Effect of Homoserine on Growth of *Mycobacterium smegmatis*: Inhibition of Glutamate Transport by Homoserine

By VENKATARAMAN SRITHARAN, COLIN RATLEDGE* AND PAUL R. WHEELER

Department of Biochemistry, University of Hull, Hull HU6 7RX, UK

(Received 21 April 1987)

Homoserine strongly inhibited growth of *Mycobacterium smegmatis* in medium containing glutamate as the sole source of nitrogen but was without effect when asparagine, alanine or glutamine was the sole nitrogen source. It was readily taken up by glutamate-grown cells, reaching an intracellular concentration of over 20 mM after 4 h incubation. The primary site of action of homoserine was deduced to be the non-competitive inhibition of glutamate transport.

INTRODUCTION

In bacteria, homoserine is a branch-point amino acid intermediate in the biosynthesis of several amino acids of the aspartate pathway. It is converted both to threonine, and thus to isoleucine, and to methionine. In addition, the precursor of homoserine, aspartate β -semialdehyde, gives rise to diaminopimelic acid and lysine (Fig. 1) (Umbarger & Davis, 1962). Thus the aspartate pathway is responsible for the synthesis of several protein amino acids as well as diaminopimelic acid used for cell wall biosynthesis. Mycobacteria generally grow well on a glycerol/salts medium containing asparagine or glutamate as the sole nitrogen source (Ratledge & Hall, 1971; Lyon *et al.*, 1967, 1974). When glutamate is used as the sole nitrogen source, it is likely that intracellular aspartic acid is derived from glutamate by transamination with oxaloacetate. Glutamate metabolism in mycobacteria has been studied in some detail (see Ratledge, 1982). Glutamate is taken up by an active transport mechanism (Yabu 1967, 1970) and is metabolized via both NAD⁺- and NADP⁺-dependent glutamate dehydrogenases whose properties in *Mycobacterium smegmatis* have been studied in some detail (Singh & Venkitasubramanian, 1977).

Ostensibly, homoserine, being both a metabolite of aspartate and a precursor of several amino acids (see Fig. 1), would be expected to have little or no inhibitory action on growth but the reverse has proved to be the case. This has previously been noted with other bacteria (Kotre *et al.*, 1973), including *Escherichia coli*, but the inhibition was sometimes incomplete and moreover in the case of *E. coli* (the other bacteria not being studied in this respect) was relieved by adding glutamate. In this work, we have studied the inhibition by homoserine of the growth of *M. smegmatis* utilizing glutamate as nitrogen source.

METHODS

Organisms and growth. *Mycobacterium smegmatis* NCIB 8548 was grown in 100 ml glycerol/salts (Ratledge & Hall, 1971) liquid medium containing either asparagine or glutamate (5 g l⁻¹) in 250 ml conical flasks at 37 °C with orbital shaking (200 r.p.m.). Except for DL-homoserine, all other amino acids were L-isomers. Additional amino acids including homoserine were added to the growth medium either before autoclaving or before inoculation.

Abbreviations: NAD⁺-GDH, NAD⁺-dependent glutamate dehydrogenase; NADP⁺-GDH, NADP⁺-dependent glutamate dehydrogenase.

Fig. 1. Biochemical relationships of glutamate and aspartate to the amino acids derived from aspartate and homoserine.

Growth was monitored by determining the dry weight of 1 ml samples (in duplicate) of growing cultures by drying the cells at 120 °C for 3 h. Cultures were harvested by filtration through Whatman no. 1 filter paper; the cells were washed with several volumes of distilled water and stored frozen at -20 °C until required.

Uptake of radioactive glutamic acid. Washed, intact cells of *M. smegmatis* were placed in tightly closed Eppendorf tubes containing, in 1 ml final volume, glycerol/magnesium/phosphate buffer (0.5 M-glycerol; 2 mM-MgSO₄; 0.1 M-KH₂PO₄/Na₂HPO₄ buffer, pH 6.8) and [U-¹⁴C]glutamic acid (20 μM; sp. act. 50 μCi mol⁻¹, 1.85 MBq mol⁻¹). After incubation at 37 °C for 15 min with continuous shaking (200 r.p.m.), the reaction was stopped by rapidly cooling the contents in ice (Lyon *et al.*, 1967; Prasad *et al.*, 1976). The cells were washed in the cold with the same buffer containing unlabelled glutamate and the radioactivity was determined by liquid scintillation counting in a scintillant containing butyl-PBD [2-(4'-t-butylphenyl)-5-(4"-biphenyl)-1,3,4-oxadiazole] and Triton N-101 in xylene.

Preparation of cell-free extract. Bacteria were thawed and suspended in 10 times their volume of 50 mM-KH₂PO₄/Na₂HPO₄ buffer, pH 8.0, containing 10 mM-dithiothreitol and 10 mM-EDTA and disrupted ultrasonically (80 W applied for 2 min followed by cooling) in a Branson Soniprobe Automatic Generator type 7533A (USA) for 6 min (g wet wt cells)⁻¹ at 0 to 4 °C. The supernatant obtained after centrifuging the extract at 20000 g for 10 min was used for the assay of glutamate dehydrogenase, homoserine dehydrogenase and aspartokinase. Protein was estimated by a modified Lowry method (Layne, 1957).

Enzyme assays. NAD⁺-GDH (EC 1.4.1.2) and NADP⁺-GDH (EC 1.4.1.4) were assayed in the direction of oxidation of glutamate with the formation of NADH and NADPH being followed at 340 nm (Corman & Inamdar, 1970). The assay mixture contained, in 1 ml, 0.5 M-Tris/HCl buffer pH 9.0, 0.4 mM-NAD⁺ or NADP⁺, 100 mM-sodium glutamate and a suitable amount of cell extract protein as enzyme source. The contents were preincubated at 37 °C and the reaction was started by adding glutamate. Enzyme activity was calculated from ΔA₃₄₀ measured over 5 min and expressed as μmol NADH or NADPH formed min⁻¹ (mg protein)⁻¹. A control lacking glutamate was run each time.

Aspartokinase (EC 2.7.2.4) was assayed by detecting the aspartate hydroxamate ($\epsilon_{520} = 350$ l mol⁻¹ cm⁻¹) formed during a 1 h incubation (Stadtman *et al.*, 1961) except that the FeCl₃ reagent was prepared as described by Truffa-Bachi *et al.* (1970) and the final colour was developed with 3 ml of this reagent. Homoserine dehydrogenase (EC 1.1.1.3) was assayed using DL-homoserine as the substrate and NADP⁺ as coenzyme (Datta & Gest, 1965). Controls were performed for both enzymes by omitting aspartate and homoserine from the respective assay mixtures. Specific activity of homoserine dehydrogenase was expressed as units (mg protein)⁻¹, where 1 U enzyme converts substrate to measured product at 1 μmol min⁻¹.

Determination of the intracellular homoserine concentration. DL-Homoserine was added at 5 mM to a 100 ml culture of *M. smegmatis* after 2 d growth on glutamate medium. After a further 4 h growth, the cells were filtered, washed three times with cold 0.9% NaCl solution and then extracted, according to the procedure of Herbert *et al.* (1971), with boiling water for 30 min and subsequently with 60% (v/v) ethanol at 4 °C for 60 min. The two extracts containing the soluble amino acids were pooled, evaporated at 50 °C and redissolved in 0.5 ml 0.5 M-Tris/HCl buffer, pH 9.0. The amount of L-homoserine in the aqueous extract was estimated by adding a sample to a cell-free extract from *M. smegmatis* containing homoserine dehydrogenase and determining the reaction velocity upon the addition of NADP⁺ and thus the substrate concentration. A control was also carried out using the soluble amino acid pool obtained from cells not exposed to homoserine.

RESULTS AND DISCUSSION

Physiological effects of homoserine on M. smegmatis

M. smegmatis grows well in glycerol/salts medium containing either asparagine or glutamate as sole nitrogen source. When DL-homoserine, at 5 mM, was added to medium containing glutamate, growth was severely restricted and did not recover even after 7 d (Fig. 2). As the same magnitude of effect was produced using 2.5 mM-L-homoserine, it was concluded that the D-isomer was without effect. All subsequent experiments used DL-homoserine. With DL-homoserine at 1 mM, the yield of bacteria after 4 d growth was still decreased by 60% (results not shown). If homoserine (5 mM, final concn) was added to the glutamate medium after 48 h, growth was again inhibited (Fig. 2).

The intracellular concentration of L-homoserine was determined in a 48 h culture of *M. smegmatis*, which had been exposed to 5 mM DL-homoserine for 4 h (see Methods), as being 5.65 μmol per 100 mg dry cells. Without exposure to homoserine, a value of 1.0 μmol per 100 mg dry cells was obtained which was attributed to the general reactivity of the soluble amino acid pool towards the crude enzyme assay system being used. Thus the difference between the two values was attributed to the presence of L-homoserine and, if a cell volume of approximately 2 μl (mg dry wt^{-1}) is assumed as a typical value for bacteria (Midgley & Dawes, 1973), this is equivalent to 23 mM.

The absence of any inhibition with medium containing asparagine or alanine as nitrogen sources (results not shown) suggested that homoserine was acting against the utilization of glutamate itself rather than on a central pathway of metabolism. However, it was felt important to substantiate such a deduction experimentally. If homoserine was inhibiting a central pathway, the most logical point would be an enzyme of the aspartate pathway of amino acid biosynthesis (see Fig. 1), i.e. aspartokinase or homoserine dehydrogenase, or both.

Aspartokinase was detected in cell extracts prepared from *M. smegmatis* incubated in glycerol/glutamate medium containing DL-homoserine (5 mM) for 9 d. (Cells were pooled from several flasks to provide enough material. Approximately 500 mg wet weight of cells could be obtained from 10 flasks.) In these extracts 0.40 μmol aspartate hydroxamate was formed h^{-1} (mg protein^{-1}). DL-Homoserine when added to the enzyme assay at 5 mM caused nearly 45% inhibition of activity; at 20 mM it gave 70% inhibition. The specific activity of the aspartokinase [0.44 μmol aspartate hydroxamate formed h^{-1} (mg protein^{-1})] was, however, almost unchanged in comparison with those recovered from cells grown on glutamate in the absence of homoserine, indicating that no repression of enzyme synthesis had occurred. Homoserine dehydrogenase, which was present in the same cell extracts at 5.9 mU (mg protein^{-1}), was unaffected by DL-homoserine at either 5 or 20 mM but its synthesis was repressed by 70% when DL-homoserine (at 5 mM) had been included in the growth medium.

Thus, as homoserine partially inhibits aspartokinase and partially represses homoserine dehydrogenase, its effects, if it were only acting on these two enzymes, should be reversed by adding into the growth medium those amino acids whose biosynthesis depend upon the two enzymes: lysine, diaminopimelic acid, methionine, threonine and isoleucine. However, no single amino acid or combination of amino acids completely reversed the effect of homoserine (data not shown). Although growth up to 80% of control rates (results not shown) was found when threonine, isoleucine and methionine were added together this was attributed to the added amino acids (each at 5 mM) reversing the effects of homoserine by acting as alternative nitrogen sources to glutamate, since the effect of homoserine was confined to cells utilizing glutamate.

We therefore conclude that homoserine, in spite of its partial inhibitory effect towards aspartokinase and repression of homoserine dehydrogenase, probably interferes with the utilization of glutamate, as the effect is manifested only in the presence of this nitrogen source.

Utilization of glutamate by M. smegmatis

Utilization of glutamate involves transport into the cells followed by either oxidation (via glutamate dehydrogenase/s) or transamination (see Fig. 1).

Fig. 2

Fig. 2. Effect of homoserine on growth of *M. smegmatis* on glycerol/glutamate medium. Growth was followed by determining in duplicate the dry weight of cells drawn from culture media periodically in the absence (Δ) and presence ($\square$) of 5 mM-DL-homoserine; DL-homoserine added at 5 mM (indicated by arrow) after 2 d growth ($\circ$). The experiment was carried out three times and the results given are for a typical experiment.

Fig. 3

Fig. 3. Uptake of L-glutamic acid by washed cells of *M. smegmatis* previously grown on glutamate (20 mM) medium. Experimental details are given in Methods. The 100% value corresponds to 0.33 μ mol glutamic acid (20500 c.p.m.). The experiment was carried out four times in duplicate and the results given are for a typical experiment.

Effect of homoserine on glutamate uptake. *M. smegmatis* possesses an active transport system for glutamate which is saturable, and has in addition a passive but non-saturable diffusion process (Yabu, 1970, 1971). The transport of glutamate into *M. smegmatis* was severely impaired by homoserine (Fig. 3). By plotting the results, as well as those for glutamate uptake in the absence of homoserine, in the usual double reciprocal manner, the inhibition kinetics of glutamic transport in the presence of homoserine was found to be non-competitive ($K_i = 150 \mu$ M for the DL-isomer, i.e. 75 μ M for the L-isomer). Increasing the glutamate concentration did not improve the uptake of glutamate significantly. The low rate of glutamate uptake remaining in the presence of homoserine was considered to be due to diffusion.

The inhibition of glutamate uptake by homoserine was specific and was unaffected by 5 mM-threonine, methionine or isoleucine (data not shown). Uptake of glutamate into washed cells of *M. smegmatis*, previously grown on asparagine or glutamine medium, was also similarly inhibited by homoserine (results not presented).

Effect of homoserine on endogenous metabolism of glutamate. *M. smegmatis* possessed activities of both NADP⁺- and NAD⁺-GDH at 90 mU (mg protein)⁻¹ and 5.7 mU (mg protein)⁻¹, respectively, in extracts from glutamate-grown cells. The two enzymes had different pH optima (8.0 for NADP⁺-GDH and 9.0 for NAD⁺-GDH) and different electrophoretic mobilities (not shown). Thus we conclude that the two GDH activities are carried by two separate proteins in *M. smegmatis*. DL-Homoserine at 5 mM inhibited NAD⁺-GDH by 40% and at 50 mM by 65% but had less effect on NADP⁺-GDH even at the highest concentrations (up to 50 mM) (data not shown). The specific inhibition of NAD⁺-GDH by homoserine could also be demonstrated after separating the GDHs by PAGE and carrying out activity staining in the presence and absence of homoserine.

Although Kotre *et al.* (1973) and Savageau *et al.* (1972) previously noted that the growth of a range of micro-organisms was inhibited by high concentrations of homoserine (15 mM), this could be reversed, at least with *E. coli* B/r, by adding glutamate to the growth medium. They

concluded that the inhibition resulted from the sensitivity of NADP⁺-GDH to homoserine. Presentation to the cells of glutamate – the product of the inhibited reaction – then relieved the inhibition. This is clearly distinct from the phenomenon we have observed here with *M. smegmatis*, where homoserine exerts its effect only when glutamate is sole nitrogen source. The evidence which we have presented would indicate that the primary cause of inhibition is the almost total prevention of glutamate uptake into the cells. Inhibition of other enzyme activities (NAD⁺-GDH and aspartokinase) and repression of enzyme synthesis (homoserine dehydrogenase) then become secondary to this main effect. The consequences of glutamate deprivation as the sole nitrogen source to the cells are then self-evident.

V.S. is supported by the Commonwealth Scholarship Commission (UK) and P.R.W. thanks the MRC for support.

REFERENCES

- CORMAN, L. & INAMDAR, A. (1970). L-Glutamate dehydrogenase. *Methods in Enzymology* **17**, 844–849.
- DATTA, P. & GEST, H. (1965). Homoserine dehydrogenase of *Rhodospirillum rubrum*. Purification, properties and feedback control of activity. *Journal of Biological Chemistry* **240**, 3023–3033.
- HERBERT, D., PHIPPS, P. J. & STRANGE, R. E. (1971). Chemical analysis of microbial cells. *Methods in Microbiology* **5B**, 210–336.
- KOTRE, A. M., SULLIVAN, S. J. & SAVAGEAU, M. A. (1973). Metabolic regulation by homoserine in *Escherichia coli* B/r. *Journal of Bacteriology* **116**, 663–672.
- LAYNE, B. (1957). Spectrophotometric and turbidimetric methods for measuring proteins. *Methods in Enzymology* **3**, 447–454.
- LYON, R. H., ROGERS, P., HALL, W. H. & LICHSTEIN, H. C. (1967). Inducible glutamate transport in mycobacteria and its relation to glutamate oxidation. *Journal of Bacteriology* **94**, 92–100.
- LYON, R. H., HALL, W. H. & MARTINEZ, C. C. (1974). Effect of L-asparagine on growth of *Mycobacterium tuberculosis* and utilization of other amino acids. *Journal of Bacteriology* **117**, 151–156.
- MIDGLEY, M. & DAWES, E. A. (1973). The regulation of glucose transport and methyl α -glucoside in *Pseudomonas aeruginosa*. *Biochemical Journal* **132**, 141–154.
- PRASAD, R., KALRA, V. K. & BRODIE, A. F. (1976). Different mechanisms of energy coupling for transport of various amino acids in cells of *Mycobacterium phlei*. *Journal of Biological Chemistry* **251**, 2493–2498.
- RATLEDGE, C. (1982). Nutrition, growth and metabolism. In *Biology of Mycobacteria*, vol. 1, pp. 186–271. Edited by C. Ratledge & J. L. Stanford. London: Academic Press.
- RATLEDGE, C. & HALL, M. J. (1971). Influence of metal ions on the formation of mycobactin and salicylic acid in *Mycobacterium smegmatis* grown in static culture. *Journal of Bacteriology* **108**, 312–319.
- SAVAGEAU, M. A., KOTRE, A. M. & SAKAMOTO, N. (1972). A possible role in the regulation of primary amination for a complex of glutamine: α -ketoglutarate amido transferase and glutamate dehydrogenase in *Escherichia coli*. *Biochemical and Biophysical Research Communications* **48**, 41–47.
- SINGH, P. & VENKITASUBRAMANIAN, T. A. (1977). Glutamate dehydrogenase of *Mycobacterium smegmatis*. *Indian Journal of Biochemistry and Biophysics* **14**, 379–381.
- STADTMAN, E. R., COHEN, G. N., LE BRAS, G. & DE ROBICHON-SZULMAJSTER, H. (1961). Feedback inhibition and repression of aspartokinase activity in *Escherichia coli* and *Saccharomyces cerevisiae*. *Journal of Biological Chemistry* **236**, 2033–2038.
- TRUFFA-BACHI, P. & COHEN, G. N. (1970). Aspartokinase I and homoserine dehydrogenase I (*E. coli*). *Methods in Enzymology* **17A**, 694–698.
- UMBARGER, E. & DAVIS, B. D. (1962). Pathways of amino acid metabolism. In *The Bacteria*, vol. 3, pp. 167–252. Edited by I. C. Gunsalus & R. Y. Stanier. New York: Academic Press.
- YABU, K. (1967). The uptake of D-glutamic acid by *Mycobacterium avium*. *Biochimica et biophysica acta* **135**, 181–183.
- YABU, K. (1970). Amino acid transport in *Mycobacterium smegmatis*. *Journal of Bacteriology* **102**, 6–13.
- YABU, K. (1971). Aspartic acid transport in *Mycobacterium smegmatis*. *Japanese Journal of Microbiology* **15**, 449–456.