Quantifying the Allosteric Properties of *Escherichia coli* Carbamyl Phosphate Synthetase: Determination of Thermodynamic Linked-Function Parameters in an Ordered Kinetic Mechanism†

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ABSTRACT: The effects of the allosteric ligands UMP, IMP, and ornithine on the partial reactions catalyzed by *Escherichia coli* carbamyl phosphate synthetase have been examined. Both of these reactions, a HCO_3^--dependent ATP synthesis reaction and a carbamyl phosphate-dependent ATP synthesis reaction, follow bimolecular ordered sequential kinetic mechanisms. In the ATPase reaction, MgATP binds before HCO_3^- as established previously for the overall reaction catalyzed by carbamyl phosphate synthetase [Raushel, F. M., Anderson, P. M., & Villafranca, J. J. (1978) *Biochemistry 17*, 5587–5591]. The initial velocity kinetics for the ATP synthesis reaction indicate that MgADP binds before carbamyl phosphate in an equilibrium ordered mechanism except in the presence of ornithine. Determination of true thermodynamic linked-function parameters describing the impact of allosteric ligands on the binding interactions of the first substrate to bind in an ordered mechanism requires experiments to be performed in which both substrates are varied even if only one is apparently affected by the allosteric ligands. In so doing, we have found that IMP has little effect on the overall reaction of either of these two partial reactions. UMP and ornithine, which have a pronounced effect on the apparent K_m for MgATP in the overall reaction, both substantially change the thermodynamic dissociation constant for MgADP from the binary E–MgADP complex, K_{ia}, in the ATP synthesis reaction, with UMP increasing K_{ia} 15-fold and ornithine decreasing K_{ia} by 18-fold. By contrast, only UMP substantially affects the K_{ia} for MgATP in the ATPase reaction, increasing it by 5-fold. These effects are independent of the concentration of the unaffected substrate, carbamyl phosphate in the ATP synthesis reaction and HCO_3^- in the ATPase reaction, and do not reflect the influence that the allosteric ligands have on the K_m for nucleotide in each case. The UMP effect on the ATPase reaction and the ornithine effect on the ATP synthesis reaction derive primarily from changes in the off-rate constant for the nucleotide.

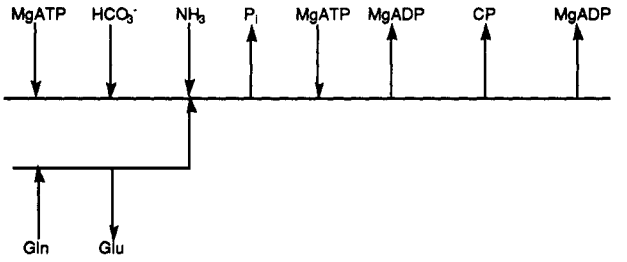
Carbamyl phosphate synthetase from *Escherichia coli* catalyzes the production of carbamyl phosphate from MgATP, HCO_3^-, and glutamine via the following reaction in vivo:

$$2MgATP + HCO_3^- + \text{glutamine} \xrightarrow{Mg^{2+}} NH_2(C{=}O)OPO_3^{2-} + 2MgADP + P_i + \text{glutamate} \quad (1)$$

In vitro carbamyl phosphate synthetase will also catalyze the synthesis of carbamyl phosphate, with ammonia rather than glutamine serving as the nitrogen source (Trotta et al., 1978; Rubino et al., 1987). Carbamyl phosphate synthetase is important physiologically since carbamyl phosphate is needed for both pyrimidine and arginine biosynthesis (Pierard & Wiame, 1964; Robin et al., 1989). The enzyme is subject to feedback control via allosteric mechanisms so that neither imbalances in the nucleotide pools nor toxic levels of ammonia occur. Specifically, ornithine is an activator, while UMP is an inhibitor of carbamyl phosphate synthetase (Pierard, 1966; Anderson & Meister, 1966b; Anderson, 1977). Ornithine and UMP achieve their effects by binding to separate allosteric binding sites on the large subunit (Trotta et al., 1971, 1974). In addition, activation by IMP has been reported (Boettcher & Meister, 1981, 1982; Kasprzak & Villafranca, 1988) with IMP apparently binding proximate to the UMP site. All of these allosteric ligands exert their primary influence on the binding of MgATP (Anderson & Meister, 1966b; Meister & Powers, 1978).

The kinetic mechanism of the complete reaction has been established to be ordered Ter–Uni–Uni–Ter ping-pong (Raushel et al., 1978) as summarized in the following diagram:

Scheme I



From this mechanism, it is clear that MgATP not only binds at two different steps in the kinetic mechanism but that MgATP binds at two different places in the active site, since two equivalents of nucleotide are bound simultaneously at one point during turnover. In the chemical mechanism, the first MgATP is responsible for the activation of HCO_3^- whereas the second MgATP is responsible for the phosphorylation of carbamate (Anderson & Meister, 1965, 1966; Powers & Meister, 1978b; Raushel & Villafranca, 1979; Post et al., 1990). These two separate reactions are reflected, respectively, in the following two partial reactions also catalyzed by car-

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bamyl phosphate synthetase in vitro:

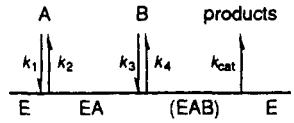
$$\text{MgATP} \xrightarrow{HCO_3^-} \text{MgADP} + P_i \quad (2)$$

$$NH_2(C{=}O)OPO_3^{2-} + \text{MgADP} \xrightarrow{Mg^{2+}} \text{MgATP} + CO_2 + NH_3 \quad (3)$$

A study of the effects of the allosteric ligands can therefore be greatly simplified by studying either of these partial reactions.

It has been concluded that the allosteric ligands principally affect the second MgATP-binding step in the overall forward reaction because these allosteric ligands appear to influence the nucleotide K_m in reaction 3 to a greater extent than they do the nucleotide K_m in reaction 2 (Anderson & Meister, 1966b; Post et al., 1990). However, this assessment has been made phenomenologically, without an explicit consideration of the consequences that the ordered nature of the kinetic mechanism has on the manifestation of the allosteric effects. The absolute dependence of reaction 2 on HCO_3^- and the ordered kinetic mechanism for the overall reaction (Raushel et al., 1978) suggest an ordered relationship between the binding of MgATP and HCO_3^- with HCO_3^- binding second. Reaction 3 probably involves an ordered addition of MgADP and carbamyl phosphate since the product release steps in the overall reaction all occur in a strictly ordered fashion (Raushel et al., 1978). However, unlike the case for reaction 2, the order cannot be presumed because carbamyl phosphate release in Scheme I occurs between the release of each MgADP, and it is not known which MgADP corresponds to the second MgATP to bind. As discussed below, the quantitative evaluation of allosteric effects in an ordered kinetic mechanism is quite dependent upon whether the influenced substrate binds first or last. Since MgATP binds both first and last in the overall reaction (reaction 1), we present herein a detailed evaluation of the influence of allosteric ligands on reactions 2 and 3 in order to more fully understand the true nature of the allosteric regulation of carbamyl phosphate synthetase. These reactions will be considered within the context of the following ordered sequential, initial velocity kinetic scheme:

Scheme II



Materials and Methods

Carbamyl phosphate synthetase was purified from *E. coli* RR1 carrying the plasmid pMC41 as described in Mullins et al. (1991). Dilithium carbamyl phosphate, potassium ATP and ADP, trisodium PEP, L-glutamine, disodium NADH, sodium NADP, ornithine-HCl, disodium UMP and IMP, and Hepes[1] free acid were purchased from Sigma. Pyruvate kinase and lactate dehydrogenase were obtained from Boehringer-Mannheim in glycerol solutions and were used without further treatment. Hexokinase and glucose-6-phosphate dehydrogenase were purchased from Boehringer-Mannheim as ammonium sulfate suspensions and were dialyzed exhaustively to remove any traces of ammonia. All other chemicals were reagent grade.

Reactions 1 and 2 were assayed at 25 °C by coupling the production of MgADP to the oxidation of NADH through the inclusion of pyruvate kinase, PEP, and lactate dehydrogenase in the assay mixture. Disappearance of NADH was followed continuously by monitoring the decrease in absorbance at 340 nm with a strip chart recorder.

For reaction 1, each cuvette contained in a final volume of 1.0 mL the following: 50 mM Hepes-KOH, pH 7.5, 100 mM KCl, 20 mM $MgCl_2$, 0.2 mM NADH, 1 mM PEP, 30 mM $NaHCO_3$, 10 mM L-glutamine, and 20 μg each of pyruvate kinase and lactate dehydrogenase. MgATP concentrations varied between 9 μM and 6 mM. Effector concentrations ranged from 0 to 15 mM. Reactions were started with the addition of 2.5–4 μg of carbamyl phosphate synthetase.

For reaction 2, each 1.0-mL assay mixture contained the following: 50 mM Hepes-KOH, pH 7.5, 100 mM KCl, 20 mM $MgCl_2$, 0.2 mM NADH, 1 mM PEP, and 12.5 μg each of pyruvate kinase and lactate dehydrogenase. MgATP concentrations varied between 2 μM and 8 mM. $NaHCO_3$ concentrations varied between 75 μM and 15 mM. Effector concentrations, if present, were 15 mM. Reactions were started with the addition of 35 μg of carbamyl phosphate synthetase. Solutions used to make the assay mixtures were prepared under N_2 with ingredients dissolved in deionized–distilled water. This solution was degassed prior to the addition of solid KOH to adjust the pH to 7.5. The intrinsic bicarbonate concentration of this assay mixture was estimated to be 200 μM, and the reported concentrations have been adjusted to include this basal concentration.

Production of MgATP in reaction 3 was measured at 25 °C by coupling to the reduction of NADP with hexokinase, glucose, and glucose-6-phosphate dehydrogenase. Initial velocities were determined from the increase in absorbance at 340 nm as a function of time using a strip chart recorder. All cuvettes contained 50 mM Hepes-KOH, pH 7.5, 100 mM KCl, 20 mM $MgCl_2$, 1 mM NADP, 10 mM glucose, 1 unit each of hexokinase and glucose-6-phosphate dehydrogenase, and the indicated concentrations of MgADP, carbamyl phosphate, and allosteric ligand. Carbamyl phosphate solutions were made immediately before use by dissolving preweighed amounts of the lithium salt into 100 mM Hepes-KOH, pH 7.5. Carbamyl phosphate concentrations varied between 50 μM and 15 mM. MgADP concentrations varied between 2 μM and 10 mM. Effectors, if present, were 15 mM. Reactions were started by the addition of 20 μg of carbamyl phosphate synthetase.

Data were fit to the following initial velocity rate expressions via nonlinear regression following the general approach outlined by Cleland (1967). Programs were written in HP Basic 2.1 as implemented on HP 9826A and HP 9836A desktop computers.

$$\frac{v}{E_t} = \frac{V_{max}[A]}{K_a + [A]} \quad (4)$$

$$\frac{v}{E_t} = \frac{V_{max}[A][B]}{K_{ia}K_b + K_b[A] + [A][B]} \quad (5)$$

$$\frac{v}{E_t} = \frac{V_{max}[A][B]}{K_{ia}K_b + K_a[B] + K_b[A] + [A][B]} \quad (6)$$

$$\frac{v}{E_t} = \frac{V_{max}([A] + Q_{ax}W_x[A][X])}{K_a^0K_{ix}^0 + K_a^0[X] + K_{ix}^0[A] + Q_{ax}[A][X]} \quad (7)$$

In eqs 4 and 7, A represents substrate, and in eqs 5 and 6, A represents the first substrate and B represents the second substrate to bind in an ordered bimolecular kinetic mechanism. X represents an allosteric ligand. The notation in eqs 4–6 follows that described by Cleland (1963). In eq 7, the notation is as described by Reinhart (1983).

[1] Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

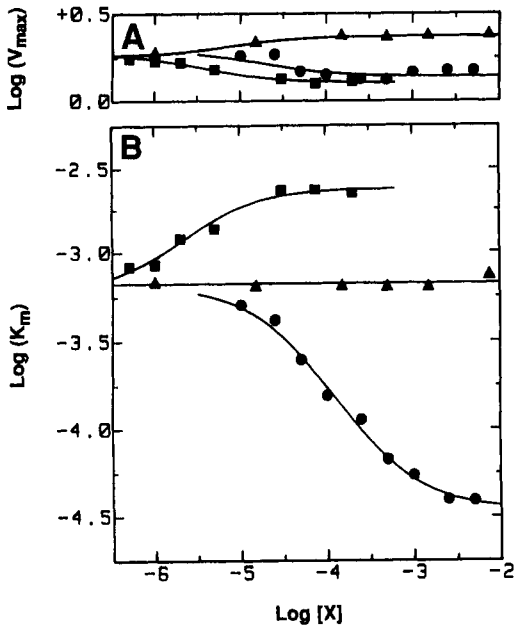


FIGURE 1: Influence of the allosteric ligands UMP (■), IMP (▲), and ornithine (●) on the apparent V_{max} (A) and K_m for MgATP (B) for the native forward reaction catalyzed by carbamyl phosphate synthetase (reaction 1 in the text). Initial velocities at each effector concentration were fit to eq 4 described in the text. Other substrates of the reaction, i.e., HCO_3^- and glutamine, were fixed at near saturating concentrations (30 and 10 mM, respectively). V_{max} was expressed in micromolecules of carbamyl phosphate formed per minute per milligram protein, and K_m was expressed in molar units, before logarithms were calculated.

RESULTS

Overall Reaction (Reaction 1)

The effects of ornithine, UMP, and IMP on reaction 1 when MgATP is the variable substrate are summarized in Figure 1. These data were obtained with all other substrates at near saturating concentrations. All three allosteric ligands have only a small effect on maximal velocity while ornithine and UMP have a substantial effect on the apparent K_m for MgATP. UMP inhibits this reaction by increasing this K_m 5-fold. Ornithine activates by lowering the K_m by over 20-fold.

These effects can be quantified by fitting the data for each allosteric ligand to eq 7, which is derived from the single-substrate, single-modifier mechanism (see discussion), with A = MgATP and X = the allosteric ligand. The parameters K_a^0, K_{ix}^0, Q_{ax}, and W_x resulting from the data pertaining to each allosteric ligand will represent the apparent Michaelis constant for MgATP in the absence of X, the apparent dissociation constant of X in the absence of MgATP, the magnitude to which K_a^0 changes when X is saturating, and the magnitude of the change in V_{max} brought about by saturation with X, respectively. Due to the ordered nature of the kinetic mechanism by which carbamyl phosphate synthetase catalyzes reaction 1, and the dual role MgATP has as a substrate, the apparent effects of the allosteric ligands shown in Figure 1B may not accurately reflect the true perturbation they engender on the individual binding interactions of substrate (see Discussion). However, from the fit of these data to eq 7, one can estimate the apparent dissociation constant of the allosteric ligand itself, K_{ix}^0, to be equal to 1.2 μM, 7.7 μM, and 0.4 mM for UMP, IMP, and ornithine, respectively.

ATPase Reaction (Reaction 2)

Although reaction 2 is a unimolecular hydrolysis, the absolute requirement for HCO_3^- (Anderson & Meister, 1966a; Mullins et al., 1991) suggests that a complete evaluation of the actions of the allosteric ligands include a consideration of the dependence of the rate on HCO_3^- concentration. In Figure 2 we present the initial velocity double-reciprocal plot pattern that results when MgATP is the variable substrate at various fixed concentrations of HCO_3^-. The data indicate that the rate of reaction is influenced by the concentration of HCO_3^- even when MgATP is saturating. Despite the fact that HCO_3^- is a nonconsumed catalytic cofactor, these data suggest that HCO_3^- does not bind first in an equilibrium ordered fashion as metal ions often do when required for catalysis (Cleland, 1970). Rather, the pattern is typical of a sequential mechanism in which both ligands must rebind each catalytic cycle. This requirement is most easily mandated by HCO_3^- binding after MgATP in an ordered manner that is consistent with the ordered kinetic mechanism of the overall reaction determined by Raushel et al. (1978).

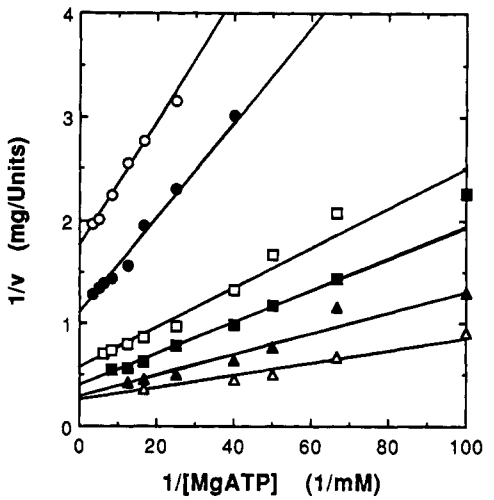


FIGURE 2: Dependence of the reciprocal of the initial velocity, v, of the HCO_3^--dependent ATPase reaction (reaction 2 in the text) on the reciprocal concentration of MgATP. HCO_3^- concentration is equal to 0.275 (○), 0.35 (●), 0.95 (□), 1.7 (■), 3.95 (▲), or 15.2 (△) mM. Lines represent the best fit of the data at each carbamyl phosphate concentration to eq 4 described in the text.

Raushel and Villafranca (1979) have concluded, on the basis of rapid quenching and isotope partitioning experiments, that the MgATP that reacts in the ATPase reaction binds first in the overall reaction. Therefore, the effects of the allosteric ligands on both MgATP and HCO_3^- can be assessed by fitting the initial velocity data to eq 6, the rate equation for a bimolecular ordered mechanism, with A = MgATP and B = HCO_3^-, and observing the effects of the inclusion of a saturating concentration (15 mM) of each allosteric ligand. The resulting best fit parameters are summarized in Table I.

We note that for Scheme II, $V_{max} = E_t k_{cat}$, $K_a = k_{cat}/k_1$, and $K_{ia} = k_2/k_1$. Consequently the rate constants k_{cat}, k_1, k_2 appearing in Scheme II can be recovered from the parameters V_{max}, K_a, and K_{ia}. The values of these rate constants are also listed in Table I.

It is apparent from these results that only UMP has a substantial effect on the ATPase reaction. UMP inhibits this reaction both by decreasing V_{max} almost 2-fold and by increasing K_{ia}, the thermodynamic dissociation constant for MgATP, over 5-fold. IMP has no effect on V_{max} but does increase K_{ia} over 2-fold, indicating that IMP inhibits the enzyme slightly at low concentrations of MgATP and HCO_3^-. Interestingly, ornithine, which dramatically decreases the apparent Michaelis constant for MgATP in the overall reaction, similarly reduces the K_a for MgATP but has only a small effect on K_{ia}. None of the allosteric ligands influences the

Table I: Kinetic Parameters for the ATPase Reaction Catalyzed by CPS[a]

	additions			
	none	UMP (15 mM)	IMP (15 mM)	ornithine (15 mM)
V_{max} (units/mg)	0.169 ± 0.008	0.089 ± 0.003	0.168 ± 0.008	0.111 ± 0.003
K_{ia} (μM)	50 ± 10	270 ± 30	120 ± 10	32 ± 4
K_a (μM)	26 ± 3	14 ± 3	34 ± 3	3.1 ± 0.4
K_b (mM)	1.52 ± 0.16	0.90 ± 0.09	1.22 ± 0.12	0.89 ± 0.08
σ	0.003	0.002	0.002	0.003
rate constants[b]				
k_{cat} (s^{-1})	0.45 ± 0.02	0.24 ± 0.01	0.45 ± 0.02	0.30 ± 0.01
$k_1 \times 10^{-4}$ (M^{-1} s^{-1})	1.7 ± 0.2	1.7 ± 0.4	1.3 ± 0.1	9.7 ± 1.3
k_2 (s^{-1})	0.85 ± 0.20	4.6 ± 1.2	1.6 ± 0.2	3.1 ± 0.6

[a] Calculated according to eq 6. Substrate designations: A = MgATP; B = HCO_3^-. Activity was measured at 25 °C and pH 7.5 in Hepes-KOH buffer containing 100 mM KCl and 20 mM excess $MgCl_2$. [b] Rate constant designations refer to Scheme II as described in the text. Values listed are calculated from the above kinetic parameters assuming an ordered mechanism.

Michaelis constant for HCO_3^- by more than 2-fold.

ATP Synthesis Reaction (Reaction 3)

To properly assess the effects of the allosteric ligands on reaction 3 it is necessary to first ascertain the kinetic mechanism. Since reaction 1 is catalyzed with an ordered substrate addition and product release (Raushel et al., 1978), it is likely that MgADP and carbamyl phosphate bind in an ordered fashion in reaction 3. However, although the chemical mechanism suggests that the MgATP produced in reaction 3 corresponds to the second ATP that binds in reaction 1, it is not known which MgADP released in reaction 1 corresponds to that ATP, and hence the order of substrate addition in reaction 3 is unknown.

Initial Velocity Studies. Initial rates were determined by varying MgADP and carbamyl phosphate concentrations. These data are shown in Figure 3A in double-reciprocal form with carbamyl phosphate as the variable substrate. The lines represent the fit of the data, obtained at each MgADP concentration, directly to eq 4. The crossover point appears to be positioned on the *Y*-axis as indicated by the essentially invariant intercept replot, whereas the slope replot is unremarkable (Figure 3B). On the other hand, the slope replot derived from double-reciprocal plots generated when MgADP is the variable substrate appears to extrapolate through the origin while the intercept replot has a significant slope (data not shown). These features are characteristic of an equilibrium ordered mechanism (Cleland, 1970) in which the first substrate to bind, A, achieves a virtual binding equilibrium with E in the steady-state. Equation 5 is the rate equation describing such an equilibrium ordered, Bi–Bi mechanism with A and B representing the first and second substrates to bind, respectively. The unsymmetrical nature of this mechanism results from the lack of a K_a[B] term in the denominator. Otherwise the rate equation for the equilibrium ordered Bi–Bi mechanism (equation 5) is identical to the rate equation for the steady-state ordered Bi–Bi mechanism (equation 6).

The above data were fit to eq 5, and the resulting parameters are shown in Table II. When these data are fit to eq 6, the additional parameter, K_a, that is necessitated by this equation is negative and has an absolute value close to zero (data not shown). Thus, we conclude that the rapid equilibrium ordered mechanism is more appropriate.

Effect of UMP and IMP. To determine the maximal effects of UMP and IMP on the complete kinetic behavior of carbamyl phosphate synthetase when catalyzing reaction 3, both MgADP and carbamyl phosphate were varied, as above, with

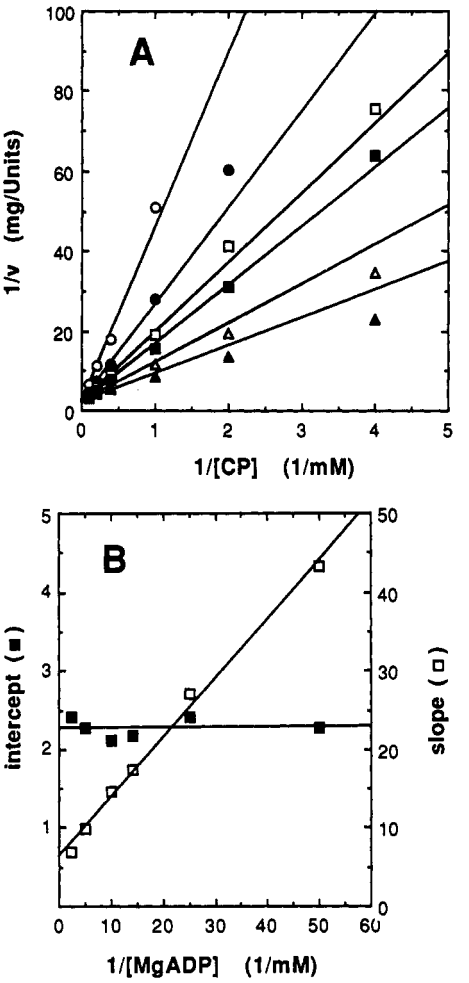


FIGURE 3: (A) Dependence of the reciprocal of the initial velocity, *v*, of the ATP synthesis reaction on the reciprocal concentration of carbamyl phosphate (CP). MgADP concentration is equal to 0.02 (○), 0.04 (●), 0.07 (□), 0.1 (■), 0.2 (△), or 0.4 (▲) mM. Lines represent the best fit of the data at each MgADP concentration to eq 4 described in the text. (B) Slope (□) and intercept (■) of the lines drawn in panel A as a function of the reciprocal of MgADP concentration.

Table II: Kinetic Parameters for the ATP Synthesis Reaction Catalyzed by CPS[a]

	additions			
	none (eq 5)	UMP (15 mM) (eq 5)	IMP (15 mM) (eq 5)	ornithine (15 mM) (eq 6)
V_{max} (units/mg)	0.40 ± 0.01	0.131 ± 0.003	0.58 ± 0.02	0.34 ± 0.01
K_{ia} (mM)	0.22 ± 0.02	3.3 ± 0.3	0.42 ± 0.03	0.012 ± 0.003
K_a (mM)				0.006 ± 0.001
K_b (mM)	1.6 ± 0.1	1.6 ± 0.1	1.8 ± 0.1	1.3 ± 0.1
σ	0.010	0.002	0.009	0.008
rate constants[b]				
k_{cat} (s^{-1})	1.07 ± 0.03	0.35 ± 0.01	1.55 ± 0.05	0.91 ± 0.03
$k_1 \times 10^{-4}$ (M^{-1} s^{-1})				15 ± 3
k_2 (s^{-1})				1.8 ± 0.5

[a] Substrate designations: A = MgADP; B = carbamyl phosphate. Activity was measured at 25 °C and pH 7.5 in Hepes-KOH buffer containing 100 mM KCl and 20 mM excess $MgCl_2$. [b] Rate constant designations refer to Scheme II as described in the text. Values listed are calculated from the above kinetic parameters assuming an ordered mechanism.

the inclusion of a saturating concentration (15 mM) of UMP and IMP, respectively. Qualitatively, the double-reciprocal plot patterns were unsymmetric with respect to substrate, i.e., similar to the patterns obtained in the absence of either al-

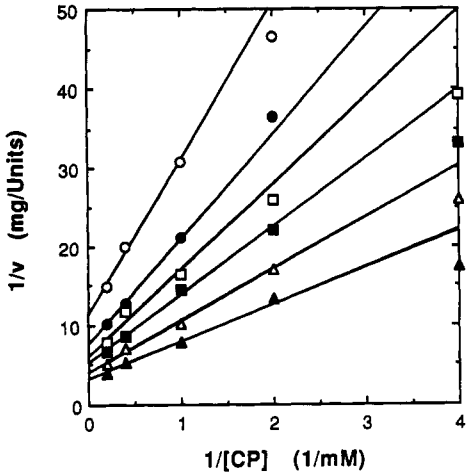


FIGURE 4: Dependence of the reciprocal of the initial velocity, v, of the ATP synthesis reaction of the reciprocal concentration of carbamyl phosphate (CP) in the presence of 15 mM ornithine. MgADP concentration is equal to 0.002 (○), 0.004 (●), 0.007 (□), 0.01 (■), 0.02 (△), or 0.07 (▲) mM. Lines represent the best fit of the data at each MgADP concentration to eq 4 described in the text.

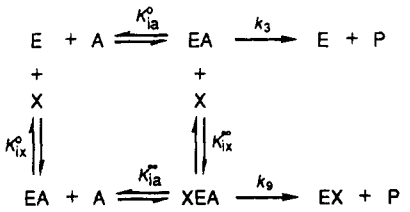
losteric ligand (data not shown). The data, when fit to eq 5, resulted in the parameters shown in Table II. Once again eq 6 did not provide a satisfactory description of the data when compared to that provided by eq 5.

Effect of Ornithine. Unlike UMP and IMP, the activation by 15 mM ornithine results in a qualitative change in the double-reciprocal plot pattern when carbamyl phosphate is the variable substrate as shown in Figure 4. The crossover point clearly occurs to the left of the Y-axis. The pattern when MgADP is the variable substrate is similar in appearance, and the slope replots from both patterns extrapolate to a Y-axis intercept significantly displaced from the origin (data not shown). These data are clearly best described by the sequential Bi–Bi rate equation (eq 6), and the parameters resulting in the best fit of these data to eq 6 are also presented in Table II.

Discussion

The simple single-substrate, single-modifier mechanism serves as a useful paradigm when considering the actions of an allosteric ligand.

Scheme III



This mechanism serves to highlight the fact that allosteric ligands can fundamentally have two different types of effects. They can alter the maximal rate of turnover, in which case $k_3 \neq k_9$ in the above mechanism, or allosteric ligands can alter the affinity with which the substrate binds to the enzyme, in which case $K_{ia}^0 \neq K_{ia}^\infty$ in the above mechanism. An allosteric ligand effects these actions by binding before and/or subsequent to the binding of substrate.

It is a defining characteristic of allosteric action that the effects of an allosteric ligand are saturable (Reinhart, 1983), and the two types of effects can be quantified by measuring the kinetic properties of the enzyme when the allosteric ligand is saturating and comparing them to the kinetic properties observed in its absence. We have previously defined two parameters, W_x and Q_{ax}, which explicitly convey the actions of an allosteric ligand on V_{max} and K_{ia}, respectively (Reinhart, 1983):

$$W_x = \frac{V^\infty}{V^0} \tag{8}$$

$$Q_{ax} = \frac{K_{ia}^0}{K_{ia}^\infty} = \frac{K_{ix}^0}{K_{ix}^\infty} \tag{9}$$

W_x and Q_{ax} quantify the actions of an allosteric ligand caused by its binding and thereby allow one to distinguish between the *actions* of an allosteric ligand and its *affinity* for the enzyme. As indicated in eq 9, the thermodynamic affinity of an allosteric ligand will be influenced by the binding of substrate in the same manner and to the same extent as the influence it has on the binding of substrate. This linkage between substrate and allosteric ligand binding is an important concept in understanding the underlying cause of an allosteric ligand's actions (Reinhart et al., 1989), and its determination establishes the need to quantify the true thermodynamic (as opposed to kinetic) impact engendered by the binding of an allosteric ligand on the binding of substrate. Q_{ax} is thereby related to the coupling free energy, ΔG_{ax}, between the allosteric ligand and the substrate ligand (Weber, 1972, 1975) by (Reinhart, 1983):

$$\Delta G_{ax} = -RT \ln Q_{ax} \tag{10}$$

where R equals the gas constant and T equals absolute temperature.

The mechanism shown in Scheme III is directly applicable to multisubstrate enzyme reactions that have a random mechanism if all of the nonvaried substrates are either very low with respect to their respective K_m values or saturating (in which case E in the scheme would represent enzyme bound with these nonvaried substrates). However, in the case of ordered kinetic mechanisms, the determination of the influence that an allosteric ligand has on the substrate interactions requires an explicit understanding of the order of addition of the various substrates so as to clearly determine what thermodynamic binding parameters, if any, are being affected. Moreover, the determination of Q_{ax}, for any mechanism, from initial velocity kinetic data requires an explicit consideration of the relationship between K_{ia}, which is a thermodynamic parameter, and K_m, which is inherently a kinetic parameter.

It is customary to interpret allosteric effects on apparent affinity, i.e., K_m, of substrate in thermodynamic terms. This is done in part because analogies are drawn to allosteric binding proteins, the most notable being hemoglobin. Often the rapid equilibrium assumption is valid to within experimental uncertainty so that such an approach is appropriate. However, when the substrate interaction being influenced is the first in an ordered sequence, such as A binding in Scheme II, then particular care must be taken in interpretation. As revealed by the steady-state rate equation for the mechanism in Scheme II, the K_m for A at a saturating concentration of B, K_a, is equal to k_{cat}/k_1 whereas the K_m for A as [B] approaches 0, K_{ia}, is equal to the true dissociation constant for A from the EA complex (i.e., k_2/k_1). Consequently, if the allosteric ligand significantly alters k_2, the apparent influence that is evident at high concentration of the *unaffected* substrate will cause the perturbation of the true K_{ia} of the *affected* substrate to be seriously under- or overestimated.

Both of the partial reactions catalyzed by carbamyl phosphate synthetase are properly considered within the context of Scheme II. Consequently, we have evaluated the influence

Table III: Allosteric Parameters for the ATPase Reaction

	UMP	IMP	ornithine
W_x	0.53 ± 0.03	0.99 ± 0.06	0.66 ± 0.03
Q_{ax}	0.19 ± 0.04	0.42 ± 0.09	1.6 ± 0.3
ΔG_{ax} (kcal/mol)	+0.98 ± 0.12	+0.52 ± 0.13	−0.26 ± 0.11

Table IV: Allosteric Parameters for the ATP Synthesis Reaction

	UMP	IMP	ornithine
W_x	0.33 ± 0.01	1.45 ± 0.06	0.85 ± 0.03
Q_{ax}	0.067 ± 0.009	0.52 ± 0.06	18 ± 5
ΔG_{ax} (kcal/mol)	+1.61 ± 0.08	+0.39 ± 0.07	−1.72 ± 0.15

of each of the allosteric ligands UMP, IMP, and ornithine as a function of the concentration of both substrates. This has enabled us to ascertain the true effects of these allosteric ligands on the thermodynamic dissociation constant of nucleotide since it is the first substrate to bind in each case. In the process, their effect on the true V_{max} of each reaction, and hence k_{cat}, was also established. We present these results as Q_{ax}, the associated coupling free energy ΔG_{ax}, and W_x for the ATPase reaction (reaction 2) and the ATP synthesis reaction (reaction 3) in Tables III and IV, respectively.

ATPase Reaction (Reaction 2). UMP and ornithine cause V_{max} for the ATPase reaction to diminish somewhat, as indicated in the value for W_x in Table III. IMP has no effect on V_{max}.

At high HCO_3^- concentrations, ornithine appears to promote the binding of ATP substantially because K_a decreases by over 8-fold. Under similar circumstances, UMP appears to also activate, since K_a decreases nearly 2-fold. However, these effects cannot be ascribed to equilibrium binding phenomena. Actually UMP is a substantial inhibitor of ATP binding, increasing K_{ia} by over 5-fold. The decrease in K_a is entirely due to the decrease in k_{cat}. Similarly, the effect on K_{ia} is entirely due to an increase in k_2. k_1 is unaffected. Ornithine, on the other hand, increases k_1 nearly 6-fold, hence the large enhancement of K_a. However, k_2 is also increased 3.6-fold, so the thermodynamic effect is only a 60% decrease in K_{ia}. IMP has a 2-fold inhibitory effect on the thermodynamic binding of ATP, which is derived from modest changes in both k_1 and k_2.

ATP Synthesis Reaction (Reaction 3). Ordinarily, a steady-state, ordered sequential kinetic mechanism involving two substrates produces a rate equation that is symmetrical in form with respect to each substrate such that a determination of the order of substrate binding cannot be made without further information. An exception to this situation occurs when the mechanism is rapid-equilibrium ordered.

A rapid-equilibrium ordered mechanism occurs when the off-rate constant for the first substrate, k_2, is much greater than k_{cat} (Cleland, 1970).[2] Under these circumstances, the rate of the reaction when B is infinite becomes independent of the concentration of A. In other words, the X-coordinate of the crossover point of the double-reciprocal plot pattern when B is the variable substrate is equal to 0. V_{max}, therefore, becomes a parameter that is independent of A when B is saturating since the Y-coordinate of the crossover point, which is equal to $(1/V_{max})(1 - k_{cat}/k_2)$ for an ordered bimolecular reaction, becomes equal to $1/V_{max}$ when $k_2 \gg k_{cat}$. The double-reciprocal plot when A is the variable substrate does not show these special characteristics; i.e., the Y-axis intercept is a function of [B], although a slope replot will extrapolate through the origin again reflecting the independence of the rate on [A] when B is saturating. Consequently, a distinction can be made between the first and second substrates to bind in this special case.

The data presented in Figure 3 conform to the pattern expected for an equilibrium-ordered mechanism with ADP binding first. The rate equation for an equilibrium-ordered mechanism (eq 5) fits the data well, and from this fit the best approximation of the true thermodynamic dissociation constant for ADP, K_{ia}, can be ascertained. UMP and IMP do not change the equilibrium-ordered appearance of the data, and therefore the effects of these allosteric ligands can be quantitatively assessed by evaluating the effect of saturating levels of these ligands on the parameters resulting from overall fit to eq 5 (Table IV). It is noteworthy that the K_m for carbamyl phosphate is affected by less than 20% by each ligand and that UMP and ornithine have substantial, and opposite, affects on K_{ia} for MgADP.

The inhibitory effect engendered by IMP stands in contrast to the previously reported activation observed for this ligand (Anderson & Meister, 1966b; Boettcher & Meister, 1982). We attribute this discrepancy to the different conditions in which we performed our kinetic assays. Specifically, the conditions employed by the above researchers included only a limiting amount of Mg^{2+}. As shown by Raushel et al. (1978, 1979), many of the apparent complexities associated with the kinetic properties of this enzyme are removed if the assays are performed with excess Mg^{2+}. In addition, we have also avoided the use of phosphate, since it has been shown to also affect the kinetic properties of the enzyme (Anderson, 1977; Powers & Meister, 1978a; Boettcher & Meister, 1981). Interestingly, IMP has little apparent effect when evaluated in phopshate buffer (Anderson & Marvin, 1970).

As shown in Figure 4, the double-reciprocal plot pattern is significantly different when ornithine is present at saturating concentrations. When carbamyl phosphate is the variable substrate in the presence of ornithine, the crossover point clearly falls to the left of the Y-axis, as it does when MgADP is the variable substrate. The slope replot derived from the pattern in which MgADP is the variable substrate extrapolates to a Y-axis intercept clearly removed from the origin. The MgADP vs carbamyl phosphate rate data in the presence of saturating ornithine fit much better to eq 6 than to eq 5 as the overall error and the individual parameter error values verify (data not shown).

This qualitative change in mechanism can be explained if one considers the defining condition of an equilibrium ordered mechanism, namely, that $k_2 \gg k_{cat}$. Ornithine has essentially no effect on V_{max} (i.e., $W_x = 0.85$, Table IV), and hence k_{cat} remains essentially constant in the presence of ornithine. On the other hand, ornithine decreases the value of K_{ia} significantly (i.e., $Q_{ax} > 1$). Given that $K_{ia} = k_2/k_1$, this effect entails a decrease in k_2 and/or an increase in k_1. Since the rapid equilibrium condition is apparently no longer met in the presence of ornithine, we conclude that ornithine achieves a lowering of K_{ia} by substantially decreasing the value of k_2, the off-rate constant for MgADP. If the entire change in K_{ia} were

[2] Knight and Cleland (1989) have pointed out that a rapid-equilibrium random mechanism can exhibit behavior that is indistinguishable from a rapid-equilibrium ordered mechanism if there exists an extreme synergism between the binding of the two ligands such that $K_a \ll K_{ia}$ (and $K_b \ll K_{ib}$). We do not favor this explanation for the rapid-equilibrium ordered behavior we observe for the ATP synthesis reaction in the absence of ornithine because the normal sequential behavior observed in the presence of ornithine would therefore be expected to be random. The random addition of carbamyl phosphate and ADP would be inconsistent with the ordered release of carbamyl phosphate and ADP observed by Raushel et al. (1978) in the full reaction with ornithine present.

derived from a change in k_2, k_{cat}/k_2 in the absence of ornithine would equal 0.03, a value sufficient to meet the rapid equilibrium condition given the error in our data.

Despite the fact that ornithine changes the kinetic mechanism of carbamyl phosphate synthetase with respect to reaction 2, true values of Q_{ax} can be obtained. The parameter K_{ia} recovered from the fit to eq 6 has the same definition as it does in eq 5, namely, the dissociation constant for the first substrate. We consider it extremely unlikely that ornithine would cause the order of addition to reverse, therefore, K_{ia} recovered from eq 6 should still refer to the dissociation constant for MgADP. The coupling parameter calculated from the ratio of these K_{ia} parameters, according to eq 9, is equal to 18 (Table IV).

If one assumes that the kinetic mechanism for reaction 3 is consistent with the order of product release in the overall reaction catalyzed by carbamyl phosphate synthetase (reaction 1), then the first MgADP released is the product of the first MgATP bound whereas the second ADP released ADP released corresponds to the second MgATP that binds with this latter pair being the nucleotides most affected by the binding of allosteric ligands. Although this ordering is unusual for kinases, which usually follow a "first on, last off" pattern, it is consistent with the emerging view that the active site of carbamyl phosphate synthetase effectively represents a composite of two reaction centers; one that catalyzes the MgATP hydrolysis driven formation of enzyme-bound carbamate from bicarbonate and either NH_3 or glutamine and a second site that catalyzes a phosphoryl transfer from the second MgATP to carbamate forming carbamyl phosphate and MgADP (Post et al., 1990). The MgADP produced from the first hydrolysis reaction might be sufficiently "sticky" so as to enable the second MgATP to bind prior to its release, but that its release proceeds more readily than does that of carbamyl phosphate.

This mechanism might lead one to anticipate that 2 equiv of MgADP would bind, one before and one after the binding of carbamyl phosphate, in reaction 3 with the second MgADP dissociating each turnover in a manner analogous to HCO_3^- in reaction 2. However, if this putative second binding of MgADP were kinetically required, it should produce a nonhyperbolic dependence on MgADP concentration when carbamyl phosphate concentration is fixed at a nonsaturating level. We have not observed such an effect; a plot of reciprocal rate versus reciprocal MgADP concentration, determined with carbamyl phosphate concentration equal to 1 mM, is linear as MgADP concentration is varied from 17 μM to 1 mM (data not shown).

We also note that we have measured k_{cat} to be 3.8 s^{-1} for reaction 1 in the presence of ornithine, a value significantly larger than the value of k_2 in reaction 3 (1.8 s^{-1}) despite the fact that the off-rate constant for ADP dissociating from an E–ADP complex would appear to be a common step in both reactions. However, k_{cat} for the forward reaction was measured in the presence of saturating glutamine, whereas glutamine was absent in our study of reaction 3. Glutamine can bind in a random fashion (relative to the binding of the other substrates in the forward reaction), and its binding appears to enhance the k_{cat} of the overall reaction since V_{max} measured with NH_3 rather than glutamine as a substrate has been found to be approximately 40% of the former value (Rubino et al., 1986; Mullins et al., 1991). The absence of glutamine in our determination of k_2 in reaction 3 could therefore explain the difference between its value and that of k_{cat} for reaction 1.

Summary. The actions of UMP and ornithine on the partial reactions catalyzed by carbamyl phosphate synthetase are consistent with the conclusion that these allosteric ligands act primarily by modifying the nucleotide affinity for the enzyme. V_{max} of each reaction is affected less than 2-fold by every allosteric ligand, with the exception of the 3-fold decrease in the V_{max} for the ATP synthesis reaction induced by UMP. (Table IV). Similarly the K_m for the other substrate in each reaction, HCO_3^- in reaction 2 and carbamyl phosphate in reaction 3, is never affected by more than 2-fold. In addition, IMP has a 2-fold or less effect on all of the parameters in both reactions. The only substantial effects are manifested by UMP on the K_{ia} for MgATP in the ATPase reaction (5-fold increase) and both UMP and ornithine on the K_{ia} for MgADP in the ATP synthesis reaction (15-fold increase and 18-fold decrease, respectively). These effects are true thermodynamic effects on the dissociation constant pertaining to the enzyme–nucleotide binary complex.

We feel that it is significant that these ligands achieve their effects by altering the off-rate constant in the two instances in which our data allow us to distinguish between on- and off-rate constant effects (i.e., UMP in the ATPase reaction and ornithine in the ATP synthesis reaction). This suggests that the structural consequences associated with the binding of the allosteric ligand might not be apparent unless one compares the properties of the substrate-bound form with or without the allosteric ligand bound. This in turn requires that structural information be obtained pertaining to the ternary complex of enzyme–substrate–allosteric ligand even in the case when the allosteric ligand is inhibitory.

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Structural Similarity of D-Aminopeptidase to Carboxypeptidase DD and β-Lactamases†

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ABSTRACT: The gene for D-aminopeptidase (*dap*) has been isolated from the bacterium *Ochrobactrum anthropi* SCRC C1-38 [Asano, Y., Nakazawa, A., Kato, Y., & Kondo, K. (1989) *J. Biol. Chem. 264*, 14233-14239] and its nucleotide sequence determined. An expression plasmid pC138DP (4.5 kb) was constructed by placing the gene downstream of the *lac* promoter of pUC19. The amount of the enzyme in the cell-free extract of *Escherichia coli* JM109/pC138DP was elevated to 288 000 units/L of culture, which is about 3600-fold over that of *O. anthropi* SCRC C1-38. The enzyme comprised about 30% of the total extractable cellular protein. The gene consisted of an open reading frame of 1560 nucleotides which specifies a protein of M_r 57 257. The deduced amino acid sequence of the enzyme showed that it is related to carboxypeptidase DD, β-lactamases, and penicillin-binding proteins. Seven mutants of the enzyme were generated by site-specific mutagenesis to explore the roles of the residues of interest, around the sequence Ser61-X_{aa}-X_{aa}-Lys64, where X_{aa} is any amino acid, since the identical sequences also appear in the penicillin-recognizing peptide hydrolases with Ser at the active sites. The mutant enzymes expressed in *E. coli* were purified to homogeneity and kinetically characterized. Replacements of the site at Ser61 and Lys64 yielded mutants showing significantly reduced V_{max} values, while most of the K_m values remained unchanged. Changes at Cys60, which is adjacent to the likely active center Ser61, to Ser and Gly resulted in the production of enzyme less sensitive to PCMB, with almost unaltered V_{max}/K_m values. The enzyme appears to be a serine peptidase rather than a thiol one. The inhibition by PCMB in the wild-type enzyme may have been caused by a formation of a mercaptide bond between Cys 60 and PCMB. Considering that D-aminopeptidase, carboxypeptidase DD (a penicillin-binding protein), and β-lactamase have a common feature in recognizing peptides containing D-amino acid and that the former two catalyze transpeptidation reactions with substrates containing D-alanyl-D-alanine moieties, we propose that the enzyme is a new member of the "penicillin-recognizing enzymes". We showed that the enzyme is actually inhibited by β-lactam compounds, such as 6-APA, 7-ACA, benzylpenicillin, and ampicillin, although they are not the substrate for the enzyme. The relationship between the primary structures and the reactions catalyzed by D-aminopeptidase and other serine hydrolases β-lactamases and carboxypeptidase DD is discussed. To our knowledge, this enzyme is the first example of an aminopeptidase with Ser at the active site.

We have been exploiting new enzymes to use as catalysts in organic synthesis (Asano et al., 1982, 1987, 1989a-d, 1990, 1991). We have previously reported on the purification, characterization, and properties of novel D-stereospecific aminopeptidase and an D-stereospecific amino acid amidase from bacterial isolates from soil, *Ochrobactrum anthropi* SCRC C1-38 (Asano et al., 1989a,b) and *O. anthropi* SCRC SV3 (Asano et al., 1989c), respectively. The former enzyme is specific toward peptides with a free D-amino acid at the NH_2 terminus and D-amino acid amides, etc., and thus was named as "D-aminopeptidase" (Asano et al., 1989b), while the latter was specific toward D-amino acid amides with bulkier substituents (Asano et al., 1989c). The enzymes can be used in

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