Published in final edited form as:

Methods Enzymol. 2009 ; 466: 83–107. doi:10.1016/S0076-6879(09)66005-5.

The impact of ions on allosteric functions in human liver pyruvate kinase

Aron W. Fenton and

Department of Biochemistry and Molecular Biology, The University of Kansas Medical Center, MS 3030, 3901 Rainbow Boulevard, Kansas City, KS 66160, Phone: (913) 588-7033, Fax: (913) 588-9896

Aileen Y. Alontaga

Department of Biochemistry and Molecular Biology, The University of Kansas Medical Center, MS 3030, 3901 Rainbow Boulevard, Kansas City, KS 66160, Phone: (913) 588-9812, Fax: (913) 588-9896

Aron W. Fenton: afenton@kumc.edu; Aileen Y. Alontaga: aalontaga@kumc.edu

Abstract

Experimental designs used to monitor the magnitude of an allosteric response can greatly influence observed values. We report here the impact of buffer, monovalent cation, divalent cation and anion on the magnitude of the allosteric regulation of the affinity of human liver pyruvate kinase (hL-PYK) for substrate, phosphoenolpyruvate (PEP). The magnitudes of the allosteric activation by fructose-1,6-bisphosphate (Fru-1,6-BP) and the allosteric inhibition by alanine are independent of most, but not all buffers tested. However, these magnitudes are dependent on whether Mg^{2+} or Mn^{2+} is included as the divalent cation. In the presence of Mn^{2+} , any change in $K_{app-PEP}$ caused by Fru-1,6-BP is minimal. hL-PYK activity does not appear to require monovalent cation. Monovalent cation binding in the active site impacts PEP affinity with minimum influence on the magnitude of allosteric coupling. However, Na^+ and Li^+ reduce the magnitude of the allosteric response to Fru-1,6-BP, likely due to mechanisms outside of the active site. Which anion is used to maintain a constant monovalent cation concentration also influences the magnitude of the allosteric response. The value of determining the impact of ions on allosteric function can be appreciated by considering that representative structures used in comparative studies have often been determined using protein crystals grown in diverse buffer and salt conditions.

Introduction

A review of recent literature demonstrates that there is a broad renewed interest in understanding the molecular basis of allosteric regulation. In our own studies we prefer to use a strict definition of allostery as the energetic coupling between two binding events on the same protein (Fenton, 2008). However, many other definitions of allosteric regulation are in widespread use. The commonality of these definitions is the propagation of an effect due to a local perturbation (most often ligand binding) through the protein. In turn, this results in change in protein structure and/or function at a site that is distinct (and often distant) from the original site of perturbation. Here we use protein structure to refer to the combined characteristics of the average protein conformation and protein dynamics. Protein dynamics is in turn defined as fluctuations within a stable protein without external

perturbations (i.e. a change in average conformation upon ligand binding is not included in a reference to “dynamic”). Of the two components of structural descriptions, crystallographic techniques have produced a wealth of understanding about protein conformation. Although protein dynamics have been recognized for many years (Cui and Karplus, 2008; Frauenfelder et al., 1988), the number of techniques that can monitor protein dynamics have been limited until recently. It follows that much of the discussion in the latest literature surge focuses on how protein dynamics (rather than more historical focuses on conformation) contribute to propagation of an allosteric signal through a protein (Formanek et al., 2006; Gunasekaran et al., 2004; Kern and Zuiderweg, 2003; Popovych et al., 2006; Swain and Gierasch, 2006; Volkman et al., 2001).

Studies of both protein conformation and dynamics (and even evolution (Lockless and Ranganathan, 1999; Pendergrass et al., 2006; Suel et al., 2003)) approach the structure/function relationship of allosteric regulation from the structural side. Therefore, building on the groundwork established by Weber (Weber, 1972), Reinhart has outlined the application of allosteric linkage to quantitatively characterize allosteric functions in enzymes undergoing steady-state turnover (Reinhart, 1983; Reinhart, 1988; Reinhart, 2004). Therefore, the theoretical basis of allosteric linkage between two ligand binding events to one protein has now been thoroughly discussed for both systems that operate at equilibrium and those that function at steady-state. The focus of the current work highlights technical aspects of data acquisition necessary to facilitate a linkage analysis. Specifically, the binding affinity of one ligand (which typically requires titration using a concentration range of this ligand) is determined over a concentration range of a second ligand (see below). If one or both ligands carry charge, a variation in the concentration of that ligand will be accompanied by a pH gradient, an ionic strength gradient, and/or a counter-ion concentration gradient, depending on experimental design. Any one of these gradients can modify protein function independent of or in concert with modifications caused by ligand binding.

We are interested in understanding the molecular basis of the allosteric regulation of human liver pyruvate kinase (L-PYK for liver isozymes in general; hL-PYK for the human protein). Pyruvate kinase (PYK) catalyzes the conversion of phosphoenolpyruvate (PEP) and MgADP to pyruvate and MgATP as the last step of glycolysis. There are four different mammalian PYK isozymes. L-PYK and the isozyme expressed in erythrocytes (R-PYK) are products of a single gene due to the use of different translation start sites and differ only by the additional 31 amino acids located at the N-terminus of R-PYK (Noguchi et al., 1987); the muscle (M₁-PYK) and “fetal” (M₂-PYK) forms of PYK are gene products from a second gene. Historically accepted allosteric regulatory features of L-PYK include: 1) allosteric activation of PEP affinity by fructose-1,6-bisphosphate (Fru-1,6-BP); 2) allosteric inhibition of PEP affinity by alanine (Ala); and 3) allosteric inhibition of PEP affinity by ATP. It is also historically accepted that binding of allosteric effectors to L-PYK alters the affinity of the enzyme for PEP without altering the catalytic rate (k_{cat}) or the affinity of the enzyme for MgADP (Blair, 1980; Hall and Cottam, 1978; Munoz and Ponce, 2003). We find the best definition for this “K-type” allostery is a comparison of how one ligand binds in the absence vs. in the presence of a second ligand (Fenton, 2008). This definition facilitates a linked-equilibrium analysis of this regulatory phenomenon.

PEP binding in the active site of PYK is in large part due to coordination with both a divalent and a monovalent cation (Figure 1) (Larsen et al., 1997). It has been historically accepted that both ions are required for enzymatic activity, as is the divalent cation bound form of ADP (Boyer, 1962; Nowak and Suelter, 1981). Given the inherent challenges due to changing counter-ion concentrations associated with the ligand titrations (introduced above), the ion requirements of PYK further complicate a study of the allosteric properties of hL-PYK. Therefore, this work presents experimental designs that have been useful in exploring

how ion types (and to a less extent concentration) impact observed allosteric responses of hL-PYK to Fru-1,6-BP and Ala.

General Strategy to assess allosteric coupling

Figure 2 represents the energy cycle that describes allosterity. The relationship between dissociation constants that defines the allosteric coupling constant (Q_{ax}) is (Weber, 1972):

$$Q_{ax} = K_{ia}/K_{ia/x} = K_{ix}/K_{ix/a} \quad \text{Equation 1}$$

If $Q_{ax} > 1$, the allosteric effector (X) causes increased affinity of the protein for A. If $Q_{ax} < 1$, the allosteric effector causes decreased affinity of the protein for A. If $Q_{ax} = 1$, there is no allosteric coupling between A and X. One established method to determine Q_{ax} is to monitor the affinity (or apparent affinity, K_{app} , derived from initial velocity techniques, see (Reinhart, 1983)) of the protein for one ligand as a function of the concentration of the second ligand (Di Cera, 1995; Reinhart, 2004). On a log-log plot, the allosteric coupling is the difference between the upper and lower plateaus (Figure 3). Because the allosteric coupling is a comparison of dissociation (or affinity) constants, it is independent of either the substrate affinity in the absence of effector or the effector affinity in the absence of substrate. Therefore, when an allosteric system is perturbed (e.g. introduction of mutations or modification of ligand), the varied experimental conditions may alter the ligand affinities, allosteric coupling or both (Fenton et al., 2003; Williams et al., 2006). This is graphically exemplified in Figure 3, in which curves A, B, and C share a common Q_{ax} value. Curve D represents a condition with an altered value of Q_{ax} .

PYK assay

Throughout this work, several components of the assay are independently varied. Therefore, a “standard assay” will be described here and used as the control in comparative studies. Variations from the standard assay will be detailed in the relevant sections. We have previously demonstrated the pH dependency of the allosteric functions of hL-PYK (Fenton and Hutchinson, 2009); with the exception of studies that vary divalent cation, pH will be maintained at pH 7.5 in the current study. Creation of the E130K mutation, protein expression, and protein purification (ammonium sulfate fractionation and DEAE-cellulose column) were as previously described (Fenton and Hutchinson, 2009; Fenton and Tang, 2009). Purified protein was stored in 10 mM MES (pH 6.8) 5 mM $MgCl_2$, 10 mM KCl and 2 mM DTT and characterized within 2 days after the last step of purification. When monovalent or divalent cations were varied, purified hL-PYK and lactate dehydrogenase were first desalted with Sephadex G-50 (Sigma) resin into the respective assay buffer before use.

Activity measurements in the “standard assay” were carried out at 30 °C using a lactate dehydrogenase coupled assay. Reactions contained 50 mM bicine pH 7.5, 10 mM $MgCl_2$, 2 mM (K)ADP, 0.1 mM (2Na)EDTA, 0.18 mM (2Na)NADH, and 19.6 U/mL lactate dehydrogenase. (K)PEP and effector concentrations were varied as indicated. Stock solutions of PEP and effectors were adjusted to pH 7.5 with KOH before addition and serial dilutions of ligands were in KCl to maintain constant K^+ concentration. K^+ concentrations from additions of KOH and from counter-ions of ligands were summed and KCl was supplemented to a total K^+ concentration of 150 mM in all assays. Due to the high affinity for Fru-1,6-BP, only low concentrations of (Na)Fru-1,6-BP are required; the Na^+ contributed by additions of this ligand are considered negligible. A solution of Ala in water has a pH near pH 7.5. Therefore, there is essentially no change in cation concentrations or ionic strength over a concentration range of Fru-1,6-BP or Ala. Independent of other sources

of ions, the cumulative Na^+ concentration (in all assays in this study) derived from EDTA, NADH, (and when applicable Fru-1,6-BP) ranges from 0.56 mM to 0.65 mM in the absence and saturating presence of Fru-1,6-BP, respectively. To minimize pipetting error, all assay components except effector and PEP were combined in a cocktail. Therefore, three additions were made to each well of a 96-well plate using a 12-channel pipettor: 1) cocktail, 2) effector, 3) PEP. All volumes added were greater than 70 μL . The enzymatic reaction was initiated with PEP and monitored at 340 nm over time in a UV-Star flat bottom 96-well plate (Greiner Bio-one) containing a total reaction volume of 350 μL . All activity readings were collected using a Molecular Devices Spectramax Plus384 spectrophotometer. 30 °C approaches the minimum temperature (ambient + 5°) requirement of the plate reader used for semi high throughput data collection.

The use of monovalent cations other than K^+ and Na^+ (these two are the cations available in commercial sources of PEP and ADP) required ion exchange. This exchange was performed by mixing (K)PEP or (K)ADP with Dowex 50WX4 resin (Sigma) in a ration of 1 mol (K)ligand to 4000g (wet weight) of washed resin. Once ligand and resin were suspended in a minimum volume of water, the suspension was allowed to equilibrate at 4°C for 30 minutes and then the acidic form of the ligand was recovered using either a syringe filter or Buchner funnel/filter paper. The acidic form of ligands were lyophilized to near dryness and stored at -20°C. Immediately before use, lyophilized samples were resuspended in water. pH was adjusted to 7.5 with measured quantities of the desired base. PEP and ADP concentrations were determined using the pyruvate kinase/lactate dehydrogenase assay and monitoring the absolute A_{340} difference (similar to (Czof and Lamprecht, 1974)). Dilutions of cation-PEP and cation-effector were in the respective cation-Cl.

Data were fit to appropriate equations using the nonlinear least-squares fitting analysis of Kaleidagraph (Synergy) software. As previously noted, the dependency of enzymatic activity on PEP concentration is biphasic (Fenton and Hutchinson, 2009). $K_{\text{app-PEP}}$ values were obtained by fitting initial rates obtained from kinetic assay to:

$$v = \frac{V_{\text{max}}[\text{PEP}]^{n_H}}{(K_{\text{app-PEP}})^{n_H} + [\text{PEP}]^{n_H}} + c[\text{PEP}], \quad \text{Equation 2}$$

Where V_{max} is the maximum velocity associated with the low PEP phase, $K_{\text{app-PEP}}$ is the concentration of substrate that yields a rate equal to one-half the V_{max} , and n_H is the Hill coefficient associated with the low PEP (i.e. high PEP affinity) phase. The response of activity to increasing concentrations of PEP is biphasic, although a maximum activity for the second phase has not been obtained (Fenton and Hutchinson, 2009). We have speculated multiple potential mechanisms that might give rise to the biphasic response. The protein may actually bind PEP to active sites with different affinities, such that the tetramer functions as a dimer of dimer. In this mechanism, c would be the $V_{\text{max}}/K_{\text{app-PEP}}$ for the low affinity PEP phase (Fenton and Hutchinson, 2009). Due to the experimental design aimed at holding cation concentrations constant, Cl^- concentration decreases as PEP concentration increases. Dilution of Cl^- , change in total anion concentration over the PEP concentration range, and/or PEP binding to some site other than the active site may give rise to the second phase. In this second speculation, c is the linear response expected due to non-specific effects caused by changing concentrations of anions.

$K_{\text{app-PEP}}$ values obtained from Eq. (2) were plotted as a function of effector concentration and fit to (Reinhart, 1983; Reinhart, 1988; Reinhart, 2004):

$$K_{app-PEP} = K_a \left(\frac{K_{ix} + [\text{Effector}]}{K_{ix} + Q_{ax} [\text{Effector}]} \right) \quad \text{Equation 3}$$

where $K_a = K_{app-PEP}$ when $[\text{Effector}] = 0$, K_x = the dissociation constant for effector when $[\text{PEP}] = 0$ and Q_{ax} = the coupling constant between PEP and the effector. Q_{ax} is related to the coupling free energy, ΔG_{ax} between PEP and effector by (Johnson and Reinhart, 1994):

$$\Delta G_{ax} = -RT \ln(Q_{ax}). \quad \text{Equation 4}$$

To gain some appreciation for the reproducibility of data collection, Figure 4 presents 9 data sets for Ala inhibition and 7 data sets for Fru-1,6-BP activation collected using 7 different protein preparations, and collected over 24 months. Fit parameters obtained from simultaneously fitting all data to Eq. (3) are presented in Table I. These parameters are in good agreement with those previously reported from the same assay conditions, with the exception that HEPES was previously used as the buffer (Fenton and Tang, 2009). In Table I, each parameter is shown $\pm$ the error estimate determined by this simultaneous fit. In addition, each data set was fit individually. The standard deviation of the equivalent parameters determined by these individual fits is also presented in Table I. Of the parameters, evaluations of K_{ix-FBP} are the least reproducible amongst data sets.

Buffers

Useful buffers should not interact with the study system. However, many common buffers are sulfate derivatives. Sulfate can mimic phosphate in some settings. Since hL-PYK binds phosphorylated ligands at both the active site and the Fru-1,6-BP binding site, sulfate based buffers may have the potential to interact with this enzyme. Consistent with this cautionary consideration, buffer effects on L-PYK properties have previously been noted by several authors (Blair, 1980).

Initial tests of the impact of buffers were performed by replacing an effector in the standard assay with the buffer being tested (noted as “effector-buffer” to distinguish from the assay-buffer). The impact of effector-buffers on $K_{app-PEP}$ was determined in different assay-buffers Figure 5A and B. The use of Tris/HCl as the effector-buffer caused an increase in $K_{app-PEP}$ independent of whether titrations were in HEPES/KOH or bicine/KOH assay-buffer. Likewise, increasing concentrations of MOPS/KOH causes $K_{app-PEP}$ to decrease in both HEPES/KOH and bicine/KOH assay-buffers. In bicine/KOH assay-buffer, HEPES/KOH also causes $K_{app-PEP}$ to decrease. Considered in isolation, this data might indicate that a number of buffers interact with hL-PYK and therefore, modify function of the protein. Since the use of bicine as the effector-buffer did not impact $K_{app-PEP}$ when assayed in HEPES assay-buffer, bicine was chosen as the assay-buffer in the control assay. However, a finding that many different buffers interact with the same protein seems questionable. Therefore, experiments were designed to challenge this interpretation.

Within the experimental design of the standard assay, the uncontrolled variables are the concentration of Cl^- and the total anionic concentration across the concentration of effector (or effector-buffer in this instance). One experimental design that would allow changes in buffer type with minimal changes in Cl^- concentration is a comparison of the impact of allosteric effectors on $K_{app-PEP}$ in different assay-buffers. Figure 5C shows that data collected in bicine/KOH, EPPS/KOH, HEPES/KOH, and Tris/bicine are equivalent within error. Tris/bicine was added since assays with Tris/HCl have higher Cl^- than other buffers systems (However, 50 mM of Tris requires high concentrations of bicine — 213 mM — to

obtain pH 7.5; therefore the total ionic strength of this assay buffer exceeded that of other buffer systems). At 50 mM Tris/HCl (Figure 5A and B) the effect on $K_{app-PEP}$ is minimal. This minor shift is also apparent in the $K_{app-PEP}$ obtained when Tris/HCl is the assay-buffer (Figure 5C). The only buffer that resulted in considerable differences from the data obtained in other buffers was MOPS (Figure 5D). Therefore, estimations of the magnitudes of allosteric functions obtained in all buffers tested, with the exception of MOPS, are comparable (e.g. results reported in Table I for the control assay vs. those reported in HEPES (Fenton and Tang, 2009)).

The contrast between the apparent impact of titrating effector-buffers on $K_{app-PEP}$ (Figure 5A and B) and the finding that changing the type of assay-buffer does not alter estimates of K_{a-PEP} or allosteric functions (Figure 5C and D) can be explained if Cl^- inhibits K_{a-PEP} . This can be appreciated by considering that titrations of $K_{app-PEP}$ with Tris/HCl (Figure 5A and B) increases Cl^- over the concentration range of the effector-buffer, while titrations with anionic buffers decrease Cl^- over this range. Given this new information, it seems likely that previously reported buffer effects on L-PYK properties (Blair, 1980) were at least in part due to uncontrolled Cl^- concentration. Due to the potential that Cl^- ions might impact PEP affinity, further studies of the effects of anions are discussed below.

Divalent cation

During the initial characterization of PEP dephosphorylation in 1934, it was recognized that Mg^{2+} was required for enzymatic activity of PYK (Boyer, 1962). However, the role of the divalent cation in allosteric regulation remains poorly understood. This lack of understanding is likely in part due to the complex nature of this system. The complexity of a protein that binds both a free divalent cation and the divalent metal complex of ADP is well recognized. However, it is less appreciated that both Fru-1,6-BP and PEP can bind divalent metal in solution (McGilvery, 1965; Nowak and Lee, 1977). Likewise, the influence that monovalent cation concentrations have on the affinity of ADP for divalent cation have been reported (Martell and Smith, 1974–1989), yet little is understood about the impact of monovalent cation concentrations on the divalent cation interaction with hL-PYK, Fru-1,6-BP, or PEP. The potential synergistic effect in the binding of divalent and monovalent cations to the protein (Reed and Cohn, 1973; Suelter et al., 1966) and the potential of competitive binding of these ligands to the enzyme add even more layers of complexity.

Due to this complexity, the experimental design used here was to replace Mg^{2+} with other divalent metals rather than titrate the divalent metal concentration (i.e. $MgCl_2$, $MnCl_2$, or $CaCl_2$). Even this approach required additional modifications to the standard assay since saturating concentration of Mn^{2+} is limiting at pH 7.5; comparisons of the allosteric regulation elicited by both Fru-1,6-BP and Ala in either Mg^{2+} or Mn^{2+} were made at pH 6.5, 6.8, and 7.0 in 50 mM HEPES buffer.

Over the narrow pH range from 6.5 to 7.0, K_{a-PEP} increases in the presence of Mg^{2+} , but not Mn^{2+} (Figure 6). At any one pH, the magnitude of the allosteric response to Ala is dependent on which divalent cation is present; this magnitude is greater when Mg^{2+} serves as the required metal compared to when Mn^{2+} serves this role. The magnitude of the response to Ala increases as pH increases when Mn^{2+} is present, but only minimally increases when Mg^{2+} is present. The allosteric response to Fru-1,6-BP is present when Mg^{2+} is the divalent cation. The magnitude of this response increases with increasing pH. However, the ability of Fru-1,6-BP to elicit an allosteric response is questionable when Mn^{2+} is the divalent cation, at any of the three buffer pH values.

The role of divalent cation in the allosteric regulation of a PYK isozyme has most thoroughly been characterized to-date in the Fru-1,6-BP activation of yeast PYK

(Bollenbach and Nowak, 2001a; Bollenbach and Nowak, 2001b; Mesecar and Nowak, 1997a; Mesecar and Nowak, 1997b), allowing a comparison with the current results. Despite the complexities caused by multiple binding events involving divalent cation, this ligand was varied in studies of yeast PYK. In the yeast enzyme, the interactions between the protein/divalent cation and PEP are different depending on whether Mg^{2+} or Mn^{2+} fills the divalent requirement. Therefore, the interaction between PEP and divalent cation may not simply be electrostatic in nature. In addition, Mg^{2+} or Mn^{2+} mediates the allosteric coupling between PEP and Fru-1,6-BP in the presence or absence of the second substrate, MgADP. In other words, Fru-1,6-BP binding to protein is coupled directly to divalent metal affinity; PEP binding to protein is coupled directly to divalent metal affinity; but Fru-1,6-BP binding and PEP binding are not directly coupled in the absence of the divalent metal cation. Collectively, results reported for the yeast enzyme are not entirely consistent with the results shown in Figure 6; primarily, Mn^{2+} supports allosteric activation by Fru-1,6-BP in the yeast PYK isozyme, but supports no, or only a marginal allosteric response by Fru-1,6-BP in hL-PYK.

The marginal response of the $K_{app-PEP}$ for L-PYK to Fru-1,6-BP in the presence of Mn^{2+} has also been reported for the rat L-PYK isozyme (Blair and Walker, 1984). Additionally reported in the rat L-PYK system, PEP has the ability to impact Fru-1,6-BP binding even in the absence of divalent cation. This second result for the rat protein is in contrast to the expectation based on studies of yeast PYK. Therefore, the role of divalent cation in the regulation of L-PYK by Fru-1,6-BP may be dissimilar to the role of this metal in the same regulation of yeast PYK.

Monovalent cation

M_1 -PYK holds the historic honor as being the first enzyme demonstrated to require a monovalent cation for activity (Kachmar and Boyer, 1953; Nowak and Suelter, 1981; Page and Di Cera, 2006). This conclusion was based on the observation that the activity present when sodium salts of ADP and PEP were used was only 1.5% of that obtained in the presence of K^+ (Boyer, 1962; Kachmar and Boyer, 1953); 8% by later estimations (Kayne, 1971). More recent studies suggest that K^+ induces a conformational change in the active site that switches substrate binding from an ordered mechanism in the absence of K^+ (ADP cannot bind until after PEP binds) to a random mechanism in the presence of K^+ (Oria-Hernandez et al., 2005). Monovalent requirements for activity have also been noted in an *E. coli* PYK isozyme (Valentini et al., 1979). Much less is known about the role of the monovalent cation in the allosteric regulation of PYK isozymes in general, and specifically of the hL-PYK isozyme.

PEP, ADP and Fru-1,6-BP carry negative charges at physiological pH. All three ligands are commercially sold as salts of sodium and/or potassium. Since potassium is likely the physiological ion and due to the historical acceptance that Na^+ does not support enzymatic activity, the potassium salt of anionic ligands have been used in our control assay. Also, due to this dependency on potassium for activity, titrations of ligands accompanied by a potassium counter-ion (and after adjustment of pH to the desired 7.5 using measured additions of KOH) have been with a serial dilution of the titrant made in a solution of KCl, such that the final K^+ concentration in all assays across the titration is constant at 150 mM.

To determine the role of the monovalent cation, potassium salts of PEP, ADP, and KOH were replaced with the sodium salts. Dilutions of Na-PEP and Na-effector were in NaCl. Na^+ supports activity in hL-PYK; activity with Na^+ as the monovalent cation is ~40–50% that of the activity observed when K^+ is present. Since activity is observed when Na^+ is present, it is unclear if Na^+ supports activity or if activity is present even in the absence of

monovalent cation. In addition, PEP affinity is slightly decreased in the presence of Na^+ vs. K^+ (Figure 7). It is apparent that Na^+ supports both Fru-1,6-BP activation and Ala inhibition of $K_{\text{app-PEP}}$. However, the magnitude of the allosteric activation elicited by Fru-1,6-BP is greatly reduced when K^+ is substituted with Na^+ .

In the presence of Na^+ vs K^+ , different allosteric responses are observed. However, based on this data alone, it is unclear if only one or both cations elicit a response. Furthermore, if only one cation elicits a response, it is unclear whether K^+ elicits a response and the Na^+ data represents no response, or vice-versa (i.e. which data might be a control with no effect from monovalent cation). To gain some insight into this question, the allosteric response to both Fru-1,6-BP and Ala were determined over different concentrations of Na^+ or K^+ (Figure 8) Using this approach, the impact of varying Cl^- and varying ionic strength should be consistent whether Na^+ or K^+ is varied. When the data in Figure 8 are examined in isolation, the lack of change over ion concentration (i.e. $Q_{\text{ax-FBP}}$ determined in Na^+ and the $Q_{\text{ax-Ala}}$ determined in K^+) might support that these conditions reflect the absence of an effect from monovalent cation (i.e. reference data). The reservation to this interpretation is that changes in the total ionic strength that accompany the ion concentration might counter effects caused by the monovalent cation to result in an apparent lack of response over the ion concentration. As monovalent cation concentration is decreased, $Q_{\text{ax-FBP}}$ determined in K^+ approaches that determined in Na^+ . However, the opposite is true when considering $Q_{\text{ax-Ala}}$, i.e. $Q_{\text{ax-Ala}}$ determined in Na^+ approaches that determined in K^+ . Therefore, based on this data alone, it is interesting to speculate that activation by Fru-1,6-BP might be impacted by K^+ , whereas the inhibition by Ala might be impacted by Na^+ . However, upon considering results with additional monovalent cations, these results must be reconsidered (below).

Based on sequence alignments and the observation that some PYK isozymes do not require K^+ for activity, it was found that replacing a glutamic acid residue near the active site of M_1 -PYK with lysine removes the requirement of this isozyme for K^+ (Laughlin and Reed, 1997). Creating the same mutation in hL-PYK (E130K) is predicted to both fulfill the role of the active site monovalent cation and to prevent binding of monovalent cations in the active site. Note that this consideration assumes some level of similarity between the catalytic mechanism of hL-PYK and that of M_1 -PYK (this assumption is not easily tested given the level of hL-PYK activity in the presence of Na^+ , therefore there is no hL-PYK comparative control without activity). Consistent with K^+ and E130K fulfilling the same functional role in determining the $K_{\text{a-PEP}}$, this value for the wild type protein and the E130K mutant protein are very similar when measured in the presence of K^+ (Figure 7). This result can be used as evidence that the impact of the K^+ on $K_{\text{a-PEP}}$ is primarily a specific effect due to binding at the active site. In contrast, $K_{\text{a-PEP}}$ is modified by introduction of E130K when measurements are performed in the presence of Na^+ . Therefore, if Na^+ binds in the active site, it does not perform the same functional role in determining $K_{\text{a-PEP}}$ as the E130K mutant or K^+ . In addition, the magnitude of the Fru-1,6-BP allosteric response for E130K measured in Na^+ continues to be reduced compared to that measured in K^+ . Therefore, it also seems likely that the impact of Na^+ on the magnitude of Fru-1,6-BP allostery results from a mechanism involving Na^+ binding outside of the active site.

Even after efforts to determine data trends as a function of varying K^+ and Na^+ and an effort to remove specific cation binding in the active site via introduction of the E130K mutant, a convincing argument cannot be made for which allosteric response to Fru-1,6-BP and Ala represents no influence by monovalent cation (i.e. a standard for comparison). Therefore, studies were expanded to consider effects of other monovalent cations. The use of monovalent cations besides K^+ and Na^+ required ion exchange to replace K^+ from both ADP and PEP with the respective cation. Note that data collected with these alternate cations were collected once and were not repeated. Activity was observed in the presence of all cations

tested. Compared to $V_{\max}$ activity in the presence of K^+ , observed activities were: Tris (25%), NH_4^+ (140%), cyclohexylammonium (25%), Li^+ (25%), Cs^+ (12%) and Rb^+ (75%). Since Tris and cyclohexylammonium are much larger than the single atom cations bound in crystal structures, these larger ions are not likely to bind in the active site. Therefore, it appears that hL-PYK does not require a monovalent cation for activity. However, it should also be noted that the potential sources of contaminating monovalent cation, including NH_4^+ carryover with protein, are sufficiently numerous to prevent an estimate of the level of contamination by non-specified monovalent cations.

Considering allsoteric responses of wild type hL-PYK in the presence of the various monovalent cations tested (Figure 7), three groups can be distinguished. Data collected in K^+ , NH_4^+ , Rb^+ , and Cs^+ show similar PEP affinities and allosteric responses. Na^+ and Li^+ constitute a second group with (as compared to the first group) reduced affinity for PEP and a reduced magnitude of the allosteric response to Fru-1,6-BP. The final group results from the use of either Tris or cyclohexylammonium as the monovalent cation; this group displays the lowest affinity for PEP, but a magnitude of allosteric activation by Fru-1,6-BP similar to that of the first group. If we return to the reasoning that Tris or cyclohexylammonium are not likely to bind in the active site, then it seems reasonable to conclude that the allosteric similarities in the first and third group represent no impact by the monovalent cation on this function. Therefore, cations in the first group increase PEP affinity, but do not play a role in the allosteric mechanism. Combining this explanation with conclusions from the study of the E130K mutant, we may suggest that the impact of Na^+ and Li^+ on the allosteric response to Fru-1,6-BP are not a result of these ions binding in the active site.

This final explanation also requires reconsideration of the effects observed in Figure 8. Based on the above argument, K^+ modifies PEP affinity, but does not alter allosteric responses. It follows that the impact of increasing K^+ on Q_{ax-FBP} (Figure 8) is a result of changes in total ionic strength and/or anion concentration (a conclusion also supported from the anion data presented below). The same total ionic strength/anion effects must be included with increasing Na^+ . Since Na^+ reduce the allosteric response for Fru-1,6-BP (discussed above), it seems likely that the lack of Q_{ax-FBP} response to increasing NaCl (Figure 8) is due to compensating effects result from ionic strength/anion enhancement and Na^+ reduction.

The collective findings in this section support that 1) hL-PYK does not likely require a monovalent cation for activity (based on the consideration that Tris and cyclohexylammonium are not expected to bind in the active site); 2) monovalent cation binding in the active site impacts PEP affinity without altering the magnitude of allosteric coupling; and 3) Li^+ and Na^+ impact the magnitude of allosteric coupling by Fru-1,6-BP due to mechanisms that occur outside of the active site cation binding site.

Anion

By maintaining constant K^+ , Cl^- concentration is left as the uncontrolled variable in the standard assay described above. Total anion concentration also decreases over the PEP concentration range; at 150 mM constant K^+ , 143 mM Cl^- decreases to 109 mM while PEP (also an anion) concentration increases from 0 to 10 mM. The requirement for monovalent cation for activity prevents a simplistic study that varies salt concentration. Instead, the experimental design most approachable with this system was to replace the KCl used to dilute ligands with other potassium salts. In this design, even though the anion concentration varies inversely to the concentration of PEP, the variation in anion concentration is the same for all monovalent anions (but not divalent anions).

In assays free of Cl^- , the magnitude of the allosteric inhibition elicited by Ala appears relatively independent of anion type, when this anion is NO_3^- , CH_3COO^- , or SO_4^{2-} (Figure 9A). In contrast, NO_3^- causes a large increase in $K_{a\text{-PEP}}$. Both the CH_3COO^- and SO_4^{2-} decreased PEP affinity in the saturating presence of Fru-1,6-BP (Note that use of SO_4^{2-} changes anionic concentration relative to assays with Cl^-). The use of SO_4^{2-} also causes a noticeable decrease in the observed Fru-1,6-BP affinity.

Experiments with KBr and KI were with a 10 mM Cl^- background derived from the use of MgCl_2 . Replacing Cl^- with Br^- or I^- increases $K_{a\text{-PEP}}$ consistent with the ionic radius of the anion (Figure 9B); F^- caused precipitation at the required concentrations. The affinity for PEP at high concentrations of Ala also increases consistent with the ionic radius of the anion. However, the affinity for PEP at high concentrations of Fru-1,6-BP seems relatively independent of whether Cl^- , Br^- , or I^- is used. Results with this series of anions might be used to support that the anion effects are specific. However, before making this conclusion, consider that pyruvate kinase is a fluorokinase (Tietz and Ochoa, 1958). Therefore, monoatomic anions likely bind in the active site. If these ions bind in the active site competitively with PEP and this is the only specific anion binding event, then the anions are not expected to alter allosteric coupling (i.e. when PEP binds, the competing anion will no longer be bound). Therefore, since anion type also alters allosteric function, a single, specific interaction in the active site is not supported. Effects caused by other anions (besides Cl^- , Br^- , and I^-) are also consistent with this conclusion. However, these data have not eliminated the possibility that anions specifically bind to a limited number of sites on the protein outside of the active site. Anion binding has not been elucidated in currently reported structures of PYK isozymes.

In as much as we can consider that the allosteric responses of the E130K mutant protein are not impacted by monovalent cation, effects on this mutant protein elicited by increasing concentration of potassium salts of various anions can be assigned to the combined effect of changes in ionic strength and anion concentration. Therefore, allosteric effects elicited by Ala and Fru-1,6-BP were determined over a concentration range of potassium-anion salts (Figure 10). In these assays PEP and effectors were diluted in water with no attempt to maintain constant ionic strength. Data trends over the ion series are consistent with the wild type data presented in Figure 9. Acetate is unique in that as the concentration of this anion increases, the allosteric activation by Fru-1,6-BP decreases, rather than increases as with all other anions; although not detailed here, potassium acetate also caused unique changes of $K_{\text{app-PEP}}$ and V_{max} relative to other anions, a finding that might indicate a unique interaction of acetate with the protein. Over the concentration range of the remaining anions, Cl^- appears to have one of the smallest effects on both the allosteric coupling elicited by Ala and on that elicited by Fru-1,6-BP; other anions elicited greater changes on one of the two allosteric effects. Therefore, Cl^- was used as a counter-ion in the standard assay.

To further characterize the impact that anions have on the observed allosteric effects in the absence of varying ionic strength, at least one non-interacting anion must be identified as a standard for comparison. However, what criteria should be used to identify such an anion is unclear. Data groups considered in conjunction with knowledge of the characterized binding site provided bases for discussion of monovalent cations; no data groups of the anion data is obvious and no anion binding site has been detailed in PYK structures. Confidence that buffers are non-interacting is based on reproducible results independent of buffer type; no such anion-independent reproducibility was identified in the anion series used, including a screen of additional anions (Figure 9C). Therefore, the results presented support that the type of anion present impacts the magnitude of the allosteric response. Although more descriptive conclusions cannot currently be made, this is the first indication that anions impact the allosteric response in hL-PYK.

Concluding Remarks

The role of PYK in glycolysis was discovered early in the history of metabolism (Boyer, 1962). This early identification can be viewed both as a benefit and a hindrance to future studies of individual PYK isozymes. The benefits include decades of studies that have detailed many structural and functional aspects of this enzyme. However, current understanding relies on many studies (including much of the current understanding about catalysis (Boyer, 1962)) that were completed before the relationship of isozymes was fully recognized. As a result, many properties have often been assumed to be common to all PYK isozymes with little or no verification. Our original assay conditions took into consideration these well accepted “knowns” regarding PYK function (Fenton and Hutchinson, 2009), including the assumption that K^+ was one of a limited number of monovalent cations that could activate PYK isozymes. However, such monovalent requirements were a result of studies primarily using the M_1 -PYK isozyme, not an L-PYK isozyme. With knowledge that monovalent cations are not required for activity, but monovalent cation (and anion) type impacts V_{max} , $K_{app-PEP}$ and the magnitude of allosteric responses, much of the divergence in kinetic parameters previously noted (Blair, 1980) may be due to uncontrolled levels of Na^+ and Cl^- in the assay systems.

It should be emphasized that in a broader study of allostery, characterizations of the impact of various ions on allosteric function should not be viewed purely as an academic endeavor. As one demonstration of the utility of understanding the impact of ions on allosteric function, consider that much of the structural comparisons relevant to allosteric systems use structures determined by X-ray crystallography. To obtain crystal growth, different enzyme complexes are often crystallized using different buffer, salt and/or pH conditions. Using hL-PYK as an example, we have shown here that changing buffer, divalent cation, monovalent cation or anion type and/or concentration can modify the allosteric response. In addition, we have previously demonstrated that the allosteric functions in this enzyme are pH dependent (Fenton and Hutchinson, 2009). The dependence of allosteric function on buffer/pH/ions requires a consideration of which structural comparisons are viable in an effort to understand changes associated with allosteric functions. In the specific example of pyruvate kinase, structural comparisons used to explain conformational changes associated with Fru-1,6-BP regulation have been with structures crystallized with Mn^{2+} bound in the active site. The difficulty in interpreting such comparisons now becomes apparent since hL-PYK does not respond to Fru-1,6-BP in the presence of Mn^{2+} . Furthermore, many past structural comparisons have been made between isozymes from different sources (including different species). In the case of PYK, the variable monovalent cation-independent activity catalyzed by rabbit M_1 -PYK (Boyer, 1962; Kachmar and Boyer, 1953; Kayne, 1971) vs. hL-PYK likely implies functional differences between the two enzymes. With variable functions between two isozymes, a structural comparison between such isozymes is at best a questionable approach to study allosteric function.

As a second example of the utility of understanding the impact of ions on allosteric function, NMR measurements have been used to determine the distances between cations in PYK (Nowak and Suelter, 1981; Villafranca and Raushel, 1982). These differences can be considered for each of the different complexes of an allosteric cycle (Figure 2). Since Mg^{2+} does not have a paramagnetic signal, Mn^{2+} has been used as the divalent cation. Multiple monovalent cation (including Tl^+) have been used as a monovalent cation with paramagnetic signal (Loria and Nowak, 1998; Villafranca and Raushel, 1982). Again, we can now appreciate that changing which monovalent or divalent cations are added to the assay system modifies the magnitudes of the allosteric response. Furthermore, the differences between divalent function in L-PYK vs. yeast PYK (discussed above) demonstrates that these magnitudes are likely unique to individual isozymes. Therefore, to facilitate data

interpretation of physical measurements such as changes in NMR-determined distances between monovalent and divalent cations, a complete characterization of allosteric responses with the alternative ions is required. Note with this consideration there is no current knowledge that directly ties physical changes with functional changes (e.g. does an effector induced change of a discrete distance between the divalent cation and K^+ result in a different magnitude of allosteric coupling depending on which divalent cation is present?).

Through the results presented here it can be appreciated that even very small changes in the system (Mg^{2+} to Mn^{2+} ; K^+ to Na^+ ; or Cl^- to Br^-) can alter the observed magnitude of the allosteric response. This conclusion highlights the necessity for quantitative studies of allosteric regulation at a functional level (Fenton, 2008; Reinhart, 2004). Information gained through these functional studies can, in turn, be used to guide structure/function correlations.

Acknowledgments

We would like to thank Josh Smith and Michael Chopade for their aid in exchanging substrate ions. This work was supported by NIH grant DK78076.

Reference list

- Blair, JB. Regulatory Properties of Hepatic Pyruvate Kinase. In: Veneziale, CM., editor. The Regulation of Carbohydrate Formation and Utilization in Mammals. University Park Press; Baltimore: 1980. p. 121-151.
- Blair, JB.; Walker, RG. Arch Biochem Biophys. Vol. 232. 1984. Rat liver pyruvate kinase: influence of ligands on activity and fructose 1,6-bisphosphate binding; p. 202-13.
- Bollenbach TJ, Nowak T. Kinetic linked-function analysis of the multiligand interactions on $Mg(2+)$ -activated yeast pyruvate kinase. Biochemistry. 2001a; 40:13097–106. [PubMed: 11669648]
- Bollenbach TJ, Nowak T. Thermodynamic linked-function analysis of $Mg(2+)$ -activated yeast pyruvate kinase. Biochemistry. 2001b; 40:13088–96. [PubMed: 11669647]
- Boyer, PD. Pyruvate Kinase. In: Boyer, PD., et al., editors. The Enzymes. Vol. 4. Academic Press; New York: 1962. p. 95-113.
- Cui Q, Karplus M. Allostery and cooperativity revisited. Protein Sci. 2008; 17:1295–307. [PubMed: 18560010]
- Czof, R.; Lamprecht, W. Pyruvate, Phosphoenolpyruvate and D-Glycerate-2-phosphate. In: Bergmeyer, HU., editor. Methods of Enzymatic Analysis. Vol. 3. Verlag Chemie Weinheim: Academic Press, Inc; New York: 1974. p. 1446-1451.
- Di Cera, E. Thermodynamic Theory of Site-Specific Binding Processes in Biological Macromolecules. Cambridge University Press; New York, NY: 1995.
- Fenton AW. Allostery: an illustrated definition for the ‘second secret of life’. Trends Biochem Sci. 2008; 33:420–5. [PubMed: 18706817]
- Fenton AW, Hutchinson M. The pH dependence of the allosteric response of human liver pyruvate kinase to fructose-1,6-bisphosphate, ATP, and alanine. Arch Biochem Biophys. 2009; 484:16–23. [PubMed: 19467627]
- Fenton AW, et al. Identification of substrate contact residues important for the allosteric regulation of phosphofructokinase from Escherichia coli. Biochemistry. 2003; 42:6453–9. [PubMed: 12767227]
- Fenton AW, Tang Q. An activating interaction between the unphosphorylated n-terminus of human liver pyruvate kinase and the main body of the protein is interrupted by phosphorylation. Biochemistry. 2009; 48:3816–8. [PubMed: 19320443]
- Formanek MS, et al. Reconciling the “old” and “new” views of protein allostery: a molecular simulation study of chemotaxis Y protein (CheY). Proteins. 2006; 63:846–67. [PubMed: 16475196]
- Frauenfelder H, et al. Conformational substates in proteins. Annu Rev Biophys Biophys Chem. 1988; 17:451–79. [PubMed: 3293595]

- Gunasekaran K, et al. Is allostery an intrinsic property of all dynamic proteins? *Proteins*. 2004; 57:433–43. [PubMed: 15382234]
- Hall ER, Cottam GL. Isozymes of pyruvate kinase in vertebrates: their physical, chemical, kinetic and immunological properties. *Int J Biochem*. 1978; 9:785–93. [PubMed: 367845]
- Johnson JL, Reinhart GD. Influence of MgADP on phosphofructokinase from *Escherichia coli*. Elucidation of coupling interactions with both substrates. *Biochemistry*. 1994; 33:2635–43. [PubMed: 8117726]
- Kachmar JF, Boyer PD. Kinetic analysis of enzyme reactions. II. The potassium activation and calcium inhibition of pyruvic phosphoferase. *J Biol Chem*. 1953; 200:669–82. [PubMed: 13034826]
- Kayne FJ. Thallium (I) activation of pyruvate kinase. *Arch Biochem Biophys*. 1971; 143:232–9. [PubMed: 5561746]
- Kern D, Zuiderweg ER. The role of dynamics in allosteric regulation. *Curr Opin Struct Biol*. 2003; 13:748–57. [PubMed: 14675554]
- Larsen TM, et al. Ligand-induced domain movement in pyruvate kinase: structure of the enzyme from rabbit muscle with Mg²⁺, K⁺, and L-phospholactate at 2.7 Å resolution. *Arch Biochem Biophys*. 1997; 345:199–206. [PubMed: 9308890]
- Laughlin LT, Reed GH. The monovalent cation requirement of rabbit muscle pyruvate kinase is eliminated by substitution of lysine for glutamate 117. *Arch Biochem Biophys*. 1997; 348:262–7. [PubMed: 9434737]
- Lockless SW, Ranganathan R. Evolutionarily conserved pathways of energetic connectivity in protein families. *Science*. 1999; 286:295–9. [PubMed: 10514373]
- Loria JP, Nowak T. Conformational changes in yeast pyruvate kinase studied by 205Tl⁺ NMR. *Biochemistry*. 1998; 37:6967–74. [PubMed: 9578583]
- Martell, AE.; Smith, RM. Critical Stability Constants. Plenum Press; New York: 1974–1989.
- McGilvery RW. Fructose 1,6-Diphosphate. Acidic Dissociation Constants, Chelation with Magnesium, and Optical Rotatory Dispersion. *Biochemistry*. 1965; 4:1924–1930.
- Mesecar AD, Nowak T. Metal-ion-mediated allosteric triggering of yeast pyruvate kinase. 1. A multidimensional kinetic linked-function analysis. *Biochemistry*. 1997a; 36:6792–802. [PubMed: 9184162]
- Mesecar AD, Nowak T. Metal-ion-mediated allosteric triggering of yeast pyruvate kinase. 2. A multidimensional thermodynamic linked-function analysis. *Biochemistry*. 1997b; 36:6803–13. [PubMed: 9184163]
- Munoz ME, Ponce E. Pyruvate kinase: current status of regulatory and functional properties. *Comp Biochem Physiol B Biochem Mol Biol*. 2003; 135:197–218. [PubMed: 12798932]
- Noguchi T, et al. The L- and R-type isozymes of rat pyruvate kinase are produced from a single gene by use of different promoters. *J Biol Chem*. 1987; 262:14366–71. [PubMed: 3654663]
- Nowak T, Lee MJ. Reciprocal cooperative effects of multiple ligand binding to pyruvate kinase. *Biochemistry*. 1977; 16:1343–50. [PubMed: 557338]
- Nowak T, Suelter C. Pyruvate kinase: activation by and catalytic role of the monovalent and divalent cations. *Mol Cell Biochem*. 1981; 35:65–75. [PubMed: 7015112]
- Oria-Hernandez J, et al. Pyruvate kinase revisited: the activating effect of K⁺ *J Biol Chem*. 2005; 280:37924–9. [PubMed: 16147999]
- Page MJ, Di Cera E. Role of Na⁺ and K⁺ in enzyme function. *Physiol Rev*. 2006; 86:1049–92. [PubMed: 17015484]
- Pendergrass DC, et al. Mining for allosteric information: Natural mutations and positional sequence conservation in pyruvate kinase. *IUBMB Life*. 2006; 58:31–8. [PubMed: 16540430]
- Popovych N, et al. Dynamically driven protein allostery. *Nat Struct Mol Biol*. 2006; 13:831–8. [PubMed: 16906160]
- Reed GH, Cohn M. Electron paramagnetic resonance studies of manganese (II)-pyruvate kinase-substrate complexes. *J Biol Chem*. 1973; 248:6436–42. [PubMed: 4354210]
- Reinhart GD. The determination of thermodynamic allosteric parameters of an enzyme undergoing steady-state turnover. *Arch Biochem Biophys*. 1983; 224:389–401. [PubMed: 6870263]

- Reinhart GD. Linked-function origins of cooperativity in a symmetrical dimer. *Biophys Chem.* 1988; 30:159–72. [PubMed: 3416042]
- Reinhart GD. Quantitative analysis and interpretation of allosteric behavior. *Methods Enzymol.* 2004; 380:187–203. [PubMed: 15051338]
- Suel GM, et al. Evolutionarily conserved networks of residues mediate allosteric communication in proteins. *Nat Struct Biol.* 2003; 10:59–69. [PubMed: 12483203]
- Suelter CH, et al. Studies on the interaction of substrate and monovalent and divalent cations with pyruvate kinase. *Biochemistry.* 1966; 5:131–9. [PubMed: 5938930]
- Swain JF, Gierasch LM. The changing landscape of protein allostery. *Curr Opin Struct Biol.* 2006; 16:102–8. [PubMed: 16423525]
- Tietz A, Ochoa S. Fluorokinase and pyruvic kinase. *Arch Biochem Biophys.* 1958; 78:477–93. [PubMed: 13618030]
- Valentini G, et al. Monovalent cations requirement of the fructose 1,6-bisphosphate-activated pyruvate kinase from *E. coli*. *Ital J Biochem.* 1979; 28:345–61. [PubMed: 399956]
- Villafranca JJ, Raushel FM. The monovalent cation site of pyruvate kinase and other enzymes: NMR investigations. *Fed Proc.* 1982; 41:2961–5. [PubMed: 7140996]
- Volkman BF, et al. Two-state allosteric behavior in a single-domain signaling protein. *Science.* 2001; 291:2429–33. [PubMed: 11264542]
- Weber G. Ligand binding and internal equilibria in proteins. *Biochemistry.* 1972; 11:864–78. [PubMed: 5059892]
- Williams R, et al. Differentiating a Ligand's Chemical Requirements for Allosteric Interactions from Those for Protein Binding. Phenylalanine Inhibition of Pyruvate Kinase. *Biochemistry.* 2006; 45:5421–9. [PubMed: 16634623]

Figure 1.

A) Schematic of the coordination between K^+ , protein bound Mg^{2+} , and phospholactate in the PEP binding site (the active site) as determined in M_1 -PYK (Larsen et al., 1997), but shown with L-PYK numbering (residue types are identical). Coordinating interactions are indicated by dashed lines. B) Comparative structure of PEP.

Figure 2.

Thermodynamic energy cycle for a protein or enzyme (E) which binds one substrate (A) and one allosteric effector (X). Each enzyme complex may be a single protein conformation, an equilibrium of a limited number of conformational substates, or an ensemble of conformational substates (a dynamic structure).

Figure 3.

Model Data. This model data demonstrates potential changes that could result from modifying the allosteric effector, mutating or covalently modifying the protein, and/or changing temperature, pH, or other solution conditions. Curve A is the reference line. The allosteric coupling (Q_{ax}) for curve A is represented by the double headed arrow. Although compared to A, B has a 10-fold decrease in effector affinity in the absence of substrate and C has a 10-fold decrease in substrate affinity in the absence of effector, A, B, and C have equivalent allosteric coupling. D has a 10 fold change in allosteric coupling as compared to A. (Reproduced with permission from (Fenton, 2008))

Figure 4.

$K_{app-PEP}$ as a function of Ala (•) or Fru-1,6-BP (□) as determined in the standard assay. Lines represent fits to Eq (3). When error bars are not apparent they are smaller than the symbols. Fit parameters are listed in Table I.

Figure 5.

The impact of buffer type on $K_{app-PEP}$ and allosteric function of wild type hL-PYK. A) $K_{app-PEP}$ determined over a concentration range of Tris/HCl ($\circ$), MOPS/KOH (Δ), or bicine/KOH ($\bullet$) in HEPES/KOH assay-buffer. B) $K_{app-PEP}$ determined over a concentration range of Tris/HCl ($\circ$), MOPS/KOH (Δ), or HEPES/KOH ($\blacksquare$), in bicine/KOH assay-buffer. In A and B, effector-buffer replaces effector in the standard assay; lines reflect data trends. C) $K_{app-PEP}$ as a function of Ala (circles) or Fru-1,6-BP (squares) determined in bicine/KOH, EPPS/KOH, HEPES/KOH, Tris/bicine and Tris/HCl. With the exception of Tris/HCl (open squares and filled circles), no effort is made to distinguish data for other buffers (closed squares and open circles). D) $K_{app-PEP}$ as a function of Ala ($\bullet$) or Fru-1,6-BP ($\square$) determined in MOPS/KOH. Lines in C and D represent fits to Eq. (3). Note different y-axis and x-axis ranges in A and B compared with C and D. When error bars are not apparent they are smaller than the symbols.

Figure 6.

The effect of divalent cation ion type on K_{a-PEP} and allosteric function of wild type hL-PYK at pH 6.5, 6.8 and 7.0. $K_{app-PEP}$ was determined over a concentration range of Ala (•) or Fru-1,6-BP (□) and in 50 mM HEPES buffer. Lines represent fits to Eq. (3). When error bars are not apparent they are smaller than the symbols. No activity was observed in the presence of Ca^{2+} . Even at pH 6.5, 10 mM Ni^{2+} or Co^{2+} are oxidized and become insoluble and are therefore were not studied.

Figure 7.

The impact of monovalent cation ion type on K_{a-PEP} and allosteric function. $K_{app-PEP}$ was determined over a concentration range of Ala (●) or Fru-1,6-BP (□) for wild type hL-PYK (WT) in the presence of a number of cations, including cyclohexylammonium (CA^+). Wild type/ Na^+ includes data collected both with commercial sodium salts of substrates and with sodium substrates obtained using ion exchange. $K_{app-PEP}$ was also determined for E130K over a concentration range of Ala (●) or Fru-1,6-BP (□) in the presence of K^+ (solid line) or of Ala (○) or Fru-1,6-BP (■) in the presence of Na^+ (dashed line) Cl^- was used as the anion. Lines represent fits to Eq. (3). When error bars are not apparent they are smaller than the symbols.

Figure 8.

The influence of monovalent cation concentration on Q_{ax} values. $Q_{\text{ax-FBP}}$ (dashed lines) and $Q_{\text{ax-Ala}}$ (solid lines) for wild type hL-PYK were determined over concentration ranges of either Na^+ ($\square$) or K^+ ($\bullet$). PEP and effectors were diluted in salt to maintain constant monovalent cation concentration. The minimum concentration of monovalent cations is determined by the contribution from substrates. Increasing concentration of monovalent cation is accompanied by increasing Cl^- concentration. However, changes in Cl^- concentration should be consistent whether Na^+ or K^+ is varied. Lines reflect data trends. When error bars are not apparent they are smaller than the symbols.

Figure 9.

The consequence of changing anion type on K_{a-PEP} and allosteric activation of wild type hL-PYK by Fru-1,6-BP (dashed line) or allosteric inhibition by Ala (solid line). A) K_2SO_4 (•), KCH_3COO (Δ), or KNO_3 (□) was used to maintain constant K^+ . B) KCl (▲), KBr (□), or KI (•) was used to maintain constant K^+ . C) $K_2B_4O_7$ (▲), K_2CO_3 (□), K_2WO_4 (•), MES/KOH (○), PIPES/KOH (■) was used to maintain constant K^+ . Assays used to collect data in both B and C were in the presence of 10 mM Cl^- , due to the use of $MgCl_2$. Lines represent fits to Eq. (3). When error bars are not apparent they are smaller than the symbols.

Figure 10.

The influence of anion concentration on Q_{ax} values. Q_{ax-FBP} (top) and Q_{ax-Ala} (bottom) for E130K hL-PYK were determined over concentration ranges of A) NO_3^- ($\square$), SO_4^{2-} ($\bullet$), or CH_3COO^- ($\triangle$) and B) Cl^- ($\triangle$), Br^- ($\bullet$), or I^- ($\square$). All anions were added as the potassium salt. PEP and effectors were diluted in water, such that ionic strength was not constant. Unlike data collected in Figure 9, Mg^{2+} was added as the respective Mg-anion salt; minimum concentration of anion was determined by this addition. Increasing concentration of anion is accompanied by increasing K^+ concentration. However, the use of the E130K mutation is expected to minimize the impact of the monovalent cation. Lines reflect data trends. When error bars are not apparent they are smaller than the symbols.

Table I

Fit parameters for data obtained in the standard assay as presented in Figure 4

Parameter ^a	Fit Value from simultaneous fit all sets	Standard deviation of fit values from individual data sets ^b
K_{a-PEP} [mM]	0.244 ± 0.003	0.03
K_{ix-FBP} [μ M]	0.068 ± 0.004	0.03
K_{ix-Ala} [mM]	0.29 ± 0.01	0.05
Q_{ax-FBP}	15.2 ± 0.4	2.7
Q_{ax-Ala}	0.074 ± 0.001	0.013

^aFrom Eq. (3)^bn=9 for Ala data and n=7 for Fru-1,6-BP data