Published in final edited form as:

ACS Chem Biol. 2016 July 15; 11(7): 1805–1809. doi:10.1021/acscchembio.6b00349.

Conjugation Strategy Strongly Impacts the Conformational Stability of a PEG-Protein Conjugate

Paul B. Lawrence, Wendy M. Billings, McKenzie B. Miller, Brijesh K. Pandey, Andrew R. Stephens, Minnie I. Langlois, and Joshua L. Price

Department of Chemistry and Biochemistry, Brigham Young University, Provo, UT 84602

Abstract

Site-specific PEGylation is an important strategy for enhancing the pharmacokinetic properties of protein drugs, and has been enabled by the recent development of many chemoselective reactions for protein side-chain modification. However, the impact of these different conjugation strategies on the properties of PEG-protein conjugates is poorly understood. Here we show that the ability of PEG to enhance protein conformational stability depends strongly on the identity of the PEG-protein linker, with the most stabilizing linkers involving conjugation of PEG to planar polar groups near the peptide backbone. We also find that branched PEGs provide superior stabilization relative to their linear counterparts, suggesting additional applications for branched PEGs in protein stabilization.

Introduction

PEGylation is broadly used to enhance the pharmacokinetic properties of protein drugs.^{1,2} Early PEGylation efforts involved non-specific modification of protein side-chains with reactive PEG electrophiles;^{3,4} more recent developments have provided many useful strategies for site-specific PEG-protein conjugation.⁵ However, all site-specific conjugation strategies are unlikely to be equally suited for a given application; for example, it is unclear whether variations in the PEG-protein linker significantly change the properties of the PEG-protein conjugate. Here we show that the PEG-protein conjugation strategy dramatically impacts the ability of PEG to enhance protein conformational stability.

Increasing protein conformational stability is a useful method⁶ for enhancing the pharmacokinetic properties of proteins because unfolded or misfolded proteins can aggregate,⁷ experience proteolytic degradation,⁸ and be recognized by antibodies more readily⁹ than are highly stable folded proteins. We recently found that protein PEGylation imparts enhanced resistance to proteolysis when PEG increases protein conformational stability.^{6,10} We have also developed a predictive structure-based method for identifying such locations within two model proteins: the WW domain of the human protein Pin 1 and the Src SH3 domain.¹⁰ We generated the required PEG-protein conjugates for these preliminary studies by attaching a short PEG oligomer to the side-chain amide nitrogen of

Supporting Information. Experimental methods, compound characterization and variable temperature CD data. The supporting information is available free of charge on the ACS Publications website.

an Fmoc-protected Asn residue, which was incorporated at desired positions within WW or SH3 via solid-phase peptide synthesis.

Our long-term goal is to identify and evaluate stabilizing PEGylation sites within larger and more therapeutically relevant proteins using this method. However, its reliance on Asn-PEGylation presents a major logistical challenge: Asn-PEG is not genetically encodable. Asn-PEGylated peptides and proteins can be prepared via solid-phase peptide synthesis¹⁰ and/or native chemical ligation,¹¹ but not via biological expression. An attractive potential alternative to Asn-PEGylation is to incorporate PEG site-specifically⁵ via chemoselective reactions¹² with genetically encodable amino acids. For example, Cys thiols can be selectively modified by conjugate addition with maleimide-based PEG reagents, among other approaches.¹³ Similarly, unnatural azide- or alkyne-bearing amino acids like azidohomoalanine¹⁴ (Aha) or propargyloxyphenylalanine¹⁵ (PrF) can be incorporated into expressed proteins,^{16,17} which can then be modified selectively with the appropriate alkyne- or azide-functionalized PEG via the copper-catalyzed azide-alkyne cycloaddition (CuAAC).^{18–20} In addition, transglutaminase enzymes can modify the amide nitrogen of a Gln side-chain with PEG, albeit without complete site-specificity.²¹ However, the Cys-PEG thioether and the PrF- and Aha-PEG triazole linkers (Figure 1) differ substantially from the Asn-PEG amide linker used in our previous studies; and though Gln-PEG more closely resembles Asn-PEG, it places the pendant PEG further from the peptide backbone than does Asn-PEG. Our structure-based method for selecting PEGylation¹⁰ sites relies on assumptions about the orientation of the PEGylated side chain relative to nearby OH groups; we wondered whether substantial changes to linker structure would alter the ability of PEG to enhance protein conformational stability.

Results and Discussion

We investigated the impact of linker structure on PEG-based protein stabilization by comparing the impact of Cys-, Aha-, PrF-, and Gln-PEGylation relative to Asn-PEGylation at a previously characterized stabilizing position within WW. To this end, we used solid-phase synthesis to prepare proteins **2**, **3**, **4**, and **5**, in which Cys, PrF, Aha, and Gln occupy position 19 of WW, respectively (Figure 1). We then prepared four Cys-PEGylated counterparts of protein **2**: (1) protein **2p-1**, in which a four-unit PEG is directly attached to the Cys thiol; (2) protein **2p-2**, in which PEG is attached to the imide nitrogen of the α -alkylthiosuccinimide linker obtained by conjugate addition of a PEG-maleimide to the Cys thiol; (3) protein **2p-3**, which differs from **2p-2** in that the PEG is connected to the amide nitrogen of a more flexible α -alkylthioacetamide linker (we note that **2p-3** is a mixture of epimers at the α -carbon of position 19 as a result of the conjugation reaction; see the supporting information for details); and (4) protein **2p-4**, which differs from **2p-2** in that the PEG is connected to the imide nitrogen of the α -alkylthiosuccinimide via a three-carbon linker. In addition, we modified the azide of the Aha side chain in **3** with a PEG alkyne via CuAAC to give Aha-PEGylated protein **3p**. Similarly, we modified the alkyne of the PrF side chain in **4** with a PEG azide to give PrF-PEGylated protein **4p**. We prepared Gln-PEGylated protein **5p** by incorporating the appropriate PEG-linked Gln monomer during solid-phase synthesis.

We used variable temperature circular dichroism (CD) to assess the stability of PEGylated variants **2p-1**, **2p-2**, **2p-3**, **2p-4**, **3p**, **4p**, and **5p** relative to their non-PEGylated counterparts. Importantly, non-PEGylated **2**, **3**, **4**, and **5** are relatively similar in stability to Asn-containing **1** (Table 1), indicating that changing Asn to Cys, Aha, PrF, or Gln at position 19 does not dramatically perturb WW stability. As described previously¹⁰ Asn-PEGylation at position 19 increases WW stability by -0.74 ± 0.02 kcal mol⁻¹ (compare **1p** vs. **1** in Table 1). In contrast, **2p-1** and **2p-2** are only slightly more stable than **2**, whereas **2p-3** and **2p-4** are each slightly less stable than **2**. The Aha-PEG triazole and Gln-PEG amide linkers in **3p** and **5p** provide nearly identical PEG-based stabilization (-0.36 ± 0.01 kcal/mol for **3p** vs. -0.37 ± 0.01 kcal/mol for **5p**; Table 1), consistent with their similar lengths and with previous observations that peptide bonds can be replaced by triazoles without dramatic disruptions to protein structure.^{18,22} In contrast, PrF-PEGylation does not substantially change WW stability (compare **4p** vs. **4** in Table 1). These observations suggest that the stabilizing impact of Asn-PEGylation is highly sensitive to the structure of the PEG-protein linker. Cys-PEGylated **2p-1**, **2p-2**, **2p-3**, and **2p-4**, and PrF-PEGylated **4p** apparently fail to recapitulate some essential structural feature of the Asn-PEGylated **1p**, whereas Aha-PEGylation (as in **3p**) and Gln-PEGylation (as in **5p**) more closely mimic the stabilizing effect of Asn-PEGylation.

Gln-PEG differs from Asn-PEG by the addition of a single methylene group between the amide linker and the peptide backbone; this subtle structural difference decreases the amount of PEG-based stabilization in **5p** by 0.37 ± 0.02 kcal/mol relative to **1p**. Previous work suggests that Asn-PEGylation stabilizes the folded conformation of **1p** by entropically favorable local desolvation of nearby amino acids on the protein surface. The longer Gln side chain may not place PEG close enough to the protein surface for optimal desolvation, leading ultimately to diminished PEG-based stabilization. Distance from the backbone might also explain why triazole-containing Aha-PEG and its isostere Gln-PEG provide similar amounts of PEG-based stabilization relative to each other, but inferior amounts of stabilization relative to the shorter Asn-PEG. To test this hypothesis, we replaced Aha in **3** with propargylglycine (PrG) to give protein **6**; we then prepared PEGylated **6p** by modifying the acetylene in **6** with a PEG-azide via CuAAC (Figure 1). A PrG-linked PEG should be one methylene unit closer to the protein backbone than an Aha-linked PEG and should therefore more closely resemble the Asn-linked PEG of **1p**. Consistent with this hypothesis, we observed that PrG-PEGylation increases the stability of **6p** by -0.59 ± 0.03 kcal/mol relative to **6**, a larger increment than observed previously for Aha-PEGylation. However, PrG-PEGylation is unlikely to be a useful alternative to Asn-PEGylation: it is not as stabilizing as Asn-PEGylation and is also not genetically encodable.

Having demonstrated the importance of linker distance from the peptide backbone, we wanted to understand the additional structural features that make Asn-PEG more stabilizing than the other linkers investigated here. Unlike its inferior isostere PrG-PEG, Asn-PEG contains a secondary amide group; we wondered whether hydrogen bonding by the amide proton plays an important role in PEG-based stabilization. Attempts to address this question via amide-to-ester mutation^{23,24} were unsuccessful due to the instability of the resulting ester protein-PEG linkage. Accordingly, we used an alternative approach, preparing protein

m1, in which *N*-methyl Asn occupies position 19, along with its PEGylated counterpart **m1p**, in which the methylated Asn nitrogen is also attached to a four-unit PEG (Figure 1). NMR analysis indicates that the Fmoc-protected *N*-methyl *N*-PEGylated Asn monomer used to prepare protein **m1p** exists as a mixture of E and Z amide stereoisomers; and we expect the same to be true within protein **m1p**. Nevertheless, variable temperature CD data for **m1p** can be fit to a simple two-state folding model without evidence of heterogeneity, suggesting that the E and Z amide stereoisomers of **m1p** must have closely similar folding free energies.

Interestingly, *N*-methylation of Asn itself increases WW stability by -0.45 ± 0.02 kcal/mol (Figure 2; compare **m1** vs. **1** in Table 1). This result is in agreement with our previous observations that appending even a single ethylene oxide unit to Asn19 in WW can provide substantial stabilization,⁶ and highlights the impact of changes to side-chain structure very close to the peptide backbone on protein conformational stability. Further elongation from the methyl group in **m1** to the four-unit PEG in **1p** provides an additional -0.30 ± 0.01 kcal/mol of stability. Subsequent *N*-methylation of the Asn-PEG amide nitrogen in **1p** gives protein **m1p** ($T_m = 63.7 \pm 0.2$ °C), which is indistinguishable from **1p** ($T_m = 63.8 \pm 0.1$ °C) in terms of stability. This result indicates that the Asn-PEG amide proton in **1p** is not an essential feature of stabilizing PEG-protein linkers. Elongating the *N*-methyl group of **m1p** to a second four-unit PEG results in *N*-branched bis-PEGylated variant **1-nbp** (Figure 1, Table 1), which is -1.19 ± 0.02 kcal/mol more stable than non-PEGylated **1** and -0.45 ± 0.02 kcal/mol more stable than mono-PEGylated **1p**. The difference in stability between **1-nbp** and **1p** cannot be attributed solely to the fact that **1-nbp** contains four more ethylene oxide units than **1p**: branched **1-nbp** is also more stable than a previously characterized derivative of **1p** that has eight ethylene oxide units instead of four.²⁵ This observation highlights the possibility that branched PEGs may be more effective at increasing protein stability than linear PEGs of similar molecular weight.

We wondered whether the favorable impact of branching on PEG-based stabilization depends on the location of the branch point at the amide nitrogen of Asn or whether a different branch point might provide similar levels of stabilization. Accordingly, we prepared protein **1-cbp** (Figure 1) in which the branch point is located at the first carbon of the appended PEG chain; the resulting linkage is a secondary amide (as in **1p**) and not a tertiary amide (as in **1-nbp** and **m1p**). C-branched bis-PEGylated protein **1-cbp** is only marginally less stable than **1-nbp** (Figure 2, Table 1), and is -0.38 ± 0.04 kcal/mol more stable than mono-PEGylated **1p**, suggesting that PEG-branching can provide substantial stabilization independent of the identity (N vs. C) or geometry (trigonal planar vs. tetrahedral) of the branch point.

The picture that emerges from these data is that the identity of the PEG-protein linker is a critical determinant of PEG-based stabilization within WW. In particular, linkers that provide the largest PEG-based increases in stability have rigid planar functional groups (amides as in **1p**, **1-nbp**, **1-cbp**, and **5p**; triazoles as in **3p** and **6p**) relatively close to the peptide backbone, but do not absolutely require the presence of an amide proton. Such linkers may be better able to disrupt the structure of water near the PEGylation site, leading

to the entropically favorable local surface desolvation we identified previously for Asn-PEGylation WW.¹⁰ We speculate that the Cys linkers in **2p-1**, **2p-2**, **2p-3**, and **2p-4** may be too flexible and that the **PrF** linker in **4p** may be too far from the protein surface for optimal stabilization via this mechanism. Indeed, the substantially larger PEG-based stabilization observed for PrG-PEGylation relative to Aha-PEGylation and for Asn-PEGylation relative to Gln-PEGylation demonstrate that moving this rigid planar functional group even one atom further from the peptide backbone can prevent the pendant PEG from providing optimal stabilization, presumably because such longer side chains are not as close to the protein-bound water of the first solvation shell and are therefore less able to participate in the stabilizing desolvation described above.

Despite their inferiority in this regard, Gln-PEG and Aha-PEG can be installed chemoselectively on encoded side-chains unlike their more stabilizing truncated counterparts Asn-PEG and PrG-PEG. The use of Gln-PEG or Aha-PEG as encodable alternatives to Asn-PEG therefore merits further investigation. Additionally, the superiority of branched vs. linear Asn-PEGs suggests important additional applications for branched PEGs in protein stabilization. In the short term, applying our predictive guidelines for Asn-PEGylation to larger therapeutic proteins may require a fragment condensation/ligation approach and/or the development of new chemoselective reactions that target Asn side chains. Alternatively, it will be interesting to see whether the rules developed previously for identifying stabilizing Asn-PEGylation sites are also useful for identifying stabilizing Aha- or Gln-PEGylation sites. More broadly, our results suggest that efforts to generate optimally stable PEG-protein conjugates must consider conjugation chemistry as an important variable.

Methods

Protein Synthesis

WW domain variants were synthesized via a solid phase synthesis, using the standard Fmoc protecting group strategy, as described in the supporting information. The syntheses of Fmoc-protected PEGylated derivatives of L-asparagine, L-cysteine, and L-glutamine are described in the supporting information. Following cleavage from the resin, WW variants were purified by reverse-phase HPLC on a C18 column using a linear gradient of water and acetonitrile with 0.1% v/v TFA. The identity of each WW domain was confirmed by matrix-assisted laser desorption/ionization time-of-flight spectrometry and purity was evaluated by analytical HPLC.

CD Measurements

CD spectra and variable temperature CD data were collected using an Aviv 420 spectropolarimeter. We fit the variable temperature CD data to obtain T_m and G_f values for each protein (Table 1), as described in the supporting information.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the NIH NIGMS (1R15GM116055-01) and by start-up funds from the Department of Chemistry and Biochemistry at Brigham Young University.

References

1. Frokjaer S, Otzen DE. *Nat Rev Drug Discov.* 2005; 4:298–306. [PubMed: 15803194]
2. Veronese, FM., editor. *PEGylated Protein Drugs: Basic Science and Clinical Applications.* Birkhauser Verlag; Basel: 2009.
3. Abuchowski A, Mccoy JR, Palczuk NC, Vanes T, Davis FF. *J Biol Chem.* 1977; 252:3582–3586. [PubMed: 16907]
4. Abuchowski A, Vanes T, Palczuk NC, Davis FF. *J Biol Chem.* 1977; 252:3578–3581. [PubMed: 405385]
5. Nischan N, Hackenberger CPR. *J Org Chem.* 2014; 79:10727–10733. [PubMed: 25333794]
6. Pandey BK, Smith MS, Torgerson C, Lawrence PB, Matthews SS, Watkins E, Groves ML, Prigozhin MB, Price JL. *Bioconjugate Chem.* 2013; 24:796–802.
7. Dobson CM. *Nature.* 2003; 426:884–890. [PubMed: 14685248]
8. Parsell DA, Sauer RT. *J Biol Chem.* 1989; 264:7590–7595. [PubMed: 2651442]
9. Ohkuri T, Nagatomo S, Oda K, So T, Imoto T, Ueda T. *J Immunol.* 2010; 185:4199–4205. [PubMed: 20817878]
10. Lawrence PB, Gavrilov Y, Matthews SS, Langlois MI, Shental-Bechor D, Greenblatt HM, Pandey BK, Smith MS, Paxman R, Torgerson CD, Merrell JP, Ritz CC, Prigozhin MB, Levy Y, Price JL. *J Am Chem Soc.* 2014; 136:17547–17560. [PubMed: 25409346]
11. Dawson PE, Muir TW, Clarklewis I, Kent SBH. *Science.* 1994; 266:776–779. [PubMed: 7973629]
12. Sletten EM, Bertozzi CR. *Angew Chem Int Ed.* 2009; 48:6974–6998.
13. Chalker JM, Bernardes GJL, Lin YA, Davis BG. *Chem Asian J.* 2009; 4:630–640. [PubMed: 19235822]
14. Ngo JT, Tirrell DA. *Acc Chem Res.* 2011; 44:677–685. [PubMed: 21815659]
15. Bundy BC, Swartz JR. *Bioconjugate Chem.* 2010; 21:255–263.
16. Johnson JA, Lu YY, Van Deventer JA, Tirrell DA. *Curr Opin Chem Biol.* 2010; 14:774–780. [PubMed: 21071259]
17. Kim CH, Axup JY, Schultz PG. *Curr Opin Chem Biol.* 2013; 17:412–419. [PubMed: 23664497]
18. Tornoe CW, Christensen C, Meldal M. *J Org Chem.* 2002; 67:3057–3064. [PubMed: 11975567]
19. Rostovtsev VV, Green LG, Fokin VV, Sharpless KB. *Angew Chem Int Ed.* 2002; 41:2596–2599.
20. Deiters A, Cropp TA, Summerer D, Mukherji M, Schultz PG. *Bioorg Med Chem Lett.* 2004; 14:5743–5745. [PubMed: 15501033]
21. Fontana A, Spolaore B, Mero A, Veronese FM. *Adv Drug Deliv Rev.* 2008; 60:13–28. [PubMed: 17916398]
22. Horne WS, Yadav MK, Stout CD, Ghadiri MR. *J Am Chem Soc.* 2004; 126:15366–15367. [PubMed: 15563148]
23. Deechongkit S, Dawson PE, Kelly JW. *J Am Chem Soc.* 2004; 126:16762–16771. [PubMed: 15612714]
24. Gao J, Kelly JW. *Protein Sci.* 2008; 17:1096–1101. [PubMed: 18434500]
25. Pandey BK, Smith MS, Torgerson C, Lawrence PB, Matthews SS, Watkins E, Groves ML, Prigozhin MB, Price JL. *Bioconjugate Chem.* 2013; 24:796–802.

Figure 1.
Amino acid sequences of PEGylated and non-PEGylated WW variants with side-chain structures at position 19 as indicated.

Figure 2. Folding free energies of WW variants in which Asn, N-Methyl-Asn, or their PEGylated derivatives occupy position 19.

Table 1Impact of Linker Structure on PEG-based Stabilization of WW.^a

Protein	T _m (°C)	G _f (kcal/mol)	G _r (kcal/mol)
1	55.9 ± 0.2	0.38 ± 0.02	—
1p	63.8 ± 0.1	−0.36 ± 0.01	−0.74 ± 0.02
1-nbp	68.1 ± 0.1	−0.81 ± 0.02	−1.19 ± 0.02
1-cbp	67.3 ± 0.3	−0.75 ± 0.04	−1.13 ± 0.04
m1	60.7 ± 0.1	−0.06 ± 0.01	—
m1p	63.7 ± 0.2	−0.36 ± 0.01	−0.30 ± 0.01
2	55.6 ± 0.4	0.42 ± 0.02	—
2p-1	56.8 ± 0.2	0.26 ± 0.01	−0.16 ± 0.02
2p-2	56.3 ± 0.2	0.34 ± 0.01	−0.08 ± 0.02
2p-3	54.9 ± 0.2	0.43 ± 0.01	0.01 ± 0.02
2p-4	53.1 ± 0.4	0.67 ± 0.02	0.24 ± 0.02
3	54.0 ± 0.2	0.55 ± 0.01	—
3p	57.7 ± 0.2	0.18 ± 0.01	−0.36 ± 0.01
4	52.3 ± 0.3	0.68 ± 0.02	—
4p	53.7 ± 0.2	0.64 ± 0.02	−0.04 ± 0.02
5	54.2 ± 0.2	0.48 ± 0.01	—
5p	58.7 ± 0.2	0.11 ± 0.01	−0.37 ± 0.01
6	52.6 ± 0.3	0.73 ± 0.03	—
6p	58.6 ± 0.1	0.14 ± 0.01	−0.59 ± 0.03

^aMelting temperatures and folding free energies, are given ± standard error at 50 μM protein concentration in 20 mM aqueous sodium phosphate, pH 7 at 333.15K.