Allosteric Effects of Carbamoyl Phosphate Synthetase from *Escherichia coli* Are Entropy-Driven[†]

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

Department of Chemistry, University of Wisconsin−La Crosse, La Crosse, Wisconsin 54601, Department of Chemistry, Texas A&M University, College Station, Texas 77843-3255, and Department of Biochemistry and Biophysics, Texas A&M University, College Station, Texas 77843-2128

Received June 3, 1996; Revised Manuscript Received July 3, 1996[⊗]

ABSTRACT: When catalyzing the formation of MgATP and carbamate from MgADP and carbamoyl phosphate, *Escherichia coli* carbamoyl phosphate synthetase (CPS) binds MgADP with a large negative change in heat capacity. The magnitude of this heat capacity change is not appreciably altered by the presence of a saturating concentration of either the allosteric activator ornithine or the inhibitor UMP despite the substantial and opposing effects these ligands have on the binding affinity for MgADP. By contrast, no detectable change in heat capacity is associated with the thermodynamic coupling between MgADP and either ornithine or UMP. The sign of the apparently constant enthalpic and entropic contributions to the coupling free energy for each of these ligands is opposite that of the coupling free energy, indicating that the observed allosteric phenomenology is in net opposed by the enthalpy of the interaction and instead arises from a change in entropy of the system. IMP produces only a very small allosteric effect as indicated by a near-zero value for the MgADP−IMP coupling free energy. However, the enthalpic and entropic contributions are individually larger in absolute value for the IMP coupling than for those pertaining to the other allosteric ligands, and entropy dominates the coupling free energy above 36 °C, causing IMP to become an activator at high temperature. In addition, the sign of the coupling enthalpy and entropy for IMP has the same sign as the coupling enthalpy and entropy produced by ornithine, suggesting that IMP and ornithine may similarly influence the enzyme at a molecular level despite binding to different allosteric sites on the enzyme. The data are consistent with a model in which the actions of the allosteric ligands arise primarily from changes in the conformational degeneracy introduced by each ligand. With this model, one can also rationalize the failure of these allosteric ligands to substantially influence k_{cat}.

Allosteric ligands affect the activity of enzymes either by perturbing catalysis, reflected in changes in k_{cat}, and/or by altering the ability of substrates to bind to the active site. Often these latter effects involve a perturbation of thermodynamic dissociation constants pertaining to binary enzyme−substrate complexes, denoted as K_{ia} for substrate ‘A’ in the notation convention proposed by Cleland (1963). Such actions of an allosteric ligand are described in an unambiguous, model-independent manner by the coupling free energy, ΔG_{ax}, between substrate and allosteric ligand, ‘X’ (Weber, 1972, 1975; Reinhart, 1983, 1988). ΔG_{ax} is defined by the following relationships:

$$\Delta G_{ax} = -RT \ln Q_{ax} \quad (1)$$

$$Q_{ax} = K^0_{ia}/K^\infty_{ia} \quad (2)$$

where K^0_{ia} represents K_{ia} determined in the absence of allosteric ligand and K^∞_{ia} represents K_{ia} determined with the concentration of allosteric ligand maintained high enough so that both enzyme species appearing in the substrate dissociation equilibrium are fully saturated with the allosteric ligand.

As expected, the coupling free energy results from component ΔH_{ax} and ΔS_{ax} terms. We have previously observed (Reinhart et al., 1989) that the sign of the coupling free energy, and hence the nature of the allosteric effect (i.e., activation or inhibition), can be established by ΔS_{ax}. In these cases, the sign of ΔG_{ax} is opposite that of ΔH_{ax}, suggesting a molecular origin of the overall allosteric phenomenon that is not dominated by electrostatic or steric effects and hence is fundamentally different from that often envisioned (Koshland, 1970).

Carbamoyl phosphate synthetase (CPS)[1] from *Escherichia coli* is an $\alpha\beta$ dimeric enzyme (Trotta et al., 1971, 1974) subject to allosteric inhibition by UMP and allosteric activation by ornithine (Pierard, 1966; Anderson & Marvin, 1968), phenomena thought to be important to the physiological regulation of pyrimidine biosynthesis and ammonia utilization, respectively (Pierard, 1966; Robin et al., 1989). Ornithine and UMP achieve their effects by binding to distinctly separate allosteric binding sites on the large subunit. IMP also binds to the UMP allosteric site, causing modest

[†] This work was supported by National Institutes of Health Grants GM 33216 (G.D.R.) and DK 30343 (F.M.R.). Much of the research reported herein was performed while B.L.B. and G.D.R. were members of the Department of Chemistry and Biochemistry at the University of Oklahoma, Norman, OK 73019.

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

[‡] Department of Chemistry, University of Wisconsin−La Crosse.

[§] Department of Chemistry, Texas A&M University.

[||] Department of Biochemistry and Biophysics, Texas A&M University.

[⊗] Abstract published in *Advance ACS Abstracts,* August 15, 1996.

[1] Abbreviations: CPS, carbamoyl phosphate synthetase; CP, carbamoyl phosphate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane.

S0006-2960(96)01305-0 CCC: $12.00 © 1996 American Chemical Society

activation or inhibition depending on the buffer conditions and temperature (Braxton et al., 1994). Although physiologically CPS catalyzes the formation of carbamoyl phosphate (CP) from 2 equiv of ATP, bicarbonate, and glutamine (also forming 2 equiv of ADP, glutamate, and P_i in the process), the allosteric effects of UMP and ornithine are also evident in the following "ATP synthesis" reaction that is catalyzed by CPS in vitro (Braxton et al., 1992):

$$\mathrm{MgADP} + \mathrm{CP} \rightarrow \mathrm{MgATP} + \mathrm{HCO_3^-} + \mathrm{NH_3} \qquad (3)$$

The reaction given in eq 3 is catalyzed exclusively by the large subunit of CPS, which means that the allosteric effects do not involve, or require, intersubunit "communication" between the allosteric ligand and the substrate ligand as is often the case with typical oligomeric allosteric enzymes. A practical consequence of this fact is that steady-state kinetics data are devoid of cooperativity (i.e., nonhyperbolic substrate saturation profiles) and thus are subject to more straightforward interpretation.

Ornithine, UMP, and IMP influence the ability of CPS to catalyze the reaction depicted in eq 3 almost exclusively by perturbing the apparent affinity the enzyme displays for MgADP (Anderson & Meister, 1966b; Braxton et al., 1992). We have concluded previously that MgADP and CP bind in an ordered fashion with MgADP binding first (Braxton et al., 1992). The mechanism appears to be rapid-equilibrium-ordered except in the presence of ornithine. For an ordered mechanism, the Michaelis constant for MgADP, obtained at saturating CP, is not a thermodynamic dissociation constant, and therefore cannot be interpreted with thermodynamic linkage arguments. However, full analysis of steady-state kinetic data does yield K_{ia}, the thermodynamic dissociation constant governing the dissociation of MgADP from the CPS−MgADP binary complex since the Michaelis constant for MgADP approaches K_{ia} as the concentration of CP approaches zero.[2]

We present in this report a thermodynamic evaluation of the influence of ornithine, UMP, and IMP on CPS as revealed by the coupling free energies between MgADP and each allosteric ligand. The component values of ΔH_{ax} and ΔS_{ax} have been estimated from a van't Hoff analysis of the temperature dependence of the corresponding coupling parameters. The results indicate that both activating and inhibitory intrasubunit couplings are entropy-driven, with the nature of the allosteric effect *opposed* rather than established by the coupling enthalpy, ΔH_{ax}.

MATERIALS AND METHODS

Dilithium carbamoyl phosphate, K-ADP, L-glutamine, Na-NADP, ornithine hydrochloride, the disodium salts of UMP and IMP, dihydroxyacetone, and Hepes free acid were purchased from Sigma. Hexokinase, glucose-6-phosphate dehydrogenase, and glycerol-3-phosphate dehydrogenase were purchased from Boehringer-Mannheim as ammonium sulfate suspensions and were dialyzed exhaustively prior to use so that all traces of ammonia were removed. Glycerokinase from *Bacillus stearothermophilus* was also supplied by Boehringer-Mannheim in Tris buffer and was used without dialysis. All other chemicals were reagent grade. Carbamoyl phosphate synthetase was purified from *E. coli* RR1 carrying the plasmid pMC41 as described previously (Mullins et al., 1991).

The ATP synthesis reaction catalyzed by *E. coli* CPS was followed by monitoring the production of MgADP in a coupled enzyme assay. In the temperature range of 10−34.4 °C, the enzymatic assays were performed in 1.0 mL of aqueous solution containing 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, carbamoyl phosphate, and allosteric ligands. The assays performed above 34.4 °C contained 50 mM Hepes−KOH, pH 7.5, 100 mM KCl, 20 mM $MgCl_2$, 0.25 mM NADH, 10 mM dihydroxyacetone, 2.5 units of glycerokinase, 1 unit of glycerol-3-phosphate dehydrogenase, and the indicated concentrations of MgADP, carbamoyl phosphate, and allosteric ligands in a total volume of 1.0 mL. CPS (5.3 mg/mL) was diluted 1:10 into glycerol-3-phosphate dehydrogenase (~5 mg/mL) which had been dialyzed into 50 mM Hepes−KOH, pH 7.5, 100 mM KCl. Each assay contained less than 20 μg/mL CPS, concentrations too low to promote self-association of the $\alpha\beta$ dimer (Anderson, 1986). For all temperatures, MgADP concentrations ranged from 2 μM to 14 mM. Carbamoyl phosphate concentrations varied between 0.05 and 15 mM. When included, the effector ligand UMP, IMP, and ornithine concentrations were equal to 15 mM, which is saturating under all concentrations of MgADP and carbamoyl phosphate examined. In order to ensure that the very labile carbamoyl phosphate did not degrade prior to its use, solutions were freshly prepared in 100 mM Hepes−KOH, pH 7.5, and stored at 4 °C for no more than 1 h before use. Approximately 5 min before rates were to be measured, the appropriate volume of this solution was added to the reaction mixture. Above 34.4 °C, reactions were initiated by addition of carbamoyl phosphate. Below 34.4 °C, the reactions were initiated by the addition of CPS. The pH of all reaction mixtures was adjusted to pH 7.5 after equilibration at the indicated temperature using a pH meter that had been calibrated at the same temperature.

Initial velocity data were fit to eq 4 if UMP, IMP, or no effector was included in the assay. In the presence of ornithine, initial velocities were fit to eq 5. Programs were written in HP Basic 2.1 as implemented on an HP 9836A desktop computer utilizing the analysis strategy recommended by Cleland (1967):

$$v = \frac{V_m[\mathrm{A}][\mathrm{B}]}{K_{ia}K_b + K_b[\mathrm{A}] + [\mathrm{A}][\mathrm{B}]} \qquad (4)$$

$$v = \frac{V_m[\mathrm{A}][\mathrm{B}]}{K_{ia}K_b + K_b[\mathrm{A}] + K_a[\mathrm{B}] + [\mathrm{A}][\mathrm{B}]} \qquad (5)$$

where v is the initial velocity, V_m is the maximal velocity obtained for a given enzyme concentration when both MgADP and CP are saturating, A = MgADP, B = CP, K_{ia} is the dissociation constant for MgADP from enzyme when CP is absent, K_a is the Michaelis constant for MgADP when

[2] If the enzyme−substrate complex isomerizes prior to the binding of the second substrate, K_{ia} can contain contributions from both the binding equilibrium constant and the isomerization equilibrium constant. However, K_{ia} determined in the manner described is equal to the same apparent dissociation constant that would be obtained from any equilibrium binding measurement which does not distinguish between the nature of the two bound states.

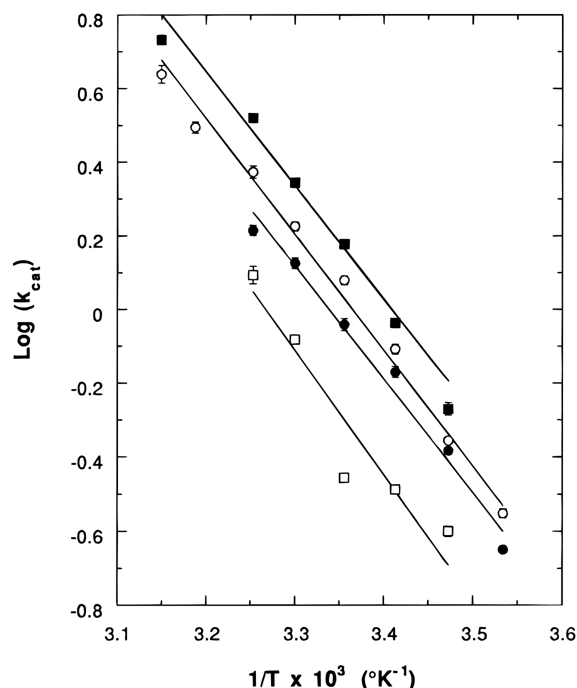


FIGURE 1: Plot of log(k_{cat}) versus reciprocal temperature expressed in degrees kelvin. Lines indicate the result of linear regression analysis of the individual data sets. k_{cat} values were calculated by dividing the V_m values obtained by fitting initial velocity kinetic data to eqs 4 or 5 described in the text by the total concentration of CPS. Symbols are defined as follows: no effector (○), 15 mM UMP (□), 15 mM IMP (■), and 15 mM ornithine (●). Error bars represent the standard error of each determination when this value is larger than the symbol.

carbamoyl phosphate is saturating, and K_b is the Michaelis constant for CP when MgADP is saturating.

RESULTS

In Figure 1, we present Arrhenius plots of log(k_{cat}) versus reciprocal temperature that pertain to the CPS-catalyzed ATP synthesis reaction (eq 3) in the absence and presence of the allosteric ligands ornithine, UMP, and IMP, respectively. When present, each allosteric ligand was fully saturating. Simple linear regression of each data set results in lines of comparable slope indicating that none of the allosteric ligands significantly affects the apparent activation energy for this reaction. The displacement of the lines indicates that the ligands do modify V_{max} somewhat, with UMP showing the greatest effect, an average reduction to one-third, consistent with our previous observations (Braxton et al., 1992).

By comparison, the effects of the allosteric ligands on K_{ia}, the dissociation constant for MgADP from the CPS−MgADP binary complex, are much more substantial as indicated by the data shown in Figure 2. This thermodynamic parameter is obtainable from steady-state kinetic analysis since MgADP is the first substrate to bind in the otherwise ordered kinetic mechanism that this enzyme obeys when catalyzing the ATP formation reaction (Braxton et al., 1992). In Figure 2, we present van't Hoff plots of log(K_{ia}) versus reciprocal temperature obtained in the absence and saturating presence of each of the allosteric ligands ornithine, UMP, and IMP. It is evident that under each of these conditions the functional dependence of log(K_{ia}) on reciprocal temperature is nonlinear. Since the slope of these plots, which conveys the standard

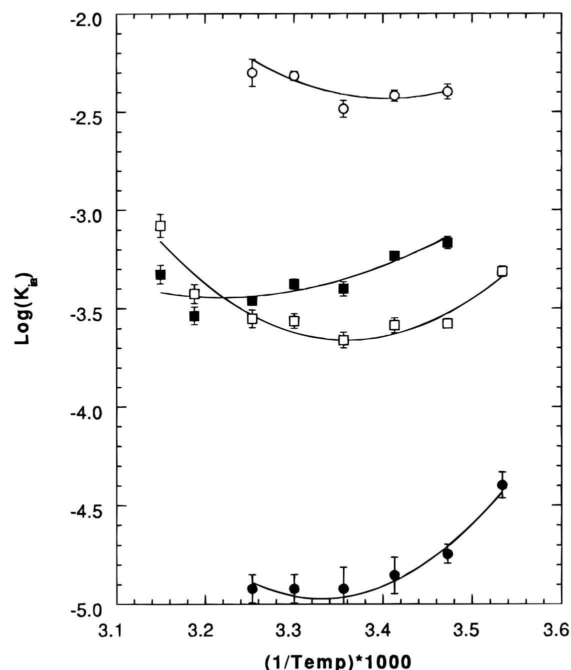


FIGURE 2: van't Hoff plots of the dissociation constant for MgADP (K_{ia}) for the MgATP synthesis reaction catalyzed by CPS in the saturating presence of ornithine (●), UMP (□), IMP (■), or no other ligand (○). Solid curved lines represent the best fit of each data set to eq 6 described in the text. K_{ia} values were determined by fitting initial velocity data obtained at various concentrations of MgADP and carbamoyl phosphate to eqs 4 or 5. Error bars represent the standard error of each determination when this value is larger than the symbol.

Table 1: Apparent Heat Capacity Changes for the Binding of MgADP to CPS[a]

bound ligand	ΔC_p [kcal/(mol·deg)]	T_h (°C)	T_s (°C)
none	−1.1 ± 0.1	24.5 ± 0.4	29.0 ± 0.6
ornithine	−1.4 ± 0.4	27.0 ± 1.8	32.0 ± 3.2
UMP	−0.9 ± 0.4	20.5 ± 1.8	24.3 ± 1.2
IMP	−0.5 ± 0.1	37.5 ± 2.8	47.9 ± 5.6

[a] Obtained by fitting the data from Figure 2 to eq 6 as described in the text.

enthalpy for the binding equilibrium in each case, is changing with temperature, these data also imply that a substantial change in heat capacity accompanies the binding of MgADP to the enzyme regardless of the presence or absence of bound allosteric ligands. The curvature evident in Figure 2 can be reasonably approximated by fitting the data to eq 6, as indicated by the curved line through each data set shown in Figure 2. Equation 6 represents the expected dependence of log(K_{ia}) on reciprocal temperature if one assumes that the change in heat capacity remains constant over the temperature range examined (Baldwin, 1986; Ha et al., 1989):

$$\log(K_{ia}) = (-\Delta C_p/2.3R)\{(T_h/T) - \ln(T_s/T) - 1\} \quad (6)$$

where ΔC_p represents the change in heat capacity for MgADP *binding*, T_h equals the temperature at which $\Delta H°$ for binding equals zero, and T_s equals the temperature at which $\Delta S°$ for binding equals zero. Values obtained from this nonlinear regression analysis for each of these parameters are presented in Table 1.

The extent to which $\Delta H°$ and $T\Delta S°$ vary over the temperature range examined can be estimated from the

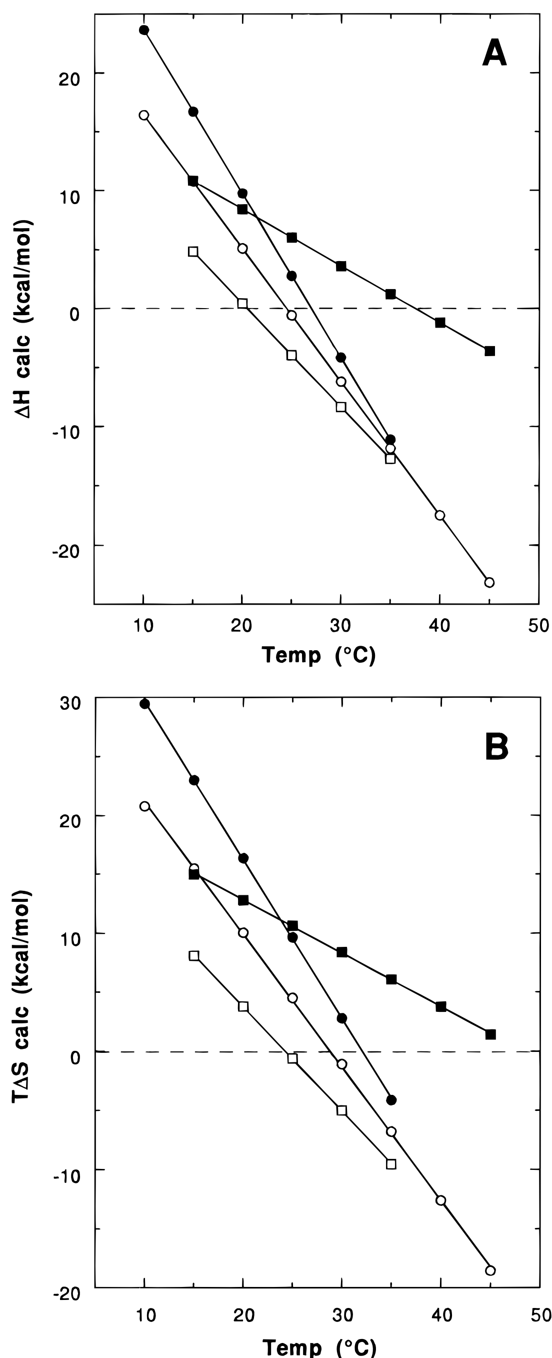


FIGURE 3: Variation in ΔH (A) and $T\Delta S$ (B) for MgADP binding to CPS as predicted by the fits of the data presented in Figure 2 to eq 6 described in the text. Symbols serve only to demarcate the lines and have the same definition as given in Figure 2.

relationships (Baldwin, 1986):

$$\Delta H^\circ = \Delta C_p(T - T_h) \quad (7)$$

$$T\Delta S^\circ = T\Delta C_p \ln(T/T_s) \quad (8)$$

The resulting linear plots of apparent van't Hoff ΔH° for MgADP binding versus temperature predicted by eq 7 and the values given in Table 1 for each of the four conditions are shown in Figure 3A. It is interesting to note that each of these lines crosses through zero (at $T = T_h$ by definition) at a value within the experimentally-accessible temperature range, and that T_h varies somewhat depending on the saturating presence of ornithine, UMP, IMP, or no other

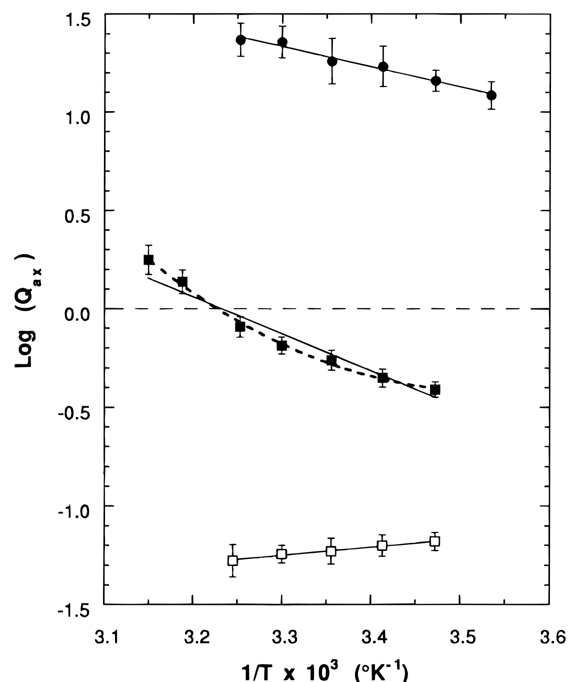


FIGURE 4: Effect of temperature on Q_{ax} for MgADP−ornithine (●), MgADP−UMP (□), and MgADP−IMP (■) interactions, presented as a plot of log(Q_{ax}) versus reciprocal temperature expressed in degrees kelvin. The data indicate that as temperature increases, UMP becomes a better inhibitor, ornithine becomes a better activator, and IMP becomes a poorer inhibitor (when temperature $<\sim 36$ °C) and a better activator (when temperature $>\sim 36$ °C). Q_{ax} was calculated using eq 2 described in the text. Error bars represent the standard error of each determination. The data pertaining to the ornithine and UMP couplings are fit to a line while the IMP coupling data have been fit both to a line (solid curve) and to eq 6 (dashed curve) as described in the text.

ligands, respectively. In all cases, the sign of ΔH° for binding is positive at temperatures below, and negative at temperatures above these cross-over temperatures. Figure 3B shows a similar relationship between $T\Delta S^\circ$ and temperature that is predicted from eq 8. Note that $T\Delta S^\circ$ changes sign when temperatures equal T_s, and that $T\Delta S^\circ$ is also positive at low temperatures and negative when $T > T_s$. Only within the relatively small temperature range between T_h and T_s will ΔH° be negative and $T\Delta S^\circ$ be positive, in which case both terms favor the binding of MgADP to the enzyme.

The coupling parameter, Q_{ax}, that quantifies the nature and extent of the influence that either ornithine, UMP, or IMP has on MgADP binding can be calculated according to equation 2 from the data presented in Figure 2. The logarithms of the results of these calculations are presented in Figure 4 as a function of reciprocal temperature. At all temperatures, log(Q_{ax}) for ornithine is positive and log(Q_{ax}) for UMP is negative, consistent with the activation and inhibition, respectively, of MgADP binding caused by these ligands. Increasing temperature, on the other hand, causes the sign of log(Q_{ax}) for IMP to change from negative to positive above approximately 36 °C, indicating that the nature of the allosteric effect switches from inhibition to activation at temperatures above 36 °C as previously reported (Braxton et al., 1994).

In contrast to the data presented in Figure 2, the data presented in Figure 4 for all three allosteric ligands exhibit much less curvature. The ornithine and UMP data were fit to the equation for a straight line as well as eq 6, and eq 6

Table 2: Thermodynamic Components of Allosteric Coupling to MgADP Binding by CPS from *E. coli*

allosteric ligand	ΔG_{ax}[a] (kcal/mol)	ΔH_{ax}[b] (kcal/mol)	$T\Delta S_{ax}$[b] (kcal/mol)	$\Delta C_{p,ax}$[c] [kcal/(mol·deg)]
ornithine	-1.75 ± 0.50	$+4.8 \pm 1.5$	$+6.5 \pm 1.5$	ND[d]
UMP	$+1.68 \pm 0.40$	-1.8 ± 1.4	-3.5 ± 1.4	ND[d]
IMP	$+0.32 \pm 0.06$	$+8.5 \pm 0.8$	$+8.2 \pm 0.8$	$+0.5 \pm 0.2$

[a] Determined from the data shown in Figure 2 using eqs 1 and 2 described in the text and letting $T = 25$ °C. [b] Evaluated for $T = 25$ °C from the data shown in Figure 4 using the linear form of the van't Hoff equation which assumes ΔH and ΔS are constant over the temperature range examined. [c] Determined from a fit to eq 6 as described in the text. [d] ND = not determined.

produced no improvement in the fit despite the fact that it has more parameters (results not shown) indicating that, over the temperature range examined, ΔH_{ax} and ΔS_{ax} do not change significantly with temperature. The apparently constant values for ΔH_{ax} and ΔS_{ax} for ornithine and UMP that can be derived from the fit to the linear van't Hoff equation (Reinhart et al., 1989) are given in Table 2.

For the coupling interaction involving IMP, some curvature is evident in the data presented in Figure 4, and not surprisingly eq 6 fits these data somewhat better than does a straight line, although only $\Delta C_{p,ax}$ is well determined by this fit. Consequently, we have estimated the values of ΔH_{ax} and $T\Delta S_{ax}$ at 25 °C from a linear fit of the data, and these values, as well as the apparent value of $\Delta C_{p,ax}$ obtained from the fit to eq 6, are also presented in Table 2. Regardless of the curvature in the IMP data, it is evident that IMP and ornithine exhibit the same sign in ΔH_{ax} and ΔS_{ax} throughout the temperature range examined despite binding to different allosteric binding sites (Boettcher & Meister, 1981, 1982) and causing different phenomenology (conveyed by ΔG_{ax}) to result from their interaction with the enzyme. Possibly the most significant aspect of the data summarized in Table 2, however, is that the sign of ΔH_{ax} is opposite that of ΔG_{ax} for UMP and ornithine at 25 °C, indicating that entropy, not enthalpy, is driving the allosteric effects at that temperature. And even though ΔH_{ax} has the same sign as ΔG_{ax} for IMP at 25 °C, the nature of the allosteric effect induced by IMP, and hence the sign of ΔG_{ax}, changes above approximately 36 °C (Braxton et al., 1994) so that entropy even dominates the actions of IMP above that temperature as well. It therefore becomes particularly important to try to understand the nature of the physical and/or chemical perturbations that are responsible for the coupling entropy change since it establishes the nature of each allosteric effect.

DISCUSSION

The physiological reaction catalyzed by CPS follows an ordered mechanism which requires that 2 equiv of MgATP be bound simultaneously (Raushel et al., 1978), thus implying two separate binding domains for nucleotide within the active site. It has been shown previously that the ATP synthesis reaction (eq 3) and the HCO_3^--dependent ATPase reactions also catalyzed by CPS each involve one of these nucleotide sites (Anderson & Meister, 1966a; Post et al., 1990). While there is some effect of allosteric ligands on the ATPase activity, allosteric perturbation of the ATP synthesis reaction most closely mimics the allosteric effects seen in the physiological reaction (Anderson & Meister, 1966b; Braxton et al., 1992). This fact, plus the simple stoichiometry, led us to focus on this reaction in the present study.

It is evident from Figure 1 that the allosteric ligands have only a minor effect on k_{cat} and that the effect that is apparent does not reflect a change in activation energy, implying little effect on transition-state stabilization. This is somewhat surprising in light of the substantial change induced by the allosteric ligands in V/K, the apparent first-order rate constant approached at low concentrations of MgADP when the combination of free enzyme with MgADP is rate-limiting. Together these observations suggest that the binding of allosteric ligands causes a substantial alteration of the structure of the active site in the absence of substrate ligands but not in their presence. It would seem, therefore, that the binding of substrate (in this case MgADP) can overcome most of the structural perturbations within the catalytic site that might otherwise influence catalytic turnover. Given the fact that ornithine and UMP in particular strongly influence the binding of MgADP in opposite directions, either the binding determinants for MgADP are quite distinct from the residues involved in catalysis subsequent to binding or the binding of substrate induces virtually the same active-site configuration regardless of the configuration that might exist prior to binding.

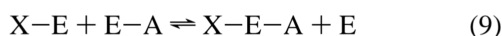
The opposing actions of ornithine and UMP also present an interesting challenge to understanding the change in heat capacity associated with MgADP binding that is evident in Figure 2. One initially attractive possibility to consider for the cause of such substantial values for ΔC_p is a possible preexisting conformational equilibrium that is shifted as a consequence of MgADP binding to the "active" form. Even if ΔH for the conformational equilibrium and ΔH for substrate binding to only one of those forms are constant over the experimental temperature range, the experimentally observed ΔH for binding can vary with temperature, giving rise to an apparent ΔC_p (Sturtevant, 1977; Ferrari & Lohman, 1994). However, the data in Figure 3A indicate that ΔC_p for MgADP binding (conveyed by the slope of the line) if anything increases in absolute value after the activator ornithine has bound, and $|\Delta C_p|$ if anything decreases after the binding of UMP. These results are inconsistent with a preexisting conformational equilibrium model since ornithine would be expected to substantially minimize $|\Delta C_p|$ by "pre-shifting" the two-state conformational equilibrium in favor of the MgADP binding form. For example, in the limit if ornithine lowered K_{ia} by shifting the two-state equilibrium entirely to the "active" form, MgADP would then be binding to only that one enzyme form with constant ΔH, and ΔC_p would therefore be equal to zero. Similarly, as an inhibitor, UMP would be expected to maximize $|\Delta C_p|$ by shifting the conformational equilibrium away from the form to which MgADP binds, thus causing the binding to fully involve the two equilibria with presumably different individual ΔH values. Although neither change in ΔC_p observed can be considered statistically significant given the error in the data (see below), it is clear that the trends predicted by the two-state model are not observed.

A commonly invoked source of negative ΔC_p in protein–ligand binding reactions is the removal of hydrophobic surfaces from exposure to the aqueous solvent upon binding (Sturtevant, 1977). One can immediately recognize that a contribution from this mechanism should be expected in this case since ADP itself is amphipathic, containing a significant

hydrophobic aromatic moiety that will presumably combine with a complementary hydrophobic region within the binding domain thereby diminishing their total exposure to water. However, the values of ΔC_p presented in Table 1 are larger than is usually observed for the binding of small ligands to proteins (Sturtevant, 1977). Indeed, the data presented in Figures 2 and 3 are remarkably similar to those reported by Record and co-workers (Ha et al., 1989) for various protein−DNA interactions. They concluded, primarily on the basis of the magnitude of ΔC_p and the number of water molecules that would therefore be involved (the latter calculated with reference to model aqueous solution−liquid hydrocarbon partitioning data), that a ligand-induced conformational change upon binding must be invoked to provide for a greater hydrophobic surface to become excluded from aqueous exposure.

Although the above conclusions regarding protein−DNA interactions have subsequently been subject to alternative interpretation (Ferrari & Lohman, 1994), it is nonetheless possible that MgADP similarly initiates a conformational change upon binding that results in removal of a large hydrophobic area from aqueous exposure. However, for this mechanism to account for the entire magnitude of ΔC_p, approximately 4000 $Å^2$ of hydrophobic surface (equivalent to an area 63 Å × 63 Å) would have to be removed from aqueous exposure as calculated by Ha et al. (1989). Moreover, this conformational change must leave the active site open for the subsequent binding of carbamoyl phosphate, since MgADP binds first in an ordered mechanism (Braxton et al., 1992), presumably ruling out a domain-closing type of mechanism similar to that proposed for many kinases (Bennett & Steitz, 1978). In addition, examination of partitioning data of model hydrophobic compounds led Sturtevant to conclude that $\Delta S/\Delta C_p = -0.26 \pm 0.05$ at 25 °C for a hydrophobic exclusion mechanism. The data from Table 1 indicate that the corresponding ratio for the binding of MgADP to CPS is much lower than this value under each condition examined. Consequently, we conclude that it is unlikely that burial of hydrophobic residues (and the accompanying change in hydration) accounts for the entire ΔC_p observed. Sturtevant noted a similar discrepancy for NAD^+ binding to yeast glyceraldehyde-3-phosphate dehydrogenase and attributed the behavior to an alteration in internal modes of vibration that were introduced by the binding of NAD^+ (Sturtevant, 1977). The combination of these two mechanisms can lead to the sign of ΔS changing as a function of temperature in the manner depicted in Figure 3B as Sturtevant discussed (Sturtevant, 1977). Of course other mechanisms could also contribute to ΔC_p such as a perturbation of one or more protonation equilibria (Eftink et al., 1983).

With respect to the actual effects of the allosteric ligands themselves, however, the thermodynamic signature is quite different. The maximum effect of an allosteric ligand on substrate binding affinity is quantitatively summarized by the coupling free energy, and in this regard, it is useful to consider that the coupling free energy is a free energy *difference* between two binding free energies. From eqs 1 and 2, it can easily be shown that ΔG_{ax} and Q_{ax} represent the standard free energy and equilibrium constant, respectively, for the disproportionation equilibrium:



$$X{-}E + E{-}A \rightleftharpoons X{-}E{-}A + E \qquad (9)$$

where the species on the left side of the equilibrium represent enzyme (E) with either allosteric ligand (X) or substrate (A) bound individually while the species on the right side represent the ternary complex with both substrate and allosteric ligand bound simultaneously to enzyme as well as free, unligated enzyme, respectively. As this equilibrium relates to the data presented in Figure 4, E represents CPS, A represents MgADP, and X represents either ornithine, UMP, or IMP. It is significant that neither free substrate nor free allosteric ligand contributes to this equilibrium. Moreover, the number of filled and unfilled binding sites on either side of the equilibrium is balanced.

Compared to Figure 2, the van't Hoff plots of the coupling constant describing the interaction between either ornithine or UMP, respectively, and MgADP shown in Figure 4 are relatively straight over the same temperature range in which considerable curvature is evident in Figure 2. Whatever the cause of ΔC_p associated with ligand binding, the comparative absence of a significant ΔC_p associated with the difference in binding to free enzyme and enzyme to which either UMP or ornithine has previously bound suggests that these contributions largely cancel. The negligible ΔC_p and the tangible ΔS associated with the coupling equilibrium (eq 9) suggest that a mechanism other than those discussed above may dominate this difference equilibrium.

Sturtevant has pointed out that of the six different mechanisms that are likely to lead to ΔC_p and ΔS associated with protein−ligand interactions, only a change in the number of quasi-isoenergetic enzyme conformations produces a finite value for ΔS but no change in the heat capacity of the system (Sturtevant, 1977). This mechanism, as it pertains to eq 9 and the data given in Table 2, would suggest that the ternary complex (X−E−A) and free enzyme (E) differ from both of the binary enzyme−ligand complexes (X−E and E−A) primarily by the size of their configurational subset populations. Specifically for ornithine, since $\Delta S_{ax} > 0$, this mechanism suggests that there are a greater number of quasi-isoenergetic conformations populated by the XEA and E enzyme forms compared to the XE and EA enzyme forms. Conversely, since $\Delta S_{ax} < 0$ for UMP, this mechanism would imply that X−E and E−A have in sum a greater conformational degeneracy compared to X−E−A and E. This possibility has particular significance since entropy, not enthalpy, is driving the corresponding coupling equilibria.

The idea that the nature of the allosteric effects in CPS might originate from ligand-induced changes in conformational degeneracy also provides a rationalization for the observation that the allosteric ligands have relatively little effect on k_{cat} while profoundly affecting K_{ia} as discussed above. A conformational substate population, particularly one that can be easily perturbed by the noncovalent binding of small ligands, likely consists of various configurations separated by energy barriers that are small relative to the available thermal energy as described by Frauenfelder and co-workers (Ansari et al., 1985). The low activation energy barriers would provide for their rapid interconversion, suggesting that the effect of these ligands would be primarily manifest on the dynamics of the enzyme structure. Cooper and Dryden (1984) have discussed how such perturbations in the relatively slow harmonic and anharmonic global motions that have been observed in proteins can easily account for allosteric effects on the binding constant of substrate of a few kilocalories per mole as seen here. Such fluctuations typically occur at frequencies of approximately

50 cm^{-1} (1.5×10^{12} s^{-1}), which are several orders of magnitude faster than rate-limiting steps involved in catalytic turnover. Hence, these perturbations would not be expected to influence k_{cat}. It might also be noted that, according to the calculations of Cooper and Dryden, entropy-driven allosteric couplings are the expected result when the influence is transmitted by protein dynamics rather than conformation per se, and they are predicted to be opposed by the coupling enthalpy as seen here (Cooper & Dryden, 1984).

We therefore propose that the species appearing in the disproportionation equilibria for CPS in combination with MgADP and its allosteric ligands differ in their internal dynamic properties and thereby contribute to the change in entropy associated with that equilibrium and that it is this perturbation by the allosteric ligands that leads to their respective effects. We note in particular that a corollary to this proposal is that localized static structural perturbations in the substrate binding domain resulting from the binding of the allosteric ligand are unlikely to provide an accurate explanation for the mechanism of action of the allosteric ligands of this enzyme.

REFERENCES

Anderson, P. M. (1986) *Biochemistry 25*, 5576−5582.

Anderson, P. M., & Meister, A. (1966a) *Biochemistry 5*, 3157−3163.

Anderson, P. M., & Meister, A. (1966b) *Biochemistry 5*, 3164−3169.

Anderson, P. M., & Marvin, S. V. (1968) *Biochem. Biophys. Res. Commun. 32*, 928−934.

Ansari, A., Berendzen, J., Bowne, S. F., Frauenfelder, H., Iben, I. E. T., Sauke, T. B., Shyamsunder, E., & Yount, R. D. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 5000−5004.

Baldwin, R. L. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 8069−8072.

Bennett, W. S., Jr., & Steitz, T. A. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 4848−4852.

Boettcher, B., & Meister, A. (1981) *J. Biol. Chem. 256*, 5977−5980.

Boettcher, B., & Meister, A. (1982) *J. Biol. Chem. 257*, 13971−13976.

Braxton, B. L., Mullins, L. S., Raushel, F. M., & Reinhart, G. D. (1992) *Biochemistry 31*, 2309−2316.

Braxton, B. L., Tlapak-Simmons, V. L., & Reinhart, G. D. (1994) *J. Biol. Chem. 269*, 47−50.

Cleland, W. W. (1963) *Biochim. Biophys. Acta 67*, 104−137.

Cleland, W. W. (1967) *Adv. Enzymol. Relat. Areas Mol. Biol. 29*, 1−32.

Cooper, A., & Dryden, D. T. F. (1984) *Eur. Biophys. J. 11*, 103−109.

Eftink, M. R., Anusiem, A. C., & Biltonen, R. L. (1983) *Biochemistry 22*, 3884−3896.

Ferrari, M. E., & Lohman, T. M. (1994) *Biochemistry 33*, 12896−12910.

Ha, J.-H., Spolar, R. S., & Record, M. T., Jr. (1989) *J. Mol. Biol. 209*, 801−816.

Koshland, D. E., Jr., Ed. (1970) *The Enzymes*, 3rd ed., Vol. 1, pp 341−396, Academic Press, New York.

Mullins, L. S., Lusty, C. J., & Raushel, F. M. (1991) *J. Biol. Chem. 266*, 8236−8240.

Pierard, A. (1966) *Science 154*, 1572−1573.

Post, L. E., Post, D. J., & Raushel, F. M. (1990) *J. Biol. Chem. 265*, 7742−7747.

Raushel, F. M., Anderson, P. M., & Villafranca, J. J. (1978) *Biochemistry 17*, 5587−5591.

Reinhart, G. D. (1983) *Arch. Biochem. Biophys. 224*, 389−401.

Reinhart, G. D. (1988) *Biophys. Chem. 30*, 159−172.

Reinhart, G. D., Hartleip, S. B., & Symcox, M. M. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 4032−4036.

Robin, J. P., Penverne, B., & Herve, G. (1989) *Eur. J. Biochem. 183*, 519−528.

Sturtevant, J. M. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 2236−2240.

Trotta, P. P., Burt, M. E., Haschemeyer, R. H., & Meister, A. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68*, 2599−2603.

Trotta, P. P., Pinkus, L. M., Haschemeyer, R. H., & Meister, A. (1974) *J. Biol. Chem. 249*, 492−499.

Weber, G. (1972) *Biochemistry 11*, 864−878.

Weber, G. (1975) *Adv. Protein Chem. 29*, 1−83.

BI961305M