ACTA CHEMICA SCANDINAVICA 25 (1971) 2311–2319

Regulation of Tryptophanase Formation in *Escherichia coli*

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(1) The formation of tryptophanase in *Escherichia coli* B/1t7, a tryptophan auxotroph, and the influence of 19 amino acids and aeration on the formation and on the activity of tryptophanase have been studied. (2) The tryptophanase formation began in the acceleration phase, and its amount rose to a maximum before the midpoint of the exponential growth phase. No tryptophanase was formed during the lag phase. (3) The rate of aeration had a marked effect on the formation of tryptophanase. When the aeration decreased, the relative amount of tryptophanase increased. (4) The effects of the amino acids were influenced by the aeration. In the aerated cultures, the most pronounced corepressors were alanine, serine, and homoserine. In the unaerated cultures, the effects of the amino acids generally decreased. Alanine and serine repressed clearly, but homoserine lost all capability to repress the formation of tryptophanase. Cysteine, however, repressed strongly in the unaerated culture. (5) The influence of the activity of tryptophanase was studied in whole cells and cells disrupted by freezing and thawing. Cysteine, lysine, and serine inhibited the reaction catalyzed by tryptophanase. Alanine inhibited only in disrupted cells, whereas phenylalanine and glycine inhibited only in whole cells.

The formation of tryptophanase (EC 4.2.1. e) in *Escherichia coli* is regulated in two ways: tryptophan induces the formation,[1,2] whereas glucose and several other substances repress it.[3–7]

Although the synthesis of enzymes sensitive to glucose can be repressed by many different substances which a cell utilizes as sources of carbon and energy,[8–10] it seems that in each case, the enzyme is repressed specifically by an intermediate metabolite whose content increases under the prevailing conditions. It has been proposed that the specific corepressor is the final product of an enzymatic reaction or a closely related compound.[11] Pyruvic acid is one of the products of the reaction catalyzed by tryptophanase, and it has been found to repress tryptophanase. Pyruvic acid may be formed from all the carbohydrates and numerous other substances, which *E. coli* is able to metabolize and which repress the formation of tryptophanase.[11,7,12]

The hypothesis of repression by an end product has not, however, been generally accepted. Okinara and Dobrogosz [13–15] believe that high energy compounds that participate in redox reactions are decisive factors in the

repression of β-galactosidase. Magasanik [8] expressed the opinion that repression of enzymes is connected with the metabolism of high energy phosphate-containing compounds and inorganic phosphate. Also ATP has frequently been mentioned as a hypothetic corepressor.[9,8,16,15]

The extent of an enzyme reaction can be more readily influenced by altering the enzyme activity directly than by changing the amount of enzyme present by induction or repression. Indole, the second product of the action of tryptophanase, strongly inhibited the decomposition of tryptophan.[1,17] Pyruvic acid does not alter the activity of tryptophanase, but several compounds related to tryptophan effect substrate competitive inhibition.[18]

In this work, an attempt is made to determine what effects amino acids added to the growth medium, and what effects variations in aeration have on the formation of tryptophanase in *E. coli* B/1t7; and furthermore, whether amino acids influence the activity of tryptophanase. The results are in accordance with the assumed significance of pyruvic acid and its aerobic metabolism in the catabolite repression of tryptophanase.

EXPERIMENTAL

Organism. The bacterial strain used in the experiments was *Escherichia coli* mutant B/1t7 [19] which was supplied by Dr. E. E. Snell (University of California, Berkeley).

Culture. The TGC medium used contained 2 g of glycerol, 1 g of casein hydrolysate, 1 g of L-tryptophan, 15 mg of $CaCl_2.6H_2O$, 0.5 mg of $FeSO_4.7H_2O$, 3 g KH_2PO_4, 5.3 g of K_2HPO_4, 1 g of $(NH_4)_2SO_4$, and 0.1 g of $MgSO_4.7H_2O$ in 1 l of distilled water. The unadjusted pH of the medium was 6.9. The GC medium was identical with the TGC medium, except that it did not contain tryptophan. Amino acids were added to the sterilized TGC medium immediately before inoculation, and the pH was readjusted to 6.9 when necessary.

The *E. coli* B/1t7 strain was preserved on TGC agar slants and transferred every second week.

The inoculum for the aerated cultures was grown at 37° in 50 ml of TGC medium in a 250 ml erlenmeyer flask, without shaking, for 15 – 17 h. The cells were separated by centrifugation, and an amount giving a turbidity corresponding to about 25 μg dry wt. of cells/ml was suspended in 200 ml of fresh TGC medium in a 500 ml erlenmeyer flask. The cells were cultivated at 30° in an E. Buhler, Sm 1 type shaker, oscillating at 240 rpm, until the turbidity of the culture had increased fivefold. 25 ml of the inoculum were diluted to 100 ml with fresh TGC medium, in which an amino acid had been dissolved, and the mixture was then grown at 30° in a 250 ml wide-mouthed erlenmeyer flask in a shaker for 4 h. An identical culture, differing only in that it contained no amino acid, was grown at the same time.

The inoculum for the unaerated cultures was grown in the GC medium. The inoculum which was not centrifuged was added to 1 l of GC medium in a 2 l erlenmeyer flask, and the culture was incubated without shaking at 30° for 16 – 19 h, until the turbidity corresponded to 25 μg dry wt. of cells/ml. The suspension was added in 95 ml portions to 250 ml flasks, to each of which had been added 5 ml of GC medium, containing an amino acid and tryptophan for induction. The culture was continued at 30° without aeration.

The bacterial growth was followed by measuring at intervals the turbidity of the cultures in a Klett-Summerson colorimeter, equipped with a No. 62 (590 – 660 nm) filter.

In the amino acid experiments, samples for tryptophanase assays were taken from the beginning of the growth to approximately the midpoint of the exponential growth phase. The cells in each sample corresponded to about 0.1 mg dry wt. The samples were centrifuged at 5000 rpm for 10 min, under low temperature conditions, in a Servall RC-2 centrifuge (rotor SS-34). The separated cells were washed once with 0.9 % NaCl solution, and suspended in 1 – 2 ml of the same solution. The cell suspensions were then stored at +4°.

Tryptophanase assays. The tryptophanase activities were determined in whole cell samples, unless otherwise mentioned, by the method described by Newton and Snell.[20] Disruption of the cells by ultrasonic waves or by freezing and thawing was found unnecessary, and led to loss of activity, even though mercaptoethanol had been added to protect the enzyme. The intensity of rosindole color was measured in a Klett-Summerson colorimeter, equipped with a No. 56 (540 – 590 nm) filter.

A unit of tryptophanase was taken to be the amount of tryptophanase, which produces 1 μmol of indole/min. The specific activity is expressed as the number of units/mg of cell protein.

Protein determinations. The protein determinations were made by the method of Lowry *et al.*[21] on samples of the same cell suspensions from which samples were taken for the tryptophanase assays. The method was found to be suitable also for determining the protein contents of the intact cells.

Reagents. The amino acids used, and their suppliers are: L-alanine, β-L-alanine, L-arginine HCl, L-aspartic acid, L-asparagine, glycine, L-cysteine HCl, L-glutamic acid, L-glutamine, L-histidine, L-leucine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tryptophan, and L-valine (Fluka); L-isoleucine (Nutritional Biochemicals Corp., Cleveland, Ohio); L-lysine HCl and L-methionine (British Drug Houses, Poole, England); and DL-homoserine (Calbiochem, Los Angeles, Calif.).

The vitamin-free acid-hydrolysated casein was from Difco Laboratories, Detroit, Mich.; and pyridoxal phosphate (98 % *purum*) and L-glutathione (98 % *puriss.*) from Fluka. The other chemicals were obtained from E. Merck or from British Drug Houses.

RESULTS

Tryptophanase activity in different phases of growth of E. coli B/1t7. The relative amount of tryptophanase rose to a maximum slightly before the midpoint of the exponential growth phase of *E. coli* B/1t7 in the aerated cultures (Fig. 1). Thereafter the specific activity of the enzyme slowly decreased. No tryptophanase is formed during the lag phase.[22] The specific activity of tryptophanase immediately decreased when growth began in all of the aerated cultures. In many cases, this decrease in activity was so large

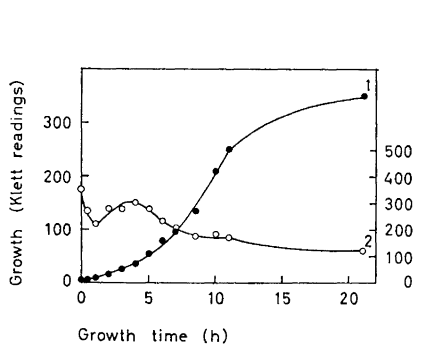

Fig. 1. The formation of tryptophanase in *E. coli* B/1t7, grown in an aerated TGC medium at 30°. 1, growth curve; 2, tryptophanase formation curve.

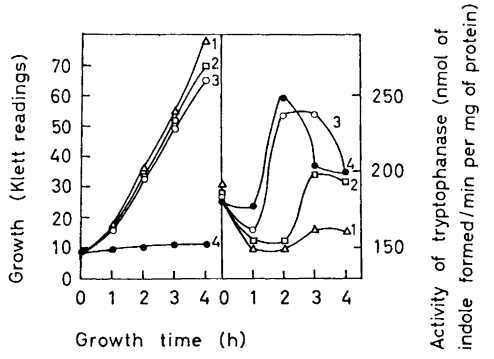

Fig. 2. The effect of aeration on the growth of *E. coli* B/1t7 (left) and on the formation of tryptophanase (right). The cells were grown at 30° in a TGC medium, aerated by mixing with a stirrer (Heidolph, type E 60). The relative rates of stirring were (1) 9:10, (2) 3:10; (3) 1:10; and (4) no stirring.

that it cannot be explained by dilution of the enzyme when bacterial growth begins, even though no new enzyme is produced; it seems that the tryptophanase was destroyed in this phase.

The cells used in the aerated culture experiments were cells whose initial growth was interrupted in the early part of the logarithmic growth phase, when the relative amount of tryptophanase in the cells was at a maximum. It seems that when inoculum cells were taken from the early growth phase, the specific activity of the enzyme tended to increase more than when taken from a later growth phase.

The effect of aeration on the formation of tryptophanase. E. coli B/1t7 was grown in both aerated and unaerated media. The growth rate in unaerated cultures was very low, at most one-fifth or one-fourth of the rate in the aerated cultures, and especially the onset of the growth was slow. Growth curves and curves depicting the variation in the specific activity in four parallel cultures, one of which was unaerated, and in three of which the stirring rates were different, are shown in Fig. 2. Increased aeration resulted in a higher growth rate, but it had a much more pronounced effect on the content of tryptophanase in the cells. In all aerated cultures, the specific tryptophanase activity decreased in the early stages of growth, and increased later, depending on the effectiveness of the aeration of the culture. No initial decrease occurred in the specific

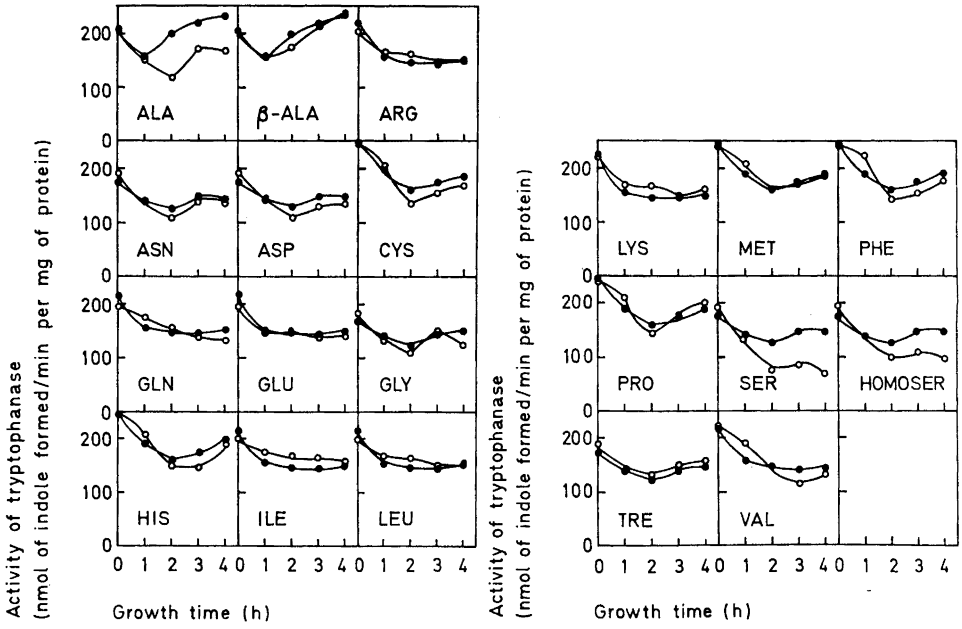

Fig. 3. The effects of amino acids on the formation of tryptophanase in *E. coli* B/1t7. The cells were grown in TGC medium at 30° with aeration in a shaker. Medium (○) contained 1 mg/ml of amino acid which was added before inoculation. Medium (●) contained no added amino acid (the control culture). The inoculation cells were grown in TGC medium with aeration.

tryptophanase activity of the unaerated cultures, but since also the growth was very slow, dilution of the enzyme could hardly have taken place.

The effects of amino acids on the formation of tryptophanase in aerated cultures. The influence of 19 amino acids on the formation of tryptophanase in *E. coli* B/1t7 cells is shown in Fig. 3. Freshly harvested inoculum cells were suspended in TGC medium, and the culture was divided into 20 portions. One of these served as a control culture, and to the others was added 1 mg/ml of the amino acid to be investigated. All cells were grown at 30° with shaking. The bacterial growth was uniform and rapid in all cultures: no lag phase was noted before growth began. In 4 h, the turbidity of the culture rose to a level five times the initial turbidity. The majority of the amino acids did not influence the rate of growth. After 4 h, the cell mass was 20 % greater in the presence of glycine, and 10 % greater in the presence of glutamic acid than in the control culture. After the same period of time, the growth was about 15 % smaller in the cultures containing alanine and cysteine than in the control culture.

Of the studied amino acids, only alanine, serine, and homoserine inhibited the formation of tryptophanase. The effects of the other amino acids were slight or transient and cannot be considered significant. In 4 h, alanine lowered the tryptophanase activity to 73 % of the activity of the control culture; serine, to 52 %; and homoserine, to 75 % (Fig. 3). The influence of serine and homoserine was also studied at higher concentrations. Serine at concentrations of 2 mg/ml and 10 mg/ml did not have any greater effect on the growth rate or on the formation of tryptophanase than it had at a concentration of 1 mg/ml. However, homoserine at a concentration of 5 mg/ml repressed the formation of tryptophanase twice as much as it did at a concentration of 1 mg/ml. Homoserine at a high concentration retarded the bacterial growth.

The effects of amino acids on the formation of tryptophanase in unaerated cultures. The cell population which had developed in 4 h in an aerated culture,

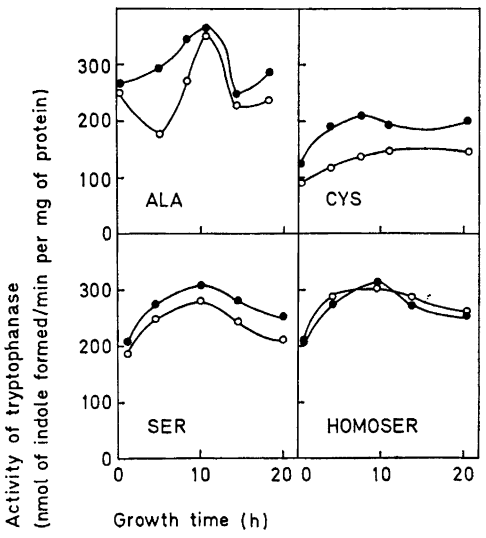

Fig. 4. The effects of amino acids on the formation of tryptophanase in *E. coli* B/1t7. The cells were grown at 30° in unaerated TGC media, each of which contained 1 mg/ml of amino acid (O), and in TGC medium that contained no added amino acid (●). The starter cells were grown in an unaerated GC medium.

required 20 h to develop in an unaerated culture. In addition to the aeration, these cultures differed from the preceding in that the initial bacterial growth took place in the GC medium, and tryptophan was added to the last culture; hence, induction took place only in this culture. The first sample tested for tryptophanase activity was taken 1 – 2 h after the addition of tryptophan. The effects of alanine, cysteine, serine, and homoserine on the formation of tryptophanase are shown in Fig. 4. Alanine clearly repressed the synthesis of tryptophanase in the unaerated culture too, but did not influence the growth as it did in the aerated culture. Cysteine, in contrast to its effects in the aerated culture, depressed both bacterial growth and tryptophanase formation, the latter strongly, in the unaerated culture. Serine repressed tryptophanase formation also in the unaerated culture, but it had no effect on the growth of *E. coli* even under these conditions. In contrast, homoserine retarded the bacterial growth, but did not affect the formation of tryptophanase. The other amino acids studied did not influence either the bacterial growth or the tryptophanase formation in the unaerated culture.

The effects of amino acids on tryptophanase activity. The influence of amino acids on the reaction catalyzed by tryptophanase was studied by adding amino acids to reaction mixtures in enzyme activity assays. The enzyme was assayed in whole cells as in the preceding experiments, and for comparison, in cells that had been disrupted by four successive freezings and thawings.

Table 1. The influence of amino acids on tryptophanase activity of *E. coli* B/1t7 cells. The reaction mixtures contained 10 μmol/ml of the amino acid to be investigated. The enzyme was assayed in whole cells and in cells that had been disrupted by freezing and thawing. The results describe activities expressed as percentages of the enzyme activity, determined in a reaction mixture containing no added acid.

Amino acid	Tryptophanase activity in percent of control value	
	Whole cells	Disrupted cells
Alanine	101	90
Arginine	94	101
Asparagine	95	94
Aspartic acid	92	99
Cysteine	55	49
Glutamine	94	97
Glutamic acid	98	96
Glycine	92	105
Histidine	99	92
Isoleucine	104	99
Leucine	110	100
Lysine	87	83
Methionine	98	95
Phenylalanine	95	104
Proline	108	105
Serine	71	77
Homoserine	107	101
Threonine	108	100
Valine	107	105

The cells were frozen at $-40°$ in an ethanol bath, and thawed in a water bath at 20°. The disruption of the cells led to about a 10 % loss of tryptophanase activity, but with only a few exceptions, the ratio of the activity to that in the control cells remained practically constant. The data in Table 1 show that alanine, cysteine, lysine, and serine inhibited the formation of indole in the tryptophanase reaction, whereas the other amino acids had no significant effect. When the results of the assays of whole and disrupted cells were compared, the greatest differences were obtained with alanine, glycine, and phenylalanine. Alanine did not inhibit the enzymatic reaction in whole cells; but in the presence of glycine and phenylalanine, the tryptophanase activity was lower when the enzyme was assayed in whole cells than in disrupted cells.

DISCUSSION

Tryptophanase is not formed during the lag phase of the growth cycle of *E. coli*, but its synthesis begins during the acceleration phase, and its relative amount rises to a maximum in the logarithmic growth phase.[22–24] A new aspect, discovered in the present study, is the decrease in the specific activity of tryptophanase in the early stages of growth (Fig. 1).

Serine repressed the formation of tryptophanase very effectively in all of the experiments (Figs. 3 and 4). The same observation has been made in most earlier studies.[7,12,28,30] Boezi and DeMoss [25] found that serine inhibits tryptophan permease, but they did not consider the lack of the inducer within the cell significant enough to account for the repression by serine. The explanation proposed for the strong repression by serine is its rapid deamination to pyruvic acid.[7,12] Catabolite repression has also been observed to influence the synthesis of L- and D-serine dehydrases.[11,31–33] Serine dehydrases are not, however, as sensitive to repression by glucose or pyruvic acid as tryptophanase.[34,11] Raunio [12] has found that addition of serine promotes the accumulation of pyruvic acid in *E. coli* cultures.

Homoserine strongly represses the formation of tryptophanase in aerated cultures of *E. coli* B/1t7 (Fig. 3). An increase in the homoserine concentration has a greater influence on the degree of repression, than an equal increase in the serine concentration has on the degree of repression. This may mean that the catabolite corepressor of tryptophanase is more readily formed from serine than from homoserine. As the repression was not observed in unaerated cultures (Fig. 4), the reactions converting homoserine into the corepressor depend on the availability of oxygen. Dobrogosz [26] has observed that some substances, which weakly repress the formation of β-galactosidase, disappear under anaerobic conditions. The effect of homoserine on the formation of tryptophanase has not been studied earlier. In *E. coli*, homoserine is involved in the metabolism of cysteine and serine, and hence in the production of pyruvic acid; but its effect was unexpectedly much greater than the effects of other amino acids, which seems to be more closely related to pyruvic acid. If, however, serine exerts a repressive effect, independent of pyruvic acid, then the effect of homoserine may be due solely to its structural similarity to serine.

Alanine strongly represses the synthesis of tryptophanase, and retards bacterial growth in aerated *E. coli* B/1t7. Because the decreased growth rate

is in most cases followed by a diminished repression,[14,35] the effects of alanine are particularly noteworthy. Alanine did not inhibit the growth of *E. coli* B/1t7 in unaerated cultures, but its repression of tryptophanase was, nevertheless, marked (Fig. 4). Pyruvic acid is a product of both deamination and transamination of alanine. The formation of deaminating alanine dehydrogenase is five to six times faster under aerobic than under anaerobic conditions.[36] This reaction is also oxidative, and may explain the greater repression of the formation of tryptophanase in aerated cultures. Beggs and Lichstein [7] found that alanine is a weak corepressor of tryptophanase formation.

Cysteine has been found to be a powerful corepressor of tryptophanase in unaerated cultures.[12] This was confirmed in the present work (Fig. 4). Cysteine desulfurase, which occurs in *E. coli*, catalyzes the decomposition of cysteine to puryvic acid, hydrogen sulfide, and ammonia. As the reaction does not consume oxygen, it is probably the most important metabolic pathway of cysteine in unaerated cultures. The toxicity of the found hydrogen sulfide probably causes the growth to slow down. In aerated cultures, cysteine had only a very weak effect.

The results obtained are not in variance with the hypothesis that pyruvic acid is a specific catabolite corepressor of tryptophanase.

Newton and Snell [20,37] showed that tryptophanase catalyzes the decomposition of cysteine and serine, and also the formation of tryptophan from indole and serine or threonine. Although serine and cysteine have a much weaker affinity for tryptophanase than for tryptophan, they, nevertheless, inhibit the decomposition of tryptophan. Alanine is not the actual substrate of tryptophanase, but it inhibits tryptophanase competitively.[18] Table 1 shows that not only serine, alanine, and cysteine, but also lysine inhibits the formation of indole. Evidently, the group decisively responsible for the inhibitory action of lysine is the amino group which is attached to the α-carbon, and which is at the same distance from the α-carbon as the amino groups of kynurenin, which has been shown to be an inhibitor of indole formation.[18] These amino groups probably become attached to the site on the enzyme where the ring nitrogen of tryptophan is bound.[38,39]

In the present study, inhibition was studied with both intact and ruptured cells. The results obtained from ruptured cells may be taken to reveal the direct effect of amino acids on the enzyme. But in the case of the intact cells, it is necessary to take into account how rapidly the amino acid is able to enter the cell, and whether it hinders the substrate from entering the cell. Alanine did not inhibit tryptophanase in intact cells, and this suggests that it is unable to penetrate the cell wall. It is true, however, that the inhibition by alanine was weak even when the cells has been ruptured. Glycine and phenylalanine, on the other hand, seemed to prevent the entry of tryptophan into the cell. Phenylalanine has also been found to inhibit tryptophan permease in some cases.[27–29]

Acknowledgements. The authors wish to express their sincere appreciation for the helpful cooperation of Professor E. E. Snell(University of California, Berkeley, U.S.A.).
This work was supported by a grant from the *Finnish Natural Science Research Council.*

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Received November 11, 1970.