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Common Structural Changes Accompany the Functional Inactivation of HPr by Seryl Phosphorylation or by Serine to Aspartate Substitution†

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Received October 9, 1989; Revised Manuscript Received November 6, 1989

ABSTRACT: Although many proteins are known to be regulated via reversible phosphorylation, little is known about the mechanism by which the covalent modification of seryl, threonyl, or tyrosyl residues alters the activities of the target systems. To address this question, modified versions of *Bacillus subtilus* HPr, a protein component of the bacterial phosphotransferase system, have been studied by ^{1}H NMR spectroscopy. Phosphorylation at Ser_{46} or a Ser to Asp substitution at this position inactivates HPr [Reizer, J., Sutrina, S. L., Saier, M. H., Stewart, G. C., Peterkofsky, A., & Reddy, P. (1989) *EMBO J. 8*, 2111–2120]. Two-dimensional spectra of these two modified proteins display nearly identical proton chemical shifts that differ significantly from those observed in the spectra of the unphosphorylated, wild-type protein and of functionally active HPr mutants. The results demonstrate that the functional inactivation of HPr brought about by the serine to aspartate mutation is accompanied by the same structural changes that occur when HPr is phosphorylated at Ser_{46}.

Reversible phosphorylation of proteins, catalyzed by protein kinases and phosphatases, is a widely used mechanism for regulation of cellular activities in both eukaryotes and prokaryotes (Krebs, 1985; Cozzone, 1988). There is, however, only one protein for which there is structural information for both its unphosphorylated and phosphorylated forms; glycogen phosphorylase has been shown by X-ray crystallographic techniques to undergo a conformational change upon phosphorylation of Ser_{14} (Sprang et al., 1988). These changes affect the subunit interface and influence the enzyme's response to allosteric regulators. Clearly, structural studies on other phosphorylated proteins are needed to provide an understanding of the mechanisms by which phosphorylation at a specific residue can so profoundly effect a protein's activity.

The phosphocarrier protein, HPr,[1] plays a central role in the PEP-dependent sugar transport system (PTS) in bacteria. It serves as a phosphoryl group acceptor and donor, accepting a phosphoryl moiety from PEP via phospho enzyme I to generate a phosphohistidyl intermediate and transferring it to any of a number of sugar permeases, the sugar-specific enzymes II and III of the PTS (Postma & Lengeler, 1985;

†This work was supported by NIH Grants DK35187 (R.E.K.) and AI21702 and AI14176 (J.R. and M.H.S.). M.W. was supported by Damon Runyon-Walter Winchell Cancer Research Fund Fellowship DRG-1007.

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[1] Abbreviations: PTS, phosphoenolpyruvate-dependent sugar phosphotransferase system; HPr, histidine-containing phosphocarrier protein of the PTS; Ser, serine; Asp, aspartic acid; PEP, phosphoenolpyruvate; ATP, adenosine triphosphate; TSP, sodium 3-(trimethylsilyl)-propionate-*2,2,3,3-d*$_4$; NMR, nuclear magnetic resonance; 2D NMR, two-dimensional nuclear magnetic resonance spectroscopy; RELAY, two-dimensional relayed coherence transfer spectroscopy; NOESY, two-dimensional NOE spectroscopy.

0006-2960/89/0428-9908$01.50/0

Reizer et al., 1988a). This "catalytic" phosphorylation at His_{15} differs from a "regulatory" phosphorylation at Ser_{46} that, in Gram-positive bacteria, is catalyzed by an ATP-dependent protein kinase, yielding HPr(Ser-P) (Deutscher et al., 1986; Reizer, 1989). Phosphorylation at Ser_{46} has a markedly inhibitory effect on the activity of the protein (Deutscher et al., 1984; Reizer et al., 1989). This regulatory phosphorylation has been proposed to give rise to a hierarchy of preferences for the utilization of PTS sugars (Deutscher et al., 1984). In addition, HPr and the HPr(Ser) ATP-dependent kinase as well as the HPr(Ser-P) phosphatase have been found in heterofermentative lactobacilli that lack the other components of the PTS, suggesting that HPr has a role in the regulation of a non-PTS-related function (Romano et al., 1987; Reizer et al., 1988b).

The three-dimensional structure for the HPr from the Gram-negative bacterium *Escherichia coli*, which is not subject to ATP-dependent phosphorylation, has been determined by two-dimensional nuclear magnetic resonance (2D NMR) techniques (Klevit & Waygood, 1986; Klevit et al., 1986). As a basis for studies involving the Gram-positive variants of HPr, we have recently determined the sequence-specific resonance assignments of the 88-residue HPr from *Bacillus subtilus* (unpublished experiments). From these analyses, we concluded that the overall folds of the proteins are very similar, and both models are currently being refined with metric matrix distance geometry techniques (Crippen, 1981). Even at the current level of refinement of the unmodified wild-type HPr structure, the proton resonance assignments are valuable for evaluating the effect of covalent modifications on the HPr structure. In this paper we use the crosspeak patterns of the 2D NMR spectra of phosphorylated and mutant HPr proteins as "fingerprints" in order to examine the relatedness of various modified protein structures.

Materials and Methods

Sample Preparation. Wild-type *Bacillus subtilus* HPr and the site-directed mutants S46D, S46A, and S46T were expressed in *Escherichia coli* and purified as previously described (Reizer et al., 1989). *B. subtilus* HPr(Ser-P) was prepared by incubation of the wild-type protein with the HPr-specific ATP-dependent kinase from *Streptococcus faecalis* as described (Deutscher et al., 1986).

NMR samples were prepared by dissolving 10–20 mg of protein in a 4-mL volume of 5 mM potassium phosphate, pH 6.9, followed by dialysis against the same buffer. The samples were lyophilized again and dissolved in 0.4 mL of 90% H_2O/10% D_2O, centrifuged to remove insoluble material, and transferred to 5-mm NMR tubes. The final protein concentration among the different HPr samples ranged from 3 to 6 mM, in 50 mM potassium phosphate buffer, pH 6.9.

NMR Spectroscopy. RELAY spectra (Bax & Drobny, 1985) were recorded on a Bruker AM-500 NMR spectrometer with a 32-ms mixing time in H_2O at 30 °C. ^1H chemical shifts were referenced to TSP at 0.0 ppm. Data were processed with the software package FTNMR (Hare Research, Woodinville, WA). In some spectra, crosspeaks near 4.75 ppm are bleached due to the presaturation used to suppress the water resonance and are indicated with boxes. These crosspeaks have been observed in other experiments in which alternate water suppression schemes have been used (data not shown).

Results

In Figure 1A, the fingerprint regions of the RELAY spectra of HPr and HPr(Ser-P) are overlaid. In this region of the two-dimensional spectrum, every amino acid residue in the protein gives rise to a single intraresidue NH–C^αH crosspeak (except for prolyl and glycyl residues). The resulting pattern of dispersed crosspeaks (the fingerprint) is characteristic of a particular protein due to the unique combinations of chemical shifts exhibited by the NH–C^αH pairs and is a direct consequence of the structure of the protein (Wüthrich, 1986). It can be seen that phosphorylation at Ser_{46} results in the perturbation of the chemical shift positions of many, but not all, of the NH–C^αH resonances. The use of RELAY spectra aided in the identification of shifted peaks since the NH–C^αH peak, the NH–C^βH peak, and the C^αH–C^βH peak(s) of a given spin system can all be correlated in one spectrum. In addition, the identifications have been confirmed by an analysis of NOESY spectra in which sequential connectivities could be observed (data not shown). Figure 2 shows the relevant sequence-specific assignments for wild-type HPr.

Table I: Nonparametric Statistical Analysis of the Chemical Shift Differences between Modified and Unmodified HPr Amide and C^αH Proton Resonances[a]

	HPr(Ser-P)	S46D	S46T	S46A
HPr(Ser-P)				
S46D	0.88			
S46T	0.46	0.43		
S46A	0.43	0.38	0.74	

[a] The deviations of the amide and C^αH proton chemical shifts of the modified proteins relative to those of the unphosphorylated, wild-type protein were measured, and the Spearman rank-order correlation coefficient (Siegal, 1956; Press et al., 1986) was calculated by pairwise comparison of these deviations for two modified proteins. A crosspeak was included in the analysis only if it exhibited at least a 0.04 ppm deviation in either dimension in any of the modified protein spectra. The same 33 crosspeaks were analyzed in each comparison ($N = 66$). For this sample size, a value of 0.31 or higher indicates that there is at least a 99% probability that the two sets of deviations are related.

A similar spectral analysis was performed on a functionally inactive mutant of *B. subtilus* HPr in which Ser_{46} was replaced with an aspartate residue (S46D) (Reizer et al., 1989). Interestingly, the resulting fingerprint pattern, shown in Figure 1B, is strikingly similar to that obtained with HPr(Ser-P). In both spectra, the same crosspeaks are affected, the directions of the changes in chemical shift position are the same, and their magnitudes are very similar. In order to assess whether any change at position 46 results in the same perturbed spectrum, 2D NMR spectra of two functionally active mutant HPrs, substituted with either threonine or alanine (S46T and S46A, respectively) (Reizer et al., 1989), were obtained. As can be seen in panels C and D of Figure 1, a number of the same crosspeaks are affected, but the overall patterns are different from those obtained for HPr(Ser-P) and S46D and appear to be more closely related to each other.

In order to test for relatedness between the spectra, a nonparametric statistical analysis was performed on the data. The input consisted of the differences in chemical shift (both magnitude and sign) between the wild-type, unphosphorylated spectrum and each of the modified spectra. Table I contains the correlation coefficients obtained for pairwise comparison of the four modified HPr spectra. The correlation coefficient of 0.88 obtained for HPr(Ser-P) and S46D shows that these two spectra are strongly related, indicating that the S46D mutation and phosphorylation at Ser_{46} have very similar effects on the structure of HPr. In addition, the spectra of the two active mutants, S46T and S46A, were well correlated with each other. Significantly weaker correlations were obtained when spectra from active versus inactive HPr proteins were compared.

The residues undergoing chemical shift perturbations in HPr(Ser-P) and S46D are indicated on the model of *B. sub-*

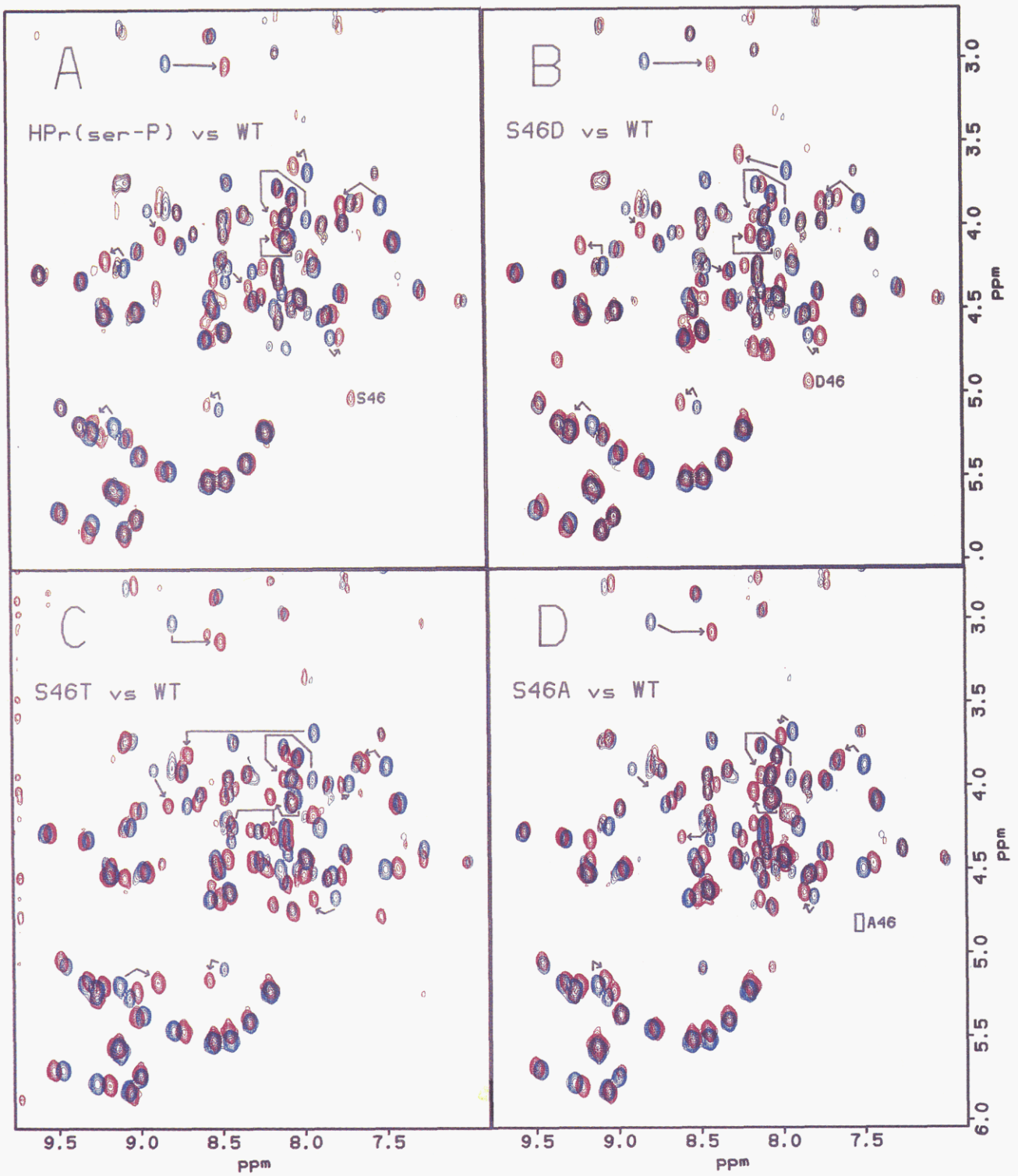

FIGURE 1: NMR spectra of native and modified forms of HPr. The fingerprint region of the RELAY spectra (Bax & Drobny, 1985) of unmodified, wild-type *B. subtilus* HPr is shown in blue in each panel. Similar spectra obtained for modified versions of HPr are overlaid in red in panels A–D. The arrows indicate crosspeaks undergoing large shifts in position relative to the unmodified protein. (A) HPr(Ser-P); (B) S46D; (C) S46T; (D) S46A. The spectrum for S46D (panel B, red) was obtained with lower decoupler power for water suppression than that used for the other spectra. This explains the presence of crosspeaks around 4.8 ppm in the S46D spectrum that are not observed in the other spectra.

tilus HPr in Figure 3. Backbone proton resonances of residues in the primary sequence near the phosphorylation site exhibit chemical shift differences in all the modified forms. Ser_{46} is at the end of a tight turn that connects a β-strand with a segment of extended secondary structure. Another segment of the backbone showing large chemical shift perturbations is residues forming the first two turns of an α-helix in the wild-type protein (Klevit & Waygood, 1986; unpublished experiments) as well as in HPr(Ser-P) and S46D (unpublished results). Finally, the ^1H chemical shifts of amino acid residues near the active site His_{15} are changed. Overall, the changes in chemical shift position for most of the affected residues are relatively large while proton resonances in other portions of the protein remain unchanged.

The schematic in Figure 3 indicates that the chemical shift changes are not purely a consequence of distance from the site

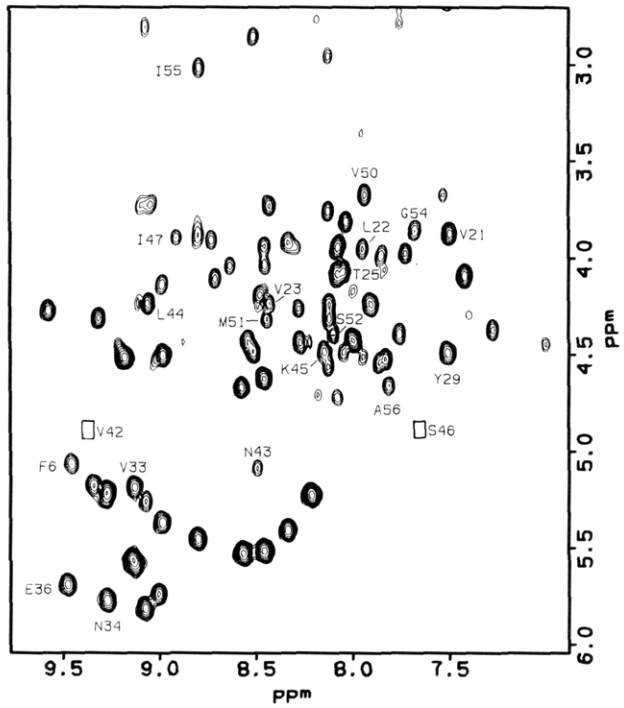

FIGURE 2: Relevant sequence-specific resonance assignments for wild-type, unphosphorylated *B. subtilus* HPr. Resonance assignments are from unpublished experiments. The same region of the RELAY spectra shown in Figure 1 is displayed.

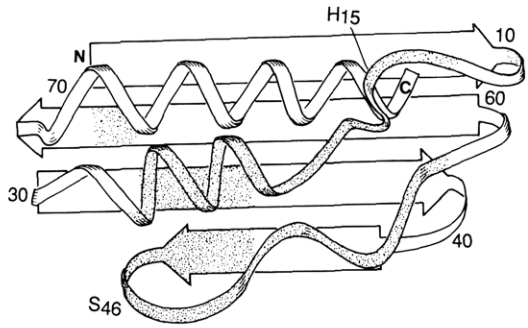

FIGURE 3: Summary of the chemical shift changes seen in HPr(Ser-P). The ribbon structure model was derived from an analysis of the sequential and long-range NOEs observed for unphosphorylated, wild-type *B. subtilus* HPr (unpublished experiments). The stippled areas indicate residues in HPr(Ser-P) exhibiting backbone proton resonances undergoing chemical shifts greater than 0.04 ppm in either dimension relative to those of wild type.

of modification but seem to follow certain pathways, along elements of secondary structure. Because the backbone chemical shift perturbations extend to localized regions of the protein far away from position 46, the changes are not purely a result of through-bond effects caused by the addition of a negative charge. It should be noted that Figure 3 is a map of the chemical shift perturbations, and conclusions cannot be made about the nature and magnitude of conformational changes at this time. The details of the structural alterations brought about by the modifications are currently being studied by an analysis of the NOESY spectra of these proteins. Preliminary findings show that the region around Ser_{46} does not undergo the extended-to-helical conformational change that was observed for glycogen phosphorylase (Sprang et al., 1988).

Discussion

There is a body of evidence to show that chemical shift and protein structure are correlated. It has been observed that backbone protons in residues involved in hydrogen-bonded structures tend to resonate progressively downfield with decreasing H-bond distance (Wagner et al., 1983). Also, the orientation of aromatic side chains can cause nearby protons to undergo chemical shift perturbations due to ring current effects (Perkins, 1982). In studies of HPr, we have noted that the chemical shifts of backbone protons are more sensitive to the secondary structure of a given residue than to the exact nature of the residue itself. For example, the amide proton resonance of residue 30, which is a threonine in *E. coli* HPr (Klevit et al., 1986), an aspartate in *B. subtilus* HPr (unpublished experiments), and an asparagine in *S. faecalis* HPr (Glaser, 1987), is the most downfield resonance in all three spectra, at ~11 ppm. Also, from the spectra presented in Figure 1, it can be seen that the substitution of an aspartate or an alanine for Ser_{46} does not appreciably change the chemical shifts of the amide or $C^{\alpha}H$ resonances of residue 46 itself.

These empirical correlations between chemical shift and structure are undoubtedly significant, yet there is currently no coherent model that can be used to accurately predict the absolute resonance position of a given proton within a protein structure. The exact chemical shift is a function of complex contributing factors that together result in determining the specific electronic environments of the individual protons within the protein. While it is this complexity that precludes an accurate prediction of chemical shift positions and their direct use in structural determinations, it is also the reason crosspeaks take on their characteristic positions within an NMR spectrum. Comparison of fingerprint crosspeak patterns is therefore an extremely sensitive and general method to compare the conformational effects of modifications and mutations on a protein structure.

In a mutational study of *E. coli* isocitrate dehydrogenase (Thorsness & Koshland, 1987), the seryl residue that becomes reversibly phosphorylated in a reaction catalyzed by isocitrate dehydrogenase–kinase–phosphatase was changed to an aspartate. As was later shown for HPr (Reizer et al., 1989) and eukaryotic translation initiation factor eIF2 (Kaufman et al., 1989), this type of substitution mutation resulted in a protein whose activity was similar to that of the phosphoserine form of the protein. However, in all three cases, both the phosphorylated and the Ser to Asp mutant forms of the proteins were inactive, leaving doubt as to whether the structural changes brought about by these two types of modifications are actually the same. The observation that the NMR spectra of the phosphorylated and the S46D mutant forms of HPr show extremely similar crosspeak patterns, which differ from those seen in the spectra of the unphosphorylated wild-type protein and of other active mutants, is strong evidence that the two modifications have very similar effects on the conformation of HPr. These results indicate that the serine to aspartate mutation may be a general method of mimicking phosphorylation at a seryl residue in not only a functional sense but a structural one as well.

Acknowledgments

We thank Reinhard Eisermann for supplying the purified *B. subtilus* HPr used to generate HPr(Ser-P), Jon Herriott for help with the statistical analysis, Grace Pârraga for useful discussions, Gary Drobny for use of the 500-MHz NMR spectrometer, and Trisha Davis, Ben Shapiro, and Ted Young for suggestions on the manuscript.

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