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Protein Labeling

One-Step Azolation Strategy for Site- and Chemo-Selective Labeling of Proteins with Mass-Sensitive Probes

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Abstract: *The chemical modification of proteins in a site-selective manner leads to many advances in various scientific fields. The major challenges with conventional N-terminal bioconjugation techniques are the lack of universal sequence compatibility and poor mass-detection sensitivity of the resulting bioconjugates. This approach efficiently analyzes proteolytic fragments and native proteins in a complex mixture. Multiple chemical steps are usually required for the site-selective synthesis of bioconjugates with enhanced mass-detection sensitivity. We present a single-step, versatile strategy for the selective modification of protein N-termini with mass boosters. The chemical tag enhances the peptide detection by multiple orders thus leading to the unambiguous analysis of the resulting bioconjugates. We demonstrate that tagging proteolytic fragments with mass sensitivity probes in a complex mixture improves the detection of resulting bioconjugates.*

Introduction

Modification of proteins at a single site with varying synthetic molecules is of high significance in the field of chemical biology, for efficient drug delivery, material science and synthesis of bio-hybrid materials.[1–5] Recently, there is a great increase in the number of methods for site-selective labeling of proteins but is limited by the requirement of particular amino acids at the N-terminus.[6–12] Such as N-terminal cysteines leading to the formation of thiazolidines with aldehydes.[13] Periodate oxidation of N-terminal serine and threonine residues[14] for oxime,[15] hydrazone,[16] Wittig,[17] Aldol[18] and Henry bioconjugation.[19] N-terminal tryptophans can be modified via Pictet-Spengler reactions.[20] Some of the methods also required multiple steps.[15,16] A recent 2 pyridinecarboxaldehyde (2PCA) method provides one-step approach for the N-terminal modification of proteins but is unable to tag proteins with proline amino acid in the second position, and doubly modify the peptide with glycine amino acid at the N-terminus, thus lack universal sequence compatibility (Figure 1 a).[21]

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Supporting information (materials, instruments, reaction procedures, azolation, stability study, mass sensitivity booster studies, and products characterization by HRMS, HPLC, and NMR spectra) and the ORCID identification number(s) for the author(s) of this article can be found under: https://doi.org/10.1002/anie.202007608.

Another major limitation with the current methods of N-terminal labeling of proteins is the low mass detection sensitivity of the resulting protein bioconjugates thus making it difficult to characterize. The low mass sensitivity of the N-terminal labeled bioconjugates is due to the blockage of the free N-terminus by hydrophobic groups.[22] This is a major issue while analyzing the conjugated proteins in a proteomic mixture. Consequently, there is a great need to develop new synthetic methodologies that can circumvent the aforementioned limitations and provide an efficient strategy for site-selective labeling of proteins with mass sensitive probes and exhibit universal sequence compatibility.

The current method to increase the detection senstivity of bioconjugates involve the addition of mass senstive probes but in a non-selective manner.[23,24] This leads to the formation of heterogeneous mixture which makes the analysis difficult (Figure 1 b). Multiple methods are usually needed to achieve all these goals, for example, one method is required for the site-selective modification and a second method for the addition of chemical tag that enhances mass sensitivity of the bioconjugate. As a result, a simple, one-step method capable of achieving all the abovementioned goals with the broad scope of structurally and chemically varied peptides and proteins would be highly beneficial. This would enable efficient analysis of the modified peptides in a complex mixture and aid in the detection of low abundant peptides.

Results and Discussion

Design of azoles for N-terminal modification

To develop a single step site-selective modification of proteins that enhances the mass sensitivity, we sought to use azolines for bioconjugation. As a key design element, we hypothesized that the nucleophilic attack of the N-terminus on 2-methyl thiol azolines would lead to the formation of a stable C–N bond at the site of conjugation under physiological conditions. The azoline group has sp2 hybridized nitrogen thus increases the ionization of peptides and enhance the mass sensitivity (Figure 1 c). This approach represents a rare example of selective N-terminal azolation of proteins with improved mass sensitivity that might offer a general strategy for efficient synthesis of azoline bioconjugates (Figure 1 c).

Among several advantages, we recognized that the azoline strategy would: 1) be chemoselective for the N-terminus rather than any other amino acid residues including lysine; 2) enhance the detection sensitivity of bioconjugates due to the high ionization of azolines; 3) labels the N-terminus of

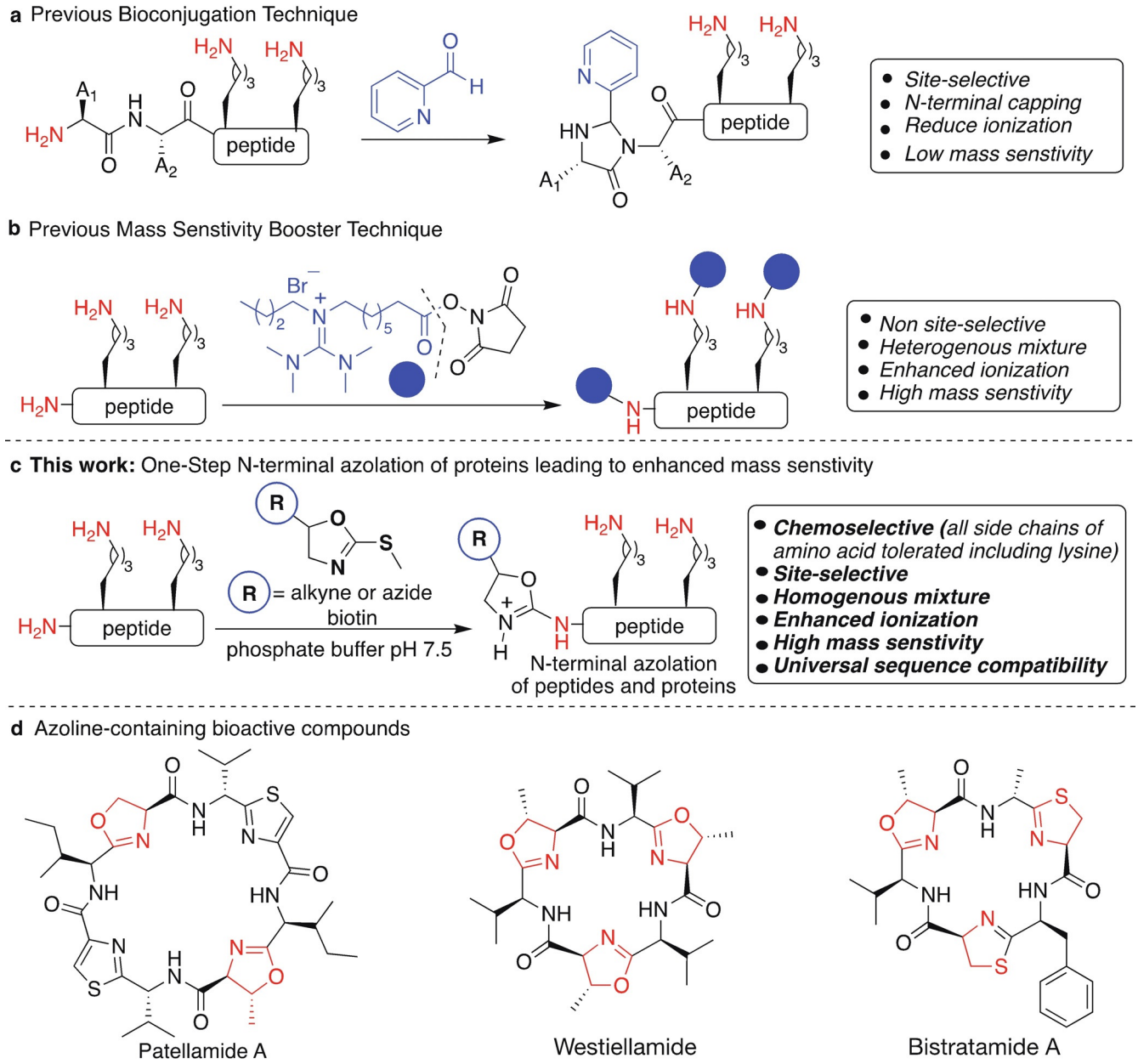

Figure 1. On step azolation for selective N-terminal labeling of biomolecules. a) Limitations of the current N-terminal 2-PCA labeling method. b) Limitations of the current mass booster techniques. c) Selective N-terminal labeling of biomolecules via azolation with mass boosters. d) Azoline-containing bioactive compounds.

proteolytic fragments in a complex mixture thus able to detect low abundant peptides (Figure 1c). This strategy introduces 1,3-azoline moiety into the bioconjugate, a privileged scaffold in the medicinal chemistry and pharmaceutical industry (Figure 1d).[25] Azoline moieties in the backbones of peptidic natural products are important structural motifs that contribute to diverse bioactivities such as protease inhibitor or cytotoxic activity.[26–28] Here in, we report the successful execution of these ideas and present a rapid, N- terminal azolation for use on a wide range of peptides and proteins under physiological conditions.

Screening of Azoline: Universal Sequence Compatibility

We initiated our study by screening a variety of heterocyclic 2-methylthio azolines such as oxazoline **1a**, imidazoline **1b** and thiazoline **1c** with a model peptide FKVCF **2a** containing multiple nucleophilic residues such as N-terminus, lysine and cysteine under physiological conditions (phosphate buffer pH 7.5, 10 mM) (Figure 2a). A treatment of peptide FKVCF **2a** (4 mM) with excess of 2-methylthio oxazoline **1a**, **Ox1** (200 mM) exclusively labeled the N-terminus to generate the oxazoline-modified peptide Ox1-FKVCF **3a** as confirmed by MS/MS analysis (Supplementary Figure 1).

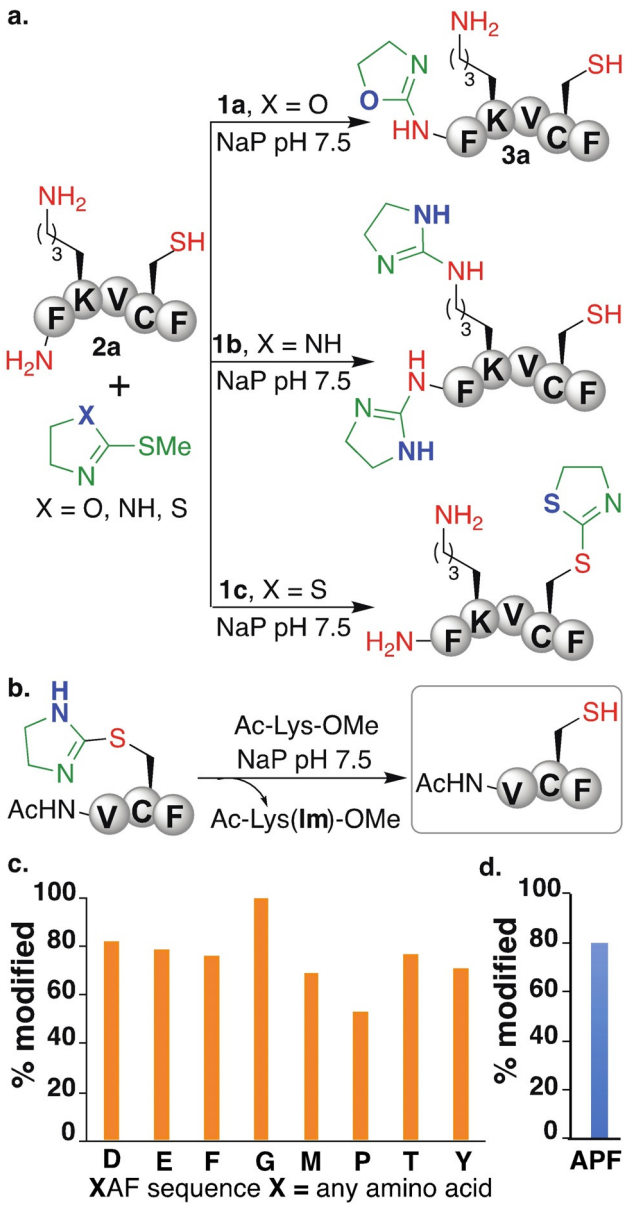

Figure 2. Site-selective peptide modification using azolines. a) Screening of various azolines such as oxazoline **1a**, imidazoline **1b**, and thiazoline **1c** to determine their chemoselectivity for peptides. b) Reversibility of azoline-cysteine adduct with lysine. c) Universal sequence compatibility of peptides with azolination. Conditions for (a), (b): 4 mM peptide, 200 mM azoline, 10 mM phosphate buffer at pH 7.5 and 37 °C. Conditions for (c): 6.25 mM peptide, 312.5 mM **1a**, **Ox1**, 10 mM phosphate buffer at pH 7.5 and 37 °C for 3 h.

High-performance liquid chromatography (HPLC) analysis showed that 82 % conversion to the N-terminal oxazoline-modified peptide **3a** was achieved in 2 h. Lysine side chains were shown to be unreactive towards 2-methylthio oxazoline **1a** because the pKa of the side chain of lysine ≈ 10.6 is higher than pKa of the N-terminus ≈ 9.6. Therefore, the side chain of lysine remained protonated under the physiological conditions thus no labeling of lysine was observed.

The reaction of peptide FKVCF **2a** (4 mM) with 2-methylthio imidazoline **1b** (200 mM) modified lysine side chain as confirmed by MS/MS analysis (Figure 2a and Supplementary Figure 1). This might be due to the ability of imidazole to act as a base and deprotonate the lysine side chain. We have confirmed this by carrying out the reaction with N-acetylated 2-methyl thioimidazoline (**Ac-1b**) that showed highly selectivity for labeling the N-terminus (Supplementary Figure 2). Interestingly, the modification of the cysteine side chain with both **1a** and **1b** was not observed under the reaction conditions although it is highly nucleophilic under physiological conditions. This is most likely due to the reversibility of the oxazoline-cysteine and imidazoline-cysteine conjugates which exhibit functionally similar thioether as the starting material **1** and can react with N-terminus or lysine to form stable products (Figure 2b). We have confirmed the reactivity with amines by carrying out the reaction of imidazoline-cysteine peptide conjugate (Im-Cys) with Ac-Lys-OMe (Figure 2b, Supplementary Figure 3). The reaction of a peptide FKVCF **2a** (4 mM) with 2-methylthio thiazoline **1c** (200 mM) modified cysteine only as confirmed by MS/MS (Supplementary Figure 4). Any modification of the N-terminus and lysine side chain was not observed due to the low electrophilicity of the 2-methylthio thiazoline **1c** (Figure 2a). The formation of small amounts of a disulfide bond between peptide FKVCF **2a** was also observed during the N-terminal oxazolation reaction. To simplify the analysis, a peptide FVKAF **2b** without cysteine was used for further studies. 2-methylthio oxazoline **1a** selectively modified the N-terminus of the peptide FVKAF **2b** and generated Ox1-FVKAF conjugate **3b** with high conversion (80 %) under the reaction conditions. The site of modification was confirmed by MS/MS analysis of Ox1-FVKAF **3b** (Supplementary Figure 5). As further validation of the reaction site-selectivity, no modification of the N-acetyl tripeptide Ac-AKF was observed with **1a**, **Ox1** under the physiological conditions (pH 7.5) and at high pH 8.5 (Supplementary Figure 6). These results suggested that the reaction proceeds via nucleophilic attack of the N-termini, which remains unprotonated at physiological conditions pH 7.5 as compared to the protonated side chain of lysine.

Next, we evaluated the effect of the N-terminal amino acids on oxazolation efficiency and chemoselectivity by screening a panel of peptides with varying reactive amino acid residues at the N-terminus. The incubation of peptides (XAF, **2c–2j** X = D, E, F, G, M, P, T, Y (6.25 mM) with 2-methylthio oxazoline **1a**, **Ox1** (312.5 mM) in 10 mM phosphate buffer at pH 7.5 for 3 h yielded N-terminus Ox1-modified peptides **3c–3j** in a quantitative manner as determined by LCMS (Figure 2c and Supplementary Figure 7). No modifications of the reactive side-chains were observed under the reaction conditions thus reaction is highly selective for the N-terminus. The N-terminal oxazolation of peptides GAF **2f** and APF **2k** with glycine at the N-terminus and proline at the second position proceeded smoothly to generate N-terminal oxazoline-modified peptides (**3f–3k**) in a quantitative manner (Supplementary Figure 8). This is in contrast to the 2PCA method resulting in the double modification with glycine at the N-terminus and no modification of a peptide with proline at the second position.[21] These studies showed that oxazolation is independent of the nature of amino acid sequence

thus exhibit universal sequence compatibility. Further, Ox1-GAF-OMe conjugate **3 f-OMe** was synthesized on a large scale and characterized by NMR (Supplementary Figure 9).

To gain a deeper understanding of reaction rates and the products formed, time-course studies on a linear peptide GAF-OMe **2 f-OMe** were undertaken. For this investigation, quantitative monitoring was carried out by injecting samples for HPLC analysis at regular time intervals. The oxazolation between tripeptide GAF-OMe **2 f-OMe** (2 mM) and 2-methylthio oxazoline **1 a** (100 mM) at pH 7.5 was monitored over 60 min (Supplementary Figure 10).

From these data, it is clear that the initial rate of the formation of Ox1-GAF-OMe conjugate **3 f-OMe** is considerably fast with 70% conversion achieved in just 10 min (Figure 3 a and Supplementary Figure 10). The rate studies of the oxazolation between tripeptide GAF-OMe **2 f-OMe** (1 mM) and 2-methylthio oxazoline **1 a** (1 mM) at pH 7.5 were monitored over 60 min and showed that the reaction follows a second-order rate constant ($k = 1.66 \times 10^{-2}\ M^{-1}S^{-1}$) (Figure 3 a, Supplementary Figure 11). The stability of protein conjugates is a major concern for bioconjugation reactions. Therefore, we evaluated the stability of oxazole-peptide conjugate Ox1-GAF-OMe **3 f-OMe** in aqueous buffers at pH ranging from 3.5 to 10.5, for 48 h at 25 °C and 37 °C. HPLC analysis showed high stability of Ox1-GAF-OMe **3 f-OMe** at varying pH conditions (Figure 3 b, Supplementary Figure 12).

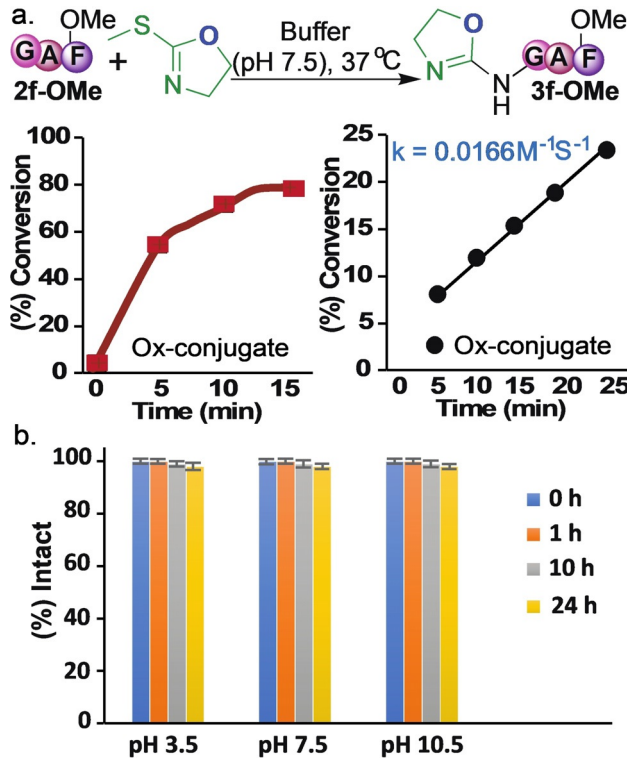

Figure 3. Rate and stability studies of oxazolation. a) Rate studies for synthesis of Ox-GAF-OMe conjugate **3 f-OMe**. The oxazolation of tripeptide GAF-OMe **2 f-OMe** (1 mM) with **1 a** (1 mM) at pH 7.5 follows a second order rate constant. Time-course study with 50 equivalents of **Ox1** (left plot), rate studies to determine the reaction rate by using 1 equiv. of **Ox1** b) pH stability of Ox-GAF-OMe conjugate **3 f-OMe** at 25 °C.

Taken together, these studies establish that oxazolation is N-terminal selective under physiological conditions, employs mild conditions, exhibits fast kinetics, gives higher yields and forms stable oxazoline-adducts thus has the potential to be an effective bioconjugation strategy.

Protein modification-reaction optimization

To determine the applicability of this reaction to proteins, we started our initial investigation on protein myoglobin Mb. Detailed optimization studies with myoglobin Mb revealed that the most efficient conditions are 2-methylthio oxazoline **1 a Ox1** in 10 mM phosphate buffer at pH 7.5 and 25 °C for 12 h (Figure 4 a and Supplementary Figure 13). Long reaction time is needed for high labeling yield (70→99%) but moderate amounts of labeling are observed even in shorter reaction time (6 h) (50–80%). The concentration of protein (0.75 mM–3 mM) did not influence the extent of modification (Figure 4 a, Supplementary Figure 13). Excellent conversion (70%) to N-terminal oxazoline-modified myoglobin Ox1-Mb was obtained at the more neutral pH as confirmed by MS/MS analysis (Figure 4 a, Supplementary Figure 13). In contrast to the NHS ester method, only the single modification product was observed despite the presence of 17 lysine residues on Mb. Low pH 6.5 reduced the conversion to modified protein (43%, Figure 4 a). An increase in pH 8.5 increased the % conversion to modified protein (52%) but also resulted in the lower selectivity, leading the small amounts of modification on the side chain of lysine (30%) (Figure 4 a, Supplementary Figure 13). The increase in the probe equivalents from 50 to 200 lead to the multiple modifications of protein Mb. High site-selectivity for the N-terminus was observed with 50 equiv of the probe (Figure 4 a).

Synthesis of functionalized Oxazoles for N-terminal modification

Next, we synthesized 2-methylthio oxazolines with a variety of functional groups to attach various synthetic groups on proteins (Figure 4 b). Compound **I** was synthesized in two steps from serine methylester (Supplementary Figure 14).

The acylation with NHS ester provided an oxazoline for attaching biotin (**Ox5**, Figure 4 b and Supplementary Figure 14). Propargylation of compound **I** introduced alkyne group on the oxazoline **Ox4**, and azidation of this compound generated azide tag on oxazoline **Ox3** (Figure 4 b and Supplementary Figure 14). Varying degree of modification of Mb with different functional group handles is dictated by the bulk of the handles. Smaller the size of the handle, higher labeling efficiency was observed, 90% labeling of Myoglobin (Mb) with Ox2 (Figure 4 d and Supplementary Figure 15). Ox5 due to its bulky nature leads to reduced labeling of Mb (40%) (Figure 4 c and Supplementary Figure 15). The reaction of myoglobin with **1 b** modified multiple lysine side chains as analyzed by HRMS and MS/MS (Supplementary Figure 16).

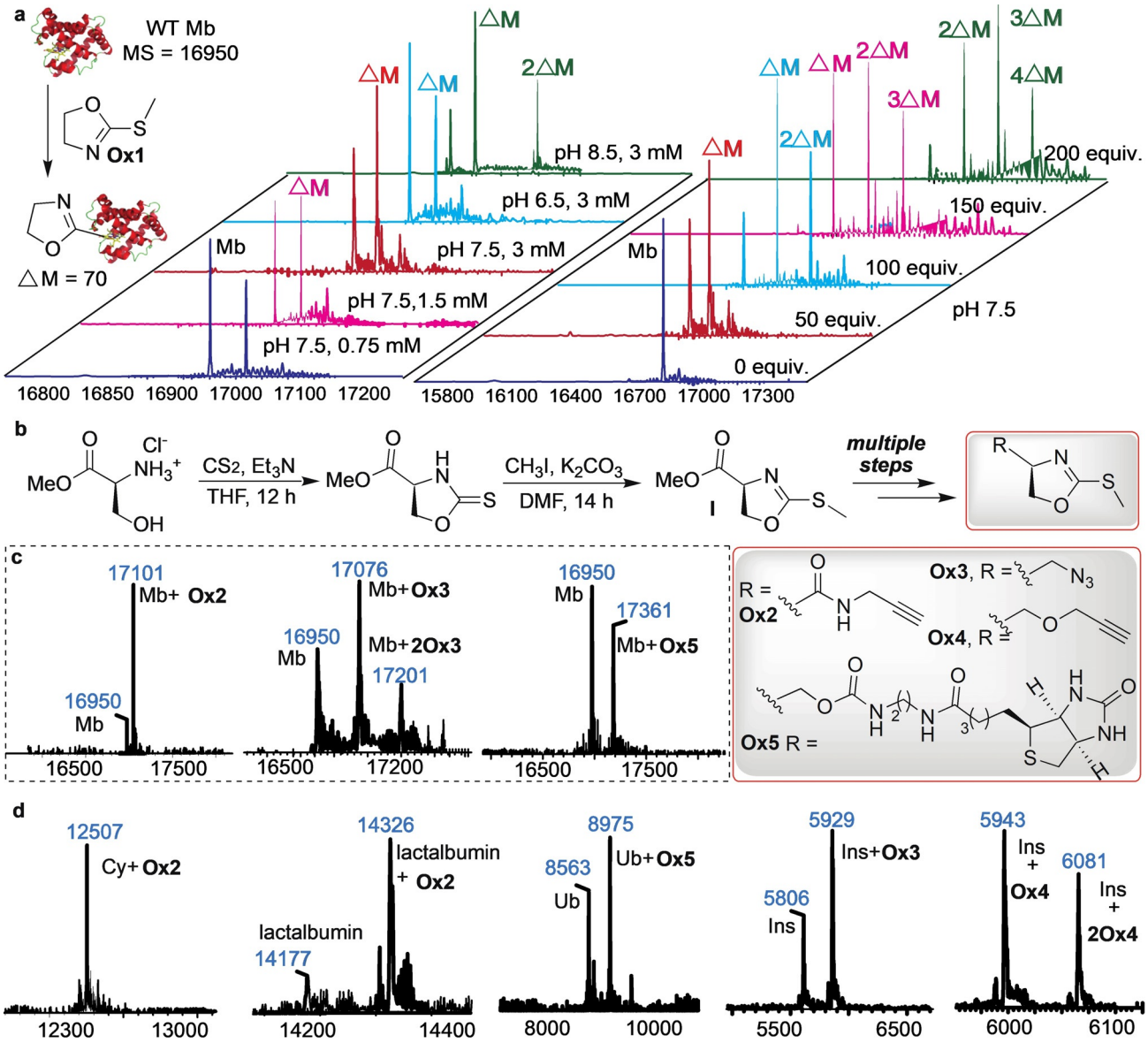

Figure 4. Site-specific attachment of oxazoline and its derivatives to a variety of different proteins. a) Optimization of protein myoglobin modification with oxazoline **1a Ox1**. b) Synthetic scheme for varying functionalized oxazoline derivatives such as alkyne, azide and biotin tags. c) Site-selective modifications of myoglobin Mb with varying oxazoline derivatives. d) Site-selective modifications of a variety of different proteins with oxazoline derivatives. Conditions for (c), (d): 3 mM protein, 50–300 equiv oxazoline, 10 mM phosphate buffer at pH 7.5 and 40 °C. The samples were incubated for 12 h in (c) and (d).

Protein structure and function is retained after modification

The UV studies on oxazoline-modified myoglobin Ox1-Mb and unmodified myoglobin Mb indicated that the secondary and tertiary structure of a protein is unaffected due to the modification under the reaction conditions (Supplementary Figure 17).

Myoglobin bioactivity assay

The activity assay of Ox1-Mb for its ability to oxidize *o*-phenylenediamine with hydrogen peroxide to 2,3-diaminophenazine[29] further confirmed that the installation of an oxazoline at the N-terminus does not alter the activity of Mb (Supplementary Figure 18). Together, these results reaffirmed that the oxazolation of a protein selectively installed probe at a single-site while conserving its structure and bioactivity.

Protein Scope for N-terminal Oxazolation

We carried out the oxazolation of a variety of proteins such as cytochrome C (Cy), alpha-lactalbumin, ubiquitin (Ub) and insulin (Ins) (3 mM) with varying functionalized oxazolines (50–300 equiv). The reactions afforded good to excellent yields (50–99 %) with excellent selectivity (Figure 4d and

Supplementary Figure 19). Notably, ubiquitin with seven lysine residues also generated single modification product at the N-terminus. Insulin with two N-termini generated double modification products due to reactions at both the N-termini (Figure 4d and Supplementary Figure 19). Varying degree of modification of proteins with different functional group handles is dictated by the bulk of the handles and accessibility to the N-terminus. Smaller the size of the handle, higher labeling efficiency was observed, >99% labeling of Cytochrome (Cy) with Ox2, >99% labeling of Insulin (Ins) with Ox2 and Ox4, 90% labeling of Myoglobin (Mb) with Ox2, and 90% labeling of Lactalbumin with Ox2 (Figure 4d and Supplementary Figure 19). Ox5 due to its bulky nature leads to reduced labeling of proteins (40–70%) (Figure 4d and Supplementary Figure 19). These examples indicated that reaction is tolerant to a wide range of molecular weights, three-dimensional structures and structurally critical disulfide bonds. Selective modification of proteins in the presence of cysteine is highly challenging due to the high nucelophilicity of the cysteine thiol group at neutral pH. The cross-reactivity with cysteine is commonly observed in various N-terminal bioconjugation methods.[30–32] No such modification of cysteine was observed during the oxazolation of protein with reduced insulin containing six free cysteines (Supplementary Figure 20). The unique feature of our approach is that it leads to the single-site N-terminal modification of proteins in the presence of highly nucleophilic cysteines under physiological conditions.

Labeling of proteolytic fragments in the complex mixture

To determine the applicability of oxazolation to simultaneously tag the N-termini of multiple peptides and proteins in a complex system, oxazolation was performed with both **Ox1** and **Ox4** on a mixture of peptides with different amino acids at the N-terminus (XAF (**2e–2f**, **2l–2n**) X = F, G, A, V, L). The results indicated full N-terminal oxazolation of all the peptides in a quantitative manner as analyzed by MS (Figure 5 and Supplementary Figure 21). The ability to modify all the N-termini of peptides in the complex mixture could be highly valuable in proteomics studies.

Oxazoline as Mass Sensitivity Booster

One of the major limitations with current methods of N-terminal modification is the difficulty in the characterization of resulting bioconjugates. MS is widely used to determine the labeling and to detect the site of modification but unfortunately unable to map a considerable part of the peptides due to the poor ionization of the labeled peptide or suppression from other ions. The poor ionization of N-terminal labeled peptides is due to the blockage of the free N-terminus with hydrophobic labels. This limitation is more pronounced in the complex mixtures. Therefore it is essential to develop methods that can enhance the detection of labeled proteolytic fragments. One way to achieve this goal is the addition of chemical tags that increases ionization of the labeled peptides

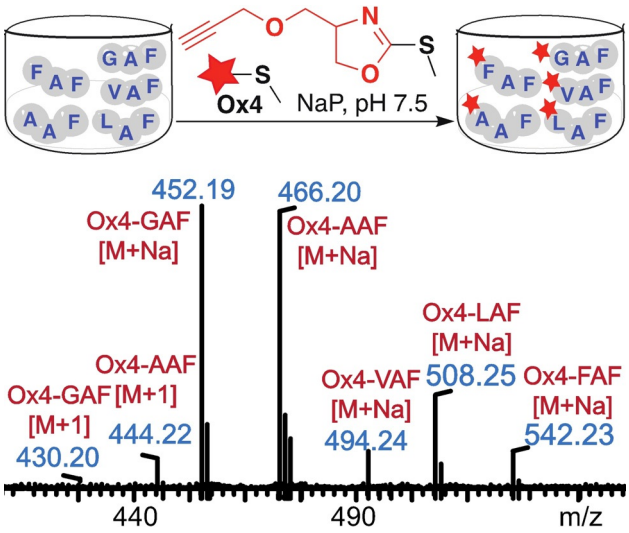

Figure 5. Selective N-terminal labelling of all the peptides by oxazoline **Ox4** in the complex mixture of a variety of peptides.

in the MS. Some strategies have been reported for enhancing the ionization ability of peptides but they are not selective and modify various amino acids thus making the analysis difficult.[23]

The unique feature of our approach is that it not only selectively labels N-terminus but also introduces the oxazoline group containing a sp^2 nitrogen thus enhances the ionization of the labeled fragments. To determine the effect of the oxazoline tag on mass sensitivity, we compared the sensitivity of the multiple unlabeled peptides with methyl ester protected C-termini and rich in hydrophobic groups (XAF-OMe (**2e**, **2fOMe**, **2lOMe–2mOMe**), X = F, G, A, V) with corresponding N-terminal oxazoline-labeled peptides (Ox-XAFOMe, X = F, G, A, V **3e**, **3fOMe**, **3lOMe–3mOMe**) by mixing equal concentrations of these peptides followed by analysis using MS (Figure 6a and Supplementary Figure 22). Insights into the impact of the oxazoline moiety on the detection sensitivity were revealed by MS analysis (Figure 6a). Peptides with oxazoline tags (Ox-XAF-OMe, **3e**, **3fOMe**, **3lOMe–3mOMe**) showed remarkable improvement in the signal enhancement irrespective of the unmodified sequence of the peptides (Figure 6a).

Inspired by these results, we investigated the mass sensitivity of proteins and their oxazoline-labeled bioconjugates. We selected cytochrome C and myoglobin to test the potential of oxazoline as a sensitivity booster. For this investigation, cytochrome C and myoglobin was digested using cyanogen bromide followed by reaction with 2-methylthio oxazoline **1a, Ox1**. The proteolytic fragments before and after the N-terminal oxazoline labeling were analyzed directly by MS without any purification (Supplementary Figure 23 and Figure 24). For unlabeled proteolytic fragments of cytochrome C, low sensitivity was observed and some fragments remained undetected (proteolytic fragment a, Supplementary Figure 23). For the labeled proteolytic fragments of cytochrome C, all the fragments were detected with significantly high mass intensities (Supplementary Figure 23). We observed huge change in the MS of the oxazoline-labeled and

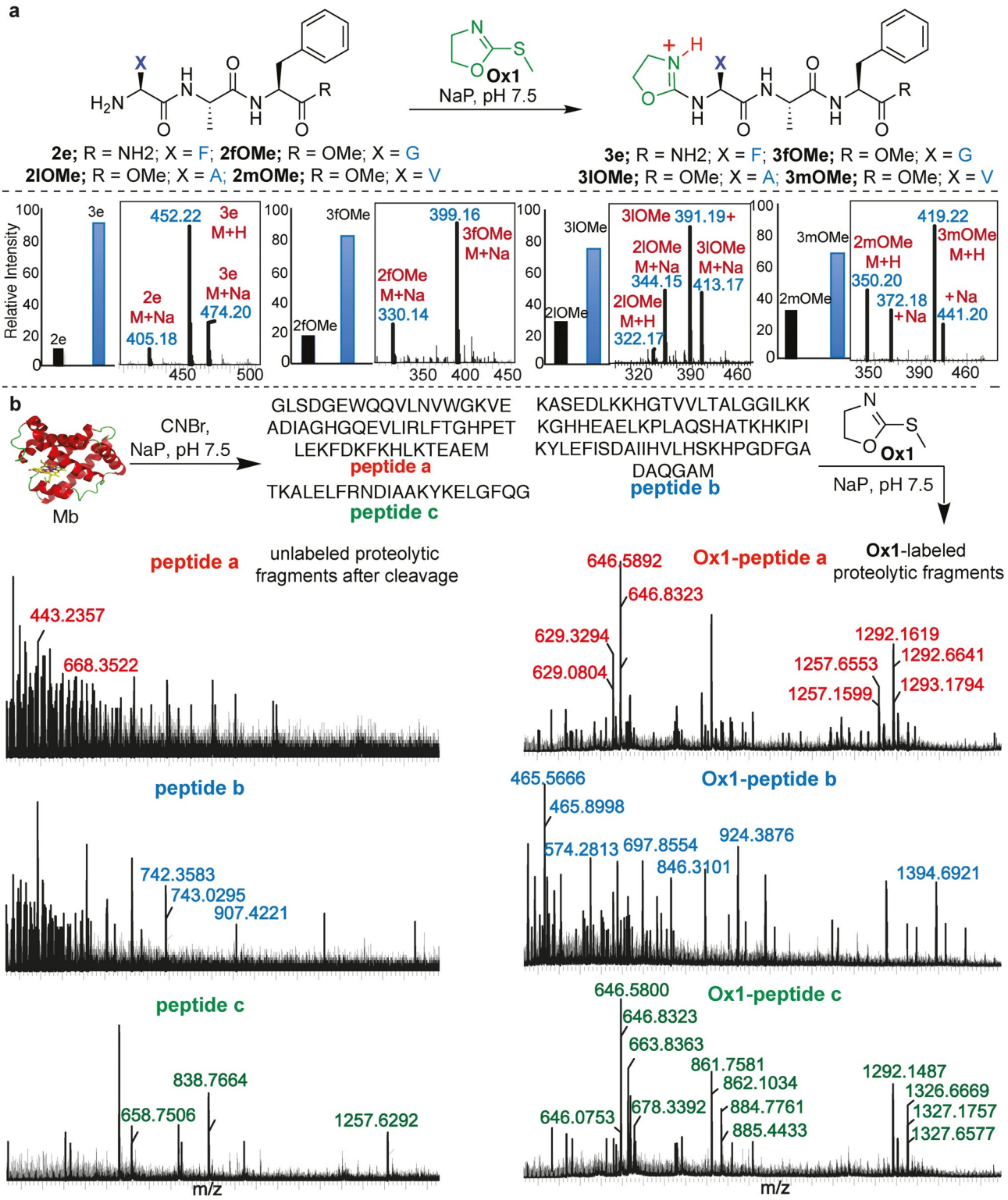

Figure 6. Tagging peptides and proteolytic fragments with mass sensitivity boosters. a) Oxazoline tagging of N-terminal peptides increases the mass detection sensitivity as compared to untagged peptides. b) Oxazoline tagging improves the detection of proteolytic fragments in a complex mixture. Proteolytic fragment **a** is barely visible after cleavage of myoglobin (left MS trace). All the proteolytic fragments of myoglobin, peptides **a**, **b**, and **c** are more ionized and clearly visible after tagging with oxazoline (right MS trace).

unlabeled myoglobin proteolytic fragments. For unlabeled proteolytic fragments of myoglobin, very poor sensitivity was observed for all the fragments (proteolytic fragments a, b and c, Figure 6b, Supplementary Figure 24). For the labeled proteolytic fragments of myoglobin, all the fragments were detected with significantly high mass intensities (Figure 6b,

Supplementary Figure 24). These studies showed improved sensitivity of the labeled proteolytic fragments in the complex mixture thus highly significant and circumvent detection limitation of the current N-terminal bioconjugation methods. The ability to modify all the N-termini of digested proteolytic fragments in the complex mixture could be highly valuable in proteomics studies. We also showed that mass sensitivity of the N-terminal modified myoglobin by Ox1 enhanced significantly as compared to the unmodified intact myoglobin (Supplementary Figure 25). Moreover, the N-terminal oxazolation method could be used to detect the unique proteolytic fragments obtained due to chemotherapy-induced cell death leading to the discovery of novel biomarkers of cell death.

Conclusion

In summary, we have developed a novel oxazolation strategy, a single-step approach for the selective labeling of proteins with mass sensitive probes under physiological conditions. This method is highly selective for N-terminus because of the increased availability of deprotonated alpha-amino groups and interference from lysine and highly nucleophilic cysteine side chains was not observed. Although some N-terminal methods are often dependent on the nature and sequence of amino acids, no such effect was observed for this method. The potency of the oxazolation is well demonstrated by the selective labeling of a variety of peptides and proteins with different amino acid compositions including reaction with proteolytic fragments in complex mixtures as shown in Figures 5 and 6. The N-terminal labeling methods often suffer from low detection sensitivity of the resulting bioconjugates due to the blockage of the free N-terminus by the hydrophobic group and multiple derivatizations are needed for the complete analysis. The N-terminal oxazolation generated bioconjugates with remarkably high detection sensitivity. This is due to the sp^2-hybridized nitrogen of the oxazoline, which enhances the ionization of the labeled peptides. This strategy provides an excellent tool for the unambiguous analysis of bioconjugates in a complex mixture. One of the major advantages of our approach is that it carries out the site-selective modification of the intact protein at the N-terminus, increases its mass sensitivity without diminishing its activity which is in contrast to other mass sensitive boosters.

The unique feature about our approach as compared to other methods for mass sensitivity booster is that it is highly selective and generates only one modified fragment rather than mixture of the multiple modified fragments generated by the use of commercial mass sensitivity reagents due to their non-selective nature. Analysis of the mass data with a mixture of differently modified fragments is highly complicated.

The N-terminal oxazoline-modified peptides and proteins exhibit high stability over a range of pH conditions, demonstrating the potential utility of this chemistry in the conjugation of pharmaceutically active compounds and biological probes. Considering the simple setup and chemoselective nature of this bioconjugation reaction with easily derivatized mass sensitivity boosters and its use in efficient tagging of proteolytic fragments from a complex mixture, we anticipate that this method will become a highly useful tool in many scientific fields.

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Conflict of interest

The authors declare no conflict of interest.

Keywords: chemoselective · mass sensitivity · N-terminal labeling · oxazolation · site-selective

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