SHORT COMMUNICATIONS

Inhibition by L-Threonine of Aspartokinase as a Cause of Threonine Toxicity to *Methylococcus capsulatus*

By M. ECCLESTON* AND D. P. KELLY

*Department of Microbiology, Queen Elizabeth College,
Campden Hill, London, W8 7AH*

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We have previously suggested that the inhibition of growth of *Methylococcus capsulatus* by L-threonine resulted from interference with early steps in the branched pathway for the biosynthesis of the aspartate-family amino acids (Eccleston & Kelly, 1972*a*). We now present evidence to suggest that aspartokinase may be the enzyme critically affected by L-threonine.

Wild-type and threonine-resistant strains of *Methylococcus capsulatus* were cultured in 250 ml of liquid medium (Eccleston & Kelly, 1972*a*) containing 10% (v/v) inocula of 24 h shaken cultures. Cultures were magnetically stirred at 37 °C in sealed 5 l Erlenmeyer flasks initially containing an atmosphere of air + CH₄ + CO₂ (20 + 9 + 1, by vol). Cultures were harvested towards the end of logarithmic growth (0.95 to 1.30 mg dry wt/ml) by centrifuging at 4 °C. Organisms were washed with 0.02 M-phosphate, pH 6.8, containing 0.03 M-β-mercaptoethanol, and resuspended in this buffer mixture. Suspensions (5 ml) were mixed with washed Ballotini no. 12 glass beads (1 g) and sonicated at maximum power for 6 × 2.5 min periods at 0 to 4 °C in an MSE 100 watt ultrasonic disintegrator. Sonicates were centrifuged at 38000 × g (30 min, 4 °C) and supernatant liquids used on the day of preparation as crude extracts for assaying aspartokinase. Protein concentration in the extracts was determined by the method of Lowry, Rosebrough, Farr & Randall (1951). Aspartokinase (ATP: L-aspartate 4-phosphotransferase, EC. 2.7.2.4) was assayed at 37 °C according to the modification by Stadtman, Cohen, Le Bras & de Robichon-Szulmajster (1961) of the method of Black & Wright (1955). Reactions were terminated by adding the acid-ferric reagent and mixtures blended by vigorous mechanical agitation. Absorbance at 540 nm was measured in a Unicam SP 600 Series II spectrophotometer against zero-time blanks. Values obtained were corrected for colour developed in controls from which aspartate was omitted. Standards were prepared using DL-aspartic acid β-hydroxamate monohydrate (Sigma Chemical Co., London) and enzyme activities calculated as nanomoles of asparthydroxamate formed/mg protein/min. Activity was proportional to extract concentration up to about 1 mg extract protein/ml assay mixture. Asparthydroxamate formation was constant for about 30 min, but then declined abruptly to about 40% of the initial rate and continued for at least a further 90 min at the lower rate. Routinely 0.2 ml volumes of extract (about 1 mg protein) and an incubation time of 30 min were used. Activity was greatest between pH 7.4 to 7.8, and pH 7.6 was used in the experiments described below. Details of the development of the optimal assay conditions are given elsewhere (Eccleston, 1973).

Mean aspartokinase activity of eleven crude extract preparations from wild-type *Methylococcus capsulatus* was 9.5 nmol/mg protein/min (extreme values, 5 and 13). Comparable activities were found in extracts of several threonine-resistant mutant strains (Eccleston &

* Present address: Department of Biological Sciences, University of Warwick, Coventry, Warwickshire, CV4 7AL.

Fig. 1. Effect of L-methionine, L-lysine, L-threonine and L-threonine + L-lysine on aspartokinase activity in crude extracts of wild-type *Methylococcus capsulatus*. Activities are expressed as percentages of uninhibited controls (determined in triplicate), corrected for blank colour development. Mean and extreme values from three separate experiments are given. ×, L-Methionine; ●, L-lysine; ○, L-threonine; ◐, L-lysine + L-threonine.

Kelly, 1972a): namely, 8.6 for strains M16 and M36 cultured in medium lacking threonine, and 9.7 or 8.8 respectively for strain M27 cultured with or without 5 mM-L-threonine in the culture medium.

Aspartokinase in wild-type extracts was strongly inhibited by L-threonine (Fig. 1), which produced half-maximal inhibition at about 0.2 mM. Concentrations of 1 mM and above produced 85 to 100% inhibition. Aspartokinase in extracts of strains M27 and M36 was comparably sensitive to L-threonine, showing 70 to 80% inhibition at 1 to 5 mM. Activity in strain M16 seemed less sensitive, 5 mM-L-threonine causing only 60% inhibition, although low concentrations produced inhibition comparable to that found with wild-type extracts.

Other amino acids were tested for their effect on wild-type *Methylococcus capsulatus* aspartokinase activity. D-Threonine had no significant effect at concentrations up to 10 mM, but L-lysine inhibited by about 56% at 5 mM and 87% at 10 mM. Equimolar mixtures of L-lysine and L-threonine over a range of concentrations were more inhibitory than either compound alone and produced an approximately additive inhibitory effect (Fig. 1). It was concluded from several determinations that 2:6-diaminopimelic acid and L-isoleucine were not significantly inhibitory at concentrations below 5 mM, although in one experiment only, 10 mM-L-isoleucine strongly depressed activity. In one experiment, L-homoserine (0.07 to 5 mM) caused about 40% inhibition at all concentrations tested.

L-Methionine stimulated aspartokinase activity about 20% at concentrations between 0.035 mM and 10 mM (Fig. 1), after correcting all data for blank absorbance values possibly resulting from side-reactions of methionine in the assay mixture (Miyajima, Otsuka & Shio, 1968). Methionine also tended to relieve inhibition caused by L-threonine. Activity relative to controls was 9.9% with 5 mM-L-threonine alone, but 33.8% when 5 mM-L-methionine was also present.

Aspartokinase catalyses the phosphorylation of aspartate to aspartylphosphate, the first intermediate in the branched pathway for the synthesis of lysine, methionine, threonine and isoleucine (Mattoon, 1967; Truffa-Bachi & Cohen, 1968). Inhibition of aspartokinase by

intermediates and end-products of this pathway has been well established as a major regulatory process in many bacteria (Datta & Gest, 1964; Tristram, 1968; Cohen, Stanier & Le Bras, 1969). As Mattoon (1967) pointed out, such inhibition could result in interference with the synthesis of the other end-products of the pathway, thus leading to an imbalance in amino acid biosynthesis. In coliform bacteria, however, this does not occur as multiple aspartokinases are present, each subject to control by different end-products (Stadtman *et al.* 1961; Cohen *et al.* 1969). In some other bacteria, including pseudomonads, *Bacillus* species, *Rhodopseudomonas capsulatus* and *Brevibacterium flavum*, concerted inhibition by lysine+threonine occurs, with neither effector alone usually being strongly inhibitory (Paulus & Gray, 1964; Burlant, Datta & Gest, 1965; Kuramitsu, 1968; Miyajima *et al.* 1968; Cohen *et al.* 1969).

Our experiments indicate that all the aspartokinase activity in *Methylococcus capsulatus* can be inhibited by L-threonine alone, thus affording an explanation of threonine toxicity to growing cultures (Eccleston & Kelly, 1972*a*). Accumulation of exogenous L-threonine into *M. capsulatus* could have reduced aspartokinase activity so much that the organisms were unable to synthesize sufficient lysine or methionine to allow normal protein synthesis and growth. We have not established whether more than one species of aspartokinase occurs in *M. capsulatus*, but it is clear that concentrations of 10 mM or above of either L-lysine or L-threonine virtually abolish activity in cell-free extracts. Failure of lysine to act also as a growth inhibitor is probably because lysine is transported into the organisms at a much lower rate than threonine (Eccleston & Kelly, 1972*b*). It was, however, observed that growth rate was further decreased when lysine was added to cultures already partially inhibited by L-threonine (Eccleston & Kelly, 1972*a*).

The stimulatory effect of L-methionine on aspartokinase in crude extracts suggests a regulatory function for this amino acid, and it is perhaps significant that partial relief of threonine-inhibition was produced by methionine: such an interaction in the intact organism could have regulatory importance. Relief by L-methionine of growth-inhibition by L-threonine (Eccleston & Kelly, 1972*a*) could also have been a result not only of depression of threonine uptake (Eccleston & Kelly, 1972*b*) but of some restoration of aspartokinase activity in the organism.

The sensitivity of growing organisms to threonine and the observation that 1.0 mM-L-threonine inhibited both growth rate and aspartokinase activity by about 85 % suggest that the activity of aspartokinase in growing organisms is a rate-limiting factor in determining maximum growth rate in batch cultures of *Methylococcus capsulatus*. The similar specific activities and comparable effect of L-threonine on the activity of aspartokinase in the threonine-resistant mutants and wild-type organisms suggests that threonine tolerance in the mutants did not result from mutations affecting aspartokinase, but was due to the decreased permeability of the mutants to amino acids, especially L-threonine (Eccleston & Kelly, 1972*b*).

This is the first reported study of aspartokinase in an obligate methylotroph, and the results provide a likely explanation of threonine toxicity in terms of biosynthetic regulatory mechanisms, such as have been obtained with some obligate chemolithotrophs and *Chlorobium thiosulfatophilum* (Kelly, 1971; Kelly, Poole & Owen, 1973).

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