Matrix-assisted laser desorption mass spectrometry of rhodopsin and bacteriorhodopsin

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ABSTRACT Matrix-assisted laser desorption ionization (MALDI) mass spectrometry has been used to obtain accurate molecular weight information for the integral membrane proteins bacteriorhodopsin and bovine rhodopsin desorbed from solubilized membrane preparations. Mass differences in the molecular weights measured for bleached and unbleached bacteriorhodopsin and rhodopsin indicate the removal of the retinal chromophores upon bleaching. The MALDI technique was also successful for determination of the major cleavage products obtained upon treatment of membrane bound rhodopsin with endoproteinase Asp-N and thermolysin. Our results indicate that the MALDI method is a useful means of obtaining accurate molecular weight information on hydrophobic proteins isolated in their native membranes.

INTRODUCTION

Since its introduction, the technique of matrix-assisted laser desorption ionization (MALDI) mass spectrometry (1, 2) has led to molecular weight measurements of a variety of biomolecules including proteins (1, 2), oligosaccharides (3), and oligonucleotides (4). The technique has been particularly successful in providing accurate molecular weight information for proteins with molecular weights up to 300 kD often present in complex mixtures of proteins, buffers, salts, etc. (5, 6). Accurate molecular weight measurements with MALDI have been used to estimate the sugar content in and drug binding to immunoglobulins (7), to determine protein subunit interactions (8), and to study protein/metal ion binding (9). A majority of the proteins examined to date have been water soluble proteins. An important, yet problematic, group of proteins are membrane bound hydrophobic proteins which includes, for example, proteins involved in cell recognition and cellular signaling. The hydrophobic nature of these proteins and their propensity for aggregation makes sample handling very difficult, often requiring manipulations in powerful and potentially destructive solvents. Only a small number of reports have addressed MALDI of hydrophobic proteins (10–12), and very few details have been published on the methods used (13).

The integral membrane protein rhodopsin is an example of a protein for which it is difficult to obtain accurate molecular weight information. The posttranslational modifications, especially glycosylation, make molecular weight estimates by gel electrophoresis or gel filtration unreliable. We have therefore selected this protein for examination by the MALDI methodology. For comparison, we have also studied bacteriorhodopsin, a similar seven α-helical, integral membrane protein which is not glycosylated. Both proteins have been subjected to molecular weight measurement both before and after bleaching to remove the retinal chromophores.

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MATERIALS AND METHODS

Purple membrane, containing bacteriorhodopsin (BR), was grown in cultures of *Halobacteruim halobium* by the method of Becker and Cassim (14) and purified by layering on a 30–60% sucrose gradient and centrifuging at 25,000 rpm for 17 h. The resulting membrane preparation had an OD 280/500 ratio of $\geq$2, indicative of high purity. Bleached purple membrane was formed by the method of Tokunaga et al. (15) by extensive irradiation with white light at 4°C in the presence of high concentrations of hydroxylamine. The retinal oxime was removed by hexane extraction.

The rod outer segments containing rhodopsin were isolated from frozen bovine retinae (Hormel, Austin, MN) according to the method of Papermaster and Dreyer (16). Bleached rhodopsin was prepared as described by Zorn and Futterman (17). Enzymatic cleavages of membrane bound bovine rhodopsin with endoproteinase Asp-N (Boehringer Mannheim, Indianapolis, IN) and thermolysin (Calbiochem Corp., La Jolla, CA) were carried out according to the methods of Palczewski et al. (18) and Nakayama and Khorana (19), respectively.

The samples for the MALDI experiment were prepared by first solubilizing the membrane preparations (3 μl) in 7 μl 99% formic acid (Sigma Chemical Co., St. Louis, MO) and 2 μl hexafluoroisopropanol (Brand-Nu Laboratories, Meriden, CT). Sonication was often required to completely dissolve the membranes. The solubilized membranes were then mixed in a 1:3 or 1:7 ratio with 50 mM sinapinic acid (Aldrich, Milwaukee, WI) in 70% formic acid. Approximately 0.3 μl of the resulting solutions were placed on the 3-mm diameter stainless steel probe tip and allowed to air dry.

The mass spectrometer consists of a conventional linear (1 m) time-of-flight instrument equipped with a nitrogen laser (Laser Science, Cambridge, MA) emitting 337-nm photons focussed to a 100 μm^2 spot providing a power density of 10 megawatts/cm^2. Mass spectra were averaged with a LeCroy (Chestnut Ridge, NY) 9400A digital oscilloscope and subsequently transferred to a Zenith 386-SX computer for storage and calibration. Low mass ions were pulsed away using deflection plates in efforts to avoid detector saturation. Mass calibration was accomplished with protein internal standards which were spotted on a different portion of the probe tip after the sample protein solution had dried. The probe was then rotated during the experiment to generate calibrant and protein signals in one spectrum. Typically 50–200 laser shots were averaged to produce a mass spectrum, although each sample lasted for 500–1,000 laser shots when rotated. The standards used were bovine insulin (MW 5733.6) (20), horse heart cytochrome *c* (12360.1) (21), and horse skeletal muscle myoglobin (16951.5) (22) obtained from Sigma Chemical Co. and *Staphylococcus aureus* V-8 protease (29991) (23) from Boehringer Mannheim. Mass calibration was carried out with software provided by Genetics Institute. With the exception of bacteriorhodopsin, the $(M + H)^+$ ion alone was used to determine the mass of the sample proteins. BR had a consistently in-

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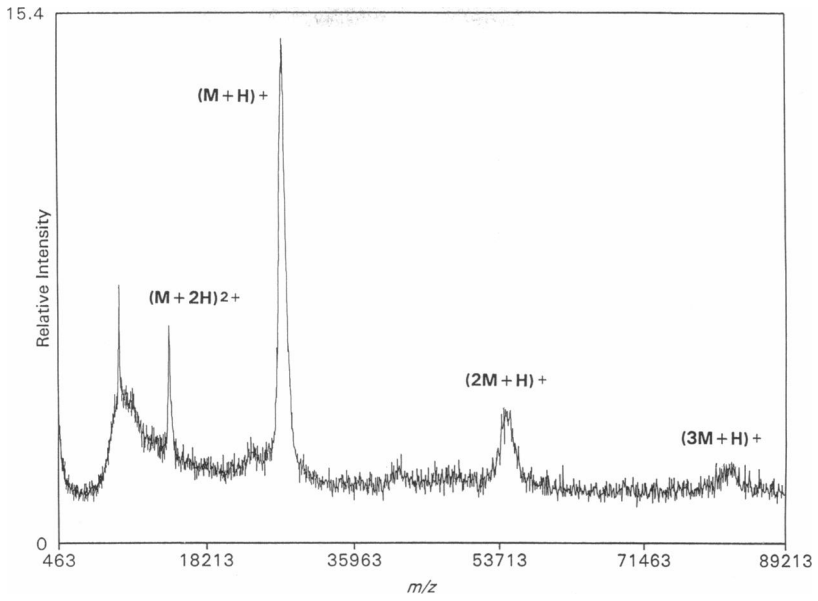

FIGURE 1 MALDI spectrum of bacteriorhodopsin desorbed from solubilized purple membranes.

tense doubly charged, $(M + 2H)^{2+}$, ion which was used with the $(M + H)^+$ ion to determine the molecular weight of the protein.

RESULTS AND DISCUSSION

All of the proteins examined in this study were present in their native membranes before solubilization in the matrix solution. Fig. 1 shows a MALDI mass spectrum of bacteriorhodopsin obtained from a concentrated solution of purple membrane. The spectrum is similar to that reported by Chait (10) and Hillenkamp (11) where the singly protonated molecular ion, $(M + H)^+$, is the dominant peak, while the doubly charged monomer, $(M + 2H)^{2+}$, singly charged dimer, $(2M + H)^+$, and singly charged trimer species, $(3M + H)^+$, are also visible. Although the baseline continues to rise at lower masses, no significant peaks are observed. The absence of other major peaks in this mass spectrum demonstrates the specificity of MALDI for proteins in complex matrices, e.g., lipid membranes. The small peak at m/z 7,648 daltons is of unknown origin and may be a minor membrane bound protein. The molecular weight measured with the aid of internal standards is 27,048 ± 74 daltons as shown in Table 1, which represents an error of 0.07% based upon the calculated molecular weight of 27,068 daltons (including retinal). The measured molecular weight indicates that the retinal prosthetic group remains in the protein through the preparation and desorption processes. The MALDI spectrum of bleached bacteriorhodopsin (6.2 pmol placed on probe) looks identical to that of BR itself, but the measured molecular weight of 26,789 ± 36 daltons (Table 1) indicates that the retinal has been removed.

Fig. 2 shows the MALDI spectrum of 1.8 pmol bovine rhodopsin desorbed from solubilized disc membranes. In addition to the singly charged protonated molecular ion, $(M + H)^+$, the doubly charged ion, $(M + 2H)^{2+}$, and the protonated dimer, $(2M + H)^+$, of rhodopsin are also observed. The measured molecular weight of 42,034 ± 73 daltons, shown in Table 1, agrees well with that calculated from the amino acid sequence including retinal and the known posttranslational modifications. The posttranslational modifications used in the molecular weight calculation include the two most abundant oligosaccharides (24), two palmityl groups (25, 26), and the acetylated NH_2-terminus (27). The resolution of the spectrometer is not adequate to resolve less abundant posttranslational modifications such as other oligosaccharides; however, the symmetry of the $(M + H)^+$ signal suggests that these components, if present, are indeed of minor abundance. The spectrum of bleached rhodopsin looks identical to that of rhodopsin; however, the mea-

TABLE 1 Molecular weight measurements of integral membrane proteins

Protein	Measured mass*	n‡	Calculated mass§	Error
Bacteriorhodopsin	27,048 ± 74	23	27,068	0.07%
Bacterioopsin	26,789 ± 36	5	26,784	0.02%
Rhodopsin	42,034 ± 73	15	42,002	0.08%
Opsin	41,748 ± 134	11	41,718	0.07%
Rhodopsin (1-329) (Asp-N cleaved)	39,826 ± 93	4	39,781	0.11%
Rhodopsin (1-240) (Thermolysin cleaved)	29,041	1	29,296	0.87%

* Measured mass ± standard deviation, in daltons. ‡n = number of protein samples measured to obtain a molecular weight. §Calculated mass in daltons.

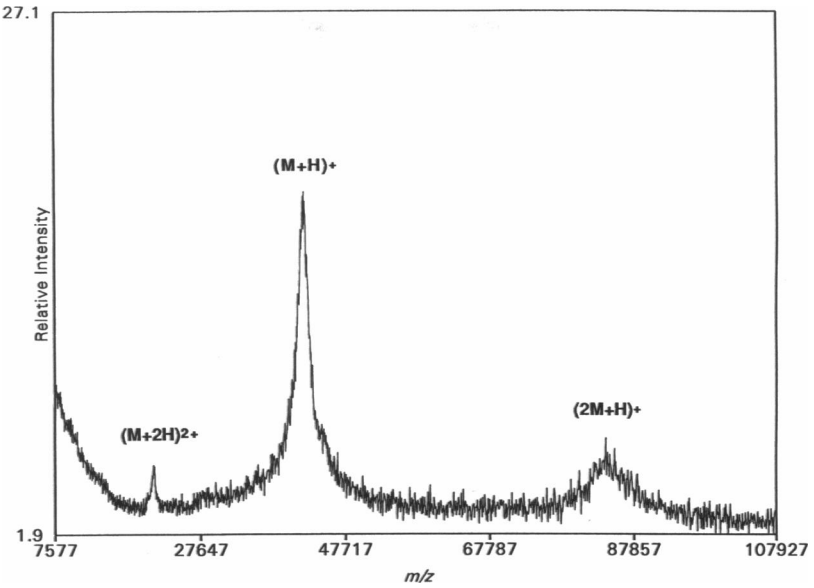

FIGURE 2 MALDI spectrum of bovine rhodopsin desorbed from solubilized rod outer segments.

sured mass (Table 1) indicates the absence of the retinal from the protein.

In the course of structural studies on rhodopsin, several enzymatic cleavage reactions have been carried out on the membrane bound protein. Cleavage with endoproteinase Asp-N at Asp 330 is predicted to remove a COOH-terminal peptide leaving a membrane bound truncated rhodopsin (1-329) of molecular weight 39,781 daltons. A MALDI mass spectrum of Asp-N cleaved rhodopsin desorbed from solubilized membranes is shown in Fig. 3. A protonated molecular ion, $(M + H)^+$, is observed at m/z 39,827 ± 93, giving a 0.1% error from the calculated value (Table 1). The doubly charged ion, $(M + 2H)^{2+}$, is present at m/z 19,961, as well as an ion at m/z 10,694 which may be due to another protein in the membrane preparation. Similar results were also obtained with the enzyme thermolysin where the data indicated a major product resulting from predicted cleavage of rhodopsin at the NH_2-terminal side of Ala 241 to give a product of mass 29,041 daltons as shown in Table 1 (the large error being due to only one spectrum being calibrated with internal standards or further cleavage of the COOH-terminal portion of this fragment as has been reported previously [28]).

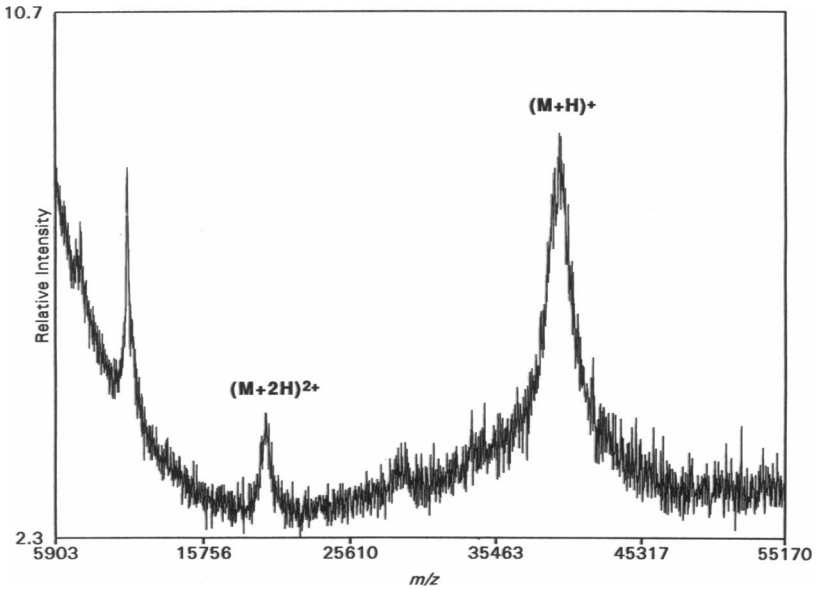

FIGURE 3 MALDI spectrum of truncated bovine rhodopsin (1-329) cleaved with endoproteinase Asp-N.

CONCLUSIONS

We have successfully mass analyzed a series of hydrophobic proteins and protein fragments desorbed from solubilized membranes by matrix-assisted laser desorption ionization mass spectrometry. This method should be applicable to other membrane bound hydrophobic proteins and hence should be extremely useful in the characterization of those proteins involved in cell signalling and cell recognition. The mass accuracy of the MALDI method has allowed the observation of small mass shifts in large proteins (e.g., loss of retinal, mass 284, from rhodopsin MW 42,002 daltons; a 0.7% change) and may be useful in examining affinity, photoaffinity, or fluorescent labeled proteins. Further studies on peptide mapping and identification of posttranslational modifications in membrane proteins are underway.

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