NEW ASPECTS OF ACETYLGLUTAMATE ACTION. COLD INACTIVATION OF FROG CARBAMYL PHOSPHATE SYNTHETASE

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Evidence for activation and/or intermediate formation previous to carbamyl phosphate synthesis with rat liver preparations, acetylglutamate and ATP has been presented (Grisolia and Towne, 1957). Metzenberg et al. (1958) reported that they had evidence (with frog carbamyl phosphate synthetase) for activation by acetylglutamate in the absence of ATP. This discrepancy together with differences shown by frog and rat liver preparations in stability with and without acetylglutamate (Caravaca and Grisolia, 1959; 1960), suggested dual effects in the mechanism of action of acetylglutamate which have now been clarified.

Table I shows evidence for intermediate formation with the very unstable rat synthetase after preincubation with acetylglutamate, but only in the presence of ATP (increasing upon HCO_3^- addition). As shown in the Table preincubation of the frog enzyme with ATP and acetylglutamate also yields increased citrulline synthesis, but the relative increase with ATP and acetylglutamate appears less marked because of the extensive activation with acetylglutamate alone. Although not illustrated, additional supporting evidence for intermediate formation requiring ATP prior to carbamyl phosphate synthesis has been obtained from preincubation experiments in which ATP was removed with glucose-hexokinase and in other experiments with charcoal (suggested by Dr. A. Kornberg).

TABLE I

EFFECT OF PREINCUBATION OF FROG AND RAT LIVER CARBAMYL PHOSPHATE SYNTHETASE WITH ACETYLGLUTAMATE, ATP, Mg^{++} AND HCO_3^-.

Reagents present during preincubation	Frog Enzyme	Rat Enzyme
	μmoles citrulline synthesized	
None	0.11	0.08
Acetylglutamate	0.28	0.08
ATP; Mg^{++}	0.13	0.08
Acetylglutamate; ATP; Mg^{++}	0.34	0.16
Acetylglutamate; ATP; HCO_3^-; Mg^{++}	0.41	0.19

Frog liver acetone fraction, Marshall et al. (1958) was lyophilized. Some protein became insoluble, raising the specific activity to that of the best and essentially homogenous preparations of Marshall et al.; they contained, however, enough ornithine transcarbamylase so that supplementation was not required.

1.6 mg of protein from frog liver preparation or 6 mg protein from lyophilized rat liver mitochondria, 100 μmoles of Tris-Cl^-, pH 7.4, and, when indicated, 10 μmoles of acetylglutamate, 4 μmoles of ATP, 10 μmoles of $MgSO_4$ and 100 μmoles of $KHCO_3$ in 1 ml, were preincubated at 38° for 5 minutes. All tubes were then cooled to 25°, and made up (at definite intervals) to 2 ml and to contain the following, expressed in μmoles: acetylglutamate, 10; ATP, 8; $KHCO_3$, 100; NH_4Cl, 50; $MgSO_4$, 25; ornithine, 10. Incubation at 25° for exactly 30 seconds.

After activation (nearly maximal by 2 min. at 30° in 2 mM acetylglutamate) the frog synthetase becomes very unstable when kept at 4° or lower. However, the enzyme is stable at 20° to 30°, as exemplified in Table II. It is of interest that the more unstable rat liver enzyme is neither activated (unless it occurs so rapidly that it can not be measured under our conditions) nor cold inactivated by acetylglutamate.

The sedimentation coefficient of our preparation extrapolated

EFFECT OF TEMPERATURE ON STABILITY OF FROG LIVER CARBAMYL PHOSPHATE SYNTHETASE WITH AND WITHOUT ACETYLGLUTAMATE.

Storage Time in hours	Stored at	Acetylglutamate added: None	1×10^{-3} M	2×10^{-3} M
		Citrulline synthesis in μmoles		
0	0°	0.27	0.51	0.58
2.5	0°	0.27	0.38	0.40
5	0°	0.26	0.27	0.28
22	0°	0.22	0.09	0.09
0	20°	0.27	0.51	0.58
2.5	20°	0.27	0.53	0.58
5	20°	0.29	0.53	0.59
22	20°	0.26	0.48	0.52

Samples containing 2 mg of the synthetase per ml in 0.05 M Tris-Cl^-, pH 7.4 and the indicated additions, were preincubated for 5 minutes at 38°, cooled and stored as shown. 1.0 ml portions were withdrawn and assayed under the conditions of Table I, except that the incubation was for 1 minute. The contaminant transcarbamylase activity was also assayed; the numbers are not recorded since there was no change in activity.

to zero protein concentration is 12×10^{-13}, in good agreement with the reported value of Marshall et al. (1958). Ultracentrifuge analysis of the enzyme preparations failed to reveal any difference between the control and the activated samples (from 2 to 10 $\times 10^{-3}$M acetylglutamate) even after 30 to 40 hours of storage at 20°. When the samples were stored at 4° however, a change in the sedimentation pattern became apparent in the acetylglutamate treated preparations, but only after protracted storage. For example, no change was noticeable at 24 hours, but after 40 hours, a peak comprising about 40% of the total area separated as a slower

moving component. The nature of the change in the sedimentation characteristics is under investigation (in collaboration with Dr. H.J. Grady). No change in viscosity was noted before or after activation by acetylglutamate nor after cold inactivation.

Two other examples of enzymatic cold inactivation have been described recently: a readily reversible type (Fincham, 1960) and a partially reversible type (Pullman et al, 1960). However in the present case, cold inactivation is induced by the co-enzyme and in contrast to the above examples no evidence for reversibility has been obtained thus far by rewarming the enzyme with and without ATP and by precipitation with ammonium sulfate.

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