Counting Sulfhydryls and Disulfide Bonds in Peptides and Proteins Using Mercurial Ions as an MS-Tag

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Organic mercurial compounds are the most specific and sensitive reagents for reaction with the sulfhydryl groups (—SH) in peptides and proteins because of the strong mercury–sulfur affinity. Using the monofunctional organic mercury ion RHg^+ as a mass spectrometry (MS)-tag has the advantages of reacting with one sulfhydryl group, offering definite mass shift, and especially stable and characteristic nonradioactive isotopic distribution. Mass spectrometric analysis of derivatized sulfhydryls in peptides/proteins is thus an alternative for precisely counting the number of sulfhydryl groups and disulfide bonds (—S—S—). Here the tags used include monomethylmercury chloride, monoethylmercury chloride, and 4-(hydroxymercuri) benzoic acid. The feasibility of this strategy is demonstrated using HPLC/ESI-MS to count —SH s and —S—S— in model peptides/proteins, i.e., glutathione, phytochelatin, lysozyme and β -lactoglobulin, which contain increasing —SH s and various —S—S— linkages. (J Am Soc Mass Spectrom 2008, 19, 1108–1113) © 2008 American Society for Mass Spectrometry

Cysteine-free sulfhydryls (—SH) of proteins are exceptionally useful in terms of both their structural and redox regulatory aspects. Some critical cysteine residues can either activate or deactivate protein functions in various physiologically important reactions and, increasingly, are being recognized as important modulators of protein function [1]. Disulfide bond (—S—S—) formation between —SH s, a posttranslational modification, is vital for the correct folding and tertiary structure stability of many secreted and membrane proteins [2]. Therefore, identification of —SH and —S—S— can give valuable information concerning the relationship between the structure and function of a protein. On the other hand, frequency analysis of amino acids in the proteomes of fully sequenced genomes shows that cysteine is one of the most rarely used amino acids in proteins across all superkingdoms [3]. Because of their ubiquity but lower abundance, —SH s provide a greater opportunity for saturating the reactive sites, resulting in more reproducible quantitative labeling and less impact on the proteins. Numerous spectrometric methods for determining the —SH s derivatized by alkylation agents have been reported [4, 5]. Although there are increasing numbers of commercially available thiol-reactive dyes, such as iodoacetamide or maleimide, the application of these dyes to proteomic studies

still has some limitations. Organic dyes often suffer from photobleaching; their molecules (~ 1 nm in diameter) are usually large in size, sometimes limiting their access to —SH s existing inside the configuration of a protein because of steric restrictions; and dye molecular rigidity might be destroyed for the sake of labeling, leading to fluorescence signal suppression. The functional groups with a selective reaction toward thiols include, but are not limited to, the iodoacetamide or maleimide moiety. Many monovalent and divalent metal ions can directly react with —SH s [6, 7] and, among them, mercurial compounds (especially organic mercurial compounds) are the most specific and sensitive reagents for reaction with —SH s because of the strong mercury–sulfur affinity [8, 9]. Although relevant reports are scarce [10–13], one realizes the potential application of organic mercurial compounds and tries to expand them to several analytical platforms. Using the monofunctional organic mercury ion RHg^+ as a mass spectrometry (MS)-tag has the advantages of reacting with one —SH , definite mass shift, and especially stable and characteristic nonradioactive isotopic distribution when monocharged derivatized peptides and proteins are monitored. Thus, mass spectrometric measurements of the —SH s derivatized by RHg^+ in peptides and/or proteins constitute an alternative strategy for counting —SH s and —S—S— after suitable reduction.

This report describes the feasibility of using high performance liquid chromatography (HPLC) coupled with electrospray ionization mass spectrometry (ESI-MS) to count the number of —SH s and —S—S— in

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peptides and proteins using RHg^+ as an MS-tag. One can easily measure the exact numbers of —SHs and —S—S— in peptides/proteins because the binding stoichiometry between RHg^+ and —SH can simply reach 1:1. Free —SHs in peptides and/or proteins are treated directly with RHg^+ to label them, whereas —S—S— in proteins are subjected to reduction by tris-(2-carboxyethyl)-phosphine (TCEP) and the resulting nascent —SHs are labeled immediately with RHg^+ (Scheme 1). The labeled proteins can be isolated by HPLC and characterized by ESI-MS. The feasibility of this strategy is demonstrated by counting —SHs and —S—S— in model peptides/proteins, i.e., glutathione (GSH), phytochelatins (PCs), lysozyme, and β -lactoglobulin, which contain increasing —SHs and various —S—S— linkages.

Experimental

Methylmercury chloride (CH_3HgCl ; >95%) and ethylmercury chloride ($\text{CH}_3\text{CH}_2\text{HgCl}$; >98%) were obtained from Alfa Aesar (Ward Hill, MA, USA), and 4-(hydroxymercuri) benzoic acid ($\text{HOOC}-\text{C}_6\text{H}_4-\text{HgOH}$) from Fluka (Buchs, Switzerland). TCEP, β -lactoglobulin, and lysozyme were purchased from Sigma-Aldrich (St. Louis, MO, USA). GSH and tris(hydroxymethyl) aminomethane (Tris) were purchased from Sino-American Technology (Shanghai, China). Ultrapure water (UPW; 18 M Ω) was prepared with a Milli-Q system (Millipore Filter Co., Bedford, MA, USA) and used throughout this study.

A solution of GSH (1 μM in 50 μL UPW) was added to small vials containing 10 μL $\text{HOOC}-\text{C}_6\text{H}_4-\text{HgOH}$ (10 μM in 1 mM NaOH solution) and 10 μL CH_3HgCl (10 μM in methanol). The vials were sealed and vibrated in the dark for 20 min to ensure a complete reaction. PC_3 was prepared as described previously [14]. PC_3 (50 μL , 15 μM) in Tris-buffer (20 mM, pH 7) was mixed with 5 μL CH_3HgCl methanol solution (30 mM) and 5 μL $\text{HOOC}-\text{C}_6\text{H}_4-\text{HgOH}$ (30 mM in 1 mM NaOH solution) and then the mixture was incubated in the dark at room temperature for 20 min. Before derivatization, the disulfide bonds in lysozyme (50 μL , 10 μM) were reduced with TCEP (10 $\times$ in excess compared to the disulfide bonds) at room temperature for 20 min. Derivatization of the nascent free —SHs by $\text{CH}_3\text{CH}_2\text{HgCl}$ and CH_3HgCl (2.5 $\times$ in excess compared to TCEP) was performed in the dark for 20 min.

Scheme 1. The —SHs and —S—S— of a peptide or protein labeled with RHg^+ .

β -Lactoglobulin (5 μM) was directly mixed with a 2.5-fold excess amount of CH_3HgCl to react with free sulfhydryl. Then the solution was heated in 8 M urea to denature the labeled β -lactoglobulin and treated in the same way as lysozyme to label disulfide bonds at room temperature for 1 h.

Isolation of the labeled peptide/protein was carried out on an Agilent 1100 series chromatographic system (Agilent Technologies, Palo Alto, CA, USA) using a Zorbax C-18 column (1.0 I.D. $\times$ 150 mm long, 3.5 μm). A gradient elution program was used to linearly increase the percentage of mobile phase B (0.3% acetic acid in acetonitrile) from 10 to 60% while decreasing the mobile phase A (0.3% acetic acid in UPW) from 90 to 40% with a flow rate of 0.05 mL/min. The HPLC was directly coupled to an Esquire-LC ESI ion trap mass spectrometer (Bruker Daltonics, Bremen, Germany). All MS experiments were performed in the positive-ion mode. The operational parameters were as follows: nebulizer, 22 psi; dry gas, 12 L/min; dry temperature, 300 $^\circ\text{C}$; capillary, -3500 V; endplate offset, -500 V; skim 1, 35.0 V; skim 2, 6.0 V; capillary exit offset, 60.0 V; octopole, 2.80 V; lens 1, -5.0 V; lens 2, -60.0 V; trap drive, 55.0; and max accumulation time, 50.00 ms.

Results and Discussion

Counting Free Sulfhydryls (—SHs)

GSH, which is a tripeptide (Glu-Cys-Gly), contains one free sulfhydryl. A typical ESI mass spectrum of GSH at m/z 308 is shown in Figure 1a. Figure 1b shows the mass spectrum of the GSH labeled by one $\text{HOOC}-\text{C}_6\text{H}_4-\text{Hg}^+$, indicating that the complex mass shifts to the higher mass region at m/z 626, 627, 628, 629, 630, 631, 632, and 633 arising from the natural isotopic distribution of Hg. The isotopic distribution observed coincides with that from theoretical calculation. Figure 1c shows that GSH labeled by CH_3Hg^+ gave a similar result. The visible characteristic isotopic distribution is of particular significance to offer solid evidence for the labeled —SH, making the MS technique a superior alternative for counting —SHs.

When peptides or proteins contain more free —SH groups, the RHg^+ also shows great efficiency as an MS-tag. PC_3 , which has the structure $(\gamma\text{-Glu-Cys})_3\text{-Gly}$, was chosen as a model peptide (Figure 2a). Although $\text{HOOC}-\text{C}_6\text{H}_4-\text{Hg}^+$ has been used to label —SHs, we always obtain incompletely derivatized PC_3 (data not shown) because of the steric hindrance arising from the close arrangement of —SHs in the molecule. Considering that the dissociation constant between CH_3Hg^+ and cysteine is as low as $10^{-20.3}$ [15], that the ratio of the dissociation constants of $[\text{CH}_3\text{Hg-GS}]$ to $[\text{HOOC}-\text{C}_6\text{H}_4\text{Hg-GS}]$ is $10^{-1.11}$ estimated by ESI-MS (data not shown), as well as the fact that CH_3Hg^+ has a much smaller size compared with $\text{HOOC}-\text{C}_6\text{H}_4-\text{Hg}^+$, CH_3Hg^+ should serve as a better MS-tag. This is evidenced by the result that the three —SHs in PC_3 have

Figure 1. ESI-MS spectra of (a) native form of GSH ($308m/z$); (b) GSH labeled by $\text{HOOC-C}_6\text{H}_4\text{-Hg}^+$, with peaks at m/z 626, 627, 628, 629, 630, 631, 632, and 633 arising from the natural isotopic distribution of Hg; (c) GSH labeled by $\text{CH}_3\text{-Hg}^+$, with peaks at m/z 520, 521, 522, 523, 524, 525, 526, and 527 arising from the natural isotopic distribution of Hg.

Figure 2. ESI-MS spectra of (a) native form of PC_3 (772 m/z); (b) PC_3 labeled by CH_3Hg^+ , where three —SHs have completely reacted with $3 \times \text{CH}_3\text{Hg}^+$, with peaks from m/z 1412.8 to m/z 1419.8 arising from the isotopic distribution of Hg. The influence of the Hg factor weight on the isotopic distribution pattern is decreasing compared with Figure 1b and c.

been completely labeled by $3 \times \text{CH}_3\text{Hg}^+$, as shown in Figure 2b.

Counting Disulfide Bonds (—S—S—)

Lysozyme from hen egg white was chosen as a model protein to demonstrate the feasibility of the proposed strategy for counting the —S—S— in a protein. The intact disulfide folding of lysozyme has been well studied [16]. Lysozyme contains eight cysteines, giving rise to four —S—S— (Cys6-Cys127, Cys30-Cys115, Cys64-Cys80, and Cys76-Cys94). As shown in Figure 3a, the intact lysozyme shows a series of multicharged ions ranging from +10 to +16. The deconvoluted MW is 14,303.9. Figure 3b shows the spectrum of completely labeled lysozyme after being reduced by TCEP, where the eight free —SHs in the reduced lysozyme were all labeled by $\text{C}_2\text{H}_5\text{Hg}^+$. Although some of the —SHs must

be “partially buried” by their surrounding amino acids, there is little impact of steric hindrance for $\text{C}_2\text{H}_5\text{Hg}^+$ and it is accessible to all the —SHs in the lysozyme because of its smaller size. Similar results obtained using CH_3Hg^+ as an MS-tag (Supplemental Figure S1b, which can be found in the electronic version of this article) suggest that the size of $\text{C}_2\text{H}_5\text{Hg}^+$ and/or CH_3Hg^+ is small enough to gain access to all —SHs, so there is no need to denature the protein before reduction. The deconvoluted MW of labeled lysozyme is 16,139.9. The shift mass discrepancy is just $8 \times \text{C}_2\text{H}_5\text{Hg}^+$, indicating that four —S—S— exist in lysozyme. It should be noted that there are different labeled intermediate products during the labeling process, such as $1 \times \text{C}_2\text{H}_5\text{Hg}^+$, $2 \times \text{C}_2\text{H}_5\text{Hg}^+$, and so forth (data not shown), suggesting that the $\text{C}_2\text{H}_5\text{Hg}^+$ labeling process is performed stepwise, but the intermediates exist only during the labeling process. Our results show

that when the organic mercury has a 10-fold molar excess compared to that of the cysteine groups, given enough reaction time (20 min to 1 h), the final product is completely labeled. Clearly, intensive studies should be carried out to obtain a detailed delineation of the dynamic process involved.

Counting Both Free Sulfhydryls and Disulfide Bonds

β -Lactoglobulin contains one free sulfhydryl (Cys121) and two disulfide bonds (Cys66–Cys160 and Cys106–Cys119) [17]. It is a good model to demonstrate our strategy. Figure 4a shows the ESI mass spectrum of intact β -lactoglobulin A (deconvoluted MW = 18,361.3) and β -lactoglobulin B (deconvoluted MW = 18,275.5). Free —SH in β -lactoglobulin is treated directly with CH_3Hg^+ for derivatization. In Figure 4b new pairs of peaks are observed that correspond to derivatized β -lactoglobulin and yield a mass shift of 215.6 Da. Then the disulfide bonds in β -lactoglobulin were reduced by TCEP and the resulting nascent —SHs were derivatized immediately by CH_3Hg^+ . Figure 4c shows that β -lactoglobulin is completely labeled by $5\times \text{CH}_3\text{Hg}^+$. Figure 4c, like Figure 3b, looks much noisier than the original spectrum of the unmodified protein. The results show that the ionizability of labeled

Figure 3. ESI-MS spectra of lysozyme without labeling and with labeling by $\text{C}_2\text{H}_5\text{Hg}^+$: (a) intact lysozyme, with the deconvoluted MW: 14,303.9; (b) the lysozyme completely labeled by $8\times \text{C}_2\text{H}_5\text{Hg}^+$, with the deconvoluted MW: 16,139.9.

Figure 4. ESI-MS spectra of β -lactoglobulin without labeling and with labeling by CH_3Hg^+ : (a) intact β -lactoglobulin A and B, their deconvoluted MWs are 18,361.3 and 18,275.5, respectively; (b) free sulfhydryl of β -lactoglobulin labeled by CH_3Hg^+ , MWs of A and B shift to 18,576.8 and 18,491.7; (c) β -lactoglobulin completely labeled by $5\times \text{CH}_3\text{Hg}^+$. The corresponding MWs of A and B are 19,438.7 and 19,353.3.

peptides/proteins decreases with increasing RHg^+ content. However, the spectra still offer enough information for precisely counting the sulfhydryls and disulfide bonds in the proteins, and their ionizability maintains the same order of magnitude as the traditional iodoacetamide derivatization (Figure S1b and c).

Conclusion

RHg^+ interacts specifically with the —SHs of peptides and/or proteins at room temperature, such that the resulting stable, soluble, and covalently bonded biocon-

jugates exhibit great potential for the application of RHg^+ as a promising alternative MS-tag for screening peptides and proteins by HPLC/ESI-MS. It provides an alternative strategy to count the number of —SHs and —S—S— involved, offering valuable information for proteomic studies. Such information is not only important to protein chemistry, but also crucial to the quantitative analysis of peptides and/or proteins when a hard ionization source mass spectrometry, such as inductively coupled plasma mass spectrometry (ICP-MS) [18, 19], is applied to overcome the relatively low ionization ability of ESI-MS for labeled peptides and/or proteins. Both quantification of a protein and locating —SHs and —S—S— in a protein with ESI-MS together with ICP-MS using RHg^+ as a tag will be the subjects of future studies.

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