MULTIPLE CONTROL OF N-ACETYLGLUTAMATE SYNTHETASE FROM PSEUDOMONAS AERUGINOSA: SYNERGISTIC INHIBITION BY ACETYLGLUTAMATE AND POLYAMINES

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SUMMARY

N-Acetylglutamate synthetase (EC 2.3.1.1), the first enzyme of arginine synthesis was shown to be under multiple control by the reaction products and the endproducts of the pathway in tenfold purified extracts from Pseudomonas aeruginosa. Synergistic inhibition of the enzyme was exerted by N-acetyl-L-glutamate and polyamines. At 0.5 mM N-acetyl-L-glutamate spermine was the most potent inhibitor, whereas spermidine, cadaverine and putrescine inhibited the enzyme to a lesser extent. Furthermore, feedback-inhibition by L-arginine was enhanced synergistically by N-acetyl-L-glutamate and CoA.

The biosynthesis of the polyamines is linked closely to the arginine pathway (outlined in Fig. 2). In Escherichia coli putrescine (1,4-diaminobutane) can be formed by two routes: by decarboxylation of L-ornithine and by decarboxylation of L-arginine, followed by cleavage of urea from agmatine (1,2). We have detected the corresponding enzyme activities in extracts from Pseudomonas aeruginosa (unpublished results). Putrescine is coupled with the propylamine moiety of decarboxylated S-adenosylmethionine to yield spermidine (N-(3-aminopropyl)-1,4-diaminobutane) (3,4). Spermine (N, N'- bis (3-aminopropyl)-1,4-diaminobutane), which commonly occurs in eukaryotes, but only in a few prokaryotes (5), has been found in P. aeruginosa (6).

Using a sensitive in vitro assay we have previously shown that in P. aeruginosa the first enzyme of arginine synthesis, N-acetylglutamate synthetase (EC 2.3.1.1), is subject to feedback inhibition by L-arginine (7). In a few other microorganisms such as E.coli (8), P.putida (9), Proteus mirabilis (10), this enzyme has been demonstrated, but its regulatory properties have not been investigated in vitro.

The purpose of the present communication is to show, that the activity of N-acetylglutamate synthetase from P. aeruginosa is under multiple control. The polyamines, which may be regarded as endproducts of the arginine pathway, and N-acetyl-L-glutamate synergistically inhibit the enzyme in vitro. Furthermore, both products of the enzymatic reaction, N-acetyl-L-glutamate and CoA, enhance the inhibition exerted by L-arginine.

MATERIALS AND METHODS

P. aeruginosa PAO 1, a wild-type strain, was grown on minimal medium P supplemented with 20 mM L-glutamate as described previously (7). Crude extracts were prepared by sonication in 0.1 M potassium phosphate buffer pH 7.0 containing 2 mM 2-mercaptoethanol.

ASSAY OF N-ACETYLGLUTAMATE SYNTHETASE. Enzyme activity was determined according to the standard method in the presence of 7 mM aminooxyacetic acid, an inhibitor of pyridoxal phosphate-dependent enzymes (7). For the experiment described in Table 1 the following modified procedure was used: 20 µl of partially purified enzyme (38 µg protein) were preincubated at 37°C for 60 s in an incubation mixture consisting of 5 µl of 1 M Tris·HCl pH 8.9, 0.07 M aminooxyacetic acid, 5 µl of 20 mM acetyl-CoA (omitted for blank values), and 10 µl of water or inhibitors as indicated. The reaction was started by addition of 10 µl of prewarmed 25 mM L-[^{14}C]glutamate (1 µCi/1µmole). After 60 s of incubation at 37°C, the reaction was stopped by addition of 10 µl of 2 M HCl. The N-acetyl-L-[^{14}C]glutamate formed was determined in an aliquot as described for the standard assay (7).

PARTIAL PURIFICATION OF N-ACETYLGLUTAMATE SYNTHETASE. Solid ammonium sulfate (0.277 g/ml) was added to the crude extract. The precipitate was dissolved in 0.01 M phosphate buffer pH 7.2 containing 2 mM 2-mercaptoethanol, desalted on Sephadex G-25 and chromatographed on a hydroxyapatite column as previously described (7). The fractions exhibiting the highest enzyme activities were pooled and concentrated by vacuum dialysis. The overall purification was approximately tenfold.

RESULTS

The activity of partially purified N-acetylglutamate synthetase was measured as a function of incubation time (Fig. 1). Under standard assay conditions a nearly linear response was obtained. In the presence of 2 mM spermidine or 20 mM putrescine, however, there was a marked deviation from linearity. The inhibitory effect of polyamines was small during the first 2.5 min of incubation and increased gradually with time. This suggested that accumulating reaction product(s) might enhance the inhibition.

In order to minimize the accumulation of products the incubation time was shortened to 60 s and L-[^{14}C]glutamate of high specific radioactivity was used as a substrate. The enzyme concentration was chosen such that only about 1 % of the L-glu-

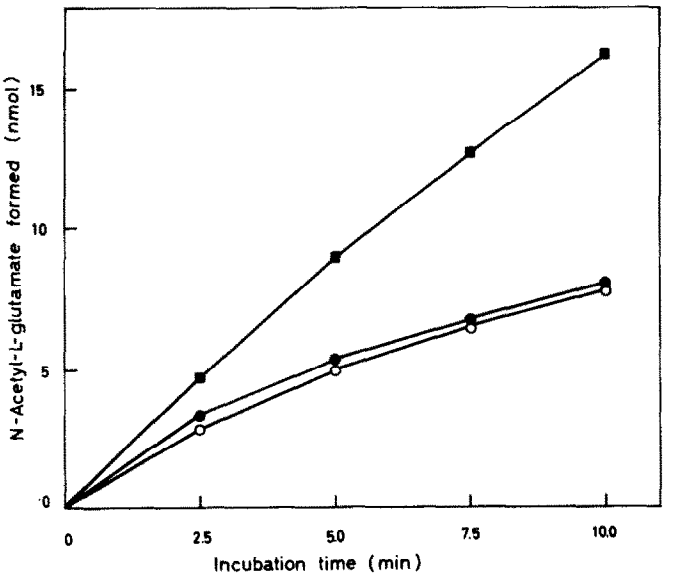

Figure 1. Kinetics of N-acetylglutamate formation in vitro in the presence and absence of polyamines. N-Acetylglutamate synthetase activity was measured by the standard procedure (7). Each tube contained 20 µg of partially purified enzyme. ■, Without polyamines; ●, with 2 mM spermidine; ○, with 20 mM putrescine.

tamate was converted to N-acetyl-L-glutamate, i.e. the final concentration of N-acetyl-L-glutamate in the assay mixture did not exceed 0.06 mM. Table 1 (a) shows that under these conditions both reaction products, CoA (at 1 mM) and N-acetyl-L-glutamate (at 0.5 mM), when added to the reaction mixture, inhibited the enzyme slightly. 10 mM Putrescine and 1 mM spermidine alone also exerted a very weak inhibition. However, the polyamines became strongly inhibitory when 0.5 mM N-acetyl-L-glutamate was added. Thus a product and an end-product exert a synergistic inhibition of the first enzyme of the pathway. On the other hand, the inhibition brought about by the simultaneous presence of 1 mM CoA and 1 mM spermidine or 10 mM putrescine corresponded to the sum of the inhibitions caused by each inhibitor alone. The effect of other polyamines was tested in the presence and absence of N-acetyl-L-glutamate (Table 1 (a)). As inhibitors, 1,3-diaminopropane and cadaverine (1,5-diaminopentane) were similar to, or slightly better than putrescine. The tetra-amine spermine was the most effective inhibitor. Methylamine, $MgCl_2$, NH_4Cl, and NaCl, at a concentration of 10 mM, had no effect. High concentrations of $MgCl_2$ (0.1 M), however, were inhibitory (7).

The inhibition pattern did not change during purification of the enzyme. The following control experiments were performed to confirm that the inhibition of the par-

tially purified enzyme by polyamines did not arise from some secondary effect. (i) A time course experiment similar to that shown in Fig. 1 was carried out with 10 mM (instead of 5 mM) L-glutamate. The inhibition by spermidine remained qualitatively unchanged. Acetyl-CoA in the reaction mixture did not disappear more rapidly when spermidine was present. Thus it is unlikely that substrate concentrations became limiting in the presence of polyamines. (ii) Degradation of N-acetyl-L-glutamate by the partially purified enzyme preparation, with or without added spermidine, could not be detected. (iii) The enzyme was preincubated at 37°C for 10 min with spermidine and either L-glutamate or acetyl-CoA. Time course experiments gave approximately the same picture as Fig. 1, indicating that the enzyme did not lose activity during incubation with polyamines.

It is noteworthy that even at high concentrations the polyamines alone did not depress the enzyme activity to zero, whereas excess L-arginine inhibits the enzyme completely (7). As demonstrated by Table 1 (b) the inhibition by L-arginine was also

TABLE I

Inhibition of N-acetylglutamate synthetase by polyamines and arginine

Inhibitor	Relative enzyme activity (%)†		
	Without addition	Addition of 0.5 mM N-acetyl-L-glutamate	Addition of 1.0 mM CoA
(a) None	100*	89	82
1 mM Spermidine	93	22	74
10 mM Putrescine	85	26	69
10 mM 1,3-Diaminopropane	74	12	ND§
10 mM Cadaverine	77	20	ND§
1 mM Spermine	70	12	ND§
(b) 0.1 mM L-Arginine	82	33	44
0.2 mM L-Arginine	27	3	5

† Enzyme activity was measured by the modified procedure (see Materials and Methods).

* 100 % activity corresponds to 2.7 nmole N-acetylglutamate formed per 60 s.

§ ND, not determined.

synergistically enhanced by N-acetyl-L-glutamate and, to a lesser extent, by CoA.

DISCUSSION

These and earlier in vitro experiments may have the following implications in vivo (see Fig. 2). Excess L-arginine inhibits N-acetylglutamate synthetase (7) as well

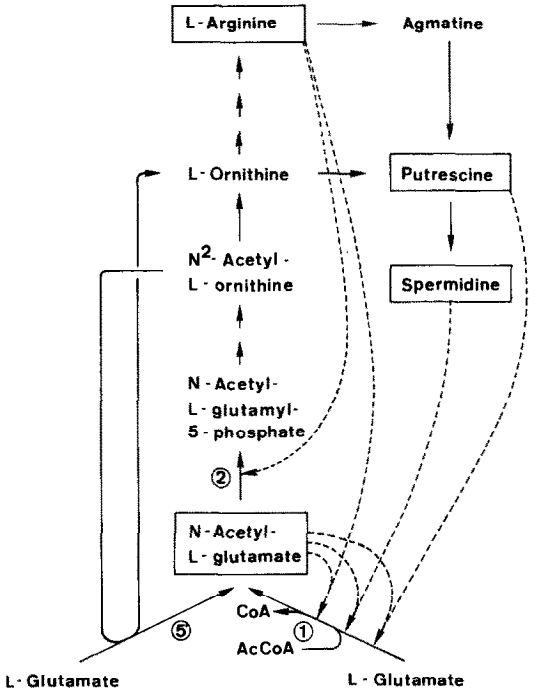

Figure 2. Arginine and polyamine synthesis in P. aeruginosa. Enzymes important to this study are: (1) N-acetylglutamate synthetase, (2) N-acetylglutamate 5-phosphotransferase, and (5') ornithine acetyltransferase. Dashed lines indicate feedback inhibition. N-Acetyl-glutamate together with arginine or a polyamine inhibits enzyme (1) in a synergistic way.

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as N-acetylglutamate 5-phosphotransferase (EC 2.7.2.8), the second enzyme of the pathway (11,12). At moderate L-arginine levels the inhibition of N-acetylglutamate synthetase depends on the concentration of N-acetyl-L-glutamate and, to a lesser extent, of CoA (Table 1). High levels of N-acetyl-L-glutamate, by synergistic action, inhibit the enzyme strongly. Under these putative conditions arginine synthesis can still take place by means of a cyclic reaction mediated by ornithine acetyltransferase (EC, 2.3.1.35, enzyme 5' in Fig. 2), which conserves the acetyl group in the pathway (12,13). When the pool size of N-acetyl-L-glutamate decreases, feedback inhibition of the first enzyme is relieved and the acetylation cycle can be replenished again.

In view of the high intracellular concentration of polyamines in Pseudomonas (3, 6), it may be assumed that a considerable portion of ornithine or arginine is used for putrescine synthesis. The polyamines do not inhibit N-acetylglutamate 5-phosphotransferase (unpublished results). By inhibiting N-acetylglutamate synthetase effectively in the presence of sufficient N-acetyl-L-glutamate, they can curtail, but do not shut off arginine synthesis. It will be of interest to see whether similar regulatory mechanisms occur in other microorganisms possessing an ornithine acetyltransferase.

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