Identification and characterization of cysteinyl exposure in proteins by selective mercury labeling and nano-electrospray ionization quadrupole time-of-flight mass spectrometry

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We describe a method for probing surface-exposed cysteines in proteins by selective labeling with *p*-hydroxymercuribenzoate (PMB) combined with nano-electrospray ionization mass spectrometric analysis (nanoESI-MS). The rapid, stoichiometric, and specific labeling by PMB of surface-exposed cysteines allows for characterization of the accessibility of the cysteines using a single MS analysis. Moreover, by taking advantage of the large mass shift of 321 Da, unique isotopic pattern, and enhanced MS signal of PMB-labeled cysteine-containing peptide fragments, the surface-exposed cysteines in proteins can be accurately identified by peptide mapping. The number and sites of reactive cysteines on the surface of human and rat hemoglobins (hHb and rHb) were identified as examples. Collision-induced dissociation tandem mass spectrometric (MS/MS) analysis of specific peptides further confirmed the selective labeling of PMB in hHb. The subtle difference between the different cysteine residues in rHb was also evaluated by multiple PMB titrations. The difference between the two cysteines in their environment may partially explain their reaction specificity. Cysteine 125 in the β unit of rHb is exposed on the surface, explaining its reactivity with glutathione. Cysteine 13 in the α subunit of rHb is much less exposed, and is located in a hydrophobic pocket, a conclusion that is consistent with the previous observation of its selective binding with dimethylarsinous acid, a reactive arsenic metabolite. The method is potentially useful for probing cysteines in other biologically important proteins and for studying proteins that are associated with conformational or structural changes induced by denaturing processes, protein modifications, protein-protein interactions and protein assemblies. Copyright © 2010 John Wiley & Sons, Ltd.

Cysteine is a moderately hydrophobic amino acid that may be exposed on a protein or be shielded by tertiary or quaternary protein structures. It can be involved in protein folding by forming disulfide bonds, maintenance of structural integrity and stability,^{1–3} formation of functional domains (e.g. catalysis, DNA binding),^{4–6} metal chelation, mediation of redox state (e.g. S-conjugation, glutathionylation, S-nitrosylation), and regulation of signal transduction (S-nitrosylation).^{7–11} The accessibility of cysteine is an important means for proteins to mediate their conformations and functions.^{1,12–15} Several studies have demonstrated that cysteines on the protein surface that are solvent-accessible are generally more reactive and biochemically important.^{13,14,16}

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For example, glutathionylation at exposed Cys124 and Cys141 of p53 caused by oxidative stress can inhibit the binding of p53 to DNA.¹³ The exposure of the buried cysteine in the α unit of human hemoglobin that is induced by the mutation of tyrosine at β 35 to phenylalanine may destabilize the hemoglobin tetramer and lead to hemolysis.¹⁶ The access to surface cysteines has been exploited as an indicator for studying protein assembly and denaturation, and force-induced conformational changes.^{17–19} Understanding the biological functions and associated conformational changes of thiol-containing proteins also benefits characterization of the spatial environment around the cysteines. Traditionally, whether cysteines are exposed on the protein surface or buried inside the protein is measured by spectrometric titration,^{16,20} fluorescence labeling,²¹ and labor-intensive point mutation of cysteines.²²

A range of cysteine-specific reagents have been applied to probe the cysteinyl reactivity in various proteins.^{23–28} *p*-Hydroxymercuribenzoate (PMB) is one of such reagents. It only labels free cysteines specifically due to the high affinity

of monovalent mercury for the nucleophilic sulfhydryl group.²⁹ Moreover, due to its bulky size and hydrophilic nature (negative charge), PMB is difficult to diffuse into the hydrophobic core of proteins, and only labels free cysteines on the protein surface. PMB labeling has been applied to identify the exposed cysteines in proteins by spectrometric titration without denaturation of proteins.^{16,20} It was also proposed to label denatured proteins for measuring the number of total cysteines on proteins.³⁰ More recently, PMB-based beads were applied to enrich cysteine-containing peptides as a complementary method for improving the sequence coverage and enhancing the identification confidence of proteins.³¹ However, no specific method based on PMB labeling and MS detection has been exploited for studying the cysteine environment in proteins.

Here we combine selective mercury labeling with mass spectrometry technology for fast identification and characterization of surface-exposed cysteines in proteins. To demonstrate the application of this method, human and rat hemoglobins were chosen as model proteins. The number and sites of cysteines in hemoglobins vary greatly from one species to another, which could lead to different tolerance to exogenous stresses. With the method developed here, the number and location of the exposed cysteines in both hemoglobin of human and rat were identified.

EXPERIMENTAL

Caution: *p*-Hydroxymercuribenzoate (PMB) is toxic, and special care should be taken.

Reagents

PMB, trypsin (TPCK treated, proteomics grade), and standard hemoglobins of human and rat (hHb and rHb^{WT}) were purchased from Sigma (St. Louis, MO, USA). Another rat hemoglobin (rHb^{F344}) was extracted from blood of an F344 rat (~16 weeks old) following a published protocol.³² Both methanol and water were of HPLC grade, and purchased from Fisher Scientific (Fair Lawn, NJ, USA). All other reagents used were of analytical grade.

Single and multiple titration of hemoglobin using PMB

Hb (5 nmol; 50 μ M in 100 μ L sodium phosphate buffer (50 mM, pH 7.0)) was reacted with 0, 1, 2, 4, 6, 12, or 24-fold molar excess of PMB by addition of stock PMB (50 mM PMB in 50 mM sodium phosphate buffer, pH 7.0) at room temperature for 10 min. The resultant mixtures were subjected to desalting using a BioSpin column (Bio-Rad Laboratories, Hercules, CA, USA) for removal of unreacted PMB. The filtrates were then diluted to a final concentration of Hb of ~2 μ M using 10% methanol/water solution containing 0.001% formic acid, and subjected to nano-electrospray ionization mass spectrometric (nanoESI-MS) analysis.

Trypsin digestion of hemoglobin

The reaction mixtures after incubation as prepared above were first centrifuged using Microcon-10 concentrators (molecular weight (MW) cutoff: 10 kDa; Millipore, Milford, MA, USA) against the phosphate buffer (50 mM, pH 7.0) to remove unreacted PMB by centrifugation at 13000 rpm and 4°C. The resultant PMB-labeled Hb was then exchanged into digestion buffer (50 mM ammonia bicarbonate, pH 7.8), followed by digestion using trypsin with a mass ratio of protein to enzyme of 30:1 for 18 h. The resultant peptide mixtures were subjected to Ziptip C18 (Millipore, Milford, MA, USA) purification. The eluent from the Ziptip C18 was directly loaded onto a nanoESI tip and subjected to nanoESI-MS analysis.

NanoESI-MS analysis of PMB-Hb reaction products

We used a previously described system with slight modification.^{32,33} Briefly, a nanoESI source (Protana, Denmark) was coupled to a QSTAR Pulsar I mass spectrometer (Applied Biosystem/MDS Sciex, Concord, Ontario, Canada). The conductive capillary tip for nanoESI (Proxeon Biosystem, Denmark) filled with samples was positioned onto the nanoESI source using a three-dimensional positioner. The system was operated in the positive ionization mode with an ionization spray voltage of 950 V, first declustering potential (DP1) of 65 V, second declustering potential (DP2) of 10 V, and focusing potential (FP) of 215 V. The instrument was routinely calibrated using standard horse skeletal apomyoglobin (Sigma-Aldrich, St. Louis, MO, USA) as an external standard. Each spectrum (*m/z* 600–1500) was collected for 2 min (120 cycles). The resulting spectra were displayed and deconvoluted using Analyst/Bioanalyst software (Applied Biosystem/MDS Sciex), and further processed using Igor Pro Software (WaveMetrics, Lake Oswego, OR, USA).

For MS/MS analysis of peptides of interest, the second quadrupole of the QSTAR system was used to select the parent ions which were fragmented into a series of product ions in the third quadrupole by applying a collision energy of 22 V, and a collision gas of 6 (absolute value). The product ions were then transferred to the time-of-flight tube and detected by a multichannel plate detector.

RESULTS AND DISCUSSION

Measurement of the number of surface-exposed cysteines in hHb

Hemoglobin is a tetramer protein consisting of two α units and two β units. A heme group is attached in each unit through non-covalent interaction. The nanoESI-MS analysis of untreated human hemoglobin (hHb, 2 μ M) showed two major peaks of intact α (peak 3, 15742 Da) and intact β units (peak 7, 16482 Da). Both intact α and β units contained one heme group (bottom trace, Fig. 1(A)). In addition, two minor peaks of apo α (peak 1, 15126 Da) and apo β units (peak 4, 15866 Da) were observed, resulting from a loss of one heme group (616 Da) from the corresponding unit during nanoESI. The measured molecular weights of these units are consistent with our previous work^{32,33} and that reported in the Swiss-Prot database.³⁴

Figure 1. Comparison of the mass spectra of hemoglobin labeled by PMB under native (A) and denatured (B) conditions. (A). Mass spectra of the native hHb and PMB-labeled native hHb showing that PMB can react with the β unit of hHb predominantly and form a 1:1 complex (β unit to PMB), suggesting the presence of two exposed cysteines for one hHb tetramer. (B) Mass spectra of the denatured hHb and PMB-labeled denatured hHb showing that PMB can react with both the α and β units of hHb and form 1:1 and 1:2 complexes, respectively (hHb unit to PMB). The peaks identified are listed in the figure with their measured molecular weights. The detailed experimental conditions are described in the Experimental section.

To measure the number of surface-exposed cysteines in native hHb, hHb (2 μ M) was reacted with excess PMB (20 μ M) in phosphate buffer (50 mM, pH 7.0) without any denaturants. The nanoESI-MS analysis of excess PMB titrated hHb showed the disappearance of the apo β unit and the intact β unit, accompanied by the appearance of two complexes, apo β -PMB (peak 6, 16187 Da) and β -PMB (peak 8, 16803 Da) (top trace in Fig. 1(A)). Both complexes have a

molecular mass 321 Da higher than the corresponding apo β unit (peak 4, 15866 Da) and intact β unit (peak 7, 16482 Da). The increase in mass by 321 Da is consistent with the binding of one PMB with one cysteine in the β unit, indicating the formation of a 1:1 complex as shown in the reaction scheme (Scheme 1). Although there are two free cysteines (β 93 and β 112) in the β unit, no peak corresponding to a complex of other stoichiometry was observed. These results suggest that

Scheme 1. PMB binding to cysteine residues exposed on the surface of native proteins induces molecular weight increase of the protein by 321 Da.

only one cysteine in the β unit is highly exposed or solvent-accessible. This cysteine can be easily targeted by the selective labeling of bulky and hydrophilic PMB molecules.

Although excess PMB was used for labeling, more than 95% of apo α (peak 1, 15126 Da) and the α unit (peak 3, 15742 Da) could not be labeled by PMB, judging from the peak heights of the apo α -PMB complex (peak 2, 15447 Da) and the α -PMB complex (peak 5, 16063 Da) (Fig. 1(A)) that represent less than 5% of the unreacted α unit. Both the complexes have a binding stoichiometry of 1:1 (one PMB molecule bound to each α unit). The results indicate that the single cysteine (α 104) in the α unit is buried and not accessible by PMB. This observation is consistent with the quaternary structure of hemoglobin in which α 104 sits at the interface between the α and β units (PDB database). With the preservation of the quaternary structure of human hemoglobin, α 104 is poorly exposed to solvent.

In contrast, when the same amount of PMB was reacted with hHb under denaturing conditions (e.g. in the presence of 4 M urea), it was found that each α unit can bind to one PMB molecule (peak 2), and each β unit can bind to two PMB molecules (peak 4, Fig. 1(B)). Therefore, one hHb molecule which contains two α and two β units can bind six PMB molecules in total under denaturing conditions. The number of PMB molecules bound to hHb is consistent with the available cysteines in each unit, indicating that excess PMB can label all the free cysteines when they are all exposed through denaturation of the protein.

Although the hHb tetramer has a total of six cysteines (two α 104, two β 93, and two β 112), under the native conditions only two β cysteines (one in each β) are the primary residues exposed on the surface. Increasing the molar ratio of PMB to hHb showed the same pattern of PMB labeling under the native conditions and support that only one cysteine in the β unit is accessible by PMB (data not shown). The measured

number of surface-exposed cysteines is also consistent with spectrometric measurement requiring multiple titrations (Supplementary Fig. S1(A), see Supporting Information). The results are also consistent with the accessible area of individual cysteines using the theoretical calculation software GETAREA,³⁵ by which an increasing order of surface area was found as α 104, β 112, and β 93.

Identification of the surface-exposed cysteinyl sites using peptide mapping

The specific sites of cysteines bound to PMB were further identified by using mass spectrometry peptide mapping. The unlabeled and PMB-labeled proteins were digested using TPCK-modified trypsin. The resultant peptide mixtures were then purified and enriched using C18 Ziptips, and further analyzed using nanoESI-MS. It was expected that 20 peptides (with expected MW >500 Da) could be generated from the trypsin digestion of hHb, as shown in Fig. 2(A). Among these 20 expected peptides, three peptides contain cysteine. In the absence of PMB labeling, only 16 peptides were observed and none of the three cysteine-containing peptides (α T8, β T7, and β T9) was detectable (bottom trace in Fig. 2(B)). The coverage of the amino acid sequence was 66%. Failure to detect these cysteine-containing peptides is consistent with previous work,³⁶ and may be caused by the oxidation and adsorption of cysteines.^{36,37} After PMB labeling of cysteines, the detection sensitivity of the cysteine-containing peptides is greatly enhanced, probably by preventing oxidation and adsorption. As shown in the top trace of Fig. 2(B), all the three PMB-labeled peptides were observed after the denatured hHb was labeled with PMB. The coverage of the amino acid sequence was enhanced to 86%. Under the nondenaturing conditions of PMB labeling, only one cysteine-containing peptide (β T7) was labeled with PMB (middle trace in Fig. 2(B)). This is consistent with the above result showing

(A)

(B)

Figure 2. Mapping of PMB-labeled native hHb and denatured hHb by trypsin digestion followed by nanoESI-MS analysis. (A) Sequence map of α and β units of hHb. Note: the sequences written in bold indicate the observed peptides. (B) Full MS spectra for the peptide mixtures obtained from trypsin digestion of unlabeled hHb, labeled native hHb, and labeled denatured hHb showing the identified peptides. The insets show the unique cluster patterns of PMB-labeled peptides. The presence of PMB-labeled β T7 and the absence of PMB-labeled β T9 and α T8 from PMB-labeled native hHb suggest that only the cysteine in β T7 (Cys93 β) is the exposed cysteine residue in hHb.

that only a single cysteine in the β unit was accessible by PMB.

Another attractive feature of PMB labeling is that PMB-labeled peptides have a specific isotopic signature. Mercury has six major isotopes ranging from 197.97 Da to 203.97 Da, with the highest intensity at 201.97 Da. When the mercury-containing PMB is attached to a peptide, such as β T7, it would change the isotopic distribution of the labeled peptide by moving the most intensive isotopic peak from the lower

MW range to the higher MW range of the isotopic cluster, and hence changing the overall pattern of the isotopic clusters. Using the proteomics software MS-Isotope,³⁸ we calculated the theoretical isotopic patterns of peptide β T7 (from hHb) with and without labeling by PMB. There are clear differences in isotopic patterns between labeled and unlabeled peptides. The relative intensity of isotopic peaks agreed well between the theoretical calculation and the experimentally measured values for PMB-labeled β T7

(Supplementary Fig. S2, see Supporting Information). Thus, such a specific signature could facilitate rapid identification of PMB-labeled peptides from the large pool of unlabeled peptides.

By scanning the whole spectrum to search for such characteristic isotopic clusters, three MS peaks associated with cysteine-containing peptides were identified (Fig. 2(B)), corresponding to α T8, β T7, and β T9, respectively. For denatured hHb with PMB labeling, three PMB-labeled peptides were all observed (top trace and its insets in Fig. 2(B)). One isotopic cluster with m/z ranging from 871 to 874 (highest intensity at m/z of 872.6) is a doubly charged species, which corresponds to PMB-labeled peptide β T7 containing Cys93 β ($^{83}\text{GTFATLSELHCDK}^{95}$). Another triply charged isotopic cluster in the range of m/z 680 to 683 (highest intensity at m/z of 681.5) corresponds to the PMB-labeled peptide β T9 ($^{105}\text{LLGNVLVCVLAHHFGK}^{120}$) containing Cys112 β . The third cluster with m/z ranging from 822 to 825 carries four positive charges (highest intensity at m/z of 823.4) and corresponds to the PMB-labeled peptide α T8 containing Cys104 α . However, for the native hHb labeled with PMB (middle trace in Fig. 2(B)), only one doubly charged cluster with m/z ranging from 871 to 874 is observed, which corresponds to β T7 containing Cys93 β . There is no other isotopic cluster corresponding to PMB-labeled β T9 or α T8. These results indicate that in the native state of hHb, only the cysteine at β 93 is exposed. The other cysteines at β 112 and α 104 can only be accessible by PMB in the presence of a denaturant such as urea. These results are consistent

with the previous report that β 93 is on the surface of the hHb tetramer.¹⁶

Confirmation of PMB labeling at cysteines by peptide sequencing using MS/MS

For the native hHb labeled with PMB, the peak with m/z of 873 (unit resolution) was selected as the precursor ion and subjected to collision-induced dissociation and time-of-flight (TOF) MS analysis. A series of ions generated during fragmentation of the precursor ions were then compared with the m/z values generated from the theoretical prediction.³⁸ The sequence ion of y_2 was observed clearly, as shown in Fig. 3. The signal for y_3 to y_{10} was not clear. Instead, the m/z values of y_3^* to y_{10}^* are all 321 Th higher than the theoretical values for the respective y_3 to y_{10} ions. There is no peak with m/z 321 Th higher than those for y_1 or y_2 . These results indicate that one PMB molecule is bound to Cys93 β in the peptide of β T7.

Application of the method in identification of exposed cysteines in rHb

With the identification of exposed cysteines in human hemoglobin, we further applied the method to characterize the exposed cysteines in rat hemoglobin (rHb). rHb contains 10 free cysteine residues, with three in each α unit and two in each β unit. First, we examined the number of exposed cysteines by using excess PMB to react with rHb followed by nanoESI-MS analysis of the mixture. As shown in the MS spectrum (bottom trace of Fig. 4), wild-type rHb (rHb^{WT})

Figure 3. MS/MS analysis of β T7²⁺ of hHb demonstrating that PMB is labeled at the third position from the C-terminal of the β T7 fragment and indicating that Cys93 β is the exposed cysteine in hHb. The precursor ions (β T7²⁺) were selected in the second quadrupole with m/z of 873 Th, fragmented by collision-induced dissociation before MS analysis. Mainly, a series of y ions are observed, but with an m/z increment of 321 Th for the y_3 to y_{10} ions, indicating that PMB is labeled at the y_3 position, which is Cys93 β .

Figure 4. The deconvoluted mass spectra obtained from nanoESI-MS analysis of the unlabeled and PMB-labeled native rHb (wild-type) showing the formation of the complexes of subunits of native rHb and PMB with a binding stoichiometry of 1:2 and indicating the presence of eight exposed cysteines in the rHb tetramer. Major peaks were identified with molecular mass and listed in the figure.

displays six major peaks corresponding to apoα' (peak 1, 15152 Da, a variant of the α unit), apoα (peak 2, 15196 Da), intact α' (peak 3, 15768 Da), intact α (peak 5, 15812 Da), apoβ (peak 7, 15848 Da), and intact β (peak 10, 16464 Da). After reaction with a 10-fold molar excess of PMB, all these major peaks are shifted to higher molecular mass positions, all with a mass increment of 642 Da (peaks 4, 6, 8, 9, 11, and 12 in top trace of Fig. 4). These results indicate the incorporation of two PMB molecules, suggesting the existence of two accessible cysteines exposed in each unit. Although three cysteines (α13, α104, and α111) are present in each α unit of rHb^{WT}, no peak corresponding to the complex of one apo/intact α unit bound with three PMB molecules is observed, indicating that one cysteine is shielded by the protein. The variant of the α unit of rHb^{WT}, the α' unit, which has the same three cysteines, displays the same mass shift pattern. Since the β unit of rHb^{WT} contains only two cysteine residues (β93 and β125), incorporation of two PMB molecules into one β unit indicates that both cysteines are reactive with PMB.

With use of a much higher molar ratio of PMB titration for rHb^{WT} (24:1), the nanoESI-MS analysis consistently suggests the presence of two surface-exposed cysteines for each observed unit of rHb (including the α unit, the α' unit and the β unit) (data not shown). Combining the information obtained from both the α and β units of rHb^{WT}, it is concluded that there are eight surface-exposed cysteines per rHb tetramer (2α and 2β units). These results are similar to those obtained by spectrophotometric titration from which about seven exposed cysteines are suggested (Supplementary Fig. S1(B), see Supporting Information).

We further examined the position of the exposed cysteines in rHb. For identification of exposed cysteinyl sites in rHb,

we used a hemoglobin extracted from frozen red blood cells of an F344 rat, labeled with excess PMB under native conditions, and analyzed the PMB-labeled sites. Theoretically, it is expected that 22 peptides (with expected MW >500 Da) could be generated from trypsin digestion of rHb, as shown in Fig. 5(A). Among these 22 expected peptides, there are four cysteine-containing peptides. Without PMB labeling, there are 16 peptides observed. Similar to hHb, none of the cysteine-containing peptides is detectable (bottom trace in Fig. 5(B)). The coverage of amino acid sequence is 65%. In comparison, up to 19 peptides including three cysteine-containing peptides (αT9, βT8, and βT11) were observed for PMB-labeled native rHb^{F344} (Fig. 5(B) and its inset). In this case, the coverage of the amino acid sequence approached over 84%. The peaks with *m/z* at 926.62 (4⁺), 741.53 (5⁺), and 618.13 (6⁺) are due to the complex of two PMB molecules with the peptide αT9 which contains Cys104α and Cys111α (¹⁰⁰FLSHCLLVTLACHHPGDFTPAMHASLDK¹²⁷), indicating that both Cys104α and Cys111α are surface-exposed. In contrast, no doubly charged cysteine-containing αT2 peptide with a PMB label was detected in the *m/z* range of 464–465 even when rHb was reacted with excess PMB (inset of Fig. 5(B)). The result indicates that the cysteine at α13 belonging to the αT2 fragment is not surface-exposed. This is consistent with the simulation of the three-dimensional structure of rHb, in which cysteine at α13 was found to locate in a hydrophobic milieu.³²

Similarly, the observed peptide ions with *m/z* of 889.76 (2⁺) and 593.51 (3⁺) correspond to the complex of one PMB and one βT8 (⁸³GTFAHLSELHCDC⁹⁵) containing Cys93β, and the peptide ions with *m/z* of 831.79 (2⁺) are due to the complex of one PMB and one βT11 (¹²¹EFTPCA-QAAFQK¹³²) containing Cys125β. These results indicate that

(A)

(B)

Figure 5. NanoESI-MS analysis of the trypsin digest from rHb^{F344} and its PMB-labeled products. (A) Sequence map of α and β units of rHb^{F344}. The sections written in bold annotate the observed peptides. (B) Full MS spectra for the peptide mixtures obtained from trypsin digestion of both unlabeled and labeled native rHb^{F344} showing the identified peptides. The insets show the unique cluster patterns of PMB-labeled peptides. The presence of ions related to α T9(PMB)₂ from α 100 to α 127, β T8(PMB) from β 83 to β 95, and β T11(PMB) from β 121 to β 132 indicates that two cysteines in the α unit (α 104 and α 111) and two cysteines in the β unit (β 93 and β 125) were labeled by PMB, providing evidence for surface exposure of these cysteines.

both cysteines at β 93 and β 125 of rHb are surface-exposed, which is consistent with the result obtained by single PMB titration followed by direct nanoESI-MS analysis in which two PMB molecules can bind to one β unit of rHb (Fig. 4).

It is surprising that one PMB-labeled isotopic cluster (peak with highest intensity at m/z of 824.76, $z = 2$) was observed

with an intensity about 5-fold higher but a molecular weight of 14 Da less than that of doubly charged PMB-labeled β T11. It does not match with any expected cysteine-containing peptides, which are the only residues labeled specifically by high affinity PMB. Recalling that the major β unit of rHb from the F344 rat was 14 Da smaller than the wild-type rHb,^{32,39} we hypothesize that the 14 Da loss in MW is due to mutation

of a single amino acid residue within the range of 121–132 (β T11) in the β unit of rHb^{F344}. By searching for mutations of wild-type rHb in the Swiss-Prot database, it was found that there were no mutations reported on cysteines at any of the five positions of rHb^{WT} (α 13, α 104, α 111, β 93 and β 125), or on other residues which would be mutated to generate new cysteines. However, there was one mutation on β 123 where ¹²³T (threonine) can be mutated to ¹²³S (serine), which could result in a lowering of the MW by 14 Da. Among eight mutations reported on both α and β units of rHb in the Swiss-Prot database, only this mutation matches the observed peak (m/z at 824.76, $z = 2$). Therefore, the peak at m/z of 824.76 (2^+) was assigned as one PMB-labeled peptide β ^{F344}T11 (¹²¹EFSP-CAQAAFQK¹³²). These results also suggest that the mutation of β 123T $\rightarrow$ β 123S in rHb may not significantly change the exposure of the neighboring cysteine (β 125).

Taken together, the eight cysteines in one tetramer of rHb (two α 104, two α 111, two β 93, and two β 125) are surface-exposed, and only the two cysteines at α 13 are shielded in a hydrophobic pocket.

Evaluation of the relative extent of accessibility to the surface cysteines

We also made use of PMB labeling followed by the MS analytical technique to evaluate the relative accessibility of the suggested surface-exposed cysteines, thereby providing information about subtle differences between surface-exposed cysteines. This was achieved by PMB titration of rHb combined with nanoESI-MS analysis. In this case, rHb^{F344} was used as an example. By assuming the sum of each unit and their PMB complexes as 100%, the relative intensity of each species was estimated from the deconvoluted mass spectra in Supplementary Fig. S3 (see Supporting Information), and plotted against the concentration of PMB. When rHb (2.0 μ M) was titrated with 2.0 μ M PMB, about 79% of the β unit was labeled by one or two PMB molecules (Fig. 6(B)). Meanwhile, only 13% of the α unit was labeled by one PMB molecule and no α unit was labeled by two PMB molecules (Fig. 6(A)). When the same amount of rHb was titrated with 4.0 μ M PMB, about 85% of the β unit was labeled by PMB, of which 55% was labeled by two PMB molecules. In contrast, only about 32% of the α unit was labeled by PMB, and mostly by one PMB. These results suggest that the two surface-exposed cysteines (Cys93 β and Cys125 β) in the β unit are much more reactive to PMB than those in the α unit (Cys104 α and Cys111 α), indicating a much higher degree of exposure for the cysteines in the β unit. These results are also consistent with the theoretical calculation of GETAREA,³⁵ by which we found that the surface areas for β 93 and β 125 are much greater than those for cysteines in the α unit.

We have identified previously two highly reactive cysteines in rHb, Cys13 α and Cys125 β . Cys13 α is a highly reactive site for binding with dimethylarsinous acid (DMA^{III}),^{32,33} a key metabolite formed in human exposure to naturally occurring arsenic in the environment.^{40,41} Cys125 β reacts specifically with an endogenous anti-oxidant, glutathione.³³ The study reported here suggests that the DMA^{III}-binding site, Cys13 α , is not exposed on the surface. Its high reactivity toward DMA^{III} is probably due to a

Figure 6. The relative intensity of the α and β units of rHb and their 1:1 and 1:2 complexes with PMB versus the titrated concentrations of PMB. The intensities were read out from Supplementary Fig. S3 (see Supporting Information).

moderately hydrophobic pocket that is suitable for entry of DMA^{III}. A preliminary molecular dynamic simulation supports this notion.³² On the other hand, the surface-exposed Cys125 β readily reacts with larger and more hydrophilic glutathione.

Previously, Gao and Wang⁴² applied methylglyoxal together with a MS/MS technique to examine the surface-exposed arginine residues in human hemoglobin. Here we combine the high sensitivity and resolution of nanoESI Q-TOF MS with the high selectivity of a monovalent mercury compound for evaluation of solvent-exposed cysteines in both hemoglobin of human and rat. We demonstrate here two advantages of PMB labeling. One is the ability to rapidly determine the number of surface-exposed cysteines by a single titration. When a protein is labeled by excess PMB, the number of surface-exposed cysteines can be derived from the number of complexed PMB per protein molecule, which can be directly read out from the deconvoluted mass spectrum because its large mass shift (321 Da per complexed PMB molecule). The other is that PMB labeling introduces a unique isotopic fingerprint to the peptides containing surface-exposed cysteine(s) in peptide mapping, which facilitates fast identification of the exposed cysteinyl sites in proteins.

PMB labeling is highly selective to the free and exposed cysteines. The high affinity is primarily derived from the electrophilic property of the mercury atom and the nucleophilic characteristics of the sulfhydryl group or its ionized form – the thiolate anion.^{23,29,43} The dissociation

constants for the mercaptide formed between cysteine and mercuric ion or methyl mercury are below fM.²⁹ The high affinity of PMB for sulfhydryl groups, together with its bulky size and hydrophilic nature, allows it to selectively bind to the cysteines on the protein surface with good stability.

In addition to the high affinity of PMB with surface-exposed cysteines, our work hints that PMB labeling can retain the conjugation information on reactive surface-exposed cysteines. It was observed that the glutathione-conjugated β unit (peak 11, MW 16756 Da) could be formed in rHb^{F344} extracted from rat red blood cells (Supplementary Fig. S3, see Supporting Information). With titration using excess PMB, no significant displacement of conjugated glutathione from the glutathione-conjugated β unit by PMB was observed. On the contrary, a complex of the β unit that contains one PMB and one conjugated glutathione (peak 13, 17077 Da) was observed. Previous work has suggested that glutathione predominantly binds to the highly reactive Cys125 β in rHb^{F344},³³ which is also a target of high affinity PMB as described in this work. These results suggest that high affinity PMB cannot displace the conjugated glutathione from surface-exposed Cys125 β . This may indicate that PMB titration probably has the capacity to retain the information on the conjugation state of surface-exposed cysteines, which is useful for the study of the cysteine redox status of various proteins that can be regulated by endogenous oxidative stress.

Cysteine is one of the highly conservative amino acids in most proteins, and often plays crucial roles in the structure and function of proteins. This is mainly derived from the redox properties of the sulfhydryl group, the ability to chelate metal ions, and to stabilize proteins by forming disulfide bonds.^{23,44} The cysteines on the protein surface are often the target of the endogenous/exogenous substrates such as glutathione, NO, and other reactive metabolites of drugs/toxins, etc.^{12–14,44,45} Modification by these substrates can lead to a range of cellular responses. We provide here a convenient approach for fast screening and identification of biochemically significant cysteines on proteins, exploring their associated conformational changes caused by various stresses, post-translational modifications, enzyme-substrate reactions, protein-protein interactions, and protein assembly, which will facilitate the detailed down-stream functional studies of these proteins.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article.

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