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Energetic Coupling between an Oxidizable Cysteine and the Phosphorylatable N-Terminus of Human Liver Pyruvate Kinase

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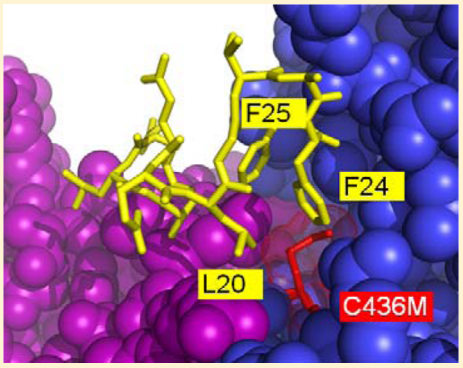
Supporting Information

ABSTRACT: During our efforts to characterize the regulatory properties of human liver pyruvate kinase (L-PYK), we have noted that the affinity of the protein for phosphoenolpyruvate (PEP) becomes reduced several days after cell lysis. A 1.8 Å crystallographic structure of L-PYK with the S12D mimic of phosphorylation indicates that Cys436 is oxidized, the first potential insight into explaining the effect of "aging". Interestingly, the oxidation is only to sulfenic acid despite the crystal growth time period of 2 weeks. Mutagenesis confirms that the side chain of residue 436 is energetically coupled to PEP binding. Mass spectrometry confirms that the oxidation is present in solution and is not an artifact caused by X-ray exposure. Exposure of the L-PYK mutations to H_2O_2 also confirms that PEP affinity is sensitive to the nature of the side chain at position 436. A 1.95 Å structure of the C436M mutant of L-PYK, the only mutation at position 436 that has been shown to strengthen PEP affinity, revealed that the methionine substitution results in the ordering of several N-terminal residues that have not been ordered in previous structures. This result allowed speculation that oxidation of Cys436 and phosphorylation of the N-terminus at Ser12 may function through a similar mechanism, namely the interruption of an activating interaction between the nonphosphorylated N-terminus with the nonoxidized main body of the protein. Mutant cycles were used to provide evidence that mutations of Cys436 are energetically synergistic with N-terminal modifications, a result that is consistent with phosphorylation of the N-terminus and oxidation of Cys436 functioning through mechanisms with common features. Alanine-scanning mutagenesis was used to confirm that the newly ordered N-terminal residues were important to the regulation of enzyme function by the N-terminus of the enzyme (i.e., not an artifact caused by the introduced methionine substitution) and to further define which residues in the N-terminus are energetically coupled to PEP affinity. Collectively, these studies indicate energetic coupling (and potentially mechanistic similarities) between the oxidation of Cys436 and phosphorylation of Ser12 in the N-terminus of L-PYK.



The primary focus of our laboratory is the characterization of the molecular mechanisms of allosteric and covalent regulation of human liver pyruvate kinase (L-PYK). However, while addressing these structure–function questions, we have noted that the apparent affinity of L-PYK for substrate phosphoenolpyruvate (PEP) becomes weaker several days after cell lysis. Our previous solution for this problem was based on a rapid purification protocol,[1] which facilitates the collection of data before the time-dependent changes occur. In fact, all activity-dependent data previously reported from our laboratory for the L-PYK protein[1−6] have been collected within 2 days of cell lysis. Our first insight into a potential mechanism for the time-dependent decrease in substrate affinity was through the observation of an oxidized cysteine (i.e., Cys436) in a protein structure determined by X-ray crystallography. We report here not only the nature of the time-dependent shift in PEP affinity and the structure that initiated insights into the mechanism of this shift but also a series of experiments that indicate that there is energetic synergy in the outcomes elicited by mutation of the oxidized residue (Cys436) and by modification of the N-terminus. The latter is consistent with Cys436 oxidation and N-terminal modification functioning through a related mechanism. Therefore, a brief review of the mechanism by which phosphorylation modifies PEP affinity will become useful.[5−7]

Of the four mammalian pyruvate kinase (PYK) isozymes, the one expressed in liver (L-PYK) provides key regulation for maintaining the balance between gluconeogenesis and glycolysis. Like all PYK isozymes, L-PYK catalyzes the transfer of phosphate from phosphoenolpyruvate (PEP) to ADP to form pyruvate and ATP. Consistent with its regulatory role in metabolism, human L-PYK is inhibited by phosphorylation at Ser12, is allosterically inhibited by alanine, and is allosterically

Received: October 1, 2012
Revised: December 4, 2012
Published: December 27, 2012

Table 1. Crystallographic Data and Model Statistics for the Structure of S12D-L-PYK in Complex with Citrate, ATP, and Fru-1,6-BP

	S12D-L-PYK·citrate·Mn·ATP·Fru-1,6-BP	C436M-L-PYK·citrate·Mn·ATP·Fru-1,6-BP
PDB entry	4IP7	4IMA
beamline	SSRL 11-1	19-ID, APS
wavelength (Å)	0.9	0.97921
space group	$P2_1$	$P2_1$
unit cell	a = 78.1 Å	a = 82.7 Å
	b = 205.1 Å	b = 204.7 Å
	c = 83.9 Å	c = 86.5 Å
	$\alpha = \gamma$ = 90.0°	$\alpha = \gamma$ = 90.0°
	β = 92.2°	β = 96.7°
resolution limits (Å)	50.0−1.8	50.0−1.95
total no. of reflections	1019737	737037
no. of unique reflections	237180	200629
completeness (%) (all data)	97.8 (93.3)[a]	97.7 (86.1)[b]
redundancy	4.3 (3.7)[a]	3.7 (2.9)[b]
$I/\sigma_{(I)}$	17.7 (1.7)[a]	14.9 (1.4)[b]
R_{sym}[c] (%)	5.5 (51.1)[a]	7.4 (51.8)[b]
no. of molecules per asymmetric unit	4	4
no. of reflections used	224885	179645
R_{free}[d]	22.6 (35.1)[a]	23.3
R_{work}[e]	18.9 (33.2)[a]	19.8
root-mean-square deviation for bond lengths (Å)	0.02	0.007
root-mean-square deviation for bond angles (deg)	2.2	1.047
Ramachandran plot (%)		
preferred	96.2	97.07
allowed	2.9	2.72
outliers	0.9	0.21
average B factor		
protein		
chain A	37.7	40.0
chain B	42.4	41.4
chain C	43.1	41.8
chain D	46.8	40.2
ligand		
citrate	32.0	35.8
adenosine	34.6	43.2
Fru-1,6-BP	26.9	31.5
metal		
Mn^{2+}	27.6	38.1
Na^{+}	39.8	
water	38.4	42.4

[a]Values in parentheses represent statistics for data in the highest-resolution shell (1.86−1.80 Å). [b]Values in parentheses represent statistics for data in the highest-resolution shell (2.02−1.95 Å). [c]$R_{sym} = \sum|I_{obs} - I_{avg}|/\sum I_{avg}$. [d]$R_{free}$ was calculated using 5% of the data. [e]$R_{work} = \sum||F_{obs}| - |F_{calc}||/\sum F_{obs}$.

activated by fructose 1,6-bisphosphate (Fru-1,6-BP). All forms of covalent and allosteric regulation result in altered PEP affinity.

L-PYK and the isozyme expressed in erythrocytes (R-PYK) are products from the same gene as a result of the use of different start sites (the other two isozymes, M_1-PYK and M_2-PYK, are products of a second gene). As a result of the use of different start sites, R-PYK contains 31 additional amino acids at the N-terminus compared to L-PYK. However, little is known about the structure of the N-terminus of either isozyme. The previously reported 2.7 Å structure of R-PYK contained a 49-residue N-terminal truncation[8] (equivalent to L-PYK without the initial 18 residues). Furthermore, additional residues were disordered such that the first ordered residue is equivalent to Gln26 of L-PYK. Therefore, the previous structure is not informative with regard to how the N-terminus interacts with the main body of the protein or how phosphorylation modulates this interaction.

Previously, we demonstrated that the S12D mutation mimics the effect of phosphorylation on L-PYK function.[5] However, there were also indications in the literature that N-terminal truncation could mimic phosphorylation.[9] Therefore, we completed a truncation series to provide additional experimental support for the hypothesis that phosphorylation of Ser12 interrupts an activating interaction between the N-terminus and the main body of the protein.[5] As further evidence that the phosphorylated N-terminus does not interact with the main body of the protein or does so with a very low binding energy, we demonstrated that a short peptide designed to mimic the nonphosphorylated N-terminus can cause the S12D protein to bind PEP with strengthened affinity.[6] Therefore, the currently available data suggest that phosphor-

dx.doi.org/10.1021/bi301341r | Biochemistry 2013, 52, 466−476

ylation of Ser12 shifts a binding equilibrium to favor less of an activating interaction between the N-terminus and the main body of L-PYK. As noted above, the summary of the studies reported here is consistent with the oxidation of Cys436 also functioning in an inhibitory fashion by shifting a binding equilibrium to favor less of the activating interaction between the N-terminus and the main body of L-PYK.

MATERIALS AND METHODS

Mutagenesis and Protein Expression and Purification. Mutagenesis of the L-PYK gene to create desired mutations was conducted with Quikchange (Stratagene). Wild-type and mutant proteins were expressed in the FF50 strain of *Escherichia coli*.[1] Cell lysis, ammonium sulfate fractionation, and DEAE-cellulose column purification were conducted as previously reported.[1] Mutant proteins for alanine-scanning mutagenesis were only partially purified using ammonium sulfate fractionation followed by protein dialysis.[5]

Crystallization. Initial crystallization conditions for S12D-L-PYK were determined by submission of the protein to the high-throughput crystallization facility at the Hauptman-Woodward Institute (Buffalo, NY).[10] Crystals of the S12D-L-PYK·Fru-1,6-BP·Mn·Na·citrate complex were grown by the vapor diffusion hanging drop method. Briefly, S12D-L-PYK was dialyzed into 10 mM MES (pH 6.8), 5 mM $MgCl_2$, 10 mM KCl, and 2 mM DL-dithiothreitol (DTT) and then diluted in the same buffer to a final concentration of 5 mg/mL. Immediately before use, 25 μL of a Na-ATP/Na-Fru-1,6-BP solution was added to 100 μL of protein, resulting in 4 mg/mL protein, 52 mM Na-ATP, and 1.3 mM Na-Fru-1,6-BP. The mother liquor solution consisted of 700 μL of 50 mM sodium citrate (pH 4.9), 26 mM $MnCl_2$, and 3−5% polyethylene glycol (PEG) 6000. The ratio of well solution to protein/Fru-1,6-BP/ATP solution was varied (well:protein, volumes in microliters): 2:2, 4:2, 2:4, and 4:4. Crystals were observed to grow over a period of 2−4 weeks at 4 °C. Crystals were cryoprotected by sequential transfer of the crystals (at 4 °C) into mother liquor solutions containing an increasing concentration of glycerol in 5% increments to a final concentration of 25%. During this transfer, the concentration of PEG 6000 was increased slightly and the concentration of other buffer components was decreased slightly such that final concentrations were 25 mM sodium citrate (pH 4.9), 8.0% PEG 6000, 12 mM $MnCl_2$, 25% glycerol, 0.5 mM MES, 0.5 mM KCl, 0.5 mM Fru-1,6-BP, and 20 mM ATP. The crystals were cryo-cooled prior to data collection by direct immersion in liquid nitrogen. Once these crystallization conditions were optimized for the S12D protein, they were also used for the C436M protein.

X-ray Diffraction Data Collection. Data on the cryo-cooled crystals at 100 K were collected at Stanford Synchrotron Radiation Laboratory beamline 11-1 (Menlo Park, CA). All data were integrated and scaled with HKL-2000.[11] Data statistics are listed in Table 1.

Structure Solution and Refinement. The initial phases for S12D-L-PYK were obtained by the method of molecular replacement using MOLREP[15] encoded in the CCP4 suite.[16] X-ray coordinates of the R-PYK monomer[8] (Protein Data Bank entry 2VGB) were used as the search model after being stripped of all water molecules, ligands, and ions. The molecular replacement solution resulted in an initial model containing a single tetramer in the asymmetric unit.

Refinement of the S12D-L-PYK model was conducted in Refmac5[17] encoded in the CCP4 suite. After each round of refinement, $F_o - F_c$ and $2F_o - F_c$ electron density maps were produced and manual model building and optimization were conducted using the model-building, map-fitting program Coot.[18] The addition of ligands, metals, and water molecules was also conducted using Coot prior to a final round of restrained refinement in Refmac5. A final round of TLS refinement was conducted after the optimal number of TLS groups for each chain was determined using the TLSMD web server (http://skuld.bmsc.washington.edu/~tlsmd/). Each L-PYK chain contains four TLS groups for a total of 16 TLS groups. This resulted in a decrease in R and R_{free} values from 19.2 to 18.9% and from 23.3 to 22.6%, respectively. Structural validation was conducted using MolProbity (http://molprobity.biochem.duke.edu/).[12] The final model statistics are summarized in Table 1. The C436M structure was refined using similar steps, only the monomer of the S12D structure was used as the search model in the initial molecular replacement.

Kinetic Assays and Data Analysis. Activity measurements were taken at 30 °C using a lactate dehydrogenase coupled assay.[4] For samples exposed to H_2O_2, the protein was added to a cocktail with all reagents necessary for activity, with the exception of PEP. H_2O_2 was added to this cocktail. After an 8 min incubation, PEP was added and activity monitored as previously described.[4] The lack of an impact on V_{max} activity confirms that activity reagents other than L-PYK were not sensitive to H_2O_2 during the brief incubation. C436A was the only mutation to largely prevent the H_2O_2-dependent shift in $K_{app\text{-}PEP}$. This was true whether proteins were used immediately after purification or if the four proteins were first diluted to similar activities and then dialyzed together against buffer with 2 mM tris(2-carboxyethyl)phosphine hydrochloride (TCEP). The latter was an experimental design to ensure that reducing agent added with protein was equivalent in all assays.

Data fitting was conducted with the nonlinear least-squares analysis of Kaleidagraph (Synergy). Fits of PEP titrations of initial rates (ν) used to obtain $K_{app\text{-}PEP}$ are as previously described.[1,4] Upon evaluation of allosteric regulation, $K_{a\text{-}PEP}$ was determined by fitting a plot of the $K_{app\text{-}PEP}$ values as a function of effector concentration to eq 1:[13]

$$K_{app\text{-}PEP} = K_a\left(\frac{K_{ix} + [\text{effector}]}{K_{ix} + Q_{ax}[\text{effector}]}\right) \quad (1)$$

where $K_a = K_{app\text{-}PEP}$ without effector, K_{ix} is the dissociation constant for effector (X) binding to the protein in the absence of substrate (A), and Q_{ax} is the allosteric coupling constant discussed in detail elsewhere.[13] Because of the current focus of how different regions of the protein impact PEP affinity, only $K_{app\text{-}PEP}$ determined in the absence of effector or $K_{a\text{-}PEP}$ values are included.

RESULTS

Time-Dependent Shift in the Affinity for PEP. While studying the regulatory properties of purified L-PYK, we have noted that the affinity of the protein for PEP becomes weaker as the protein ages for days (Figure 1A). Although the data in Figure 1 show a transition in $K_{app\text{-}PEP}$ after only 1 day, the exact time for the transition can vary considerably from 1 to 4 days (data not shown), but protein purified and stored in the presence of 2 mM TCEP produces a constant $K_{app\text{-}PEP}$ for up to 7 days. (Recovery of "fresh" protein properties from long-term storage has now been accomplished by plunge freezing in thin-

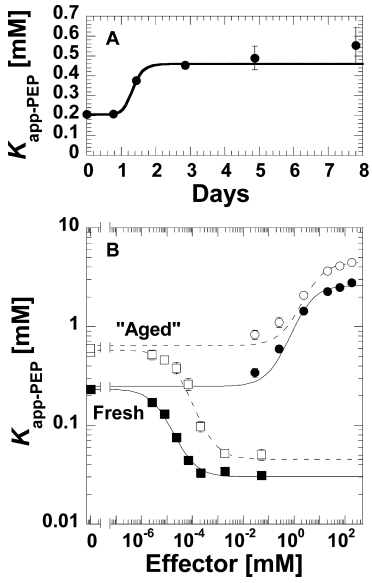


Figure 1. Influence of time and aging on the affinity of L-PYK for PEP. (A) The initial velocity derived $K_{app\text{-}PEP}$ for wild-type L-PYK as a function of storage time after purification (36 h after cell lysis is required for purification). The line represents the trend in the data. (B) Influence of aging on allosteric properties elicited by Fru-1,6-BP (squares) and alanine (circles). The influence of aging appears to primarily impact PEP affinity because the allosteric properties (the distance between the plateaus at low and high effector concentrations) is relatively unchanged for freshly purified (filled symbols) vs aged (empty symbols) enzyme. Lines represent the best fits to eq 1.

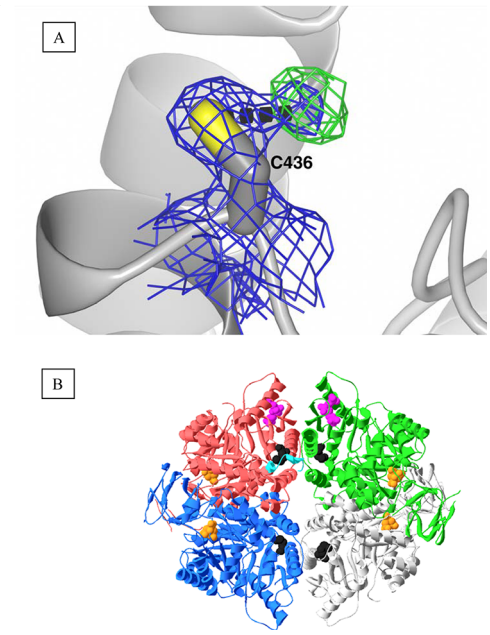


Figure 2. (A) Aberrant, unmodeled density adjacent to Cys436. The $2F_o - F_c$ density rendered at 1.2σ and the $F_o - F_c$ density rendered at 2.5σ are illustrated as blue and green mesh, respectively. The dashed black line indicates a distance of 2.3 Å from the center of the unmodeled density to the sulfur of C436. (B) Spatial relationship of Cys436 (black space fill) to other structural features. The four subunits of the L-PYK homotetramer are colored red, green, blue, and gray. The N-terminus of the red subunit is colored cyan as determined by the C436M structure reported in this study. Citrate in the active site is shown as orange space fill. Fru-1,6-BP is displayed in the top two subunits as magenta space fill but (although present in the structure) is not displayed in the two bottom subunits.

walled polymerase chain reaction tubes followed by rapid thawing following a published method.[14]) The change in PEP affinity due to "aging" appears to cause only slight changes in the magnitudes of the allosteric functions (Figure 1B).

Oxidation of Cysteine 436 to Sulfenic Acid. We originally initiated crystallographic studies of L-PYK to characterize the structure of the N-terminus, which is not present and/or disordered in the previous structure[8] [an attempt to identify a previously proposed allosteric ATP binding site was a second goal, but the obtained structures provided little insight into the location of this ATP site (see the Supporting Information)]. This effort resulted in a 1.8 Å structure of the S12D enzyme (Table 1). Of particular interest, this structure presents evidence that Cys436 is partially oxidized to the sulfenic derivative (Figure 2). This partial oxidation is observed as density at an intermediate length between a hydrogen-bonded water molecule and a covalently linked hydroxyl group, a result consistent with the averaging of oxidized and nonoxidized side chains. Sulfenic acids are not typically stable. Instead, a sulfenic acid derivative is often rapidly further oxidized by reacting with additional oxygens, resulting in sulfinic and sulfonic derivatives (Supporting Information). With this in mind, it is interesting that even though crystals used in this study required 2−3 weeks to grow, no evidence of oxidation beyond the sulfenic state was observed.

Confirmation of Oxidation of Cys436. Mass spectrometry was used to confirm that Cys436 can be oxidized in purified protein in solution (Table 2). The observed mass is consistent with the sulfonic acid form of Cys436; sulfenic acids often further oxidize to sulfonic acid during mass spectrometry analysis.[15] The sensitivity of $K_{app\text{-}PEP}$ to a brief incubation with low concentrations of H_2O_2 is also consistent with protein oxidation (Figure 3). These observations are also evidence that the oxidation in the structure is not solely an artifact caused by exposure to X-rays, although exposure may have contributed to oxidation.

The location of this regulatory oxidation at Cys436 is supported by the fact that much higher concentrations of H_2O_2 are required before the C436A mutant protein shows reduced PEP affinity (Figure 3); the impact of this mutation on apparent PEP affinity is presented below. To confirm the specificity, C338A was shown to maintain H_2O_2 sensitivity (Supporting Information). C338A is a control protein with a mutant that also removes a cysteine residue but at a location distant from the N-terminal region in the structure. Therefore, the loss of sensitivity to oxidation in the C436A mutant protein is not a general effect caused by a global modification of all cysteine residues in the protein (six per subunit).

Several reports in the literature indicate roles for various PYK isozymes in oxidative stress.[16−24] Characterization of cysteine oxidation in L-PYK in vivo was not our goal. However, we did find it reasonable to test the potential impact of oxidation of cysteine residues reported in the literature. Anastasiou et al. reported that M_2-PYK is oxidized at the cysteine position equivalent to Cys370 in human L-PYK.[17] This site of oxidation was determined by mutating three cysteine residues that reside in a subunit−subunit interface in the M_2-PYK protein. In our study, to test the potential regulatory role of oxidation of Cys370 in L-PYK, we mutated this residue to alanine. The mutant protein has a $K_{app\text{-}PEP}$ (0.223 ± 0.006 mM) similar to

dx.doi.org/10.1021/bi301341r | *Biochemistry* 2013, 52, 466−476

Table 2. Cys436-Containing Peptides and Their Modifications Detected by Sequest Analysis of Tandem Mass Spectra[a]

Peptide sequence	MH+	z	P (pep)	XC	ΔCn
AAPLSRDPTEVTAIGAVEAAFKC@CAAAIIVLTT	3323.82	**3**	1.01E-05	**3.72**	0.36
AAPLSRDPTEVTAIGAVEAAFKC#C#AAAIIVLTTTGR	3704.27	3	2.81E-12	8.21	0.58
C@C@AAAIIVLTTTGR	1489.70	2	7.17E-05	2.68	0.35
C#C#AAAIIVLTTTGR	1507.80	2	1.24E-06	3.93	0.42

[a]L-PYK was subjected to iodoamide alkylation prior to its overnight treatment with trypsin at 37 °C. The resulting peptides were analyzed by HPLC−MS/MS on an LTQ-FT mass spectrometer. C# stands for carboxymethylcysteine; C@ stands for cysteinesulfonic acid. Tandem mass spectra were searched against a human protein database using the Sequest algorithm and the following parameters for protein ID: peptide probability of random match < 10^{-3}, XC ≥ 1.9, 2.5, or 3.5 for z = 1, 2, or 3, respectively, and ΔCn ≥ 0.1. Peptides containing oxidized Cys are highlighted in bold. Because C#-denoted cysteines were reactively modified by alkylation, they were not irreversibly oxidized.

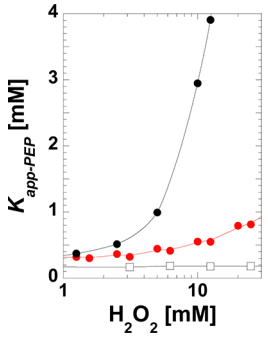


Figure 3. Sensitivity of $K_{app\text{-}PEP}$ for the wild-type (black circles) and C436A (red circles) L-PYK proteins to a short (8 min) incubation with H_2O_2. Exposure to concentrations of up to 100 mM did not alter V_{max} activity (data not shown). Wild-type protein (squares) in the presence of 2 mM DTT was included as a control. Data collection was replicated by multiple individuals using different protein preparations. Although general features were maintained in replicates (i.e., the wild-type protein being much more sensitive to H_2O_2 than the C436A mutant), there was considerable variability between protein preparations in the $K_{app\text{-}PEP}$ determined for a given protein at a given H_2O_2 concentration. A representative comparison is included here.

Table 3. $K_{a\text{-}PEP}$ Values for Forms of L-PYK with Cys436 Mutations

protein	$K_{a\text{-}PEP}$[a]
wild-type	0.240 ± 0.002
C436A	0.372 ± 0.002
C436S	0.802 ± 0.004
C436D	0.804 ± 0.002
C436N	0.703 ± 0.004
C436M	0.112 ± 0.001
C436T	0.768 ± 0.006
C436H	0.345 ± 0.004

[a]Fit parameter from eq 1.

that of the wild-type protein. Upon exposure to H_2O_2 (Supporting Information), the C370A protein continues to show a response consistent with an oxidation event that modifies $K_{app\text{-}PEP}$. Therefore, it appears that Cys370 is not the residue that responds to oxidation in L-PYK.

If the oxidation event responsible for weaker PEP affinity is truly the sulfenic acid derivatization of Cys436, then it seems reasonable to anticipate that this modification is reversible. However, despite this prediction, we have not to date successfully demonstrated reversibility using either DTT or TCEP additions.

Characterization of the C436M Protein. Energetic coupling between position 436 and the PEP binding site was confirmed by replacing cysteine at position 436 with a number of different residues (Table 3). None of the mutant proteins retained wild-type $K_{a\text{-}PEP}$. Of the mutant proteins, almost all substitutions caused weaker PEP affinity. C436A and C436H caused the smallest changes in $K_{a\text{-}PEP}$. C436S, C436D, C436N, and C436T caused larger reductions in PEP affinity. We are unaware if there is a single amino acid type that acts as a good mimic for oxidized cysteine in the same manner that glutamic acid and aspartic acid mimic phosphorylated serine. However, on the basis of the speculation from the summary of this study (i.e., that oxidation shifts the equilibrium of the binding interaction between the N-terminus and the main body of the protein), the only requirement for mimicry in this setting may be the ability to disrupt interactions that contribute positively to binding, hence the decreased PEP affinity in most mutants. The corollary is that C436M, the only mutant protein with a $K_{app\text{-}PEP}$ lower than that of the wild-type protein, may stabilize the interaction between the N-terminus and the main body of the protein.

The C436M protein was crystallized using the same crystallization conditions used earlier to obtain the structure of the S12D protein. Several N-terminal residues (residues 18−24) that were not ordered in previous structures are ordered in the structure of the C436M protein. Specifically, the side chains of residues Leu20, Phe24, and Phe25 all make favorable van der Waals interactions with methionine at position 436 (Figure 4).

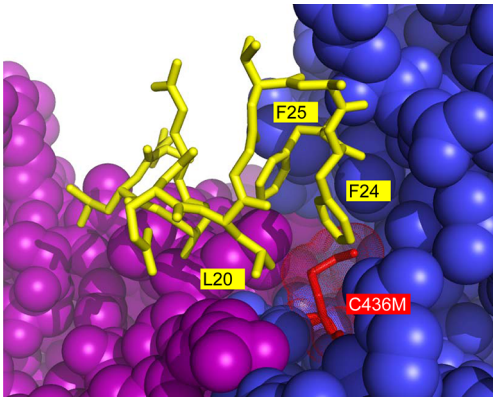
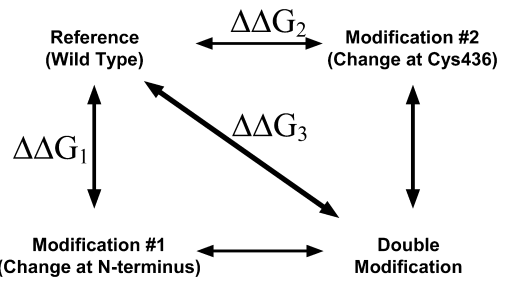


Figure 4. N-Terminal residues (yellow sticks) that become ordered as a result of the C436M (red stick) mutation. Specifically, residues 18−24 (QELGTAF) are ordered as a result of the mutation. The side chains of residues Leu20, Phe24, and Phe25 from the N-terminus interact with methionine at position 436 of the same subunit (blue). The newly ordered residues cross a subunit interface to interact with a second subunit (purple).

dx.doi.org/10.1021/bi301341r | *Biochemistry* 2013, 52, 466−476

Table 4. Additivity of Mutant Cycles



reference		modification #1			modificaiton #2			double modification		
	$K_{a\text{-PEP}}$ (mM)	description	$K_{a\text{-PEP}}$[b] (mM)	$\Delta\Delta G_1$[a] (kcal/mol)	description	$K_{a\text{-PEP}}$ (mM)	$\Delta\Delta G_2$[a] (kcal/mol)	calculated $\Delta\Delta G_1 + \Delta\Delta G_2$ (kcal/mol)	$K_{a\text{-PEP}}$ (mM)	$\Delta\Delta G_3$[a] (kcal/mol)
wild type	0.240 ± 0.002	Δ2−10	0.605 ± 0.004	0.55 ± 0.01	C436S	0.802 ± 0.004[c]	0.726 ± 0.008	1.28 ± 0.02	1.139 ± 0.003	0.938 ± 0.007
					C436N	0.703 ± 0.004[c]	0.647 ± 0.008	1.20 ± 0.02	1.016 ± 0.005	0.869 ± 0.008
		S12D	0.552 ± 0.007	0.50 ± 0.01	C436S	0.802 ± 0.004[c]	0.726 ± 0.008	1.23 ± 0.02	0.89 ± 0.01	0.79 ± 0.01
					C436N	0.703 ± 0.004[c]	0.647 ± 0.008	1.15 ± 0.02	0.95 ± 0.01	0.83 ± 0.01
					C338A	0.93 ± 0.01	0.82 ± 0.01	1.32 ± 0.02	2.09 ± 0.02	1.30 ± 0.01

[a] ΔG is the change in free energy associated with PEP binding. $\Delta\Delta G$ is the difference in ΔG of PEP binding of modified protein relative to that of the wild-type enzyme. Conversion of $K_{a\text{-PEP}}$ to ΔG is based on the generally accepted view that PYK isozymes function as rapid equilibrium systems.[30] As such, the free energy associated with PEP binding was calculated by the equation $\Delta G = -RT \ln K_{a\text{-PEP}}$. [b] Values from ref 12. [c] Values from Table 2.

dx.doi.org/10.1021/bi301341r | Biochemistry 2013, 52, 466−476

Energetic Synergy of the N-Terminus and Cys436. Because the C436M mutation causes ordering of additional N-terminal residues, these N-terminal residues directly interact with the side chain of residue 436, and oxidation of Cys436 and phosphorylation of the N-terminus[5] both result in weaker affinity for the substrate, we considered that oxidation and phosphorylation might function through similar mechanisms. Such a shared mechanism would result in energetic synergy between outcomes resulting from modifications at the phosphorylation site and modifications at the oxidation site. The potential of this energyetic synergy was tested using mutant cycle analysis. This approach has previously been useful in testing if two different regions of a protein work synergistically in an allosteric mechanism.[25] Mutant cycle analysis can be understood conceptually by considering our proposed mechanism for phosphorylation, i.e., that a modification at the N-terminus (i.e., phosphorylation, the S12D phosphorylation mimic, or N-terminal truncation[5]) disrupts an activating interaction between the N-terminus and the main body of the protein.[5] Oxidation of Cys436 on the main body of the protein may also interrupt this N-terminus−main body interaction. Therefore, introducing both a mutation that mimics Cys436 oxidation [i.e., C436S or C436N (see Table 3)] and a modification at the N-terminus that mimics phosphorylation (S12D or an N-terminal truncation[5]) will not introduce any further change compared to that introduced by adding only one of the two modifications. "Disrupts an interaction" is not intended to strictly imply removal of an all-or-none interaction, and this scenario may be better visualized as a shift in a binding equilibrium for N-terminal binding, in which the addition of phosphorylation or oxidation shifts the equilibrium more to the unbound state. Therefore, introducing both a mutation that mimics Cys436 oxidation and a modification at the N-terminus that mimics phosphorylation might minimally shift the binding equilibrium compared to that caused by adding only one of the two modifications. In an all-or-none view of N-terminal binding or a shift in a binding equilibrium, this energetic synergy can be identified at the free energy level as nonadditivity. In contrast, if the two modifications elicit their respective effects on PEP affinity through independent mechanisms, it should be expected that the sum of the change (relative to that of the wild type) caused by each modification alone will be equivalent to the change observed in the doubly modified enzyme (Table 4).

Mutant cycle analysis was completed for all combinations of C436S or C436N versus Δ2−10 (the protein without residues 2−10) or S12D. As a control for an example of additivity (i.e., no energetic coupling), a mutant cycle analysis was also completed between S12D and C338A, a mutation located at a site distant from the N-terminal region in the structure. Via comparison of the values in each row that are contained in columns 9 and 11 of Table 4, it is evident that the sum of the effects caused by S12D and C338A is equivalent to the effects caused when both mutants are added together. Therefore, these two mutations do not alter PEP affinity via perturbing the same energetic mechanism. However, for each combination of S12D or Δ2−10 with either C436S or C436N, there is a difference of 0.33−0.44 kcal/mol between the sum of changes caused by the individual modification (calculated $\Delta\Delta G_1 + \Delta\Delta G_2$) compared to the changes caused by the simultaneous addition of both mutations together ($\Delta\Delta G_3$). As described above, this non-additivity indicates energetic synergy between modifications of the two regions of the protein. In turn, energetic synergy between these regions is consistent with the idea that introducing mutations at the Cys436 position modifies PEP affinity through a mechanism (or component of that mechanism) similar to that caused by introducing modifications at the N-terminus. On the basis of our previous work,[5] this similar mechanism is likely a modification-dependent shift in the equilibrium of the interaction between the N-terminus and the main body of the protein. However, because the effect of a modification to the N-terminus in isolation is not completely equivalent in magnitude to the influence of a mutation of Cys436 (i.e., in Table 4, $K_{a\text{-PEP}}$ values of 0.552 and 0.605 for modifications of the N-terminus vs $K_{a\text{-PEP}}$ values of 0.703 and 0.802 for modifications of C436), we suggest similar but not completely equivalent outcomes.

Alanine-Scanning Mutagenesis of the N-Terminus for the Impact on PEP Affinity. An alanine scan across the N-terminus was initiated for two reasons: (1) to confirm that residues identified by structural studies as interacting with the methionine at position 436 contribute to N-terminal function (i.e., evidence that the observed interactions are not an artifact caused by the introduced mutation) and (2) to further detail individual residues that contribute to N-terminal functions. Of the unstructured N-terminal residues from the previous crystal structure,[8] MEGPAGYLRRASVAQLTQELGTAFF..., non-alanine/non-glycine residues in the N-terminus were mutated to alanine as the best mimic of side chain removal. The resulting series of mutant proteins each contain a single alanine substitution. Initially, the impact of these individual alanine substitutions on the apparent affinity of the enzyme for PEP was evaluated (Figure 5A). This apparent affinity was treated as a true K_d and converted to a free energy value. Using these free energy values, the mutant-dependent deviation from that of the wild type was plotted as a function of the position of the mutated residue. For any residue at which an alanine or glycine is present in the wild-type protein, the wild-type value is used (i.e., no difference from that of the wild type) in the graph. Interestingly, there is an apparent periodicity. In this periodicity, S12A and Q18A and, to a lesser extent, T22A cause strengthened PEP affinity. L20A and F24A and, to a lesser extent, L16A contribute to weakened PEP affinity. Considered in isolation, this periodicity could conceivably be consistent with a helical structure in which side chain removal on one side of the helix removes critical interactions between the N-terminus and the main body of the protein (Supporting Information). In contrast, side chain removal on the opposite side of the helix might remove hydrophilic residues that interact with water, favoring a shift in the propensity of the helix to bind to the protein. The residues ordered in the C436M structure are consistent with this periodicity, although the structure is not exactly helical in nature. Residues 20 and 24 are both interacting with the mutated methionine at position 436, and side chains of residues 18 and 22 are orientated toward solvent.

The previously completed truncation series indicated the region including residues 7−10 as being significant for binding of the nonphosphorylated N-terminus with the main body of the protein.[5] Therefore, the lack of effects caused by removing side chains of residue 2 or 4 is consistent with a lack of an effect due to truncation (residue 1 was not probed, and residues 3, 5, and 6 are either Ala or Gly in the wild-type protein and were not mutated). However, one surprise in the data in Figure 5A was the relatively small magnitude of the reduced affinity for PEP caused by mutations in the region from residue 7 to 10. We considered that each residue in this region might contribute

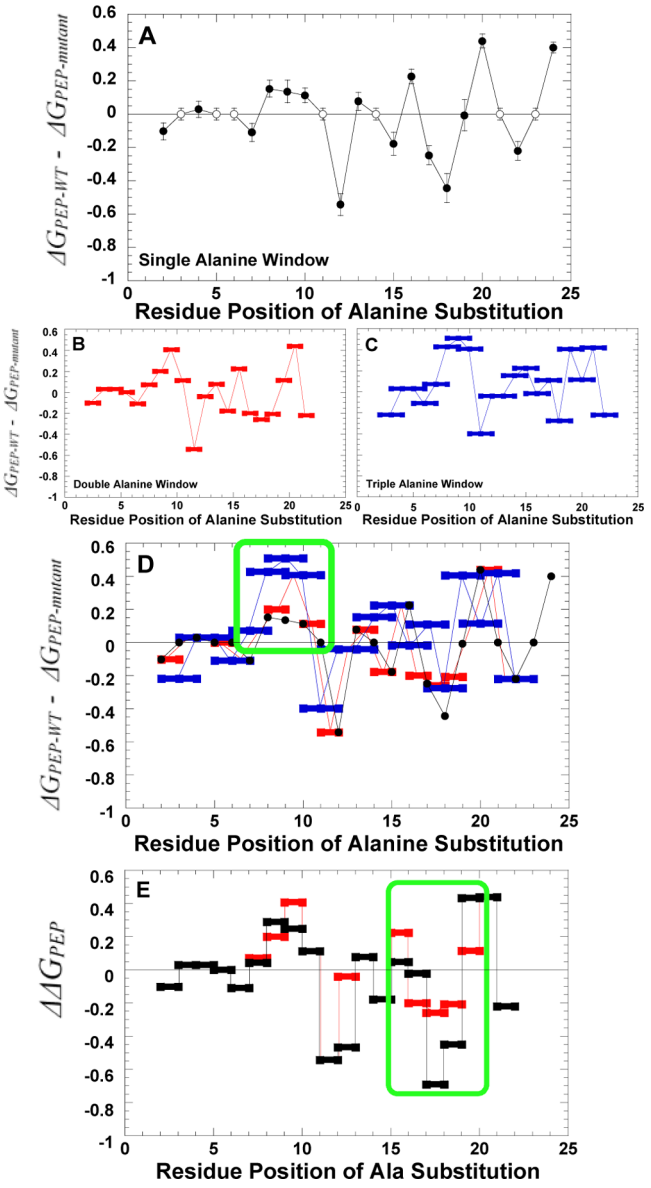


Figure 5. Impact of an N-terminal alanine scan on the ΔG associated with $K_{a\text{-PEP}}$. (A) Impact of single residues substituted with alanine. Filled circles represent a comparison between measurements for the mutant protein and the wild-type protein. Residues that are alanine or glycine in the wild-type structure were not probed. Therefore, the wild-type data used to represent $K_{a\text{-PEP}}$ at these positions are represented by empty circles to clearly distinguish data for mutant proteins. (B) Impact of substituting two consecutive positions, both with alanine. (C) Impact of substituting three consecutive positions with alanine. (D) Overlay of the three data sets included in panels A–C. In all panels, data are compared to those of the wild-type protein. A positive deviation from zero on the *y*-axis indicates reduced PEP affinity; a negative value is improved PEP affinity. Panel D includes both the sum of effects caused by individual mutations [sum of $\Delta\Delta G$ values for the two single alanine mutations (black)] and the difference between the wild-type value and that observed for the protein with both alanine substitutions of interest inserted simultaneously [$\Delta\Delta G$ values for double-alanine window mutations (red)]. As a means of simplifying panels, error bars are included in only panel A. However, error estimates are included for all data in the Supporting Information. Green boxes are included in panels D and E to emphasize areas of interest.

a fraction of the binding energy between the N-terminus and the main body of the protein. As such, mutating a single residue (i.e., Figure 5A) would have an only slight effect on N-terminal binding and, thus, indirectly have an only slight effect on PEP affinity. In contrast, the previous deletion series would represent an additive effect; e.g., removal of residue 10 also removes residues 2–9. To test this possibility, we conducted a second alanine scan in which two consecutive residues were replaced with alanine. Again, the impact on PEP affinity was determined (Figure 5B). A third alanine scan was also repeated using a window of three consecutive alanine residues (Figure 5C). Outcomes of all three versions of the alanine scans are overlaid in Figure 5D.

Consistent with the possibility that the binding energy for the nonphosphorylated N-terminus is spread over many interactions, combining consecutive alanine replacements highlights the region containing residues 7–10. To fully appreciate this additivity, consider that the data for mutations with three consecutive alanine replacements (blue points in Figure 5D) in the regions from residue 7 to 10 do not overlap with any of the data for single-alanine replacements (black data). This is in contrast to examples like the data surrounding residue 20 in which any mutation combination that includes a modification of residue 20 is approximately equivalent (i.e., data in black overlap data in blue). The comparison of data from various sizes of alanine "windows" is then fully consistent with the conclusion from the previous truncation series and suggests that each residue in the range of positions 7–10 functions in an independent manner to add to the overall binding energy.

Driven by the data in hand, we also questioned if there are regions in the N-terminus that function as a unit. In such interactions, it is expected that modification of any one residue in the "unit" would alter the interaction of the entire unit. Therefore, the impact of introducing two mutations simultaneously into the unit should not be as drastic as the sum of changes caused by introducing the mutations individually, i.e., nonadditivity. As exemplified above for mutations that modify Cys436 and the N-terminus, mutant cycle analysis can be used to identify nonadditivity and therefore energetic synergy. However, this double-mutant cycle analysis should be considered with some caution in this new application. We are unaware if mutant cycle analysis has previously been used with consecutive residue positions. Alanine, albeit our best mimic of side chain removal, is known to change secondary structure propensity toward helix formation. Therefore, mutational analysis cannot consider an alanine substitution as merely the removal of the original side chain (loss of function) but must keep in mind the potential of gain of function that could result from alanine-driven helix formation. These considerations are also the basis of why we chose not to extend the nonadditivity analysis to the three consecutive alanine data. Nonetheless, using the data from panels A and B of Figure 5, Figure 5E considers when the combination of two consecutive alanine replacements results in a change in PEP affinity that is not equivalent to the sum of the effects caused by the two substitutions made independently in two different proteins. This nonadditivity analysis identifies only one region with three or more consecutive positions, residues 15–20. In turn, the energetic synergy indicated by nonadditivity is consistent with residues 15–20 functioning as a unit.

Our exercise to provide data consistent with regions of the N-terminus that function as a unit (i.e., nonadditivity) and regions in which each residue contributes individually (i.e.,

dx.doi.org/10.1021/bi301341r | *Biochemistry* 2013, 52, 466–476

additivity) should not overshadow the overall conclusion that many residues in the N-terminus contribute (both positively and negatively) to the overall PEP affinity. In particular, recall that the two goals of the alanine scan were (1) to confirm that residues identified by structural studies to interact with the methionine at position 436 contribute to N-terminal function (i.e., evidence that the observed interactions are not an artifact caused by the introduced mutation) and (2) to further detail individual residues that contribute to N-terminal functions. Leu20 and Phe24, two residues identified to directly interact with methionine at position 436, were included in the group of residues that impact N-terminal function. Therefore, both goals of the alanine scan were accomplished.

DISCUSSION

As introduced above, this study was initiated with a structural indication of a potentially oxidizable cysteine that could provide an explanation for an observed time-dependent shift in the substrate affinity displayed by a purified protein. However, the summation of the studies used to detail these initial observations has resulted in an advancement of our knowledge about the structure−function relationship of the N-terminus in regulation by oxidation and phosphorylation. In addition, clarification of the binding orientation of Fru-1,6-BP and the absence of bound ATP were secondary conclusions drawn from the new structure (Supporting Information).

Phosphorylation. Despite a long-standing understanding of the region of L-PYK that is phosphorylated in response to glucagon, mechanistic studies that aim to understand how this phosphorylation results in weaker PEP affinity have only recently been initiated.[5−7] It now seems likely that the Leu20 region of the N-terminus must interact with Cys436 before additional contacts involving residues 7−10 can be made. Therefore, modification of Cys436 may result in changes to a larger region of the N-terminus−main body interactions than modification of the Ser12 position. This would be consistent with the fact that modification of the N-terminus and Cys436 did not result in completely equivalent magnitudes in the changes each caused in PEP affinity and that the C436M mutation causes order of some but not all N-terminal residues. All data continue to be consistent with the proposal that phosphorylation interrupts an activating interaction between the N-terminus and the main body of the protein.

Oxidation. We now have sufficient data to speculate that oxidation of Cys436 functions through shifting a binding equilibrium to favor less of an activating interaction between the N-terminus and the main body of the protein. However, this oxidation may perturb a slightly different set of interactions as compared to phosphorylation.

Opposite to phosphorylation, it is the potential physiological role (if any) of this modification that is uncharacterized, and only speculation about this role can be entertained at this time. PYK transcription is activated by oxygen restriction in both tissues that express the L- and R-PYK isozymes and those that express the M_1 and M_2 isozymes.[18−20] A potential metabolic rationale is that glycolysis is induced when oxygen is restricted, because glycolysis (as opposed to oxidative phosphorylation) is the primary source of cellular energy under these conditions. As additional support, upregulation of M_1-PYK increases tolerance to hypoxic stress in neuronal cells,[21] whereas a mutation in R-PYK augments oxidative stress in erythroleukemia cells.[22] Given this strong evidence of the upregulation of PYK protein concentration caused by hypoxia, it may at first appear paradoxical that the enzymatic activity of a R-PYK protein is diminished upon exposure to oxygen radicals,[23] a condition associated with hypoxia.[24] However, this may be rationalized when considering that (1) much of the cellular injury during tissue hypoxia occurs during reoxygenation (i.e., reperfusion leads to oxygen-derived free radicals[26,27]) and (2) reversible oxidation can be a form of protein regulation.[28] Therefore, although increased levels of PYK might be elicited in the absence of oxygen, rapid inhibition of the enzymatic activity of PYK may be necessary upon reoxygenation. This acute response might be possible through reversible protein oxidation. Not only would inhibition of PYK activity by oxidation be beneficial to prevent further pyruvate formation and overloading mitochondrial functions upon reoxygenation, but inhibition of lower glycolysis may force carbon flux through the pentose phosphate pathway to facilitate NADPH production. NADPH is used in the reduction of the glutathione redox control system that is, in turn, used to combat oxidative stress (such as that caused by overloaded mitochondria upon reoxygenation after hypoxyia) in the cell. This hypothesis has gained considerable support even during the time this report was being prepared.[16,17,29]

Despite this potential scenario, we have provided no evidence that oxidation of Cys436 occurs in vivo. Therefore, at most we can review several observations that are consistent with regulatory oxidation. (1) The change in $K_{app\text{-}PEP}$ caused by oxidation is also the outcome of other forms of PYK regulation, including phosphorylation and allosteric effectors.[1,5] (2) There is a general growing consensus that reversible protein oxidation can be a physiologically important form of regulation.[28,30] Cysteine oxidation is reversible until a second oxygen is added to the sulfur group, and reversibility is one of the criteria that supports a regulatory role of an oxidation event.[28] Cys426 in the current structure is modified with a single oxygen, despite the weeks required for crystal growth and X-ray exposure. This raises the speculation that some unique aspect of the protein prevents further oxidation, a property that could facilitate the reversibility of a physiologically important regulation. (3) Cys436 is the only oxidized cysteine in the structure, a specificity that is necessary to be consistent with regulation. (4) Although it is currently unclear how regulation is conserved among isozyme in a protein family,[31,32] Cys436 is conserved within mammalian liver PYK isozymes, as well as in other mammalian isozymes (Supporting Information). If regulation is conserved within a protein family, then oxidation of the equivalent of Cys436 in other isozymes may also contribute a regulatory response to oxidative stress to tissues other than the liver. Identification of oxidation of Cys436 using in vivo techniques will be required before further consideration of a physiological role of this oxidation will be warranted.

Implications for Future Studies. Although we have not yet elucidated the complete list of N-terminus−main body interactions, we have successfully identified a partial list. Furthermore, on the basis of extensive data reported in this study, two strategies can be suggested for obtaining the remaining uncharacterized N-terminus−main body interactions. The additive effects of simultaneously introducing C436M with S12A and Q18A may aid in causing the N-terminus to be further ordered in structural studies. Alternatively, a computational approach may be used to search the surface of the protein for potential N-terminal (i.e., residues 7−10) binding sites, while considering the interactions identified in our C436M structure.

■ ASSOCIATED CONTENT

Supporting Information

Other notable observations regarding the allosteric ATP binding site, a helical wheel representation of residues 11−24, other notable observations regarding citrate binding in the active site, other notable observations regarding the Fru-1,6-BP binding site, and a table with all data used to generate Figure 5. This material is available free of charge via the Internet at http://pubs.acs.org.

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Funding

This work was supported by National Institutes of Health Grant DK78076.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

Portions of this research were conducted at the Stanford Synchrotron Radiation Laboratory, a national user facility operated by Stanford University on behalf of the U.S. Portions of this research were conducted at the Advanced Photon Source. Department of Energy Office of Basic Energy Sciences. The Stanford Synchrotron Radiation Laboratory Structural Molecular Biology Program is supported by the Department of Energy Office of Biological and Environmental Research and by the National Institutes of Health National Center for Research Resources, Biomedical Technology Program, and the National Institute of General Medical Sciences. We thank Dr. Aileen Alontaga for her efforts to recover oxidized protein by addition of DTT and The Mass Spectrometry/Proteomics Core at The University of Kansas Medical Center (directed by Dr. Anotonio Artigues) for mass detection of oxidized cysteine.

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