Production of L-Asparaginase II by *Escherichia coli*

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L-Asparaginase II was synthesized at constant rates by *Escherichia coli* under anaerobic conditions. The enzyme was produced optimally by bacteria grown between pH 7 and 8 at 37°C. Although some enzyme was formed aerobically, between 100 and 1,000 times more asparaginase II was produced during anaerobic growth in media enriched with high concentrations of a variety of amino acids. Bacteria grown under these conditions should provide a rich starting material for the large-scale production of the enzyme. No single amino acid specifically induced the synthesis of the asparaginase, nor did L-asparaginase, even when it was used as the only source of nitrogen. The enzyme was produced at lower rates in the presence of sugars; glucose was the most inhibitory.

Deamidation of L-asparagine by extracts of *Escherichia coli* was first reported in 1957 by Tsuji (28). *E. coli* was later shown to produce two distinct asparaginases (L-asparaginase amido-hydrolase, EC 3.5.1.1) which differ in a number of properties, perhaps most significantly in their markedly different affinities for asparagine (5, 24, 27). The enzyme with the greater affinity, asparaginase II, appears to be located in the periplasmic space between the bacterial plasma membrane and the cell envelope (6). This asparaginase has been purified to apparent homogeneity (H. Whelan and J. C. Wriston, Jr., *Federation Proc.*, p. 586, 1968).

Since the demonstration (2, 3) that an L-asparaginase is the component in guinea pig serum which inhibits the growth of certain tumors (16, 17), there has been considerable interest in asparaginases from various sources. Asparaginase II of *E. coli* even more effectively inhibits the growth of transplantable mouse and rat tumors in vivo (5, 20, 26); it is also active against lymphoma in the dog (14, 22) and against human lymphoblastic leukemia (15, 21). This asparaginase, which interferes with the incorporation of amino acids into tumor protein in cell cultures (4), also inhibits the synthesis of protein under the direction of bacteriophage f2 RNA in cell-free extracts of *E. coli* (25). Asparaginase I from *E. coli* is inactive against tumor growth and does not interfere with protein synthesis in microbial extracts (26). Asparaginase I has the lower affinity for asparagine.

Whereas the production of asparaginase I is unaffected by the conditions of growth, the amount of asparaginase II in the bacterial cell varies greatly. In this paper, we report some of the conditions which control the production of asparaginase II.

**MATERIALS AND METHODS**

For most of the experiments, we used *E. coli* K-12 wild type. We also used strain 22-64, which lacks citrate synthase (10), and strain 309-1, which lacks α-ketoglutarate dehydrogenase (B. D. Davis et al., *Federation Proc.*, p. 211, 1959); both are mutants of the W strain and were obtained from B. D. Davis. The strain of *E. coli* B was obtained from J. Teller of Worthington Biochemical Corp., Freehold, N.J. Mutants of K-12 which are unable to convert asparagine to aspartic acid were isolated after mutagenesis with ethylmethanesulfonic acid (18). After treatment with the mutagen, cells were diluted and grown overnight on membrane filters (Millipore Co., New Bedford, Mass.) overlying solid growth media. These filters were transferred to a detection medium, solidified with 2% agar (Difco), containing 10 mM asparagine and 20% sucrose. After incubation for 6 hr at 37°C, the membrane filters bearing the colonies were removed, and the underlying detection medium was carefully blotted for 1 min with Whatman no. 1 filter paper in order to absorb amino acids. After drying, these papers were dipped in 0.5% ninhydrin in acetone. The conversion of asparagine to aspartic acid was indicated by a blue spot given by aspartic acid corresponding to the position of a colony. Blue spots were distinguishable from the brownish background given by unconverted asparagine. Absence of a blue spot indicated the position of a colony with impaired conversion. Eleven mutants were isolated from 1,500 colonies.

Bacteria were grown aerobically on a rotary shaker in 250-ml Erlenmeyer flasks containing a volume of 20 ml of culture medium. Anaerobic growth was in jars (Torsion Balance Co., Clifton, N.J.) under a mixture of 90% nitrogen and 10% carbon dioxide gas at a pressure of 3 psi. Bacteria were always adapted to experimental conditions of growth for at least five
generations. Cells were grown at 37 C, since growth at either higher or lower temperatures yielded lower amounts of asparaginase II.

For aerobic growth, the mineral-salts medium A of Davis and Mingioli (7) was used without citrate. For anaerobic growth, the same medium was supplemented with 2.4 mg of L-cystine per liter (11). Additions to the minimal medium are indicated in the legends to tables and figures. Tryptone, nutrient broth, casein hydrolysate, and yeast extract were Difco products.

Growth was measured turbidimetrically at a wavelength of 570 nm with a Coleman Junior spectrophotometer. One optical density unit corresponded to 0.93 mg of protein per ml, as determined, after lysis of the cells, by the method of Lowry et al. (19) with egg white lysozyme as standard. For determination of asparaginase II, 15-ml samples were pipetted into ice; the cells were collected by centrifugation at 14,000 × g, washed twice, and finally suspended in 0.8 ml of 0.1 M NaCl buffered at pH 7.8 with 50 mM tris(hydroxymethyl)aminomethane-chloride. Cells were lysed by treatment with ethylenediaminetetraacetic acid (EDTA) and lysozyme. The suspension was made 1 mM in EDTA and kept at 0 C for 8 min. After the addition of magnesium acetate to a concentration of 1 mM, 1 mg of egg white lysozyme (Worthington Biochemical Corp.) was added to give a final volume of 1 ml. The samples were incubated for 15 min at 37 C. Samples (0.01 ml) of these lysates were assayed for asparaginase II either directly or after dilution with buffered saline, in a final volume of 0.06 ml, by following the conversion of 14C-asparagine to aspartic acid with rapid chromatography on ion-exchange paper (Ecteola, Whatman ET81) (6). The specific enzymatic activity is given as micromoles of aspartic acid produced per hour per optical density unit of the culture. At the low concentration of asparagine used in this assay (0.1 mM), asparaginase I does not contribute significantly to the products formed (6, 26).

RESULTS

Formation of asparaginase II in standing cultures. Schwartz et al. (26) observed that asparaginase II was formed in standing cultures during the transition from aerobic to anaerobic growth. The time course of the appearance of the enzyme under these conditions is shown in Fig. 1. Little enzyme was produced by cells during aerobic growth. As soon as the aeration was discontinued, the specific activity increased exponentially until a plateau was reached by 40 min. The form of these kinetics suggested that a factor, perhaps oxygen, inhibitory to the production of the asparaginase was disappearing during the period following the cessation of aeration. These considerations led us to study the formation of enzyme under strict anaerobiosis.

It was important to establish beforehand that the enzyme is stable under aerobic conditions. Sulfhydryl reagents did not affect the enzyme in vitro. Incubation of asparaginase at pH 8 with 10 mM p-hydroxymercuribenzoate or with any of the alkylation agents, iodoacetate, iodoacetamide, or N-ethylmaleimide, also at 10 mM, did not inhibit the enzyme. Asparaginase II was not inactivated by exposure to air. When the bacteria were aerated after a period of anaerobic growth, the formation of the enzyme stopped. Even so, enzyme previously formed in the absence of air remained at a constant concentration in the aerated culture.

The production of asparaginase in standing cultures depended upon protein synthesis. Cells were first grown aerobically. The addition of chloramphenicol at the time when the aerations was discontinued prevented the appearance of the enzyme. Dependence of enzyme formation on protein synthesis indicated that its appearance resulted from de novo synthesis rather than from the conversion of a preexisting but inactive protein precursor.

Roberts et al. (23) recently reported that, when E. coli is grown for 12 to 16 hr with shaking, there is a greater yield of asparaginase in air than in an atmosphere of nitrogen. It was concluded that anaerobiosis depressed the formation of asparaginase II. The supply of oxygen is likely to be limiting to cultures at high cell densities.
even when shaken in air. Thus, as these workers have also shown (23), under their conditions of cultivation, the highest concentration of asparaginase appeared in cells as the bacteria approached the end of growth.

Formation of asparaginase II during anaerobic growth. The experiments with standing cultures suggested that asparaginase II might be produced continuously and at high rates by bacteria adapted to anaerobic conditions of growth. Bacteria produced asparaginase II at a constant rate for many generations during growth in anaerobic jars. Under these conditions, formation of the enzyme was stimulated greatly by the enrichment of the mineral-salts-glucose medium with tryptone, yeast extract, or casein hydrolysate (Table 1).

Similar results were obtained with all of the strains of E. coli tested. Strains B and W under various conditions of growth had about three times as much asparaginase as strain K12. Nevertheless, formation of the enzyme in all strains was stimulated to the same extent by anaerobiosis.

Since casein hydrolysate was as effective as tryptone or yeast extract, we attempted to determine whether any single amino acid or group of amino acids would stimulate the production of asparaginase. Supplementation of the minimal medium with asparagine did not stimulate the production of the enzyme (Table 2). Furthermore, the addition of asparagine or glutamine together with the casein hydrolysate did not enhance enzyme synthesis. Commercial casein hydrolysate contains substances other than amino acids. Nevertheless, amino acids alone were sufficient to bring about the increased synthesis of the asparaginase. As shown in Table 2, supplementation of the minimal medium with a mixture of 17 amino acids resulted in the production of the enzyme at almost the same rate as that seen with casein hydrolysate. Supplementation of the minimal medium with any one of the groups of amino acids, however, did not significantly increase enzyme production. When two or more groups of amino acids were combined, more enzyme was produced. The results for a few of the combinations are also shown in Table 2.

Moreover, supplementation with either a mixture of vitamins (biotin, niacin, pantothenic acid, thiamine, and riboflavin, each at a concentration of 1 μg/ml) or with purine and pyrimidine bases (adenine, uracil, guanine, and cytosine, each at 1 mg/ml) did not increase the rate of enzyme synthesis. Casamino Acids also contain a substantial amount of salt; we observed no effect of sodium chloride, however, on the synthesis of the enzyme in concentrations up to 5%. Another component of commercial casein hydrolysate is iron. The addition of ferric chloride did not influence the production of the enzyme in concentrations from 0.25 to 1 μg/ml either in the presence or in the absence of the amino acid mixture.

As with a number of other catabolic enzymes, the formation of asparaginase II was inhibited by the addition of sugars, particularly glucose. When xylose, maltose, or galactose was used in place of glucose, enzyme production was inhibited to a lesser extent. Galactose allowed the highest rate of enzyme synthesis of all the

| TABLE 2. Effect of amino acids on the formation of asparaginase II by E. coli during anaerobic growth* |
|-------------------------------------------------|----------------|
| Addition                                        | Specific activity |
| None                                           | 0.4            |
| Casein hydrolysate                             | 3.4            |
| Asparagine                                     | 0.5            |
| Casein hydrolysate and asparagine              | 3.5            |
| I                                              | 0.3            |
| II                                             | 0.3            |
| III                                            | 0.4            |
| IV                                             | 0.4            |
| I + II                                         | 0.8            |
| II + III                                       | 0.6            |
| I + II + III                                   | 1.4            |
| I + II + IV                                    | 1.5            |
| I + II + III + IV                              | 3.2            |

* Bacteria were grown in anaerobic jars in minimal medium containing 1% glucose with additional supplementation as indicated.

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sugars tested. No matter what carbon source was used, however, enzyme synthesis was enhanced by increasing concentrations of amino acids (Table 3). Under all conditions, the addition of glucose inhibited the production of the enzyme. This inhibition is presumably an example of catabolite repression. The greatest amounts of enzyme were obtained in the absence of any sugar when high concentrations of amino acids were added, although the growth rate under these conditions was low. It can be estimated that, when asparaginase II was produced maximally, it constituted about 0.1% of the bacterial cell protein, as the specific activity of the purified enzyme is approximately 20,000 μmoles of ammonia produced per hr per mg of protein (J. C. Wriston, Jr., personal communication).

During both aerobic and anaerobic growth, E. coli was capable of using asparagine as its sole source of nitrogen. Asparagine could not be used as the sole source of carbon, under either aerobic or anaerobic conditions of growth. The rate of entry of the amino acid into E. coli appeared to be relatively slow, because the growth rate of an auxotroph lacking asparagine synthetase (Cedar and Schwartz, unpublished data) was dependent on the concentration of asparagine below 200 μg/ml. For most amino acids, the range of concentrations which limits the growth rate of E. coli is two or three orders of magnitude lower than this. Little asparaginase II was formed either aerobically or anaerobically when asparagine was the sole source of nitrogen (Table 4).

In 1943, Gale (8) discussed the effect of growth at various pH values on the formation of a number of activities in suspensions of intact E. coli. Formation of some of the deaminases studied was stimulated at values above pH 8, whereas decarboxylases were produced during growth under acidic conditions. Asparaginase II was synthesized at optimal rates by bacteria grown between pH 7 and 8.

Formation of asparaginase II by mutants of E. coli. Changes in the concentrations of enzymes catalyzing the reactions of the citric acid cycle are known to occur in bacteria during anaerobic growth (12, 15). For example, α-ketoglutarate dehydrogenase is not produced (1). Mutant 309-1, which lacks this enzyme, and mutant 22-64, lacking citrate synthase, were tested for the production of asparaginase II. Growth of these mutants aerobically was similar to anaerobic growth of the wild type inasmuch as the citric acid cycle was no longer intact. Nevertheless, asparaginase II was not produced by either mutant during aerobic growth; production of the enzyme during growth in anaerobic jars was similar to that of the W strain wild type.

We have not yet been successful in isolating mutants lacking asparaginase II. Mutants that were identified by a defect in their ability to convert asparagine to aspartic acid when grown as colonies on solid media were found to grow at about one-fifth the rate of the wild-type bacteria in anaerobic jars. A small amount of asparaginase II, however, was formed in standing cultures and during the slow anaerobic growth. Thus, these mutants did not selectively lack asparaginase II, but rather were impaired in their ability to grow under anaerobic conditions. The nature of the defect in these mutants was not investigated further.

Table 3. Effect of the carbon source on the formation of asparaginase II by E. coli during anaerobic growth

<table>
<thead>
<tr>
<th>Casein hydrolysate added (%)</th>
<th>Specific activity</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Glucose</td>
<td>Galactose</td>
<td>No sugar added</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>0.4 (0.34)</td>
<td>0.88 (0.34)</td>
<td>(0)</td>
<td></td>
</tr>
<tr>
<td>0.2</td>
<td>3.2 (0.34)</td>
<td>5.2 (0.34)</td>
<td>(0)</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>6.4 (0.38)</td>
<td>11.2 (0.38)</td>
<td>12 (0.22)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>9.2 (0.43)</td>
<td>14 (0.43)</td>
<td>15.6 (0.30)</td>
<td></td>
</tr>
</tbody>
</table>

* Bacteria were grown in anaerobic jars in minimal medium with casein hydrolysate at the concentrations indicated. Sugars, when added, were present at a concentration of 1%. Specific growth rates (the reciprocal of the doubling time in hours multiplied by the natural logarithm of 2) under the various conditions are indicated in parentheses.

Table 4. Production of asparaginase II when asparagine served as the sole source of nitrogen

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<th>Growth medium</th>
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<tr>
<td></td>
<td>Aerobic</td>
</tr>
<tr>
<td>A-N + asparagine</td>
<td>0.01</td>
</tr>
<tr>
<td>A</td>
<td>0.02</td>
</tr>
<tr>
<td>A + casein hydrolysate</td>
<td>0.05</td>
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* Bacteria were grown under both aerobic and anaerobic conditions in minimal medium A with the omission of ammonium sulfate (A-N) but containing 0.1% asparagine. For comparison, cells were also grown in complete medium A, and in medium A supplemented with 0.2% casein hydrolysate. Galactose was added to all media at a concentration of 1%.

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**DISCUSSION**

Anaerobiosis affects the formation of a number of enzymes other than asparaginase II, and many of these are involved in the degradation of amino acids. Stephenson and Gale showed that the deamination of glutamate (27) and of serine (9) was enhanced in bacterial suspensions after growth under anaerobic conditions. Glucose was also found to inhibit the production of these activities. Better characterized was the catabolic L-threonine deaminase described by Wood and Gunsalus (30) and later studied by Umbarger and Brown (29); Umbarger and Brown distinguished this enzyme from the biosynthetic deaminase which appears during both aerobic and anaerobic growth and which functions in the biosynthesis of isoleucine. We know of no studies on the effect of anaerobiosis on glutaminase production.

The appearance of specific deaminases during anaerobic growth may offset the loss of oxidative deamination reactions which are used aerobically. Halpern and Umbarger (13) observed that the formation of aspartase, the enzyme catalyzing the deamination of aspartic acid to fumarate and ammonia, is dependent upon the addition of amino acids and is enhanced under anaerobic conditions (9; Marcus and Halpern, personal communication). The production of the enzyme is also inhibited by glucose (9). Hirsch et al. (15) showed that a fumarate reductase distinct from succinate dehydrogenase is formed during anaerobic growth when glycerol and fumarate are provided as carbon sources. These two reactions may constitute a terminal deaminating pathway in the fermentation of amino acids for which no specific deaminases exist, since it is likely that much of their amino nitrogen must pass through aspartate after transamination with oxalacetic acid. Asparaginase would serve to feed asparagine from the environment directly into this pathway. A final decision on the function of asparaginase II, however, awaits the isolation of mutants which lack the enzyme.

Neither its substrate, asparagine, nor its products, aspartic acid and ammonia, appear to act specifically to induce the formation of asparaginase II. Amino acids generally stimulated the production of the enzyme, the rate depending both on the variety and on the total amounts of amino acids added. These results might reflect a system of control involving either a specific inducer, which is derived in some way from the metabolism of amino acids during anaerobic growth, or multiple inducers, no one of which is available in sufficient concentration to result in the formation of the asparaginase when only a few amino acids are added. Alternatively it seems to us more reasonable to consider that a common metabolite acts as a repressor of asparaginase synthesis, and that the utilization of amino acids during anaerobiosis results in a decrease in the concentration of this repressing metabolite. We can speculate that a single mechanism controls the formation during anaerobic growth of a number of enzymes involved in amino acid degradation. Amino acids are encountered as mixtures in nature, and there might be no advantage in regulating the synthesis of each enzyme individually.

The use of asparaginase II in the treatment of human neoplastic disease makes it important that the enzyme be produced in large quantities. The content of the asparaginase varies widely in different strains of *E. coli* (5); however, in all strains the enzyme is localized in the periplasmic region between the plasma membrane and cell envelope, and its production is stimulated more than 100-fold when the bacteria are grown anaerobically under conditions where amino acids are utilized as carbon source. Bacteria grown under these conditions should provide a rich starting material for the large-scale production of the enzyme.

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2048

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J. BACTERIOL.


