Effect of Amino Acids and Purine Bases on Thiamine Synthesis by *Escherichia coli*¹

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Phenylalanine and histidine, out of several amino acids tested by the addition to the growth medium of *Escherichia coli*, showed stimulatory effects on thiamine production by the cells. In order to know the mechanism of phenylalanine action, it was compared with the adenine effect in which adenine added to the growth medium caused a decrease in thiamine production of *E. coli* accompanying with the increase of thiamine synthesizing activity from hydroxymethylpyrimidine and thiazole. Phenylalanine had also a similar elevating effect on thiamine synthesizing activity from hydroxymethylpyrimidine and thiazole in spite of the increase in cellular thiamine contents. The phenylalanine effect could be abolished by a simultaneous thiazole addition at a physiological concentration for the organism in contrast with the adenine effect, which was arrested by hydroxymethylpyrimidine addition. The phenylalanine effect is supposed to be concerned with the synthesis of thiazole moiety of thiamine. The regulation of thiamine biosynthesis in *E. coli* was also discussed.

Recent several isotopic studies for clarifying the precursors of pyrimidine and thiazole moieties of thiamine have revealed that some amino acids as well as formate and acetate effectively incorporated into the thiamine molecule in several organisms (1-7). However, these active compounds except for sulfur-containing amino acids had no appreciable stimulatory effects on *de novo* synthesis of thiamine in growing, or resting cells of *E. coli* (8). This finding is well understandable from the observations that thiamine biosynthesis is regulated by repression-derepression mechanism in *Aerobacter aerogenes* (9), in *Salmonella typhimurium* (10) and *E. coli* (14). In these reports thiamine added to the growth medium of the test organisms caused repression in thiamine synthesis, while adenosine or adenine in the growth medium led to the derepression of thiamine synthesis and to the

¹ Following abbreviations are used: hydroxymethylpyrimidine, 2-methyl-4-amino-5-hydroxymethylpyrimidine; thiazole, 4-methyl-5- β -hydroxyethylthiazole.

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lowering of the cellular thiamine contents.

The present paper deals with a stimulatory effect of some aromatic amino acids, especially phenylalanine, on thiamine production of *E. coli* and elevation of thiamine synthesis from hydroxymethylpyrimidine and thiazole by the washed cell suspensions of the organism grown in the medium supplemented with phenylalanine. The effect of adenine was also investigated.

EXPERIMENTAL

1. Organism and Culture Media

A wild strain of *E. coli* ATCC 9637 was used for this work. The basal medium used was that of Davis and Mingioli (11). The inoculum was grown overnight at 37° in 10 ml of the basal medium.

2. Chemicals

Hydroxymethylpyrimidine and thiazole were kindly supplied by Dr. S. Yurugi, Takeda Chemical Industries, Osaka. Amino acids used are all L-form and obtained from commercial sources. Adenine hydrochloride and other purine and pyrimidine bases are the products of Kokoku Pulp Co., Ltd., Tokyo. Takadiastase was purchased from Sankyo Co., Ltd., Tokyo.

3. Growth of *E. coli* in Liquid Media

One drop of the inoculum was inoculated in 25 ml of the basal medium with or without the supplements indicated, followed by incubation for 17-18 hours at 37° with constant shaking. After incubation, each 5 ml of the cultures was used for growth measurement turbidimetrically, and was calculated to the dry weight of the cells. The cells in the remaining cultures were harvested by centrifugation, washed with a saline solution and resuspended in 5 ml of 0.1 M acetate buffer (pH 4.1). It was used for thiamine assay.

4. Thiamine Assay

Thiamine in the cells was extracted by heating for 15 minutes at 85° in a water bath. This was followed by incubation with Takadiastase at 45° to convert thiamine phosphates into thiamine. The enzyme reaction was stopped by adding 10 % metaphosphoric acid and centrifuged to remove denatured protein. Thiamine in the supernatant was determined fluorometrically by the thiochrome method after adsorption on permutite using a Shimazu fluorometer (12).

5. Thiamine Synthesis from Hydroxymethylpyrimidine and Thiazole by Washed Cell Suspensions

Washed cell suspensions of *E. coli* were prepared as follows: one milliliter of the inoculum was inoculated in 200 ml of the basal medium with or without the supplements. After incubation for 17 hours at 37° with constant shaking, the cells were harvested at stationary phase by centrifugation, washed once with sterile saline solution and resuspended in 3 to 5 ml of sterile saline to obtain a cell suspension of 15-20 mg dry weight per ml. The reaction mixture consisted of 20 mM Tris-maleate buffer (pH 7.0), each 10 μ M hydroxymethylpyrimidine (13.9 μ g) and thiazole (14.3 μ g), 0.2 % glucose, 0.9 % NaCl and 1 ml of the washed cell suspension in a total volume of 10 ml. After incubation for 1 hour at 37° with constant

shaking, an aliquot (8 ml) was pipetted into a centrifuge tube and the pH was adjusted to 4.5 with *N* HCl. Further procedure was followed by the method for thiamine assay described above. The amount of thiamine formed from hydroxymethylpyrimidine and thiazole were calculated by subtracting initial contents in the cells from total amounts of thiamine detected.

RESULTS

1. Effect of Phenylalanine and Histidine in the Growth Medium on Cellular Thiamine Contents

Several aliphatic amino acids except for sulfur-containing ones failed to increase thiamine content per unit weight of the cells. However, phenylalanine and histidine, among the aromatic and heterocyclic amino acids tested at the concentrations indicated, enhanced cellular thiamine contents (Table 1). The thiamine produced in the cultures was found in the cells, but scarcely detected in the medium.

TABLE 1
Effects of Aromatic and Heterocyclic Amino Acids Added to the Growth Medium on Thiamine Contents of E. coli

Addition to medium	Cells produced	Amount of thiamine		Ratio
		Total	Per mg cells	
<i>M</i>	<i>mg</i>		<i>mμg</i>	<i>per cent</i>
None	16.9	475	28.1	100.0
Tryptophan (1×10^{-2})	18.3	400	21.9	78.0
Phenylalanine (1×10^{-2})	16.0	725	45.3	161.1
Tyrosine (1×10^{-2})	20.0	550	27.5	97.9
Histidine (1×10^{-3})	13.6	500	36.8	130.9
Proline (1×10^{-2})	26.8	475	17.7	62.9

The optimal concentrations of phenylalanine and histidine in the reaction mixtures were 10 and 1 mM, respectively and 10 mM histidine inhibited the growth as shown in Table 2. The phenylalanine effect was a little higher than that of histidine and the cellular thiamine content was one and a half or twice as much as that of the non-supplemented.

TABLE 2
Effects of Concentrations of Phenylalanine and Histidine on Thiamine Contents in the Cells

Addition to medium	Cells produced	Amount of thiamine		Ratio
		Total	Per mg cells	
<i>M</i>	<i>mg</i>		<i>mμg</i>	<i>per cent</i>
None	17.1	525	30.7	100.0
Phenylalanine (1×10^{-3})	17.6	550	31.2	101.6
Phenylalanine (1×10^{-2})	17.1	800	46.8	152.3
Phenylalanine (2×10^{-2})	10.1	375	37.1	120.8
Histidine (1×10^{-4})	17.1	525	30.7	100.0
Histidine (1×10^{-3})	15.9	575	36.2	117.9
Histidine (1×10^{-2})	No growth			

The additional effects of various amino acids at the concentrations indicated in the presence of 10 mM phenylalanine were given in Table 3. Except for methionine there was no increase in thiamine contents in all the cases.

TABLE 3
*Additional Effects of Various Amino Acids on Thiamine Contents
in the Presence of Phenylalanine*

Addition to medium	Cells produced	Amount of thiamine	
		Total	Per mg cells
<i>M</i>	<i>mg</i>		<i>μg</i>
Phenylalanine (1×10^{-2})	18.3	1,000	54.7
+ Glycine (1×10^{-3})	19.4	1,050	54.2
+ Alanine (1×10^{-3})	19.9	1,000	51.5
+ Valine (1×10^{-3})	18.5	850	45.9
+ Leucine (1×10^{-3})	18.4	650	35.3
+ Isoleucine (1×10^{-3})	18.3	900	49.2
+ Serine (1×10^{-3})	19.0	1,000	52.6
+ Threonine (1×10^{-3})	20.2	1,100	54.5
+ Cystine (1×10^{-3})	12.0	200	16.7
+ Methionine (1×10^{-3})	18.5	1,500	81.1
+ Aspartic ac. (1×10^{-3})	19.4	1,100	56.7
+ Glutamic ac. (1×10^{-3})	19.4	875	45.1
+ Lysine (1×10^{-3})	17.8	875	49.2

2. Effects of Purine and Pyrimidine Bases in the Growth Medium on Cellular Thiamine Contents

Since adenosine lowered cellular thiamine contents in *A. aerogenes* (9), and *S. typhimurium* (10), the same tendency was expected in *E. coli*. The effects of several purine and pyrimidine bases added to the mixtures at 1 mM concentrations were therefore examined. As is shown in Table 4, adenine and inosine effectively reduced the thiamine contents in the cells, while adenosine and pyrimidine bases showed no effects at 1 mM concentrations. The effects of hydroxymethylpyrimidine and thiazole added to the mixture at several concentrations were also investigated.

TABLE 4
Effects of Purines and Pyrimidines on Thiamine Contents

Addition to medium	Cells produced	Amount of thiamine	
		Total	Per mg cells
$1 \times 10^{-3} M$	<i>mg</i>		<i>μg</i>
None	17.3	475	27.5
Adenine	17.0	275	16.2
Guanine	28.8	600	20.8
Hypoxanthine	17.5	350	20.0 (15.9) ^a
Adenosine	10.1	400	39.6 (19.6) ^a
Inosine	16.6	150	9.0 (5.1) ^a
Aminoimidazolecarboxamide	17.7	350	19.8 (8.6) ^a
AMP	18.9	450	23.8
Cytosine	17.1	450	26.3
Uracil	17.6	475	27.0
Thymine	17.0	475	27.9

^a The value after addition of $10^{-2} M$.

TABLE 5
Effects of Hydroxymethylpyrimidine and Thiazole Added to the Medium on Thiamine Contents

Addition to medium	Cells produced	Amount of thiamine	
		Total	Per mg cells
<i>M</i>	<i>mg</i>	<i>μg</i>	
None	16.8	475	28.3
Hydroxymethylpyrimidine (1×10^{-7})	16.9	575	34.0
Hydroxymethylpyrimidine (1×10^{-6})	16.9	600	35.5
Hydroxymethylpyrimidine (1×10^{-5})	16.8	650	38.7
Hydroxymethylpyrimidine (1×10^{-4})	16.9	625	37.0
Thiazole (1×10^{-7})	17.0	450	26.5
Thiazole (1×10^{-6})	17.3	450	26.0
Thiazole (1×10^{-5})	17.3	475	27.5
Thiazole (1×10^{-4})	17.3	500	28.9

As is shown in Table 5, 0.1 to 100 μM hydroxymethylpyrimidine in the mixture elevated cellular thiamine contents to the same extents, while the same amounts of thiazole had no effect. It is of interest in regulation of thiamine synthesis in *E. coli* as later discussed in this paper that only the pyrimidine moiety of thiamine, but not thiazole, stimulated thiamine production though the presence of both compounds elevated much more thiamine content than with hydroxymethylpyrimidine alone. On the other hand, the adenosine effect in *A. aerogenes* is thought to be due to the inhibition of hydroxymethylpyrimidine synthesis leading to the lowered thiamine contents in the cells (9). The addition of hydroxymethylpyrimidine to the medium seems to overcome this inhibition, causing cellular thiamine synthesis in the cells. That this is also the case in *E. coli*, is shown in Table 6, in which 0.1 μM hydroxymethylpyrimidine added to the mixture with adenine brought about the elevated thiamine content to the normal level but it was not the case with thiazole, even at 100 times concentration.

TABLE 6
Abolishment by Hydroxymethylpyrimidine of Adenine Effect on Cellular Thiamine Contents

Addition to medium	Thiamine produced
<i>M</i>	<i>μg/mg cells</i>
None	23.7
Adenine (1×10^{-3})	12.7
Adenine+hydroxymethylpyrimidine (1×10^{-8})	12.5
Adenine+hydroxymethylpyrimidine (1×10^{-7})	21.7
Adenine+thiazole (1×10^{-7})	14.0
Adenine+thiazole (1×10^{-5})	14.0

3. Mechanism of Phenylalanine Action

Since hydroxymethylpyrimidine could overcome the adenine effect of reducing cellular thiamine content, the effects of adding hydroxymethylpyrimidine or thiazole on phenylalanine effect was investigated. As is shown in Table 7, the elevated cellular thiamine contents by phenylalanine were brought back to the control level with 0.1 μM thiazole, but not with hydroxymethylpyrimidine. This suggests that phenylalanine is concerned somehow with the synthesis of thiazole

TABLE 7
Effects of Thiazole on Cellular Thiamine Content Elevated by Phenylalanine

Addition to medium	Cells produced	Amount of thiamine	
		Total	per mg cells
<i>M</i>	<i>mg</i>		<i>mμg</i>
None	17.7	500	28.2
Phenylalanine (1×10^{-2})	17.7	1,150	65.2
+thiazole (1×10^{-8})	17.6	900	51.1
+thiazole (1×10^{-7})	17.6	600	34.1
+thiazole (1×10^{-6})	17.6	550	31.2
+thiazole (1×10^{-5})	17.7	500	28.2
+thiazole (1×10^{-4})	17.7	450	25.4
+hydroxymethylpyrimidine (1×10^{-7})	17.8	1,150	64.6
+hydroxymethylpyrimidine (1×10^{-4})	17.7	1,150	65.0

portion of thiamine in analogy to the effect of adenine which inhibits the synthesis of pyrimidine portion. However, phenylalanine had a paradoxical effect compared with that of adenine: phenylalanine increased cellular thiamine contents, whereas adenine reduced them.

4. *Thiamine Synthesis from Hydroxymethylpyrimidine and Thiazole by Washed Cell Suspensions of E. coli Grown in a Medium Supplemented with Adenine or Phenylalanine*

In order to know more precise action of phenylalanine, thiamine synthesizing activities from hydroxymethylpyrimidine and thiazole by non-growing cell suspensions of *E. coli* grown in a medium supplemented with or without adenine or phenylalanine were compared as described in the Method. The resting cells as well as growing cells of *E. coli* can easily synthesize thiamine from hydroxymethylpyrimidine and thiazole added to the medium in the presence of glucose (13). The stimulation of thiamine synthesis by resting cells of *S. typhimurium* preincubated with adenosine was also reported (10). As shown in Table 8, it was found that phenylalanine (5 mM) increased the thiamine synthesizing activities from hydroxymethylpyrimidine and thiazole about twice as much as that of the non-supplemented. The degree is a little lower than that detected with adenine. The effect of adenine on stimulation of thiamine synthesis by washed cell suspensions appeared to be due to derepression in thiamine synthesis. Although phenylalanine had an opposite effect to adenine in respect of the cellular thiamine contents, the effect by phenylalanine might be due to derepression in thiamine synthesis. As might be expected from the result of thiazole effect on thiamine contents of the cells, thiazole added

TABLE 8
Thiamine Synthesis from Hydroxymethylpyrimidine and Thiazole by Cell Suspensions of E. coli

Addition in incubation	Amount of thiamine per mg cells	Thiamine formed	Thiamine formed per mg cells
<i>M</i>	<i>mμg</i>	<i>mμg</i>	<i>mμg</i>
None	22.2	3,340	210
+adenine (2×10^{-3})	11.1	10,200	570
+phenylalanine (5×10^{-3})	34.0	6,800	400

TABLE 9
Abolishment by Thiazole of Phenylalanine Effect on Elevation of Thiamine Synthesis

Addition in incubation	Amount of thiamine per mg cells	Thiamine formed	Thiamine formed per mg cells
<i>M</i>	<i>mμg</i>	<i>mμg</i>	<i>mμg</i>
None	15.7	5,100	220
+ phenylalanine (1×10^{-2})	60.0	11,800	1,000
+ phenylalanine (1×10^{-2}) and thiazole (1×10^{-7})	21.9	5,950	270
+ phenylalanine (1×10^{-2}) and thiazole (1×10^{-6})	22.9	6,100	290

at a physiological concentration for bacterial growth to the medium with phenylalanine, abolished the elevation of thiamine synthesizing activities from hydroxymethylpyrimidine and thiazole by the resting cells (Table 9). This indicates that the derepression in thiamine synthesis induced by phenylalanine was arrested by thiazole.

DISCUSSION

In the course of study of the effects on thiamine synthesis by *E. coli* of several amino acids added to the growth medium, we found that phenylalanine as well as histidine specifically elevated cellular thiamine contents. It is well known that sulfur containing amino acids had some stimulatory effect on thiamine synthesis as efficient precursors in the synthesis of the thiazole moiety of thiamine (8). However, it was unexpected that phenylalanine and histidine showed such stimulatory effect on *de novo* thiamine synthesis as much as or much more than cysteine and methionine, as they do not seem to be precursors of the pyrimidine or thiazole moiety of thiamine. In further experiments, it was shown that the compounds structurally similar to phenylalanine such as β -phenylethylamine and β -phenylethylalcohol had no effect on thiamine production. From the results obtained in studying the phenylalanine effect compared with that of adenine apart from histidine action which needs to be checked, the elevation by phenylalanine of thiamine contents in the cells accompanied with an increase in thiamine synthesizing activities from hydroxymethylpyrimidine and thiazole by the resting cells preincubated with phenylalanine. In addition, the phenylalanine effect was abolished by simultaneous addition of thiazole at a low concentration ($0.1 \mu M$) which is physiological for the growth of *E. coli*. These results favour to give an idea that the stimulation of thiamine production by phenylalanine may be due to derepression of thiamine synthesizing enzymes caused by inhibition of thiazole synthesis by the compound in contrast with the conception of adenine. As already mentioned, thiamine synthesis is regulated by the ultimate product in the thiamine synthesis pathway and it is derepressible by adenine which caused reduction of thiamine contents in the cells by inhibiting the synthesis of the pyrimidine moiety of thiamine. The paradoxical effect of phenylalanine on elevating cellular thiamine contents is not fully understandable. However, recalling the data given in Table 5 in which a surplus

supply of hydroxymethylpyrimidine increases thiamine production in the cells, but not thiazole and also the conception by Moyed (9) that thiazole synthesis is controlled by thiazole itself rather than by the ultimate product, thiamine, it is possible to assume that phenylalanine may compete with an unknown precursor in thiazole synthesis pathway and this competition may reduce a thiazole pool in the cells which leads to derepress thiamine synthesizing system and increase thiamine contents as a result of derepression.

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