Kinetic Studies of Bovine Liver Carbamoyl Phosphate Synthetase

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A thorough study of initial-rate data has been made on carbamoyl phosphate synthetase from bovine liver. On the basis of the results the order of substrate binding to the enzyme is ATPMg followed by HCO_3^- , ATPMg and NH_4^+ . A model for the enzymic mechanism is proposed, and the rate equations describing it are presented. Details of the derivation of the initial-rate equation for the kinetic mechanism proposed have been deposited as Supplementary Publication SUP 50032 (6 pages) at the British Library, Lending Division (formerly the National Lending Library for Science and Technology), Boston Spa, Yorks. LS23 7QB, U.K., from whom copies may be obtained on the terms indicated in *Biochem. J.* (1973), 131, 5.

Carbamoyl phosphate synthetase (EC 2.7.2.5) prepared from mammalian liver mitochondria (Metzenberg *et al.*, 1957) and frog liver mitochondria (Marshall *et al.*, 1958) has been shown to catalyse the reaction:

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

Only very limited studies on the kinetic mechanisms of frog liver (Fahien & Cohen, 1964) and rat liver carbamoyl phosphate synthetase (Kerson & Appel, 1968; Guthöhrlein & Knappe, 1969) have been carried out. Kinetic mechanisms have been proposed for the enzymic reaction on the basis of these incomplete studies and the present paper, along with an accompanying paper on product-inhibition studies (Elliott & Tipton, 1974*b*), presents the results of a full kinetic study of the carbamoyl phosphate synthetase-catalysed reaction.

Experimental

Materials

Water was distilled from an all-glass still and deionized for experiments involving variation of Mg^{2+} and HCO_3^- concentrations. Pyruvate kinase and lactate dehydrogenase (pig muscle) in 50% glycerol, ATP, NADH (grade II) and phosphoenolpyruvate were obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. *N*-Acetyl-L-glutamic acid was obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K. and

was recrystallized from hot water before use. All other reagents were obtained from BDH Chemicals Ltd., Poole, Dorset, U.K. and were the highest grade of purity available.

Preparation of carbamoyl phosphate synthetase

Carbamoyl phosphate synthetase was purified from bovine liver as described elsewhere (Elliott & Tipton, 1973) by a modification of the method of Guthöhrlein & Knappe (1968). All enzyme preparations used had specific activities of between 0.8 and 1.5 units/mg of protein (1 unit equivalent to the production of 1 μmol of ADP/min at 30°C) and contained less than 1% ATPase* (EC 3.6.1.3) and adenylate kinase (EC 2.7.4.3) activity.

Assay of enzyme activity

Carbamoyl phosphate synthetase activity was assayed by coupling ADP formation to NAD^+ formation with pyruvate kinase and lactate dehydrogenase (Fahien & Cohen, 1964). The assay system contained enzyme, 20 μg of pyruvate kinase, 20 μg of lactate dehydrogenase, 2.5 mM-phosphoenolpyruvate and 0.2 mM-NADH with the requisite concentrations of NH_4Cl , ATP, MgSO_4 and KHCO_3 in 50 mM-glycylglycine adjusted to pH 7.6 with KOH. The assay mixture was preincubated for 5 min in a 30°C water bath to allow the temperature to equilibrate and any endogenous pyruvate and ADP to be removed. The reaction was started by addition of *N*-acetyl-L-glutamate (adjusted to pH 7.6 with KOH) to give a final volume of 1 ml. Formation of NAD^+ was followed spectrophotometrically by the decrease in absorbance at 340 nm with a Gilford 240 spectrophotometer connected to a Rikadenki

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* Abbreviation: ATPase, adenosine triphosphatase.

10-in (25 cm) chart recorder (giving a maximum useful full-scale deflexion of 0.02 absorbance unit). The spectrophotometer cuvette holder was water-jacketed at 30°C.

The amounts of ATP and MgSO_4 required to give known concentrations of ATPMg complex and free Mg^{2+} were calculated on the basis of stability constants determined under the assay conditions. The values of the stability constants, determined by using 8-hydroxyquinoline (Burton, 1959), were

$$K_{\text{stability (real)}} = \frac{[\text{ATPMg}]}{[\text{ATP}]_{\text{free}} \cdot [\text{Mg}^{2+}]_{\text{free}}} = 30000 \text{ M}^{-1}$$

and

$$K_{\text{stability (apparent)}} = \frac{[\text{ATPMg}]}{[\text{ATP}]_{\text{free}} ([\text{Mg}^{2+}]_{\text{free}} + [\text{Mg}^{2+}]_{\text{buffer}})} = 7600 \text{ M}^{-1}$$

When the concentration of KHCO_3 was varied KCl was added to the assay mixture to keep the ionic

strength constant. Endogenous HCO_3^- was removed from the assay mixture by extensively degassing all solutions before use. Where possible degassing was carried out on the acid form of the solutions, which were then brought to the correct pH with a freshly prepared solution of KOH.

Results

Initial-rate data were obtained for each substrate (including *N*-acetyl-L-glutamate and Mg^{2+} as 'substrates') at various concentrations of the other substrates. Initially the non-variable substrates were kept at concentrations well above the previously determined K_m values (Kerson & Appel, 1968), i.e. 5 mM-ATPMg, 100 mM- KHCO_3 , 10 mM- NH_4Cl , 10 mM-*N*-acetyl-L-glutamate and 1 mequiv. of free Mg^{2+} /litre. Figs. 1–10 show the reciprocal plots obtained under these conditions. Most combinations gave patterns

Fig. 1. Double-reciprocal plots of the variation of initial velocity against HCO_3^- (a) and ATPMg (b) concentrations

Details of the assay method and conditions are given in the text. In (a) the concentrations of ATPMg were 0.5 mM (○), 0.7 mM (■), 1.0 mM (△), 2.0 mM (●) and 5.0 mM (□). In (b) the concentrations of HCO_3^- were 5.0 mM (○), 7.0 mM (■), 10.0 mM (△), 20.0 mM (●) and 100.0 mM (□).

Fig. 2. Double-reciprocal plots of the variation of initial velocity against *N*-acetyl-L-glutamate (a) and ATPMg (b) concentrations

Details of the assay method and conditions are given in the text. In (a) the concentrations of ATPMg were 0.5 mM (○), 0.7 mM (■), 1.0 mM (△), 2.0 mM (●) and 5.0 mM (□). In (b) the concentrations of *N*-acetyl-L-glutamate were 50 μM (○), 70 μM (■), 100 μM (△), 200 μM (●) and 1 mM (□).

Fig. 3. Double-reciprocal plots of the variation of initial velocity against HCO_3^- (a) and NH_4^+ (b) concentrations

Details of the assay method and conditions are given in the text. In (a) the concentrations of NH_4^+ were 0.25 mM ($\circ$), 0.33 mM ($\blacksquare$), 0.5 mM ($\triangle$), 1.0 mM ($\bullet$) and 5.0 mM ($\square$). In (b) the concentrations of HCO_3^- were 1.0 mM ($\circ$), 1.25 mM ($\blacksquare$), 1.4 mM ($\triangle$), 2.5 mM ($\bullet$) and 5.0 mM ($\square$).

Fig. 4. Double-reciprocal plots of the variation of initial velocity against HCO_3^- (a) and *N*-acetyl-L-glutamate (b) concentrations

Details of the assay method and conditions are given in the text. In (a) the concentrations of *N*-acetyl-L-glutamate were 50 μM ($\circ$), 70 μM ($\blacksquare$), 100 μM ($\triangle$), 200 μM ($\bullet$) and 1 mM ($\square$). In (b) the concentrations of HCO_3^- were 3.0 mM ($\circ$), 3.5 mM ($\triangle$), 5.0 mM ($\square$), 7.0 mM ($\bullet$), 12.0 mM (Δ) and 102.0 mM ($\blacksquare$).

on reciprocal plots that intersected on or near the $1/[S]$ axis. However, when the concentration of ATPMg was varied at various fixed free Mg^{2+} concentrations the lines intersected on the $1/v$ axis (Fig. 8). This pattern is indicative of a mechanism where ATPMg binds to the enzyme after Mg^{2+} , which binds to the enzyme in equilibrium (see, e.g., Cleland, 1970). This situation may be described by the equation

$$v = \frac{V}{1 + \frac{K_m^{\text{ATPMg}}}{[\text{ATPMg}]} + \frac{K_s^{\text{Mg}^{2+}} K_m^{\text{ATPMg}}}{[\text{Mg}^{2+}] [\text{ATPMg}]}}$$

In addition, under these conditions, when the NH_4^+ concentration was varied at a series of ATPMg or *N*-acetyl-L-glutamate concentrations, families of apparently parallel lines were observed in reciprocal plots (Figs. 9 and 10). However, it was difficult to decide whether these lines were truly

parallel or were in fact weakly converging. Such a distinction is essential for establishing the kinetic mechanism operative, and a series of experiments were carried out to establish which was the case.

When the concentration of KHCO_3 was decreased from 100 mM to 5 mM (i.e. from about 20 times K_m to about K_m) there was no effect on the type of pattern observed when the NH_4^+ concentration was varied at a number of ATPMg concentrations. Decreasing the concentration of KHCO_3 did, however, result in an intersecting pattern of reciprocal plots when the NH_4^+ concentration was varied at a number of *N*-acetyl-L-glutamate concentrations (Fig. 11). As decreasing the concentration of KHCO_3 from what appears to be saturating to about K_m causes the parallel lines to become intersecting, it may be concluded that HCO_3^- binds to the enzyme between

Fig. 5. Double-reciprocal plots of the variation of initial velocity against Mg^{2+} (a) and HCO_3^- (b) concentrations

Details of the assay method and conditions are given in the text. In (a) the concentrations of HCO_3^- were 5 mM (○), 7 mM (■), 10 mM (Δ), 20 mM (●) and 100 mM (□). In (b) the concentrations of Mg^{2+} were 0.2 mM (●), 0.25 mM (□), 0.33 mM (▲), 0.5 mM (○) and 1.0 mM (■).

N-acetyl-L-glutamate and NH_4^+ (see, e.g. Elliott & Tipton, 1974a).

To test whether the reciprocal plots with respect to NH_4^+ at various ATPMg concentrations were in fact weakly converging the experiments were repeated with substrate concentrations down to an order of magnitude lower than those previously used. The reciprocal plots so obtained were non-linear, but appeared to converge (Fig. 12). Non-linear reciprocal plots with respect to ATPMg have been observed previously by Fahien & Cohen (1964) and might be expected because of the stoichiometry of the reaction (see, e.g., Elliott & Tipton, 1974a). When ATPMg was varied in the absence of NH_4^+ , ADP formation still occurred (Fahien & Cohen, 1964) but the reciprocal plot was linear. It was found that both *N*-acetyl-L-glutamate and $KHCO_3$ were required for this reaction. This effect could account for the observed departure from linearity of the

Fig. 6. Double-reciprocal plots of the variation of initial velocity against NH_4^+ (a) and Mg^{2+} (b) concentrations

Details of the assay method and conditions are given in the text. In (a) the concentrations of Mg^{2+} were 0.2 mM (▲), 0.25 mM (○), 0.33 mM (■), 0.5 mM (Δ) and 1.0 mM (●). In (b) the concentrations of NH_4^+ were 0.5 mM (○), 0.7 mM (■), 1.0 mM (Δ), 2.0 mM (●) and 5.0 mM (□).

reciprocal plots at low NH_4^+ concentrations since the partial reaction

may then be proceeding at a significant rate compared with the total reaction.

If one of the other substrates binds to the enzyme between the two molecules of ATPMg then at an infinite concentration of that substrate a reciprocal plot against ATPMg should be linear (see Elliott & Tipton, 1974a). Figs. 13–15 show reciprocal plots for *N*-acetyl-L-glutamate, HCO_3^- and NH_4^+ as the varied substrates at a number of ATPMg concentrations. The secondary plots of the intercepts on the $1/v$ axis against $1/[ATPMg]$ are equivalent to reciprocal plots at an infinite concentration of the other substrate. Of the three secondary plots only that for HCO_3^- was linear, suggesting that only HCO_3^- binds to the enzyme between the two ATPMg molecules.

Table 1 shows the kinetic constants obtained from the reciprocal plots.

Discussion

Marshall *et al.* (1961) showed that carbamoyl phosphate synthetase from frog liver would bind one molecule of *N*-acetyl-L-glutamate in the absence

of other substrates. If it is assumed that the bovine liver enzyme would show the same effects then the following mechanism will account for the results presented above:

where $A = \text{ATPMg}$, $B = \text{HCO}_3^-$, $G = N\text{-acetyl-L-glutamate}$, $M = \text{Mg}^{2+}$, $N = \text{NH}_4^+$, $P = \text{phosphate}$, $Q = \text{ADP}$, $R = \text{carbamoyl phosphate}$ and $E' = \text{active enzyme}$.

Fig. 7. Double-reciprocal plots of the variation of initial velocity against *N*-acetyl-L-glutamate (a) and Mg^{2+} (b) concentrations

Details of the assay method and conditions are given in the text. In (a) the concentrations of Mg^{2+} were 0.2 mM ($\circ$), 0.25 mM ($\blacksquare$), 0.33 mM ($\triangle$), 0.5 mM ($\bullet$) and 1.0 mM ($\square$). In (b) the concentrations of *N*-acetyl-L-glutamate were 50 μM ($\circ$), 70 μM ($\blacksquare$), 100 μM ($\triangle$), 200 μM ($\bullet$) and 1 mM ($\square$).

Fig. 8. Double-reciprocal plots of the variation of initial velocity against Mg^{2+} (a) and ATPMg (b) concentrations

Details of the assay method and conditions were given in the text. In (a) the concentrations of ATPMg were 0.5 mM ($\circ$), 0.7 mM ($\blacksquare$), 1.0 mM ($\triangle$), 2.0 mM ($\bullet$) and 5.0 mM ($\square$). In (b) the concentrations of Mg^{2+} were 0.2 mM ($\bullet$), 0.25 mM ($\square$), 0.33 mM ($\triangle$), 0.5 mM ($\circ$), 1.0 mM ($\blacksquare$) and 5.0 mM ($\triangle$).

Fig. 9. Double-reciprocal plots of the variation of initial velocity against ATPMg (a) and NH_4^+ (b) concentrations

Details of the assay method and conditions are given in the text. In (a) the concentrations of NH_4^+ were 0.5 mM ($\circ$), 0.7 mM ($\blacksquare$), 1.0 mM ($\triangle$), 2.0 mM ($\bullet$) and 5.0 mM ($\square$). In (b) the concentrations of ATPMg were 0.5 mM ($\circ$), 0.7 mM ($\blacksquare$), 1.0 mM ($\triangle$), 2.0 mM ($\bullet$) and 5.0 mM ($\square$).

The random-order segment may be assumed to be in equilibrium as Fahien *et al.* (1964) found that the dissociation constants for *N*-acetyl-L-glutamate and ATPMg from frog liver carbamoyl phosphate synthetase were the same as their respective K_m values in the absence of NH_4^+ . The reciprocal plot for ATPMg at various Mg^{2+} concentrations (Fig. 8) indicates that Mg^{2+} binds to the enzyme in equilibrium before ATPMg [as did the results of Marshall *et al.* (1961) for the frog liver enzyme].

Fig. 10. Double-reciprocal plots of the variation of initial velocity against *N*-acetyl-L-glutamate (a) and NH_4^+ (b) concentrations

Details of the assay method and conditions are given in the text. In (a) the concentrations of NH_4^+ were 0.1 mM ($\circ$), 0.2 mM ($\blacksquare$), 0.5 mM ($\triangle$) and 5.0 mM ($\bullet$). In (b) the concentrations of *N*-acetyl-L-glutamate were 50 μM ($\circ$), 70 μM ($\blacksquare$), 100 μM ($\triangle$), 200 μM ($\bullet$) and 1 mM ($\square$).

Since this equilibrium binding does not appear to be destroyed by high concentrations of HCO_3^- it is reasonable to postulate a steady-state isomerization step between the binding of ATPMg and HCO_3^- (see Elliott & Tipton, 1974a). In addition the existence of such an isomerization would account for the observation that the reciprocal plots do not yield patterns indicative of compulsory-order equilibrium binding when HCO_3^- is varied at several Mg^{2+} concentrations. *N*-Acetyl-L-glutamate must also bind randomly with respect to both Mg^{2+} and ATPMg since reciprocal plots do not give rise to patterns of lines that intersect on the $1/v$ axis with this substrate.

Fig. 11. Double-reciprocal plots of the variation of initial velocity against NH_4^+ (a) and *N*-acetyl-L-glutamate (b) concentrations

The conditions were identical with those used for Fig. 10 except that the 100mM- KHCO_3 was replaced by 5mM- KHCO_3 and 95mM-KCl. In (a) the concentrations of *N*-acetyl-L-glutamate were 50 μM ($\circ$), 70 μM ($\blacksquare$), 100 μM ($\triangle$), 200 μM ($\bullet$) and 1 mM ($\square$). In (b) the concentrations of NH_4^+ were 70 μM ($\circ$), 100 μM ($\blacksquare$), 200 μM ($\triangle$), 500 μM (Δ) and 5 mM ($\bullet$).

The initial-rate equation for the proposed mechanism has been obtained by using the method of Cha (1968) in combination with that of Vol'kenshtein & Gol'dshtein (1966):

Fig. 12. Double-reciprocal plots of the variation of initial velocity against ATPMg (a) and NH_4^+ (b) concentrations

The conditions were identical with those used in Fig. 9 except that lower ranges of ATPMg (a) and NH_4^+ (b) concentrations were used. In (a) the concentrations of NH_4^+ were 0.2mM ($\circ$), 0.5mM ($\triangle$) and 10.0mM ($\square$). In (b) the concentrations of ATPMg were 33 μM ($\circ$), 50 μM ($\triangle$), 100 μM ($\square$) and 5mM ($\bullet$).

where K_m^{A1} and K_m^{A2} are the theoretical K_m values for the first and second ATPMg molecules to bind and *A*, *B*, *G*, *M* and *N* represent the concentrations of the substrates and activators as defined above. This equation is cumbersome, containing twenty-four terms in the denominator, but if it is assumed that

$$v = \frac{V}{1 + \frac{K_m^{A1} + K_m^{A2}}{A} + \frac{K_m^G}{G} + \frac{K_m^B}{B} + \frac{K_m^N}{N} + \frac{K_m^{A1}K_m^G}{AG} + \frac{K_m^{A1}K_m^B}{AB} + \frac{K_m^{A1}K_m^N}{AN} + \frac{K_m^GK_m^B}{GB} + \frac{K_m^{A1}K_m^GK_m^B}{AGB} + \frac{K_m^{A2}K_m^GK_m^B}{A^2GB} + \frac{K_m^{A1}K_m^M}{AM} + \frac{K_m^{A1}K_m^B}{AB} + \frac{K_m^{A2}K_m^B(K_m^N + K_m^N')}{ABN} + \frac{K_m^{A1}K_m^M K_m^G K_m^B}{AMGB} + \frac{K_m^{A2}K_m^G K_m^B K_m^N}{A^2GBN} + \frac{K_m^{A1}K_m^{A2}K_m^B}{A^2MB} + \frac{K_m^{A1}K_m^{A2}K_m^B K_m^N}{A^2MBN} + \frac{K_m^{A1}K_m^{A2}K_m^B K_m^N'}{A^2MBN} + \frac{K_m^{A1}K_m^{A2}K_m^G K_m^B K_m^N}{A^2MGBN} + \frac{K_m^{A1}K_m^{A2}K_m^G K_m^B K_m^N'}{A^2MGBN} + \frac{K_m^{A1}K_m^{A2}K_m^G K_m^B K_m^N'}{A^2MGBN}}$$

Fig. 13. Variation of initial velocity with the concentration of *N*-acetyl-L-glutamate

The conditions were identical with those used in Fig. 2 except that a lower range of ATPMg concentrations was used. (a) Double-reciprocal plots at a series of fixed ATPMg concentrations: 33 μM ($\blacksquare$), 40 μM ($\triangle$), 50 μM ($\blacktriangle$), 67 μM ($\circ$), 100 μM ($\bullet$), 200 μM ($\square$), 500 μM ($\boxplus$), 1 mM (∇) and 5 mM ($\triangledown$). (b) Secondary plot of the intercepts from (a) against the reciprocal of the ATPMg concentration.

Fig. 14. Variation of initial velocity with the concentration of HCO_3^-

The conditions were identical with those used in Fig. 1 except that a lower range of ATPMg concentrations was used. (a) Double-reciprocal plots at a series of fixed ATPMg concentrations: 33 μM ($\circ$), 40 μM ($\blacksquare$), 50 μM ($\triangle$), 67 μM ($\bullet$), 100 μM ($\square$), 200 μM ($\blacktriangle$), and 5 mM (∇). (b) Secondary plot of the intercepts from (a) against the reciprocal of the ATPMg concentrations.

N-acetyl-L-glutamate and Mg^{2+} are saturating (i.e. at such high concentrations that all terms containing their concentrations become negligible, cf. Cleland, 1970) the equation may be reduced to:

$$v = \frac{V}{1 + \frac{K_m^{A1} + K_m^{A2}}{A} + \frac{K_m^B}{B} + \frac{K_m^N}{N} + \frac{(K_m^{A2} + K_m^{A2'})K_s^B + K_s^{A1}K_m^{B'}}{AB} + \frac{K_s^{A2}K_m^N}{AN} + \frac{K_s^{A2}K_s^B(K_m^N + K_m^{N'})}{ABN} + \frac{K_s^{A1}K_m^{A2}K_s^B}{A^2B} + \frac{K_s^{A1}K_s^{A2}K_s^B K_m^N}{A^2BN}}$$

This is the equation which describes the simplified form of the mechanism ignoring activator binding:

Other authors seem to have considered a study involving all substrates and activators to be too complex, and so have kept at least one substrate and

one activator constant throughout their experiments. This approach has in fact complicated the

Fig. 15. Variation of the initial velocity with the concentration of NH_4^+

The conditions were identical with those used in Fig. 9 except that a lower range of ATPMg was used. (a) Double-reciprocal plots at a series of fixed ATPMg concentrations: 33 μM (○), 40 μM (■), 50 μM (△), 67 μM (●), 100 μM (□), 200 μM (▲) and 5 mM (▼). (b) Secondary plot of the intercepts from (a) against the reciprocal of the ATPMg concentration.

Table 1. Apparent K_m values for substrates and activators of bovine liver carbamoyl phosphate synthetase

Apparent K_m values (obtained from the replots of the intercepts from reciprocal plots) at infinite concentration of the stated substrates with the concentrations of the other substrates being high, i.e. 5 mM-ATPMg, 10 mM- NH_4Cl , 100 mM- KHCO_3 , 10 mM-*N*-acetyl-L-glutamate, 1 mequiv./l of Mg^{2+} .

Infinite substrate	K_m^{AG} (μM)	$K_m^{\text{Mg}^{2+}}$ (mM)	K_m^{ATPMg} (mM)	$K_m^{\text{HCO}_3^-}$ (mM)	$K_m^{\text{NH}_4^+}$ (mM)
<i>N</i> -Acetyl-L-glutamate	—	0.18	0.80	4.5	0.25
Mg^{2+}	80	—	0.71	3.4	0.25
ATPMg	90	*	—	10	0.76
HCO_3^-	50	0.19	0.50	—	0.40
NH_4^+	125	0.19	0.52	4	—

* $K_m^{\text{Mg}^{2+}}$ at infinite concentration of ATPMg is negligible as it is equal to half the enzyme concentration (Tipton, 1974).

suggestion that HCO_3^- bound to the enzyme before NH_4^+ , as ATPase activity existed in the presence of HCO_3^- but absence of NH_4^+ , they postulated a number of mechanisms. If it is assumed that the kinetic properties of the rat and bovine liver enzymes are similar (as are their physical properties and K_m values for substrates) then a more complete study would have possibly ruled out all the mechanisms that these workers proposed.

The only other kinetic analysis of mammalian liver carbamoyl phosphate synthetase was performed by Kerson & Appel (1968) who attempted to show the dependence of the reaction rate on ATP and Mg^{2+} concentrations. They found that when ATP and Mg^{2+} were varied in equimolar amounts a sigmoid plot of v against ATP or ATPMg concentration was obtained, but if Mg^{2+} was in 5 mequiv./l excess then the curve was hyperbolic. We have also observed this pattern of behaviour, but when the concentrations of free Mg^{2+} and ATPMg were calculated, and the free Mg^{2+} concentration held constant, essentially hyperbolic plots of v against ATPMg were obtained. [Any deviations due to the second-order nature of the reaction occur at a concentration far below those used by Kerson & Appel (1968).] The explanation of this phenomenon is that both ATPMg and free Mg^{2+} are required by the enzyme and the concentrations of the various species are related by the equation:

$$K_{\text{stability}} = \frac{[\text{ATPMg}]}{[\text{ATP}]_{\text{free}} \cdot [\text{Mg}^{2+}]_{\text{free}}}$$

Thus at low concentrations of total ATP and Mg^{2+} only a small proportion of the total ATP will be in the form of ATPMg and there will also be a low concentration of free Mg^{2+} . As the total concentrations rise then so do the proportion of the total ATP as ATPMg

situation as the use of all the substrates and activators allows a greater distinction between mechanisms. Guthöhrlein & Knappe (1969) only varied HCO_3^- and NH_4^+ concentrations in an attempt to clarify the 'bicarbonate-activation' step. On the basis of results showing no essentially irreversible step between binding of HCO_3^- and NH_4^+ , and the

Fig. 16. Calculated graphs for the variation of initial velocity against ATP concentration at a 1:1 ratio of $\text{ATP}/\text{Mg}^{2+}$ in the presence and absence of additional Mg^{2+}

The additional Mg^{2+} concentrations used were 0 (■), 1.0 mM (○) and 5.0 mM (▲). The values were calculated as described in the text.

and the concentration of free Mg^{2+} . Fig. 16 shows the expected curves when plotting v against ATP with equimolar ATP and Mg^{2+} with a value of 60000M^{-1} for the ATPMg stability constant, 0.7 mM for K_s^{ATPMg} and 0.5 mequiv./l for $K_s^{\text{Mg}^{2+}}$ (obtained from Fig. 8). This ignores the facts that Mg^{2+} will bind to the buffer [phosphate was used by Kerson & Appel (1968)] and that free ATP may inhibit the reaction, both factors tending to make the curve more sigmoid.

The most extensive kinetic investigation of carbamoyl phosphate synthetase previously undertaken was carried out by Fahien & Cohen (1964) on the frog liver enzyme. There do appear to be some differences between the frog and bovine liver enzymes in that frog liver carbamoyl phosphate synthetase appears to be inhibited by NH_4^+ at low ATPMg concentrations and reciprocal plots against ATPMg were non-linear even in the absence of NH_4^+ . However, it would appear that their task of proposing a kinetic mechanism would have been considerably eased if the effects of variations in HCO_3^- concentrations had also been studied.

The mechanism that has been proposed in the present paper is the simplest one that is fully consistent with the results obtained. Any more complex model would involve the postulation of various

abortive complexes or significant contributions to the overall rate by partial reactions.

Supplementary information

Details of the derivation of the initial-rate equation for bovine liver carbamoyl phosphate synthetase that is given in this paper have been deposited with the British Library, Lending Division (formerly the National Lending Library for Science and Technology), Boston Spa, Yorkshire LS23 7QB, U.K. for storage (supplementary Publication SUP 50032).

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