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Identification of Reactive Cysteines in a Protein Using Arsenic Labeling and Collision-Induced Dissociation Tandem Mass Spectrometry

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Trivalent arsenicals have high affinity for thiols (such as free cysteines) in proteins. We describe here the use of this property to develop a collision-induced dissociation (CID) tandem mass spectrometry (MS/MS) technique for the identification of reactive cysteines in proteins. A trivalent arsenic species, dimethylarsinous acid (DMA^{III}), with a residue mass (103.9607) and mass defect distinct from the normal 20 amino acids, was used to selectively label reactive cysteine residues in proteins. The CID fragment ions of the arsenic-labeled sequences shifted away from the more abundant normal fragments that would otherwise overlap with the ions of interest. Along with the internal and immonium ions, the arsenic-labeled fragment ions served as MS/MS signatures for identification of the binding sites and for assessment of the relative reactivity of individual cysteine residues in a protein. Using this method, we have identified two highly reactive binding sites in rat hemoglobin (Hb): Cys-13α and Cys-125β. Cys-13α was bound to DMA^{III} in the Hb of rats fed with arsenic, and this binding was responsible for arsenic accumulation in rat blood, while Cys-125β was found to bind to glutathione in rat blood. This study revealed the relative reactivity of the cysteines in rat Hb in the following decreasing order: Cys-13α $\gg$ Cys-111α $>$ Cys-104α and Cys-13α $\gg$ Cys-125β $>$ Cys-93β. Arsenic-labeling is easy and fast for identification of active binding sites without enzymatic digestion and acid hydrolysis, and useful for characterization and identification of metal binding sites in other proteins.

Keywords: hemoglobin • arsenic • mass defect • labeling • proteomics • red blood cells • protein binding site • glutathione

Introduction

Ligand binding is crucial to the conformation, function, and activity of many proteins. For example, many enzymes are metal-containing proteins or require cofactors to function properly.[1–4] The importance of protein binding has led to the continued development of a wide range of techniques to study binding affinity, stoichiometry, and specific binding sites.[5–9] Tremendous advances in mass spectrometry (MS) techniques[10–17] have made studies of proteomes possible in recent years.

Recent advances in proteomics have highlighted the identification and characterization of protein biomarkers, post-translational modifications, and protein interactions with both macro and small molecules.[10,11,18–20] One of the most challenging aspects is to identify the binding site when ligand−protein interaction is labile. Such labile ligand−protein complexes may dissociate prior to or during MS identification because of pH change, and/or protein conformational change. Previous studies often used enzymatic digestion of proteins before using mass spectrometry to identify the binding sites of protein modifications.[10,11,21–24] These approaches, however, are not suitable for labile covalent bindings and low-affinity, noncovalent bindings due to the breakdown of the native conformation of proteins or incompatible enzymatic digestion conditions. The chemical cleavage methods generate MS protein/peptide ladders rapidly and improve protein sequencing,[25,26] but they often use strong acids, which may induce substrate rearrangement or eliminate labile substrates, leading to false identification or failure to identify the specific binding sites.

Recent advances in top-down MS techniques provide promising means for direct fragmentation of intact proteins in the gas phase without need for prior enzymatic digestion.[20,27–31] Of particular interest are infrared multiphoton dissociation (IRMPD) and electron capture dissociation (ECD) developed

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for FT-ICR/MS. The main advantages include potential 100% sequence coverage and improved identification of post-translational modifications.[20,28,29,31] However, the limited availability of large, expensive FT-ICR/MS instruments is a hurdle facing their wide application.

A common gas-phase fragmentation technique, collision-induced dissociation (CID), has been widely applied to sequencing of biopolymers such as peptides, oligonucleotides, and oligosaccharides, and to the identification of binding sites.[24,30,32–37] However, it has limited capability for direct identification and characterization of a large protein because it is very difficult to generate enough ions to cover the whole protein; this becomes even more difficult as the size of proteins increases. Furthermore, there are numerous overlaps of mass clusters of fragment ions in the low mass-to-charge ranges. The common atoms normally present in proteins, such as ^{12}C (12.0000), ^{1}H (1.0078), ^{14}N (14.0031), and ^{16}O (15.9949), have near unit mass, except that ^{32}S (31.9721) has a mass defect of −0.0279. Therefore, protein fragments with different amino acid compositions but the same nominal mass cannot be easily resolved by the common CID MS/MS. This problem of narrow distribution of mass defects has been documented previously.[38–41] For example, CID fragmentation of the α unit of rat hemoglobin (rHb) can theoretically produce up to 380 fragments that are distributed within a window of 100 mass units (m/z range of 300−400), and as many as 12 fragment ions are expected at a single nominal mass. The overlap becomes more severe with the increase of the protein mass. For large proteins (over 1 million Da), resolving these mass fragments is challenging even using the highest resolution MS currently available.

To overcome this problem, we have developed an arsenic labeling technique for identification of protein binding sites using CID MS/MS. Trivalent arsenic species can bind with high affinity to reactive cysteines in proteins.[42–44] Arsenic has a single isotope (74.9216 amu), and its mass defect from the nearest integer value is −0.0784 amu, which is distinct from the mass defect of other elements commonly present in proteins. The labeling of arsenic to the protein increases the negative mass defect, and shifts the labeled fragment ions away from the unlabeled fragment ions. The unique arsenic signature allows easy and fast identification of the specific arsenic-labeled fragment ions (internal and immonium ions) from a large number of other fragment ions that are otherwise overlapping. Although the arsenic−sulfur bond can be hydrolyzed in solution under nonphysiological conditions, it is more stable in the gas phase, which preserves the sequence information.

To demonstrate the proof of principle, we show here the identification of a highly reactive cysteine-13 residue in the α chain of rat hemoglobin that preferentially binds to a trivalent dimethyl arsenic species. We further show that this method is able to assess the relative reactivity of individual cysteine residues and to identify the specific glutathionylation site in rat hemoglobin.

Experimental Procedures

(**Caution:** The arsenic compounds used in this study are toxic and should be handled with care.)

Materials. Iododimethylarsine [$(CH_3)_2AsI$] was obtained from Dr. W. R. Cullen (University of British Columbia, Vancouver, BC, Canada). It was synthesized according to the established procedure,[45,46] and was kept at −20 °C. A dilute solution of the precursor was freshly prepared using deionized water to form dimethylarsinous acid [DMA^{III}, $(CH_3)_2AsOH$]. Methanol, formic acid, ammonium acetate, ammonium hydroxide, and water (all HPLC grade) were purchased from Fisher Scientific (Fair Lawn, NJ). All other reagents used in the experiments were of analytical grade.

Preparation of Rat Erythrocytes. Erythrocyte samples of rats were obtained from Dr. Samuel Cohen (University of Nebraska Medical Center, Omaha, NE). The rats were treated with sodium arsenate (iAs^V), monomethylarsonic acid (MMA^V), or dimethylarsinic acid (DMA^V), as described in detail elsewhere.[44] Rat erythrocytes were separated from plasma by centrifugation at 3200 rpm at 4 °C for 10 min. The resulting erythrocytes were lysed with hypotonic solution. The lysate was centrifuged at 12 000 rpm at 4 °C for 10 min, and the supernatant was collected for analysis of arsenic−hemoglobin complexes formed *in vivo*. In parallel, control erythrocytes were obtained from rats fed with normal diet without arsenic. The samples were prepared using the same procedures as described above.

Mass Spectrometric Identification of Hemoglobin−Arsenic Complexes in Rat Erythrocytes. A QSTAR Pulsar i mass spectrometer (Applied Biosystems/MDS Sciex, Concord, ON, Canada) equipped with a nanoelectrospray ionization (nanoESI) source (Protana, Denmark) was used for analysis of the supernatants described above. The supernatants of erythrocyte lysates were first desalted using a BioSpin-6 column (Bio-Rad Laboratories, Hercules, CA). Immediately prior to nanoESI-MS analysis, one portion of the desalted protein fraction was diluted 10 times with aqueous ammonium acetate solution (5 mM, pH 7.0). The resulting solutions were then loaded onto a nanoelectrospray capillary tip (Protana, Denmark) and subjected to nanoESI-MS analysis with the TOF-MS scan mode. The nanoelectrospray voltage was 1100 V, the declustering potential (DP1) was 30 V, the focusing potential (FP) was 200 V, and the collision-induced dissociation energy (CID) was 2 V. The instrument was routinely calibrated using standard horse skeletal apomyoglobin (Sigma, St. Louis, MO). The Analyst QS/Bioanalyst software (Applied Biosystems/MDS Sciex) was used for data acquisition and analysis. The mass spectra were smoothed using the binomial smoothing function provided in the IgorPro Software (WaveMetrics, Lake Oswego, OR).

Another portion of the desalted protein was diluted with an aqueous solution containing 10% methanol and 0.002% formic acid (pH 5.5) prior to nanoESI-MS analysis. Under these acidic conditions, the hemoglobin tetramer was dissociated to monomers. The instrument parameters were nanoelectrospray voltage of 1100 V, DP1 of 65 V, FP of 215 V, and CID of 3 V, which provided good sensitivity for detection of monomers.

CID MS/MS Analysis of Protein Complexes. The CID MS/MS experiments were conducted when the solution at pH 5.5 was infused and a continuous stable electrospray was maintained for a period of 2 h. The parent ions of the protein and its arsenic complex were selected in the second quadrupole of the QSTAR system and were fragmented in the third quadrupole, a collision-induced dissociation (CID) cell. The collision energy was 110 V, and the flow of the collision gas (nitrogen) was setting 6 (absolute value). In MS/MS mode, the instrument

Scheme 1. Sequences of Rat Hemoglobin α Chain and the Synthesized Peptides

Rat hemoglobin, α chain:

P1

1 VLSADDKTNI KNCWGKIGGH GGEYGEEALQ RMFAAFPTTK

13

41 TYFSHIDVSP GSAQVKAHGK KVADALAKAA DHVEDLPGAL

P2 111

81 STLSDLHAHK LRVDPVNFKF LSHCLLVTLA CHHPGDFTPA

104 P3

121 MHASLDKFLA SVSTVLTSKY R

Peptide synthesis:

P1: NH_2-NIKNCWGKI-COOH (cys-13 α)

P2: NH_2-FLSHCLLVT-COOH (cys-104 α)

P3: NH_2-VTLACHHPG-COOH (cys-111 α)

was calibrated by a peptide mixture provided by Applied Biosystems (Foster City, CA).

DMA^{III} Labeling of Hemoglobin for Identification of Binding Sites. Rat hemoglobin was first extracted from erythrocytes of control rats by lysis of the erythrocytes as described previously.[42] The hemoglobin concentration in the lysate was measured by Drabkin's method.[47] The lysate was then mixed with 100-fold molar excess of DMA^{III} in 20 mM ammonium acetate buffer (pH 7.0) at room temperature for 1 h. The resultant solution was subjected to BioSpin-6 column centrifugation to remove the salt and the unbound DMA^{III}. The filtrate was then diluted and subjected to nanoESI-MS and MS/MS analyses.

Peptide Synthesis and DMA^{III} Labeling. Three cysteine-containing peptides with sequences adapted from the rat hemoglobin α unit, containing free amine and carboxy groups (Scheme 1) at each end were synthesized at the Alberta Peptide Institute (Edmonton, AB, Canada) using an Applied Biosystems Model 430A Peptide Synthesizer with t-Boc and/or Fmoc Na-Protection and HBTU Chemistry. The peptides were cleaved from the resin and the side-chain protecting groups were removed with hydrogen fluoride or trifluoroacetic acid. The cleaved peptides were purified by HPLC and the purity of the peptides was also determined by HPLC (>95%). The amino acid composition was determined using a Beckman 6300 amino acid analyzer, and molecular weights were determined using electrospray mass spectrometry with a relative deviation from the theoretical values of less than 0.01%. The peptide sequences were also determined using the HP G1005A protein sequencer.

The three synthetic peptides (5 μM) were prepared in aqueous solution (1 mM ammonium acetate, pH 7.0), and incubated with excess DMA^{III} (100 times more concentrated than the peptides) at room temperature for 1 h. The reaction solution was then subjected to MS and CID MS/MS analyses. The parameters for MS and MS/MS analysis using the QSTAR system were the same as those for analysis of the lysates of rat erythrocytes with the exception of the collision energy (30−60 V) and collision gas flow rate (setting 5).

Results

Binding of DMA^{III} to Rat Hemoglobin *in Vivo*. We first used nanoESI-MS to examine the *in vivo* formation of arsenic−hemoglobin complexes in rats because of its capability of detecting tetramer hemoglobin (Hb) that contains 2 α and 2 β units at near neutral conditions.[48,49] The erythrocyte samples from the control rats fed with normal diet and from the rats fed with arsenic-supplemented diet were analyzed and compared. A mass spectrum from the erythrocytes of the control rats (Figure 1, bottom trace) shows the presence of tetramer (T representing $\alpha_2\beta_2$, charges ranging from 16 to 18), dimer (D representing $\alpha\beta$), and monomer Hb (α and β) species. A mass spectrum from the lysate of the erythrocytes from the treated rats shows similar patterns (Figure 1, upper trace). However, spectral peaks associated with the α units in the erythrocytes of the treated rats show consistent shifts of m/z by 104. These include monomer, dimer (D), and tetramer (T) species containing the α units. The mass shift of 104 Da in the α unit indicates the binding of one DMA^{III} [$(CH_3)_2AsOH$, FW 122] to the α unit of rat Hb, with the loss of one H_2O molecule.[43] These results of *in vivo* arsenic−Hb complex formation in the rats are consistent with those of our previous *in vitro* experiments in which trivalent arsenic species were incubated with either purified rat Hb or rat erythrocytes.[43]

To improve the sensitivity and resolution of nanoESI-MS for detecting arsenic−Hb binding, we further optimized the electrospray conditions to pH of 5.5 (with 0.002% formic acid) and methanol concentration of 10%. Under these conditions, the Hb tetramers are dissociated to monomers (with the heme group intact) (Figure 2). A comparison of mass spectra from the analyses of erythrocytes of the control rats (bottom trace) and of the rats fed iAs^V (upper trace) shows consistent peaks at [α + 104]. This corresponds to the binding of DMA^{III} to the α unit with the loss of one H_2O molecule. Further decrease of pH to 4.5 resulted in partial dissociation of DMA^{III} from the α unit of Hb; a lower ratio of DMA^{III}-bound α unit to the unmodified α unit was observed (data not shown). Therefore, our subsequent experiments were conducted with samples diluted in 10% methanol and 0.002% formic acid (pH 5.5) immediately prior to nanoESI-MS analyses. These conditions constitute a compromise for observing the binding of arsenic to the α units of Hb while maximizing the sensitivity and resolution of MS analysis.

We have analyzed erythrocyte samples from 30 control rats (fed normal diet) and 90 treated rats that were fed a diet supplemented with 200 μg/g iAs^V, MMA^V, and DMA^V. The distinct mass spectral shift of 104 Da is consistent throughout all the arsenic-fed rats. The results demonstrate that, regardless whether rats are fed with iAs^V, MMA^V, or DMA^V, the arsenic species bound to rat Hb is DMA^{III}. This finding is consistent with the fact that rats are able to metabolize iAs^V, MMA^V, and DMA^V to DMA^{III} and trimethylarsine oxide (TMAO). DMA^{III} is an intermediate metabolite that is highly reactive, forming complexes with Hb. Therefore, DMA^{III}−Hb complexes are present in the erythrocytes of rats fed with all three test arsenic species.

Identification of DMA^{III} Binding to a Cysteine in Rat Hb. To explain the above observations of DMA^{III} binding preferentially to the α unit of rat Hb, we hypothesized that DMA^{III} binds to a particular cysteine in the α unit of rat Hb. To test this hypothesis, we used collision-induced dissociation (CID) tandem mass spectrometry to identify the binding site of DMA^{III} in rat Hb. We selected multiply charged ions of Hbα−DMA^{III} (m/z = 1769.6, 9^+) and Hbα (m/z = 1758.0, 9^+) (Figure 2), and monitored the fragment ions produced from CID. Figure 3 demonstrates the CID MS/MS analysis of the

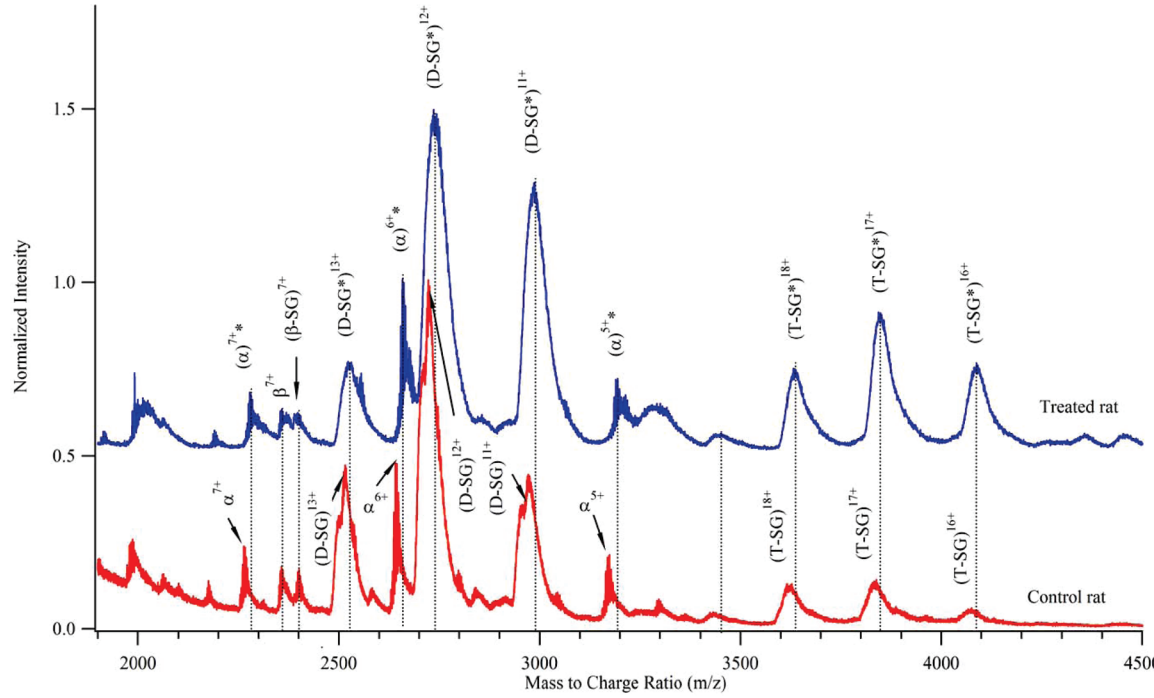


Figure 1. Multiply charged nanoESI-MS spectra of the erythrocyte lysates from rats fed with normal diet (control) or iAs^V-supplemented diet (treated rats) showing that DMA^{III} is specifically bound to rHbα, forming a complex with stoichiometry of 1:1. Nanoelectrospray was conducted with sample solutions at neutral conditions (pH 7.0). T denotes tetramer (α2β2), D denotes dimer (αβ), and -SG represents glutathione conjugate. Peaks labeled with asterisks represent DMA^{III}-containing species.

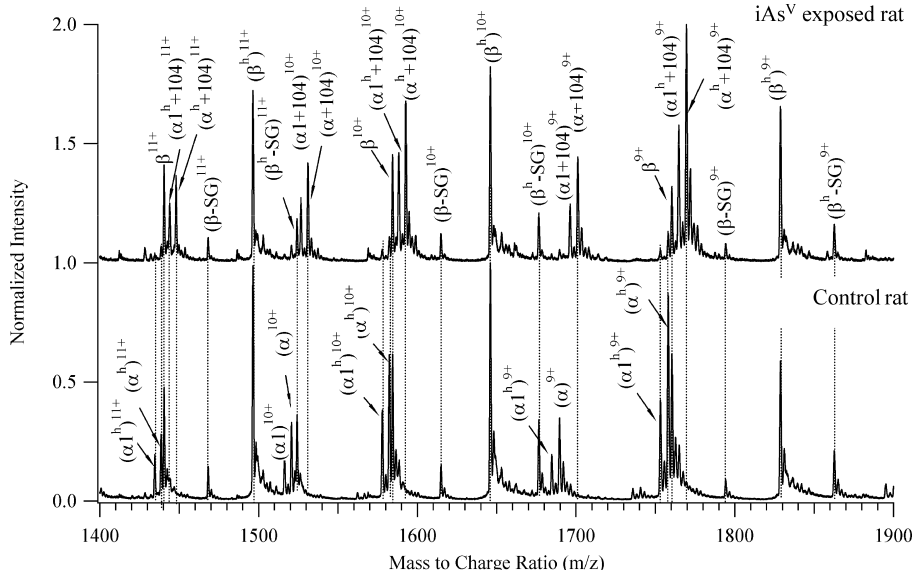


Figure 2. Multiply charged nanoESI-MS spectra of the erythrocyte lysates from rats fed with normal diet (control) or iAs^V-supplemented diet (treated rats) showing that DMA^{III} is specifically bound to rHbα, forming a complex with stoichiometry of 1:1. Nanoelectrospray was conducted with sample solution at pH 5.5. α and β indicate α and β units without the heme group, and α^h and β^h represent α and β units with the heme group intact.

Hbα−DMA^{III} complexes formed in the rat treated with iAs^V and the Hbα from the control rat. Two characteristic fragment ions with m/z of 136.942 and 179.978 are observed from the Hbα−DMA^{III} complex (dashed blue trace in Figure 3), but are absent from the control rat (solid red trace in Figure 3). These two fragment ions are associated with the DMA^{III}-tagged cysteine residues (Scheme 2). The fragment of the parent ions at other charge states showed the same characteristic ions. No DMA^{III}-tagged immonium ions of other amino acids were observed. These results indicate that DMA^{III} is selectively bound to a cysteine residue.

We have further confirmed the results of DMA^{III} binding to a cysteine residue by examining the fragment ions of DMA^{III} complexes with cysteine and glutathione (GSH, a cysteine-containing tripeptide). The CID MS spectra confirm the expected fragment ions of 136.941 from the DMA^{III}−cysteine complexes, as well as 136.941 and 179.983 from DMA^{III}−GSH complexes (spectra not shown). The formation of the second

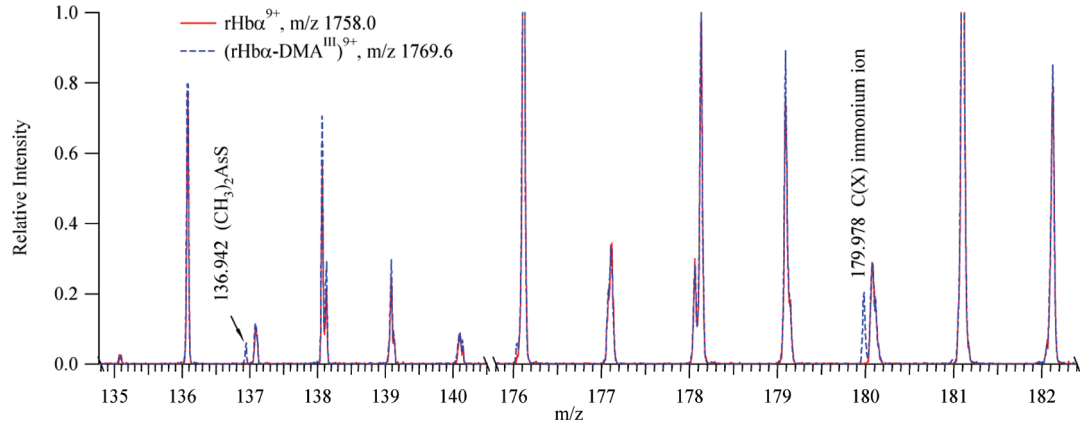
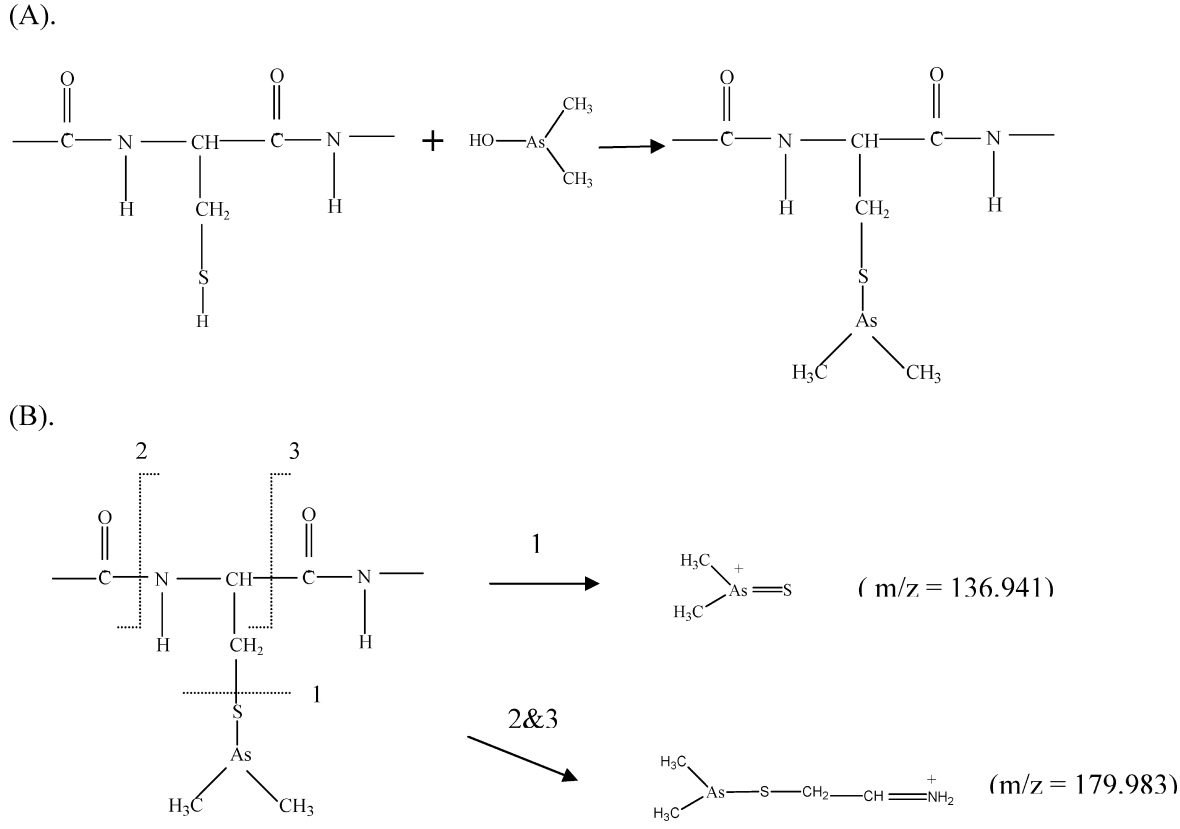


Figure 3. Comparison of the CID MS/MS spectra of the parent ions of the rHbα−DMA^{III} complex (m/z = 1769.6, 9$^+$) and rHbα (m/z = 1758.0, 9$^+$) showing that two characteristic ions with amu of 136.942 and 179.978 are uniquely present in the MS/MS spectrum of the rat Hbα−DMA^{III} complex. This suggests that DMA^{III} is selectively bound to the cysteine residue in rHb of rats fed arsenic-supplemented diet. For MS/MS analysis, the collision energy (CE) was 110 eV and the CID gas setting was 6. The m/z range for monitoring was 130−210. The preparation for the nanoelectrospray solution and other detailed MS and MS/MS conditions are described in the Experimental Procedures. C(X) denotes that the cysteine residue contains a DMA^{III} tag [(CH3)2As-].

Scheme 2. DMA^{III} Binding to Cysteine and the Characteristic Fragment Ions under Collision-Induced Dissociation of the DMA^{III}−Cysteine Complex

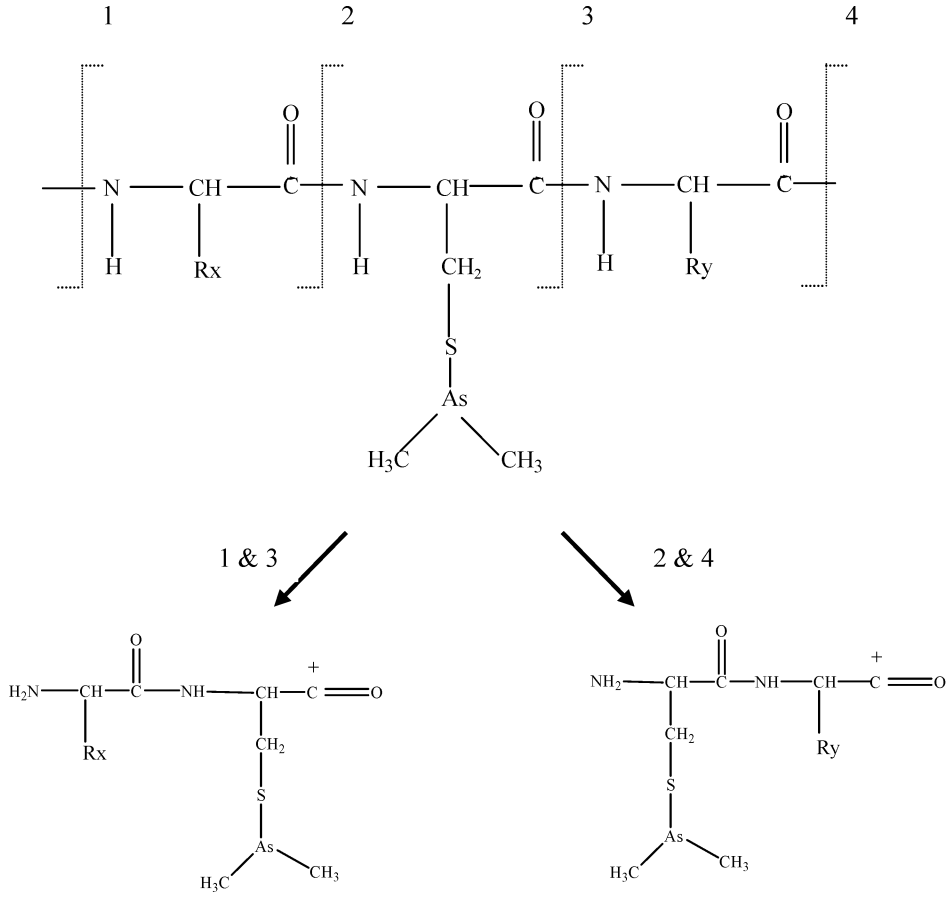


fragment ion (immonium ion: 179.983) requires the breakage of the neighboring peptide bond as shown in Scheme 2B. These results confirm that DMA^{III} is selectively bound to a cysteine residue in the protein by forming an arsenic−sulfur bond.

Identification of the Specific Cysteine α13 Bound to DMA^{III}. Although each α unit of rat Hb contains three cysteines (α13, α104, and α111) and each β unit contains two cysteines (β93 and β125), the MS evidence shows that DMA^{III} binds only to one cysteine in the α unit (Figures 1 and 2). To identify which of these cysteines in the α unit of rat Hb is the reactive binding site for DMA^{III}, we have utilized CID MS/MS and accurate mass measurement to determine the specific cysteine bound to DMA^{III}.

The principle of this technique is described below with rat erythrocytes as an example. Binding of DMA^{III} to rat Hb leads to an increase of 103.9607 mass unit in the protein, which has a unique mass defect of −0.0393 from the unit mass. This large negative mass defect is not present in unmodified proteins. Upon fragmentation, the DMA^{III}-tagged internal ions show a negative mass shift from the common fragment ions of a

Scheme 3. Generation of Characteristic Internal Dipeptide Ions Containing the DMA^{III} Tag under Collision-Induced Dissociation of the DMA^{III}−Protein Complex



protein. Accurate mass measurements of these ions provide information specific to the local sequence of each cysteine residue. As an example, the formation of DMA^{III}-tagged internal dipeptide ions is illustrated in Scheme 3. For each nonterminal cysteine residue in a protein, there are two possible internal dipeptide ions tagged with DMA^{III} (Scheme 3). Their molecular weights are the sum of 189.961 (DMA^{III} binding to a cysteine residue) and the molecular weight of the neighboring amino acid. Therefore, the expected internal dipeptide ion species containing the DMA^{III} tag and each of the three cysteine residues (Cys-13, Cys-104, and Cys-111) in the rat Hb α unit can be obtained (Table 1 in Supporting Information). The presence of a particular pair of internal dipeptide ions with a DMA^{III} tag provides information on the specific cysteine residue that is bound to DMA^{III}. The absence of the DMA^{III} tagged internal dipeptide ions of other cysteines suggests the absence of binding to those cysteines. A comparison of these DMA^{III} tagged ions indicates which specific cysteine residue is highly reactive to DMA^{III}. The fragmentation analysis of the rat Hb α unit (without the DMA^{III}-tag) under the same conditions can be examined as a negative control. Because small internal ions usually give strong response and better resolution, internal dipeptide ions were chosen for the purpose of identifying the binding sites. Larger internal peptide ions with a DMA^{III} tag are not listed but they were used to further support the identification.

Figure 4 shows the CID MS/MS spectrum of the *in vivo* DMA^{III}−Hbα complex superimposed with that of the Hbα. These were obtained from the analyses of erythrocyte samples from arsenic-treated rats and control rats. Two strong internal dipeptide ions of NC and CW with the DMA^{III} tag were observed with corresponding *m*/*z* of 322.020 and 394.057 (dashed blue trace in Figure 4). Internal ions with a loss of NH_3 (304.998 and 377.032), CO (294.034 and 366.066), and a simultaneous loss of NH_3 and CO (277.002 and 349.038) were also observed for each internal dipeptide ion containing the DMA^{III} tag (Table 1 in Supporting Information). These same internal dipeptide ions with DMA^{III} tag are absent in the MS/MS spectrum of the Hb samples from the control rat (solid red trace in Figure 4). Because only Cys-13α has N and W as the neighboring amino acid residues (as shown in Scheme 1), the results indicate that DMA^{III} is bound to Cys-13α.

A number of internal ions of higher molecular weight that contain the cysteine−DMA^{III} tag were also observed from fragmentation of DMA^{III}−Hbα complexes (Table 1 in Supporting Information). Internal tripeptide ions with DMA^{III} tags were observed with *m*/*z* of 433.082, 451.090, and 508.085, corresponding to internal ions of DMA^{III}-tagged KNC, CWG, and NCW, respectively. An internal pentapeptide ion of 676.217 amu was also observed, the possible result of two DMA^{III}-tagged fragments, KNCWG and NCWGK, after a loss of both CO and NH_3. These internal peptide ions with DMA^{III} tags are associated with Cys-13α in the protein sequence context, supporting that Cys-13α is the *in vivo* reactive binding site. Fragments of parent ions at other charge states also showed results consistent with this conclusion.

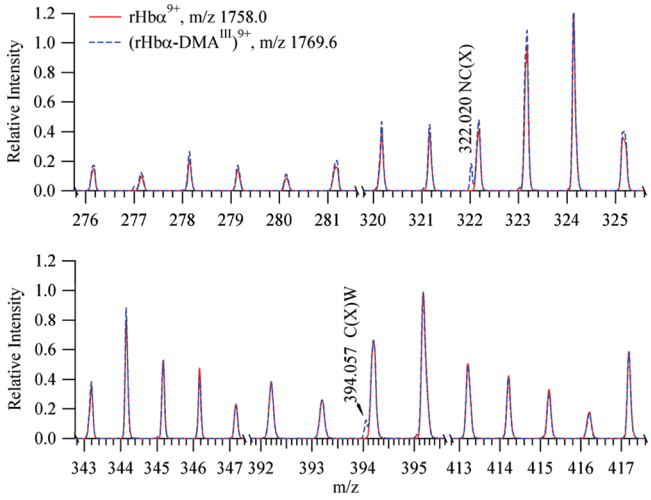


Figure 4. Comparison of the MS/MS spectra of the parent ions of the rHbα−DMA^{III} complex (m/z = 1769.6, 9$^+$) and rHbα (m/z = 1758.0, 9$^+$) in the m/z range of 250−420. It shows that two characteristic internal dipeptide ions with DMA^{III} tag at 322.020 and 394.057 amu are uniquely present in the MS/MS spectrum of the rHbα−DMA^{III} complex (in treated rats) but not in that of rHbα itself (in control rats). The experimental conditions are the same as in Figure 3. C(X) denotes that the cysteine residue contains a DMA^{III} tag [(CH3)2As-].

Furthermore, although more than 90% of the α unit of rat hemoglobin (rHb) in the sample chosen for CID MS/MS analysis formed a covalent 1:1 complex with DMA^{III} *in vivo* (Figure 2), no internal dipeptide ions with DMA^{III} tags were associated with Cys-104α (HC, CL) or Cys-111α (AC, CH) (Figure 4 and Table 1 in Supporting Information). To confirm that the absence of these internal ions is not due to differential production by CID fragmentation, we synthesized a [rHbα−(DMA^{III})$_3$] complex by incubating rHb with 100-fold molar excess of DMA^{III} and examined this complex under the same CID MS/MS conditions. Figure 5 shows the CID MS/MS spectrum supporting the α unit bound to three DMA^{III} molecules [rHbα−(DMA^{III})$_3$]. The DMA^{III}-tagged internal ions are summarized in Table 1 (Supporting Information). In the presence of three DMA^{III} molecules simultaneously bound to the α chain of rHb, the DMA^{III}-tagged internal dipeptide ions associated with Cys-13α, Cys-104α, and Cys-111α were all observed. The internal dipeptide ions of HC or CH with the DMA^{III} tag (345.045 amu from Cys-104α and Cys-111α) have much stronger signals (~3-fold) than those of NC with the DMA^{III} tag (from Cys-13α). The intensity of CL with the DMA^{III} tag at 321.064 is higher than that of DMA^{III}-tagged NC internal ions. The observed signal of the AC internal ion with the DMA^{III} tag (279.015, associated with Cys-111α) is very weak, but the tripeptide ACH with the DMA^{III} tag shows a strong signal, and its intensity is similar to that of the NC internal ion with the DMA^{III} tag. These results indicate that the internal ions from all three cysteine residues can be produced under the same conditions. Therefore, the absence of DMA^{III}-tagged internal ions of HC/CH, CL, AC, and ACH in the erythrocytes of rats is due to the absence of DMA^{III} binding to these cysteines *in vivo*, and not due to the inability to produce the internal ions from these species. Taken together, these results suggest that Cys-13α of the native rHb is the most reactive binding site for DMA^{III} *in vivo*.

Confirmation of the Binding Site by Synthesized Peptides. The identification of the reactive binding site Cys-13 in the α chain of rat Hb is further supported by the evidence from three synthetic peptides encompassing each of the cysteine residues in the α chain of rat Hb. The cysteine residue is located in the center of each peptide (Scheme 1). To produce a sufficient amount of peptide−DMA^{III} complex for MS/MS analysis, each synthesized peptide was incubated *in vitro* with 100-fold molar excess amount of DMA^{III}.

The nanoESI-MS analysis of the reaction mixture of these peptides with excess DMA^{III} demonstrates that all three peptides are able to react with DMA^{III} and form peptide−DMA^{III} complexes with stoichiometry of 1:1 (Figure 6 in Supporting Information). In a further CID MS/MS analysis of the peptide−DMA^{III} complexes with a collision energy of 50 V, two cysteine-bound characteristic fragment ions with m/z of 136.942 and 179.983 were clearly observed for all three peptide−DMA^{III} complexes (data not shown), which supports that DMA^{III} is bound to cysteine (Scheme 2). With a collision energy of 30 V, the two specific ions with m/z of 136.93 and 179.97 were also observable, but less intense (Figure 7 in Supporting Information). Internal peptide ions with a DMA^{III} tag for each peptide were clearly observed, and most of them are listed in Table 2 (Supporting Information). For peptide 1 (P1), which has a homologous sequence adapted from a local sequence of Cys-13α, there was a pair of internal dipeptide ions at m/z of 322.028 and 394.078 (Table 2 in Supporting Information), which correspond to NC and CW with the DMA^{III} tag. These peaks were absent in the MS/MS spectra of peptide 1 without the DMA^{III} complex. The presence of the internal dipeptide ions of NC and CW with the DMA^{III} tag is associated with DMA^{III} binding to peptide 1. This pair of internal dipeptide ions with the DMA^{III} tag is consistent with the results of MS/MS fragmentation analysis of the *in vivo* DMA^{III}−Hbα complex (Figure 4). Likewise, the paired presence of two internal ions of DMA^{III}-tagged HC and CL with m/z of 345.034 and 321.065 is due to DMA^{III} binding to peptide 2 (Table 2 in Supporting Information) and the paired presence of internal ions of DMA^{III}-tagged CH and AC with m/z of 345.038 and 279.028 is due to DMA^{III} binding to peptide 3 (Table 2 in Supporting Information). These data suggest that DMA^{III}-tagged peptides can provide characteristic internal ions that are dependent on the local sequences neighboring the cysteine residues. Therefore, the present technique is able to identify DMA^{III} binding to any of these sites.

Assessing the Relative Reactivity of Individual Cysteines Using Arsenic Labeling. Having demonstrated the identification of arsenic binding sites in a protein, we further show that the arsenic labeling technique can be used to evaluate the relative reactivity of individual cysteine residues in the protein.

By labeling the protein with increasing amounts of DMA^{III} and by monitoring the resulting tagged di- and tripeptide internal ions, we have found that rat Hbα could bind to 1, 2, or 3 DMA^{III} molecules, depending on the molar excess of DMA^{III} (Figure 8 in Supporting Information). At a lower concentration of DMA^{III}, the rHb−DMA^{III} complex has a 1:1 stoichiometry (dashed orange trace); Cys-13α is the only binding site of DMA^{III}, as evidenced by the presence of a pair of internal dipeptide ions at m/z of 322.034 and 394.060. Internal ions corresponding to DMA^{III} binding to Cys-104α (321.075 for CL and 345.040 for HC) and Cys-111α (416.066 for ACH and 345.040 for CH) were observed only after an increase of molar excess of DMA^{III} (to 10-fold), to form the

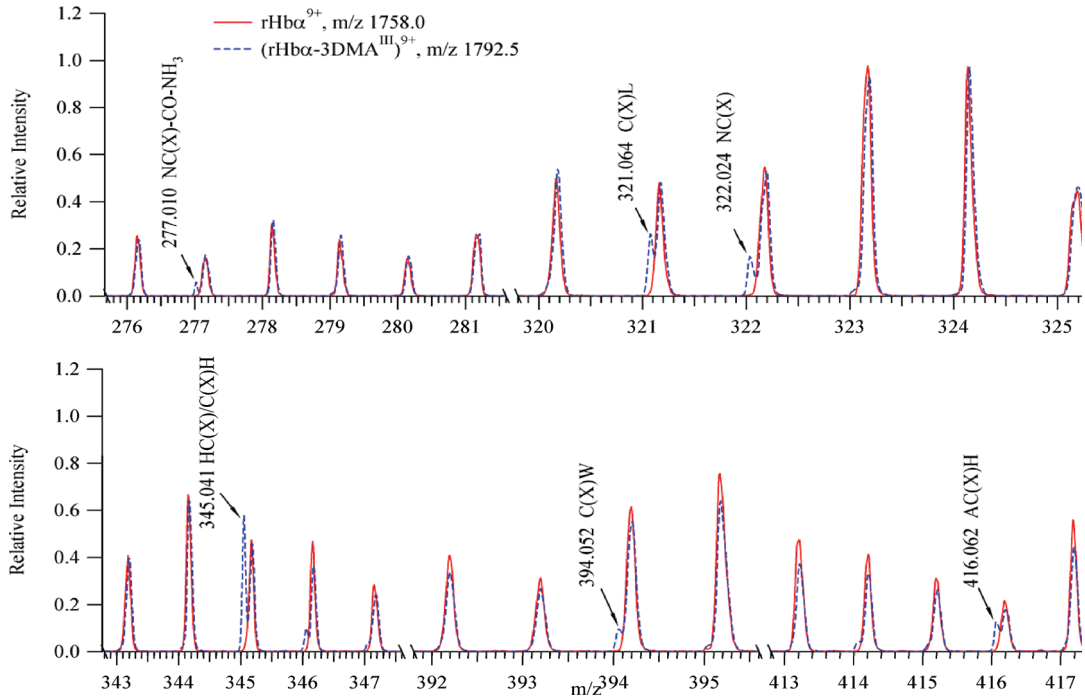


Figure 5. Comparison of the MS/MS spectra of rHbα unit (*m*/*z* 1758.0, 9+) and its *in vitro* complex with three DMA^{III} molecules (*m*/*z* 1792.6, 9+). The rHb−DMA^{III} complex was formed by incubating rHb purified from the erythrocyte lysate of control rats with 100-fold molar excess of DMA^{III}. The internal dipeptides at amu of 322.024 (NC-DMA^{III}) and 394.052 (DMA^{III}-CW) are due to DMA^{III} binding to Cys-13α. The internal peptides at amu of 416.062 [AC(DMA^{III})H] and 345.041 (DMA^{III}-CH) suggest DMA^{III} binding to Cys-111α. The internal dipeptides at amu of 321.064 (DMA^{III}-CL) and 345.041 (HC-DMA^{III}) suggest DMA^{III} binding to Cys-104α. The preparation of the *in vitro* rHb complex with excess DMA^{III} and the nanoESI-MS and MS/MS conditions were described in detail in the Experimental Procedures. C(X) denotes that the cysteine residue contains a DMA^{III} tag [(CH3)2As-].

rHb−(DMA^{III})$_2$ complex (the 1:2 stoichiometry) (dashed green trace). There was no increase in the intensities of the internal dipeptide ions corresponding to DMA^{III} binding to Cys-13α (322.034 and 394.060), suggesting that Cys-13α was already saturated by DMA^{III}. Likewise, only the intensities of the ions corresponding to DMA^{III} binding to Cys-104α (321.075 for CL and 345.040 for HC) increased with further increase of DMA^{III} molar excess to 100-fold (dotted blue trace). There was no change in the intensities of the ions corresponding to DMA^{III} binding to Cys-111α (416.066 for ACH). These results suggest that Cys-111α was saturated by DMA^{III} before Cys-104α binds to DMA^{III} to form the rHb−(DMA^{III})$_3$ complex (the 1:3 stoichiometry). Taken together, these results suggest that the relative reactivity of cysteine residues is in the order of Cys-13α ≫ Cys-111α > Cys-104α in rat Hb. Using the same technique, we have also observed that the reactivity of Cys-125*β* was greater than that of Cys-93*β* in rat hemoglobin (data for *β* chain not shown).

Identification of the Sites of Protein Binding to Glutathione. In addition to identifying the sites of protein binding to arsenic, we further extended the arsenic labeling and CID MS/MS technique to identify the sites of protein binding to glutathione. Cellular glutathione (GSH) can conjugate with proteins by forming mixed disulfides, a process called glutathionylation. This process can serve to regulate enzyme activity, and is also involved in many signal transduction pathways.[21] In rat erythrocytes, GSH conjugation with the rat Hb *β* chain (1:1 stoichiometry) was observed as shown in Figure 2. With excess DMA^{III} labeling *in vitro*, the complex of *β* chain with only one DMA^{III} showed that DMA^{III} was bound to Cys-125*β* (neighboring amino acid residues being P and A). The complex of *β* chain with one DMA^{III} and one glutathione indicated that DMA^{III} was bound to Cys-93*β* (neighboring amino acid residues of H and D), and thus, glutathione was bound to Cys-125*β*. The complex of *β* chain with two DMA^{III} showed that the DMA^{III}-tagged internal ions were associated with both cysteines, Cys-93*β* and Cys-125*β* (Figure 9 in Supporting Information). These results suggest that glutathione is mainly bound to Cys-125*β*. Without glutathione binding, Cys-125*β* is more reactive to DMA^{III}. But glutathione blocks the Cys-125*β*, so DMA^{III} can only bind to Cys-93*β*.

We have also achieved direct identification of the GSH binding site in rat Hb by examining the dipeptide internal ions containing the GSH tag. We found that glutathione was bound to Cys-125*β*, consistent with the result obtained by the arsenic labeling method described above. The signals of the dipeptide internal ions containing GSH are much lower than those of the DMA^{III}-tagged ions. Thus, the method of using DMA^{III} labeling is more sensitive.

Discussion

The arsenic labeling CID MS/MS method presented here made use of the unique mass defect of arsenic, the high selectivity of arsenic to cysteine residues, and the excellent stability of arsenic−sulfur bonds in the gas phase. The arsenic signature in the fragment ions led to the successful identification of a highly reactive binding site in rHb, Cys-13α, for the reactive arsenic metabolite DMA^{III}. To our knowledge, no report has made use of CID MS/MS to identify a binding site in such a large protein (~16 kDa, Hb monomer). This method does not require hydrolysis or enzymatic digestion of the proteins,

avoiding potential problems of dissociation of labile metal-protein complexes in solution.

Because of the unique mass defect introduced by arsenic labeling, the requirement for mass resolution is not stringent, and identification of the binding sites can be achieved even at moderate mass resolution (~5000 at m/z of 500). Arsenic is not naturally present in proteins; therefore, the arsenic-labeled fragment ions have low background, which allows the detection of protein modifications at low frequencies. Compared with other top-down methods, the present approach is not limited by the requirement of a large, expensive FT-ICRMS instrument, nor is special fragmentation equipment such as ECD or IRMPD necessary. This method is also potentially useful for the generation of cysteine-containing sequence tags for identification of proteomes, which would be complementary to bottom-up approaches by which the cysteine-containing peptides are very difficult to identify.

This method based on arsenic labeling has also been demonstrated to be useful in the qualitative evaluation of the reactivity of individual cysteine residues in the proteins, and the identification of the endogenous protein complexes with glutathione. The method has potential for the identification of the reactive cysteines in other endogenous and exogenous conjugation and post-translational modification (particularly for those that cannot be detected when enzyme or chemical cleavage is involved). Because of the unique chemical characteristics of the thiol group in the cysteine residue, such as nucleophilicity, redox activity, and metal binding properties,[1] cysteine-containing proteins are often very active targets attacked by endogenous or exogenous compounds, and therefore modified. The evaluation of the individual reactivity of the cysteine residue is critical for understanding the structure and function of a large number of proteins that serve as redox switches and sensors for cellular oxidative stress, and take part in the regulatory and single transduction pathways.[1]

Arsenic is a ubiquitous environmental contaminant and has posed major public health concerns around the world. The mechanisms by which arsenic causes various health effects remain unclear.[50] The present method may be used to study arsenic−protein interactions that are involved in the pathways of arsenic metabolism, transport, signal transduction, cell cycle control, and DNA repair. The finding of the highly reactive Cys-13 in the α chain of rHb has been shown to be associated with the accumulation of arsenic in rat blood and toxicity in rat bladder.[42]

Our MS/MS analyses of erythrocytes from rats fed arsenic show that DMA^{III} is preferentially bound to Cys-13α, not the other cysteines, in rats (Figures 3, Figure 4, and Table 1 in Supporting Information). When rat Hb was reacted with excess DMA^{III} *in vitro* to achieve DMA^{III} binding to all the three cysteines (Cys-13α, Cys-104α, and Cys-111α), the MS/MS analyses under the same conditions showed the expected fragment ions corresponding to the binding of DMA^{III} to the three cysteines (Figure 5 and Table 1 in Supporting Information). These results suggest that the failure to observe the arsenic-containing fragment ions for cysteines other than Cys-13α is probably not due to poor cleavage at those sites, but instead is due to the lack of DMA^{III} binding to these cysteines *in vivo*. It is worth considering the possibility that the exhaustively modified protein assumes a conformation different from that with a low degree of modification and that the difference in conformation may alter the preference for cleavage. In addition, previous work has shown variability in relative cleavage efficiencies between different amino acid combinations in doubly protonated gas phase peptides.[51] With respect to this consideration, we have shown that MS/MS analyses of DMA^{III} complexes with three synthetic peptides encompassing the cysteines were able to obtain the expected fragment ions for their identification (Figure 6, Figure 7, and Table 2 in Supporting Information). These results confirm that the method is able to identify binding sites and to assess relative reactivity.

The results on the preferential binding of DMA^{III} to Cys-13α in treated rats are most relevant to the hemoglobin molecules that were already synthesized before entering the erythrocytes and, thus, do not indicate the effect of arsenic binding to cysteines during *de novo* synthesis of hemoglobin. Erythrocytes themselves do not have nuclei, and they do not synthesize proteins by themselves. The hemoglobin has already been synthesized before the red blood cells mature and enter the blood stream. We collected blood from the arteries of rats that were fed arsenic for 1−15 weeks; the matured erythrocytes already contained fully assembled hemoglobin, not the precursors undergoing protein synthesis in the bone marrow. Therefore, if arsenic was bound to cysteines prior to Hb synthesis, these arsenic−cysteine complexes would not be present in the rat blood samples we have analyzed here.

DMA^{III} was shown to bind predominantly to Cys-13α in Hb of arsenic-treated rats (Figures 1 and 2), although rat Hb has three cysteines in the α chain (Cys-13α, Cys-104α, and Cys-111α) and two cysteines in the β chain (Cys-93β and Cys-125β). In contrast, glutathione was found to bind predominantly to the β chain (Cys-125β) (Figure 1 and Supporting Information Figure 9). The observed preferential binding may be related to the specific conformation of the protein and the local environment of the cysteine residues in the protein. A preliminary molecular modeling analysis suggests that Cys-13α is located in a hydrophobic pocket that is favorable for binding with DMA^{III}.[42] From the observation that Cys-125β binds to glutathione but not DMA^{III} *in vivo*, it is possible that Cys-125β has a hydrophilic environment that is more suitable for binding to glutathione than to DMA^{III}.

The application of the arsenic labeling method to the identification of reactive cysteines in hemoglobin (a highly abundant protein) demonstrates the proof of concept and feasibility. For a typical analysis, 5−10 pmol hemoglobin is sufficient to obtain the required MS/MS information for identification of the binding sites. Further improvement in sensitivity could be achieved by using a relatively higher collision energy or higher flow rate of collision gas, which could help generate small fragment ions (m/z below 1000 Da). It is conceivable to extend the method of arsenic labeling and CID MS/MS to studies of other proteins.

The unique mass defect of arsenic and the affinity of trivalent arsenic compounds to thiols makes dimethylarsinous acid a useful label for characterizing the relative reactivity of cysteines in proteins. The arsenic-labeled fragment ions serve as tandem mass spectral signatures for identification of the arsenic binding sites in the protein. Analyses of rat erythrocytes reveal the preferential binding of Cys-13α of rat hemoglobin to dimethylarsinous acid and Cys-125β to glutathione.

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Supporting Information Available: Tables showing major internal ions observed from CID MS/MS analysis for the rat hemoglobin−DMA^{III} complexes; mass spectra showing characteristic internal peptides from the synthetic peptide−DMA^{III} complexes; and mass spectra showing glutathione binding to the β chain of rat hemoglobin. This material is available free of charge via the Internet at http://pubs.acs.org.

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