Growth Inhibition of *Acetobacter aceti* by L-Threonine and L-Homoserine: the Primary Regulation of the Biosynthesis of Amino Acids of the Aspartate Family

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SUMMARY

Growth of *Acetobacter aceti* NCIB8554 was inhibited by L-threonine and L-homoserine. Inhibition was relieved by L-aspartate, L-methionine or L-isoleucine. Aspartokinase was inhibited by L-threonine or L-lysine and concertedly inhibited by the two together. L-Methionine or L-isoleucine reversed the joint inhibition. Homoserine dehydrogenase was inhibited by L-threonine. *Acetobacter aceti* possesses one aspartokinase and a separate homoserine dehydrogenase. Neither enzyme is subject to repression by amino acids of the aspartate family.

INTRODUCTION

Growth of many organisms is inhibited by common metabolites (Ingram & Jensen, 1973; Rittenberg, 1969); study of this inhibition may provide useful information about the regulation of metabolism.

In the biosynthesis of the aspartate family of amino acids in bacteria, the two primary points of control are aspartokinase (AK) and homoserine dehydrogenase (HSD) (Cohen, 1971). The syntheses of lysine, methionine, threonine and isoleucine are affected by control of AK, whilst those of the last three are also influenced by control of HSD (Cohen, 1971).

In *Escherichia coli* K12 there are three separately regulated AK isoenzymes, two of which also carry HSD activity (Cohen, 1971) but many other procaryotes have AK and HSD as single separate enzymes; the AK is then subject to concerted feedback inhibition by two of the end products of the pathway and the HSD is sensitive to threonine (Cohen, Stanier & Le Bras, 1969).

In this study, the growth inhibition of *Acetobacter aceti* by threonine and homoserine has been described and the primary regulation of the aspartate family examined.

METHODS

Growth of organism. *Acetobacter aceti* NCIB8554 was grown at 30 °C with ethanol (11 mM) as the carbon source and ammonium sulphate (7.6 mM) as the nitrogen source, in a medium buffered with 0.1 M-phosphate buffer, pH 5.5, and containing the following salts per litre of distilled water: MnSO₄·4H₂O, 0.01 g; NaCl, 0.01 g; FeCl₃, 0.01 g; CuSO₄·5H₂O, 0.01 g; CaCl₂·2H₂O, 0.01 g; ZnSO₄·7H₂O, 0.01 g; and MgSO₄·7H₂O, 0.2 g. The ethanol, buffer and salts were sterilized separately and combined after cooling. Growth experiments were performed in either 25 ml tubes containing 5 ml medium and orbitally shaken at 350 rev./min or 500 ml baffled flasks containing 50 ml medium orbitally shaken at 120 rev./min. The

growth rates in the two systems were similar. Overnight cultures were used for inoculation and growth was observed by measuring the extinction at 546 nm using an Eppendorf spectrophotometer (1 cm light path). Organisms used for enzyme assays were grown in a fermenter aerated at a rate of 1 vol. air/vol. liquid/h and stirred at 1000 rev./min.

Enzyme preparation. Bacteria suspended in 0.1 M-K₂HPO₄ with 0.5 mM-ethylene diamine tetraacetic acid and 3 mM-mercaptoethanol (solution A) were disrupted using a Branson sonifier (Branson Instruments, Inc., U.S.A.) at a power setting of 6 A for ten 15 s periods.

The extract was centrifuged at 45000 *g* for 10 min and the supernatant fluid fractionated with ammonium sulphate. The precipitates were collected by centrifugation at 27000 *g* for 15 min, suspended in solution A, and dialysed against this solution.

Enzyme assays. AK (ATP:L-aspartate 4-phosphotransferase, EC. 2.7.2.4) was assayed by the method of Truffa-Bachi & Cohen (1970). HSD (L-homoserine:NADP⁺ oxidoreductase, EC. 1.1.1.3) was assayed by the method of Datta & Gest (1970). Enzyme units are defined as that amount of enzyme giving rise to 1 μ mol DL-aspartic acid β -hydroxamate/h (AK), or 1 μ mol reduced NADP/min (HSD). Specific activities are expressed as enzyme units/mg protein. Protein was measured by the Biuret method (Layne, 1957).

Column chromatography. A column (30 $\times$ 2.8 cm) was packed with the anion exchange cellulose DE52 (Whatman, Carl Bittman, Basel), equilibrated in 10 mM-phosphate buffer pH 7.5 containing 5 mM-potassium aspartate and 2 mM-ethylene diamine tetraacetic acid. A 20 to 80 % (w/v) ammonium sulphate preparation of the crude extract was applied to the column and eluted with the buffer and a KCl gradient (0.1 to 1.0 M). Fractions (9 ml) were collected and assayed for the two enzymes as described above and for protein by measuring the extinction at 280 nm.

Uptake experiments. A 2.5 ml portion of exponentially growing bacteria ($E_{546\text{ nm}}^{1\text{ cm}} = 0.25$; 56 μ g dry wt/ml) were added to L-[U-¹⁴C]threonine (specific activity 208 mCi/mM) giving a final threonine concentration of 2 μ M. Samples (0.25 ml) were taken, filtered (0.45 μ m pore size) and washed four times with 2.5 ml growth medium (30 °C). The filters were placed in vials and dried. Scintillation fluid (10 ml) containing 4 g 2,5-diphenyloxazol (PPO) and 250 mg 2,2'-*p*-phenylen-bis-(4-methyl-5-phenyloxazol) (dimethyl-POPOP)/l toluene was added to each vial and the samples counted using a Beckman LS-250 counter system. Threonine uptake was linear over the 10 min period tested. The counting efficiency was about 80 %.

Chemicals. The L-isomers of amino acids were used throughout except for diaminopimelic acid which was a mixture of LL, DD and *meso* isomers. Methionine, lysine, isoleucine, homoserine and threonine were obtained from Fluka, Buchs, Switzerland; monosodium aspartate and diaminopimelic acid from Sigma; ATP, NAD and NADP from Boehringer, Mannheim, Germany; PPO and dimethyl-POPOP from Merck, Darmstadt, Germany. L-[U-¹⁴C]threonine was obtained from New England Nuclear Corp., Boston, U.S.A.

RESULTS

The effect of the aspartate family of amino acids on growth

Acetobacter aceti was grown with aspartate, homoserine, threonine, diaminopimelic acid, lysine, methionine or isoleucine, at concentrations ranging from 0.1 to 20 mM. Only threonine and homoserine inhibited growth. Threonine inhibited completely at 1 mM but had no significant effect at 0.4 mM. Homoserine was only slightly inhibitory at 1 mM but inhibited completely at 10 mM.

Methionine (0.2 mM), aspartate (5 mM) or isoleucine (5 mM) prevented inhibition if added

Fig. 1. The effect of (a) methionine, (b) aspartate and (c) isoleucine in preventing the growth inhibition of *A. aceti* by 1 mM-threonine. ○, Control without threonine; ●, cultures containing 1 mM-threonine; numbers refer to mM concentrations of (a) methionine, (b) aspartate and (c) isoleucine.

to cultures at the same time as 1 mM-threonine (Fig. 1). If added 2.5 h after the threonine, methionine (10 mM), aspartate (50 mM) or isoleucine (40 mM) restored the growth rate to near that of uninhibited controls after a lag of about 2 h; lower concentrations took longer. Lysine or diaminopimelic acid had no effect.

Each amino acid of the aspartate family was tested at 10 mM concentration for its effect on the growth inhibition caused by 5 mM-homoserine. Aspartate, isoleucine or methionine prevented inhibition, lysine and diaminopimelic acid did not.

Uptake of threonine

The rate of threonine uptake by *A. aceti* was 295 pmol/min/mg dry wt and was unaffected by methionine, isoleucine or aspartate at concentrations 200 times that of the threonine.

Separation of AK and HSD

When a crude extract was fractionated with ammonium sulphate most of the AK sedimented between 20 and 40% (w/v) saturation whilst HSD sedimented between 40 and 60% (w/v) saturation (Fig. 2a). The two enzymes were also separated by ion-exchange chromatography (Fig. 2b), the HSD being eluted before the AK. The threonine sensitivity of AK in each of the 5% (w/v) ammonium sulphate fractions was the same.

Enzyme inhibition

AK was assayed in the 20 to 40% (w/v) ammonium sulphate fraction in the presence of each of the amino acids of the aspartate family (1 to 20 mM); all combinations of two amino acids, each 10 mM, were also tested. Only lysine, threonine or both together inhibited enzyme activity (Fig. 3). Threonine at 10 mM caused 90% inhibition and 10 mM-lysine inhibited by about 33%, but in combination, only 1 mM-lysine and 0.3 mM-threonine were needed to give 90% inhibition. Methionine and isoleucine stimulated enzyme activity slightly; homoserine and diaminopimelic acid had no effect (Fig. 3). The inhibition caused

Fig. 2. The separation of AK and HSD (a) by ammonium sulphate fractionation and (b) by ion exchange chromatography (each fraction contained 9 ml). $\circ$, AK; $\bullet$, HSD; $\blacksquare$, protein.

Fig. 3. The effect of amino acids on AK activity in a 20 to 40 % (w/v) ammonium sulphate fraction. ●, Methionine; △, isoleucine; ▲, homoserine; □, diaminopimelic acid; ○, lysine; ■, threonine; ■, lysine plus 0.3 mm-threonine.

Fig. 4. The effect of methionine and isoleucine on the concerted inhibition of AK by 1 mm-threonine plus 0.1 mm-lysine. ○, Methionine; ●, isoleucine.

by 1 mm-threonine plus 0.1 mm-lysine (90 %) was reduced by increasing concentrations of either methionine or isoleucine (Fig. 4); aspartate was without effect.

Maximal inhibition (60 %) of HSD in the 40 to 60 % (w/v) ammonium sulphate fraction was reached with 10 mm-threonine; no other amino acid of the aspartate family or combinations of two of these affected enzyme activity. The HSD was NADP specific.

Enzyme repression

Repression was examined by growing the organism in a fermenter to mid-exponential growth and then taking a control sample. Threonine (0.5 mM), homoserine (2 mM) or other test amino acids (10 mM) were added and samples taken 2 and 4 h later. AK and HSD were measured in dialysed crude extracts and differences of over 20 % from the control were regarded as significant.

AK was not repressed in any of the eight situations tested. The specific activity of the AK increased by 20 and 27 % when aspartate or methionine, respectively, were present in the culture. The AK always displayed the same degree of sensitivity to threonine or threonine plus lysine. HSD decreased to 71 % that of the control 2 h after threonine addition but the specific activity at 4 h was up to 80 % of the control and the result is not thought to be significant.

DISCUSSION

The growth inhibition of *A. aceti* 8554 by threonine has been shown to be due to feedback effects on the enzymes of the biosynthetic pathway leading from aspartate. The AK was inhibited by lysine and threonine and concertedly by both, but 100 % inhibition was not found (Fig. 3).

Inhibition of growth by threonine is probably due to a shortage of methionine in the organism, since methionine at very low concentrations prevented growth inhibition (Fig. 1*a*) and methionine *in vitro* reduced the concerted feedback inhibition by lysine plus threonine (Fig. 4). Isoleucine is thought to overcome growth inhibition solely by its effect on the concerted inhibition of AK (Fig. 4), thereby allowing the production of methionine to continue. The role of aspartate in overcoming growth inhibition cannot be explained in these terms, nor could any effect on transport be shown. Homoserine inhibited growth but had no effect on AK. Serine and valine also inhibited growth of *A. aceti* 8554, and isoleucine can prevent their inhibition; the serine inhibition is also prevented by methionine or aspartate (O'Sullivan, unpublished results). The growth inhibition by homoserine may, then, have a biochemical basis related to these amino acids.

Acetobacter aceti appeared to possess only one AK and one HSD, as judged by the single peak of activity found by ammonium sulphate fractionation and ion exchange chromatography (Fig. 2) and by the uniform sensitivity of the AK to threonine and to threonine plus lysine in all repression experiments and ammonium sulphate fractions. AK and HSD were not subject to repression by amino acids under the conditions tested. Since HSD was inhibited only weakly by threonine and not at all by any other amino acid of the aspartate family, its importance as a control point in the biosynthesis of threonine, isoleucine and methionine is questionable. The chief regulatory mechanism of the biosynthesis of amino acids of the aspartate family in *A. aceti* seems, therefore, to be the concerted feedback inhibition of the AK.

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