Obfuscation of Allosteric Structure–Function Relationships by Enthalpy–Entropy Compensation

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ABSTRACT The pH and temperature dependence of the allosteric properties of phosphofructokinase (PFK) from *Bacillus stearothermophilus* have been studied from 5 to 9 and 6 to 40°C, respectively. Throughout this pH and temperature range the allosteric ligands MgADP and phospho(*enol*)pyruvate (PEP) have no effect on k_{cat}. The dissociation constants of the substrate, fructose 6-phosphate, and the allosteric ligands, as well as the absolute value of the coupling free energies between these ligands, all increase when the pH is raised, indicating that the inhibition by PEP and the activation by MgADP increase despite each ligand's somewhat lower affinity. However, the constituent coupling enthalpies and entropies substantially diminish in absolute value as pH is increased, suggesting that the magnitudes of molecular perturbations engendered by the binding of allosteric ligands do not correlate with the magnitudes of the functional consequences of those perturbations. Temperature and pH exert their influence on the observed allosteric behavior by changing the relative contributions made by the largely compensating ΔH and $T\Delta S$ terms to the coupling free energy.

INTRODUCTION

The allosteric properties of K-type regulatory enzymes present a unique opportunity to study the factors that bear on protein–ligand condensation reactions since by definition the binding of allosteric ligands perturbs the subsequent binding of the substrate ligand. For example, the allosteric ligands phospho(*enol*)pyruvate (PEP) and MgADP affect the activity of *Bacillus stearothermophilus* phosphofructokinase (PFK) by perturbing the affinity with which the substrate fructose 6-phosphate (Fru-6-P) binds to the active site. PFK from *B. stearothermophilus*, like its analog from *Escherichia coli*, has one allosteric binding domain where both allosteric modifiers bind (Evans and Hudson, 1979). We have previously observed that changing temperature can change the magnitude of the inhibition of Fru-6-P binding by PEP (Tlapak-Simmons and Reinhart, 1994) and the nature as well as magnitude of the allosteric effect observed by MgADP (Braxton et al., 1994). It is possible that these effects could be caused by temperature-induced changes in the protonation state of either the enzyme or the ligand which in turn leads to altered binding properties. We therefore present herein a comprehensive evaluation of the effects of both pH and temperature on the allosteric properties of PFK from *B. stearothermophilus* to address these possibilities.

The influence of pH on the properties of PFK from a variety of sources has been extensively studied, and the nature of this influence varies considerably. Rabbit muscle PFK loses its allosteric and cooperative responses at pH values above pH 8 (Bock and Frieden, 1974, 1976a, b; Bock et al., 1975; Frieden et al., 1976; Paetkau and Lardy, 1967). Reinhart (1985) found that decreasing the pH did not affect the binding or the inhibition by MgATP of rat liver PFK but did affect the affinity of the enzyme for Fru-6-P. The pH dependence of the allosteric properties of *E. coli* PFK have been examined by Deville-Bonne et al. (1991). A group apparently needs to be deprotonated for the enzyme to be catalytically active. This group has a pK of 7.1 in the absence of allosteric effectors and 6.6 in the presence of the activator (GDP), and has been proposed to be Asp-127 (carboxyl group) which is near the active site (Shirakihara and Evans, 1988). Likewise, deprotonation is also essential for PFK from *Lactobacillus bulgaricus* to be fully active (Le Bras et al., 1991).

In this study we have examined the influence of pH on the allosteric activation and inhibition of *B. stearothermophilus* PFK by MgADP and PEP, respectively, using a thermodynamic linked-function approach (Weber, 1972, 1975; Reinhart, 1985, 1988). This approach allows a separate assessment to be made of the effects of pH on ligand binding and the action of the allosteric ligands, respectively. The latter effect can be evaluated by the influence of pH on the coupling free energy between each allosteric ligand and Fru-6-P. By varying temperature as well, one can further estimate effects on the enthalpy and entropy components of this free energy parameter.

MATERIALS AND METHODS

Materials

Biochemicals and buffers were purchased from Sigma Chemical Co. (St. Louis, MO). The coupling enzymes were purchased from Boehringer

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Mannheim (Indianapolis, IN) as ammonium sulfate suspensions. The other reagents used were high purity, and deionized, distilled water was used throughout the experiment.

PFK preparation

Phosphofructokinase (PFK) from *Bacillus stearothermophilus* was purchased from Sigma in lyophilized form. PFK was prepared for use by dissolution in 50 mM MOPS, 10 mM KCl, 10 mM $MgCl_2$, 0.1 mM EDTA, 1 mM ATP, and 1 mM dithiothreitol, at pH = 7.0. The coupling enzymes, aldolase, triosephosphate isomerase, and glycerin-3-phosphate dehydrogenase were purchased as ammonium sulfate suspensions from Boehringer Mannheim and dialyzed extensively in the 3-(*N*-morpholino)propanesulfonic acid (MOPS) buffer solution before use.

Enzyme activity determination

The kinetic assays were started by adding 10 μl of PFK, which had been diluted appropriately to an assay mixture measuring 1 ml. The assay mixture for the pH and temperature studies contained 10 mM of each of the following: sodium acetate (pK_h = 4.7), 2-[*N*-morpholino] ethanesulfonic acid (MES) (pK_h = 6.1), MOPS (pK_h = 7.2), N0(2-hydroxyethyl)piperazine-*N*′-(3-propanesulfonic acid) (EPPS) (pK_h = 8.0), and 2-[cyclohexylamino] ethanesulfonic acid (CHES) (pK_h = 9.3) in addition to 0.1 mM EDTA, 14 mM $MgCl_2$, 100 mM KCl, 2 mM dithiothreitol (DTT), 0.2 mM NADH, 3 mM ATP, 50 μg glycerin-3-phosphate dehydrogenase, 5 μg triosephosphate isomerase, 250 μg aldolase, 1 mM creatine phosphate and 10 μg/ml creatine phosphokinase. The above buffer was used for the Fru-6-P–PEP interactions, but for the Fru-6-P–MgADP interactions 10 mM KCl was present in the buffer instead of 100 mM KCl. Creatine phosphokinase and creatine phosphate were only added to the assays performed in the absence of ADP. The pH was varied from 5 to 9 as indicated and adjusted with either HCl or KOH to the appropriate value after the temperature had equilibrated. Stock solutions of Fru-6-P (disodium), phospho(*enol*)pyruvate (trisodium), and ADP (dipotassium) were also adjusted to the proper pH at the indicated temperature. Steady-state rates were measured after the completion of any slow pre-steady-state transients (Reinhart and Lardy, 1980). The rates were determined by observing the oxidation of NADH at 340 nm using a Beckman DU spectrophotometer updated with Gilford electronics and strip-chart recorder. Rates are expressed as units per milligram of protein. One unit is defined to be equal to the formation of 1 μmol of fructose-1,6-bisphosphate per minute.

The concentration of Fru-6-P producing one-half maximal velocity under each particular set of conditions, K_a, was determined from the *x* axis intercept of the Hill plot resulting from a least-squares linear regression of data plotted as $\log[v/(V_{max} - v)]$ versus [Fru-6-P], where V_{max} is maximal velocity estimated from a direct plot of v versus log[Fru-6-P] and v is the reaction rate. The apparent K_a values for Fru-6-P as a function of MgADP and PEP concentrations were determined at pH 5 to 9 and temperatures between 5 and 40°C. Most of the assay conditions were repeated at least once on a different day to detect operational error. PEP and MgADP have no influence on V_{max} at all temperatures and pH values examined (data not shown).

Protein determination

The protein concentration was determined colorimetrically by reaction with BCA (Pierce, Rockford, IL) using bovine serum albumin as a standard. Both samples and standards contained 10% buffer to correct for possible interference by the buffer reagents.

Linkage analysis

The notation we use has been previously defined (Johnson and Reinhart, 1994, 1997). To summarize briefly, the ligands are designated with the subscripts h, a, x, and y for the protons, Fru-6-P, MgADP, and PEP, respectively. The dissociation constants of Fru-6-P, MgADP, and PEP in the absence of any other ligands are designated as K^o_{ia}, K^o_{ix}, and K^o_{iy}, respectively. [This notation is a modification of the notation proposed by Cleland (1963) in which the terms K_a and K_{ia} were used to distinguish between the Michaelis constant and the thermodynamic dissociation constant, respectively, of substrate "A."] The dissociation constants of these ligands may be altered if one or more other ligands are bound to the enzyme. The bound ligands will be denoted in the subscript following "/". Thus $K_{ia/xh}$ refers to the dissociation constant for Fru-6-P dissociating from PFK with MgADP bound at the effector site of the protonated enzyme. $K_{ix/ah}$ refers to MgADP dissociating from the effector site with Fru-6-P and protons bound. Since all of the experiments reported in this paper were performed with a saturating concentration of MgATP, all parameters should include a "b" after the slash in their notation to be completely consistent with the notation used previously (Johnson and Reinhart, 1994, 1997).

The different dissociation constants pertaining to the ligands under investigation are not all independent, since they are related by the principles of thermodynamic linkage (Wyman, 1964, 1967; Weber, 1972, 1975) and the various coupling parameters that quantify the influence that two ligands can have on each other's binding (Weber, 1975; Reinhart, 1983, 1988). The thermodynamic linkage of the binding of ligands A and X, for example, is given by

$$\frac{K^o_{ia}}{K_{ia/x}} = \frac{K^o_{ix}}{K_{ix/a}} = Q_{ax} \tag{1}$$

These ratios define the coupling constant, Q_{ax}, which quantitatively describes the magnitude of the allosteric effect that results from the binding of the effector ligand. Note that $Q_{ax} > 1$ for activators, and $Q_{ax} < 1$ for inhibitors. If $Q_{ax} = 1$ there is no effect of the allosteric ligand on the binding of substrate, and vice versa.

The coupling constant Q_{ax} is related to the corresponding coupling free energy (ΔG_{ax}) and its constituent enthalpy (ΔH_{ax}) and entropy (ΔS_{ax}) at constant temperature (T), by the following expressions (Reinhart et al., 1989):

$$\Delta G_{ax} = -RT\ln(Q_{ax}) = \Delta H_{ax} - T\Delta S_{ax} \tag{2}$$

It is also evident from Eqs. 1 and 2 that these parameters directly relate to the following disproportionation equilibrium:

$$\text{X–E} + \text{E–A} \rightleftharpoons \text{E} + \text{X–E–A} \tag{3}$$

where X–E and E–A represent enzyme with allosteric ligand and substrate bound respectively, E represents free enzyme with neither ligand bound, and X–E–A represents a ternary complex of enzyme with both ligands bound simultaneously. Similar relationships and equilibria pertain to the inhibition by PEP.

RESULTS

We have demonstrated previously that the rapid-equilibrium assumption is valid as it pertains to Fru-6-P binding to *B. stearothermophilus* PFK during steady-state turnover (Tlapak-Simmons and Reinhart, 1994) and that the variation of the apparent Michaelis constant for Fru-6-P (K_a) with MgADP is described by the following relationship:

$$K_a = K^o_{ia}\left[\frac{K^o_{ix} + [X]}{K^o_{ix} + Q_{ax}[X]}\right] \tag{4}$$

A virtually identical relationship holds for PEP (substituting Y for X in the notation) where K_a now represents the $K_{0.5}$ when Fru-6-P binding curves exhibit positive cooperativity.

By fitting data obtained at various pH values and temperatures to Eq. 4 we can therefore examine the effect of pH and temperature on each of the parameters K^o_{ia}, K^o_{ix}, K^o_{iy}, Q_{ax}, and Q_{ay}.

The effect that pH has on K^o_{ia}, K^o_{ix}, and K^o_{iy} at 25°C can be seen in Fig. 1. These data in turn can be fit to an equation analogous to Eq. 4 to ascertain the coupling between each ligand (Fru-6-P, MgADP, or PEP, respectively) and protons. The solid curves in Fig. 1 represent the best fit in each case.

It can be seen from the data presented in Fig. 1 that pH has in general only a small influence on the dissociation constants for each ligand in the absence of other ligands. For each ligand the coupling is >1, implying that protonation increases the affinity for each ligand, although for Fru-6-P the increase is only 30% and for MgADP is ~60%. The affinity for PEP increases somewhat more, ~threefold at the low pH limit. The apparent pK_h for PFK is similarly perturbed to the same extent by the binding of each ligand. The pK_h in the absence of ligands is ~7.0 when Fru-6-P and PEP bind, and slightly higher (7.6) when MgADP binds, suggesting possibly a different protonation influences the binding of this ligand. van't Hoff analyses of the temperature dependencies of these dissociation constants are unremarkable in that they exhibit varying degrees of heat capacity, as is normally observed in protein–ligand binding reactions (data not shown). The dissociation constants determined at 25°C for the various ligated forms of *B. stearothermophilus* PFK are presented in Table 1. The pK_h values derived from these data vary insignificantly with temperature (data not shown).

The coupling parameters between Fru-6-P and either MgADP (Q_{ax}) or PEP (Q_{ay}) are influenced in significant ways by both temperature and pH. In Fig. 2 we present van't Hoff plots of the variation in either Q_{ax} (Fig. 2 *A*) or Q_{ay} (Fig. 2 *B*) with reciprocal temperature at each of several different pH values ranging from pH 5 to pH 9. At each pH value both coupling parameters generate van't Hoff plots well described by straight lines, indicating a reasonably constant ΔH over the temperature range examined (5–40°C except for pH 5). The slopes vary systematically with pH, with the values becoming smaller in absolute value with increasing pH. At low pH the values of $\log(Q_{ax})$ transition in a continuous manner from positive to negative values as reciprocal temperature increases, indicating that MgADP becomes an inhibitor of Fru-6-P binding at low temperatures (Braxton et al., 1994). The "crossover" temperature is clearly pH dependent, and at low temperature MgADP will become an inhibitor as pH is dropped as well. The overall small absolute values of $\log(Q_{ax})$ indicate that MgADP has a fairly small effect throughout this range of temperature and pH.

TABLE 1 Ligand dissociation constants from *B. stearothermophilus* PFK at 25°C with MgATP concentration equal to 3 mM

Ligand	Other Saturating Ligands	Designation	K_d (μM)
Fru-6-P	—	K^o_{ia}	30.7 ± 0.4
″	MgADP	$K_{ia/x}$	17.7 ± 0.3
″	PEP	$K_{ia/y}$	(21.6 ± 1.2) × 10^3
″	H^+	$K_{ia/h}$	23.9 ± 0.9
″	MgADP, H^+	$K_{ia/hx}$	24.1 ± 1.0*
″	PEP, H^+	$K_{ia/hy}$	(1.5 ± 0.06) × $10^{3\#}$
MgADP	—	K^o_{ix}	29.4 ± 3.5
″	Fru-6-P	$K_{ix/a}$	17.0 ± 2.0
″	PEP	$K_{ix/h}$	16.9 ± 2.9
″	Fru-6-P, H^+	$K_{ix/ah}$	17.1 ± 2.9*
PEP	—	K^o_{iy}	119.2 ± 7.9
″	Fru-6-P	$K_{iy/a}$	(83.9 ± 7.3) × 10^3
″	H^+	$K_{iy/h}$	33.4 ± 3.2
″	Fru-6-P, H^+	$K_{iy/ah}$	(1.46 ± 0.15) × $10^{3\#}$

* pH = 5.0.
\# pH = 5.5.

The effect of PEP by comparison is quite substantial and consistently inhibitory [$\log(Q_{ay}) < 0$] throughout the temperature and pH range examined. The slopes of the van't Hoff plots, which are positive, increase as pH increases. The influence of pH on the magnitude of inhibition at any temperature examined is substantial, causing the coupling parameter to diminish (i.e., the extent of inhibition to increase) by over an order of magnitude as pH is increased from 5.5 to 9. The coupling parameters involving each allosteric ligand, including protons, determined at 25°C are listed in Table 2.

The essentially linear van't Hoff plots can yield estimates of the contributions made by ΔH and $T\Delta S$ at 25°C to the coupling free energy as a function of pH for each allosteric ligand. These results, along with the corresponding coupling free energy values, are presented for MgADP and PEP in Fig. 3, *A* and *B*, respectively. In Fig. 3 *A* it is evident that as pH increases both ΔH_{ax} and $T\Delta S_{ax}$ significantly diminish in size. This change occurs primarily between pH 7 and pH 8, whereas the observed decrease in ΔG_{ax} occurs at pH values

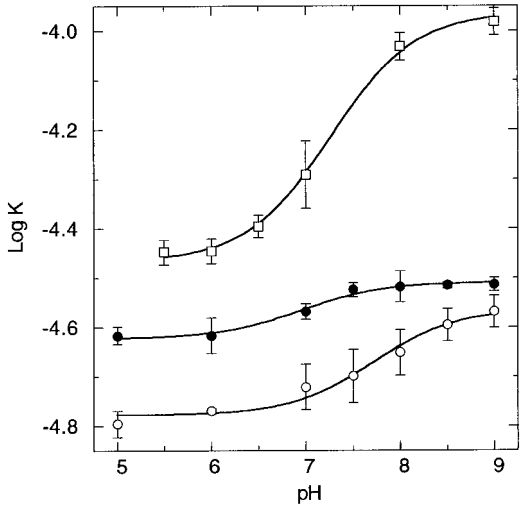

FIGURE 1 Variation of logarithm of K^o_{ia} (●), K^o_{ix} (○), and K^o_{iy} (□) as a function of pH at 25°C. The curves were obtained by fitting each data set to an equation of the form presented in Eq. 4 as described in the text.

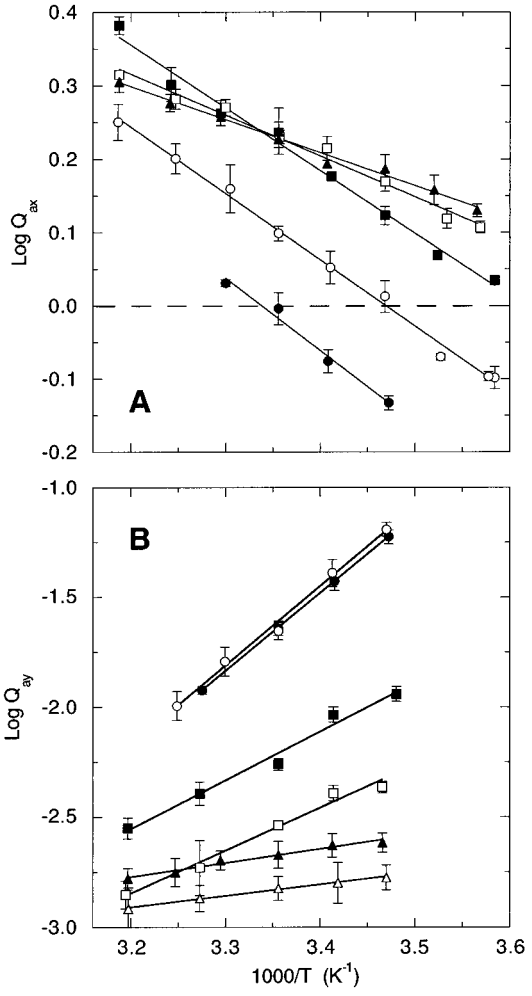

FIGURE 2 van't Hoff analyses of the coupling constant at various pH values between MgADP–Fru-6-P (*A*) and PEP–Fru-6-P (*B*). The pH values in *A* are equal to: 5 (●), 6 (○), 7 (■), 8 (□), 9 (▲). In *B* the pH values equal: 5.5 (●), 6 (○), 6.5 (■), 7 (□), 8 (▲), 9 (△).

<pH 7. Thus the pH dependence of the observed allosteric effect (ΔG_{ax}) is due to the small difference in the magnitude of the pH-dependent change in ΔH_{ax} compared to $T\Delta S_{ax}$ and may, therefore, appear shifted relative to the principal effects on ΔH_{ax} and $T\Delta S_{ax}$. Also, inspection of Figs. 2 *A* and 3 *A* suggests that the influence of temperature is primarily one of displacement of the $T\Delta S_{ax}$ curve relative to the ΔH_{ax} curve, which remains invariant with temperature within the range of temperature values examined.

ΔH_{ay} and $T\Delta S_{ay}$ associated with the inhibition by PEP display a very large influence of pH, decreasing by over 10 kcal/mol when pH is decreased from pH 9 to pH 5 (Fig. 3 *B*). ΔG_{ay}, by comparison, decreases by only 3 kcal/mol over this same pH range because of the high degree of compensation that occurs between the ΔH_{ay} and $T\Delta S_{ay}$ values.

DISCUSSION

Despite the fact that pH has no influence on the inaction of the allosteric ligands toward k_{cat}, the influence of pH on the allosteric properties of PFK from *B. stearothermophilus* is quite significant. Although the data suggest only a small effect on the binding of substrate and allosteric ligands, the perturbation of the energetics underlying the allosteric interactions is quite substantial.

TABLE 2 Coupling constants (*Q*) quantifying the interactions among Fru-6-P, MgADP, PEP, and protons on *B. stearothermophilus* PFK at 25°C with [MgATP] = 3 mM

Interacting Ligands	Other Saturating Ligand	Designation	Q
Fru-6-P–MgADP	—	Q_{ax}	1.73 ± 0.01
″	H^+	$Q_{ax/h}$	0.99 ± 0.02*
Fru-6-P–PEP	—	Q_{ay}	(1.42 ± 0.08) × 10^{-3}
″	H^+	$Q_{ay/h}$	(2.28 ± 0.10) × 10^{-2}#
Fru-6-P–H^+	—	Q_{ah}	1.28 ± 0.05
″	MgADP	$Q_{ah/x}$	0.73 ± 0.13*
″	PEP	$Q_{ah/y}$	20.6 ± 2.5#
MgADP–H^+	—	Q_{hx}	1.74 ± 0.21
″	Fru-6-P	$Q_{hx/a}$	0.99 ± 0.13*
PEP-H^+	—	Q_{hy}	3.56 ± 0.24
″	Fru-6-P	$Q_{hy/a}$	57.2 ± 6.3#
Fru-6-P–MgADP-H^+	—	Q_{ahx}	2.20 ± 0.28*
Fru-6-P–PEP–H^+	—	Q_{ahy}	0.104 ± 0.009#

* pH = 5.0.
# pH = 5.5.

MgADP interactions

At 25°C increasing pH decreases the binding affinity by ~60% (Fig. 1). At all temperatures and pH values within the ranges examined, the effect of MgADP on the affinity displayed by PFK for Fru-6-P is also small. The largest value for this coupling observed was a 2.5-fold increase at 40°C (log Q_{ax} = 0.4) as shown in Fig. 2 *A*, which represents a coupling free energy of −0.5 kcal/mol. At any given pH value, decreasing temperature lowers this value, and at low pH the coupling free energy proceeds systematically through a value of 0 kcal/mol (indicative of no allosteric affect) to positive values (indicative of inhibition) as previously noted (Braxton et al., 1994). The temperature at which this crossover occurs is a function of pH, equaling ~35°C at pH 6 and ~25°C at pH 5.

Upon initial examination, the effect of pH appears to be variable, with increasing pH causing an increase in Q_{ax} at high temperature and a decrease in Q_{ax} at low temperature. However, the nature of the relationship between the temperature effects and pH effects on the coupling free energy is more clearly revealed by the effect of pH on the individual ΔH_{ax} and $T\Delta S_{ax}$ terms deduced from the van't Hoff plots presented in Fig. 2 *A*. At each pH examined the van't Hoff plot is quite linear, indicating that both ΔH_{ax} and ΔS_{ax} are essentially constant over the temperature range examined. Increasing the pH causes a significant drop in the value of both of these parameters from pH 7 to pH 8.5 such that ΔH_{ax} and $T\Delta S_{ax}$ change by almost, but not quite, the same extent as shown in Fig. 3 *A*. It is the small disparity in the

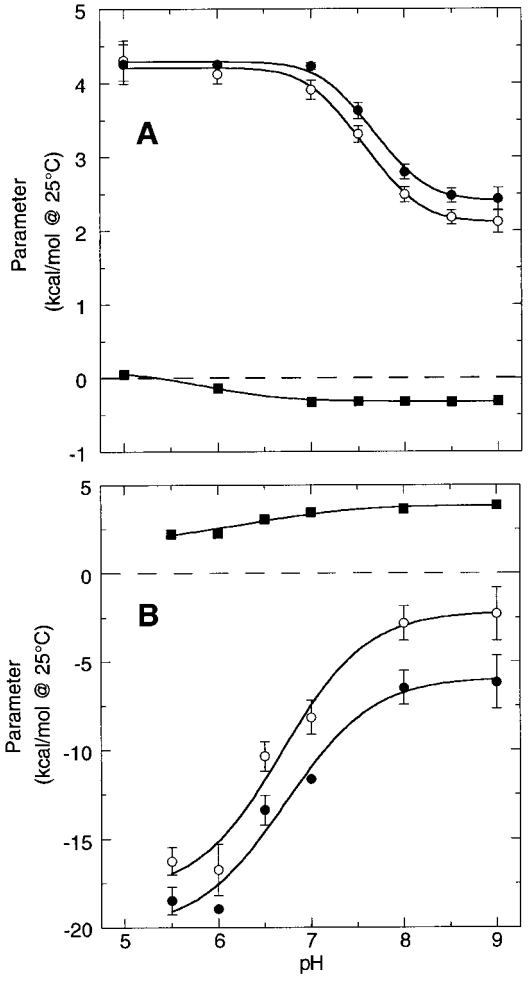

FIGURE 3 Parameters determined from the van't Hoff plots as a function of pH. (*A*) MgADP–Fru-6-P interaction. (■), ΔG_{ax}; (○), ΔH_{ax}; (●), $T\Delta S_{ax}$. (*B*) PEP–Fru-6-P interaction. ΔH_{ay} (○), $T\Delta S_{ay}$ (●), ΔG_{ay} (■)

values of ΔH_{ax} and $T\Delta S_{ax}$, rather than the comparatively large drop in their individual values, that causes the observed pH-dependent change in the coupling free energy and hence the allosteric effect by MgADP. Changing temperature results only in a vertical displacement of the $T\Delta S_{ax}$ curve in Fig. 3 *A*, and given the close proximity of the curves, increasing temperature causes the curves to cross over at increasing pH values, thus increasing the temperature at which the sign of ΔG_{ax}, and hence the nature of the allosteric effect, changes. This phenomenon also causes the apparent pH-dependence of ΔG_{ax} (the *observed* allosteric effect) to appear shifted relative to the direct effect observed on its ΔH_{ax} and ΔS_{ax} components.

It would appear, therefore, that protonation causes a significant structural perturbation, indicated by the ~2-kcal increase in ΔH_{ax} (and $T\Delta S_{ax}$) but that the functional consequence of this perturbation is almost completely negated by the enthalpy–entropy compensation that results. The small change in allosteric effectiveness with protonation that is seen is due to the very small degree of incompleteness in the enthalpy–entropy compensation, but this small disparity between ΔH_{ax} and $T\Delta S_{ax}$ is enough to cause the sign of ΔG_{ax} to change from negative to positive at temperatures >25°C when the enzyme is protonated.

Changing temperature alone does not produce its crossover effect by changing either ΔH_{ax} or ΔS_{ax}. Consequently, we conclude that the temperature effect is not the result of a temperature-induced change in the protonation state of the enzyme, but rather a direct manifestation of the normal temperature dependence of free energy as conveyed by the relationship given in Eq. 2.

PEP interactions

The effects of temperature and pH on the allosteric influence of PEP is even more pronounced quantitatively, although qualitatively the effects are similar to those observed for MgADP. pH has a somewhat larger effect on the binding of PEP, causing the K_d for PEP to increase threefold upon deprotonation at 25°C (Fig. 1). The effect of temperature on Q_{ay} is much more pronounced, as indicated by the much steeper slopes in Fig. 2 *B*, yet linear van't Hoff relationships are maintained at all pH values. As shown in Fig. 3 *B*, both ΔH_{ay} and $T\Delta S_{ay}$ decrease by over 10 kcal/mol at 25°C as pH is lowered. Yet despite these large changes, the coupling free energy decreases by little over 2 kcal/mol over the same range because of the high degree of compensation between the enthalpic and entropic contributions. The compensation is somewhat less at high pH, which leads to the greater coupling free energy at high pH, despite the fact that the coupling components, ΔH_{ax} and $T\Delta S_{ax}$, are individually much smaller at high pH.

OVERALL CONCLUSIONS

These data emphasize the fact that structural changes and functional changes associated with allosteric effects may not directly correlate. The absolute value of the enthalpy and entropy terms that comprise the coupling free energies describing the actions of the allosteric ligands of *B. stearothermophilus* PFK are individually much larger at low pH, yet the absolute value of the coupling free energies are much larger (and hence the allosteric effects are much larger) at high pH. Protonation clearly influences the physical basis for the interaction between the two bound ligands. The magnitude of change in ΔH_{ay} associated with the PEP inhibition, for example, is equivalent to at least five new H-bonds being formed at low pH. Whatever the physical or structural basis for the coupling interaction, it is therefore likely to be more evident at low pH despite the diminished allosteric effect that is observed at low pH. It is also important to note that the change in charge resulting from changing pH does not just change ΔH_{ax}. $T\Delta S_{ax}$ changes to nearly the same extent, leading to nearly perfect compensation.

It is also clear from these data that protonation of *B. stearothermophilus* PFK cannot be considered as favoring an allosterically "inhibited form" or "active form" of the

enzyme as a two-state model might predict. At low pH all of the ligands examined—a substrate, an activator, and an inhibitor—bind more tightly. At low pH PEP is a weaker inhibitor and MgADP is a weaker activator—even becoming an inhibitor at high temperatures. Instead, the significance of pH is that upon protonation the allosteric couplings lead to more pronounced *changes* in both ΔH and $T\Delta S$ for Fru-6-P binding.

It should be noted, however, that both the inhibition by PEP and the activation by MgADP are established by the entropy component of the coupling free energy regardless of pH. In other words, the signs of the coupling free energy and the coupling enthalpy are opposite one another under all conditions we have examined except when MgADP becomes an inhibitor at low temperature, indicating that the enthalpy associated with the couplings favors an allosteric effect opposite of what is observed. This is another contrast with PFK from *E. coli*, which exhibits enthalpy-dominated couplings for PEP and MgADP (Johnson and Reinhart, 1994, 1997), suggesting fundamentally different mechanisms might be responsible for transmitting the allosteric "communication" between sites. Entropy-dominated allosteric couplings have also been observed for PFK from rat liver (Reinhart et al., 1989), and carbamoyl phosphate synthetase from *E. coli* (Braxton et al., 1996).

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