Articles

Allosteric Dominance in Carbamoyl Phosphate Synthetase†

B. L. Braxton,‡ Leisha S. Mullins,§ Frank M. Raushel,‡,§ and Gregory D. Reinhart*,‡

Department of Biochemistry and Biophysics, Texas A&M University, College Station, Texas 77843-2128, and Department of Chemistry, Texas A&M University, College Station, Texas 77843-3255

Received August 28, 1998; Revised Manuscript Received December 2, 1998

ABSTRACT: A linked-function analysis of the allosteric responsiveness of carbamoyl phosphate synthetase (CPS) from *E. coli* was performed by following the ATP synthesis reaction at low carbamoyl phosphate concentration. All three allosteric ligands, ornithine, UMP, and IMP, act by modifying the affinity of CPS for the substrate MgADP. Individually ornithine strongly promotes, and UMP strongly antagonizes, the binding of MgADP. IMP causes only a slight inhibition at 25 °C. When both ornithine and UMP were varied, models which presume a mutually exclusive binding relationship between these ligands do not fit the data as well as does one which allows both ligands (and substrate) to bind simultaneously. The same result was obtained with ornithine and IMP. By contrast, the actions of UMP and IMP together must be explained with a competitive model, consistent with previous reports that UMP and IMP bind to the same site. When ornithine is bound to the enzyme, its activation dominates the effects when either UMP or IMP is also bound. The relationship of this observation to the structure of CPS is discussed.

The two primary allosteric ligands of carbamoyl phosphate synthetase (CPS[1]) from *Escherichia coli*, UMP, and ornithine act by binding to separate allosteric binding sites located in the allosteric domain at the carboxy-terminus of the large subunit of the heterodimeric enzyme (*1*, *2*). These ligands increase and decrease, respectively, the K_m for nucleotide in both the complete forward reaction (the formation of carbamoyl phosphate from 2 MgATP, HCO_3^-, and glutamine) and the partial back reaction (the formation of MgATP from MgADP and carbamoyl phosphate) (*3*−*5*). IMP is also an allosteric ligand of CPS, but its effect is comparatively small (*5*, *6*), and the nature of the effect is dependent on temperature (*7*). Under conditions of high P_i and limiting Mg^{2+} concentrations, IMP appears to allosterically activate CPS (*3*). However, at 25 °C IMP inhibits the binding of substrate nucleotides slightly when assayed in the presence of excess free Mg^{2+} and in the absence of P_i (*5*).

Kinetic studies, proteolytic cleavage protection experiments, and equilibrium dialysis binding studies on CPS suggest that each of these allosteric ligands alters not only the affinity of CPS for MgATP and MgADP but also its affinity for other allosteric ligands (*8*−*10*). Specifically, both IMP and ornithine diminish affinity for UMP, while IMP encourages ornithine binding. Of course, the converse is true in that UMP inhibits binding of both IMP and ornithine while ornithine increases affinity for IMP. Inhibition of IMP binding by UMP is reportedly due to the ligands competing for the same binding site (*8*, *11*, *12*).

These effects have previously been explained with reference to a two-state model in which UMP binds only to the inactive enzyme form, and ornithine and IMP, then thought to be an activator exclusively, bind only to the active enzyme form (*8*, *13*). Although such a model is able to describe qualitatively some of the regulatory features of CPS, it does not provide a quantitative description of the energetics of all of the mutual interactions that occur between various ligands bound at these multiple sites. Two-state models also fail to adequately account for the temperature-dependent effects of IMP. Most fundamentally, however, the presumption that inhibiting and activating ligands cannot bind simultaneously has not been explicitly tested. For these reasons we embarked on the present study in which the actions of the allosteric ligands are quantitatively evaluated in combination using a linkage analysis (*14*−*19*). In addition, the simultaneous effects of these ligands can provide potentially important and unique insights into the mechanism which underlies their actions.

The three-dimensional structure of *E. coli* CPS has been solved by X-ray crystallography (*20*, *21*). The structure reveals several notable features, including in partcular the existence of three different active sites, separated from each other by more than 40 Å, which all contribute uniquely to the overall reaction 22. The first active site, located on the small subunit, catalyzes the hydrolysis of glutamine to form glutamate and ammonia. At the second site the ammonia reacts with carboxy phosphate (formed by the reaction of MgATP with HCO_3^-) to produce carbamate, MgADP, and

† This work was supported by National Institutes of Health Grants GM 33216 (G.D.R.) and DK 30343 (F.M.R.).

* To whom correspondence should be addressed. E-mail: gdr@tamu.edu.

‡ Department of Biochemistry and Biophysics.

§ Department of Chemistry.

[1] Abbreviations: CPS, carbamoyl phosphate synthetase; HEPES, 4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid.

10.1021/bi982097w CCC: $18.00
Published on Web 01/15/1999

P_i. Finally, the carbamate reacts at the third active site with a second MgATP to form carbamoyl phosphate and MgADP. It is evident from various kinetic and mechanistic studies (22) that the partial reactions catalyzed by the enzyme, the glutaminase activity, the HCO_3^--dependent ATP′ase activity, and the carbamoyl phosphate-dependent ATP-synthesis activity, are catalyzed by these three active sites, respectively. Significantly, from the point of view of the present study, the domain on the large subunit, on which are located both allosteric binding sites, is immediately adjacent to the third active site. This structural relationship may explain why the allosteric ligands have a greater influence on the carbamoyl phosphate-dependent ATP synthesis reaction than on the other two partial reactions (*5*).

MATERIALS AND METHODS

Dilithium carbamoyl phosphate, potassium ADP, L-glutamine, sodium NADP, ornithine-HCl, the disodium salts of UMP and IMP, and Hepes free acid were purchased from Sigma. Hexokinase and glucose-6-phosphate dehydrogenase were purchased from Boehringer-Mannheim as ammonium sulfate suspensions and were dialyzed exhaustively prior to use to remove all traces of ammonia. All other chemicals were reagent grade. Carbamoyl phosphate synthetase was purified from *E. coli* RR1 carrying the plasmid pMC41 as described previously (*23*).

Enzymatic assays were performed for the ATP synthesis reaction in 1.0 mL of buffer containing 50 mM HEPES−KOH, pH 7.5, 100 mM KCl, 20 mM $MgCl_2$, 0.5 mM carbamoyl phosphate, 1 mM NADP, 10 mM glucose, 1 unit each of hexokinase, and glucose-6-phosphate dehydrogenase, and the indicated concentrations of MgADP and allosteric ligands. MgADP concentrations ranged from 4 μM to 25 mM. To ensure stability of the very labile carbamoyl phosphate, the reaction mix was kept on ice until approximately 5 min before rates were measured. Reactions were initiated by addition of 25 μg of CPS if both ornithine and IMP were included, while 38 μg of CPS was used for all other conditions. Synthesis of MgATP at 25 °C was monitored by following the reduction of NADP at 340 nm as described previously (*24*). Initial rates were determined at various substrate concentrations and fit to the Michaelis−Menten equation from which the apparent Michaelis constant for MgADP, K_a, was determined. For these experiments, carbamoyl phosphate concentration was 0.5 mM so that K_a would approximate K_{ia} since MgADP binds first in an ordered kinetic mechanism (*5*).

Notation. In the equations and parameters discussed below, the following notation will be used. The substrate MgADP will be denoted by the letter "A", and the allosteric ligands ornithine, UMP, and IMP will be denoted by the letters "X", "Y", and "Z", respectively. Parameters pertaining to each of these ligands contain the corresponding letter in lower case as a subscript. The subscripts for thermodynamic dissociation constants are preceded by the letter "i" to be consistent with the notation proposed by Cleland (*25*). Dissociation or Michaelis constants determined in the absence of other three ligands (of the four considered in this study) are denoted by a superscript of "o". When a dissociation constant is determined with other ligands always bound to the enzyme, the bound ligands are indicated following a slash ("/") in the subscript. Thus K^o_{ix} refers to the dissociation constant of ornithine in the absence of MgADP, UMP, or IMP; $K_{iy/x}$ refers to the dissociation constant of UMP when ornithine is saturating; $K_{a/xz}$ refers the Michaelis constant for MgADP when ornithine and IMP are saturating; etc.

Coupling parameters are denoted by a "Q" followed by subscripts denoting the pertinent ligands. Coupling parameters between 2 ligands describe the nature and magnitude of a K-type allosteric effect, and they are defined as the ratio of a dissociation constant for one ligand divided by the dissociation constant for that ligand in the saturating presence of the second ligand. Two-ligand coupling parameters can also be determined when a third ligand is continually bound to its own (third) site. In such a case, the bound ligand is again denoted following a slash in the subscript. For example, $Q_{ax/z}$ refers to the coupling parameter between MgADP and ornithine with IMP always bound. Finally, when 3 ligands can bind simultaneously, a 3-ligand coupling parameter describes their mutual influence. This parameter corresponds to a ratio in which the numerator is the product of the dissociation constants for each ligand in the absence of the others, and the denominator is equal to the product of the actual dissociation constants that the fully ligated enzyme would exhibit if the ligands dissociated sequentially. For a more detailed discussion of linkage analysis involving multiple ligands, see refs *17, 26*.

Analysis. The interactions of each combination of substrate and two allosteric ligands with CPS were evaluated in the following manner, as illustrated for the ligands MgADP, ornithine, and UMP. For any given UMP concentration, K_a's for MgADP were determined at several ornithine concentrations. Each K_a was determined from a nonlinear regression fit to the Michaelis−Menten equation of initial velocity rates measured at eight MgADP concentrations ranging from 0.2- to at least 3-times the pertinent K_a. For each UMP concentration, K_a was measured in this manner for at least 10 concentrations of ornithine. When [UMP] = 0, the K_a's determined at various ornithine concentrations were analyzed using eq 1 and resulted in values for K^o_a, K^o_{ix}, and Q_{ax}. Equation 1 is derived from the single-substrate, single-modifier kinetic mechanism provided the substrate or allosteric ligand is in binding equilibrium in the steady state (*17*).

$$K_a = K^o_a\left(\frac{K^o_{ix} + [X]}{K^o_{ix} + Q_{ax}[X]}\right) \quad (1)$$

Each comparable set of K_a's measured with [UMP] ≠ 0 were also fit to eq 1 yielding apparent values for $K^o_a{}'$, $K^o_{ix}{}'$, and Q'_{ax}. (The prime notation indicates that the values are apparent since the concentration of Y is nonzero.)

The entire matrix of K_a values determined at all concentrations of ornithine and UMP was fit to the following equations:

$$K_a = K_a^o\left(\frac{K_{iy}^o[X] + K_{ix}^o[Y] + Q_{xy}[X][Y] + K_{ix}^o K_{iy}^o}{K_{iy}^o Q_{ax}[X] + K_{ix}^o Q_{ay}[Y] + Q_{axy}[X][Y] + K_{ix}^o K_{iy}^o}\right) \quad (2)$$

$$K_a = K_a^o\left(\frac{K_{iy}^o[X] + K_{ix}^o[Y] + K_{ix}^o K_{iy}^o}{K_{iy}^o Q_{ax}[X] + K_{ix}^o Q_{ay}[Y] + K_{ix}^o K_{iy}^o}\right) \quad (3)$$

$$K_a = K_a^o\left(\frac{K_{iy}^o[X] + K_{ix}^o[Y] + K_{ix}^o K_{iy}^o}{K_{iy}^o Q_{ax}[X] + K_{ix}^o K_{iy}^o}\right) \quad (4)$$

Equation 2 represents the general linked-function relationship derived from a rapid-equilibrium mechanism in which each ligand, A, X, and Y, can bind to a separate binding site (*17*, *18*). Equation 3 is derived from eq 2 by setting $Q_{xy} = Q_{axy} = 0$, and therefore represents a mechanism in which the allosteric ligands X and Y cannot bind simultaneously, for example, if X and Y bind competitively to a single site. Equation 4 is derived from eq 2 if $Q_{ay} = Q_{xy} = Q_{axy} = 0$ as would be the case if Y cannot bind simultaneously with either X or A, such as might be the case if X and A bind to one "state" of the enzyme (e.g., an "active state") and Y binds exclusively to a different state (e.g., an "inhibited state").

The goodness of fit to eqs 2 through 4 was evaluated in two ways: first by comparison of the statistical variance of each fit; and second by comparison of $K_a^{o\prime}$, $K_{ix}^{o\prime}$, and Q'_{ax} to the values predicted by the fits to eqs 2−4. The dependence of $K_a^{o\prime}$, $K_{ix}^{o\prime}$, and Q'_{ax} on [Y] predicted by eq 2 is given by the following (18):

$$K_a^{o\prime} = K_a^o\left(\frac{K_{iy}^o + [Y]}{K_{iy}^o + Q_{ay}[Y]}\right) \quad (5)$$

$$K_{ix}^{o\prime} = K_{ix}^o\left(\frac{K_{iy}^o + [Y]}{K_{iy}^o + Q_{xy}[Y]}\right) \quad (6)$$

$$Q'_{ax} = \frac{(K_{iy}^o + [Y])(K_{iy}^o Q_{ax} + Q_{axy}[Y])}{(K_{iy}^o + Q_{ay}[Y])(K_{iy}^o + Q_{xy}[Y])} \quad (7)$$

Similarly, the dependence of $K_a^{o\prime}$, $K_{ix}^{o\prime}$, and Q'_{ax} on [Y] predicted by eqs 3 and 4 is given by eqs 5−7 provided the coupling values not appearing in eqs 3 and 4 are set equal to 0.

Nonlinear regression programs were written either in HP Basic 2.1, as implemented on an HP 9836A desktop computer, or in the C programming language and run on a Silicon Graphics Personal Iris workstation. The programs used the fitting strategy described by Cleland (*27*).

RESULTS

The effects of the presence of two allosteric ligands on the ATP synthesis reaction catalyzed by *E. coli* CPS were investigated. The ATP synthesis reaction was chosen because the magnitudes of the allosteric effects on the binding of MgADP are similar to those for the full forward reaction, yet this reaction is technically easier to study because of its simpler stoichiometry (*5*).

The kinetic mechanism for the ATP synthesis reaction is ordered with MgADP binding before carbamoyl phosphate. For such a mechanism, the Michaelis constant for the first substrate to bind, K_a, is completely unrelated to a thermodynamic dissociation constant when the second substrate is saturating, since under these circumstances $K_a = k_{cat}/k_1$ where k_1 is equal to the second-order rate constant for A binding to the enzyme. When the second substrate is present at low concentration, however, K_a approaches the value of K_{ia}, the thermodynamic dissociation constant associated with the binding of A to free enzyme. In the analyses of the results that follow, we have assumed that $K_a \approx K_{ia}$ since we fixed the concentration of carbamoyl phosphate to no more than half of its Michaelis constant value, which in turn represents a lower limit to the operational steady-state dissociation parameter of carbamoyl phosphate which is approached only when MgADP is saturating.

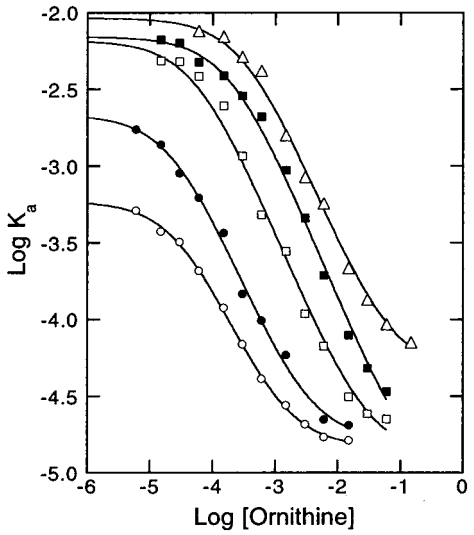

FIGURE 1: K_a for MgADP versus ornithine concentration at several fixed concentrations of UMP equal to 0 (○), 0.0068 (●), 0.02 (□), 0.1 (■), and 5.0 mM (△). Curves were generated by fitting the K_a values at each UMP concentration to eq 1, generating apparent values for K_a^o, K_{ix}^o, and Q_{ax}, as described in the text.

MgADP−Ornithine−UMP Coupling. The effect of UMP on the activation by ornithine is presented in Figure 1, where K_a as a function of ornithine is plotted for a few of the several concentrations of UMP actually examined. Lines are drawn corresponding to fits of the data at each UMP concentration to eq 1. The apparent dissociation constants for MgADP and ornithine, $K_a^{o\prime}$ and $K_{ix}^{o\prime}$, as well as the coupling between MgADP and ornithine, Q'_{ax}, resulting from each of these fits are shown in Figure 2 as a function of UMP concentration. As UMP concentration increases, $K_a^{o\prime}$ increases in accordance with UMP's expected inhibitory effect on MgADP-binding, $K_{ix}^{o\prime}$ for ornithine increases indicating that UMP antagonizes ornithine's affinity, and Q'_{ax} increases indicating that UMP increases the *extent* to which ornithine lowers K_a. Note that the effect of UMP on all three parameters, $K_a^{o\prime}$, $K_{ix}^{o\prime}$, and Q'_{ax}; is saturable. This feature implies that a quaternary complex of CPS with MgADP, ornithine, and UMP bound simultaneously can form when each ligand is present at high concentration.

Figure 2 also presents a comparison of the observed parameters $K_a^{o\prime}$, $K_{ix}^{o\prime}$, and Q'_{ax}, as a function of UMP concentration to those predicted by eqs 2−4. The ability of eqs 2−4 to describe the mutual effects of ornithine and UMP on the K_a for MgADP can be compared by evaluating how

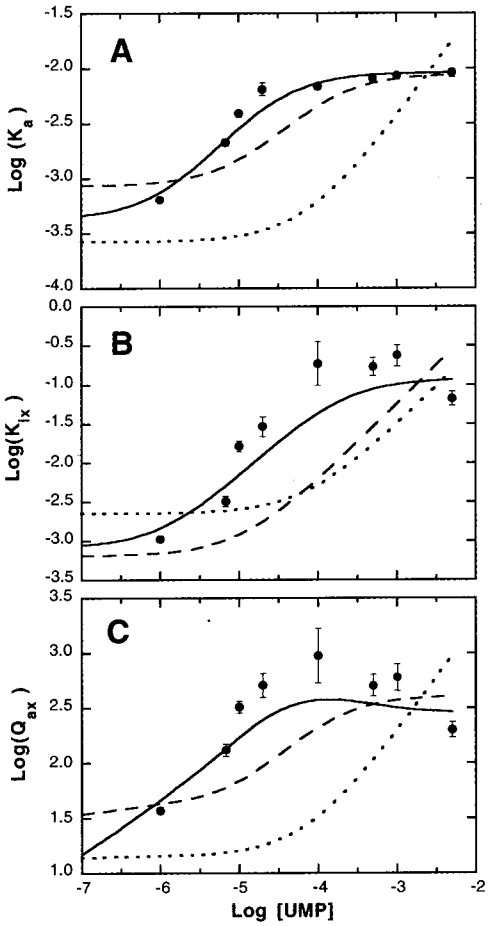

FIGURE 2: Replot of the apparent values of K_a^o, K_{ix}^o, and Q_{ax} determined at several concentrations of UMP as described in Figure 1 and presented in panels A, B, and C, respectively. Curves represent the behavior of these parameters predicted by fitting the data at all UMP concentrations to either eq 2 (—), eq 3 (- - -), or eq 4 (— — —) as described in the text.

well each predicts the dependence of $K_a^{o\prime}$, $K_{ix}^{o\prime}$, and Q'_{ax}, on UMP concentration using eqs 5−7. Clearly the parameters are predicted better by eq 2 than by eqs 3 and 4. The failure of eqs 3 and 4 to adequately describe the MgADP−ornithine−UMP interaction also implies that there is indeed a quaternary complex involving the binding of both UMP and ornithine formed within the concentration range of ligands examined. Models in which ornithine and UMP bind exclusively to different enzyme forms (such as "active" or "inhibited") are not justified by these data.

The values of the three intrinsic dissociation constants, K_a^o, K_{ix}^o, and K_{iy}^o; that result from the fit to eq 2 are presented in Table 1. The four coupling parameters, Q_{ax}, Q_{ay}, Q_{xy}, and Q_{axy}, are presented in Table 2. The other parameters listed in these two tables are derived from these 7 independent parameters as discussed below.

MgADP−Ornithine−IMP Coupling. In a similar way, the effect of IMP on ornithine activation was evaluated. In Figure 3, the K_a for MgADP is plotted as a function of ornithine concentration at several IMP concentrations. The lines correspond to fits at each IMP concentration to eq 1. In Figure 4, the apparent parameters $K_a^{o\prime}$, $K_{ix}^{o\prime}$, and Q'_{ax}, at each IMP concentration obtained from these fits are compared to those predicted by the allosteric models described by eqs 2−4 as related by eqs 5−7 (substituting Z for Y in the notation). As observed previously, as IMP concentration increases, $K_a^{o\prime}$ increases slightly indicating that IMP inhibits the binding of MgADP to a small extent under these conditions. Interestingly, $K_{ix}^{o\prime}$ for ornithine decreases somewhat demonstrating that IMP promotes ornithine binding despite the fact that it inhibits MgADP-binding. IMP also has a small effect on the apparent coupling between MgADP

Table 1: Ligand Dissociation Constants from *E. coli* CPS at 25 °C with Carbamoyl Phosphate Concentration Equal to 0.5 MM

ligand	other saturating ligands	designation[a]	K_d (mM)
MgADP		K_{ia}^o	0.43 ± 0.05
	Orn	$K_{ia/x}$	0.016 ± 0.003
	UMP	$K_{ia/y}$	9.2 ± 1.7
	Orn, UMP	$K_{ia/xy}$	0.033 ± 0.017
Ornithine		K_{ix}^o	0.82 ± 0.15
	MgADP	$K_{ix/a}$	0.031 ± 0.006
	UMP	$K_{ix/y}$	120 ± 50
	MgADP, UMP	$K_{ix/ay}$	0.42 ± 0.15
UMP		K_{iy}^o	$(1.2 \pm 0.2) \times 10^{-3}$
	MgADP	$K_{iy/a}$	0.027 ± 0.006
	Orn	$K_{iy/x}$	0.18 ± 0.08
	MgADP, Orn	$K_{iy/ax}$	0.37 ± 0.13

[a] A = MgADP, X = Ornithine, Y = UMP.

Table 2: Coupling Constants (Q) Which Quantify the Interactions between MgADP, Ornithine, and UMP on *E. coli* CPS at 25 °C

interacting ligand	other saturating ligand	designation[a]	Q
MgADP−ornithine		Q_{ax}	27 ± 4
	UMP	$Q_{ax/y}$	280 ± 150
MgADP−UMP		Q_{ay}	0.047 ± 0.007
	ornithine	$Q_{ay/x}$	0.50 ± 0.27
ornithine-UMP		Q_{xy}	0.0068 ± 0.0030
	MgADP	$Q_{xy/a}$	0.072 ± 0.025
MgADP−ornithine−UMP		Q_{axy}	0.090 ± 0.025

[a] A = MgADP, X = Ornithine, Y = UMP.

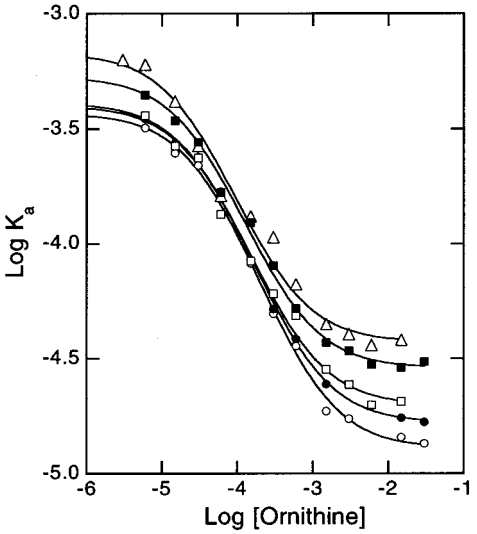

FIGURE 3: K_a for MgADP versus ornithine concentration at several fixed concentrations of IMP equal to 0 (○), 0.01 (●), 0.3 (□), 5.0 (■), and 15 mM (△). Curves were generated by fitting the K_a values at each IMP concentration to eq 1, generating apparent values for K_a^o, K_{ix}^o, and Q_{ax}, as described in the text.

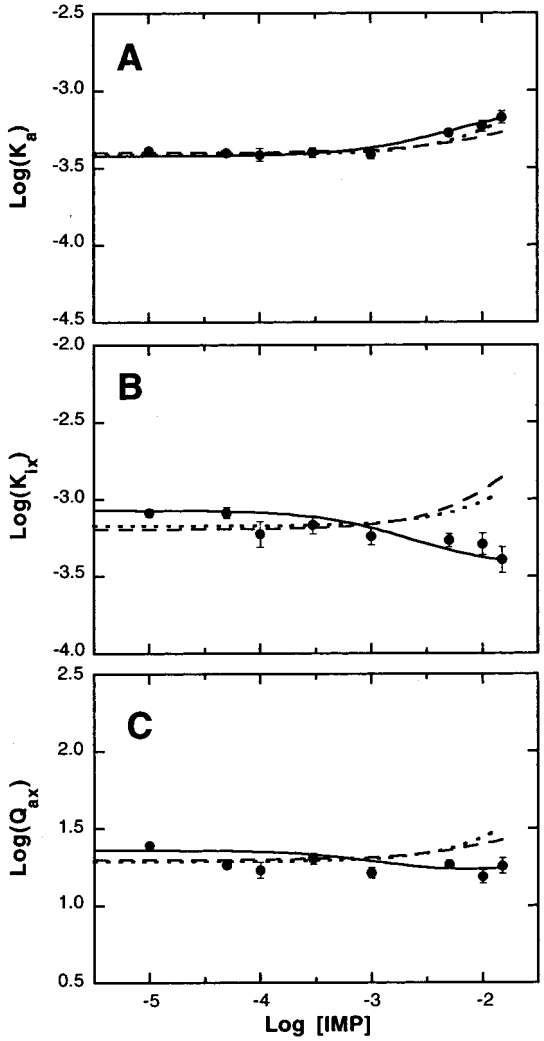

FIGURE 4: Replot of the apparent values of K_a^o, K_{ix}^o, and Q_{ax} determined at several concentrations of IMP as described in Figure 3 and presented in panels A, B, and C, respectively. Curves represent the behavior of these parameters predicted by fitting the data at all IMP concentrations to either eq 2 (—), eq 3 (- - -), or eq 4 (— — —) as described in the text.

and ornithine, Q'_{ax}, indicating that IMP causes ornithine to become less activating. It is once again evident that eq 2 fits significantly better than eqs 3 and 4, primarily because of its ability to accommodate the decrease in $K_{ix}^{o\prime}$.

The dissociation constants obtained from the fit to eq 2 are presented in Table 3, and the coupling parameters are presented in Table 4.

MgADP−UMP−IMP Coupling. The effect of IMP on UMP inhibition is shown in Figure 5 where K_a, as a function of UMP concentration, is plotted for several IMP concentrations. The lines correspond to fits to an equation analogous to eq 1 with suitable substitutions in the notation.

The data were fit to equations based on eqs 2, 3, and 4 and two other models: one in which both X and Y are competitive inhibitors of K_a but not competitive with respect to each other ($Q_{ax} = Q_{ay} = Q_{axy} = 0$, $Q_{xy} \neq 0$), and another in which the relationship between all three ligand pairs was competitive ($Q_{ax} = Q_{ay} = Q_{axy} = Q_{xy} = 0$). Of all the models, only the one which describes UMP and IMP as mutually competitive but neither competitive versus MgADP (equation 3) was able to fit the data.

Table 3: Ligand Dissociation Constants from *E. coli* CPS at 25 °C with Carbamoyl Phosphate Concentration Equal to 0.5 mM

ligand	other saturating ligands	designation[a]	K_d (mM)
MgADP		K_{ia}^o	0.38 ± 0.01
	Orn	$K_{ia/x}$	0.017 ± 0.001
	IMP	$K_{ia/z}$	0.80 ± 0.13
	Orn, IMP	$K_{ia/xz}$	0.043 ± 0.010
ornithine		K_{ix}^o	0.85 ± 0.07
	MgADP	$K_{ix/a}$	0.036 ± 0.003
	IMP	$K_{ix/z}$	0.36 ± 0.07
	MgADP, IMP	$K_{ix/az}$	0.019 ± 0.004
IMP		K_{iz}^o	3.5 ± 1.4 [b]
	MgADP	$K_{iz/a}$	7.4 ± 3.1
	Orn	$K_{iz/x}$	1.5 ± 0.6
	MgADP, Orn	$K_{iz/ax}$	3.8 ± 1.6

[a] A = MgADP, X = Ornithine, Z = IMP. [b] See also value in Table 5 and text for a discussion of the apparent discrepancy.

Table 4: Coupling Constants (Q) Which Quantify the Interactions between MgADP, Ornithine, and IMP on *E. coli* CPS at 25° C

interacting ligands	other saturating ligand	designation[a]	Q
MgADP−Ornithine		Q_{ax}	23 ± 1
	IMP	$Q_{ax/z}$	19 ± 5
MgADP−IMP		Q_{az}	0.47 ± 0.08
	ornithine	$Q_{az/x}$	0.38 ± 0.09
ornithine−IMP		Q_{xz}	2.4 ± 0.4
	MgADP	$Q_{xz/a}$	1.9 ± 0.4
MgADP−ornithine−IMP		Q_{axz}	21 ± 3

[a] A = MgADP, X = Ornithine, Z = IMP.

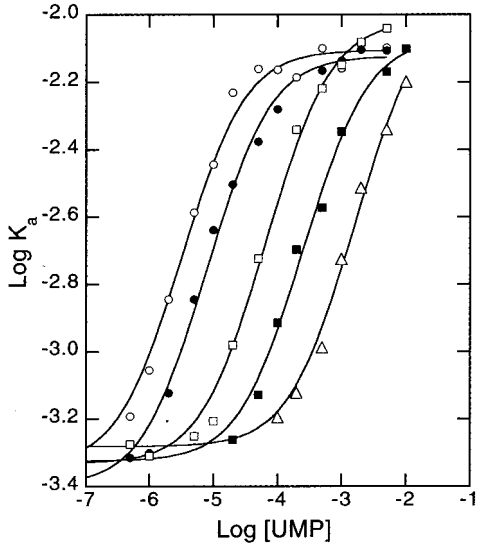

FIGURE 5: K_a for MgADP versus UMP concentration at several fixed concentrations of IMP equal to 0 (○), 0.0625 (●), 0.25 (□), 1.0 (■), and 5.0 mM (△). Curves were generated by fitting the K_a values at each IMP concentration to an equation analogous to eq 1, generating apparent values for K_a^o, K_{iy}^o, and Q_{ay}, as described in the text.

The apparent dissociation constants for MgADP and UMP, $K_a^{o\prime}$ and K_{iy}^o, as well as the coupling between MgADP and UMP, Q'_{ay}, were determined at several IMP concentrations and are represented in Figure 6. Note that the effect of IMP on K_{iy}^o gives no evidence of being saturable as expected for a strictly competitive relationship. The lines in Figure 6

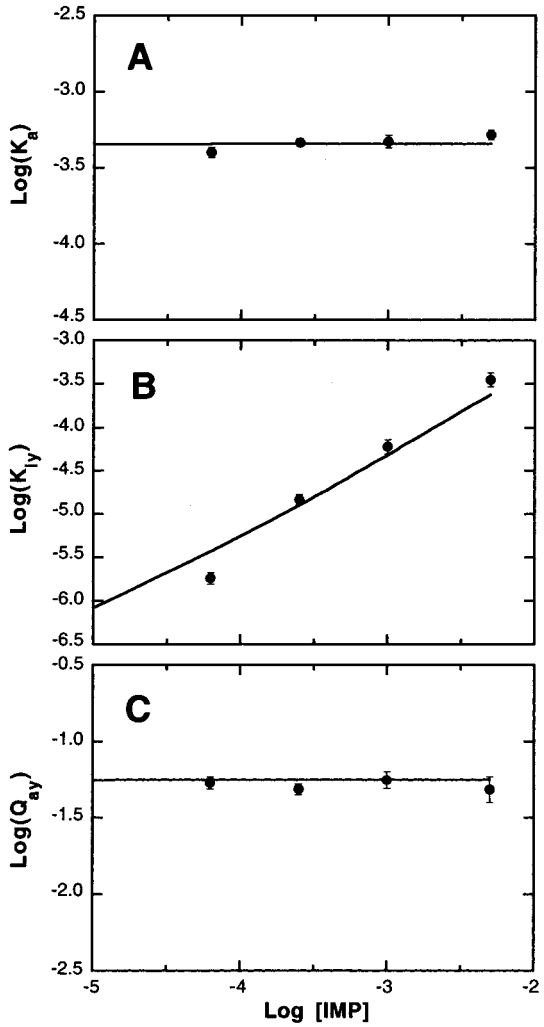

FIGURE 6: Replot of the apparent values of K_a^o, K_{iy}^o, and Q_{ay} determined at several concentrations of IMP as described in Figure 5 and presented in panels A, B, and C, respectively. Curves represent the behavior of these parameters predicted by fitting the data at all IMP concentrations to an equation analogous to eq 3 as described in the text.

represent values of $K_a^o{}'$, K_{iy}^o, and Q'_{ay} as a function of IMP concentration predicted by eqs 5−7 and the values for the parameters derived from the fit of all the data to eq 3 (with X = Y and Y = Z in the notation). Equation 3 is able to describe the data well, and we conclude that UMP and IMP cannot bind simultaneously, which is consistent with the two ligands competing for the same binding site on CPS. The parameters that result from the fit to eq 3 are presented in Tables 5 and 6.

DISCUSSION

Linkage relationships between 3 ligands that can bind simultaneously allow for the calculation of dissociation constants of individual ligands for every occupancy circumstance of the other two binding sites from the 7 fundamental, independent parameters appearing in eq 2 and the definitions of the coupling parameters. These values are also given in Tables 1 and 3 for MgADP−ornithine−UMP and MgADP−ornithine−IMP, respectively. The fewer parameters resulting from eq 3 for MgADP−UMP−IMP appear in Table 5.

Table 5: Ligand Dissociation Constants from *E. coli* CPS at 25 °C with Carbamoyl Phosphate Concentration Equal to 0.5 mM

ligand	other saturating ligands	designation[a]	K_d (mM)
MgADP		K_{ia}^o	0.45 ± 0.06
	UMP	$K_{ia/y}$	8.1 ± 1.6
	IMP	$K_{ia/z}$	0.45 ± 0.09
UMP		K_{iy}^o	(0.75 ± 0.18) × 10^{-3}
	MgADP	$K_{iy/a}$	0.014 ± 0.004
IMP		K_{iz}^o	0.016 ± 0.004[b]
	MgADP	$K_{iz/a}$	0.016 ± 0.005

[a] A = MgADP, Y = UMP, Z = IMP. [b] See also value in Table 3 and text for a discussion of the apparent discrepancy.

Table 6: Coupling Constants (Q) Which Quantify the Interactions between MgADP, UMP, and IMP on *E. coli* CPS at 25 °C

interacting ligands	designation[a]	Q
MgADP−UMP	Q_{ay}	0.055 ± 0.008
MgADP−IMP	Q_{az}	0.99 ± 0.15

[a] A = MgADP, Y = UMP, Z = IMP.

Likewise, coupling parameters between each pair of ligands can be altered by the binding of the third ligand to its site, and these modified coupling parameters can also be calculated from identities that exist between these parameters as described previously (*17*, *26*). These values are present in Tables 2, 4, and 6.

The values presented in Tables 1 and 2 therefore summarize the interactions of CPS with substrate MgADP and both allosteric ligands ornithine and UMP. These values indicate that the dissociation constant for MgADP when both ornithine and UMP are bound ($K_{ia/xy}$ = 0.033 mM) is almost as low as it is when ornithine alone is bound ($K_{ia/x}$ = 0.016 mM) and is substantially different than when UMP alone is bound ($K_{ia/y}$ = 9.2 mM). Similarly, from Table 2, we can see that the inhibitory coupling between MgADP and UMP is diminished to only 0.5 when ornithine is bound whereas the activation of MgADP binding by ornithine is enhanced 10-fold when UMP is bound. These results clearly suggest that the allosteric effects of ornithine *dominate* those induced by UMP on the binding of MgADP (as substrate for the MgATP synthesis reaction) when these two mutually antagonistic ligands are "forced" onto the enzyme simultaneously by high free ligand concentrations.

In addition, these data allow for the first time the direct quantitation of the coupling between ornithine and UMP, given by the coupling constant Q_{xy}. These two ligands antagonize each other's binding strongly (Q_{xy} = 0.0068) and to a significantly greater extent than do ADP and UMP. This antagonism is mitigated by an order of magnitude, however, when ADP is bound ($Q_{xy/a}$ = 0.072). In fact, the coupling between each pair of ligands is increased by an order of magnitude when the third ligand is present, leading to UMP becoming nearly ineffectual when ornithine is bound ($Q_{ay/x}$ = 0.5) and ornithine to be a much stronger activator when UMP is bound ($Q_{ax/y}$ = 280).

It is of little surprise that ornithine similarly dominates the actions of IMP, as summarized in Tables 3 and 4, since the effects of IMP alone are so small. The dissociation constant for MgADP when both ornithine and IMP are bound, $K_{ia/xz}$, is almost an order of magnitude lower than

that for MgADP in the absence of allosteric ligands. It is interesting to note, however, that the presence of IMP mitigates the effect of ornithine by approximately the same amount as UMP does despite the fact that UMP has a much larger inhibitory effect by itself than does IMP; that is, $K_{ia/xy} \approx K_{ia/xz}$. This feature leads to an ironic distinction in the effect of UMP and IMP on the MgADP−ornithine coupling, Q_{ax}. When UMP is saturating, the coupling between MgADP and ornithine ($Q_{ax/y}$) is increased 10-fold; that is, ornithine is a much more effective activator; whereas the MgADP−ornithine coupling when IMP is bound, $Q_{ax/z}$, is actually diminished slightly relative to Q_{ax}. It is also interesting that, despite the opposite effects that ornithine and IMP have individually, they do not antagonize each other's binding but rather promote each other's binding, albeit by only 2-fold ($Q_{xz} = 2.4$).

The data we present in Figures 5 and 6 are fully consistent with previously published conclusions that UMP and IMP bind competitively (*8*, *11*, *12*). Only the model (eq 3) that does not allow for the simultaneous binding of both allosteric ligands fits the data adequately. Both IMP and UMP bind individually to a site separate from those to which ornithine and MgADP bind, and the competitive relationship between IMP and UMP suggests that these two ligands bind to identical or overlapping sites, consistent with the picture emerging from the X-ray structures of *E. coli* CPS (*20*, *21*).

Each matrix of assays described in Figures 1, 3, and 5 was performed and analyzed independently. The consistency of the analysis between these data sets can be evaluated by comparing the parameters that are determined in common. Generally the agreement is very good. Thus, the values of K^{o}_{ia} determined in each of the three sets of experiments agree and are all equal to 0.4 mM. K^{o}_{ix} values given in Tables 1 and 3 are both equal to 0.8 mM, and K^{o}_{iy} values from Tables 1 and 5 agree reasonably well with a value of 1 μM. Likewise the values for the coupling parameters Q_{ax} and Q_{ay} are approximately equal to 25 and 0.05, respectively, in both of their independent determinations.

The one exception to this general pattern of consistency involves the dissociation constant for IMP, K^{o}_{iz}. In Table 3 this parameter has a value of 3.5 mM, whereas in Table 5 K^{o}_{iz} is given as 16 μM, a 200-fold discrepancy. Of these two values, we give somewhat greater credence to the smaller value because it results from the direct competition between IMP and UMP for binding. Hence the measurable consequence of IMP binding is large, as seen in Figures 5 and 6. By contrast, the larger value appearing in Table 3 is determined from the very small effect that IMP has on the binding of MgADP and ornithine as evident in Figure 4. We note also that the standard error for this value is 40%, and the low precision of this estimate casts doubt on its accuracy as well. However, the high-affinity interaction evident in Figure 5 and Table 5 has no discernible effect on the binding of MgADP ($Q_{az} = 1$ in Table 6), so we cannot rule out the possibility that effects of IMP binding evident in Figures 3 and 5 derive from separate interactions with the enzyme.

Relationship to Structure. The three-dimensional crystal structure of carbamoyl phosphate synthetase has recently been solved to high resolution in the presence of bound MnADP (*20*). The positive allosteric site was further occupied by ornithine while phosphate was bound to the protein where the phosphoryl group of either IMP or UMP would normally bind. Thus far, only the structure of the ornithine-bound form of the protein has been determined to high resolution. Therefore, it is not possible to speculate with confidence on the structural differences between CPS in the absence of the allosteric ligands and the changes incurred upon binding of ornithine and/or UMP to the protein. Nevertheless, the available structure does suggest a possible conduit through which the modulation of the ADP-binding site within the carbamoyl phosphate domain might be influenced by the binding of ornithine. The structure of the ADP-binding site and its relationship to the ornithine and UMP sites is presented in Figure 7. The bound ornithine interacts with amino acids from the allosteric domain (residues 933−1073) as well as the carbamoyl phosphate domain (residues 553−933). The key interactions appear to be the ion pair between the δ-amino group of ornithine and the side chain carboxylates of Glu-783 and Glu-892. Moreover, the carbonyl oxygen of Glu-783 serves as a direct ligand to the essential monovalent cation, K^+. An additional ligand to the bound K^+ is the side chain carboxylate of Glu-761, which has been found to also hydrogen bond to the two ribose hydroxyl groups of the bound ADP. There is thus a direct electrostatic connection between the bound ornithine and ADP at the active site. The δ-amino group of ornithine is ~12 Å from the ribose group of the bound ADP.

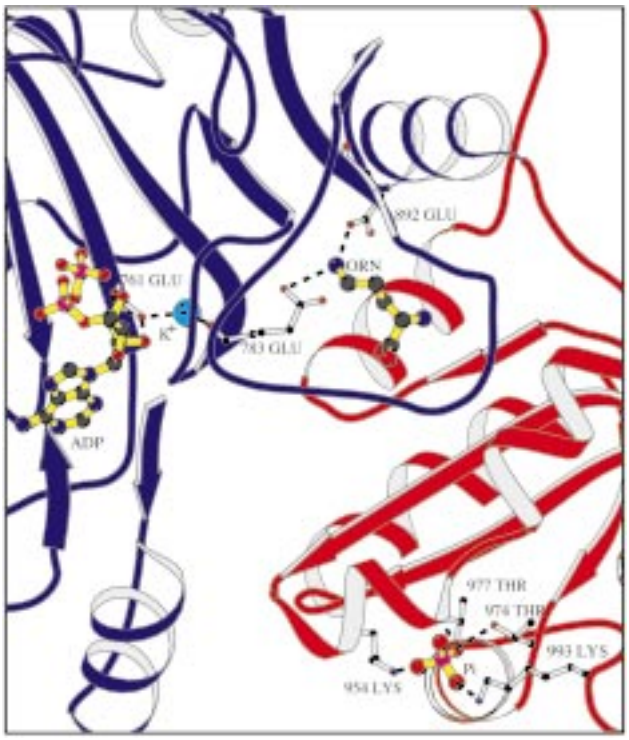

FIGURE 7: Binding sites for the allosteric ligands of CPS (*20*). The part of the figure depicted in blue shows a portion of the binding site for ADP within the carbamoyl phosphate domain. The part of the figure illustrated in red shows the relative positions for the binding of ornithine and of UMP (shown with a bound P_i) within the allosteric domain.

The bound phosphate within the IMP/UMP site is ~24 Å away from the bound ADP and thus much further from the active site than is the ornithine. The primary interactions between the phosphate and the protein originate from the side chains of 2 lysine residues (Lys-954 and Lys-993) and 2 threonine residues (Thr-974 and Thr-977). The allosteric

effects exerted on the bound ADP are dominated by ornithine when this ligand and UMP are bound simultaneously to the protein. Therefore, the direct electrostatic connection between the ornithine-binding site and the active site may allow the effects of ornithine to overwhelm whatever changes are induced by the binding of UMP. A structural explanation for this observation is not available at the present time, although previous analysis of the energetics of the allosteric responsiveness of CPS has suggested that ornithine and UMP may achieve their effects primarily through perturbations of the dynamics of the protein structure (*24*).

Regardless of the mechanism by which the allosteric ligands of CPS achieve their effects, these results strongly argue against the value of modeling the mechanism with only two states. First of all, both activating and inhibiting ligands can bind simultaneously, and even quaternary complexes with MgADP, ornithine, and UMP all bound can be formed. Moreover, each ligand exhibits a discrete affinity for each state of ligation. For example, MgADP binds with 6 different affinities depending on the identity and occupancy of the other ligand-binding sites. Also, unexpected coupling relationships are also evident. For example, the binding of the activator ornithine diminishes the antagonism between the inhibitor UMP and the substrate MgADP. Any mechanism proposed to explain the allosteric properties of *E. coli* CPS must acknowledge this complexity.

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BI982097W