

Identifying Electrostatic Interactions Controlling pH-switching in Myristoylated Hisactophilin

by

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Author's declaration

This thesis consists of material all of which I authored or co-authored: see Statement of Contributions included in the thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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Statement of Contributions

Fitting of denaturation curves was done in collaboration with Luis B.P. Socas. NMR titration experiments of the D57S, GGGG, and SGGY hisactophilin mutants were done by Luis B.P. Socas. The left panel of Figure 4.6 was made by Luis B.P. Socas.

Growth, purification and experimental characterization of the H89G hisactophilin mutant was done by undergraduate student Benjamin Emmanuel.

Abstract

Myristoyl-switching in proteins is an essential form of functional regulation that controls fundamental biological processes such as signal transduction, protein-membrane interactions, and viral infection. In this form of functional regulation, the reversible switching of a saturated C₁₄ fatty-acyl chain covalently attached to the N-terminus of a protein switches between two states: 1) a sequestered state where the myristoyl group is buried in a hydrophobic environment and 2) a state with increased solvent accessibility where the myristoyl group is available for interaction with binding partners. Myristoyl-switching controls protein function by modulating affinity for membrane and protein binding partners, depending on the accessibility of the hydrophobic myristoyl group.

Hisactophilin is a membrane binding protein found in *Dictyostelium discoideum* responsible for binding and bundling actin in a pH-dependent manner, largely driven by the reversible exposure of its myristoyl group. This protein's myristoyl switch is controlled by an intramolecular network of electrostatic-hydrophobic interactions; at low pH ~1.5 protons are bound by some of the many ionizable groups, resulting in a conformational shift where the sequestered myristoyl group is made more accessible for insertion into cellular membranes. Through a combination of implicit solvent molecular dynamics simulations and experimental methods, residues D57, H89 and H91 were hypothesized to be the residues controlling myristoyl-switching in hisactophilin. Mutation of these residues indicates that the proposed mechanism of pH-switching in hisactophilin is not fully correct. Design and experimental characterization of follow-up mutants indicates that pH-switching may be controlled through an alternative mechanism. Further investigating the molecular mechanisms of myristoyl-switching in this protein will provide valuable insight into how hydrophobic-electrostatic networks regulate function in allosteric proteins.

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Dedication

This thesis is dedicated to my mom and dad for always encouraging me to stay true to myself and pursue the meaningful things I enjoy in life.

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Chapter 1: Introduction

1.1 Protein engineering and design: the future of protein science

Proteins are one of the four fundamental biological macromolecules that facilitate the complex processes underlying life. While the self-assembling nature of phospholipids and the capacity for storage of information within strands of nucleic acids are undeniably spectacular, it is proteins that hold the most remarkable potential for advantageous applications in the everyday lives of humans. Present applications of proteins range from catalyzing industrial-scale reactions to produce valuable and fine chemicals to immunizing humans to protect against deadly infections.^{1,2} Proteins are already being used to address the challenges human society faces today, and the scope of applications for these complex molecules will continue to broaden as our understanding of the fundamental nature of proteins develops. Emerging prospects for real-world applications of proteins include, but are not limited to, biological nanomaterials and integration into renewable energy generation methods.^{3,4} Proteins hold tremendous promise for addressing many pressing challenges, but to unlock the full potential of these molecules we must continue developing our understanding of their fundamental nature to the point where protein function can be intentionally designed through thoughtful crafting of three-dimensional folds and complexes. Then, with a thorough understanding of the relationship between protein form and function, protein engineering and design can reach its full potential.

A prominent challenge in protein engineering and design is manipulation of the complex allosteric networks controlling the functions of many natural proteins.⁵ Allostery refers to the process in which binding of a ligand at one site of a protein results in structural changes at a distal site, often affecting the functional status of the protein.⁶ These binding interactions are typically transmitted between the ligand binding and distal site through subtle structural rearrangements throughout the protein structure, involving both covalent and non-covalent interactions. Much of our understanding of allosteric networks has come from the study of model protein systems possessing allosteric

properties. It is critical to continue investigating the nature of allosteric networks in a variety of model protein systems to develop a comprehensive and applicable understanding of this ubiquitous phenomenon. This thesis will use the model protein hisactophilin to investigate the fundamental structure-function relationship of electrostatic-hydrophobic allosteric communication networks in proteins.

1.2 Hisactophilin is a myristoylated membrane and actin binding protein

Fascinins are actin binding and bundling proteins involved in cell movement through reorganization of the cytoskeleton.⁷ These proteins fold into the conserved β -trefoil structure, consisting of a six-strand β -barrel capped by three β -hairpins^{8,9} (**Figure 1.1**). This fold consists of three repeated β - β - β -loop- β motifs, giving β -trefoil proteins pseudo-3-fold symmetry. Hisactophilin is a small and unusual 117 residue actin and membrane binding β -trefoil protein from the eukaryotic organism *Dictyostelium discoideum*¹⁰ (**Figure 1.1**). The small size and availability of structural and functional characterization for hisactophilin make it an attractive candidate for experimental structure-function studies, and as will be discussed below, hisactophilin's structure encodes several complex functions that are of fundamental interest.

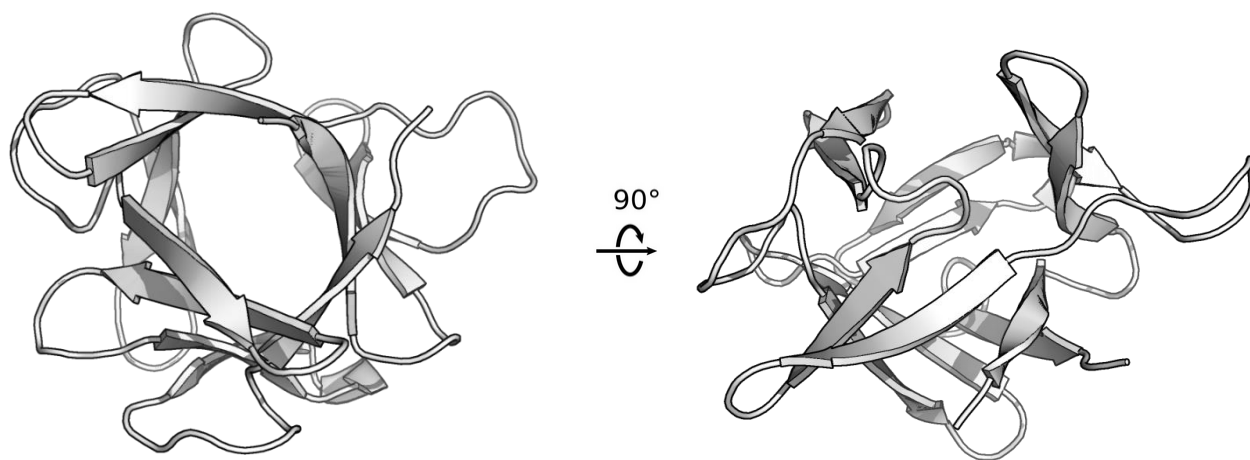


Figure 1.1: Hisactophilin is a 13.4 kDa β -trefoil protein. The β -trefoil fold is characterized by approximate 3-fold symmetry and consists of a six-strand β -barrel motif capped by a triangular array formed by 3 β -hairpins. This fold contains three repeated β - β - β -loop- β trefoil motifs.

The natural function of hisactophilin is to bind and bundle actin as part of cellular movement and to protect cells experiencing osmotic stress through fortification of the actin cytoskeleton.^{10,11} As part of its actin binding function, hisactophilin also reversibly binds to the inner leaflet of the plasma membrane, positioning the protein near to its substrate. Hisactophilin's actin and membrane binding functions are likely partially dependent on intermolecular interactions between substrates and some of hisactophilin's many charged residues, including 31 histidines (**Figure 1.2**). It is for this tremendous histidine content and affinity for actin that the protein was appropriately named hisactophilin. Considering the unusually high histidine content, it may be unsurprising to learn that hisactophilin's actin binding function is pH dependent. When intracellular pH is high, the majority of hisactophilin molecules are localized in the cytoplasm.¹² However, when intracellular pH decreases in response to external stimulus, hisactophilin localizes to the plasma membrane where it can perform its actin binding functions. To facilitate actin binding, hisactophilin binds and anchors itself to the inner leaflet of the plasma membrane, partially driven by electrostatic interactions between 10 basic residues surrounding the N-terminus and the negatively charged heads of phospholipid groups on the membrane. Like several other membrane binding proteins, however, electrostatic interactions alone are insufficient to achieve the necessary affinity for functionally relevant levels of membrane binding.^{13,14,15} An additional membrane localization signal is required for proper binding function of hisactophilin.

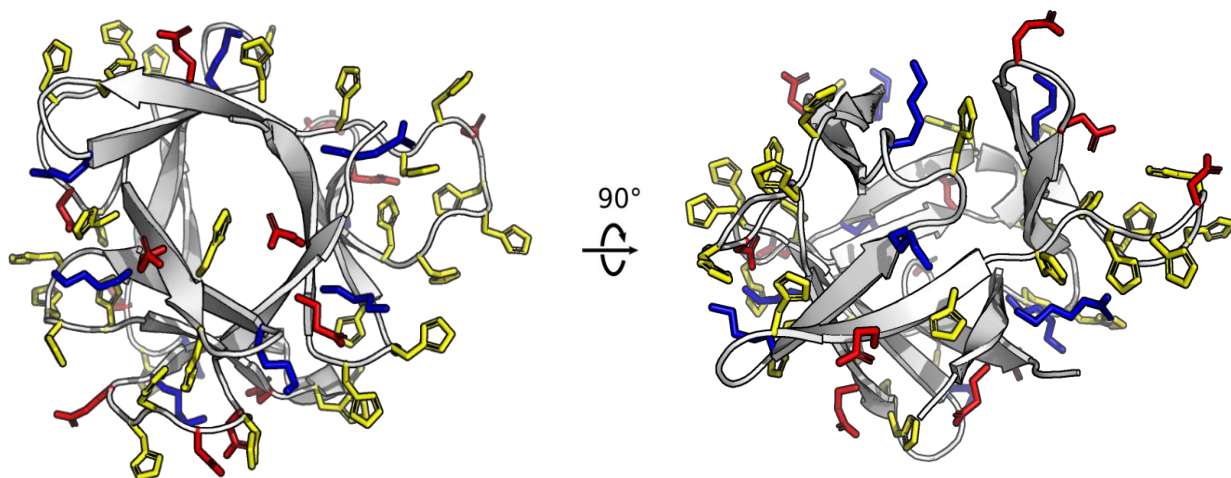


Figure 1.2: Hisactophilin has a high proportion of ionizable residues distributed throughout the protein structure. The 31 histidine sidechains are shown in yellow, 1 arginine and 7 lysine side chains are shown in blue, and 6 aspartic and 7 glutamic acid side chains are shown in red.

Hisactophilin undergoes a co-translational modification referred to as N-myristoylation. This process involves the covalent addition of a saturated C_{14} fatty acyl chain to the N-terminal glycine of the protein following excision of the preceding methionine.¹⁶ Hydrophobic interactions between the myristoyl group and the plasma membrane, in combination with electrostatic interactions between basic residues and the plasma membrane, are required for proper membrane binding by hisactophilin.¹⁷ The myristoyl group plays a crucial role not only in membrane binding, but also in the pH-dependent functional regulation of these binding interactions. Details of the myristoylation modification and the involvement of the myristoyl group in pH dependent regulation of hisactophilin's membrane binding function will be discussed in the sections to follow.

1.3 Myristoylation is a common and essential protein modification

The function of many proteins is modified by co- or post-translational modifications that impart new functional properties to the molecules they affect. Protein lipidation is one class of modifications, encompassing a variety of chemical moieties including both

saturated and unsaturated lipids of varying chain length and structure.¹⁶ The protein modification of interest in this work is N-myristoylation, which can generally be described as the covalent addition of a saturated C₁₄ fatty acyl chain to the N-terminal glycine of a protein. Myristoylation is a common modification affecting up to 2% of proteins in any eukaryotic proteome and considering that myristoylated proteins are often involved in signal transduction, it is no surprise to learn that this modification is essential for proper cellular function.¹⁸

The myristoylation reaction is catalyzed by a family of enzymes termed N-myristoyltransferases, which recognize specific sequence motifs on their substrate proteins. The details of what is sufficient for recognition by N-myristoyltransferases has been studied through a variety of approaches, initially with short peptides,¹⁹ and more recently with structural descriptions of the NMT active site.^{20,21} Based on the short peptide studies, the proposed consensus sequence for recognition by NMTs is [G¹X²X³X⁴S/T⁵X⁶X⁷X⁸], where X represents any of the 20 natural amino acids.²² However, as is often the case, exceptions to this consensus sequence are known (including hisactophilin whose N-terminal sequence is [G¹N²R³A⁴F⁵K⁶]). The structurally informed substrate recognition criteria proposed by Castrec et. al. provides a more accurate description of the importance of sequence context for recognition by NMTs.²⁰

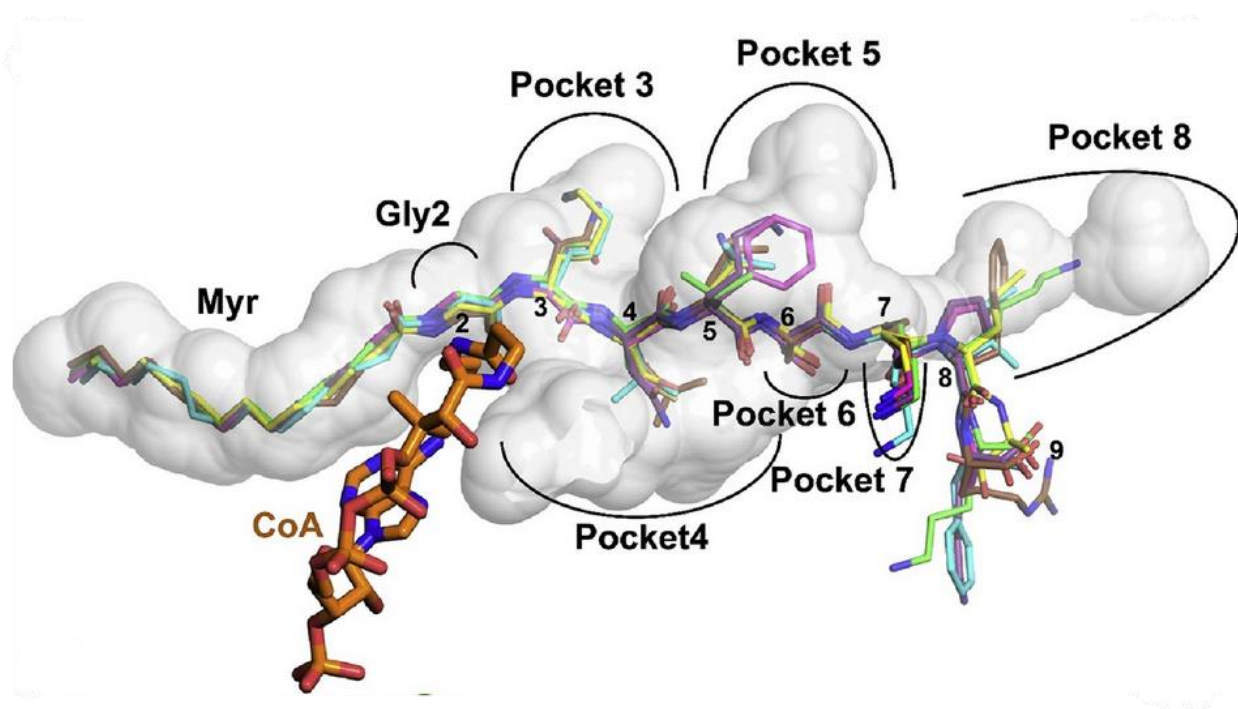


Figure 1.3: The active site of hNMT1 consists of several defined pockets involved in substrate recognition. The active site of hNMT1 co-crystallized with several peptide substrates and its substrate myristoyl-CoA is shown. The size each pocket defines the structural features of substrates that are able to be accommodated within the hNMT1 active site. Figure taken from [18].

The active site of human NMT1 consists of several defined pockets^{18,20} (**Figure 1.3**). The first pocket recognizes the linear hydrocarbon chain of the myristoyl-CoA substrate, followed by a second pocket that is involved in recognition of the N-terminal glycine residue that will be used as the site of myristoylation. The size of the third pocket gives a preference for small and medium sized residues, whereas pockets 4 and 5 are larger and can accommodate most residues. The fifth pocket is narrow and constricted, preferring smaller residues such as serine. However, as Castrec et. al. wisely points out, this set of criteria describes only global recognition patterns and cannot account for every NMT substrate known.¹⁸

Myristoylation greatly affects the physical properties of hisactophilin, influencing not only thermodynamic stability but also kinetic stability.^{23,24} Addition of a myristoyl group increases the thermodynamic stability of hisactophilin at all pHs, ranging from a stabilizing effect of 3.15 to 1.13 kcal/mol at limiting high and low pHs, respectively. While myristoylation increases thermodynamic stability of hisactophilin, it has the opposite effect on the protein's kinetic stability. Myristoylation increases the unfolding and folding rates of hisactophilin by decreasing the energy barrier between the transition state and the folded and unfolded states, respectively.²⁴ This smoothing of the folding energy landscape results from formation of non-native hydrophobic interactions between the myristoyl group and hydrophobic residues in hisactophilin.

In addition to hisactophilin, the functions of many myristoylated proteins are known. For example, recoverin is an EF hand protein found in mammalian retinal cells involved in light signal transduction whose function is dependent on its myristoyl group.²⁵ Likewise, anchoring of the HIV-1 matrix protein to host cell membranes is dependent on its hydrophobic myristoyl group.²⁶ Interestingly, despite their structural variety and diverse functions, the reversible membrane binding affinity of each of these proteins is controlled by the same fundamental process: myristoyl-switching.

1.4 Myristoyl-switching controls protein function

Myristoylation is often tightly coupled to the regulation of protein function through a process known as myristoyl-switching. In this form of function regulation, the myristoyl reversibly switches between two states: 1) a sequestered state where the myristoyl is buried in a hydrophobic area of the protein, and 2) an accessible state where the myristoyl is made more solvent accessible to facilitate interactions with binding partners (**Figure 1.4**). The function of myristoyl-switching proteins is controlled through this process by modulation of affinity for binding partners, depending on the accessibility of the myristoyl.²⁷

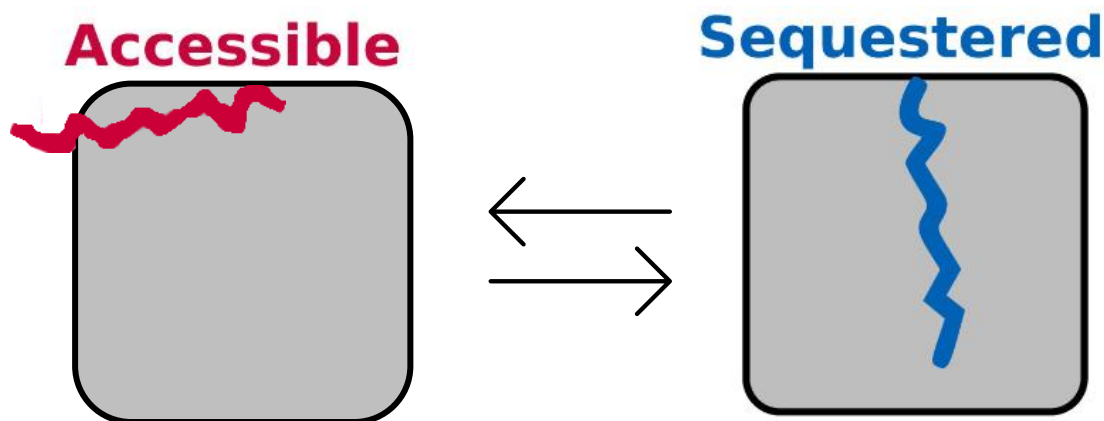


Figure 1.4: Simplified myristoyl-switching schematic. Many myristoylated proteins undergo switching, in which the myristoyl (red and blue lines) reversibly switches between a sequestered (inactive) and accessible (active) state. This process controls protein function through modulation of binding affinity depending on the accessibility of the myristoyl. Figure adapted from [29].

Switching of the myristoyl group is typically triggered by interaction with some type of effector. A variety of myristoyl-switching effectors are known: Ca^{2+} binding in recoverin²⁵, guanosine triphosphate binding in ARF1 GTPase²⁸, protein trimerization in the HIV-1 matrix protein²⁶, and pH in hisactophilin²³, among others. The H^+ binding, i.e. pH-dependent myristoyl switch of hisactophilin presents an especially interesting opportunity to investigate a fundamental question in protein chemistry: how do ionizable surface residues interact with hydrophobic core residues to control protein function? It is believed these sorts of interactions are what control switching in hisactophilin, and so this model system will be of great use in furthering understanding of the fundamental nature of electrostatic-hydrophobic networks in proteins.

1.5 Hisactophilin has a pH-dependent myristoyl-switch

The membrane and actin binding functions of hisactophilin are controlled by pH. This pH-dependent functional regulation is manifested in the protein's myristoyl switch, whereby the myristoyl reversibly switches between two different locations in the protein: 1) a sequestered state where the myristoyl is buried within a hydrophobic binding pocket in the core of the protein, and 2) an accessible state where the myristoyl has increased solvent accessibility, positioning it for insertion into the plasma membrane (**Figure 1.5**) (for the sake of variety, the terms “switching,” “pH-switching,” and “myristoyl-switching” will be used interchangeably throughout the text when referring to the reversible pH-dependent change of hisactophilin's myristoyl group between the sequestered and accessible states). Because of this switching phenomenon, the stabilization hisactophilin receives from myristoylation is highly pH-dependent (**Figure 1.6**). At pHs above the pK_{switch} of 6.95, hisactophilin molecules mainly occupy the sequestered state in which the myristoyl participates in hydrophobic interactions with many core residues.²³ However, as pH decreases below the pK_{switch} , the equilibrium shifts towards an accessible state where the myristoyl no longer occupies the core. The question that arises from these observations is this: how are pH-sensitive electrostatic interactions on the protein surface communicated to the hydrophobic myristoyl group? Significant amounts of experimental and computational work have been done to try to ascertain the molecular mechanisms of pH-switching in hisactophilin, with the purpose of deepening our understanding of the functional nature of electrostatic-hydrophobic interactions in proteins.

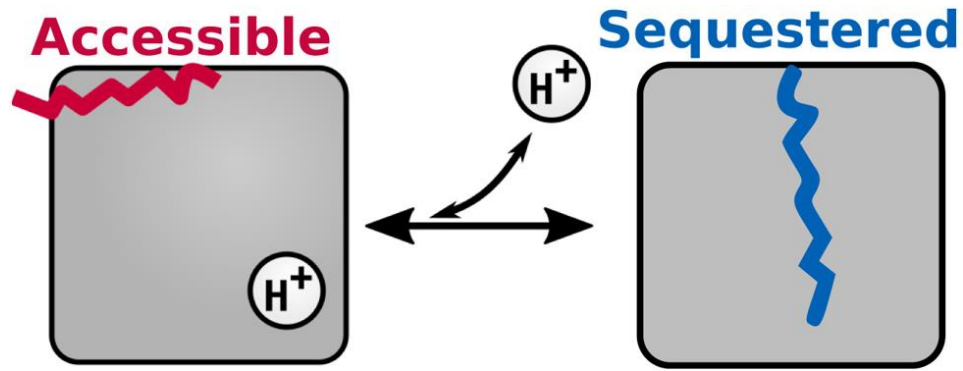


Figure 1.5: Simple pH-dependent myristoyl-switching schematic. The myristoyl (red and blue lines) reversibly switches between a sequestered state (right) and an accessible state (left) upon uptake or release of a proton. The function of hisactophilin is regulated by pH through modulation of affinity for binding partners depending on the accessibility of the myristoyl. Figure taken from [29].

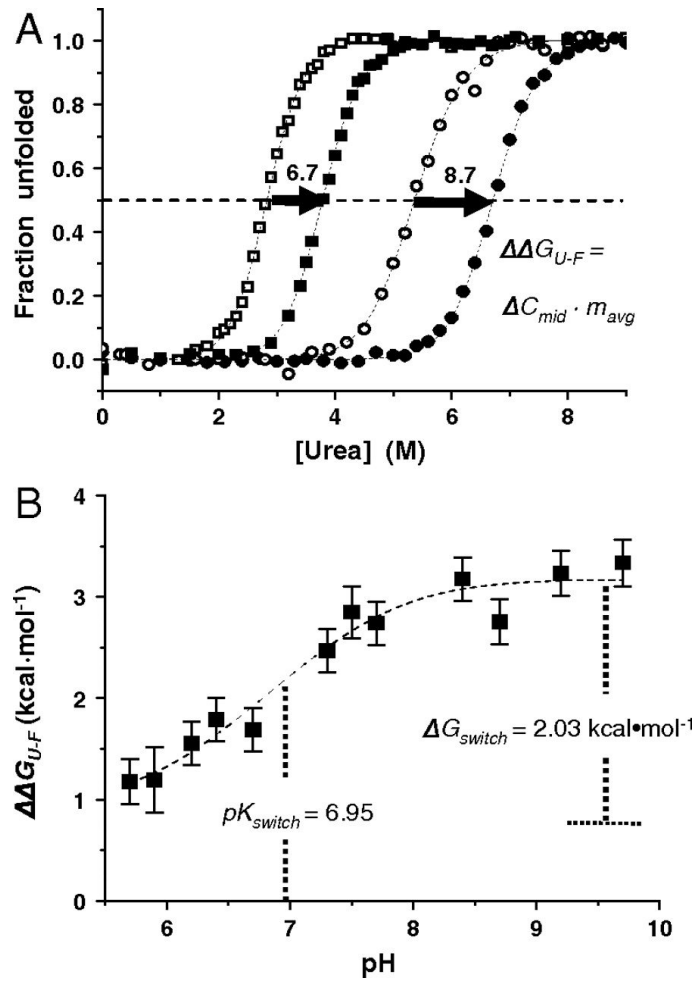


Figure 1.6: Stabilization from myristoylation is strongly pH dependent in hisactophilin.

A) Hisactophilin receives greater stabilization from myristoylation at high pH compared to low pH. Open symbols show denaturation curves of non-myristoylated wildtype hisactophilin and closed symbols denaturation of the myristoylated protein. Circles and squares indicate measurements made at pH 8.7 and 6.7, respectively. A greater increase in stabilization upon myristoylation is seen at high pH, reflecting the increased number of proteins occupying the sequestered state at high pH. B) Difference in stability between the non-myristoylated and myristoylated protein between limiting high and low pHs. Figure taken from [23].

Due to the abundant ionizable groups in hisactophilin, it is difficult to determine exactly which residues are sensing pH-changes to control switching. However, it is known that

the switch from the sequestered to the accessible state is associated with the reversible uptake of ~1.5 protons.²³ It is likely some of the 31 histidine residues spread throughout the protein's structure are responsible for proton uptake as pH decreases, suggested by the pK_{switch} of 6.95 being close to the pK_a of histidine sidechains (~6.04). Further support for the importance of histidines in controlling pH-switching is the widespread changes in $pK_{a\text{app}}$ of histidines between the sequestered and accessible state, increasing from 6-6.5 to 7-7.6 in the accessible state.²³ This increased $pK_{a\text{app}}$ of ionizable groups at low pH favours protonation and the switch to the accessible state. Despite the insight into the number of ionizable groups involved in switching, it remains to be determined which of the 31 histidine residues are critical for pH-switching in hisactophilin. However, past experimental findings highlight certain regions of the protein structure as being of potential functional importance.

1.6 A proposed pH-dependent myristoyl-switching mechanism in hisactophilin

Variable temperature nuclear magnetic resonance (NMR) studies of hisactophilin have identified regions of the protein that are likely linked to pH-switching.²⁹ Temperature coefficients are a measure of how sensitive a given nucleus' chemical shift is to changes in temperature.³⁰ Nuclei within structured regions of proteins tend to show decreased temperature dependence of chemical shift compared to nuclei within disordered regions such as loops (**Figure 1.7**). Temperature coefficients can be measured for protein variants under different conditions in a thermodynamic cycle to extract information related to a specific process under study.³¹ For example, temperature coefficients of myristoylated and non-myristoylated hisactophilin can be measured at high and low pH to identify residues that couple myristoyl movement to protonation (**Figure 1.8, top**). Performing this cycle analysis on wildtype hisactophilin identifies regions of the protein that experience increased levels of local stability in the accessible and sequestered states (**1.8, bottom**), with the most notable increase in local stability in the region spanning residues 89-95 when in the accessible state. The increased local stability in this part of the protein suggests it may experience some form of stabilization associated with switching.

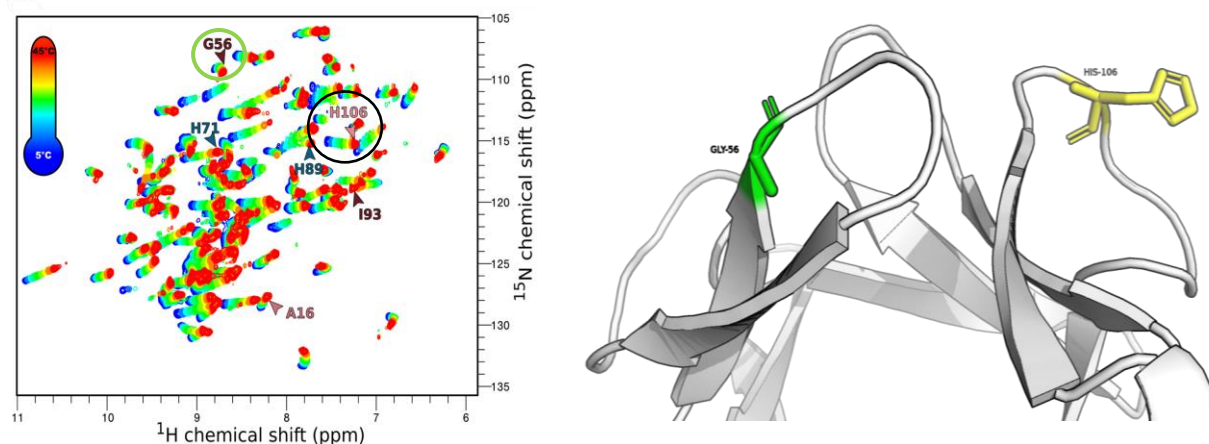


Figure 1.7: Temperature dependence of chemical shifts is determined by structural context. Left: Variable temperature ^1H - ^{15}N HSQC spectra of myristoylated hisactophilin at pH 6.2. Amides in structured regions such as beta sheets (e.g. G56) show decreased peak movement with temperature compared to amides in flexible regions such as loops (e.g. H106). Right: representative residues in structured (G56) and unstructured (H106) regions of hisactophilin are shown in green and yellow, respectively. Figure adapted from [29].



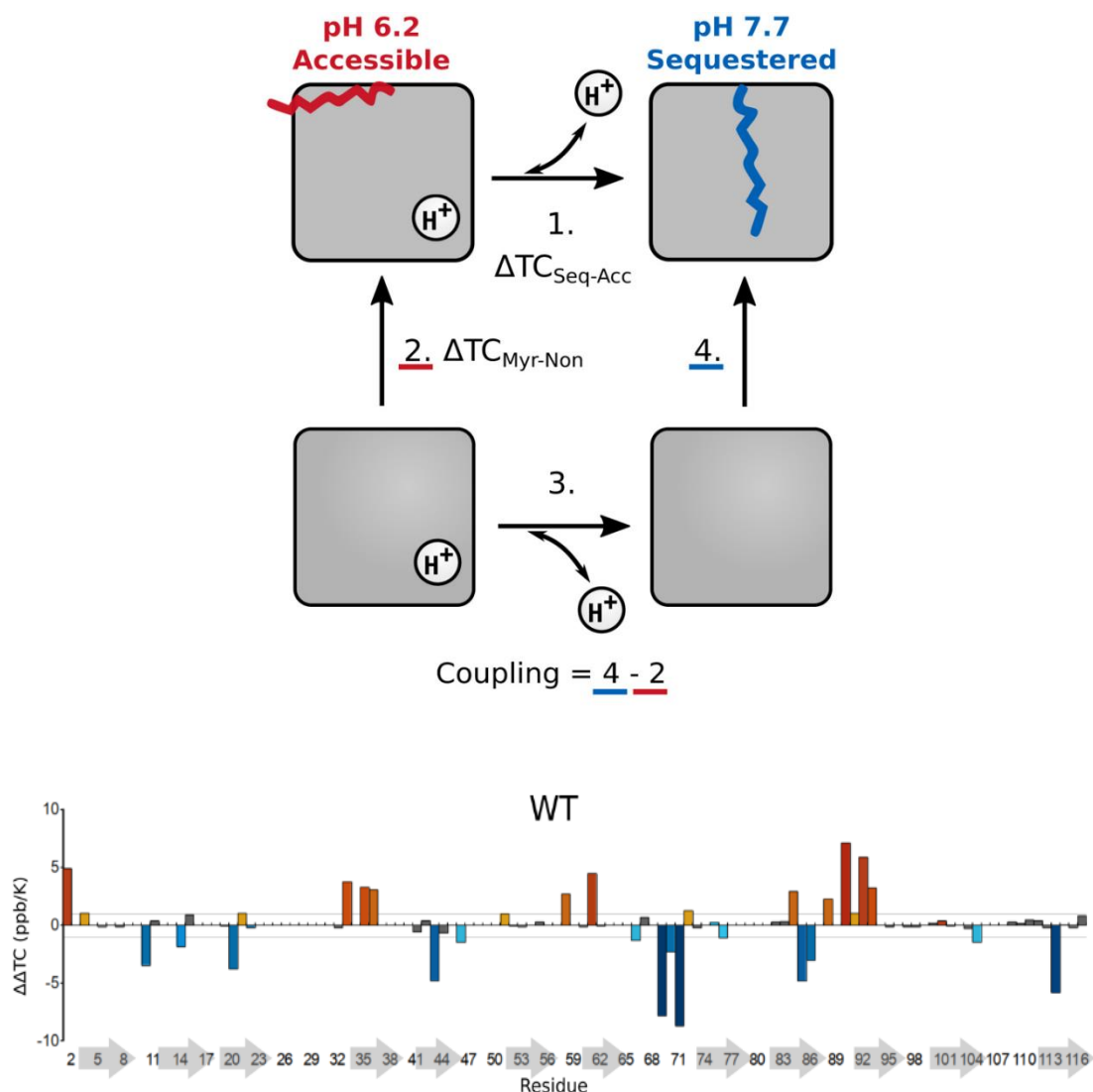


Figure 1.8: Thermodynamic cycle analysis of hisactophilin reveals regions of local stabilization associated with pH-switching. Top: thermodynamic cycle schematic. Temperature coefficients are measured for the non-myristoylated and myristoylated protein at both pH 6.2 and pH 7.7. The difference between the measured temperature coefficients of the two species at one pH ($\Delta TC_{myr-non}$) represents contribution to measured temperature coefficients coming from myristoylation at the respective pH. By taking the difference between the $\Delta TC_{myr-non}$ values at high and low pH ($\Delta\Delta TC$), residues whose temperature coefficients are affected by both myristoylation and pH changes (i.e. switching) are identified. Top: results of temperature coefficient thermodynamic cycle analysis of wildtype hisactophilin. Several regions are locally stabilized in the accessible (red) and sequestered (blue) states. Figure adapted from [29].

To complement the temperature-dependent NMR experiments on hisactophilin, constant pH molecular dynamics simulations were conducted to investigate how the structure of the protein changes from the sequestered to the accessible state.²⁹ Generalized Born with simple switching model (GBSW) simulations done in implicit solvent identified two energetic minima corresponding to the sequestered and accessible states. When comparing the frequencies of electrostatic contact formation seen in the two states, the most prominent differences are between residues D57 and H91, and to a lesser extent D57 and H89, suggesting that interactions between these residues may be of functional importance²⁹ (**Figure 1.9**).

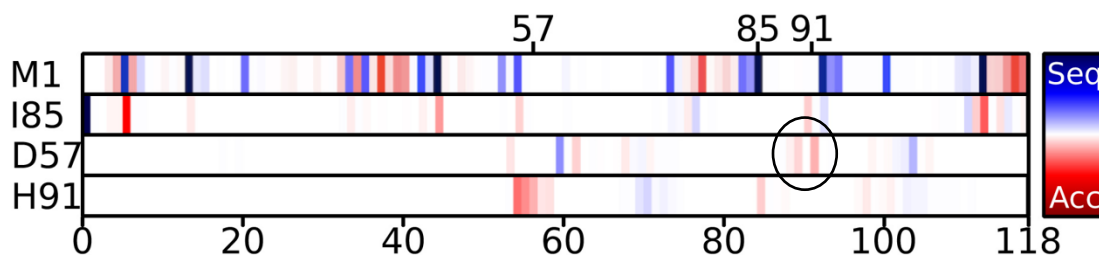


Figure 1.9: Implicit solvent GBSW simulations identify prominent pair-wise contact differences between the sequestered and accessible states in hisactophilin. Contacts observed more frequently in the sequestered and accessible states are shown in blue and red, respectively. M1 = myristoyl group. The most prominent contact differences seen in the accessible state are between D57, H91, and to a lesser extent, H89 (circled). Simulations were done using the CHARMM22 forcefield with implicit solvent constant pH molecular dynamics algorithm and an implicit solvent GBSW model. 100 20 ns replicates were performed at pH 6, 7, and 8. Figure taken from [29].

The differences in electrostatic contact frequencies between the sequestered and accessible states seen in the simulations provide a possible explanation for the temperature-dependent NMR data. Regions with increased local stability at low pH could be explained by the pH-dependent formation of favourable electrostatic interactions between an acidic sidechain and a protonated histidine imidazole group, such as D57 and

H89/H91. Additionally, it is known that the myristoyl binding pocket is highly sensitive to the contacts made by hydrophobic residues, so it is plausible that subtle rearrangement of core residues caused by movement of residues on the surface could be a factor controlling switching. Thus, a possible mechanism for pH-dependent myristoyl-switching in hisactophilin was developed²⁹ (**FIGURE 1.10**).

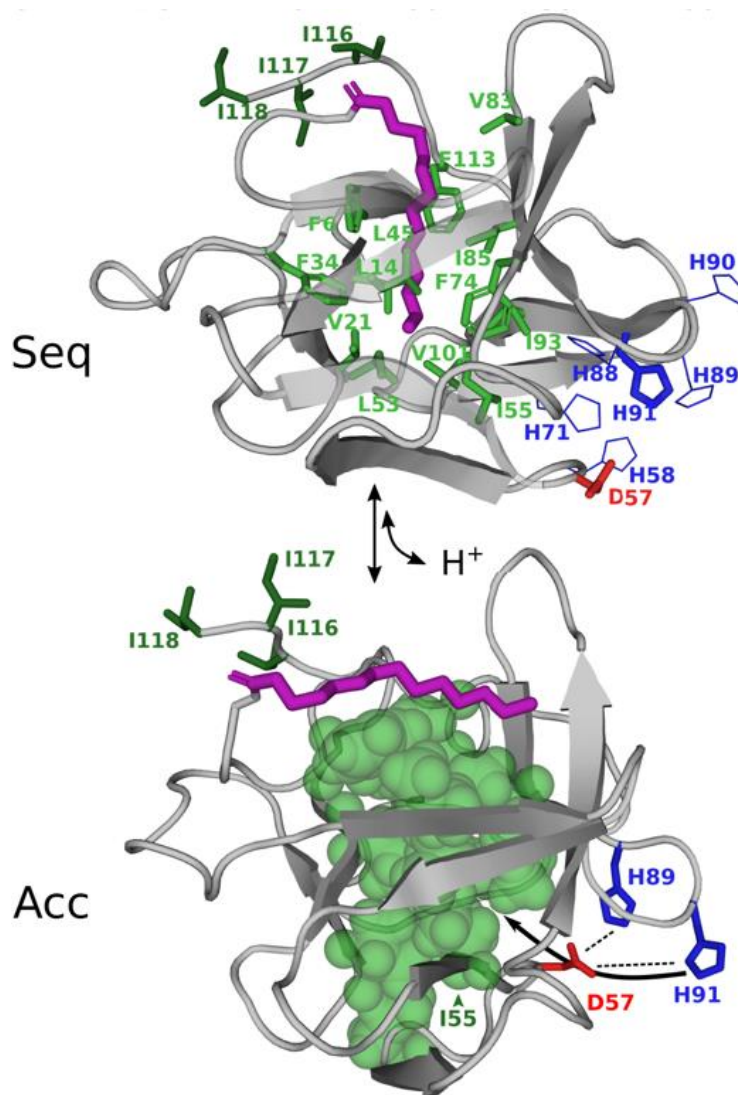


Figure 1.10: Proposed molecular mechanism of pH-dependent myristoyl-switching in hisactophilin. The myristoyl is shown in magenta and core residues in green. At high pH H89 and H91 have increased pKa values due to unfavourable electrostatic repulsion and remain neutral with the myristoyl sequestered in the core (Seq). At low pH, H89 and H91 have

decreased pKa values due to the nearby aspartic acid and become protonated to form favourable electrostatic interactions with D57. H91 is repositioned towards I55 when interacting with D57 at low pH, perturbing myristoyl binding pocket geometry. Perturbation of binding pocket geometry forces the myristoyl to exit the core and occupy the accessible state. Figure taken from [29].

This proposed mechanism for pH-switching in hisactophilin provides a testable hypothesis. The prediction is that removing D57, H89 or H91 will prevent formation of the electrostatic interactions at low pH thought to drive the switch to the accessible state. Consequently, variants of hisactophilin in which these residues are mutated should be functionally insensitive to pH changes and remain in the sequestered state. The first step in testing this hypothesis was to design a set of hisactophilin mutants suitable for expression and experimentation.

1.7 Research objectives

The objective of this research project is to experimentally test and subsequently refine a hypothesized mechanism of pH-dependent myristoyl-switching in the protein hisactophilin. This goal will be achieved through design and experimental characterization of hisactophilin mutants using a variety of techniques.

Chapter 2: Design of Hisactophilin Point Mutants to Test pH-switching Hypothesis

2.1 Considerations in the rational design process

Mutation studies are a foundational approach for probing structure-function relationships in proteins. Altering the chemical identity of any given amino acid residue through mutation and measuring how protein function is affected allows one to make inferences about the functional roles that residue may play. In hisactophilin, it is hypothesized that the formation of electrostatic interactions at low pH between residues D57, H89 and H91 are the primary force controlling pH-dependent myristoyl-switching²⁹; these three residues are an excellent example of a system where mutation studies can directly test a hypothesis to provide insight into the molecular mechanisms of protein function.

As a first step in testing the above hypothesis, an initial set of hisactophilin point mutants was designed. These mutants had to meet two general criteria: 1) the residues of interest (D57, H89, H91) must be mutated from ionizable residues to non-ionizable residues, and 2) the mutations should minimally destabilize the protein. In this way, the mutations will alter ionizable residues involved in switching while avoiding introducing other changes to the protein structure or stability. Thankfully, there is great diversity in the chemical properties of the twenty natural amino acids to choose from when making mutant protein variants. However, it is important to carefully consider which mutations one makes and how they may impact the stability and folding of the protein; if the mutant protein's structure or stability are significantly altered relative to the wildtype protein its useability in experimental procedures may be limited.

Rational design of mutant proteins involves considering a variety of information sources to guide the selection of mutant residue identities. For the design of the hisactophilin point mutants, four sources of information were considered to decrease the likelihood of selecting an unfavourable mutation:

- 1) A multiple sequence alignment of the fascin proteins to identify residue preferences at each position in hisactophilin, as highly conserved residues tend to be stabilizing (**Figure 2.1**).
- 2) Residues in structurally equivalent positions of fascins with solved structures to identify alternative residues that are known to exist in the same structural context as the residues of interest in hisactophilin (**Figure 2.2, Table 2.1, Table 2.2**).
- 3) D57, H89 and H91 are all present in β -turns in hisactophilin. D57 is residue i in a type I β -turn, and H89 and H91 are residues $i+1$ and $i+3$ in a type I' β -turn, respectively. Considering this, the preferred residues in each position for type I and type I' β -turns were taken into consideration (**Table 2.3**).
- 4) Mutant stability predictions were made with the aim of minimizing changes in protein stability coming from the mutations. The stability predictors MAESTRO³² and FoldX³³ were used for this purpose (**Table 2.4**).

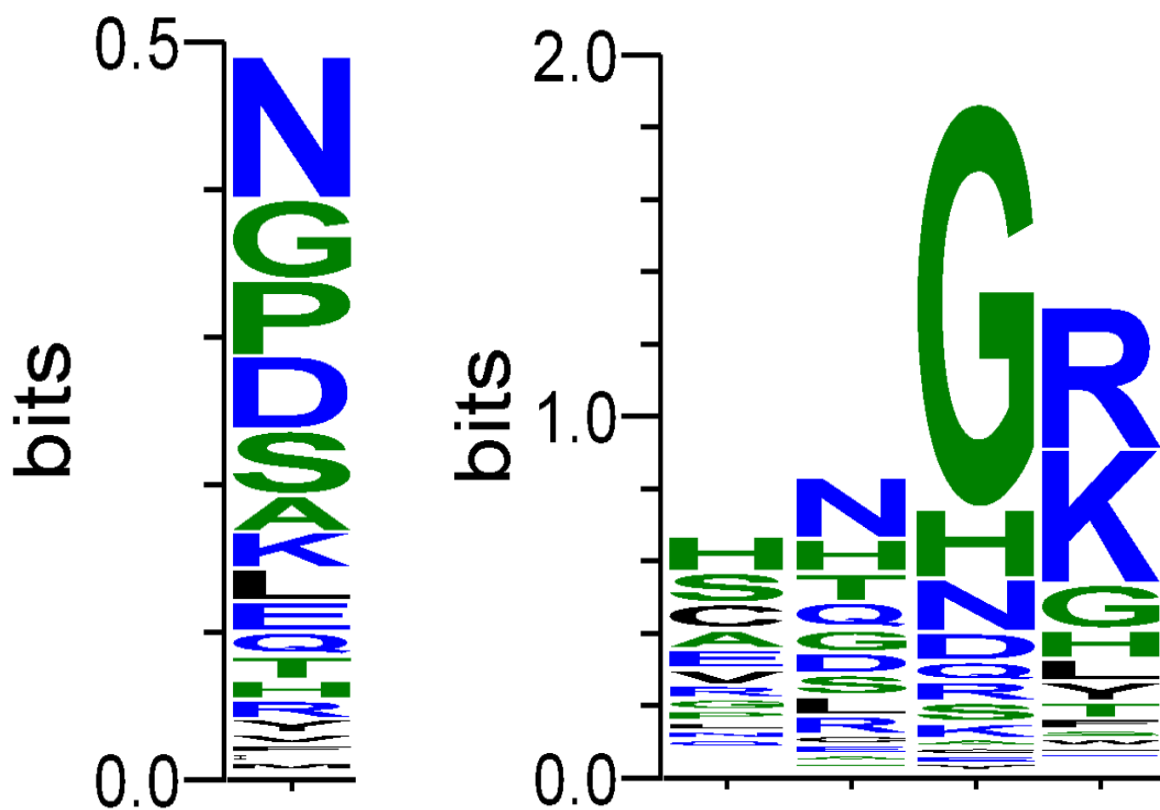


Figure 2.1: Fascin consensus logos for positions equivalent to D57 (left logo) and the β 9-10 turn consisting of H88, H89, H90 and H91 (right logo) in hisactophilin. Variable residue identity is seen in each position: upward position and relative size indicates level of representation in the multiple sequence alignment. Sequence alignment was acquired from the conserved domain database.³⁴

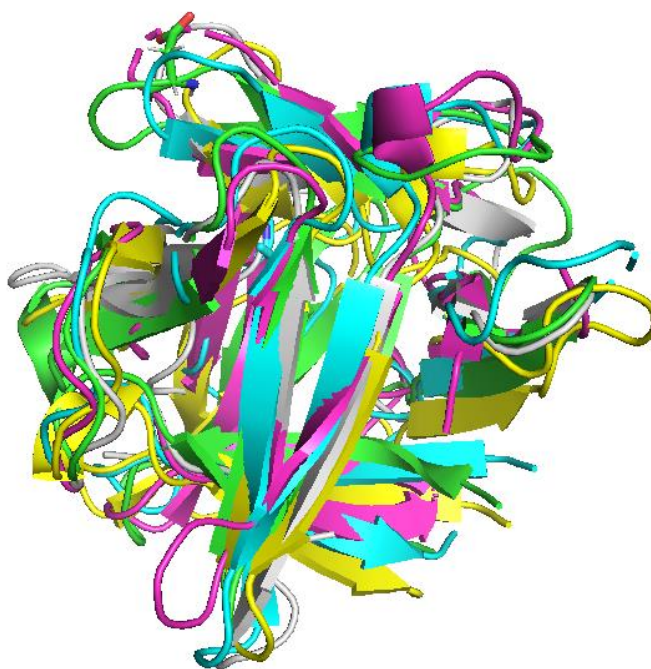


Figure 2.2: Structural alignment of hisactophilin (1HCD) with the four domains of human fascin (1DFC). The alignment was manually inspected in PyMOL to determine which residues were in positions structurally equivalent to D57, H89 and H91. D57 is residue *i* in a type I β -turn. H89 and H91 are residues *i*+1 and *i*+3 in a type I' β -turn, respectively. Summaries of the residue composition of structurally equivalent turns are shown in Tables 1 and 2.

Table 2.1: D57 structurally equivalent residues in the three β 2-3 turns in the three Trefoil repeat of each of the four subdomains of human fascin protein. Residues in position i of type I turns are indicated in purple (D57*: D57 of hisactophilin)). N/A indicates that the turn was unmodeled in the structural model and no turn classification could be assigned.

β 2-3 Turn Structural Equivalents in Symmetric Trefoil Repeats (D57)												
Protein	Turn 1				Turn 2				Turn 3			
	i	i+1	i+2	i+3	i	i+1	i+2	i+3	i	i+1	i+2	i+3
His.	E	G	E	A	D*	H	K	Q	D	H	H	G
Domain 1	N/A				D	K	D	G	N/A			
Domain 2	N/A				N/A				G	P	S	G
Domain 3	N/A				T	A	T	G	K	K	N	G
Domain 4	N/A				G	S	D	S	D	H	A	G

Table 2.2: H89 and H91 structurally equivalent residues in the three β 1-2 turns in the three Trefoil repeats of each of the four subdomains of human fascin protein. Residues in position i+1 of type I' turns are indicated in purple (H89*: H89 of hisactophilin). Residues in position i+3 of type I' turns are indicated in red (H91*: H91 of hisactophilin). N/A indicates that the turn was unmodeled in the structural model and no turn classification could be assigned.

β 1-2 Turn Structural Equivalents in Symmetric Trefoil Repeats (H89, H91)												
Protein	Turn 1				Turn 2				Turn 3			
	i	i+1	i+2	i+3	i	i+1	i+2	i+3	i	i+1	i+2	i+3
His.	H	H	G	H	H	G	G	K	H	H*	H	H*
Domain 1	N/A				N/A				N/A			
Domain 2	N/A				Q	D	Q	R	D	C	E	G
Domain 3	A	A	N	E	N/A				A	S	N	G
Domain 4	N/A				N	D	G	A	V	G	G	R

Table 2.3: Under- and over-represented residues in common β -turn types. (+): over-represented in respective β -turn type. (-): under-represented in respective β -turn type. Adapted from [35].

		i	i+1	i+2	i+3
Type I	(+)	cPghStND	PSEK	whSTNDe	wcGn
	(-)	IVLmAfyWQERK	IVLmFywcqGhtn	IVLmAPG	ivlmqPERk
Type II	(+)	qP	Pek	GN	mcqSt
	(-)	sD	ivLywcGst	IVLmAFywqPSTdERK	ilPgn
Type I'	(+)	Fst	GNd	Gn	yqr
	(-)	Q	ivpt	ivlapsterk	p
Type II'	(+)	St	G	stN	mg
	(-)	Vlafqpter	p	ivlp	N/A

Table 2.4: Mutant stability predictions obtained using the MAESTRO webserver and FoldX BuildModel function. Green and red indicate mutations predicted to be stabilizing or destabilizing, respectively.

	MAESTRO	FoldX
Mutation	$\Delta\Delta G$ (kcal/mol)	$\Delta\Delta G$ (kcal/mol)
D57N	0.243	-0.312
D57S	0.378	0.121
D57G	0.817	-1.616
D57A	0.189	0.652
H89Q	-0.539	-0.514
H89G	-0.181	-0.002
H89S	-0.798	-0.781
H89N	-0.831	-0.204
H91Q	0.692	0.016
H91G	1.466	-0.37
H91S	1.045	-1.49
H91N	0.87	0.305
H91A	1.101	-0.221
H91Y	-0.025	-0.417

Table 2.5: Summary of rational design considerations used to inform selection of D57, H89 and H91 mutations in hisactophilin. Green and red indicate favourable or unfavourable mutations based on the information being considered, respectively.

Mutation	Consensus	Structural equivalents	β -turn preference	Favoured by FoldX	Favoured by MAESTRO
D57N	✓	x	✓	✓	x
D57S	✓	x	✓	x	x
D57G	✓	✓	✓	✓	x
D57A	✓	x	x	x	x
H89Q	x	x	x	✓	✓
H89G	✓	x	✓	✓	✓
H89S	✓	x	x	✓	✓
H89N	✓	x	✓	✓	✓
H91Q	x	x	✓	x	x
H91G	✓	x	x	x	✓
H91S	✓	x	x	x	✓
H91N	x	x	x	x	x
H91A	✓	✓	x	x	✓
H91Y	✓	x	✓	✓	✓

Informed by the summary of the rational design considerations shown in **Table 2.5**, a set of four point mutants were chosen experimental characterization. The expression test results for hisactophilin mutants D57S, H89G and H91Y are shown in **Figure 2.3** (D57G not shown – expression was comparable to the other mutants shown). The high expression level of the mutant proteins seen in **Figure 2.3** suggests the mutations selected through rational design were well chosen. With a set of point mutants that expressed well, experimental characterization of switching function in the mutants is possible, as described next.

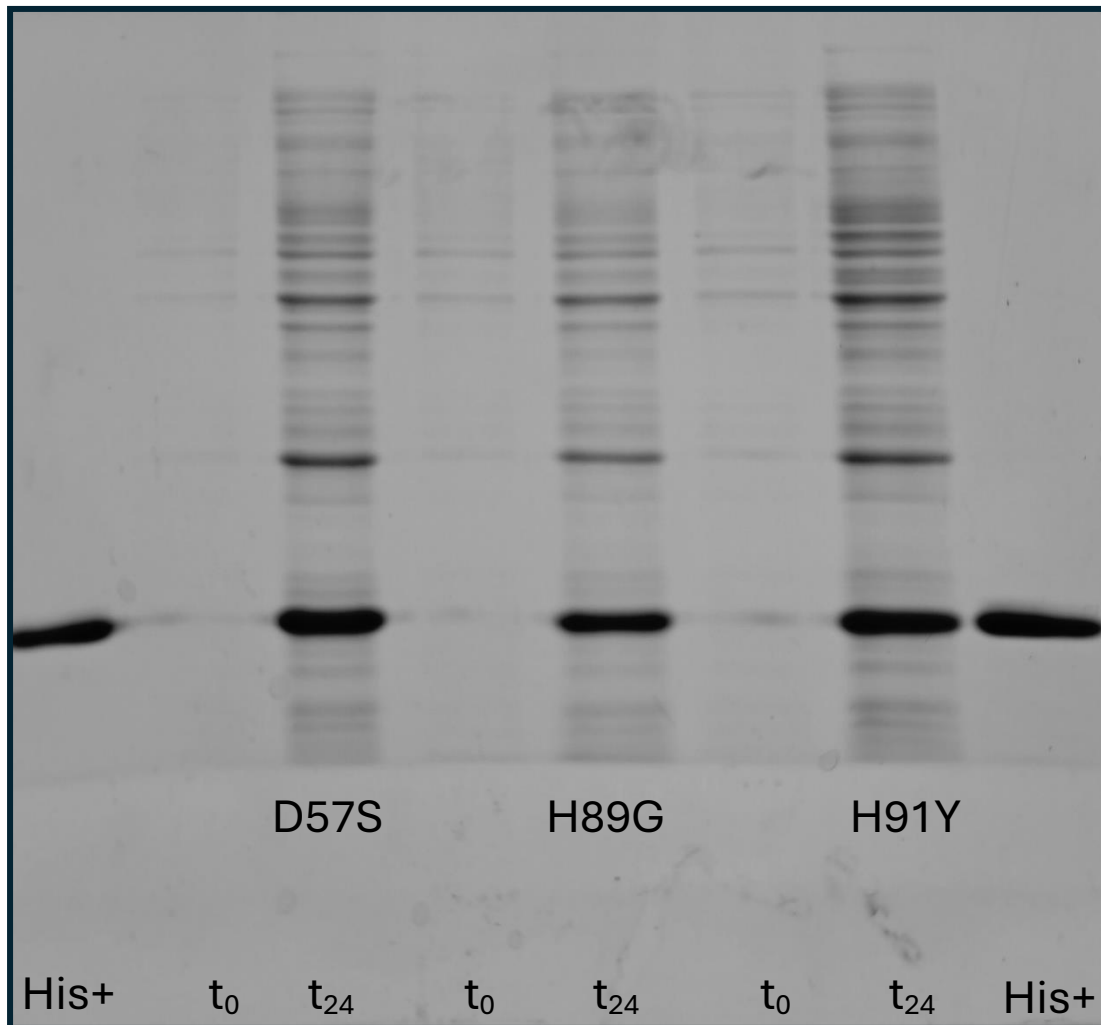


Figure 2.3: Point mutants selected through the rational design process overexpress well based on SDS-PAGE analysis of whole cell protein content. His+: hisactophilin standard. t₀: cellular protein content pre-induction. t₂₄: cellular protein content after induction with 1 mM IPTG and expression at 25°C for 24 hours.

Chapter 3: Experimental Methods

3.1 Protein expression

Wildtype and mutant hisactophilin proteins were expressed in *E. coli* BL21 cells co-transformed with pHW1 (mutant hisactophilin gene, ampicillin resistance) and pHV738 (hNMT1, *E. coli* methionyl aminopeptidase, kanamycin resistance). pHW1 mutagenesis was done by SynBio Technologies (New Jersey, USA). Chemically competent *E. coli* BL21 carrying pHV738 were transformed with pHW1 expression vectors by heat shock and subsequently streaked onto LB agar plates containing the appropriate antibiotics. Individual colonies of transformed bacteria were used to inoculate 10mL LB cultures which were then grown overnight at 37C with shaking at 225 rpm. The following day, 1L LB culture flasks were inoculated using an entire 10mL overnight culture. 1L cultures were grown at 37C with shaking at 225 rpm. Sodium myristate was added to the cultures (final concentration of 20 mM) once an OD₆₀₀ of ~0.25 was reached, followed by induction of protein expression with 1 mM IPTG at an OD₆₀₀ of ~0.7. After induction, the cultures were incubated at 25C with shaking at 225 rpm overnight, or for approximately 20 hours. Cells were harvested by centrifugation at 5000 xg for 12 minutes at 4C. If the collected cell pellets were not used immediately following centrifugation, they were flash frozen in liquid nitrogen and stored at -80C.

3.2 Cell lysis and immobilized metal affinity chromatography

3L cell pellets were resuspended in approximately 50mL of binding buffer (50 mM Tris, 100 mM NaCl, pH 8) with 1 mM PMSF and MgCl₂ added. A few crystals of DNaseI were introduced to the resuspension followed by stirring at room temperature until homogenous. Cells were lysed in an Emulsiflex-C5 cell disruptor (Avestin, Ottawa, Ontario) at a pressure of ~20000 psi. Following lysis, the solution was centrifuged at 20000 rpm for 22 minutes at 4C, twice. The prepared lysate was then loaded onto a pre-equilibrated nickel affinity column at 3 mL/min, followed by binding buffer until the A₂₈₀ returned to its baseline value. Next, wash buffer (50 mM sodium phosphate, 100 mM

NaCl, pH 6.3) was run for at least one hour or until the A_{280} returned to baseline. Bound protein was eluted by switching to a low pH elution buffer (50 mM formate, 100 mM NaCl, pH 3.5). The elution fraction was dialysed in 4L mqH₂O for four hours three times at room temperature, or at 4°C if left overnight. After buffer exchange, the protein was concentrated to a volume of 2-5 mL in a 50 mL Amicon stirred cell containing a 3 kDa cutoff membrane filter. Finally, the concentrated protein was centrifuged at 15000 xg for 12 minutes at room temperature to remove particulates in preparation for the following purification step. Prepared concentrated protein aliquots were either used immediately or flash frozen in liquid nitrogen and stored at -80°C until needed.

3.3 High-performance liquid chromatography

Mixed hisactophilin species were separated by reverse-phase C₁₈ high-performance liquid chromatography using a Waters C₁₈ µbondapak column in a radial compression cartridge adjusted to ~1000 psi. All solvents were filtered through a Whatman grade 32 filter paper and degassed under vacuum for at least 30 minutes at room temperature. The column was equilibrated by running 30/70 mqH₂O+0.1% TFA (A)/acetonitrile+0.1% TFA (B) at 10 mL/min for 20 minutes, followed by 98/2 A/B for 25 minutes. 1 mL aliquots of concentrated protein were injected for each run and separated using a linear gradient from 98/2 to 38/62 A/B over 60 minutes. Eluted myristoylated and non-myristoylated fractions were pooled separately and dialyzed in pH 4 mqH₂O for four hours at room temperature, twice, or at 4°C if left overnight. The protein was then buffer exchanged into 20 mM ammonium carbonate (pH unadjusted) twice for four hours each at room temperature, or at 4°C if left overnight, before being concentrated in an Amicon stirred cell with a 5 kDa cutoff membrane and lyophilized in 2 mg aliquots.

3.4 Chemical denaturation experiments

Lyophilized protein was redissolved to a concentration of 45 µM in 500 mM MES pH 6.2 or potassium phosphate pH 7.7. The 45 µM protein stock was diluted ten-fold into Eppendorf tubes containing volumes of water and urea corresponding to the desired

denaturant concentration (ranging from 0-9M), giving a final protein and buffer concentration of 4.5 μ M and 50 mM in the prepared samples, respectively. Samples were equilibrated in a 25°C water bath for at least 3 hours following their preparation. Protein unfolding was monitored through intrinsic tyrosine fluorescence with an excitation wavelength of 277 nm and emission scans from 290-350 nm using a Fluorolog 311 fluorimeter (Horiba, Japan). Accurate denaturant concentrations were measured by refractometry. Denaturation curve data was fit to a binomial extrapolation model using Origin with m_1 and m_2 fixed to 2.03 and 0.072, respectively:

$$Y = (Y_N + S_N x) - ((Y_N + S_N x) - (Y_U + S_U x)) \frac{e^{\frac{-(m_1 C_{mid} - m_2 C_{mid}^2) + m_1 x - m_2 x^2}{RT}}}{1 + e^{\frac{-(m_1 C_{mid} - m_2 C_{mid}^2) + m_1 x - m_2 x^2}{RT}}}$$

where Y is the observed signal, Y_N is the native signal, S_N is the slope of the native baseline, Y_U is the unfolded signal, S_U is the slope of the unfolded baseline, x is concentration of denaturant, C_{mid} is the midpoint of unfolding, R is the gas constant and T is temperature in Kelvin.

3.5 1D-¹H NMR pH titrations

Lyophilized protein was redissolved in 55 mM potassium phosphate, pH 10 to a concentration of 8 mg/mL (~0.6 mM) with 10% D₂O and 1 mM DSS. 1D-¹H spectra were measured on a Bruker Avance 600 MHz spectrometer equipped with a TXI probe at 297 K. pH adjustments were made using 0.2 M HCl and NaOH stocks. For each pH adjustment, the protein sample was transferred from the NMR tube to an Eppendorf tube using an extended glass pipette. P1 pulse widths were remeasured after every pH adjustment. The number of scans acquired varied depending on the quality of the sample (precipitation was often observed as the pH was lowered).

Chapter 4: Experimental Measurement of pH-switching Function in Hisactophilin Mutants

4.1 Introduction

The functional status of myristoyl-switching in hisactophilin mutants was evaluated using two complementary techniques: chemical denaturation cycles and 1-dimensional proton nuclear magnetic resonance (1D- ^1H NMR). Chemical denaturation experiments are based on the pH-dependent stabilization that is seen in the wildtype protein coming from switching of the myristoyl with pH²³ (**Figure 1.6**); if switching is “broken,” this pH-dependent stabilization will be diminished compared to the wildtype protein. The second method, 1D- ^1H NMR, monitors pH-sensitive changes in the chemical shifts of multiple internal probes, including the terminal methyl protons of the myristoyl and other protons in the protein such as the methyl of I85. Similar to the principle of the chemical denaturation experiments, if the myristoyl pH-switching is impaired, this will result in a decreased sensitivity of chemical shifts to pH. By considering the outputs of the two methods and comparing the results to that of the wildtype protein, inferences about the functional status of switching in the mutant proteins can be made.

Chemical denaturation is a well-established biophysical method for evaluating the stability of proteins.³⁶ This method monitors a change in some property (e.g. fluorescence, ellipticity, absorbance) as the fraction of unfolded proteins in equilibrated samples increases with denaturant concentration.²³ Commonly used denaturants are urea and guanidine hydrochloride (GdnHCl), with urea being preferred, when possible, to avoid the effects of GdnHCl on ionic strength. An example of the change in tyrosine fluorescence intensity of hisactophilin samples incubated in increasing concentrations of urea is shown in **Figure 4.1**. Plotting the change in intensity of the emission maximum at 306 nm as a function of urea concentration produces a characteristic sigmoidal denaturation curve. The single transition in denaturation experiments of hisactophilin fits well to a two-state transition between folded and unfolded proteins^{23,47} (**Figure 4.1**). Fitting of denaturation curve data is used to determine the midpoint of chemical denaturation, C_{mid} ,

and the Gibbs free energy of protein unfolding in water, $\Delta G_{\text{unfolding}}$. This measurement of protein stability is a valuable tool in studies with hisactophilin, as the protein's stability is highly sensitive to changes in pH.

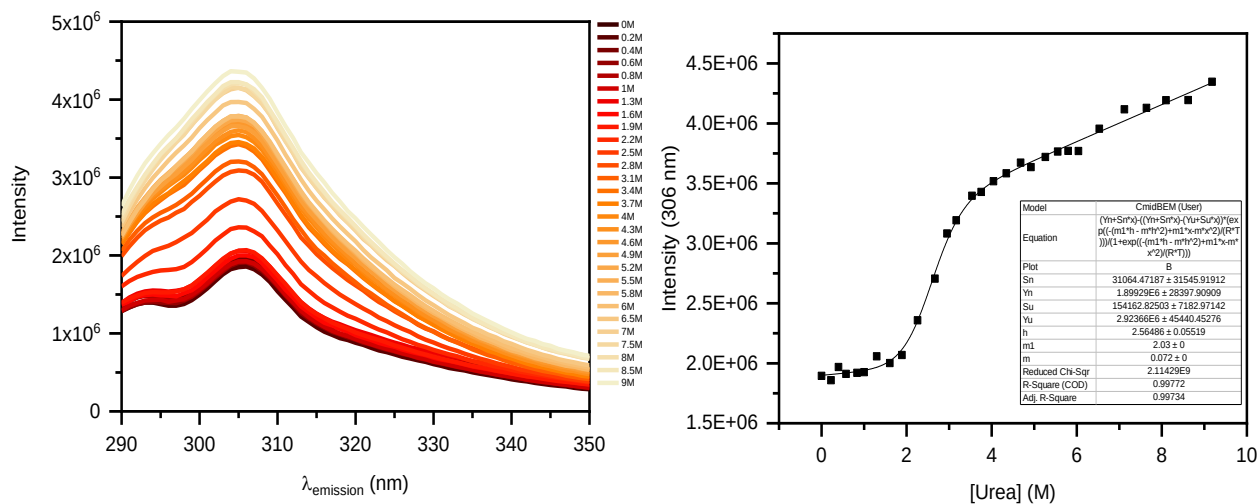


Figure 4.1 Fluorescence-monitored protein unfolding in chemical denaturants measures protein stability. Left: overlaid emission spectra (excitation wavelength = 277 nm) of myristoylated hisactophilin H106S at pH 6.2 in increasing concentrations of urea. The emission maximum at 306 nm increases as the protein unfolds. Right: plotting the emission intensity at 306 nm as a function of urea concentration produces a sigmoidal denaturation curve which is then fit to a binomial extrapolation model to determine the midpoint of unfolding and the related free energy of unfolding (see section 3.4 for binomial extrapolation

Chemical denaturation studies of hisactophilin mutants can provide insight into the functional status of myristoyl-switching. In the wildtype protein, myristoylation stabilizes the protein to a greater extent at high pH compared to low pH (**Figure 1.6, Figure 4.2**). This differential stabilization at high and low pH results from switching of the myristoyl; at high pH the myristoyl is sequestered in the protein core, forming favourable contacts with hydrophobic residues throughout the core. In contrast, at low pH the myristoyl is in the accessible state and no longer participates in hydrophobic interactions with core residues to the same extent as when in the sequestered state, resulting in decreased stability compared to the sequestered state (**Figure 1.6, Figure 4.2**). By measuring the stabilization caused by myristoylation at high and low pH, the extent to which the myristoyl group responds to changes in pH can be determined. In mutants with fully broken switches, the difference in stabilization from myristoylation should be the same at all pHs, as the myristoyl no longer undergoes a change in environment with pH.²⁹

Because there are factors contributing to protein stability other than myristoylation (e.g. ionization of residues that are not involved in myristoyl-switching), pH-dependent changes in the stability of the non-myristoylated protein must also be considered to determine the changes in stability coming from switching of the myristoyl (**Figure 4.2**). The difference in $\Delta G_{\text{unfolding}}$ between the myristoylated and non-myristoylated protein at any pH yields the level of stabilization coming from myristoylation at that pH ($\Delta\Delta G_{\text{myr-non, pH x}} = \Delta G_{\text{myr, pH x}} - \Delta G_{\text{non-myr, pH x}}$). As mentioned above, the $\Delta\Delta G_{\text{myr-non}}$ value is greater at high pH than low pH, reflecting the different locations the myristoyl occupies at the two pHs (**Figure 1.6, Figure 4.2**). The difference between the $\Delta\Delta G$ values at high and low pH corresponds to the free energy of switching from the sequestered to the accessible state, ΔG_{switch} ($\Delta G_{\text{switch}} = \Delta\Delta G_{\text{high pH}} - \Delta\Delta G_{\text{low pH}}$). The ΔG_{switch} value is a useful reporter of switching function; if there is no longer a measurable free energy of switching then it can be inferred that switching of the myristoyl has been decoupled from changes in pH.

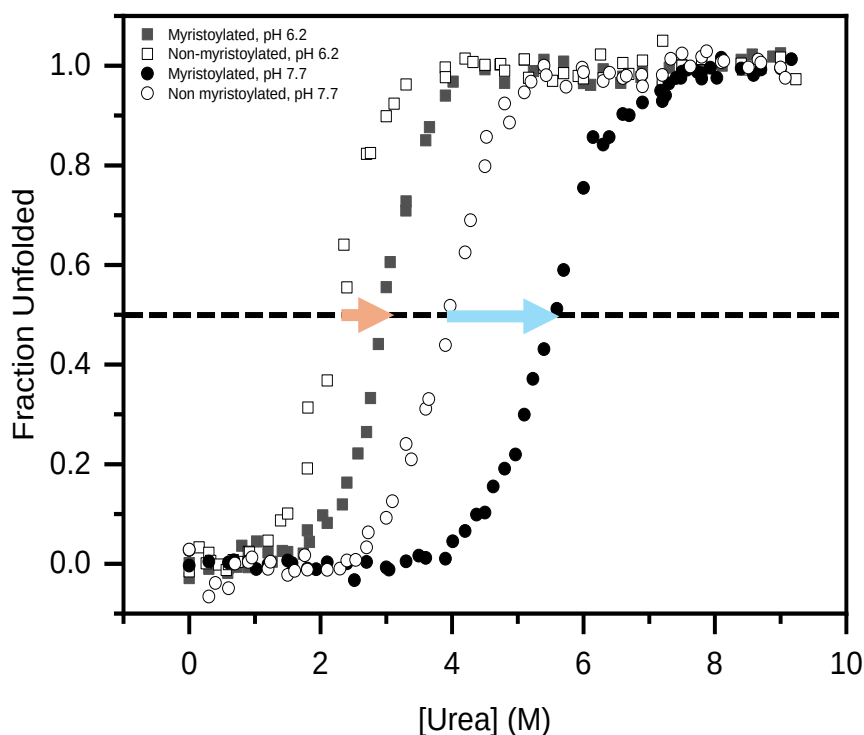


Figure 4.2: Myristoylation imparts greater stabilization to wildtype hisactophilin at high pH compared to low pH. Urea denaturation curves are shown for the myristoylated protein (closed symbols) and non-myristoylated protein (open symbols) at pH 7.7 (circles) and pH 6.2 (squares). Myristoylation increases protein stability at both pHs, with a more significant increase at high pH due to sequestration of the myristoyl. The difference in midpoints of unfolding (the concentration of urea at which $\Delta G_{\text{unf}} = 0$ kcal/mol, or half of protein molecules are unfolded) between the myristoylated and non-myristoylated proteins at low and high pH are indicated by red and blue arrows, respectively.

4.2 Point mutant chemical denaturation results

Chemical denaturation experiments with the point mutants suggest that mutation of D57, H89, or H91 only minorly affect pH-switching in hisactophilin (**Figure 4.3**) The ΔG_{switch} values of D57G, H89G, and H91Y are 1.06 ± 0.05 , 0.82 ± 0.04 , 0.83 ± 0.06 kcal/mol, respectively. Comparing these values to the wildtype ΔG_{switch} of 0.93 ± 0.04 kcal/mol suggests that movement of the myristoyl group in the mutants is still coupled to changes in pH. The persistence of switching function upon mutation of H89 or H91 could, in principle, be explained by some form of functional compensation between neighbouring histidines in the beta turn if electrostatic interactions with D57 are what control switching. However, the fact that mutation of D57 does not abolish switching suggests pH-dependent electrostatic interactions involving this residue may not be of mechanistic importance, as was originally proposed. Oddly, mutation of D57 seems to increase the pH-dependence of switching, meaning a smaller change in pH is needed to reach the same level of accessibility as in the wildtype protein. To provide further verification of these unexpected findings, complementary 1D- ^1H NMR experiments for evaluating switching function in the mutants were conducted in parallel with the chemical denaturation.

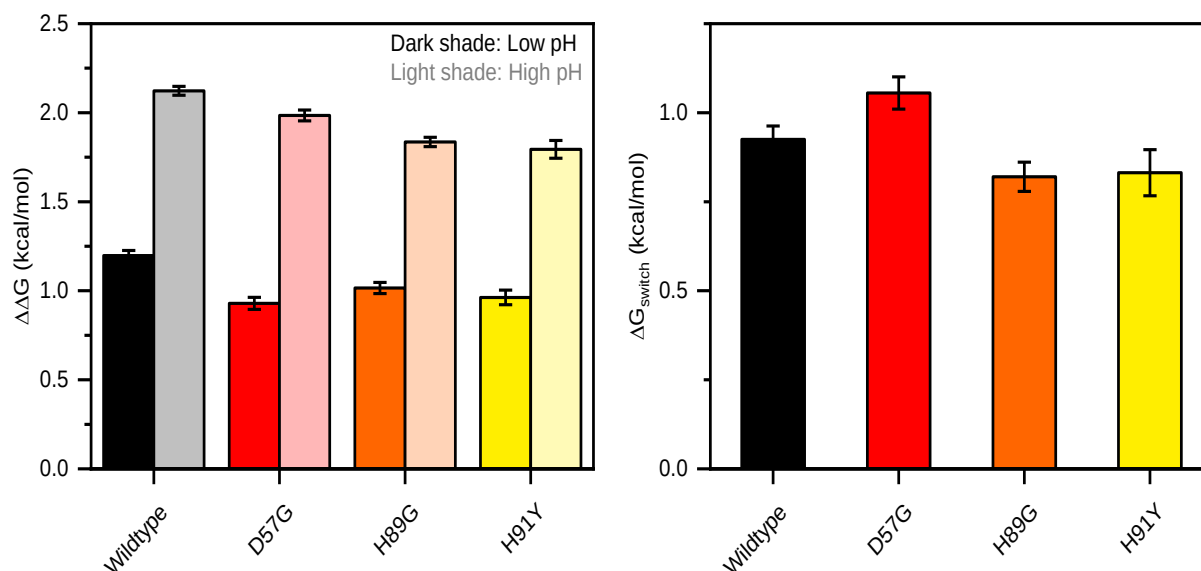


Figure 4.3 Chemical denaturation of hisactophilin point mutants suggests pH-switching is still functional. Left: The difference in free energy of unfolding between myristoylated and non-myristoylated hisactophilin variants at pH 6.2 (red) and pH 7.7 (blue) are shown. Right: the difference in stabilization coming from myristoylation at high and low pH (ΔG_{switch}) is shown. Notably, the point mutants do not show significantly different ΔG_{switch} values compared to wildtype hisactophilin.

4.3 Point mutant 1D-¹H NMR pH titrations: Introduction

NMR is a powerful technique that reports on the local magnetic and chemical environments of spin-active nuclei within a sample. Proteins are often grown in specialized media that serve to label the proteins with spin active nuclei such as ¹⁵N and ¹³C, enabling more complex NMR experiments to be carried out. However, a significant amount of valuable information can be obtained from unlabeled protein samples simply by measuring the chemical shifts of the abundant spin-active proton nuclei. **Figure 4.4** displays a 1D-¹H NMR spectrum of myristoylated wildtype hisactophilin at pH 10.34, well above the midpoint of switching. Notably, several peaks are well isolated from the bulk of the spectrum, most importantly the peak corresponding to the protons on the terminal methyl group of the myristoyl. The up-field position of this peak at high pH is likely the result of the myristoyl's position in the core when sequestered, where its proximity to the ring currents of several aromatic core residues causes significant shielding from the external magnetic field of the spectrometer (**Figure 4.5**). By monitoring the chemical shifts of the myristoyl methyl and other well-resolved peaks as the sample's pH is decreased, information related to the pH-dependent movement of the myristoyl group can be obtained.

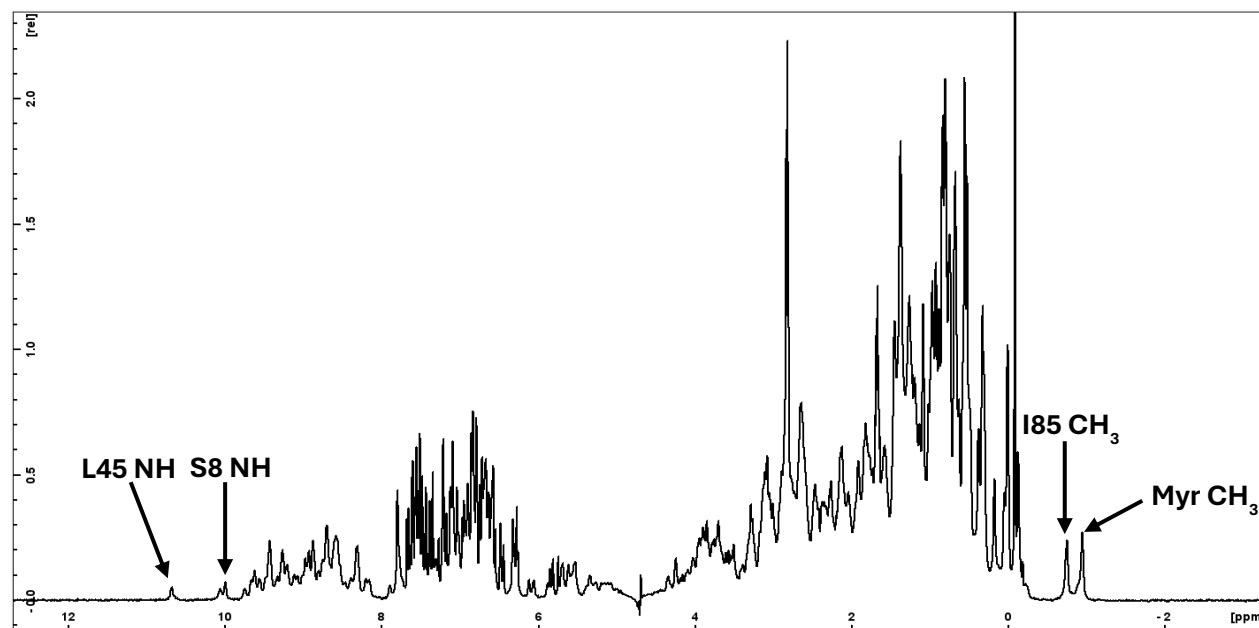


Figure 4.4: Several informative peaks in the 1D- ^1H NMR spectrum of myristoylated hisactophilin are well-resolved at high pH. Down-field amide peaks and up-field methyl proton peaks are labeled on a spectrum of myristoylated wildtype hisactophilin in 50 mM potassium phosphate acquired at pH 10.34, 298K.

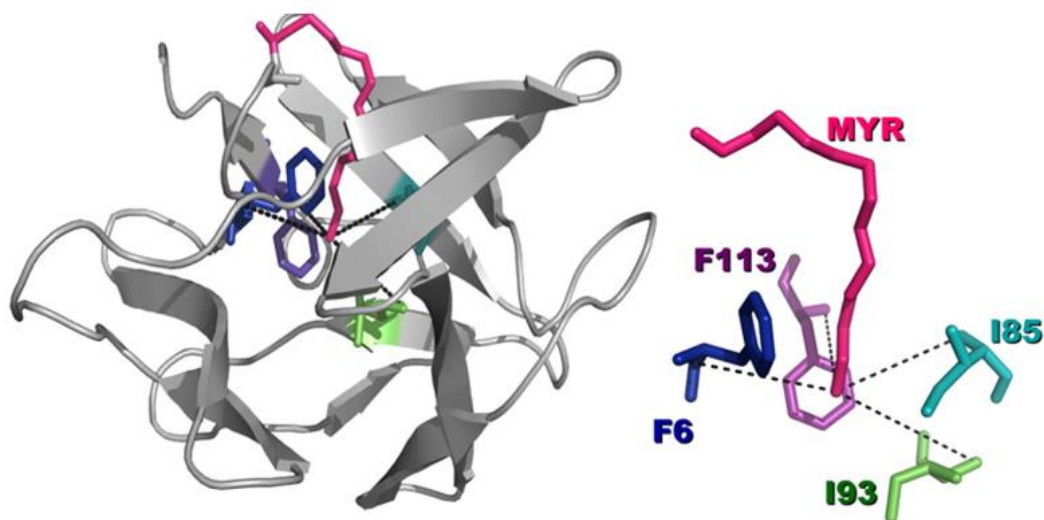


Figure 4.5: Hisactophilin's myristoyl packs against aromatic core residues when sequestered. Left: A structural model of hisactophilin is shown with the myristoyl (magenta) modeled into the structure based on experimental NOE restraints. Right: a close-up of the interactions the terminal methyl of the myristoyl makes with the core residues F6, F113, I85 and I93. Figure taken from [23].

As the pH of the NMR sample is decreased, the peak that is representative of the population weighted average of positions sampled by the myristoyl methyl moves down-field (**Figure 4.6**). This movement is reflective of the increased number of protein molecules sampling the accessible state at low pH, in which the myristoyl methyl is no longer shielded by the ring currents of the aromatic core residues. It stands to reason then, that in mutants with broken myristoyl switches the pH-dependence of the myristoyl methyl's chemical shift should be diminished compared to the wildtype protein.

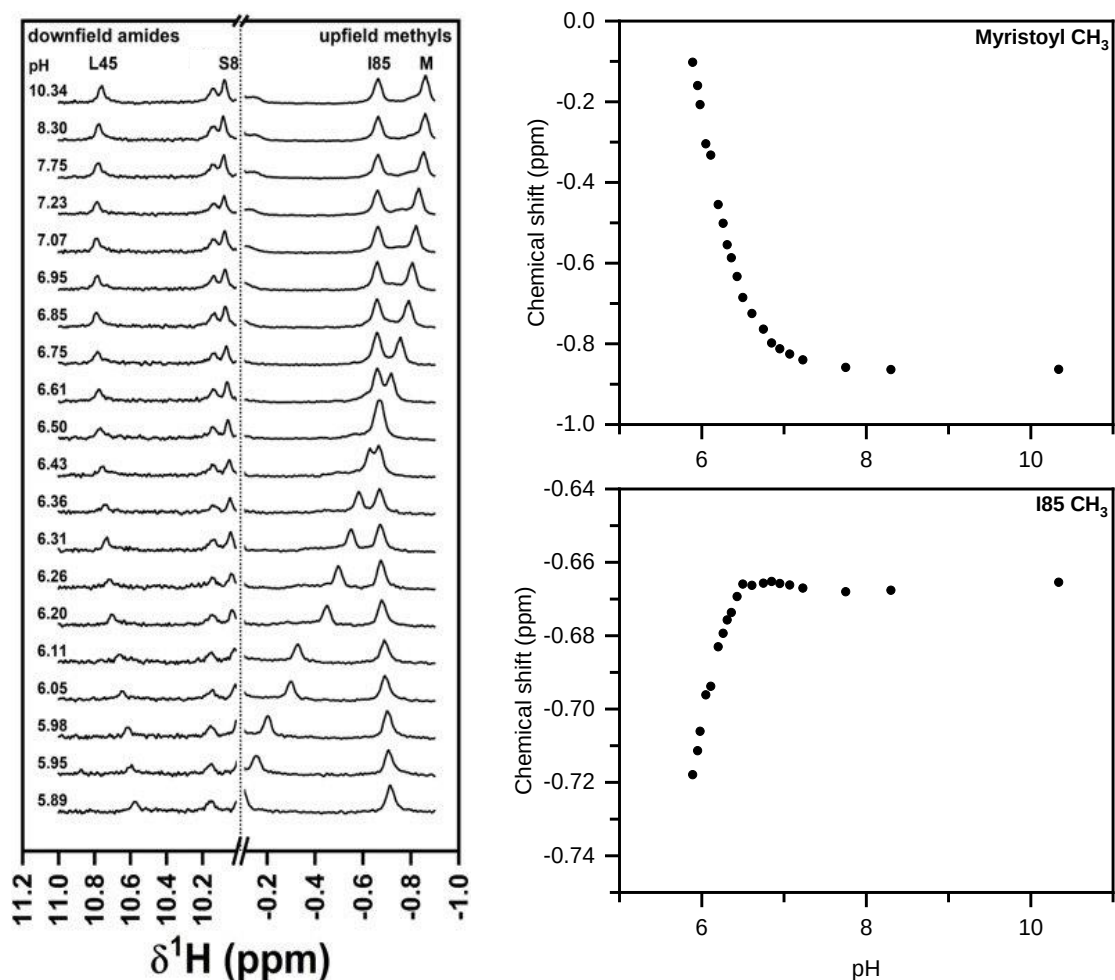


Figure 4.6: Wildtype hisactophilin myristoyl and I85 methyl proton chemical shifts are pH-dependent because of the myristoyl switch. Left: down- and up-field portions of wildtype myristoylated hisactophilin 1D-¹H NMR spectra obtained throughout a pH titration (M=myristoyl). Plotting the chemical shifts of the myristoyl and I85 methyl proton peaks as a function of pH produces the titration plots shown on the right. Significant movement of the myristoyl methyl proton peak occurs as the pH is decreased past the midpoint of switching (6.95), reflective of the increased number of myristoyl groups occupying the accessible state. The I85 methyl peak also shows movement with pH, although to a lesser extent than the myristoyl peak.

4.3.1 Point mutant $1D$ - ^1H NMR pH titrations: results

The results from the $1D$ - ^1H NMR titrations suggest that the myristoyl group of the mutants responds to pH in a manner similar to the wildtype protein (**Figure 4.7**). A slight difference in peak movement with pH is seen for H89G and H91Y, suggesting mutation of these residues may have a minor impact on the pH-dependence of switching. However, as seen **in Figure 4.7**, the titrations with D57G and D57S look highly similar to that of the wildtype protein. These results are in good agreement with the chemical denaturation experiments which also indicate mutation of D57, H89 and H91 does not fully break myristoyl switching in hisactophilin. Interestingly, both sets of data suggest that mutation of H89 and H91 slightly decrease the pH-dependence of switching, whereas mutation of D57 seemingly increases it. However, the difference between the wildtype, D57G and D57S pH titration profiles is subtle, and so replicate experiments should be performed before interpreting these results with confidence. Regardless, based on the proposed mechanism of switching it was expected that mutation of D57 would fully decouple myristoyl-switching from pH changes; based on the chemical denaturation and NMR data presented above it is clear that this is not the case. Therefore, it is likely that the originally proposed mechanism of switching outlined in section 1.6 is not fully correct and requires refinement with consideration of the newly available data.

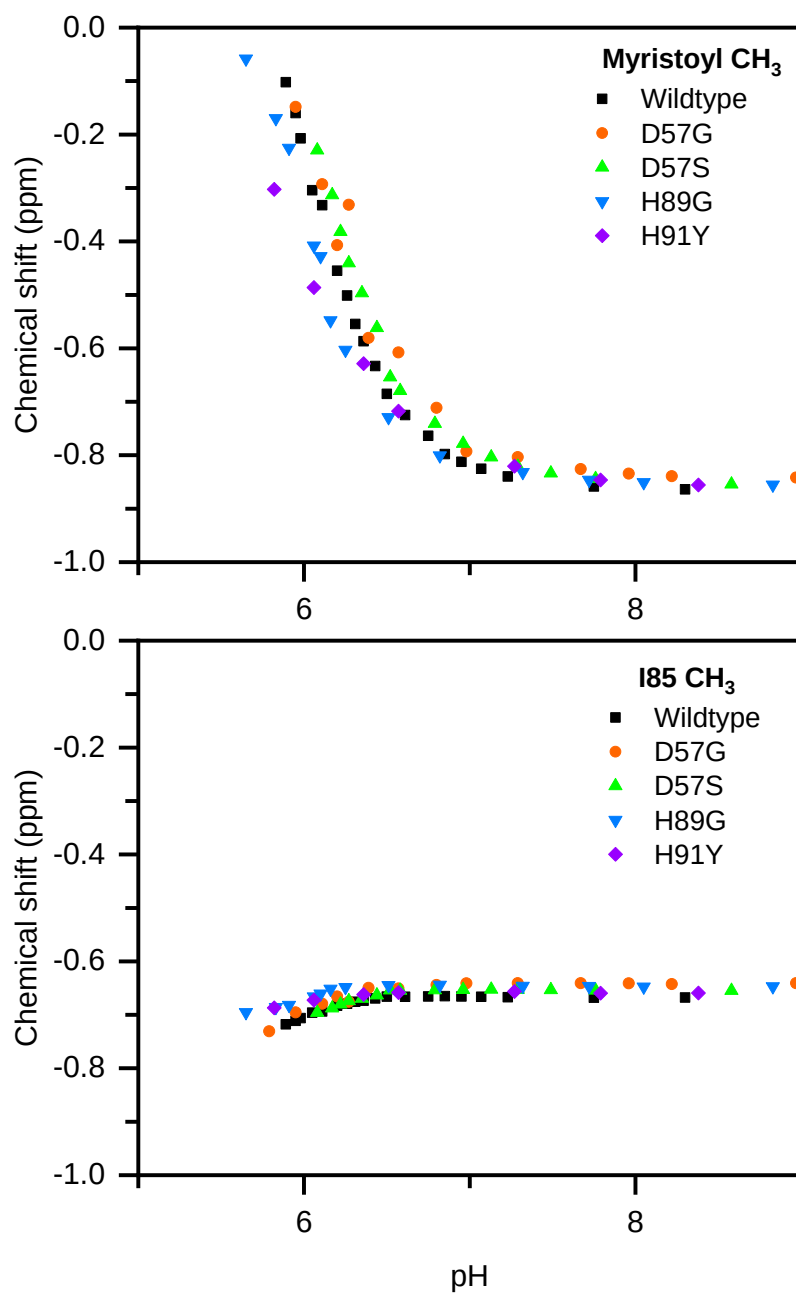


Figure 4.7: Hisactophilin point mutant pH-dependence of chemical shift is similar wildtype. The pH-dependent movement of the myristoyl CH₃ proton peak and I85 CH₃ proton peak is shown for the wildtype protein and point mutants. D57G and D57S show similar peak movement, whereas H89 and H91 show slightly decreased pH-dependence.

4.4 Thoughts on a refined pH-switching mechanism

Based on the experimental data presented above, it seems reasonable to conclude that stabilizing electrostatic interactions involving D57 are not of mechanistic importance for pH-switching in hisactophilin. It is curious though, that mutation of H89 and H91 slightly decreases the pH-dependence of switching, as seen in **Figure 4.3** and **Figure 4.7**. Additionally, the mutant H90G has previously been subjected to the same chemical denaturation experiments described above and was found to have an effect on switching similar to that of H89G and H91Y.²⁹ Considering that mutation of H89, H90 and H91 all have the same minor effect on switching, it may be the case that intra-turn interactions amongst the four histidines in the β 9-10 turn are of importance. In this case, it would be repulsive interactions amongst the protonated histidine side chains at low pH that control switching, rather than formation of favourable stabilizing interactions with an acidic side chain. It is of interest to note, also, that the core residues I85 and I93 are on the β strands connected by the β 9-10 turn (**Figure 4.8**). It has been demonstrated previously through experiment that the position of the myristoyl is highly sensitive to the stereochemistry of I85 in the core²⁹; mutation of I85 to leucine prevents entry of the myristoyl into the core. It may be the case that structural rearrangement of the β 9-10 turn when the histidines are protonated is coupled to switching through subtle movement of the β strands on either side of the turn that could result in steric clashing between the myristoyl and core residues, forcing it to exit the core.

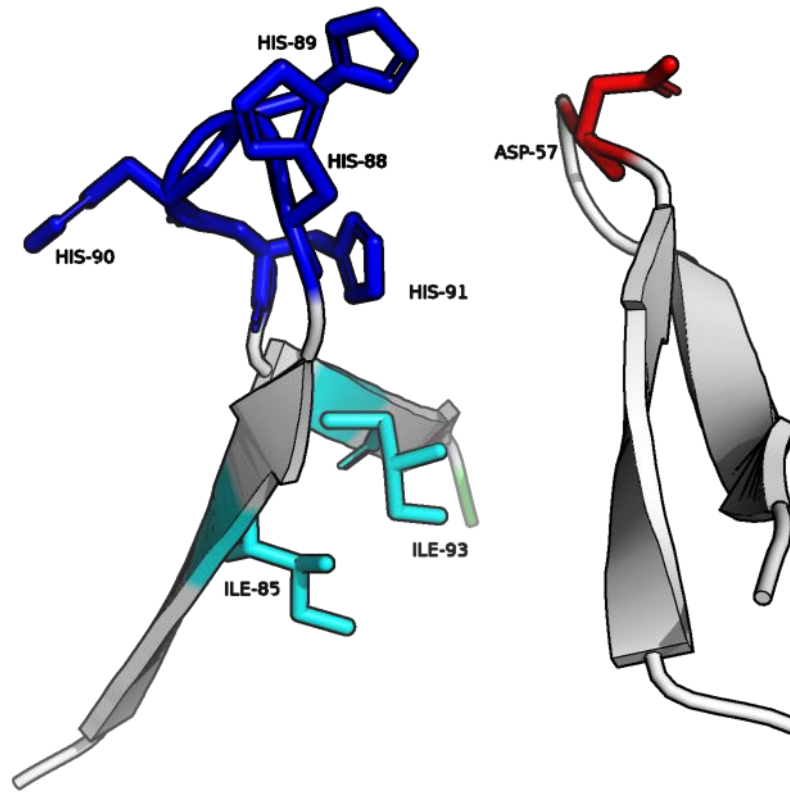


Figure 4.8: Structural context of hisactophilin ionizable residues of interest in this work.

D57 is positioned nearby the four histidines of the β 9-10 turn. It is possible that removal of the negatively charged side chain of D57 in the mutants D57G and D57S explains the slightly increased pH-dependence of switching observed experimentally. Removal of the negative charge may make the local net charge more positive at low pH as the histidines are protonated, leading to increased repulsion amongst the four histidines. Notably, the four histidines are in the turn that connects the β -strands I85 and I93. Subtle movement of these core residues caused by movement of the histidines in the turn when protonated may be sufficient to disrupt myristoyl binding pocket geometry, leading to switching.

The suspected functional role of electrostatic repulsion between positively charged histidines may explain the unexpected results seen with the D57G mutant. Removal of a negative charge near the cluster of four histidines makes the local net charge of that region more positive, meaning it may be easier to reach the level of repulsion needed for

switching to occur. The more readily achieved repulsion may explain why a smaller decrease in pH is needed for the D57 mutants to reach the same level of accessibility as wildtype (**Figure 4.3, Figure 4.7**). However, it is also thought that proximity to an acidic sidechain raises the pK_a of the histidines,²⁹ making these two ideas difficult to reconcile. Therefore, additional experiments are needed to investigate the role of charge repulsion in the mechanism of pH-switching.

The proposed repulsive electrostatic interactions may be an alternative explanation to the increased local stability of the beta turn region identified in the temperature dependent NMR experiments (**Figure 1.8**); repulsive interactions between the four histidines in the beta turn may decrease the number of possible conformations that can be sampled by the side chains, serving to rigidify the beta turn and surrounding regions of the protein. Based on the proposed mechanism for pH-switching in hisactophilin had radically changed from one based on favourable electrostatic interactions to an electrostatic repulsion-based mechanism. Of course, without experimental testing to falsify or support this new hypothesis it will remain only an idea.

A set of multi mutants in which electrostatic repulsion between the four histidines is no longer possible was designed to test the repulsion-based pH-switching hypothesis. The prediction is that when all four histidines are mutated, rearrangement of the $\beta 9-10$ turn at low pH caused by electrostatic repulsion amongst the charged side chains will no longer be possible and therefore the subtle alteration of core geometry suspected to accompany this rearrangement will no longer occur. Additionally, a histidine residue outside of the $\beta 9-10$ turn was mutated as a control. There is no experimental evidence to suggest that H106 plays a role in switching, and so to validate the suspicion that histidines in certain regions of the protein are of functional importance this histidine was mutated with the expectation that no change in switching function will occur.

4.5 Design of hisactophilin multi-mutants to test the refined switching hypothesis

A rational design approach similar to that described in Chapter 2 was used in the design of the multi-mutants. The same criteria used to evaluate the H89 and H91 point mutants was used to evaluate potential multi-mutant sequences, as the four histidines H88, H89, H90 and H91 form a β -turn.

Table 5.1: Multi-mutant stability predictions obtained using the MAESTRO webserver and FoldX BuildModel function. Green and red indicate mutations predicted to be stabilizing or destabilizing, respectively.

Mutation	MAESTRO	FoldX
	$\Delta\Delta G$ (kcal/mol)	$\Delta\Delta G$ (kcal/mol)
H88G/H89G/H90G/H91G	0.079	-3.459
H88S/H89G/H90G/H91S	-0.242	-3.094
H88S/H89G/H90G/H91Y	-0.743	-3.597
H88F/H89G/H90G/H91Y	-1.241	-1.83
H88S/H89G/H90G/H91L	-0.67	-4.055
H88S/H89S/H90G/H91L	-0.653	-2.457

Rational design led to selection of the mutants H88G/H89G/H90G/H91G (GGGG), H88S/H89G/H90G/H91Y (SGGY), and D57G/H88S/H89G/H90G/H91Y (GSGGY) for experimental characterization. Next, an appropriate mutation for the control histidine H106 was selected.

At this point it is known that mutation of H89 and H91 has a minor effect on the pH-dependence of switching in hisactophilin. To confirm that these effects are not seen upon mutation of any of the 31 histidines in the protein, the residue H106 was chosen as a control. Variable-pH NMR indicates that the chemical shift of the backbone amide proton of H106 responds to pH similarly in both the myristoylated and non-myristoylated protein, suggesting this residue is likely not involved in switching (**Figure 4.9**). Because this residue is found in a loop rather than a β -turn like the previously mutated residues,

β -turn residue preferences cannot be considered in the selection of the mutation for H106. However, the fascin consensus sequence and stability predictors can still be of use.

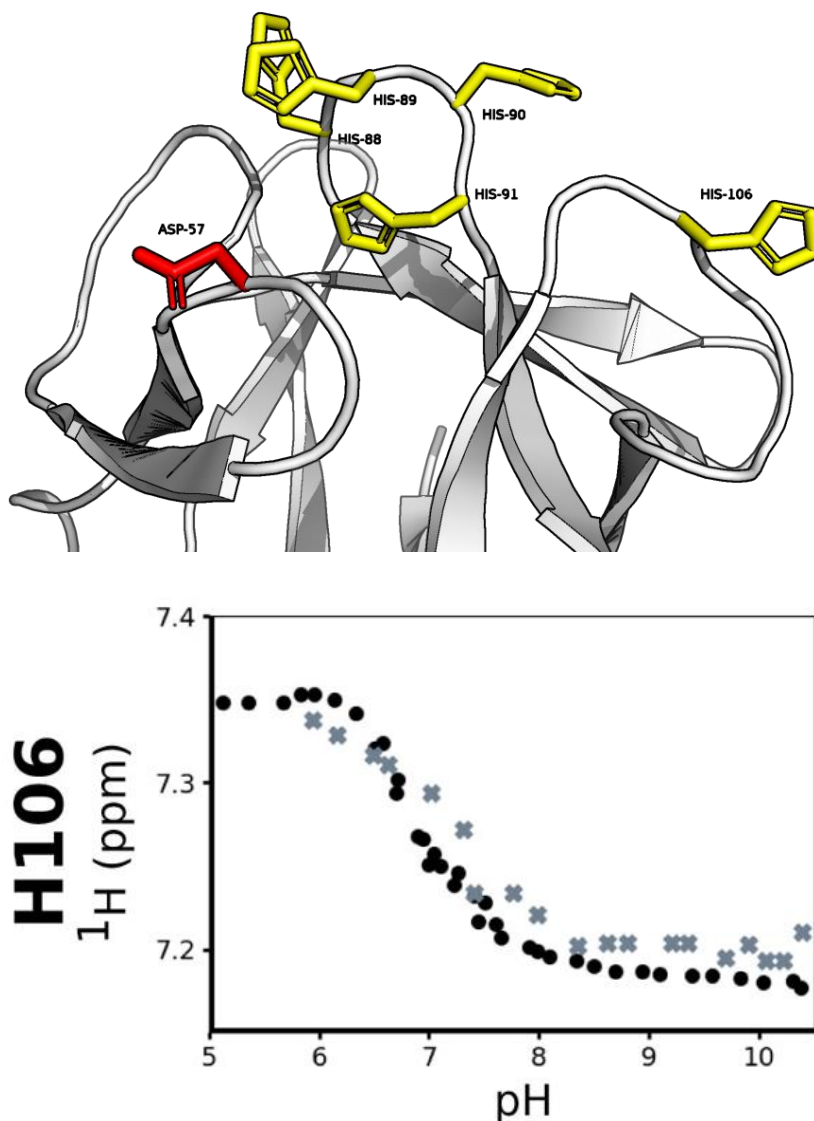


Figure 4.9: H106 is predicted to not be involved in myristoyl-switching in hisactophilin.

Top: Structural model of hisactophilin (1HCD) displaying side chains of residues of interest in this work. Bottom: variable pH NMR titration measurement of H106 backbone amide proton chemical shift. The backbone amide proton chemical shift of H106 behaves similarly in the myristoylated and non-myristoylated protein, suggesting it is likely not involved in switching. Figure adapted from [29].

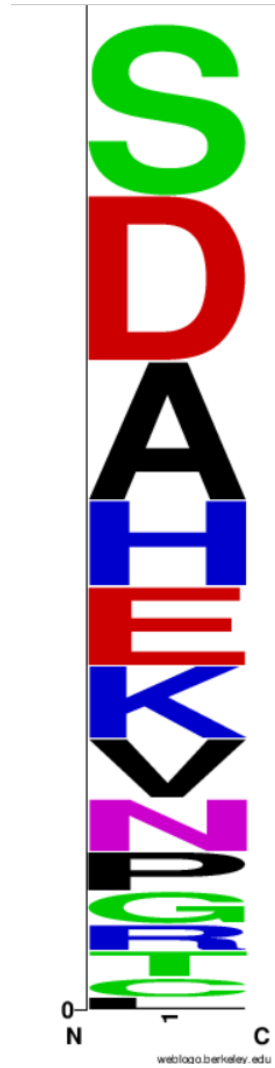


Figure 4.10: Fascin consensus logo displaying preferred residues in positions equivalent to H106 of hisactophilin. Multiple sequence alignments were acquired from the conserved domain database.

Table 5.2: H106 control mutant stability predictions obtained using the MAESTRO webserver and FoldX BuildModel function. A modified version of the generation script used by the inverse folding model ProteinMPNN was provided by Shawn Reeves of the Kalyaanamoorthy lab to provide additional predictions of how favourable a given mutation is relative to the wildtype residue. Green and red indicate mutations predicted to be stabilizing or destabilizing, respectively.

	MAESTRO	FoldX	Protein MPNN
Mutation	$\Delta\Delta G$ (kcal/mol)	$\Delta\Delta G$ (kcal/mol)	$\Delta\text{Log}_{\text{Likelihood}}$
H106S	0.252	-0.979	1.173
H106G	0.227	-1.4	1.057

Based on the stability predictor results and available consensus information, the mutation H106S was selected. The multi-mutants GGGG, SGGY and GSGGY, as well as the control mutant H106S, were characterized experimentally and shown to express to a high level (**Figure 4.11**) (GGGG not shown). With this acceptable level of overexpression, experimental characterization of the multi-mutants can proceed.

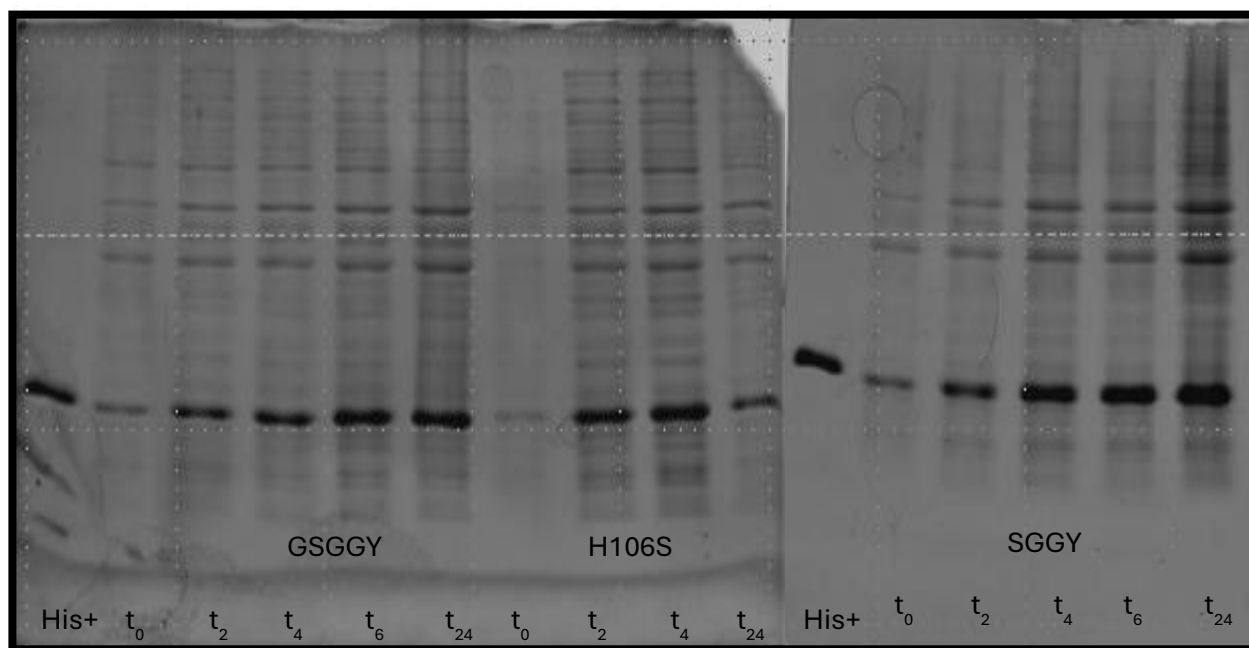


Figure 4.11: Mutants selected through the rational design process overexpress well based on SDS-PAGE analysis of whole cell protein content. His+: hisactophilin standard. t_0 : cellular protein content pre-induction. t_{24} : cellular protein content after induction with 1 mM IPTG and expression at 25°C for 24 hours.

4.5.1 Multi-mutant chemical denaturation experiments

The expectation based on the newly proposed repulsion-based switching mechanism was that removal of all four histidines in the $\beta 9$ -10 turn will prevent the switch from the sequestered to the accessible state. Delightfully, chemical denaturation experiments with the multi mutants seem to support the idea that it is interactions amongst charged histidines controlling pH-switching in hisactophilin.

Chemical denaturation measurements indicate that the ΔG_{switch} of GGGG is the lowest seen yet compared to wildtype, with a value of 0.30 ± 0.03 kcal/mol (**Figure 4.12**). Notably, the $\Delta \Delta G$ at low pH resembles that of wildtype at high pH, suggesting that the protein may be broken in the sequestered state as was predicted. The addition of the D57G

mutation to SGGY seems to restore some pH-dependence of switching compared to GGGG alone, with the GSGGY mutant having a ΔG_{switch} of 0.37 ± 0.08 kcal/mol. This finding further supports the idea that local charge repulsion is an important factor in switching, as removal of the negative charge from that region in both D57G and GSGGY may promote the repulsive interactions amongst positively charged histidine side chains at low pH. However, the fact that the D57G mutation increases pH-dependence of switching even when repulsion amongst the four histidines in the $\beta 9-10$ turn are not present in the GSGGY mutant suggests electrostatic interactions between other positively charged residues in that region of the protein may also be of functional importance.

The control mutant H106S was selected to verify that some histidines in the protein are not involved in switching. Previous NMR experiments provided no evidence for involvement of H106 in switching, and so it is expected that mutation of this residue should have no impact on switching. Indeed, the mutant H106S has a ΔG_{switch} of 0.95 ± 0.05 kcal/mol, very close to the wildtype ΔG_{switch} of 0.93 ± 0.04 kcal/mol (**Figure 4.12**). The similarity in switching energetics between wildtype and H106S indicates that this histidine likely plays no role in driving the switch from the sequestered to accessible state, as was predicted based on the experimental findings.

Chemical denaturation analysis of the multi mutants GGGG and GSGGY indicate that mutation of the four histidines in the $\beta 9-10$ turn significantly decreases the pH-dependence of switching in hisactophilin. These findings support the proposed electrostatic repulsion-based switching mechanism described in section 4.4. However, a single experiment is insufficient to thoroughly test a hypothesis. As with the point mutants described above, $1\text{D-}^1\text{H}$ NMR pH titrations were carried out with the multi mutants to increase confidence in the conclusions being made about the importance of repulsive electrostatic interactions in pH-switching.

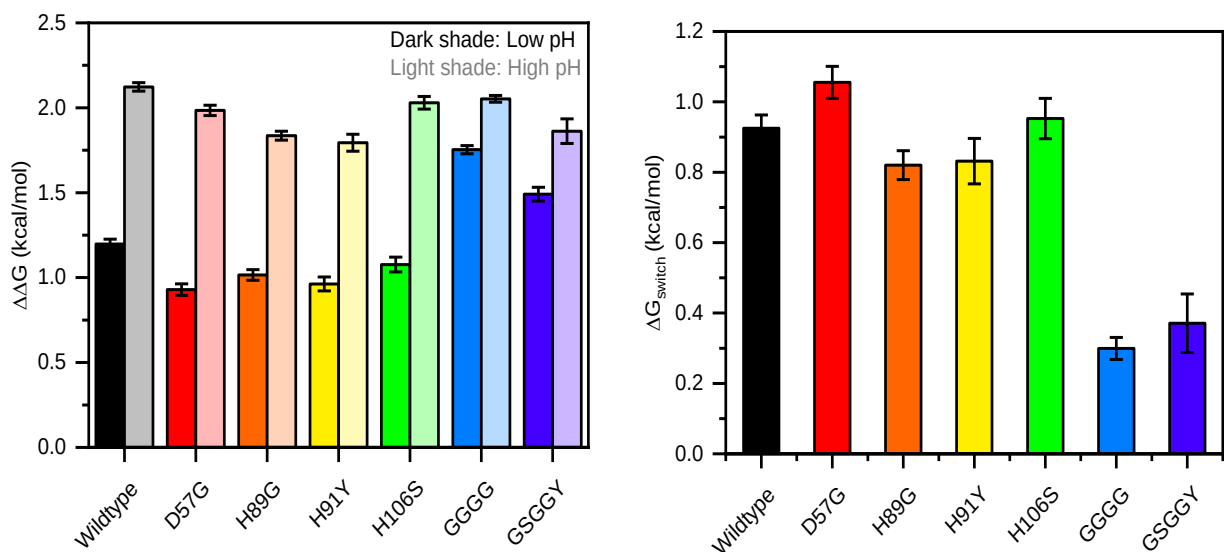


Figure 4.12: Chemical denaturation experiments with hisactophilin multi-mutants show significantly decreased pH-dependence of switching compared to wildtype. Left: $\Delta\Delta G = (\Delta G_{\text{unfolding, myr}} - \Delta G_{\text{unfolding, non-myr}})$. $\Delta\Delta G$ values are acquired at pH 6.2 (low pH) and pH 7.7 (high pH), corresponding to the accessible and sequestered states in wildtype. The $\Delta\Delta G$ of wildtype is greater at high pH than low pH due to the stabilizing effect of the myristoyl being sequestered. The multi mutants GGGG and GSGGY show a significantly increased $\Delta\Delta G$ at low pH compared to wildtype. Right: $\Delta G_{\text{switch}} = (\Delta\Delta G_{\text{high pH}} - \Delta\Delta G_{\text{low pH}})$. ΔG_{switch} values represent the free energy difference associated with the differential position of the myristoyl at high and low pH. ΔG_{switch} values are significantly decreased in the multi mutants GGGG and GSGGY relative to wildtype. A summary of fit parameters for individual denaturation curves can be found in the appendix.

4.5.2 Multi mutant $1D-^1H$ NMR pH titrations

The $1D-^1H$ NMR pH titrations once again provide valuable verification of the chemical denaturation findings. Chemical denaturation experiments with GGGG and GSGGY suggest that GGGG is the most broken compared to wildtype, with GSGGY having a slightly restored pH-dependence of switching compared to GGGG. These findings are

replicated well in the NMR experiments (**Figure 4.13**). GGGG and SGGY (chemical denaturation experiments have not yet been done with SGGY) methyl proton peaks show the least movement with pH compared to wildtype, suggesting these mutations significantly decreased the pH-dependence of switching in hisactophilin. In agreement with the chemical denaturation experiments, GSGGY methyl peak sensitivity to pH is intermediate between GGGG and wildtype.

It is difficult to interpret the effect of the H106S mutation when considering both the chemical denaturation data and the NMR titration profiles. The chemical denaturation data suggest the H106S mutation has no significant effect on switching, but the NMR titration profile of this mutant suggests it has an effect similar to H89G. Experimental replicates should be acquired to increase confidence in the conclusions about the effect of the H106S mutation.

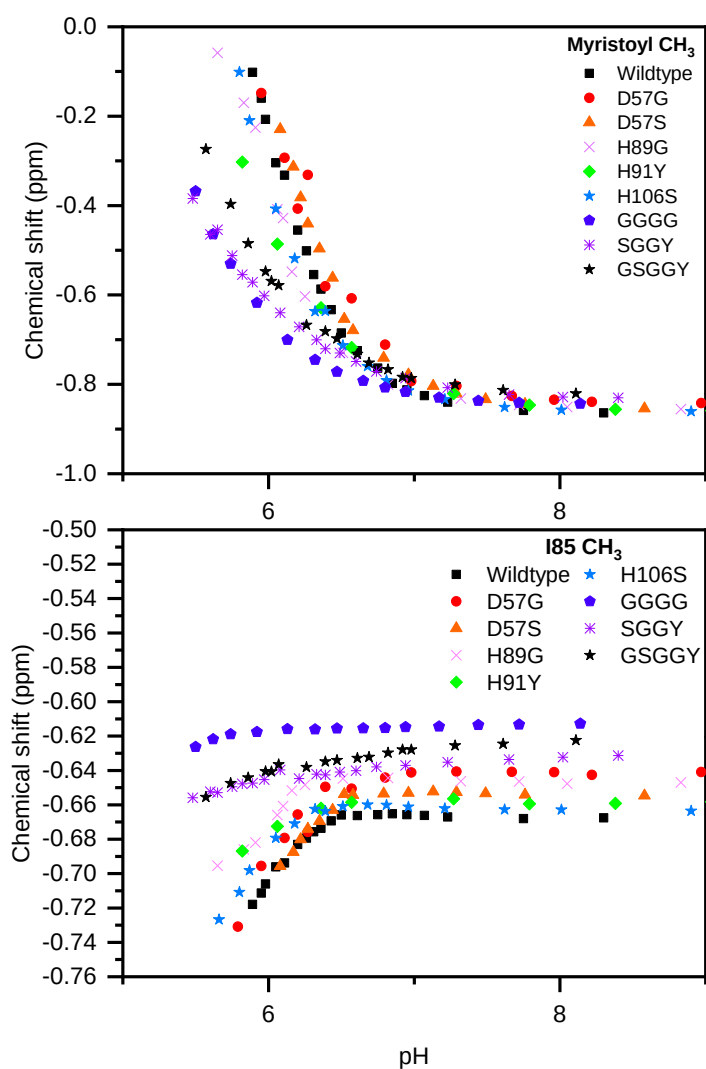


Figure 4.13: pH-dependence of chemical shift in multi mutant methyl proton peaks is significantly diminished compared to wildtype hisactophilin. The pH-dependent changes in chemical shift of the myristoyl CH₃ proton peak and I85 CH₃ proton peak are shown. Hisactophilin multi-mutants GGGG, SGGY, and GSGGY, in which H88, H89, H90 and H91 are mutated, shown significantly diminished pH-dependence compared to wildtype.

Chapter 5: Discussion and Future Research: **Moving Towards a Mechanistic Understanding of** **pH-switching in Hisactophilin**

5.1 Interpretations of the experimental results

Based on previous experimental and computational studies it was proposed that the formation of favourable electrostatic interactions between D57 and H89/H91 at low pH are what control reversible pH-dependent myristoyl-switching in hisactophilin.²⁹ The work described here refutes this claim and indicates that the originally proposed mechanism of pH-switching is not fully correct. Mutation of D57 does not break switching in hisactophilin, and surprisingly appears to increase the pH-dependence of switching. However, mutation of H89 and H91 does indeed minorly decrease the pH-dependence of switching, as was expected.

It has been demonstrated experimentally that mutating H89, H90, or H91 alone slightly decreases the pH-dependence of myristoyl-switching in hisactophilin.²⁹ Interestingly, however, mutating all four of H88, H89, H90 and H91 together shows a substantial effect on switching function (as seen with the GGGG and SGGY mutants). There are several plausible explanations for these observations:

- 1) It is the combined effect of repulsive interactions amongst the four histidines in the turn when protonated that is of importance for switching and therefore mutating only one is insufficient to break pH-switching.
- 2) H88 is the only histidine of functional importance in the β 9-10 turn. Without mutating H88 in isolation, it can not be said with certainty that it is not only the removal of H88 that is producing the significant breaking of switching seen in the multi-mutants.
- 3) There is functional redundancy between the four histidines. If it is the case that favourable electrostatic interactions between a single histidine residue in the turn and some acidic residues are what drive switching, neighbouring histidines may be able to compensate for mutation of single histidines in the β 9-10 turn. Only when all four

histidines are removed, and functional compensation is no longer possible, is a significant effect on switching function seen.

Without additional experimentation, it will be difficult to determine which, if any, of the above postulates is correct. Fortunately, there are several sensible experimental routes to choose from to continue moving towards a mechanistic understanding of pH-switching in hisactophilin.

5.2 Additional mutants to investigate the role of charge repulsion in pH-switching

Experimentation with additional hisactophilin mutants may be able to help in distinguishing between the possible explanations for the experimental results described here. A single point mutant in which H88 is mutated is the most straightforward route for testing the hypothesis that H88 is the key histidine in the β 9-10 turn. Likewise, point mutants in which positive charge is introduced to the β -turn or surrounding structure could serve to test the hypothesis that local charge repulsion and the subtle structural changes that accompany it are what control pH-switching. For example, mutating some of the histidines in β 9-10 turn or D57 to lysine or arginine would be predicted to increase the pH-dependence of switching, as fewer groups would need to be ionized to reach the same local net charge.

At this point, it is known that removing the four histidines H88, H89, H90 and H91 together is sufficient to substantially impair switching function in hisactophilin. These findings beg the question of whether introduction of a similar cluster of ionizable groups elsewhere in the protein may be able to restore the pH-dependence of switching to wildtype values. This endeavour will likely not be straightforward and will benefit from the information obtained from additional mutant studies, such as those described above.

5.3 Liposome-based binding assays for evaluating membrane binding affinity

Considering that one of the primary functions of the myristoyl group switching to its accessible state is to promote anchoring of the protein to cellular membranes, it is of interest to have an assay which can evaluate affinity of hisactophilin mutants for model membranes. Liposomes are versatile and commonly used membrane-mimetics, coming in a variety of user-defined sizes ranging from small (20-100 nm), to large (>100 nm), and up to giant (>1000 nm).³⁸ Depending on the method of preparation, liposomes can have either a single bilayer (unilamellar) or several concentric bilayers (multilamellar). Additionally, the lipid composition of the liposomes used is at the discretion of the user, providing opportunity to explore the influence of lipid properties on protein-membrane binding interactions.

Two biophysical techniques commonly used for measuring protein-membrane binding interactions are surface-plasmon resonance (SPR) and isothermal titration calorimetry (ITC).³⁹ In principle, either of these methods should be suitable for studying interactions between hisactophilin mutants and liposomes. However, as there is precedence for the use of ITC in hisactophilin membrane binding studies, this method seems like a logical first choice.¹⁷ Liposome binding studies will provide additional insight into functional effects of the various mutations described throughout this work. It is predicted that mutants such as GGGG and SGGY, which are thought to be broken in the sequestered state, should have decreased affinity for liposomes compared to hisactophilin variants with functional pH-switches.

5.4 Design of a novel pH-switch in a non-switching architecture

The ultimate goal of this project is to demonstrate a comprehensive understanding of pH-switching mechanisms through the design of a novel pH-switching protein. In experimentally testing hypotheses of pH-switching mechanisms in hisactophilin, the first steps of this endeavour have been taken. Additionally, a suitable scaffold for introduction of pH-switching into has already been identified.

ThreeFoil is a 3-fold symmetric, ultra-stable β -trefoil protein designed by a previous member of the Meiering lab.⁴⁰ Sharing the same fold as hisactophilin, this protein is an ideal candidate for the reverse engineering a pH-dependent myristoyl switch (**Figure 5.1**). However, ThreeFoil lacks the structural features needed not only for switching, but at a more basic level also the features needed for sequestration of a myristoyl group. ThreeFoil is missing the myristoyl binding pocket seen in hisactophilin, primarily because of the bulkier aromatic core residues and more compact packing of ThreeFoil's core.⁴¹ Considering this, the first task in designing a scaffold for a pH-switch is to create a myristoyl binding pocket in ThreeFoil. Thankfully, the identity of core residues in a protein of the same fold that possesses a myristoyl binding pocket is known.

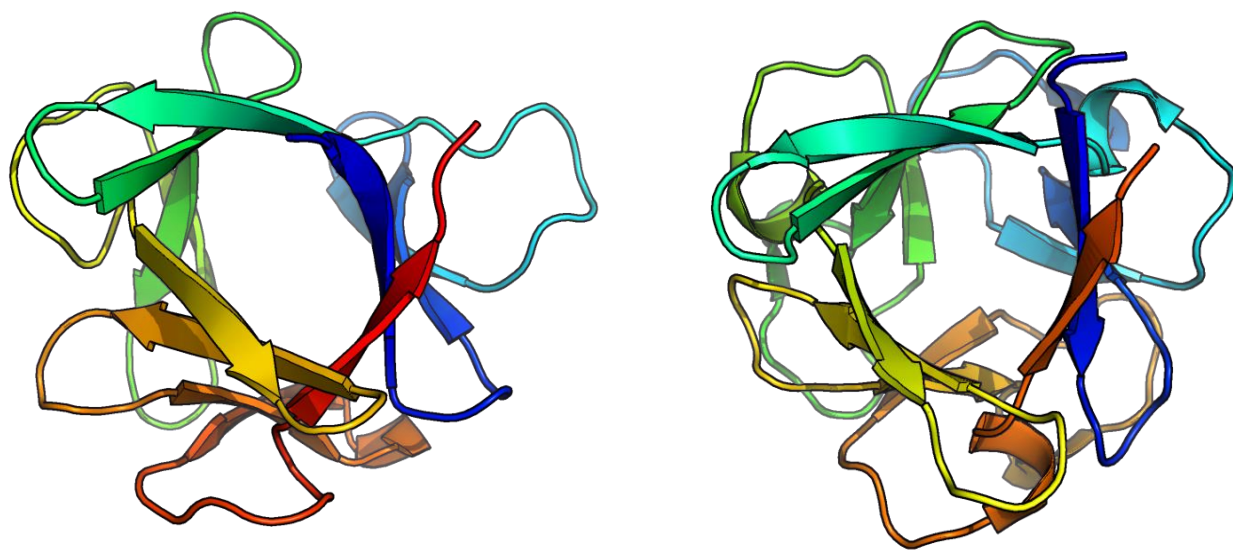


Figure 5.1: ThreeFoil is an extremely stable, 3-fold symmetric β -trefoil sharing the same fold as hisactophilin. Left: Structure of hisactophilin (1HCD). Right: Structure of ThreeFoil (3PG0). ThreeFoil lacks the myristoyl-binding pocket seen in the core of hisactophilin. Modifications of ThreeFoil's architecture will be made to introduce a myristoyl-binding pocket into the protein with the intention of eventually introducing pH-dependent switching of the myristoyl, as seen in hisactophilin.

In an effort to create space in the core of ThreeFoil for sequestration of a myristoyl group, the hydrophobic core residues of ThreeFoil will be replaced with those of hisactophilin. It is immediately clear that introducing a hole in the core of a protein will likely introduce instability, and so along with the coreswap mutations, several mutations previously identified to stabilize the fold will need to be introduced.⁴³

In addition to having a suitable binding pocket for a myristoyl group, the matter of having the protein be myristoylated presents an additional challenge. ThreeFoil is not naturally N-myristoylated, and so an additional consideration is the design of a N-myristoyltransferase recognition motif into this protein. Various recognition motifs for substrates of the N-myristoyltransferases have been proposed as discussed in section 1.3, and no single sequence motif is most obviously the best choice for introduction into ThreeFoil. However, it is known that hisactophilin is myristoylated by hNMT1, and so it stands to reason that the N-terminal sequence of hisactophilin may be sufficient for recognition by the enzyme when placed into other proteins.

Introduction of a myristoyl binding pocket and hNMT1 recognition sequence into ThreeFoil will prepare a scaffold for introduction of pH-switching into once the mechanism of pH-switching in hisactophilin has been fully revealed. By continuing to study pH-switching in hisactophilin, the understanding of how electrostatic interactions on the surfaces of proteins communicate with hydrophobic core residues will progress and open up new avenues for applications in protein engineering and design endeavours.

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Appendix

		Binomial Extrapolation Fit Parameters										
		Mutant	C _{mid}	Error	Y _n	Error	Y _u	Error	S _n	Error	S _u	Error
Low pH	Myr	Wildtype	2.96	0.03	2.27E+06	26934.26	3.81E+06	48259.85	-6564.43	22877.18	164676.3	7058.065
		D57G	2.39286	0.03965	2.43E+06	34484.16	3.27E+06	33250.48	-85236.7	33611.16	132399.9	5497.799
		H89G	2.48483	0.03267	1.86E+06	14323.72	2.70E+06	35864.5	-19730.2	15131.07	107614.6	6369.898
		H91Y	3.4679	0.04177	3.04E+06	21985.99	3.97E+06	62133.11	56751.23	14828.41	184378.3	9448.765
		H106S	2.54834	0.04657	1.89E+06	25626.17	2.93E+06	37711.61	20258.08	28646.8	154464.5	5910.57
		GGGG	2.74985	0.04258	2.01E+06	28838.74	3.13E+06	54032.97	75415.47	23883	180906.2	8439.295
		GSGGY	3.38686	0.09856	2.70E+06	50165.93	3.10E+06	83183.06	80836.17	29395.8	208943.4	12689.22
	Non-myr	Wildtype	2.27	0.05	2.46E+06	30307.31	3.42E+06	38381.72	-24543.1	37047.07	132461.2	5961.536
		D57G	1.8547	0.03431	3.73E+06	18791.19	4.96E+06	34052.66	-28040.7	33712.35	174575.5	6072.096
		H89G	1.84278	0.02759	3.17E+06	16891.86	4.20E+06	22027.88	-9176.05	27442.57	168959.5	3810.911
		H91Y	2.85919	0.05005	3.80E+06	22351.19	4.51E+06	40922.63	37805.42	22857.03	207306.2	6392.302
		H106S	1.91709	0.04002	3.01E+06	27314.98	4.22E+06	32987.58	-90551.2	45379.59	187473.4	5325.07
		GGGG	1.72326	0.07716	2.92E+06	32046.91	3.66E+06	36170.89	98678.24	58037.39	202592.3	6413.133
		GSGGY	2.45193	0.08883	3.80E+06	34752.01	4.33E+06	65369.23	63572.67	34881.71	214188.7	10501.95
High pH	Myr	Wildtype	5.49605	0.03924	2.04E+06	20110.27	4.00E+06	174311.9	45539.32	8119.447	117428.2	21642.6
		D57G	4.80473	0.05244	2.10E+06	17701.68	3.04E+06	119813.1	40611.84	7867.587	118718	15206.48
		H89G	5.29641	0.04428	1.61E+06	13776.06	2.62E+06	151290.4	23376.41	5137.991	85363.53	20676.32
		H91Y	5.59711	0.07462	2.50E+06	18809.81	3.57E+06	284860.2	39733.83	7000.55	97187.56	37266.59
		H106S	4.95604	0.05687	1.65E+06	19590.53	2.77E+06	130530	41652.93	8810.262	104183.7	16578.22
		GGGG	5.15877	0.051	1.74E+06	34181.09	3.21E+06	177326.8	54541.62	12797.16	137670.9	22448.22
		GSGGY	5.63632	0.09116	2.58E+06	17006.4	3.36E+06	229041.9	59352.34	7092.068	123717.6	27882.02
	Non-myr	Wildtype	3.93178	0.05359	2.27E+06	23465.16	2.90E+06	76697.73	2513.528	13784.95	168704.7	10607.53
		D57G	3.41182	0.03909	3.56E+06	17163.98	4.05E+06	55246.16	15286.53	12097.93	239459.2	7874.169
		H89G	4.00568	0.03956	2.42E+06	17534.85	2.95E+06	66090.43	39581.4	9988.643	199499.1	9786.409
		H91Y	4.26355	0.05907	3.49E+06	26899.33	4.00E+06	95211.22	25314.47	13908.41	178309.6	13197.39
		H106S	3.52135	0.05401	2.42E+06	22709.88	3.02E+06	54935.75	26893.05	15291.5	168892	8007.954
		GGGG	3.66775	0.05043	2.33E+06	30819.93	3.16E+06	66467.93	101345.9	20800.23	251597.7	9787.993
		GSGGY	4.22543	0.09174	3.83E+06	26347.69	3.96E+06	99110.67	33201.38	16085.42	242050.2	13146.6

$$m_1 = 2.03$$

$$m_2 = 0.072$$