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Amide-to-*E*-Olefin versus Amide-to-Ester Backbone H-Bond Perturbations: Evaluating the O−O Repulsion for Extracting H-Bond Energies

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There is growing interest in deciphering the contributions of backbone−backbone hydrogen bonding (H-bonding) to protein folding energetics.[1] Since backbone H-bonds are formed between main chain amides (Figure 1A), they can be perturbed by replacing the amide bond of interest in a protein with an isostructural moiety which has reduced or lacks H-bonding capacity. Currently, the most convenient approach to perturb backbone H-bonding is to replace amides with esters (Figure 1A).[2] An amide-to-ester (A-to-E) mutation eliminates the H-bond donor (N−H) and weakens the H-bond acceptor (C═O). A-to-E mutations are conservative in that the *trans* conformation of the linkage is maintained, as well as the ϕ, ψ dihedral angle preference of the flanking substructure. One concern with this approach is the possible electrostatic repulsion introduced between the O replacing the NH and carbonyl oxygen of the acceptor amide (Figure 1A, red line). The magnitude of this O−O repulsion is unclear, which complicates the extraction of H-bond energies from A-to-E perturbation thermodynamic data.[1d,2c,d] It has been proposed that an amide-to-*E*-olefin (A-to-O) mutation is the ideal peptide bond perturbation.[3] An A-to-O mutation in a protein eliminates one H-bond donor (NH) and one H-bond acceptor (CO) without introducing electrostatic repulsions. However, this strategy has rarely been realized due to the difficulties associated with the stereospecific synthesis of alkene-containing isosteres and incorporating them into proteins. Recently, our group has reported a convenient protocol for the preparation of the Phe−Phe *E*-olefin dipeptide isostere and its incorporation into proteins.[4] Herein, we report perturbation of the Phe22−Phe23 amide bond in the Pin WW domain employing both A-to-O and A-to-E mutations (Figure 1B). An energetic comparison of the ester mutant and *E*-olefin mutant enables us to quantify the repulsive O−O interaction introduced by A-to-E mutations and to establish the H-bond energy.

The Pin WW domain, the ligand binding domain of the human Pin 1 protein, is one of the smallest and best-studied β-sheet proteins.[2c,5,6] It is a 34-residue polypeptide that folds into a twisted three-stranded antiparallel β-sheet structure (Figure 1B). Mutational studies show that the Pin WW domain is highly tolerant to side chain mutations at nearly every position.[6] Taking advantage of this fact, we carried out this study with the Val22Phe/Tyr23Phe variant of the Pin WW domain, because we had already prepared the Phe−Phe *E*-olefin dipeptide isostere required to perform the A-to-O mutation. The V22F/Y23F mutant is slightly destabilized compared to wild-type (wt) Pin, with the folding free energy lower by 0.7 kcal/mol (Table 1). Far-UV circular dichroism spectroscopy, fluorescence spectroscopy, and NMR (vide infra, Figure 2) data show that the double mutant reversibly folds into the same β-sheet structure as the wt WW domain (Supporting Information).

The NH of F23 makes a H-bond to the carbonyl of R14 (Figure 1B), while the CO of F22 is exposed to solvent, according to solution and solid state structural data.[7] This particular H-bond formed by F23 and R14 was perturbed by applying A-to-E and A-to-O mutations to the amide bond comprising F22 and F23. Both

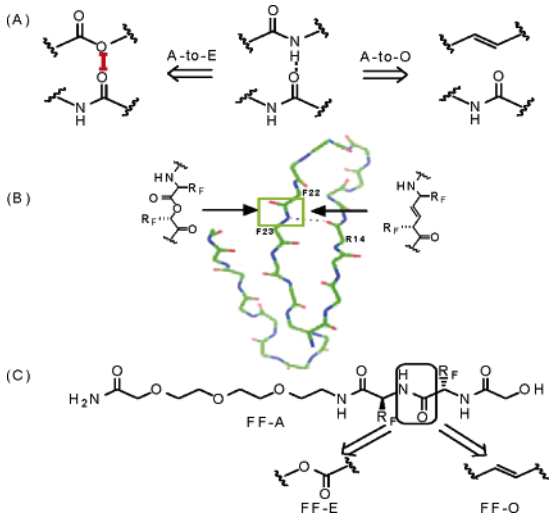

Figure 1. (A) The backbone A-to-E mutation eliminates a H-bond donor and weakens the acceptor. The A-to-O mutation eliminates both the H-bond donor and acceptor. (B) The backbone amide perturbation strategy in the Pin WW domain, wherein a H-bond donor is removed using both A-to-E and A-to-O mutations. (C) Structure of the Phe−Phe dipeptide derivatives designed to evaluate the desolvation energy differences between the amide, ester, and *E*-olefin derivatives. PEG fragments were appended to increase water solubility.

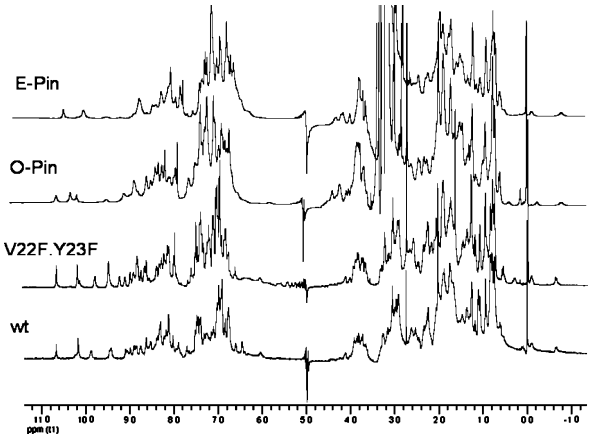

Figure 2. ^{1}H NMR spectra (500 MHz, 283 K) of the wt Pin WW domain and variants thereof. Spectra of all variants exhibit two characteristic upfield-shifted native state resonances, assigned below in the text. Protein samples were prepared in 20 mM sodium phosphate buffer, pH 7.2, H_2O/D_2O 9:1 (v/v); 1.0 M TMAO was added to the buffer of E-Pin and O-Pin to ensure complete folding.

ester and olefin-containing Pin WW domain variants, referred to as E-Pin and O-Pin hereafter, were prepared by manual solid-phase peptide synthesis utilizing the Boc/benzyl strategy. The ester bond in E-Pin was introduced by using the α-hydroxy acid equivalent of phenylalanine-23 as a building block.[2c] The Phe−Phe *E*-olefin

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Table 1. Thermodynamic Parameters of the Pin WW Domain Variants

protein	T_m[a] (°C)	*m* value[b] (kcal/(mol·M))	ΔG_f[b] (kcal/mol)	$\Delta\Delta G_f$ (kcal/mol)
wt Pin	59.8	1.04	-3.3 ± 0.1	0
V22F/Y23F	45.9	0.84	-2.6 ± 0.1	0.7 ± 0.1[c]
O-Pin	27.6	1.51	-1.8 ± 0.1	0.8 ± 0.1[d]
E-Pin	20.0	1.55	-1.3 ± 0.1	1.3 ± 0.1[d]

[a] Calculated by fitting thermal denaturation curves. [b] Calculated by fitting chaotrope denaturation curves. [c] For the control protein, $\Delta\Delta G_f = \Delta G_{f,V22F/Y23F} - \Delta G_{f,wt}$. [d] For the O-Pin and E-Pin, $\Delta\Delta G_f = \Delta G_{f,mut} - \Delta G_{f,V22F/YF23F}$.

dipeptide isostere was incorporated into the O-Pin chain in place of F22−F23 according to the reported protocol.[4] HF cleavage afforded the crude peptides, which were purified by RP-HPLC and characterized by MALDI-TOF mass spectrometry.

The backbone mutants were subjected to spectroscopic analysis to ensure that they adopt a normally folded structure. Both E-Pin and O-Pin exhibit a far-UV CD maximum at 227 nm (Figure S1) and a fluorescence emission maximum at 342 nm upon excitation at 295 nm (Figure S2), characteristics of the three-stranded β-sheet structure of the wt and the V22F/Y23F WW domains. Most importantly, the ^{1}H NMR spectra of E-Pin and O-Pin display dispersed resonances in both the amide and aliphatic regions, similar to those of wt and V22F/Y23F Pin WW domains (Figure 2). The spectra of the mutants also exhibit two small upfield resonances (−0.1, −0.6 ppm) originating from the Pro37 C^γ protons shielded by Trp11 and the N26 C^β proton shielded by Phe25, respectively, interactions characteristic of the folded state (Figure S5). The E-Pin and O-Pin variants, eliminating one H-bond in the hydrophobic core, are less stable than V22F/Y23F Pin as indicated by the melting temperature (T_m) and the folding free energy (ΔG_f) derived from chaotrope-induced denaturation curves (Table 1, Figure S3). O-Pin exhibits a 0.8 kcal/mol decrease in stability, while E-Pin is 1.3 kcal/mol less stable than the V22F/Y23F variant.

The strength of the proposed O−O repulsion (ΔG_{O-Orep}, Figure 1A) can be estimated by the following equation (for a detailed derivation, see the Supporting Information):

$$\Delta G_{O-Orep} = \Delta\Delta G_{f,E-Pin} - \Delta\Delta G_{f,O-Pin} - 0.5(\Delta G_{t,COO} - \Delta G_{t,-CHCH-})$$

where, $\Delta\Delta G_{f,E-Pin}$ and $\Delta\Delta G_{f,O-Pin}$ represent the folding free energy difference for E-Pin and O-Pin compared with Pin WW domain variant V22F/Y23F; $\Delta G_{t,COO}$ and $\Delta G_{t,-CHCH-}$ represent the desolvation energy of the ester bond and *E*-olefin bond, respectively. The desolvation energies can be estimated from the water-to-octanol transfer free energies, with octanol mimicking a protein interior.[8] To measure the relevant transfer free energies in the context of the F22−F23 substructure in the Pin WW domain, a Phe−Phe dipeptide derivative, FF-A (Figure 1C), and the corresponding ester (FF-E) and *E*-olefin (FF-O) isosteres were synthesized. The molecules include a short PEG fragment attached to the core structure to ensure sufficient water solubility so that their partitioning between water and octanol can be accurately evaluated. Measured partition coefficients of FF-A, FF-E, and FF-O remained constant over a broad concentration range (10 μM to 1 mM), indicating that aggregation was not occurring. The transfer free energies, calculated from their partition coefficients, are −1.4, −1.9, and −2.4 kcal/mol for FF-A, FF-E, and FF-O, respectively. On the basis of these data, the term ($\Delta G_{t,COO} - \Delta G_{t,-CHCH-}$) is calculated to be 0.5 kcal/mol. Therefore, the equation for ΔG_{O-Orep} is solvable, affording a value of 0.3 kcal/mol, which allows us to calculate the perturbed H-bond strength to be 1.3 kcal/mol.[1d]

The determined value of the O−O repulsion here is small, consistent with some previously reported data allowing a rough estimate of ΔG_{O-Orep}.[9] However, a larger value of 2.6 kcal/mol for the O−O repulsion free energy has been reported by comparing the binding affinity of vancomycin for two peptidomimetic compounds, where an amide bond is replaced by an ester and ketomethylene.[10] A value of 2.6 kcal/mol may be an overestimate of the repulsion energy because it was not corrected for solvation/desolvation energies. In addition, the ketomethylene moiety is more flexible than an amide bond; therefore, conformational alterations are possible. It is also possible that the O−O interaction in a folded β-sheet protein might be different from that observed in vancomycin/peptidomimetic complexes.

In summary, we report backbone perturbations in a β-sheet protein employing A-to-E and A-to-O mutations. Both mutations, deleting the same H-bond in the hydrophobic core, lead to a pronounced decrease in protein stability. The folding free energies of the ester and olefin mutants, together with the transfer free energies measured on relevant model compounds, afford an estimation of 0.3 kcal/mol for the O−O electrostatic repulsion term in the context of a β-sheet H-bond network. The determined value of ΔG_{O-Orep} should enable more accurate H-bond strength measurements utilizing A-to-E mutations. From this data, the H-bond between F23 and R14 in the Pin WW domain is determined to be worth 1.3 kcal/mol.

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Supporting Information Available: Procedures for protein synthesis, far-UV CD and fluorescence spectroscopy of the WW variants, and chaotrope denaturation curve acquisition and data extraction. This material is available free of charge via the Internet at http://pubs.acs.org.

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