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Mass Spectrometric Analysis of Cyanogen Bromide Fragments of Integral Membrane Proteins at the Picomole Level: Application to Rhodopsin

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Advances in time-of-flight mass spectrometry allow unit mass resolution of proteins and peptides up to about 6000 Da molecular weight. Identification of larger proteins and study of their posttranslational or experimental modifications by mass analysis is greatly enhanced by cleavage into smaller fragments. Most membrane proteins are difficult to mass analyze because of their high hydrophobicity, typical expression in low quantities, and because the detergents commonly used for solubilization may be deleterious to mass analysis. Cleavage with cyanogen bromide is beneficial for analysis of membrane proteins since the methionine cleavage sites are typically located in hydrophobic domains and cleavage at these points reduces the size of the hydrophobic fragments. Cyanogen bromide also gives high cleavage yields and introduces only volatile contaminants. Even after cleavage membrane proteins often contain fragments that are difficult to chromatograph. Matrix-assisted laser desorption ionization mass spectrometry (MALDI MS) is capable of analyzing complex mixtures without chromatography. We present a MALDI MS method that quickly and reliably identifies the cyanogen bromide fragments and posttranslational modifications of reduced and alkylated bovine rhodopsin from as little as 30 pmol of rhodopsin in detergent-solubilized retinal rod disk membranes, using 1–5 pmol of digest per sample. The amino acid sequences of some of the peptides in the digest were confirmed by post source decomposition MS analysis of the same samples. The method appears to be general and applicable to the analysis of membrane proteins and the protein composition of membrane preparations. © 2001 Academic Press

Key Words: **matrix-assisted laser desorption ionization (MALDI); integral membrane proteins; rhodopsin; cyanogen bromide; protein digests; protein identification.**

Integral membrane proteins are important targets for analysis since they are the receptors for signaling networks that control physiology and sensory mechanisms and integral membrane proteins are also receptors for specific adhesion mechanisms that control development and repair of multicellular organisms. The development of matrix-assisted laser desorption ionization (1) and electrospray ionization (2) for biological mass spectrometry has revolutionized many aspects of analytical biochemistry. Although, this revolution has yet to decisively spread to integral membrane proteins. Integral membrane proteins present many difficulties for mass spectrometry because their high hydrophobicity causes solubility problems, and reagents that are used for solubilization and purification often interfere with mass spectrometry.

Bacteriorhodopsin and rhodopsin are the principal integral membrane proteins that have been characterized extensively via mass spectrometry, due to the relative ease of purification of these proteins. Rhodopsin makes up over 90% of the protein content in washed retinal rod outer segment (ROS)[2] membranes

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[2] Abbreviations used: ACHA, α-cyano-4-hydroxycinnamic acid; CNBr, cyanogen bromide; DHB, 2,5-dihydroxybenzoic acid; DTT, dithiothreitol; LC/ESI/MS, liquid chromatography–electrospray ionization–mass spectrometry; LDAO, lauryldimethylamine oxide; MALDI MS, matrix-assisted laser desorption/ionization–mass spectrometry; ROS, rod outer segments; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TCA, trichloroacetic acid; TFA, trifluoroacetic acid; GPCR, G protein-coupled receptor; PSD, postsource decay.

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(3, 4). ROS membranes contain about 40% rhodopsin and 60% lipids by weight (5, 6), which is a rather typical lipid-to-protein ratio for biological membranes. In contrast, most integral membrane proteins are expressed in complex mixtures in cells at much lower quantities and thus require extensive purification for study. Bacteriorhodopsin is even easier to isolate for study than rhodopsin, but bacteriorhodopsin occurs in a crystalline membrane, which contains an unusually low fraction of lipid and is not a typical case. A number of mass spectrometry methods have been developed to study bacteriorhodopsin (7–10). We have chosen to focus our present efforts on optimizing protein biochemical methods for mass spectrometric analyses of vertebrate rhodopsin that are also compatible with gel filtration and gel electrophoresis. Rhodopsin is a member of the large G protein-coupled receptor (GPCR) superfamily. The GPCR superfamily is particularly important because these proteins are the targets of a large fraction of pharmaceuticals and genomic analysis indicates that perhaps 5% of the proteins coded by the human genome are GPCRs (11).

Functional interactions of membrane proteins in signaling or adhesion pathways are often studied via photo cross-linking of peptides or radioligands to the proteins (12–14). Detailed structural information can be provided by MS analysis of the cross-linking sites (13, 15). In order to localize cross-links and other modifications with currently available mass analysis technology, it is essential to cut the protein into smaller peptides, using chemical and/or enzymatic cleavage. Purification and analysis of membrane proteins must usually occur in detergents to facilitate solubilization, penetration of enzymes and other reagents, chromatography, aliquoting, and accurate concentration determinations; however, most commonly used detergents tend to interfere with mass spectral analysis (16). Membrane proteins and their hydrophobic fragments tend to adhere to glass, plastic, or the surfaces of chromatographic supports, which can result in very significant sample losses upon manipulation. Fortunately, the detergent lauryldimethylamine oxide (LDAO) has been found to be a favorable detergent for effective solubilization and gel filtration analysis of rhodopsin and other hydrophobic proteins. LDAO can be largely removed, if needed, and most importantly LDAO does not interfere with MALDI MS (16).

For the reasons described above, mass spectrometric analysis of membrane protein digests requires enhanced methods that are highly sensitive and that can tolerate contamination. There have been few studies that have been able to mass identify fragments of membrane proteins, most have not been able to identify all fragments, had low sensitivity and/or incomplete sequence coverage. Orlando *et al.* (17) reported a FAB/MS/MS method capable of identifying nine of the ten expected cyanogen bromide fragments of bacteriorhodopsin, and provided sequence information on six of the peptides. After trypsin digestion and HPLC in the detergent octyl glucoside and MALDI analysis, Barnidge *et al.* (18) was able to recover all but one large hydrophobic fragment of rhodopsin. Trypsin cleaves largely near the membrane surfaces and yields highly hydrophobic peptides that are difficult to recover from chromatography. LeCoutre *et al.* (19) reported a method capable of identifying all the cyanogen bromide fragments of OmpA, the most abundant outer membrane protein in a crude extract of *Escherichia coli* membranes. Recently, it was reported (9) that all of the cyanogen bromide peptides of rhodopsin could be detected and sequenced by LC/MS, but this method required rather large amounts (4–6 nmol) of starting material. Methods that require affinity purification and/or two-dimensional gel electrophoresis before mass spectrometry that accurately identify membrane proteins of low copy number in cells have been reported (20, 21). We report a simple method employing TCA concentration, ethanol extraction of contaminants, and CNBr digestion. When starting with as little as 30 pmol of rhodopsin in detergent-solubilized membranes it is sufficient to spot 1–5 pmol of digest on a MALDI sample well to obtain 99% sequence coverage.

MATERIALS AND METHODS

Bovine retinal ROS membranes were prepared under dim red light (4, 22) and washed with water containing EDTA or 4 M urea to strip off surface-bound proteins. Rhodopsin concentrations were determined by UV/vis spectroscopy, using an extinction coefficient of 42,700 M^{-1} cm^{-1} at 500 nm or 72,590 M^{-1} cm^{-1} at 280 nm (23). Typically 125 μg of rhodopsin in washed ROS was dissolved in 1 mL 1% LDAO under dim red light, after which a difference optical absorption spectrum was measured before and after bleaching with white light to determine rhodopsin concentrations (24). Most aliquots were reduced with dithiothreitol (~15 mM DTT) for 1 h and alkylated with iodoacetamide (~100 mM) for 2–3 h. Some samples were reduced with 5 mM DTT and alkylated with 15 mM 5-iodoacetamido fluorescein (Molecular Probes) for later SDS–PAGE analysis of the efficiency of CNBr cleavage. Samples were gassed with nitrogen or argon, tightly capped, and tumbled during these incubations.

TCA precipitation is a useful method for recovering and concentrating proteins after chromatography in detergent (25) and for removing reagents used for protein chemistry. Rhodopsin irreversibly aggregates after TCA precipitation and becomes insoluble in anything but neat TFA or formic acid. Formic acid was not used to avoid partial formylation of serines and threonines. Fortunately, the CNBr reaction proceeds

efficiently in 80% TFA/20% water. Aliquots (10–100 μL) of 3–5 μM rhodopsin dissolved in LDAO were precipitated with 10% TCA and spun on a tabletop centrifuge at 14K rpm for 2 min. The pellet was washed by bath sonication in 95% ethanol and the washed precipitate was collected by centrifugation to remove lipids, TCA, and other reagents. The ethanol wash was repeated three times, after which the pellet was dissolved in 100% TFA, containing 500 M excess CNBr per methionine in the sample. Distilled water was then added to make the final TFA concentration 80%. Samples to be digested were flushed with argon and sonicated in a programmable bath sonicator intermittently (0.2 Hz, 1-s pulses) in the dark for 24 h during digestion. Digests were then diluted threefold with distilled water and freeze–dried. Small portions were dissolved in SDS (at room temperature) for PAGE analysis. Tricine gels were purchased from BioRad, and gel electrophoresis was performed as previously described (26). Digests were dissolved in a minimum amount (20–50 μL) of 50/50 water/acetonitrile and freeze–dried again to remove traces of TFA and CNBr. This latter step was repeated two to three times, and it was very helpful to obtain suitable matrix crystals for MALDI MS. A dilution series of the digest to be analyzed was made in 20/80 water/acetonitrile before mixing 1:1 to 3:1 with saturated MALDI matrix in 20/80 water/acetonitrile (α-cyano-4-hydroxycinnamic acid, or 2,5-dihydroxybenzoic acid). Typically, 0.1 to 0.3 μL of matrix/sample mixture was spotted per well. Using the above procedure, we found that 10 μL of 3 μM rhodopsin starting material (30 pmol) was the minimum amount of rhodopsin required to obtain 99% sequence coverage. Fresh and high-purity (99%) DHB and ACHA are recommended. Chemicals were purchased from Sigma/Aldrich Chemical Co., except for LDAO (Calbiochem). High-purity (>99%) cyanogen bromide should be used fresh to reduce tryptophan oxidation (27). MALDI TOF spectra were performed on Bruker BiFlex III or Perceptive Biosystems Voyager RP. Typical accelerating voltages were 20 kV, and delayed extraction times on the BiFlexIII ranged from 100 to 400 ns. Spectra were averaged over 100 to 300 laser shots.

RESULTS

Rhodopsin is perhaps the best-studied member of the vast GPCR superfamily. The X-ray structure of rhodopsin was recently reported (28), which reveals new features of its structure and confirms many aspects long anticipated (5). GPCRs span the membrane bilayer seven times and Fig. 1 shows a diagram of the transmembrane topology of bovine rhodopsin together with the amino acid sequence. The 16 methionines, which are the sites of CNBr cleavage, are highlighted, as are the 10 cysteines, which are important in the mass spectral analysis. Cysteines 321 and 322 are palmitoylated in the native protein. We find, consistent with a recent report (9), that it appears to be essential to alkylate cysteines to optimize recovery of peptides in mass spectral analysis. Asparagines 2 and 15 bear N-linked glycosylation, which generate mass heterogeneity, due to differing numbers of sugars at these positions. Chemical cleavage of methionines with CNBr (29) is typically quite efficient, although reduced cleavage efficiency is reported for methionines followed by serine or threonine (30) and there is one Met-Ser and one Met-Thr in the bovine rhodopsin sequence.

The several posttranslational modifications, as well as solubility problems with many of the peptides, complicate complete analysis of rhodopsin digestion products. To minimize the number of fragments and increase the ease of accurately mass analyzing digests, it is important to optimize the cleavage conditions to minimize missed cleavages. SDS–PAGE in tricine gels was found to be a useful method for routinely assessing the fraction of rhodopsin that is cleaved; however, protein staining on PAGE gels strongly favors detection of high-molecular-weight species. To more accurately measure CNBr digestion, we labeled rhodopsin sulfhydryl groups with iodofluorescein after DTT reduction and observed the digests by fluorescence on the gels. Figure 2 shows fluorescein-labeled, CNBr-digested rhodopsin and undigested fluorescein-labeled rhodopsin on 16.5% tricine SDS–PAGE gels, which are able to resolve small peptides. Seven of the CNBr fragments of rhodopsin contain cysteine residues and can be labeled by alkylation with iodo-fluorescein. Gels were imaged with a BioRad Fluor-S MultiImager. Figure 2 shows a fluorescence emission image of a tricine gel, using broad band UV excitation centered at 302 nm. Lane 3 contains about 50 pmol of fluorescently labeled, undigested rhodopsin. The monomer (MW ~40 kDa) is predominately detected in lane 3, with faint noncovalent multimers of rhodopsin present at higher molecular weights. Lane 1 contains 280 pmol of a synthetic fluorescein-labeled peptide standard of approximately 2400 Da. Lane 2 contains about 300 pmol of fluorescently labeled, CNBr-digested rhodopsin and shows that no undigested rhodopsin can be detected. The estimated detection limit at this exposure level is about 10 pmol. Some of the smaller fragments (<4000 Da) in Lane 2 run so closely on the gels that they could not be clearly resolved. The highest molecular weight clearly detected in Lane 2 migrates below 10,000 Da. This band is most likely fragment 87–143 (MW ~6800) with one or two cysteines tagged with iodofluorescein. Essentially complete delipidation and removal of detergent through TCA precipitation and washing by sonication in ethanol was necessary to achieve good resolution on the tricine gels.

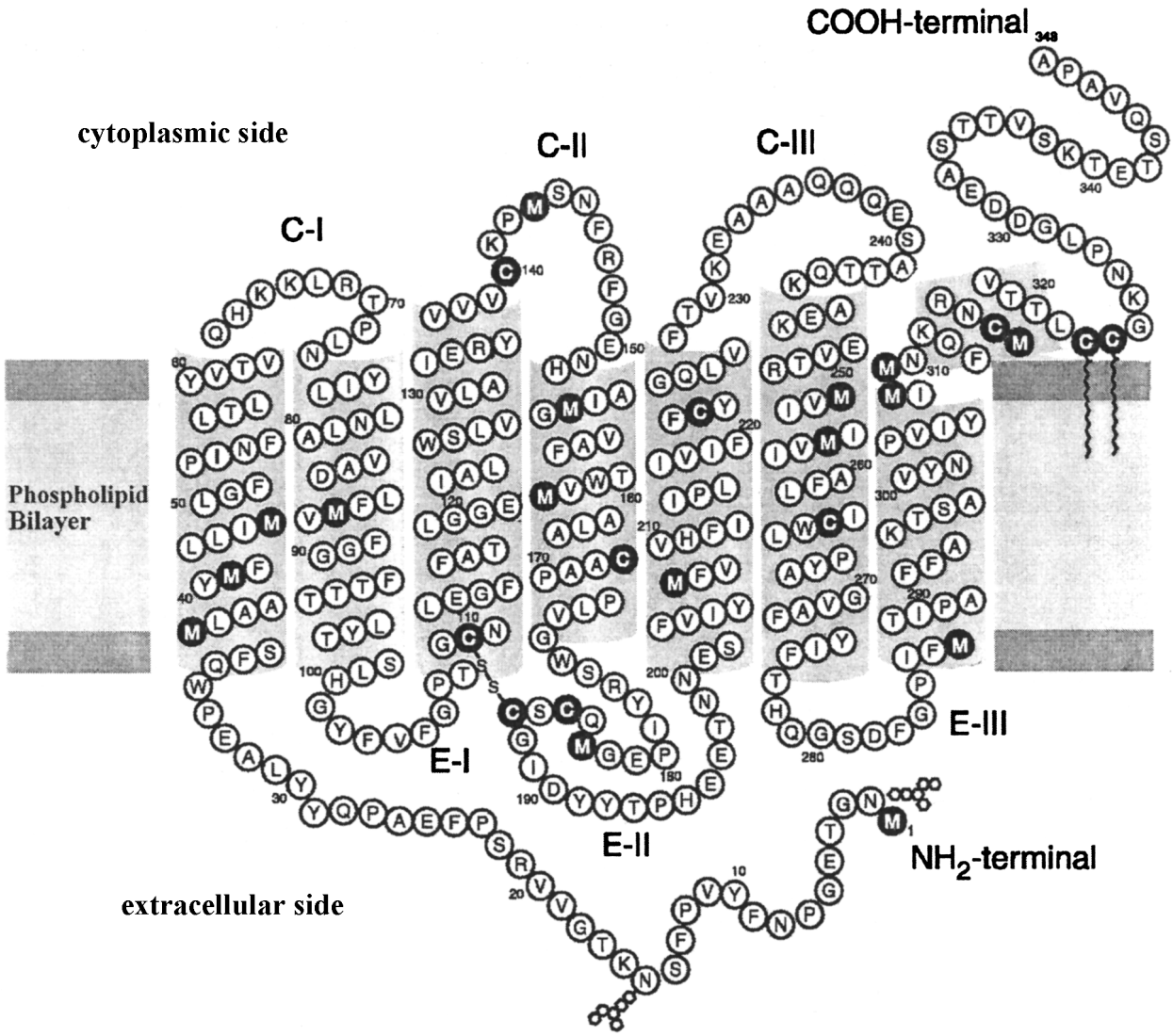

FIG. 1. Transmembrane topological model of rhodopsin with highlighted methionines, which are cleaved by CNBr, and highlighted cysteines, which are best alkylated for these studies. This figure is schematically reflective of the organization of the recently reported rhodopsin crystal structure (28).

LDAO completely solubilizes but does not denature dark-adapted rhodopsin (24) and LDAO was also found to be compatible with the mass spectral methods used in the present work. LDAO-solubilized rhodopsin (10–100 μL, 3–5 μM) was reduced with DTT and the sulphydryls alkylated for most experiments. Iodoacetic acid was previously reported to give poor detection of cysteine-containing fragments in LC/ESI/MS (9); therefore reduction of disulfides with tributyl phosphine and sulfydryl alkylation with vinyl pyridine in 50% *n*-propanol was recommended. We found that this latter method irreversibly aggregated the rhodopsin and thus interfered with gel filtration analysis of undigested rhodopsin. Reduction and alkylation were found to go to completion with DTT and the nonionic sulfhydryl alkylation reagent iodoacetamide, if ROS were solubilized in LDAO or SDS detergent. We found that gel filtration analysis was important for monitoring protein labeling in other experiments and for monitoring digestion with CNBr, and thus we used the DTT/iodoacetamide method for reduction, alkylation, and analysis of the proteins before digestion.

Prior to CNBr digestion, aliquots were usually subjected to 10% TCA precipitation and several steps of washing with ethanol, during bath sonication, to remove TCA, detergent, lipids, and reducing and alkylating reagents. TCA precipitation was not required to obtain MALDI spectra; however, it served to concentrate dilute rhodopsin in pellets in microfuge tubes after centrifugation and thus facilitated the recovery of

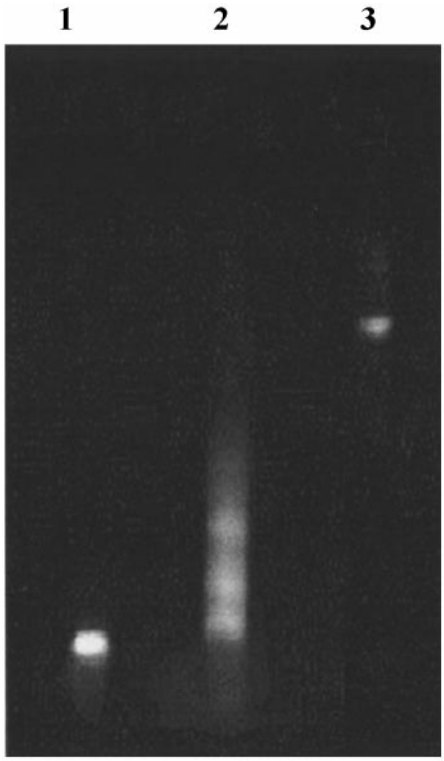

FIG. 2. SDS–PAGE analysis of fluorescein labeled proteins and peptides investigated in this study on a 16.5% tricine gel with fluorescence detection, as explained under Materials and Methods. Lane 1, 280 pmol of fluorescein-labeled peptide standard, MW ~2400; lane 2, CNBr digest of fluorescein-labeled rhodopsin; lane 3, fluorescein-labeled undigested rhodopsin.

small amounts of protein material. Contaminants, such as detergents, lipids, and reducing and alkylating reagents, could be tolerated if the starting concentration of rhodopsin was high, but once the protein concentrations reach the low micromolar range it was essential to concentrate by TCA precipitation and remove nonprotein contaminants by ethanol washing to obtain MALDI spectra. Proteins solubilized in other detergents (such as octyl glucoside) will TCA precipitate, and contaminants can then be removed by ethanol washing. Proteins solubilized in SDS will not TCA precipitate without an RNA carrier (23), and therefore SDS was not used. While contaminants were not always detrimental to MALDI spectra, we found that they severely interfered with LC-ESI-MS of the digests, if not removed (data not shown). Washed TCA pellets were dissolved in 100% trifluoroacetic acid containing 500 M excess of CNBr relative to protein methionines. After dissolution, distilled water was added to 20%, and tubes were flushed with nitrogen and capped. The added water was found to be essential for efficient CNBr digestion. The digestion tubes were sonicated intermittently in a water bath overnight (~24 h) in the dark. MALDI analyses indicated that sonication helped foster cleavage to completion (data not shown), presumably by enhancing the access of the reagents to the bonds to be cleaved. Samples were diluted 2–3× with water after digestion and freeze–dried. Several subsequent cycles of dissolution of the digests in a minimum amount (10–20 μL) of 50/50 water/acetonitrile, and freeze–drying, more completely removed volatile contaminants, improved MS resolution, and concentrated small sample amounts. MALDI sample preparation is discussed further under Materials and Methods. We found that starting with 10 μL of a 3 μM rhodopsin solution solubilized in 1% LDAO, containing ~30 pmol rhodopsin, was sufficient material for sample workup to obtain mass spectra covering 99% of rhodopsin's sequence, missing only two single methionines. A total of 1–5 pmol of the 30 pmol sample was needed for MALDI, leaving the remainder for other analyses.

A portion of a MALDI MS spectrum (acquired on the Bruker Biflex III) of a CNBr digest of rhodopsin is shown in Fig. 3. This spectrum was acquired near the laser intensity threshold for obtaining signals to maximize resolution and mass accuracy. This spectrum contains 91% sequence coverage. The matrix, α-cyano-4-hydroxycinnamic acid (ACHA), consistently yielded higher resolution than 2,5-dihydroxybenzoic acid (DHB). Ion intensities were quite variable from spot to spot on the sample plate, as is often seen in MALDI. To greatly reduce chemical noise produced by matrix ions, and to obtain the best resolution and intensity for all fragments, the deflector was set to 400 and acquisition conditions were optimized for a broad range. Markedly improved resolution and mass accuracy could be obtained on any single ion of interest by optimizing the laser power, extraction delay, and second-stage extraction voltage for its particular mass range and searching for sweet spots on the sample. We could obtain monoisotopic mass resolution on rhodopsin peptides up to about 5300 Da using ACHA as a matrix, as shown in Fig. 4. Rhodopsin CNBr fragments larger than 5300 were usually observed as their average mass.

The masses that were attributed to rhodopsin, together with their corresponding theoretical rhodopsin CNBr fragment masses, are shown in Table 1. Internal calibration with monoisotopic masses of selected rhodopsin CNBr fragments resulted in the observed masses shown in Table 1. The entire sequence was identified except for fragments 1 and 309, which are the remains of single methionines. These methionine fragments must be present in the digests, because their neighboring fragments are detected, but they are obscured by matrix ions even without the use of a low mass gate. Many peaks that were detected with significant signal to noise ratio do not match rhodopsin CNBr fragments. These unassigned peptide peaks are not labeled in the spectra and are not listed in Table 1.

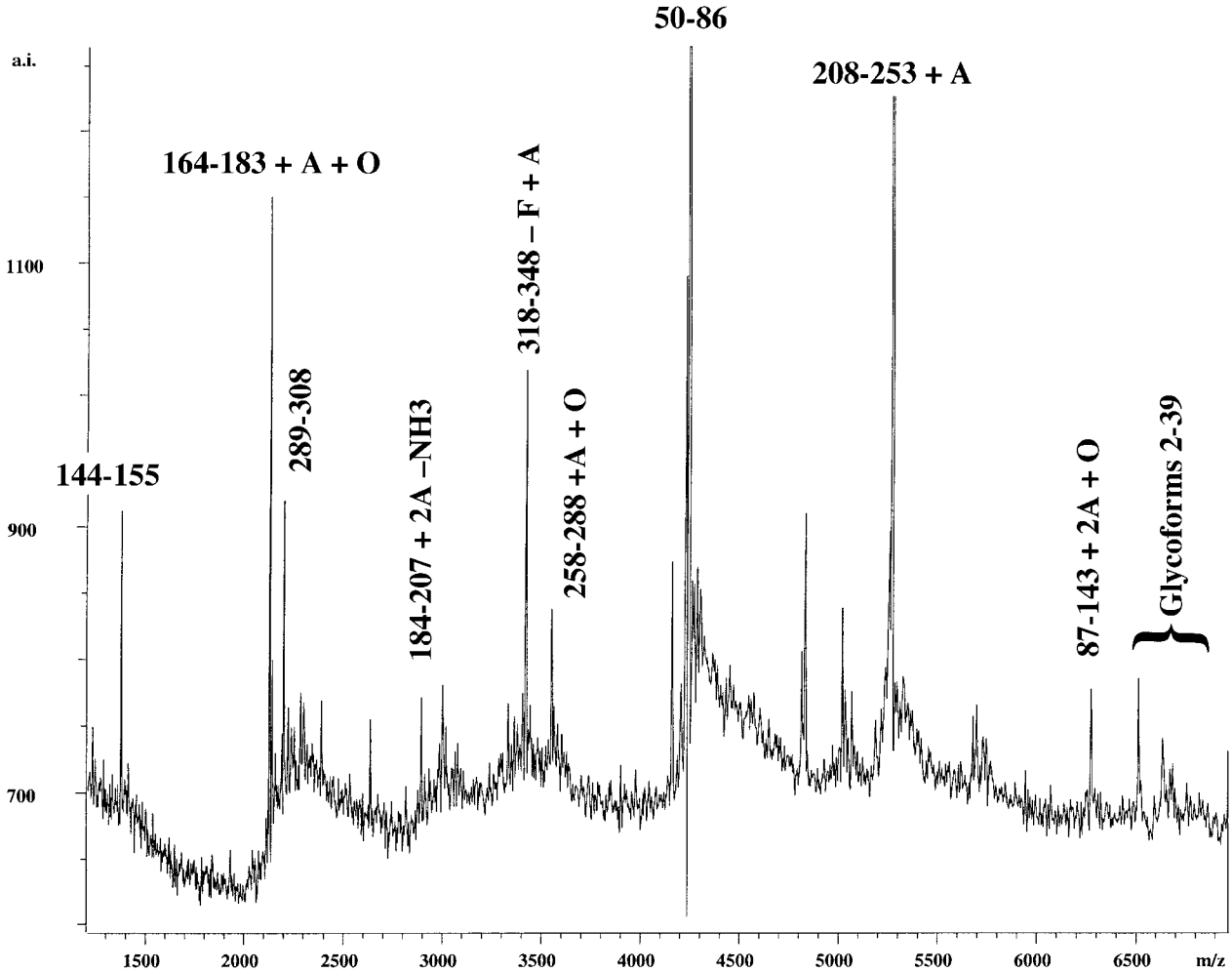

FIG. 3. Delayed extraction and reflected MALDI spectrum of 2 pmol of reduced and thiol alkylated rhodopsin in α-cyano-4-hydroxycinnamic acid.

The masses of the unknown peaks do not match *expected* incomplete cleavage products of rhodopsin to within 0.05% error. Some of the unidentified peaks might be explained by modified incompletely cleaved fragments of rhodopsin, if CNBr intermediates (33), oxidation, fragmentation, and/or neutral losses are posited. Further analysis is ongoing to identify unknown peaks.

We found that fragments containing cysteines tend to yield substantially better sensitivity after reduction and alkylation of the sulfhydryl groups, consistent with the LC/MS results of Ball *et al.* (9). The sample shown in Fig. 3 is from 2 pmol of a CNBr digest from reduced and alkylated rhodopsin spotted in the sample well. Differences between the the unreduced and unalkylated vs reduced and alkylated spectra included mass shifts of +57 due to the addition of one acetamide for fragments 164–183, 208–253, and 258–288, which contain one cysteine, and mass shifts of +114 due to the addition of two acetamides for fragments 87–143 and 184–207, which contain two cysteines.

Palmitoyl groups are bound to cysteines by thioester linkages in fragment 318–348, and are labile to removal by transesterfication with DTT. Removal of fatty acids was not driven to completion under the analysis conditions for the sample shown in Fig. 3. If a high enough fresh DTT concentration (40 mM) is used, both cysteines can be deacylated and then completely alkylated. Fragment 318–348 contains one deacylated and acetamide alkylated cysteine and one remaining palmitoyl group. All four possible forms of the 318–348 fragment have been observed in different experiments (as listed in Table 1). Fragment 184–207 is primarily detected as a pyroglutamate. Many glycoforms of fragment 2–39 are detected. The most intense glycoform is approximately 6501.8 molecular weight, as previously reported (9). This glycosylation adds 2194.7 Da to the unmodified 4307.1-Da molecular weight of fragment

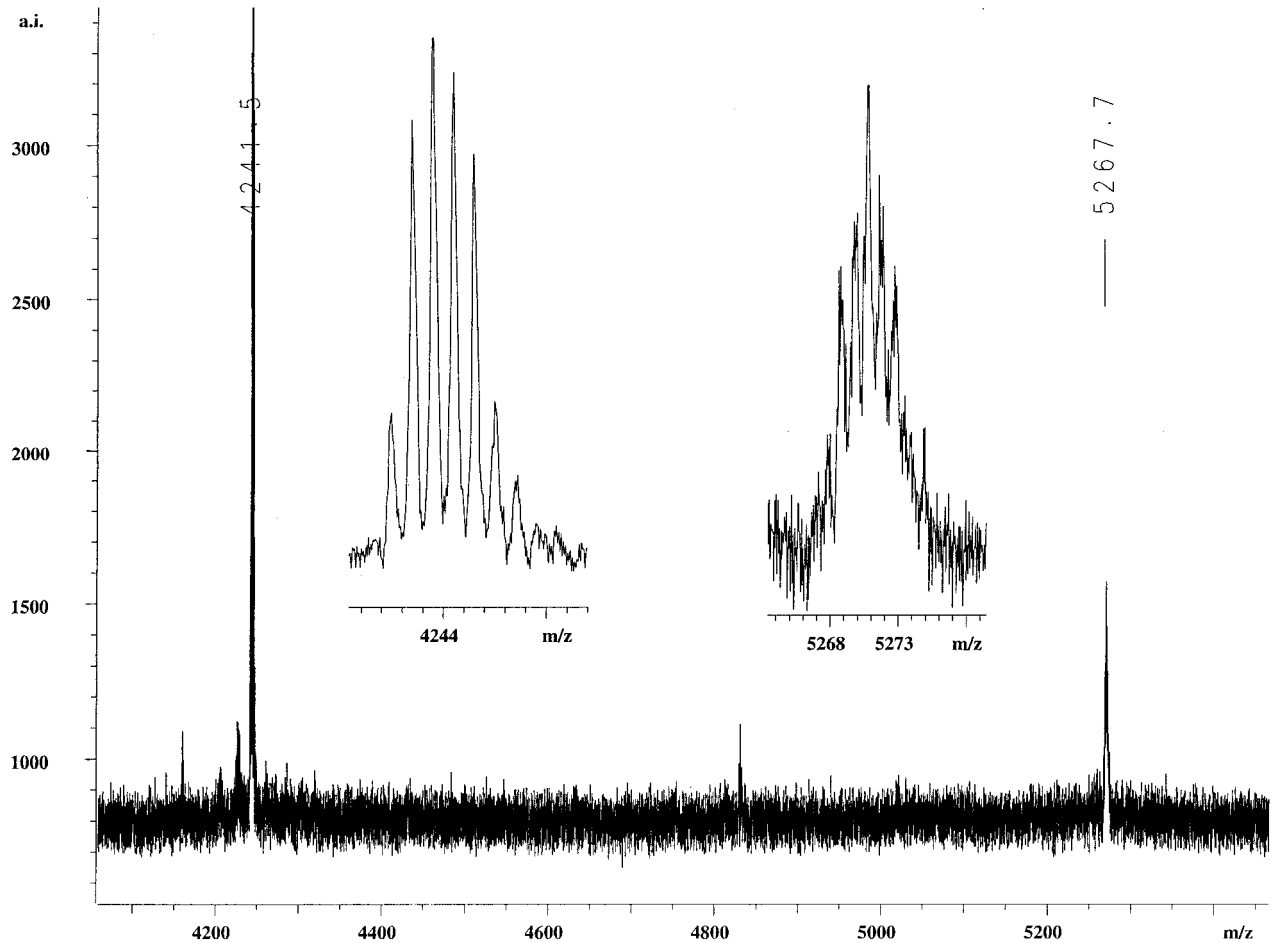

FIG. 4. Unsmoothed (to clearly display monoisotopic resolution of the insets) reflected and delayed extraction MALDI spectrum of 4 pmol of reduced and thiol alkylated rhodopsin in α-cyano-4-hydroxycinnamic acid.

2–39. Often, an almost equally intense ion is found at 6298 molecular weight (spectrum not shown), which corresponds to one *N*-acetylglucosamine less than the major glycoform. This rhodopsin CNBr fragment contains one of the core oligosaccharides with three mannose and two *N*-acetylglucosamines, which is the basis for addition of sugars to form heterogeneous glycoproteins (34).

Tryptophan-containing fragments (2–39, 87–143, 164–183, 258–288), show a mixture of oxidized (+16) and unoxidized products. As previously reported (27), the use of fresh CNBr reduced tryptophan oxidation (results not shown). It is important to note that fragments containing cysteines and no tryptophan did not show detectable oxidized products.

Sensitivity was better in linear mode than in reflector mode, which indicates there was significant metastable ion decay before reflection. Figure 5 was acquired in linear mode with the matrix DHB and displays the fragments 254–257 (427.1 Da), 40–44 (520.3 Da), 45–49 (588.4 Da), 156–163 (878.4 Da), and 310–317 (1049 Da). Typically, it was not possible to obtain the MH+ ion of the lowest mass fragments 254–257, and 40–44 in reflector mode, whereas the larger fragments 45–49, 156–163, and 310–317 were easy to observe in reflector mode. The lowest mass fragments, 254–257 and 40–44, were observable with the matrix DHB, but not ACHA. Figure 5 was acquired with the low mass gate set to 200 Da and calibrated internally with the monoisotopic mass of a matrix dimer and the monoisotopic mass of fragment 310–317 at 1049.5 Da. Although not shown in this particular figure, the lower mass fragments often ionize as Na^+ and K^+ adducts, and these adducts were useful in assigning fragments 254–257, 40–44, and 45–49.

Postsource decay (PSD) fragmentation carried out at higher laser power can identify sequences by their "mass finger print" (35). Figure 6 displays, for example, a PSD spectrum of fragment 310–317, derived from the parent ion of molecular weight 1051.74 Da (the calibration of this particular spectrum is slightly high for all masses). Primarily "b- and y-ions" (36) are detected

TABLE 1

Rhodopsin Cyanogen Bromide Fragments

Observed mass	Theoretical mass		Sequence identification	Ion type
	Monoisotopic	Average		
427.1[a]	427.3	427.6	254–257	MH+
449.3[a]	449.3	449.6	254–257	Na+
520.3[a]	520.3	520.6	40–44	MH+
588.4[a]	588.4	588.8	45–49	MH+
610.5	610.4	610.8	45–49	Na+
626.5	626.4	626.8	45–49	K+
862.4[a]	862.4	863.0	156–163	MH+
878.4[a]	878.4	879.0	156–163 + O	MH+
1049.5[a]	1049.5	1050.2	310–319 + A	MH+
1107.4	1107.6	1108.5	40–49 (−30)	MH+
1374.6[a]	1374.7	1375.5	144–155	MH+
2054.0	2054.1	2055.4	164–183	MH+
2110.9[a]	2111.1	2112.5	164–183 + A	MH+
2127.1[a]	2127.1	2128.5	164–183 + O, A	MH+
2198.3[a]	2198.2	2199.6	289–308	MH+
2220.9[a]	2221.2	2221.6	289–308	Na+
2398.6	2299.2	2300.8	289–309 (−30)	MH+
2778.6	2778.2	2780.0	184–207 − NH3	MH+
2892.2[a]	2892.2	2894.1	184–207 + 2A − NH3	MH+
2909.1[a]	2909.2	2911.1	187–207 + 2A	MH+
3124.3	3123.5	3125.4	318–348 − 2F	MH+
3237.6[a]	3237.5	3239.6	318–348 − 2F + 2A	MH+
3418.8[a]	3418.7	3420.9	318–348 − F + A	MH+
3536.7	3532.8	3535.1	258–288 + A	MH+
3548.5[a]	3548.8	3551.1	258–288 + A + O	MH+
3604.1	3599.9	3602.3	318–348 (+2F)	MH+
4224.1[a]	4224.4	4227.1	50–86 − NH3	MH+
4241.5	4241.4	4244.1	50–86	MH+
5267.4[a]	5267.8	5271.1	208–253 + A	MH+
6274.8[a]	6273.2	6277.3	87–143 + 2A + O	MH+
6300.2	6294.8	6298.6	2–39 + G	MH+
6314.3[a]	6310.8	6314.6	2–39 + G + O	MH+
6502.3	6497.8	6501.8	2–39 + G	MH+
6516.9[a]	6513.8	6517.8	2–39 + G + O	MH+

Note. Peptide masses matched to within 0.05% of the masses expected for the cyanogen bromide digest of rhodopsin. Cysteines were alkylated with iodoacetamide. O, oxygen; W, water; A, acetamide; NH3, ammonia; F, palmitoyl; G, glycosylations; −30, CNBr intermediate (33).

[a] Low masses calibrated internally with monoisotopic DHB matrix dimer (309.061) and fragment 310–319 + A (1049.495) and/or fragment 144–155 (1374.655). High masses were calibrated internally with monoisotopic masses of fragment 310–319 + A, and fragment 50–86 (4241.425).

and are labeled accordingly. In addition, immonium ions for N, K, Q, F, and R are detected between 70 and 129 Da, supporting the composition of this peptide. While PSD was not the primary focus of the method development targets in this paper, it was also used to confirm the identities of the sodiated fragment 254–257 (449.3 Da) and fragment 144–155 (1374.6 Da).

DISCUSSION

Investigation of the function of eukaryotic integral membrane proteins and their site-specific mutations necessitates the use of eukaryotic expression systems to obtain proper folding and posttranslational modifications. Eukaryotic membrane proteins are typically expressed in very low levels and thus the study of these proteins requires the use of radioactive or fluorescently labeled ligands for functional detection. Bovine rhodopsin and bacteriorhodopsin are the only integral membrane proteins that have been directly sequenced by mass spectrometry and their posttranslational modifications fully analyzed. The sequences of other membrane proteins are most often deduced indirectly from their cDNA, and many of their posttranslational modifications are not measured directly. Methods that increase sensitivity are highly desirable to extend the capabilities of mass spectrometry to study membrane proteins. Mass spectrometric detection limits are often quoted as 50 fmol or lower for highly purified proteins (1, 37). For most proteins, background from contamination raises detection limits in practice, and picomole amounts are often required for mass spectrometric detection (16, 38–40). One microliter of 1 μM protein solution yields 1 pmol of protein. In order to obtain even low micromolar concentrations, most membrane proteins have to be purified in detergent and concentrated. Optimization of reduction and alkylation yield and cleavage methods is also essential to achieve high sensitivity in mass spectrometric detection of membrane protein fragments. The fluorescein labeling and cyanogen bromide digestion of rhodopsin depicted in the tricine gel in Fig. 2, demonstrates that the digestion methods used in this study are effective. TCA precipitation and ethanol washing serves to rapidly concentrate dilute protein solutions and to remove protein modification reagents, lipids, and detergents which may be detrimental to mass spectral analysis. Sample losses in chromatography tend to be large for hydrophobic fragments of membrane proteins. Chromatography was avoided in the present studies by the use of MALDI, and the removal of contaminants led to improvements in sensitivity, enabling detection of all cyanogen bromide digestion product peptides from 10 μL of 3 μM rhodopsin. As little as 1 pmol of the digest could be spotted, using the MALDI MS method described in this paper, and still obtain 99% sequence coverage. Partial peptide sequences could be obtained on the same samples using PSD. In general, the fragments having the highest relative intensity have lower masses, the lowest detection limits, and higher numbers of charged residues in the sequence. Some of the fragments of rhodopsin show sub-picomole detection limits. Ion intensities similar to those seen in Fig. 3 are typically found in many spectra; however, 5–10× variation in ion intensity was common for all ions. The older Perceptive Biosystems RP MALDI, without delayed ion extraction, was also capable of yielding 99% sequence coverage (albeit with much less resolution

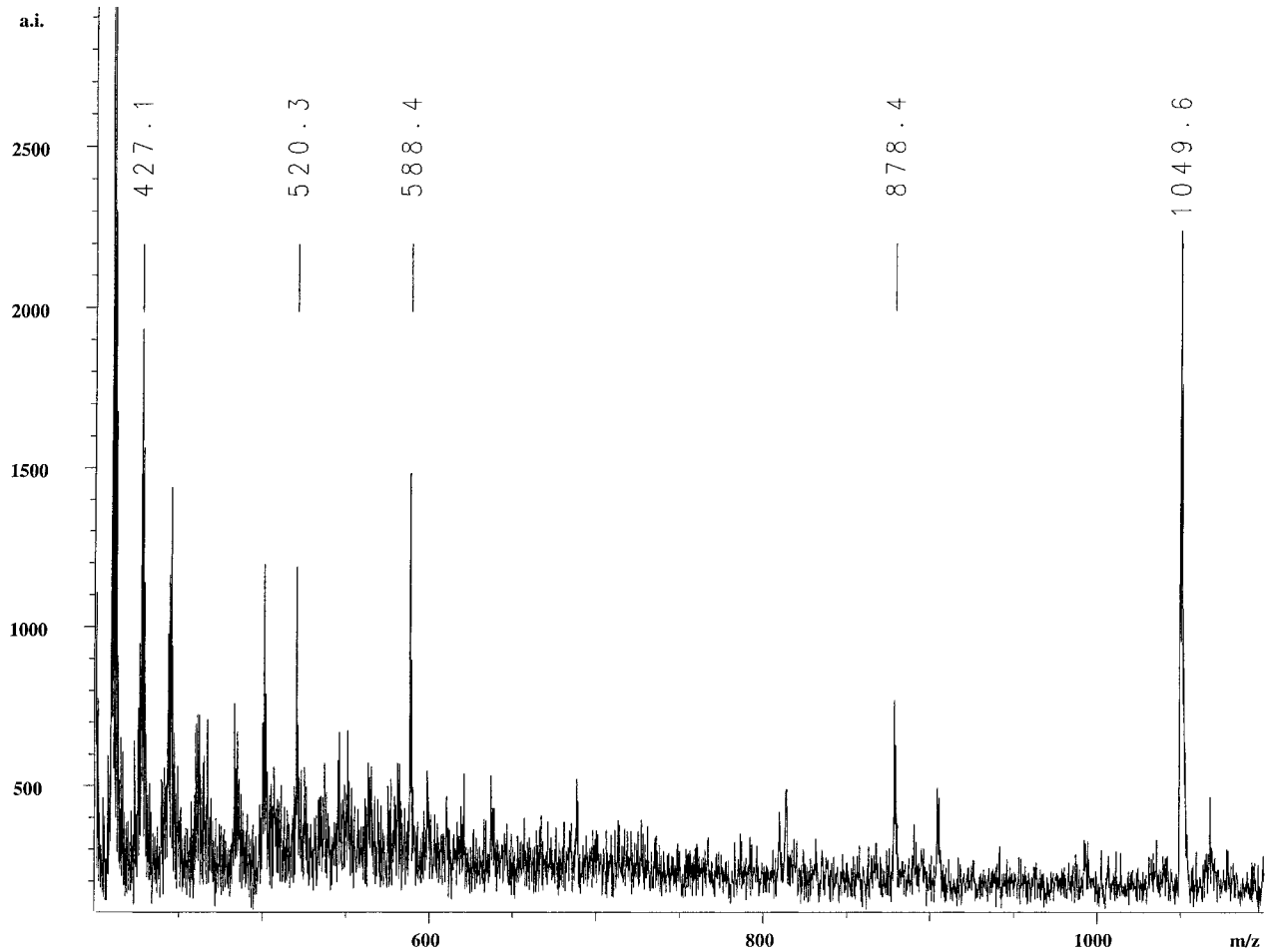

FIG. 5. Linear mode, delayed extraction MALDI spectrum of 4 pmol of reduced and thiol alkylated rhodopsin in 2,5-dihydroxybenzoic acid.

and mass accuracy and much greater uncertainty of ion assignment) with 1-pmol samples (data not shown).

Ion suppression and concomitant loss or reduction of ion signals is a common phenomenon in MS of protein/peptide mixtures, and thus separation of mixtures by liquid chromatography before MS is desirable. Reverse-phase LC-MS is an excellent method for achieving this goal if the mixture contains well-behaved proteins/peptides in sufficient quantity. Ball *et al.* (9) report an LC/MS method for identifying most of the bovine rhodopsin CNBr fragments during a 90-min chromatographic run. The Ball method required 6 nmol (~250 μg) of starting material, which is 200-fold more material than the 30 picomole required for the MALDI method described here. A later paper by Abloncy *et al.* (27), using the Ball *et al.* method (9), reported similar results for rat rhodopsin with a similar quantity of starting material (approximately 4 nmol/175 μg). MALDI analysis is very fast, and the data analysis is relatively straightforward, since MALDI produces primarily singly charged ions and thus no spectral deconvolution is required.

Since rhodopsin is 90+% pure in the rod outer segments, and the rhodopsin CNBr fragments have been previously characterized (9, 27), the primary focus of this work was to optimize biochemical methods to observe all rhodopsin CNBr fragments by mass spectrometry at the picomole level. It was difficult to obtain the 300 ppm instrument specified mass accuracy for external calibration over the broad range of 300–7000 Da. This difficulty with mass accuracy is most likely because the large number of fragments to assign, and the laser threshold for these hydrophobic peptides was significantly higher than the synthetic standards used for external calibration. In this regard, the calibration of the LC-MS method reported by Ball *et al.* is significantly easier, since the external calibration of an ion trap (or quadrapole mass filter) of peptide ions produced by electrospray does not suffer from the inherent kinetic energy differences produced by laser desorption. Ball *et al.* reported average masses for fragments greater than 2000 Da, while with the Bruker Biflex III was able to obtain monoisotopic mass resolution up to about 5300 Da. Multiple internal calibrations using

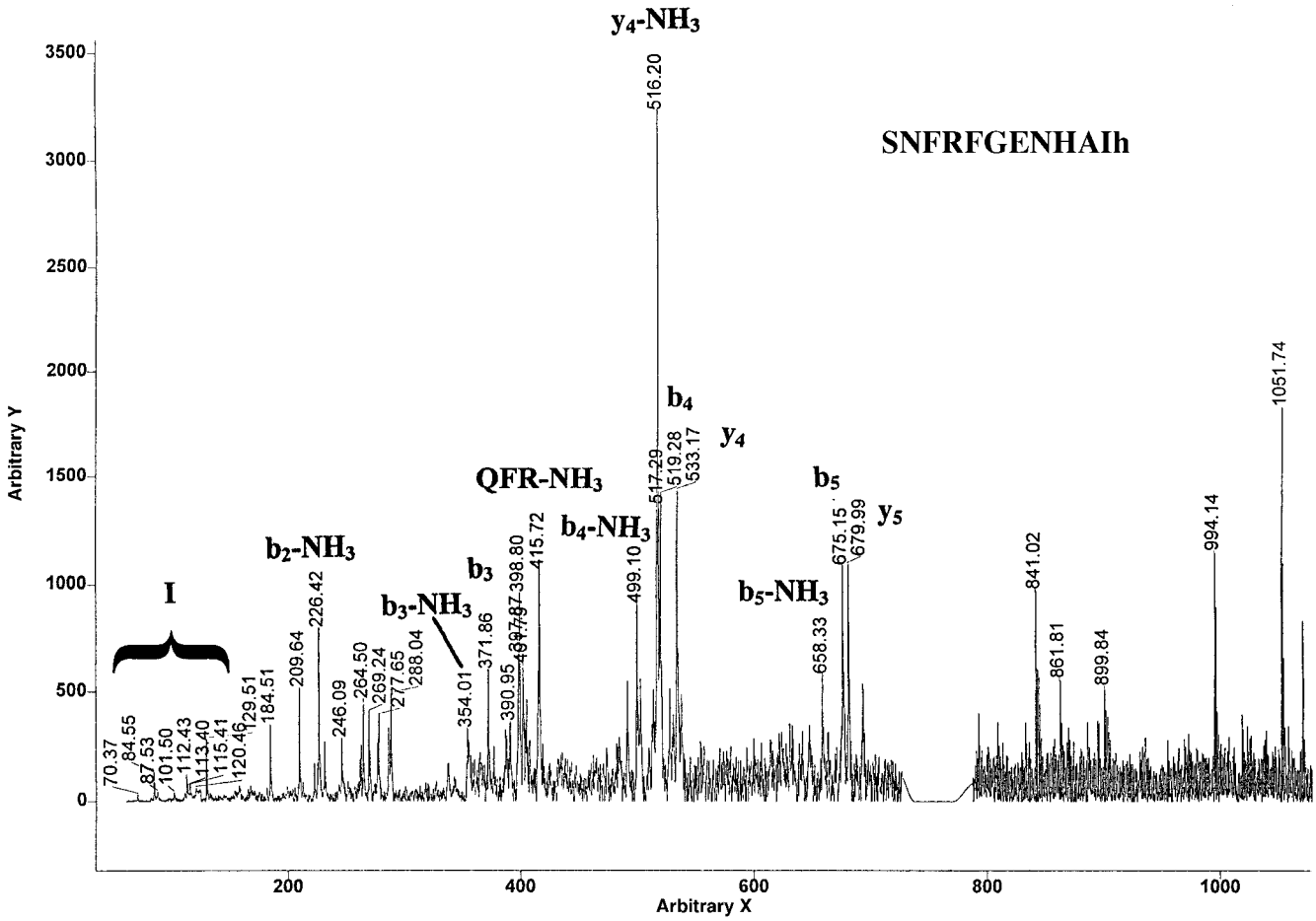

FIG. 6. MALDI postsource decay (PSD) spectrum of fragment 310–317. Standard one-letter amino acid residue abbreviations are used for the peptide sequence (h, homoserine lactone) and fragment ions are labeled accordingly (36).

monoisotopic masses of CNBr fragments attributed to rhodopsin were required to obtain the best mass accuracy. All of the internally calibrated masses in Table 1 have mass accuracies <100 ppm, except for fragment 254–257. The mass error on the fragment attributed to the 254–257 peptide of almost 500 ppm, calls its identification into question. Its sodium adduct, on the other hand, had no significant mass error, and was mass fingerprinted by PSD (data not shown). PSD analysis was not the focus of this work, but future PSD analysis of unknowns not mass matched to expected CNBr fragments of rhodopsin seems feasible, and may lead to their identification.

CONCLUSION

Sensitivity improvements and the essentially full sequence coverage of rhodopsin reported in this biochemical method for detergent-solubilized retinal rod membrane digests is a significant step toward practical MS analysis of membrane proteins. This method may also be useful for mass spectrometric studies of other membrane systems containing numerous different proteins without fractionation. Membrane proteins have been difficult to analyze and most membrane proteins occur in highly complex mixtures in low quantities in native membranes, requiring gel filtration, gel electrophoresis, antibody precipitation, chromatography or other purification in detergent prior to the application of the mass analysis protocol presented.

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