PRELIMINARY NOTES

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A pyrimidine-specific carbamate kinase in Neurospora

The biosynthesis of arginine and pyrimidines in Neurospora has been postulated by DAVIS[1] to involve two distinct modes of carbamyl phosphate synthesis. He has presented evidence for a carbamate kinase (ATP:carbamate phosphotransferase, EC 2.7.2.2) activity in Neurospora which normally serves only the arginine pathway, and this would imply the existence of a separate pyrimidine-specific enzyme. In addition, CHARLES AND BROADBENT[2] have observed that uridine inhibits the growth of two argininеless mutants, and that arginine inhibits the growth of pyr 3 strain 1298 when these organisms are grown on carbon dioxide. It is suggested that these mutants are responding to carbon dioxide by using excess carbamyl phosphate produced by the alternate pathway, and they are, therefore, subject to regulation by the endproduct of that pathway. Further support for separate modes of carbamyl phosphate synthesis has been provided by studies of β-alanine inhibition in Neurospora[3]. β-Alanine appears to interfere with carbamyl group metabolism, probably at the carbamate kinase step, since inhibition is reversed by various carbamyl compounds. However, a differential inhibitory effect of β-alanine upon certain pyr mutants has been observed. When grown under conditions leading to decreased endogenous levels of arginine, these strains were unaffected by 0.005 M β-alanine, a concentration otherwise sufficient to cause 50% inhibition of growth. This result may be explained by the derepression of an arginine-specific carbamate kinase, again implying the existence of a separate, pyrimidine-specific mode of carbamyl phosphate synthesis.

We have now obtained direct evidence for a pyrimidine-specific carbamate kinase in Neurospora. Carbamate kinase levels in acetone powder extracts were found to increase as much as 9-fold above normal levels when a pyrimidineless strain of Neurospora was grown with limiting amounts of uracil. A partially purified carbamate kinase activity was also observed to increase as much as 5-fold under the same conditions. The enzyme preparations were obtained from normal and pyrimidine-depleted cultures in the following manner. Pyrimidineless strain pyr 4 was allowed to grow with shaking in 2-l Fernbach flasks at 37° for 45 h, a time at which carbamate kinase activity had reached its peak in the unstarved cultures. Duplicate flasks contained 700 ml of Fries basal medium supplemented with either 170 mg or 350 mg uracil. The mycelia were filtered off, washed briefly with water, and treated as described previously for the preparation of acetone powders[4]. Unless noted otherwise, all fractionation was carried out at 4°. After storing overnight at −10° over P_2O_5, 2-g portions of acetone powder were extracted with 20 ml of 0.1 M Tris sulfate buffer, pH 6.6, containing 0.1 mM β-mercaptoethanol, 0.1 mM EDTA, and 20% glycerol (v/v) for 1 h. The suspension was centrifuged at 15 000 × g for 20 min and the supernatant was dialyzed against the same buffer mixture for 1 h. The preparation was next treated with protamine and charcoal. To 20 ml of the solution, 0.9 ml of 2.7% protamine solution was added dropwise with stirring. After standing for 20 min, 60 mg of Norite A was added and the mixture was stirred for 10 min. The

suspension was then centrifuged for 20 min at 25 000 × *g*. The light straw-colored supernatant was next treated with 5 g of finely ground ammonium sulfate to achieve 40% saturation, the pH was adjusted to 7.0 with 2 M NH_4OH and the suspension was stirred for 30 min. The preparation was then centrifuged at 25 000 × *g* for 20 min, and the supernatant was brought to 50% saturation by the slow addition of 1.25 g ammonium sulfate. After stirring for 30 min, the suspension was centrifuged at 25 000 × *g* for 30 min and the precipitate was taken up in 10 ml of 0.02 M Tris sulfate, pH 7.5, and frozen at −20°. The various fractions were dialyzed for 2 h against 0.01 M Tris sulfate, pH 7.5, and then immediately assayed for carbamate kinase activity by measurement of the incorporation of [^{14}C]bicarbonate into acid-non-volatile citrulline as described previously[4]. The purification procedure as outlined in Table I yielded an approximately 8-fold purified preparation, based upon

TABLE I

PURIFICATION OF CARBAMATE KINASE FROM *Neurospora crassa*

Assay of enzyme activity was carried out by measurement of [^{14}C]bicarbonate incorporation into citrulline in a coupled reaction with excess ornithine carbamyl transferase and ornithine[5]. The reaction mixture contained ornithine, 5 mM; $MgSO_4$, 2 mM; NH_4Cl, 3 mM; NaF, 2 mM; ATP, 2 mM; $NaH^{14}CO_3$, 2 mM; specific activity $5 \cdot 10^5$ counts/min per μmole; and potassium phosphate buffer (pH 8.5), 50 mM; in a total volume of 1.0 ml. Samples were incubated for 30 min at 25°, deproteinized with perchloric acid, and mixed with an equal volume of 2 M HCl–ethanol (1:1, v/v). Volumes of 0.25 ml were plated on stainless-steel planchets, evaporated to dryness and counted at infinite thinness.

Fraction	*Protein (mg/ml)*	*Specific activity (units/mg protein*)*	*Yield (%)*
Acetone powder extract	8.4	0.53	---
Protamine charcoal fraction	0.76	0.59	83.5
Ammonium sulfate fraction 40–50% saturation	5.2	4.0	32.7

* One unit is that amount of activity which causes the formation of 1 mμmole of acid-non-volatile citrulline per min.

the acetone powder extract. The purified enzyme displayed an absolute requirement for ATP and Mg^{2+}. Glutamine at various levels was able to partially replace substrate NH_4^+; with glutamine as nitrogen donor the rate of synthesis of citrulline in the coupled reaction was approximately one-fourth that with NH_4^+. K_m values of $2.94 \cdot 10^{-3}$ M for bicarbonate and $5.37 \cdot 10^{-4}$ M for NH_4^+ were obtained.

A comparison of the specific activities of carbamate kinase isolated from normal and starved cultures of the mutant is presented in Table II. It may be seen that growth on limiting uracil leads to a 3-fold derepression of carbamate kinase in crude extracts in Expt. 1 and to a 9-fold derepression in Expt. 2. The same is true for the partially purified enzyme. In Expt. 1, limiting uracil results in a 3-fold increase in activity, and in Expt. 2 a 5-fold increase is observed. The magnitude of these increases in carbamate kinase activity after pyrimidine starvation is similar to that reported by DAVIS for an argineneless mutant lacking ornithine carbamyl

TABLE II

EFFECT OF LIMITING URACIL UPON THE LEVELS OF CARBAMATE KINASE IN NEUROSPORA STRAIN pyr 4
Growth conditions are included in the text.

Growth supplement	*Carbamate kinase activity*			
	Expt. 1		*Expt. 2*	
	Acetone powder extract	*40–50% saturation $(NH_4)_2SO_4$ fraction*	*Acetone powder extract*	*40–50% saturation $(NH_4)_2SO_4$ fraction*
2.2 mM uracil	0.19	1.12	0.69	2.26
4.5 mM uracil	0.07	0.74	0.08	0.45

transferase which was starved for arginine[6]. However, he was unable to demonstrate any effect of limiting pyrimidine upon the levels of carbamate kinase in pyrimidine-less mutants. It would therefore appear that the enzyme we are measuring is different, at least in part, from that isolated by DAVIS. It is also noteworthy that the K_m for NH_4^+ which we have determined is smaller by two orders of magnitude than that reported by DAVIS, whereas the K_m values for bicarbonate are essentially the same. The optimal pH also appears significantly different. A value of 7.5 was obtained in the present work whereas DAVIS reported 8.1 to 8.3.

Since our assay procedures are comparable, we conclude that the differences we have observed are due to a second carbamate kinase, pyrimidine-specific, which is preferentially isolated by our purification procedure.

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1 R. H. DAVIS, *Biochim. Biophys. Acta*, 107 (1965) 54.
2 H. P. CHARLES AND J. A. BROADBENT, *Nature*, 201 (1964) 1004.
3 R. L. HERRMANN, AND C. W. WHITE, *Biochim. Biophys. Acta*, 124 (1966) 233.
4 R. L. HERRMANN, M. F. LOU AND C. W. WHITE, *Biochim. Biophys. Acta*, 121 (1966) 79.
5 B. LEVENBERG, *J. Biol. Chem.*, 237 (1962) 2590.
6 R. H. DAVIS, *Science*, 142 (1963) 1652.

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