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Impact of Site-Specific PEGylation on the Conformational Stability and Folding Rate of the Pin WW Domain Depends Strongly on PEG Oligomer Length

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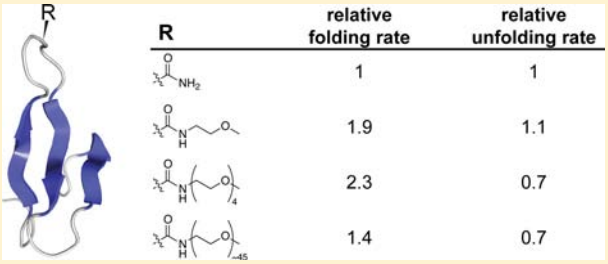
Supporting Information

ABSTRACT: Protein PEGylation is an effective method for reducing the proteolytic susceptibility, aggregation propensity, and immunogenicity of protein drugs. These pharmacokinetic challenges are fundamentally related to protein conformational stability, and become much worse for proteins that populate the unfolded state under ambient conditions. If PEGylation consistently led to increased conformational stability, its beneficial pharmacokinetic effects could be extended and enhanced. However, the impact of PEGylation on protein conformational stability is currently unpredictable. Here we show that appending a short PEG oligomer to a single Asn side chain within a reverse turn in the WW domain of the human protein Pin 1 increases WW conformational stability in a manner that depends strongly on the length of the PEG oligomer: shorter oligomers increase folding rate, whereas longer oligomers increase folding rate and reduce unfolding rate. This strong length dependence is consistent with the possibility that the PEG oligomer stabilizes the transition and folded states of WW relative to the unfolded state by interacting favorably with side-chain or backbone groups on the WW surface.



INTRODUCTION

Covalent attachment of polyethylene glycol (PEG) oligomers to nucleophilic amino acid side chains on protein surfaces (i.e., PEGylation) has been used for more than thirty years as a strategy for protecting proteins and protein drugs from aggregation, proteolytic degradation, and recognition/neutralization by antibodies.[1−5] PEGylated forms of many protein drugs are now available, including Adagen (PEGylated adenosine deaminase),[6] Neulasta (PEGylated granulocyte colony stimulating factor),[7] Pegintron (PEGylated interferon-α2b),[8] Pegasys (PEGylated interferon-α2a),[9] and Oncaspar (PEGylated L-asparaginase),[10] among others.[11] The increased serum half-life, decreased aggregation propensity, and decreased immunogenicity of these PEGylated drugs relative to their non-PEGylated counterparts allows them to be administered less frequently and with fewer side effects, thereby enhancing patient compliance and quality of life.[12]

The beneficial pharmacokinetic effects of protein PEGylation are thought to derive primarily from the increased hydrodynamic volume of the PEGylated protein (which prevents the protein from being filtered out of the serum in the glomerular capillaries of the kidneys)[1−5] and the large size of the PEG oligomer, which shields the protein from proteases, antibodies, and aggregation. However, recent reports indicate that shorter PEG oligomers can also confer beneficial pharmacokinetic properties, despite their small size.[13,14] For example, attachment of a short PEG oligomer comprised of three ethylene oxide units to the C-terminus of glucose-dependent insulinotropic polypeptide (GIP) inhibits degradation of GIP by dipeptidylpeptidase IV even though the PEGylation site is far removed from the binding site of the protease.[15] Moreover, mice treated with PEGylated GIP prior to an intraperitoneal glucose injection have significantly reduced serum glucose concentration relative to mice treated with unmodified GIP. The short three-unit PEG oligomer should not be large enough to prevent renal filtration or to act as a steric shield, implying that a distinct mechanism may account for the enhanced pharmacokinetic properties of PEGylated GIP in vivo.

One possible mechanism is that the PEG oligomer increases protein conformational stability. Many of the pharmacokinetic problems encountered by protein drugs (aggregation, proteolytic degradation, recognition/neutralization by antibodies) are fundamentally related to protein conformational stability (i.e., the difference in free energy between the folded and unfolded conformations of a protein).[16−18] Protein aggregation accelerates when the protein folding energy landscape allows significant population of unfolded, misfolded, or partially folded conformations.[19] Additionally, unfolded or partially

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folded proteins are especially susceptible to proteases,[20−22] and elicit immune responses more readily than do fully folded proteins.[23,24] Increasing the conformational stability of proteins could therefore be an additional general strategy for enhancing the pharmacokinetic properties of protein therapeutics. If PEGylation consistently led to an increase in conformational stability of protein drugs, the beneficial effects of protein PEGylation should be extended and enhanced.

However, the impact of PEGylation on the protein conformational stability is difficult to predict. PEGylation increases the conformational stability of several proteins, including a designed helix bundle,[25−27] lipase,[28,29] trypsin,[30−32] α-chymotrypsin,[33−35] ribonuclease,[31] horseradish peroxidase,[36] laccase,[37] endostatin,[38] insulin,[39,40] the src SH3 domain,[41] and some interferons.[42,43] In contrast, PEGylation decreases the conformational stability of cytochrome C[44] and bovine serum albumin,[45] and does not change the conformational stability of subtilisin Carlsberg,[46,47] catalase,[31] L-asparaginase,[31] brain derived neurotrophic factor,[48] human factor VIIa,[49] or α-chymotrypsinogen.[50] Conflicting reports indicate that PEGylation can increase or decrease the conformational stability of granulocyte colony stimulating factor[51,52] and of lysozyme.[29,53−55] The molecular basis for these differences is unclear, in part because of (1) variations in the methods used to characterize protein conformational stability and (2) nonspecific PEGylation strategies that generate heterogeneous mixtures of protein−PEG conjugates, which differ in the number and location of the attached PEG oligomers.

We seek a deeper understanding of the impact of PEGylation on protein folding energetics with the goal of developing general guidelines for using PEGylation to increase the conformational stability and ultimately to further enhance the pharmacokinetic properties of PEGylated protein drugs. Here we use a small protein, the WW domain of the human protein Pin 1 (hereafter called WW), as a model system for understanding how PEGylation generally impacts the conformational stability of β-sheet proteins (Figure 1A). We chose WW as a model system because its protein folding energy landscape has been extensively characterized and because its small size facilitates the direct chemical synthesis of homogeneous site-specifically PEGylated variants.[56−59]

Recent work indicates that attaching a short PEG oligomer (comprising four ethylene oxide units) to the side-chain amide nitrogen of an Asn residue within the N-terminal reverse turn of WW increases WW conformational stability in a manner that does not depend strongly on the identities of the side chains near the PEGylation site.[60] Here we show that PEGylation at this position stabilizes WW primarily by increasing its folding rate, consistent with simultaneous stabilization of the transition state and the native state of WW by similar amounts. The extent of stabilization depends on the length of the attached PEG oligomer, with PEG oligomers shorter than three units substantially less able to increase WW conformational stability, possibly because the three-unit PEG oligomer engages in specific stabilizing interactions with WW surface side chains that are inaccessible to the shorter oligomers. We find that a 45-unit PEG oligomer also increases WW stability by a substantial amount, suggesting that what we learn from the shorter three- and four-unit oligomers in our model system will apply to longer PEG oligomers of the kind typically found in many therapeutically relevant PEGylated proteins.

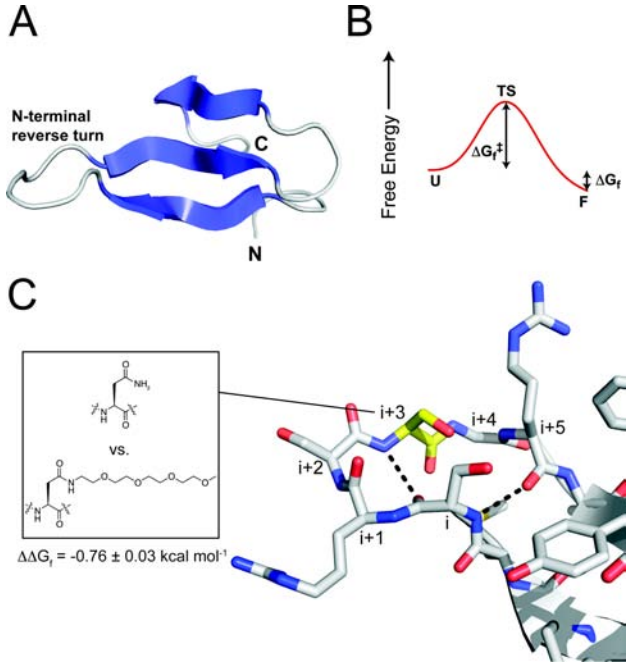

Figure 1. (A) Ribbon diagram of the WW domain of the human protein Pin 1 (PDB: 1PIN, ref 59). β-strands are shown in blue; reverse turns in gray. (B) Reaction coordinate energy diagram for WW folding, showing unfolded ensemble **U**, folded state **F**, transition state **TS**, folding activation energy $\Delta G_f^{\ddagger}$, and folding free energy ΔG_f. (C) Energetic consequences of PEGylating an Asn residue at the *i*+3 position of the N-terminal reverse turn of WW. In this stick representation of the N-terminal reverse turn, main-chain hydrogen bonds are represented by black dashes and the *i*+3 position is highlighted in yellow. All structures were rendered in Pymol.

EXPERIMENTAL PROCEDURES

Protein Synthesis. PEGylated proteins **6PEG$_1$**, **6PEG$_2$**, **6PEG$_3$**, **6PEG$_4$**, **6PEG$_4$-A**, **6PEG$_4$-L**, **6PEG$_4$-F**, **6PEG$_4$-F,T**, **6PEG$_8$**, and **6PEG$_{45}$** and non-PEGylated proteins **6**, **6-A**, and **6-L** were synthesized as C-terminal acids by microwave-assisted solid-phase peptide synthesis,[61] using a standard Fmoc Nα protection strategy. Amino acids were activated by 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU, purchased from Advanced ChemTech) and *N*-hydroxybenzotriazole hydrate (HOBt, purchased from Advanced ChemTech). Fmoc-Gly-loaded Wang LL resin was purchased from EMD Biosciences. Fmoc-protected α-amino acids (with acid-labile side-chain protecting groups) were purchased from Advanced ChemTech, except for Fmoc-Asn(PEG4)-OH, which was synthesized as described previously,[60] and Fmoc-Asn(PEG1)-OH, Fmoc-Asn(PEG2)-OH, and Fmoc-Asn(PEG3)-OH, Fmoc-Asn(PEG8)-OH, and Fmoc-Asn(PEG45)-OH, which were synthesized using analogous methods, as described in the Supporting Information. Piperidine, *N*,*N*-diisopropylethylamine (DIEA), and *N*-methyl pyrrolidinone (NMP) were purchased from Advanced ChemTech.

Acid-labile side-chain protecting groups were globally removed and proteins were cleaved from the resin by stirring 50 μmol resin for ∼4 h in a solution of phenol (250 mg), water (250 μL), thioanisole (250 μL), ethanedithiol (125 μL), and triisopropylsilane (50 μL) in trifluoroacetic acid (TFA, 4 mL). Proteins were purified by preparative reverse-phase high-performance liquid chromatography (HPLC) on a C18 column

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Table 1. Folding Free Energies, Folding and Unfolding Rates, and Changes in Folding and Unfolding Activation Energies for 6, 6PEG$_4$, and Other PEGylated WW Variants[a]

protein	T_m (°C)	ΔG_f (kcal/mol)	$\Delta\Delta G_f$ (kcal/mol)	k_f ($\times 10^3$ s^{-1})	k_f ratio	$\Delta\Delta G_f^{\ddagger}$ (kcal/mol)	k_u ($\times 10^3$ s^{-1})	k_u ratio	$\Delta\Delta G_u^{\ddagger}$ (kcal/mol)
6[b]	56.1 ± 0.2	0.40 ± 0.03		3.8 ± 0.2			6.9 ± 0.5		
6PEG$_1$	59.4 ± 0.3	0.06 ± 0.03	−0.34 ± 0.04	7.2 ± 0.3	1.9 ± 0.2	−0.42 ± 0.05	7.9 ± 0.5	1.1 ± 0.1	−0.08 ± 0.07
6PEG$_2$	59.6 ± 0.4	0.04 ± 0.04	−0.37 ± 0.05	6.3 ± 0.4	1.6 ± 0.1	−0.33 ± 0.06	6.6 ± 0.5	1.0 ± 0.1	0.03 ± 0.07
6PEG$_3$	62.9 ± 0.5	−0.32 ± 0.06	−0.72 ± 0.07	9.3 ± 0.9	2.5 ± 0.3	−0.60 ± 0.08	5.8 ± 0.8	0.8 ± 0.1	0.12 ± 0.10
6PEG$_4$	63.3 ± 0.1	−0.36 ± 0.01	−0.76 ± 0.03	8.8 ± 0.1	2.3 ± 0.2	−0.56 ± 0.04	5.1 ± 0.1	0.74 ± 0.06	0.20 ± 0.05
6PEG$_8$	62.0 ± 0.3	−0.20 ± 0.03	−0.61 ± 0.04	6.8 ± 0.3	1.8 ± 0.1	−0.39 ± 0.05	5.0 ± 0.3	0.72 ± 0.07	0.22 ± 0.07
6PEG$_{45}$	60.8 ± 0.4	−0.08 ± 0.04	−0.48 ± 0.05	5.4 ± 0.3	1.4 ± 0.1	−0.24 ± 0.05	4.8 ± 0.3	0.70 ± 0.07	0.24 ± 0.07

[a]Tabulated data are given as mean ± standard error at 60 °C for 50 μM solutions of WW variants in 20 mM sodium phosphate buffer (pH 7). $\Delta\Delta G_f$, $\Delta\Delta G_f^{\ddagger}$, and $\Delta\Delta G_u^{\ddagger}$, and folding and unfolding rate ratios for PEGylated proteins (**6PEG$_1$**, **6PEG$_2$**, **6PEG$_3$**, **6PEG$_4$**, **6PEG$_8$**, and **6PEG$_{45}$**) are relative to non-PEGylated protein **6**. [b]Data for **6** are from ref 64. [c]Folding free energy data were derived from variable temperature CD experiments. Folding and unfolding rates and activation energies were derived from laser temperature jump kinetic experiments. Data for these experiments appear in Supporting Information figures S115−S122.

Table 2. Folding Free Energies, Folding and Unfolding Rates, and Changes in Folding and Unfolding Activation Energies for 6, 6PEG$_4$, and Their Sequence-modified Derivatives[a]

protein	T_m(°C)	ΔG_f (kcal/mol)	$\Delta\Delta G_f$ (kcal/mol)	k_f ($\times 10^3$ s^{-1})	k_f ratio	$\Delta\Delta G_f^{\ddagger}$ (kcal/mol)	k_u ($\times 10^3$ s^{-1})	k_u ratio	$\Delta\Delta G_u^{\ddagger}$ (kcal/mol)
6[b]	56.1 ± 0.2	0.40 ± 0.03		3.8 ± 0.2			6.9 ± 0.5		
6PEG$_4$[c]	63.3 ± 0.1	−0.36 ± 0.01	−0.76 ± 0.03	8.8 ± 0.1	2.3 ± 0.2	−0.56 ± 0.04	5.1 ± 0.1	0.74 ± 0.06	0.20 ± 0.05
6-F[b]	52.1 ± 0.3	0.80 ± 0.04		1.2 ± 0.1			4.2 ± 0.6		
6PEG$_4$-F[c]	59.4 ± 0.1	0.06 ± 0.01	−0.74 ± 0.04	4.35 ± 0.05	3.5 ± 0.4	−0.83 ± 0.08	4.8 ± 0.1	1.1 ± 0.2	−0.1 ± 0.1
6-F,T[b]	47.7 ± 0.5	1.16 ± 0.09		0.9 ± 0.3			5.3 ± 1.7		
6PEG$_4$-F,T[c]	55.7 ± 0.2	0.48 ± 0.02	−0.68 ± 0.10	2.40 ± 0.06	2.6 ± 0.8	−0.6 ± 0.2	5.0 ± 0.2	0.9 ± 0.3	0.0 ± 0.2

[a]Tabulated data are given as mean ± standard error at 60 °C for 50 μM solutions of WW variants in 20 mM sodium phosphate buffer (pH 7). $\Delta\Delta G_f$, $\Delta\Delta G_f^{\ddagger}$, and $\Delta\Delta G_u^{\ddagger}$, and folding and unfolding rate ratios for PEGylated proteins (**6PEG$_4$-F,T**, **6PEG$_4$-F**, and **6PEG$_4$**) are relative to the corresponding sequence-matched unmodified proteins (**6F,T**, **6-F**, and **6**, respectively). [b]Data for these proteins are from ref 64. [c]Folding free energy data for **6PEG$_4$-F,T**, **6PEG$_4$-F**, and **6PEG$_4$** were derived from variable temperature CD experiments. Folding and unfolding rates and activation energies for **6PEG$_4$-F,T**, **6PEG$_4$-F**, and **6PEG$_4$** were derived from laser temperature jump kinetic experiments performed herein. Data for these experiments appear in Supporting Information figures S115−S122.

using a linear gradient of water in acetonitrile with 0.1% v/v TFA. Proteins were characterized by electrospray-ionization time-of-flight mass spectrometry (Supplementary Figures S82−S101) and protein purity was confirmed by analytical HPLC (Supplementary Figures S102−S114).

Circular Dichroism. Variable temperature CD measurements were made with an Aviv 420 Circular Dichroism Spectropolarimeter at 227 nm, from 0.2 to 94.2 °C (at 2 °C intervals), with 120 s equilibration time between data points and 30 s averaging times, using quartz cuvettes with a path length of 0.1 cm (Supplementary Figures S115−S126). Protein concentrations were determined spectroscopically based on tyrosine and tryptophan absorbance at 280 nm in 6 M guanidine hydrochloride in 20 mM sodium phosphate.[62] The melting temperature and free energy of folding data in Tables 1 and 2 were obtained by globally fitting the variable temperature CD and the laser temperature jump data (see below) to equations for two-state thermal unfolding transitions (see Supporting Information for details).

Laser Temperature Jump Experiments. Protein solutions (50 μM in 20 mM sodium phosphate, pH 7) were each subjected to a rapid laser-induced temperature jump of ∼10−11 °C at each of several temperatures using a nanosecond laser temperature jump apparatus as described previously.[57,63] Following each temperature jump, the approach of the protein to equilibrium at the new temperature (i.e., relaxation) was monitored using the fluorescence decay of a Trp residue in the protein as a probe. Relaxation traces (along with relevant variable temperature CD data, see above) were then globally fit to equations for two-state thermal unfolding transitions (see Supporting Information for details), which allowed us to calculate the folding and unfolding rate constants and activation energies shown in Tables 1 and 2 (see Supporting Information Figures S115−S122).

Proteolysis Assay. Protein solutions (50 μM in 20 mM sodium phosphate buffer, pH 7) were incubated at room temperature with proteinase K (10 μg/mL) for up to 90 min or with Pronase (5 μg/mL) for up to 360 min. Degradation of the full-length protein in the presence of protease was monitored by analytical HPLC, using integrated peak area to calculate the amount of the full-length peptide still remaining at each of several time points. Protein half-lives were calculated by fitting relative integrated peak areas as a function of time to a monoexponential decay equation (see Supporting Information for details, Figures S127−S130).

RESULTS AND DISCUSSION

The WW domain is an extensively characterized[56−58] β-sheet protein that contains three antiparallel β-strands connected by two reverse turns (Figure 1A).[59] The N-terminal reverse turn adopts an unusual conformation consisting of a four-residue type II β-turn within a larger six-residue hydrogen-bonded loop. The folding free energy landscape of Pin WW can be approximated as a simple one-step reaction-coordinate diagram in which the unfolded ensemble proceeds through a high-energy transition state to the folded conformation without

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passing through discrete intermediates (Figure 1B).[57] The conformational stability (or folding free energy, ΔG_f) of WW is therefore defined as the difference in free energy between the unfolded ensemble and the folded state (Figure 1B). ΔG_f is related to the folding equilibrium constant K_f ($\Delta G_f = -RT \ln K_f$), which is in turn related to the folding rate k_f and the unfolding rate k_u ($K_f = k_f/k_u$). These relationships indicate that an increase in protein conformational stability can occur due to an increase in folding rate, a decrease in unfolding rate, or both.

Protein **6** is a previously characterized[64,65] variant of WW in which the *i*+3 position of the six-residue N-terminal reverse turn of the native WW domain is Asn (T_m = 56.1 ± 0.2 °C). PEGylation of the *i*+3 Asn gives protein **6PEG$_4$** (T_m = 63.3 ± 0.1 °C), which is −0.76 ± 0.03 kcal mol^{-1} more stable than **6** at 60 °C (Figure 1C, Table 1).[60] Temperature jump kinetic experiments performed herein reveal that the increased thermodynamic stability of **6PEG$_4$** relative to **6** comes predominantly from an increase in folding rate with a small decrease in unfolding rate (Table 1). At 60 °C, **6PEG$_4$** folds 2.3 ± 0.2 times faster than **6**, and unfolds 1.4 ± 0.1 times slower than **6**. One explanation for these observations is that the four-unit PEG oligomer simultaneously reduces the free energies of the WW transition state and folded state by similar amounts, possibly due to stabilizing interactions between PEG and side-chain or backbone groups on the protein surface. If so, changing the length of the PEG oligomer should change the stabilizing impact of PEGylation: shorter oligomers might not be long enough to make key stabilizing contacts, whereas longer oligomers might be able to make additional stabilizing contacts.

To test this hypothesis, we varied the length of the PEG oligomer attached to the *i*+3 Asn in the N-terminal reverse turn of WW. In proteins **6PEG$_1$**, **6PEG$_2$**, **6PEG$_3$**, and **6PEG$_8$**, the four-unit PEG oligomer of **6PEG$_4$** has been replaced by an oligomer that is one, two, three, and eight units long, respectively (Figure 2). In protein **6PEG$_{45}$**, the discrete four-unit oligomer of **6PEG$_4$** has been replaced by a polydisperse monomethoxy PEG oligomer with an average molecular weight of 2000 g mol^{-1}, corresponding to an average length of 45 ethylene oxide units.

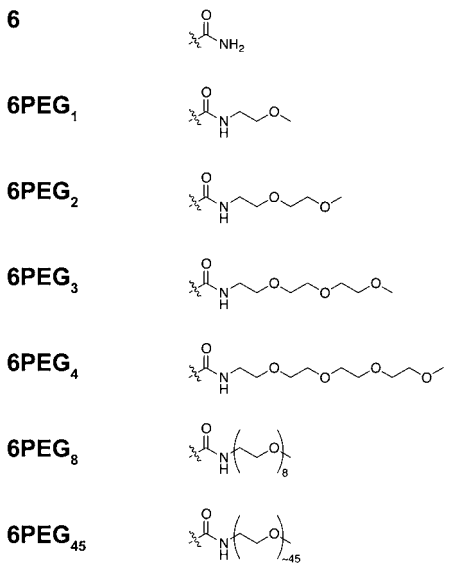

Figure 2. Structures of PEG oligomers in PEGylated proteins **6PEG$_1$**, **6PEG$_2$**, **6PEG$_3$**, **6PEG$_4$**, **6PEG$_8$**, and **6PEG$_{45}$**.

Protein **6PEG$_3$** (T_m = 62.9 ± 0.5 °C) is approximately as stable as **6PEG$_4$**, and is −0.72 ± 0.07 kcal mol^{-1} more stable than non-PEGylated **6**, suggesting that the loss of one ethylene oxide unit does not substantially diminish the stabilizing impact of PEGylation (Table 1). However, proteins **6PEG$_2$** (T_m = 59.6 ± 0.4 °C) and **6PEG$_1$** (T_m = 59.4 ± 0.3 °C) are each substantially less stable than **6PEG$_4$**, though they are still −0.37 ± 0.05 and −0.34 ± 0.04 kcal mol^{-1} kcal mol^{-1} more stable than non-PEGylated **6**, respectively. Extending the four-unit PEG oligomer by additional ethylene oxide units has a less pronounced effect. Protein **6PEG$_8$** (T_m = 62.0 ± 0.3 °C) is slightly less stable than **6PEG$_4$**, but is −0.61 ± 0.04 kcal mol^{-1} more stable than non-PEGylated **6**. Protein **6PEG$_{45}$** (T_m = 60.8 ± 0.4 °C), which harbors the longest PEG oligomer investigated here, is less stable than **6PEG$_4$**, but remains −0.48 ± 0.05 kcal mol^{-1} more stable than non-PEGylated **6**.

As with overall conformational stability, WW folding rate depends on the length of the PEG oligomer attached to the *i*+3 Asn in the N-terminal reverse turn (Table 1). At 60 °C, **6PEG$_4$** and **6PEG$_3$** have similar folding rates that are each substantially faster than the folding rate of non-PEGylated **6**, indicating that the four- and three-unit PEG oligomers decrease the folding activation energy of **6PEG$_4$** and of **6PEG$_3$** by −0.56 ± 0.04 and −0.60 ± 0.08 kcal mol^{-1}, respectively, relative to **6**. Proteins **6PEG$_2$** and **6PEG$_1$** still fold more rapidly than non-PEGylated **6**, but fold substantially more slowly than their longer counterparts **6PEG$_4$** and **6PEG$_3$**. Extending the PEG oligomer by additional ethylene oxide units also decreases WW folding rate. Proteins **6PEG$_8$** and **6PEG$_{45}$** each fold faster than non-PEGylated **6**, but substantially more slowly than **6PEG$_4$** and **6PEG$_3$**.

WW unfolding rate also depends on the length of the PEG oligomer at the *i*+3 Asn of the N-terminal reverse turn of WW (Table 1). The unfolding rates of **6PEG$_1$** and **6PEG$_2$** are indistinguishable from that of non-PEGylated **6**. Extending the PEG oligomer to three-units decreases the unfolding rate of WW, but adding ethylene oxide units beyond this point appears not to have an effect: the unfolding rates of **6PEG$_3$**, **6PEG$_4$**, **6PEG$_8$**, and **6PEG$_{45}$** are indistinguishable.

The reduced conformational stability, reduced folding rate, and increased unfolding rate of **6PEG$_1$** and **6PEG$_2$** relative to **6PEG$_3$** is consistent with the presence of a long-range stabilizing interaction between PEG and specific protein side-chain or backbone groups that are accessible to PEG oligomers at least three units long, but not to the one- and two-unit oligomers. However, the ability of a single ethylene oxide unit to stabilize **6PEG$_1$** by −0.34 ± 0.04 kcal mol^{-1} relative to **6** may indicate the presence of short-range interactions between the first ethylene oxide unit of PEG and residues within the N-terminal reverse turn of WW. The reduced conformational stability, reduced folding rate, and unchanged unfolding rate of **6PEG$_8$** and **6PEG$_{45}$** relative to **6PEG$_3$** could indicate that the extra ethylene oxide units in these longer oligomers do not engage in additional stabilizing interactions with backbone or side-chain groups on the WW surface, but instead increase the conformational entropy of the WW unfolded state. This change would lower the free energy of the unfolded state relative to the folded state and the transition state, resulting in decreased conformational stability and decreased folding rate for the WW variants harboring longer oligomers relative to **6PEG$_3$** or **6PEG$_4$**.

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Previous reports may provide some insight into whether or not the PEG oligomer engages in short-range interactions with residues within the N-terminal reverse turn. As reported previously,[60] changing the identities of side-chains at the *i* and *i*+5 positions of the N-terminal reverse turn of WW (Figure 1C) does not substantially change the impact of PEGylation on WW conformational stability. Kinetic temperature jump experiments performed herein confirm this observation (Table 2). Replacing the *i* position Ser in **6PEG$_4$** with Phe gives protein **6PEG$_4$-F**, which folds 3.5 ± 0.4 times faster than its non-PEGylated counterpart **6-F** at 60 °C. This 3.5-fold acceleration in folding rate is larger than was observed for **6PEG$_4$** vs **6** (Table 2). However, the unfolding rates of **6PEG$_4$-F** and **6-F** are not significantly different (whereas **6PEG$_4$** unfolds substantially more slowly than **6**), so that overall, the difference in stability between **6PEG$_4$-F** and **6-F** ($\Delta\Delta G$ = −0.74 ± 0.04 kcal mol^{-1}) is about the same as the difference in stability between **6PEG$_4$** and **6**. These results suggest that the PEG oligomer does not interact strongly with the side-chain at the *i* position of the N-terminal reverse turn or, alternatively, that the interaction between the PEG and the *i* position Ser in **6PEG$_4$** is about as strong as the interaction between PEG and the *i* position Phe in **6PEG$_4$-F**.

Similarly, replacing the *i*+5 position Arg of **6PEG$_4$-F** with Thr gives protein **6PEG$_4$-F,T**, which is −0.68 ± 0.10 kcal mol^{-1} more stable than its non-PEGylated counterpart **6-F,T** at 60 °C (Table 1). Protein **6PEG$_4$-F,T** folds 2.6 ± 0.8 times faster than **6-F,T**, whereas the unfolding rates of **6PEG$_4$-F,T** and **6-F,T** are indistinguishable. Additional substitutions at the *i*+5 position have a similarly small impact on the PEG-associated increase to WW conformational stability. Replacing the *i*+5 position Arg in **6PEG$_4$** with Ala or Leu gives proteins **6PEG$_4$-A** and **6PEG$_4$-L**, which are −0.96 ± 0.12 kcal mol^{-1} and −0.68 ± 0.11 kcal mol^{-1} more stable, respectively, than their non-PEGylated counterparts **6-A** and **6-L**. These results suggest that the PEG oligomer does not interact strongly with amino acid side-chains at the *i*+5 positions of the N-terminal reverse turn, but do not preclude long-range interactions with WW surface residues outside the N-terminal reverse turn.

The optimal PEG oligomer length for increasing conformational stability appears to be three to four units long, which is much smaller than the large polydisperse PEG polymers used in most therapeutically relevant protein−PEG conjugates. However, this short oligomer still has significant impact on the proteolytic stability of WW. The half-life of **6PEG$_4$** is 3.4 ± 0.3 times greater than that of non-PEGylated **6** in a solution of proteinase K (Figure 3A), and is 3.6 ± 0.3 times greater than that of **6** in a solution of Pronase (Figure 3B). These observations are consistent with reports that a three-unit PEG oligomer increases the proteolytic stability of a small peptide hormone.[15] It is possible that the protective effect observed here stems more from the increased conformational stability of **6PEG$_4$** relative to **6** than from the ability of the four-unit PEG oligomer to shield the WW surface from proteolytic attack. If so, it should be possible to use conformationally stabilizing PEGylation to enhance the proteolytic protection already imparted by larger conventional PEG oligomers.

■ CONCLUSIONS

Protein PEGylation is already an effective method for reducing the proteolytic susceptibility, aggregation propensity, and immunogenicity of protein drugs.[1−5,12,18] However, these major pharmacokinetic challenges are related to protein

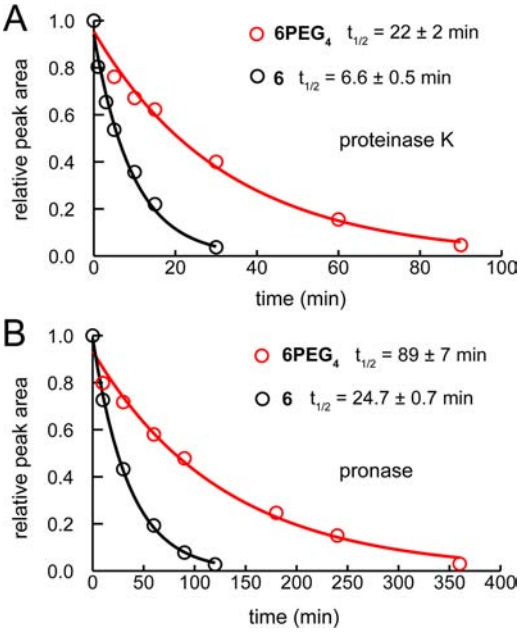

Figure 3. Proteolysis of **6** and **6PEG$_4$** (50 μM protein concentration in 20 mM sodium phosphate buffer, pH 7) by (A) proteinase K (10 μg/mL) or (B) Pronase (5 μg/mL) as monitored by HPLC. Data points for **6PEG$_4$** and **6** are shown as red and black circles, respectively, and each represent the average of three replicate experiments. Solid lines represent fits of the data to a monoexponential decay function, which was used to calculate the indicated half-lives.

conformational stability,[16−22] and we believe that the beneficial effects of protein PEGylation would be further enhanced if PEGylation consistently led to an increase in protein conformational stability. However, the rules that govern PEG-mediated increases to protein conformational stability are incompletely understood. Here we have shown that the increase in stability associated with PEGylating a single Asn side chain in the N-terminal reverse turn of WW comes primarily from an increase in protein folding rate in a manner that does not depend strongly on the identity of side chains at the *i* and *i*+5 positions of the PEGylated reverse turn, but that does depend strongly on the length of the PEG oligomer, with an optimal oligomer length of three to four ethylene oxide units. This length dependence suggests that the PEG oligomer stabilizes the transition and folded states of WW relative to the unfolded state by interacting favorably with surface residues in WW that are outside of the PEGylated reverse turn. Efforts to identify these specific PEG−protein interactions are underway.

In the context of these observations, it is interesting to consider why PEGylation of surface-exposed residues in other proteins does not always lead to increased conformational stability.[29,31,44−51,54,55] It seems likely that the structural characteristics of a PEGylation site influence the degree to which PEGylation at that site can stabilize the protein. If long-range interactions with surface residues contribute strongly to the stabilizing impact of PEGylation in WW, it should be possible to identify these interactions, and apply them to increase the conformational stability and folding rate of other proteins, in research and therapeutic settings. It is true that WW is much smaller than many of the PEGylated proteins whose pharmacokinetic properties we ultimately hope to improve. However, recent efforts to increase WW conformational stability via glycosylation have been successfully applied

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in two larger proteins,[64] providing hope that insights gained from WW PEGylation will be useful in larger, more therapeutically relevant proteins.

The PEG oligomers we used in this study are much smaller than the polydisperse PEGs used in many clinically available PEGylated protein drugs. However, recent reports have shown that site-specific PEGylation of peptides and proteins with shorter monodisperse PEG oligomers can have substantial pharmacokinetic benefits.[13,14] We believe such benefits would be further enhanced if these short PEG oligomers also increased protein conformational stability. Moreover, the three-, four-, eight-, and forty-five-unit oligomers all increase WW stability by substantial amounts, indicating that the short oligomers are also reasonably good predictors of the impact of longer oligomers on protein conformational stability.

■ ASSOCIATED CONTENT

Supporting Information

Complete experimental methods, HPLC and ESI-TOF characterization of proteins, variable temperature CD data, laser temperature jump relaxation data, and proteolysis assay data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Abuchowski, A., Vanes, T., Palczuk, N. C., and Davis, F. F. (1977) Alteration of Immunological Properties of Bovine Serum-Albumin by Covalent Attachment of Polyethylene-Glycol. *J. Biol. Chem. 252*, 3578−3581.

(2) Abuchowski, A., Mccoy, J. R., Palczuk, N. C., Vanes, T., and Davis, F. F. (1977) Effect of Covalent Attachment of Polyethylene-Glycol on Immunogenicity and Circulating Life of Bovine Liver Catalase. *J. Biol. Chem. 252*, 3582−3586.

(3) Harris, J. M., and Chess, R. B. (2003) Effect of pegylation on pharmaceuticals. *Nat. Rev. Drug Discovery 2*, 214−221.

(4) Veronese, F. M., and Mero, A. (2008) The Impact of PEGylation on Biological Therapies. *Biodrugs 22*, 315.

(5) Jevsevar, S., Kunstelj, M., and Porekar, V. G. (2010) PEGylation of therapeutic proteins. *Biotechnol. J. 5*, 113−128.

(6) Hershfield, M. S., Buckley, R. H., Greenberg, M. L., Melton, A. L., Schiff, R., Hatem, C., Kurtzberg, J., Markert, M. L., Kobayashi, R. H., Kobayashi, A. L., and Abuchowski, A. (1987) Treatment of Adenosine Deaminase Deficiency with Polyethylene Glycol-Modified Adenosine Deaminase. *New Engl. J. Med. 316*, 589−596.

(7) Molineux, G. (2004) The Design and Development of Pegfilgrastim (PEG-rmetHuG-CSF, Neulasta). *Curr. Pharm. Des. 10*, 1235−1244.

(8) Wang, Y.-S., Youngster, S., Grace, M., Bausch, J., Bordens, R., and Wyss, D. F. (2002) Structural and biological characterization of pegylated recombinant interferon alpha-2b and its therapeutic implications. *Adv. Drug Delivery Rev. 54*, 547−570.

(9) Rajender Reddy, K., Modi, M. W., and Pedder, S. (2002) Use of peginterferon alfa-2a (40 KD) (Pegasys) for the treatment of hepatitis C. *Adv. Drug Delivery Rev. 54*, 571−586.

(10) Graham, M. L. (2003) Pegaspargase: a review of clinical studies. *Adv. Drug Delivery Rev. 55*, 1293−1302.

(11) Alconcel, S. N. S., Baas, A. S., and Maynard, H. D. (2011) FDA-approved poly(ethylene glycol)-protein conjugate drugs. *Polym. Chem. 2*, 1442−1448.

(12) Fishburn, C. S. (2008) The pharmacology of PEGylation: Balancing PD with PK to generate novel therapeutics. *J. Pharm. Sci. 97*, 4167−4183.

(13) Zhang, L., Klein, B. D., Metcalf, C. S., Smith, M. D., McDougle, D. R., Lee, H.-K., White, H. S., and Bulaj, G. (2012) Incorporation of Monodisperse Oligoethyleneglycol Amino Acids into Anticonvulsant Analogues of Galanin and Neuropeptide Y Provides Peripherally Acting Analgesics. *Mol. Pharmaceutics 10*, 574−585.

(14) Li, L., Crow, D., Turatti, F., Bading, J. R., Anderson, A.-L., Poku, E., Yazaki, P. J., Carmichael, J., Leong, D., Wheatcroft, M. P., Raubitschek, A. A., Hudson, P. J., Colcher, D., and Shively, J. E. (2011) Site-Specific Conjugation of Monodispersed DOTA-PEGn to a Thiolated Diabody Reveals the Effect of Increasing PEG Size on Kidney Clearance and Tumor Uptake with Improved 64-Copper PET Imaging. *Bioconjugate Chem. 22*, 709−716.

(15) Gault, V. A., Kerr, B. D., Irwin, N., and Flatt, P. R. (2008) C-terminal mini-PEGylation of glucose-dependent insulinotropic polypeptide exhibits metabolic stability and improved glucose homeostasis in dietary-induced diabetes. *Biochem. Pharmacol. 75*, 2325−2333.

(16) Krishnamurthy, R., and Manning, M. C. (2002) The stability factor: importance in formulation development. *Curr. Pharm. Biotechnol. 3*, 361−371.

(17) Hermeling, S., Crommelin, D., Schellekens, H., and Jiskoot, W. (2004) Structure-Immunogenicity Relationships of Therapeutic Proteins. *Pharm. Res. 21*, 897−903.

(18) Frokjaer, S., and Otzen, D. E. (2005) Protein drug stability: A formulation challenge. *Nat. Rev. Drug Discovery 4*, 298−306.

(19) Dobson, C. M. (2003) Protein folding and misfolding. *Nature 426*, 884−890.

(20) Daniel, R. M., Cowan, D. A., Morgan, H. W., and Curran, M. P. (1982) A correlation between protein thermostability and resistance to proteolysis. *Biochem. J. 207*, 641−644.

(21) Parsell, D. A., and Sauer, R. T. (1989) The structural stability of a protein is an important determinant of its proteolytic susceptibility in Escherichia coli. *J. Biol. Chem. 264*, 7590−7595.

(22) Imoto, T., Yamada, H., and Ueda, T. (1986) Unfolding rates of globular proteins determined by kinetics of proteolysis. *J. Mol. Biol. 190*, 647−649.

(23) Thai, R., Moine, G., Desmadril, M., Servent, D., Tarride, J.-L., Menez, A., and Leonetti, M. (2004) Antigen Stability Controls Antigen Presentation. *J. Biol. Chem. 279*, 50257−50266.

(24) Ohkuri, T., Nagatomo, S., Oda, K., So, T., Imoto, T., and Ueda, T. (2010) A Protein's Conformational Stability Is an Immunologically Dominant Factor: Evidence That Free-Energy Barriers for Protein Unfolding Limit the Immunogenicity of Foreign Proteins. *J. Immunol. 185*, 4199−4205.

(25) Shu, J. Y., Lund, R., and Xu, T. (2012) Solution Structural Characterization of Coiled-Coil Peptide−Polymer Side-Conjugates. *Biomacromolecules 13*, 1945−1955.

(26) Shu, J. Y., Tan, C., DeGrado, W. F., and Xu, T. (2008) New Design of Helix Bundle Peptide−Polymer Conjugates. *Biomacromolecules 9*, 2111−2117.

(27) Jain, A., and Ashbaugh, H. S. (2011) Helix Stabilization of Poly(ethylene glycol)−Peptide Conjugates. *Biomacromolecules 12*, 2729−2734.

(28) Baillargeon, M., and Sonnet, P. (1988) Polyethylene glycol modification *Candida rugosa* lipase. *J. Am. Oil Chem. Soc. 65*, 1812−1815.

(29) Longo, M. A., and Combes, D. (1999) Thermostability of modified enzymes: a detailed study. *J. Chem. Technol. Biot. 74*, 25−32.
(30) Gaertner, H. F., and Puigserver, A. J. (1992) Increased Activity and Stability of Poly(Ethylene Glycol)-Modified Trypsin. *Enzyme Microb. Technol. 14*, 150−155.
(31) Monfardini, C., Schiavon, O., Caliceti, P., Morpurgo, M., Harris, J. M., and Veronese, F. M. (1995) A Branched Monomethoxypoly-(Ethylene Glycol) for Protein Modification. *Bioconjugate Chem. 6*, 62−69.
(32) Chiu, K., Agoubi, L. L., Lee, I., Limpar, M. T., Lowe, J. W., and Goh, S. L. (2010) Effects of Polymer Molecular Weight on the Size, Activity, and Stability of PEG-Functionalized Trypsin. *Biomacromolecules 11*, 3688−3692.
(33) Topchieva, I. N., Efremova, N. V., Khvorov, N. V., and Magretova, N. N. (1995) Synthesis and Physicochemical Properties of Protein Conjugates with Water-Soluble Poly(Alkylene Oxides). *Bioconjugate Chem. 6*, 380−388.
(34) Castellanos, I. J., Al-Azzam, W., and Griebenow, K. (2005) Effect of the covalent modification with poly(ethylene glycol) on alpha-chymotrypsin stability upon encapsulation in poly(lactic-co-glycolic) microspheres. *J. Pharm. Sci. 94*, 327−340.
(35) Rodriguez-Martinez, J. A., Solá, R. J., Castillo, B., Cintron-Colon, H. R., Rivera-Rivera, I., Barletta, G., and Griebenow, K. (2008) Stabilization of alpha-Chymotrypsin Upon PEGylation Correlates With Reduced Structural Dynamics. *Biotechnol. Bioeng. 101*, 1142−1149.
(36) Garcia, D., Ortéga, F., and Marty, J.-L. (1998) Kinetics of thermal inactivation of horseradish peroxidase: stabilizing effect of methoxypoly(ethylene glycol). *Biotechnol. Appl. Biochem. 27*, 49−54.
(37) Lopez-Cruz, J. I., Viniegra-Gonzalez, G., and Hernandez-Arana, A. (2006) Thermostability of native and pegylated Myceliophthora thermophila laccase in aqueous and mixed solvents. *Bioconjugate Chem. 17*, 1093−1098.
(38) Nie, Y., Zhang, X., Wang, X., and Chen, J. (2006) Preparation and Stability of N-Terminal Mono-PEGylated Recombinant Human Endostatin. *Bioconjugate Chem. 17*, 995−999.
(39) Hinds, K. D., and Kim, S. W. (2002) Effects of PEG conjugation on insulin properties. *Adv. Drug Delivery Rev. 54*, 505−530.
(40) Yang, C., Lu, D. N., and Liu, Z. (2011) How PEGylation Enhances the Stability and Potency of Insulin: A Molecular Dynamics Simulation. *Biochemistry 50*, 2585−2593.
(41) Meng, W., Guo, X., Qin, M., Pan, H., Cao, Y., and Wang, W. (2012) Mechanistic Insights into the Stabilization of srcSH3 by PEGylation. *Langmuir 28*, 16133−16140, DOI: 10.1021/la303466w.
(42) Dhalluin, C., Ross, A., Leuthold, L. A., Foser, S., Gsell, B., Muller, F., and Senn, H. (2005) Structural and biophysical characterization of the 40 kDa PEG-interferon-alpha(2a) and its individual positional isomers. *Bioconjugate Chem. 16*, 504−517.
(43) Basu, A., Yang, K., Wang, M., Liu, S., Chintala, R., Palm, T., Zhao, H., Peng, P., Wu, D., Zhang, Z., Hua, J., Hsieh, M.-C., Zhou, J., Petti, G., Li, X., Janjua, A., Mendez, M., Liu, J., Longley, C., Zhang, Z., Mehlig, M., Borowski, V., Viswanathan, M., and Filpula, D. (2006) Structure-Function Engineering of Interferon-β1b for Improving Stability, Solubility, Potency, Immunogenicity, and Pharmacokinetic Properties by Site-Selective Mono-PEGylation. *Bioconjugate Chem. 17*, 618−630.
(44) Garcia-Arellano, H., Valderrama, B., Saab-Rincon, G., and Vazquez-Duhalt, R. (2002) High temperature biocatalysis by chemically modified cytochrome c. *Bioconjugate Chem. 13*, 1336−1344.
(45) Plesner, B., Fee, C. J., Westh, P., and Nielsen, A. D. (2011) Effects of PEG size on structure, function and stability of PEGylated BSA. *Eur. J. Pharm. Biopharm. 79*, 399−405.
(46) Yang, Z., Williams, D., and Russell, A. J. (1995) Synthesis of Protein-Containing Polymers in Organic-Solvents. *Biotechnol. Bioeng. 45*, 10−17.
(47) Yang, Z., Domach, M., Auger, R., Yang, F. X., and Russell, A. J. (1996) Polyethylene glycol-induced stabilization of subtilisin. *Enzyme Microb. Technol. 18*, 82−89.
(48) Callahan, W. J., Narhi, L. O., Kosky, A. A., and Treuheit, M. J. (2001) Sodium chloride enhances the storage and conformational stability of BDNF and PEG-BDNF. *Pharm. Res. 18*, 261−266.
(49) Plesner, B., Westh, P., and Nielsen, A. D. (2011) Biophysical characterisation of GlycoPEGylated recombinant human factor VIIa. *Int. J. Pharmaceut. 406*, 62−68.
(50) Rodríguez-Martínez, J. A., Rivera-Rivera, I., and Griebenow, K. (2011) Prevention of benzyl alcohol-induced aggregation of chymotrypsinogen by PEGylation. *J. Pharm. Pharmacol. 63*, 800−805.
(51) Popp, M. W., Dougan, S. K., Chuang, T. Y., Spooner, E., and Ploegh, H. L. (2011) Sortase-catalyzed transformations that improve the properties of cytokines. *Proc. Natl. Acad. Sci. U.S.A. 108*, 3169−3174.
(52) Natalello, A., Ami, D., Collini, M., D,ÄôAlfonso, L., Chirico, G., Tonon, G., Scaramuzza, S., Schrepfer, R., and Doglia, S. M. (2012) Biophysical Characterization of Met-G-CSF: Effects of Different Site-Specific Mono-Pegylations on Protein Stability and Aggregation. *Plos One 7*, e42511.
(53) Nodake, Y., and Yamasaki, N. (2000) Some properties of a macromolecular conjugate of lysozyme prepared by modification with a monomethoxypolyethylene glycol derivative. *Biosci., Biotechnol., Biochem. 64*, 767−774.
(54) So, T., Ueda, T., Abe, Y., Nakamata, T., and Imoto, T. (1996) Situation of monomethoxypolyethylene glycol covalently attached to lysozyme. *J. Biochem. 119*, 1086−1093.
(55) Gokarn, Y. R. (2003) Hydrodynamic Behavior and Thermal Stability of a PEGylated Protein: Studies with Hen Egg Lysozyme, Ph.D. Dissertation, University of New Hampshire.
(56) Koepf, E. K., Petrassi, H. M., Sudol, M., and Kelly, J. W. (1999) WW: An isolated three-stranded antiparallel β-sheet domain that unfolds and refolds reversibly; evidence for a structured hydrophobic cluster in urea and GdnHCl and a disordered thermal unfolded state. *Protein Sci. 8*, 841−853.
(57) Jäger, M., Nguyen, H., Crane, J. C., Kelly, J. W., and Gruebele, M. (2001) The Folding Mechanism of a β-sheet: The WW Domain. *J. Mol. Biol. 311*, 373−393.
(58) Jäger, M., Dendle, M., and Kelly, J. W. (2009) Sequence determinants of thermodynamic stability in a WW domain—An all-β-sheet protein. *Protein Sci. 18*, 1806−1813.
(59) Ranganathan, R., Lu, K. P., Hunter, T., and Noel, J. P. (1997) Structural and functional analysis of the mitotic rotamase Pin1 suggests substrate recognition is phosphorylation dependent. *Cell 89*, 875−886.
(60) Price, J. L., Powers, E. T., and Kelly, J. W. (2011) N-PEGylation of a reverse turn is stabilizing in multiple sequence contexts unlike N-GlcNAcylation. *ACS Chem. Biol. 6*, 1188−1192.
(61) Murray, J. K., and Gellman, S. H. (2005) Application of microwave irradiation to the synthesis of 14-helical β-peptides. *Org. Lett. 7*, 1517−1520.
(62) Edelhoch, H. (1967) Spectroscopic determination of tryptophan and tyrosine in proteins. *Biochemistry 6*, 1948−1954.
(63) Ballew, R. M., Sabelko, J., Reiner, C., and Gruebele, M. (1996) A single-sweep, nanosecond time resolution laser temperature-jump apparatus. *Rev. Sci. Instrum. 67*, 3694−3699.
(64) Culyba, E. K., Price, J. L., Hanson, S. R., Dhar, A., Wong, C. H., Gruebele, M., Powers, E. T., and Kelly, J. W. (2011) Protein native-state stabilization by placing aromatic side chains in N-glycosylated reverse turns. *Science 331*, 571−575.
(65) Price, J. L., Powers, D. L., Powers, E. T., and Kelly, J. W. (2011) Glycosylation of the enhanced aromatic sequon is similarly stabilizing in three distinct reverse turn contexts. *Proc. Natl. Acad. Sci. U. S. A. 108*, 14127−14132.