Published in final edited form as:

Angew Chem Int Ed Engl. 2017 June 12; 56(25): 7074–7077. doi:10.1002/anie.201700860.

A pH Switch for β -Sheet Folding

Jordan M. Anderson and Niels H. Andersen

University of Washington, Seattle, WA 98105

Abstract

Protein design advancements have led to biotechnological strategies based on more stable and more specific structures. Herein we present a 6-residue sequence (HPATGK) that acts as a stable structure-nucleating turn at physiological and higher pH but is notably unfavorable for chain direction reversal at low pH. When placed into the turn of a beta-sheet, this leads to a pH switch of folding. Using a standard 3-stranded β -sheet model, the WW domain, it was found that the pH switch sequence insertion caused minimal change at pH 8 but a $\sim 50^\circ\text{C}$ drop in the melting temperature (T_m) was observed at pH 2.5: $G_F = >11.3\text{ kJ/mol}$. Using the strategies demonstrated in this paper, the redesign of β -sheets to contain a global, or local, pH dependent conformational switch should be possible.

pH Switch

With biotechnology looking for more ways to control the conformation of proteins, we present a simple mutation to create a pH switchable β -sheet. Using a six-residue sequence in the turn nucleating hairpin of a β -sheet, global folding can be controlled. With the sheet being fully folded at physiologic pH and unfolded at low pH (pH 2.5).

Keywords

Conformational Switch; β -Sheets; pH Switch; Protein Design; Protein Folding

Main Text

More precise control of the active folded structure of a protein is the basis of many bioengineering strategies, typically either by controlling the equilibrium between folded and unfolded or by effecting a specific change in structure. Conformational switches give the scientist the ability to design a protein that can be reversibly turned from an inactive structure to an active state. These structural switches have found possible applications including hydrogels^[1–3], biosensors^[4,5] and even drug delivery^[6]. Much of this relies on using natural structures that are controlled by post-translational modifications^[7], photoactive cofactors^[8,9], or binding to metal ions^[10,11], small molecules^[12] or even other proteins and peptides^[13,14]. These solutions can require drastic redesign or insertion of relatively large protein domains^[15,16], or protein backbone modifications^[17] that are not amenable to protein synthesis by standard over-expression methods. Herein we describe a β -turn pH switch that uses a sequence of six canonical amino acids, which at low pH can destabilize a β -sheet, or specific region, while having notably high stability at physiological or higher pHs.

Using specific sequences of amino acids, the conformational space of the backbone, in conjunction with a collection of H-bonds can cause a kink, reversing backbone direction. The chain direction reversal often occurs at a β -turn locus. While β -turns are best known in hairpins, there are several common turn sequences^[18,19] that are not consistent with β -hairpin formation. In our view, nascent hairpins are structural motifs that can still form when excised from the protein context and typically have stable β -turns. These have been found to be present at the point of fold nucleation for numerous proteins; the N-terminal hairpin of ubiquitin^[20,21] being a notable example. Thus, the control of nascent hairpin formation can serve to control the folding of complete protein structures. In this report, we use a small protein, the WW domain. This three-stranded sheet has both a nucleating and non-nucleating hairpin and is a common motif in studies of the folding of β -sheets^[22], including numerous examples of the effects of turn replacements.^[23]

Herein, we demonstrate such structural switching with a [4:6] β -turn sequence, HPATGK, that is highly structured and stable at basic pH and unstructured at acidic pH. When this sequence is placed into the nucleating hairpin of a β -sheet it can be used to switch the folding of a protein on and off. The use of all natural L-amino acids, means it is also possible to easily incorporate the sequence into proteins synthesized by over-expression not only by solid phase peptide synthesis (SPPS). This recently discovered pH switch was found during a mutational study on the site specific amino acid requirements of different types of turns in β -hairpins. In a reference β -hairpin system, NMR studies indicated that the mole fraction of the folded state went from $\chi_F = 0.90$ at pH 8 to < 0.1 at pH 2.5^[24]: $G_F = 11$ kJ/mol.

Methods

All peptides were prepared using standard, microwave assisted, Fmoc-SPPS (CEM Liberty Blue synthesizer). CD samples were obtained by diluting stock solutions of UV verified (Trp and Tyr $\epsilon_{280} = 5690$ and $1280 \text{ M}^{-1} \text{ cm}^{-1}$ respectively) concentrations to a $30 \mu\text{M}$

polypeptide concentration in 20 mM potassium phosphate buffer (KPhos). CD spectra were taken from 5–95 °C, at 10 °C intervals. Melting temperatures (T_m , the temperature at which molar fraction of the folded state (χ_F) is 0.50) were calculated by monitoring the maximum of the Trp/Trp exciton couplet at 228 nm. Additional information and spectra can be found in the supporting information. The T_m can be determined with greater precision than individual χ_F values since there appears to be some variance in the $[\theta]_{228}$ value associated with the fully-folded state and its temperature dependence.

NMR samples were ~1 mM in 20 mM KPhos with 10% D₂O as a lock signal and 0.1 mM 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS) as an internal standard. NMR ¹H chemical shift assignments were made using the usual combination of intra-residue connectivities from TOCSY (dipsi2esgpph) spectra and vicinal (and cross-strand) backbone connectivities from NOESY (noesyegpph) spectra. Chemical shift deviations (CSDs, defined as the observed signal – random coil value) were calculated using an in-house algorithm (found at <http://andersenlab.chem.washington.edu/CSDb/>). The CSDs of diagnostic sites were used to calculate an alternative set χ_F values at 300K; these were calculated by dividing the CSDs by a value that represents 100% folded. More specifically, for the E4 H_N, W7 H_ε3, Y15 H_N, Y16 H_α, Y16 H_N, Y17 H_N, N18 H_β3, K23 H_N, R24 H_α, P29 H_γ3 and W26 H_ε3 sites, the CSDs in the control WW domain (the **Cntrl** sequence in panel A of Figure 1) at 280K were taken as representative of the 99% folded CSDs. More information of what interactions cause each diagnostic CSD can be found in the supporting information. Mole fraction in the folded state (χ_F) was calculated by $\chi_F = (\text{CSD}_{\text{observed}} / \text{CSD}_{\text{Cntrl}}) * 0.99$, which can then be converted into $G_F = -RT * \ln(\chi_F / (1 - \chi_F))$.

Results and Discussion

The WW domain is a three-stranded β -sheet that is commonly used for folding studies due to its small size, ease of synthesis, and the ease of measuring its folding dynamics. It is known that turn 1 (T1) is the nucleating turn of this sheet^[25]. The WW_{st29}^[25] system was designed in-house, starting as a truncated version of the wild type protein Pin1 with the inclusion of a Trp/Trp aromatic cluster flanking T1.^[25,26] The cross-strand Trp/Trp cluster also reports the stability of the overall structure as the amplitude of a circular dichroic exciton couplet with maximum at 228 nm.^[27] In addition to this spectroscopic feature, the WW domain has numerous specific and well-dispersed structuring chemical shifts that can be quantitated by proton NMR. These are reported as chemical shift deviations (CSDs). These backbone H_N and H_α CSDs confirm that all the peptides examined herein have a common three-stranded β -sheet structure. Additionally, there are large side chain CSDs due to structuring: upfield shifted W7 H_ε3 (–2.5 ppm), N18 H_β3 (–3 ppm), upfield shifted P29 H_γ3 (–1.8 ppm) and downfield shifted W26 H_ε3 (+0.5 ppm).

The WW domain that functions as our control (**Cntrl**) in this study has a NPATGR [4:6]- β -turn^[25] in position T1 (Sequences of each peptide are shown in Figure 1A). By CD, the measured T_m is 76.9 °C at pH 8 and 48.7 °C at pH 2.5; NMR measures of the extent of folding indicate a G_F of 2.4 kJ/mol for acidification at 300K. It is not uncommon for there to be some amount of pH dependence to WW domain fold stability, due to changes in the sidechain protonation states. Figure 1C shows the CD melting curves of **Cntrl** in black

(pH 8 and pH 2.5, solid and dashed lines respectively), even with the 28 °C difference in T_m , at both pHs the folded state is appearing to be fully populated at 5 °C. Upon replacing this turn with the minimally changed HPATGK (**p1-pHSW**), CD spectra reveal little change in the T_m (70.6 °C, Figure 1C, purple solid line), or in the χ_F measured by NMR ($\Delta G_F = -0.7$ kJ/mol, 300K), at pH 8. On the other hand, when the His sidechain of the turn is protonated (pH 2.5), peptide **p1-pHSW** becomes almost completely unfolded, with a T_m of < 5 °C and a $\sim 350,000^\circ \text{ dm}^2 \text{ mol}^{-1}$ (67 %) // CD units I always think $^\circ \text{ cm}^2 \text{ d mol}^{-1}$ /// reduction in the exciton couplet maximum in the CD at 5 °C, an acidification ΔG_F of >11.3 kJ/mol at 300 K as measured by NMR. The folded state population (χ_F) of **p1-pHSW** at 300K is 0.26 at pH 2.5; even a cursory examination of the NMR (see Figure S1) reveals an unfolded peptide rather than a protein fold - overwhelming evidence of a pH switchable β -sheet. ^1H NMR of the backbone H_N s and H_α s confirms that peptides **Cntrl** and **pHSW** (at pH 8) show the same folded structure, with all diagnostics mentioned previously (Supporting Table S2).

All our data indicates that this pH switching is the result of a single protonation of the His residue within the β -turn. CD spectra were collected at multiple pHs. A plot of $[\theta]_{228}$ at 25 °C (Supporting Figure S7), displays an inflection point at pH ~ 4.8 on the acid side of the standard pKa of the His sidechain. The conformational switching mechanism, from enhanced to decreased stability upon protonation, is likely the cause of the shift in the apparent His pKa to a more acidic than usual value.

This pH switch design works well because the first hairpin ($\beta 1$ -T1- $\beta 2$) is the fold-nucleating hairpin for this WW domain. When a non-nucleating hairpin is disrupted in a β -sheet, it could have an effect of only destabilizing that specific region of a structure. This could possibly be used to switch on/off a certain region of a larger structure while maintaining the folded state of the rest of the protein.

In this type of WW domain, structuring at T2 is known to not be necessary for sheet nucleation, this is even more the case in the version used here. When the pH switch β -turn is placed into T2 of the WW domain (**p2-pHSW**), there is little to no change at high pH by both CD and NMR. Both the T_m (80.8 °C) and χ_F (0.96, 300K), reveal a stable folded state essentially unchanged from that of the control sequence.

At low pH, there is a reduction ($\sim 190,000^\circ$, 33 %) in the magnitude of the CD exciton maximum; this is a much smaller loss than was observed with **p1-pHSW** (see Fig. 1 panel C) and is attributed to a drop in the stability of the global fold due to undocking of the third β -strand. By NMR, it is obvious that there is a change in the structure. Figure 2 shows the H_N CSDs for **Cntrl** and **p2-pHSW** at pH 8 and 2.5. Although **p2-pHSW** shows the same well folded WW domain structure at pH 8, at pH 2.5 only hairpin 1 ($\beta 1$ -T1- $\beta 2$) remains well-structured with no significant structuring shifts evident for residues in T2 or $\beta 3$. If the NMR diagnostics are separated by β -strand, at pH 8 all strands have fraction folded values of $\chi_F = 0.99 - 0.96$, whereas at pH 2.5 $\chi_F = 0.75, 0.74$ and 0.14 for β -strands $\beta 1, \beta 2$ and $\beta 3$, respectively. Since T2 is placed in the non-nucleating position, acidification only disrupts $\beta 3$. Both turns in our control WW domain system are [4:6]-hairpins in their most native version. To test whether this pH-switching strategy is limited to structures with a fold-nucleating [4:6]-hairpin turn, we placed the HPATGK sequence into longer connecting

loops. This was accomplished within the current structural motif by increasing the sequence length of the connector between $\beta 1$ and $\beta 2$. Specifically, we used glycines to increase the loop length. Two examples are shown in Figure 3, with either 2 or 4 glycines (**G2_pHSW** and **G4_pHSW** respectively) on either side of the β -turn. In these circumstances the pH switch sequence, when at a turn-favoring pH, acts to reverse the backbone direction favoring a structured motif and decreasing the entropy of the unfolded state of the loop. Although, there is a gradual decrease in the mole fraction folded of the β -sheet as the loop is elongated at pH 8, hairpin 1 retains its fold-nucleating role and significant pH switching of the WW structure remains. There is a ~ 40 °C decrease in the CD T_m for both loop insertions upon protonation (Figure 3). At pH 8, **G2_pHSW** is still fully folded at 5 °C, but with a reduction in the T_m to 51.1 °C. **G4_pHSW** at the high pH shows both a reduction in T_m and in the fraction in the folded state at 5 °C, this is expected, because of the additional segmental motion that the extra 8 flexible loop residues add to the β -strand. Figure 3, besides confirming the pH switch function of HPATGK within longer loops, also provides some insights into unstructured loop length effects in this system. The exciton couplet magnitude, which reflects WW domain formation, is larger for **G2_pHSW** and **G4_pHSW** at pH 2.5 than is observed for **pHSW**. Likely due to the added flexibility in the loop that allows the aromatic cluster to achieve a more optimal geometry, altering the maximum CD couplet. Peak broadening due to slow equilibria between folded and unfolded states complicates the NMR analyses for these systems. Although **G4_pHSW** has an extensively broadened NMR and cannot be investigated, **G2_pHSW** still shows all the diagnostics of a three-stranded sheet (Figure S4), with a χ_F of 0.87 at pH 8 and 300 K. At pH 2.5, the NMR spectra of both loop species could not be assigned.

As a design note, the addition of a turn-flanking Trp/Trp aromatic cluster provides ~ 7 kJ/mol of fold stabilization in many hairpin systems. In systems where it might also be advantageous to stabilize the folded state of a specific hairpin, the full WHPATGKW should be considered. While the added stabilization is less for some WW domains,^[25,26] a ~ 4 kJ/mol stabilization was found in the system used here. To confirm that the pH switching is not an exclusive property of the WHPATGKW, we have also examined WW domains lacking the added Trp/Trp cluster. A comparable folded state ($\chi_F=0.96$, pH 8, 300K) and pH switching was observed (see Supporting Material for details) for a mutant containing -MHPATGKV- in place of the Trp-flanked turn.

As a greater understanding of protein structure has led to biotechnological advances, taking advantage of how these structures are controlled can add a new level of precision to the engineering of protein activities. Herein we have demonstrated that a unique sequence, predominantly an Asx to His mutation within a [4:6] turn, can change the a turn within a β -sheet into a pH-dependent conformational switch. Using the HPATGK turn sequence, within the nucleating hairpin of a β -sheet, one can cause the larger structure to be unfolded at low pH but fully folded at high pH. Although this study uses a change from pH 2.5–8, the elucidation of the His pKa in the turn, 4.8 (Supporting Figure S7), shows that most of the loss in fold stability occurs over the 3 – 6.5 pH range. This has high physiological significance. For instance, the difference in pH from the lower stomach to the small intestine is from ~ 2.5 to ~ 7 respectively.^[28] If a classic [4:6] turn is not tolerated at the position of

mutation, it was found that adding two or four glycines on either side (**G2_pHSW** and **G4_pHSW**) provides 10 – 14 residue loops that still function as a pH switch, these could be used as loop and β -turn replacements in other β -sheets. Our pH dependent conformational switch is small, uses natural amino acids and operates under mild conditions. It should provide a new level of control for the structuring of β -sheet containing proteins.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

These studies were supported by grants from the University of Washington.

References

1. Schneider JP, Pochan DJ, Ozbas B, Rajagopal K, Pakstis L, Kretsinger J. J Am Chem Soc. 2002; 124:15030–15037. [PubMed: 12475347]
2. Ozbas B, Kretsinger J, Rajagopal K, Schneider JP, Pochan DJ. Macromolecules. 2004; 37:7331–7337.
3. Haines LA, Rajagopal K, Ozbas B, Salick DA, Pochan DJ, Schneider JP. J Am Chem Soc. 2005; 127:17025–17029. [PubMed: 16316249]
4. Vallée-Bélisle A, Plaxco KW. Curr Opin Struct Biol. 2010; 20:518–526. [PubMed: 20627702]
5. Frommer WB, Davidson MW, Campbell RE. Chem Soc Rev. 2009; 38:2833–41. [PubMed: 19771330]
6. Reja RM, Khan M, Singh SK, Misra R, Shiras A, Gopi HN. Nanoscale. 2016; 8:5139–5145. [PubMed: 26876788]
7. Bah A, Vernon RM, Siddiqui Z, Krzeminski M, Muhandiram R, Zhao C, Sonenberg N, Kay LE, Forman-Kay JD. Nature. 2015; 519:106–9. [PubMed: 25533957]
8. Harper SM, Neil LC, Gardner KH. Science. 2003; 301:1541–1544. [PubMed: 12970567]
9. Fan HY, Morgan SA, Brechun KE, Chen YY, Jaikaran ASI, Woolley GA. Biochemistry. 2011; 50:1226–1237. [PubMed: 21214273]
10. Ambroggio XI, Kuhlman B. J Am Chem Soc. 2006; 128:1154–1161. [PubMed: 16433531]
11. Cerasoli E, Sharpe BK, Woolfson DN. J Am Chem Soc. 2005; 127:15008–15009. [PubMed: 16248623]
12. Dagliyan O, Shirvanyants D, Karginov AV, Ding F, Fee L, Chandrasekaran SN, Freisinger CM, Smolen GA, Huttenlocher A, Hahn KM, et al. Proc Natl Acad Sci. 2013; 110:6800–6804. [PubMed: 23569285]
13. Magliery TJ, Wilson CGM, Pan W, Mishler D, Ghosh I, Hamilton AD, Regan L. J Am Chem Soc. 2005; 127:146–157. [PubMed: 15631464]
14. Johnsson N, Varshavsky A. Proc Natl Acad Sci U S A. 1994; 91:10340–4. [PubMed: 7937952]
15. Radley TL, Markowska AI, Bettinger BT, Ha JH, Loh SN. J Mol Biol. 2003; 332:529–536. [PubMed: 12963365]
16. Cutler TA, Mills BM, Lubin DJ, Chong LT, Loh SN. J Mol Biol. 2009; 386:854–868. [PubMed: 19038264]
17. Renner C, Moroder L. ChemBioChem. 2006; 7:868–878. [PubMed: 16642526]
18. Hutchinson EG, Thornton JM. Protein Sci. 1994; 3:2207–2216. [PubMed: 7756980]
19. Motta A, Reches M, Pappalardo L, Andreotti G, Gazit E. Biochemistry. 2005; 44:14170–14178. [PubMed: 16245933]
20. Searle MS, Williams DH, Packman LC. Nat Struct Biol. 1995; 2:999–1006. [PubMed: 7583674]

21. Bofill R, Simpson ER, Platt GW, Crespo MD, Searle MS. J Mol Biol. 2005; 349:205–221. [PubMed: 15876378]
22. Jäger M, Nguyen H, Crane JC, Kelly JW, Gruebele M. J Mol Biol. 2001; 311:373–393. [PubMed: 11478867]
23. Fuller, Aa, Du, D., Liu, F., Davoren, JE., Bhabha, G., Kroon, G., Case, Da, Dyson, HJ., Powers, ET., Wipf, P., et al. Proc Natl Acad Sci U S A. 2009; 106:11067–72. [PubMed: 19541614]
24. Anderson JM, Jurban B, Huggins KNL, Shcherbakov AA, Shu I, Kier B, Andersen NH. Biochemistry. 2016; 55:5537–5553. [PubMed: 27601214]
25. Kier BL, Anderson JM, Andersen NH. J Am Chem Soc. 2014; 136:741–749. [PubMed: 24350581]
26. Jäger M, Dendle M, Fuller Aa, Kelly JW. Protein Sci. 2007; 16:2306–2313. [PubMed: 17766376]
27. Arl-aryl Biopolymers 2016
28. Maurer JM, Schellekens RCA, Van Rieke HM, Wanke C, Iordanov V, Stellaard F, Wutzke KD, Dijkstra G, Van Der Zee M, Woerdenbag HJ, et al. PLoS One. 2015; 10:1–17.

Figure 1.

(A) Sequences of the WW domains, with summaries of NMR and CD data. ⁽¹⁾ NMR reveals structuring in $\beta 1$ and $\beta 2$ but not in $\beta 3$. (B) Cartoon schematic of a global and local pH switch. (C) CD spectra comparing the fold stability of **p1-pHSW**, **p2-pHSW** and **Cntrl** at both high and low pH.

Figure 2.

H_N NMR data of Cntrl and p2-pHSW as a plot of structuring shifts (CSDs) of backbone HN sites. along the entire sequence.

Figure 3.
Complete CD melts at two pH values for WW domains with HPATGK-containing loops of varying length.