3816 *Biochemistry* **2009,** *48,* 3816–3818
DOI:10.1021/bi900421f

BIOCHEMISTRY
including biophysical chemistry & molecular biology
Rapid Report

An Activating Interaction between the Unphosphorylated N-Terminus of Human Liver Pyruvate Kinase and the Main Body of the Protein Is Interrupted by Phosphorylation†

Aron W. Fenton* and Qingling Tang

Department of Biochemistry and Molecular Biology, The University of Kansas Medical Center, Kansas City, Kansas 66160

Received March 11, 2009. Revised Manuscript Received March 24, 2009

ABSTRACT: The initial 26 amino acids of human liver pyruvate kinase (L-PYK) are not present/observed in the crystal structure. This region includes Ser12, the site of hormone-dependent phosphorylation. Truncating the N-terminus of L-PYK mimics the effects of phosphorylation by causing a decrease in apparent phosphoenolpyruvate (PEP) affinity. An N-terminus truncation series was used to map the minimum number of residues that could be removed to result in the decrease in apparent PEP affinity. Results are consistent with a mechanism by which phosphorylation at Ser12 interrupts an activating interaction of N-terminal residues (including those at positions 7−10) with the main body of the protein, as a means of inhibiting substrate affinity.

Hormonal regulation of pyruvate kinase (PYK) plays an important role in maintaining glycolysis/gluconeogenesis homeostasis in liver. Hormone-induced changes in liver PYK (L-PYK) occur over days (*1*−*3*), but L-PYK activity changes within minutes of intravenous injections of insulin or glucagon (*4*−*7*). This acute regulation is mediated by phosphorylation/dephosphorylation of the enzyme (*8*,*9*). Phosphorylation of L-PYK at Ser12 by cyclic-AMP dependent protein kinase (in response to increased glucagon) decreases the affinity of L-PYK for its substrate phosphoenolpyruvate (PEP) (*10*−*19*).

More is understood about the physiological consequence of L-PYK phosphorylation than the molecular/atomic mechanism by which this covalent modification impacts the protein's affinity for substrate. An X-ray crystallographic structure of PYK from human erythrocyte (R-PYK) has been reported (*20*). L-PYK and R-PYK are produced from the same gene as a result of alternate start sites; R-PYK has a 31 amino acid N-terminal extension compared to L-PYK (*21*). To obtain reproducibility in crystal growth, the N-terminus of R-PYK used in structural studies was truncated by 49 amino acids. Therefore, the protein used in those structural studies is equivalent to L-PYK minus the initial 18 N-terminal amino acids. Additional N-terminal residues in the L/R-PYK structure were not ordered, such that the residue equivalent to Gln26 of L-PYK is the first observed amino acid. On the basis of the relative location of Gln26 in the L/R-PYK structure and the potential length of the extended N-terminus sequence, the N-terminus is not thought to interact directly with the active site. Therefore, it is unknown where, or how, the N-terminal 26 residues of L-PYK — with or without the phosphorylation of Ser12 — interact with the main body of the protein. We report here a systematic study of the N-terminus of human L-PYK — with and without a mimic of the phosphorylated Ser12 (i.e., S12D).

In this work, we take the lack of observable change in protein function upon N-terminal residue removal to be consistent with the absence of an interaction (in the full-length protein) between the probed residues and the main body of the protein. As with any negative evidence, the lack of an observed effect cannot be interpreted as the nonexistence of such an effect. However, increasing the number of protein properties monitored increases the likelihood of identifying any one property that is dependent on the deleted region. The allosteric properties of L-PYK are ideal as functions that are sensitive to changes in many areas of the protein. Allostery requires the effector to bind at a site distinct (and often distant) from the active site. Upon binding of the effector, multiple pathways of interaction (linked-reactions) within the protein are responsible for the overall observed allosteric coupling between the effector site and the active site (*22*). We refer to the combination of these multiple pathways as the allosteric mechanism. The allosteric mechanism for any one effector likely involves many regions of the protein. Therefore, monitoring the allosteric response of two different effectors, fructose-1,6-bisphosphate (Fru-1,6-BP or FBP; activator) and alanine (inhibitor), likely reports potential structural (conformational and/or dynamic) changes of a high percentage of the L-PYK protein. Thus, rather than focus solely on the initial velocity derived apparent affinities for the substrate PEP ($K_{app\text{-}PEP}$) as a means of monitoring the impact of modifications of the N-terminus, allosteric functions were also determined.

†This work was supported by NIH Grant DK78076.
*To whom correspondence should be addressed. Telephone: (913) 588-7033. Fax: (913) 588-9896. E-mail: afenton@kumc.edu.

 Published on Web 3/25/2009

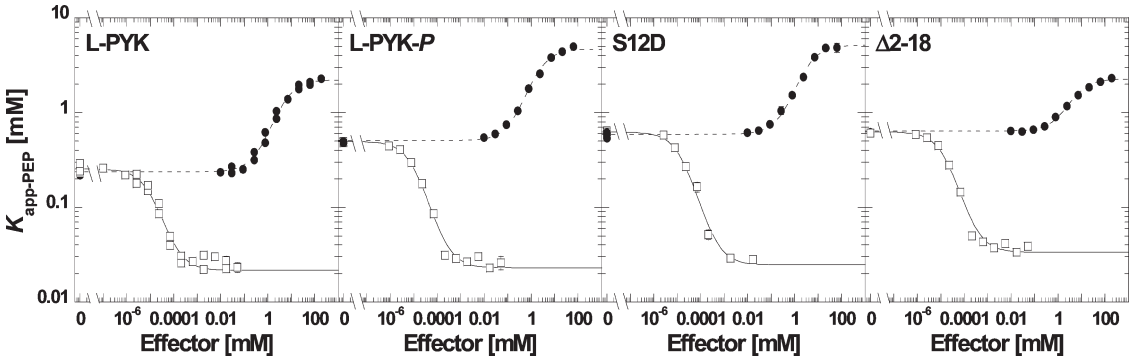

FIGURE 1: $K_{app\text{-}PEP}$ as a function of Fru-1,6-BP (□), or alanine (●) at pH 7.5. Proteins were purified using DEAE cellulose as previously described (*ref25*). Lines represent the best fits to eq 2; fit parameters are in Supporting Information. When error bars are not apparent, they are smaller than data point symbols.

Allosteric function was analyzed using a linkage-analysis (*22*−*25*). To facilitate a linkage-analysis, $K_{app\text{-}PEP}$ values were determined over a concentration range of allosteric effector, fructose-1,6-bisphosphate or alanine inhibitor. On a log−log plot of $K_{app\text{-}PEP}$ vs effector concentration (Figure 1), the affinity for the substrate in the absence of effector ($K_{a\text{-}PEP}$) determines the vertical placement. On the same plot, the affinity for effector determines horizontal placement (although the midpoint of the transition is not a binding value for the effector). Finally, the difference between plateaus (one plateau at low effector concentration and one plateau at high effector concentration) is dictated by the allosteric coupling (Q_{ax}) between substrate and effector binding to protein.

As can be seen by comparing the left *y*-intercept, phosphorylation (phosphorylated L-PYK labeled L-PYK-*P*) causes a decrease in the affinity of the enzyme for PEP in the absence of effector (an increase in $K_{a\text{-}PEP}$) (Figure 1 and Supporting Information). This result is consistent with previous reports (*10*−*14*). This change is accompanied by an increase in the magnitude of the allosteric coupling of activation by Fru-1,6-BP. However, the magnitude of the allosteric coupling of the inhibition by alanine appears unresponsive to phosphorylation. Since enzymatic phosphorylation is never 100% efficient, mutants that mimic this covalent modification can be useful as an experimental tool. The introduction of S12D or S12E mutations mimics the effects of phosphorylation (Figure 1 and Supporting Information). S12D was therefore used as a phosphorylation mimic (phoshporylation^{mimic}).The truncated R-PYK subunit used in the previous structural study (*20*) is equivalent to L-PYK without the initial 18 N-terminal amino acids. L-PYK that lacks the initial 18 residues (except the initial Met; labeled Δ2−18) demonstrates a $K_{a\text{-}PEP}$ similar to that of L-PYK-*P*. In addition, removal of the initial 18 amino acids of L-PYK reduces the allosteric coupling elicited by alanine; this finding does not mimic the response of L-PYK-*P* (see below). The decreased inhibition by alanine can best be appreciated in Figure 1 by comparing the right y-intercept of graphs for L-PYK-*P* and Δ2−18 data.

Historically, subtilisin treatment, rather than molecular techniques, was used to remove the N-terminus of L-PYK (*26*). However, we found that when the Δ2−18 protein was treated with subtilisin, an increase in $K_{a\text{-}PEP}$ was observed (Supporting Information). Therefore, subtilisin treatment does more than just remove the phosphorylatable Ser12; this treatment was not used further in this study.

Following the example provided by Bondos and coworkers to study regions of a protein that are not ordered in a crystallographically determined structure (*27*), a series of truncation mutations was introduced to remove N-terminal residues in the presence and absence of S12D. Removing the initial 10 N-terminal residues had no impact on $K_{a\text{-}PEP}$ when the S12D mutation was present. In the absence of this phosphorylation^{mimic}, removal of the initial six N-terminal residues also had little influence on $K_{a\text{-}PEP}$. Sequential removal of N-terminal residues in the absence of phosphorylation^{mimic}, starting at the seventh position, caused $K_{a\text{-}PEP}$ to increase to a level similar to that caused by the addition of phosphorylation. There is no further impact on $K_{a\text{-}PEP}$ upon removal of additional residues beyond position 10.

To this point, data are consistent with a phosphorylation-dependent modulation of the interaction between the N-terminus and the main body of the protein. However, the unique allosteric response of Δ2−18 to alanine might signify that the N-terminus plays a role in alanine inhibition, even in the presence of phosphorylation. A potential role for the phosphorylated N-terminus in inhibition by alanine (and therefore, the observed difference in alanine inhibition when comparing L-PYK-*P* and Δ2−18) would require an interaction of this terminus with the main body of the protein. However, it is also possible that different regions of the N-terminus impact $K_{a\text{-}PEP}$ vs allosteric inhibition by alanine ($Q_{ax\text{-}Ala}$). As seen in Figure 2, the impact of truncations on $Q_{ax\text{-}Ala}$ begins at or shortly after position 12 (the site of phosphorylation) and progresses as additional residues are removed. Therefore, the regions of the N-terminus that impact $K_{a\text{-}PEP}$ and that impact $Q_{ax\text{-}Ala}$ are not equivalent.

Taken together, these results do not identify a function that requires residues 1−6 to interact with the main body of the protein in the presence or in the absence of phosphorylation. Since the dependencies of $K_{a\text{-}PEP}$ and $Q_{ax\text{-}Ala}$ on the N-terminus map to different regions of this initial amino acid sequence, our results do not identify a function (and therefore a required interaction with the main body of the protein) for the initial 12 residues when the N-terminus is phosphorylated. However, in the absence of phosphorylation, the presence of residues 7−10 enhances PEP

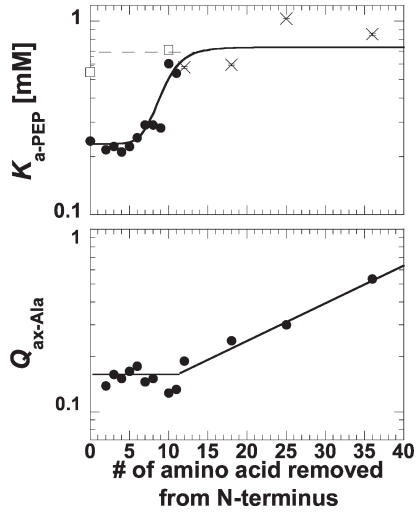

FIGURE 2: $K_{a\text{-}PEP}$ and $Q_{ax\text{-}Ala}$ as a function of the number of N-terminal residues removed. Truncations are either in the presence of S12D (the phosphorylation^{mimic}) (□), without modification to Ser12 (●), or with the Ser12 position removed (×). Proteins were partially purified by ammonium sulfate fractionation. $Q_{ax\text{-}Ala}$ values were obtained by fitting data such as that exemplified in Figure 1 to eq 2. The $K_{a\text{-}PEP}$ for Δ2−9 is not reproducible; therefore, $Q_{ax\text{-}Ala}$ for this truncated protein is not included. Lines represent data trends. When error bars are not apparent for $Q_{ax\text{-}Ala}$ data, they are smaller than data point symbols.

affinity (i.e., activation). This result, in turn, is consistent with a mechanism by which phosphorylation at Ser12 interrupts the interaction of N-terminal residues (including positions 7−10) with the main body of the protein. This interaction must be energetically coupled with PEP binding in the active site, that is, an activating energetic coupling. Therefore, interruption of this activating N-terminal/main-body interaction (by phosphorylation or by truncation) gives rise to the apparent phosphorylation-dependent inhibition of PEP affinity.

Unlike truncations in the presence of the phosphorylation^{mimic}, in the absence of phosphorylation, progressive removal of residues from 7 to 10 incrementally impact the $K_{a\text{-}PEP}$. This observation necessitates some level of interaction between the unphosphorylated N-terminus and the main body of the protein. The removal of each sequential residue in this region likely reduces the stability of the interaction between the N-terminus and the main body of the protein. Therefore, the N-terminus with sequential deletions of residues 7−10 likely interact with the main body of the protein less often than the N-terminus of the full-length protein, but the interaction is not completely abandoned until the N-terminus is either further truncated, or phosphorylated.

On the basis of the conclusion that phosphorylation functions by disrupting an activating interaction between the nonphosphorylated N-terminus (including residues 7−10) and the main body of the protein, future structure/function studies can focus on understanding the mechanism by which the unphosphorylated N-terminus decreases $K_{a\text{-}PEP}$.

SUPPORTING INFORMATION AVAILABLE

Materials and Methods; sequence alignment of human L-PYK and human R-PYK N-terminal residues; the structure of pyruvate kinase; reaction time for maximum phosphorylation; supporting observations and implications for R-PYK; subtilisin cleavage of L-PYK; primers for mutagenesis; and parameters from linkage analysis of DEAE purified proteins. This material is made available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

1. Weber, G., Stamm, N. B., and Fisher, E. A. (1965) *Science 149* 65–67.
2. Fleig, W. E., Geerling, I., Roben, H., and Ditschuneit, H. (1984) *Biochim. Biophys. Acta 805*, 165–173.
3. Yamada, K., and Noguchi, T. (1999) *Biochem. J. 337*(1), 1–11.
4. Taunton, O. D., Stifel, F. B., Greene, H. L., and Herman, R. H. (1974) *J. Biol. Chem. 249*, 7228–7239.
5. Greene, H. L., Taunton, O. D., Stifel, F. B., and Herman, R. H. (1974) *J Clin Invest 53*, 44–51.
6. Stifel, F. B., Taunton, O. D., Greene, H. L., and Herman, R. H. (1974) *J. Biol. Chem. 249*, 7240–7244.
7. Taunton, O. D., Stifel, F. B., Greene, H. L., and Herman, R. H. (1972) *Biochem. Biophys. Res. Commun. 48*, 1663–1670.
8. El-Maghrabi, M. R., Haston, W. S., Flockhart, D. A., Claus, T. H., and Pilkis, S. J. (1980) *J. Biol. Chem. 255*, 668–675.
9. Engstrom, L. (1978) *Curr. Top. Cell Regul. 13*, 28–51.
10. Ekman, P., Dahlqvist, U., Humble, E., and Engstrom, L. (1976) *Biochim. Biophys. Acta 429*, 374–382.
11. Ishibashi, H., and Cottam, G. L. (1978) *J. Biol. Chem. 253* 8767–8771.
12. Blair, J. B. ((1980)) Regulatory Properties of Hepatic Pyruvate Kinase, in *The Regulation of Carbohydrate Formation and Utilization in Mammals* (Veneziale, C. M., Ed.), pp 121−151, University Park Press, Baltimore..
13. El-Maghrabi, M. R., Claus, T. H., McGrane, M. M., and Pilkis, S. J. (1982) *J. Biol. Chem. 257*, 233–240.
14. Ljunstrom, O., Berglund, L., and Engstrom, L. (1976) *Eur. J. Biochem. 68*, 497–506.
15. Inoue, H., Noguchi, T., and Tanaka, T. (1986) *Eur. J. Biochem. 154*, 465–469.
16. Humble, E. (1980) *Biochim. Biophys. Acta 626*, 179–187.
17. Pilkis, S. J., El-Maghrabi, M. R., Coven, B., Claus, T. H., Tager, H. S., Steiner, D. F., Keim, P. S., and Heinrikson, R. L. (1980) *J. Biol. Chem. 255*, 2770–2775.
18. Hjelmquist, G., Andersson, J., Edlund, B., and Engstroom, L. (1974) *Biochem. Biophys. Res. Commun. 61*, 559–563.
19. Edlund, B., Andersson, J., Titanji, V., Dahlqvist, U., Ekman, P., Zetterqvist, O., and Engstoom, L. (1975) *Biochem. Biophys. Res. Commun. 67*, 1516–1521.
20. Valentini, G., Chiarelli, L. R., Fortin, R., Dolzan, M., Galizzi, A., Abraham, D. J., Wang, C., Bianchi, P., Zanella, A., and Mattevi, A. (2002) *J. Biol. Chem. 277*, 23807–23814.
21. Noguchi, T., Yamada, K., Inoue, H., Matsuda, T., and Tanaka, T. (1987) *J. Biol. Chem. 262*, 14366–14371.
22. Fenton, A. W. (2008) *Trends Biochem. Sci. 33*, 420–425.
23. Reinhart, G. D. (2004) *Methods Enzymol. 380*, 187–203.
24. Weber, G. (1972) *Biochemistry 11*, 864–878.
25. Fenton, A. W., and Hutchinson, M. (2009) *Arch. Biochem. Biophys. 484*, 16–23.
26. Bergstrom, G., Ekman, P., Humble, E., and Engstrom, L. (1978) *Biochim. Biophys. Acta 532*, 259–267.
27. Liu, Y., Matthews, K. S, and Bondos, S. E. (2008) *J. Biol. Chem. 283*, 20874–20887.