Influence of MgADP on Phosphofructokinase from *Escherichia coli.* Elucidation of Coupling Interactions with Both Substrates†

Jason L. Johnson and Gregory D. Reinhart*,‡

Department of Chemistry and Biochemistry, University of Oklahoma, Norman, Oklahoma 73019

Received October 6, 1993; Revised Manuscript Received December 22, 1993®

ABSTRACT: A comprehensive assessment is presented of the mutual influence that MgADP, MgATP, and fructose 6-phosphate (Fru-6-P) have on each other's binding to phosphofructokinase (PFK) from *E. coli.* When virtually any combination of these ligands binds to PFK it produces a significant perturbation in the intrinsic tryptophan fluorescence intensity and/or polarization which not only provides a means to follow binding in titration experiments but which also underscores the fact that more than two different enzyme conformations result from the binding of these ligands. When MgATP is saturating, the binding of MgADP to the allosteric site increases the affinity the enzyme subsequently displays for Fru-6-P. However, in the absence of MgATP, MgADP can bind to both the allosteric site and the nucleotide portion of the active site, with the latter antagonizing the binding of Fru-6-P to an extent that leads to an overall inhibition of Fru-6-P binding by MgADP. MgADP binding at the allosteric site also inhibits the binding of MgATP, indicating that under many circumstances MgADP should be more properly viewed as an inhibitor rather than an activator of *E. coli* PFK. After quantifying all of the 20 dissociation constants and 11 coupling parameters between ligand pairs pertinent to this three-ligand system, the more significant coupling parameters have been further characterized by examining their variation with temperature to establish the apparent enthalpy and entropy contributions to the corresponding coupling free energies. For both activating and inhibitory couplings, the enthalpy and entropy terms have the same sign as the coupling free energy. The presence of a third bound ligand alters the magnitude of the coupling free energies in ways that are dictated by changes in the corresponding coupling entropies.

Escherichia coli PFK[1] (EC 2.7.1.11) is a tetrameric allosteric enzyme that catalyzes the M ATP-dependent phosphorylation of Fru-6-P to form fructose-1,6-bisphosphate and MgADP. MgATP and Fru-6-P bind in a random fashion, with each antagonizing the other's binding in a saturable manner (Berger & Evans, 1991; Deville-Bonne et al., 1991b; Johnson & Reinhart, 1992). MgATP induces positive cooperativity in the Fru-6-P binding profile, with Hill numbers reaching a maximum of 3.6 at pH 8.0 when MgATP is saturating (Blangy et al., 1968; Berger & Evans, 1991; Johnson & Reinhart, 1992). MgATP, in contrast, binds in a hyperbolic fashion when Fru-6-P is saturating, but exhibits substrate inhibition at low concentrations of Fru-6-P (Blangy et al., 1968; Kundrot & Evans, 1991; Johnson & Reinhart, 1992), behavior which derives at least partially from interactions between different active sites of the tetramer (Johnson & Reinhart, 1992).

Physiological regulation of *E. coli* PFK has been thought to result at least in part from allosteric inhibition by PEP and allosteric activation by MgADP. Each alters the apparent affinity of the enzyme for Fru-6-P, while the maximal rate of turnover remains essentially unaffected (Blangy et al., 1968). Originally, the concerted transition theory was used to describe the actions of these ligands (Monod et al., 1965; Rubin & Changeux, 1966; Blangy et al., 1968). More recently, the limitations of the strict application of a two-state model have become increasingly apparent (Lau & Fersht, 1987; Kundrot & Evans, 1991; Deville-Bonne et al., 1991a; Johnson & Reinhart, 1992; Zheng & Kemp, 1992). Nonetheless, interpretation of structures determined by X-ray crystallography has generally focused on rationalizing the principal differences between "active" and "inhibited" forms (Shirakihara & Evans, 1988; Rypniewski & Evans, 1989; Evans, 1992). In particular, MgADP binding has been presumed to generate the "active" form by virtue of its facilitating effect on Fru-6-P binding when MgATP is saturating.

Significantly missing from these discussions of regulatory mechanism has been a consideration of the effects that MgADP might have on the binding of MgATP, the "other" substrate of PFK. It is well known that MgADP exhibits competitive product inhibition versus MgATP as expected for a random, sequential kinetic mechanism (Blangy et al., 1968). But the question of what effect the binding of MgADP to the allosteric site has on the binding of MgATP at the active site has not previously been addressed.

In this report, we present a complete assessment of the binding interactions between Fru-6-P, MgATP, and MgADP with *E. coli* PFK, which were determined using both enzyme activity and intrinsic fluorescence to monitor each ligand's binding in the presence and absence of the other ligands. Surprisingly, MgADP was found to be a more profound *inhibitor* of MgATP binding than it is an activator of Fru-6-P binding even after accounting for its competitive product inhibition. In addition, the binding of MgADP to the nucleotide portion of the active site antagonizes the binding

† Supported by Grants GM 33216 from the National Institutes of Health and HRO-025 from the Oklahoma Center for the Advancement of Science and Technology.

* Author to whom correspondence should be addressed.

‡ Recipient of an Established Investigator Award from the American Heart Association.

® Abstract published in *Advance ACS Abstracts,* February 1, 1994.

[1] Abbreviations used: PFK, phosphofructokinase; Fru-6-P, fructose 6-phosphate; PEP, phospho(*enol*)pyruvate; BCA, bicinchoninic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; EPPS, *N*-(2-hydroxyethyl)piperazine-*N*′-(3-propanesulfonic acid); DTT, dithiothreitol.

0006-2960/94/0433-2635$04.50/0

of Fru-6-P so that, when fully saturating in the absence of MgATP, MgADP actually inhibits the binding of Fru-6-P.

This assessment has been elaborated further by monitoring the variation of several of the ligand–ligand interactions with temperature. van't Hoff analyses of these data indicate that the net coupling free energies responsible for PFK's allosteric responses are always smaller in absolute value than the corresponding coupling enthalpy, indicating that enthalpy–entropy compensation is occurring as observed previously for the allosteric activation of beef heart NAD^+-dependent isocitrate dehydrogenase by MgADP and the allosteric inhibition of rat liver phosphofructokinase by MgATP (Reinhart et al., 1989). In the present case, which entails both activation and inhibition couplings within the same enzyme, the coupling free energies are always dominated by the enthalpy term in contrast to the results obtained previously with rat liver PFK (Reinhart et al., 1989).

MATERIALS AND METHODS

Materials. Phosphofructokinase was purified from *E. coli* K12, carrying the wild-type pfkA gene, obtained as a frozen paste from Grain Products Corp. All chemical reagents used in buffers, PFK purification, and fluorescence and enzymatic assays were of analytical grade, purchased from either Sigma, Fisher, or Aldrich. The Matrex Gel Blue A-agarose resin for affinity chromatography was purchased from Amicon Corp. Creatine phosphate, creatine kinase, the potassium salts of ADP and Fru-6-P, and the sodium salt of ATP were obtained from Sigma. The coupling enzymes aldolase, triosephosphate isomerase, and glycerol-3-phosphate dehydrogenase in ammonium sulfate suspensions were purchased from Boehringer Mannheim. Coupling enzymes were dialyzed extensively against a buffer consisting of 50 mM MOPS–KOH, 100 mM KCl, 5 mM $MgCl_2$, and 100 mM EDTA at pH 7.0. Deionized distilled water was used throughout.

PFK Purification. PFK was purified via a modification of the method of Kotlarz and Buc (1982) as described previously (Johnson & Reinhart, 1992).

Enzyme Activity Determination. Activity measurements were carried out in 1.0 mL of an EPPS buffer adjusted to pH 8.0 at the appropriate temperature and containing 50 mM EPPS, 10 mM $MgCl_2$, 10 mM NH_4Cl, 0.1 mM EDTA, 2 mM DTT, 0.2 mM NADH, 250 μg of aldolase, 50 μg of glycerol-3-phosphate dehydrogenase, and 5 μg of triosephosphate isomerase. At low concentrations of MgATP, 1 mM creatine phosphate and 10 μg/mL creatine kinase served as an MgATP regenerating system. To initiate the enzymatic reaction, 10 μL of suitably diluted PFK was added to the mixture. The steady-state reaction rate was determined by monitoring the decrease in absorbance at 340 nm with respect to time, i.e., the oxidation of NADH, on a strip chart-recorder tracing after the disappearance of any slow, pre-steady-state transients. A unit of activity is described as the amount of enzyme required to produce 1 μmol of fructose 1,6-bisphosphate per minute.

Protein Determination. Protein determinations were accomplished using the BCA Protein Assay Reagent (Smith et al., 1985). Absorbance at 278 nm, using $\epsilon_{278} = 0.6$ cm^2 mg^{-1} (Kotlarz & Buc, 1977), agreed with BCA-determined protein concentrations.

Fluorescence Measurements. Steady-state intensity and anisotropy (or polarization) of the intrinsic PFK fluorescence was measured with an ISS Model K2 multifrequency-phase fluorometer. A xenon arc lamp provided the excitation source for intensity measurements, while the 300-nm line of a Spectra-Physics Model 2045 argon ion laser was used in anisotropy measurements. The excitation beam from the laser was passed through a 2-mm-thick Schott WG-290 filter to remove the 275-nm line also produced by this laser in the "deep-UV" mode. In both cases, emission was collected through a 2-mm-thick Schott WG-345 cut-on filter and a Corning 7-54 band-pass filter. All titration experiments were performed in 50 mM EPPS–KOH (pH = 8.0), 10 mM $MgCl_2$, 10 mM NH_4Cl, and 0.2 mM EDTA, with a blank correction for the buffer made in both intensity and anisotropy studies. MgADP titrations were performed in a 0.4- × 0.4-cm cuvette to minimize inner filter effects in intensity measurements, while all other studies were conducted in a 1- × 1-cm cuvette. PFK subunit concentration was equal to 0.36 μM for all steady-state fluorescence measurements.

Measurements of Ligand Binding. When both substrates are present, the average Fru-6-P dissociation constant can be obtained from the concentration of Fru-6-P required to produce half-maximal kinetic activity since Fru-6-P achieves binding equilibrium during steady-state turnover (Johnson & Reinhart, 1992). MgATP does not achieve a rapid-binding equilibrium, however, so its thermodynamic binding behavior in the presence of Fru-6-P cannot be assessed directly from kinetic measurements (Johnson & Reinhart, 1992). When the enzyme is not active due to the absence of either MgATP or Fru-6-P, either the intensity and/or the polarization of the intrinsic fluorescence resulting from the single tryptophan moiety present in each subunit has been found to be responsive to ligand binding. As seen in Figure 1A, the fluorescence intensity responds significantly to each of the three ligands. Fru-6-P causes a 33% drop, while MgADP binding promotes a similar, though slightly smaller (28%), change. MgATP causes the emission to increase to a small (9%), but nonetheless significant, extent. When MgADP binds after MgATP, a large decrease in intensity results; however, the reverse situation, when MgATP is introduced after MgADP has bound, produces no detectable change in fluorescence intensity. It is similarly difficult to observe MgADP binding after Fru-6-P; however, Fru-6-P binding after MgADP does produce a significant drop of about 7%. All of these changes in emission intensity are mimicked by corresponding changes in fluorescence lifetime (Johnson & Reinhart, 1994).

The pattern of ligand-induced changes is quite different when fluorescence polarization of the intrinsic tryptophan is monitored as indicated in Figure 1B. In contrast to their similar effects on intensity, Fru-6-P and MgADP cause divergent changes to the intrinsic polarization, with Fru-6-P causing a substantial decrease and MgADP causing a small increase. The polarization when both Fru-6-P and MgADP are bound is intermediate, thereby allowing MgADP binding to be observed easily after Fru-6-P binding. MgATP binding causes a larger increase in polarization that proceeds to decrease upon the binding of MgADP, although MgATP binding after MgADP has bound only causes a small change in the intrinsic tryptophan polarization.

Quantifying the Allosteric Actions of MgADP. Since we wish to consider the effects of MgADP on the binding of both substrates, Fru-6-P and MgATP, there are three relevant ligands to consider. We designate these ligands as A, B, and X for Fru-6-P, MgATP, and MgADP, respectively. Dissociation constants of these ligands from *E. coli* PFK determined in the absence of any other ligands are designated K_{ia}°, K_{ib}°, K_{ix1}°, and K_{ix2}°, where the designations "1" and "2" in the last two parameters signify the dissociation of MgADP exclusively from the allosteric site and the nucleotide portion

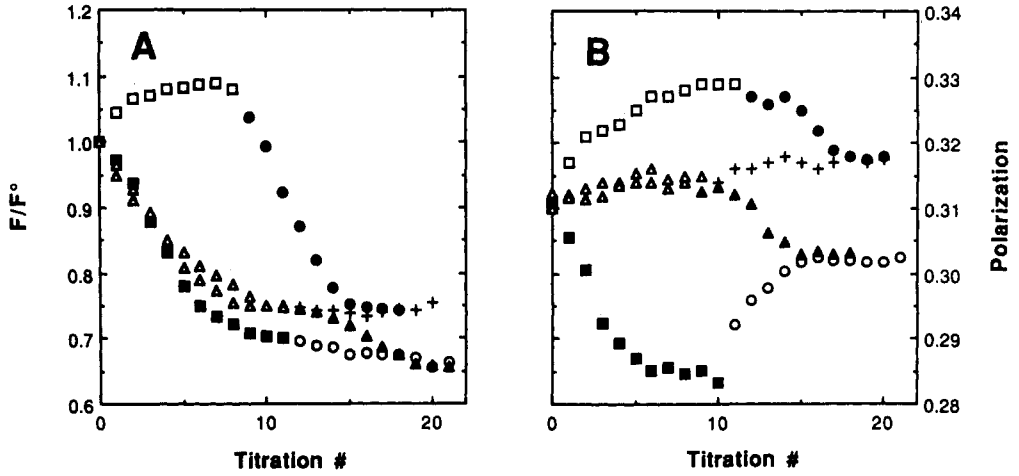

FIGURE 1: Changes in the intrinsic tryptophan fluorescence intensity (A) and polarization (B) accompanying the addition of ligands. *E. coli* PFK was first titrated with either Fru-6-P (■), MgADP (△), or MgATP (□). Subsequently, titrations were performed in which MgADP was added following saturation with Fru-6-P (○) or MgATP (●), as well as those in which Fru-6-P (▲) or MgATP (+) was added following saturation with MgADP. Intensity data are normalized to the fluorescence intensity in the absence of ligands ($F°$). Titration no. represents a constant multiple increase in concentration of the corresponding ligand.

of the active site, respectively. In general, the dissociation constants of these ligands will be altered if one or both of the other ligands are bound to the enzyme. Bound ligands are denoted in the subscript following a "/". Consequently, $K_{ia/x1}$ refers to the dissociation constant of Fru-6-P dissociating from PFK with MgADP bound to the allosteric site, $K_{ix2/a}$ refers to MgADP dissociating from the active site with Fru-6-P bound, $K_{ib/ax1}$ pertains to the MgATP dissociation when Fru-6-P is bound and MgADP is bound to the allosteric site, etc.

A total of 20 different dissociation constants pertaining to these three ligands can thereby be defined; however, they are not all independent. Rather, these dissociation constants are related by the principles of thermodynamic linkage (Wyman, 1948, 1964, 1967; Weber, 1972, 1975) and the various coupling parameters that quantify the reciprocal influence that two ligands have on each other's binding (Weber, 1975; Reinhart, 1983, 1988). For example, in order to quantify the overall effect of MgADP, binding to its allosteric site, on the binding of Fru-6-P it is necessary to determine the thermodynamic coupling between the two ligands, designated Q_{ax1}, which is defined as follows:

$$Q_{ax1} = \frac{K^{\circ}_{ia}}{K_{ia/x1}} = \frac{K^{\circ}_{ix1}}{K_{ix1/a}} \quad (1)$$

Equation 1 expresses the fact that thermodynamic linkage recognizes that the effect that the binding of A has on the subsequent binding of X (to site 1) must be equal to the impact that the binding of X has on the subsequent binding of A. The magnitude of this mutual effect is given by the coupling constant Q_{ax1}. Other coupling constants, for example, between MgATP and MgADP (designated Q_{bx1}) and between Fru-6-P and MgATP (designated Q_{ab}), are defined analogously. To denote a coupling between two ligands while the third ligand is saturating, a slash mark is followed by the letter designation for the saturating ligand. For example, the coupling between Fru-6-P and MgADP (at the effector site) when MgATP is saturating is represented by $Q_{ax1/b}$ and is defined as follows (Reinhart, 1988):

$$Q_{ax1/b} = \frac{K_{ia/b}}{K_{ia/bx1}} = \frac{K_{ix1/b}}{K_{ix1/ab}} \quad (2)$$

In total, 11 two-ligand coupling parameters can be defined from the 20 dissociation constants defined above.

Finally, not all of these pair-wise coupling parameters are fully independent, but rather they are related to the two three-ligand coupling parameters that can in principle be quantified in this system, Q_{abx1} and Q_{axx}, which correspond to the two fully-ligated species that can exist. A three-ligand coupling parameter is formally defined as the product of the dissociation constants of each of the three ligands individually divided by the product of the actual dissociation constants that together produce free enzyme from the fully-ligated form (Reinhart, 1983, 1988). As discussed in detail elsewhere (Reinhart, 1983, 1988), these three-ligand coupling parameters are related to the two-ligand coupling parameters as follows:

$$Q_{abx1} = Q_{ab}Q_{ax1}Q_{bx1/a} = Q_{ab}Q_{bx1}Q_{ax1/b} = Q_{ax1}Q_{bx1}Q_{ab/x1} \quad (3)$$

$$Q_{axx} = Q_{ax1}Q_{ax2}Q_{xx/a} = Q_{ax1}Q_{xx}Q_{ax2/x1} = Q_{ax2}Q_{xx}Q_{ax1/x2} \quad (4)$$

Several of the two-ligand coupling parameters were determined by measuring the dependence of the apparent dissociation constant for one ligand on the concentration of the second ligand and fitting these data to an equation analogous to eq 5 as described previously (Reinhart, 1983;

$$K_{0.5} = K^{\circ}_{ia}\left(\frac{K^{\circ}_{ix1} + [X]}{K^{\circ}_{ix1} + Q_{ax1}[X]}\right) \quad (5)$$

Reinhart, 1985; Reinhart & Hartleip, 1987; Reinhart & Hartleip, 1992; Braxton et al., 1992; Symcox & Reinhart, 1992). The notation in eq 5 specifically pertains to Fru-6-P and MgADP binding to the active site and allosteric site, respectively. The apparent dissociation constant for Fru-6-P is designated $K_{0.5}$ reflecting its determination from the concentration of Fru-6-P that produces one-half saturation.

Data Analysis. Dissociation parameters were obtained from titration experiments in which binding was monitored by changes in reaction rate or intrinsic fluorescence intensity or anisotropy. Unweighted data were fit directly to appropriate binding functions using either the commercial program ENZFITTER (Elsevier-Biosoft) or custom nonlinear regression programs that followed the general approach outlined by Cleland (1967). Custom programs were written either in the C programming language and run on a Silicon Graphics Personal Iris workstation or in HP BASIC and run on a

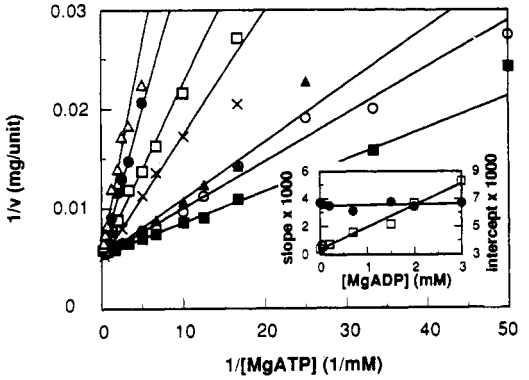

FIGURE 2: Double-reciprocal plot of initial velocity (v) versus MgATP concentration of the PFK-catalyzed reaction at the following MgADP concentrations: 0 mM (■), 0.05 mM (○), 0.2 mM (▲), 0.7 mM (×), 1.5 mM (□), 2 mM (●), and 3 mM (△). Inset shows the corresponding slope (□) and intercept (●) replots.

Hewlett-Packard 9836A desktop computer. Resulting dissociation constants were fit to eq 5 and other equations described in the text assuming constant relative error when weighting the data during the analysis.

Error Evaluation. The values of dissociation constants and coupling constants are reported ± the standard error which reflects the precision of the respective determination. Realistically, this represents a lower limit to the accuracy of the value. Different enzyme preparations yield somewhat different values. Although no attempt was made to systematically assess the range of such variation in each parameter, we conservatively estimate, based upon periodic assessment, that the quoted values are accurate to better than ±50%.

RESULTS

MgADP–Fru-6-P Interactions. MgADP exhibits competitive product inhibition of MgATP binding, with a slope inhibition constant, K_{is}, equal to 290 μM when Fru-6-P is equal to 4 mM as shown in Figure 2. Since this concentration of Fru-6-P is well in excess of its dissociation constant even with MgATP saturating (Johnson & Reinhart, 1992), Fru-6-P was fully saturating under all MgATP concentrations used when obtaining these data. When Fru-6-P is saturating MgADP binds to the allosteric binding domain with a dissociation constant equal to 2.4 μM (see Figure 3A). Consequently, the K_{is} determined by these data is equal to the dissociation constant of MgADP from the nucleotide portion of the active site while both Fru-6-P and MgADP remain bound to the active site and the allosteric site, respectively, and this constant is designated $K_{ix2/ax1}$ according to the definitions summarized above.

When one monitors the binding of MgADP directly by following the changes it induces in the intrinsic fluorescence of the enzyme, the results shown in Figure 3 are obtained. The data in Figure 3A were obtained with Fru-6-P concentration equal to 2 mM, which is also fully saturating. Although the intensity shows little variation under these conditions, the anisotropy of the intrinsic tryptophan fluorescence changes substantially, indicating a dissociation constant equal to 2.4 μM. The value of free MgADP concentration utilized in this determination was corrected for the amount bound to the enzyme, which was not negligible relative to total MgADP concentration at the low end of the concentration range examined. Hill plot analysis indicates that no cooperativity is evident in this binding profile. We conclude, therefore, that these data reflect the binding of MgADP to the allosteric site when Fru-6-P is bound, with the dissociation constant

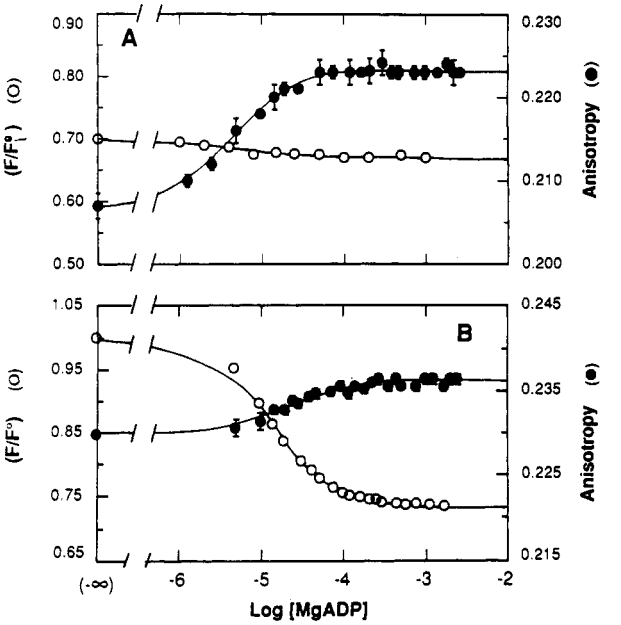

FIGURE 3: Determination of dissociation constants for MgADP in the absence of MgATP by monitoring either intrinsic tryptophan fluorescence intensity (○) or anisotropy (●). (A) [Fru-6-P] = 2 mM. (B) [Fru-6-P] = 0.

designated $K_{ix1/a}$. Note further how both fluorescence intensity and polarization are completely constant at MgADP concentrations exceeding 100 μM despite the fact that MgADP is binding to the active site at these concentrations according to the data in Figure 2. This observation suggests that the intrinsic fluorescence of *E. coli* PFK is specifically responsive to MgADP binding at the allosteric site. This conclusion is consistent with the observation made by Berger & Evans (1991) that when MgADP binding to the allosteric site is blocked via the site-directed mutation of Lys213 to Ala213 (Lau & Fersht, 1987) the intrinsic fluorescence intensity is not altered by the binding of MgADP to the active site of that mutant.

The data in Figure 3B were obtained in the absence of Fru-6-P. As indicated in Figure 1, both fluorescence intensity and anisotropy change in response to MgADP binding. On the basis of the conclusion just reached we can ascribe these changes to the binding of MgADP to the allosteric site. Significantly, the binding is hyperbolic, as confirmed by Hill plot analysis (Hill, 1910; data not shown), with an apparent dissociation constant equal to 13.1 μM. This result implies that either the binding of MgADP to the active site occurs with a dissociation constant substantially different from 13.1 μM or that the binding of MgADP to these two sites occurs independently, which would mean that the coupling between ADP binding to the two sites, Q_{xx}, is approximately equal to 1. Note that significant cooperativity in the MgADP binding profile is predicted if the two dissociation constants are of comparable magnitude and $Q_{xx} \neq 1$, despite the fact that only one binding is being directly detected.[2] Since cooperativity is not observed, the apparent dissociation constant indicated by these data would be equal to K^{o}_{ix1} in either case. We prefer the latter explanation, i.e., that $Q_{xx} = 1$, because of the data shown in Figure 4 as discussed below.

[2] This situation is analogous to an enzyme that binds substrate in rapid equilibrium at both the active site and an allosteric site. The rate equation for this case is given by the single-substrate, single-modifier equation (Reinhart, 1983) when the modifier does not affect V_{max} and both substrate and modifier ligands are identical. The equation reduces to a hyperbolic function only if the coupling parameter equals 1 or 0.

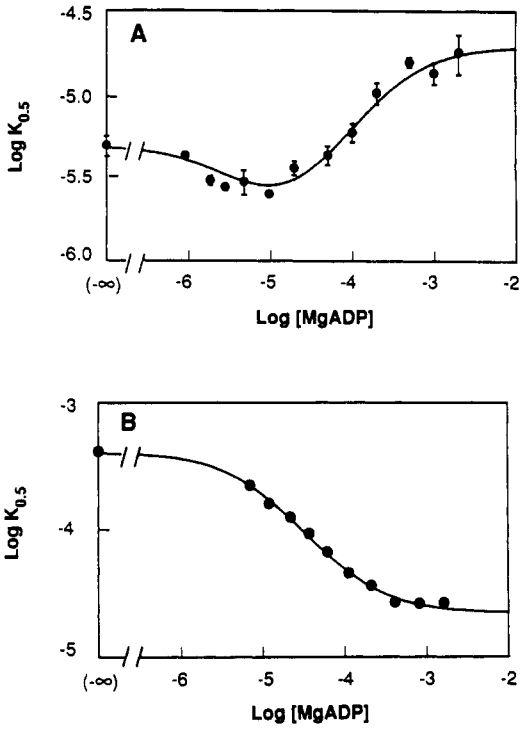

FIGURE 4: (A) Logarithm of the $K_{0.5}$ for Fru-6-P as a function of the logarithm of MgADP concentration in the absence of MgATP. $K_{0.5}$ values were determined from titrations of intrinsic fluorescence. The solid line represents the best nonlinear regression fit of the data to eq 10 as described in the text. (B) The logarithm of $K_{0.5}$ for Fru-6-P as a function of the logarithm of MgADP concentration when [MgATP] = 3 mM. $K_{0.5}$ values were determined from titrations of enzyme activity. The solid line represents the best fit of the data to eq 5 described in the text.

The data presented in Figure 3 represent the binding of MgADP at two extremes of Fru-6-P concentration, saturation and zero. Further insight regarding the interaction of MgADP with its two binding sites, and the interaction between the binding of MgADP and that of Fru-6-P, is obtained by following the binding of MgADP at an intermediate concentration of Fru-6-P, specifically the concentration of Fru-6-P that produces 1/2 saturation, i.e., $K_{0.5}$. A plot of $K_{0.5}$ for Fru-6-P as a function of MgADP concentration is presented in Figure 4A. It is immediately obvious that MgADP binding is biphasic when monitored in this way. At low concentrations MgADP causes $K_{0.5}$ for Fru-6-P to decrease, consistent with the expected activation produced by the binding of MgADP to the allosteric site. At higher concentrations of MgADP, however, the $K_{0.5}$ for Fru-6-P increases in a saturable manner, finally achieving a plateau value that is greater than its value in the total absence of MgADP. It seems clear that the second phase is due to the interaction of MgADP at the active site and the antagonism toward Fru-6-P binding that evidently results from that interaction. When Fru-6-P is at intermediate concentration, i.e., when $K_{0.5}$ for Fru-6-P is the dependent variable, the manifestation of this antagonism is also moderate and is evident within the concentration range of MgADP employed. This suggests that the intrinsic dissociation constant for MgADP from the active site in the absence of Fru-6-P, K°_{ix2}, is much closer to the value of K°_{ix1} than is $K_{ix2/ax}$.

The data in Figure 4A can be analyzed with reference to the equation that describes the binding of a ligand "X", such as MgADP, to two different sites, both of which affect the binding affinity of substrate "A", solved for the concentration of substrate producing half-saturation, $K_{0.5}$ (Reinhart, 1983):

$$K_{0.5} = K^{\circ}_{ia}\left[\frac{K^{\circ}_{ix1}K^{\circ}_{ix2} + (K^{\circ}_{ix1} + K^{\circ}_{ix2})[X] + Q_{xx}[X]^2}{K^{\circ}_{ix1}K^{\circ}_{ix2} + (Q_{ax1}K^{\circ}_{ix2} + Q_{ax2}K^{\circ}_{ix1})[X] + Q_{axx}[X]^2}\right] \quad (6)$$

This equation can be simplified by introducing the values for K°_{ix1}, Q_{xx}, $K_{ix1/a}$, and $K_{ix2/ax}$, determined from the data shown in Figures 2 and 3 as described above, in the following manner:

$$Q_{ax1} = \frac{K^{\circ}_{ix1}}{K_{ix1/a}} = \frac{13.1}{2.4} = 5.5 \quad (7)$$

$$Q_{axx} = Q_{ax1}Q_{ax2}Q_{xx/a} = Q_{ax1}\left(\frac{K^{\circ}_{ix2}}{K_{ix2/ax}}\right) = \left(\frac{5.5}{290}\right)K^{\circ}_{ix2} = 0.019K^{\circ}_{ix2} \quad (8)$$

Substituting eqs 7 and 8 into eq 6 yields eq 9:

$$K_{0.5} = K^{\circ}_{ia}\left[\frac{13.1K^{\circ}_{ix2} + (13.1 + K^{\circ}_{ix2})[X] + [X]^2}{13.1K^{\circ}_{ix2} + (5.5K^{\circ}_{ix2} + 13.1Q_{ax2})[X] + 0.019K^{\circ}_{ix2}[X]^2}\right] \quad (9)$$

When the data presented in Figure 4A are fit to eq 9, the value of Q_{ax2} is very poorly determined (standard error > 500%). This is not surprising when one considers the fact that the term $K^{\circ}_{ix1}Q_{ax2}[X]$ appearing in the denominator of eq 6 represents the enzyme form with Fru-6-P bound and MgADP bound exclusively to the active site. This enzyme form is not produced to any appreciable extent since MgADP binds to the active site with an affinity more than 2 orders of magnitude less than its affinity for the allosteric site when Fru-6-P is bound as discussed above. If we further simplify eq 9 by omitting this term, eq 10 results. When the data in Figure

$$K_{0.5} = K^{\circ}_{ia}\left[\frac{13.1K^{\circ}_{ix2} + (13.1 + K^{\circ}_{ix2})[X] + [X]^2}{13.1K^{\circ}_{ix2} + 5.5K^{\circ}_{ix2}[X] + 0.019K^{\circ}_{ix2}[X]^2}\right] \quad (10)$$

4A are fit to eq 10 the overall fit actually improves, confirming our conclusion that the term containing Q_{ax2} is not justified by the data. The values of K°_{ia} and K°_{ix2} that result from this fit are 4.5 and 12.0 μM, respectively, and the curve appearing in Figure 4A corresponds to eq 10 using these values. This value for K°_{ia} compares favorably with the value of 6.8 μM previously reported (Johnson & Reinhart, 1992). Note that MgADP displays nearly identical affinities for the allosteric site and the active site in the absence of Fru-6-P (13.1 μM and 12.0 μM, respectively). Since this conclusion is based in part upon the assumption that $Q_{xx} = 1$, we also fit the data to eq 10 modified to include Q_{xx} in the X^2 term in the numerator, as shown in eq 6, so that its value could float. Once again, the overall fit was worse and the value of Q_{xx} poorly determined (standard error 50%) though approximately equal to 1 nonetheless. Hence, the inclusion of this parameter (i.e., assuming $Q_{xx} \neq 1$) is not justified by these data. The overall coupling constant, Q_{axx}, is therefore equal to 0.226 according to eq 8 and the value of K°_{ix2} determined by the fit.

Finally, in Figure 4B we present the variation of $K_{0.5}$ for Fru-6-P with MgADP when MgATP is saturating (3 mM). Values of $K_{0.5}$ for Fru-6-P at concentrations of MgADP ranging from 0 to 1.6 mM were determined, as summarized in Figure 4B at 25 °C. Since MgATP is saturating, MgADP cannot bind to the active site, thus removing any ambiguity

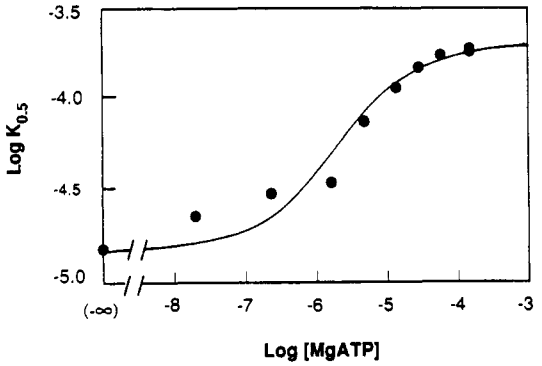

FIGURE 5: Influence of MgATP on $K_{0.5}$ for MgADP in the absence of Fru-6-P. $K_{0.5}$ was determined by monitoring the MgADP-induced quenching of PFK's intrinsic fluorescence as shown in Figure 3. The line represents the best fit of the data to eq 5 described in the text.

in the analysis. Fitting these data to an equation analogous to eq 5 yields the values 420 μM, 140 μM, and 18.3 for the dissociation and coupling constants $K_{ia/b}$, $K_{ix1/b}$, and $Q_{ax1/b}$, respectively. Note how the activation by MgADP is substantially more pronounced when MgATP is saturating as indicated by the fact that $Q_{ax1/b} > Q_{ax1}$. However, since $K_{ix1/b} > K^{\circ}_{ix1}$, it is also clear that MgADP simultaneously antagonizes the binding of MgATP.

MgADP–MgATP Interactions. The value of 13.1 μM determined for K°_{ix1}, the dissociation constant of MgADP from the allosteric site in the absence of Fru-6-P and MgATP determined as described above (Figure 3B), is substantially different from the value of 140 μM observed for $K_{ix1/b}$, the dissociation constant of MgADP determined via kinetic measurements with MgATP saturating (Figure 4B). This discrepancy arises from an antagonistic coupling between MgADP and MgATP. The coupling constant, Q_{bx1}, can be determined directly in a more accurate manner by monitoring the change in the $K_{0.5}$ for MgADP, measured via the quenching of PFK's intrinsic fluoresence, that results from varying the concentration of MgATP as shown in Figure 5. Nonlinear regression of these data to eq 5 indicates that Q_{bx1} is equal to 0.072. The apparent value of the dissociation constant for MgATP, K°_{ib}, resulting from this fit does not equal the true value of K°_{ib} because of the competitive relationship between MgADP and MgATP binding at the active site (Figure 2). Instead, K°_{ib} was determined by titrating MgATP in the absence of MgADP and monitoring the concomitant intensity increase, as shown in Figure 1A. The value of 0.025 μM obtained in this manner was reported previously (Johnson & Reinhart, 1992). Note that the apparent dissociation constant for MgADP approaches a value of 190 μM at high concentration of MgATP in reasonable agreement with the value of $K_{ix1/b}$ determined above.

Finally, the influence that MgADP bound to the effector site has on the thermodynamic binding of MgATP in the presence of Fru-6-P cannot be assessed directly with kinetic measurements since MgATP does not achieve a binding equilibrium during steady-state catalytic turnover (Johnson & Reinhart, 1992). However, this coupling can be inferred from the other measured couplings as described below.

Remaining Interactions. The values of dissociation constants and coupling parameters just described are tabulated in Tables 1 and 2, respectively. From these values other dissociation constants can be determined as indicated in eq 1. We have previously described in detail the coupling between the substrates Fru-6-P and MgATP (Johnson & Reinhart, 1992), and for completeness we also include in Tables 1 and 2 the values for $K_{ib/a}$ and Q_{ab} that were previously determined. Knowing the value of Q_{ab} allows the calculation of the three-ligand coupling constant Q_{abx} according to eq 3. With this parameter determined, the coupling between MgADP and MgATP in the saturating presence of Fru-6-P ($Q_{bx1/a}$) and the coupling between Fru-6-P and MgATP when MgADP is saturating ($Q_{ab/x1}$) may also be calculated from the identities included in eq 3. $Q_{ax2/x1}$ is in turn derived from Q_{axx}, Q_{ax1}, and Q_{xx} according to eq 4.

The remaining two-ligand coupling parameters that exist in this three-ligand system, Q_{ax2}, $Q_{xx/a}$, and $Q_{ax1/x2}$, are mutually interdependent and can only be determined if we assume that $Q_{xx/a}$ is approximately equal to 1, which is suggested by the following argument. When Fru-6-P is

Table 1: Ligand Dissociation Constants from *E. coli* PFK for all Combinations of Fru-6-P, MgATP, and MgADP

ligand	other saturating ligands	designation	value (μM)
Fru-6-P		K°_{ia}	4.5 ± 0.3[a]
	MgATP	$K_{ia/b}$	420 ± 20
	$MgADP_{eff}$[b]	$K_{ia/x1}$	1.2 ± 0.2
	$MgADP_{act}$[b]	$K_{ia/x2}$	110 ± 32
	$MgADP_{act}$, $MgADP_{eff}$	$K_{ia/xx}$	20 ± 9
	MgATP, $MgADP_{eff}$	$K_{ia/bx1}$	23 ± 1
MgATP		K°_{ib}	0.025 ± 0.004[c]
	Fru-6-P	$K_{ib/a}$	1.7 ± 0.3[d]
	$MgADP_{eff}$	$K_{ib/x1}$	0.35 ± 0.07
	Fru–6-P, $MgADP_{eff}$	$K_{ib/ax1}$	7.0 ± 2.1
$MgADP_{eff}$		K°_{ix1}	13 ± 3
	Fru-6-P	$K_{ix1/a}$	2.4 ± 0.3
	MgATP	$K_{ix1/b}$	190 ± 7[e]
	$MgADP_{act}$	$K_{ix1/x2}$	13 ± 3
	Fru-6-P, MgATP	$K_{ix1/ab}$	10 ± 1
	Fru-6-P, $MgADP_{act}$	$K_{ix1/ax2}$	2.4 ± 0.3
$MgADP_{act}$		K°_{ix2}	12 ± 1
	Fru-6-P	$K_{ix2/a}$	290 ● 13
	$MgADP_{eff}$	$K_{ix2/x1}$	12 ± 1
	Fru-6-P, $MgADP_{eff}$	$K_{ix2/ax1}$	290 ± 13

[a] A value of 6.8 μM was reported by Johnson & Reinhart (1992). [b] $MgADP_{eff}$ refers to MgADP binding to PFK's effector site, whereas $MgADP_{act}$ refers to active site binding. [c] As reported by Johnson & Reinhart (1992). [d] MgATP does not achieve rapid equilibrium during enzymatic turnover (K_m = 49 μM). [e] This value was directly measured by titrating intrinsic fluorescence; however, a value of 140 μM was obtained from a fit of the data shown in Figure 5 to eq 5.

Table 2: Coupling Interactions between Fru-6-P, MgATP, and MgADP When Bound to *E. coli* PFK at 25 °C

coupled ligands	other saturating ligand	desig-nation	value
Fru-6-P-$MgADP_{eff}$[a]		Q_{ax1}	5.5 ± 1.1
	MgATP	$Q_{ax1/b}$	18.3 ± 1.1
	$MgADP_{act}$[a]	$Q_{ax1/x2}$	5.5 ± 2.0
MgATP–$MgADP_{eff}$		Q_{bx1}	0.072 ± 0.008
	Fru-6-P	$Q_{bx1/a}$	0.24 ± 0.06
Fru-6-P–MgATP		Q_{ab}	0.015 ● 0.001
	$MgADP_{eff}$	$Q_{ab/x1}$	0.050 ± 0.014
Fru-6-P–$MgADP_{act}$		Q_{ax2}	0.041 ± 0.012
	$MgADP_{eff}$	$Q_{ax2/x1}$	0.044 ● 0.013
$MgADP_{eff}$–$MgADP_{act}$		Q_{xx}	1[b]
	Fru-6-P	$Q_{xx/a}$	1[b]
Fru-6-P–MgATP–$MgADP_{eff}$		Q_{abx1}	0.020 ± 0.003
Fru-6-P–$MgADP_{eff}$–$MgADP_{act}$		Q_{axx}	0.23 ± 0.05

[a] $MgADP_{eff}$ refers to MgADP binding to PFK's effector site, whereas $MgADP_{act}$ refers to active site binding. [b] Values of 1 are only inferred by the nature of the data, so that errors were not determined.

saturating and PFK is titrated with MgADP, MgADP will first bind to the allosteric site with a dissociation constant equal to 2.4 μM. Subsequently, MgADP binds to the active site with a dissociation constant equal to 290 μM, increased from 12 μM due to either antagonism from the Fru-6-P bound or the first MgADP bound or both. When MgADP binds to the active site, no change in fluorescence intensity or anisotropy is observed. In all other cases when a coupled ligand binds after MgADP binds to the allosteric site, either one or both of these fluorescence properties change (Figure 1). Also, in the absence of Fru-6-P, MgADP binds to both sites independently since $Q_{xx} = 1$. These observations suggest, but do not prove, that MgADP binds independently to the allosteric and active sites when Fru-6-P is bound, i.e., that $Q_{xx/a} = 1$. Assuming this to be the case, the remaining parameters can be determined from the identities given in eq 4.

All of the two-ligand and three-ligand coupling constants, that together provide a comprehensive picture of all of the possible interactions between the three ligands, Fru-6-P, MgATP, and MgADP, including those interactions resulting from MgADP binding to the nucleotide portion of the active site, are listed in Table 2. From the definitions of these coupling constants, and the values of the dissociation constants just enumerated, the remaining dissociation constants can be readily determined and are listed in Table 1.

van't Hoff Analysis of Coupling Free Energies. The coupling free energies corresponding to the coupling constants involving the substrates and MgADP binding to the allosteric site appearing in Table 2 are readily obtained, as illustrated for the MgADP–Fru-6-P interaction in eq 11

$$\Delta G_{ax1} = -RT \ln(Q_{ax1}) \tag{11}$$

where R equals the gas constant and T equals absolute temperature. At constant temperature, net enthalpy, ΔH_{ax1}, and entropy, ΔS_{ax1}, components of this coupling free energy can be ascertained from the variation in Q_{ax1} with temperature, provided ΔH_{ax1} remains relatively constant, according to the standard van't Hoff relation (Reinhart et al., 1989):

$$\log Q_{ax1} = -\left(\frac{\Delta H_{ax1}}{2.3R}\right)\frac{1}{T} + \frac{\Delta S_{ax1}}{2.3R} \tag{12}$$

By plotting values of $\log(Q_{ax1})$ as a function of reciprocal temperature, ΔH_{ax1} can therefore be determined from the slope of the resulting line and values for ΔS_{ax1} from the intercept.

Values of Q_{ax1} and $Q_{ax1/b}$ for the MgADP–Fru-6-P interaction in *E. coli* PFK were determined at temperatures ranging from 5 to 35 °C (data not shown). In both cases (± MgATP), a straight line with positive slope fits the data well, indicating that Q_{ax1} and $Q_{ax1/b}$ decrease with increasing temperature. Weighted linear regression of these data provides the thermodynamic values that give rise to the activation of Fru-6-P binding by MgADP at the allosteric site, and vice versa, in the saturating presence and absence of MgATP, as shown in Table 3.

Similar analysis was performed on the MgATP–MgADP interaction in the absence of Fru-6-P (data not shown). In this case, values of Q_{bx1} increase with increasing temperature, indicative of a positive sign for ΔH_{bx1}. Weighted linear regression of these data provides the values for ΔH_{bx1} and ΔS_{bx1} given in Table 3.

van't Hoff analysis of the Fru-6-P–MgATP antagonistic coupling Q_{ab} involved measuring $K_{0.5}$ for Fru-6-P as a function of MgATP concentration at various temperatures. In addition, K_{ia}^{o} at each temperature was directly measured by monitoring the quenching of PFK's intrinsic fluorescence. As is true for the case of the MgATP–MgADP coupling, Q_{ab} increases with temperature to provide a linear van't Hoff plot. Values for the coupling enthalpy and entropy deriving from a weighted linear regression fit to eq 14 are also provided in Table 3.

Table 3: Thermodynamic Parameters Pertaining to Allosteric Couplings in *E. coli* PFK

coupling	ΔG at 25 °C (kcal/mol)	ΔH (kcal/mol)	$T\Delta S$ at 25 °C (kcal/mol)
Q_{ax1}	−1.0 ± 0.1	−3.1 ± 1.5	−2.1 ± 1.5
Q_{bx1}	+1.5 ± 0.1	+1.7 ± 0.4	+0.2 ± 0.4
Q_{ab}	+2.5 ± 0.2	+8.5 ± 1.3	+6.0 ± 1.3
$Q_{ax1/b}$	−1.7 ± 0.1	−2.0 ± 0.5	−0.3 ± 0.5
$Q_{bx1/a}$	+0.8 ± 0.1	+2.8 ± 1.2	+2.0 ± 1.2
$Q_{ab/x1}$	+1.8 ± 0.1	+9.6 ± 0.9	+7.8 ± 0.9

The values of $Q_{bx1/a}$, the coupling constant between MgATP and MgADP when Fru-6-P is saturating, and $Q_{ab/x1}$, the coupling constant between Fru-6-P and MgATP when MgADP is saturating at the effector site, were deduced from those just mentioned through the combined application of the identities embodied in eqs 3, 4, and 11. Free energy, enthalpy, and entropy associated with ligand interactions are governed by the same identities that relate coupling constants (eqs 3 and 4), only the parameters are additive rather than multiplicative given the logarithmic conversion of Q to ΔG (eq 11). These values are also presented in Table 3.

DISCUSSION

The similar decrease in fluorescence intensity caused by the binding of Fru-6-P at the active site and MgADP at the effector site has previously been interpreted as supporting the notion that both ligands stabilize a common active or "R" form (Deville-Bonne & Garel, 1992). The quite different results obtained when monitoring fluorescence polarization indicate that this is not the case. The conformations resulting from the binding of Fru-6-P at the active site and MgADP at the effector site are distinct as monitored by the intrinsic tryptophan.

As directly demonstrated in Figure 4A, in the absence of MgATP the combined effects of MgADP binding to the active site as well as to the effector site result in overall *inhibition* of Fru-6-P binding. This result brings into question interpretations of the mechanism of action of MgADP that have been proposed previously based upon the results of X-ray crystallography. Specifically, Shirakihara and Evans (1988) have determined the structure of *E. coli* PFK with MgADP bound to both active and allosteric sites (along with fructose 1,6-bisphosphate bound to the active site) and, presuming that this structure represented the active "R" form, interpreted various structural details as being responsible for the putative high affinity for Fru-6-P. It is now apparent that the binding affinity of Fru-6-P would increase if MgADP were absent.

Only when MgATP is bound does MgADP binding result exclusively in the commonly appreciated activation of Fru-6-P binding, although the actions of MgADP do not completely relieve the antagonism that exists between MgATP and Fru-6-P in this circumstance since $K_{ia/bx1}$ is still about 4-fold greater than K_{ia}^{o} as shown in Table 1. In other words, MgADP activates unambiguously only an enzyme that displays less than its optimum affinity for Fru-6-P even after MgADP binds, a result that further argues that the actions of MgADP cannot be adequately explained by a simple two-state model.

MgADP binding at the effector site also profoundly inhibits the binding of MgATP whether or not Fru-6-P is bound, as

revealed by the fact that both Q_{bx1} and $Q_{bx1/a}$ are less than 1. Although this antagonism will probably not prevent MgATP from binding under physiological conditions, given that $K_{ib/ax1}$ = 7.0 μM, this observation again presents a challenge to the existing interpretations of structural data (Evans, 1992) which have not focused on this consequence of MgADP binding.

Despite the fact that MgADP binding at the allosteric site antagonizes MgATP binding to the active site, it has no influence on MgADP binding at the active site (and vice versa) since we have concluded that Q_{xx} and $Q_{xx/a}$ are both equal to 1. A plot of steady-state intensity and polarization as a function of MgADP concentration when Fru-6-P is saturating (Figure 3A) clearly shows that MgADP elicits no response from the tryptophan fluorescence when interacting at the active site suggesting that a minimal conformational perturbation in the vicinity of the tryptophan results from this interaction. Since MgADP binds to the active site with much lower affinity after Fru-6-P and MgADP have previously bound to the active and allosteric sites, respectively (Figure 2), the antagonism that gives rise to this lower affinity most likely results from a direct intra-active-site interaction between Fru-6-P and MgADP. This interaction resembles the intra-active-site interaction that is responsible in part for the antagonism evident between Fru-6-P and MgATP (Johnson & Reinhart, 1992).

The distinctions in intrinsic fluorescence properties exhibited by *E. coli* PFK when bound to Fru-6-P, MgATP, and MgADP enable the coupling parameters between any two of these ligands to be quantified in both the absence and presence of the third ligand. For example, the influence of MgADP on Fru-6-P affinity is approximately three times greater in the presence of MgATP than in its absence: $Q_{ax1/b}$ = 18.3 versus Q_{ax1} = 5.5. The coupling constant between Fru-6-P and MgATP, Q_{ab}, was previously found to be equal to 0.015 (Johnson & Reinhart, 1992). When MgADP is saturating at the effector site, the MgATP antagonism of Fru-6-P affinity is somewhat less ($Q_{ab/x1}$ = 0.056). The coupling parameter between MgATP and MgADP in the absence of Fru-6-P, Q_{bx1}, is equal to 0.095 but this antagonism is mitigated when Fru-6-P is saturating, although MgADP still inhibits MgATP binding allosterically by approximately 4-fold ($Q_{bx1/a}$ = 0.23).

The van't Hoff analyses reveal further insights into the perturbations of these coupling interactions elicited by the third ligand. All of the coupling free energies listed in Table 3 are the consequence of opposing enthalpy and entropy contributions. In all cases the sign of ΔG and ΔH are the same, indicating that the allosteric effects measured by the corresponding coupling parameter are dominated by the enthalpy term. The entropy terms serve in each case to mitigate the absolute value of the ΔG. It is particularly noteworthy, however, how the presence of the third ligand acts to effect a change in the coupling free energy. In the case of the activating interaction, i.e., ΔG_{ax1}, the presence of MgATP causes the Fru-6-P–MgADP activation to increase as noted above. However, despite the fact that the activation is enthalpy driven, the coupling enthalpy actually decreases in absolute value, as does the coupling entropy, when MgATP binds. The increased activation is realized only because the impact of MgATP on ΔS_{ax1} is greater than its effect on ΔH_{ax1} so that a larger difference between ΔH_{ax1} and $T\Delta S_{ax1}$ results from MgATP binding.

The effects of the third ligand on the inhibitory interactions are directly analogous. In both inhibitory interactions, i.e., ΔG_{bx1} and ΔG_{ab}, the presence of the third ligand, Fru-6-P and MgADP, respectively, causes the inhibition to decrease. In all cases, however, the third ligand achieves its effects because it has a greater effect on the coupling entropy. Although the coupling free energies are smaller after the third ligand binds, the coupling enthalpies are actually larger. The smaller free energies result from the fact that the coupling entropies are increased to a greater extent than are the coupling enthalpies, so that a smaller difference between ΔH and ΔS occurs.

These results underscore once again the importance of both entropic and enthalpic contributions to a coupling free energy that in turn establishes the nature and magnitude of an allosteric effect. This point is also supported by the different responses displayed by the fluorescence intensity and polarization of the intrinsic tryptophan. Features other than simple static structural perturbations are involved in, or at least correlate with, the transmission of allosteric effects in this enzyme. In particular, the polarization changes apparent in Figure 1B suggest that the flexibility of the tryptophan is subject to perturbation by these ligands. Since it has been previously proposed that perturbation of the dynamic properties of a protein may be a motif by which allosteric ligands achieve their effects by contributing to the coupling entropy (Copper & Dryden, 1984; Reinhart, et al., 1989), we present in the following paper (Johnson & Reinhart, 1994) a detailed examination of the nature of the ligand-induced changes in the local motion of the tryptophan in an effort to shed light on the possible role such factors may play in the allosteric actions of MgADP in *E. coli* PFK.

REFERENCES

Berger, S. A., & Evans, P. R. (1991) *Biochemistry 30*, 8477–8480.

Blangy, D., Buc, H., & Monod, J. (1968) *J. Mol. Biol. 31*, 13–35.

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

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.

Deville-Bonne, D., Bourgain, F., & Garel, J.-R. (1991a) *Biochemistry 30*, 5750–5754.

Deville-Bonne, D., Laine, R., & Garel, J.-R. (1991b) *FEBS Lett. 290*, 173–176.

Deville-Bonne, D., & Garel, J.-R. (1992) *Biochemistry 31*, 1695–1700.

Evans, P. R. (1992) *Proc. Robert A. Welch Found. Conf. Chem. Res: Regulation of Proteins by Ligands 36*, 39–56.

Hill, A. V. (1910) *J. Physiol. London 40*, iv–vii.

Johnson, J. L., & Reinhart, G. D. (1992) *Biochemistry 31*, 11510–11518.

Johnson, J. L., & Reinhart, G. D. (1994) *Biochemistry* (following paper in this issue).

Kotlarz, D., & Buc, H. (1977) *Biochim. Biophys. Acta 484*, 35–48.

Kotlarz, D., & Buc, H. (1982) *Methods Enzymol. 90*, 60–70.

Kundrot, E. E., & Evans, P. R. (1991) *Biochemistry 30*, 1478–1484.

Lau, F., & Fersht, A. (1987) *Nature 326*, 811–812.

Monod, J., Wyman, J. L., & Changeux, J. P. (1965) *J. Mol. Biol. 3*, 318–356.

Reinhart, G. D. (1983) *Arch. Biochem. Biophys. 225*, 389–401.

Reinhart, G. D. (1985) *Biochemistry 24*, 7166–7172.

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

Reinhart, G. D., & Hartleip, S. B. (1987) *Arch. Biochem. Biophys. 258*, 65–76.

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

Reinhart, G. D., & Hartleip, S. B. (1992) *Arch. Biochem. Biophys. 296*, 224–230.

Rubin, M. M., & Changeux, J. P. (1966) *J. Mol. Biol. 21*, 265–274.
Rypniewski, W. R., & Evans, P. R. (1989) *J. Mol. Biol. 207*, 805–821.
Shirakihara, Y., & Evans, P. R. (1988) *J. Mol. Biol. 204*, 973–994.
Symcox, M. M., & Reinhart, G. D. (1992) *Anal. Biochem. 206*, 394–399.
Smith, D. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J., & Klenk, B. C. (1985) *Anal. Biochem. 150*, 76–85.
Weber, G. (1972) *Biochemistry 11*, 864–878.
Weber, G. (1975) *Adv. Protein Chem. 29*, 1–83.
Wyman, J. (1948) *Adv. Protein Chem. 4*, 407–531.
Wyman, J. (1964) *Adv. Protein Chem. 19*, 223–286.
Wyman, J. (1967) *J. Am. Chem. Soc. 89*, 2202–2218.
Zheng, R.-L., & Kemp, R. G. (1992) *J. Biol. Chem. 267*, 23640–23645.