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Stapling of two PEGylated side chains increases the conformational stability of the WW domain *via* an entropic effect†

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Hydrocarbon stapling and PEGylation are distinct strategies for enhancing the conformational stability and/or pharmacokinetic properties of peptide and protein drugs. Here we combine these approaches by incorporating asparagine-linked *O*-allyl PEG oligomers at two positions within the β-sheet protein WW, followed by stapling of the PEGs *via* olefin metathesis. The impact of stapling two sites that are close in primary sequence is small relative to the impact of PEGylation alone and depends strongly on PEG length. In contrast, stapling of two PEGs that are far apart in primary sequence but close in tertiary structure provides substantially more stabilization, derived mostly from an entropic effect. Comparison of PEGylation + stapling *vs.* alkylation + stapling at the same positions in WW reveals that both approaches provide similar overall levels of conformational stability.

Introduction

Conjugating polyethyleneglycol to proteins (*i.e.* PEGylation) is a well-known strategy for improving the stability and pharmacokinetic properties of peptide and protein drugs.[1–3] The benefits of PEGylation are thought to derive mostly from the large hydrodynamic radius of PEG,[4,5] which increases the serum half-life of the PEG–protein conjugate relative to its non-PEGylated counterpart by slowing renal clearance. PEG also shields proteins from aggregation, proteolysis, and antibody-epitope recognition. Early PEGylation efforts used low-specificity reactions that generated heterogeneous mixtures of PEG–protein conjugates differing in the location and number of PEGylation sites. Many of the resulting PEG–protein conjugates bound less strongly to their targets or had diminished *in vitro* enzymatic activity, presumably because PEG shielded the proteins from substrates or binding partners.

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More recently, advances in chemoselective biorthogonal reactions[6–13] and chemical protein synthesis[14] have enabled conjugation of a single PEG to any arbitrary position within a protein. These site-specific PEG–protein conjugates tend to retain more *in vitro* activity than their non-specifically PEGylated counterparts.[15] However, aside from avoiding active sites or binding interfaces, there are few guidelines for selecting the best PEGylation site(s) for a given protein. Instead, efforts to this end generally rely on screening many prospective sites to identify those that provide optimal pharmacokinetic benefits.[16,17]

We have previously proposed that optimal PEGylation sites will be characterized by substantial PEG-based increases to protein conformational stability. Evidence for this hypothesis includes the observation that PEGylated variants of the β-sheet protein WW are more resistant to proteolysis if PEG is placed at locations where it increases protein conformational stability.[18] PEG-based stabilization of proteins depends strongly on the location of the PEGylation site and derives from an entropic effect that likely involves the release of water molecules from the protein surface to bulk solvent.[18] Simultaneous PEGylation at two such stabilizing sites leads to even higher levels of conformational stability and proteolytic resistance. For example, a WW variant modified with two four-unit monomethoxy PEGs at positions 16 and 19 is more stable than either of its mono- or non-PEGylated counterparts.[18] We wondered whether connecting these two PEGs covalently might confer additional stability on the resulting macrocyclic PEG–protein conjugate relative to its acyclic doubly PEGylated counterpart.

Macrocyclization is a well-known strategy for enhancing peptide and protein conformational stability. By preorganizing[19–22] an unfolded peptide in a conformation that resembles the folded state, macrocyclization "pre-pays" a portion of the energetic cost of folding, thereby stabilizing the folded peptide relative to its unfolded counterpart. Peptide macrocyclization strategies include azobenzene photoisomerization;[23,24] C–H activation;[25,26] oxidation of thiols to disul-

fides;[27,28] sulfur or selenium alkylation;[29–31] azide–alkyne cycloaddition;[32–34] formation of lactams[35,36] or oximes;[37,38] and olefin metathesis, often called hydrocarbon stapling, which has been used in side-chain[39–42] and backbone contexts.[43–45]

Results and discussion

We sought to combine the advantages of hydrocarbon stapling and PEGylation by preparing WW variant **16/19-44**, in which Asn residues at positions 16 and 19 have each been modified with a four-unit *O*-allyl PEG (Fig. 1, $m = n = 4$). Variable temperature circular dichroism (CD) experiments reveal that **16/19-44** is −0.68 ± 0.02 kcal mol^{-1} more stable that its non-PEGylated counterpart **16/19-00**, due to a favorable entropy term (Table 1, Fig. 2). On-resin ring-closing metathesis of **16/19-44**, followed by cleavage and HPLC purification resulted in stapled peptide **s16/19-44** (Fig. 1). Mass spectrometry confirmed the identity of the desired product, which has a molecular weight that is smaller than that of non-stapled **16/19-44** by an amount corresponding to the molecular weight of ethylene (which is lost during the ring-closing metathesis reaction; see ESI†). Stapled **s16/19-44** is only marginally more stable than non-stapled **16/19-44** (Table 1; Fig. 2).

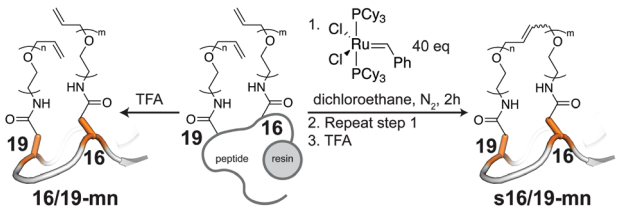

Fig. 1 Preparation of WW variants with Asn-linked olefin-terminated PEGs at positions 16 and 19 in their stapled (**s16/19-*mn***) and non-stapled (**16/19-*mn***) forms.

Positions 16 and 19 are only three residues apart within the same reverse turn; we wondered whether stapling might be more stabilizing at shorter PEG lengths. To test this hypothesis, we systematically decreased the length of the PEGs at positions 16 and/or 19 to generate WW variants **s16/19-34**, **s16/19-33**, **s16/19-23**, **s16/19-22**, **s16/19-12**, **s16/19-11** along with their non-stapled counterparts. PEG-stapled **s16/19-23** is the most stable of these variants (compare T_m values in Table 1) and is also more stabilized by stapling than any of the other stapled variants (Fig. 2). In **s16/19-23**, the staple-based stabilization of −0.29 ± 0.02 kcal mol^{-1} comes from a favorable entropic component ($-T\Delta\Delta S_f$ = −2.3 ± 0.6 kcal mol^{-1}), offset by a weaker unfavorable enthalpic component (Table 1; Fig. 2). Presumably, the five-unit PEG staple in **s16/19-23** provides a level of preorganization that is compatible with the relative locations of positions 16 and 19 in the folded conformation of WW. Shorter and more rigid PEG staples might limit the ability of these side-chains to adopt their native conformations, resulting in staple-based destabilization (compare data for **s16/19-11** *vs.* **16/19-11** and **s16/19-22** *vs.* **16/19-22** in Table 1 and Fig. 2). In contrast, longer and more flexible PEG staples might provide insufficient preorganization and therefore only marginally impact WW stability (compare data for **s16/19-44** *vs.* **16/19-44** in Table 1 and Fig. 2).

We next wondered whether PEG-based stapling would be more or less stabilizing than conventional hydrocarbon stapling. To address this question, we prepared WW variants **h16/19-22** and **h16/19-23**, in which Asn residues at positions 16

Table 1 Folding free energies of WW variants that are PEGylated *vs.* PEG-stapled or alkylated *vs.* hydrocarbon-stapled at positions 16 and 19[a]

					Relative to **16/19-00**				Impact of stapling		
WW variant	*m*	*n*	T_m (°C)	ΔC_p (kcal mol^{-1} K^{-1})	$\Delta\Delta G_f$ (kcal mol^{-1})	$\Delta\Delta H_f$ (kcal mol^{-1})	$-T\Delta\Delta S_f$ (kcal mol^{-1})	$\Delta\Delta C_p$ (kcal mol^{-1} K^{-1})	$\Delta\Delta G_f$ (kcal mol^{-1})	$\Delta\Delta H_f$ (kcal mol^{-1})	$-T\Delta\Delta S_f$ (kcal mol^{-1})
16/19-00	0	0	58.9 ± 0.2	−0.34 ± 0.08	—	—	—	—	—	—	—
16/19-11	1	1	64.9 ± 0.3	−0.52 ± 0.03	−0.43 ± 0.03	8.5 ± 0.7	−8.9 ± 0.8	−0.18 ± 0.08	—	—	—
s16/19-11	1	1	62.4 ± 0.2	−0.49 ± 0.02	−0.25 ± 0.02	7.7 ± 0.5	−8.0 ± 0.5	−0.16 ± 0.08	0.17 ± 0.03	−0.8 ± 0.8	0.9 ± 0.8
16/19-12	1	2	66.5 ± 0.1	−0.34 ± 0.07	−0.68 ± 0.02	2.0 ± 0.6	−2.7 ± 0.7	−0.01 ± 0.10	—	—	—
s16/19-12	1	2	65.8 ± 0.2	−0.52 ± 0.02	−0.51 ± 0.02	7.9 ± 0.7	−8.4 ± 0.6	−0.19 ± 0.08	0.17 ± 0.02	5.8 ± 0.8	−5.7 ± 0.8
16/19-22	2	2	68.0 ± 0.1	−0.62 ± 0.02	−0.80 ± 0.02	3.9 ± 0.3	−4.7 ± 0.5	−0.29 ± 0.08	—	—	—
s16/19-22	2	2	69.9 ± 0.1	−0.62 ± 0.02	−0.92 ± 0.02	5.7 ± 0.6	−6.6 ± 0.6	−0.28 ± 0.08	-0.12 ± 0.02	1.7 ± 0.6	−1.8 ± 0.5
16/19-23	2	3	67.7 ± 0.1	−0.64 ± 0.02	−0.75 ± 0.02	4.8 ± 0.4	−5.6 ± 0.6	−0.30 ± 0.08	—	—	—
s16/19-23	2	3	71.7 ± 0.1	−0.61 ± 0.02	−1.04 ± 0.02	6.8 ± 0.6	−7.8 ± 0.6	−0.28 ± 0.08	-0.29 ± 0.02	2.0 ± 0.6	−2.3 ± 0.6
16/19-33	3	3	67.5 ± 0.2	−0.39 ± 0.12	−0.77 ± 0.03	2.0 ± 1.1	−2.8 ± 1.2	−0.05 ± 0.14	—	—	—
s16/19-33	3	3	70.1 ± 0.2	−0.65 ± 0.02	−0.92 ± 0.02	6.5 ± 0.8	−7.5 ± 0.8	−0.32 ± 0.08	-0.15 ± 0.04	4.5 ± 1.3	−4.6 ± 1.3
16/19-34	3	4	67.2 ± 0.1	−0.62 ± 0.02	−0.72 ± 0.01	4.1 ± 0.4	−4.8 ± 0.6	−0.29 ± 0.08	—	—	—
s16/19-34	3	4	71.4 ± 0.1	−0.62 ± 0.01	−0.96 ± 0.02	8.2 ± 0.6	−9.2 ± 0.6	−0.28 ± 0.08	-0.25 ± 0.02	4.1 ± 0.6	−4.4 ± 0.6
16/19-44	4	4	66.5 ± 0.1	−0.63 ± 0.02	−0.68 ± 0.02	3.2 ± 0.4	−3.9 ± 0.6	−0.30 ± 0.08	—	—	—
s16/19-44	4	4	69.8 ± 0.1	−0.58 ± 0.02	−0.84 ± 0.02	7.8 ± 0.6	−8.6 ± 0.6	−0.24 ± 0.08	-0.16 ± 0.02	4.6 ± 0.6	−4.7 ± 0.6
h16/19-22	2	2	67.5 ± 0.2	−0.74 ± 0.02	−0.78 ± 0.03	3.6 ± 0.9	−4.4 ± 0.9	−0.41 ± 0.08	—	—	—
hs16/19-22	2	2	78.9 ± 0.1	−0.64 ± 0.02	−1.61 ± 0.04	9.0 ± 0.8	−10.6 ± 0.8	−0.30 ± 0.08	-0.84 ± 0.04	5.4 ± 1.0	−6.2 ± 1.0
h16/19-23	2	3	57.4 ± 0.3	−0.76 ± 0.03	0.14 ± 0.03	−0.6 ± 1.0	0.7 ± 1.0	−0.43 ± 0.08	—	—	—
hs16/19-23	2	3	73.1 ± 0.2	−0.60 ± 0.02	−1.06 ± 0.04	9.5 ± 0.9	−10.5 ± 0.9	−0.26 ± 0.08	−1.20 ± 0.04	10.1 ± 1.2	-11.3 ± 1.2

[a] Folding free energies are given ±standard error in kcal mol^{-1} at the melting temperature of control compound **16/19-00** (332 K) in 20 mM sodium phosphate buffer (pH 7).

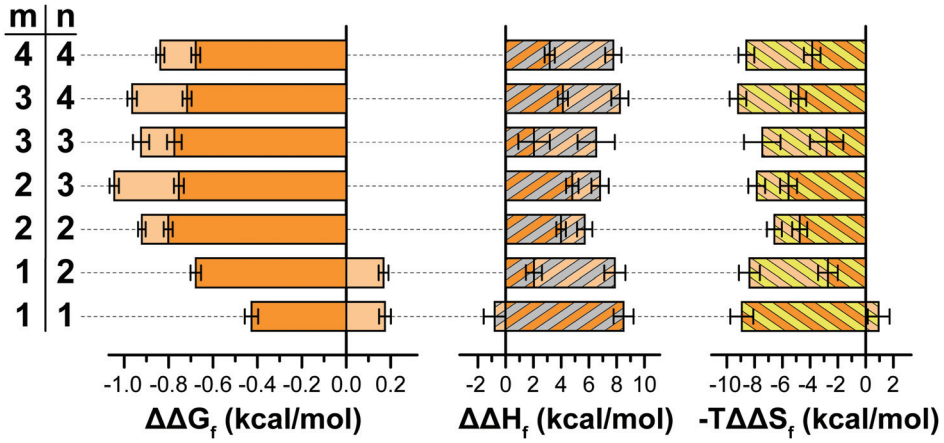

Fig. 2 Changes in folding free energy ($\Delta\Delta G_f$, open bars) due to PEGylation (orange) *vs.* stapling (light orange) of WW variants at positions 16 and 19, along with the enthalpic ($\Delta\Delta H_f$, overlaid gray stripes) and entropic ($-T\Delta\Delta S_f$, overlaid yellow stripes) components of $\Delta\Delta G_f$ in kcal mol^{-1} ± standard error in 20 mM sodium phosphate buffer (pH 7) at the melting temperature of control compound **16/19-00** (332 K).

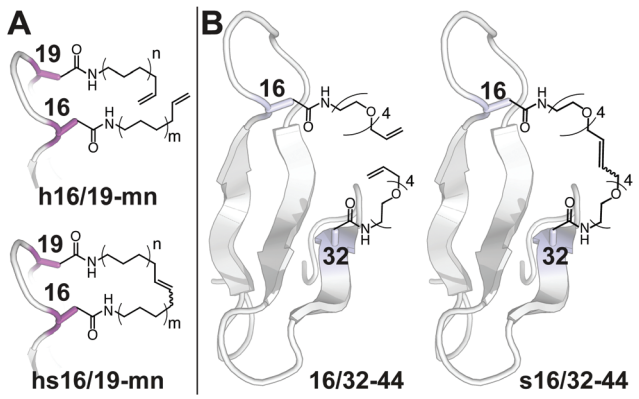

Fig. 3 (A) WW variants with Asn-linked olefin-terminated hydrocarbon chains at positions 16 and 19 in their stapled (**hs16/19-*mn***) and non-stapled (**h16/19-*mn***) forms. (B) WW variants with four-unit Asn-linked PEGs at positions 16 and 32 in their stapled (**s16/32-44**) and non-stapled (**16/32-44**) forms.

and 19 have each been modified with olefin-terminated hydrocarbons of the same length as the PEG-chains in **16/19-22** and **16/19-23**, respectively (Fig. 3A). We also prepared their stapled counterparts **hs16/19-22** and **hs16/19-23** as described above. Alkylated variant **h16/19-22** is −0.78 ± 0.03 kcal mol^{-1} more stable than unmodified **16/19-00**; stapling stabilizes **hs16/19-22** by an additional −0.84 ± 0.04 kcal mol^{-1} relative to **h16/19-22** (Table 1; Fig. 4). In contrast, **h16/19-23** is moderately less stable than **16/19-00** ($\Delta\Delta G_f$ = 0.14 ± 0.03), but stapling stabilizes **hs16/19-23** by −1.20 ± 0.04 kcal mol^{-1} relative to **h16/19-23** (Table 1; Fig. 4). In both cases, staple-based stabilization comes from a favorable entropic term, offset by an unfavorable enthalpic term, which is similar to what we observed for PEG-stapling.

Though unrelated to stapling, the high stability of **h16/19-22** (T_m = 67.5 ± 0.2 °C) relative to **h16/19-23** (T_m = 57.4 ± 0.3 °C) is striking given the subtle structural difference between the two variants (*i.e.* four methylene groups). Alkylation increases ΔH_f and decreases the $-T\Delta S_f$ of **h16/19-22** by 3.6 ± 0.9 kcal mol^{-1} and −4.4 ± 0.9 kcal mol^{-1}, respectively, relative to **16/19-00**, making the folded conformation of **h16/19-22** more stable than the unfolded ensemble by −0.78 ± 0.03 kcal mol^{-1} at 332 K. Adding four methylene groups to hydrocarbon chain at position 19 reverses these trends (compare **h16/19-23** *vs.* **h16/19-22**: $\Delta\Delta H_f$ = −4.2 ± 1.1 kcal mol^{-1}, $-T\Delta\Delta S_f$ = 5.1 ± 1.2 kcal mol^{-1}, $\Delta\Delta G_f$ = 0.91 ± 0.04 kcal mol^{-1}), such that

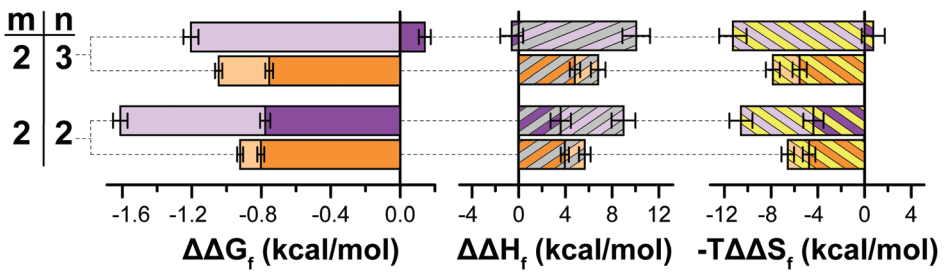

Fig. 4 Changes in ΔG_f, ΔH_f (overlaid gray stripes), and $-T\Delta S_f$ (overlaid yellow stripes), due to alkylation (purple) *vs.* hydrocarbon stapling (light purple) of WW variants at positions 16 and 19 with oligomer length as indicated. Experimental conditions are as described in Fig. 2. Analogous values for PEGylation (orange) *vs.* PEG stapling (light orange) are shown for comparison.

h16/19-23 is slightly less stable than **16/19-00**. It is possible that the additional four carbons allow the position 19 hydrocarbon chain in **h16/19-23** to interact with a non-polar group on the WW surface that is inaccessible to the shorter chain in **h16/19-22**. Such an interaction would be consistent with the observed favorable difference in enthalpy between **h16/19-23** and **h16/19-22**; the larger offsetting change in the entropic term could reflect a concomitant loss of conformational entropy. The similar ΔC_p values for **h16/19-23** and **h16/19-22** suggest that the addition of four methylene groups does not substantially change how much non-polar surface area is buried in the folded conformation of each variant.

The magnitude of staple-based stabilization is much greater for alkylated **hs16/19-22** (-0.84 ± 0.04 kcal mol^{-1}) and **hs16/19-23** (-1.20 ± 0.04 kcal mol^{-1}) than for PEGylated **s16/19-22** (-0.12 ± 0.02 kcal mol^{-1}) and **s16/19-23** (-0.29 ± 0.02 kcal mol^{-1}), respectively, relative to their unstapled counterparts (Table 1, Fig. 4). However, the overall impact of PEGylation + stapling relative to **16/19-00** (-0.92 ± 0.02 kcal mol^{-1} for **s16/19-22**; -1.04 ± 0.02 kcal mol^{-1} for **s16/19-23**) more closely approximates that of alkylation + stapling (-1.61 ± 0.04 for **hs16/19-22**; -1.06 ± 0.04 for **hs16/19-23**), suggesting the possibility of using PEG stapling and hydrocarbon stapling in similar contexts. Indeed, turbidity-monitored aggregation experiments (see ESI† for details) revealed that **s16/19-23** and **hs16/19-23** are similarly resistant to aggregation, which is consistent with their similar thermal stability (compare their T_m values in Table 1).

We wondered whether the modest staple-based stabilization of **s16/19-23** relative to **16/19-23** could reflect the proximity of positions 16 and 19 not only in tertiary structure but also in primary sequence: the entropic cost of bringing these positions into close proximity in the folded state might already be small, thus limiting the impact of staple-based preorganization. In contrast, we would expect stapling to be more impactful between two positions that are close in tertiary structure but far apart in primary sequence. Positions 16 and 32 are sixteen residues apart in sequence, but their side-chain β-carbons are only 9 Å apart in the folded structure of the parent WW protein from which the WW variants herein were derived.[46]

We explored this possibility by preparing WW variant **16/32-44**, in which Asn residues at positions 16 and 32 have each been modified with an olefin-terminated four-unit PEG (Fig. 3B). We also prepared its stapled counterpart **s16/32-44**, along with non-PEGylated control compound **16/32-00**. PEGylated **16/32-44** is -0.74 ± 0.06 kcal mol^{-1} more stable than its non-PEGylated counterpart **16/32-00**. Stapling *via* olefin metathesis stabilizes **s16/32-44** by an additional -0.72 ± 0.06 kcal mol^{-1} relative to non-stapled **16/32-44** (Table 2, Fig. 5). This staple-based stabilization is more than double what we observed previously for **s16/19-23** relative to **16/19-23**, consistent with the hypothesis that stapling is most beneficial for locations that are distant in primary sequence, but close in tertiary structure.

The enthalpies of transfer of nonpolar molecules to water tend to have strong positive temperature dependencies (*i.e.*, heat capacities: $\Delta C_p = \partial \Delta H / \partial T$), which increase linearly with nonpolar surface area.[47] These observations reflect the limited translational and orientation freedom experienced by first shell water molecules around a nonpolar solute *vs.* in pure bulk water: applied energy must "melt" this ice-like shell prior

Table 2 Folding free energies of WW variants that are PEGylated *vs.* PEG-stapled or alkylated *vs.* hydrocarbon-stapled at positions 16 and 19[a]

					Relative to **16/19-00**				Impact of stapling		
WW variant	m	n	T_m (°C)	ΔC_p (kcal mol^{-1} K^{-1})	$\Delta\Delta G_f$ (kcal mol^{-1})	$\Delta\Delta H_f$ (kcal mol^{-1})	$-T\Delta\Delta S_f$ (kcal mol^{-1})	$\Delta\Delta C_p$ (kcal mol^{-1} K^{-1})	$\Delta\Delta G_f$ (kcal mol^{-1})	$\Delta\Delta H_f$ (kcal mol^{-1})	$-T\Delta\Delta S_f$ (kcal mol^{-1})
16/32-00	0	0	44.4 ± 0.2	−0.44 ± 0.12	—	—	—	—	—	—	—
16/32-44	4	4	54.0 ± 0.2	−0.64 ± 0.05	−0.74 ± 0.06	3.2 ± 1.3	−3.9 ± 1.3	−0.20 ± 0.13	—	—	—
s16/32-44	4	4	70.6 ± 0.3	−0.52 ± 0.02	−1.46 ± 0.07	13.0 ± 1.3	−14.5 ± 1.3	−0.08 ± 0.13	−0.72 ± 0.06	9.8 ± 1.2	−10.6 ± 1.1

[a] Folding free energies are given ± standard error in kcal mol^{-1} at the melting temperature of control compound **16/32-00** (318 K) in 20 mM sodium phosphate buffer (pH 7).

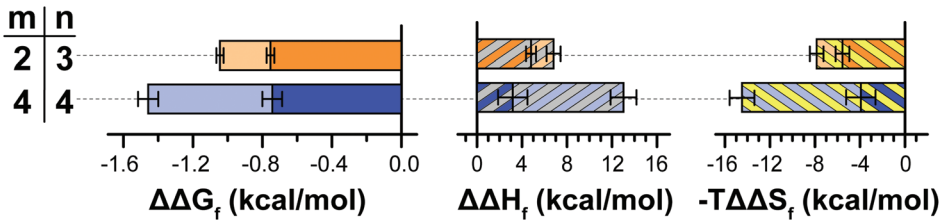

Fig. 5 Changes in ΔG_f, ΔH_f (overlaid gray stripes), and $-T\Delta S_f$ (overlaid yellow stripes), due to PEGylation (blue) *vs.* stapling (light blue) at positions 16 and 32 within WW, with oligomer length as indicated. Experimental conditions are as described in Fig. 2. Analogous values for PEGylation (orange) *vs.* stapling (light orange) at positions 16 and 19 are shown for comparison.

to increasing overall temperature of solution; the heat capacity of the system is therefore higher after transfer of the non-polar molecule to water (*i.e.* the transfer process has a large positive ΔC_p). By analogy, large positive ΔC_p values are associated with aqueous processes that involve increased exposure of nonpolar surface area to water. Similarly, large negative ΔC_p values are associated with processes driven by the hydrophobic effect, which involve sequestering nonpolar surface area from water (*e.g.*, protein folding).

We wondered whether the ΔC_p values for the PEGylated/stapled WW variants in Table 1 might provide some insight into the origin of the overall stabilization associated with PEGylation and stapling. The impact of PEGylation + stapling on the folding free energy (ΔG_f) and heat capacity (ΔC_p) values for WW variants **s16/19-*mn*** and **s16/32-44** (relative to their non-PEGylated non-stapled counterparts **16/19-00** and **16/32-00**, respectively) appear in Fig. 6. $\Delta\Delta G_f$ varies somewhat linearly with $\Delta\Delta C_p$; the most negative $\Delta\Delta C_p$ values are associated with the most negative $\Delta\Delta G_f$ values. This observation suggests that PEGylation + stapling decreases the amount of solvent-exposed surface area in the folded conformation of WW, thereby stabilizing it relative to the unfolded conformation. $\Delta\Delta G_f$ and $\Delta\Delta C_p$ values for alkylated + stapled **hs16/19-*mn*** variants relative to their unmodified counterpart **16/19-00** follow this trend less closely, which could reflect the fact that alkylation + stapling has a smaller impact on the non-polar surface area of WW than does PEGylation + stapling owing to the non-polar character of the hydrocarbon staple. In contrast, $\Delta\Delta C_p$ and $\Delta\Delta G_f$ data for **s16/32-44** relative to **16/32-00** do not agree with this trend, suggesting the possibility that PEGylation + stapling at these positions increases WW stability *via* an effect that does not rely strongly on burial of non-polar surface area.

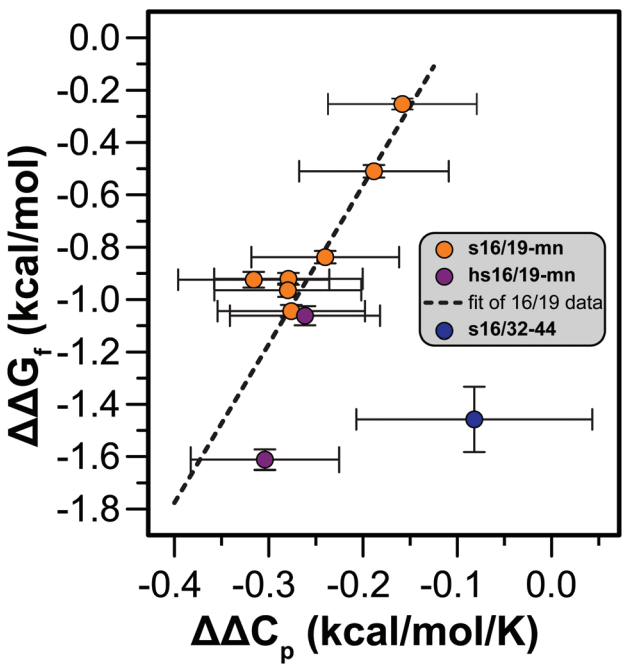

Fig. 6 $\Delta\Delta G_f$ *vs.* $\Delta\Delta C_p$ of WW variants **s16/19-*mn*** (orange), **hs16/19-*mn*** (purple), and **s16/32-44** (blue) *vs.* their unmodified counterparts. Dotted line represents the fit of the $\Delta\Delta G_f$ *vs.* $\Delta\Delta C_p$ data for **s16/19-*mn*** and **hs16/19-*mn*** to a linear function with slope = 6.1 ± 1.5 K (p = 0.005); intercept = 0.6 ± 0.4 kcal mol^{-1} (p = 0.137); R^2 = 0.7057; F = 16.8 (p = 0.005).

Conclusion

Here we compared the impact of PEG stapling *vs.* hydrocarbon stapling on the conformational stability of the β-sheet protein WW. For two sites that are close in both primary sequence and tertiary structure, we found that PEGylation and stapling both increase WW conformational stability *via* an entropic effect and that varying the length of the stapled PEGs can optimize the impact of subsequent stapling. The overall impact of PEGylation + stapling on WW conformational stability is similar to that of hydrocarbon modification + stapling; though hydrocarbon stapling tends to account for a great proportion of this overall stabilization than does PEG stapling. Finally, we found that PEG stapling contributes more to WW conformational stability when the two PEG sites are far apart in primary sequence but close in tertiary structure. Our results highlight the possibility of combining the stabilizing impact of hydrocarbon stapling with the conformational and pharmacokinetic benefits of PEGylation in the future development of peptide and protein drugs.

Experimental section

All WW variants were synthesized as C-terminal acids on Fmoc-Gly-Wang LL resin (EMD Biosciences) by microwave-assisted solid phase peptide synthesis using a standard Fmoc Nα protection strategy. As described previously.[48] All Fmoc-protected amino acids were purchased from Advanced Chem Tech, except for the modified non-natural asparagine derivatives with PEG or hydrocarbon chain on the residue, which were synthesized as described in the ESI.† Unstapled WW variants were cleaved from resin and purified by preparative reverse-phase high-performance liquid chromatography (HPLC) on a C18 column using a linear gradient of water in acetonitrile with 0.1% v/v trifluoroacetic acid. Peptide identity was confirmed by electrospray ionization time-of-flight mass spectrometry. For stapled WW variants, ring-closing olefin metathesis was performed on resin using the first-generation Grubbs ruthenium complex prior to cleavage and purification as described in the ESI.† Conformational stability of stapled and unstapled WW variants was assessed by variable-temperature circular dichroism spectropolarimetry; melting temperatures and folding free energy, enthalpy, and entropy values were derived from global fits of the CD data to a two-state folding model.

Conflicts of interest

There are no conflicts of interest to declare.

Acknowledgements

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