Carbamoyl-Phosphate Synthetase: An Example of Effects on Enzyme Properties of Shifting an Equilibrium between Active Monomer and Active Oligomer†

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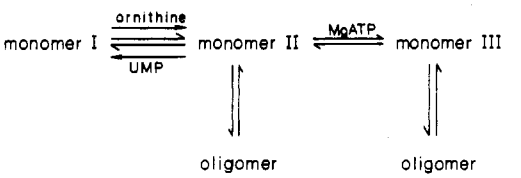
ABSTRACT: Carbamoyl-phosphate synthetase from *Escherichia coli* is subject to allosteric activation by ornithine, allosteric inhibition by uridine 5′-phosphate (UMP), and reversible concentration-dependent self-association. Positive allosteric effectors, magnesium adenosine 5′-triphosphate (MgATP), K^+, and inorganic phosphate facilitate association. The purpose of this study was to determine the state of association of carbamoyl-phosphate synthetase in the presence and absence of different substrates and effectors and to consider the basis for the observed effects of enzyme concentration on specific activity. Studies employing gel filtration chromatography have shown that when the concentration of carbamoyl-phosphate synthetase is low (<0.01 mg/mL), the enzyme exists as monomer under all conditions, including the presence of UMP in phosphate buffer and the presence of all substrates plus ornithine (conditions that support maximal catalytic activity). At higher enzyme concentrations (e.g., >0.01 mg/mL) the specific activity increases with increasing enzyme concentration when MgATP is nonsaturating but is independent of enzyme concentration when MgATP is saturating or when ornithine is present with MgATP being either saturating or nonsaturating. These results indicate that the catalytic activity of this enzyme is not directly linked to oligomer formation. The theoretical properties and possible significance of a generalized model of enzyme association–dissociation in which the active monomeric form, in equilibrium with another monomeric form, is specifically subject to self-association but the different states of association have the same specific activity, are discussed. This generalized model together with the results with carbamoyl-phosphate synthetase suggests that the effects characteristic of associating enzyme systems may arise in some circumstances through processes in which different states of association do not necessarily have different catalytic activities and/or ligand-binding properties.

Carbamoyl-phosphate synthetase from *Escherichia coli* is subject to allosteric activation by ornithine (also by IMP[1] and NH_4^+) and to allosteric inhibition by UMP (Anderson & Meister, 1966; Pierard, 1966; Anderson & Marvin, 1968, 1970). Ornithine and UMP act by decreasing or increasing, respectively, the concentration of MgATP required for half-maximal velocity. Studies in our laboratory and by Meister and colleagues (Anderson & Marvin, 1970; Trotta et al., 1974; Powers et al., 1980) have shown that the enzyme is subject to self-association, which is differently affected by the presence of allosteric effectors, MgATP, and K^+ and is dependent upon enzyme concentration. Positive allosteric effectors, P_i, MgATP, and K^+ facilitate association.

Earlier studies in our laboratory provided evidence that the effects of MgATP and allosteric effectors on carbamoyl-phosphate synthetase are related to changes in the equilibrium between different monomeric states, which can be influenced by self-association as illustrated in Scheme I (Anderson & Marvin, 1970). This scheme was initially based upon the observation that the rate of inhibition of the enzyme by SH reagents is greatly decreased by the presence of ornithine or, of particular significance, by increasing enzyme concentration and that both of these effects can be prevented by UMP. Key features of this scheme include (1) a preexisting equilibrium between monomers I and II, (2) shifting of the preexisting equilibrium from monomer I to monomer II by positive allosteric effectors and/or MgATP, (3) shifting of the preexisting equilibrium in favor of monomer I by UMP, a negative allosteric effector, (4) a substrate binding site available in monomer II, but not in monomer I, for binding MgATP, (5)

Scheme I



induced formation of monomer III by binding of MgATP as substrate to monomer II, and (6) self-association of monomers II and III, but not of monomer I, to oligomer. Oligomer is now known to reflect reversible dimer and tetramer formation (see below). Subsequent studies involving reacting-enzyme gel filtration chromatography, solid-phase immobilization of a form of the enzyme considered to be monomeric, and effects of enzyme concentration provided evidence that monomer III and the oligomeric forms derived from monomer III are catalytically active (Anderson, 1977a). These results were interpreted to indicate that catalytic activity is not directly linked to oligomer formation; i.e., a monomeric form of the enzyme is as active as oligomer.

Analytical ultracentrifugation and other studies by Trotta et al. (1974) and, more recently, by Powers et al. (1980) have clearly shown that the enzyme exists in equilibrium among monomeric, dimeric, and tetrameric forms. Important features of a scheme for self-association proposed by Powers et al. (1980) are (1) that the presence of UMP results in dimer formation but prevents further association to tetramer and (2)

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[1] Abbreviations: UMP, uridine 5′-phosphate; ATP, adenosine 5′-triphosphate; IMP, inosine 5′-phosphate; P_i, inorganic phosphate; CTP, cytidine 5′-triphosphate; ADP, adenosine 5′-diphosphate; EDTA, ethylenediaminetetraacetic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane.

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that the presence of MgATP, K^+, NH_4^+, ornithine, and/or IMP facilitate tetramer formation, the extent of tetramer formation being dependent upon enzyme concentration. Powers et al. (1980) also showed that when MgATP is nonsaturating, an increase in specific activity is observed as the enzyme concentration is increased beyond 0.01 mg/mL (a constant, lower specific activity is observed at different, lower enzyme concentrations). When MgATP is saturating, a higher but constant specific activity is observed at all enzyme concentrations. On the basis of these observations, it was concluded that a linkage exists between self-association and catalytic activity and that the observed increase in specific activity is a direct result of oligomer formation; i.e., an oligomeric form of the enzyme is more active than monomer.

The results of our studies and those by Powers et al. (1980) are generally in agreement except for the conclusions concerning the relative activities of monomer and oligomer. A question also exists concerning the state of association of the enzyme at lower concentrations in the presence of UMP. The studies in our laboratory leading to the conclusion that a monomeric form of the enzyme is fully active and that oligomer formation is incidental to formation of monomers II and III and is not required for catalytic activity (Scheme I) were based, in part, upon previous indications that the enzyme exists as a monomer in the presence of P_i and UMP at low enzyme concentrations (Anderson & Marvin, 1970; Trotta et al., 1974), a supposition that needs to be clarified. Powers et al. (1980) have established that the enzyme exists only as dimer in the presence of UMP at higher enzyme concentrations, but it has not been established whether or not the UMP-stabilized dimer dissociates to monomer at lower enzyme concentrations. Earlier studies in our laboratory utilizing sucrose density gradient centrifugation techniques suggest, in retrospect, that the enzyme probably does, in fact, dissociate to monomer in the presence of UMP at lower enzyme concentration (Anderson & Marvin, 1970).

The above considerations, as well as recent studies in our laboratory on the role of subunit association–dissociation in the enzyme CTP synthetase (Anderson, 1983), have prompted us to reassess the effects of subunit association–dissociation on the catalytic and regulatory properties of carbamoyl-phosphate synthetase. The present study was initiated to provide additional supporting documentation regarding the state of association of fully active enzyme at lower enzyme concentrations and the state of association of the enzyme in the presence of UMP at lower enzyme concentrations. More importantly, the basis for the effects of enzyme concentration on specific activity is considered. The specific point of interest is the interpretation of the observed increase in specific activity with increasing enzyme concentration that is accompanied by an increase in the proportion of the enzyme as oligomer. The results of this and our previous study (Anderson, 1977a), as well as the results reported by Powers et al. (1980), appear to be consistent with the view that the effects of carbamoyl-phosphate synthetase concentration on specific activity are due to mass-action effects on the state of association analogous to that depicted in Scheme I and that an oligomeric form of the enzyme is not more active than monomer. Theoretical considerations and possible significance of a generalized and simplified model of association–dissociation of enzymes that incorporates key features of Scheme I are also presented and discussed. A preliminary report of this work has been published (Anderson, 1984).

Materials and Methods

Carbamoyl-phosphate synthetase was isolated from *E. coli* B by the procedure described by Anderson et al. (1970), as modified by Matthews and Anderson (1972) and by Trotta et al. (1974). The enzyme was stored at 4 °C as a precipitated suspension in a solution containing 0.4 g/mL $(NH_4)_2SO_4$, 0.2 M potassium phosphate buffer, pH 7.8, 1 mM EDTA, and 10 mM ornithine. Enzyme samples were obtained from the suspension by centrifuging and dissolving the resulting pellet of enzyme in the appropriate buffer. For kinetic studies the enzyme was placed in 0.1 M potassium phosphate buffer, pH 7.8, by passing an appropriate volume through a small Sephadex G-25 column equilibrated with this buffer. Enzyme concentration was determined by measurement of A_{280} (Wellner et al., 1973). Sephadex G-200, Sephadex G-25, and Blue Dextran 2000 were products from Pharmacia. Other biochemicals of the highest quality available were purchased from Sigma Chemical Co.

Gel filtration chromatography was carried out at 22 °C on a column (2.5 × 112 cm) of Sephadex G-200 equilibrated with the appropriate solution at pH 7.8. Enzyme concentration refers to the concentration of enzyme in the 4-mL sample loaded onto the column. A flow rate of 24 mL/h (5.6 mL/fraction) was maintained by use of a peristaltic pump. The elution volume of enzyme (V_e) was determined by appropriate enzyme activity measurements [formation of ADP from ATP or incorporation of $[^{14}C]HCO_3^-$ into [^{14}C]carbamoyl phosphate (Anderson et al., 1970)] or by measurement of A_{280} when elution was carried out in the presence of sodium barbital buffer. The column volume accessible to solvent (V_i) was determined by measuring the elution volume of NH_4Cl, which was measured with Nessler's reagent (Lang, 1958). The void volume of the column (V_0) was established by measuring the elution volume of Blue Dextran 2000 (A_{640}). The elution properties of the enzyme are expressed as the distribution coefficient (K_d) defined as $K_d = (V_e - V_0)/(V_i - V_0)$.

Carbamoyl-phosphate synthetase activity was determined by measuring the rate of formation of [^{14}C]carbamoyl phosphate in reaction mixtures that contained $[^{14}C]HCO_3^-$ [5 mM, (5–20) × 10^6 cpm], glutamine (10 mM), an ATP regenerating system composed of phosphoenolpyruvate (3 mM) and pyruvate kinase (30 units/mL), and other components at pH 7.8 as indicated in volumes of 0.4–1.0 mL at 17 °C. Reaction times varied from 30 s (high enzyme concentration) to 30 min (very low enzyme concentration), and product formation was found to be linear with time. [^{14}C]Carbamoyl phosphate was determined as previously described (Anderson et al., 1970). Specific activity is expressed as micromoles per hour per milligram of enzyme.

Results and Discussion

Gel Filtration Chromatography. Ultracentrifugation studies by Powers et al. (1980) established conditions under which carbamoyl-phosphate synthetase exists predominantly as monomer, dimer, or tetramer. As shown in Figure 1, these different states of association can be clearly distinguished by gel filtration chromatography on Sephadex G-200. The different components in the elution buffers and the corresponding expected states of association are indicated in the legend to Figure 1. Distribution coefficients for the enzyme under different conditions (different enzyme concentrations and different components in the elution buffer) are listed in Table I. The elution profiles from which the distribution coefficients were determined for each different elution buffer were similar to those illustrated in Figure 1. Experiments 1, 5, and 6, column A, in Table I correspond to conditions under which the enzyme exists as monomer, dimer, and tetramer, respectively. The elution position for monomer is based upon the

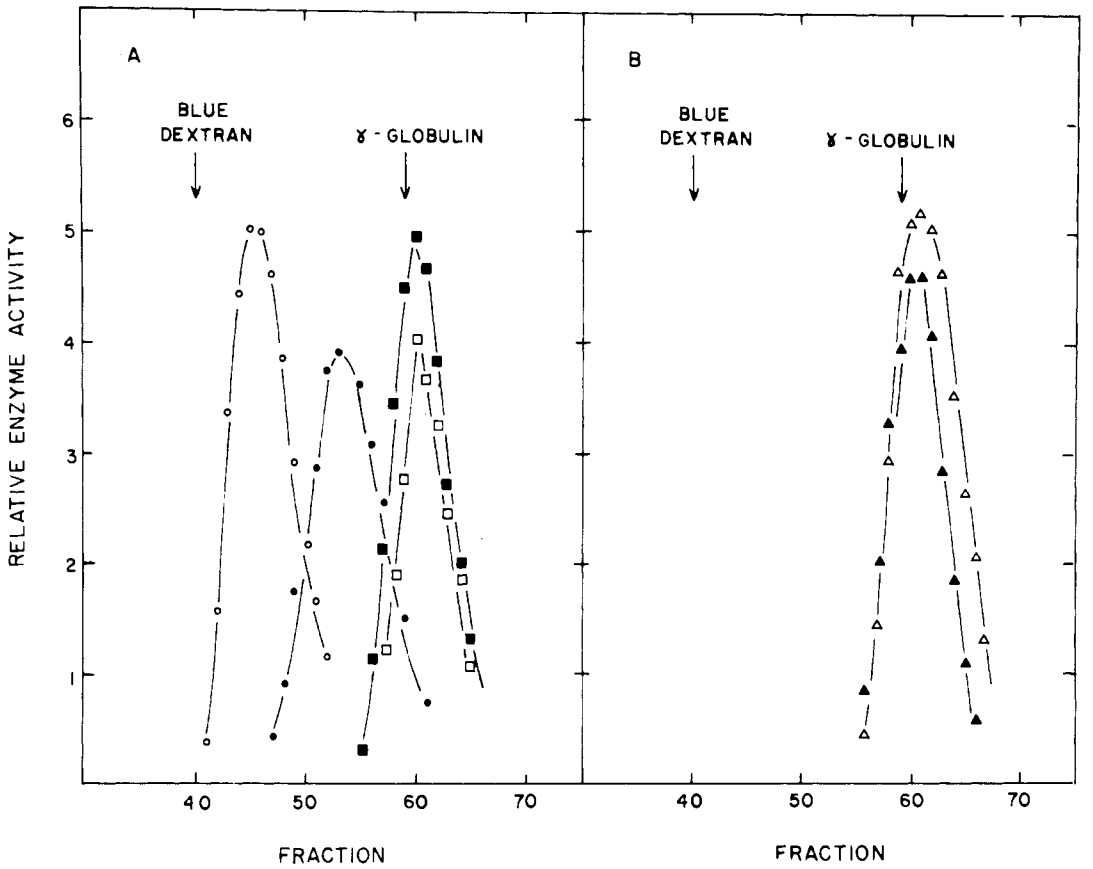


FIGURE 1: Gel filtration chromatography of carbamoyl-phosphate synthetase on Sephadex G-200. The elution positions of Blue Dextran 2000 (void volume) and γ-globulin ($M_r \simeq 160000$) are indicated by arrows. The buffer components, initial enzyme concentration, and expected state of association (if known) corresponding to each elution pattern are as follows: (A) 5 mM ornithine and 0.2 M potassium phosphate, pH 7.8, 8 mg/mL, tetramer (○); 1 mM UMP and 0.2 M potassium phosphate, pH 7.8, 8 mg/mL, dimer (●); 1 mM UMP and 0.2 M potassium phosphate, pH 7.8, 0.0016 mg/mL (■); 4 mM ornithine and 0.1 M potassium phosphate, pH 7.8, 0.0018 mg/mL (□); (B) 0.03 M sodium barbital, pH 7.8, and 0.001 M EDTA, 3 mg/mL, monomer (△); 0.04 M NaHepes, pH 7.8, 0.1 M KCl, 0.01 M ATP, 0.014 M $MgSO_4$, 0.01 M glutamine, 0.01 M ornithine, and 0.01 M $NaHCO_3$, 0.0016 mg/mL (▲). Other conditions and procedures are described under Materials and Methods.

Table I: Distribution Coefficients for Elution of Carbamoyl-Phosphate Synthetase from Sephadex G-200 under Different Conditions

column	expt	components present during chromatography[a]		enzyme concn (mg/mL)	K_d
		buffer (mM)	other components (mM)		
A	1	sodium barbital (30)	NaCl (100), EDTA (0.5)	3.0	0.28
	2	potassium phosphate (200)	UMP (1)	0.0016	0.27
	3	potassium phosphate (200)	UMP (1)	0.032	0.23
	4	potassium phosphate (200)	UMP (1)	1.5	0.19
	5	potassium phosphate (200)	UMP (1)	8.0	0.18
	6	potassium phosphate (200)	ornithine (5)	8.0	0.07
	7	NaHepes (40)	KCl (100), ATP (10), $MgSO_4$ (14), glutamine (10), ornithine (10), $NaHCO_3$ (10)	0.0016	0.27
B	1	NaHepes (40)	NaCl (100)	1.9	0.31
	2	NaHepes (40)	KCl (100)	0.97	0.27
	3	NaHepes (40)	KCl (100), $MgSO_4$ (20)	0.97	0.31
	4	NaHepes (40)	KCl (100), ornithine (4)	0.97	0.28
	5	NaHepes (40)	KCl (100), ornithine (4)	2.6	0.22
	6	NaHepes (40)	KCl (100), ornithine (4), $MgSO_4$ (20)	0.97	0.28
	7	NaHepes (40)	KCl (100), ornithine (4), $MgSO_4$ (20)	2.6	0.23
	8	NaHepes (40)	KCl (100), UMP (1)	0.97	0.32
	9	NaHepes (40)	KCl (100), UMP (1)	2.6	0.25
	10	NaHepes (40)	KCl (100), UMP (1), $MgSO_4$ (20)	0.97	0.30
	11	NaHepes (40)	KCl (100), UMP (1), $MgSO_4$ (20)	2.6	0.25
	12	potassium phosphate (100)	ornithine (4)	0.0018	0.29
	13	potassium phosphate (100)	ornithine (4)	2.91	0.09
	14	potassium phosphate (100)	ATP (10), $MgSO_4$ (10), glutamine (10), $NaHCO_3$ (10)	0.0016	0.33

[a] Numbers in parentheses indicate millimolar concentrations of buffer or components in buffer.

findings by Powers et al. (1980) and Trotta et al. (1974) that the enzyme exists only as monomer in the absence of K^+, phosphate, and other substrates and allosteric effectors, even at high enzyme concentration. Consequently, elution of the enzyme at this position represents a limiting value, and any kind of association, particularly as proposed by Powers et al. (1980) in which the phosphate-induced dimer is visualized as an end-to-end association of monomers, should be reflected in a reduced K_d value. The fact that this elution position is slightly later than that of γ-globulin is also consistent with a

monomer molecular weight of about 160 000. The changes in K_d values with different conditions (e.g., the presence or absence of K^+, phosphate, or ornithine) are consistent with changes in $s_{20,w}$, which have been reported as discussed above (Anderson & Marvin, 1970; Trotta et al., 1974; Powers et al., 1980).

The most important result that is apparent from the data in Figure 1 and in Table I is that at low enzyme concentrations the enzyme elutes at a position corresponding to monomer, regardless of which components are present in the elution buffer. This includes the presence of either UMP, ornithine, or all substrates plus ornithine (all in the presence of potassium phosphate buffer). The presence of all substrates plus ornithine represents conditions under which the enzyme is fully active (Anderson & Marvin, 1970; also, see Figures 2 and 3). In this latter case (reacting-enzyme gel filtration chromatography) the concentration of ADP in the fractions that eluted shortly after the enzyme activity was measured and estimated to be at a level consistent with full activity during chromatography (these fractions had been exposed to the enzyme prior to elution for a period of time dependent upon the flow rate). This observation, together with the fact that the parameter utilized to measure elution volume was enzyme activity, supports the conclusion that at low enzyme concentrations the enzyme exists as monomer (monomer III in Scheme I) under conditions where the enzyme displays full catalytic activity. This conclusion is in agreement with the results of reacting-enzyme ultracentrifugation studies reported by Powers et al. (1980), in which the enzyme was shown to sediment as monomer at low enzyme concentrations under conditions that result in maximal catalytic activity (substrates being saturating).

The presence or absence of excess Mg^{2+} does not appear to significantly affect the state of association (Table I).

The elution of carbamoyl-phosphate synthetase at low enzyme concentration in the presence of UMP and potassium phosphate buffer at the position corresponding to monomer (Figure 1) and the observed changes in K_d values with enzyme concentration under these conditions (Table I) clearly reflect dissociation of dimer to monomer. Our earlier studies showed that carbamoyl-phosphate synthetase could be immobilized to a solid support with little loss in activity when UMP was present and the enzyme concentration was 0.003 mg/mL (Anderson, 1977a). The present studies confirm the assumption that the enzyme was predominantly monomer (monomer I in Scheme I) before immobilization, thus strengthening the argument that the enzyme was immobilized as monomer and that a monomeric form of the enzyme is, therefore, fully active.

Effect of Carbamoyl-Phosphate Synthetase Concentration on Specific Activity. As shown in Figure 2A, the specific activity of carbamoyl-phosphate synthetase increases with increasing enzyme concentration above 0.01 mg of enzyme/mL when the concentration of MgATP is 1.5 mM but is not affected when the MgATP concentration is saturating [40 mM in the absence of ornithine; see Anderson and Meister (1966) and Anderson and Marvin (1968, 1970)]. The same results were obtained by Powers et al. (1980), who interpreted this finding as evidence that oligomer is more active than monomer, since reacting enzyme centrifugation studies had shown that in the presence of substrates the enzyme exists as monomer at enzyme concentrations below 0.01 mg/mL and self-association occurs at higher enzyme concentrations. However, the MgATP concentration in the reacting-enzyme centrifugation experiments carried out by Powers et al. (1980) was saturating.

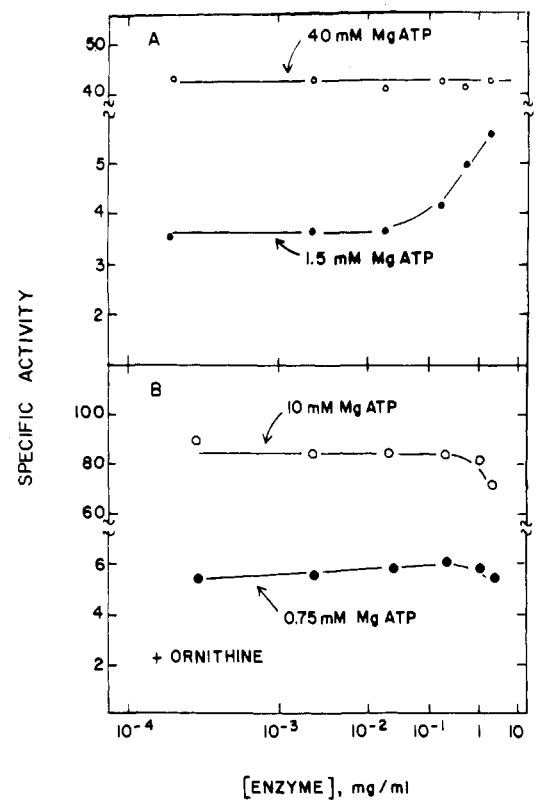


FIGURE 2: Effect of carbamoyl-phosphate synthetase concentration on specific activity. (A) The reaction mixtures contained 0.1 M potassium phosphate buffer, pH 7.8, and either 1.5 or 40 mM MgATP as indicated. (B) The reaction mixtures contained 0.1 M potassium phosphate buffer, pH 7.8, 5 mM ornithine, and either 0.75 or 10 mM MgATP as indicated. Other components of the reaction mixture and assay procedures are described under Materials and Methods.

A decrease in specific activity should, therefore, be observed with decreasing enzyme concentration even when MgATP is saturating if monomer is, in fact, less active than oligomer. The studies by Powers et al. (1980) and our results indicate that this is not the case; instead, a constant, maximal specific activity is observed at all enzyme concentrations. These results are consistent with Scheme I, however, even though oligomer is not considered to be catalytically more active than monomer. When MgATP is nonsaturating, the specific activity increases with increasing enzyme concentration because as a result of oligomer formation the equilibrium among the different enzyme forms is shifted away from monomer I, which cannot bind MgATP, in favor of enzyme forms that bind MgATP. When MgATP is saturating, the enzyme exists predominantly as monomer III in equilibrium with oligomeric forms of monomer III. Even though the proportion of the enzyme existing as oligomer would increase with increasing enzyme concentration, the specific activity would not increase, since monomer III and oligomers of monomer III have the same specific activity.

The presence of ornithine greatly reduces the concentration of MgATP required for half-maximal activity (Anderson & Marvin, 1968, 1970; see Figure 3). When ornithine is present and the concentration of MgATP is low enough (0.75 mM) that maximal activity is not attained, the specific activity is not significantly affected when the enzyme concentration is increased above 0.01 mg/mL (Figure 2B); in fact, a small decrease is observed, similar to that obtained when the MgATP concentration is saturating (10 mM in the presence of ornithine). The results of previous studies (Anderson & Marvin, 1970; Trotta et al., 1974; Powers et al., 1980) as well as those reported here provide substantial evidence that under these

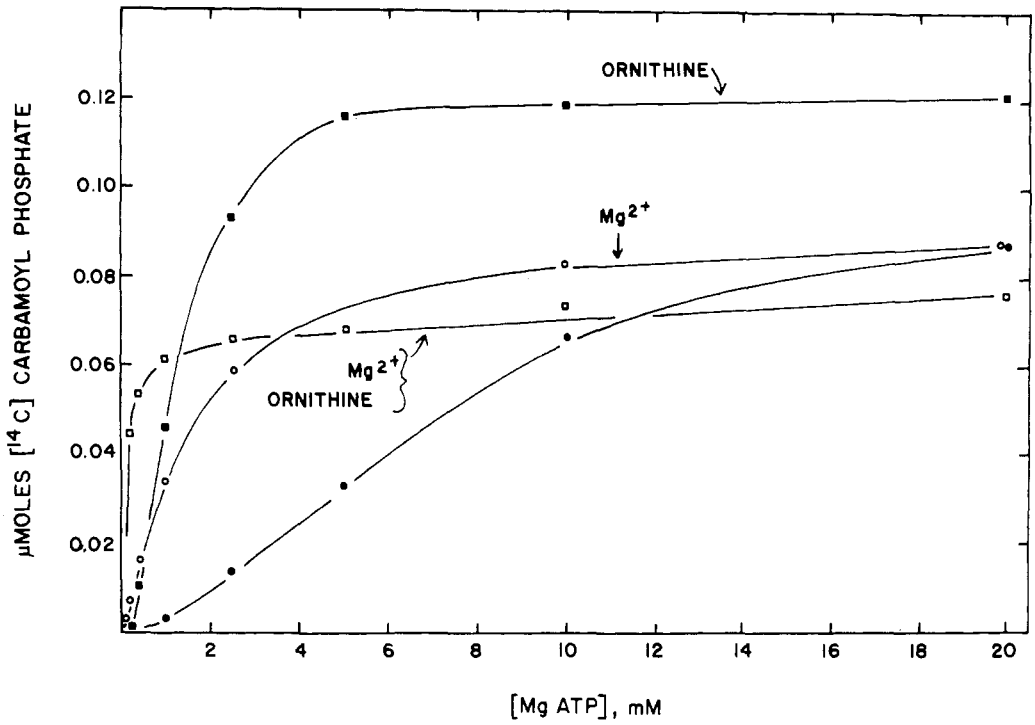


FIGURE 3: Carbamoyl-phosphate synthetase activity as a function of MgATP concentration. The reaction mixtures contained glutamine, [^{14}C]HCO_3^-, phosphoenolpyruvate and pyruvate kinase as indicated under Materials and Methods, ATP and $MgSO_4$ in equimolar proportions as indicated, 0.1 M KCl, 0.1 M Tris buffer, pH 7.8, 0.01 mg/mL enzyme, and 5 mM ornithine and/or 20 mM $MgSO_4$ where indicated in addition.

conditions the enzyme undergoes association from monomer to predominantly tetramer over the range of enzyme concentrations employed in these experiments. The results in Figure 2B provide further support for Scheme I. Thus, the presence of ornithine would result in displacement of the monomer I–monomer II equilibrium in favor of monomer II. Consequently, the specific activity would not be significantly affected by increasing enzyme concentration because monomer I, which cannot bind MgATP, is no longer a component of the system, analogous to the situation when ornithine is absent but MgATP is saturating, as described above. In contrast, if maximal specific activity was directly dependent upon oligomer formation, an increase in specific activity would be expected with increasing enzyme concentration in the presence of ornithine whether or not MgATP was saturating, particularly above 0.1 mg/mL.

The above experiments and those by Powers et al. (1980), as well as previous related studies (Anderson & Marvin, 1970; Anderson, 1977a), were carried out with equimolar concentrations of Mg^{2+} and ATP, since the allosteric effects and the effects of enzyme concentration are more readily apparent under these conditions. However, it has been established that (1) carbamoyl-phosphate synthetase has a specific binding site for free Mg^{2+}, which is essential for catalytic activity, (2) the presence of excess Mg^{2+} significantly reduces the concentration of ATP required for half-maximal activity, and (3) the presence of both ornithine and excess Mg^{2+} eliminates the apparent cooperativity observed for MgATP and results in a very low apparent K_m for MgATP (Raushel et al., 1978, 1979, 1983). These kinetic effects are illustrated in Figure 3. As discussed previously the marked sigmoidal relationship between MgATP concentration and various properties of the enzyme, including catalytic activity, may be primarily a function of free Mg^{2+} concentration, which affects the concentration of free ATP and of MgATP as well as the proportion of binding sites for free Mg^{2+} on the enzyme that are filled (Anderson, 1977a). These observations together with the conclusion that oligomer

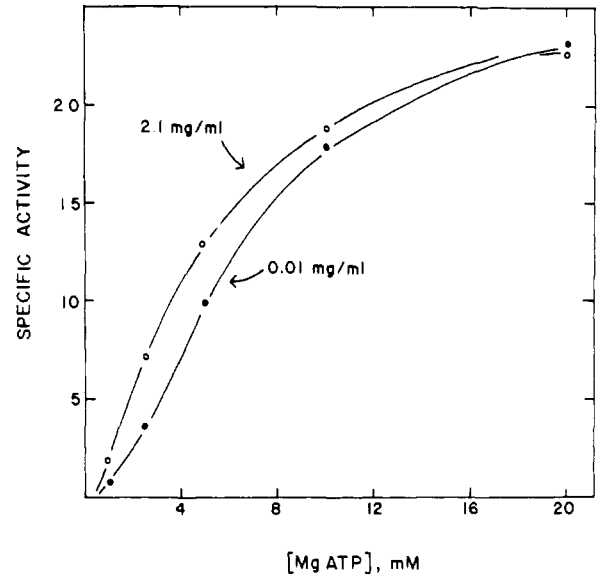
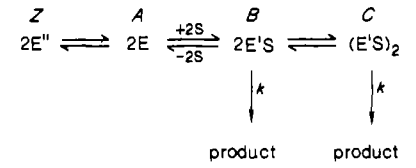


FIGURE 4: Specific activity of carbamoyl-phosphate synthetase as a function of MgATP concentration—effect of enzyme concentration. Reaction conditions were the same as described in Figure 3, except the reaction mixtures contained enzyme as indicated.

formation is not required for full catalytic activity indicate that the regulatory properties of this allosteric enzyme are not mediated through site–site interactions (Anderson, 1977a).

Our previous conclusion that oligomer formation was not required for catalytic activity or manifestation of allosteric effects was interpreted as evidence that the catalytic and regulatory functions of carbamoyl-phosphate synthetase are not dependent upon oligomer formation (Anderson, 1977a). In fact, the results of the present study and those of Powers et al. (1980) indicate that this is not exactly correct. Although apparently not directly dependent upon oligomer formation, the regulatory properties and the apparent catalytic activity can be affected by enzyme concentration, as predicted by

Scheme II



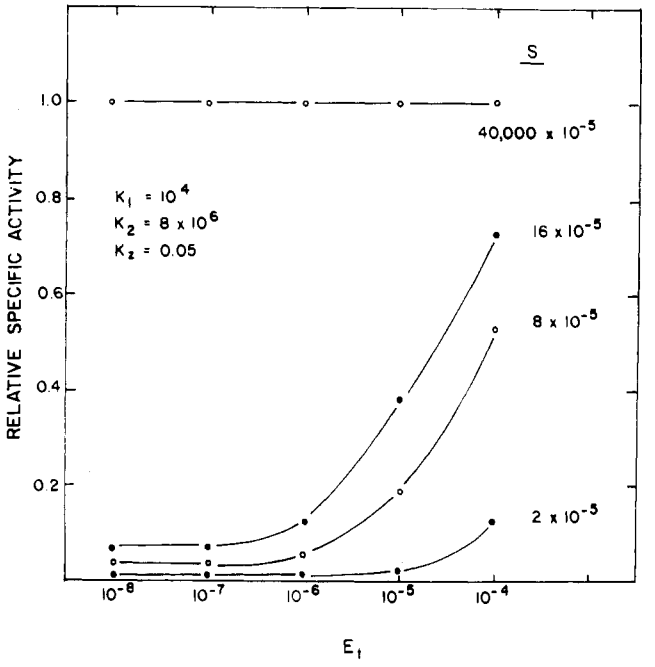


FIGURE 5: Calculated effects of enzyme concentration on relative specific activity as a function of substrate concentration based on Scheme II and eq 3 under Results and Discussion. Values for E_t and S represent molar concentrations. Assigned, constant values of K_1, K_2, and K_z are as indicated.

Scheme I. This is illustrated, for example, by the effect of enzyme concentration on the specific activity as a function of MgATP concentration as shown in Figure 4. A higher enzyme concentration results in a decrease in the concentration of MgATP required for half-maximal activity.

Supporting Theoretical Considerations. Since several key features of Scheme I, which incorporates properties specific to carbamoyl-phosphate synthetase, may be significant for other enzyme systems, a generalized and simplified model (Scheme II) is considered for the purpose of analyzing the possible effects of these features on enzyme properties. The determining characteristic features in Scheme II include the following: (1) an equilibrium preexists between two monomeric forms with ligand binding only to one monomeric form, (2) a ligand-induced conformational change occurs upon binding of ligand to the monomer, (3) only the monomeric form to which ligand is bound can associate (to dimer), and (4) most importantly, oligomer (dimer) and monomer from which it is derived are equally catalytically active. Assuming rapid equilibrium between all species, expressions for the concentration of the catalytically active monomer (E'S) and for velocity can be derived as follows. To simplify the expressions, the concentrations of each of the enzyme forms are defined as the capitalized letters written just above the different enzyme forms in Scheme II. Ligand (substrate) concentration is defined as S. The three equilibrium (association) constants are then defined as

$$K_z = A/Z \qquad K_1 = B/AS \qquad K_2 = C/B^2$$

and the total enzyme concentration is

$$E_t = Z + A + B + 2C$$

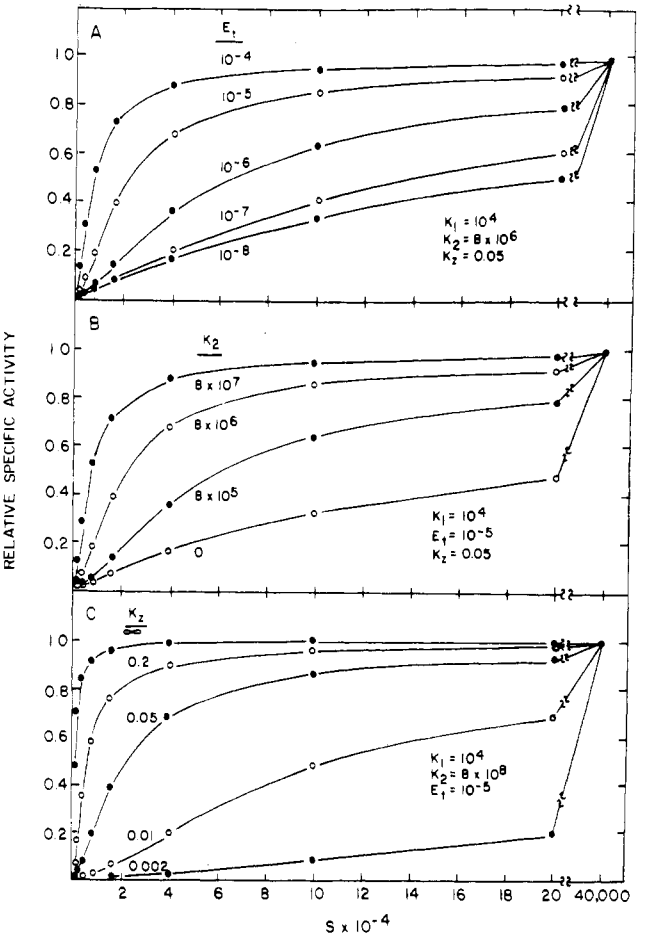


FIGURE 6: Calculated effects of substrate concentration on relative specific activity based on Scheme II and eq 3 under Results and Discussion. Values for E_t and S represent molar concentrations. (A) As a function of total enzyme concentration. Assigned, constant values of K_1, K_2, and K_z are as indicated. (B) As a function of the value of the monomer–dimer association constant, K_2. Assigned, constant values for K_1, K_z, and E_t are as indicated. (C) As a function of the value of the equilibrium constant, K_z, for the preexisting equilibrium between two monomeric forms. Assigned, constant values for K_1, K_2, and E_t are as indicated.

where E_t = total enzyme concentration. These four equations can be rearranged to give eq 1, a quadratic equation for B

$$-(2K_1K_2S)B^2 + (-1 - (1/K_z) - K_1S)B + K_1SE_t = 0 \quad (1)$$

$$B = \{-(-1 - 1/K_z - K_1S) \pm [(-1 - 1/K_z - K_1S)^2 + 8K_1^2K_2S^2E_t]^{1/2}\}/-4K_1K_2S \quad (2)$$

in terms of S, E_t, and equilibrium constants. The solution for eq 1 using the quadratic formula is given by eq 2. Assuming that monomer and dimer have the same specific activity, the velocity is

$$v = kB + 2kC = k(B + 2K_2B^2) \quad (3)$$

where B can be calculated from eq 2. The rate constant k is arbitrarily set at 1 and v is expressed as relative specific activity in the caluclations for Figures 5 and 6.

With hypothetical but reasonable values for the various association constants, the effects of enzyme concentration on relative specific activity at different concentrations of substrate were calculated by using eq 3. As shown in Figure 5, when substrate is saturating, maximum specific activity is attained at all enzyme concentrations, i.e., whether or not enzyme exists as oligomer. This is because the preexisting monomeric equilibrium is displaced away from the inactive form when

substrate is saturating. When substrate is nonsaturating, the lower level of activity reflects the equilibrium distribution between inactive and active monomer. The specific activity is constant at lower enzyme concentrations but increases at higher enzyme concentrations where association occurs because formation of active dimer from active monomer shifts the equilibrium distribution away from inactive monomer; the concentration of active monomer plus active dimer is increased. [These hypothetical effects of enzyme concentration are qualitatively similar to the results obtained experimentally with carbamoyl-phosphate synthetase, equating S with MgATP (Figure 2 and Powers et al., 1980).] A similar associating mechanism has been proposed for the polypeptide glucagon (Gratzer & Beaven, 1969). The glucagon monomer exists as an equilibrium between coiled and structured forms. Only the structured monomeric form can associate, and the associated state is considered to act as a thermodynamic trap for the structured form.

If dimer in Scheme II is considered to be catalytically more active than monomer, considerations analogous to those in the above paragraph yield similar results, except that the specific activity decreases with decreasing enzyme concentration even when substrate is saturating. The decrease in specific activity might not be observed experimentally if the value of K_2 is extremely high such that virtually all enzyme with substrate bound exists as dimer (oligomer), even at very low enzyme concentrations. [This situation does not appear to be the case with carbamoyl-phosphate synthetase (Figure 1, Table I, and Powers et al., 1980).]

Several additional features of mechanisms such as Scheme II are of significance. First, as shown in Figure 6A, calculations using eq 3 show that as a result of the association–dissociation process ligand binding is sigmoidal and affected by enzyme concentration. [An analogous situation is observed for carbamoyl-phosphate synthetase (Figure 4).] The sigmoidal nature of ligand binding is dependent upon K_2 and becomes hyperbolic when $K_2 = 0$. Second, the "apparent K_m" (concentration of substrate that gives half-maximal activity) can be significantly lower than the actual dissociation constant ($1/K_1$) as the result of enzyme subunit association; this effect increases as the value of K_2 increases as illustrated in Figure 6B,C and is simply the result of displacement of the E + S $\rightleftharpoons$ E′S equilibrium in favor of E′S due to the removal of a proportion of E′S as the result of association to $(E'S)_2$. This effect is more significant if K_z is very high, i.e., absence of a preexisting equilibrium; for example, assuming K_z = infinity, $E_t = 10^{-5}$, and $1/K_1 = 1 \times 10^{-4}$, the apparent K_m has values of 6×10^{-6} and 1×10^{-4} when $K_2 = 8 \times 10^6$ and 0, respectively. Luther et al. (1985) recently reported that the apparent K_m value for fructose 6-phosphate in the reaction catalyzed by phosphofructokinase is dependent upon enzyme concentration, reflecting an effect of self-association analogous to the effects described here. Finally, the feature of a preexisting equilibrium between inactive and active forms that is affected by allosteric effectors provides a simple mechanism for greatly influencing the "sigmoidicity" and apparent K_m through relatively small changes in the equilibrium constant (K_z), as illustrated in Figure 6C. For example, in the case of carbamoyl-phosphate synthetase, K_z (or a corresponding equilibrium constant or set of equilibrium constants) could be near infinity in the presence of ornithine and a very low value in the presence of UMP.

Theoretical treatments of enzymes subject to reversible association–dissociation, in which the state of association influences enzyme activity and the state of association is affected by substrates and/or allosteric ligands, have been primarily based upon the assumption that different states of association that are in equilibrium with each other have different specific activities or affinities for ligands; an effect of enzyme concentration on specific activity is considered as evidence for such a system (Frieden, 1981; Kurganov, 1982; Neet, 1980). The considerations presented in this paper suggest that the effects characteristic of associating enzyme systems may arise in some circumstances through processes in which different states of association do not necessarily have different catalytic activities and/or ligand binding properties. Instead, the controlling influence may be that association serves simply to increase the concentration of an active (or inactive) species by mass-action effects, analogous to the mechanism illustrated by Scheme II, above, and by Scheme I for carbamoyl-phosphate synthetase. Preliminary investigations in our laboratory suggest that the effects of enzyme concentration on the catalytic and regulatory properties of CTP synthetase (Anderson, 1983) may also be effected, in part, by a mechanism similar to that described by Scheme II, except that the preexisting equilibrium is between inactive monomer and active dimer.

Registry No. UMP, 58-97-9; MgATP, 1476-84-2; L-ornithine, 70-26-8; carbamoyl-phosphate synthetase, 37233-48-0.

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