In Situ Maleimide Bridging of Disulfides and a New Approach to Protein PEGylation

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ABSTRACT: The introduction of non-natural entities into proteins by chemical modification has numerous applications in fundamental biological science and for the development and manipulation of peptide and protein therapeutics. The reduction of native disulfide bonds provides a convenient method to access two nucleophilic cysteine residues that can serve as ideal attachment points for such chemical modification. The optimum bioconjugation strategy utilizing these cysteine residues should include the reconstruction of a bridge to mimic the role of the disulfide bond, maintaining structure and stability of the protein. Furthermore, the bridging chemical modification should be as rapid as possible to prevent problems associated with protein unfolding, aggregation, or disulfide scrambling. This study reports on an in situ disulfide reduction-bridging strategy that ensures rapid sequestration of the free cysteine residues in a bridge, using dithioleimides. This approach is then used to PEGylate the peptide hormone somatostatin and retention of biological activity is demonstrated.

A common approach to manipulating the properties of protein therapeutics involves their derivatization via amino acid selective bioconjugation protocols. Functional moieties regularly attached to proteins in this manner include fluorophores or radiolabels for imaging applications and polymers such as poly(ethylene glycol) (PEG) to increase in vivo stability, solubility, and reduce immunogenicity. The majority of bioconjugation methods currently employed use the nucleophilic residues lysine or cysteine for functionalization with electrophilic reagents. A drawback with lysine modification is that numerous lysine residues are commonly accessible on the protein surface, and thus, such reactions often afford mixtures of products. In contrast, cysteine has a relatively low natural abundance and when present is often tied up in a disulfide bridge. The introduction of a cysteine by mutagenesis is routinely used to provide a single point of attachment; however, in some situations the mutagenesis may not be practical or the resultant mutant may have undesirable properties such as susceptibility to dimerization or disulfide scrambling. An alternative strategy is to release active cysteine residues by reduction of native disulfide bonds. It is notable that many potential protein therapeutics contain accessible disulfides, which serve to afford increased stability to the protein structures. Herein lies the challenge, as by cleaving such disulfide bonds, this stabilizing effect is lost. A solution is to deploy reagents that serve to rebridge the two cysteine residues, mimicking the role of the disulfide bond, and thus retaining the structure and function of the proteins. To this end, we have recently reported on the application of bromomaleimides for the bridging of a reduced disulfide, incorporating a rigid two-carbon spacer between the two cysteine thiols of the peptide hormone somatostatin (Figure 1a, reagent 1).

This bridging protocol and the related procedure described by Broccolini and co-workers both still suffer a potential limitation. They require the disulfide bond to be initially cleaved with reducing agents to afford the two free cysteines to which the bridging reagent is then added. There is a risk that in the time that has elapsed between disulfide cleavage and completion of the bridging event structurally sensitive proteins may have started to unfold. Even if the opening of a disulfide bond has no immediate negative effects on the protein structure, free thiols can lead to aggregation and disulfide scrambling. To prevent this problem, the optimum bridging strategy must limit the time the cysteines are free before they are captured by the reagent. Our strategy to achieve this involves developing new bridging reagents that can be used in tandem with a reducing agent, such that as the free cysteines are revealed they are immediately sequestered in a bridge. Herein, we report on a selection of new maleimide-based reagents for disulfide bridging and reveal the first examples of an in situ procedure using these reagents. We also describe new reagents for the PEGylation of disulfides and demonstrate retention of biological activity of bridged somatostatin analogues.

Somatostatin is a cyclic peptide that plays a key role in regulating the endocrine system by inhibiting the release of various hormones, including growth hormone, insulin, and secretin.

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Somatostatin cannot be used clinically, because it has a short half-life in vivo; however, stable analogues have been developed and are widely employed in the treatment of acromegaly and gastroenteropancreatic tumors. Somatostatin contains a single disulfide bridge between Cys3 and Cys14, which serves to maintain the beta-turn that has been identified as the binding motif for the interaction with a family of G-protein coupled receptors (somatostatin receptors 1-5).

Having shown previously that dibromomaleimide would efficiently bridge the disulfide of somatostatin, we perceived that dichloromaleimide and diodomaleimide would exhibit similar but distinct reactivity in this reaction. Treatment of somatostatin with TCEP followed by dichloro- and diodomaleimide did indeed lead to effective bridging (Figure 1), and comparison with dibromomaleimide led to the following order of reactivity: diiodomaleimide > dibromomaleimide > dichloromaleimide (Figure 1b). This order of reactivity, which was further confirmed by stopped-flow measurements (see supplementary Table 1 in the Supporting Information), suggests that the rate-limiting step for this conjugate addition-elimination sequence involves loss of the leaving group. Thus, the iodides as the best leaving groups lead to the fastest reaction.

The susceptibility of dithiomaleimides to be cleaved by thiols illustrated that thiol-exchange reactions were facile in these systems. This highlighted to us the potential of dithiomaleimides as new disulfide bridging reagents. Thus, we synthesized dimercaptoethanomaleimide and dithiophenomaleimide to evaluate their reactivity. Treatment of somatostatin with TCEP followed by 1 equiv of dimercaptoethanomaleimide led to 68% yield of bridged compound after 1 h (Figure 1c). Prolonged reaction times did not improve the isolated yield of product, which suggested that an equilibrium mixture had been formed as a consequence of competition between the aliphatic thiols in the peptide and mercaptoethanol. By simply increasing the amount of dimercaptoethanomaleimide to 10 equiv, we observed complete conversion to bridged somatostatin after 1 h. In contrast, the use of thiophenol as an improved leaving group led to complete bridging after 10 min with just 1 equiv of reagent (Figure 1d), at a rate comparable to that of dibromomaleimide.

These results demonstrate the potential for various halomaleimides and thiomaleimides to serve as cysteine reactive reagents and to form maleimide bridged disulfides. Our next goal was to test whether any of these reagents would be suitable for the in situ disulfide cleavage-bridging sequence. Crucially, the reagents would have to be tolerant of the presence of the reducing agent TCEP. In a series of test reactions, we found that the halomaleimides showed a greater reactivity toward TCEP than the dithiomaleimides (see supplementary Table 2 in the Supporting Information), suggesting that the dithiomaleimides would be more likely to be suitable for an in situ protocol. For comparison, we chose to trial both the dibromomaleimide and dithiophenomaleimide in in situ studies (Figure 2a). The maleimides (5 equiv) were thus added to somatostatin, followed by varying quantities of TCEP. In the case of dibromomaleimide, somatostatin bridging was observed to up to 60% bridged somatostatin (Figure 2b). Notably, some unreacted somatostatin remained, suggesting that the TCEP-dibromomaleimide side reaction was impeding the desired sequence. Furthermore, the formation of side products was observed, which appear, by MS analysis, to be somatostatin-maleimide-TCEP conjugates. In contrast, treatment of somatostatin with dithiophenomaleimide led to near-quantitative bridging on the addition...
of 3–5 equiv of TCEP (Figure 2c). This bridging reaction is complete within 20 min (Figure 2d), illustrating the efficiency of the reaction sequence. Clearly, by tempering the reactivity of the maleimide to TCEP cross reactivity, selectivity for the desired disulfide reduction-bridging sequence had been obtained. Dithiophenolmaleimide 5 therefore represents, to our knowledge, the first reagent reported for the efficient in situ bridging of disulfides.

We also reasoned that, in place of TCEP, thiol reducing agents could be employed in this in situ bridging. However, at pH 6.2, the pH at which reduced somatostatin is maintained in solution, we observed negligible disulfide cleavage with mercaptoethanol or DTT. Selenols, however, are known to catalyze disulfide cleavage, and we found that benzeneselenol could be used effectively with dithiophenolmaleimide to carry out the in situ bridging (Figure 3). In this reaction, it is the thiophenol released from the maleimide that serves as the reducing agent in conjunction with the selenol. Control reactions showed that the selenol was unable to reduce the disulfide in the absence of the thiophenolmaleimide (see Supporting Information).

Stabilized analogues of somatostatin are clinically important due to the short in vivo half-life of the peptide itself, and thus, we aimed to synthesize a new PEGylated somatostatin employing this methodology (Figure 4a). We synthesized an N-PEG dibromomaleimide 7 and an N-PEG dithiophenolmaleimide 8, via single-step procedures utilizing a Mitsunobu reaction. The dibromomaleimide-PEG was inserted into somatostatin by the sequential protocol of disulfide reduction followed by addition of the bridging reagent (conditions a), while the dithiophenolmaleimide-PEG was inserted by the in situ disulfide reduction-bridging protocol (conditions b). In both reactions, the PEGylated somatostatin was formed near-quantitatively in under 20 min.

To test whether the bridging modification had a deleterious effect on the activity of the resultant somatostatin analogues, we tested the simple maleimide-bridged analogue 6, the PEGylated
analogue 9, and the fluorescein-labeled analogue reported by us previously in a signaling assay. G-protein gated inwardly rectifying potassium (GIRK) channels are activated by seven helix receptors coupling to the inhibitory family of G-protein and specifically by the direct binding of the Gβγ subunit to the channel subunits. In the pituitary, the activation of GIRK channels via somatostatin receptors (SSTR) contributes to the inhibition of hormone release. To model this system, we
transiently transfected human SSTR2 into HEK293 cells stably expressing Kir3.1 and Kir3.2 and examined for current activation after agonist application using whole-cell patch clamping. All three analogues were able to activate currents in a manner comparable to that of unmodified somatostatin (Figure 4b). The response was inhibited by incubation with pertussis toxin demonstrating receptor-mediated activation of inhibitory G-proteins and by tertiapin a relatively specific inhibitor of GIRK channels (Figure 4c).

## CONCLUSION

We have found that a selection of dihalo- and di thi omaleimides provide reagents of tunable reactivity for the chemical modification and bridging of disul phide bonds. Via an improved bridging protocol, we have shown that dithi phenomaleimides can be effectively employed in situ with a reducing agent to ensure rapid sequestration of the free cysteine residues. This will serve to limit the problems associated with reducing disulphide bonds to afford free cysteines for bioconjugation, such as protein unfolding, aggregation, and disulphide scrambling. We have also described new reagents for the PEGylation of proteins, a process known to increase in vivo stability and solubility and reduce immunogenicity of protein therapeutics, and demonstrated that PEGylated and fluorescent somatostatin analogues retain agonist activity. We suggest that the use of bridging reagents via in situ protocols will preclude many of the previous limitations of disulphide modification, broadening the scope of proteins that can be manipulated in this manner.

## ASSOCIATED CONTENT

### Supporting Information.

Full experimental procedures and data are provided. This material is available free of charge via the Internet at http://pubs.acs.org.

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### REFERENCES

(1) Lundblad, R. L. (2005) Chemical reagents for protein modification, 3rd ed., CRC Press, Boca Raton.
(2) Hermanson, G. T. (1996) Bioconjugate techniques, Academic Press, London.
(3) Veroneze, F. M., and Pasut, G. (2005) PEGylation, successful approach to drug delivery. Drug Discovery Today 10, 1451–1458.
(4) Fodje, M. N., and Al-Karadaghi, S. (2002) Occurrence, conformational features and amino acid propensities for the pi-helix. Protein Eng. 15, 353–358.
(5) Volkin, D. B., Mach, H., and Middaugh, C. R. (1997) Degradative covalent reactions important to protein stability. Mol. Biotechnol. 8, 105–122.
(6) Jones, M. W., Mantovani, G., Ryan, S. M., Wang, X. X., Brayden, D. J., and Haddleton, D. M. (2009) Phosphine-mediated one-pot thiol–ene “click” approach to polymer-protein conjugates. Chem. Commun. 5272–5274.
(7) Brochini, S., Balan, S., Godwin, A., Choi, J. W., Zloh, M., and Shaunak, S. (2006) PEGylation of native disul phide bonds in proteins. Nat. Protoc. 1, 2241–2252.
(8) Baker, J. R., Tedaldi, L. M., Smith, M. E. B., and Nathan, R. (2009) Bromomaleimides; new reagents for the selective and reversible modification of cysteine. Bioconjugate Chem. 6583–6585.
(9) Smith, M. E. B., Schumacher, F. F., Ryan, C. P., Tedaldi, L. M., Papaioannou, D., Waksman, G., Caddick, S., and Baker, J. R. (2010) Protein modification, bioconjugation, and disulphide bridging using bromomaleimides. J. Am. Chem. Soc. 132, 1960–1965.
(10) Baker, J. R., Caddick, S., and Smith, M. E. B. (2009) Patent applications; 0913965.0 - Reversible covalent linkage of functional moieties, 0913967.6 - Functionalisation of solid substrates, and 0914321.5 - Thiol protecting group, United Kingdom.
(11) Brochini, S., Godwin, A., Balan, S., Choi, J. W., Zloh, M., and Shaunak, S. (2008) Disulphide bridge-based PEGylation of proteins. Adv. Drug Delivery Rev. 60, 3–12.
(12) Balan, S., Choi, J. W., Godwin, A., Teo, I., Laborde, C. M., Heidelberger, S., Zloh, M., Shaunak, S., and Brochini, S. (2007) Site-specific PEGylation of protein disulphide bonds using a three-carbon bridge. Bioconjugate Chem. 18, 61–76.
(13) Shaunak, S., Godwin, A., Choi, J. W., Balan, S., Pedone, E., Vijayaraman, D., Heidelberger, S., Teo, I., Zloh, M., and Brochini, S. (2006) Site-specific PEGylation of native disulphide bonds in therapeutic proteins. Nat. Chem. Biol. 2, 312–313.
(14) Petersen, M. T. N., Jonson, P. H., and Petersen, S. B. (1999) Amino acid neighbours and detailed conformational analysis of cysteines in proteins. Protein Eng. 12, 535–548.
(15) Eldjarn, L., and Pihl, A. (1957) The equilibrium constants and oxidation-reduction potentials of some thiol-disulphide bonds. Via an improved oxidation-reduction potentials of some thiol-disulphide bonds. Bioconjugate Chem. 6583–6585.
(16) (1995) Somatostatin and its receptors, John Wiley and Sons Ltd, Chichester.
(17) Freda, P. U. (2002) Somatostatin analogs in acromegaly. J. Clin. Endocrinol. Metab. 87, 3013–3018.
(18) Oberg, K. (2009) Somatostatin analog octreotide LAR (R) in gastro-entero-pancreatic tumors. Expert Rev. Anticancer Ther. 9, 557–566.
(19) Miyazaki, A., Tsuda, Y., Fukushima, S., Yokoi, T., Vantus, T., Bokonyi, G., Szabo, E., Horvath, A., Keri, G., and Okada, Y. (2008) New cyclic somatostatin analogues containing a pyrazinone ring: Importance of Tyr for antiproliferative activity. Bioorg. Med. Chem. Lett. 18, 6199–6201.
(20) Chow, Y. L., and Naguib, Y. M. A. (1984) [2 + 2] photo cycloadditions of dichloromaleimide and dichloromaleic anhydride to cyclic olefins. J. Chem. Soc., Perkin Trans. J 1165–1171.
(21) Dubernet, M., Caubert, V., Guillard, J., and Viaud-Massuard, M. C. (2005) Synthesis of substituted bis(heteroaryl)maleimides. Tetrahedron 61, 4585–4593.
(22) Lynch, D. M., and Crovetti, A. J. (1972) Reactions of dichloromaleimides with alcohols, phenols, and thiols. J. Heterocycl. Chem. 9, 1027–8.
(23) Singh, R., and Maloney, E. K. (2002) Labeling of antibodies by in situ modification of thiol groups generated from selenol-catalyzed reduction of native disulphide bonds. Anal. Biochem. 304, 147–156.
(24) Hibino, H., Inanobe, A., Furutani, K., Murakami, S., Findlay, I., and Kurachi, Y. (2010) Inwardly rectifying potassium channels: their structure, function, and physiological roles. Physiol. Rev. 90, 291–366.
(25) Patel, Y. C. (1999) Somatostatin and its receptor family. Front. Neuroendocrinol. 20, 157–198.