ACETYLGLUTAMATE KINASE: A FEEDBACK-SENSITIVE ENZYME OF ARGININE BIOSYNTHESIS IN NEUROSPORA

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Summary: A radioactive assay was developed for the arginine-synthetic enzyme, acetylglutamate kinase (EC 2.7.2.8). Activity of the enzyme was demonstrated in crude extracts of Neurospora mycelium. Precipitation with ammonium sulfate, resulting in separation of the enzyme from an inhibitor, was initially required to detect activity. Most preparations are only partially sensitive to arginine, with maximal inhibition achieved at an effector concentration of 0.5 mM. The enzyme is activated about 10% by 1 mM lysine or citrulline, while 1 mM ornithine stimulates activity by 75%.

The compartmentation of the arginine pool (1,2) and the mitochondrial location of some arginine biosynthetic enzymes (3) of Neurospora makes the question of feedback inhibition in the pathway especially interesting. Feedback inhibition of ornithine synthesis by arginine was postulated on physiological grounds earlier (4). Ornithine synthesis begins with formation of acetylglutamate from glutamate by an acetyl-CoA-dependent enzyme. However, in Neurospora, acetylglutamate is also regenerated in a transfer of the acetyl group of N^{α}-acetylornithine to glutamate, which completes a cycle in the reaction liberating ornithine. It was expected, therefore, that a key feedback-sensitive enzyme would be acetylglutamate kinase, which transforms acetylglutamate to N-acetyl-γ-glutamyl phosphate. Earlier attempts to demonstrate the enzyme in Neurospora (5) were unsuccessful. A dependable assay for the kinase was sought to determine its sensitivity to arginine and to facilitate studies of repression and localization.

MATERIALS AND METHODS: Mycelium of *Neurospora crassa* was grown in Vogel's medium N in 700-ml shaken cultures (6) and extracted by grinding with sand and

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0.05 M Tris-HCl buffer, pH 7.5, containing 0.2 mM dithiothreitol and 0.1 mM EDTA (7). After centrifugation, the supernatant was passed through Sephadex G-25 to remove small molecules. Fractionation was performed by adding, at 0 C, a saturated solution of ammonium sulfate containing 0.2 mM dithiothreitol. Precipitates were dissolved in extraction buffer and passed through Sephadex G-25 before use. The strains of Neurospora used were 74A (wild-type) or arg-12^s (low ornithine transcarbamylase). The latter strain has 2-3 times the normal specific activity for arginine enzymes when it is grown on minimal medium.

The low activity of the enzyme in Neurospora led us to base the assay on use of radioactive acetyl-[^{14}C]glutamic acid. The product of the reaction, N-acetyl-γ-[^{14}C]glutamyl phosphate, is converted as it is formed to N-acetyl-γ-[^{14}C]glutamyl hydroxamate in the presence of hydroxylamine (8). The product is isolated by ion exchange and counted in a toluene-Triton-X-100 scintillation fluid. Conditions of assay and isolation of product are given in Table 1 and Figure 1.

Acetyl-γ-[^{14}C]glutamic acid was prepared by acetylation of uniformly labelled [^{14}C]glutamic acid (New England Nuclear Corp, 209 mCi/mmole) with acetic anhydride (9) and purified by paper chromatography. For use as a standard, N-acetyl-γ-[^{14}C]glutamyl hydroxamate was generated in an acetylglutamate kinase reaction mixture (8) containing partially purified E. coli enzyme (10). Unlabelled γ-glutamyl hydroxamate was purchased from Sigma Chemical Co.

RESULTS: Figure 1 illustrates the processing of reaction mixtures by which the product of the reaction is isolated, based on the method of Chabas et al. (11), but optimized by continuous elution with 0.2 N formic acid. The identity of peak C as N-acetyl-γ-glutamyl hydroxamate was confirmed by several criteria. Its failure to bind Dowex-50 H^+ indicates the lack of a free amino group. Color formation with $FeCl_3$ indicates the presence of the hydroxamate group. Elution from AG-1 formate resin between glutamate and acetylglutamate agrees

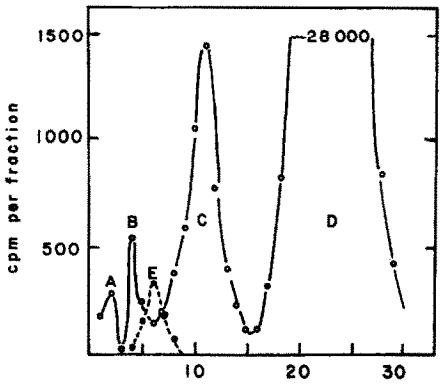

Figure 1. Isolation of acetyl-γ-glutamyl hydroxamate by anion exchange chromatography. Reaction mixture supernatant (0.2 ml) was first passed through a column of Dowex 50 X-8 (H^+-form, 200-400 mesh) containing 1.0 ml resin and eluted with 2.0 ml water to remove glutamate. The eluate was transferred to a column of AG-1 X-8 resin (formate-form, 200-400 mesh, 7 x 55 mm) and eluted with 0.2 M formic acid. Fractions (2.0 ml) were collected and counted. For routine work, the fractions corresponding to peak C were pooled and evaporated before counting. From the column were eluted, in sequence, a labile non-enzymatic product (A), a stable non-enzymatic product (B), acetyl-γ-glutamyl hydroxamate (C) and acetylglutamate (D). Peak E is the position at which glutamate would be eluted, if present.

Table 1. Essential components of reaction mixture

Omissions	Radioactivity (cpm) after incubation for: 60 min	120 min
None*	8188	12078
Enzyme	1460	1452
ATP	1312	1413
Hydroxylamine**	4781	6063
$MgCl_2$	1319	1360
1/2 enzyme	4653	9355

*The complete system (60 µl) contained Tris-HCl, 7.6 µmoles; acetyl-[^{14}C]glutamate, 0.12 µmole, 1.2 x 10^5 cpm; ATP, 0.9 µmole; $MgCl_2$, 1.8 µmole; hydroxylamine HCl (neutralized with NaOH), 12 µmoles; and 20 µl of enzyme preparation containing 0.36 mg protein from arg-12^s (40-55% ammonium sulfate fraction). The pH was 7.0. Reactions were run at 30 C and were stopped with 0.24 ml 95% ethanol. Tubes were covered, heated at 90 C for 1 min, centrifuged and the radioactivity corresponding to the area of peak C, Fig. 1, was determined.

**Hydroxylamine added at end of enzyme incubation.

with its intermediate acidity. In paper chromatography and high-voltage electrophoresis, the mobility of labelled material in peak C in comparison to acetylglutamate, glutamate, and glutamyl-γ-hydroxamate is consistent with its being N-acetyl-γ-glutamyl hydroxamate.

Attempts to demonstrate the kinase reaction in crude Neurospora extracts failed initially. These extracts also inhibited the E. coli kinase. Because the inhibitory agent was heat-labile and stable to dialysis, ammonium sulfate fractionation of Neurospora extracts was undertaken. Activity of the enzyme was optimal in a fraction precipitating between 40 and 55% saturation. A lesser yield of activity resulted if ammonium sulfate concentration of the fraction was extended beyond 60%. This indicated that the inhibitory agent was similar to that of yeast (12). After optimizing the reaction conditions, activity could be detected in unfractionated extracts.

Using the 40-55% ammonium sulfate fraction, we studied the requirements for the Neurospora kinase reaction (Table 1). The reaction demonstrated a sharp optimum for hydroxylamine (0.2 M). Product formation declined to 63% at 0.1 M and to 34% at 0.4 M. If hydroxylamine is withheld during the reaction, N-acetyl-γ-glutamyl phosphate evidently accumulates, as shown by hydroxamate formation when hydroxylamine is later added (Table 1). Reactions were linear with time up to about 90 minutes, and with the amount of enzyme. The

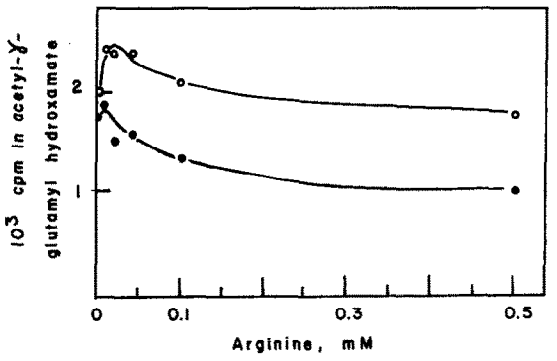

Figure 2. The effect of arginine on enzyme activity of a 40-55% ammonium sulfate fraction (upper curve), and of an unfractionated extract (lower curve).

Table 2. Effect of arginine and related compounds on activity.*

Addition to reaction mixture:	Net Radioactivity (cpm) in acetyl-γ-glutamyl hydroxamate	
	Expt. I	Expt. II
None	1182	4941
Arginine (1mM)	766	4939
Lysine (1mM)	1294	5706
Citrulline (1mM)	1374	5311
Ornithine (1mM)	1995	8835

*Unfractionated extract was used in both experiments; 272 μg protein from wild type 74A in the first assay, 350 μg from arg-12^{s} in the second. Incubation times were 90 and 94 min, respectively.

concentration of N-acetylglutamate used was not saturating, and the apparent K_m of the Neurospora enzyme, like that of enzymes from other sources (10), is probably over 5 mM.

A preliminary test of arginine and related compounds on the activity was made. Inhibition by arginine varied among extracts: some revealed a completely desensitized activity. About 40% inhibition was found most frequently. A number of arginine concentrations were tested for two preparations; each is maximally inhibited at 0.5 mM (Fig. 2). Curiously, an activation was observed with the lowest arginine concentrations tested, giving way to progressive inhibition as the arginine concentration is increased. To test whether the activating or inhibitory effects are specific for arginine, its effect was compared to those of lysine, citrulline and ornithine. The results (Table 2) show that the last three are all activating at 1 mM, ornithine being the most potent. Activation of the enzyme is thus a less specific phenomenon, and occurs irrespective of the arginine sensitivity

of a given preparation. Inhibition, on the other hand, is brought about only by arginine. While the significance of activation by ornithine is not clear, the range of arginine concentrations which exert submaximal inhibition might indicate the arginine concentrations at the site in the cell at which the kinase is located. Localization studies and tests of repression will be reported in another communication.

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