Published in final edited form as:
J Am Chem Soc. 2009 October 7; 131(39): 14081–14087. doi:10.1021/ja9047575.

Controlling Peptide Folding with Repulsive Interactions between Phosphorylated Amino Acids and Tryptophan

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Abstract

Phosphorylated amino acids were incorporated into a designed β-hairpin peptide to study the effect on β-hairpin structure when the phosphate group is positioned to interact with a tryptophan residue on the neighboring strand. The three commonly phosphorylated residues in biological systems, serine, threonine, and tyrosine, were studied in same β-hairpin system. It was found that phosporylation destabilizes the hairpin structure by approximately 1.0 kcal/mol regardless of the type of phosphorylated residue. In contrast, destabilization due to glutamic acid was about 0.3 kcal/mol. Double mutant cycles and pH studies are consistent with a repulsive interaction as the source of destabilization. These findings demonstrate a novel mechanism by which phosphorylation may influence protein structure and function.

Introduction

Phosphorylation of proteins is ubiquitious in cellular processes as a regulatory control. It is estimated that about one third of all human proteins are phosphylated.[1] Phosphorylation is a part of intracellular signal transduction pathways that modulate cellular proliferation, macromolecule production, and gene expression.[1] Abnormal phosphorylation can be the cause or the result of many diseases, including cancer and Alzheimer's disease.[1] Yet, how phosphorylation alters a protein's function is still poorly understood and the effect appears to differ from protein to protein.[2] Currently the most effective method for studying how phosphorylation effects protein structure and function is by comparison of crystal structures of phosphorylated and unphosphorylated proteins.[2,3] However, not all proteins crystallize easily, such as membrane and instrincally disordered proteins, and a large amount of homogenous modified protein is also required for crystallography, thus giving an incomplete picture of how phosphorylation affects the structure and function of proteins. The study of phosphorylated residues on structure in smaller model systems is warranted to obtain a clearer understanding of how this post-translational modification can affect structure.

Recently, a body of information on how phosphorylation affects local protein structure has emerged from studies in model peptides systems. Doig and co-workers have shown that phosphoserine strongly stabilizes α-helical peptides when positioned near the N-terminus, or when positioned to make a favorable salt bridge within the helix.[4,5] Fujitani and coworker observed a stabilization of α-helix structure from a peptide fragment excised from H^+/K^+ ATPase through phosphorylation of tyrosine.[6] In another native protein model, it has also been shown that phosphorylation of serine and threonine can have a destabilizing affect within α-helix system, particularly with phosphothreonine.[7,8] The DeGrado laboratory has used phosphorylation as a molecular switch to promote the self assembly of de novo designed helical

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Supporting Information. NMR assignments and NOEs.

bundles.[9] Work from the Zondlo laboratory has elucidated that the proline rich regions of the naturally disordered Tau peptide when phosphorylated adopts a polyproline II helix structure.[10] Suau and coworkers observed a structural transition in the intrinsically disordered carboxy-terminal domain from histone H1 linker protein, where α-helical character was decreased and β-sheet character increased depending on the extent of phosphorylation.[11] Excised peptides from phophosphorylated proteins have also exhibit conformational changes upon phosphorylation to various loop structures.[12–14] It is becoming apparent that phosphorylation is a versatile post-translational modification that can induce a wide variety of structural changes.

Stabilization or destabilizaion via charge-charge interactions with a phosphorylated residue are to be expected. Phopshorylated residues can also induce structural changes via metal binding.[15–18] However, to our knowledge, no one has investigated whether phosphorylation can result in a repulsive interaction with a hydrophobic or aromatic group that could affect peptide structure. Herein we present the destabilizing effect of phosphorylated amino acids due to repulsive interactions with tryptophan (Trp) within a designed β-hairpin peptide. These studies help to further delineate the range of structural changes that may occur due to phosphorylation.

Results and Discussion

System Design

A set of 12-residue peptides designed to autonomously fold into β-hairpins in aqueous solution was used to investigate the effect of phosphorylation on a sidechain-sidechain interaction with a cross-strand Trp residue on structure. Features that influence folding include the turn sequence, the β-sheet propensity of the strand residues, and the sidechain-sidechain interactions.[19] All of the β-hairpins peptides contain the sequence VNGK to promote favorable type I' turn which has been shown to promote in β-hairpin structure.[20–23] The **SW-1** peptide system was designed to study the effect of phosphorylation on a modestly folded β-hairpin sequence containing a serine residue in position 2 that is directly cross strand from a tryptophan in position 11 on the non-hydrogen bonded (NHB) face of the hairpin (Figure 1a). The NHB face is defined as the face of the peptide displaying side chains from the non-hydrogen bonded residues in a two stranded β-sheet.[24] On the NHB face the side chains of residues are oriented closer to cross strand residue side chains thus giving a larger contribution to hairpin stability through side chain-side chain interactions than those residues on the hydrogen bonded (HB) face.[24] Subsequently, serine was replaced with threonine and tyrosine at position 2 and their phosphorylated analogs to determine the significance of the phosphorylated residue (Figure 1b). Unfolded control peptides **1–9** were synthesized in which each peptide consisted of either residues 1–7 composing of the N-terminal arm and turn of the β-hairpin or residues 6–12 consisting of the C-terminal arm and turn (Figure 1c). Cyclic peptides **10–14** were synthesized as fully folded controls for each of the β-hairpins. Cyclization was achieved by a disulfide bond between cysteine residues at the N and Ctermini of the peptides (Figure 1d).

SW-1 Peptide Structural Studies

NMR spectroscopy was used to determine to what extent the **SW-1** peptide and the phosphoserine containing peptide **pSW-1** fold into β-hairpin structures. Downfield shifting of ≥ 0.1 ppm of the α-protons (Hα) along the peptide backbone relative to unfolded values indicates β-hairpin structure.[25] **SW-1** was found to have a majority of Hα shifts above 0.1 ppm compared to the unstructured controls, except for the two terminal residues which are typically frayed in β-hairpins and Asn 6 which is located within the turn of the hairpin (Figure 2a). This Asn is typically upfield shifted from the unstructured control value due to its conformation in the turn. Downfield shifting of backbone amide hydrogens in HB positions relative to random

coil values also indicates β- sheet structure, which is seen for valine 3, valine 5, and isoleucine 10 (Figure 2b). NHB residues threonine 4, threonine 9 and tryptophan 11 are also significantly downfield shifted. NOESY data further confirmed that this peptide forms the predicted β-hairpin structure (See Supporting Information).

The **pSW-1** peptide was synthesized with a phosphoserine replacing the serine in **SW-1** and characterized by NMR spectroscopy to study the effect of phosphoserine on the β-hairpin structure. The Hα shifts of **pSW-1** are not as downfield shifted as **SW-1** peptide, many of which are < 0.1 ppm, indicating that incorporation of a phosphoserine causes a destabilization of the β-hairpin structure in this sequence (Figure 2a). A decrease in amide backbone shifts is also seen at the hydrogen-bonded positions when compared to **SW-1** (Figure 2b). NOESY data of **pSW-1** show no long distance NOEs, indicating little to no β-hairpin structure. The lack of long distance NOE's is indicative of a highly dynamic peptide which may still sample a β-hairpin conformation but spends most of its time in an unstructured state.

Circular Dichroism (CD) experiments were also performed to confirm that there is a loss of structure with incorporation of phosphoserine (Figure 2c). Since the **SW-1** peptide is only modestly folded, there is a large negative signal at 197 nm typical of random coil but there is also shoulder at 215 nm that indicates β-sheet structure. This shoulder at 215 nm for **pSW-1** is not observed and a larger random coil signal at 197 nm is present which is consistent with the NMR data, reinforcing that **pSW-1** has little defined structure.

The extent of folding to a β-hairpin by **SW-1** and **pSW-1** peptides was quantified using two methods. The first method utilizes the extent of Hα downfield shifting relative to random coil controls and fully folded control as previously described (see Experimental Procedures). The second method utilizes the extent of the diastereotopic glycine Hα splitting located in the turn of the hairpin relative to glycine Hα splitting observed in the fully folded control (see Experimental Procedures). **SW-1** was found to be 40% folded and **pSW-1** only 10% folded using both methods (Table 1). The extent of destabilization due to phosphoserine was calculated to be approximately 1.3 kcal/mol. The destabilization that occurs when the phosphoserine is incorporated in this hairpin is believed to result from an unfavorable interaction between the phosphate group and the tryptophan indole ring directly across from it. This destabilization may be caused by repulsion of the negatively charged phosphate and the electron rich indole ring of cross-strand tryptophan, or through steric clash between the large phosphate group and the tryptophan, or a combination of both.

pH Studies

To determine whether the destabilization was caused by electrostatic repulsion of the phosphate group with the electron rich indole ring of tryptophan or a steric clash of the phosphate group with the tryptophan a pH study was performed on **SW-1** and **pSW-1**. By varying the pH and thus the charge on the phosphate group, a change in fraction folded will indicate whether there is an electronic component to the destabilization. A comparison of the fraction folded verses pH for both **SW-1** and **pSW-1** is given in Figure 3. It was observed that as the pH increases, the **SW-1** fraction folded is constant, while the **pSW-1** fraction folded decreases. Interestingly, the fraction folded at pH 2.5 and 4.7 is unchanged for **pSW-1,** which correlates to a −1 charge on phosphate, while at pH 7.4 the fraction folded is lower and the phosphate group now has a −2 charge, and a higher fraction folded is observed at pH 1.2 where the phosphate group is uncharged. As the charge state of the phosphoserine increases, the degree of folding decreases, indicating that the charge of the phosphate is a predominant contributor to the destabilization of the β-hairpin. However, sterics appear to play some role in the destabilization as **pSW-1** is less folded than **SW-1** even in a neutral charge state of the phosphoserine.

Double Mutant Studies

To determine whether the phosphoserine-tryptophan interaction is the major destabilizing interaction, a double-mutant cycle was performed (Figure 4)[25]. In the double-mutant cycle, both of the interacting residues are mutated individually and together. The single mutants, B and C, disrupt the side-chain-side-chain interaction in A, but may result in other changes that affect the stability of the β-hairpin, such as the β-sheet propensity. The double mutant, D, corrects for all unintended changes that affect the β-hairpin stability. Thus, the sum of the stabilities of peptides A and D minus those of the single mutants, B and C, provides the side-chain-side-chain interaction of phosphoserine and tryptophan. The peptides **SW-1** and **pSV-1** were used as individual mutants and **SV-1** was synthesized as the double mutant. **pSV-1** contains a phosphoserine at position 2 and a valine at position 11 to replace the tryptophan and **SV-1** contains a serine at position 2 and a valine at position 11. The fraction folded and ΔG of folding for all the peptides in the double-mutant cycle are given in Table 2. These studies were preformed in a pD 4 buffer solution due to low solubility of **SV-1** and **pSV-1** at pD 7. The interaction of the phosphoserine-tryptophan was calculated to be a destabilization of 0.5 kcal/mol, which is the same as the overall destabilization between **SW-1** and **pSW-1** at pH 4, therefore indicating that this interaction is the direct cause of the destabilization of β-hairpin. Indeed, it is interesting to note that phosphorylation has no impact whatsoever on folding of the **SV-1/pSV-1** peptides, which provides further support that the nature of the destabilization is electronic rather than steric.

EW-1 and QW-1 Peptide Studies

To further investigate the electrostatic destabilization of hairpin, the peptide **EW-1** was study with a glutamic acid at position 2 replacing the phosphoserine. The peptide **QW-1** was used as a neutral analog for direct comparison to **EW-1**. NMR characterization showed that **QW-1** is in fact more stable than **EW-1** at pH 7: **EW-1** is 24% folded while **QW-1** is 50% folded (Figure 5a, Table 1). Comparison of the CD spectra of **EW-1** and **QW-1** in pH 7 phosphate buffer also indicates that **QW-1** is more folded than **EW-1** (Figure 5b). This again shows that placing a negatively charged species cross strand from tryptophan in this hairpin system is destabilizing. A pH study demonstrates that **EW-1** exhibits pH dependence similar to **pSW-1** (Figure 6). At lower pH, **EW-1** becomes more folded, just as with **pSW-1**. In contrast, **QW-1** is independent of pH. Interestingly, when protonated, glutamic acid is more stabilizing than glutamine. The same was not true for **pSW-1** and **SW-1**, which may suggest a greater role for steric repulsion in the case of **pSW-1** than **EW-1**.

TW-1 Peptide Studies

To determine if phosphothreonine, another commonly phosphorylated amino acid, has the same effect as phophoserine, the **TW-1** peptide system was designed with the same β-hairpin scaffold as **SW-1**, but with threonine positioned directly cross strand from tryptophan as in the **SW-1** peptide (Figure 1). NMR analysis of the Hα chemical shifts of **TW-1** and **pTW-1** indicate that destabilization occurs when Thr is phosphorylated (Figure 7a) to a similar extent as was observed for **SW-1** and **pSW-1. TW-1** is about 40% folded while **pTW-1** is only 12% folded, giving a destabilization of approximately 0.9 kcal/mol (Table 1). The CD spectra of **TW-1** and **pTW-1** corroborate the NMR data, indicating that **pTW-1** is less stable than **TW-1,** with **TW-1** having a more pronounced shoulder at 215 than **pTW-1** and **pTW-1** having a larger minima at 197 nm (Figure 7b).

YW-1 Peptide Studies

The effect of tyrosine phosphorylation within the β-hairpin model system was also explored. NMR analysis of **YW-1** indicates that incorporation of the tyrosine cross-strand from the tryptophan stabilizes the hairpin (Figure 8a), which is about 82% folded. The incorporation of

phosphotyrosine results in a destabilization of 1.0 kcal/mol of the hairpin, which is about 60% folded (Table 1). CD spectra confirmed the change in stability between **YW-1** and **pYW-1** showing a strong β-sheet minimum at 215 nm and no random coil minima at 197 nm for **YW-1** while **pYW-1** has a smaller minimum at 215 nm than **YW-1** and a minimum at 197 nm (Figure 8b).

The larger degree of folding of **YW-1** and **pYW-1** relative to their **SW-1** and **TW-1** analogs is due to a favorable interaction between tyrosine and the tryptophan. It has been previously shown that aromatic groups cross-stand in NHB positions of β-hairpins have a stabilizing effect via edge-face aromatic interactions.[26,27] NMR characterization of the aromatic region of **YW-1** suggests that Tyr2 interacts with Trp11 in an edge-face interaction as well, with the ortho-proton of Tyr2 directed towards the face of Trp11. The ortho and meta hydrogens on the tyrosine are not equivalent, with one ortho proton significantly upfield shifted (Figure 9). The observation of two sets of ortho and meta protons indicates restricted rotation of the aromatic ring of Tyr. In the less folded **pYW-1,** the ortho-proton on Tyr is shifted about half as much as in **YW-1**. The lesser extent of aromatic proton shifting is consistent with these residues interacting less in the destabilized hairpin.

Since the **YW-1** hairpin has a very high stability due to the favorable contacts of the tyrosine, the overall hairpin stability may not be directly destabilized by the interaction of the phosphotyrosine with tryptophan. To address this issue, a double mutant cycle was performed as described above, where valine replaces tryptophan in the appropriate mutant peptides. As mentioned above, the double mutant cycle was performed at pH 4 due to solubility limitations of the valine-containing peptides at pH 7. The double mutant study found that the destabilization due to the interaction between phosphotyrosine and tryptophan is approximately 0.4 kcal/mol at pH 4 (Table 3). This destabilization is close to the total energy lost upon phosphorylation of Tyr at pH 4 (ie **pYW-1** relative to **YW-1**), which is 0.6 kcal/mol.

Discussion

The studies described above demonstrate destabilization of β-hairpin structure upon phosphorylation of a residue (Ser, Thr, Tyr) when it is cross-strand from Trp. Experimental measurement of an unfavorable interaction can be more difficult than measuring a favorable interaction. The β-hairpin system, however, allows for an intramolecular method of measuring an unfavorable interaction between naturally occurring residues. A pH dependence was demonstrated, in which protonation of the acidic residue increases the stability of the hairpin, consistent with a repulsive electrostatic interaction. A similar pH dependent destabilization of the folded state was found for Glu across from Trp, although the magnitude of destabilization was less. Substituting different phosphorylated residues (serine, threonine, tyrosine) in the β-hairpin peptide sequence has a similar destabilizing effect of 0.9 – 1.3 kcal/mol on the β-hairpin structure at pH 7, in which the phosphate is a dianion. This is interesting because tyrosine analog **YW-1** is considerably more stable than **TW-1** and **SW-1**, yet the amount of destabilization caused by incorporation of phosphorylated residue is roughly the same. In contrast, the magnitude of the repulsive Glu-Trp interaction is 0.3 kcal/mol, which is similar to the magnitude of the phosphoSer-Trp interaction at pH 4, in which the phosphate is a monoanion. It seems that in this hairpin system the phosphate-aromatic interaction is not significantly dependent of the residue that the phosphate group is attached; the charge state has a larger influence on the magnitude of destabilization.

These results are all consistent with a repulsive electrostatic interaction as the driving force for destabilization of the folded state that may be described as an unfavorable anion-π interaction, [28] although a repulsive charge-hydrophobic interaction cannot be ruled out at this time. Such unfavorable interactions in a natural occurring system have not previously been investigated.

This type of interaction represents a novel mechanism by which phosphorylation may destabilize a particular protein structure, change its structure, or prevent a protein binding event. This effect on structure is significant given the ubiquitous nature of protein phosphorylation in signal transduction and aberrant phosphorylation in many disease states. Although Trp is not an extremely common amino acid, the same type of repulsive interaction can be expected with Phe, albeit likely weaker, as has been observed in other comparisons of Trp and Phe interactions in peptide model systems.[29,30] Since poly-phosphorylation is common in proteins, one can envision an additive destabilizing effect of multiple phosphorylations. This sort of additive effect has been proposed in the partial unfolding of ankyrin repeats 1 and 2 of the tumor suppressor $p19^{INK4d}$ upon phosphorylation,[31] and induction of folding upon hyperphosphorylation of tau protein in Alzheimer's disease, for example.[10]

Lawrence and coworkers have used a similar repulsive interaction to design reporter peptides for protein tyrosine kinases that produce a fluorescent signal upon phosphorylation of tyrosine.[32] These tyrosine kinase substrates contain a tyrosine that quenches the fluorophore pyrene in close proximity through an aromatic interaction. Upon phosphorylation of the tyrosine, a fluorescent signal is generated due to the disruption of the aromatic interaction, allowing these peptides to act as a fluorescence reporter of tyrosine kinases. It was hypothesized that the charged and bulky phosphate in combination with a less electron rich π system of phosphotyrosine was responsible for the disruption of the tyrosine-pyrene interaction. These fluorescence reporter peptides appear to undergo a similar repulsive electrostatic interaction as is observed in **YW-1** and **pYW-1,** in which the π-π interaction is disrupted by phosphorylation.

Conclusion

We have shown that incorporation of phosphorylated residues cross strand from tryptophan in a β-hairpin results in the destabilization of the hairpin structure. The nature of this interaction is primarily due to an unfavorable interaction between of the negatively charged phosphate group and the electron rich indole ring of tryptophan. The magnitude of the destabilization is dependent on pH and ranges from 0.4 kcal/mol to approximately 1 kcal/mol. These findings demonstrate a novel mechanism by which phosphorylation may influence protein structure and function and have implications for phosphorylation-dependent signaling. Further studies are currently being conducted investigating the position-dependence of the destabilizing phosphate-tryptophan as well as the effect of multiple phosphorylations in alternative β-hairpin systems.

Experimental Procedures

Synthesis and Purification of peptides

Peptides were synthesized by automated solid phase peptide synthesis on an Applied Biosystems Pioneer Peptide Synthesizer using Fmoc protected amino acids on a PEG-PAL-PS resin. Fmoc-[N]-protected and Benzyl-[O]-protected phosphoserine, phosphothreoine, and phosphotyropsin were purchased from AnaSpec. Activation of amino acids was performed with HBTU, HOBT in the presence of DIPEA in DMF. Peptide deprotection was carried out in 2% DBU (1,8 diazabicyclo[5.4.0]undec-7-ene), 2% piperidine in DMF for approximately 10 minutes. Extended cycles (75 minutes) were used for each amino acid coupling step. All control peptides where acetylated at the N-terminus with 5% acetic anhydride, 6% lutidine in DMF for 30 min. Cleavage of the peptide from the resin was performed in 95:2.5:2.5 Trifluoroacetic acid (TFA): Ethanedithiol (or Triisopropylsilane (TIPS)): water for 3 hours. Ethanedithiol was used as a scavenger in for sulfur containing peptides. TFA was evaporated and cleavage products were precipitated with cold ether. The peptide was extracted into water and lyophilized. It was then purified by reverse phase HPLC, using a Vydac C-18

semipreparative column and a gradient of 0 to 100% B over 40 minutes, where solvent A was 95:5 water:acetonitrile, 0.1% TFA and solvent B was 95:5 actonitrile:water, 0.1% TFA. After purification the peptide was lyophilized to powder and identified with ESI-TOF mass spectroscopy.

Cyclization of peptides

Cyclic control peptides were cyclized by oxidizing the cysteine residues at the ends of the peptide via stirring in a 10 mM phosphate buffer (pH 7.5) in 1% DMSO solution for 9 to 12 hours. The solution was lyophilized to a powder and purified with HPLC using the method described above.

CD Spectroscopy

CD spectroscopy was performed on an Aviv 62DS Circular Dichroism Spectrophotometer. Spectra were collected from 260 nm to 185 nm at 25°C, 1 sec scanning.

NMR Spectroscopy

NMR samples were made to a concentration of 1 mM in D_2O buffered to pD 4.0 (uncorrected) with 50 mM NaOAc-d3, 24 mM AcOH-d4, 0.5 mM DSS, or pD 7.0 (uncorrected) with 50 mM $KPOD_4$, 0.5 mM DSS. Samples were analyzed on a Varian Inova 600-MHz instrument. One dimensional spectra were collected by using 32-K data points and between 8 to 128 scans using 1.5 second presaturation. Two dimensional total correlation spectroscopy (TOCSY) and nuclear Overhauser spectroscopy (NOESY) experiments were carried out using the pulse sequences from the chempack software. Scans in the TOCSY experiments were taken 16 to 32 in the first dimension and 64 to 128 in the second dimension. Scans in the NOESY experiments were taken 32 to 64 in the first dimension and 128 to 512 in the second dimension with mixing times of 200 to 500msec. All spectra were analyzed using standard window functions (sinbell and Gaussian with shifting). Presaturation was used to suppress the water resonance. Assignments were made by using standard methods as described by Wüthrich.[33] All experiments were run at 298.15 K (Hα shift) or 293.15K (double mutant and pH studies).

Determination of Fraction Folded

To determine the unfolded chemical shifts, 7-mers were synthesized as unstructured controls and cyclic peptides were synthesized for fully folded. The chemical shifts for residues in the strand and one turn residue were obtained from each 7-mer peptide. The chemical shifts of the fully folded state were taken from the cyclic peptides. The fraction folded on a per residue bases was determined from Equation 1.

$$\text{Fraction Folded} = [\delta\text{obs} - \delta 0]/[\delta 100 - \delta 0], \quad [1]$$

where δobs is the observed CαH chemical shift, δ100 is the CαH chemical shift of the cyclic peptides, and δ0 is the CαH chemical shift of the unfolded 7-mers. The overall fraction folded for the entire peptide was obtained by averaging the fraction folded of resides Val3, Lys8, and Ile10. These residues are in hydrogen bonded positions have been shown to be the most reliable in determining fraction folded.34 The overall fraction fold was also determined using the extent of CαH glycine splitting observed in the turn residue Gly10 given in Equation 2.

$$\text{Fraction Folded} = [\Delta\delta_{\text{Gly Obs}}]/[\Delta\delta_{\text{Gly 100}}], \quad [2]$$

where $\Delta\delta_{\text{Gly Obs}}$ is the difference in the glycine CαH chemical shifts of the observed, and $\Delta\delta_{\text{Gly 100}}$ is the difference in the glycine CαH chemical shifts of the cyclic peptides.

The ΔG of folding at 298 K for the peptides was calculated using Equation 3 where *f* is the fraction folded.

$$\Delta G = -RT\ln(f/(1-f)), \quad [3]$$

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We gratefully acknowledge support from the National Institutes of Health (GM 071589) and the National Science Foundation (CHE-0716126).

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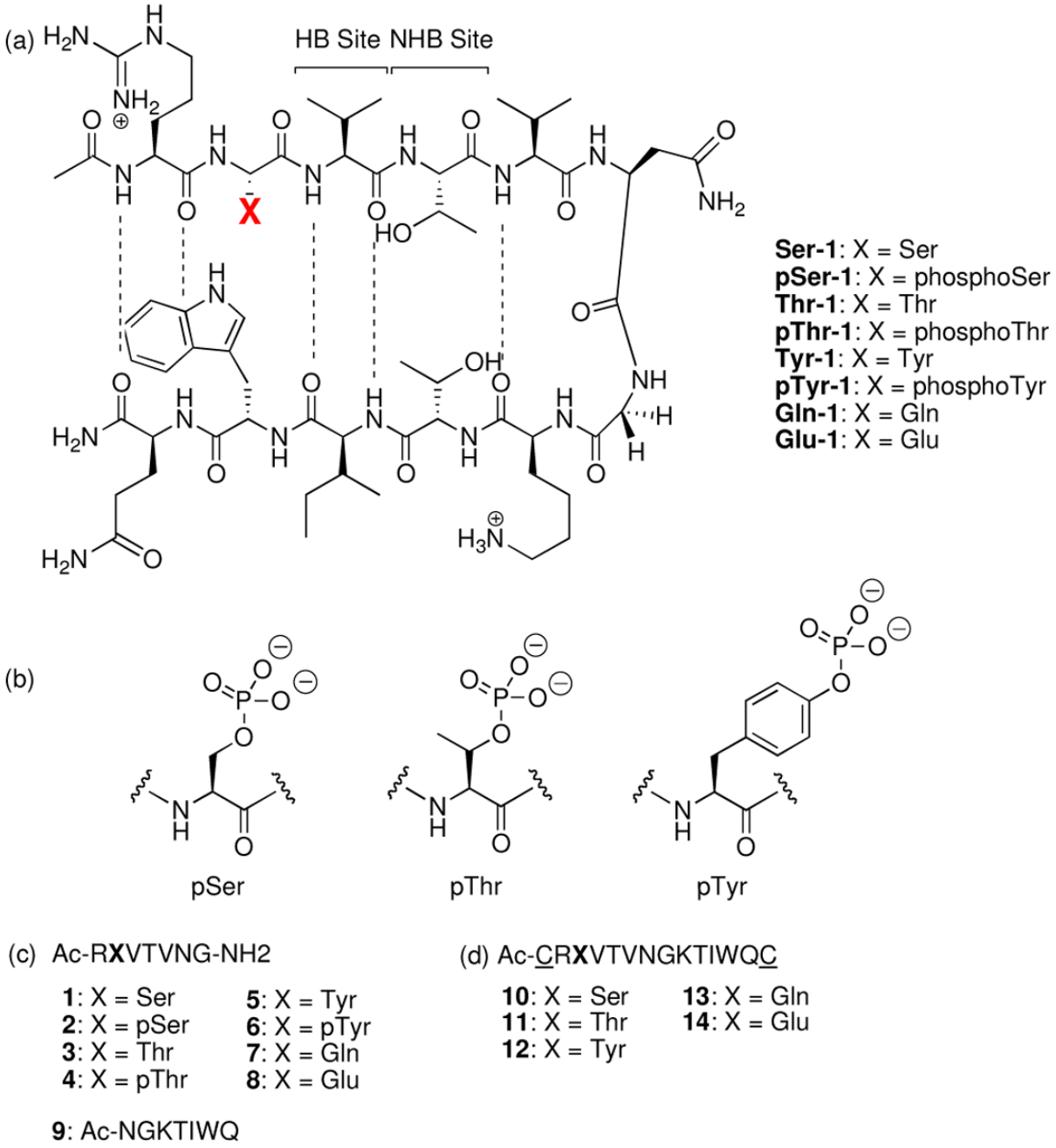


Figure 1.

(a) Schematic diagram of the designed β-hairpin peptides. Interstrand hydrogen bonding and relative orientation of the side chains are indicated. (b) Structure of the phosphorylated amino acids. (c) Sequences of unstructured control peptides. (d) Sequences of the cyclic control peptides for the fully folded state. The underline residues form a disulfide bond.

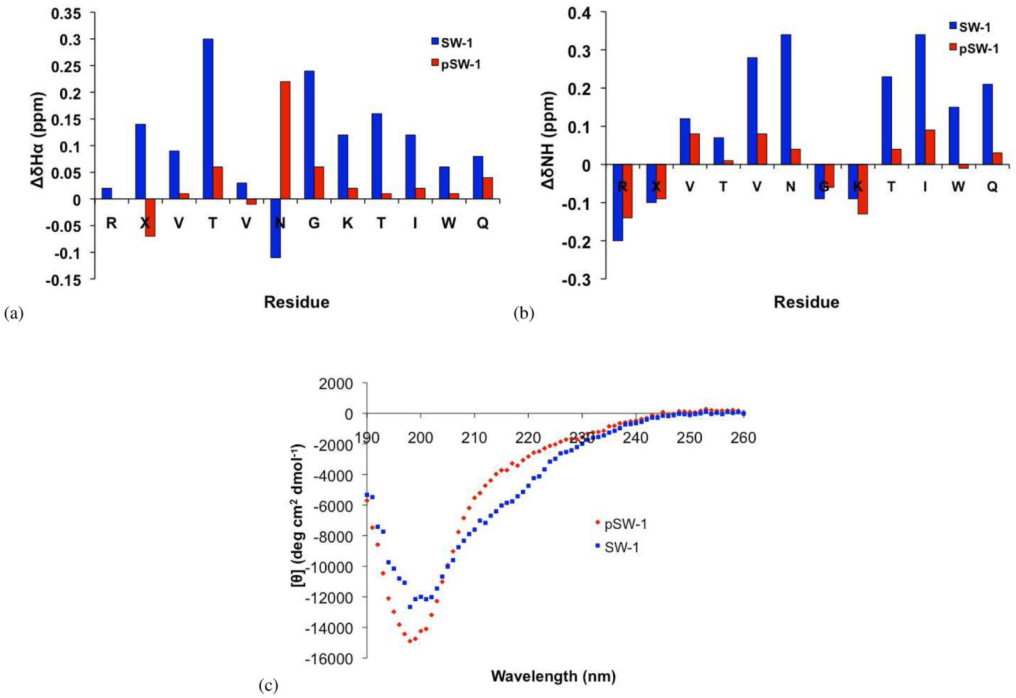


Figure 2.

(a) Hα chemical shift differences: **SW-1** (blue bars, X = serine) and **pSW-1** (red bars, X = phosphoserine) from random coil peptides in pD 7.0 buffer. The Gly bars reflect the Hα separation in the hairpin. (b) Backbone amide chemical shifts of **SW-1** and **pSW-1**. (c) Circular dichroism spectra comparison of **SW-1** (blue) and **pSW-1** (red) at 25°C in 10 mM sodium phosphate pH 7.0 buffer.

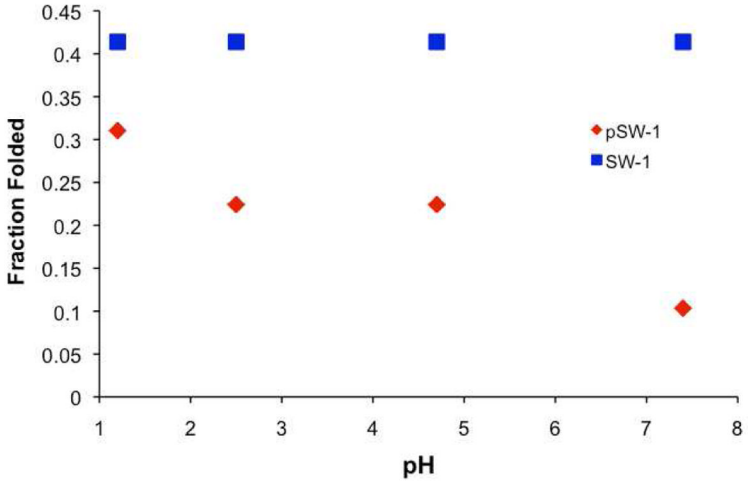
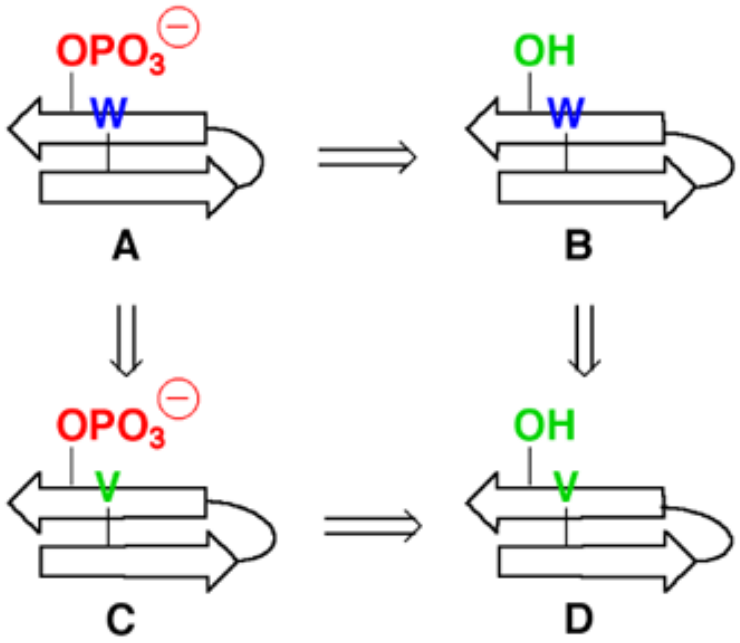


Figure 3.
pH study of fraction folded of **SW-1** (blue) and **pSW-1** (red). Fraction folded was determined by NMR glycine splitting in buffers with the pD indicated at 20 C.



$$\Delta G_A - \Delta G_B - \Delta G_C + \Delta G_D = \Delta\Delta G_{(pX\text{-}W)}$$

Figure 4.
Double-mutant cycle diagram for interaction between cross-strand phosphoserine and tryptophan. The cross-strand interaction between phosphoserine and tryptophan is determined by subtracting the stability of *B* and *C* from *A* and *D*.

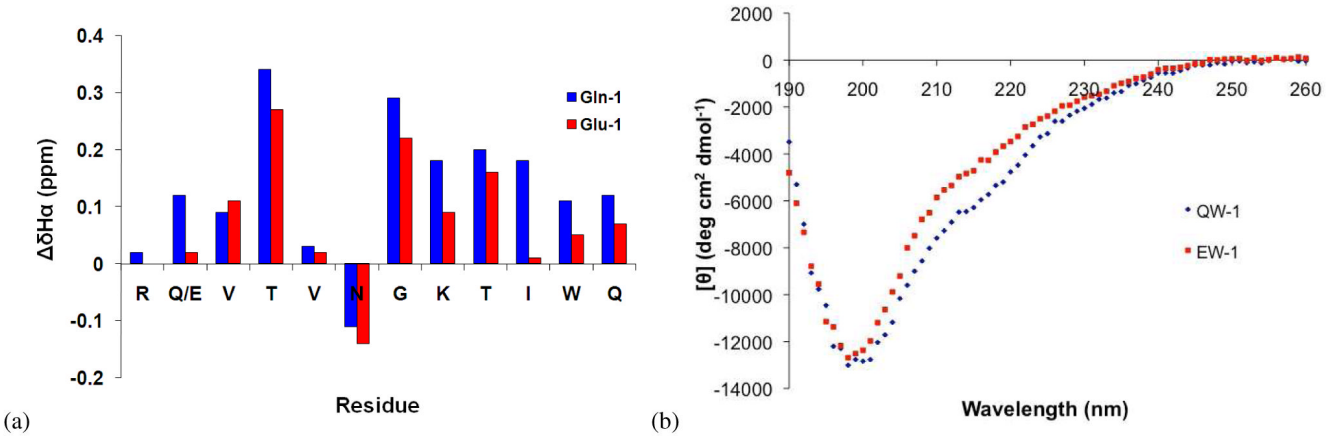


Figure 5.

(a) Hα chemical shift differences: **QW-1** (blue bars) and **EW-1** (red bars) from random coil peptides. Values calculated from data obtained at 25 °C, 50 mM potassium phosphate-*d2*, pD 7.0 The Gly bars reflect the Hα separation in the hairpin. (b) Circular dichroism spectra comparison of **SW-1** (blue) and **pSW-1** (Red) at 25°C in 10 mM sodium phosphate pH 7.0 buffer.

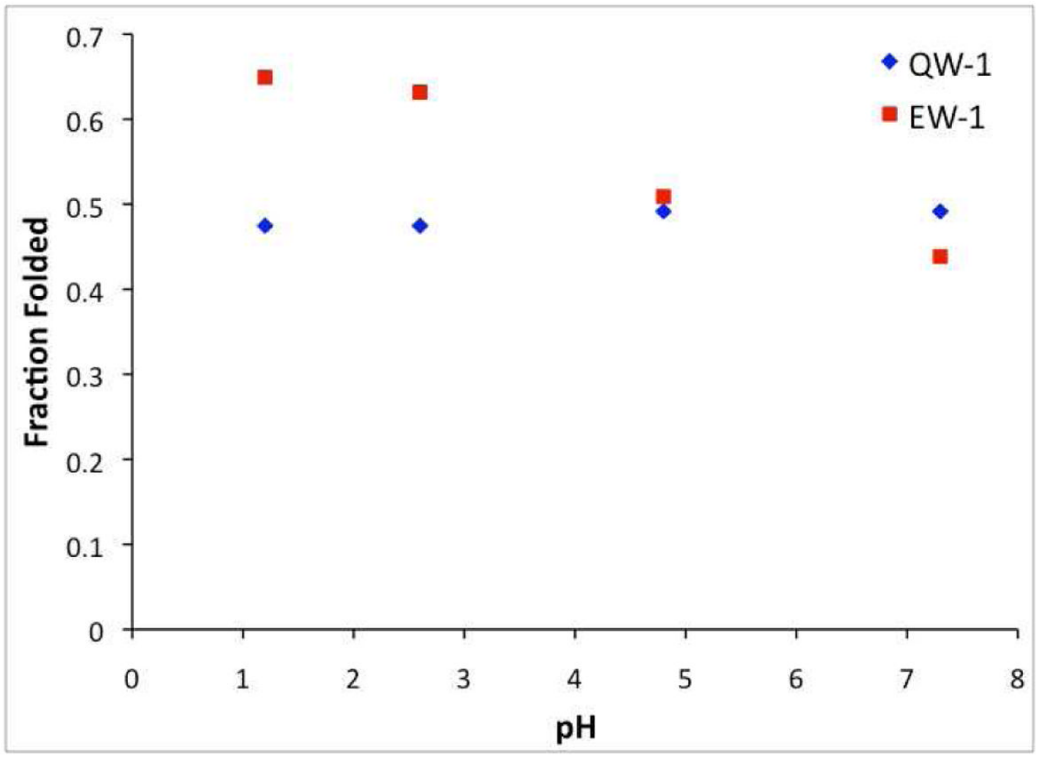


Figure 6.
pH study of fraction folded of **QW-1** (blue) and **EW-1** (red). Fraction folded was determined by NMR glycine splitting in buffers with the pH indicated at 20 C.

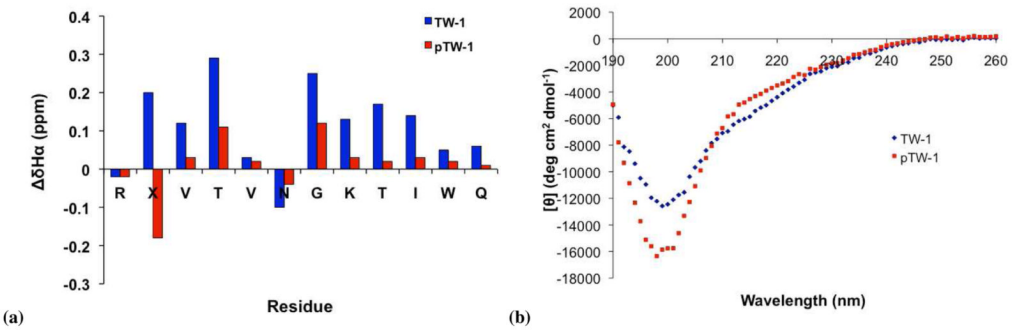


Figure 7.

(a) Hα chemical shift differences: **TW-1** (blue bars) and **pTW-1** (red bars) from random coil peptides. Residue X is either threonine(**TW-1)** or phosphothreonine (**pTW-1)**. Values calculated from data obtained at 25 °C, 50 mM potassium phosphate-*d2*, pD 7.0 The Gly bars reflect the Hα separation in the hairpin**. (b)** Circular dichroism spectra comparison of **TW-1** (blue) and **pTW-1** (red) at 25°C in 10mM sodium phosphate pH 7.0 buffer.

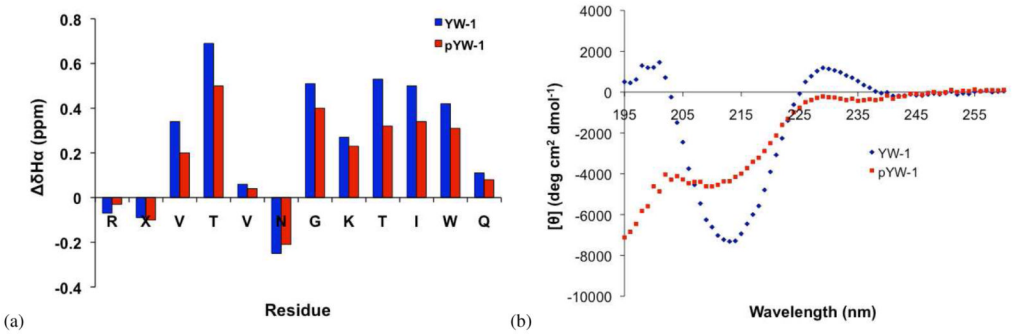


Figure 8.

(a) Hα chemical shift differences: **YW-1** (blue bars) and **pYW-1-1** (red bars) from random coil peptides. Residue X is either tyrosine (**YW-1)** or phosphotyrosine (**pYW-1)**. Values calculated from data obtained at 25 °C, 50 mM potassium phosphate-*d2*, pD 7.0 The Gly bars reflect the Hα separation in the hairpin. (b) Circular dichroism spectra of **YW-1** (blue) and **pYW-1** (red) at 25°C in 10mM sodium phosphate pH 7.0 buffer.

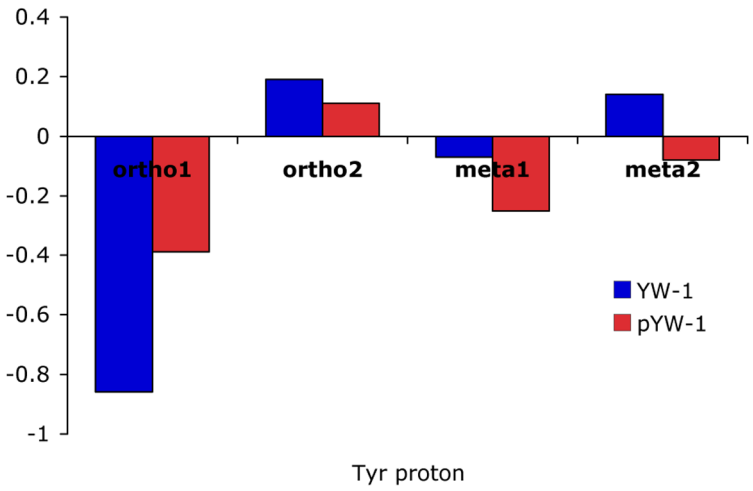


Figure 9.
Upfield shifting of Tyr protons in **YW-1** and **pYW-1** relative to random coil values determined from control peptides **5** and **6**.

Table 1

Fraction folded and ΔG of folding for beta-hairpin peptides. Values calculated from data obtained at 25 °C, 50 mM potassium phosphate-*d2*, pD 7.0 (uncorrected), referenced to DSS.

Peptide	Fraction Folded (Gly Splitting)[a]	Fraction Folded (Hα)[b]	ΔG Folding (kcal/mol)	ΔΔG (pXW-1 – XW-1)
SW-1	0.41 (±0.01)	0.40 (±0.04)	0.23 (±0.05)	
pSW-1	0.10 (±0.01)	0.07 (±0.02)	1.53 (±0.05)	1.3
QW-1	0.49 (±0.01)	0.50 (±0.1)	−0.03 (±0.09)	
EW-1	0.39 (±0.01)	0.3 (±0.2)	0.28(±0.09)	0.31
TW-1	0.43 (±0.01)	0.40 (±0.06)	0.2 (±0.1)	
pTW-1	0.21 (±0.01)	0.12 (±0.06)	1.1 (±0.2)	0.9
YW-1	0.88 (±0.02)	0.82 (±0.07)	−0.90(±0.04)	
pYW-1	0.62 (±0.02)	0.5 (±0.3)	0.1 (±0.2)	1.0

[a] Error determined by chemical shift accuracy on NMR spectrometer.

[b] Average of the Hα values from Val3, Val5, Orn8, and Ile10. The standard deviation is in parentheses.

Table 2

Double Mutant Cycle Data for **pSW-1** and mutants at pH 4.[a]

	Peptide	Fraction Folded[b]	ΔG_f (kcal/mol)	$\Delta\Delta G_{(pXW)}$ (kcal/mol)
A	**pSW-1**	0.23 (±0.01)	0.74 (±0.05)	
B	**SW-1**	0.41 (±0.01)	0.21 (±0.05)	
C	**pSV-1**	0.22 (±0.02)	0.75 (±0.05)	
D	**SV-1**	0.22 (±0.02)	0.75 (±0.05)	
				0.53

[a] Conditions: 20 °C, 50 mM sodium acetate-*d*, pD 4.0.

[b] ΔG of folding was calculated from the fraction folded using equation 3. Error determined by chemical shift accuracy on NMR spectrometer.

Table 3

Double mutant Cycle data for **pYW-1** and mutants at pH 4.[a]

	Peptide	Fraction Folded[b]	ΔG_f (kcal mol^{-1})	$\Delta\Delta G_{(pXW)}$ (kcal mol^{-1})
A	**pYW-1**	0.72 (±0.01)	−0.56 (±0.05)	
B	**YW-1**	0.88 (±0.01)	−1.16 (±0.05)	
C	**PTyrV-1**	0.33 (±0.01)	0.42 (±0.05)	
D	**TyrV-1**	0.40 (±0.01)	0.24 (±0.05)	
				0.42

[a] Conditions: 20 °C, 50 mM sodium acetate-*d*, pD 4.0 (uncorrected).

[b] ΔG of folding was calculated from the fraction folded using equation 3. Error determined by chemical shift accuracy on NMR spectrometer.