Regulation of Homoserine Transacetylase in Whole Cells of *Bacillus polymyxa*  

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The levels of homoserine transacetylase (EC 2.3.1.31) in *Bacillus polymyxa* grown in minimal medium can vary over a 40-fold range, depending on whether methionine limits growth or is present in excess. This suggests that the synthesis of the enzyme is under repressive control by methionine or one of its metabolites.

The stability of homoserine transacetylase in growing cells was measured after repression of further synthesis by the addition of methionine. At 30°, the enzyme was stable for 2 hours, whereas at 37° it decayed with a half-life of 40 min. This contrasts with the striking instability in cell-free extracts described in the preceding paper (Wyman, A., and Paulus, H. (1975) J. Biol. Chem. 250, 3897-3903).

The properties of homoserine transacetylase were also studied in cells of *B. polymyxa* that had been made permeable to small molecules by treatment with toluene. They differed in two important respects from those of the enzyme in cell-free extracts described in the preceding paper: the enzyme was relatively stable, with a half-life of 15 min at 37°, and responded in a sigmoid manner to increasing concentrations of the inhibitors L-methionine and S-adenosylmethionine. These observations suggest that homoserine transacetylase is an oligomeric protein within the bacterial cell but dissociates into monomers in cell-free extracts.

When *B. polymyxa* was transferred at 39° from a rich medium to one without amino acids, growth resumed only very slowly. The growth lag after shift-down was not observed at 37° or in the presence of methionine or cystathionine. This phenomenon appears to be due to a need for derepression of homoserine transacetylase upon shift-down which is thwarted at 39° by the rapid thermal inactivation of the enzyme. A possible physiological function of the striking thermolability of the first enzyme in methionine biosynthesis is discussed.

In the preceding paper (1), we described the purification and properties of homoserine transacetylase from *Bacillus polymyxa*. The enzyme was found to be subject to multivalent feedback inhibition by L-methionine and S-adenosylmethionine and exhibited some rather unusual properties, such as extreme instability even at moderate temperatures, and the absence of cooperativity in the interaction with substrates and inhibitors which was due to its monomeric structure. In this paper, we first examine the regulation of synthesis of homoserine transacetylase and then compare its stability and response to feedback inhibitors in vitro and within the cell. Finally, we describe a physiological consequence of the instability of homoserine transacetylase in growing cultures of *B. polymyxa*.

**Experimental Procedure**

*Materials:* The sources of materials were described in the preceding paper (1).

*Bacterial Strains and Growth Conditions:* The methionine auxotroph, *Bacillus polymyxa* M4 (this strain is identical with *B. polymyxa* ATCC 27850) was derived from *B. polymyxa* ATCC 25901 and grown as described earlier (1). The prototrophic strain, *B. polymyxa* ATCC 25901, was grown in the same medium except that methionine was omitted. Growth was monitored in a Klett-Summerson photoelectric colorimeter with a No. 42 filter. One Klett unit corresponded to about 10⁸ cells per ml.

*For the shift-down experiments,* *B. polymyxa* ATCC 25901 was grown at 37° in the rich medium of Stanly et al. (2). When the culture had reached late exponential phase, samples were diluted 100 fold into minimal medium (1) at the temperature and with supplements as indicated. All cultures (20 ml) were incubated in 250-ml nephelometric culture flasks on a New Brunswick model G-25 rotary shaker.

*Preparation of Extracts:* All steps were carried out at 0-2°. Cells of *B. polymyxa* were suspended in 3 volumes of Buffer S (80 mM potassium phosphate, pH 7.0, 1 mM EDTA, 1 mM 2-mercaptoethanol, and 20% ethylene glycol) and disrupted in a French pressure cell at 8,000 psi. The extract was centrifuged at 100,000 × g for 1 hour and dialyzed against Buffer S for about 15 hours.
Toluene Treatment—A sample (40 ml) of a culture of *B. polymyxa* M4, which had been subjected to methionine starvation for about 2 days (1), was treated as follows at room temperature: the cells were collected by centrifugation at 8,000 × g for 5 min and resuspended in 1 ml of Buffer T (0.1 M triethanolamine hydrochloride, pH 7.8, 5 mM MgCl₂, 2 mM EDTA, and 0.1 mM dithiothreitol). Toluene (0.01 ml) was added and the suspension was gently agitated for 10 min and again centrifuged at 8,000 × g for 5 min. The pellet was resuspended in 1 ml of ice-cold Buffer T containing 50% glycerol and could be stored at −20°C for several days with only a slight loss of homoserine transacetylase activity.

Enzyme Assays—Homoserine transacetylase in cell-free extracts was measured either by Assay I or Assay II as described previously (1). Enzyme assays in toluene-treated cells were done by the homoserine-O-acetylhomoserine exchange reaction (Assay II) in order to avoid possible enzymic destruction of acetyl-CoA. The assay conditions were slightly modified, primarily by including Mg²⁺ to prevent cell lysis. This had no significant effect on homoserine transacetylase activity. The incubation mixtures (0.2 ml) contained 0.5 mM L-[^3]Cl homoserine, 1 mM O-acetylhomoserine, 0.1 M triethanolamine hydrochloride, pH 7.8, 1 mM EDTA, 5 mM MgCl₂, 0.1 mM dithiothreitol, and 12.5% glycerol. On account of the greater stability of homoserine transacetylase in toluene-treated cells (cf. Fig. 3 below), incubation could be carried out for 10 min at 25°C. Under the conditions of the assay, no cell lysis occurred and all enzyme activity remained associated with material that sedimented in 5 min at 8,000 × g.

RESULTS

Repression of Homoserine Transacetylase by Methionine—The specific activity of homoserine transacetylase in crude extracts was about 1 unit per g of protein both in the prototrophic strain, *Bacillus polymyxa* ATCC 25901, and in the methionine auxotroph, *B. polymyxa* M4, when grown at 30°C in minimal medium supplemented with an excess of methionine (0.1 mM). During growth in a methionine-free medium, the level of homoserine transacetylase in the prototrophic strain reached about 6 units per g of protein. The growth of the methionine auxotroph was limited by methionine at concentrations below 0.1 mM. Under these conditions, growth stopped as the supply of methionine was depleted and, at the same time, homoserine transacetylase levels rose rapidly and reached a maximum of 37 units per g of protein about 2 hours after the onset of starvation (Fig. 1).

Stability of Homoserine Transacetylase in Growing Cells—The effect of temperature on the stability of homoserine transacetylase in vivo could be assessed by measuring the decay of enzyme activity after further synthesis had been repressed by the addition of methionine. Cultures of *B. polymyxa* ATCC 25901, grown to mid-exponential phase in minimal medium either at 30°C or at 37°C, were treated with 0.1 mM L-methionine. Cell-free extracts were prepared from samples of the cultures taken at intervals after the addition of methionine and were assayed for homoserine transacetylase activity. Fig. 2 shows that at 30°C the total amount of homoserine transacetylase activity remained constant for at least 2 hours, whereas at 37°C enzyme activity decayed rapidly with a half-life of 40 min.

Properties of Homoserine Transacetylase in Toluene-treated Cells—When homoserine transacetylase activity was measured in cells of *B. polymyxa* that had been made permeable by treatment with toluene, it was found that the reaction rate was more rapid at 25°C than at 8°C and nearly constant with time for 30 min (Fig. 3). This was in striking contrast with the behavior of the enzyme in cell-free extracts where the enzyme was strikingly unstable, especially at the higher temperatures (Fig. 3, inset). Direct measurement of the stability of homoserine transacetylase in toluene-treated cells showed that at 25°C and 31°C no activity was lost in 10 min, whereas at 37°C the enzyme had a half-life of about 10 min (Fig. 4). Homoserine transacetylase activity in toluene-treated cells, like that in cell-free extracts (1), was inhibited by L-methionine and S-adenosylmethionine in a synergistic manner (Fig. 5). However, while the inhibitor saturation curves in cell-free extracts were strictly hyperbolic (1), a high degree of cooperativity was observed in toluene-treated cells. Thus, when the
FIG. 3. Time course of the homoserine transacetylase reaction in toluene-treated cells. The amount of ["Clhomoserine-O-acetyl-homoserine exchange by toluene-treated cells of Bacillus polymyxa M4 (1.7 mg) during incubation for different times at (O) 8°C and (■) 25°C was measured by Assay II. A similar experiment was done with a crude extract of B. polymyxa M4 (inset). To permit comparison, activities are normalized to the amount of cells from which the extract was derived.

FIG. 4. Stability of homoserine transacetylase in toluene-treated cells. Toluene-treated cells of B. polymyxa M4 were suspended in Buffer T containing 12.5% glycerol at a concentration of 20 mg per ml and were incubated at (■) 25°C, (●) 31°C, or (○) 37°C. Samples were removed at various times and assayed for homoserine transacetylase activity by Assay II.

data for inhibition by methionine were represented as a Hill plot, a slope close to 2 was obtained (Fig. 5C).

Growth Inhibition during Shift-down—The transfer of B. polymyxa ATCC 25901 from a medium rich in amino acids to minimal medium was accompanied by a considerable growth lag, especially at higher temperatures. As shown in Fig. 6A, when such a shift-down was done at 30°C, growth did not resume for about 20 hours. On the other hand, if the culture was kept at 37°C for as little as 2 hours after shift-down and then transferred to 39°C, growth resumed considerably earlier, in spite of the fact that the growth rate was slightly greater at 39°C than at 37°C. The addition of L-methionine also reduced the lag period after shift-down at 39°C, even at concentrations (10^-6 and 10^-5 M) much below that required (10^-4 M) to satisfy the growth requirement of the methionine auxotroph, B. polymyxa M4 (Fig. 6B). Cystathionine, but not homoserine or O-acetyl-homoserine, had a similar effect (Fig. 6C).

DISCUSSION

Growth in the presence of methionine led to a 5-fold reduction in the levels of homoserine transacetylase in Bacillus polymyxa ATCC 25901. Conversely, when the methionine auxotroph, B. polymyxa M4, was starved for the amino acid, the specific activity of the enzyme rose nearly 40-fold (Fig. 1). These observations suggest that the synthesis of homoserine transacetylase in B. polymyxa is under repressive control by methionine or one of its metabolites. A similar kind of regulation of homoserine transsuccinylase has been observed in Salmonella typhimurium (3) and Escherichia coli (4).

The repression of homoserine transacetylase synthesis by methionine allowed an estimation of the half-life of the enzyme in vivo. At 30°C, the total amount of homoserine transacetylase in the bacterial culture remained constant for more than 2 hours after further synthesis had been stopped by addition of methionine, whereas at 37°C the enzyme decayed with a half-life of about 40 min (Fig. 2). This contrasts sharply with the behavior of the enzyme in cell-free extracts where its half-life was only 15 min at 0°C and considerably less at 25°C (1). Nevertheless, even in growing cells, homoserine transacetylase must be considered unusually labile, since the activity of most bacterial enzymes in vivo is lost only very slowly at 37°C (5). It should be noted that the optimal temperature for growth of B. polymyxa is near 37°C.

In order to elucidate the basis for the large difference in stability of homoserine transacetylase in growing cultures and in cell-free extracts, we investigated the properties of the enzyme in cells of B. polymyxa that had been made permeable
to small molecules by treatment with toluene. The results (Fig.
4) showed that homoserine transacetylase was only slightly less
stable in toluene-treated cells than in vivo (half-lives of 15 min
and 40 min, respectively, at 37°C) and very much more stable
than in cell-free extracts (1). Another striking difference
between the behavior of homoserine transacetylase in toluene-
treated cells and cell-free extracts was the sigmoid response to
increasing concentrations of methionine and S-adenosylmethi-
onine in the former (Fig. 5),¹ whereas in cell-free extracts
inhibitor saturation curves were strictly hyperbolic (1). This
suggested that in the cell, unlike in extracts (1), homoserine
transacetylase exists as an oligomeric protein. If the oligomeric
form of the enzyme were more stable than the monomer, this
would account for the greater stability of homoserine transace-
tylase in toluene-treated and in growing cells. The failure to
detect oligomeric forms of the enzyme in cell-free extracts (1)
could have been due to disaggregation resulting from the
dilution that accompanies cell disruption. In fact, the concen-
tration of homoserine transacetylase was about 1000 times
higher during assay in toluene-treated cells than in crude
extracts.² It would not be surprising if such an enormous
difference in concentration were to affect the behavior of a
dissociating system. This illustrates the value of toluene-
treated cells as an experimental system for the study of enzyme
regulation which permits assays to be done at physiological
enzyme concentrations, far above those amenable to assay in
cell-free systems.

An interesting property of B. polymyxa is its inability to
resume growth when transferred at 39°C from a rich medium to a
minimal medium without amino acids. This effect was not
observed at 37°C nor in the presence of methionine or cystathio-
nine (Fig. 6). In contrast, when a culture already growing in
minimal medium was transferred from 37 to 39°C, growth
continued without a lag at a slightly faster rate. The following
explanation is suggested by our earlier observations that the
first enzyme of methionine biosynthesis, homoserine transace-
tylase, is repressed during growth on methionine and that the
enzyme is also unusually temperature-sensitive: upon transfer
from an amino acid rich to a minimal medium, homoserine
transacetylase and the other enzymes of methionine biosynthe-
sis must be derepressed to provide an adequate supply of the
amino acid for growth. However, enzyme synthesis itself
requires methionine, and derepression is therefore an autocata-
lytic process whose initiation depends upon the functioning of
the low residual enzyme levels present during growth in rich
medium (or upon proteolysis) to provide catalytic amounts of
methionine. Because homoserine transacetylase is tempera-
ture-sensitive, there will be a critical temperature above which
it can no longer provide the necessary amounts of precursor,
and growth therefore cannot resume in the absence of exoge-
nous methionine. This interpretation is supported by the

¹Sigmoid inhibition curves could also arise as an artifact if the
inhibitor were destroyed or adsorbed by other proteins in the incubation
mixture. In the case of methionine, these possibilities can be ruled out
because the amino acid exerts its inhibitory effect at relatively high
concentrations (above 10 mM) and is not likely to be converted to other
substances in the absence of ATP. In order that an adsorption artifact
could account for the observed degree of sigmoidicity, it would be
necessary that about 5 mM methionine be adsorbed to other proteins.
Because the protein concentration in the incubation mixtures was only
1.5 mg per ml, it is very unlikely that the required amount of
methionine (5 mM or 0.75 mg per ml) could have been adsorbed.
²The intracellular protein concentration is about 150 mg per ml
whereas enzyme assays in crude extracts were carried out at a protein
concentration of less than 0.1 mg per ml.
observation that catalytic amounts of methionine permit growth resumption at 39° (Fig. 6B). Moreover, the fact that cystathionine is also effective rules out the possibility that it is derepression per se of the methionine biosynthetic enzymes which is temperature-sensitive. For some of these enzymes are also required for cystathionine utilization. A more rigorous test of our model could be based on the prediction that O-acetylhomoserine, but not homoserine itself, should also eliminate the growth lag upon shift-down. However, both substances were found to inhibit the resumption of growth at 39° (Fig. 6C). The failure to observe the predicted result is probably due to the absence of a specific transport system for O-acetylhomoserine. Extracellular hydrolysis of the ester and uptake of homoserine would explain why both homoserine and O-acetylhomoserine caused growth inhibition.\(^3\)

Methionine biosynthesis can also be a limiting factor in the growth of other bacterial species. Upon shift-down from rich to minimal medium, Proteus morganii exhibited a long growth lag which could be eliminated by supplementing the minimal medium with amino acids, among which L-methionine was the most effective (6). Unfortunately, the effect of temperature on the growth lag has not been studied in this case. However, temperature has a striking effect on methionine biosynthesis in E. coli. When E. coli, growing in minimal medium at 37°, was transferred to a higher temperature (40−45°), the growth rate decreased immediately unless methionine was added, and experiments in vivo and in vitro showed this effect to be due to the reversible inactivation of homoserine transsuccinylase (7, 8). Because this phenomenon was observed in five different strains of E. coli, it was suggested that it may provide some undisclosed selective advantage (7). It is therefore interesting that the first enzyme in the methionine pathway is also the most heat-labile biosynthetic enzyme in B. polymyxa, with the only difference that in this case inactivation is irreversible. The evolution of an identical temperature-sensitive step in two such unrelated organisms is remarkable and indeed suggests regulatory significance and survival value. Since methionine participates more centrally in macromolecular synthesis than any other metabolic product (except, perhaps, adenine nucleotides), temperature sensitivity of methionine biosynthesis might assure the simultaneous arrest of the synthesis of several types of macromolecules, thereby preventing their imbalance. That this might indeed be the case is suggested by the observation that exposure of E. coli to 45° interferes with the initiation of cell division and thus leads to synchronization (9), a phenomenon which was subsequently shown to be due to methionine starvation brought about by thermal inactivation of homoserine transsuccinylase (10).

\(^3\)Growth inhibition by L-homoserine is probably due to its rapid conversion to L-threonine which inhibits homoserine dehydrogenase and thereby further limits methionine biosynthesis (M. Brenner and H. Paulus, unpublished observations).
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