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Allosteric regulation of human liver pyruvate kinase by peptides that mimic the phosphorylated/dephosphorylated N-terminus

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Abstract

An advantage of studying allosteric regulation over covalent modification is that allostery allows the experimentalist to vary the concentration of effector, thereby allowing independent quantification of effector binding and allosteric coupling. In turn, this capacity allows the use of effector analogues to determine which regions of the effector contributes to effector binding and which contributes to allosteric regulation. Like many other proteins, human liver pyruvate kinase (hL-PYK) is regulated by phosphorylation. The phosphorylation of hL-PYK occurs on Ser12 of the N-terminus. Phosphorylation appears to interrupt an interaction (distant from the active site) between the N-terminus and the main body of the protein. Since this interaction increases the affinity of hL-PYK for the substrate (phosphoenolpyruvate, PEP), phosphorylation dependent interruption of the N-terminus/main-body interaction results in an antagonism of PEP binding. Due to the advantages of studying an allosteric system, we detail a protocol to express and purify N-terminal peptides of hL-PYK using a SUMO-fusion system. We further demonstrate that these peptides act as allosteric regulators that modulate the affinity of hL-PYK for PEP.

Keywords

Allostery; pyruvate kinase; hL-PYK; linked equilibrium; N-terminus phosphorylation

1. Introduction

Although covalent modification and allosteric regulation of a protein often result in similar regulatory consequences (e.g. impact on protein affinity for substrate), there is an experimental advantage to the study of allosteric effectors. Our working definition of allosteric regulation is how one ligand (A) binds to a protein (E) in the presence vs. absence of a second ligand (X) (1). This definition defined a thermodynamic energy cycle (Figure 1). It also defines the allosteric coupling constant (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 pre-bound, 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 pre-bound. Since Q_{ax} is a ratio, the magnitude of this allosteric coupling is

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independent of the magnitude of any one ligand dissociation constant. It follows that, 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. Several studies have now utilized this knowledge in combination with an analogue series to detail which region(s) of the effector contributes to ligand binding and which region(s) contribute to determining the magnitude of the allosteric coupling, Q_{ax} (2–5). Although mutations are commonly used to mimic covalent modifications (e.g. phosphorylation (6–8)), such studies do not distinguish if 1) the modification alters the ability of the modified protein region to interact with other regions of the protein (i.e. an internal binding event between different regions of the protein), vs. 2) the mutation has little effect on the internal binding event, but modifies the ability of this binding event to modify protein function (i.e. allosteric effect).

Although used under many different names, chemical fragmentation and fragment complementation have often been employed to identify functional roles for increasingly small chemical moieties. Such fragmentation studies include: 1) the use of substrate analogues to study enzymatic mechanisms; 2) the use of effector analogues (referenced above) to study allosteric effectors (2–5); 3) the use of "fragment-based" screening in conjunction with structural determination to identify ligand binding sites on protein surfaces (9); 4) the addition of small molecules to rescue mutant side chain function (chemical rescue) (10); and 5) the complementation of protein fragments to recover protein activity (11–14). However, Carty *et al* was the first to propose that a protein derived phosphopeptide could regulate glycogen phosphorylase in the absence of being covalently linked with the main body of the protein (15) (Note: The results supporting this early work have now been questioned (16).). Following this suggestion, the use of peptides (with and without mutations that mimic covalent modification) as allosteric effectors is the focus of the current work.

Like glycogen phosphorylase, human liver pyruvate kinase (hL-PYK) is regulated by phosphorylation. Pyruvate kinase isozymes, including hL-PYK, catalyze the final step in glycolysis:

$$\text{Phosphoenolpyruvate (PEP)} + \text{ADP} \rightleftarrows \text{Pyruvate} + \text{ATP}.$$

Regulation of hL-PYK maintains glucose homeostasis, preventing hyperglycemia and hypoglycemia. This regulation is accomplished both by the phosphorylation of the N-terminus at Ser 12 (17–19), and by allosteric regulation by fructose-1,6-bisphosphate (Fru-1,6-BP), Ala, and ATP (20). Introducing the S12D mutation mimics the impact of phosphorylation, decreasing PEP affinity to an extent comparable to the effect caused by phosphorylation (18). We have previously used a truncation series to outline which regions of the N-terminus are energetically coupled with PEP binding in the active site (18). Truncating residues 1–6 have little impact on PEP affinity. In contrast, sequentially removing residues 7–10 decreases PEP affinity, to an extent equivalent to that caused by phosphorylation. However, this effect is not apparent when S12D has already been introduced. Taken together, these observations are consistent with an activating interaction between the main-body (all parts of the protein except the N-terminus) and the unphosphorylated N-terminus. Phosphorylation may simply interrupt this activating interaction. Unfortunately, the first 26 residues of hL-PYK are not defined in the currently available crystal structure (21). Therefore, it remains unclear where or how the N-terminus of hL-PYK interacts with the main-body of the protein.

To further elucidate which regions of the N-terminus contribute to "binding" to the main-body of the protein and which contribute to energetic coupling with the active site, we

would like to use the advantages available via an allosteric system. This approach requires that the N-terminus be added as a peptide that is not covalently linked to the main-body of the protein. Therefore, the ability of N-terminal peptides to allosterically modify the affinity of S12D-hL-PYK (hL-PYK with the S12D mutation) for PEP was tested. The magnitude of allosteric coupling, Q_{ax}, was measured by determining ligand affinity of the protein for PEP over a concentration range of the peptide effector (1, 22, 23). From the onset of our experimental design, we expected that high concentrations of peptides would be required for binding; binding between peptide and main-body was expected to be much weaker due to the removal of an entropic contribution (i.e removal of covalent attachment). Therefore, this study required large quantities of peptides. This need for high peptide concentration (at high purity) challenged the cost effectiveness of commercial peptide synthesis. Furthermore, our focus on regulation required a convenient purification tag that does not modify the regulatory properties of the peptide. Therefore, we report the use of an *E. coli* SUMO-fusion system to express and purify peptides of interest (See note 1). This expression system has two advantages; high yields and specific tag cleavage using SUMO-protease (24); the latter property allows the removal of the purification tag. A detailed method for the purification of the peptides and the use of these peptides as allosteric effectors is described in this chapter. Based on this proof-in-principle study demonstrating allosteric regulation by the N-terminal peptides, future work can use modified peptides in an effort to map which regions of the N-terminus of hL-PYK contribute to binding and which regions participate in allosteric coupling.

2. Materials

6XHis-SUMO-tagged peptides were constructed for the ease of purification and tag removal. Other systems such as 6XHis-tagged systems are also commonly used for the purification of proteins and peptides. However, in hL-PYK we observed that the introduction of the 6XHis-tag to the N-terminus of the protein changes the $K_{app\text{-}PEP}$, (See note 2). Since this might indicate that the 6XHis-tag modifies the interaction of the N-terminus with the main-body of the protein (i.e. the property we wish to study), this tag was not used in further studies. Based on the potential for specific site cleavage for tag removal, we choose to use the SUMO expression system (Life Sensors) to express peptides.

2.1. Peptide Purification

1. Resuspension Buffer: 25 mM HEPES/KOH pH 7.5, 100 mM NaCl 10 mM imidazole, 10% glycerol, 5 mM β-mercaptoethanol, and 0.1 % Triton-X
2. Wash Buffer: 25 mM HEPES/KOH pH 7.5, 100 mM NaCl, 10 mM imidazole, and 5 mM β-mercaptoethanol
3) Elution Buffer: 25 mM HEPES/KOH pH 7.5, 100 mM NaCl, 300 mM imidazole, 5 mM β-mercaptoethanol
4) Dialysis Buffer: 25 mM HEPES/KOH pH 7.5, 100 mM NaCl, and 5 mM β-mercaptoethanol

2.2 Enzymatic Assay

1. Assay Buffer: A 10× assay buffer was prepared with 500 mM bicine, 100 mM $MgCl_2$, 1 mM EDTA. The pH of the buffer was adjusted to pH 7.5 using KOH and stored at 4 °C.
2. 100 mM NADH (Sigma) stock solution was made and stored at −20 °C.

3. L-lactic dehydrogenase (LDH) (Type III bovine heart) from Calzyme laboratories, Inc., was dialyzed in 50 mM Bicine pH 7.5, 10 mM $MgCl_2$, 0.1 mM EDTA and 100 mM KCl buffer. The dialyzed LDH was stored at 4 °C until use.
4. 471 mM stock of ADP(Na) was prepared and the pH of the solution was adjusted to pH 7.5 and stored at −20 °C
5. Potassium-PEP (Chem-IMPEX International) was used to prepare 480 mM stock solution, pH of the solution was adjusted to 7.5 using KOH and the stock solution of PEP was stored at −20 °C. The PEP solution was diluted serially (1:2) in 147 mM KCl to obtain the working concentrations.
6. The effector (peptides prepared as described later) was serially (1:2) diluted in a buffer to maintain constant K^+ of 150 mM in the final reaction.

3.1 Construction and Expression of SUMO peptides

1. The N-terminal peptides were created by first cloning the human liver pyruvate kinase (*l-pyk*) from pLC11 (20, 21) into the pE-SUMOpro Kan vector (Life sensors), a vector that contains the 6XHis-SUMO gene immediately in front of the multiple cloning site. BbsI and SacI restriction sites were added at the 5'- and 3'-ends of the *l-pyk* gene, respectively, using PCR. PCR product cleaved with both BbsI and SacI was cloned into BsaI-SacI cleaved pE-SUMOpro Kan vector. The constructed plasmid containing the *l-pyk* gene was named pSUMO-LPYK.
2. Once cloned, a second construct (pSUMO-LPYK/Δ1–5) was created by deleting codons for the initial 5 residues of hL-PYK. This deletion was based on our observation that the initial 5 N-terminal amino acids do not impact PEP affinity (18). Both pSUMO-LPYK and pSUMO-LPYK/Δ1–5 were used in further steps.
3. Stop codons were introduced at different locations in the *l-pyk* coding sequence of pSUMOLPYK and pSUMO-LPYK/Δ1–5 in order to obtain desired peptides, Figure 3. Termination sites introduced at position 13 were chosen based on the observation that residues 7–10 in the N-terminus are energetically coupled with PEP binding (18). Termination at position 18 was based on the hL-PYK residues removed from the protein used in crystallization studies (21). Termination at position 25 was chosen because residue position 26 was the first observable residue in the crystallized protein (21). Termination at position 36 removes the initial helix in the N-terminal helix-turn-helix observed in the crystal structure.
4. The SUMO-peptide gene constructs were transformed into BL21(DE3) *E. coli* cells (Stratagene) for expression.
5. Cultures were grown to a OD_{600} of 0.6 before inducing protein expression by the addition of 1 mM IPTG. After induction, cultures were allowed to grow for 4 hours (See note 3).

3.2 Purification of N-terminal Peptides

1. Resuspend pellet in resuspension Buffer.
2. Lyce the cells using sonication.
3. Centrifuge the protein sample at 9681xg (9000 rpm in Sorvall SS-34 rotor) for 15 minutes.
4. Collect cell lysate and add 0.1 % protamine sulfate. Mix for approximately 1 hr at 4 °C. (See Note 4)

5. Centrifuge at 23426xg (14,000 rpm in Sorvall SS-34 rotor) for 10 minutes. Collect the supernatant. A clear solution should be obtained. Repeat the procedure if required. Continue with the supernatant.
6. Nickel resin (Biorad) should be washed and pre-equilibrated with wash buffer. To promote protein binding to the Ni-resin, mix the supernatant from step 3.2.5 with 500 μL of Ni-resin for approximately 2 hours at 4 °C.
7. Pour the resin into a column using gravity flow. Collect the flow-through and store until the purification is complete. Wash the resin with wash buffer with a minimum of 20 column volumes or until the absorbance is less than 0.1.
8. Elute the protein with elution buffer (i.e. 300 mM imidazole). Add 1 mL of the elution buffer on the resin. Incubate for 5 minutes. Collect elutions (See note 5).
9. Check the fractions on an SDS gel for purity (Figure 3). Pool fractions with highest purity and dialyze in dialysis buffer to remove traces of imidazole (See note 6).
10. Add 0.5 mL of 5 mg/mL 6XHis-SUMO protease (24) and mix at 4 °C (See note 7). The SUMO protease used in this step has been modified to contain an N-terminal 6XHis-tag. This modification aids in removing the protease purified peptides.
11. To allow the cleaved 6XHis SUMO-tag and the 6XHis-SUMO protease to bind the Ni-resin, allow the protein sample to mix with the 500 μL Ni-resin (50% slurry provided by the manufacturer) and pour the resin on the column using gravity flow, collect the flow-through.
12. Cleaved peptides run through the column and the 6XHis-SUMO-tag and 6XHis-SUMO protease bind to the column. Wash the resin with a minimum volume of dialysis buffer.
13. Check protein fraction by SDS gel and MALDI analysis (Figure 3).
14. Measure the absorbance at 214 nm for the amide bonds and a peptide-specific calculated extinction coefficient (25) to measure the concentration of the purified peptide.

3.3 Assay to test the peptides as effectors

1. Expression of hL-PYK in FF50 *E. coli* and the purification of hL-PYK from this expression system have previously been described (20). Our hypothesis of hL-PYK regulation is that phosphorylation interrupts an activating interaction between the non-phosphorylated N-terminus and the main-body of the protein (18). Therefore, all tests for interaction of isolated peptides reported in the current study were with the S12D-hL-PYK mutant protein. This mutation mimics phosphorylation and is expected to prevent competition between binding of the isolated peptide and binding of the covalently attached N-terminus.
2. For the enzymatic assays, a cocktail was prepared (See note 8) from the stock solutions as described above (3.96 mL of 10× screening-assay buffer, 87 μl of 100 mM NADH, 8 μl of LDH, 198 μl of 400 mM ADP, 6 mg of fresh DTT, a volume of S12D hL-PYK that results in a ΔA_{340}/min equal to ~0.015 in the final in-well assay (determined at the beginning of each day), and distilled water to a final volume of 22 mL). Both PEP and effector solutions were serially diluted to obtain the required concentrations.
3. Figure 5 show that all designed N-terminal peptides increase the apparent affinity of the S12D-hL-PYK protein for PEP. Initial velocities as a function of substrate concentration were fit to as described earlier (23)

4. The $K_{app\text{-}PEP}$ values are plotted with varying concentration of the effector and fitted to the equation,

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

where $K_a = K_{app\text{-}PEP}$ in the absence of effector, K_{ix} is the dissociation constant for effector (X) in the absence of substrate, and Q_{ax} is the coupling constant as introduced in the earlier section (1, 22, 26, 27).

All the N-terminal peptides show an allosteric regulation when added as effectors to the S12D-hL-PYK protein (Figure 5). S12D mutation mimics the phosphorylated protein, both the mutation and the covalent modification reduce affinity for the substrate PEP. The increased PEP apparent affinity of S12D-hL-PYK upon the addition of the N-terminal peptides emphasizes the energetic coupling between the N-terminus of the protein and PEP affinity. In addition to the initial screening at a single concentration of peptide (Figure 5), a quantitative analysis of peptide affinity and allosteric coupling between peptide and substrate binding can be considered. Figure 6 exemplifies this quantitative approach for the AA 6–17 peptide. With increasing concentration of peptide the PEP affinity of the S12D protein approaches that for the wild type protein (23). Restated, the loss of affinity due to the phosphorylation mimic is rescued by the addition of a non-covalent N-terminal peptides. Furthermore, this is not a non-specific effect due to high concentrations of peptide; although only initial data have been collected for the impact of the AA 6–17 peptide containing the S12D mutant (Figure 6), it is clear that if this peptide elicits any impact on the affinity of the protein for PEP, it does so only at much higher peptide concentrations.

Using the quantitative approach exemplified in Figure 6, the utility of creating peptide that mimic covalent modification can be appreciated. Consider, that we now have a working hypothesis that phosphorylation interrupts an activating interaction between the N-terminus and the main body of the hL-PYK protein (18). Furthermore, residues 7–10 are required for regulation by the N-terminus. In the full length protein it might be possible to mutate residues 7–10 to determine which replacement residues and at which residue positions modify PEP affinity. However, this approach would not distinguish if a mutation alters PEP affinity by altering the binding between the N-terminus and the main body of the protein, or by altering the energetic coupling (Q_{ax}). As presented in Figure 6, Q_{ax} is the distance between the horizontal plateau at low peptide concentration and the horizontal plateau approached at high peptide concentration (1, 22, 26, 27). In contrast to making mutations in the full length protein, mutating residues 7–10 in the peptide AA 6–17 will allow a systematic partitioning of how each of these residue contribute to N-terminal/main body binding and to allosteric coupling between N-terminal and PEP binding. This general strategy should find broad application in the study of mechanisms by which proteins are regulated by covalent modification.

4. Notes

1. There are other systems for the expression and purification of peptides in bacterial system (28, 29).
2. The addition of 6XHis-tag modified the affinity for PEP when tested using a single pass assays. Table 1 show various designs which were attempted. The sequence in bold represents the WT sequence for the hL-PYK gene.
3. Longer growth times after induction caused degradation of the peptides (Figure 7).

4. Protamine sulfate is used to precipitate the nucleic acids. It is important to obtain a clear solution after the centrifugation step.
5. Additional purity of the sample/elutions can be obtained by using an imidazole gradient.
6. Sample dialysis removes traces of imidazole so that when subjected to the Ni–column for the second time, the cleaved 6XHis-SUMO protein and the 6XHis-SUMO protease will bind to the column. In the second Ni–column step, all proteins except the cleaved peptides should bind to the Ni–resin. The desired peptide will be in the flow-through after the second Ni-column (Figure 4).
7. At this step we have left the samples stored at 4 °C overnight with no adverse effects.
8. Preparation of a reaction cocktail reduces the number of additions to a well in the 96-well plate in an effort to minimize pipetting error. In the current study, only three additions (cocktail including hL-PYK, peptide effector, and PEP) were added to each well.

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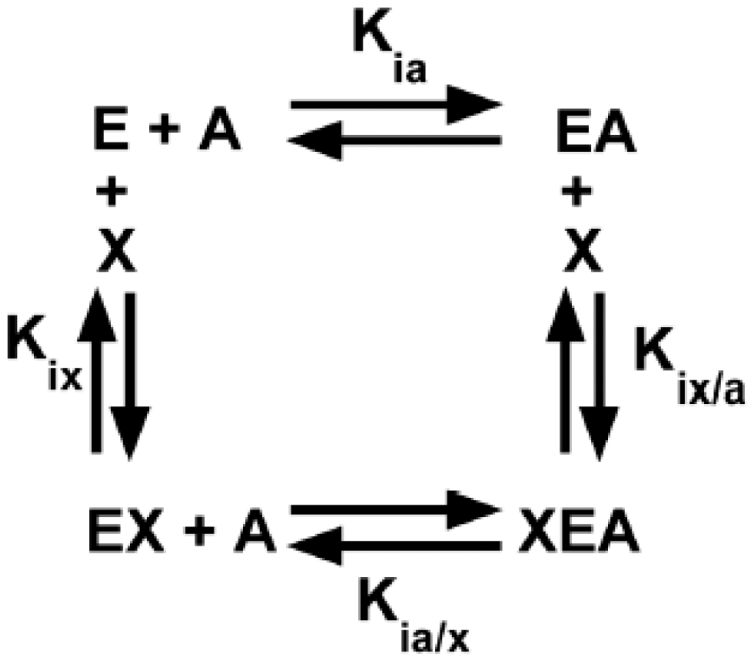

Figure 1.
Thermodynamic cycle defines the binding enzyme (E) to substrate (A) and allosteric effector (X).

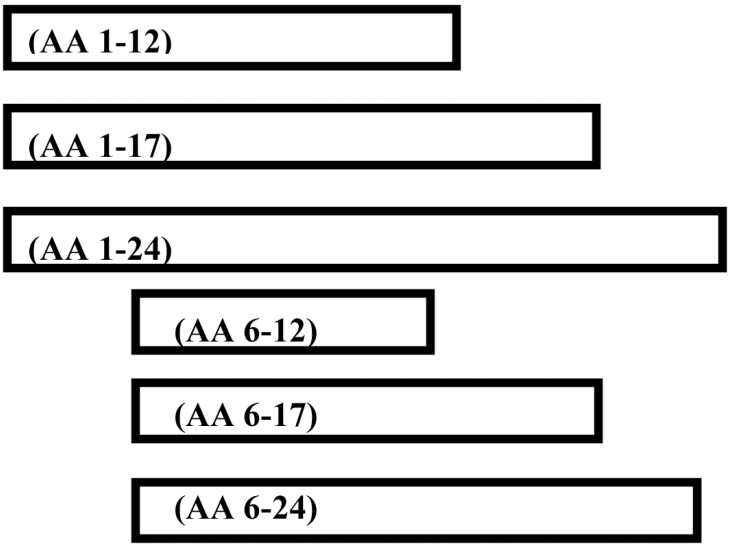

Figure 2.
A representation of the peptides tested in Figures 5 and 6 as allosteric effectors of hL-PYK. Peptides are labeled by the residue positions of hL-PYK represented in the peptide.

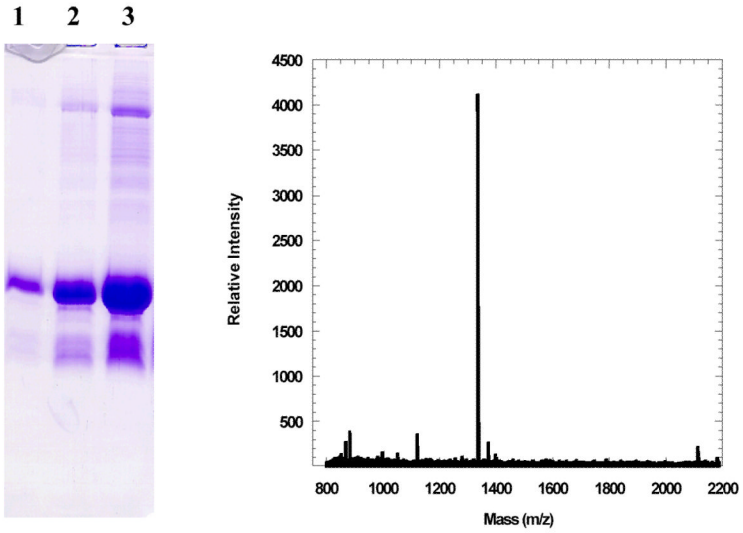

Figure 3.

The purity of the purified peptides analyzed using SDS-PAGE gel and MALDI. Panel A: shows a gel with purified protein from cells grown with a 4-hour induction time; lanes 1, 2, and 3 represent fractions 1, 2, and 3 for the elution of His-Sumo-AA 6–17 from the initial Ni-column step. Panel B: MALDI data for purified peptide AA 6–17.

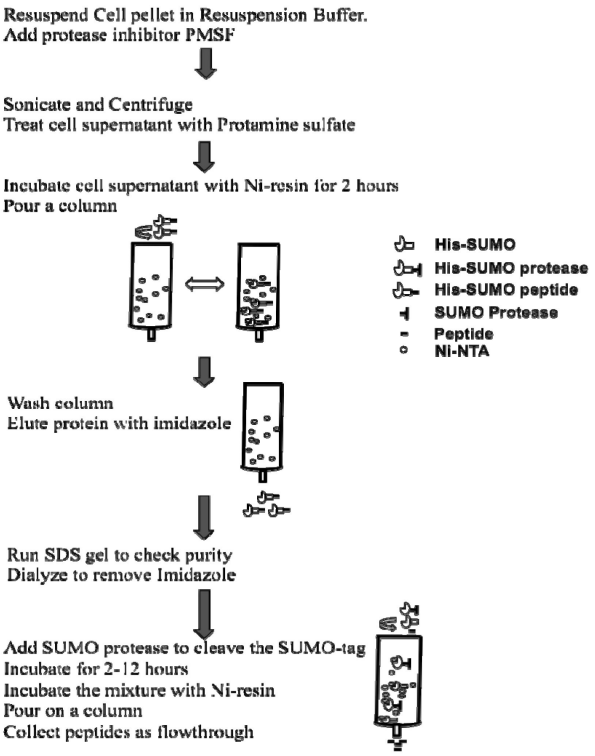

Figure 4.
Flow chart representing the peptide purification steps.

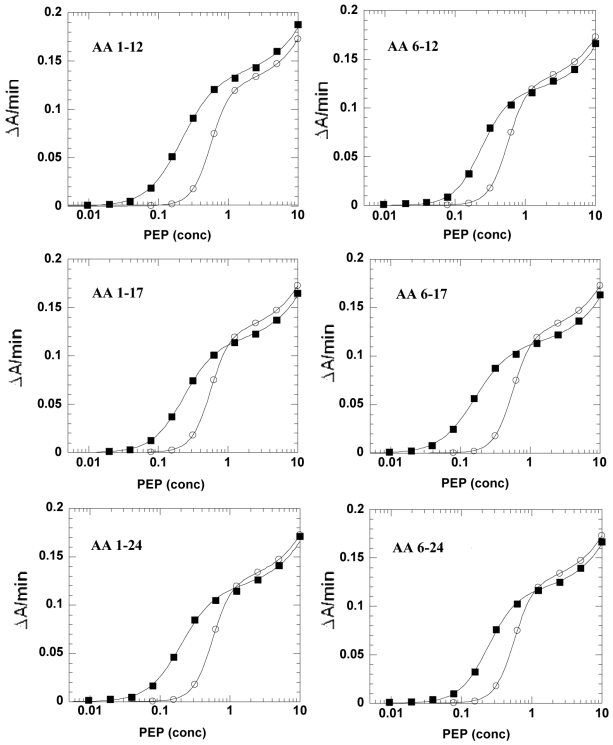

Figure 5.

An initial screen for the effect of N-terminal peptides on S12D-hL-PYK. In each panel, open circles (◦) represent the S12D protein with no effector. Symbol filled squares (■) represents the data for PEP binding for S12D protein in the presence of peptide as labeled, AA 1–12, AA 6–12, AA 1–17, AA 1–24, AA 6–24 and AA 6–17. All the peptide used in this screen increases the PEP affinity of the S12D-hL-PYK protein.

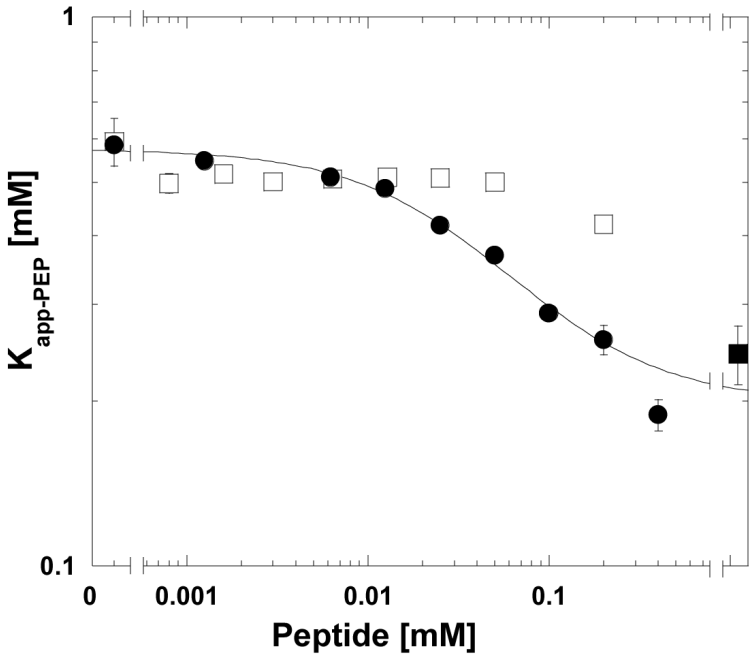

Figure 6.

$K_{app\text{-}PEP}$ vs peptide concentration to monitor the effect of N-terminal peptide on the affinity of PEP. Filled circles are for the AA6–17 peptide. Open square are for the AA6–17-S12D control peptide. The wild type $K_{app\text{-}PEP}$ (solid square) data point (in the absence of effector) was included in the fit (representing the "infinite concentration") for the response to the AA 6–17 peptide.

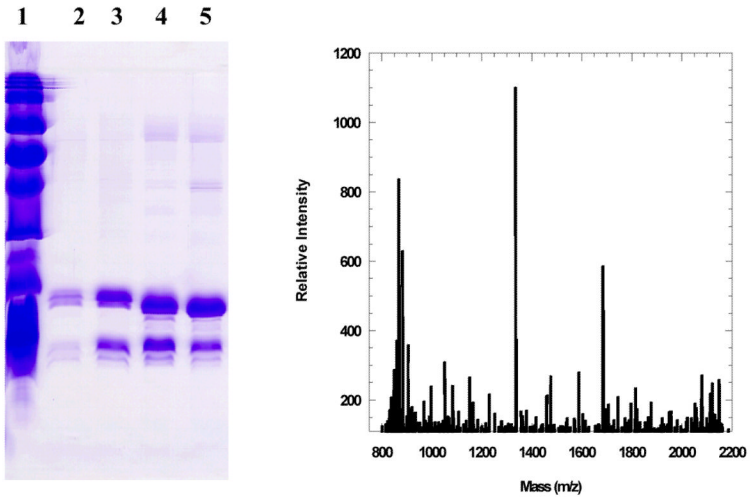

Figure 7.

A longer time of induction show degradation of peptides. Panel A shows a gel with purified protein from cells grown with 24-hour induction. Lane 1 is the high molecular weight protein marker. lanes 2, 3, 4, and 5 represent elutions of His-Sumo-AA 1–12, His-Sumo-AA 1–17, His-Sumo-AA 1–24, and His-Sumo-AA 1–35 respectively. Panel B: MALDI data for the AA1–25 with 24 hour induction show degradation of peptide.

Table 1

Different construct for hL-PYK-N terminal peptides with the 6XHis tag, compared to the wild type N-terminal sequence[a].

	Sequence
1	MDHHHHHHGGGG M E G P A G Y L R R A S V A Q L T Q…
2	MDHHHHHHGG M E G P A G Y L R R A S V A Q L T Q…
3	MDHHHHHH M E G P A G Y L R R A S V A Q L T Q…
4	MHHHHHH M E G P A G Y L R R A S V A Q L T Q…
Wild Type	**M E G P A G Y L R R A S V A Q L T Q** …
5	MDHHHHHH G G P A G Y L R R A S V A Q L T Q…
6	MDHHHHHH G P A G Y L R R A S V A Q L T Q…
7	MDHHHHHH P A G Y L R R A S V A Q L T Q…
8	MDHHHHHH A G Y L R R A S V A Q L T Q…
9	MDHHHHHH G G Y L R R A S V A Q L T Q…
10	MDHHHHHH G Y L R R A S V A Q L T Q…
11	MDHHHHHH Y L R R A S V A Q L T Q…

[a]Each of these modifications slightly perturbed the $K_{app\text{-}PEP}$ as compared to that of the wild type protein. Based on a single assay, the smallest perturbation was the #3 construct that shifted the $K_{app\text{-}PEP}$ to 0.34 mM. However, regulation by phosphorylation only shifts $K_{app\text{-}PEP}$ from 0.2 to 0.6 mM. Therefore, the impact of adding the 6Hix-tag caused a change that was on the order of half that caused by phosphorylation. Proteins with these labels were not considered further.