Effect of Allosteric Effectors and Adenosine Triphosphate on the Aggregation and Rate of Inhibition by *N*-Ethylmaleimide of Carbamyl Phosphate Synthetase of *Escherichia coli**

Paul M. Anderson and Sally V. Marvin†

ABSTRACT: Sucrose density gradient centrifugation studies showed that the sedimentation constant of carbamyl phosphate synthetase from *Escherichia coli* B ranges from 8.5 to 15.3 with increasing enzyme concentration. The increase in the sedimentation constant is facilitated by inosine monophosphate or ornithine (positive allosteric effectors) and, to a lesser extent, by adenosine triphosphate and $MgCl_2$, but is prevented by uridine monophosphate (a negative allosteric effector). A monomer–oligomer equilibrium is considered to account for these large changes in *s* value. The formation of oligomer (*s* value of about 15) apparently is not required for catalytic activity or for manifestation of the positive allosteric effects of ornithine or inosine monophosphate, since a minimal *s* value is observed in the presence of substrates and ornithine or inosine monophosphate at low enzyme concentration. The enzyme is inhibited by reaction with *N*-ethylmaleimide. The rate of inhibition decreases with increasing enzyme concentration, as compared with the rate of inhibition at the same enzyme concentration in the presence of uridine monophosphate. The presence of ornithine prevents inhibition at all enzyme concentrations.

The rate of inhibition of enzyme activity by *N*-ethylmaleimide is greatly increased by the addition of adenosine triphosphate–$MgCl_2$ and $NaHCO_3$. Ornithine does not protect against inhibition in the presence of these substrates, but reduces the concentration of adenosine triphosphate–$MgCl_2$ required to give a maximal effect. The addition of uridine monophosphate eliminates the effect of adenosine triphosphate–$MgCl_2$, apparently by preventing the binding of adenosine triphosphate–$MgCl_2$. The results indicate that the conformational state of the enzyme which exists in the presence of uridine triphosphate is different from that which predominates in the presence of ornithine; thus, in the presence of ornithine the enzyme is able to aggregate reversibly at higher enzyme concentrations, the enzyme activity is not inhibited appreciably by *N*-ethylmaleimide, and the effect of adenosine triphosphate–$MgCl_2$ on the rate of inhibition by *N*-ethylmaleimide is enhanced, whereas in the presence of uridine monophosphate the aggregation of the enzyme is not observed, the enzyme activity is inhibited by *N*-ethylmaleimide, and the effect of adenosine triphosphate–$MgCl_2$ on the rate of inhibition by *N*-ethylmaleimide is prevented. The binding of adenosine–$MgCl_2$ apparently results in the formation of a conformational state which is different from that which exists in the presence of either uridine monophosphate alone or ornithine alone.

Carbamyl phosphate synthetase from *Escherichia coli* B catalyzes the following reaction (Anderson and Meister, 1965):

$$2ATP + HCO_3^- + \text{L-glutamine} + H_2O \xrightarrow{K^+,\ Mg^{2+}} 2ADP + P_i + \text{carbamyl phosphate} + \text{L-glutamate} \quad (1)$$

The enzyme activity is subject to feedback inhibition by UMP, a pyrimidine nucleotide, and is activated by ornithine and by IMP and other end products of purine nucleotide biosynthesis (Anderson and Meister, 1966; Pierard, 1966; Anderson and Marvin, 1968). Activation by ornithine provides a mechanism for reversing feedback inhibition by UMP; this assures a continued supply of carbamyl phosphate when it is needed for arginine biosynthesis, since the rate of biosynthesis of ornithine will increase when the supply of arginine is decreased (Pierard, 1966). Activation by IMP and other purine nucleotides would result in an increased rate of carbamyl phosphate synthesis and, hence, pyrimidine nucleotide formation (Anderson and Meister, 1966).

The mechanism whereby ornithine, IMP, or UMP exerts their respective effects has not been studied. The presence of ornithine or IMP decreases the concentration of ATP required to give half-maximal velocity, whereas the presence of UMP has the opposite effect, suggesting that the affinity of the enzyme for ATP might be altered by these allosteric effectors (Anderson and Meister, 1966; Anderson and Marvin, 1968). At relatively high concentrations of enzyme, the rate of sedimentation in sucrose density gradients is dependent on which allosteric effector is present; the values of the sedimentation constants suggest that the molecular weight of the enzyme in the presence of ornithine or IMP is twice that of the enzyme in the presence of UMP (Anderson and Marvin, 1968).

In this paper evidence for a monomer–oligomer equilibrium which is altered by the presence of the different allosteric effectors or ATP is presented. The effects of enzyme concentration and of ATP, IMP, UMP, and/or ornithine on the rate of inactivation of carbamyl phosphate synthetase by *N*-ethylmaleimide have also been studied and have provided information concerning the significance of the monomer–

* From the Department of Chemistry, Southern Illinois University, Carbondale, Illinois 62901. *Received August 20, 1969.* This work was supported in part by U. S. Public Health Service Grant AM11443.

† Present address: Peace Corps, Care of American Embassy, Ankara, Turkey.

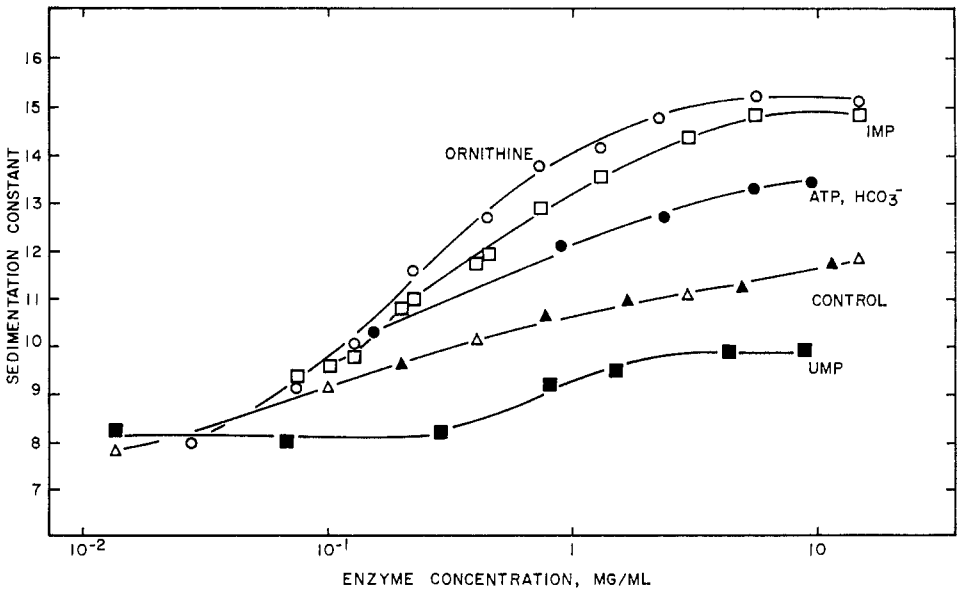


FIGURE 1: Variation of the sedimentation constant with carbamyl phosphate synthetase concentration. The *s* values were determined as described in the text; where indicated the gradients also contained ornithine (5 mM), IMP (5 mM), UMP (5 mM) or ATP (20 mM; all solutions containing ATP also contained equimolar concentrations of $MgCl_2$), and $NaHCO_3$ (10 mM). The enzyme concentration which is expressed as milligrams per milliliter was the concentration of the enzyme in the 0.1-ml aliquot which was placed on the gradient. The open triangles represent *s* values obtained at 11° instead of 17°.

oligomer transition in manifesting the regulatory properties of the enzyme.

Materials and Methods

Carbamyl phosphate synthetase was isolated from *E. coli* B by modification of the procedure described by Anderson and Meister (1965) (Anderson and Marvin, 1968; Anderson *et al.*, 1969). *E. coli* B cell paste (washed, three-fourths log phase, grown on enriched medium) was purchased from Grain Processing Corp.

[^{14}C]$NaHCO_3$ was obtained from New England Nuclear Corp. Ornithine, cysteine, glutathione, glutamine, catalase (beef liver), ATP (disodium salt), UMP, IMP, *N*-ethylmaleimide, and EDTA were purchased from Sigma Chemical Co.

Sedimentation constants were determined by sucrose density gradient centrifugation as described by Martin and Ames (1961). The sucrose gradients (5–20%) containing 0.15 M potassium phosphate buffer (pH 7.8), in a total volume of 4.9 ml, were stored overnight at 4° before use. The volume of sample layered on the top of the column was 0.1 ml; in addition to carbamyl phosphate synthetase of appropriate concentration in 0.15 M potassium phosphate buffer (pH 7.8), this solution also contained beef liver catalase (0.02 mg, $s = 11.3$ S (Martin and Ames, 1961)) as the reference protein. Centrifugation was carried out at 17° in a Spinco Model L preparative ultracentrifuge for 7 hr at 46,000 rpm with a SW50.1 rotor. Unless stated otherwise carbamyl phosphate synthetase was located in the fractions collected by determining the catalytic activity in an appropriate aliquot by measuring the rate of formation of ADP; in some cases the enzyme was also located by measuring the protein concentration by the method of Lowry *et al.* (1951). Catalase was located in the fractions by measuring by spectrophotometric means the rate of decomposition of H_2O_2 by aliquots of the fractions (Luck, 1965).

Carbamyl phosphate synthetase activity was determined by measuring the rate of ADP formation. The aliquot containing enzyme was added to a reaction mixture containing ATP (6 μmoles), $MgCl_2$ (6 μmoles), glutamine (3 μmoles), $NaHCO_3$ (3 μmoles), KCl (30 μmoles), and Tris-HCl buffer (30 μmoles, pH 8.2) at 37° to give a final volume of 0.3 ml and the ADP formed after 10 min at 37° was determined by coupling with lactate dehydrogenase and pyruvate kinase as previously described (Anderson and Meister, 1966). In some cases the carbamyl phosphate synthetase activity was determined by measuring the rate of formation of [^{14}C]carbamyl phosphate from [^{14}C]$NaHCO_3$ as previously described (Anderson and Meister, 1966).

Enzyme concentration was determined from its absorbance at 280 mμ. The enzyme absorbance at 280 mμ (10-mm path length) was 1.5/mg of enzyme per ml in 0.1 M potassium phosphate buffer (pH 7.8); this value was established by determining the dry weight of enzyme in an aliquot of a solution in which the absorbance at 280 mμ was known.

Results

Sedimentation Studies. Values of 15.2, 15.7, and 10.1 were previously reported for the sedimentation constant of carbamyl phosphate synthetase in the presence of ornithine, IMP, and UMP, respectively (Anderson and Marvin, 1968). These large changes in the sedimentation constant could result from an equilibrium between a monomeric and an oligomeric form of the enzyme, the former predominating in the presence of UMP and the latter predominating in the presence of ornithine or IMP. An estimation of the molecular weight

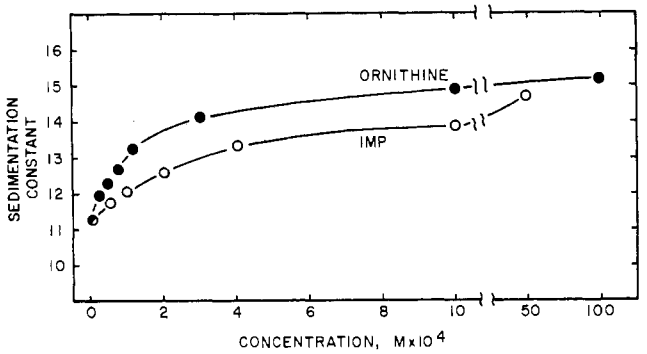


FIGURE 2: Variation of the sedimentation constant with ornithine or IMP concentration. The *s* values were determined as described in the text, except that the gradients also contained IMP or ornithine in the concentrations given. The concentration of the enzyme added to the top of the gradient was 1.9 mg/ml.

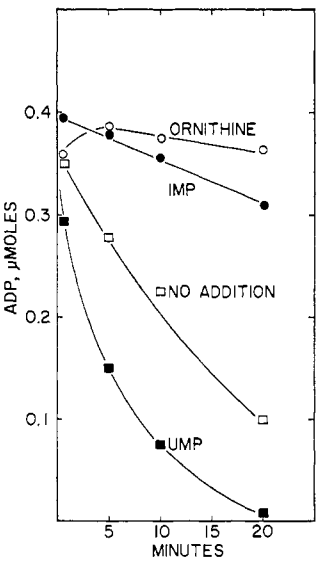


FIGURE 3: Effect of ornithine, IMP, or UMP on the rate of inhibition of carbamyl phosphate synthetase by *N*-ethylmaleimide. The reaction mixtures contained enzyme (13 mg/ml), potassium phosphate buffer (0.2 M, pH 7.6), EDTA (0.4 mM), *N*-ethylmaleimide (8.6 mM), and also ornithine (20 mM), IMP (14 mM), or UMP (14 mM) where indicated in a total volume of 35 μl at 17°. At the indicated times a 5-μl aliquot was removed and added to 0.2 ml of a solution containing potassium phosphate buffer (0.2 M, pH 7.8), ornithine (10 mM), and cysteine (10 mM) at 4° to stop the reaction. The enzyme activity in a 10-μl aliquot of each of these solutions was then determined by measuring the μmoles of ADP formed in 10 min as described in the text.

from the above *s* values gave values of 410,000 and 210,000 in the presence of IMP and UMP, respectively (Anderson and Marvin, 1968).

As shown in Figure 1, the sedimentation constant is dependent on the concentration of enzyme, particularly in the presence of ornithine or IMP; only one peak of enzyme (as measured by catalytic activity or protein concentration) was observed in the fractionated gradients from which the data in Figure 1 was obtained. These results would be expected if a monomeric and an oligomeric form of the enzyme were in rapid equilibrium with each other (Gilbert, 1967). The *s* value appears to approach a minimum of about 8.5 at very low enzyme concentrations and a maximum of about 15.3 in the presence of ornithine or IMP when the concentration of the enzyme applied to the gradient is greater than 5 mg/ml. The presence of UMP prevents the formation of a significant amount of enzyme species with an *s* value larger than 10.3; the *s* value increases from a minimum of 8.5 at low enzyme concentration (less than 0.2 mg/ml) to 10.3 for enzyme concentrations greater than 3 mg/ml. These large changes in *s* value are considered to result from a change in the state of aggregation of the enzyme; that a reversible conformational change would account for these observations does not seem likely.

In the absence of allosteric effectors or substrates the *s* value of the enzyme also increases with increasing enzyme concentration (Figure 1), but the change is not as pronounced as that observed in the presence of ornithine or IMP.

As shown in Figure 1, the presence of ATP and $NaHCO_3$ in the gradients results in an intermediate increase in the *s* value with increasing enzyme concentration. Similar results were obtained in the absence of added bicarbonate, but endogenous bicarbonate was probably present in sufficient concentration to prevent demonstration of a possible bicarbonate requirement.

The effect of ATP is dependent on the presence of magnesium ions. In an experiment carried out as described in Figure 1 with an initial enzyme concentration of 4.2 mg/ml *s* values of 13.4 and 11.0 were obtained when the gradients contained 0.02 M ATP with or without equimolar $MgCl_2$, respectively.

The addition of ATP to gradients containing ornithine or IMP resulted in a decrease in the observed *s* value from that observed in the presence of ornithine or IMP alone, and the *s* value obtained when both ATP and UMP were present in the gradients was significantly larger than the *s* value observed when only UMP was present. In one experiment which was carried out as described in Figure 1 and in which ATP (20 mM), $MgCl_2$ (20 mM), and $NaHCO_3$ (10 mM) were present in all gradients the *s* values obtained when ornithine (5 mM), IMP (5 mM), or UMP (1 mM) was also present in the gradient were 14.3, 14.2, and 11.6, respectively; the concentration of enzyme added to the top of the gradients was 3.0 mg/ml. The peak of enzyme activity obtained in the fractionated gradients after centrifugation was broader than usual.

Glutamine had no effect on the *s* value of the enzyme when the concentration of the enzyme added to the gradients was 3.0 mg/ml.

The concentration of enzyme applied to the top of the gradient which was required in order to observe a significant increase in the sedimentation constant was about 0.1 mg/ml (Figure 1). This is considerably larger than the concentration of 0.02 mg of enzyme/ml normally used in the catalytic assay. An experiment was carried out as described in Figure 1 in which the gradients contained the same final concentrations of ATP, $MgCl_2$, $NaHCO_3$, glutamine, and potassium phosphate buffer as are used in the catalytic assay; after centrifugation the enzyme was located in the gradients by measuring the rate at which aliquots of the fractions catalyzed the formation of [^{14}C]carbamyl phosphate from [^{14}C]$NaHCO_3$. An *s* value of 10.2 was obtained for the enzyme when the concentration of the enzyme applied to the gradient was 0.23 mg/ml. This experiment was repeated using gradients which also contained ornithine (5 mM) or IMP (5 mM); the sedimentation constants

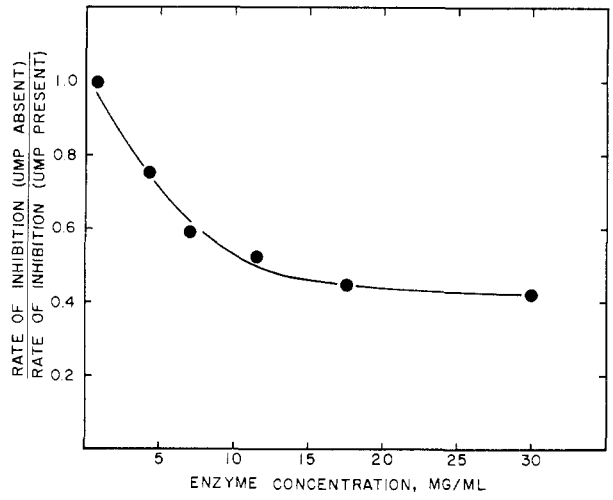


FIGURE 4: Effect of enzyme concentration on the rate of inhibition of carbamyl phosphate synthetase by *N*-ethylmaleimide. Aliquots of appropriate volumes were removed at 0.5-min intervals for 6 min from reaction mixtures containing potassium phosphate buffer (0.15 M, pH 7.6), EDTA (0.4 mM), *N*-ethylmaleimide (5 mM), and enzyme at 17° and added to a solution of appropriate volume at 4° containing potassium phosphate buffer (0.1 M, pH 7.5) and ornithine (0.02 M) to stop the reaction. An aliquot of each of these solutions was then assayed for enzyme activity by measuring the μmoles of ADP formed in 10 min as described in the text. A linear decrease in activity with time was obtained for each enzyme concentration and the rate of inhibition was expressed as per cent decrease in enzyme activity per min; the rates of inhibition when the enzyme concentration was 0.6, 6.9, 17.4, and 30 mg/ml were 8.2, 6.9, 5.9, and 3.0%/min, respectively. The rate of inhibition was also determined when the reaction mixtures contained UMP (10 mM) by the same procedure and the ratio of the rates of inhibition in the presence or absence of UMP calculated.

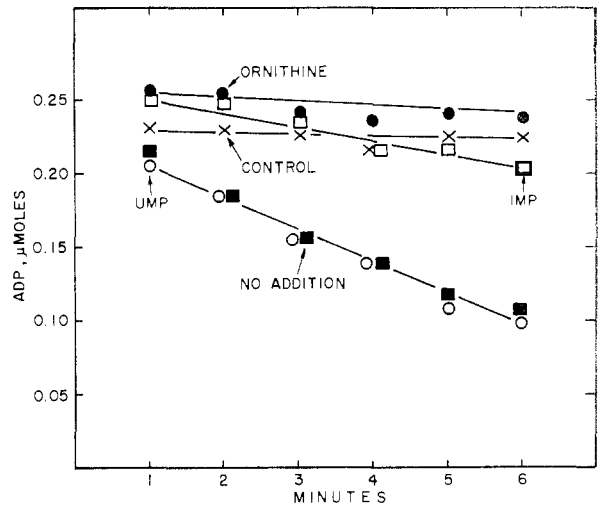


FIGURE 5: Effect of ornithine and IMP on the rate of inhibition of carbamyl phosphate synthetase by *N*-ethylmaleimide at low enzyme concentration. The reaction mixtures contained potassium phosphate buffer (0.14 M, pH 7.5), EDTA (0.4 mM), *N*-ethylmaleimide (5 mM), and enzyme (0.04 mg/ml) in a final volume of 2.8 ml at 17°. At the indicated times a 0.1-ml aliquot was removed and the enzyme activity in this aliquot determined immediately by measuring the μmoles of ADP formed in 10 min as described in the text, except that the standard assay mixture also contained glutathione (3 μmoles) to react with the excess *N*-ethylmaleimide. The control was an identical reaction mixture without *N*-ethylmaleimide. Where indicated the reaction mixtures contained ornithine (10 mM), IMP (10 mM), or UMP (1 mM).

obtained were 10.6 and 10.4, respectively. Consequently, it would appear that under catalytic conditions where considerable activation by ornithine or IMP is observed the presence of ornithine or IMP does not result in a significant increase in the sedimentation constant; *i.e.*, the formation of the oligomeric state of the enzyme (*s* value of about 15.0) is not required for catalytic activity or for manifestation of the positive allosteric effects of ornithine or IMP.

It would be expected that during the course of centrifugation of the enzyme in the presence of all substrates, as described above, a certain quantity of substrate would be converted to product. The concentration of the enzyme undergoes a five- to tenfold dilution by the time centrifugation is terminated and a given area of the gradient through which the enzyme migrates is probably occupied by enzyme of maximum concentration for less than 60 min under the conditions used; assuming a maximum specific activity of 60 μmoles of carbamyl phosphate formed per hr/mg of enzyme at 17° (see Figure 9), these considerations indicate that a major portion of the enzyme would exist in the presence of excess substrate during the entire course of centrifugation and the concentration of the products which accumulate would approximate the concentration of products formed in the standard assay procedure.

The effect of ornithine and IMP concentration on the rate of sedimentation of the enzyme is shown in Figure 2.

Inhibition by N-Ethylmaleimide. The catalytic activity of carbamyl phosphate synthetase is rapidly inactivated by incubation with *N*-ethylmaleimide. As shown in Figure 3, the rate of inhibition is greatly reduced in the presence of ornithine or IMP as compared to the rate in the presence of UMP. In the absence of allosteric effectors an intermediate rate of inhibition is observed which is dependent on the concentration of enzyme. At low enzyme concentrations (less than 0.5 mg/ml, see Figure 4) the rate of inhibition in the absence of UMP is the same as the rate in the presence of UMP. The ratio of the rate of inhibition in the absence of UMP to that in the presence of UMP as a function of enzyme concentration is shown in Figure 4. A possible explanation of these data could be that the site(s) at which *N*-ethylmaleimide reacts is no longer available in the oligomeric form of the enzyme. Thus, complete protection could be obtained at high enzyme concentration in the presence of ornithine (or IMP), but not in their absence, since the sedimentation data indicate that even at high concentrations the enzyme does not exist entirely in the oligomeric form in the absence of ornithine or IMP. According to this interpretation, ornithine (or IMP) should not significantly affect the rate of inhibition at low enzyme concentration where the oligomeric form of the enzyme does not exist even in the presence of ornithine (see Figure 1). However, as shown in Figure 5, the presence of ornithine or, to a lesser extent, IMP also decreased the rate of inhibition at very low enzyme concentrations as compared to the rate of inhibition in the presence of UMP, indicating that the protective effect of ornithine is obtained under conditions where the oligomeric form of the enzyme is not observed.

The effect of ornithine concentration at several enzyme concentrations is shown in Figure 6. The concentration of orni-

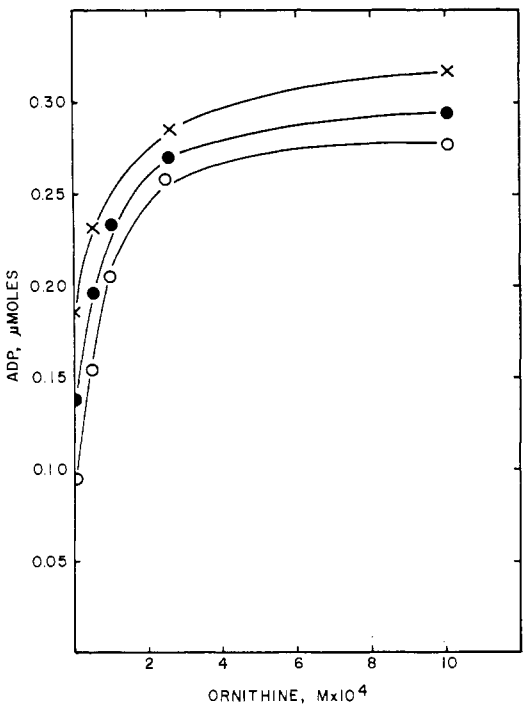


FIGURE 6: Effect of ornithine concentration on the rate of inhibition of carbamyl phosphate synthetase by *N*-ethylmaleimide. The reaction mixtures contained potassium phosphate buffer (0.15 M, pH 7.5), EDTA (0.4 mM), ornithine as indicated, *N*-ethylmaleimide (5 mM), and enzyme. After 3 min at 17° the reaction was stopped by adding an aliquot of a solution at 4° containing potassium phosphate buffer (0.1 M, pH 7.8), ornithine (20 mM), and glutathione (5 mM). A 50-μl aliquot of these diluted solutions was assayed for enzyme activity by measuring the μmoles of ADP formed in 10 min as described in the text. The experiment was carried out using three different enzyme concentrations. The concentration of enzyme, volume of the reaction mixtures, and the volume of solution used to stop the reactions, respectively, were: (×-×) 20 mg/ml, 35 μl, and 4.5 ml; (●—●) 4 mg/ml, 70 μl, and 1.8 ml; (○—○) 0.8 mg/ml, 350 μl, and 1.5 ml.

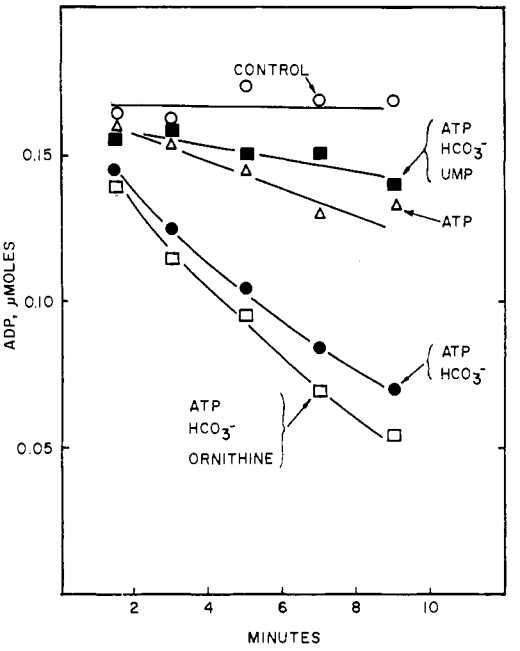


FIGURE 7: Effect of ATP on the rate of inhibition of carbamyl phosphate synthetase by *N*-ethylmaleimide. The reaction mixtures contained potassium phosphate buffer (0.15 M, pH 7.5), EDTA (0.5 mM), *N*-ethylmaleimide (0.5 mM), enzyme (3.3 mg/ml), and where indicated ATP (2 mM; all solutions containing ATP also contained equimolar concentrations of $MgCl_2$), $NaHCO_3$ (20 mM), ornithine (10 mM), or UMP (10 mM) in a final volume of 145 μl at 17°. At the indicated times a 10-μl aliquot was removed and added to 0.4 ml of a solution containing potassium phosphate buffer (0.1 M, pH 7.8) and ornithine (10 mM) at 4° to stop the reaction. The enzyme activity in 50-μl aliquots of these solutions was determined by measuring the μmoles of ADP formed in 10 min as described in the text. The control was an identical reaction mixture without *N*-ethylmaleimide. The decrease in activity observed in the absence of ATP under the above conditions was approximately the same as that shown above for the reaction mixture which contained ATP, $NaHCO_3$, and UMP.

thine required to reduce the rate of inhibition by one-half is 7×10^{-5} M at all three enzyme concentrations.

Effect of ATP on the Rate of Inhibition of Carbamyl Phosphate Synthetase by N-Ethylmaleimide. The rate of inhibition of carbamyl phosphate synthetase is greatly increased in the presence of ATP. This is demonstrated by the experiment described in Figure 7; the concentration of *N*-ethylmaleimide required to obtain an appreciable rate of inhibition in the presence of ATP was much less than that required in the absence of ATP (see Figure 3). The addition of ornithine, which protects the enzyme against inhibition by *N*-ethylmaleimide in the absence of ATP, increases the rate of inhibition as compared with that observed in the presence of ATP alone, and the addition of UMP, which increases the rate of inhibition by *N*-ethylmaleimide in the absence of ATP, greatly decreases the rate of inhibition as compared to that observed in the presence of ATP alone. The effect of ATP is stimulated by the addition of $NaHCO_3$. In the absence of glutamine or ammonia carbamyl phosphate synthetase catalyzes the slow bicarbonate-dependent hydrolysis of ATP to ADP and inorganic phosphate (Anderson and Meister, 1966). Determination of the specific activity for this reaction under the conditions described in Figure 7 (0.15 M potassium phosphate buffer, pH 7.5; 5×10^{-4} M EDTA; 17°) yielded a value of 0.017 μmole of ATP hydrolyzed per min/mg of enzyme when the concentrations of ATP and $MgCl_2$ were 2 mM, respectively, and the concentration of $NaHCO_3$ was 10 mM, indicating that the adenosine triphosphatase activity is greatly reduced in the presence of phosphate buffer and that a significant portion of the ATP present in the experiments described in Figures 7 and 8 was not hydrolyzed during the course of the reactions with *N*-ethylmaleimide.

The effect of ATP concentration on the rate of inhibition is shown in Figure 8. The approximate concentration of ATP required to give a half-maximal effect is decreased in the presence of ornithine and greatly increased in the presence of UMP, indicating that the apparent affinity of the enzyme for ATP is markedly affected by the presence of allosteric effectors.

As shown in Figure 8, the concentration of ATP required to give a half-maximal effect is not significantly altered by the presence of IMP. We have found that IMP also has no effect on the catalytic activity of carbamyl phosphate synthetase in the presence of phosphate buffer at 17°, the conditions used in the experiment described in Figure 8. The effects of ornithine,

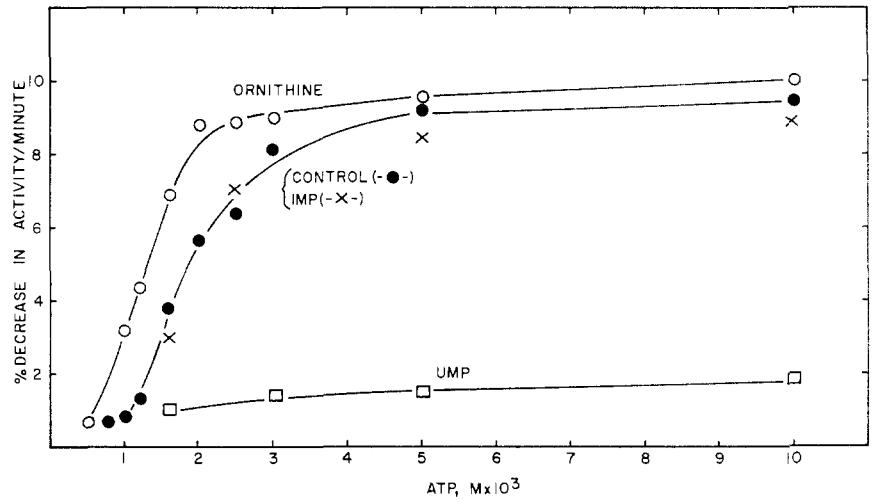


FIGURE 8: Effect of ornithine, IMP, and UMP on the rate of inhibition of carbamyl phosphate synthetase by *N*-ethylmaleimide as a function of ATP concentration. Reaction mixtures containing potassium phosphate buffer (0.15 M, pH 7.5), EDTA (0.5 mM), enzyme (6 mg/ml), $MgCl_2$ in concentrations that were equimolar with ATP, ATP as shown, and where indicated ornithine (10 mM), IMP (10 mM), or UMP (1 mM) were incubated with *N*-ethylmaleimide (0.62 mM) at 17° in a final volume of 145 μl. At 1-min intervals for 6 min a 20-μl aliquot was removed from each reaction mixture and added to 0.7 ml of a solution containing potassium phosphate buffer (0.1 M, pH 7.8), ornithine (10 mM), and glutathione (5 mM) at 4° to stop the reaction. The enzyme activity in 50-μl aliquots of these solutions was determined by measuring the μmoles of ADP formed in 10 min as described in the text. A linear decrease in activity with time was obtained for each reaction mixture. The rate of decrease in enzyme activity is expressed as per cent decrease in enzyme activity per minute.

IMP, or UMP on catalytic activity under these conditions are described in Figure 9. It can be seen that the activating effect of IMP which is observed in Tris-HCl buffer at 37° (Anderson and Meister, 1966; Anderson and Marvin, 1968) is negligible or actually reversed in the presence of phosphate buffer at 17°. As shown, however, the allosteric effects of ornithine and UMP are observed in phosphate buffer at 17°, the conditions under which the sedimentation and inhibition studies reported above were carried out.

Discussion

The studies presented above show that several properties of carbamyl phosphate synthetase are grossly affected by the presence of the allosteric effectors and that these effects are dependent on which allosteric effector is present. The results provide evidence that the enzyme can aggregate reversibly in the presence or absence of ornithine or IMP, but not in the presence of UMP. However, manifestation of the positive allosteric effects of ornithine or IMP apparently is not dependent on the formation of the oligomeric form of the enzyme, since the different effects of UMP and ornithine or IMP on catalytic activity and on the rate of inhibition by *N*-ethylmaleimide are obtained under conditions where oligomer is not formed.

The data suggest that the enzyme exists in a conformational state in the presence of ornithine which is different from that which exists in the presence of UMP. Thus, in the presence of ornithine the enzyme is able to aggregate reversibly at higher enzyme concentrations, the enzyme activity is not inhibited appreciably by *N*-ethylmaleimide, and the effect of ATP on the rate of inhibition by *N*-ethylmaleimide is enhanced, whereas in the presence of UMP the enzyme is not able to aggregate at high enzyme concentrations, the enzyme activity is inhibited at an appreciable rate by reaction with *N*-ethylmaleimide, and the effect of ATP on the rate of inhibition by *N*-ethylmaleimide is virtually eliminated. The results of the inhibition studies also suggest that the enzyme exists in a conformational state in the presence of ATP or ATP plus ornithine which is different from that which predominates in the presence of either ornithine alone or UMP alone, and that the binding of ATP to enzyme is greatly affected by the presence of ornithine or UMP.

The results can be summarized by a scheme (Figure 10) in which the enzyme, for the reasons cited above, is considered to exist in at least three different conformational states (designated monomer-I, monomer-II, and monomer-III, respectively) and that the enzyme is not able to aggregate appreciably when it exists in the conformational state which predominates in the presence of UMP (monomer-I). As one possible pathway for the interconversion between monomer-I and monomer-II it is postulated that these two conformational states are in equilibrium with each other and that UMP binds preferentially to monomer-I while ornithine binds preferentially to monomer-II.

In the presence of excess ornithine the equilibrium between monomer-I and monomer-II would be shifted largely in favor of monomer-II. Under these conditions, the equilibrium between monomer-II and oligomer in the scheme in Figure 10 would shift toward oligomer with increasing enzyme concentration according to the law of mass action; the presence of UMP would prevent the formation of oligomer since little monomer-II would exist in the presence of excess UMP. The sedimentation data show that the *s* value also increases with increasing enzyme concentration in the absence of ornithine or IMP. This would be expected if the transition from monomer-I to monomer-II and the polymerization of monomer-II to oligomer were not dependent on the binding of an allosteric effector or substrate; the equilibrium would be shifted from monomer-I and monomer-II to oligomer with increasing enzyme concentration, as observed.

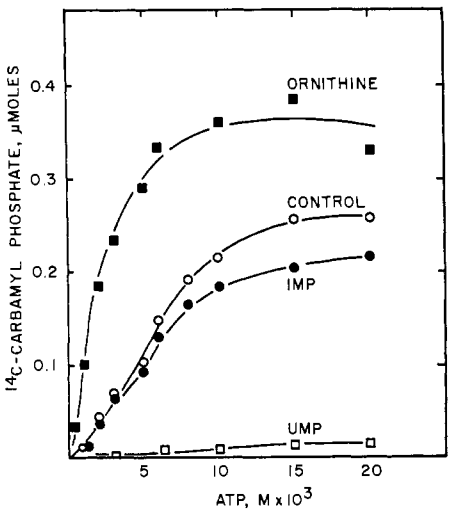


FIGURE 9: Effect of IMP, UMP, and ornithine on carbamyl phosphate synthetase activity in potassium phosphate buffer at 17° as a function of ATP concentration. The reaction mixtures contained $MgCl_2$ in concentrations that were equimolar with ATP, [^{14}C]-$NaHCO_3$ (20 μmoles, 600,000 cpm), potassium phosphate buffer (0.1 M, pH 7.8), and enzyme (0.03 mg/ml) in a final volume of 1.0 ml. Where indicated the reaction mixtures also contained ornithine (5 mM), UMP (1 mM), or IMP (5 mM). The [^{14}C]carbamyl phosphate synthesized after incubation for 10 min at 17° was determined as described in the text.

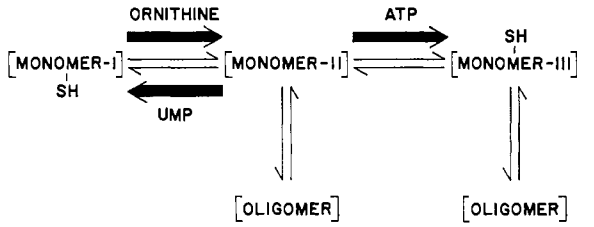


FIGURE 10: Scheme proposed as a possible mechanism for the allosteric effector and ATP–$MgCl_2$ dependent inhibition by *N*-ethylmaleimide and aggregation of carbamyl phosphate synthetase.

The effect of the allosteric effectors on the rate of inhibition of enzyme activity by reaction with *N*-ethylmaleimide is depicted in the scheme in Figure 10 as being due to the presence of an SH group in monomer-I which can react with *N*-ethylmaleimide to give a decrease in catalytic activity, but which is not available for reaction in monomer-II (or oligomer). Thus, the presence of ornithine would protect against inhibition at all enzyme concentrations and the maximum rate of inhibition would occur in the presence of UMP. The ratio of the rate of inhibition in the absence of allosteric effectors to that in the presence of UMP decreases with increasing enzyme concentration. This would be expected according to the argument presented above since the relative amount of monomer-I present in the absence of allosteric effectors would decrease with increasing enzyme concentration; in the presence of UMP monomer-I would predominate at all enzyme concentrations. That the rates of inhibition in the presence or absence of UMP are the same at low enzyme concentrations would indicate that the postulated equilibrium between monomer-I and monomer-II strongly favors monomer-I in the absence of allosteric effectors or substrates.

The binding of ATP to the enzyme apparently results in the increased susceptibility of a functional group which reacts with *N*-ethylmaleimide to give a decrease in catalytic activity. In the scheme in Figure 10 this functional group is illustrated as another SH group, although there is no evidence to show that it is not the same group (or groups) which react with *N*-ethylmaleimide in the presence of UMP. The presence of excess UMP eliminates this effect. This is accounted for by assuming that ATP binds only to monomer-II, resulting in the formation of monomer-III which is more susceptible to inhibition by *N*-ethylmaleimide. The addition of ornithine would increase the concentration of monomer-II, thus increasing the apparent affinity of the enzyme for ATP, as observed. This could also account for the positive allosteric effect of ornithine and the negative allosteric effect of UMP on catalytic activity.

Two of the observations discussed above, *i.e.*, the effects of enzyme concentration in the absence of allosteric effectors on the observed *s* value of the enzyme and on the rate of inhibition by reaction with *N*-ethylmaleimide, are consistent with and most simply explained by the suggestion that there is an equilibrium between monomer-I and monomer-II in the scheme in Figure 10. Such a system would be analogous to the model proposed by Monod *et al.* (1965) in which two or more conformational states of the enzyme differing in their affinity for allosteric effectors or the appropriate substrates are considered to be in equilibrium with each other; the action of positive allosteric effectors can be explained by assuming that they bind preferentially to the conformational state which binds substrate preferentially (monomer-II, above). However, these effects of enzyme concentration in the absence of allosteric effectors could also be explained with some additional assumptions by a scheme in which the formation of monomer-I or monomer-II is considered to occur as a result of the binding of UMP or ornithine, respectively, to a different conformational state of the enzyme which exists in the absence of allosteric effectors (Koshland and Neet, 1968).

As illustrated in Figure 10 the reaction of the enzyme with *N*-ethylmaleimide is presumed to be with an SH group or groups on the enzyme; however, it remains to be established that this is the site of action, since *N*-ethylmaleimide has been shown to react in certain cases with other amino acid residues (Vallee and Riordan, 1969).

The *s* value of the enzyme in the presence of UMP increases from 8.5 to a plateau of 10.3 with increasing enzyme concentration. A possible explanation for this observation might simply be that the ability of the monomeric unit to associate is greatly reduced, but not eliminated, in the presence of UMP. Approximate molecular weight calculations based on these *s* values and the maximum *s* value of 15.2 observed in the presence of ornithine yield figures of 390,000, 216,000, and 162,000, respectively (Martin and Ames, 1961). Studies currently being carried out have shown that the enzyme can apparently be broken down into two different subunits in the presence of guanidine hydrochloride; tentative estimates of the molecular weight of each subunit have yielded values of 50,000 and 160,000, respectively (P. M. Anderson and S. Matthews, 1969, unpublished data). The possibility that the monomeric unit of the enzyme is composed of two such nonidentical subunits

which can dissociate at low enzyme concentration is being investigated. Accurate molecular weight determinations will be required, however, for elucidation of the nature of the monomer–oligomer transition.

Binding studies are currently being carried out and the preliminary results are consistent with the scheme in Figure 10; the presence of ATP or ornithine greatly decreases the apparent affinity of the enzyme for UMP, and the presence of UMP decreases the apparent affinity of the enzyme for ornithine (P. M. Anderson and J. Demetriou, 1969, unpublished data).

It is apparent from these studies that the observed properties of this enzyme are very dependent on the temperature, enzyme concentration, buffer used, and the presence of substrates or allosteric effectors, and that results obtained under different conditions should be compared with appropriate caution.

Acknowledgments

We thank Mrs. Patricia Wasmund for her skillful assistance in preparing the purified enzyme used in this work.

References

Anderson, P. M., and Marvin, S. V. (1968), *Biochem. Biophys. Res. Commun. 32*, 928.

Anderson, P. M., and Meister, A. (1965), *Biochemistry 4*, 2803.

Anderson, P. M., and Meister, A. (1966), *Biochemistry 5*, 3157, 3164.

Anderson, P. M., Wellner, V. P., Rosenthal, G. A., and Meister, A. (1969), *Methods Enzymol.* (in press).

Gilbert, G. A. (1967), *Anal. Chim. Acta 38*, 275.

Koshland, D. E., Jr., and Neet, K. E. (1968), *Ann. Rev. Biochem. 37*, 359.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.

Luck, H. (1965), *in* Methods of Enzymatic Analysis, Bergmeyer, H. U., Ed., New York, N. Y., Academic.

Martin, R. G., and Ames, B. N. (1961), *J. Biol. Chem. 236*, 1372.

Monod, J., Wyman, J., and Changeaux, J. (1965), *J. Mol. Biol. 12*, 88.

Pierard, A. (1966), *Science 154*, 1572.

Vallee, B. L., and Riordan, J. F. (1969), *Ann. Rev. Biochem. 38*, 733.

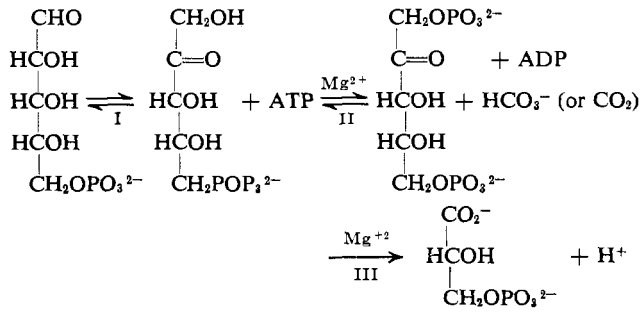
Spinach 5-Phosphoribose Isomerase. Purification and Properties of the Enzyme*

Alan C. Rutner

ABSTRACT: Spinach leaf 5-phosphoribose isomerase has been purified 2800-fold to a homogeneous state free of 5-phosphoribulokinase and ribulose diphosphate carboxylase. The isomerase has a sedimentation coefficient ($s_{20,w}$) of 4.10 S and a molecular weight by sedimentation equilibrium of 53,200 ($\bar{v}$ = 0.749). The pure enzyme catalyzes the isomerization of 1.21×10^5 moles of D-ribose 5-phosphate to D-ribulose 5-phosphate/min per mole of enzyme at pH 7.1 and 37° and the K_m for ribose 5-phosphate is 4.6×10^{-4} M. The enzyme is weakly inhibited by high concentrations of inorganic phosphorus, adenosine monophosphate, adenosine diphosphate, adenosine triphosphate, and *p*-mercuribenzoate. No evidence has been found for the existence of more than one form of 5-phosphoribose isomerase in spinach leaves.

The isolation and partial characterization of 5-phosphoribose isomerase (D-ribose 5-phosphate ketol-isomerase EC 5.3.1.6) from photosynthetic organisms has been reported by a number of investigators (Axelrod and Jang, 1954; Hurwitz *et al.*, 1956; Anderson *et al.*, 1968; Pon and Knowles, 1968). This enzyme catalyzes the first reaction (reaction I) of the carboxylative phase of the photosynthetic carbon reduction cycle. Reaction II is catalyzed by 5-phosphoribulokinase and reaction III by ribulose diphosphate carboxylase. Although the enzyme has been known for many years and the mechanism of the reaction has been studied (McDonough and Wood, 1961; Rose, 1962) little is known concerning the physical properties of the isomerase.

The initial objective of this investigation was to devise a



* From the Department of Biochemistry, New York University School of Medicine, New York, New York 10016. *Received August 21, 1969.* This investigation was supported by Research Grants AM-12946 and AM-09117 from the National Institutes of Health, U. S. Public Health Service.