Activation of Carbamoyl Phosphate Synthase by *N*-Acetyl-L-Aspartate

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Carbamoyl phosphate synthase from liver of both rat and frog, normally dependent on *N*-acetyl-L-glutamate (on the basis of K_m and physiological concentrations) as an activator, was shown to be activated by high concentrations of *N*-acetyl-L-aspartate. However, the high concentrations of *N*-acetyl-L-aspartate required for activation produce non-competitive inhibition. Similarly, high concentrations of *N*-acetyl-L-glutamate, in very large excess of the amount required to activate the enzyme, inhibit. The limit for *N*-acetyl-L-glutamate as an impurity in *N*-acetyl-L-aspartate was found to be less than 1 in 5000 parts, far below the 1 in 250 parts needed to produce the activation observed with *N*-acetyl-L-aspartate.

The carbamoyl phosphate synthase from both rat and frog liver mitochondria (Grisolia & Cohen, 1953; Metzenberg *et al.*, 1958) is dependent on Ac-Glu. Carbamoyl phosphate synthase I [ATP-carbamate phosphotransferase (dephosphorylating), EC 2.7.2.5] has a high specificity for Ac-Glu, which acts as an allosteric effector (Metzenberg *et al.*, 1958; Grisolia & Raijman, 1964). Early studies indicated that for a compound to activate the synthase it required a glutamic acid portion and a carbonyl group on the amino moiety (Grisolia & Cohen, 1953). A later study, however, indicated that 2-acetoxyglutarate can also activate the enzyme (Schooler *et al.*, 1963), thus slightly altering the assumptions concerning specificity.

Another Ac-Glu analogue, Ac-Asp, which exists at very high concentrations in the brain (Tallan *et al.*, 1954), was thought to have only a buffering effect, if any, in brain metabolism (McIntosh & Cooper, 1965). However, it has been found to activate the phosphate-insensitive glutaminase of both brain and liver (Katunuma *et al.*, 1967) and to replace the Ac-Glu required by carbamoyl phosphate synthase from a land snail (Tramell & Campbell, 1970). McIntosh & Cooper (1965) did not detect citrulline synthesis in extracts of rat brain. Although they found a small activation of the liver enzyme by Ac-Asp, they stated that Ac-Asp does not serve as a cofactor for citrulline synthesis.

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§ Abbreviations: Ac-Glu, *N*-acetyl-L-glutamate; Ac-Asp, *N*-acetyl-L-aspartate; $\text{NH}_2\text{CO-Asp}$, *N*-carbamoyl-aspartate.

Materials and Methods

Experimental details and enzyme preparations are described in the legends to figures. Ac-Asp, $\text{NH}_2\text{CO-Asp}$, Ac-Glu and other reagents were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. Ac-Asp was also prepared by the method of Tallan (1963).

Ion-exchange-chromatographic determination of Ac-Glu as a contaminant of Ac-Asp was carried out as follows. Weighed amounts of Ac-Asp were dissolved in 0.1 M-HCl. Portions were then transferred to borosilicate ampoules and 5 ml of 6 M-HCl was added. The ampoules were sealed, maintained at 110°C for 22 h and the contents then evaporated to dryness. The dry materials were then dissolved in 0.1 M-HCl and measured amounts analysed on an amino acid analyser (Hamilton, 1963). All glassware was baked at 625°C for 4 h before use. Deionized water (conductivity 18×10^6 mho/cm and <1 part per 10^9 total amino acid contamination) was used. The 6 M-HCl was distilled once in an all-glass apparatus; 0.1 M-HCl was prepared from 6 M-HCl and deionized water.

Results and Discussion

As illustrated in Fig. 1, Ac-Asp at high concentrations markedly activates carbamoyl phosphate synthase I from rat and frog liver. Interestingly, in both cases the activation does not reach the maximum activity that can be obtained with Ac-Glu. As the concentrations of Ac-Asp needed to activate the enzyme are high it was crucial to exclude traces of Ac-Glu in the Ac-Asp, which could account for the activation. Therefore the Ac-Asp used was analysed

Fig. 1. Effects of *N*-acetyl-L-aspartate on rat liver (a) and frog liver (b) carbamoyl phosphate synthase

Rat liver carbamoyl phosphate synthase I was prepared by the method of Guthöhrlein & Knappe (1968) as far as the first $(\text{NH}_4)_2\text{SO}_4$ -fractionation step, with the modification of substituting 5 mM-dithiothreitol for mercaptoethanol and KCN. Frog liver carbamoyl phosphate synthase I was prepared by the method of Marshall *et al.* (1958). Assays were performed spectrophotometrically (Chabas *et al.*, 1972). The pH value of all components was adjusted to 7.4 with KOH before use in the assay. Velocity (*v*) is in arbitrary units. The enzyme added generally gave a maximum velocity with Ac-Glu of approx. 0.1–0.25 E_{340} unit/min.

for possible impurities (Hamilton, 1963, see the Materials and Methods section). The amount of Ac-Glu in Ac-Asp as an impurity was less than 0.01 % for the preparation made by us and less than 0.02 % in the preparation from Sigma Chemical Co. These values are uncorrected for any glutamic acid that might have been introduced in the course of the analytical procedures. Such corrections are usually of the order of 0.2–0.3 nmol/sample, which in this case is less than 0.003 %. If applied, the concentrations of contaminants would be even lower than those noted. Analytical runs on unhydrolysed Ac-Asp showed complete absence of free glutamic acid.

Although both Ac-Glu and Ac-Asp activate the enzyme, it appears that Ac-Asp also inhibits the enzyme in a non-competitive manner with respect to Ac-Glu activation. The possibility that Ac-Asp acts as both an activator at the Ac-Glu binding site and as an inhibitor at another site(s), because of the effect of high salt concentration or some other mechanism causing a decrease in the maximum velocity, would explain the results of Fig. 1.

Another analogue, $\text{NH}_2\text{CO-Asp}$, was also investigated as a possible activator; *N*-carbamoylglutamate activates the enzyme at about 10 times the concentration required for the same degree of activation by Ac-Glu (Grisolia & Cohen, 1953). Any possible effect of $\text{NH}_2\text{CO-Asp}$ could also have other implications, since another carbamoyl phosphate synthase, which does not require Ac-Glu activation and uses glutamine in preference to NH_3 , has been shown to catalyse the first step in pyrimidine biosynthesis (Jones, 1971). The land snail (Tramell & Campbell, 1970)

has a hybrid glutamine- and Ac-Glu-dependent carbamoyl phosphate synthase which is involved in both the urea cycle and pyrimidine biosynthesis and is subject to regulation by both Ac-Glu and pyrimidines.

$\text{NH}_2\text{CO-Asp}$, at the same concentrations as Ac-Asp used, does not activate but inhibits carbamoyl phosphate synthase I (Fig. 2). Although high salt inhibits the enzyme, its effect has been shown to be competitive with Ac-Glu (Kennedy & Grisolia, 1965), so the mixed nature of the inhibition indicates that such salt effects cannot entirely account for the inhibition. The high concentration of the dipotassium salt of Ac-Asp used would act to produce a competitive-inhibition component and is, of course, a factor in the overall inhibition. However, the other components produce an effect which indicates that $\text{NH}_2\text{CO-Asp}$ binds to a site for which Ac-Glu does not compete. Thus Ac-Asp acting as both an activator and a non-competitive inhibitor would give the kinetics of Fig. 1. Whether the activation by Ac-Asp would give the same maximum velocity found for Ac-Glu if it did not also inhibit is not possible to determine, because the concentrations required for activation and inhibition are so close. Though $\text{NH}_2\text{CO-Asp}$ does not function as an activator at the concentrations used here, perhaps it would at much higher concentrations (solubility precludes testing this). As mentioned above, *N*-carbamoylglutamate is about one-tenth as effective as Ac-Glu (Grisolia & Cohen, 1953).

The proposed explanation for the action of Ac-Asp on carbamoyl phosphate synthase I is supported by the analogous behaviour of very high concentrations

Fig. 2. Effect of N-carbamoylaspartate on carbamoyl phosphate synthase I from rat liver (a) and frog liver (b)

Assays were performed as indicated in the legend of Fig. 1. Ac-Glu concentration was varied in the absence (●) or presence of 50mm- (■) or 0.1M- (○) or 0.15M- (□) DL-NH₂CO-Asp. Velocity (*v*) is in arbitrary units.

Fig. 3. Effect of very high concentrations of Ac-Glu on carbamoyl phosphate synthase I

Assays were done as indicated in the legend of Fig. 1 with various concentrations of Ac-Glu.

Table 1. Amount of Ac-Glu in Ac-Asp required to explain the kinetic data

Values in parentheses are the percentages of Ac-Glu in Ac-Asp. For details see the text.

Source of enzyme	Concn. of Ac-Asp used (M)	Calculated concn. of Ac-Glu (mm)	
		Neglecting inhibition	Including inhibition
Frog	0.1	0.12 (0.12)	0.4 (0.40)
	0.15	0.12 (0.08)	0.71 (0.47)
Rat	0.1	0.25 (0.25)	0.42 (0.42)
	0.05	0.19 (0.38)	0.22 (0.44)

liver carbamoyl phosphate synthase I shown in Fig. 1 if the inhibition is greater for the frog enzyme (as is the case with NH₂CO-Asp) whereas the activation is the same for both.

If Ac-Asp is an inhibitor containing a small impurity of Ac-Glu, we would obtain data similar to those in Fig. 1. From the data of Figs. 1 and 2 and by assuming that the inhibition caused by Ac-Asp is the same as that caused by NH₂CO-Asp the amount of Ac-Glu that would produce the same activity as a given amount of Ac-Asp was calculated (Table 1); at saturating concentrations of Ac-Glu the velocity obtained in the presence of a fixed concentration of either NH₂CO-Asp or Ac-Asp is approximately the same. We assumed equal inhibition for the D and L

forms, as α -oxoglutarate, Ac-Asp and *N*-carbamoyl-DL-aspartate inhibit approximately equally at the same concentration when the activator site is saturated with Ac-Glu. This appears to be a rather non-specific inhibition by high concentrations of these dibasic acids. For example, the same activation is produced by 50mM-Ac-Asp as by 0.19mM-Ac-Glu or by 0.22mM-Ac-Glu in the presence of 50mM-NH₂CO-Asp; this would correspond to 0.38 and 0.44% Ac-Glu contamination in Ac-Asp respectively. Since, as indicated above, the contamination is less than 0.02% Ac-Glu in the Ac-Asp used, it could not account for the activation. Therefore Ac-Asp must act as both an activator and a non-competitive inhibitor.

Although the activation of liver carbamoyl phosphate synthase by Ac-Asp appears to have no physiological significance, the fact that Ac-Asp activates this enzyme, glutaminase (Katunuma *et al.*, 1967) and the land snail carbamoyl phosphate synthase (Tramell & Campbell, 1970), suggests that it may have a role(s) akin to that of Ac-Glu in activating or inhibiting other metabolically important enzymes. In this regard, it is noteworthy that a number of carbamoyl phosphate synthases have been discovered in animal tissues in recent years. The mitochondrial enzyme and snail enzymes that use Ac-Glu can also use Ac-Asp, whereas the glutamine-dependent synthase can use neither (Jones, 1971; H. J. Forman & S. Grisolia, unpublished work). It would also be interesting to know if the recently discovered (Vekich *et al.*, 1973) carbamoyl phosphate synthase from mammary tissue, which does not require Ac-Glu, can be affected by Ac-Asp.

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