A Kinetic Study of Carbamyl Phosphate Synthetase*

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Carbamyl phosphate synthetase prepared from frog liver has been shown to catalyze Reactions 1, 2, and 3.

\[
\begin{align*}
\text{ATP} + \text{HCO}_3^- + \text{acetylglutamate} \quad \text{Mg}^{++} & \quad \rightarrow \quad \text{ADP} + \text{P}_i + \text{"active CO"}^+ \\
\text{ATP} + \text{"active CO"}^+ + \text{NH}_4^+ + \text{acetylglutamate} & \quad \rightarrow \quad \text{H}_2\text{NCOO}^{-} + \text{ADP} \\
\text{Over-all:} \quad 2 \text{ATP} + \text{HCO}_3^- + \text{NH}_4^+ + \text{acetylglutamate} \quad \text{Mg}^{++} & \quad \rightarrow \quad 2 \text{ADP} + \text{P}_i + \text{"active CO"}^+ + \text{H}_2\text{NCOO}^{-}
\end{align*}
\]

While it is clear from the stoichiometry of the over-all reaction (Reaction 3) that 2 moles of ATP are utilized per mole of carbamyl phosphate formed (1), equilibrium dialysis experiments indicated that only 1 mole of ATP was bound per mole of enzyme in the absence of acetylglutamate\(^1\) (2). In order to gain more information about the nature of ATP binding by carbamyl phosphate synthetase, kinetic and other studies were carried out. In this paper, evidence will be presented which shows that there are two sites for ATP binding on the enzyme, of which one is independent of the binding of other substrates and the other site requires prior binding of acetylglutamate.

EXPERIMENTAL PROCEDURE

Enzymes and Substrates—Frog liver carbamyl phosphate synthetase was prepared by the method of Marshall, Metzenberg, and Cohen (3). This method yields a preparation free of ATPase and myokinase. All other enzymes and organic reagents were purchased from Sigma Chemical Company with the exception of acetylglutamate, which was obtained from the H. M. Chemical Company.

Stock solutions of reagents were prepared at pH 7.5 as their potassium salts. DPNH was stored in dry form at 4°, and fresh solutions were made on the day that they were used.

Initial Velocity Measurements—As can be seen from Reactions 1 and 2, 1 mole of ADP is formed from each mole of ATP broken down in each of the partial reaction steps. In the over-all reaction (Reaction 3), 2 moles of ADP are formed. The possibility of measuring Reactions 1 and 3 by coupling these to an ADP-utilizing system seemed feasible. (Reaction 2, the only reversible step, cannot be measured independently in the direction of ADP formation because the nature of the intermediate, “active CO\(^{2-}\)” is unknown.) It was found possible to couple Reactions 1 and 3 with pyruvate kinase and lactic dehydrogenase as follows.

\[
\begin{align*}
\text{ADP} + \text{phosphoenolpyruvate} & \quad \rightarrow \quad \text{pyruvate} + \text{ATP} \\
\text{Pyruvate} + \text{DPNH} + \text{H}^+ & \quad \rightarrow \quad \text{lactate} + \text{DPN}^+ \\
\text{Carbamyl phosphate synthetase} & \text{was thus assayed by measuring the rate of DPNH oxidation spectrophotometrically at 340 nm.}
\end{align*}
\]

It was demonstrated that the substrates required for the carbamyl phosphate synthetase reaction would not interfere with the quantitative determinations of ADP concentrations as measured by the amount of DPNH oxidized. In the presence of the carbamyl phosphate synthetase system, the rate of oxidation of DPNH, after a brief initial lag, was found to be linear and directly proportional to the amount of carbamyl phosphate synthetase added. This rate was used as the initial rate in the kinetic studies. No reaction was observed in the absence of either carbamyl phosphate synthetase or acetylglutamate.

In all of these measurements, the reactions were started by the addition of carbamyl phosphate synthetase. In order to prevent enzyme denaturation during the course of a series of measurements, stock solutions of carbamyl phosphate synthetase in 0.05 \(\text{M}\) glycylglycine, pH 7.5, were prepared freshly each day and maintained at 0°. Under such conditions, there was no decrease in enzyme activity over a period of 3 to 4 hours.

A solution containing 1 \(\times 10^{-2}\) \(\text{M}\) \(\text{MgCl}_2\), 2.5 \(\times 10^{-3}\) \(\text{M}\) phosphoenolpyruvate, 5 \(\times 10^{-2}\) \(\text{M}\) glycylglycine, 1.6 \(\times 10^{-4}\) \(\text{M}\) DPNH, 0.1 \(\text{M}\) \(\text{KHCO}_3\), 0.1 \(\text{M}\) KCl, 0.01 \(\text{M}\) acetylglutamate, 5 \(\times 10^{-2}\) \(\text{M}\) ATP, 1 \(\times 10^{-3}\) \(\text{M}\) \(\text{NH}_4\)Cl, lactic dehydrogenase (sufficient to convert 4 pmoles of pyruvate to lactate per minute), and pyruvate kinase (sufficient to convert 0.6 pmole of phosphoenolpyruvate to pyruvate per minute) was used for a standard assay of carbamyl phosphate synthetase activity. These concentra-
suitable for the rapid, stoichiometric determinations of ADP. The pyruvate, DPNH, KCl, and MgCl₂ used were established to be suitable for the rapid, stoichiometric determinations of ADP.

Assays were performed in a cuvette with a light path of 1 cm. A Beckman DU spectrophotometer coupled to a Brown recorder was used in the assay systems. Measurements were made in the interval represented by the first few per cent of the reaction following the brief initial lag period. The slit width was normally held at less than 0.3 mm.

The kinetic data presented in the figures of this paper represent the results of several determinations and have all been corrected to the same enzyme concentration of $1.3 \times 10^{-3}$ mg per ml. Different enzyme concentrations were used when necessary to adjust the velocity to the limits of the assay.

Concentrated stock solutions of the enzymes used in the experiments were stored in ammonium sulfate. The concentrations of enzymes used in the assay were such that one could, by dilution of the stock solutions, obtain an ammonium ion concentration equal to $2 \times 10^{-3}$ M. In these experiments, stock solutions of the enzyme were diluted to concentrations 15 to 20 times greater than were needed. The diluted enzyme preparations were then dialyzed extensively in 0.05 M glycylglycine, pH 7.5, at 4°C. Appropriate dilutions of the dialyzed enzyme solutions were then used in the assay systems.

**Determination of Protein**—Protein was determined by measuring the absorbance at 280 mμ. The extinction coefficient at 280 mμ, based on dry weight of sample, was found to be 0.96 ml mg⁻¹ cm⁻¹ in 0.02 M Tris-chloride buffer, pH 7.5, at 23°C.

**RESULTS**

**Kinetics in Presence of High Substrate Concentrations**—Michaelis constants for the substrates and activators of this enzyme have previously been measured by assaying the rate of carbamyl phosphate production in a system coupled with ornithine transaminase. As will be seen, the data obtained can be represented by equations derived for those simpler mechanisms. While it is recognized that kinetic analysis of a system as complicated as carbamyl phosphate synthetase will at best serve only to select between alternative mechanisms, the absence of definitive information on the nature of the intermediates of the system, by the use of more classical methods, necessitated this approach. However, the kinetic data presented in this paper were found to be consistent with the data obtained from previous nonkinetic studies of this enzyme. They are also consistent with nonkinetic data which will be presented in the accompanying paper (4).

The concentrations of ATP referred to in the present studies represent the concentrations of total magnesium-ATP complex as calculated from the constants reported by Kuby and Noltmann (5).

The kinetic constants were derived either graphically (by making secondary plots of the data) or by a series of approximations of the best values that would agree with the experimentally determined points.

**KINETIC THEORY**

The initial velocities in these experiments were measured at various concentrations of ATP, acetylglutamate, and ammonium ions. The data were obtained by varying the concentrations of one or two of these components and keeping the others at constant and sufficiently high levels so that their concentrations would not be rate-limiting.

The kinetic mechanisms derived for systems which involve one or two substrates were applied to the carbamyl phosphate synthetase system. As will be seen, the data obtained can be represented by equations derived for those simpler mechanisms. While it is recognized that kinetic analysis of a system as complicated as carbamyl phosphate synthetase will at best serve only to select between alternative mechanisms, the absence of definitive information on the nature of the intermediates of the system, by the use of more classical methods, necessitated this approach. However, the kinetic data presented in this paper were found to be consistent with the data obtained from previous nonkinetic studies of this enzyme. They are also consistent with nonkinetic data which will be presented in the accompanying paper (4).

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FIG. 1 (left). Double reciprocal plots of initial velocity versus acetylglutamate concentration at various constant levels of ATP, in the presence of a high and constant ammonium chloride concentration ($1 \times 10^{-3}$ M). The concentrations of ATP used were: A, $4 \times 10^{-4}$ M; B, $6 \times 10^{-4}$ M; C, $8 \times 10^{-4}$ M; D, $1 \times 10^{-3}$ M; E, $2 \times 10^{-3}$ M; F, $5 \times 10^{-3}$ M. All other constituents of the standard assay mixture were present in the concentrations as given in the text. Experiments were performed at 23°C in 0.05 M glycylglycine, pH 7.5.

FIG. 2 (right). Double reciprocal plots of initial velocity versus acetylglutamate concentration at various constant levels of ammonium chloride, in the presence of a constant and high ATP concentration ($5 \times 10^{-3}$ M). The concentrations of ammonium chloride used were: A, $4 \times 10^{-4}$ M; B, $8 \times 10^{-4}$ M; C, $1 \times 10^{-3}$ M; D, $2 \times 10^{-3}$ M; E, $1 \times 10^{-2}$ M. Other experimental conditions were identical with those described in the legend of Fig. 1.
With the use of comparable concentrations of substrates and acetylglutamate, the values of the Michaelis constants were found to be similar when determined by the rate of formation of ADP.

The results obtained when the concentrations of ATP and acetylglutamate are varied in the presence of high and constant amounts of ammonium, bicarbonate, and magnesium ions are shown in Fig. 1. It can be seen that while the apparent maximal velocities vary as a function of the concentration of ATP, the apparent Michaelis constant of acetylglutamate is independent of the ATP concentration. A replot of the data shown in Fig. 1 revealed that the Michaelis constant of ATP was independent of the concentration of acetylglutamate. Data of this type have been previously obtained for enzymatic systems which involve two substrates (6). The data can be represented by the equation

$$ v = \frac{V_{f}[E_0]}{1 + \frac{K_A}{[A]} + \frac{K_{A'}}{[B]} + \frac{K_c}{[C]}} \tag{1} $$

In Equation 1, $v$ is the initial rate; $V_f$ is the maximal velocity of the forward reaction; $K_A$ and $K_{A'}$ are the Michaelis constants for acetylglutamate and ATP, respectively; and $[A]$, $[B]$, and $[E_0]$ are the concentrations of acetylglutamate, ATP, and total enzyme, respectively. A secondary plot of the data of Fig. 1 (according to Equation 1) gives, by graphical analysis, the following values for the constants: $V_f[E_0]$, 2.8 μM per minute; $K_A$, $1 \times 10^{-3}$ M; and $K_{A'}$, $5 \times 10^{-4}$ M.

When the concentration of ATP is maintained at a constant and high level, the initial velocity varies as a function of the concentration of acetylglutamate and ammonium ions in a manner that can be represented by Equation 2, which is identical in form with Equation 1.

$$ v = \frac{V_{f}[E_0]}{1 + \frac{K_A}{[A]} + \frac{K_{A'}}{[B]} + \frac{K_c}{[C]}} \tag{2} $$

These data are shown in Fig. 2. The values of $K_A$ and $V_{f}[E_0]$ obtained are the same as those obtained from the data of Fig. 1.

The results obtained from varying the concentrations of ATP and ammonium ions at fixed high levels of acetylglutamate ($1 \times 10^{-2}$ M) are shown in Fig. 3. It can be seen that the apparent Michaelis constant of ATP is dependent upon the concentration of ammonium ions and that the apparent Michaelis constant of ammonium ions is dependent upon the concentration of ATP. These data can be represented by the equation

$$ \frac{[E_0]}{v} = \frac{1}{V_f} \left(1 + \frac{K_{A'}}{[B]} + \frac{K_c}{[C]}\right) \tag{3} $$

By rearrangement of Equation 3 and making secondary plots of the data, once can obtain the values of $V_{f}[E_0]$ and $K_{A'}$ at an infinite concentration of C, and the value of $K_c$ at an infinite concentration of $B$ (7). The values of $K_{A'}$ and $K_c$ thus obtained are $5 \times 10^{-4}$ and $6.6 \times 10^{-4}$ M, respectively. The value of $V_{f}[E_0]$ is 2.8 μM per minute. These values are the same as those obtained for these constants in the data presented in Figs. 1 and 2.

**Kinetics in Absence of Ammonium Ions—**In the absence of ammonium ions, the initial rate was found to vary with the concentration of ATP and acetylglutamate in the manner shown in Fig. 4. These data can be represented by an equation which is identical in form with Equation 1. In absence of ammonium ions, $v_f$ is 1 μM per minute, $K_A$ is $1 \times 10^{-3}$ M, and $K_{A'}$ is $1.8 \times 10^{-4}$ M.

**Kinetics at Low ATP Concentrations—**Figs. 1, 2, 3, and 4 show the results obtained when the concentration of ATP is high and near the value of the Michaelis constant, $K_A$. When the concentration of ATP is lower, deviations from linearity are noted in the Lineweaver-Burk plots (8). Fig. 5 shows the results obtained in the absence of ammonium ions at various concentrations of acetylglutamate. Similar curves are shown in Fig. 6, where the concentration of acetylglutamate is varied in the presence of a high and constant amount of ammonium ions.

At given constant concentrations of acetylglutamate and
ammonium ions, or in the absence of ammonium ions, the rate of ATP hydrolysis over a wide range of ATP concentrations can be represented by the empirical equation

\[ v = \frac{V + a/B}{1 + b/(B^2) + c/(B^3)} \]  

where \( v, a, b, \) and \( c \) are constants and \( [B] \) is the concentration of ATP. The constants \( V \) and \( a \) are directly proportional to enzyme concentration. This type of rate equation results if there are two kinetically significant binding sites, of which one can be an active site and the other an activating site, or both can be active sites. Further, the two active sites can be either different and independent or identical and interact in pairs (9-11).

The stoichiometry of the carbamyl phosphate synthetase reaction would suggest that at least in the presence of ammonium ions both sites are active.

**DISCUSSION**

The kinetic data presented in this paper are consistent with the concept that there are two kinetically significant ATP-binding sites on frog liver carbamyl phosphate synthetase. It is known from equilibrium dialysis experiments (2) that in the presence of magnesium ions 1 mole of ATP is bound per mole of enzyme in the absence of other substrates and acetylglutamate. Therefore, binding of ATP at a second site must be dependent upon prior binding of other substrates or acetylglutamate. Since two kinetically significant ATP-binding sites are discernible in the absence of ammonium ions, binding of ATP at the second site is not dependent upon prior binding of this ion. It is also known from equilibrium dialysis experiments (2) that acetylglutamate can be bound to the enzyme in the absence of substrates.

On the basis of the kinetic data presented in this paper, equilibrium dialysis experiments, and data presented in the accompanying paper, one can propose the following mechanism for carbamyl phosphate synthetase.

**MECHANISM I**

In Mechanism I, A is acetylglutamate, B is ATP, C is ammonium ion, and \( E^* \) is an altered form of the enzyme. It is assumed that \( P_1 \) is inorganic phosphate, \( P_2 \) is ADP, and \( P_3 \) is carbamyl phosphate. The sequence of addition of bicarbonate ions to the enzyme is not shown. It is known that bicarbonate ions are necessary for the hydrolysis of ATP in the absence of ammonium ions (1). Therefore, bicarbonate ions probably add to the enzyme at sometime during the first four steps of Mechanism I.

The complete steady state derivation of the rate equation for
Mechanism I would result in an extremely complicated equation. However, Mechanism I can be tested with the kinetic data presented in this paper.

In the absence of ammonium ions, Mechanism I simplifies to Mechanism II (reaction in the absence of ammonium ions).

\[
\begin{align*}
E + A & \xrightarrow{k_1} EA \\
E + B & \xrightarrow{k_2} EB \\
EA + B & \xrightarrow{k_3} EAB \\
EB + A & \xrightarrow{k_4} EAB
\end{align*}
\]

Mechanism II

In the presence of a high and constant amount of acetylglutamate, Mechanism II is further simplified because only Steps IIc, IIe, IIf, and IIg are involved.

In the absence of ammonium ions, it is impossible to determine whether ATP is active at both sites or whether binding of ATP at the second site results in activation of the reaction. The forms of the rate equation for Steps IIc, IIe, IIf, and IIg are identical for either case. The steady state derivation of the equation for these steps yields Equation 5 (9).

\[
v = \frac{V_1 (1 + K_{B^2})/[B]}{(1 + K_{B^2}/(1 - V_f/V_f)/[B]))}
\]

In Equation 5,

\[
V_f = k_{21} [E_2]
\]

\[
V_1 = \frac{k_{12} [E_2]}{(1 + K_{EAB}/K_{EAB^2})}
\]

\[
K_B = \frac{K_{EAB}}{(1 + K_{EAB}/K_{EAB^2})}
\]

\[
K_{B^2} = \frac{(K_{EAB})(K_{EAB^2} B') [1 - k_{12}/k_{21}]}{(K_{EAB} + 1/K_{EAB})}
\]

\[
K_{EAB} = \frac{k_2}{k_1}
\]

\[
K_{EAB^2} = \frac{k_3}{k_2}
\]

\[
K_{EAB^2} = \frac{k_4}{k_3}
\]

Table I: Variation of kinetic constants as function of acetylglutamate concentration in absence of ammonium ions

<table>
<thead>
<tr>
<th>Acetylglutamate concentration</th>
<th>(K_B)</th>
<th>(K_{B^2})</th>
<th>(v_1)</th>
<th>(V_f)</th>
</tr>
</thead>
<tbody>
<tr>
<td>mM</td>
<td>mM</td>
<td>mM</td>
<td>(\mu)</td>
<td>(\mu)</td>
</tr>
<tr>
<td>10</td>
<td>0.18</td>
<td>0.18</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>3</td>
<td>0.10</td>
<td>0.18</td>
<td>0.4</td>
<td>0.80</td>
</tr>
<tr>
<td>1</td>
<td>0.07</td>
<td>0.18</td>
<td>0.15</td>
<td>0.50</td>
</tr>
</tbody>
</table>
If the Michaelis constant, $K_B$, calculated from Equation 5 is an equilibrium constant, then the constant $K_{EAB'}$ must be infinitely high and the rate constant $k_1$ must be small compared to the rate constant $k_4$. Thus

$$K_B = \frac{K_{EAB}}{(1 + K_{EAB})/K_{EAB'}} = K_{EAB} = \frac{k_4}{k_3} \frac{[EA]}{[B]}$$

and

$$v_1 = \frac{k_3 [E_A]}{(1 + K_{EAB})/K_{EAB'}} = k_{17} [E_A]$$

Furthermore, if $K_{EAB'}$ is infinitely high, ATP is bound to a kinetically significant second site only after ATP is bound to the first site.

If the constant $K_B$ is equal to the equilibrium constant $K_{EAB}$ and the constant $V_1$ is equal to $k_{17} [E_A]$, then one can use Equation 6. On this basis, a plot of the apparent reciprocal values of $V_1$ versus the apparent reciprocal negative values of $K_B$ (obtained at different concentrations of acetylglutamate) should intersect at the point where the negative concentration of $B$ is equal to the reciprocal of the dissociation constant $K_{EB}$ (12). While it is difficult to evaluate accurately the constants $V_1$ and $K_B$ from the data of Fig. 5, and while these constants give only two points for each curve in the replot of Fig. 8, the value of $5 \times 10^{-2}$ M for the constant $K_{EB}$ can be estimated from Fig. 8. A secondary plot of the reciprocal values of $V_1$ from Table I versus the reciprocal of the concentration of acetylglutamate allows one to estimate the value of the constant $K_{EAB}$.

It is thus possible to estimate from these plots the following values of the dissociation constants: $K_{EAB} = 4 \times 10^{-5}$ M; $K_{EAB} = 1.8 \times 10^{-4}$ M; $K_{EB} = 5 \times 10^{-4}$ M; and since $(K_{EBA} K_{EB}) = (K_{EAB}) (K_{EA})$, then $K_{EA} = 1 \times 10^{-3}$ M.

In previous measurements of these constants by equilibrium dialysis (2) at $6^\circ$, the following values were obtained: $K_{EA} = 1.8 \times 10^{-6}$ M; $K_{EB} = 4 \times 10^{-6}$ M; and $K_{EAB} = 2 \times 10^{-4}$ M. Other measurements of these constants are reported in the accompanying paper (4).

In the accompanying paper (4), data are also presented which are consistent with the concept that ATP does not add to the second site in the absence of ATP on the first site (that is, $K_{EAB'}$ is infinitely high) and that in the absence of ammonium ions the Michaelis constant $K_B'$ is equal to the equilibrium constant $K_{EAB'}$ and is numerically equal to the equilibrium constant $K_{EAB}$. Therefore,

$$K_B' = K_{EAB'} = \frac{[EAB][B]}{[EADD']} = K_{EAB} = \frac{[EA][B]}{[EAD]}$$

It thus follows that the rate constant $k_{31}$ must be small compared to $k_{30}$ and that the rate constant $k_{17}$ must be small compared to $k_{15}$. Thus the original expression for $K_B'$,

$$K_B' = K_{EAB} K_{EAB'} [1 - k_{31}/k_{30}] / (K_{EAB} + 1) / K_{EAB'}$$

can be simplified to

$$K_B' = K_{EAB} K_{EAB'} k_{30} / k_{31} = [EAB] [B] / [EADD']$$

In the presence of ammonium ions, Mechanism I simplifies to Mechanism III (reaction in the presence of ammonium ions).

$$E + A \stackrel{k_1}{\rightarrow} EA \quad (IIIa)$$

$$E + B \stackrel{k_2}{\rightarrow} EB \quad (IIIb)$$

FIG. 7 (left). Double reciprocal plots of velocity versus ATP concentration in the presence of a constant and high concentration of ammonium ions ($1 \times 10^{-2}$ M) and at various constant levels of acetylglutamate. The concentrations of acetylglutamate used were: $A, 4 \times 10^{-4}$ M; $B, 1 \times 10^{-3}$ M; $G, 1 \times 10^{-2}$ M. The solid lines $A$, $B$, and $C$ were calculated by using Equation 6 and the values given in Table III. Other experimental conditions were identical with those described in the legend of Fig. 1.

FIG. 8 (right). Secondary plots of the kinetic constants $K_B$ and $V_1$ given in Table I.

$\frac{1}{(\text{ATP})(\text{mM})^{-1}}$
If in the presence of ammonium ions the concentration of acetylglutamate is maintained at a high level, Mechanism III is further simplified because only Steps IIIc, IIIe, IIIf, and IIIg are involved. Steps Ih, Ii, and Ij need not be considered, since when the concentration of acetylglutamate is high these steps are equivalent to Step IIIg.

If the concentrations of both ammonium ions and acetylglutamate are maintained at a high level, Mechanism III is further simplified since only Steps IIIc, IIIe, IIIf, and IIIg are involved. The steady state derivation of the rate equation for Steps IIIc, IIIf, and IIIg results in an equation identical in form with Equation 5. Under these conditions, $V_i$, $K_B$, $K_B'$, and $V_f$ refer to the values of these constants in the presence of ammonium ions. If the rapid equilibrium assumption is valid in the presence of ammonium ions (as it was in the absence of ammonium ions), then the rate from Step IIIg should vary with respect to the concentrations of acetylglutamate, ATP, and ammonium ions according to Equation 7.

$$v = \frac{V_i}{\left(1 + \frac{K_{B\text{app}}}{[A]} + \frac{K_{B\text{app}}}{[B]} + \frac{K_{B\text{app}}}{[A][B]} + \frac{[C]}{K_i}\right)} \tag{7}$$

In Equation 7, all of the constants have the same definition as used in Equation 6 and

$$K_i = \frac{[EAB][C]}{[EABC]}$$

Thus, when the concentration of acetylglutamate is maintained high in the presence of ammonium ions, the apparent value of $K_B$ in Equation 5 is

"$K_B$ app" = \frac{K_{EB\text{app}}}{(1 + [C])/K_i}

and the apparent value of $V_i$ is

"$V_i$ app" = \frac{V_i}{(1 + [C])/K_i}

From Equation 5 and the data of Fig. 6, it can be seen that, in the presence of high concentrations of acetylglutamate, ammonium ions increase the values of $V_i$ and $K_B'$ but decrease the values of $V_f$ and $K_B$. The values of these constants at various concentrations of ammonium ions are shown in Table II and reveal that when the concentration of ammonium ions does not exceed $2 \times 10^{-3} \text{ M}$ the influence of ammonium ions on the constants $K_B$ and $V_i$ can be represented by Equation 8.

$$v = \frac{V_i}{1 + K_B/[B] + [C]/K_i} \tag{8}$$
In Mechanism IV, free enzyme, $E$, is not present since ATP is maintained at a concentration higher than the value of $K_B$ ($k_4/k_3$) in the presence of ammonium ions. Under these conditions the reciprocal plots are linear. The rate can be represented by Equation 9,

$$v = \frac{E_b}{V_f} \left( 1 + \frac{K_A}{[A]} \right) \left( 1 + \frac{K_B'}{[B]} + \frac{K_C'}{[C]} \right)$$

where

$$K_A = K_{EA} = \frac{k_3}{k_1}$$

$$V_f = \frac{k_{11}k_{10}}{(k_a + k_0)}$$

$$K_B' = \frac{(k_0 + k_{12})}{k_{12}k_4} (V_f)$$

$$K_C = \frac{k_{11} + k_{10}}{k_4} (V_f)$$

In deriving this equation, it is assumed that the total concentration of enzyme combined with acetylglutamate can be represented by $["EA"]$, which is equal to $[EA] + [EAB] + [EABC] + [E*] + [E*B'] = \frac{[EB][A]}{K_A}$

and the total amount of enzyme, $[E_b]$, is equal to $[E_b] = [EB] + ["EA"] - [EB]$.

Thus acetylglutamate can be considered to be acting as an activator rather than as a substrate. Prior binding of the enzyme with acetylglutamate is thus necessary for the binding of ammonium ions and binding of ATP to a kinetically significant second site. From Equation 9, it can be seen that when the concentration of $A$ is held high, the rate must vary as a function of the concentration of $B$ and $C$ in the manner given by Equation 3; and when the concentrations of $B$ or $C$ are held high, Equation 9 reduces to the form of Equation 1. Thus acetylglutamate can be bound to the enzyme in the absence of ATP and that the dissociation and Michaelis constants for acetylglutamate are identical. Consequently this mechanism does not apply to carbamyl phosphate synthetase.

While ammonium ions are necessary for the synthesis of carbamyl phosphate, they inhibit ADP production at Steps 1g and H. If one assumes that in the presence of ammonium ions the rates of these steps are negligible compared to the rate of Step If, then the rate of carbamyl phosphate production (or the rate of ADP production) in the presence of ammonium ions can be approximated by Mechanism III with the exclusion of Step IIIg.

In this mechanism ATP and acetylglutamate in the presence of high concentrations of bicarbonate ions form an activated enzyme complex, $EAB$. The complete steady state derivation of the rate equation for this mechanism would result in an extremely complicated equation. The equation can be simplified, however, if one assumes that the equilibria involved in Steps IIIa through IIIc adjust rapidly compared to Steps IIIe and IIIf. This is not an unreasonable assumption, since Steps IIIa through IIIc have been shown to be consistent with a rapid equilibrium assumption. The concentration of $EAB$ would thus be

$$[EAB] = \frac{[E_b]}{\left( 1 + \frac{K_A}{[A]} + \frac{K_B}{[B]} + \frac{K_AK_B}{[A][B]} \right)}$$

The over-all rate can then be expressed by the equation

$$v = \frac{V_f}{1 + \frac{K_A}{[A]} + \frac{K_B}{[B]} + \frac{K_AK_B}{[A][B]} \left( 1 + \frac{K_B'}{[B]} + \frac{K_C'}{[C]} \right)}$$

Since Equation 10 refers to the rate only in the presence of ammonium ions, $K_A = K_{EA}$, $K_B = K_{EB}$, $K_AK_B = K_{EAK_EB}$, and $K_B'$ and $K_C'$ have the values previously defined when they were used in Equation 9.

If the numerical values of the constants obtained from the data in the presence of ammonium ions are used (see Table IV), then this equation accurately describes the results found. When the concentration of ATP exceeds $K_B$, Equation 10 simplifies to Equation 9. When the concentrations of acetylglutamate and ammonium ions are high, Equation 9 becomes

$$v = \frac{V_f}{1 + \frac{K_B}{[B]} + \frac{K_B'}{[B]} + \frac{K_BK_B'}{[B]^2}}$$

The kinetic pattern of the data presented in Figs. 1, 2, and 3 is similar to that reported by Frieden for glutamic dehydrogenase (14). Frieden explored various mechanisms involving the addition of three substrates and found that only a steady state mechanism involving a sequential order of addition would fit his data.

By use of the equation derived by Frieden (14) for the glutamic dehydrogenase system, it can be seen that if the mechanism of action of carbamyl phosphate synthetase is similar, acetylglutamate could be bound to its kinetically active site only in the presence of ATP. However, it has been shown by equilibrium dialysis (2) and in experiments to be reported in the accompanying paper (4) that acetylglutamate can be bound to the enzyme in the absence of ATP and that the dissociation and Michaelis constants for acetylglutamate are identical. Consequently this mechanism does not apply to carbamyl phosphate synthetase.

While ammonium ions are necessary for the synthesis of carbamyl phosphate, they inhibit ADP production at Steps 1g and H. If one assumes that in the presence of ammonium ions the rates of these steps are negligible compared to the rate of Step If, then the rate of carbamyl phosphate production (or the rate of ADP production) in the presence of ammonium ions can be approximated by Mechanism III with the exclusion of Step IIIg.

In this mechanism ATP and acetylglutamate in the presence of high concentrations of bicarbonate ions form an activated enzyme complex, $EAB$. The complete steady state derivation of the rate equation for this mechanism would result in an extremely complicated equation. The equation can be simplified, however, if one assumes that the equilibria involved in Steps IIIa through IIIc adjust rapidly compared to Steps IIIe and IIIf. This is not an unreasonable assumption, since Steps IIIa through IIIc have been shown to be consistent with a rapid equilibrium assumption. The concentration of $EAB$ would thus be

$$[EAB] = \frac{[E_b]}{\left( 1 + \frac{K_A}{[A]} + \frac{K_B}{[B]} + \frac{K_AK_B}{[A][B]} \right)}$$

The over-all rate can then be expressed by the equation

$$v = \frac{V_f}{1 + \frac{K_A}{[A]} + \frac{K_B}{[B]} + \frac{K_AK_B}{[A][B]} \left( 1 + \frac{K_B'}{[B]} + \frac{K_C'}{[C]} \right)}$$

Since Equation 10 refers to the rate only in the presence of ammonium ions, $K_A = K_{EA}$, $K_B = K_{EB}$, $K_AK_B = K_{EAK_EB}$, and $K_B'$ and $K_C'$ have the values previously defined when they were used in Equation 9.

If the numerical values of the constants obtained from the data in the presence of ammonium ions are used (see Table IV), then this equation accurately describes the results found. When the concentration of ATP exceeds $K_B$, Equation 10 simplifies to Equation 9. When the concentrations of acetylglutamate and ammonium ions are high, Equation 9 becomes

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In Fig. 9 the rate of ADP production in the absence of ammonium ions has been subtracted from the rate in the presence of ammonium ions. It can be seen that the resulting curve agrees with a theoretical curve calculated by using Equation 10a.

In the absence of ammonium ions the complex EAB is hydrolyzed. Ammonium ions thus act as an uncompetitive inhibitor by converting this unstable complex to a more stable complex. As pointed out earlier in this paper, the kinetic data presented in this paper were based on linear rates which followed a brief initial lag. It is conceivable that this initial lag represents the time necessary to accumulate amounts of EAB complex to steady state levels.

It has been demonstrated that when all of the constituents of the carbamyl phosphate synthetase reaction are present at saturating concentrations, the ratio $V_f/V_1$ exceeds a value of 10. This is consistent with stoichiometric measurements, which revealed that the ratio of ATP utilized to carbamyl phosphate produced was always slightly greater than 2 (1).

The Michaelis constants for ATP, $K_{d'}$; acetylglutamate, $K_d$; and ammonium ions, $K_c$, reported in the present study agree well with previous measurements of these constants performed at 37° when the rate of carbamyl phosphate produced was measured (2). Discrepancies can be explained on the basis of a temperature difference of the two systems as described in the accompanying paper (4).

**SUMMARY**

Kinetic properties of frog liver carbamyl phosphate synthetase have been studied. Kinetic equations previously derived for one-, two-, and three-substrate reactions and for reactions involving two substrates and an activator were applied to the data. The kinetic data are consistent with the following previously known facts about the enzyme: (a) acetylglutamate is an activator and not a substrate; (b) 2 moles of adenosine triphosphate are utilized for each mole of carbamyl phosphate produced; and (c) adenosine triphosphate can be bound to the enzyme in the absence of acetylglutamate.

The kinetic data lead to the following conclusions. (a) There are two discernible, kinetically active ATP-binding sites of the enzyme. ATP can add to the "first site" of the enzyme in the absence of acetylglutamate, but acetylglutamate increases the dissociation constant of ATP at this site. A second ATP-binding site requires prior binding of acetylglutamate. In the presence of ammonium ions and in the presence of saturating amounts of acetylglutamate, these two sites are kinetically indistinguishable. (b) Adenosine diphosphate can be produced by two different steps. Ammonium ions inhibit the velocity of one step but accelerate a second, which is associated with carbamyl phosphate synthesis. (c) In the presence of ammonium ions the Michaelis constant of acetylglutamate has the same numerical value as the dissociation constant of the enzyme-acetylglutamate complex, and the Michaelis constant of ATP at the "first site" is equal to the dissociation constant of the enzyme-ATP complex. In the absence of ammonium ions, the Michaelis constants for ATP at both sites are identical and are equal to the dissociation constant of the enzyme-acetylglutamate-ATP complex. The dissociation constant of the enzyme-ATP complex in the absence of ammonium ions has also been estimated. (d) A mechanism for the reaction catalyzed by carbamyl phosphate synthetase has been proposed, based on kinetic data.

**Addendum**—As previously stated, the steady state derivation of the rate equation for Mechanism I would be extremely complex. In this paper an attempt has been made to explain the data obtained with an essentially empirical equation (Equation 10), which involves steady state and rapid equilibrium assumptions. As demonstrated in the simpler case, which occurs in the absence of ammonium ions (Mechanism II), Michaelis and dissociation constants for ATP and acetylglutamate are identical. On the assumption of a rapid equilibrium, a mechanism can be proposed which also explains the rates observed in the presence of ammonium ions. In the presence of saturating amounts of acetylglutamate this mechanism is

$$EA + B = EAB \xrightarrow{k_1} EA + P_1 + P_2$$  \hspace{1cm} (Va)

$$EAB + B = EABB' \xrightarrow{k_2} EA + P_1 + P_2$$  \hspace{1cm} (Vb)

$$EAB + C = EABC$$  \hspace{1cm} (Vc)
Under these conditions Equation 11 simplifies to 11b.

\[ v = \frac{V_1}{(1 + [C]/K_{EABB'C}) + [C]/K_{EABB'C}} \]

With Equation 11 and the data of Fig. 3, the values of the constants have been calculated to be as follows:

- \( K_{EABC} = 7 \times 10^{-4} \text{ M} \)
- \( K_{EABB'C} = 2 \times 10^{-3} \text{ M} \)
- \( K_{EABCB'} = 5 \times 10^{-3} \text{ M} \)

Thus, the values of the constants are:

- \( V_1 = 1.0 \mu \text{M min}^{-1} \)
- \( V_f = 2.8 \mu \text{M min}^{-1} \)

REFERENCES

A Kinetic Study of Carbamyl Phosphate Synthetase
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