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# Stabilizing and Destabilizing Effects of Phenylalanine → $F_5$-Phenylalanine Mutations on the Folding of a Small Protein

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Strategies that stabilize protein folding patterns and/or protein associations are useful for achieving biomolecular engineering goals. Amino-acid residues bearing fluorinated side chains have been explored for this purpose,[1] and several groups have reported that fluorination of saturated carbon atoms leads to enhanced tertiary and/or quaternary structural stability.[2] This stabilization has usually been attributed to enhanced hydrophobicity of fluorocarbons relative to hydrocarbons, and, where appropriate, to a preference for fluorocarbon−fluorocarbon interaction relative to fluorocarbon−hydrocarbon interaction. Little attention has been paid to the impact of fluorinated *aromatic* side chains on protein structure.[3] Phe → $F_5$-Phe mutation at buried sites could stabilize protein folding patterns via two distinct mechanisms. First, the mutant could benefit from the enhanced hydrophobicity of $F_5$-Phe.[4] Second, if the Phe side chain contacts other aromatic side chains in the native state, then aromatic−aromatic attraction (e.g., from quadrupolar interactions) could be enhanced in the mutant; the optimal $C_6H_6/C_6F_6$ stacking energy is estimated to be −3.7 kcal/mol.[5] On the other hand, the modest increase in size resulting from H → F replacements (molecular volumes: $C_6H_6$, 106 Å$^3$; $C_6F_5H$, 141 Å$^3$)[6] could cause destablization if steric repulsions develop. Because fluoroalkyl substitutions have stabilized higher-order protein structure in many cases,[1,2] it is important to evaluate the effects of fluoroaromatic substitutions.

Here we document the impact of Phe → $F_5$-Phe mutations on the conformational stability of the chicken villin headpiece subdomain (cVHP), a 35-residue module that adopts a discrete tertiary structure.[7] The nonpolar core features a cluster of three Phe side chains, from residues 6, 10, and 17 (Figure 1a). Examination of all Phe → $F_5$-Phe mutants involving the three core residues has allowed us to survey the effect of aryl → fluoroaryl replacement in several specific contexts and thus acquire a general appreciation of such modifications.

Backbone thioester exchange (BTE)[8] was used to compare conformational stabilities among all possible Phe/$F_5$-Phe combinations at positions 6, 10, and 17. BTE is implemented by replacing a single backbone amide linkage in a polypeptide with a thioester (i.e., a single α-amino-acid residue is replaced by an α-thio-acid residue). Conformational stability can then be probed by monitoring a thioester−thiol exchange process: equilibration between a full-length molecule and peptide fragments that cannot form a complete tertiary core.[9] Mixing t-cVHP (Figure 2) with the small thiol indicated in Figure 1c, for example, induces equilibration with a thioester/thiol pair that corresponds to N-terminal and C-terminal fragments of the protein (Figure 1b). The equilibrium constant ($K_{BTE}$) provides insight on the free energy of tertiary structure formation. We have previously shown that BTE is very useful for studying small protein mutagenesis,[8] which frequently causes incomplete folding; the conformational stability of partially folded structures can be challenging to quantify via traditional methods[10] but is readily assessed with BTE.

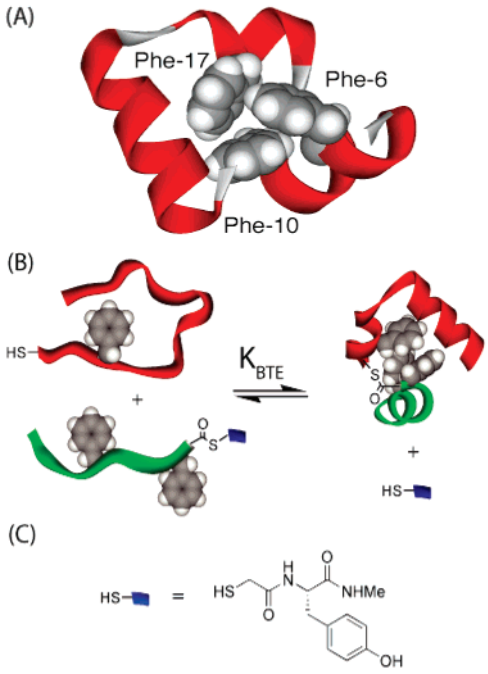


***Figure 1.*** (A) Ribbon diagram of folded cVHP,[6d] with helical backbone segments in red. The side chains of residues Phe-6, Phe-10, and Phe-17 are shown in space-filling format; all other side chains are omitted. Graphic B shows the backbone thioester exchange (BTE) process for t-cVHP (sequence in Figure 2). The thioester−thiol pair on the left is composed of fragments that contain residues 1−10 (green) and 11−35 (red), while the pair on the right contains full-length t-cVHP and a small thiol, the structure of which is shown in graphic C.

To implement BTE, we replaced the amide bond between Phe-10 and Gly-11 of cVHP with a thioester (Figure 2). Splitting the backbone at this point separates the first α-helical segment from the other two α-helices, which prevents tertiary folding. Preliminary studies, however, revealed that the cVHP-derived thioester was irreversibly depleted by acylation of a lysine side chain. This problem was eliminated by replacing all five lysine residues in cVHP with arginine. In order to evaluate the cumulative effects of the five Lys → Arg mutations plus the backbone amide → thioester replacement on cVHP conformational stability in the native state, we used classical methods to compare the conformational stability of three molecules: (1) cVHP, (2) the mutant with the five Lys → Arg modifications but a purely amide backbone (m-cVHP), and (3) the hybrid polypeptide containing the five side-chain modifications along with the thioester replacement in the backbone (t-cVHP) (Figure 2a). These three have similar circular dichroism (CD) signatures,[6] and all three molecules displayed cooperative denaturation upon addition of guanidinium chloride. Extrapolation of these data to zero GdmCl concentration[11] provided estimates of

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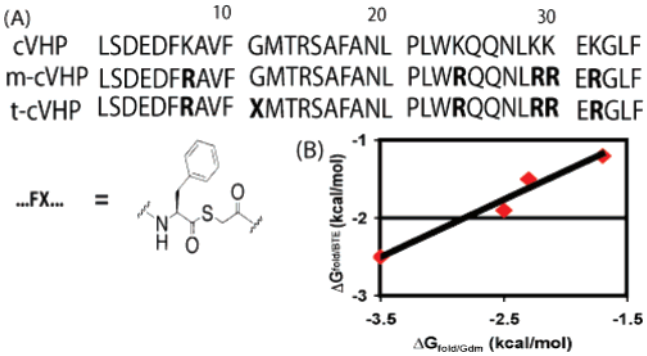


***Figure 2.*** (A) Sequence comparison for cVHP, m-cVHP, and t-cVHP; (B) correlation of $\Delta G_{fold/Gdm}$ determined for four polypeptides (cVHP and the three single Phe → $F_5$-Phe mutants at position 6, 10, or 17) and $\Delta G_{fold/BTE}$ determined for an analogous set of four thioester-containing molecules (t-cVHP and the three single Phe → $F_5$-Phe mutants at position 6, 10, or 17). Linear regression was used to determine the line of best fit.

***Table 1.*** Comparison of $\Delta G_{fold/BTE}$ for VHP-Derived Thioesters[a]

| peptide | $\Delta G_{fold/BTE}$ |
|---|---|
| t-cVHP | −1.9 |
| t-cVHP (Phe$_6$ → f$_5$-Phe) | −1.2 |
| t-cVHP (Phe$_{10}$ → f$_5$-Phe) | −2.5 |
| t-cVHP (Phe$_{17}$ → f$_5$-Phe) | −1.5 |
| t-cVHP (Phe$_{6,10}$ → f$_5$-Phe) | −1.7 |
| t-cVHP (Phe$_{6,17}$ → f$_5$-Phe) | −1.2 |
| t-cVHP (Phe$_{10,17}$ → f$_5$-Phe) | −1.2 |
| t-cVHP (Phe$_{6,10,17}$ → f$_5$-Phe | −1.1 |

[a] Values are reported in kcal/mol. We estimate the error in the BTE measurements to be ca. ±0.1 kcal/mol.

the free energies of folding ($\Delta G_{fold/Gdm}$). The five Lys → Arg mutations cause a moderate diminution of tertiary structural stability ($\Delta G_{fold/Gdm}$ = −3.3 kcal/mol for cVHP vs −2.5 kcal/mol for m-cVHP). The amide → thioester change, however, causes very little additional change in stability ($\Delta G_{fold/Gdm}$ = −2.2 kcal/mol for t-cVHP), consistent with previous BTE results.[8] These findings suggest that insights gathered via BTE analysis of t-cVHP and related molecules are relevant to the folding of analogues containing pure amide backbones (i.e., proteins).

HPLC was used to measure $K_{BTE}$ for t-cVHP (Figure 1b). By initiating thioester-thiol exchange from each side of the equation shown in Figure 1b, we could show that equilibrium was attained within 2.5 h (pH 7).[6] Tertiary folding occurs in t-cVHP under the reaction conditions, as verified by CD, but not in the other thioester−thiol pair because residues 1−10 and residues 11−35 reside in different molecules. $K_{BTE}$ can be used to calculate the free energy of tertiary folding ($\Delta G_{fold/BTE}$) in the full-length thioester, as previously described.[8] For t-cVHP, $\Delta G_{fold/BTE}$ = −1.9 kcal/mol, which is similar to $\Delta G_{fold/Gdm}$.

$\Delta G_{fold/BTE}$ was determined for all variants of t-cVHP containing one, two, or three Phe → $F_5$-Phe replacements at residues 6, 10, and/or 17 (Table 1). Each variant appeared to adopt a stable tertiary fold ($\Delta G_{fold/BTE}$ < 0), but only the Phe-10 → $F_5$-Phe mutant, displayed greater conformational stability than does t-cVHP. The range of $\Delta G_{fold/BTE}$ values (1.4 kcal/mol) is significant because it is comparable to the conformational stability of t-cVHP. We compared our VHP thioester results with data from a more traditional approach in an effort to validate the BTE method. Figure 2b shows a comparison of $\Delta G_{fold/BTE}$ for t-cVHP and its three single Phe → $F_5$-Phe mutants with $\Delta G_{fold/Gdm}$ for m-cVHP and its three single Phe → $F_5$-Phe mutants. The linear correlation shows that the conformational stability trend indicated for the thioester series by BTE faithfully mirrors that of the full-fledged polypeptides. In both series the Phe-10 → $F_5$-Phe mutant is most stably folded.[12]

Our systematic examination of all $F_5$-Phe variants involving core Phe residues in a small folded protein has revealed a specific mutation that enhances tertiary structural stability. Six of the seven $F_5$-Phe-containing mutants, however, proved to be *less* stably folded than the version containing the three native Phe residues. This trend differs from that seen with fluoroalkyl side chain substitutions, which have been stabilizing in several different systems.[2] Our observations suggest that it is difficult for the cVHP tertiary fold to take advantage of stabilization mechanisms that become available when $F_5$-Phe is introduced. The destabilizing effects could reflect steric repulsions that arise from the increase in side-chain volume and/or from minor conformational rearrangements that might be necessary for harnessing improved side-chain interactions. Identifying the precise mechanisms of destabilization induced by each mutation pattern may be difficult or impossible, as is also generally the case with stabilizing mutations. Nevertheless, our results are valuable in the context of protein engineering because they show that introduction of fluoroaryl side chains can stabilize folded conformations, but that stabilization is not a general effect of Phe → $F_5$-Phe mutation.

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**Supporting Information Available:** Experimental details, CD analysis, chemical denaturation data, and HPLC chromatograms. This material is available free of charge via the Internet at http://pubs.acs.org.

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