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Effector Analogues Detect Varied Allosteric Roles for Conserved Protein−Effector Interactions in Pyruvate Kinase Isozymes[†]

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ABSTRACT: The binding site for allosteric inhibitor (amino acid) is highly conserved between human liver pyruvate kinase (hL-PYK) and the rabbit muscle isozyme (rM_1-PYK). To detail similarities/differences in the allosteric function of these two homologues, we quantified the binding of 45 amino acid analogues to hL-PYK and their allosteric impact on affinity for the substrate, phosphoenolpyruvate (PEP). This complements a similar study previously completed for rM_1-PYK. In hL-PYK, the minimum chemical requirements for effector binding are the same as those identified for rM_1-PYK (i.e., the L-2-aminopropanaldehyde substructure of the effector is primarily responsible for binding). However, different regions of the effector determine the magnitude of the allosteric response in hL-PYK vs rM_1-PYK. This finding is inconsistent with the idea that allosteric pathways are conserved between homologues of a protein family.

All pyruvate kinase isozymes catalyze the conversion of phosphoenolpyruvate and ADP to pyruvate and ATP as the last step in glycolysis. However, various isozymes, including mammalian enzymes, differ in regulatory properties. The pyruvate kinase isozyme from human liver (hL-PYK)[1] has decreased affinity for PEP when allosterically inhibited by *Ala*. In contrast, *Ala* binding to the isozyme from rabbit muscle (rM_1-PYK) elicits minimal change in the affinity for PEP (*1*). Instead, rM_1-PYK has decreased affinity for PEP when allosterically inhibited by *Phe*. Despite their different functional outcomes, *Ala* and *Phe* competitively bind to rM_1-PYK, indicating that they bind to the same site on the protein. Thus, the differential effects of *Ala* on PEP binding by rM_1-PYK and hL-PYK provide a means for investigating how allosteric function can vary between homologous proteins.

Previously, we identified the amino acid binding site of rM_1-PYK by cocrystallization with *Ala* (Figure 1; PDB 2G50) (*1*). *Ala* binding in the equivalent site of hL-PYK has been confirmed by mutagenesis (e.g., H476L as shown in Supporting Information). Although hL-PYK and rM_1-PYK share 67% identity and 81% similarity overall, all of the residue side chains in the amino acid binding site are completely conserved in these two proteins. The two nonconserved protein residues in this binding site have their backbone atoms exposed to the effector.

We report here how 45 different amino acid analogues bind to and allosterically regulate hL-PYK. This analogue series parallels that previously used in the study of amino acid inhibition of rM_1-PYK (*1*). Using the analogue series, we distinguished the chemical moieties of the effector required for binding to hL-PYK and those that determine the magnitude of the allosteric response. A comparison of these properties in the hL-PYK and rM_1-PYK (*1*) systems supports that effector binding, but not allosteric function, is conserved between the two homologues.

MATERIALS AND METHODS

Materials. The potassium salts of ADP and PEP were purchased from Chem-Impex International, Inc. NADH was from Sigma. L-Lactic dehydrogenase (type III bovine heart) was purchased from Calzyme Laboratories, Inc. Other buffer components were from Fisher Scientific and Sigma. The pLC11 plasmid encoding hL-PYK was obtained as a gift from Drs. Andrea Mattevi and Giovanna Valentini (*2*).

Since the low solubility and low affinity for some analogues (listed by supplier) may have prevented an observed allosteric effect, the highest concentrations used to determine allosteric responses are shown in brackets, and those used in competitive binding (when completed) are in italicized brackets. The L-forms of *Ala* [559 mM], *Arg* [559 mM], *Asn* [50.3 mM], *Asp* [6.50 mM], *Cys* [83.80 mM], *Gln* [119 mM], *Gly* [977.5 mM], *Glu* [12.3 mM], *His* [81 mM], *Ile* [61.4 mM], *Leu* [56.4], *Lys* [559 mM], *Met* [167 mM], *Phe* [83.8 mM], *Pro* [559 mM], *Ser* [559 mM], *Thr* [265 mM], *Trp* [22.3 mM], *Tyr* [1.7 mM], and *Val* [111.7 mM] were purchased from Fisher Scientific. Ethanolamine [81 mM], ethylamine [40 mM] [*13 mM*], and isopropylamine [80 mM] [*30 mM*] were purchased from Sigma. (*S*)-(+)-2-Phenylglycine [8.4 mM], L-homophenylalanine HCl [1 mM], 2-aminoisobutyric acid [559 mM], butylamine [42 mM] [*13 mM*], D/L-2-aminocaprylic acid [0.15 mM], 4-nitro-L-phenylalanine [3.8 mM], and *O*-methyl-L-tyrosine [18.8 mM] were from Aldrich. L-Homoserine [95 mM], L-(+)-2,3-diaminopropionic acid [56 mM], L-(+)-2-aminobutyric acid [559 mM], propionic acid [20.1 mM] [*7.7 mM*], L-alanine methyl ester HCl [100 mM] [*29 mM*], L-norvaline [69.3 mM], L-norleucine [0.44 mM], *N*-methyl-L-alanine [559 mM], D-alanine

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[1]Abbreviations: PYK, pyruvate kinase; rM_1-PYK, the pyruvate kinase isozyme found in rabbit brain and muscle; hL-PYK, the pyruvate kinase isozyme expressed in human liver; PEP, phosphoenolpyruvate.

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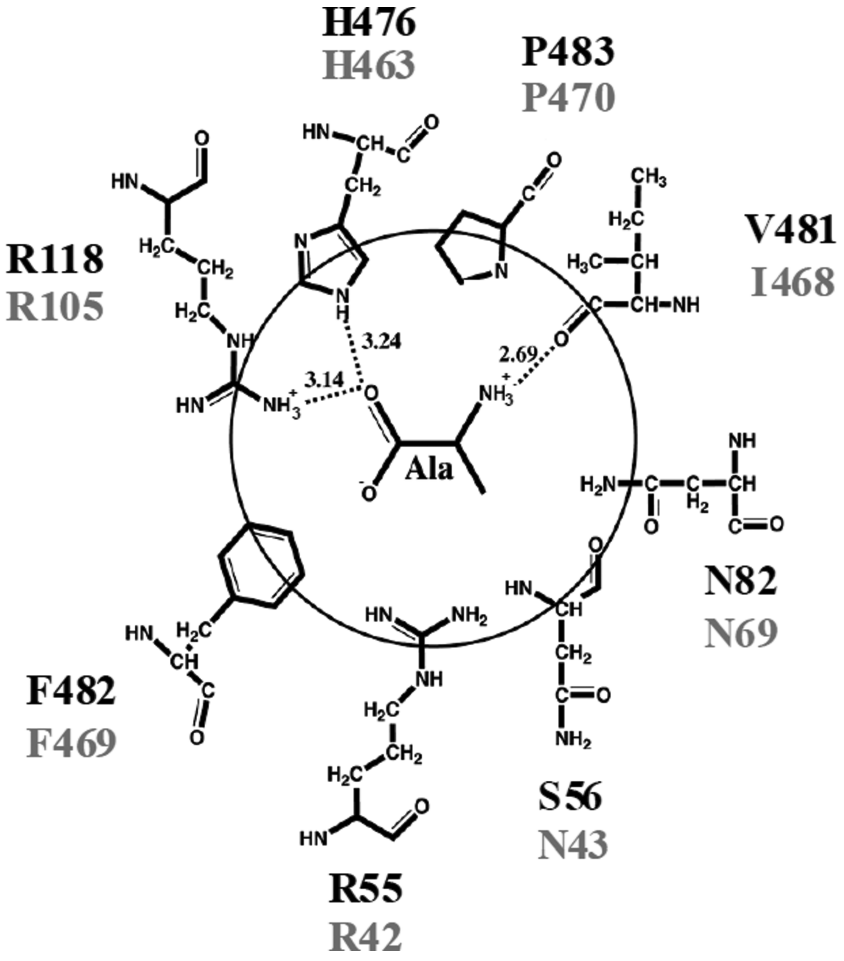

FIGURE 1: Schematic representation of the amino acid binding site of rM_1-PYK with bound Ala (*1*). The residues of hL-PYK and rM_1-PYK are in black and gray, respectively. Note that the rotomeric position of H476 is not firmly established (*9*).

[559 mM], 3-cyclohexyl-L-alanine [12.7 mM], D/L-2-aminoheptanoic acid [10.5 mM], and L-alaninol [160 mM] [*40 mM*] were obtained from Fluka. *N*-Formyl-L-alanine [15.3 mM] [*10 mM*] and *N*-acetyl-L-alanine [20.5 mM] [*10 mM*] were purchased from MP Biomedicals. D-Phenylalanine [67 mM] and (*S*)-(+)-2-amino-2-methyl-3-phenylpropanoic acid [38.50 mM] were purchased from Acros Organics.

Mutagenesis and Protein Expression and Purification. Mutagenesis of the hL-PYK gene to create the H476L gene was with Quikchange (Stratagene). Wild type and H476L were expressed in the FF50 strain of *Escherichia coli* (*3*). Cell lysis, ammonium sulfate fractionation, and DEAE-cellulose column purification were carried out as previously reported (*3*). Purified proteins were used in all studies with amino acid analogues.

Kinetic Assays. Activity measurements were carried out at 30 °C using a lactate dehydrogenase coupled assay (*4*). Reactions were in 350 μL of bicine buffer containing 50 mM bicine/KOH, 5 mM $MgCl_2$, 0.1 mM EDTA, 0.18 mM NADH, 19.6 units/mL lactate dehydrogenase, and 5 mM ADP at pH 7.5. PEP and effector ligand concentrations were varied as indicated. PEP and effector ligand stock solutions were adjusted to pH 7.5 with KOH before addition, and dilutions were in KCl to maintain constant total K^+ concentration of 150 mM in all assays (*4*). The enzymatic reaction was initiated with PEP and monitored at 340 nm over time. Data were collected in a 96-well plate using a Molecular Devices Spectramax Plus384 spectrometer. Initial rates were collected from the linear portion of the progress curve.

Data Analysis. Throughout this work, "K-type" heterotropic allostery is defined to occur "when one ligand binds to a protein differently in the absence, versus the presence, of a second ligand", given that the two ligands bind at different locations on the protein (*5*). As previously discussed (*5*−*7*), this linked-equilibrium view of allostery defines allosteric coupling (Q_{ax}) as a ratio of binding constants:

$$Q_{ax} = \left(\frac{K_{ia}}{K_{ia/x}}\right) = \left(\frac{K_{ix}}{K_{ix/a}}\right) \quad (1)$$

where K_{ia} = the dissociation constant for the first ligand, A, binding to the protein in the absence of the second ligand, X; $K_{ia/x}$ = the dissociation constant for A binding to the protein with X prebound; K_{ix} = the dissociation constant for X binding to the protein in the absence of A; and $K_{ix/a}$ = the dissociation constant for molecule X binding to the protein with A prebound. $Q_{ax} = 1$ means no allosteric response, $Q_{ax} > 1$ defines allosteric activation, and $Q_{ax} < 1$ defines allosteric inhibition. Since Q_{ax} is a ratio, the magnitude of this allosteric coupling is independent of the magnitude of any one ligand dissociation constant. Therefore, individual atom−atom interactions between atoms from protein residues and atoms from chemical moieties of the ligand may contribute uniquely to ligand binding vs allostery.

Data fitting was with the nonlinear least-squares analysis of Kaleidagraph (Synergy) software. Fits of PEP titrations of initial rates (v) used to obtain $K_{app\text{-}PEP}$ are as previously described (*3*, *4*). Although the potassium concentration used throughout ligand

concentration range was kept constant, a control experiment was included to ensure that other counterions and/or nonspecific effects of the various *Ala* analogues were not contributing to the observed regulation. For this control, the impact of the analogues on the affinity of H476L for PEP was monitored. H476L completely removes an *Ala* elicited response but maintains an affinity for PEP similar to that of wild-type hL-PYK (see Supporting Information). Responses of wild-type protein to analogues were corrected by dividing the $K_{app\text{-}PEP}$ vs analogue response for wild type by the $K_{app\text{-}PEP}$ vs analogue response for H476L (i.e., subtraction of the respective free energies).

The magnitude of Q_{ax} is measured by plotting the $K_{app\text{-}PEP}$ values as a function of effector concentration and fit to eq 2 (*7*):

$$K_{app\text{-}PEP} = K_a\left(\frac{K_{ix} + [effector]}{K_{ix} + Q_{ax}[effector]}\right) \quad (2)$$

where $K_a = K_{app\text{-}PEP}$ when [effector] = 0. In several cases, it was not possible to obtain formation of the ternary substrate−enzyme−effector complex (i.e., formation of the upper plateau when plotting $K_{app\text{-}PEP}$ as a function of effector analogue concentration) within the working concentration range of effector analogues. When sufficient data cannot be collected to define the upper plateau, Johnson and Reinhart (*8*) demonstrated that eq 2 simplifies to

$$K_{app\text{-}a} = K_a\left(1 + \frac{[analogue]}{K_{ix\text{-}analogue}}\right) \quad (3)$$

This simplified equation is equivalent to competitive binding between A and X. Therefore, when the upper plateau could not be obtained, data were fit to eq 3 as a means of evaluating effector analogue binding. Data for effector analogues fit in this manner can be used to evaluate which regions of the effector contribute to effector binding but give no insights into the region of the effector that contributes to eliciting the allosteric response. To highlight this distinction, parameters obtained from fits to eq 2 and 3 are segregated in Table 1.

When an amino acid analogue failed to elicit an allosteric response, the ability of that compound to bind competitively with *Ala* to hL-PYK was used to test for binding. In this approach $K_{ix\text{-}Ala}$ was determined (as described above using fits to eq 2) at varying concentrations of analogue (see Supporting Information). If competitive binding is observed, a K_{ix} value for the analogue could be determined by fitting such data to the competitive equation, i.e., same form as eq 3. However, we did not identify any analogues that showed competitive binding with Ala that did not also elicit sufficient influence on $K_{app\text{-}PEP}$ to allow an evaluation of K_{ix} via fitting the allosteric response to eq 2 or 3. Our discussion will consider quantitative comparisons of fit parameters (Table 1).

Due to the relatively low affinity of hL-PYK for *Ala*, our studies used very high ligand concentrations. Two controls can be considered to gain confidence that the results represented herein are not due to nonspecific effects. First, we can consider the response of wild-type hL-PYK to very high concentrations of *Ala* (see Supporting Information). Once *Ala* concentrations are sufficiently high to saturate the effector binding site (i.e., the upper plateau is obtained at 25 mM), $K_{app\text{-}PEP}$ is not further responsive to very high (up to 500 mM) concentrations of *Ala*. Therefore, it does not appear that very high concentrations of *Ala* alter $K_{app\text{-}PEP}$ due to nonspecific effects. It follows that when mutant proteins bind *Ala* with affinities lower than the wild-type protein, *Ala* concentrations up to 100 mM can be used to evaluate binding and allosteric properties. Second, *Ala* analogues (and/or counterions associated with those analogues), as opposed to *Ala*, may promote nonspecific binding effects. H476L binds PEP with a similar affinity as the wild-type protein (see Supporting Information). However, the $K_{app\text{-}PEP}$ of H476L shows no response to *Ala* up to the 100 mM concentration range used. It follows that any response of this mutant protein to high concentrations of an *Ala* analogue must be due to nonspecific effects. Therefore, allosteric responses of wild-type protein to analogues smaller than *Ala* (i.e., compounds that represent a fractionation of the *Ala* molecule) were corrected by dividing the $K_{app\text{-}PEP}$ vs analogue response for wild type by the $K_{app\text{-}PEP}$ vs analogue response for H476L (i.e., subtraction of the respective free energies).

RESULTS AND DISCUSSION

Minimum Requirement for Effector Binding. The first question to be addressed with the analogue series is which chemical moieties of the effector are the minimum required for binding to hL-PYK. Since the amino and carboxyl groups of the effector were required for binding to rM_1-PYK, modification of these regions of the effector were first considered. Complete removal of the carboxyl group of the effector (ethylamine *vs Ala*) or replacement of this moiety with either a methyl (isopropylamine *vs Ala*) or a methyl alcohol (alaninol *vs Ala*) group reduces binding sufficiently to prevent formation of the upper plateau within the working analogue concentration range. However, L-alaninol and isopropylamine bind to hL-PYK with similar affinities, indicating that the hydroxyl oxygen contributes little to binding affinity. Therefore, we can speculate that the carbonyl oxygen must contribute to effector binding. Like rM_1-PYK (*1*), hL-PYK is also regulated by L-alanine methyl ester, indicating that the effector (and allosteric response) does not require a charge on the carboxylate group (i.e., a charge on the carboxylate group is not required for binding or allosteric functions). Overall, this data trend follows that observed for rM_1-PYK, with the exception that there is less distinction between the binding affinities of hL-PYK for ethylamine vs isopropylamine and alaninol.

Also similar to the finding in the rM_1-PYK system (*1*), complete removal of the amino group (propionic acid) prevents binding of the effector to hL-PYK. Although the addition of larger chemical moieties at the amino position (*N*-formyl-L-ala and *N*-acetyl-L-ala) prevented an allosteric response, the addition of a methyl and a cyclic group leads to a minimal decrease in both binding affinity and allosteric inhibition (*N*-methyl-L-ala and *Pro vs Ala*). Both of the respective analogues contain secondary rather than primary amines, indicating that the primary amine of standard amino acids is not required for binding or allosteric functions. Of these, the regulation by *N*-methyl-L-ala shows the most contrast with the results obtained for rM_1-PYK; this analogue did not elicit an allosteric response in the muscle protein. However, since rM_1-PYK is allosterically inhibited by *Pro* (*1*, *9*), differences between the two pyruvate kinase isozymes appear limited to the ability to accommodate various chemical moieties attached to the amino group, rather than one isozyme selecting against any additions to the effector amino group.

Table 1: Fit Parameters for the Allosteric Response Caused by Different Amino Acid Analogues

fit parameters from eq 2			fit parameters from eq 3	
	allosteric response			
commercial name	K_{ix} (mM)	Q_{ax}	commercial name	K_{ix} (mM)
L-*Ala*	0.33 ± 0.01	0.100 ± 0.001		
L-*Phe*	3.9 ± 0.3	0.39 ± 0.01		
		Carboxyl Modifications		
L-alanine methyl ester[b]	1.90 ± 0.05	0.120 ± 0.002	ethanolamine[b]	50 ± 20
			ethylamine[b,c]	40 ± 10
			isopropylamine[b,c]	25 ± 2
			L-alaninol[b,c]	25 ± 1
			butylamine[b,c]	–[a]
		Amino Modifications		
N-methyl-L-ala[b]	1.1 ± 0.2	0.16 ± 0.01	propionic acid[b,c]	–[a]
L-*Pro*	1.40 ± 0.04	0.063 ± 0.001	*N*-formyl-L-ala[b,c]	–[a]
			N-acetyl-L-ala[b,c]	–[a]
		Chiral Carbon Modifications		
D-alanine[b]	10 ± 2	0.37 ± 0.02	D-phenylalanine[b]	–[a]
2-aminoisobutyric acid[b]	11 ± 2	0.16 ± 0.01	(*S*)-(+)-2-amino-2-methyl-3-phenylpropionic acid[b]	–[a]
		Side Chain Modifications		
Gly[b]	23 ± 2	0.26 ± 0.01	L-homoserine	10 ± 4
L-*Cys*	0.101 ± 0.003	0.124 ± 0.002	L-*Ile*[e]	31 ± 8
L-*Ser*	4.6 ± 0.4	0.24 ± 0.01	L-norvaline	29 ± 3
L-(+)-2,3-diaminopropionic acid	8 ± 3	0.15 ± 0.05	L-*Asn*	–[a]
L-(+)-2-aminobutyric acid (2AB)	0.43 ± 0.02	0.05 ± 0.01	L-*Asp*	–[a]
L-*Val*	7.6 ± 0.4	0.090 ± 0.004	L-*Leu*	–[a]
L-*Thr*	19.6 ± 0.7	0.160 ± 0.003	L-*Glu*	–[a]
L-*Met*	21 ± 2	0.35 ± 0.01	L-norleucine	–[a]
O-methyl-L-tyr	3.8 ± 0.6	0.20 ± 0.04	2-aminoheptanoic acid	–[a]
			2-aminocaprylic acid	–[a]
			L-*His*	–[a]
			L-*Arg*	–[a]
			L-*Lys*	–[a]
			3-cyclohexyl-L-alanine	–[a]
			(*S*)-(+)-2-phenylglycine	–[a]
			4-nitro-L-phenylalanine	–[a]
			L-homophenylalanine	–[a]
			L-*Tyr*	–[a]
			L-*Trp*	–[a]

[a] $K_{a\text{-PEP}}$ was not responsive to the amino acid analogue within the working concentration defined in Materials and Methods. [b] Corrected using H476L as described in Supporting Information. [c] Binding tested by competition with *Ala* binding. When no K_{ix} value is listed, no competitive binding was detected.

However, it appears that effector amino nitrogen is required for binding to hL-PYK, consistent with both results from effector analogue studies of rM_1-PYK and the predicted contribution based on how the effector coordinates to the protein (Figure 1).

Since both the amino nitrogen and the carbonyl oxygen (above) are required for binding and removal of additional moieties (*Ala vs Gly*, ethanolamine, ethylamine, and butylamine) greatly reduces affinity, the L-2-aminopropanaldehyde substructure appears to be the primary requirement for effector binding to hL-PYK. This same L-2-aminopropanaldehyde substructure was found to be required for effector binding to rM_1-PYK.

Determinant of Allostery. The second question to be addressed in this study is which region of the effector elicits the allosteric response. With regard to the modification introduced at the amino and carboxyl groups (discussed above), all effector analogues that bind to hL-PYK elicit an allosteric response. In addition, the effects of additions to the chiral carbon and additions of side chain atoms beyond the C_β of *Ala* were considered. However, due to the lack of a strong data trend, little can be concluded regarding the accommodation of additional chemical moieties at the chiral carbon of the effector. In contrast to the lack of response in rM_1-PYK (*1*), hL-PYK shows inhibition by 2-aminoisobutyric acid and D-alanine. These analogues bind to hL-PYK with different affinities and elicit different magnitudes of allosteric coupling as compared to *Ala* (i.e., the L-form of alanine). The fact that these analogues bind and

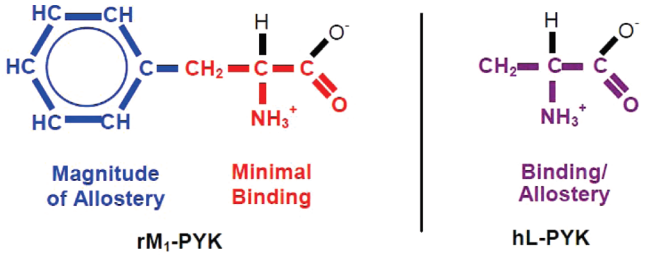

FIGURE 2: Atoms of the effector required for binding and that determine the magnitude of the allosteric response. Although these two properties map to different regions of the effector for regulation of rM_1-PYK (*1*), the same separation of function is not apparent in the regulation of hL-PYK.

influence PEP affinity greatly differs from the lack of binding in the rM_1-PYK isozyme. In contrast to the small *Ala* analogues, only the L-form of *Phe* (not D-phenylalanine) elicits an allosteric response. Replacement of the α-hydrogen of L-*Phe* with a methyl group ((*S*)-(+)-2-amino-2-methyl-3-phenylpropionic acid vs L-*Phe*) also eliminates an allosteric response, which is in contrast to the minimal effect on coupling caused by the methyl group substitution to *Ala* (2-aminoisobutyric acid *vs Ala*). Therefore, although there is no obvious data trend for analogues with additions at the chiral carbon, the data consistently indicate that all effector analogues that bind to hL-PYK elicit an allosteric response.

Since *Pro* and *Ala* are the only two amino acid effectors that have been cocrystallized with any of the pyruvate kinase isozymes (*1*, *9*), a full appreciation for how large effector side chains interact with the binding site is currently lacking. Nonetheless, effector analogues can be used to describe the functional roles of the effector side chain. Many types of chemical moieties can be added to the C_β without preventing the allosteric responses (*Ser*, *Cys*, L-(+)-2,3-diaminopropionic acid, 2-aminobutyric acid, *Pro*, *Val*, *Thr*, *Met*, and homoserine). Like rM_1-PYK, the effector site of hL-PYK is capable of accommodating amino acids much larger than *Ala* (e.g., *O*-methyl-L-tyr). These larger analogues elicit allostery, but there is a moderate trend indicating that binding affinity is reduced as the side chain increases in hydrophobic bulk (*O*-methyl-L-tyr is the exception). Also, not all side chains allow an allosteric response (*Asp* and *Asn*; no attempt was made to distinguish if these two ligands fail to bind or bind but fail to elicit a response). *Ala*, 2-aminobutyric acid, *Cys*, *Pro*, and *Val* elicit the largest magnitude of allosteric inhibition (i.e., smaller Q_{ax} values; Table 1); this maximum effect in hL-PYK is greatly reduced relative to the maximum inhibition in the rM_1-PYK system (*1*). Of the analogues for which a full analysis of allosteric coupling was obtained, *Phe* elicits the smallest antagonism of PEP affinity (i.e., Q_{ax} value closest to 1). Therefore, the nature of the effector side chain can modify the magnitude of the allosteric response, relative to *Ala*. However, the chemical moieties required to elicit an allosteric inhibition are included in *Ala*. Overall, it appears that the L-2-aminopropanaldehyde substructure of the effector that is required for binding is also the primary determinant of the allosteric regulation.

Summary. Several conclusions can be drawn from the use of amino acid analogues in this study. (1) Similar to rM_1-PYK, the extent of allosteric regulation of hL-PYK is dependent on the effector chemistry (an inconsistency with those two-state models that assume all-or-none allostery). (2) The L-2-aminopropanaldehyde substructure of the amino acid is primarily responsible for effector binding to both rM_1-PYK and hL-PYK (Figure 2). (3) Although the length of the hydrophobic side chain determines the magnitude of the allosteric coupling in rM_1-PYK, the primary allosteric determinants in the hL-PYK system are the amino and carboxyl groups of the effector. Consequently, there is a sharp contrast between the two isozyme systems: Although the determinants of effector affinity and the magnitude of the allosteric response were separate moieties of the effector in the rM_1-PYK, this separation of function was not apparent for hL-PYK inhibition.

The contrasting effector moieties that determine the magnitude of the allosteric coupling in hL-PYK vs rM_1-PYK have important implications for the common assumption of conserved allosteric function within protein families. Sequence-based evolution/coevolution of allostery within a family of homologues intrinsically assumes that this regulatory property is conserved within that family. There is growing concern that this assumption is not valid (*5*, *10*−*15*). The observation that different regions of the amino acid inhibitor elicit the allosteric response in hL-PYK vs rM_1-PYK strengthens this concern.

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SUPPORTING INFORMATION AVAILABLE

A competitive binding example, a high ligand concentration control, and a table including all analogue structures. This material is available free of charge via the Internet at http://pubs.acs.org.

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