FOR THE RECORD

Toward quantification of protein backbone–backbone hydrogen bonding energies: An energetic analysis of an amide-to-ester mutation in an α -helix within a protein

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Abstract

Amide-to-ester backbone mutagenesis enables a specific backbone–backbone hydrogen bond (H-bond) in a protein to be eliminated in order to quantify its energetic contribution to protein folding. To extract a H-bonding free energy from an amide-to-ester perturbation free energy ($\Delta G_{\text{folding,wt}} - \Delta G_{\text{folding,mut}}$), it is necessary to correct for the putative introduction of a lone pair–lone pair electrostatic repulsion, as well as for the transfer free energy differences that may arise between the all amide sequence and the predominantly amide sequence harboring an ester bond. Mutation of the 9–10 amide bond within the V9F variant of the predominantly helical villin headpiece subdomain (HP35) to an ester or an *E*-olefin backbone bond results in a less stable but defined wild-type fold, an attribute required for this study. Comparing the folding free energies of the ester and *E*-olefin mutants, with correction for the desolvation free energy differences (ester and *E*-olefin) and the loss of an *n*-to- π^* interaction (*E*-olefin), yields an experimentally based estimate of +0.4 kcal/mol for the O–O repulsion energy in an α -helical context, analogous to our previous experimentally based estimate of the O–O repulsion free energy in the context of a β -sheet. The small O–O repulsion energy indicates that amide-to-ester perturbation free energies can largely be attributed to the deletion of the backbone H-bonds after correction for desolvation differences. Quantitative evaluation of H-bonding in an α -helix should now be possible, an important step toward deciphering the balance of forces that enable spontaneous protein folding.

Keywords: villin headpiece subdomain; O–O repulsion; *n*-to- π^* interaction; amide-to-ester mutation; amide-to-*E*-olefin mutation; backbone hydrogen bonding

Accurate quantification of the forces governing protein folding and the nonadditive interactions between these, if any, must be understood before a rigorous understanding of protein folding can be achieved (Anfinsen 1973; Dill 1990; Deechongkit et al. 2004b). Backbone–backbone

H-bonding is a characteristic feature of all well-defined protein structures. Statistical data reveals that a folded protein forms 1.1 H-bonds per residue, the majority of which (~70%) are between backbone amide bonds (Myers and Pace 1996). The backbone amide carbonyl can accept two H-bonds through its two oxygen lone pairs, whereas the amide NH can only donate one H-bond. The energetic contribution of backbone–backbone H-bonding to protein folding is only beginning to become clear (Rose et al. 2006), partly owing to the recently introduced methodology enabling amide-to-ester (A-to-E) and amide-to-*E*-olefin (A-to-O) mutations to be introduced

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that eliminate specific backbone H-bonds (Koh et al. 1997; Blankenship et al. 2002; Deechongkit et al. 2004a; Yang et al. 2004).

A-to-E mutagenesis can be conveniently accomplished by replacing the α -amino acid donating the NH of the H-bond of interest with its α -hydroxy acid equivalent during stepwise solid phase peptide synthesis or semisynthesis (Blankenship et al. 2002; Spengler et al. 2007). Methodology for the facile synthesis of the nineteen α -hydroxy acid counterparts of the α -amino acids has been recently reported (Deechongkit et al. 2004c). Therefore, an ester moiety can be introduced at the desired backbone position while maintaining side-chain structure and configuration. A-to-E variants of proteins less than 50 residues in length are conveniently prepared by stepwise solid phase peptide synthesis. A-to-E variants of proteins up to a few hundred amino acids can be conveniently prepared by native chemical ligation or expressed protein ligation, wherein the coupling fragment bearing the A-to-E mutation is prepared by solid phase peptide synthesis. At present, utilization of A-to-O backbone mutagenesis is more challenging because dipeptide isosteres need to be chemically synthesized using methodology that is not yet efficient enough (Wang et al. 2003; Fu et al. 2005) to make all the mutations in a protein that one would want to make.

An A-to-E mutation eliminates the amide NH–H-bond donor and renders the ester carbonyl a weaker, but functional acceptor. The A-to-O mutation deletes both the H-bond acceptor and the donor, eliminating as many as three backbone H-bonds in a folded protein structure (Fig. 1A; Powers et al. 2005). A comparison of the folding free energies of the A-to-E and A-to-O backbone mutants to the all amide sequence, with appropriate corrections, allows estimation of the H-bond energies.

Our understanding of which corrections need to be made to convert a perturbation free energy from an amide-to-ester mutant ($\Delta G_{\text{folding,wt}} - \Delta G_{\text{folding,A-to-E mut}}$) into a H-bond energy is still in its infancy (Deechongkit et al. 2004a; Wang et al. 2006). The extent to which one needs to correct for the differences in transferring an amide, ester, or *E*-olefin bond from a solvated unfolded ensemble to a folded ensemble is determined by the extent to which the amide bond or its variants are desolvated in the folding process, and the extent to which proximal side chains contribute. An A-to-E mutation is also thought to introduce a new electrostatic repulsion perturbation (Fig. 1A, left), which may need to be corrected for. This so-called O–O repulsion is between the lone pairs of the ester oxygen atom replacing the amide NH and the lone pairs on the proximal amide carbonyl oxygen that served as the H-bond acceptor in the all-amide sequence. The magnitude of the O–O repulsion, especially in a helix, is unclear, and determining this is

Figure 1. (A) Illustration of backbone H-bond elimination with A-to-E and A-to-O mutations, with the red line highlighting the potential O–O repulsion in the ester mutant protein. (B) Experimental design for estimating the O–O repulsion energy in HP35. The structure of HP35 displays three helices shown in the ribbon presentation (PDB ID: 1YRF). The backbone atoms are shown in stick presentation with carbons in green, hydrogen atoms in white, nitrogen atoms in blue, and oxygen atoms in red. The NH of the 9-10-amide is H-bonded (the thick broken line in blue), while the CO is not H-bonded, but is the acceptor of an *n*-to- π^* interaction (the magenta arrow) from the carbonyl oxygen of the preceding residue. A-to-E mutation of the 9-10-amide deletes the indicated backbone–backbone H-bond and introduces an O–O repulsion, while the A-to-O mutation deletes the same backbone–backbone H-bond and also eliminates the *n*-to- π^* interaction. (C) Schematic illustration of the *n*-to- π^* interaction in the context of an α -helix. The magenta arrow highlights the lone pair of O_{i-1} donating electron density into the subsequent anti-bonding carbonyl π^* orbital.

the main focus of this report. The *n*-to- π^* interaction between the carbonyl oxygen atom of the peptide bond (O_{i-1}) and the subsequent carbonyl carbon (Fig. 1B,C) is energetically significant in stabilizing helical structures and its loss in the case of A-to-O, but not A-to-E, will have to be corrected for (Hinderaker and Raines 2003; Hodges and Raines 2006; Horng and Raines 2006; Raines 2006). Based on the relative binding affinity of vancomycin for two peptidomimetic compounds, where an amide bond is replaced by an ester and ketomethylene group, the O–O repulsion has been estimated to be 2.6 kcal/mol (McComas et al. 2003). A computational study of a polyalanine peptide harboring backbone mutations (ester vs. ketomethylene) estimated the O–O repulsion energy to range between 1.5 to 2.4 kcal/mol (Cieplak and Surmeli 2004). However, it is questionable whether the ketomethylene group can be used as an amide bond mimic, considering their distinct conformational preferences. Recently, we quantified the O–O repulsion energy

in a β -sheet by replacing an amide bond in the Pin WW domain with an ester and an *E*-olefin moiety, which closely mimic the size and conformation of the amide bond. The O–O repulsion energy was estimated to be only $\approx +0.3$ kcal/mol in the context of an antiparallel β -sheet structure (Fu et al. 2006), after correcting for desolvation free energy differences. It is important to note that we estimated the O–O repulsion energy without correction for the loss of the *n*-to- π^* interaction (Fu et al. 2006) because it does not appear to be a significantly stabilizing force in β -sheet structures (Hinderaker and Raines 2003).

Here, we report that the O–O repulsion energy in the context of an α -helix is $+0.4$ kcal/mol. The results show that an A-to-E mutation in an α -helix yields a comparable O–O repulsion energy to that observed in a β -sheet. The small O–O repulsion energy indicates that the measured A-to-E perturbation energy, with correction for transfer free energy differences, largely reflects the intrinsic energy of the eliminated backbone H-bond.

Results and Discussion

The villin headpiece from the F actin-binding domain of super villin has a C-terminal subdomain composed of 35 amino acids (HP35), which folds spontaneously into a three helix tertiary structure surrounding a hydrophobic core composed of three phenylalanine residues (Fig. 1B; McKnight et al. 1996). The small size, well-defined structure, and high thermodynamic stability of HP35 have resulted in this subdomain being widely used for protein folding studies (Bi et al. 2007). The crystal structure of HP35 shows that the NH group of Phe10 forms a H-bond with the CO of Phe6 (Fig. 1B), while the CO of Val9 does not engage in H-bonding and is solvent exposed (Fig. 1B; Chiu et al. 2005). Mutation of the amide bond linking Val9 and Phe10 to an ester or an *E*-olefin eliminates a single H-bond. While the A-to-E mutation may result in O–O repulsion, the A-to-O mutation does not introduce this potential electrostatic repulsion. Recent literature reveals that the *n*-to- π^* interaction between the carbonyl oxygen atom of the peptide bond (O_{i-1}) and the subsequent carbonyl carbon is stabilizing in helical structures (Fig. 1B,C; Hinderaker and Raines 2003; Hodges and Raines 2006; Horng and Raines 2006; Raines 2006). The *n*-to- π^* interaction has been estimated to be -0.7 kcal/mol (Raines 2006). The A-to-O mutation in the α -helical context, with the carbonyl deleted, eliminates this stabilizing force, while the A-to-E mutation does not. A comparison of the folding free energies of the ester mutant and the *E*-olefin mutant, with correction for the elimination of the *n*-to- π^* interaction in the A-to-O mutant and allowance for the desolvation energy differences (ester vs. *E*-olefin), allows us to experimentally

estimate the O–O repulsion energy in the ester mutant in the context of an α -helix.

A concise synthetic route for the preparation of the Phe–Phe *E*-olefin dipeptide isostere was previously reported by our group (Fu et al. 2005). To utilize this dipeptide isostere for this study, a side-chain mutation, V9F, was introduced into wild-type HP35. Molecular modeling indicates that the V9F mutation can easily be accommodated without introducing steric clashes. Circular dichroism and 1D NMR spectroscopic analysis of the HP35–V9F variant indicates that it adopts a native tertiary structure (Fig. 2A,B, respectively). Thermal denaturation analysis yields a thermal melting temperature (T_m) of 71°C for V9F HP35, in close agreement with that of WT HP35 (69°C) (Kubelka et al. 2003). The thermodynamic stability of HP35 V9F was extracted from guanidine hydrochloride denaturation curves, which yields a folding free energy ΔG_f of -3.3 kcal/mol (Table 1), identical to that exhibited by WT HP35 (McKnight et al. 1996).

The 9–10 A-to-E and A-to-O backbone mutants of HP35 were synthesized using stepwise solid-phase peptide synthesis employing the Boc/benzyl strategy (Schnolzer et al. 1992). The amide bond linking Phe9 and Phe10 was mutated to an ester and an *E*-olefin moiety, and these mutants were denoted as HP35 V9F 9–10-ester and HP35 V9F 9–10-olefin, respectively. Far-UV circular dichroism spectroscopy analysis revealed that both backbone mutants adopt a predominantly α -helical structure, consistent with the characteristic minima at 208 nm and 222 nm (Fig. 2A). The molar ellipticity is slightly less negative for the HP35 9–10-ester and HP35 9–10-olefin variants relative to the all amide sequences, presumably owing to the deletion of an amide bond. 1D proton NMR spectra of the backbone mutants exhibit sharp and well-dispersed resonances similar to that of HP35, indicating well-folded native-like structures (Fig. 2B). According to the thermal and chaotrope denaturation studies (Fig. 2C), both HP35 V9F 9–10-ester and HP35 V9F 9–10-olefin exhibit apparent two-state folding/unfolding behavior. Therefore, the folding free energies (Table 1) were easily quantified by fitting the guanidine hydrochloride denaturation curves.

As expected, deletion of the backbone H-bond in the HP35 V9F 9–10-ester and HP35 V9F 9–10-olefin leads to lower thermal and thermodynamic stability than that exhibited by the all amide sequence HP35–V9F. The T_m for HP35 V9F 9–10-ester and HP35 V9F 9–10-olefin are $>10^\circ\text{C}$ lower than that for the HP35 V9F sequence. In terms of the folding free energies (ΔG_f), HP35 V9F 9–10-ester and HP35 V9F 9–10-olefin are less stable than HP35 V9F by 1.5 kcal/mol and 1.6 kcal/mol, respectively (Table 1). To extract the O–O repulsion energy, the desolvation energy difference (ester vs. *E*-olefin) and

Figure 2. Spectroscopic and thermodynamic analysis of wild-type HP35 and its V9F variants. (A) Circular dichroism spectra of HP35 and its variants exhibit minima at 208 and 222 nm, characteristics of α -helical structures. (B) 1D ^1H -NMR spectra display sharp and well-dispersed resonances for the backbone mutants (HP35 V9F 9-10-ester and HP35 V9F 9-10-olefin), indicating well-folded native-like structures. (C) Guanidine hydrochloride denaturation curves of HP35 variants, exhibiting cooperative transitions. Curve-fitting according to the two-state model allows quantification of the folding free energies of the HP35 variants.

Table 1. Thermodynamic characterization of HP35 variants

	T_m ($^{\circ}\text{C}$)	C_m (M)	m value (kcal/[mol·M])	ΔG_f (kcal/mol)
HP35 V9F	71	3.4	0.98	-3.3 ± 0.1
HP35 V9F 9-10-ester	58	1.7	0.99	-1.8 ± 0.1
HP35 V9F 9-10-olefin	48	1.6	0.99	-1.7 ± 0.1

the energy of the n -to- π^* interaction need to be corrected for using the following equation (Fu et al. 2006):

$$\Delta G_{\text{O-Orep}} = \Delta G_{f, \text{HP35 V9F 9-10-ester}} - \Delta G_{f, \text{HP35 V9F 9-10-olefin}} - 0.5\Delta\Delta G_{\text{desolvation, ester vs. olefin}} - \Delta G_{n\text{-to-}\pi^*}$$

where, $\Delta G_{\text{O-Orep}}$ represents the O–O repulsion energy; $\Delta G_{f, \text{HP35 V9F 9-10-ester}}$ and $\Delta G_{f, \text{HP35 V9F 9-10-olefin}}$ represent the folding free energies of the HP35 V9F 9-10-ester mutant, and the HP35 V9F 9-10-olefin mutant, respectively; $\Delta\Delta G_{\text{desolvation, ester vs. olefin}}$ represents the desolvation energy of an ester minus that of an *E*-olefin moiety (see below); a factor of 0.5 is included in the desolvation energy term to reflect the fact that only half of 9-10 linkage (–O– of the ester and –C= of the olefin) is buried upon HP35 folding, while the other half (–CO– of the ester and –C= of the olefin) of the functional group remains solvated. $\Delta G_{n\text{-to-}\pi^*}$ represents the energy of the n -to- π^* interaction, which is eliminated in the A-to-O mutation, but not in the A-to-E mutation. Considering that the backbone desolvation energy may depend on the neighboring side chains (Connelly et al. 1993), we previously measured the relevant desolvation energy difference between an ester and an *E*-olefin moiety in the Phe–Phe dipeptide context to be +0.5 kcal/mol (Fu et al. 2006). Briefly, an uncharged Phe–Phe dipeptide derivative and its isosteres harboring an ester or *E*-olefin linkages were synthesized. The structures of these compounds enabled their water/octanol partition coefficients to be measured. Octanol is commonly used as a protein interior mimic (Wimley et al. 1996); therefore, the water-to-octanol transfer free energies were used to estimate the desolvation energy differences associated with partial burial of the 9-10 amide, ester, and *E*-olefin linkers during HP35 folding. The value of –0.7 kcal/mol reported by Raines (2006) is used to correct for the lack of a stabilizing n -to- π^* interaction in the A-to-O variant. Solving the equation for $\Delta G_{\text{O-Orep}}$ affords an experimentally based estimate of +0.4 kcal/mol, nearly identical to the value of +0.3 kcal/mol for the O–O repulsion in the context of an antiparallel β -sheet.

A survey of protein structures reveals that the average distance of backbone–backbone H-bonds, measured from the hydrogen atom of the amide NH to the amide carbonyl oxygen, is only slightly longer in α -helices (2.06 Å) than

in β -sheets (1.96 Å). Given the electrostatic nature of the O–O repulsion, it is perhaps not surprising that comparable O–O repulsion energies are observed in α -helices and β -sheets. The magnitude of the O–O repulsion energy is consistently small in both the α -helix and β -sheet context, indicating that the perturbation free energy associated with an A-to-E mutation in a protein is largely a consequence of backbone hydrogen bond energies after correction for transfer free energy differences. With the estimated value of the O–O repulsion and the desolvation energy difference between an amide and an ester now available, the H-bonding energy can be estimated from the A-to-E perturbation free energy data according to the following equation:

$$\Delta G_{\text{H-bond}} = \Delta G_{\text{f, HP35}} - \Delta G_{\text{f, HP35 V9F9-10-ester}} - 0.5\Delta\Delta G_{\text{desolvation, amide vs. ester}} + \Delta G_{\text{O-Orep}}$$

We previously determined the desolvation energy difference between an amide and an ester ($\Delta\Delta G_{\text{desolvation, amide vs. ester}}$) to be 0.5 kcal/mol in the Phe–Phe dipeptide context (Fu et al. 2006). Thus, the energy of the H-bond formed between F10 and F6 is estimated to be -1.4 kcal/mol, which is in close agreement with previously reported peptide bond enthalpy values (0.9–1.3 kcal/mol) measured by calorimetric studies (Scholtz et al. 1991; Lopez et al. 2002). It is noteworthy that the H-bond formed by F10 and F6 in HP35 is located at the end of the helix. Solvent accessibility and backbone flexibility at this particular H-bond may be different from those in the middle of α -helices. Further experiments will be needed to quantitatively evaluate the context dependent H-bonding energies in α -helical structures.

Materials and Methods

Chemical synthesis of HP35 variants

The all-amide sequences of HP35 were synthesized on an ABI 433A peptide synthesizer employing Fmoc/t-Bu chemistry. The backbone mutants were synthesized manually through Boc/Benzyl chemistry according to a previously published procedure (Schnolzer et al. 1992). The ester mutant HP35-ester was synthesized using unprotected L-(–)-3-phenyllactic acid as a building block. The E-olefin moiety is introduced into HP35-olefin by incorporating the pre-synthesized Phe–Phe E-olefin dipeptide isostere. The fully deprotected polypeptides were purified by reverse phase HPLC to be >95% in purity and the chemical identity confirmed by ESI mass spectrometry.

Characterization of HP35 variants

Circular dichroism spectra of all protein samples were collected using an Aviv model 202SF circular dichroism spectrometer equipped with a cell holder with a Peltier temperature controller (Hellma). Far-UV CD spectra were recorded from 200 to 250 nm at 2°C. The peptide sample was dissolved in 20 mM sodium

phosphate (pH 7.0). Thermal denaturation was monitored at 222 nm. The temperature range utilized was from 2°C to 98°C with a 2°C step size and a 90 s equilibration time. The signal was averaged for 30 s at each temperature. After the highest temperature was reached, the sample was cooled to 2°C and another full CD spectrum was measured to ensure that folding was reversible. The fraction of unfolded HP35 was determined using the baseline extrapolation method. The T_m value was determined from the fraction-unfolding curve (the T_m value was taken to be the temperature at which the fraction unfolded = 0.5).

The folding free energies were quantified through guanidine hydrochloride denaturation experiments. Two solutions were prepared. The first being 10 μ M protein in 20 mM sodium phosphate buffer at pH 7.0, and the second being 10 μ M protein in 7 M GuHCl (20 mM sodium phosphate buffer at pH 7.0). The second solution was added to the first in steps while the protein concentration remained constant. The denaturation process was monitored by recording the ellipticity at 222 nm. The denaturation curves were analyzed assuming two-state behavior using a previously described method (Pace et al. 2001).

The wild-type and the backbone variants of HP35 were analyzed by ^1H NMR using data collected on a Bruker Avance 500 MHz spectrometer. The spectra were acquired at 10°C with 500 μ M protein in 20 mM sodium phosphate buffer at pH 7.0 (10% D_2O). Water suppression was achieved using the Watergate pulse sequence.

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