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Distinguishing the Chemical Moiety of Phosphoenolpyruvate That Contributes to Allostery in Muscle Pyruvate Kinase

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Supporting Information

ABSTRACT: A series of substrate analogues has been used to determine which chemical moieties of the substrate phosphoenolpyruvate (PEP) contribute to the allosteric inhibition of rabbit muscle pyruvate kinase by phenylalanine. Replacing the carboxyl group of the substrate with a methyl alcohol or removing the phosphate altogether greatly reduces substrate affinity. However, removal of the carboxyl group is the only modification tested that removes the ability to allosterically reduce the level of Phe binding. From this, it can be concluded that the carboxyl group of PEP is responsible for energetic coupling with Phe binding in the allosteric sites.

Any heterotropic allosteric mechanism that alters substrate affinity must involve alterations in the substrate binding site. In turn, these changes must be energetically coupled to changes in the allosteric binding site. This energetic coupling is the origin of reciprocity; i.e., the impact that the effector has on substrate affinity must equal the effect that the substrate has on the affinity of the protein for the effector.[1−3] Given the required role of the active site in allosteric regulation, it is surprising that substrate binding sites (active sites) are infrequently studied for their role(s) in heterotropic allosteric mechanisms.[4] In particular, this study was initiated to identify which region of a substrate is required for allosteric function. We previously demonstrated that only a subset of the interactions between the protein and allosteric inhibitor dictate the observed allosteric response in rabbit muscle pyruvate kinase (M_1-PYK).[13] Similarly, individual chemical moieties of the substrate may contribute uniquely to ligand affinity (and/or catalysis) versus allostery.

The affinity of M_1-PYK for its substrate, phosphoenolpyruvate (PEP), is allosterically inhibited by phenylalanine (Phe). The coordination of substrates to M_1-PYK has been well-defined by cocrystallography studies,[7−13] as illustrated by the schematic in Figure 1. The requirement for monovalent and divalent cations is well characterized,[5,6] and the locations of these ions in the active site are clearly visible in a number of PYK structures (Figure 1). With regard to understanding which interactions in the PEP binding site contribute to allostery,

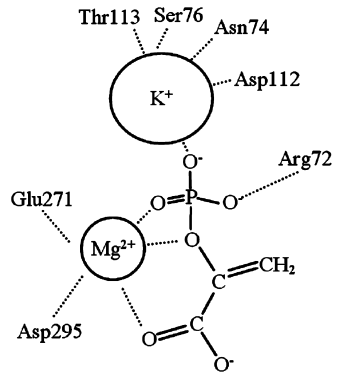

Figure 1. Schematic of the coordination among K^+, the protein-bound divalent cation, and PEP, as determined by the interaction between M_1-PYK and phospholactate.[8] Coordinating interactions are indicated by dashed lines.

Kayne and Price published ligand binding results that appear to be internally inconsistent. Phe binding was reported to be dependent on the concentration of divalent cation.[16,17] This would indicate allosteric coupling between Phe and Mg^{2+} and, because of the importance of the divalent metal−PEP coordination, would likely indicate a role for the divalent cation in Phe−PEP coupling. In contrast, the same researchers provided evidence that M_1-PYK's affinity for Phe is dependent on PEP concentration even in the absence of a divalent metal cation.[16] This second observation would not support a role for the divalent cation in Phe−PEP coupling. There is no evidence that the monovalent cation plays a role in allostery. The few mutations that have previously been reported in (or adjacent to) the active site of M_1-PYK have not contributed to an understanding of what regions of this site participate in allosteric mechanisms.[14,15] Finally, PEP analogues have been extensively used to study substrate specificity (Supporting Information), but not to study allostery. To test which chemical moieties of PEP are important in the allosteric regulation of M_1-PYK, the ability of a series of substrate analogues to inhibit Phe binding has been tested.

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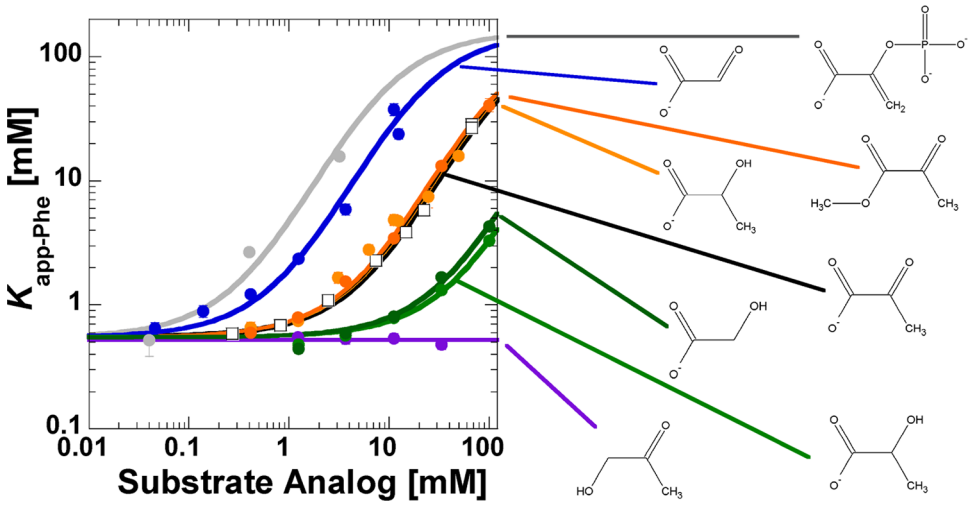

Figure 2. $K_{app\text{-}Phe}$ values determined by titrating protein fluorescence, plotted as a function of PEP or PEP analogue concentration. The ligands were pyruvate (empty black squares), PEP (gray), glyoxylate (blue), D-lactate (dark orange), methyl pyruvate (light orange), L-lactate (light green), glycolate (dark green), and hydroxyacetone (purple). Lines represent fits to eq 2 as described in the Supporting Information. Note that assay conditions are different from those used to collect data in Figure 3.

To monitor allosteric function, the binding of one ligand (i.e., Phe) must be assessed over a concentration range of a second ligand (i.e., PEP or a PEP analogue). The affinity of M_1-PYK for amino acid ligands has often been determined using ligand titration of intrinsic protein fluorescence.[16,17] However, Oberfelder and Lee have cautioned that in this system, changes in fluorescence intensity are not proportional to the extent of ligand−protein complex formation; i.e., the midpoint of a titration of protein fluorescence with effector does not reflect the dissociation constant.[18] Here, the midpoint ($K_{app\text{-}Phe}$) is reported as a reflection of the apparent affinity for the effector, and this apparent affinity is determined over a PEP or PEP analogue concentration range. At high PEP concentrations, the solubility limit of Phe prevents complete saturation with this effector. Because of these complications and to add confidence that monitoring apparent amino acid affinity is a viable approach to monitoring allosteric function, the allostery between Phe and PEP was compared with that between Ala and PEP (Supporting Information). In a manner qualitatively consistent with reciprocity, PEP decreases the protein's affinity for Phe but only minimally impacts Ala affinity.[13] Therefore, determining the apparent affinity of M_1-PYK for Phe (using titrations of protein fluorescence) over a PEP concentration range appears to be a valid qualitative method of monitoring allosteric function in this protein.

Nonphosphorylated PEP analogues (i.e., pyruvate analogues) bind in active sites of PYK isozymes.[7,9] The ability of the nonphosphorylated analogues to allosterically impact $K_{app\text{-}Phe}$ was investigated (Figure 2). Because several of these analogues were commercially available only as sodium salts, data shown in Figure 2 were collected in the presence of an additional 200 mM Na^+, relative to the assay used for the data in Figure 3.[13] This addition causes a slight decrease in the apparent affinity for Phe. For comparison, only a limited number of data points were determined for PEP concentrations under this assay condition. Even though pyruvate binds with a weaker affinity than PEP, this reaction product is the reference for the nonphosphorylated analogues. With the exception of hydroxyacetone, all nonphosphorylated analogues tested allosterically reduce Phe affinity, confirming that these analogues can elicit

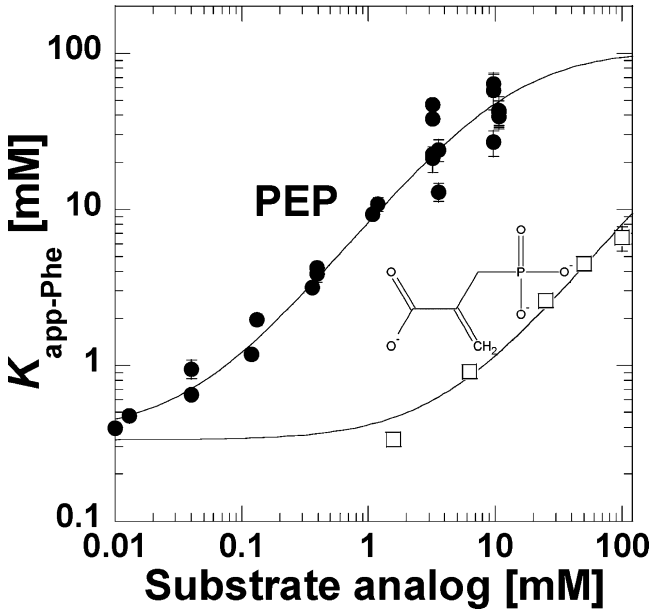

Figure 3. $K_{app\text{-}Phe}$ values determined by titrating protein fluorescence, plotted as a function of PEP (●) or PEP analogue (□) concentration. Lines represent fits to eq 2 as described in the Supporting Information.

an allosteric response. The affinities of M_1-PYK for these analogues display considerable variability (horizontal curve placement). Glyoxylate binds tighter than pyruvate; D-lactate and methyl pyruvate bind with an affinity similar to that of pyruvate, and L-lactate and glycolate bind with an affinity weaker than that of pyruvate. These data support the idea that the removal of the phosphate moiety greatly reduces ligand affinity. Also, results obtained with methyl pyruvate indicate that a charge on the carboxyl group is not necessary for allosteric function. The lack of a response to hydroxyacetone is discussed below.

The oxygen that bridges the PEP's carbon backbone and phosphate group (the carbonyl oxygen in pyruvate) was not modified in the analogues studied in Figure 2. To probe the allosteric role of the bridging oxygen, the acrylate analogue

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(Figure 3) of PEP was created. Replacing the bridging oxygen with a carbon in this analogue reduces the affinity for the protein (Figure 3). However, this compound elicits an allosteric response.

The nonphosphorylated analogue series and the acrylic analogue have effectively probed all regions of the PEP molecule to determine that PEP's binding affinity is largely determined by the carboxyl oxygens and phosphate groups of the ligand. This finding is not at all surprising given the binding coordination and the limited reactive groups on this small substrate (Figure 1).

To identify which moiety of the substrate is important for allosteric function, consider that modifications of all regions of PEP, except the carboxyl group, continue to allow binding and an allosteric response. Therefore, we can consider the carboxyl group as the necessary moiety for allosteric function. Hydroxyacetone is the only analogue included here that does not have a carboxyl moiety. However, because of the absence of both the phosphate and the carboxyl group, the lack of a response by $K_{app\text{-}Phe}$ to an increasing hydroxyacetone concentration is expected to be caused by a failure of hydroxyacetone to bind to the active site. Methyl pyruvate also includes a modification of the carboxyl moiety, but it retains both oxygen atoms. In Figure 2, it is clear that this molecule continues to elicit an allosteric response similar to that caused by pyruvate. Therefore, it appears that the presence of oxygen atoms in the carboxyl group is more important to allosteric function than the ability of this moiety to carry charge. Although unsuccessful, multiple attempts have been made to identify analogues that can bind sufficiently to be used to probe the carboxyl moiety (Supporting Information). Nonetheless, data presented here are consistent with the oxygen atoms in the carboxyl moiety playing a role in allostery.

Despite the atomic-level conclusion that the carboxyl oxygens of PEP contribute to allosteric function, one should resist the temptation to further extrapolate this result to conclude that some change in the substrate−carboxyl/divalent cation interaction is modified in the allosteric mechanism. Indeed, the crystal structure with the substrate bound details an interaction between PEP and the divalent cation as the only observed coordination with the substrate's carboxyl moiety. However, removal of this interaction that contributes positively to PEP binding is not the only means of reducing PEP affinity. Alternatively, Phe binding might induce a structural change in the protein that results in the introduction of some protein property that contributes negatively to PEP binding (i.e., increased steric hindrance, increased hydrophobicity, and/or modified active site dynamics). Nonetheless, these considerations do not subtract from the overall conclusions of this work that (1) PEP affinity is primarily determined by the phosphate and carboxylic acid moieties and (2) the carboxyl group of the substrate is responsible for allosteric function.

■ ASSOCIATED CONTENT

Supporting Information

Materials and methods, Phe titrations, and inhibition of the affinity of Ala and Phe by PEP. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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