Energetic Implications for Protein Phosphorylation

CONFORMATIONAL STABILITY OF HPr VARIANTS THAT MIMIC PHOSPHORYLATED FORMS

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The HPr protein from Bacillus subtilis is a key protein in the phosphoenolpyruvate-sugar transport system. HPr has two biological phosphorylation sites. The active site histidine is transiently phosphorylated in the phosphotransferase reaction while phosphorylation of serine 46 diminishes the activity of HPr. Here, we use protein engineering and equilibrium protein folding experiments to determine if the two phosphorylation events are energetically coupled. Our approach is to use structural mimics of the two phosphorylated forms of HPr, where histidine 15 is replaced by a negatively charged glutamate and serine 46 is changed to an aspartate, both alone and in combination. The thermodynamic analysis of the differences in conformational stability between the single and double mutants shows that the two phosphorylation sites are not energetically coupled in the HPr protein. We also show that single mutants of the active site histidine residue can have dramatic effects on the conformational stability of HPr. Combined with structural information, the method employed here will be of general use in unraveling the biological effects of phosphorylation on protein activity.

The regulation of a cellular process through reversible protein phosphorylation is ubiquitous in biology. First observed in glycogen phosphorylase (1), protein phosphorylation has now been described in both eukaryotes and prokaryotes. Protein phosphorylation controls many metabolic pathways and events in biological chemistry including gene transcription, muscle contraction, cell division and growth, and even learning and memory. Despite the diverse number of systems that show reversible protein phosphorylation, surprisingly little information is available regarding the structural and energetic consequences of this important reaction (2). Two examples where crystal structures are known for proteins in their phosphorylated and unphosphorylated forms are glycogen phosphorylase (3) and isocitrate dehydrogenase (4). In these two cases, the mechanism by which protein activity is altered by protein phosphorylation is different. In GP,1 phosphorylation promotes the formation of ordered structure near the site of the target serine residue causing long range conformational changes that affect the function of the enzyme. In isocitrate dehydrogenase, on the other hand, the serine residue that is the target of phosphorylation is at the N-cap position of an α-helix, phosphorylation only produces small local changes in the structure of the protein, and the activity of the enzyme is reduced through an electrostatic steric mechanism where the phosphate group prevents the binding of the anionic substrate.

For several other cases, the structural changes in a protein upon phosphorylation can be inferred from other biochemical studies even though we do not have direct crystallographic structures for both forms of the protein. One of these cases is the cyclic AMP-dependent protein kinase. In this case, it appears that phosphorylation causes large scale conformational changes across domain interfaces, thus relaying the phosphorylation event through conformational changes that allow proper substrate binding (5–7).

As scarce as the detailed structural descriptions of the effects of phosphorylation are, there is even less known about the energetic effects of phosphorylation. Specifically, does phosphorylation of a protein alter the conformational stability of a protein, and does this have functional consequences for the protein? Recently, the structural and energetic effects of serine phosphorylation of the bacterial protein HPr have been reported (8). Phosphorylation of the regulatory serine residue produces only local changes in structure while increasing the conformational stability of the protein by ~0.7 kcal mol⁻¹. Here we extend these studies to determine if phosphorylation of HPr at the regulatory site (Ser-46) is energetically coupled to the phosphorylation event at the active site (His-15).

The HPr protein plays a central role in the sugar transport system in bacteria (see Fig. 1). In Gram-positive bacteria, the key regulatory protein is histidine-containing protein (HPr), a small phosphoryl transfer protein that can be phosphorylated either on the “catalytic” histidine (His-15) by the scheme shown in Fig. 1 or on a regulatory serine (Ser-46) by an ATP-dependent protein kinase (9). HPr(Ser(P) has a reduced ability (~100-fold) (see Ref. 11) to accept a phosphate from EI-P (10), and the replacement of Ser-46 with Asp (S46D) mimics the functional activity (11) and the structural and energetic consequences of serine phosphorylation (8).

Histidine phosphorylation, although not as common as serine, threonine, and tyrosine phosphorylation, has now been shown in prokaryotes (12–14) and eukaryotes (15, 16), both as protein activity regulators (13, 15) and as intermediates in enzymatic catalysis (12, 14). Histidine phosphorylation is also a key feature of the PTS in bacteria (Fig. 1). All the phosphoprotein forms of the PTS proteins are on histidine residues. For HPr, phosphorylation occurs on the N³ atom of His-15, the N-cap residue of the first α-helix (see Fig. 2). A recent NMR characterization of the phosphohistidine form of HPr (17) shows that the structural consequences of phosphorylation are...
localized to the end of this helix. Therefore, the two protein phosphorylation events on HPr, on the regulatory Ser-46, and the catalytic His-15 sites show common features. Each site of phosphorylation is located at the N-cap of an α-helix, and each phosphorylation event only produces small changes in the local structure of the protein in the vicinity of the phosphorylated residue.

Here we use protein engineering to investigate the energetic coupling between regulatory and active site phosphorylation residues in HPr. Since the Ser(P)-46 form of the protein has the same structural, functional, and energetic properties as the S46D mutant (8, 11), we have used a similar approach to mimic the short lived His(P)-15 form of the protein by the mutation H15E. We investigate the consequences of these mutations, both alone and in combination, on the conformational stability of the HPr protein. We also report the effects of several mutations of the active site His-15 residue on the conformational stability. In a general sense, we address the question of how the information is transferred from a regulatory protein phosphorylation site to the “active site,” in this case some 15–20 Å away, in the absence of large or small scale conformational changes in the structure of the protein.

**EXPERIMENTAL PROCEDURES**

*Bacillus subtilis* HPr was prepared as described previously (11, 18). The bsHPr variants, H15A, H15E, S46D, and the double mutant H15E/S46D were generous gifts of Jonathan Reizer and Milton Saier, Jr. All proteins were stored as lyophilized powders at −20 °C and dissolved in the appropriate buffer just prior to use. All buffers and urea were of the highest grade available.

The conformational stability of each protein was determined through the analysis of urea denaturation curves as described previously (19, 20). The equilibrium unfolding reaction was monitored by CD at 222 nm using an Aviv 62DS spectropolarimeter with a temperature control and stirring unit. Urea denaturation experiments were performed at 25 °C in buffered solutions containing either 10 mM sodium citrate (pH 3.5), 10 mM sodium acetate (pH 4.8), 10 mM sodium phosphate (pH 7.0), or 10 mM sodium borate (pH 9.0). The urea solutions were freshly prepared daily, and the concentration of the urea stock solution was determined by refractometry (21).

Analysis of the urea denaturation curves follows the procedures outlined by Pace (21) and Santoro and Bolen (22). This analysis assumes a two-state equilibrium folding reaction and employs the linear extrapolation method described by

$$\Delta G_{obs} = \Delta G^\circ - m \cdot [\text{urea}]$$

(Eq. 1)

where $\Delta G^\circ$ is the conformational free energy in the absence of urea, and $m$ is the gradient of change of the conformational free energy with molar urea concentration. A single urea denaturation curve can be fit, using nonlinear least squares procedures by

$$m_{obs} = \frac{(N_0 + a_o[\text{urea}]) + (D_0 + a_d[\text{urea}]) \exp \left(\frac{m}{RT} \cdot [\text{urea}] - C_{mul}\right)}{1 + \exp \left(\frac{m}{RT} \cdot [\text{urea}] - C_{mul}\right)}$$

(Eq. 2)

to afford $m$ and $C_{mul}$, the concentration of urea at the midpoint of the transition. The other parameters in Equation 2 describe the linear pre- and posttransition base lines for the urea denaturation curves. A complete description of this procedure has been described (19).

To facilitate comparisons between urea denaturation curves for different protein variants, the observed CD spectroscopy signal was converted to fraction of native protein ($f_N$) using

$$f_N = \frac{m_{obs} - (D_0 + a_d[\text{urea}])}{(N_0 + a_o[\text{urea}]) - (D_0 + a_d[\text{urea}])}$$

(Eq. 3)

where the linear pre- and posttransition base lines define the CD spectroscopy signals for the native and unfolded forms of the protein.

The difference in conformational stability between variant proteins under identical solution conditions can be described by

$$\Delta C_{mul} = \Delta C_{mul}$$

(Eq. 4)

where $\langle m \rangle$ is the average $m$-value at the given pH and $\Delta C_{mul}$ is the difference in midpoints of the two urea denaturation curves being compared.

**RESULTS**

The HPr proteins undergo a reversible unfolding-refolding transition, and the equilibrium behavior adheres to a simple two-state reaction between the folded and unfolded protein (19, 20). Furthermore, the conformational stability of the wild-type protein has been determined over a wide range of temperature and urea concentrations (19), and it appears that the simple linear extrapolation method for the analysis of solvent denaturation curves can be used to adequately describe the effects of urea on the stability of the HPr proteins (20). Here we use urea denaturation experiments to determine the differences in conformational stability between the wild-type protein and a number of variants. This allows us to determine how structural mimics of protein phosphorylation alter the energetics of the HPr structure. By investigating a number of pH values, we can also determine the importance of the protonation state of the active site His-15 residue on the stability of HPr and its variants.
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The best-fit of Equation 2 to the data. The best fit values of \( G_m \) and \( m \) for these variants are collected and given in Table I. The errors on each data point are approximately the size of the symbols.

Urea denaturation curves for each of the five HPr proteins, wild type, H15A, H15E, S46D, and H15E/S46D, were performed at four different pH values. Representative curves are shown in Fig. 3 for one set of data at pH 7. In these curves, the raw CD spectroscopy signal has been converted to the fraction of native protein as a function of the molar concentration of urea using Equation 3. This facilitates a comparison of the data from different protein variants. It is clear that the five proteins show different behavior toward urea denaturation, with H15E as the least stable and S46D and H15A as the most stable proteins at pH 7.0. This experiment was repeated at least two times for each protein at each of the four pH values. The results of the analysis of these data, using Equations 1 and 2, are given in Table I. For each pH, the \( m \)-value, the dependence on pH, is presented in Table I. The errors on each data point are approximately the size of the symbols.

**DISCUSSION**

A molecular description of the effects of protein phosphorylation, both in terms of structural changes and energetic changes, will assist us in understanding the regulation of a number of biochemical processes. For the few cases where detailed structure information is known, or can be inferred, there appear to be several mechanisms by which protein phosphorylation can mediate a change in the activity of a protein. In a recent review, Johnson and Barford (2) have discussed five possible results of protein phosphorylation, providing examples of each based on high resolution structures. 1) Phosphorylation may act in an allosteric fashion where local conformational changes near the site of phosphorylation cause conformational changes that are transmitted through subunit interfaces to the active site. 2) Phosphorylation may reduce the affinity of a protein for anionic ligands through an electrostatic blocking
process without the need for conformational changes in the protein. 3) Phosphorylation may order loops or residues in alternative conformations that are now competent for ligand binding. 4) Phosphorylation may alter protein-protein interactions through changes in the surface properties of the protein. 5) Phosphorylation may do nothing to the protein. These “silent phosphorylation” events generally do not alter the conformational stability of a protein. The biological function for this type of protein phosphorylation remains unknown.

These observations have clearly provided a wealth of information on the mechanisms of regulation by protein phosphorylation; however, they are limited to providing only a structural view of protein phosphorylation. It is possible that phosphorylation may alter the conformational stability, either globally or locally, of the protein and that this may in turn regulate protein activity. Protein phosphorylation, cast in this light, can be thought of as a single site mutational event; for example, a serine residue being replaced by a phosphoserine residue. Mutational effects on protein activity and stability are well known, and mutations can alter global conformational stability as well as local flexibility either with or without structural changes (24). In some cases, the analysis of single mutants, or a series of mutants at a particular position, can provide valuable information on the role that the residue contributes to the conformational stability of a protein. Mutational effects can also be used to explore the energetics of the interaction between two residues, even if the residues are not involved in a direct contact. A powerful way to assess this interaction energy is through double mutant cycles (25–27). If the two residues of interest are energetically coupled, the change in conformational stability brought about through mutation at one site will depend on the identity of the residue at the second site. Here we use this approach to determine if the two sites of phosphorylation of bsHPr are energetically coupled despite being separated some 15–20Å in the three-dimensional structure.

Our results indicate that at pH 7 and 9, there is no energetic coupling between sites 46 and 15 in bsHPr ($\Delta G_{\text{int}} \approx 0$). The $pK_a$ of His-15 is 5.4 (17) so at pH 4.8, His-15 is predominately in the protonated form. At this pH, we still find that the interaction energy is essentially zero. Further evidence for the independence of these two sites comes from determinations of the $pK_a$ for His-15 in wild type and the S46D variant (17). The $pK_a$ for His-15 is independent of the identity of residue 46, consistent with our finding that these two sites are not energetically coupled. Therefore, we conclude that the phosphorylation of HPr on Ser-46 does not alter the electrostatic environment around His-15.

The single mutations at His-15 provide further insight into the environment around the active site of HPr. His-15 is located at the N terminus of the first helix in HPr (Fig. 2) and serves as the helix N-cap. There is a preference for certain amino acids to be at N-cap position of a helix in proteins (28, 29) and peptides (30). These preferences are different than those found in interior positions of a helix since the ends of a helix have two unique features, unsatisfied backbone hydrogen bond donors (N terminus) or acceptors (C terminus) and a natural helix macrodipole resulting in a net positive charge at the N terminus and a net negative charge at the C terminus (31). These two factors lead to discrete preferences for residues with side chains that have charged groups and hydrogen bond acceptors. In a recent study on the effects of N-cap mutations at Ser-46 in Escherichia coli HPr (32) and a more limited study in bsHPr (8), we observed a good correlation between the changes in conformational stability between mutations, $\Delta(\Delta G)$, ob-

**FIG. 4.** Double mutants cycles for the Ser-46 and His-15 variants. The numbers are changes in conformational free energy, $\Delta G$, expressed in kcal mol$^{-1}$. The $\Delta G_{\text{int}}$ value is the interaction energy between the two sites. The three cycles represent data from different pH values. A, pH 4.8; B, pH 7.0; and C, pH 9.0. The errors on the changes in conformational free energies are given in Table I.

**FIG. 5.** The conformational free energy for the His-15 variants as a function of the pH of the solution calculated using Equation 4 from the data in Table I. Listed above each bar is the charged state of the residue at position 15. In comparing all the data, the general trend for the stability of the proteins with position 15 substitutions is Ala $>$ His$^0$ $>$ Glu$^-$ $>$ His$^+$. 

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served in the proteins with those obtained in model alanine-based helical peptides (30). For the mutations we have at His-15, the case is not as simple. Instead of the order of stability found in model peptide helices His<sup>0</sup> > Glu<sup>−</sup> > Ala > His<sup>+</sup>, we find Ala > His<sup>0</sup> > Glu<sup>−</sup> > His<sup>+</sup>, that is, the alanine substitution is more stable than expected based on simple N-capping preferences. This is evidence that the active site of HPr, like many other active sites, has unique structural and energetic features and is not simply the N terminus of solvent-exposed helix.

One important question regarding the current strategy is the extent to which we can mimic the phosphorylated form of a protein with an amino acid residue with an acidic side chain. For the Ser(P)-46 form of H<sub>S</sub>Pr, the answer appears definitive; S<sub>46D</sub> mimics Ser(P)-46 in structure, activity, and conformational stability (8, 11). For His(P)-15, however, the evidence is not as clear. We do not have unequivocal structural and energetic data that suggest that H15E is a good mimic of His(P)-15 for the H<sub>S</sub>Pr protein. One limitation with the phosphohistidine form of the protein is the instability of the phosphorus—nitrogen bond (17). This short lived, phosphorylated form of HPr is not amenable to thermodynamic characterization. If we assume that H15E captures at least some of the properties of His(P)-15, there are some interesting comparisons that can be made between the mimics of the phosphorylated forms of b<sub>S</sub>Pr. For the regulatory serine phosphorylation site, the Ser(P)-46 (and S<sub>46D</sub>) form of the protein is more stable than the wild-type protein. However, in as much as H15E mimics His(P)-15, we find a reversal in the conformational stability; H15E (and hence His(P)-15) is less stable than the wild-type protein. That is, phosphorylation at the regulatory site stabilizes the protein while phosphorylation at the catalytic site destabilizes the protein. The biological ramifications of this result are intriguing, but only speculative without further study.

Our results suggest that there is not direct energetic coupling between the regulatory and active sites in b<sub>S</sub>Pr. Together with the recent structural descriptions of the Ser(P)-46 form of the protein, we can now eliminate several of the possible mechanisms by which protein phosphorylation regulates the activity of b<sub>S</sub>Pr. The most likely explanation, taking into consideration all we know about the structure and stability of the various forms of HPr, is that serine phosphorylation prevents binding of EI to the Ser(P)-46 form of HPr due to steric and/or electrostatic effects. Introduction of a negative charge at position 46, either through serine phosphorylation or by the mutation of serine to aspartate, must alter the interaction surface, preventing EI from binding and transferring a phosphate group to His-15. Further studies are underway to provide a detailed description of the EI-HPr complex.

In conclusion, we have demonstrated that the protein engineering approach and the use of double mutant cycles can provide valuable information about the energetic coupling of two sites in a protein, including the specific case of protein phosphorylation. Together with detailed structural information, this approach should be general and should help to shed some light on the mechanism of regulation by protein phosphorylation.

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