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Evidence that the Catalytic and Regulatory Functions of Carbamyl-Phosphate Synthetase from *Escherichia coli* Are Not Dependent on Oligomer Formation†

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ABSTRACT: Carbamyl-phosphate synthetase from *Escherichia coli* is an allosteric enzyme which undergoes reversible association reactions in phosphate buffer. The positive allosteric effectors, ornithine, inosine 5′-monophosphate (IMP), and ammonia, facilitate oligomer formation, whereas uridine 5′-monophosphate (UMP), a negative effector, prevents or decreases oligomer formation. When the enzyme is immobilized by reaction with activated Sepharose, under conditions where the enzyme exists only as a monomer, nearly full catalytic activity is retained and the effects of ornithine, IMP, and UMP on the catalytic activity as a function of MgATP concentration are not significantly altered. Gel-filtration chromatography on Sephadex G-200 of catalytic quantities of the enzyme in the presence of all substrates showed that the elution volume was the same as that measured for the enzyme under conditions where it is known to exist in the monomer form. The specific activity of the enzyme does not increase when the concentration of the enzyme is increased 100-fold from a concentration at which the enzyme exists as monomer to a level at which the enzyme exists predominantly as oligomer. These results indicate that the monomer form of the enzyme is the principle active species and that oligomer formation is not directly related to enzyme activity or enzyme regulation.

Carbamyl-phosphate synthetase from *Escherichia coli* is an allosteric enzyme composed of two nonidentical subunits of different molecular weight (Anderson and Meister, 1966; Matthews and Anderson, 1972; Trotta et al., 1971, 1974). The enzyme is subject to feedback inhibition by UMP[1] and is activated by ornithine and also by IMP if phosphate is absent (Anderson and Meister, 1966; Pierard, 1966; Anderson and Marvin, 1970). The allosteric effectors and all substrates, except L-glutamine, apparently bind to site(s) on the heavy subunit; the light subunit functions as a glutamine-binding subunit, releasing NH_3, which binds and reacts at a site on the heavy subunit (Trotta et al., 1971).

The enzyme undergoes reversible association reactions in the presence of phosphate buffer (Anderson and Marvin, 1970; Trotta et al., 1974). The enzyme exists as a monomer when the concentration of the enzyme is as low as the concentrations normally employed in kinetic assays. Oligomer formation is dependent on enzyme concentration and is facilitated by the presence of the positive allosteric effectors, ornithine, IMP, and ammonia, and also by the substrate MgATP, whereas the presence of UMP, a negative allosteric effector, prevents or decreases the formation of oligomer.

When the existence of this monomer–oligomer equilibrium was first reported, it was suggested that allosteric regulation could possibly be explained in terms of the state of association of the enzyme, i.e., that oligomer formation was necessary for full activity (Anderson and Marvin, 1968). More recent studies have suggested the alternative scheme shown in Figure 2 in which the monomer is the principle active species and allosteric regulation is the result of stabilization of different conformational states of the monomer by different allosteric effectors; according to this scheme, oligomer formation is not directly related to enzyme activity or enzyme regulation, but is simply facilitated by formation of the active conformational states of the enzyme (Anderson and Marvin, 1970). As pointed out by Trotta et al. (1974), however, sufficient evidence has not been reported which would clearly establish the state of association of the active form of the enzyme and thus distinguish between these two possible schemes. The purpose of the present study was to obtain evidence that the catalytic activity of this enzyme and the allosteric effects of ornithine, UMP, and IMP are not dependent on oligomer formation. A preliminary report of this work has been published (Anderson, 1976).

Material and Methods

Carbamyl-phosphate synthetase was isolated from *E. coli* B by the procedure described by Anderson et al. (1970), as modified by Matthews and Anderson (1972). Sepharose 4B, Sephadex G-200, and Blue Dextran 2000 were products from Pharmacia. Cyanogen bromide was obtained from Aldrich Chemical Co. All other biochemical reagents were obtained from Sigma Chemical Co.

Carbamyl-phosphate synthetase was immobilized by reaction with activated Sepharose 4B. The Sepharose was activated by reacting 1 g of cyanogen bromide with Sepharose 4B

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[1] Abbreviations used are: UMP and IMP, uridine and inosine 5′-monophosphates; ADP and ATP, adenosine di-, and triphosphates; EDTA, (ethylenedinitrilo)tetraacetic acid.

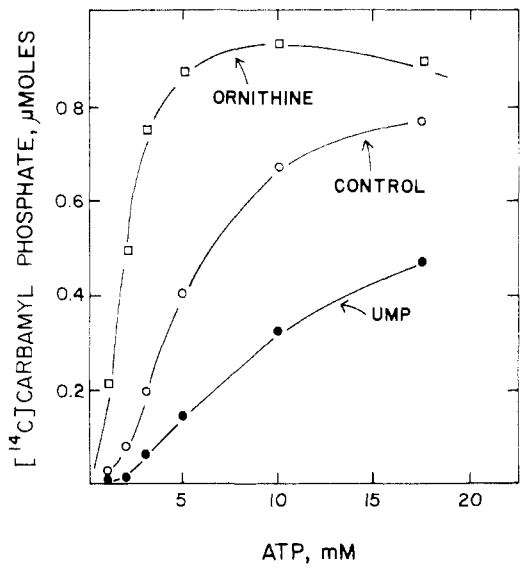

FIGURE 1: Activity of immobilized carbamyl-phosphate synthetase as a function of MgATP concentration in the presence or absence of ornithine or UMP. The reaction mixtures contained glutamine (5 mM), [^{14}C]-$NaHCO_3$ (20 mM, 4.5×10^5 cpm), ATP as indicated, $MgCl_2$ in concentrations that were equimolar with ATP, potassium phosphate buffer (0.07 M, pH 7.8), Sepharose-enzyme complex (equivalent to about 11 μg of enzyme and 0.15 ml of packed Sepharose), and, where indicated, ornithine (0.3 mM) or UMP (0.03 mM) in a final volume of 1 ml. The reaction was carried out at 37 °C for 30 min with continuous reciprocal shaking and with stirring of the contents of the tubes at 3-min intervals. The reaction was stopped by addition of 1 ml of cold 2 mM UMP followed immediately by low-speed centrifugation at 4 °C. The micromoles of [^{14}C]carbamyl phosphate in 1 ml of the supernatant was then determined as described previously (Anderson et al., 1970).

(20 ml of packed Sepharose suspended in 80 ml of H_2O) essentially as described by Cuatrecasas (1970). The activated Sepharose was filtered and washed with 300 ml of cold 0.2 M potassium phosphate buffer, pH 7.8, and then added to 200 ml of a solution containing 1.6 mg of carbamyl-phosphate synthetase (8 μg/ml), 0.2 M potassium phosphate buffer, pH 7.8, and 1 mM UMP at 17 °C. The suspension was stirred rapidly for 10 min at 17 °C and then slowly overnight at 4 °C. The suspended Sepharose–enzyme complex was then filtered and washed with 7 volumes of cold 0.2 M potassium phosphate buffer, pH 7.8, and suspended in an equal volume of this buffer (40 ml total volume).

Gel-filtration chromatography was carried out at 19 °C on a column (0.9 × 29 cm) of Sephadex G-200 equilibrated with 0.1 M potassium phosphate buffer, pH 7.8, containing 0.5 mM EDTA, and other components, as indicated in Table I. The volume of the enzyme sample added to the column was 0.4 ml. The elution volume was accurately determined by weighing the eluate in each fraction beginning at the point when all of the sample had entered the gel. Fractions of about 0.5 ml were collected at a flow rate of about 3 ml/h. The void volume of the column, V_0, was determined by measuring the elution volume of Blue Dextran 2000 (located by measuring its absorbance at 640 nm); the total volume accessible to solvent, V_i, was determined by measuring the elution volume of DNP-alanine (located by measuring its absorbance at 330 nm); the elution volume of the enzyme, V_e, was determined by measuring the rate of formation of ADP or [^{14}C]carbamyl phosphate when an appropriate sample of each fraction was assayed as previously described (Anderson et al., 1970). The volumes in each of these cases correspond to the volume having the highest concentration of the respective component. The distribution coefficient, K_d, is defined as $K_d = (V_e - V_0)/(V_i - V_0)$.

TABLE I: Distribution Coefficients for Carbamyl-Phosphate Synthetase Subjected to Gel-Filtration Chromatography under Different Conditions, Including the Presence of All Substrates.

Expt	Initial Enzyme Concn (mg/ml)	Additional Components in Buffer	K_d
1	0.1		0.26
2	0.1	UMP (5 mM)	0.27
3	1.0	Ornithine (5 mM)	0.08
4	0.1[a]	Ornithine (10 mM), $NaHCO_3$ (10 mM), Glutamine (10 mM), ATP (10 mM), $MgSO_4$ (10 mM)	0.26

[a] All substrates were present in the column as the enzyme was eluted. That the enzyme was catalytically active during the elution was established by showing that ADP was present in fractions collected after the enzyme had been eluted. The enzyme was located in the eluted fractions of this experiment by measuring the rate of formation of [^{14}C]carbamyl phosphate.

Results

Properties of Immobilized Carbamyl-Phosphate Synthetase. The enzyme was immobilized by reaction with cyanogen bromide activated Sepharose 4B under conditions where the enzyme is known to exist as a monomer, i.e., low enzyme concentration and presence of UMP (Anderson and Marvin, 1970; Trotta et al., 1974). The rationale for these experiments was that if the enzyme was immobilized as a monomer, thus preventing subsequent oligomer formation, the enzyme activity and/or the effects of ornithine and UMP on the activity would be prevented or markedly altered if either of these processes were dependent on oligomer formation. This approach is similar, but not analogous, to the techniques of attaching protein subunits to an insoluble matrix employed by others to investigate the activity of protein subunits which normally exist as oligomers (Chan, 1976; Green and Toms, 1973); in these studies, a very low concentration of cyanogen bromide and a relatively high concentration of protein were utilized for the purpose of increasing the probability that only one subunit of an oligomeric protein would become covalently attached to the Sepharose. A much higher concentration of cyanogen bromide was used for activation of the Sepharose in the present study, since the objective was to assure rapid and complete immobilization and to minimize the possibility of interaction between immobilized enzyme molecules. Accomplishment of the latter objective was also facilitated by the fact that the enzyme concentration was very low during the reaction leading to immobilization.

The results of several experiments involving immobilization of carbamyl-phosphate synthetase, as described above, indicated that immobilization results in little loss of enzyme activity. About 80% of the total enzyme units initially added were normally recovered as immobilized enzyme activity. The enzyme did not bind to Sepharose, which had not been activated by treatment with cyanogen bromide. When assayed as described in Figure 1, the enzyme activity was linearly related to the quantity of Sepharose–enzyme complex present and to time over a period of 40 min. The enzyme activity of the Sepharose–enzyme complex was not due to activity of free enzyme released from the Sepharose during incubation with

TABLE II: Effect of Enzyme Concentration on Enzyme Activity in the Presence or Absence of Ornithine at Saturating or Nonsaturating Levels of MgATP.[a]

	Relative Specific Activity			
	20 mM MgATP		5 mM MgATP	
Enzyme Concn (mg/ml)	No Ornithine (%)	10 mM Ornithine (%)	No Ornithine (%)	10 mM Ornithine (%)
0.01	100 (41)	100 (62)	100 (21)	100 (67)
0.1	89	88	101	95
1.0	83	79	107	75

[a] The reaction mixtures contained ATP, $MgSO_4$, ornithine and enzyme as indicated, [^{14}C]$NaHCO_3$ (20 mM, 11 × 10^6 cpm), L-glutamine (10 mM), and potassium phosphate buffer (0.1 M, pH 7.8) in a final volume of 1.0 ml. The reaction was allowed to proceed at 17 °C for 1 (20 mM MgATP) or 2 min (5 mM MgATP), at which time the reaction was stopped and the micromoles of [^{14}C]carbamyl phosphate synthesized was determined as previously described (Anderson et al., 1970). The results in each column are expressed as specific activity relative to a value of 100% for the specific activity at the lowest enzyme concentration. The actual specific activity (μmol of carbamyl phosphate synthesized h^{-1} (mg of enzyme at 17 °C)$^{-1}$) for the lowest enzyme concentration in each column is indicated in parentheses.

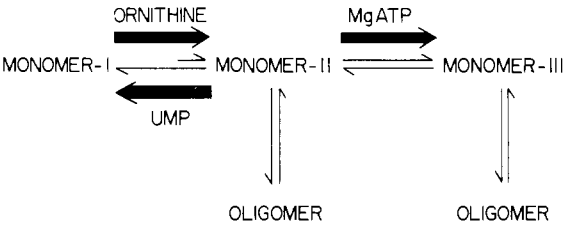

FIGURE 2: Scheme previously proposed for explaining the effect of allosteric effectors on carbamyl-phosphate synthetase activity. Monomers I and II are viewed as being in equilibrium with each other, the equilibrium strongly favoring monomer I. Monomer I cannot readily aggregate to give oligomer. MgATP binds only or predominantly to monomer II, inducing a conformational change to give monomer III, the catalytically active form of the enzyme. UMP binds only to monomer I. Ornithine binds only to monomer II, thus shifting the equilibrium in favor of monomer II. UMP and ornithine are considered to act as allosteric effectors, therefore, by shifting the equilibrium away from or toward monomer II, respectively. The monomer form of the enzyme is composed of two nonidentical polypeptide chains. Evidence for this scheme has been previously reported (Anderson and Marvin, 1970; Foley et al., 1971; Matthews and Anderson, 1972).

substrates, since all activity remained associated with the Sepharose after the Sepharose–enzyme complex was removed from a reaction mixture by filtration and subsequently washed with phosphate buffer. The enzyme could not be dissociated from the Sepharose–enzyme complex by washing with 1 M potassium phosphate buffer, pH 7.8, at 17 °C.

As shown in Figure 1, the effect of MgATP concentration, in the presence or absence of UMP or ornithine, on the activity of the immobilized enzyme was nearly the same as that observed for the free enzyme (Anderson and Meister, 1966; Anderson and Marvin, 1968). Assuming that the immobilized form of the enzyme was, in fact, that of the monomer, these results support the view that oligomer formation is not required for catalytic activity or for manifestation of the allosteric effects of ornithine and UMP on the effect of MgATP concentration on catalytic activity.

Gel-Filtration Chromatography of Carbamyl-Phosphate Synthetase in the Presence of All Substrates. The results of these studies are shown in Table I. Experiments 1 and 2 were carried out under conditions where the enzyme is known to exist as a monomer, i.e., low initial enzyme concentration and/or the presence of UMP, whereas experiment 3 was carried out under conditions where the enzyme is known to exist as oligomer, i.e., higher initial enzyme concentration and presence of ornithine (Anderson and Marvin, 1970; Trotta et al., 1974). When chromatography was carried out in the presence of all substrates plus ornithine using the lower enzyme concentration (experiment 4), the elution volume was unchanged from that observed in experiment 1; i.e., the enzyme eluted in the same position as the monomeric form of the enzyme. These results directly support the conclusion that catalytic activity and the effect of ornithine, a positive allosteric effector, on the catalytic activity are not functions of the state of aggregation of the enzyme.

Effect of Enzyme Concentration on Catalytic Activity. The studies described above support the view that the enzyme is catalytically active as a monomer. The effect of oligomer formation on the level of catalytic activity was investigated by determining the specific activity of the enzyme as a function of enzyme concentration. The results in Table II show that, in the presence of saturating levels of MgATP (20 mM), the specific activity did not increase when the enzyme concentration was increased 100-fold from a concentration at which the enzyme exists as monomer to a level at which the enzyme exists predominantly as oligomer. In fact, a small decrease in specific activity was observed. According to the scheme in Figure 2, under these conditions (saturating MgATP), the concentration of monomer I (inactive species) would be very low regardless of the enzyme concentration. The observation (Table II) that the additional presence of ornithine had little effect on these results would, therefore, be expected.

At a lower, nonsaturating, level of MgATP, the concentration of monomer I would be appreciable at low enzyme concentration. At high enzyme concentration, the proportion of monomer I would decrease as a result of mass action (the equilibrium between nomoner I and II would be shifted towards monomer II and oligomers of monomer II). Thus, an increase in specific activity might be expected as the enzyme concentration is increased. As shown in Table II, an increase in specific activity was, in fact, observed with increasing enzyme concentration when the enzyme was assayed in the presence of 5 mM MgATP. This effect, as would be expected, is not observed if ornithine is present, since the presence of ornithine would shift the equilibrium away from monomer I at all enzyme concentrations.

Discussion

Ligand-induced changes in the state of oligomerization of proteins are a potentially important regulatory mechanism and have been shown to be the basis for sigmoid reaction kinetics resulting from cooperative binding of ligands in some regulatory enzyme systems (Dunne and Wood, 1975; Hammes and Wu, 1974). Since it has been clearly established that carbamyl-phosphate synthetase is subject to reversible oligomer formation at high enzyme concentration and that the degree of oligomerization is affected by the presence of different ligands, the possibility that oligomer formation is directly related to the regulatory properties of this enzyme or is required for catalytic activity has been considered (Anderson and Marvin, 1968; Trotta et al., 1974; Powers and Meister, 1976). One reason for this consideration was the fact that the presence of

UMP, a negative allosteric effector, inhibits oligomer formation, whereas the positive allosteric effectors or the substrate MgATP facilitate oligomer formation (Anderson and Marvin, 1968, 1970). The data in Table I of this paper, however, clearly indicate that the enzyme exists predominantly in the monomeric form under conditions normally employed in the kinetic assays used for measuring catalytic activity, i.e., low enzyme concentration and all substrates plus ornithine, a positive allosteric effector, present. The data in Table II further show that the level of enzyme activity is not substantially affected by the state of aggregation of the enzyme. In addition, when the enzyme is attached to a solid matrix under conditions where the enzyme is known to exist only as monomer, thus presumably preventing or decreasing the extent of oligomer formation, full activity and normal kinetic and regulatory properties are retained, including the sigmoid relationship between activity and MgATP concentration. The data presented in this paper, therefore, indicate that the formation of oligomer is not directly related to the regulatory or catalytic properties of carbamyl-phosphate synthetase.

The results of these studies are consistent with and provide additional evidence for the scheme shown in Figure 2. According to this scheme, the allosteric effectors act by stabilizing different conformational states of the monomeric unit of the enzyme. The activity of the monomeric unit is not appreciably affected by aggregation. The fact that formation of the active conformational state(s) facilitates oligomer formation at high enzyme concentration is incidental to the catalytic and regulatory mechanisms of the enzyme. Although the monomeric form of this enzyme is composed of two nonidentical polypeptide chains, the allosteric effects appear to be restricted to the larger of the two polypeptide chains (Trotta et al., 1971; Anderson and Carlson, 1975). The regulatory properties of this allosteric enzyme, therefore, do not appear to be mediated through either protomer or subunit interactions.

Previous studies have shown that there is a marked sigmoid relationship between MgATP concentration and various properties of the enzyme, such as catalytic activity, rate of reaction of certain unique SH groups on the enzyme with SH reagents, or decomposition of an enzyme–cyanate complex (Anderson and Meister, 1966; Anderson and Marvin, 1970; Foley et al., 1971; Anderson and Carlson, 1975). These studies have also provided evidence that the allosteric effectors act by altering the apparent affinity of the enzyme for MgATP. The scheme in Figure 2 does not provide an explanation for the sigmoid relationship between MgATP concentration and the various parameters cited above. Preliminary studies in this laboratory have indicated, however, that the sigmoid relationships may simply be due to a change in the ratio of free ATP to MgATP as the total concentration of ATP and Mg^{2+}, present in equimolar concentration, is varied. When the experiments are carried out in the presence of excess Mg^{2+}, normal hyperbolic kinetics are observed. A similar explanation for the apparent allosteric activation of hexokinase by MgATP has been reported by Purich and Fromm (1971). Thus, a regulatory scheme or kinetic mechanism for carbamyl-phosphate synthetase may not have to account for the sigmoid relationships which have been observed with respect to MgATP.

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