# Binding of Allosteric Effectors to Carbamyl-Phosphate Synthetase from *Escherichia coli*†

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ABSTRACT: The binding of ornithine and inosine 5′-monophosphate (IMP), positive allosteric effectors, and of uridine 5′-monophosphate (UMP), a negative allosteric effector, to carbamyl-phosphate synthetase from *Escherichia coli* was studied by the technique of equilibrium dialysis. The monomeric form of the enzyme has one binding site for each of the three allosteric ligands. The binding of UMP is inhibited by ornithine, IMP, MgATP, and ammonia (also a positive allosteric effector). Bicarbonate, L-glutamine, and adenosine 5′-triphosphate (ATP) ($Mg^{2+}$ absent) had no effect on the binding of UMP. The affinity of the enzyme for UMP was increased if phosphate buffer was replaced by 2-amino-2-hydroxymethyl-1,3-propanediol (Tris) buffer. The binding of ornithine was inhibited by UMP and ammonia, enhanced by MgATP, MgADP, and IMP, and not affected by bicarbonate, L-glutamine, or ATP ($Mg^{2+}$ absent). Ornithine and ammonia probably bind to the same site on the enzyme. The binding of IMP is facilitated by ornithine and ammonia, but is inhibited by MgATP or ATP, indicating that adenine nucleotides can also bind to the IMP binding site. The results of these binding studies are consistent with a scheme previously proposed in which the allosteric effectors function by stabilizing one or the other of two different conformational states of the enzyme which are in equilibrium with each other (Anderson, P. M., and Marvin, S. V. (1970), *Biochemistry 9*, 171). According to this scheme, binding of the substrate MgATP is greatly facilitated when the enzyme exists in the conformational state stabilized by the positive allosteric effectors.

Carbamyl-posphate synthetase from *Escherichia coli* is an allosteric enzyme which catalyzes the following overall reaction (Anderson and Meister, 1965, 1966a, and 1966b):

$$2\ ATP + HCO_3^- + H_2O + \text{L-glutamine} \xrightarrow{Mg^{2+},K^+} NH_2CO_2PO_3^{2-} + \text{L-glutamate} + 2\ ADP + P_i$$

The enzyme is subject to feedback inhibition by UMP[1] and is activated by ornithine, ammonia, and IMP (Anderson and Meister, 1966b; Pierard, 1966; Anderson and Marvin, 1968; Trotta et al., 1974). Previous studies have indicated that the allosteric effectors act by altering the apparent affinity of the enzyme for MgATP and have provided evidence for the scheme shown in Figure 9 (Anderson and Marvin, 1970; Foley et al., 1971; Matthews and Anderson, 1972; Anderson and Carlson, 1975; Anderson, 1977). According to this scheme, the monomeric form of the enzyme is considered to be capable of existing in at least three different conformational states. Monomer I has little or no affinity for MgATP and does not readily aggregate to give oligomer. Monomers I and II are in equilibrium with each other, the equilibrium favoring monomer I. Monomer I has one or more allosteric binding sites for UMP and monomer II has allosteric binding site(s) for ornithine, ammonia, and IMP, as well as one or more binding sites for MgATP. The binding of MgATP results in the formation of monomer III, the catalytically active form of the enzyme. Monomers II and III are capable of aggregating to give oligomers, a reaction which does not significantly affect the catalytic and regulatory properties of the enzyme. Ornithine, ammonia, and IMP are considered to act as positive allosteric effectors, therefore, by shifting the equilibrium towards monomer II, thus increasing the apparent affinity of the enzyme for MgATP.

In the present study, the binding properties of three of the allosteric effectors, UMP, ornithine, and IMP, were explored by the technique of equilibrium dialysis. The results show that there is a single binding site per monomeric unit of the enzyme for each of these ligands, and that the effects of other ligands (allosteric effectors or adenine nucleotides) on the binding of each of these effectors are consistent with the scheme shown in Figure 9. A preliminary report of this work has appeared (Anderson, 1976).

## Materials and Methods

Carbamyl-phosphate synthetase was isolated from *Escherichia coli* B by the modified procedure described by Matthews and Anderson (1972). [$^3$H]Ornithine, [$^{14}$C]UMP, and [$^{14}$C]IMP were obtained from New England Nuclear Corp. The purity of each of the labeled compounds was confirmed by paper chromatography using several appropriate solvent systems. Glass beads (1-mm diameter) were obtained from Sargent Welch. Bio-Solv (BBS-3) solubilizer and Fluoralloy were obtained from Beckman Instruments, Inc. All biochemicals were obtained from Sigma Chemical Co. and all other chemicals were reagent grade.

The dialysis membrane used to separate the two chambers in the equilibrium dialysis apparatus was prepared from Visking cellulose dialysis tubing (1-in. flat width) obtained from A. H. Thomas Co. The dialysis tubing was boiled for 10 min in a 5% $Na_2CO_3$ solution containing 50 mM EDTA and then rinsed exhaustively in deionized water. The tubing was cut into small square sections and stored at 4 °C in 50% ethanol. Just prior to use, the sections were rehydrated by soaking in the buffer used for equilibrium dialysis.

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[1] Abbreviations used are: MAT, β,γ-methyleneadenosine 5′-triphosphate; ATP and ADP, adenosine tri- and diphosphates; UMP and IMP, uridine and inosine 5′-monophosphates; $P_i$, inorganic phosphate; EDTA, (ethylenedinitrilo)tetraacetic acid; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol.

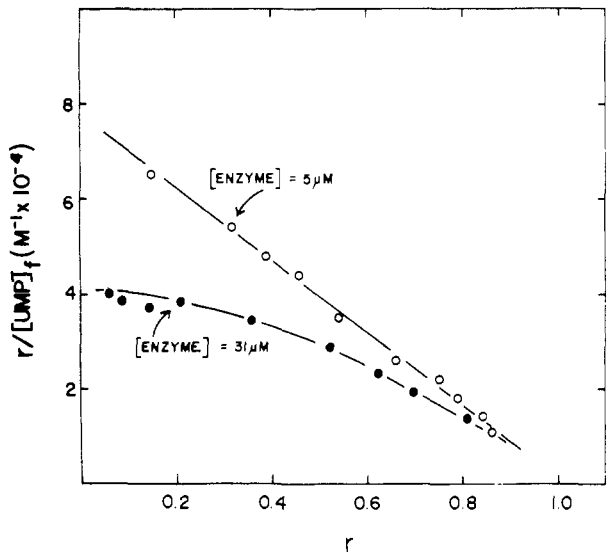


FIGURE 1: Binding of UMP as a function of enzyme concentration. The range of [$^{14}$C]UMP concentrations was 2.7–82 $\mu$M and the total radioactivity in the two dialysis chambers of each cell was 16 000 cpm. The enzyme concentration in the chamber containing enzyme was 5 and 31 $\mu$M, as indicated above.

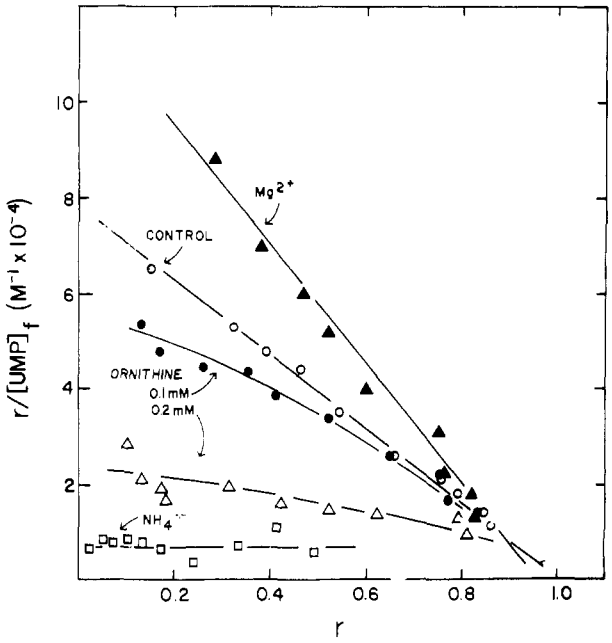


FIGURE 2: Effect of allosteric effectors and $Mg^{2+}$ on UMP binding. Where indicated, $Mg^{2+}$ (0.5 mM), ornithine (0.1 or 0.2 mM), or ammonia (40 mM) was also present in the two chambers after equilibration. The enzyme concentration was 5 $\mu$M. Other conditions were as described in Figure 1.

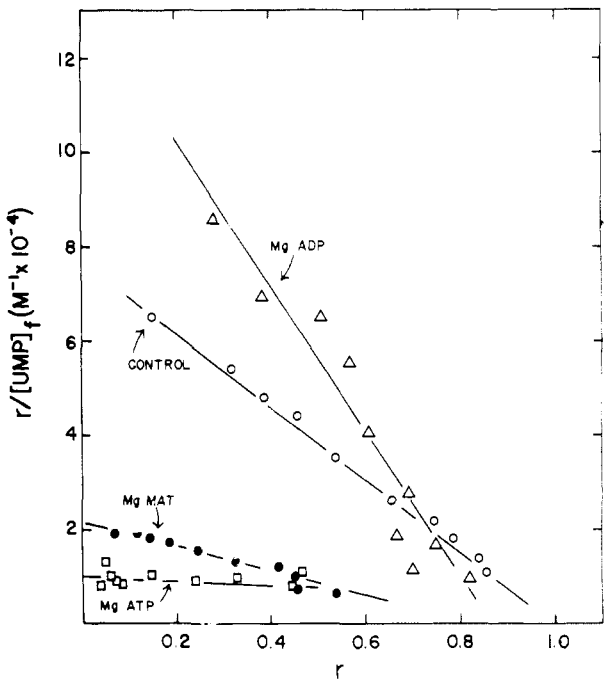


FIGURE 3: Effect of adenine nucleotides on UMP binding. Where indicated, MgADP (10 mM), MgATP (10 mM), or MgMAT (10 mM) was also present in the two chambers after equilibration. The enzyme concentation was 5 $\mu$M. Other conditions were as described in Figure 1.

Binding studies were carried out by equilibrium dialysis using dialysis cells similar in construction to those described by Englund et al. (1969). The diameter of each cylindrical chamber was 8 mm, and the depth from the dialysis membrane to the back of each chamber was 2 mm. The capacity of each chamber was 100 $\mu$l. Samples were added and removed from the chambers with a 100-$\mu$l Hamilton syringe fitted with a Chaney adapter. An 80-$\mu$l solution containing enzyme in potassium phosphate buffer (0.12 M, pH 7.7) was added to one chamber and an 80-$\mu$l solution containing various concentrations of the radioactive ligand in the same buffer was added to the other chamber. Other ligands whose effects on the binding of the radioactive ligand were to be tested were initially present in the solution containing enzyme. A small glass bead was placed in each chamber, and the chambers were rotated (30 rpm) around an axis perpendicular to the plane of the cylindrical chamber to facilitate equilibration. The dialysis was carried out at 17 °C for 4 h (equilibration was found to be complete for all ligands after 2.5 h). At the end of the 4-h equilibration period, 50-$\mu$l samples were withdrawn from each chamber and added to glass scintillation vials containing 150 $\mu$l of an 0.01 M solution of the ligand (nonradioactive) being tested and 10 ml of toluene containing 5% BBS-3 solubilizer and Fluoralloy. The radioactivity of the sample in the vials was measured with a liquid scintillation spectrometer.

The binding data were analyzed according to the following equation described by Scatchard (1949)

$$r = k\left(\frac{r}{L_f}\right) - n$$

where $r$ is the number of moles of ligand bound per mole of enzyme, $n$ is the extrapolated number of ligand binding sites per mole of enzyme, $k$ is the dissociation constant, and $L_f$ is the concentration of ligand which is not bound. If the ligand binding sites are noninteracting, plots of $r/(L_f)$ vs. $r$ should be linear and can be used for determining $r$ and $k$; if positive cooperativity exists between ligand binding sites, the plot of $r/(L_f)$ vs. $r$ will be concave downward (Hammes and Wu, 1974; Koshland, 1970). All straight-line plots were treated by the method of least-squares to obtain best fit to the data. A value of 180 000 was used for the molecular weight of the monomeric unit of the enzyme (Matthews and Anderson, 1972; Trotta et al., 1974).

## Results

*Binding of UMP.* The results of these studies are shown in Figures 1–3. When the enzyme concentration was low (5 $\mu$M or less), linear Scatchard plots were obtained (Figure 1). The

dissociation constant calculated from these data is $1.3 \times 10^{-5}$ M. Extrapolation of these data yields a limiting value of one for the number of sites per monomeric unit of the enzyme.

As shown in Figure 1, at a high enzyme concentration the Scatchard plot was not linear. As the total concentration of UMP increases, the slope and extrapolated value for the *r* intercept approach the values obtained at low enzyme concentration, suggesting the apparent existence of some form of positive cooperativity at high enzyme concentration (Koshland, 1970).

Ornithine and ammonia, both positive allosteric effectors, inhibited the binding of UMP (Figure 2). The presence of IMP, also a positive allosteric effector, had an effect similar to that of ornithine. Binding of UMP was completely inhibited if the concentrations of ornithine or IMP were high (e.g., $10^{-3}$ M).

MgATP and its analogue, MgMAT, also inhibited the binding of UMP (Figure 3). The presence of $Mg^{2+}$ was required for these effects. Certain other properties of this enzyme, which are dependent on the presence of MgATP, have also been shown to require the additional presence of bicarbonate, e.g., bicarbonate-dependent ATPase activity, MgATP- and bicarbonate-dependent availability of a unique SH group for reaction with SH reagents, and MgATP- and bicarbonate-dependent dissociation of a carbamylated enzyme derivative (Anderson and Meister, 1966a; Anderson and Marvin, 1970; Foley et al., 1971; Anderson and Carlson, 1975). The binding of MgATP and bicarbonate, presumably, is followed by cleavage of ATP and activation of bicarbonate. The effect of MgATP on UMP binding is apparently not bicarbonate dependent, however. Bicarbonate alone had no effect on the binding of UMP, and the additional presence of bicarbonate did not significantly alter the effect of MgATP (or MgMAT) on the binding of UMP. When additional bicarbonate was present, considerable cleavage of ATP occurred during the 4-h equilibration, as expected, due to the bicarbonate-dependent ATPase activity. Although endogenous bicarbonate was certainly present in the dialysis-binding solutions, it is unlikely that the levels were high enough to saturate the enzyme. Bicarbonate-dependent cleavage of ATP is apparently not required, since MAT, which cannot undergo cleavage, also inhibited binding of UMP.

MgADP facilitated the binding of UMP (Figure 2). However, this effect is probably an artifact due to the high concentration of phosphate buffer. The stability constant for the MgADP complex is lower than that of the MgATP complex, and considerable dissociation of the MgADP complex would occur because phosphate would effectively compete for the $Mg^{2+}$ (Wood et al., 1966). Free $Mg^{2+}$ (or $MgPO_4$) facilitated the binding of UMP to an extent similar to that observed with MgADP (Figure 2). Therefore, the effect of MgADP may actually be the result of the presence of an appreciable concentration of free $Mg^{2+}$, or more likely $MgPO_4$. As shown below, MgADP does inhibit the binding of UMP in the absence of phosphate.

The presence of glutamine (or cyanate, which reacts specifically with an SH group at the glutamine-binding site) did not affect the binding of UMP.

*Binding of UMP in the Absence of Phosphate Buffer.* Several properties of carbamyl-phosphate synthetase are affected by the presence of phosphate. The ability of the enzyme to undergo self-association is dependent on the presence of phosphate, and the sedimentation coefficient of the monomeric form of the enzyme is increased by the addition of phosphate (Trotta et al., 1974). The activating effect of IMP is not observed in phosphate buffer (Anderson and Marvin, 1970), and the maximum inhibitory effect of UMP is enhanced in phosphate buffer (Anderson, unpublished observations).

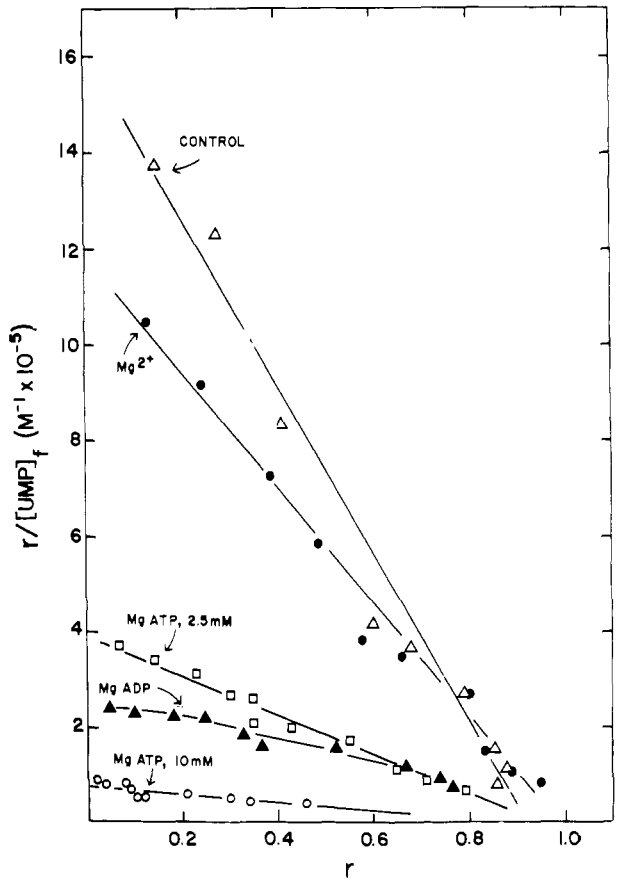


FIGURE 4: Effect of adenine nucleotides and $Mg^{2+}$ on UMP binding in Tris buffer. The range of [$^{14}$C]UMP concentrations was 0.3–11.5 $\mu$M and the total radioactivity in the two dialysis chambers of each cell was 16 000 cpm. The enzyme concentration was 2.7 $\mu$M. Where indicated, $Mg^{2+}$ (2 mM), MgADP (10 mM), or MgATP (2.5 or 10 mM) was also present in the two chambers after equilibration. The experiment was carried out as described in the text, except that the 0.12 M phosphate buffer was replaced by 0.1 M Tris buffer, pH 7.8, containing 0.1 M KCl.

As shown in Figure 4, the binding of UMP was greatly enhanced if phosphate was not present. Under these conditions, the dissociation constant is $6 \times 10^{-6}$ M; this value is in close agreement to the value of $5 \times 10^{-6}$ M obtained from kinetic studies on the effect of UMP concentration on enzyme activity in Tris buffer at 37 °C (Anderson and Meister, 1966b). As shown, MgATP, MgADP, and, to a lesser extent, free $Mg^{2+}$ inhibited binding. ATP or ADP in the absence of $Mg^{2+}$ had no effect. Ammonia, ornithine, and IMP also inhibited the binding of UMP in the absence of phosphate buffer.

*Binding of Ornithine.* The binding of ornithine was considerably enhanced by the presence of MgADP, MgMAT, and IMP, and was inhibited by the presence of UMP (Figure 5). Bicarbonate (10 mM), glutamine (10 mM), or free $Mg^{2+}$ (5 mM) had little or no effect on the binding of ornithine under these conditions. $Mg^{2+}$, however, was required for the effects of ADP and MAT. The effect of MgATP on ornithine binding could not be tested directly, since the relatively high concentration of enzyme required for these studies and the presence of significant levels of bicarbonate would result in nearly complete hydrolysis of the ATP during the course of the experiment.

In the absence of other ligands, the Scatchard plot for or-

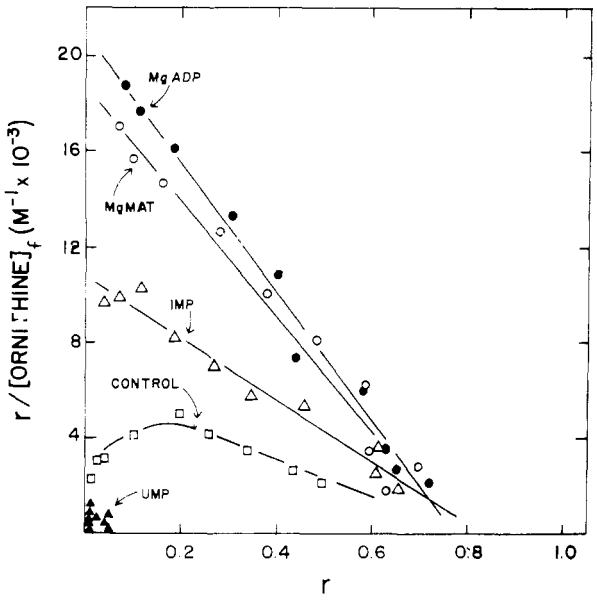


FIGURE 5: Binding of ornithine in the presence or absence of allosteric effectors and adenine nucleotides. The range of [$^3$H]ornithine concentrations was 5–350 $\mu$M and the total radioactivity in the two dialysis chambers was 32 000 cpm. The enzyme concentration was 13.7 $\mu$M. Where indicated, UMP (1 mM), IMP (5 mM), MgMAT (20 mM), or MgADP (10 mM) was also present in the two chambers after equilibration.

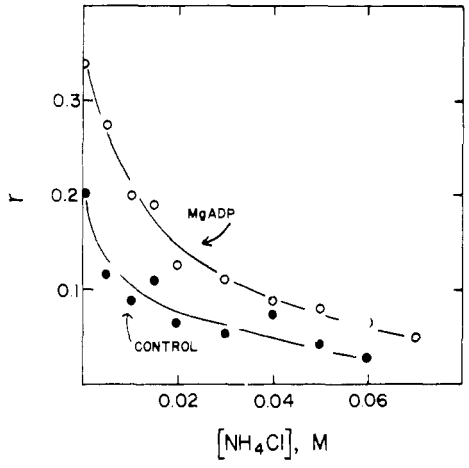


FIGURE 6: Effect of ammonia concentration in the presence or absence of MgADP on the binding of ornithine. This study was carried out at a fixed concentration of [$^3$H]ornithine (40 $\mu$M). The concentration of $NH_4Cl$ was varied as indicated. The concentration of MgADP in the two chambers after equilibration was 10 mM. Other conditions were as described in Figure 5.

nithine was nonlinear, the shape of the curve suggesting the existence of some form of apparent positive cooperativity (Figure 5). The dissociation constant, calculated from the slope of the "linear" portion of the curve, is $1.3 \times 10^{-4}$ M, and the value calculated from the slope of the line, obtained with IMP present, is $7.4 \times 10^{-5}$ M. These values are in the same range as the value of about $1 \times 10^{-4}$ M ornithine required to cause an intermediate change in the sedimentation coefficient of the enzyme (in the presence of ornithine the enzyme exists in a conformational state which favors aggregation (Anderson and Marvin, 1970)). The data indicate that there is also one binding site for ornithine per monomeric unit of the enzyme, although, for reasons which are not presently understood, the extrapolated value for the $r$ intercept was less than one in these studies.

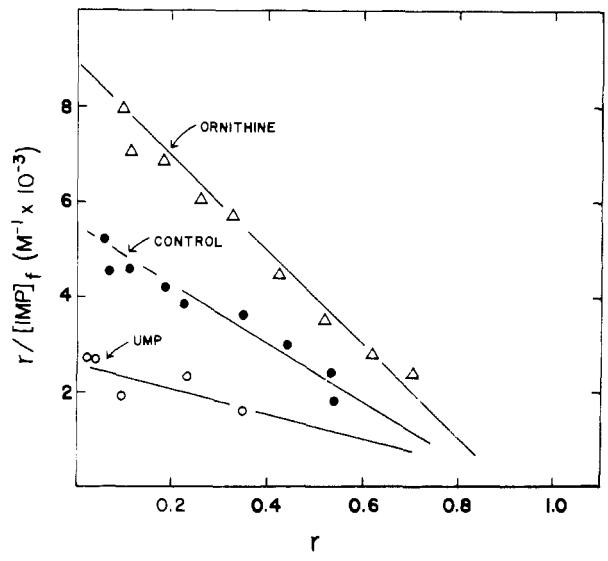


FIGURE 7: Binding of IMP in the presence or absence of allosteric effectors. The range of [$^{14}$C]IMP concentrations was 8.3–302 $\mu$M (8.3–227 $\mu$M when UMP was present) and the total radioactivity in the two dialysis chambers of each cell was 18 000 cpm. The enzyme concentration was 28 $\mu$M. Where indicated, ornithine (2 mM) or UMP (1 mM) was also present in the two chambers after equilibration.

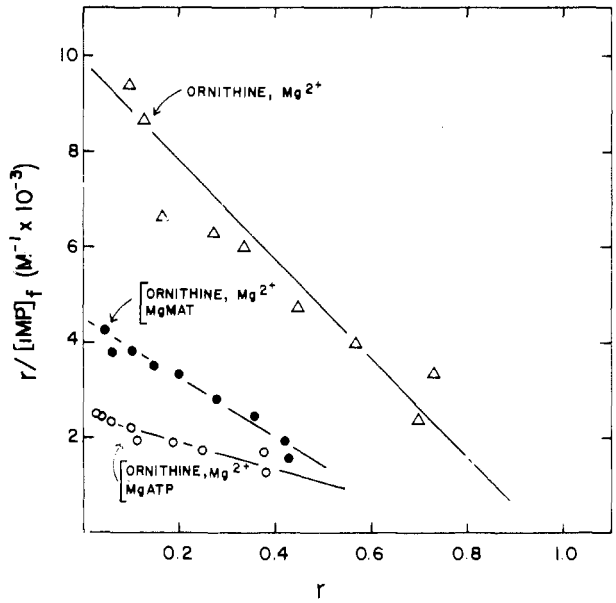


FIGURE 8: Effect of adenine nucleotides on IMP binding in the presence of ornithine and $Mg^{2+}$. Ornithine (2 mM) and $MgSO_4$ (5 mM) were present in all experiments. Where indicated, MgMAT (10 mM) or MgATP (10 mM) was also present in the two chambers after equilibration. Other conditions were as described in Figure 7.

Ammonia, also a positive allosteric effector for this enzyme, inhibited the binding of ornithine. The effect of ammonia concentration on the binding of ornithine at a fixed concentration of ornithine is shown in Figure 6. The additional presence of MgADP, which facilitated ornithine binding (Figure 5), did not significantly alter the inhibitory effect of ammonia. These results suggest that MgADP, ornithine, and ammonia all bind to the same conformational state of the enzyme and that ammonia and ornithine compete for the same binding site.

*Binding of IMP.* The binding of IMP was described by a linear Scatchard plot (Figure 7). There appears to be one binding site for IMP per monomeric unit of the enzyme, al-

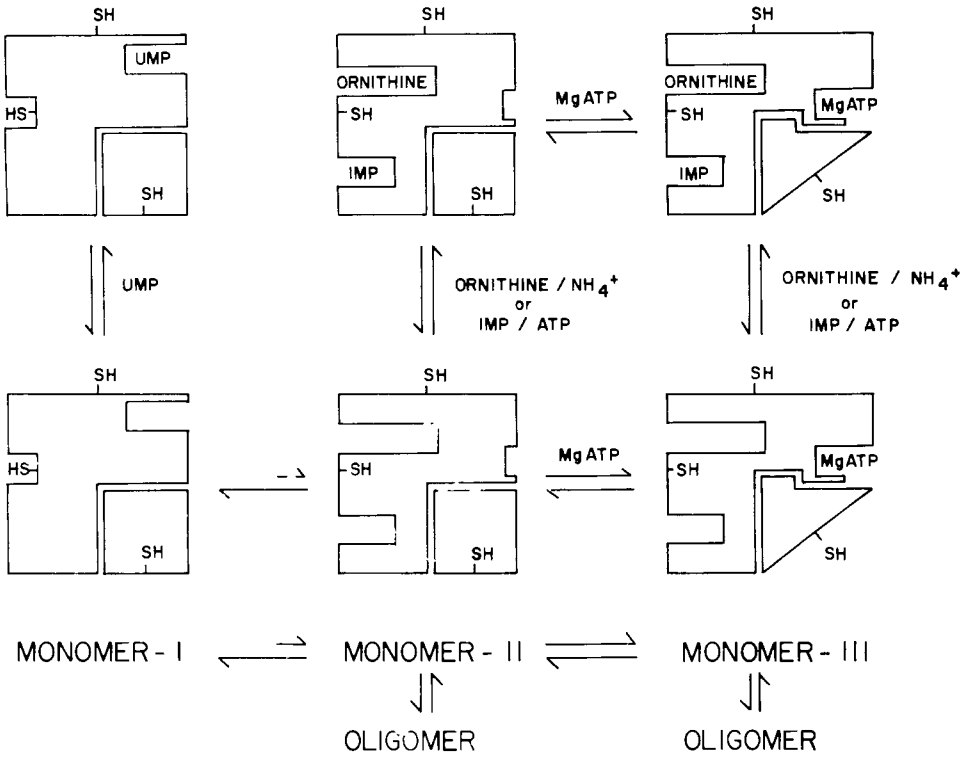


FIGURE 9: Scheme illustrating the effects of the allosteric effectors and MgATP on the conformational state of carbamyl-phosphate synthetase. The abbreviated scheme shown in the lower portion of the figure was previously proposed as a possible mechanism for the allosteric effector- and MgATP-dependent aggregation and inhibition by *N*-ethylmaleimide of the enzyme (Anderson and Marvin, 1970). The scheme in the upper portion of the figure summarizes or illustrates in more detail the conditions under which three unique SH groups are available for reaction with SH reagents (Foley et al., 1971), the fact that the enzyme is composed of one light and one heavy subunit (Matthews and Anderson, 1972; Trotta et al., 1971), the location of the three unique SH groups with respect to distribution between subunits (Matthews and Anderson, 1972), the subunit conformational changes which are thought to occur, and the binding of the allosteric effectors and MgATP, as described in the text. The availability of an SH group for reaction with SH reagents is illustrated by its location outside the enclosures representing the different conformational states of the enzyme. Although not shown, at least one other unique SH group is known to be present in the glutamine binding site on the light subunit (Pinkus and Meister, 1972; Anderson and Carlson, 1975).

though, as with ornithine, the extrapolated value for the *r* intercept was somewhat less than one. The dissociation constant calculated from the control data in Figure 7 is $1.6 \times 10^{-4}$ M. This value is close to the value of $2 \times 10^{-4}$ M determined from the effect of IMP concentration on the catalytic activity of enzyme in Tris buffer at 37 °C (Anderson and Meister, 1966b). As would be expected from the above studies, binding of IMP was facilitated by ornithine and inhibited by UMP (Figure 7). Ammonia (40 mM) had an effect similar to that of ornithine. Excess $Mg^{2+}$ also facilitated binding of IMP to a small extent, and this effect was observed whether or not ornithine was present (Figures 7 and 8).

MgMAT and MgATP were effective inhibitors of IMP binding (Figure 8) in the presence of ornithine and excess $Mg^{2+}$. Similar results were obtained when free MAT or ATP was present in the absence of ornithine or $Mg^{2+}$, suggesting that MAT and ATP (and their $Mg^{2+}$ complexes) can bind at the IMP binding site.

## Discussion

Protomer or subunit interactions are apparently not involved in the mechanism whereby the allosteric and regulatory properties of this carbamyl-phosphate synthetase are manifested (Anderson, 1977). Instead, the allosteric effectors appear to act by stabilizing one or the other of at least two different conformational states of the monomeric form of the enzyme, as illustrated in the scheme shown in Figure 9 and as discussed above. The results of the binding studies presented in this paper are consistent with this scheme and are summarized along with other structural properties of the enzyme in the scheme shown in the upper portion of Figure 9. This scheme also illustrates the fact that the monomeric unit of the enzyme is composed of two nonidentical polypeptide chains, that three different conformational states of the enzyme can be identified by the availability of different SH groups for reaction with SH reagents, and that the two conformational states labeled monomer I and monomer II are in equilibrium with each other (Anderson and Marvin, 1970; Foley et al., 1971; Matthews and Anderson, 1972; Trotta et al., 1971).

The binding studies reported in this paper indicate that there is one binding site per monomeric unit of the enzyme for each of the three allosteric effectors tested. With reference to Figure 9, the results of the previous studies, upon which this scheme is based, indicate that UMP binds preferentially to monomer I, and the positive allosteric effectors (ornithine, IMP, and ammonia) and the substrate MgATP bind preferentially to monomer II. The finding that ornithine, ammonia, IMP, MgATP, and MgMAT inhibit the binding of UMP would, therefore, be expected, since the presence of one or more of these ligands would shift the equilibrium away from monomer I. Likewise, the finding that UMP inhibits the binding of ornithine and IMP would also be expected, since the presence of UMP would shift the equilibrium away from monomer II.

According to the scheme in Figure 9, it would be anticipated that the binding of any one of the positive allosteric effectors would be facilitated by the presence of any one of the other positive allosteric effectors if there were separate binding sites for each effector. This appears to be the case for IMP and or-

nithine; IMP facilitates the binding of ornithine, and both ornithine and ammonia facilitate the binding of IMP. The presence of ammonia, however, inhibits the binding of ornithine. This could be due to stabilization by ammonia of a different conformational state which has little or no affinity for ornithine. A more likely explanation, however, is that ammonia and ornithine compete for the same binding site on monomer II. The facts that the effect of ammonia concentration on the binding of ornithine is not significantly altered when MgADP (which facilitates ornithine binding) is also present and that the binding of IMP (which also facilitates ornithine binding) is facilitated by ammonia support this explanation.

The presence of adenine nucleotides facilitates the binding of ornithine and inhibits the binding of UMP. These effects are dependent on the presence of $Mg^{2+}$, but do not appear to be affected by bicarbonate. These results indicate that one or more MgATP binding sites are present when the enzyme is in the monomer-II conformation, but do not establish whether or not there is an allosteric site (or sites) in addition to the catalytic site (or sites) for MgATP. The finding that IMP binding was inhibited by adenine nucleotides and that this inhibition was not dependent on $Mg^{2+}$ suggests that adenine nucleotides can bind at the IMP binding site and that this site is not the same as the one or more other adenine nucleotide binding sites described above. This conclusion is consistent with the observation that a number of different purine nucleotides are capable of activating this enzyme (Anderson and Meister, 1966b). Additional evidence that ATP can bind to the IMP binding site has been reported by Powers and Riordan (1975) who showed that both IMP and ATP protect the enzyme against inhibition by phenylglyoxal, a reagent which reacts selectively with arginine residues.

The binding of ornithine is not described by a linear Scatchard plot. The Scatchard plot of the binding of UMP when the enzyme concentration is high is also not linear. For proteins with multiple binding sites, nonlinear (concave downward) Scatchard plots are usually considered to be due to positive cooperativity between binding sites (Koshland, 1970; Hammes and Wu, 1974). Although it is possible that this explanation could apply to carbamyl-phosphate synthetase for the binding of ornithine (an appreciable portion of the enzyme would exist as oligomer under the conditions of the experiment), it would not account for the nonlinear (concave downward) result obtained for the binding of UMP to this enzyme. The proposed scheme in Figure 9, however, does provide a possible explanation for both results. The concentration of monomer I relative to that of monomer II will decrease as the enzyme concentration increases, due to the equilibrium between monomers I and II and oligomer formation from monomer II (Anderson and Marvin, 1970). The binding of UMP under these conditions will result in a shift in the equilibrium back to monomer I, thus increasing the number of available UMP binding sites. Likewise, the binding of ornithine will result in complete conversion of monomer I to monomer II and/or oligomer, thus increasing the number of available ornithine binding sites. A form of positive cooperativity would, therefore, be obtained, but the apparent cooperativity would not be the result of interactions between binding sites. If this explanation is correct, a similar kind of apparent cooperativity might also have been expected for the binding of IMP. The fact that the Scatchard plot for IMP binding appears to be linear could be due to the fact that a very high enzyme concentration was required for these experiments, and under these conditions a very small proportion of the enzyme would be present as monomer I.

The allosteric effectors and all substrates, except L-glutamine, apparently bind to sites on the heavy subunit of carbamyl-phosphate synthetase; the light subunit of the enzyme functions as a glutamine-binding subunit, catalyzing the hydrolysis of glutamine to give ammonia, which reacts with an activated form of bicarbonate on the heavy subunit (Trotta et al., 1971). Significant interaction between these two different subunits and their catalytic sites have been demonstrated. The evidence for this interaction comes from studies which have shown that the interaction of MgATP with bicarbonate on the heavy subunit alters the conformation and the catalytic properties of the light subunit, and that the binding of glutamine or certain analogues of glutamine to the glutamine-binding site on the light subunit results in a change in the catalytic properties of the heavy subunit (Anderson and Carlson, 1975). It has been previously noted, however, that the binding of the allosteric effectors to the heavy subunit does not appear to have a direct or significant effect on the properties of the glutamine-binding site or of the glutamine-binding subunit, indicating that the allosteric effects of these compounds are not the result of interaction between the two subunits (Anderson and Carlson, 1975). The fact that the presence of glutamine does not affect the binding of the allosteric effectors, as reported in this paper, supports this interpretation and provides additional evidence that the two different conformational states (monomers I and II), which have been shown to exist in the presence of UMP and ornithine (or IMP), respectively, are probably related to conformational changes in the heavy subunit only.

## Acknowledgment

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# Ligand Modification of Corpus Luteum Mitochondrial Cytochrome P-450 Spectra and Cholesterol Monooxygenation: An Assay of Enzyme-Specific Inhibitors†

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ABSTRACT: Absorbance changes in the spectrum of cytochrome P-450 were related to the inhibition of [26-$^{14}$C]cholesterol oxidation to [$^{14}$C]isocaproate and pregnenolone in mitochondria from bovine corpus luteum produced by two types of ligands. Nitrogenous inhibitors, such as aminoglutethimide, elicit an absorption maximum at about 427 nm and a minimum at about 393 nm (type II), while steroidal inhibitors, such as (20*R*)-20-(*p*-tolyl)-5-pregnene-3$\beta$,20-diol (20-tolyl-pregnenediol), cause difference spectra with maximum at about 420 nm and minimum at about 390 nm (reverse type I). The magnitude of spectral change and the amount of inhibition of pregnenolone synthesis by aminoglutethimide are closely correlated at concentrations ranging from 5 to 750 $\mu$M and by the model steroid, 20-tolyl-pregnenediol, at concentrations from 0.5 to 25 $\mu$M. The responses are concentration dependent and linear over the range of effective concentrations. The concentrations of inhibitors for the half-maximal inhibition of pregnenolone biosynthesis are identical with the concentrations producing half-maximal spectral changes within experimental error. Displacement of substrate from cytochrome P-450 and/or stabilization of the redox potential subsequent to the ligation of heme iron is proposed as the specific mechanism of cholesterol side chain cleavage inhibition. Finally, together, the two procedures offer a sensitive, specific, and accurate means of screening inhibitors of the cholesterol side chain cleavage system.

Studies on the spectral properties of cytochrome P-450 (P-450) have shown that binding of substrates can be determined spectrophotometrically with ease and precision (Narasimhulu et al., 1965). The sites of interaction cannot yet be specified, but at least three types of induced spectral changes associated with the membrane-bound P-450 have been recognized (Schenkman et al., 1972).

The first type, type I, is caused by the addition of substrates to the oxidized form of P-450; it is characterized by an absorption maximum at about 390 nm and a minimum at 420 nm in difference spectrum. These ligands are associated with an EPR[1]-measurable conversion of the low-spin form of cytochrome P-450 to the high-spin form (Mitani and Horie, 1969). Conversely, the addition of certain reaction products causes formation of a peak at about 420 nm and a trough at 392 nm in the difference spectrum and is termed reverse type I (RT I). This spectral change has been attributed to the interaction of lipid soluble compounds at both the type I site and at another site on the cytochrome P-450 molecule (Schenkman et al., 1972). A third type of ligand, usually a nitrogenous base, interacts with cytochrome P-450 and causes a ferrihemochrome spectral change with an absorption maximum between 425 and 435 nm and a minimum at about 390 nm (type II). The type I and nitrogenous compounds are found to displace carbon monoxide from the reduced hemoprotein suggesting interaction with the heme (Symms and Juchau, 1973; Schenkman et al., 1967).

Gigon et al. (1969) with liver microsomes demonstrated that type I ligands usually increase the initial rate of reduced P-450 carbon monoxide complex formation, while type II compounds generally decrease it. Similarly, compounds of reverse type I class also decrease the rate of P-450 reduction. With bovine corpus luteum cytochrome P-450, their effectiveness correlates with their spectral binding affinity (McIntosh et al., 1973) but, with adrenocortical mitochondria containing a mixture of P-450 cytochromes, spectral effects of ligands are not consistent. The protein from the corpus luteum is a spectrally homogeneous protein (Užgiris et al., 1975; McIntosh et al., 1971).

The results reported here show that: (1) a good correlation exists between the intensity of ligand-induced spectral change and the amount of inhibition of pregnenolone[2] synthesis in

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[1] Abbreviations used: EPR, electron paramagnetic resonance; P-450$_{CL}$, corpus luteum mitochrondrial cytochrome P-450; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid; Mann, mannitol.

[2] Trivial names used are: cholesterol, 5-cholesten-3$\beta$-ol; 20$\alpha$-hydroxycholesterol, 5-cholesten-3$\beta$,20$\alpha$-diol; pregnenolone, 3$\beta$-hydroxy-5-pregnen-20-one; 16$\alpha$-chloropregnenolone, 16$\alpha$-chloro-3$\beta$-hydroxy-5-pregnen-20-one.