N-Acetylglutamate Synthase of Escherichia coli Regulation of Synthesis and Activity by Arginine

THOMAS LEISINGER AND DIETER HAAS†
From the Mikrobiologisches Institut, Eidgenössische Technische Hochschule, CH-8006 Zürich, Switzerland

SUMMARY

N-Acetylglutamate synthase, the first enzyme of arginine biosynthesis, was stabilized in crude extracts from Escherichia coli. At 4°C the enzyme lost less than 5% of activity per day. L-Arginine repressed the formation of N-acetylglutamate synthase. Under conditions of genetic or physiological derepression, a specific activity of approximately 50 nmol per min per mg of protein was measured. No activity (i.e. less than 0.2 nmol per min per mg of protein) could be detected in extracts from cells grown under conditions of repression, whereas an intermediate level was found in cells cultivated on minimal medium. In a 6-fold purified preparation L-arginine inhibited the enzyme. Of 11 precursors and analogues of arginine tested only O-(L-norvalyl-5)-isourea inhibited N-acetylglutamate synthase as strongly as L-arginine.

N-Acetylglutamate synthase (acetyl-CoA:L-glutamate N-acetyltransferase, EC 2.3.1.1), the first enzyme of arginine biosynthesis, catalyzes the acetyl-CoA-dependent acetylation of the amino group of glutamic acid. This reaction was first demonstrated in crude extracts of Escherichia coli by Maas et al. (1). Further studies on the N-acetylglutamate synthase from this organism have been hindered by the instability of the enzyme in crude extracts and by the lack of a specific in vitro assay. Vyas and Maas (2) therefore used resting cell suspensions as a source of enzyme and measured N-acetylglutamate synthase formation in a bioassay. Making use of this system they demonstrated feedback inhibition as well as repression of the enzyme by arginine. We have shown that in crude extracts of Pseudomonas aeruginosa the enzyme was sufficiently stable to allow the development of a specific and sensitive in vitro assay (3). The synthesis of N-acetylglutamate synthase in P. aeruginosa is constitutive and the activity of the enzyme is inhibited by arginine and some arginine analogues (3, 4).

Whereas most of the enzymes of arginine biosynthesis in E. coli have been purified and characterized (5), the properties of the first enzyme of the pathway are largely unknown. The instability of N-acetylglutamate synthase observed in earlier investigations has prevented in vitro studies on the regulation of the activity of this enzyme by arginine. Similarly, it has not been feasible to explore a possible involvement of N-acetylglutamate synthase in the repression process, a regulatory function suggested for the feedback-sensitive enzymes of several other biosynthetic pathways (6). We now have adapted the assay previously described (3) to the measurement of N-acetylglutamate synthetase of E. coli. The enzyme was stabilized in cell extracts allowing us to study the regulation of its synthesis and activity by arginine. A preliminary report of some of these results has appeared elsewhere (7).

MATERIALS AND METHODS

Chemicals—L-[U-14C]Glutamic acid and [γ-32P]ATP were obtained from New England Nuclear Corp. (Boston), acetyl-CoA from Nutritional Biochemicals Corp. (Cleveland), and L-arginine-HCl, l-ornithine-HCl as well as N-acetyl-L-glutamic acid from Fluka (Buchs, Switzerland). d-Arginine-HCl, L-glutamic acid, L-canavanine sulfate, and L-2-amino-4-guanidinobutyric acid were purchased from Calbiochem, N-acetyl-L-ornithine, amino-oxyacetic acid-55 HCl, L-arginine hydroxamate, bovine serum albumin, dithiothreitol, and phenylmethylsulfonyl fluoride from Sigma (St. Louis, Mo.). L-2-Chloro-5-guanidinovaleric acid was prepared according to Hamilton and Ortíz (8). L-Indosine-HCl-H2O (L-2-amino-6-amidinobenzoic acid) was received from Dr. M. P. Hegarty (C.S.I.R.O. Division of Tropical Pastures, Cunningham Laboratory, St. Lucia, Queensland, Australia). L-N1-(1-Iminoethyl)ornithine was kindly supplied by Dr. J. P. Scannell (Hoffmann-La Roche Inc., Nutley, N.J.) and O-(L-norvalyl-5)-isourea by Dr. G. Müller and Prof. Dr. H. Zähner (Institut für Mikrobiologie der Universität, Tübingen, Germany).

Ducteria—The Escherichia coli strains used include the K12 strain 619 (his-, ile-, met-, Tn10) (9), strain 619/14 (LBGB 4481), a canavanine-resistant derivative of 619 with derepressed levels of the arginine biosynthetic enzymes, and strain 901, an arginine auxotroph derived from 619. The arginine auxotrophy of strain 901 is caused by a mutation in argC (N-acetylglutamate-5-semialdehyde dehydrogenase) having a polar effect on the expression of argE and argH (10). The arginine auxotroph 90A23R3, an argA- (N-acetylglutamate synthetase) strain, is derived from E. coli W (11).

Growth Conditions and Preparation of Cell Extracts—Cells were grown in Fernbach flasks containing 1 liter of Medium E (19) with the required amino acid supplements at a concentration of 100 μg per ml. The flasks were incubated on a rotary shaker (180 rpm) at 37°C. The cells were harvested during the exponential phase of growth (approximately 8 X 10⁸ cells per ml), washed once with 0.9% NaCl solution and stored at -20°C. Under these conditions the cells were stable for up to 6 months.

* This work was supported by the Swiss National Foundation for Scientific Research (Project No. 3.717.72).
† Present address, Department of Genetics, Monash University, Clayton, Victoria 3168 Australia.

1 Culture collection of Mikrobiologisches Institut, Eidgenössische Technische Hochschule, Zürich, Switzerland.
conditions, the activity of N-acetylglutamate synthase remained unchanged for at least 18 months.

Cell extracts were prepared by suspending 2 g of frozen cells in 6 ml of 10 mM potassium phosphate buffer, pH 6.5, containing 15% (v/v) glycerol, 1 mM EDTA, 1 mM dithiothreitol, 2 mM phenylmethylsulfonyl fluoride (extraction buffer), and disrupting them at 5° by six 15-s treatments with a Branson S-75 Sonifier at power setting No. 7. All subsequent operations were carried out at 0-4°. Cell debris was removed by centrifugation at 40,000 × g for 20 min. The supernatant was passed through a Sephadex G-25 column, equilibrated with the same buffer.

Enzyme Assays—N-Acetylglutamate synthase was assayed as described (3), with the following modifications: the reaction mixture contained, in a final volume of 50 μl: 10 mM L-[14C]glutamic acid (adjusted to pH 9 with KOH, specific activity 0.2 μCi per μmol), 4 μM acetyl-CoA, 200 mM Tris-HCl, pH 9, 7 mM α-ketoglutarate, 1.5% (v/v) glycerol, and protein from cell extracts. The enzyme activity in crude extracts was stimulated (20%) by magnesium ions. Therefore, the incubation mixture contained 10 mM MgCl2 for activity measurements in crude preparations. Standard incubation was at 25° for 5 min, after which time the reaction was terminated by the addition of 100 μl of 0.3 M HCl. The N-acetyl[14C]glutamate formed was then determined as described earlier (3). Blank values were obtained by incubating a mixture with all of the components except acetyl-CoA. L-[14C]Glutamic acid for the N-acetylglutamate synthase assay was purified by paper chromatography (3). One enzymatic unit is defined as that amount of enzyme which catalyzes the formation of 1 nmol of N-acetylglutamate per min under standard conditions as well as with other glycerol concentrations in the assay mixture (compare Fig. 2). Formation of N-acetylglutamate depended in a linear manner on the amount of protein in the assay provided that not more than approximately 15% of [14C]glutamate was converted to N-acetylglutamate.

RESULTS

Stability of Enzyme—In cell extracts prepared as described under “Materials and Methods” N-acetylglutamate synthase was reasonably stable, losing less than 5% of its activity per day at 4°. At room temperature the enzyme was unstable. When glycerol was omitted from the extraction buffer, the enzyme activity dropped rapidly after extraction and could not be detected after 12 hours at 4°. Inclusion of the protease inhibitor phenylmethylsulfonyl fluoride (16) in the extraction buffer exerted a stabilizing effect on N-acetylglutamate synthase but was without effect when added at 200 mg per liter to the growth medium.

Assay—Under standard assay conditions the acetyl-CoA-dependent formation of labeled derivatives other than N-acetyl[14C]glutamate from [14C]glutamate cannot be excluded a priori. However, evidence in support of N-acetylglutamate as the main labeled product formed was obtained when we detected approximately the same amount of N-acetylglutamate either by the standard assay or by a specific enzymatic assay using N-acetylglutamate 5-phosphotransferase. In an incubated reaction mixture we determined 18.7 nmol of N-acetylglutamate by assaying the 14C radioactivity after treatment with Dowex 50-W (standard assay) and 21.1 nmol of N-acetylglutamate with the N-acetylglutamate 5-phosphotransferase reaction. An additional indication for the specificity of the standard N-acetylglutamate assay was obtained with the argA strain 39A23R3. This strain was grown under conditions leading to derepression of the arginine biosynthetic enzymes (Medium E with 16 μg of N2-acetylornithine per ml). When an extract of these cells was assayed by the standard method we detected no formation of N-acetyl[14C]glutamate.

In the presence of 2 mM arginine, N-acetylglutamate synthase activity was drastically reduced but not completely inhibited. The low residual activity could not be reduced further by higher arginine concentrations. Irrespective of the N-acetylglutamate assay used, it amounted to about 12% of the uninhibited activity. From this observation we conclude that the residual N-acetylglutamate synthase activity, observed in the presence of arginine, is not due to some interfering side reactions.

The standard incubation conditions in the N-acetylglutamate synthase assay were chosen on the basis of the following considerations: between pH 8 and 9 enzyme activities were higher in Tris buffer than in phosphate buffer, N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid, sodium 5,5 diethylbarbiturate HCl, or glycine-NaOH. With Tris buffer, we observed a pHi optimum at 9.0. Glycerol, which is essential for the stability of the enzyme in the extraction buffer, was included, at 15% (v/v), in the standard incubation mixture. It exerts a slight depression of the enzyme activity and alters the feedback properties of the enzyme (compare Fig. 2). Aminoxyacetic acid (7 mM) inhibits arginine decarboxylase (17) but has no influence on the N-acetylglutamate synthase activity. Under standard incubation conditions the concentrations of acetyl-CoA and [14C]glutamate were not critical; the formation of product increased only slightly (less than 10%) when their concentrations were doubled. Using the standard incubation mixture, the enzyme was less stable at 37° or 30° than at 25°. Under standard conditions as well as with other glycerol concentrations in the assay mixture (compare Fig. 2) the enzyme activity was a linear function of time up to 10 min of incubation. Formation of N-acetylglutamate depended in a linear manner on the amount of protein in the assay provided that not more than approximately 15% of [14C]glutamate was converted to N-acetylglutamate.

Partial Purification of N-Acetylglutamate Synthetase by Sedimentation—When crude extracts were prepared with extraction buffer containing 10 mM magnesium acetate and centrifuged for 1 hour at 110,000 × g, approximately 50% of the enzyme activity was found in the pellet. Under these ionic conditions, the bulk of the enzyme sedimented at 40 to 50 S as determined by centrifugation through gradients of 25 to 40% (v/v) glycerol. If magnesium was omitted or Tris buffer was substituted for phosphate buffer, the enzyme could not be sedimented by centrifugation at 110,000 × g for 4 hours. Although it is not clear whether the enzyme undergoes a magnesium-induced association with itself or with some other macromolecules, the observed effect allowed the development of a procedure for a partial purification of the enzyme by centrifugation. It consisted of two centrifugation steps and resulted in an approximately 6-fold purification of the enzyme with 40% recovery of the initial activity. The first centrifugation was done in the presence of 10 mM magnesium acetate and led to a sediment with about 50% of the initial enzyme activity. This pellet was dissolved in extraction buffer containing 0.1 mM EDTA, incubated overnight at 4°, and subjected to a second centrifugation at 110,000 × g for 4 hours. Over 80% of the activity recovered in the pellet of the first centrifugation were found in the supernatant of the second centrifugation.

3 D. Haas and T. Leisinger, Eur. J. Biochem., in press.
Inhibition of N-Acetylglutamate Synthase by Arginine and Arginine Analogues—A 6-fold purified preparation of the enzyme in extraction buffer containing 0.1 mM EDTA was used for studying its inhibition by arginine, precursors of arginine, and various analogues. The substrate concentrations used in the inhibition experiments were saturating for crude extracts.

L-Arginine, O-(L-norvalyl-5)-isourea (18), and indospicine (19) were the most potent inhibitors of N-acetylglutamate synthase. As shown in Fig. 1, they caused a 50% inhibition at about 0.02 mM (O-(L-norvalyl-5)-isourea and L-arginine) and at 0.2 mM (indospicine), respectively. L-Ornithine and L-citrulline as well as N-arginine, L-canavanine, L-homoarginine, L-arginine hydroxamate, L-2-amino-4-guanidinobutyric acid, L-2-chloro-5-guanidinovaleric acid were poor inhibitors, i.e. at 2 mM they depressed the enzyme activity in the range of 0 to 18%. L-N6-(1-Iminomethyl)-ornithine (20) was an intermediate inhibitor bringing about a 45% reduction of enzyme activity at 2 mM. It is notable that canavanine, a potent growth inhibitor of *Escherichia coli* (21), produced no inhibition of the enzyme at all. O-(L-norvalyl-5)-isourea and indospicine, the two arginine analogues inhibiting *E. coli* N-acetylglutamate synthase are also inhibitors of the corresponding enzyme in *Pseudomonas aeruginosa* (4).

It was observed that inhibition by L-arginine and O-(L-norvalyl-5)-isourea reached a plateau value at about 15% residual activity, which could not be reduced further by increasing the inhibitor concentrations (Fig. 1). As explained earlier, the residual activity observed at high inhibitor concentrations was due to N-acetylglutamate synthase activity. In an effort to establish whether this residual activity represented a physiologically important property of the enzyme or whether it was due to the assay conditions, we tested the inhibition of N-acetylglutamate synthase by arginine at various concentrations of glycerol. Fig. 2 shows that the residual enzyme activity in the presence of 10 mM L-arginine was strongly dependent on the glycerol concentration in the incubation mixture. The enzyme activity in the absence of L-arginine, on the other hand, was only slightly depressed by raising the glycerol concentration in the assay. A similar modification of the feedback properties of the enzyme was observed as a function of the pH in the assay. With Tris buffer, pH 7.2, the residual activity in the presence of 1 mM L-arginine amounted to 4% whereas with Tris buffer, pH 9.0, it was 16% (data not shown).

**Table I**

| Strain of *E. coli* | Addition to Medium E (concentration) | Specific activity |
|---------------------|-------------------------------------|-------------------|
|                     | N-Acetylglutamate synthase | N2-acetylornithine 5-amino-transferase |
| mg/ml               | umol/mg protein                |                   |
| 619                  | L-Arginine (100)               | 0.9               |
| 619                  | None                            | 6.2               |
| 619/14               | L-Arginine (100)               | 12.5              |
| 961                  | L-Ornithine (100)              | 15.5              |

*Regulation of N-Acetylglutamate Synthase Formation by Arginine—In *E. coli* the formation of the arginine biosynthetic enzymes is repressed noncoordinately by the end product of the pathway. For seven out of the eight arginine biosynthetic enzymes the repression ratio has been established. It differs depending on the enzyme examined and is in the range between 3 and approximately 500 (22). As quantitative information about the control by L-arginine of N-acetylglutamate synthase is lack-
ing, we determined the specific activity of this enzyme in relation to regulatory conditions. Table I lists the specific activities of N-acetylglutamate synthase and, for comparison, of N\textsuperscript{2}-acetylornithine 5-aminotransferase in the argR\textsuperscript{-} strain 619, grown on minimal medium with and without l-arginine. These growth conditions lead to repression and to partial derepression, respectively of the arginine biosynthetic enzymes. Derepressed enzyme levels were obtained either genetically, by growing the argR\textsuperscript{-} strain 619/14 on minimal medium with l-arginine, or physiologically, by growing the arginine auxotroph 961 on minimal medium with l-ornithine, which in this organism is converted at a growth restricting rate to l-arginine. In repressed cells N-acetylglutamate synthase was not detectable (i.e., the specific activity was less than 0.2 unit per mg of protein). The derepressed enzyme levels amounted to approximately 50 units per mg of protein, which results in a repression ratio of greater than 250 (Table I).

Fig. 3 shows the kinetics of repression of N-acetylglutamate synthase. In an exponentially growing culture of strain 961 derepression (growth on l-ornithine) was followed by repression (addition of l-arginine). The specific activity of the enzyme was measured during early repression. Within the limits of the experimental error, the specific activity followed the pattern expected for the dilution of the enzyme by exponential growth. Thus, the absence of measurable enzyme activity in steady state repressed cells is probably due to the arrest of N-acetylglutamate synthase formation and not to inactivation of the enzyme.

Acknowledgment—We should like to thank Miss V. Kurer for excellent technical assistance and Dr. L. Roberts for reading the manuscript.

REFERENCES
1. MAAS, W. K., NOVELLI, G. D., AND LIPMANN, F. (1953) Proc. Nat. Acad. Sci. U. S. A. 39, 1004-1008
2. VYAS, S., AND MAAS, W. K. (1963) Arch. Biochem. Biophys. 100, 542-546
3. HAAS, D., KURER, V., AND LEISINGER, T. (1972) Eur. J. Biochem. 31, 290-295
4. HAAS, D., AND LEISINGER, T. (1972) J. Gen. Microbiol. 73, xiii
5. VOGEL, H. J. (1970) Methods Enzymol. 17A, 249-251
6. GOLDBERGER, R. F. (1974) Science 183, 810-816
7. HAAS, D., AND LEISINGER, T. (1974) Pathol. Microbiol. 40, 140-141
8. HAMILTON, P. B., AND ORTIZ, P. J. (1955) Biochem. Prep. 4, 76-78
9. BAUMBERG, S., BACON, D. F., AND VOGEL, H. J. (1965) Proc. Nat. Acad. Sci. U. S. A. 53, 1029-1032
10. BAUMBERG, S., AND ASHCROFT, E. (1971) J. Gen. Microbiol. 69, 365-373
11. VOGEL, H. J. (1961) in Control Mechanisms in Cellular Processes (Bonner, D. M., ed) pp. 23-65, The Ronald Press Company, New York
12. VOGEL, H. J., AND BONNER, D. M. (1956) J. Biol. Chem. 218, 97-106
13. VOGEL, H. J., AND JONES, E. E. (1970) Methods Enzymol. 17A, 200-264
14. LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L., AND RANDALL, R. J. (1951) J. Biol. Chem. 193, 265-275
15. SATCHELL, D. P. N., AND WHITE, G. F. (1970) Anal. Biochem. 36, 396-400
16. PROUTY, W. F., AND GOLDBERG, A. L. (1972) J. Biol. Chem. 247, 3341-3352
17. WILSON, O. H., AND HOLDEN, J. T. (1969) J. Biol. Chem. 244, 2737-2742
18. KÖNIG, W. A., KNIEFEL, H., BAYER, E., MÜLLER, G., AND ZÄHRINGER, H. (1973) J. Antibiot. 26, 44-50
19. LEISINGER, T., HAAS, D., AND HEGARTY, M. P. (1972) Biochim. Biophys. Acta 262, 214-219
20. SCANNELL, J. P., AX, H. A., PRUESS, D. L., WILLIAMS, T., DEMNY, T. C., AND STEMPHEL, A. (1972) J. Antibiot. 25, 179-184
21. SCHWARTZ, J. H., AND MAAS, W. K. (1960) J. Bacteriol. 79, 794-799
22. VOGEL, R. H., MCLELLAN, W. L., HIRVONEN, A. P., AND VOGEL, H. J. (1971) in Metabolic Pathways. Metabolic Regulation (VOGEL, H. J., ed) 3rd Ed, Vol. 5, pp. 483-488, Academic Press, New York