Anal. Chem. **2000,** *72,* 5655−5658

A Robust, Detergent-Friendly Method for Mass Spectrometric Analysis of Integral Membrane Proteins

Martine Cadene and Brian T. Chait*

The Rockefeller University, New York, New York, 10021

Recent breakthroughs in the high-resolution structural elucidation of ion channels and transporters are prompting a growing interest in methods for characterizing integral membrane proteins. These methods are proving extremely valuable in facilitating the production of X-ray diffraction-grade crystals. Here we present a robust and straightforward mass spectrometric procedure that utilizes matrix-assisted laser desorption/ionization to analyze integral membrane proteins in the presence of detergents. The utility of this method is illustrated with examples of high-quality mass spectral data obtained from membrane proteins for which atomic resolution structural studies are ongoing.

Although it has been predicted that 20−30% of all open reading frames in eubacterial, archaean, and eukaryotic organisms encode integral membrane proteins,[1,2] relatively few high-resolution structures have been obtained for this class of protein (PDB[3]). This paucity of data is related to the difficulty in handling integral membrane proteins. Because of their amphiphilic nature, exposure of naked membrane proteins to an aqueous environment often leads to aggregation and precipitation.

To assist in obtaining atomic resolution structures of integral membrane proteins, it is beneficial to have rapid, reliable methods for verifying the primary structure of the protein constructs, monitoring their stability, and elucidating their topology and domain structure.[4−6] Each of these steps requires a method that can provide the appropriate analytical information in the presence of lipids or detergents. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-MS) provides a fast, accurate means of protein characterization and has become a much-valued tool for the measurement of soluble proteins.[7] However, MALDI-MS has been applied much less frequently to integral membrane proteins, notwithstanding studies that clearly demonstrate its considerable potential for this purpose.[8−10] The previous statement also holds true for electrospray ionization (ESI) mass spectrometry.[11−15] Reasons for this infrequent utilization of mass spectrometry for studying integral membrane proteins include the aforementioned difficulties in maintaining protein solubility during the preparation of the MS samples and problems related to the suppressive effects of detergents on the mass spectrometric response.

Here we present a robust, reliable method for the accurate mass spectrometric analysis of membrane proteins in detergents or in membrane lipids. We describe how membrane proteins can be maintained in soluble form while transferring them to a medium compatible with MALDI-MS and how interference by the remaining detergent or lipid can be minimized.

MATERIAL AND METHODS

Materials. The proteins used as calibrants in this study were bovine insulin, bovine ubiquitin, and horse apomyoglobin, all from Sigma, St. Louis, MO. Membrane proteins presented here are the potassium channel from *Streptomyces lividans* (KcsA), the glycerol 3-phosphate transporter from *Escherichia coli* (G3P), and bovine rhodopsin. Full-length histidine-tagged KcsA overexpressed in *E. coli*[16] was provided by Dr. Roderick McKinnon at the Rockefeller University, New York. Histidine-tagged G3P expressed in *E. coli* was provided by Dr. Da-Neng Wang at NYU Medical Center, New York. Details of the purification will be published elsewhere. Wild-type bovine rhodopsin provided by Dr. Thomas Sakmar at the Rockefeller University, New York, was expressed and purified as follows. COS-1 cells were transiently transfected with an expression vector encoding wild-type rhodopsin (pMT-4) using Lipofectamine Plus Reagent (Gibco/BRL Life Technologies, Rockville, MD).[17] The cells were harvested after 48 h and treated with 11-

(1) Wallin, E.; von Heijne, G. *Protein Sci.* **1998**, *7*, 1029−38.
(2) Stevens, T. J.; Arkin, I. T. *Proteins* **2000**, *39*, 417−20.
(3) Berman, H. M.; Westbrook, J.; Feng, Z.; Gilliland, G.; Bhat, T. N.; Weissig, H.; Shindyalov, I. N.; Bourne, P. E. *Nucleic Acids Res.* **2000**, *28*, 235−42.
(4) Chait, B. T. *Structure* **1994**, *2*, 465−7.
(5) Cohen, S. L. and Chait, B. T. *Annu. Rev. Biophys. Biomol. Struct.*, in press.
(6) Cohen, S. L. *Structure* **1996**, *4*, 1013−6.
(7) Stults, J. T. *Curr. Opin. Struct. Biol.* **1995**, *5*, 691−8.
(8) Schey, K. L.; Papac, D. I.; Knapp, D. R.; Crouch, R. K. *Biophys. J.* **1992**, *63*, 1240−3.
(9) Rosinke, B.; Strupat, K.; Hillenkamp, F.; Rosenbusch, J.; Dencher, N.; Kruger, U.; Galla, H. J. *J. Mass Spectrom.* **1995**, *30*, 1462−8.
(10) Schnaible, V.; Michels, J.; Zeth, K.; Freigang, J.; Welte, W.; Buhler, S.; Glocker, M. O.; Przybylski, M. *Int. J. Mass Spectrom. Ion Processes* **1997**, *169/170*, 165−77.
(11) Schindler, P. A.; Van Dorsselaer, A.; Falick, A. M. *Anal. Biochem.* **1993**, *213*, 256−63.
(12) Le Maire, M.; Deschamps, S.; Moller, J. V.; Le Caer, J. P.; Rossier, J. *Anal. Biochem.* **1993**, *214*, 50−7.
(13) Hufnagel, P.; Schweiger, U.; Eckerskorn, C.; Oesterhelt, D. *Anal. Biochem.* **1996**, *243*, 46−54.
(14) Whitelegge, J. P.; Gundersen, C. B.; Faull, K. F. *Protein Sci.* **1998**, *7*, 1423−30.
(15) Barnidge, D. R.; Dratz, E. A.; Jesaitis, A. J.; Sunner, J. *Anal. Biochem.* **1999**, *269*, 1−9.
(16) Doyle, D. A.; Morais, Cabral J.; Pfuetzner, R. A.; Kuo, A.; Gulbis, J. M.; Cohen, S. L.; Chait, B. T.; Mackinnon, R. *Science* **1998**, *280*, 69−77.

10.1021/ac000811l CCC: $19.00
Published on Web 10/21/2000

cis-retinal and solubilization buffer.[18] Immunoaffinity purification of the recombinant pigment was carried out as described with final elution in 50 mM NaCl, 0.5 mM *N*-dodecylmaltoside (DDM; Anatrace, Maumee, OH). Following purification, the sample was concentrated ~5-fold using a Centricon-30 filtration unit (Amicon, Beverly, MA). An equal volume of 100 mM Tris-HCl, pH 6.8, 50 mM NaCl was added and the sample further concentrated using a Microcon-30 filtration unit (Amicon). The A_{500} of the final sample was ~2.1. To remove N-linked carbohydrates, rhodopsin was incubated for 2 h at 37 °C in the dark with *N*-glycosidase F (Boehringer Mannheim, Indianapolis, IN) at a ratio of 0.04 unit/μg of protein. The protein was subsequently bleached by addition of hydroxylamine hydrochloride (pH 7.0) at a final concentration of 10 mM and incubation in ambient light for 2 h at room temperature.

The detergents were *N*-decylmaltoside (DM; Pierce), *N*-dodecylmaltoside, *N*-octylglucoside (NOG; Pierce), *N*,*N*-dimethyldodecylamine-*N*-oxide (LDAO; Pierce, Rockford, IL), *N*-dodecylphosphocholine (FOS-12; Pierce), and *N*-dodecyl-*N*,*N*-dimethyl-3-ammonio-1-propanesulfonate (Zwittergent 3−12, Roche, Sommerville, NJ). The MALDI matrix α-cyano-4-hydroxycinnamic acid (4HCCA; Sigma) was purified by HCl precipitation prior to use. All organic solvents and water were HPLC grade. Formic acid (88%) and isopropyl alcohol (IPA) were from Fisher Scientific (Springfield, NJ).

MALDI-MS Sample Preparation. 4HCCA matrix was prepared as a saturated solution in a 3:1:2 (v/v/v) mixture of formic acid/water/IPA (here referred to as FWI). For experiments with KcsA, a 19 μM solution of protein solubilized in 10 mM LDAO was diluted 1:20 in matrix solution. G3P sample (11 μM in 2 mM DDM) and rhodopsin (15 μM in 10 mM DM) were diluted 1:20 in matrix solution containing respectively 10 and 25 nM of apomyoglobin calibrant. Proteins were analyzed within 10 min of dilution in matrix solution to prevent adventitious formylation by the formic acid component of the matrix solution.

For MALDI-MS analysis, a modified thin-layer method[19,20] of sample preparation was implemented. Saturated 4HCCA matrix was prepared in a 1:2 water/acetonitrile solvent mixture and further diluted 4-fold with IPA. About 20 μL of diluted matrix solution was applied over the whole gold surface of the sample plate (area 50 × 50 mm). The organic solvents were allowed to dry in ambient air until only traces of moisture were left on the plate. The matrix was then gently wiped with a tissue, leaving behind a faint layer of 4HCCA only visible as a yellowish reflection when the plate was examined at an angle. We will refer to this preparation as the "ultrathin layer".

A small aliquot (0.5 μL) of protein−matrix solution was spotted onto the sample plate pretreated to form the ultrathin layer. A white opaque polycrystalline film of matrix begins to form at the spot within seconds. As soon as the crystalline film appeared homogeneous, the excess liquid was removed by vacuum aspiration. The spot was then washed for a few seconds with 2−4 μL of 0.1% aqueous TFA.

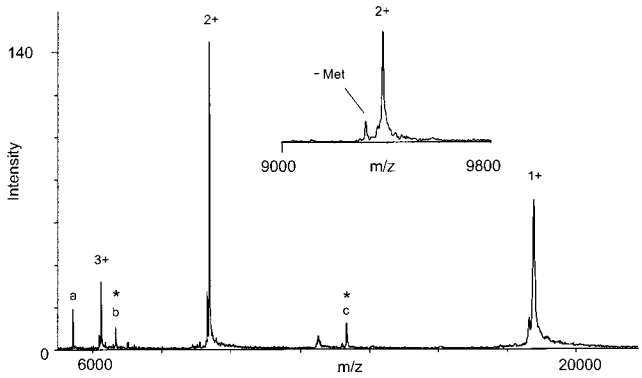

Figure 1. MALDI-MS of the potassium channel KcsA. Concentrations before 1:20 dilution in 4HCCA matrix: KcsA 19 μM; LDAO 10 mM. The numbers indicate the charge states of the protein. Letters represent the following: (a) chymotryptic fragment [126−170] of KcsA, $z = 1$; (b) chymotryptic fragment [1−125] of KcsA, $z = 2$; (c) chymotryptic fragment [1−125] of KcsA, $z = 1$. Peaks labeled with an asterisk (*) were used for internal calibration.

MALDI-MS Instrumentation and Data Collection. All spectra were acquired using a MALDI time-of-flight mass spectrometer Voyager-DE STR (PE Biosystem, Foster City, CA) operating in linear, delayed extraction mode. This instrument is equipped with a nitrogen laser delivering pulses of ultraviolet light (wavelength 337 nm) at 3 Hz to the matrix spot. Spectra from 200 individual laser shots were averaged (using 2-ns data channel width) with software provided by the manufacturer. The spectra were smoothed, calibrated, and analyzed using the program M-over-Z (http://www.proteometrics.com and http://prowl.rockefeller.edu).

RESULTS AND DISCUSSION

The membrane proteins presented here were analyzed directly in the presence of the detergent used to maintain solubility during purification. Since ionic detergents have proven to be incompatible with mass spectrometry,[9,20,21] only nonionic and zwitterionic detergents were selected for solubilization. As seen in Figures 1−3, proteins analyzed in this fashion yielded high-quality MALDI-MS spectra over a wide molecular mass (MM) range (18−53 kDa). We have similarly obtained useful mass spectral data of an integral membrane protein covalent dimer with MM ~70 kDa (data not shown).

KcsA (Figure 1) and G3P (Figure 2) were expressed in *E. coli* as homogeneous species, without posttranslational modifications other than the partial loss of the N-terminal methionine. To take advantage of the higher resolution found at lower m/z range, protein masses were determined on multiply charged ions ($z = 2$) bracketed with the appropriate internal calibrant peaks. The matrix 4HCCA was chosen for its ability to produce a high number of charge states.[22] Using this procedure, the MMs of KcsA and G3P were determined with an accuracy <100 ppm as shown in Table 1.

In contrast to KcsA and G3P, bovine rhodopsin was expressed in the eukaryotic COS cell system, which allows for posttranslational modifications. Native rhodopsin is known to be N-glycosy-

(17) Marin, E. P.; Krishna, A. G.; Zvyaga, T. A.; Isele, J.; Siebert, F.; Sakmar, T. P. *J. Biol. Chem.* **2000**, *275*, 1930−6.
(18) Zvyaga, T. A.; Fahmy, K.; Sakmar, T. P. *Biochemistry* **1994**, *33*, 9753−61.
(19) Xiang F.; Beavis, R. C. *Rapid Commun. Mass Spectrom.* **1994**, *8*, 199−204.
(20) Beavis, R. C.; Chait, B. T. *Methods Enzymol.* **1996**, *270*, 519−51.
(21) Beavis, R. C.; Chait, B. T. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 6873−7.
(22) Beavis, R. C.; Chaudhary, T.; Chait, B. T. *Org. Mass Spectrom.* **1992**, *27*, 156−8.

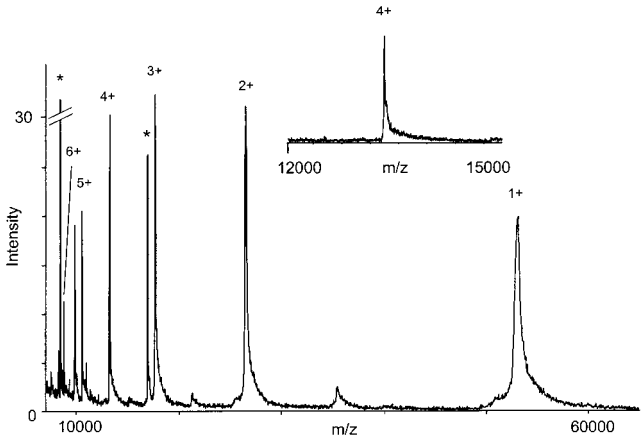

Figure 2. MALDI-MS of G3P transporter. Concentrations before 1:20 dilution in 4HCCA matrix: G3P 11 μM; DDM 2 mM. The numbers indicate the charge states of the protein. Peaks labeled with an asterisk (*) designate singly and doubly charged apomyoglobin and were used for internal calibration.

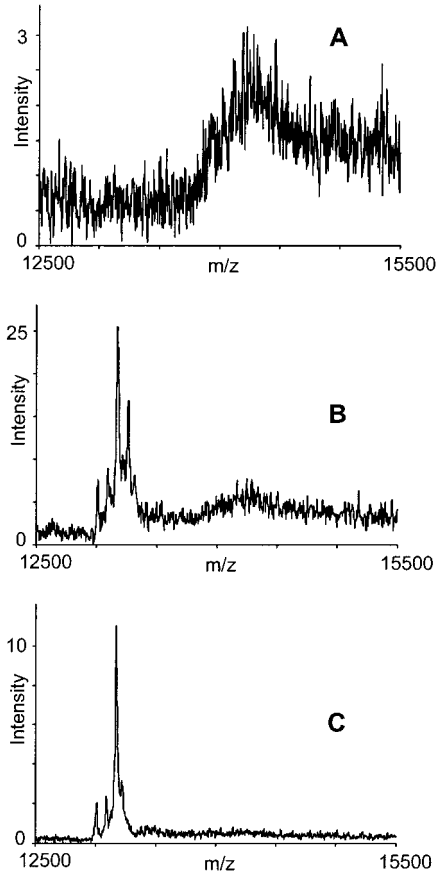

Figure 3. MALDI-MS of rhodopsin. Concentrations before 1:20 dilution in 4HCCA matrix: rhodopsin 15 μM; DDM 10 mM. For the sake of clarity, the display is limited to the region of the spectra encompassing triply charged rhodopsin species: (A) glycosylated rhodopsin; (B) deglycosylated rhodopsin; (c) deglycosylated opsin (i.e., rhodopsin that has been treated to remove the retinal moiety).

lated on asparagine residues 2 and 15[23] and palmitoylated on two cysteine residues at the C-terminus of the protein.[24] As can be seen from Figure 3A, recombinant rhodopsin displayed a high level of heterogeneity consistent with multiple posttranslational modifications. The heterogeneity of native rhodopsin has previously been observed by MALDI-MS[8,25] and ESI-MS.[14] Thus, for example, the glycosylated N-terminal tryptic peptide 1−16 of native rhodopsin exhibited a distribution extending over 1200 Da.[25] Although individual components of recombinant rhodopsin were not resolved in the present experiment, the width of the distribution (>5000 Da) is remarkable (Figure 3A). To remove the carbohydrate portion of the molecule, we deglycosylated the protein with *N*-glycosidase F. The resulting deglycosylated rhodopsin (Figure 3B) exhibited a cluster of narrow partially resolved peaks, each of which could be readily assigned to the degree of palmitoylation and the partial loss of the retinal chromophore (Table 1). Upon complete cleavage of the retinal Schiff's base through hydroxylamine bleaching, the remaining heterogeneity could be attributed to the presence of 0, 1, or 2 palmitates (Figure 3C). The accuracy achieved with this inhomogeneous sample was in the range of 200 ppm (Table 1).

The spectra in Figures 1−3 were obtained with relatively limited amounts of protein sample. A total of 5−10 pmol of protein was dissolved in the matrix solution, $^1/_{20}$ of which was then submitted to analysis. The sensitivity of the method is sufficiently high so that we can routinely analyze small single crystals of membrane proteins, corresponding to quantities that are generally <10 pmol (data not shown). We note, however, that the sensitivity for membrane proteins is typically 10 times lower than that for soluble proteins of similar MM.

A key factor in achieving a high signal-to-noise ratio was the careful choice of detergent prior to analysis. We and others[9,26,27] have found that many nonionic and a few zwitterionic detergents are compatible with mass spectrometry (Table 2). Optimal results were obtained by solubilizing the protein in a nonionic detergent at a minimal concentration above the critical micellar concentration. Matrix solvent conditions were optimized to favor membrane protein solubility. A high percentage of formic acid in the matrix solution was found to be advantageous[28−31] and produced no significant formylation unless the protein was left in prolonged contact with FWI. Thorough removal of nonprotein components was also important for obtaining high-quality spectra. This cleanup was achieved through use of the ultrathin-layer sample preparation method. A protein−matrix polycrystalline film quickly forms over the ultrathin layer, leaving most of the detergent and other components in the liquid droplet. Removing this excess liquid by vacuum aspiration greatly improved the signal quality. The

(23) Fukuda, M. N.; Papermaster, D. S.; Hargrave, P. A. *J. Biol. Chem.* **1979**, *254*, 8201−7.
(24) Papac, D. I.; Thornburg, K. R.; Bullesbach, E. E.; Crouch, R. K.; Knapp, D. R. *J. Biol. Chem.* **1992**, *267*, 16889−94.
(25) Barnidge, D. R.; Dratz, E. A.; Sunner, J.; Jesaitis, A. J. *Protein Sci.* **1997**, *6*, 816−24.
(26) Gharahdaghi, F.; Kirchner, M.; Fernandez, J.; Mische, S. M. *Anal. Biochem.* **1996**, *233*, 94−9.
(27) Bornsen, K. O.; Gass, M. A.; Bruin, G. J.; von Adrichem, J. H.; Biro, M. C.; Kresbach, G. M.; Ehrat, M. *Rapid Commun. Mass Spectrom.* **1997**, *11*, 603−9.
(28) Cohen, S. L.; Chait, B. T. *Anal. Chem.* **1996**, *68*, 31−7.
(29) Baldwin, M. A.; Wang, R.; Pan, K.-M.; Hecker, R.; Stahl, N.; Chait, B. T.; Prusiner, S. B. In *Techniques in Protein Chemistry IV*; Angeletti, R. H., Ed.; Academic Press: San Diego, CA, 1993; pp 41−5.
(30) Martin de Llano, J. J.; Jones, W.; Schneider, K.; Chait, B. T.; Manning, J. M.; Rodgers, G.; Benjamin, L. J.; Weksler, B. *J. Biol. Chem.* **1993**, *268*, 27004−11.
(31) Schey, K. L. *Methods Mol. Biol.* **1996**, *61*, 227−30.

Table 1. Molecular Mass Determination of Proteins

figure no.	obsd mass (Da)	bracketed[a] charge state(s)	interpretation	theor mass (Da)	ΔM[b] (Da)
1	18 778.0	2	KcsA	18 777.8	0.2
	18 645.9	2	KcsA − Met	18 646.6	−0.7
2	53 017.2	4, 5, 6	G3P	53 013.2	4.0
3B	39 048.5	3, 4	opsin, no palmitate	39 047.7	0.8
	39 283.0	3, 4	opsin, 1 palmitate	39 286.1	−3.1
	39 306.7	3, 4	rhodopsin, no palmitate	39 313.1	−6.4
	39 531.0	3, 4	opsin, 2 palmitates	39 524.6	6.4
	39 559.3	3, 4	rhodopsin, 1 palmitate	39 552.5	6.8
	39 791.9	3, 4	rhodopsin, 2 palmitates	39 791.0	0.9
3C	39 049.5	3, 4	opsin, no palmitate	39 047.7	1.8
	39 291.5	3, 4	opsin, 1 palmitate	39 286.1	5.3
	39 528.9	3, 4	opsin, 2 palmitates	39 524.6	4.3

[a] Charge states bracketed by calibration peaks. [b] ΔM = observed mass − theoretical mass.

Table 2. Recommended Detergents and Concentrations for Use with MALDI-MS

detergent	mass (Da)	cmc[a] (mM)	max deterg conc in final MALDI matrix−protein soln (mM)[b]
	nonionic		
decylmaltoside	482.6	1.8	0.5
dodecylmaltoside	510.0	0.17	0.5
octylglucoside	292.4	19	1.5
	zwitterionic		
lauryldimethylamine oxide	229.4	1.5	0.5
Zwittergent 3−12	335.6	2−4	0.25
N-dodecylphosphocholine	351.5	1.5	0.25

[a] Critical micellar concentration, cmc, values provided by Anatrace, Inc. and Calbiochem, Inc. [b] Concentration of detergent after dilution of the protein sample into the matrix solution ($^1/_{20}$ in the present study). Concentrations exceeding these values will prevent proper formation of matrix−sample cocrystal.

polycrystalline film was further washed with dilute TFA solution to remove residual detergent. Thin-layer methods have the added advantage of presenting a homogeneous matrix−protein cocrystal surface, allowing for reproducible data acquisition over the entire surface area.[19,20] The present method allows soluble proteins to be analyzed concomitantly with membrane proteins, allowing for straightforward internal calibration.

CONCLUSION

The present MALDI-MS procedure has proven useful for the analysis of a variety of integral membrane proteins with molecular masses up to 70 kDa. We have successfully analyzed ion channels, metabolite transporters, and receptors, containing between 2 and 12 transmembrane domains. The procedure consistently produces high-quality spectra with a resolution matching closely that obtained for soluble proteins. The method is sensitive, robust, and easy to implement.

ACKNOWLEDGMENT

We thank João Morais-Cabral (KcsA), Joanne Lemieux (G3P), and Wing-Yee Fu (rhodopsin) for supplying protein. This work was supported by a grant from the National Institutes of Health (RR00862 from the National Center for Research Resources).

Received for review July 13, 2000. Accepted September 6, 2000.

AC000811L