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SULFHYDRYL INHIBITORS AND ENZYME SUBSTRATE INTERACTIONS OF CARBAMYL PHOSPHATE SYNTHETASE

WILLIAM B. NOVOA AND SANTIAGO GRISOLIA

The Department of Biochemistry, University of Kansas Medical Center, Kansas City, Kan. 66103 (U.S.A.)

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SUMMARY

1. The indispensable cofactor for carbamyl phosphate synthetase (ATP: carbamate phosphotransferase (dephosphorylating), EC 2.7.2.5), *N*-acetyl-L-glutamate, markedly accelerates inactivation of this enzyme by the disulfide 5,5′-dithiobis(2,2′-nitrobenzoate) (DTNB). This effect has been used to examine the interaction of the enzyme with its different substrates and cofactors. The following results have been obtained.

2. ATP in the presence of Mg^{2+} is the only substrate to protect against the inactivation. This protection is considerably enhanced if K^+ is present. HCO_3^- augments the protective effect of ATP and K^+, but provides no protection by itself. ADP does not protect against the inactivation.

3. Mg^{2+} has been found to increase the rate of inactivation. This effect is particularly marked when acetyl glutamate is present. In the absence of Mg^{2+}, the enzyme appears to bind acetyl glutamate at two sites, the second of which is detectable only at high concentrations of acetyl glutamate. Furthermore, inactivation is much slower at all except high levels of cofactor. In contrast, when Mg^{2+} is present, the rate of inactivation is much greater, and the second binding site for acetyl glutamate is not detectable.

4. The significance of these findings for the enzymatic reaction has been tested by determining the K_m for acetyl glutamate in solutions containing either a slight excess of ATP over Mg^{2+}, or a large excess of Mg^{2+} over ATP. When unbound Mg^{2+} is low, the K_m for acetyl glutamate is doubled in value, and the highest levels of acetyl glutamate produce an inhibition of the reaction. When the level of free Mg^{2+} is high, there is a lower K_m for acetyl glutamate, and no inhibition by acetyl glutamate. If the inhibitory effects of high acetyl glutamate concentration in the plot with low Mg^{2+} concentration are ignored, the extrapolated maximum velocity is identical in both cases.

5. None of the other substrates of the enzyme produce any significant effect upon the inactivation.

6. The inactivation of the enzyme by organic mercurials, which is reduced

Abbreviation: DTNB, 5,5′-dithio-bis(2,2′-nitrobenzoic acid).

instead of increased by acetyl glutamate, has been reinvestigated in the light of the above results. Mg^{2+} reduces the protection by acetyl glutamate, but does not reduce inactivation in the absence of cofactor. Comparison of protection by acetyl glutamate against a variety of organic mercurials has revealed that the cofactor protects against mercurials in a manner which is independent of the nature of the organic portion of the inhibitor.

INTRODUCTION

The enzyme carbamyl phosphate synthetase (ATP: carbamate phosphotransferase (dephosphorylating), EC 2.7.2.5) which catalyzes the following reaction:

$$ATP + NH_4^+ + HCO_3^- \rightarrow \text{carbamyl phosphate} + 2\,ADP + P_i$$

requires the cofactor *N*-acetyl-L-glutamate for activity[1]. It has been shown that this cofactor, alone or in combination with substrates, will markedly affect the stability of the enzyme[2,3].

Previous studies on the inactivation of the enzyme by sulfhydryl reagents have shown that acetyl glutamate will greatly increase inactivation by reagents such as DTNB, but that ATP will prevent this effect[4,5]. These findings suggested that inactivation by this reagent could be used to examine the interaction of the enzyme with its other substrates or cofactors. The results obtained, as well as some possible interpretations, are presented here.

MATERIALS

Frog liver carbamyl phosphate synthetase was prepared as described by RAIJMAN AND GRISOLIA[6]. The enzyme preparation was carried to the acetone precipitate stage and then lyophilized for storage.

The reagents used were standard commercial products.

METHODS

Carbamyl phosphate synthetase was assayed as described previously[4]. Protein was determined by the procedure of MOKRASCH AND MCGILVERY[7]. The inactivation studies were carried out as follows: solutions containing enzyme and the appropriate combination of acetyl glutamate and substrate were incubated in a water bath for 5 min or longer to allow temperature equilibration. The DTNB was added, incubation was continued for the interval indicated, and then samples of 0.1 ml were withdrawn for assay of enzyme activity. In all cases, enzyme activity is expressed as the μmoles of citrulline synthesized by these samples of 0.1 ml.

In experiments designed to show the effect of K^+, all reagents were neutralized with Tris. In all other cases, KOH was used to neutralize reagents.

TABLE I

EFFECT OF K^+ ON ATP PROTECTION AGAINST DTNB

Each tube contained 0.41 mg of enzyme in 2.0 ml containing 100 μmoles of Tris buffer (pH 7.4), and the following additions where indicated: 10 μmoles acetyl glutamate, 0.01 μmoles DTNB, 8 μmoles ATP, 20 μmoles $MgSO_4$, 100 μmoles NaCl, 100 μmoles KCl, and 100 μmoles $NaHCO_3$. After incubation for 25 min at 30°, samples of 0.1 ml were taken for assay of enzyme activity.

Additions	*Citrulline synthesized (μmoles)*
None	0.31
Acetyl glutamate, DTNB	0.09
Acetyl glutamate, DTNB, ATP, $MgSO_4$	0.19
Acetyl glutamate, DTNB, ATP, $MgSO_4$, NaCl	0.20
Acetyl glutamate, DTNB, ATP, $MgSO_4$, KCl	0.29
Acetyl glutamate, DTNB, ATP, $MgSO_4$, $NaHCO_3$	0.14

RESULTS

Factors influencing protection by ATP

The protective effect of ATP against the acetyl glutamate-induced DTNB inactivation, which has been demonstrated previously[5], is a complex phenomenon when examined closely. Table I shows that ATP alone only prevents about half the inactivation. Addition of KCl, however, gives essentially complete protection against DTNB, an effect which cannot be produced by NaCl. Interestingly, although $NaHCO_3$ produces a slight decrease in the protection against DTNB, the most complete protection is provided by a combination of ATP, Mg^{2+}, K^+, and HCO_3^-. Since KCl augments protection considerably, and since HCO_3^- is present in all solutions exposed to the air, the protective effect of added $KHCO_3$ is best shown when the period of incubation with DTNB is extended for several hours. Table II shows that under these conditions $KHCO_3$ provides a protection against DTNB which is significantly greater than that of a similar solution containing KCl instead of the $KHCO_3$. The control

TABLE II

EFFECT OF HCO_3^- ON ATP PROTECTION AGAINST DTNB

Each tube contained in a volume of 2 ml: 0.42 mg protein, 100 μmoles Tris buffer (pH 7.4), and where indicated: 10 μmoles acetyl glutamate, 8 μmoles ATP, 20 μmoles $MgSO_4$, 100 μmoles $KHCO_3$, 100 μmoles KCl, and 0.01 μmole DTNB. After incubation for 3 h at 30°, samples of 0.1 ml were taken for assay of enzyme activity.

Additions to preincubation mixture						*Citrulline found (μmoles)*
Acetyl glutamate	*DTNB*	*ATP*	*$MgSO_4$*	*$KHCO_3$*	*KCl*	
						0.34
+		+	+	+		0.34
+	+	+	+	+		0.31
+	+	+	+		+	0.23
+	+					0.01
+	+	+		+		0.01

TABLE III

FAILURE OF ADP TO PROTECT AGAINST DTNB

Each tube contained in a volume of 2 ml: 0.29 mg of enzyme, 100 μmoles of Tris buffer (pH 7.4), and where indicated: 10 μmoles acetyl glutamate, 0.01 μmole DTNB, 10 μmoles ADP, and 20 μmoles $MgSO_4$. Samples were incubated for 25 min at 30°, at which time samples of 0.1 ml were taken for assay of enzyme activity.

Additions	*Citrulline synthesized (μmoles)*
None	0.41
Acetyl glutamate, DTNB	0.11
Acetyl glutamate, DTNB, ADP	0.08
Acetyl glutamate, DTNB, ADP, $MgSO_4$	0.09

containing ATP, Mg^{2+}, and HCO_3^- but not DTNB, shows no more activity than the control containing enzyme alone. Therefore, it is unlikely that contaminating ammonium salts could have formed enough carbamyl phosphate, during the incubation, to interfere with the assay. Table II also shows that protection by ATP is, as expected, dependent upon the presence of Mg^{2+}.

The protective effect of ATP is unchanged over a concentration range of 0.001 0.016 M.

Effect of ADP upon DTNB inactivation

Table III shows that ADP, in contrast to ATP, will not protect against DTNB inactivation. Addition of Mg^{2+} does not alter this behavior.

Before it can be concluded that the enzyme–ADP complex is not resistant to DTNB inactivation, it must be shown that the complex exists under similar conditions. The clearest demonstration of this is the fact that ADP will influence the thermal inactivation of carbamyl phosphate synthetase, as shown in Table IV. In the absence

TABLE IV

EFFECT OF ADP ON THE THERMAL STABILITY OF CARBAMYL PHOSPHATE SYNTHETASE

Each tube contained 1.0 mg of enzyme and 100 μmoles of Tris buffer (pH 7.4 at 37 °) in 2.0 ml of solution, and the following additions as indicated: 10 μmoles acetyl glutamate, 20 μmoles $MgSO_4$, and 10 μmoles ADP. Samples were taken for assay of enzyme activity before and after incubation for 5 min at 52°.

Additions	*Citrulline synthesized (μmoles)*	
	Before heating	*After heating*
None	0.50	0.37
Acetyl glutamate	0.50	0.29
Acetyl glutamate, ADP	0.45	0.17
Acetyl glutamate, $MgSO_4$	0.48	0.22
Acetyl glutamate, ADP, $MgSO_4$	0.45	0.38

* Since the pH of the Tris buffer during heating should have dropped only to about 7.0, no adjustment was made for the effects of temperature change.

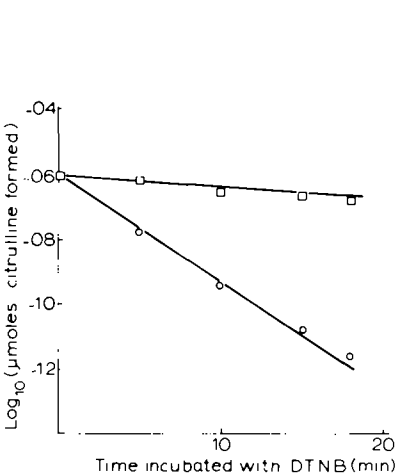
of Mg^{2+}, ADP increases the rate of inactivation, while in its presence ADP provides protection. In either case, interaction of ADP with the enzyme is evident. The fact that ADP inhibits the synthetase competitively against ATP, with a K_i of $1 \cdot 10^{-3}$ M, is also consistent with such a conclusion.

The increase in acetyl glutamate-induced thermal inactivation produced by addition of Mg^{2+} has been shown before by CARAVACA AND GRISOLIA[2] and will be discussed elsewhere in this paper.

The influence of Mg^{2+} upon acetyl glutamate-induced DTNB inactivation

Although Mg^{2+} is required for the protective effect of ATP, it will accelerate the rate of enzyme inactivation when ATP is absent. The presence of Mg^{2+} typically doubles the rate of inactivation, even when acetyl glutamate is not present. For example, in a typical experiment, addition of Mg^{2+} increased the amount of inactivation from 5 to 11% when acetyl glutamate was not present, and from 29 to 59% when the cofactor was present. Because so little inactivation occurs in the absence of acetyl glutamate, it has been found most advantageous to study the effect of Mg^{2+} by examining its influence upon the cofactor-induced inactivation.

The action of Mg^{2+} is best illustrated by a double reciprocal plot of the rate of inactivation *versus* the acetyl glutamate concentration. It has been shown that the acetyl glutamate-induced thermal inactivation of carbamyl phosphate synthetase is half maximal at an acetyl glutamate concentration close to its K_m value[8]. A modification of this technique has been used by CITRI AND ZYK[9] in studying the substrate



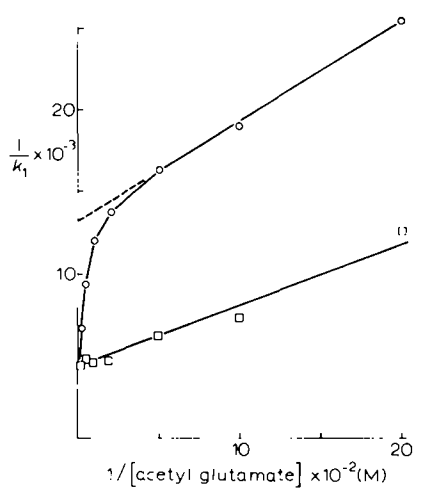


Fig. 1. Kinetics of inactivation of carbamyl phosphate synthetase by DTNB. Tubes contained the following in 2.0 ml: 0.39 mg enzyme, 100 μmoles Tris buffer (pH 7.4), and 0.01 μmole DTNB. Other additions were: □, none; ○, 10 μmoles acetyl glutamate. Solutions were incubated at 30°, and at the intervals indicated, samples of 0.1 ml were withdrawn for determination of enzyme activity.

Fig. 2. Effect of Mg^{2+} upon the acetyl glutamate-induced inactivation. First-order constants, obtained as described in the text, are expressed in sec^{-1}. Each tube contained 0.39 mg of enzyme, 100 μmoles of Tris buffer (pH 7.4), 0.01 μmole of DTNB, and the molarity of acetyl glutamate indicated in 2.0 ml. Other additions were: □, none; ○, 20 μmoles $MgSO_4$. All incubations were for 8 min at 30°.

analog-induced thermal inactivation of penicillinase. These investigators used a double reciprocal plot of the first-order constant for the rate of inactivation *versus* the substrate analog concentration to obtain a constant equivalent to the K_m for a Lineweaver Burk plot. However, they pointed out that such a constant is not necessarily an equilibrium constant, as the enzyme may remain in an altered and unstable conformation for some time after the substrate analog has dissociated from it.

The kinetics of inactivation of carbamyl phosphate synthetase can be seen in Fig. 1, which shows a plot of the logarithm of the enzyme activity remaining against the time of incubation with DTNB. The straight line, which is produced till about 70% of the activity has been lost, is characteristic of first-order kinetics. Therefore, first-order constants for the rate of inactivation could be calculated by using the integrated form of the equation for a first-order reaction. A double reciprocal plot of the rate of inactivation, obtained in this manner, *versus* the acetyl glutamate concentration is shown in Fig. 2. The enzyme was incubated with DTNB for an interval of only 8 min, so no tube showed more than 66% loss of activity. It can be seen that in the presence of Mg^{2+}, the rate of inactivation is much greater at most levels of acetyl glutamate, and there appears to be only one type of binding site for the cofactor. The constant has a value of $8.6 \cdot 10^{-4}$ M. In contrast, in the absence of Mg^{2+}, there is much less inactivation, except at the highest levels of acetyl glutamate. In addition, there appear to be two kinds of sites, one with a constant of $4.7 \cdot 10^{-4}$ M, and a second with a constant of $2.5 \cdot 10^{-2}$ M.

The relationship of these findings to the enzymatic reaction has been examined in the following manner: Although the enzymatic reaction cannot be carried out in the absence of Mg^{2+}, it can be carried out with either ATP or Mg^{2+} in excess. A Lineweaver Burk plot of the effect of acetyl glutamate on the reaction velocity, in which the effect of excess Mg^{2+} is contrasted with the effect of excess ATP, can be seen in Fig. 3. Adding an excess of about 1 μmole of ATP over the Mg^{2+}, reduces the free Mg^{2+} level to about $4 \cdot 10^{-4}$ M, if a value of $1 \cdot 10^4$ is used for the association constant of the

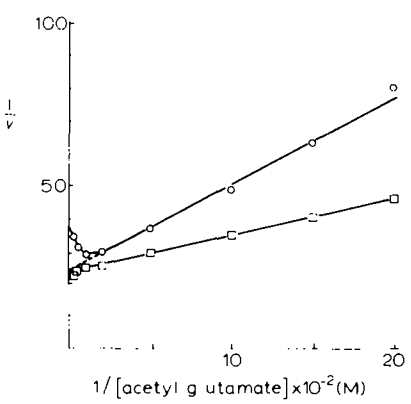


Fig. 3. Effect of low and high free Mg^{2+} levels upon the K_m of acetyl glutamate for the carbamyl phosphate synthetase reaction. Enzyme velocities are expressed as the μmoles of citrulline synthesized in 15 min at 38°, and acetyl glutamate concentrations are in molarities. Each tube contained the following in 2.0 ml: 0.022 mg of enzyme, 100 μmoles Tris buffer (pH 7.4), 10 μmoles acetyl glutamate, 10 μmoles ornithine, 100 μmoles $KHCO_3$, 50 μmoles NH_4Cl, and 10 units ornithine transcarbamylase. Other additions were: ○, 8 μmoles ATP and 7 μmoles $MgSO_4$; ●, 8 μmoles ATP and 27 μmoles $MgSO_4$.

complex[10]. On the other hand, adding an excess of about 20 μmoles of Mg^{2+} over the ATP present, gives a free Mg^{2+} level of about 0.01 M in a 2-ml volume. The K_m for acetyl glutamate, which is about $5 \cdot 10^{-4}$ M in the presence of high Mg^{2+} concentration, is increased to about $1 \cdot 10^{-3}$ M under conditions of low free Mg^{2+} concentration. In addition, when the concentration of Mg^{2+} is low, high acetyl glutamate levels inhibit by binding at a site with a K_i of about $2 \cdot 10^{-2}$ M as determined by the method of DIXON AND WEBB[11]. This inhibitory site appears to be identical to the second binding site for acetyl glutamate revealed by DTNB inactivation in Fig. 2, as both require high levels of acetyl glutamate, and as neither site is detectable at high levels of Mg^{2+}.

Influence of other substrates upon the rate of enzyme inactivation by DTNB

Neither NH_4^+ nor HCO_3^- produce any major effect upon the rate of inactivation, whether or not acetyl glutamate is present. However, HCO_3^- does produce a slight but consistent increase in the rate of inactivation.

Acetyl phosphate, which is a substrate for the synthesis of ATP from ADP by carbamyl phosphate synthetase[12], fails to produce any significant effect upon the inactivation by DTNB.

TABLE V

EFFECT OF Mg^{2+} ON INACTIVATION OF CARBAMYL PHOSPHATE SYNTHETASE BY MERCURIBENZOATE

Each tube contained the following components in a final volume of 2.0 ml: 0.5 mg of enzyme, 50 μmoles Tris buffer (pH 7.4), and where indicated: 10 μmoles acetyl glutamate, 20 μmoles $MgSO_4$, and 0.02 μmole mercuribenzoate. Solutions were incubated for 5 min after which samples of 0.1 ml were withdrawn for assay of enzyme activity.

Additions	*Citrulline found (μmoles)*
None	0.35
Mercuribenzoate	0.03
Mercuribenzoate, acetyl glutamate	0.13
Mercuribenzoate, Mg^{2+}	0.03
Mercuribenzoate, acetyl glutamate, Mg^{2+}	0.07

Inactivation of carbamyl phosphate synthetase by organic mercurials

Although acetyl glutamate accelerates the inactivation by DTNB, MARSHALL *et al.*[13] have reported that it protects against inactivation by mercuribenzoate. Subsequent investigation of the effect of acetyl glutamate upon inactivation by sulfhydryl reagents showed a wide range of results[5]. Reagents such as *N*-ethylmaleimide and iodoacetamide were similar to DTNB, while mercuriphenyl sulfonate and $AgNO_3$ were similar to mercuribenzoate. The results obtained with Mg^{2+}, described previously, indicated that it might be appropriate to reinvestigate this phenomenon.

The effect of Mg^{2+} upon inactivation by mercuribenzoate can be seen in Table V. The primary effect of Mg^{2+} is to decrease protection by acetyl glutamate. Since Mg^{2+} reverses the action of acetyl glutamate on mercuribenzoate inactivation, a study similar to that shown in Fig. 2 could not be duplicated for this reagent. However, it has already been shown by MARSHALL *et al.*[13] that protection by acetyl glutamate in

TABLE VI

INACTIVATION OF CARBAMYL PHOSPHATE SYNTHETASE BY ORGANIC MERCURIALS WITH DIFFERENT STRUCTURES

Each tube contained the following components in a final volume of 2.0 ml: 0.6–0.7 mg enzyme, 50 μmoles Tris buffer (pH 7.4), and where indicated: 10 μmoles acetyl glutamate, and 0.03 μmole mercurial. Solutions were incubated at 30° for 5 min, after which samples were withdrawn for assay of enzyme activity.

Organic mercurial	*Activity remaining after incubation*		
	With acetyl glutamate (%)	*Without acetyl glutamate* (%)	*Difference* (%)
p-Mercuribenzoate	9	52	−43
Phenylmercuric chloride	9	39	−30
p-Aminophenylmercuric acetate	7	55	−48
Methylmercuric iodide	9	69	−60
3-Chloromercuri-2-methoxypropyl urea	9	47	−38

the absence of Mg^{2+}, is complete at a concentration of $5 \cdot 10^{-4}$ M, and is not altered when the level of cofactor is increased to 0.01 M. This finding has been duplicated in our laboratory. Not surprisingly, ATP and Mg^{2+} had no influence on the inactivation of carbamyl phosphate synthetase by mercuribenzoate[5].

The behavior of organic mercurials was further investigated by inactivating carbamyl phosphate synthetase with a series of reagents in which the organic moiety differed. These included ionic and nonionic, aromatic or aliphatic, and hydrophobic or hydrophilic residues. The results can be seen in Table VI. It is apparent that the nature of the organic portion of mercurials has only a slight effect upon acetyl glutamate protection against inactivation.

DISCUSSION

The results obtained show that the greatest protection against DTNB inactivation of carbamyl phosphate synthetase is provided by a combination of ATP, Mg^{2+}, K^+, and HCO_3^-. This can most readily be interpreted as resulting from the existence of an enzyme complex with the composition of *E* ATP Mg^{2+} K^+-HCO_3^-. This could possibly be the activated *E*–"CO_2" complex suggested by METZENBERG *et al.*[14] as an intermediate in the catalytic reaction. This assumption is strengthened by the fact that no protection is provided, either by HCO_3^- in the absence of ATP, or by ADP, a substance which closely resembles ATP and presumably binds to the enzyme at the same site.

It has been shown by MARSHALL *et al.*[13] that K^+ will alter the kinetic behavior of carbamyl phosphate synthetase when the substrate NH_4^+ is present at low concentrations. Apparently, the enzyme has a requirement for a monovalent cation, which can be filled by either K^+ or NH_4^+. Therefore, it is not surprising to find that K^+ will influence the rate of DTNB inactivation.

The effects of Mg^{2+} upon carbamyl phosphate synthetase, which have been described, suggest that this metal interacts with the enzyme directly, in addition to

forming an ATP Mg^{2+} chelate which serves as a substrate for the reaction. In this connection, it is interesting to note that MARSHALL *et al.*[13] have reported that carbamyl phosphate synthetase has a requirement for Mg^{2+} in amounts greater than needed to complex with ATP. A similar result has also been published for the enzyme from rat liver[15]. The effect of Mg^{2+} upon the interaction of acetyl glutamate with the enzyme differs, depending upon whether the level of cofactor is high or low. The data on DTNB inactivation from Fig. 2, as well as the data on enzyme kinetics from Fig. 3, both show that the Mg^{2+}-deficient enzyme exhibits a second, high dissociation constant, site for binding acetyl glutamate. This site cannot be detected in the presence of excess Mg^{2+}. A somewhat similar phenomenon has been observed with the thermal inactivation of carbamyl phosphate synthetase. It has been shown by CARAVACA AND GRISOLIA[2] that acetyl glutamate will accelerate the rate of thermal inactivation of the enzyme. Subsequently FAHIEN *et al.*[16] demonstrated that much higher levels of acetyl glutamate decrease the inactivation, apparently by binding at the second site for acetyl glutamate. In contrast to these studies, the second site for acetyl glutamate cannot be detected by inactivation with mercuribenzoate[13].

The best indication of the significance of this second binding site to the catalytic activity of the enzyme is the data obtained from enzyme kinetics in the presence of high and low levels of Mg^{2+}. It is obvious that binding at the second site is inhibitory to catalytic activity. Furthermore, if the points obtained at inhibitory levels of acetyl glutamate are neglected, the extrapolated maximum velocity for the enzyme is identical whether or not an excess of Mg^{2+} is present. Thus, the second site for binding cofactor is probably a non-specific site which is not involved in the catalytic reaction, and which disappears when Mg^{2+} is present.

Examination of the data in Fig. 2 shows that, if values at high acetyl glutamate concentration are ignored, the main effect of Mg^{2+} is to increase the maximal rate of inactivation by DTNB. There is also an increase in the K for acetyl glutamate. In contrast, the effect of Mg^{2+} upon the kinetics of the enzyme is to decrease the K_m for acetyl glutamate, but not to change the V_{max}. Typically, in enzyme systems where a cofactor must be bound to the enzyme before the substrate is bound, the K_m for the cofactor will decrease as the concentration of the substrate is raised[17]. An interpretation which is consistent with the above findings is that both acetyl glutamate and Mg^{2+} bind to the enzyme, independently of each other, to produce a cooperative conformational change in the enzyme. This is necessary before the enzyme can form the activated E "CO_2" complex which has been postulated as an intermediate in the reaction. The change produced by Mg^{2+} upon the enzyme seems to reinforce the effects of acetyl glutamate, although it eliminates the second, noncatalytically functional site for acetyl glutamate. This explanation implies that the binding of Mg^{2+} to the enzyme may be essential for catalytic activity. Since carbamyl phosphate synthetase has an absolute requirement for a Mg^{2+} ATP complex as substrate, it is experimentally difficult to ascertain the extent of the requirement for free Mg^{2+}. However, it has been shown that a 2–3-fold excess of ATP over Mg^{2+} produces almost complete inhibition of the enzyme[13], an effect which has been duplicated in this laboratory. This is consistent with the possibility that free Mg^{2+} is indispensable for catalytic activity. However, inhibition by uncomplexed ATP could produce the same result.

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