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Complete chemical modification of amine and acid functional groups of peptides and small proteins

Casey J. Krusemark, Brian L. Frey, Lloyd M. Smith, and Peter J. Belshaw

Summary

The chemical modification of protein thiols by reduction and alkylation is common in the preparation of proteomic samples for analysis by mass spectrometry (MS). Modification at other functional groups has received less attention in MS-based proteomics. Amine modification (Lys, N-termini) by reductive dimethylation or by acylation (e.g. iTRAQ labeling) has recently gained some popularity in peptide-based approaches (bottom-up MS). Modification at acidic groups (Asp, Glu, C-termini) has been explored very minimally. Here, we describe a sequential labeling strategy that enables complete modification of thiols, amines, and acids on peptides or small intact proteins. This method includes (1) the reduction and alkylation of thiols, (2) the reductive dimethylation of amines, and (3) the amidation of acids with any of several amines. This chemical modification scheme offers several options both for the incorporation of stable isotopes for relative quantification and for improving peptides or proteins as MS analytes.

Keywords

mass spectrometry (MS); stable isotope labeling; acylation; dimethylation; amidation; proteomics; protein derivatization; peptide derivatization

1. Introduction

The chemical modification of peptides or proteins prior to MS-analysis is generally carried out for any of three purposes: (1) to attach an affinity handle to purify a subset of the proteome, (2) to incorporate stable isotope labels for relative quantification of two or more samples, and/or (3) to modify the physiochemical properties of peptides and proteins to improve them as MS analytes or to facilitate upstream sample preparation (e.g. chromatography). In this chapter, we present a detailed protocol for the complete modification of amines and acids on peptides or small intact proteins (Scheme 1). Given that these are very common functional groups, this approach presents useful possibilities for incorporating labels for quantification and for broadly modifying the properties of proteins or peptides in proteomic samples (1). We have shown that modifying proteins in this manner can significantly affect charging during electrospray ionization mass spectrometry (ESI-MS) and even reveal insights into the ESI mechanism (2, 3). We are currently exploiting this modification strategy for peptides, including tryptic digests of yeast lysate samples, to increase ionization efficiency and charge states during ESI.

The primary consideration when choosing chemical reactions for the modification of proteins and peptides in MS analysis is the purity of the transformation. Reactions should proceed to completion and modify only targeted functional groups. Incomplete modifications and side products produce additional complexity that can hinder analysis considerably. This is more problematic for the modification of intact proteins, as side

reactions and missed modifications are quickly compounded. For example, the modification of a protein with 10 targeted sites leads to over 1000 possible products ($2^n - 1$) when the transformation is not complete at each site (4). Additionally, a transformation with an efficiency of 95% at each site would produce only 60% (0.95^{10}) of the desired product. Other considerations in choosing modification reactions may include generality, cost, ease of use, and the retention of desired properties for MS analysis. Ideally, reactions should enable facile modification with any of several functionalities at low cost without complicating MS analysis.

We have developed conditions for the modification of intact proteins in part to facilitate “top down” proteomics, where proteins are analyzed intact, as opposed to the “bottom-up” approach, where proteins are digested into peptides. There are several advantages to the analysis of intact proteins. The increased complexity generated by proteolysis is avoided. Measurement of the intact mass of a protein can contribute significantly to its identification. Also, post-translational modifications can be more accurately located and assigned to distinct protein forms. The “top-down” approach in MS, however, has seen limited implementation partially due to the physiochemical properties of many intact proteins, which can present problems of insolubility, limited charging in MS, and poor separation in chromatography (5, 6). Chemical modification may be able to address these issues. Furthermore, the development of methods for modification of intact proteins can enable quantitative proteomics on whole proteins through labeling with stable isotopes. Labeling with a mixture of two isotopic versions of the same reagent has the additional application that the number of functional groups in a protein can be determined by the mass difference between the heavy and light forms (Fig. 1). This information, together with the intact mass, can be used to uniquely identify a protein from a protein database or genetic sequences, which can obviate the need for MS/MS sequencing (1). This approach for protein identification has been applied with metabolic isotopic labeling in cell culture (7, 8).

We have previously demonstrated the generality of global amine and acid labeling on 4 small proteins (< 17 kDa) (1). Further work may be required for the modification of larger proteins, as the possibilities of side products and missed modifications will increase with increasing protein size. Also, large proteins may present difficulties for complete modification due to the increased likelihood of higher order structure and incomplete denaturation. However, in the previous work, we did find that the smallest protein tested, ubiquitin (8.6 kDa), was the most difficult to modify at the acidic functional groups. This may have been due to the relatively high density of acidic groups or otherwise related to the particular sequence of this protein. We anticipate that the presented conditions would be directly applicable for modification of a low-mass proteomic mixture (9).

We have found modifying peptides to be generally easier than modifying intact proteins due to their smaller size and more limited secondary and tertiary structure. Thus, the reaction protocols developed herein for proteins also worked well for peptide labeling. However, we have further optimized the reaction and cleanup conditions specifically for peptide modification to produce a robust protocol for bottom-up proteomics applications.

1.1. Amine modification

Chemical modification of amines in peptides has seen several applications in MS-based proteomics. Generally, these have been limited to guanidination, acylation, and reductive alkylation reactions. Guanidination of peptides with O-methylisourea has been used to increase signal in MALDI-MS and also to incorporate stable isotopes for quantitative bottom-up proteomics (10). However, we experienced difficulty in achieving complete guanidination on intact proteins.

Acylation of amines with active esters has seen several proteomic applications involving either modification of peptides or intact proteins. Two notable examples include iTRAQ® labeling of peptide amines (11) and ICPL™ for intact proteins (12), both of which employ stable isotopes to enhance relative quantification of protein abundance. Acylation of amines is an attractive modification reaction in that several activated esters are commercially available (typically *N*-hydroxysuccinimide (NHS) esters) or are easily prepared from carboxylic acids. While acylation of amines proceeds efficiently with activated esters, our experience and additional studies have shown that selective amine acylation is not possible and that hydroxyl groups of tyrosine, serine, and threonine are acylated as well (1, 12–14). The *O*-acylations can be selectively removed, but harsh conditions are required, such as alkaline hydrolysis, lysis with high concentrations of hydroxylamine, or high temperatures. These conditions are less than desirable for proteomic analysis due to side reactions or loss of post-translational modifications (14). Presumably, the iTRAQ reagent avoids this complication due to the electron withdrawing nature of *N*-methylpiperazine acetic acid esters, which likely hydrolyze readily at neutral pH. An additional drawback of amine modification via acylation is the loss of the amine basic group that is important for charging in positive mode MS, the typical mode for proteomic analysis. This can be avoided by including a non-nucleophilic basic group, such as a tertiary or quaternary amine, in the activated ester. The preparation of such an active ester is not trivial, and including a basic site in the active ester reduces the generality of this approach.

The reductive alkylation of peptide amines with formaldehyde has been gaining popularity for proteomic applications, primarily as a means to incorporate stable isotopes for quantitative proteomics (15). Also, dimethylation significantly enhances the signal of a_1 ions in collisionally activated dissociation (CAD) (16). A drawback of the approach has been side products and incomplete modification for reactions using the common reductant sodium cyanoborohydride (16, 17). By modifying the procedures of Means and Feeney(18), we have developed conditions using pyridine-borane as the reductant that enable complete dimethylation of amines to yield modified peptides and whole proteins with excellent purity (Fig. 2) (1). In addition to high purity, the benefits of this reaction include the ease of modification, the preservation of the amine group for MS charging, and the inexpensive availability of stable isotope forms of formaldehyde. The reaction is limited in generality, since other aldehydes react much less efficiently than formaldehyde. Recently, however, complete reductive alkylation of an intact protein was demonstrated with the dialdehyde, glutaraldehyde (19).

1.2. Acid modification

The modification of peptide carboxylic acid groups has seen more limited application in MS proteomics. Esterification of acidic groups using anhydrous, acidic methanol or ethanol has been employed to incorporate stable isotopes, to prevent preferential cleavage at acidic sites in CAD, and also to eliminate copurification of carboxylic acid-containing peptides with phosphopeptides during immobilized metal affinity chromatography (IMAC) (20–22). The esterification reaction is limited to short chain alcohols that will solubilize peptides and is not applicable to proteins, which are often poorly soluble in alcohols (23). Amidation of carboxylic acids by reaction with amines has been used classically for the determination of protein acids by hydrolytic amino acid analysis (24), but it has not seen application in MS-based proteomics. A study by Tanaka and coworkers on the amidation of peptide acids with ammonia and a water-soluble carbodiimide found incomplete transformations and the dehydration of peptides due to intramolecular cyclization reactions (25). Another study by Xu et al. reported excellent reaction efficiency for carbodiimide coupling of a particular amine, 1-(2-pyrimidyl) piperazine, to peptide acid groups for a few model peptides (26).

We have developed conditions for the complete modification of peptide and protein carboxylic acids in high purity (1). Our approach involves the capping of both thiol and amine nucleophilic groups on the protein prior to the acid coupling reaction in order to prevent intramolecular cyclization (Scheme 1). The spectra in Fig. 3 demonstrate the high purity of modified protein obtained from this method. The modification of carboxylic acids by amidation has several advantages as a proteomic labeling reaction. The main advantage is its generality. We have demonstrated efficient coupling with a variety of primary amines (Figs. 4 and 5). Primary amines are widely commercially available. Additionally, carboxylic acids do not contribute to charging in positive mode MS (2), as so their modification does not hinder MS detection. As with esterification, amidation can limit preferential cleavage at acidic sites in CAD (25). Importantly, we found that amidation does not complicate MS/MS sequencing by CAD, electron transfer dissociation (ETD), or infrared multiphoton dissociation (IRMPD) (1).

2. Materials

2.1. Thiol-modification and digestion

1. Protein alkylation buffer: 6 M guanidine-HCl, 60 mM NH_4HCO_3 . Prepare freshly before use.
2. 500 mM stock solution of dithiothreitol (DTT) in water
3. Iodoacetamide (IAA)
4. Sequencing-grade modified trypsin (Promega)
5. Ultra-CleanTM SPE C18 4 ml cartridge (Grace/Alltech)

2.2. Amine modification

1. Amine modification buffer: 6 M guanidine-HCl, 300 mM triethanolamine buffer, pH 7.5. Prepare freshly before use.
2. 8 M pyridine- BH_3 in pyridine
3. Methanol
4. d0 formaldehyde, (formalin, 37% in water)
5. d2 formaldehyde, (Cambridge Isotope Laboratories)

2.3. Acid amidation

1. DMSO (see Note 1)
2. (7-Azabenzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate (PyAOP, Applied Biosystems)
3. N-methylmorpholine (NMM)
4. Desired amine HCl salts such as those listed in 2.3.1
5. Aqueous solution of 0.1% formic acid
6. Aqueous solution of 0.1% trifluoroacetic acid (TFA)
7. Chloroform

¹We have found that “wet” DMSO works as well or better than dry DMSO. Simply use a bottle of standard, reagent grade DMSO. No special precautions were taken to limit water absorption from air—the solvent was not distilled, did not contain a water adsorbent or a septum cap, and was not blanketed with inert gas after each use.

8. 5K MWCO Amicon Ultra centrifugal filter device (Millipore)
9. C18 Ultramicrospin columns (The Nest Group)

2.3.1. Amine HCl salts

1. Glycine methyl ester HCl (Aldrich)
2. d₃ glycine methyl ester HCl, prepared from d₃ methanol as previously described (27)
3. Benzylamine HCl
4. Propargylamine (Aldrich)
5. 3-Azidopropylamine, prepared as previously described (28)
6. (4-Aminobutyl)trimethylammonium dichloride (C4 Quat), prepared as described in (1)
7. 5-(dimethylamino)amylamine (C5 Tert, Matrix Scientific)
8. N,N-diisopropyl-1,5-pentanediamine (C5 isoTert, Alfa Aesar)
9. N,N-diethyl-1,4-butanediamine (C4 ethylTert, Alfa Aesar)
10. 4-(3-aminopropyl)morpholine (C3 Morph, Alfa Aesar)
11. 4-(2-aminoethyl)morpholine (C2 Morph, Aldrich)
12. 4-(2-aminoethyl)pyridine (C2 Pyridine, Alfa Aesar)
13. 4-(1-pyrrolidino)butylamine (C4 Pyrrolidine, Alfa Aesar)
14. 1 M HCl in ether (Alfa Aesar)
15. Acetone

2.4 HPLC purification

1, Mobile Phase A: 0.5% TFA in water

1. Mobile Phase B: 0.5% TFA in acetonitrile

3. Methods

3.1. Protein modification

3.1.1. Thiol alkylation

1. Dissolve protein sample in protein alkylation buffer to 5 mg/ml concentration.
2. Add 500 mM DTT stock to 20 mM concentration.
3. Heat at 37°C for 1 hour to reduce disulfide bonds.
4. Dilute solution to 2.5 mg protein/ml with protein alkylation buffer.
5. Add solid IAA to 25 mM concentration.
6. Incubate at room temperature for 1.5 hours in the dark, e.g. in foil covered tube.
7. Purify protein by RP-HPLC on a semi-preparative C18 reversed phase column using with a gradient of 15–95% B over 25 min (See Note 2).
8. Remove acetonitrile from collected fractions by rotary evaporation.
9. Freeze protein solution and lyophilize to obtain pure labeled protein.

3.1.2. Amine methylation

1. Dissolve thiol-alkylated protein or protein lacking thiols in amine modification buffer to 1.25 mg/ml. Be certain that the proteins are well dissolved and heat gently at 50°C if needed.
2. Dilute sample to 1 mg/ml with methanol.
3. Add pyridine-BH₃ complex to 30 mM.
4. Add formaldehyde (d₀ or d₂) to 20 mM.
5. Sonicate reactions for one minute in water bath sonicator to incorporate pyridine-BH₃.
6. Incubate reaction at room temperature for 2 hours.
7. Purify by reverse phase HPLC and lyophilize, as described in 3.1.1.7–3.1.1.9.

3.1.3. Acid amidation

1. Dissolve thiol-alkylated, amine dimethylated protein in DMSO to 1.5 mg/ml concentration.
2. Heat solution (50°C) and sonicate until dissolved.
3. Add glycine methyl ester HCl (d₀ or d₃) to 1 M and *N*-methylmorpholine (NMM) to 750 mM. Heat solution and sonicate if necessary to dissolve. (See Notes 3, ⁴, and ⁵)
4. Add solid PyAOP to 40 mM.
5. Allow reactions to proceed at room temperature for 2 hours.
6. Quench with an equal volume of water.
7. Purify by reverse phase HPLC, as described in 3.1.1.7–3.1.1.9. (See Note 6)
8. If reaction components (typically tripyrrolidinophosphine oxide) co-elute with modified protein on RP-HPLC, remove small molecular weight species with a molecular weight cut-off filter device prior to HPLC. Dilute the reaction mixture 10–20-fold in protein alkylation buffer and centrifuge over the MWCO filter to concentrate. Repeat dilution and centrifugation until contaminants are removed.

3.1.4. MS analysis

1. Dissolve purified, lyophilized protein in 49.5/49.5/1% H₂O/methanol/formic acid.

²While we found quick HPLC gradients to be the most convenient way to purify proteins between reaction steps, we also explored acetone or ethanol precipitation. This approach provided clean products, but there were significant losses of protein and often carryover of guanidinium salts. There are several, potentially higher throughput options for the purification of the high molecular weight proteins from the small molecules used for modification. These include dialysis/molecular weight cut-off filters, reverse phase spin columns, and more quantitative precipitation with sodium deoxycholate/trichloroacetic acid (29).

³We found these weakly basic, buffered conditions to be critical to achieving high purity transformations. Using amine-free bases or a stronger base, such as triethylamine, in the place of NMM gave poor purity products.

⁴If using other amines, the ratio of amine HCl salt to NMM base should be determined empirically to optimize efficiency of labeling. The typical ratio of amine HCl to NMM with more basic amines is 1 M amine HCl to 500 mM NMM, which was used for benzyl amine and 3-azidopropylamine (Fig. 4B and 4D). A ratio of 500 mM amine HCl and 250 mM NMM was used for propargyl amine labeling (Fig. 3C).

⁵If using a highly polar amine that is poorly soluble in DMSO, a minimum amount of water should be added to solubilize. In the case of (4-aminobutyl)trimethylammonium dichloride (Fig. 4A), 500 mM of this di-HCl salt and 350 mM NMM were used with up to 10% H₂O added.

⁶Proteins modified with glycine methyl ester should be quickly reduced in volume and frozen to minimize ester hydrolysis while in acidic HPLC buffers.

2. Analyze by MS method of choice, e.g. direct electrospray or LC-MS.

3.2. Peptide modification

3.2.1. Thiol alkylation—Peptides or proteins containing cysteine residues are reduced and alkylated with DTT and IAA according to any standard procedure, such as the one outlined in section 3.1.1. Proteins are then digested enzymatically to produce peptides. Subsequent purification with a C18 stationary phase is necessary to remove the excess DTT and IAA before proceeding with the methylation and amidation reactions.

3.2.2. Amine methylation

1. Dissolve thiol-alkylated peptides in 50:50 methanol:H₂O containing 40 mM formaldehyde, 60 mM pyridine-BH₃ complex, and 50 mM NMM to a peptide concentration of 2 mg/ml.
2. Incubate reaction at room temperature for 1–2 hours.
3. Evaporate to dryness in a vacuum centrifuge, which removes most of the excess reagents.

3.2.3. Acid amidation

1. Dissolve thiol-alkylated, amine dimethylated peptides in DMSO to 1.5 mg/ml concentration.
2. In a separate tube, prepare the following reagent mixture (note that the amounts are for 100 µg of peptides; scale accordingly). Dissolve 30 µmoles of the mono- or di-HCl salt of the desired amine in 5 µl of water and 2.6 µl of NMM. If the amine does not completely dissolve, heat the solution or add an additional 1–2 µl of water (see Note 7).
3. Transfer the peptide/DMSO solution into the amine reagent mixture and mix thoroughly. The resulting amine and NMM concentrations are 450 and 350 mM, respectively.
4. Add solid PyAOP to 60 mM.
5. Allow reaction to proceed at room temperature for 2 hours.
6. Quench with 0.1% aqueous formic acid to dilute DMSO to 5% (v/v). (See Note 8)
7. Extract the quenched reaction with chloroform (double the sample volume). Shake vigorously for 2 minutes, with periodic venting. Transfer most of the aqueous layer to a new tube with another aliquot of chloroform, and shake again. Transfer most of the aqueous layer to a new tube.
8. Evaporate to dryness in a vacuum centrifuge.
9. Dissolve peptides in 0.1% aqueous TFA to 0.3 mg/ml and purify by C18 solid-phase extraction. Elute with 75% acetonitrile, 0.1% formic acid.
10. Evaporate to dryness or dilute into a suitable (LC)-MS solvent.

⁷The amidation works best when employing hydrochloride salts of the amines. If attaching a diamine, then the di-HCl salt should be used except when one of the amine groups has a pK_a <8. In that case, we have found that the mono-HCl salts give better yields with the NMM buffer conditions used here.

⁸Acidification of the solution keeps peptides protonated, which helps retain them in the aqueous phase during chloroform extraction.

3.3. Preparation of amine HCl Salts

1. Add a stoichiometric amount of 1 M HCl in ether to the amine-free base.
2. Remove solvent by rotary evaporation.
3. Dissolve in acetone.
4. Remove solvent by rotary evaporation.

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Figure 1. Relative quantification of protein samples and determination of the number of protein functional groups

(A) ESI-FTMS spectrum of 15+ ions of thiol-alkylated lysozyme dimethylated with either D_0 or D_2 formaldehyde and mixed in a 1:1 ratio. (B) ESI-FTMS spectrum of 16+ ion of thiol-alkylated, dimethylated lysozyme amidated with either D_0 or D_3 glycine methyl ester and mixed in a 1:1 ratio. Spacing of peaks indicates the numbers of functional groups present in the protein (from ref. 1, reprinted by permission from American Chemical Society, copyright 2008).

Figure 2. ESI-FTMS spectra of (A) thiol-alkylated RNase A, (B) thiol-alkylated lysozyme, (C) thiol-alkylated, amine dimethylated RNase A, and (D) thiol-alkylated, amine dimethylated lysozyme

The expanded spectra show a cluster of peaks due to the natural isotopic distribution of the completely modified protein product. If the reaction did not go to completion, peaks would be observed at lower m/z values, but no such peaks are present (from ref. 1, reprinted by permission from American Chemical Society, copyright 2008).

Figure 3. ESI-FTMS spectra of (A) thiol-alkylated, amine dimethylated, acid amidated (with glycine methyl ester) RNase A and (B) thiol-alkylated, amine dimethylated, acid amidated (with glycine methyl ester) lysozyme

As in Figure 2, the near absence of peaks at lower m/z values indicates that the reactions have gone to completion (from ref. 1, reprinted by permission from American Chemical Society, copyright 2008).

Figure 4. ESI-FTMS spectra of thiol-alkylated, amine dimethylated, acid amidated lysozyme amidated with the indicated amines
(from ref. 1, reprinted by permission from American Chemical Society, copyright 2008).

Reaction Efficiencies for Amidation of Neurotensin

Figure 5. The reaction efficiency for various amines used to amidate the peptide neurotensin (pyroELYENKPRRPYIL)

Efficiencies were calculated from the peak intensities of the doubly-amidated product, the singly-amidated product and the starting material (dimethylated peptide) obtained by direct injection MS. The results are given as a percentage of the total number of acid sites, which means that the singly-amidated product was counted as one site reacted and one site not reacted. Different forms of the amine were used for modification as indicated (free base, mono-HCl, and/or di-HCl). Greater than 99% reaction efficiency was obtained for all amines in at least one of the forms, except for the case of pyrrolidine, which gave poor results for the di-HCl salt and the other forms were not tested.

Scheme 1.
Protein and peptide modification strategy