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Combining Fluorescence Detection and Mass Spectrometric Analysis for Comprehensive and Quantitative Analysis of Redox-Sensitive Cysteines in Native Membrane Proteins

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Monobromobimane (MBB) is a lipophilic reagent that selectively modifies free cysteine residues in proteins. Because of its lipophilic character, MBB is capable of labeling cysteine residues in membrane proteins under native conditions. Reaction of MBB with the sulfhydryl groups of free cysteines leads to formation of highly fluorescent derivatives. Here we describe a procedure for the detection and relative quantitation of MBB-labeled cysteines using fluorescence and mass spectrometric analyses, which allow determination of free cysteine content and unambiguous identification of MBB-modified cysteine residues. We have applied this approach to the analysis of the free and redox-sensitive cysteine residues of a large membrane protein, the sarcoplasmic reticulum Ca^{2+} release channel with a molecular mass of 2.2 million Da. Labeling was performed under physiologic conditions where the channel complex is in its native environment and is functionally active. The purified MBB-labeled channel complex was enzymatically digested, and the resulting peptides were separated by reversed-phase high-performance chromatography. MBB-labeled peptides were detected by fluorescence and identified by matrix-assisted laser desorption/ionization (MALDI) mass spectrometry. Under MALDI conditions, partial photolytic fragmentation of the MBB−peptide bound occurred, thus allowing convenient screening for the MBB-modified peptides in the MS spectrum by detection of the specific mass increment of 190.07 Da for MBB-modified cysteine residues. Modification of the peptides was further confirmed by tandem mass spectrometric analysis, utilizing sequencing information and the presence of the specific immonium ion for the MBB-modified cysteine residues at *m/z* 266.6. Quantitative information was obtained by comparison of both fluorescence and MS signal intensities of MBB-modified peptides. Combination of fluorescence with MS detection and analysis of MBB-labeled peptides supported by a customized software program provides a convenient method for identifying and quantifying redox-sensitive cysteines in membrane proteins of native biological systems. Identification of one redox-sensitive cysteine (2327) in the native membrane-bound sarcoplasmic reticulum Ca^{2+} release channel is described.

Proteins contain free cysteines whose oxidation regulates a wide variety of cellular functions.[1−3] Thiol redox state and protein activity are dependent on O_2 tension and glutathione redox potential and are modified by reactive oxygen species such as superoxide anion (O_2^-) or hydrogen peroxide**.** Reactive oxygen species, which are produced during normal cellular function, act as second messenger molecules to control a wide variety of cellular mechanisms including transcription, inflammation, and contractility, but when produced in excess impose on cells an oxidative stress that can lead to lipid peroxidation, DNA and protein damage, and finally cell death.[1−3] Determination of mechanisms involved in redox modification of cellular components has therefore become an important topic in biology and medicine.

Thiol derivatization is commonly used to study the function of free thiols in proteins. Two of the best-known classes of reagents are *N*-maleimide and acyl halides, both of which under controlled conditions yield highly specific sulfhydryl group modifications.[4] Recently, isotopically labeled, thiol-specific reagents have been introduced to derivatize free thiols. This facilitates mass spectrometric detection and identification of the labeled cysteine residues in proteins.[5] The use of isotopically labeled reagents allows rapid and facile detection of labeled peptides, based on the presence of doublets in the mass spectra, corresponding to "heavy" and "light"

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isotopic isomers of the reagent used for protein modification. This approach was recently applied for identification of oxidant-sensitive cysteines.[6,7] A common drawback in all methods, relying solely on mass spectrometric detection, is the suppression effect, a well-known phenomenon in MS, when ion signals can be suppressed by other molecules present in the sample. As a result, mass spectrometry alone may not be able to quantify or even to detect the presence of all molecular species containing labeled cysteines. A second, independent, detection method using a radioactive or fluorescent thiol reactive reagent can be complementary. Several examples of fluorescent labeling reagents suitable for mass spectrometric analysis were reported in the literature.[8−10]

In this study, we describe a combined fluorescent and mass spectrometric detection and analysis procedure for quantifying and identifying free cysteine residues in proteins under native conditions, using monobromobimane (MBB). MBB is a lipophilic reagent that selectively modifies free cysteine residues in proteins through reaction with sulfhydryl groups, leading to formation of highly fluorescent derivatives.[11,12] MBB has been found to be useful for labeling both small and large molecules in biological systems.[13] Using model peptides, we have developed and evaluated an approach that utilizes two complementary techniques, HPLC with fluorescence detection followed by mass spectrometry. Fluorescence and mass spectrometry in MS mode is used for the detection and quantitation of MBB-labeled peptides, while tandem mass spectrometry (MS/MS) allows unambiguous identification of MBB-labeled cysteines by sequencing. For the mass spectrometric detection of MBB-containing peptides, we use MBB-specific mass increments due to photolysis in the MS mode and specific immonium ions for MBB-labeled cysteine residues in the MS/MS mode. For the mass spectrometric quantitation, we developed a statistical program to analyze the changes of the abundances of all ion signals, permitting the quantitation of coeluting MBB-labeled peptides, which therefore cannot be distinguished by fluorescence detection.

This approach has been employed to quantify and identify redox-sensitive cysteines of the large skeletal muscle sarcoplasmic reticulum (SR) Ca^{2+} release channel also known as ryanodine receptor (RyR1). The RyR1 ion channels, which regulate the efflux of calcium ions from the sarcoplasmic reticulum to initiate muscle contraction, are composed of four RyR1 560 kDa peptide subunits and four small 12 kDa FK506 binding proteins (FKBP) for a total molecular mass of ~2200 kDa.[14−16] Numerous endogenous effectors ranging from small molecules (Ca^{2+} and Mg^{2+}, ATP) to proteins (kinases, calmodulin) regulate RyR1 activity. RyR1 can also serve as a redox sensor[17] since its activity and the number of reduced cysteines (free thiols) depend on two physiologic determinants of cellular redox state—oxygen tension (pO_2) and reduced (GSH) or oxidized (GSSG) glutathione.[18] Approximately 160 of the 404 cysteines in the tetrameric RyR1 channel complex were labeled by MBB in the presence of GSH in ambient air. In the experiments described here, MBB-labeled RyR1s were purified and digested, and the MBB-labeled peptides were analyzed using a combination of fluorescence and mass spectrometric detection and analyses. The identification of one redox-sensitive cysteine residue is described.

MATERIALS AND METHODS

Preparation of SR Membranes. Rabbit skeletal muscle SR membranes enriched in RyR1 were prepared in the presence of protease inhibitors as described previously.[16] Briefly, a crude SR membrane fraction was obtained as a 3000−90000*g* pellet that was resuspended in buffered 0.6 M KCl to dissociate contaminating contractile proteins, layered on a linear sucrose gradient of 20 and 45% sucrose, and centrifuged. Membranes enriched in RyR1[16] sedimented at 37−41% sucrose, were collected, flash-frozen in small aliquots in liquid nitrogen, and stored at −135 °C before use.

MBB Labeling, Purification, and Digestion of RyR1. SR membranes containing RyR1 were reacted for 150 min at 30 °C with 5 mM reduced (GSH) or 5 mM oxidized (GSSG) glutathione (Sigma-Aldrich, St. Louis, MO) in buffer A (0.15 M NaCl, 20 mM NaPipes, pH 7 buffer containing 100 μM EGTA, 100 μM Ca^{2+}, and protease inhibitors). Membranes were sedimented by centrifugation at 90000*g* for 30 min, resuspended in buffer A containing 50 μM GSH or GSSG and 3 mg/mL protein, reacted for 1 h at 24 °C in the dark with an excess of MBB (1 mM), and solubilized by the addition of 2 volumes of a 1.5 M NaCl, 20 mM NaPIPES, pH 7.0 buffer containing 2.2% CHAPS, 7.5 mg/mL soybean phosphatidylcholine (Avanti Polar Lipids, Alabaster, AL), and protease inhibitors. After incubation for 60 min at 24 °C and 30 min on ice, nonsolubilized material was removed by centrifugation at 50000*g* for 25 min. MBB-labeled RyR1s were purified on five sucrose gradients using a Beckman SW28 rotor. The supernatant fractions (3 mL/gradient) were placed on top of five linear 7.5−15% (w/w) sucrose gradients containing 1 M NaCl, 20 mM NaPipes, pH 7.0, and 0.6% CHAPS and centrifuged for 16 h at 120000*g* at 2 °C. To determine the position of MBB-labeled RyR1 in sucrose gradients, SR membranes were reacted in parallel with the RyR-specific probe [^{3}H]ryanodine (3 nM) (Dupont NEN, Boston, MA), solubilized with CHAPS, placed on a separate gradient, and centrifuged. The position of the [^{3}H]-labeled RyR1 was determined by liquid scintillation counting of sucrose gradient fractions. Fractions containing MBB-labeled RyR1 were collected and concentrated using a Centriprep 50 filter device (Amicon). Aliquots were taken for determination of protein concentration using BCA reagent (BioRad Laboratories). The degree of MBB modification was determined, using a standard (linear) fluorescence curve prepared by reacting MBB with reduced glutathione.[13] The number of MBB-modified cysteines/RyR1 subunit was calculated using a molecular mass of 565 000 Da. Concentrated

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samples of five gradients (0.6−0.7 mL) were diluted with 2 volumes of 50 mM $(NH_4)CO_3$ and digested with immobilized trypsin (Pierce) (50−75 μL packed beads/mL of sample). To account for possible changes in the kinetics of proteolytic digestion based on oxidation, samples were digested for 3−5 days at 24 °C. SDS−PAGE of the samples followed by silver staining confirmed completion of digestion.

Separation of Peptides from MBB-Labeled RyR1. Tryptic peptides were separated by reversed-phase HPLC on HP1100 (Agilent Technologies), equipped with a fluorescence detector (G1321A), using a Vydac 218TP54 (C_{18}, 5 μm, 250 × 4.6 mm) column (The Separations Group). The HPLC was performed using a 60-min linear gradient of 5−65% acetonitrile in 0.1% TFA at 1 mL/min. Fluorescence was monitored with an excitation wavelength of 370 nm and an emission wavelength of 480 nm. Chromatographic fractions (1 mL) were collected, lyophilized, and reconstituted in 10 μL of 0.1% TFA, 50% acetonitrile, and an aliquot was mixed with an equal volume of a saturated solution of α-cyano-2-hydroxycinnamic acid in 0.1% TFA and 50% acetonitrile, and applied to a matrix-assisted laser desorption/ionization (MALDI) target plate.

Mass Spectrometric Analyses of MBB-Labeled RyR1 peptides. Peptides separated off-line were analyzed on a 4700 Proteomics Analyzer MALDI-TOF/TOF mass spectrometer from Applied Biosystems. To assign the MBB-modified peptide peaks in the MALDI MS spectra, the MS-Fit program (Protein Prospector, MS Facility, UCSF) was used. Additionally, spectra were screened for the presence of ion signals 190.07 ± 0.1 Da apart, using a software program, which we developed. Peaks matching the theoretically predicted masses for MBB-modified peptides were selected for MS/MS analysis by MALDI-tandem time-of-flight (TOF/TOF). The fragment ion spectra were manually matched to the predicted peptide fragmentation spectra generated by MS-Product (Protein Prospector, UCSF).

MBB Labeling and Mass Spectrometric Analysis of a Model Peptide. Myc MBB-modified peptides were prepared by reacting equimolar amounts of Myc peptide (UNC Protein Chemistry Facility, Chapel Hill, NC) and MBB in 50 mM phosphate buffer, pH 7.2, for 30 min at room temperature. After Poros R2 cleanup, the MBB-modified peptide was analyzed on a 4700 Proteomics Analyzer MALDI-TOF/TOF mass spectrometer from Applied Biosystems (Framingham, MA),[19] operating in reflectron mode, or on an Applied Biosystems QSTAR quadrupole TOF instrument by nanoelectrospray ionization (ESI) using Proxeon (Odense, Denmark) nanospray needles under the conditions previously described.[20,21] Liquid chromatography (LC) coupled on-line to the QSTAR (LC−MS) was performed using a capillary LC system (HP1100, Agilent, Palo Alto, CA) equipped with a homemade splitter providing a flow rate of ~200 nL/min [20,22] to the QSTAR's ESI interface. A Dionex (Sunnyvale, CA) capillary C_{18} column with 75-μm i.d. (PepMap, 3-μm particle size) was used with a 30-min linear gradient of 5−90% acetonitrile in 0.1% formic acid. LC tandem MS analysis was performed in a data-dependent manner, providing automated switching between the MS and MS/MS mode based on an intensity threshold for the peptide ion.

Quantifying the Changes of MBB-Modified Peptides by Mass Spectrometry. We considered two samples with mass spectrometric sets of data $\{((m/z))_i^1, s_i^1\}$ and $\{((m/z))_i^2, s_i^2\}$, where index i denotes the peptide signal in each set, $((m/z))_i$ denotes the mass-to-charge ratio of this peptide, and s_i is the intensity value of the signal detected. Our goal was to find differences between these two sets of data, i.e., to find those peptides i, whose signals s_i^1 and s_i^2 differed significantly between the two sets. To address this issue, we considered the logarithms of signal intensity ratios of sets 1 and 2:

$$r_i = \log \frac{s_i^1}{s_i^2} \tag{1}$$

Under the assumption that these ratios r_i are distributed normally, then the significance of the signal intensity difference between samples 1 and 2 is given by

$$P(Z) = \frac{1}{\sqrt{2\pi}} \exp\left(-\frac{Z^2}{2}\right) \tag{2}$$

where Z is the normalized Gaussian distribution of r-values.

This approach was used in an automatic algorithm for comparing two sets of MALDI-MS data from chromatographic fractions. Signals with $P(Z) < 0.05$ were considered as significantly different.

RESULTS AND DISCUSSION

Fluorescence Detection and Mass Spectrometric Analysis of MBB-Modified Peptides from the Ryanodine Receptor. The analytical challenge of identifying MBB-modified peptides by mass spectrometry is the specific detection of those peptides in a highly complex peptide mixture, such as the proteolytic digestion of the ryanodine receptor where over 1000 tryptic peptides are expected. Since products of MBB modification are fluorescent, this physicochemical property can be used for specific detection of MBB-modified peptides by HPLC with fluorescence detection and fraction collection. The skeletal muscle ryanodine receptor contains 101 cysteines/RyR1 subunit (100 cysteines/565 kDa RyR1 peptide[23] and 1 cysteine/FK506 binding protein[24]). According to the MBB fluorescence standard curve and fluorescence determination of the intact purified RyR1, each RyR1 subunit bound an average of 35−40 MBB, when the receptor was pretreated with 5 mM reduced glutathione in ambient air. This result is in good agreement with a previous report.[18] To determine the MBB-modified cysteines, MBB-labeled RyR1 was completely digested with trypsin and the peptides obtained were separated by HPLC with on-line fluorescence detection (Figure 1A). The fluorescence-containing fractions represented less than half of the fractions with

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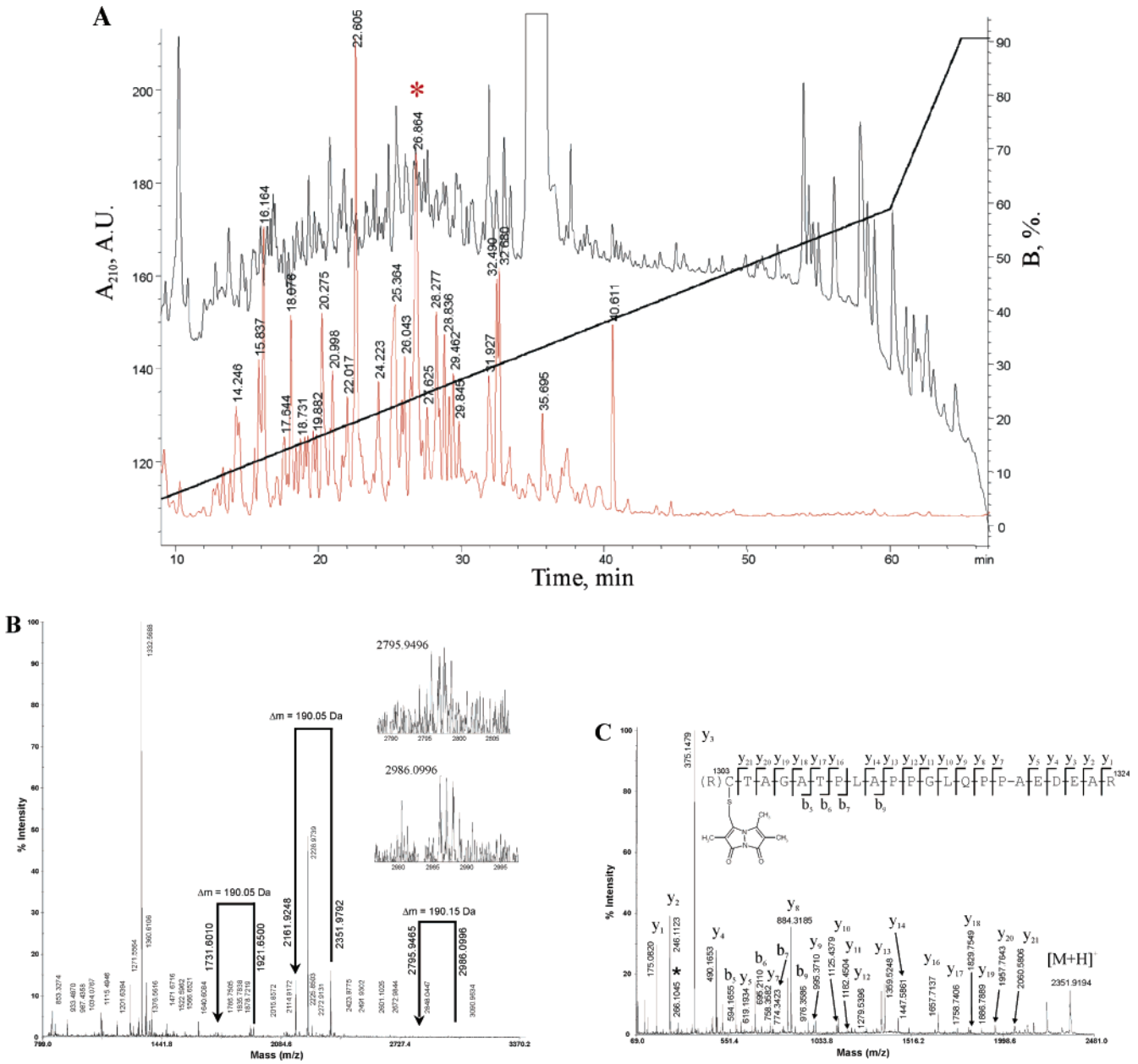

Figure 1. HPLC separation and mass spectrometric analysis of MBB-modified peptides from RyR1 after tryptic digestion. Prior to labeling with 1 mM MBB, SR membranes were reacted with 5 mM GSH as described in Materials and Methods. (A) HPLC chromatogram showing the absorbance profiles at 210 nm and the fluorescence signal with excitation and emission wavelengths of 370 and 480 nm, (red line), respectively. The right abscisses illustrates the percentage of the mobile phase B. (B) MALDI-MS spectrum of a fluorescent chromatographic fraction (* in A) showing ion signals with the mass shift of 190.07 Da, corresponding to the presence of both MBB-modified and photocleaved forms of peptides in the HPLC fraction. (C) MALDI-MS/MS spectrum of the precursor ion at *m*/*z* 2351.98 confirms the identity of the MBB-modified peptide from RyR1. The fragment ions in the spectrum corresponding to sequence-specific b_i- and y_i-ions are annotated, where the index *i* correlates to the peptide bond dissociated counted from the N-terminus for b-ions and C-terminus for y-ions. Ion marked with the asterisk at *m*/*z* = 266.1 can be assigned to the cysteinyl-bimane immonium ion.

significant UV absorption signal, demonstrating the usefulness of specific detection for MBB-labeled peptides.

Fractions exhibiting fluorescence signals were lyophilized and subsequently analyzed by mass spectrometric analysis using MALDI-MS and MALDI tandem MS analyses**.** A typical mass spectrum of one chromatographic fraction (* in Figure 1A) and an MS/MS spectrum with CID fragmentation of the MBB-modified peptide at m/z 2351.98 are shown in panels A and C in Figure 1, respectively. Prominent ion signals in MS spectrum and sequence-specific fragment ions (y- and b-ions) of the MBB-modified peptide confirm the compatibility of MBB modification with both types of mass spectrometric analysis using MALDI as ionization mode.

MS and MS/MS Signature Ions To Detect MBB-Modified Peptides. Interestingly, each signal from the MBB-modified peptides in the MALDI-MS spectrum was accompanied by a signal corresponding to the unmodified cognate peptides, thus forming a doublet of signals 190.07 ± 0.1 Da apart (Figure 1B). Moreover, the ratio of intensities of the doublet signals for each chromatographic fraction, i.e., each spot on the MALDI target, was

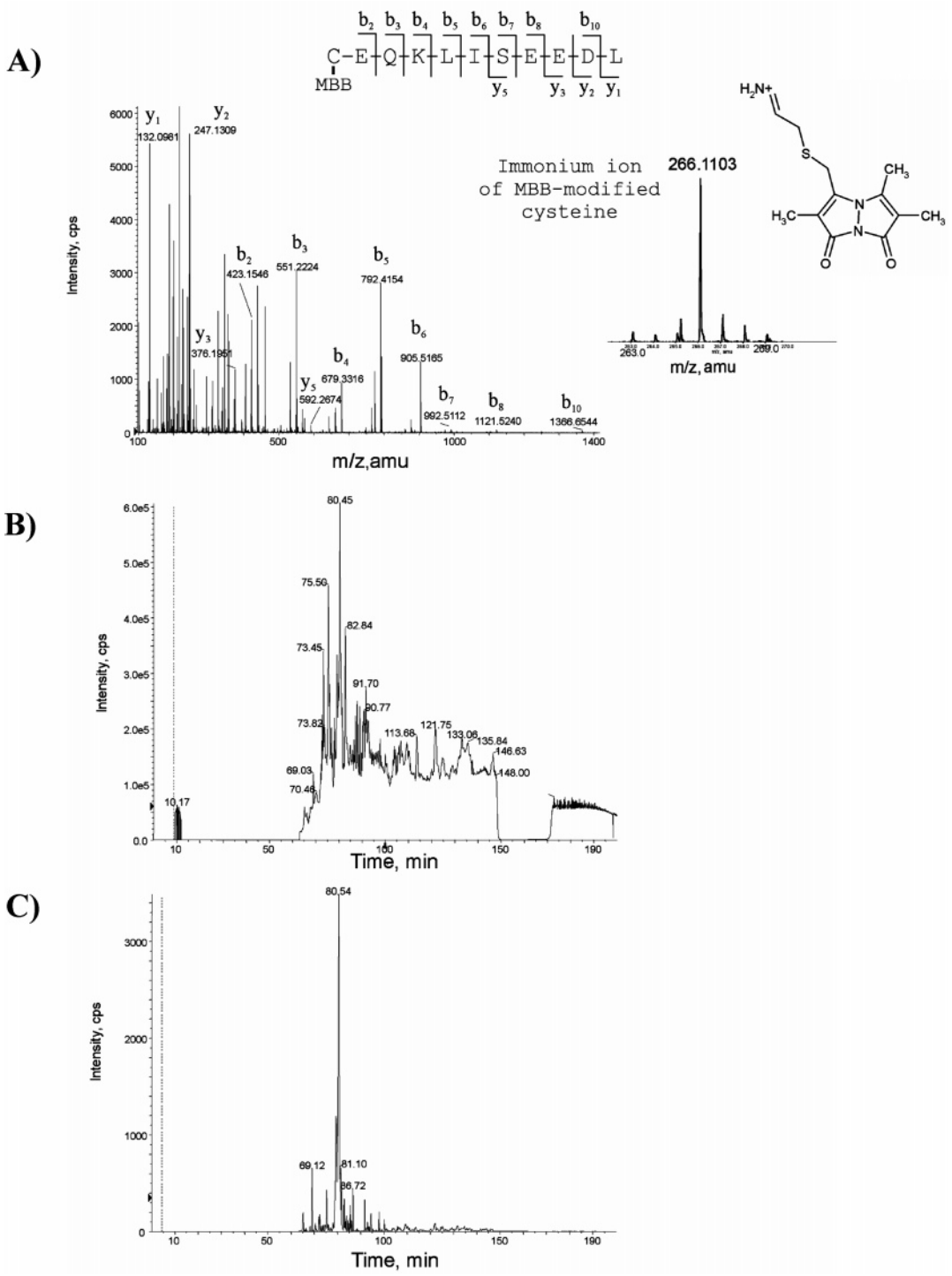

Figure 2. Mass spectrometric analysis of MBB-modified c-myc peptide (CEQKLISEEDL) by LC−MS/MS. (A) The MS/MS spectrum was acquired at retention time 80 min of the LC run. The inset shows the immonium ion of MBB-modified cysteine residue. (B) The total ion chromatogram for LC−MS analysis. (C) The extracted ion chromatogram for the immonium ion of MBB−cysteine (*m*/*z* 266.1) showing the presence of this ion in the spectra at the retention time 80 min.

approximately the same across the entire data set. The original cognate unmodified peptides (which were apparently present in the digest mixture because of incomplete reaction of the cysteine residues with MBB) usually were found in the preceding chromatographic fractions (data not shown). Examination of the fractions containing MBB-modified peptides did not reveal the presence of the unmodified form when ESI-MS was used. Taking all these facts together, we concluded that the bond between the sulfur atom of the cysteine residue and the bimane group is photocleavable under MALDI conditions. The laser-induced photolysis of MBB-modified peptides, which results in the occurrence of peptides 190.07 Da apart in the same spectrum, can thus be used as an internal ion signature for the detection of MBB-modified peptides in MALDI-MS analysis.

Taking advantage of this mass spectrometric signature of MBB modification in MALDI-MS mode, we developed a software

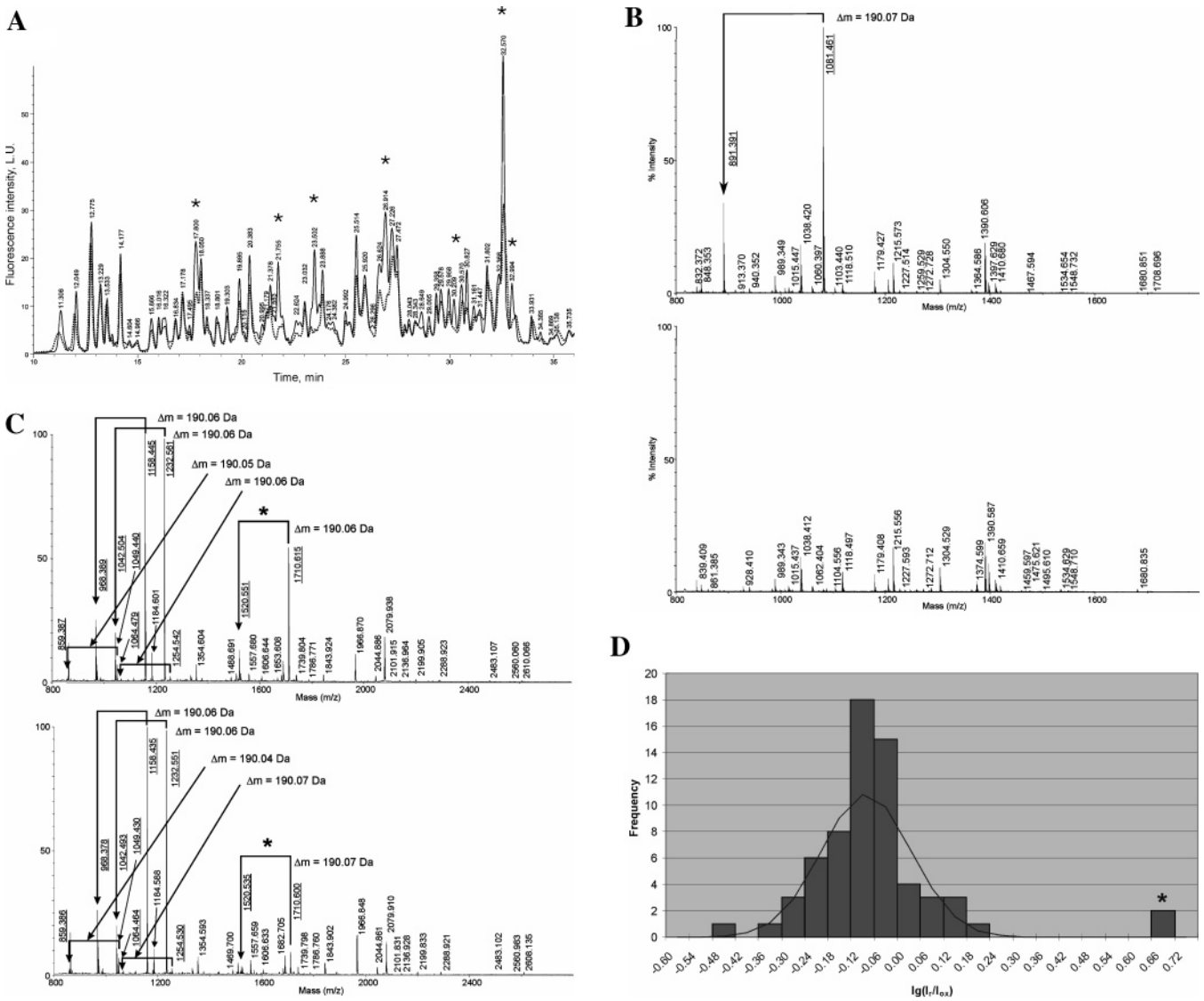

Figure 3. Identification of redox-sensitive cysteine(s) in tryptic digests of the RyR1 receptor. (A) HPLC fluorescence chromatograms of tryptic digests of RyR1 after labeling with MBB under physiological reducing and oxidizing conditions. Asterisks mark fluorescent peaks that are substantially different between the two samples. (B) MALDI-MS analysis of LC fractions with retention time of 23−24 min. Shown are the spectra of the two fractions obtained under reducing (top) and oxidizing (bottom) conditions. The ion signal that corresponds to MBB-modified peptide is observed only in the LC fraction from the sample obtained under reducing conditions. (C) MALDI-MS analysis of LC fractions containing multiple MBB-modified peptides derived from purified RyR1 labeled with MBB under reducing (top) and oxidizing (bottom) conditions. (D) Statistical analysis of the logarithms of the intensity ratios (bottom) reveals only one peptide with a redox-sensitive cysteine (marked by asterisk).

algorithm that allowed automatic screening of MBB-modified peptides based on doublets 190.07 Da apart. Applying this algorithm, signals corresponding to MBB-modified, cysteine-containing peptides were found even in complex mixtures and at low ion intensities (see inset of Figure 1B). For comparison, a manual examination of the data using Protein Prospector was performed. No additional tryptic MBB-modified peptides were detected beyond those already identified by our software algorithm. This demonstrates the usefulness of the program and the value of this signature ion for detecting MBB-modified peptides in an automated and comprehensive manner. Furthermore, since no prior knowledge about the protein sequence is required for screening of ion doublets 190.07 Da apart, this signature seems to be useful for proteomic-wide applications.

In addition, we examined the MALDI-MS/MS spectra in order to find ions characteristic of MBB modification, which can be used for automated detection of MBB-modified peptides. Close inspection of the MS/MS spectra revealed the presence of an abundant fragment ion at m/z 266.1 (Figure 1C, marked by asterisk), which can be assigned to the ion $H_2N{=}R$, where R is the amino acid residue of the MBB-modified cysteine, the "cysteinyl-bimane" immonium ion. This cysteinyl-bimane immonium ion was detected in several different MBB-modified peptides, indicating that it is not peptide specific.

We also evaluated the use of the MS/MS signature ion for detection of MBB-modified peptides by ESI-mass spectrometry coupled to on-line LC−MS, using a model peptide. We have found that peptides containing the MBB moiety can be located in the chromatogram by selecting the mass of the labeled peptide and generating an extracted ion chromatogram (EIC).[20,22] Figure 2A shows the MS/MS spectrum of a model MBB-modified peptide (c-myc), obtained by reversed-phase LC−ESI-MS/MS. The spec-

trum shows sequence-specific fragment ions (b- and y-ion) localizing the modification unambiguously to the cysteinyl residue. The cysteinyl-bimane immonium ion was detected at m/z 266.10 Da (Figure 2A, inset). Figure 2B and C demonstrate the usefulness of the MBB-cysteinyl immonium ion at m/z 266.1 as a signature ion to detect the MBB-modified peptide. Figure 2B shows the total ion chromatogram, while Figure 2C shows the EIC for m/z 266.1. The MS/MS spectrum shown in Figure 2A is from the peak eluting at 80 min.

The above example demonstrates that the characteristic MBB immonium ion can be utilized in on-line LC-nanoESI-MS/MS experiments for detecting MBB-modified peptides. Since most fluorescence detectors are not compatible with capillary HPLC, preselection of MBB peptide-containing fractions is not an option. In this case, MALDI-MS and MALDI-MS/MS are the methods of choice and furthermore these techniques are less time-consuming and are easier to automate than LC−nanoESI-MS/MS approaches.

Determination of the Redox-Sensitive Cysteine Residues of RyR1 by a Combination of Off-Line HPLC with Fluorescence Detection and Mass Spectrometric Analyses. In addition to revealing MBB-modified peptides in chromatographic fractions, HPLC with on-line fluorescence detection can provide quantitative information about the redox status of cysteine residues, thus allowing the identification of redox-sensitive cysteine residues. In this study, we have applied the combined approach of fluorescence detection and mass spectrometric analysis of MBB-modified peptides to determine redox-sensitive cysteine residues in rabbit RyR1. Sarcoplasmic membranes containing RyR1 were treated with MBB under different physiologic oxidative conditions, by preincubating the membranes with either reduced or oxidized glutathione. Figure 3A shows superimposed HPLC fluorescence chromatograms of RyR1 peptides obtained from the two samples labeled with MBB under the different oxidative conditions. Several additional peaks (marked by asterisks in Figure 3A) were observed in the sample modified under reducing conditions. The differentially fluorescent-labeled peptide peaks are attributed to peptides containing redox-sensitive cysteine residues in RyR1.

We compared by MALDI-MS one of the LC fractions that exhibited a differential fluorescence under reducing and oxidizing conditions. Figure 3B shows the MALDI-MS spectra of the LC fractions with a retention time of 23.502 min (Figure 3A). A peak doublet 190.07 ± 0.1 Da apart was observed only in the sample generated under reducing conditions. This indicates that the peptide was responsible for the higher fluorescence signal of the fraction and that only this peptide contained redox-sensitive cysteine residues. Its presence resulted in a strong fluorescent signal for the sample labeled with MBB under reducing conditions and negligible intensity for the oxidized sample.

In other, more complex cases, both reduced and oxidized HPLC fractions exhibited fluorescence, and the MALDI-MS spectra of such fractions often showed multiple doublets (Figure 3C). To determine which MBB-modified peptide(s) contained redox-sensitive cysteine residues, we compared the peak intensities of the doublets between the two samples. Due to suppression effects, quantitative analysis by mass spectrometry without internal standards is challenging. However, since both samples are derived from the same protein preparation and are processed identically, they should only differ by the presence or absence of MBB-modified peptides. Thus, a direct comparison of ion intensity of the same peaks occurring in both MS spectra should be possible.

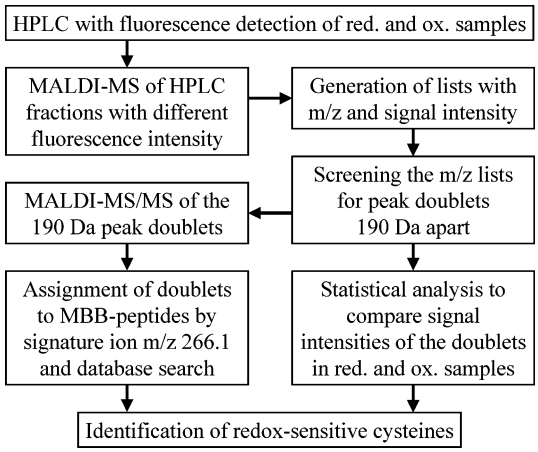

Figure 4. Analytical scheme for automated determination of the redox-sensitive cysteines.

To distinguish between changes in ion intensities due to instrumental and experimental variations, and changes due to the different oxidative status of the cysteine residues, the intensity ratios of all ion signals at the same m/z value (±0.2 Da) present in two MS spectra were determined. The frequency distribution of the logarithms of the ratios is shown as a histogram and was used to derive a Gaussian distribution reflecting the intensity changes based on instrumental and experimental variations (Figure 3D). Only two peptides were noticeably outside the main population of the Gaussian distribution, with logarithm of ion intensity ratios of 0.64 for both peptides, which is four times the value of standard deviation of the distribution (∗ in Figure 3D). These ratios are, therefore, significantly different and thus represent the cause of the difference in the fluorescent intensities.

The two peptides corresponded to the 190.07 Da peak doublet, which was observed in the MS spectra (m/z 1710.62 and 1520.55, see ∗ in Figure 3C) and was confirmed by MS/MS analysis as the MBB-modified peptide (aa 2317−2330) and its photocleavage product containing the cysteine residue 2327 (data not shown). The result shows that analysis of the ion intensity ratios can provide a reliable index for the identification of redox-sensitive cysteine residues in HPLC fractions that contain more than one MBB-labeled peptide.

Automated Analysis of Redox-Sensitive Cysteines. We have developed an analytical scheme and software for completely automated detection and identification of redox-sensitive cysteines (Figure 4). Chromatographic fractions exhibiting differential fluorescence are selected for MALDI-MS analysis. Data files, containing mass (m/z) and intensity values of peaks, are then extracted from raw MALDI-MS data files (in this case, the T2D files from the ABI TOF/TOF mass spectrometer) using customized macros implemented in Data Explorer program (ABI). Next, these mass-intensity lists are screened for the presence of doublets of signals 190.07 Da apart, corresponding to MBB-modified peptides, using a software program we developed.

To confirm the MBB modification site and identify redox-sensitive cysteines, mass lists of ion signals 190.07 Da apart are downloaded for automatic acquisition and interpretation of MS/

MS spectra. The presence of the cysteinyl-bimane immonium ion in the MS/MS spectrum confirms the assignment of MBB-modified peptides, and the accurate localization of the MBB modification is performed using the GPS Explorer search engine (ABI). A Software program for automated signal intensity comparison, including the statistical analysis for detecting redox-sensitive cysteines using the algorithm described above, is currently available only as a stand-alone program. However, the entire method could be fully automated as a result-dependent analysis within the GPS Explorer Workstation software.

CONCLUSION

The present work describes a combination of fluorescence and mass spectrometric analysis for the determination of redox-sensitive cysteines in proteins modified with MBB, a reagent that allows the labeling of membrane proteins under native conditions. LC fractions containing peptides with MBB-labeled redox-sensitive cysteines are readily detectable by the difference in fluorescence intensity between reduced and oxidized samples, thus allowing their preselection for subsequent MS analysis. The mass spectrometric detection of MBB-modified peptides is facilitated by two signature ions: the mass increment for MBB of 190.07 Da and the immonium ion of MBB-modified cysteine residues at m/z 266.1. This approach has been shown to be useful for identifying redox-sensitive cysteine residues in the massive skeletal muscle Ca^{2+} release channel complex.

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