Product Inhibition Studies on Bovine Liver Carbamoyl Phosphate Synthetase

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A study of the product-inhibition patterns of carbamoyl phosphate synthetase from bovine liver is reported. Inhibition by adenosine, AMP and inorganic ions is also reported. The results are in agreement with the previously proposed model in which the order of substrate binding is ATPMg, followed by HCO_3^- , ATPMg and NH_4^+ . The order of product release on the basis of the reported results is carbamoyl phosphate, followed by ADPMg, ADPMg and inorganic phosphate.

Carbamoyl phosphate synthetase (EC 2.7.2.5) from mammalian liver mitochondria has been shown to catalyse the reaction (Metzenberg *et al.*, 1957):

N-Acetyl-L-glutamate (Hall *et al.*, 1958), Mg^{2+} and K^+ (Marshall *et al.*, 1961) have been shown to be required for enzymic activity.

An accompanying paper (Elliott & Tipton, 1974b) has described the first full study of initial-rate data for carbamoyl phosphate synthetase from bovine liver, although there have been some incomplete studies on the enzyme from other sources (Fahien & Cohen, 1964; Kerson & Appel, 1968; Guthöhrlein & Knappe, 1969). The model proposed for binding of the substrates was:

In the present paper results are presented which confirm this order of substrate binding to the enzyme, and which determine the order of product release.

Experimental

Materials

Glutamate dehydrogenase, ADP, carbamoyl phosphate and α -oxoglutarate were obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. All other materials were as described in Elliott & Tipton (1974b).

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Preparation of enzymes

The preparation of carbamoyl phosphate synthetase used was the same as that described previously (Elliott & Tipton, 1973).

Ornithine carbamoyl transferase (EC 2.1.3.3) was prepared from rat liver by the method of Schimke (1962).

Assay of carbamoyl phosphate synthetase activity

Two assay methods were required for the study of product inhibition, as both assays for carbamoyl phosphate synthetase activity involved indirect measurement of one of the products (ADP or carbamoyl phosphate). For studies involving inhibition by carbamoyl phosphate or inorganic phosphate the spectrophotometric double coupled assay (Fahien

& Cohen, 1964), described in the accompanying paper (Elliott & Tipton, 1974b), was used.

Carbamoyl phosphate is unstable in aqueous solution, having a half-life of approx. 30 min at 0°C. Thus a stock solution of carbamoyl phosphate could not be kept for any useful period of time. This problem was overcome by adding accurately weighed amounts of solid lithium carbamoyl phosphate to the cuvette just before assaying the enzyme activity. Fortunately the apparent K_i value obtained was high enough to make weighing of the solid inhibitor practicable. A second complication with the use of carbamoyl phosphate was that it was found to be contaminated with 3% (mol/mol) NH_4^+ [assayed by using glutamate dehydrogenase by the method of Tabor (1970)], and thus allowances had to be made

Fig. 1. Inhibition of the activity of carbamoyl phosphate synthetase by carbamoyl phosphate as a function of ATPMg concentration

Details of the assay method and conditions are given in the text. (a) A Dixon (1953) plot of data obtained at the following ATPMg concentrations: 0.25 mM (○), 0.33 mM (■), 0.5 mM (Δ), 1.0 mM (●) and 5.0 mM (□). (b) A double-reciprocal plot of data obtained at the following carbamoyl phosphate concentrations: 26.7 mM (○), 20.0 mM (■), 13.4 mM (Δ), 6.7 mM (●) and 0 (□).

when varying the concentration of NH_4^+ at various carbamoyl phosphate concentrations.

Experiments involving inorganic phosphate in this system presented problems as phosphate complexes Mg^{2+} , especially in the presence of NH_4^+ , to form MgHPO_4 and $\text{Mg}(\text{NH}_4)\text{PO}_4$, both of which are highly insoluble in water. No precipitation occurred if the concentration of phosphate was less than 50 mM, NH_4^+ less than 5 mequiv./l and free Mg^{2+} less than 1 mequiv./l. Also the ionic strength of potassium phosphate in the assay at pH 7.6 is 2.74 times its molarity, and so KCl was added to keep the ionic strength constant.

For studies involving inhibition by ADP the spectrophotometric assay could not be used and so for these experiments formation of carbamoyl phosphate was coupled to formation of citrulline with

Fig. 2. Inhibition of the activity of carbamoyl phosphate synthetase by carbamoyl phosphate as a function of HCO_3^- concentration

Details of the assay method and conditions are given in the text. (a) A Dixon (1953) plot of data obtained at the following HCO_3^- concentrations: 2.0 mM (○), 3.3 mM (■), 5.0 mM (Δ), 10.0 mM (●) and 100.0 mM (□). (b) A double-reciprocal plot of data obtained at the following carbamoyl phosphate concentrations: 26.7 mM (○), 20.0 mM (■), 13.4 mM (Δ), 6.7 mM (●) and 0 (□).

ornithine carbamoyl transferase (Marshall *et al.*, 1958). Incubations were performed at 30°C for 10 min (a period over which the assays were linear) in a volume of 1 ml containing, in addition to carbamoyl phosphate synthetase and substrates, 1 unit of ornithine carbamoyl transferase (1 unit is the amount of enzyme required to form 1 μmol of citrulline/min at 30°C), 15 mM-L-ornithine and 50 mM-glycylglycine, adjusted to pH 7.6 with KOH. Duplicate incubations were performed with and without *N*-acetyl-L-glutamate, and the difference in citrulline formed was taken as the enzyme activity. (The spectrophotometric assay showed that there was no carbamoyl phosphate synthetase activity in the absence of *N*-

Fig. 3. Inhibition of the activity of carbamoyl phosphate synthetase by carbamoyl phosphate as a function of NH_4^+ concentration

Details of the assay method and conditions are given in the text. (a) A Dixon (1953) plot of data obtained at the following NH_4^+ concentrations: 0.67 mM ($\circ$), 1.0 mM ($\blacksquare$), 1.33 mM ($\triangle$), 2.0 mM ($\bullet$) and 5.0 mM ($\square$). (b) A double-reciprocal plot of data obtained at the following carbamoyl phosphate concentrations: 26.7 mM ($\circ$), 20.0 mM ($\blacksquare$), 13.4 mM ($\triangle$), 6.7 mM ($\bullet$) and 0 ($\square$).

Fig. 4. Inhibition of the activity of carbamoyl phosphate synthetase by inorganic phosphate as a function of ATPMg concentration

Details of the assay method and conditions are given in the text. (a) A Dixon (1953) plot of data obtained at the following ATPMg concentrations: 0.25 mM ($\circ$), 0.33 mM ($\blacksquare$), 0.5 mM ($\triangle$), 1.0 mM ($\bullet$) and 5.0 mM ($\square$). (b) A double-reciprocal plot of data obtained at the following inorganic phosphate concentrations: 50.0 mM ($\circ$), 37.5 mM ($\blacksquare$), 25.0 mM ($\triangle$), 12.5 mM ($\bullet$) and 0 ($\square$).

acetyl-L-glutamate.) The reaction was stopped by addition of 0.5 ml of 30% (v/v) HClO_4 and the citrulline formed was assayed by the method of Archibald (1944) as modified by Guthöhrlein & Knappe (1968).

Results and Discussion

Kinetic data were obtained for each substrate at various concentrations of each product. The non-variable substrates were kept at concentrations of about the previously determined K_m values (Kerson & Appel, 1968), i.e. 0.5 mM-ATPMg, 10 mM- KHCO_3 ,

1 mM- NH_4Cl , with high concentrations of the activators, i.e. 10 mM-N-acetyl-L-glutamate and 1 mequiv. of free Mg^{2+} /litre. Figs. 1–9 show the reciprocal plots and Dixon (1953) plots obtained from these data.

When the concentration of ATPMg was increased to 5 mM very little inhibition by phosphate with respect to NH_4^+ or HCO_3^- was seen. At 100 mM- KHCO_3 the inhibition by phosphate appeared uncompetitive with respect to NH_4^+ . Table 1 summarizes the inhibition patterns and apparent K_i values obtained.

The observation that phosphate inhibits competitively with respect to ATPMg is consistent with ATPMg being the first substrate to bind to the enzyme, and phosphate being the last product to be

Fig. 5. Inhibition of the activity of carbamoyl phosphate synthetase by inorganic phosphate as a function of HCO_3^- concentration

Details of the assay method and conditions are given in the text. (a) A Dixon (1953) plot of data obtained at the following HCO_3^- concentrations: 1.2 mM ($\circ$), 1.6 mM ($\blacksquare$), 2.2 mM ($\triangle$), 5.2 mM ($\bullet$) and 100.2 mM ($\square$). (b) A double-reciprocal plot of data obtained at the following inorganic phosphate concentrations: 50.0 mM ($\circ$), 37.5 mM ($\blacksquare$), 25.0 mM ($\triangle$), 12.5 mM ($\bullet$) and 0 ($\square$).

released. Mixed inhibition by carbamoyl phosphate with respect to all substrates is indicative of carbamoyl phosphate being the first product to be released from the enzyme. Inhibition by ADPMg therefore appears to be anomalous at first sight as it would be expected to be uncompetitive with respect to all substrates if both molecules are released between carbamoyl phosphate and phosphate. The non-linearity of the Dixon plots would be expected as two molecules of ADPMg are released and so the

Fig. 6. Inhibition of the activity of carbamoyl phosphate synthetase by inorganic phosphate as a function of NH_4^+ concentration

Details of the assay method and conditions are given in the text. (a) A Dixon (1953) plot of data obtained at the following NH_4^+ concentrations: 0.25 mM ($\circ$), 0.33 mM ($\blacksquare$), 0.5 mM ($\triangle$), 1.0 mM ($\bullet$) and 5.0 mM ($\square$). (b) A double-reciprocal plot of data obtained at the following inorganic phosphate concentrations: 50.0 mM ($\circ$), 37.5 mM ($\blacksquare$), 25.0 mM ($\triangle$), 12.5 mM ($\bullet$) and 0 ($\square$).

kinetic equation may contain an $[\text{ADPMg}]^2$ term (unless there is an essentially irreversible step between the release of the two molecules) (see Elliott & Tipton, 1974a).

Adenosine and AMP were both found to be competitive inhibitors of the enzyme with respect to ATPMg (see Fig. 10) and mixed inhibitors with respect to NH_4^+ and HCO_3^- . This suggests that ADPMg may also compete with ATPMg for the same binding site on the enzyme and so ADPMg inhibition will show both competitive and uncompetitive effects with respect to ATPMg, and mixed and uncompetitive effects with respect to NH_4^+ and HCO_3^- . The overall effect will thus be one of mixed inhibition with respect to all substrates.

The observation that phosphate inhibition with respect to NH_4^+ becomes uncompetitive at saturating

Fig. 7. Inhibition of the activity of carbamoyl phosphate synthetase by ADPMg as a function of ATPMg concentration

Details of the assay method and conditions are given in the text. (a) A Dixon (1953) plot of data obtained at the following ATPMg concentrations: 0.5 mM (○), 0.7 mM (■), 1.0 mM (Δ), 2.0 mM (●) and 5.0 mM (□). (b) A double-reciprocal plot of data obtained at the following ADPMg concentrations: 4.0 mM (○), 3.0 mM (■), 2.0 mM (Δ), 1.0 mM (●) and 0 (□).

Fig. 8. Inhibition of the activity of carbamoyl phosphate synthetase by ADPMg as a function of HCO_3^- concentration

Details of the assay method and conditions are given in the text. (a) A Dixon (1953) plot of data obtained at the following HCO_3^- concentrations: 2.5 mM (○), 3.3 mM (■), 5.0 mM (Δ), 10.0 mM (●) and 100.0 mM (□). (b) A double-reciprocal plot of data obtained at the following ADPMg concentrations: 4.0 mM (○), 3.0 mM (■), 2.0 mM (Δ), 1.0 mM (●) and 0 (□).

HCO_3^- concentration indicates that HCO_3^- binds to the enzyme before NH_4^+ (see Elliott & Tipton, 1974a). This is the same order of binding that was proposed on the basis of initial-rate data (Elliott & Tipton, 1974b).

If the proposed mechanism for binding of Mg^{2+} and *N*-acetyl-L-glutamate (Elliott & Tipton, 1974b) is correct then inorganic phosphate should be an uncompetitive inhibitor of the reaction with respect to both activators. Unfortunately it would not be possible to interpret the results of such experiments since, as stated above, inorganic phosphate complexes Mg^{2+} in the assay system and so it is not possible to determine accurately both inorganic phosphate and free Mg^{2+} concentrations. Kennedy & Grisolia (1965) found that frog liver carbamoyl phosphate synthetase was inhibited by high ionic strength, and that the

alkali metal chlorides appeared to inhibit competitively with respect to *N*-acetyl-L-glutamate with the inhibition increasing as the cationic size decreased (but the hydrated ionic radii increased), i.e. $\text{Li}^+ > \text{Na}^+ > \text{K}^+$. We have observed similar effects on the bovine liver enzyme, and further we have observed that the nature of the anion is important. K^+ is required for carbamoyl phosphate synthetase activity (Marshall *et al.*, 1961) and we have found an apparent K_m of 16 mequiv./l (at 2 mM-ATPMg, 25 mequiv. of HCO_3^- /litre, 0.5 mequiv. of NH_4^+ /litre, 1 mequiv. of Mg^{2+} /litre and 10 mM-*N*-acetyl-L-glutamate) for the bovine liver enzyme. At high concentrations of KCl, KBr and KI the reaction is inhibited (Fig. 11) and, although the apparent K_m for activation is the same

Fig. 9. Inhibition of the activity of carbamoyl phosphate synthetase by ADPMg as a function of NH_4^+ concentration

Details of the assay method and conditions are given in the text. (a) A Dixon (1953) plot of data obtained at the following NH_4^+ concentrations: 0.25 mM ($\circ$), 0.33 mM ($\blacksquare$), 0.5 mM ($\triangle$), 1.0 mM ($\bullet$) and 5.0 mM ($\square$). (b) A double-reciprocal plot of data obtained at the following ADPMg concentrations: 4.0 mM ($\circ$), 3.0 mM ($\blacksquare$), 2.0 mM ($\triangle$), 1.0 mM ($\bullet$) and 0 ($\square$).

Fig. 10. Inhibition of the activity of carbamoyl phosphate synthetase by AMP as a function of ATPMg concentration

Details of the assay method and conditions are given in the text. (a) A Dixon (1953) plot of data obtained at the following ATPMg concentrations: 0.25 mM ($\circ$), 0.33 mM ($\blacksquare$), 0.5 mM ($\triangle$), 1.0 mM ($\bullet$) and 5.0 mM ($\square$). (b) A double-reciprocal plot of data obtained at the following AMP concentrations: 2.0 mM ($\circ$), 1.5 mM ($\blacksquare$), 1.0 mM ($\triangle$), 0.5 mM ($\bullet$) and 0 ($\square$).

Table 1. Product-inhibition patterns for carbamoyl phosphate synthetase

The product-inhibition patterns were obtained at 10 mM-*N*-acetyl-L-glutamate, 1 mequiv. of Mg^{2+} /litre and approximately K_m values of the non-variable substrates, i.e. 0.5 mM-ATPMg, 10 mM- KHCO_3 and 1 mM- NH_4Cl . The K_i values quoted are apparent K_i values under these conditions obtained from the point of intersection of Dixon (1953) plots. K_i values for ADPMg are very approximate owing to non-linearity of the Dixon (1953) plots.

Product	Substrate					
	ATPMg		HCO_3^-		NH_4^+	
	Inhibition pattern	K_i (mM)	Inhibition pattern	K_i (mM)	Inhibition pattern	K_i (mM)
ADPMg	Mixed and non-linear	1	Mixed and non-linear	0.6	Mixed and non-linear	1.3
Carbamoyl phosphate	Mixed	10	Mixed	13	Mixed	19
Inorganic phosphate	Competitive	25	Mixed	60	Mixed	70
					Uncompetitive at saturating HCO_3^-

Fig. 11. Effect of K^+ salts on the activity of carbamoyl phosphate synthetase

A double coupled spectrophotometric assay was used with 50mM-Tris-glycylglycine, pH 7.6, 0.2mM-NADH, 2.5mM-phosphoenolpyruvate (tricyclohexylammonium salt), 20 μ g of pyruvate kinase, 20 μ g of lactate dehydrogenase, 10mM-N-acetyl-L-glutamate (Tris salt), 2mM-ATPMg (Tris salt), 25mM-HCO₃⁻ (Tris salt), 1mequiv. of free Mg²⁺/litre and 0.5mequiv. of NH₄⁺/litre. (a) Double-reciprocal plot of initial velocity against the concentration of KCl. (b) Double-reciprocal plot of initial velocity against the concentrations of different K⁺ salts: KCl ($\square$), KBr ($\bullet$) and KI (Δ).

for each salt, inhibition is greatest by KI and least by KCl. Thus any inhibition observed by phosphate with respect to N-acetyl-L-glutamate may be complicated by non-specific salt inhibition.

On the basis of this and an accompanying paper (Elliott & Tipton, 1974b) the overall kinetic mechanism may be written as follows:

References

- Archibald, R. M. (1944) *J. Biol. Chem.* **156**, 121-142
 Dixon, M. (1953) *Biochem. J.* **55**, 170-171
 Elliott, K. R. F. & Tipton, K. F. (1973) *FEBS Lett.* **37**, 79-81
 Elliott, K. R. F. & Tipton, K. F. (1974a) *Biochem. J.* **141**, 789-805

where A = ATPMg, B = HCO₃⁻, G = N-acetyl-L-glutamate, M = Mg²⁺, N = NH₄⁺, P = inorganic phosphate, Q = ADPMg, R = carbamoyl phosphate and E' = active enzyme.

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- Elliott, K. R. F. & Tipton, K. F. (1974b) *Biochem. J.* **141**, 807-816
 Fahien, L. A. & Cohen, P. P. (1964) *J. Biol. Chem.* **239**, 1925-1934
 Guthöhrlein, G. & Knappe, J. (1968) *Anal. Biochem.* **26**, 188-191
 Guthöhrlein, G. & Knappe, J. (1969) *Eur. J. Biochem.* **8**, 207-214

- Hall, L. M., Metzenberg, R. L. & Cohen, P. P. (1958) *J. Biol. Chem.* **230**, 1013–1021
- Kennedy, J. & Grisolia, S. (1965) *Biochim. Biophys. Acta* **96**, 102–113
- Kerson, L. A. & Appel, S. H. (1968) *J. Biol. Chem.* **243**, 4279–4285
- Marshall, M., Metzenberg, R. L. & Cohen, P. P. (1958) *J. Biol. Chem.* **233**, 102–105
- Marshall, M., Metzenberg, R. L. & Cohen, P. P. (1961) *J. Biol. Chem.* **236**, 2229–2237
- Metzenberg, R. L., Hall, L. M., Marshall, M. & Cohen, P. P. (1957) *J. Biol. Chem.* **229**, 1019–1025
- Schimke, R. T. (1962) *J. Biol. Chem.* **237**, 459–468
- Tabor, C. W. (1970) *Methods Enzymol.* **17A**, 955