydroxamate that is produced when aspartate is incubated in the presence of ATP and hydroxylamine. The assay mixture contained, in a total volume of 1 ml; L-aspartate (pH 8.0 with Tris base) 100 mM, MgCl₂ 20 mM, ATP (pH 8.0 with Tris base) 20 mM, hydroxylamine (pH 8.0 with Tris base) 400 mM, Tris-HCl buffer (pH 8.0) 40 mM and enzyme solution. Reaction was run at 37°C for 1 hr and then stopped by the addition of 1.5 ml of FeCl₃ reagent.⁴¹ After centrifugation, the optical density was measured at 540 nm. Enzyme activity is expressed as optical density units $\times 1000$. A blank reaction mixture which contained all components except L-aspartate served as a control.

The activity of homoserine dehydrogenase [Homoserine: NADP oxidoreductase (1.1.1.3)] was determined by a modification of the method of Black and Wright.⁵¹ Rate of increase of optical density at 340 nm which was caused by the reduction of NADP in the presence of L-homoserine and the enzyme was used as the measure for homoserine dehydrogenase activity. The assay mixture contained, in a total volume of 3 ml; 2-amino-2-methyl-1,3-propanediol-HCl buffer (pH 9.25) 133 mM, L-homoserine 8.1 mM, NADP 0.133 mM and enzyme solution. The reaction was carried out at a room temperature. A blank reaction mixture which contained all components except L-homoserine served as a control.

Determination of protein. Protein concentration in a enzyme solution was estimated from the optical density measurements at 280 nm and 260 nm.⁶¹

Growth experiment. Growth experiments were carried out as described previously.²¹

RESULTS

1. Effect of AHV or thialysine on the growth

The effect of AHV or thialysine on the growth of KY 9159, the parent strain, and the mutants derived from KY 9159 was studied (Fig. 3). In KY 9159, 2 mg/ml of AHV or thialysine reduced the maximum growth by 70 or 40%, respectively. In the mutant KY 10290 which was derived from KY 9159 and produced 0.6 mg/ml of L-threonine in medium A, 2 mg/ml of AHV hardly reduced the growth, whereas 8 mg/ml of AHV reduced the maximum growth by 30%. The mutant KY 10484 derived from KY 10290 showed higher resistance to AHV and higher productivity of L-

FIG. 3. Growth Inhibition of Mutants of *C. glutamicum* by AHV or Thialysine.

●—●, KY 9159 (Met⁻); ○—○, KY 10290 (Met⁻, AHV^R); △—△, KY 10484 (Met⁻, AHV^R); ▲—▲, KY 10440 (Met⁻, AHV^R, S-Lys^R).

Cells were inoculated in the minimal medium and in the minimal medium supplemented with increasing concentration of AHV or thialysine.

threonine (2.3 mg/ml in medium A) than KY 10290. The growth of KY 10484 decreased by only 15% with addition of 20 mg/ml of AHV. On the other hand, these AHV-resistant mutants showed much more susceptibility to the growth inhibition by thialysine than the parent strain, KY 9159; their growth decreased by 60~80% with addition of only 100 μ g/ml of thialysine. A thialysine-resistant mutant, KY 10440, derived from KY 10484, produced about 9.5 mg/ml of L-threonine in medium A. (Under a more favorable condition, KY 10440 produced about 14 mg/ml of L-threonine.²¹) KY 10440 showed AHV resistance comparable to KY 10484 and thialysine resistance comparable to KY 9159.

Another AHV resistant mutant KY 10230, derived from KY 9159, was also more sensitive to thialysine than KY 9159. A thialysine-resistant mutant KY 10251, derived from KY 10230, produced both 9 mg/ml of L-threonine and 5.5 mg/ml of L-lysine. KY 10251 was more resistant to thialysine than KY 9159 or KY 10440.

2. Homoserine dehydrogenases of the parent strain and AHV-resistant mutants

AHV-resistant mutants produced a large amount of L-threonine and their L-threonine

enzymes might have been less sensitive to regulation by L-threonine. Therefore, the effect of L-threonine on the homoserine dehydrogenase activities in the parent strain and AHV-resistant mutants were examined. As shown in Fig. 4, homoserine dehydrogenases in KY 10484

FIG. 4. Effect of L-Threonine on Homoserine Dehydrogenase Activity.

Except for the addition of L-threonine as indicated, the assay was carried out as described in the text. Each reaction mixture contained 0.3 ml of enzyme solution (0.6 mg protein).

●—●, KY 9159; ▲—▲, KY 10484; ■—■, KY 10440.

and KY 10440 were found to be slightly less sensitive to feedback inhibition by L-threonine than KY 9159. The enzyme of KY 9159 was completely inhibited by 7.5×10^{-4} M of L-threonine, whereas the concentration of L-threonine required for complete inhibition of the activities in KY 10484 and in KY 10440 was approximately 10^{-2} M. The enzyme in KY 10484 was slightly more susceptible to inhibition by L-threonine than that in KY 10440.

These results might not fully account for

L-threonine production in the AHV resistant mutants, because homoserine dehydrogenases of the mutants were still under the control by L-threonine. However, such a genetical alteration in the enzyme may be least a cause of L-threonine production in the mutants.

3. Aspartokinases of the parent strain and AHV- or thialysine-resistant mutants

a) *Effect of L-threonine and L-lysine on aspartokinases.* Effect of L-threonine and L-lysine on aspartokinase activities was studied with KY 9159, KY 10484 and KY 10440 (Table I). The activity of aspartokinase in KY 9159 was inhibited to a extent of 20% by 10 mM of L-lysine and stimulated to a extent of 70~80% by 10 mM of L-threonine. In the presence of 10 mM of both L-threonine and L-lysine, the activity was inhibited by approximately 80%. The enzyme in KY 10484 was subject to stimulation by L-threonine and inhibition by L-threonine plus L-lysine by about the same degree as KY 9159, although inhibition of the activity by L-lysine was rather stronger in KY 10484 than in KY 9159. On the contrary, aspartokinase activity of KY 10440 was not inhibited by L-lysine alone. Besides, L-threonine stimulated the activity by approximately 300% at a concentration of 10 mM. In the presence of 1 mM of both L-threonine and L-lysine, the activity in KY 10440 was slightly activated, whereas the activities in KY 9159 and KY 10484 were inhibited by 15~25%. L-Threonine and L-lysine added simulta-

TABLE I. EFFECT OF L-THREONINE AND L-LYSINE ON ASPARTOKINASE ACTIVITY

Except for the addition of L-lysine and L-threonine as indicated, the assay was carried out as described in the text. Each reaction mixture contained 0.3 ml of enzyme solution (7.5 mg protein).

L-Threonine added μ moles/ml	L-Lysine added μ moles/ml	Relative activity, %		
		KY 9159	KY 10484	KY 10440
—	—	100	100	100
1	—	119	116	174
10	—	176	166	295
—	1	86	75.1	115
—	10	77.5	59.6	117
1	1	86.2	77.9	128
10	10	22.1	21.5	31.3

neously at a concentration of 10 mM inhibited the enzyme activity in KY 10440 by about 70%.

b) *Effect of ammonium sulfate and L-threonine on aspartokinase.* Shioo *et al.*⁷⁾ has reported that SO_4 ion significantly stimulated aspartokinase activity in *Brevibacterium flavum*. They selected a thialysine-resistant mutant which was genetically desensitized to the concerted feedback inhibition by L-lysine and L-threonine and showed that in the presence of ammonium sulfate L-threonine slightly activated the enzyme of the mutant but partially inhibited the parental enzyme. In the absence of the salt it was an activator of both enzymes.

The data in Table I suggested that aspartokinase in KY 10440 might be partially desensitized to the concerted feedback inhibition. In order to more clarify the genetical alterations of the enzymes in the mutants the effect of ammonium sulfate and L-threonine on the activities of aspartokinases was studied with KY 9159, KY 10484 and KY 10440. As shown in Table II, ammonium sulfate activated the enzymes of all strains, and the degree of activation was greater in KY 10440 than KY 10484 or KY 9159. The effect of L-threonine on the activities of aspartokinases in the presence of ammonium sulfate is shown in Fig. 5. In the absence of ammonium sulfate, the enzymes in all strains were activated by L-threonine as already shown in Table I. In the presence of 600 mM of ammonium sulfate, the enzyme in KY 9159 was inhibited to a extent of 20% by 20 mM of L-threonine, whereas the enzyme in KY 10440 was activated to a extent

FIG. 5. Effect of Ammonium Sulfate on Aspartokinase Activity in the Presence or Absence of L-Threonine.

Except for the addition of ammonium sulfate and L-threonine as indicated, the assay was carried out as described in the text. Each reaction mixture contained 0.3 ml of enzyme solution (8.7 mg protein).

●—●, in the presence of 20 mM of L-threonine, with KY 9159; ○—○, in the absence of L-threonine, with KY 9159; ▲—▲, in the presence of 20 mM of L-threonine, with KY 10484; △—△, in the absence of L-threonine, with KY 10484; ■—■, in the presence of 20 mM of L-threonine, with KY 10440; □—□, in the absence of L-threonine, with KY 10440.

of 15~20% under the same condition. The enzyme in KY 10484 was hardly affected by L-threonine in the presence of 600 mM of ammonium sulfate.

The effect of L-lysine on aspartokinase activity in the presence of 10 mM of L-threonine and 600 mM of ammonium sulfate was tested with each strain (Fig. 6). The degree of inhibition by L-lysine was about the same in KY 9159 and KY 10484. However, the enzyme in KY 10440 was less susceptible to the inhibition by L-lysine than KY 9159 or KY

TABLE II. EFFECT OF AMMONIUM SULFATE ON ASPARTOKINASE ACTIVITY

Except for the addition of ammonium sulfate as indicated, the assay was carried out as described in the text. Each reaction mixture contained 0.3 ml of enzyme solution (8.7 mg protein). Aspartokinase activity is expressed as optical density units $\times 1000$. Relative activity is expressed as percentage of the activity in the absence of ammonium sulfate.

Strain No.	Ammonium sulfate (600 mM) added		Ammonium sulfate omitted	
	Activity	Relative activity, %	Activity	Relative activity, %
KY 9159	231	218	106	100
KY 10484	706	183	386	100
KY 10440	452	337	134	100

FIG. 6. Effect of L-Lysine on Aspartokinase Activity in the Presence of L-Threonine (10 mM) and Ammonium Sulfate (600 mM).

Except for the addition of L-lysine, L-threonine and ammonium sulfate as indicated, the assay was carried out as described in the text. Each reaction mixture contained 0.3 ml of enzyme solution (8.7 mg protein). Relative activity is expressed as percentage of the activity in the absence of L-lysine and L-threonine.

▲—▲, KY 9159; ■—■, KY 10484; ●—●, KY 10440.

10484; the concentration of L-lysine required for 50% inhibition was approximately 10 times larger in KY 10440 (1 mM) than in KY 9159 and KY 10484.

4. Homoserine dehydrogenase and aspartokinase in KY 10251

Homoserine dehydrogenase and aspartokinase in another AHV- and thialysine-resistant mutant KY 10251 which produced both L-threonine and L-lysine were compared with those in the parent strain KY 9159 and KY 10230.

The activity of homoserine dehydrogenase in KY 10251 was inhibited by L-threonine slightly more weakly than that in KY 9159 (Table III). The degree of inhibition by L-threonine in KY 10251 was about the same as that in KY 10440 shown in Fig. 4. Aspartokinase activity of KY 10251 was inhibited by only 5% in the presence of 10 mM of both L-threonine and L-lysine (Table IV). Under the same condition, the enzyme in KY 10230 as well as in KY 9159 was inhibited by about 80%.

DISCUSSION

Aspartokinase and homoserine dehydro-

TABLE III. EFFECT OF L-THREONINE ON HOMOSERINE DEHYDROGENASE ACTIVITIES IN KY 10251 AND KY 9159

Except for the addition of L-threonine as indicated, the assay was carried out as described in the text. Each reaction mixture contained 0.3 ml of enzyme solution (0.6 mg protein).

Concentration of L-threonine (M)	Relative activity, %	
	KY 9159	KY 10251
—	100	100
10^{-4}	63.1	100
5×10^{-4}	10.6	56.3
10^{-3}	0.1	27.7
10^{-2}	0	0.5

TABLE IV. EFFECT OF L-THREONINE AND L-LYSINE ON ASPARTOKINASE ACTIVITY IN KY 10230 AND KY 10251

Except for the addition of amino acids as indicated, the assay was carried out as described in the text. Each reaction mixture contained 0.3 ml of enzyme solution (8.4 mg protein).

L-Threonine added μ moles/ml	L-Lysine added μ moles/ml	Relative activity, %	
		KY 10230	KY 10251
—	—	100	100
—	10	75	107.3
10	—	119	111
10	10	21.4	95

genase were studied in L-threonine-producing and L-threonine- and L-lysine-producing mutants. The data summarizing the characterizations of the mutants and the parent strain are shown in Table V. Although homoserine dehydrogenases in all mutants which were resistant to AHV and able to produce L-threonine were less sensitive to the inhibition by L-threonine than that in the parent strain, these mutants were still subject to regulation by L-threonine ("partially sensitive"). As described above, such a genetical alteration in the enzyme may be at least a cause of L-threonine production in these mutants. Other factors such as a permeability change might be required for a better explanation of high AHV resistance and L-threonine productivity in the mutants.

AHV-resistant mutants, KY 10484 and KY

TABLE V. SUMMARY OF CHARACTERIZATION OF THE MUTANT IN *C. glutamicum*

Strains	Production of		AHV ^{b)} resistance	S-Lys ^{c)} resistance	Aspartokinase ^{d)}	Homoserine ^{d)} dehydrogenase
	L-threonine (mg/ml)	L-lysine (mg/ml)				
KY 9159 (Met ⁻)	— ^{a)}	— ^{a)}	S	s	sensitive	sensitive
KY 10484 (Met ⁻ , AHV ^R)	2.3	— ^{a)}	R	ss	sensitive partially	partially sensitive
KY 10440 (Met ⁻ , AHV ^R , S-Lys ^R)	9.5	— ^{a)}	R	s	sensitive	partially sensitive
KY 10230 (Met ⁻ , AHV ^R)	3.7	— ^{a)}	R	ss	sensitive	partially sensitive
KY 10251 (Met ⁻ , AHV ^R , S-Lys ^R)	9.0	5.5	R	r	insensitive	partially sensitive

^{a)} L-Threonine or L-lysine was not detected on paperchromatography.

^{b)} S; sensitive to AHV R; resistant to AHV.

^{c)} s; The growth was inhibited by 30~40% in the presence of 2 mg/ml of thialysine but hardly inhibited by 100 µg/ml of thialysine.

ss; The growth was inhibited by 50~80% in the presence of 100 µg/ml of thialysine.

r; The growth was not reduced in the presence of 2~5 mg/ml of thialysine.

^{d)} 'Partially sensitive' means that the susceptibility of the enzyme activity to the inhibition by L-threonine (in homoserine dehydrogenase) or the concerted feedback inhibition by L-threonine and L-lysine (in aspartokinase) decreased as compared with the parent strain.

'Insensitive' means that the enzyme was insensitive to the feedback inhibition.

10230 were much more susceptible to the growth inhibition by thialysine than the parent strain, KY 9159. The susceptibilities of aspartokinases in KY 10484 and KY 10230 to the concerted feedback inhibition by L-threonine and L-lysine were comparable to that in KY 9159, though the inhibitory effect of L-lysine alone on the enzyme was slightly stronger in KY 10484 than in KY 9159 or KY 10230. In KY 10484 and KY 10230, threonine producers, intracellular level of L-threonine might excessively increased. Consequently, aspartokinases *in vivo* would be subject to inhibition at much lower concentration of L-lysine in KY 10484 and KY 10230 than in KY 9159. Thialysine added externally would mimic L-lysine as a feedback inhibitor and act on aspartokinase at lower concentration in KY 10484 and KY 10230 than in KY 9159. Thus, thialysine would be more inhibitory to the growth in KY 10484 and KY 10230 than in KY 9159.

Aspartokinase in KY 10440 was 'partially' desensitized to the concerted feedback inhibition by L-threonine and L-lysine. The resistance to thialysine in KY 10440 was recovered

to the level comparable to that in the parent strain KY 9159. The amount of L-threonine produced in KY 10440 increased by about three times as much as that in the parental thialysine-sensitive mutant, KY 10484. On the other hand, KY 10251 derived from a AHV-resistant and thialysine-sensitive mutant, KY 10230, was more resistant to thialysine than KY 9159 and its aspartokinase activity was insensitive to the concerted feedback inhibition by L-threonine and L-lysine. Moreover, KY 10251 produced L-lysine besides L-threonine. Homoserine dehydrogenases in both KY 10440 and KY 10251 were 'partially sensitive to the inhibition by L-threonine by about the same degree. Therefore, the difference in the susceptibility to thialysine and in L-lysine production between KY 10440 and KY 10251 would be mainly due to the difference in the susceptibility of aspartokinase to the concerted feedback inhibition by L-threonine and L-lysine. Furthermore, higher level of L-threonine production in AHV- and thialysine-resistant mutants as compared with the parent AHV-resistant mutants would be brought about by the genetical desensitization

in aspartokinase to the end product inhibition.

KY 10251 seemed to be the same type as AHV- and thialysine-resistant mutants of *B. flavum* reported by Nakamori and Shiio,⁸⁾ which produced both L-threonine and L-lysine. In their mutant too, aspartokinases were insensitive to the concerted feedback inhibition and homoserine dehydrogenases were partially sensitive to the inhibition by L-threonine. The characteristics in KY 10440 seemed to be unique in that its threonine enzymes were both partially sensitive to the inhibition by the end products, nevertheless it produced over 10 mg/ml of L-threonine.

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