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Mass spectrometric analysis of integral membrane proteins at the subpicomolar level: Application to rhodopsin

Zsolt Ablonczy [a], Rosalie K. Crouch [a], Daniel R. Knapp [b,*]

[a] *Department of Ophthalmology, Medical University of South Carolina, 171 Ashley Avenue, Charleston, SC 29425, USA*
[b] *Department of Cell and Molecular Pharmacology and Experimental Therapeutics, Medical University of South Carolina, 171 Ashley Avenue, Charleston, SC 29425, USA*

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Abstract

Integral membrane proteins are among the most interesting molecules for biomedical research, as some of the most important cellular functions are inherently tied to biological membranes. One such example is the vast expanse of receptors on cell surfaces. However, due to difficulties in the biochemical purification and structure/function analysis of membrane proteins, caused by their hydrophobic or amphophilic nature, membrane proteins are still much less studied than soluble proteins. Our laboratory has successfully developed and applied a methodology for the mass spectrometric analysis of integral membrane proteins. Here, we present an improvement in the sensitivity of detection made possible by the advancement of mass spectrometric instrumentation and refinement of the chromatographic analysis. Subpicomolar samples of bovine rhodopsin purified from native membranes were successfully analyzed, obtaining complete sequence coverage and the detection and localization of posttranslational modifications. Therefore, it is demonstrated that the detection limits and sequence coverage for soluble and membrane proteins can be comparable. The methodology presented here allows mass spectrometric analysis of subpicomolar levels of photopigments or other integral membrane proteins either from their native membranes or as products of expression systems.

Keywords: Rhodopsin; Opsin; Pigment; Membrane protein; G protein-coupled receptor; Mass spectrometry; HPLC; Protein mapping; Protein identification; Protein sequencing; LTQ

1. Introduction

Mass spectrometry (MS) is the most promising method for the analysis of protein covalent structure [1–5]. The general algorithm for sequencing normally starts with enzymatic or chemical fragmentation of intact proteins followed by reversed-phase HPLC fractionation. These steps result in a peptide mixture that is resolved in time and has peptide fragments of a suitable length for sequencing by MS. The general algorithm is relatively easy to apply to soluble proteins, but integral membrane proteins often undergo irreversible aggregation, adhere to sample handling surfaces, and bind to the chromatographic columns due to their amphophilic nature. It is often possible, though, to observe a few fragments from the soluble regions of the analyzed membrane protein [6,7]. However, the full structural characterization (including posttranslational and chemical modifications) is much more difficult because the steps in the analytical procedure have to be adapted to the chemical nature of integral membrane proteins. We have previously reported a methodology, which was successfully applied to the analysis of different integral membrane and membrane-associated proteins [8,9]. Here, we present an improved methodology, which increases detection sensitivity by three orders of magnitude and also increases sequence coverage, made possible by advances in chromatographic and mass spectrometric instrumentation technologies.

One specific group of integral membrane proteins, the large G protein-coupled receptor superfamily [10,11], has

* Corresponding author. Tel.: +1 843 792 5830; fax: +1 843 792 2475.
E-mail address: knappdr@musc.edu (D.R. Knapp).

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been extensively studied. The best characterized member of this family of proteins is rhodopsin, the photopigment of the dim-light sensitive retinal rod cells [12]. Rhodopsin has become a model for the G protein-coupled receptor superfamily, because it is reasonably stable and easily isolated, as rhodopsin constitutes 90% of the membrane protein content of the retinal outer segments. The protein can easily be extracted with high yields in the native membrane form [13–15] and has also been successfully expressed in recombinant form in several eukaryotic cell lines retaining its main physical and biological properties [16–19] that can be examined by reliable assays and spectroscopy [20,21]. Both the native and the recombinant forms have successfully been analyzed by mass spectrometry before [8]. Although the previous analysis opened the way for the study of structural consequences of site directed mutagenesis of rhodopsin [22,23] and the examination of other low abundance rod and cone photopigments [24], a mass spectrometric detection limit for integral membrane proteins, comparable to that of soluble proteins, has not been previously reported.

The MS analysis of integral membrane proteins developed and applied in our laboratory is based on the reduction and alkylation of cysteine residues solubilized in mild non-ionic detergents. Then the proteins are precipitated with acetone, dissolved in neat trifluoroacetic acid (TFA), and cleaved with cyanogen bromide (CNBr). After sample drying (for the removal of the acid and the remaining CNBr) and subsequent dilution (to achieve the required solvent initial compositions), the cleavage mixture is resolved by reversed-phase HPLC. With this methodology, the entirety of bacteriorhodopsin [9,25]; bovine, rat, mouse, porcine and salamander rhodopsin [8,9,22,26,27]; bovine, rat and human aquaporin 0 [28–30]; and the gecko green cone opsin (unpublished data) have been mapped and sequenced by electrospray ionization mass spectrometry. The method was also used for the analysis of rhodopsin and α-transducin molecular interactions [31] and for crosslinking experiments to obtain three-dimensional information on rhodopsin [32]. The procedures are simple and straightforward, and 0.1–1 nmol of initial sample is adequate for the analysis (several washes are still performed for the removal of contaminants and reagents from the sample). The procedure is also versatile in that it can be applied to integral membrane proteins both from their native membranes and recombinant expression systems.

Since the initial development of the method, new approaches have also been published [33]. These methods are based on trypsin digestion prior to mass spectrometric analysis. Trypsin is widely used for the digestion of soluble proteins. These methods have the potential for being more uniform, as they are basically the same for membrane and soluble proteins. However, CNBr-based fragmentation procedures might still be better suited for integral membrane proteins, as the membrane spanning regions of these proteins contain relatively few of the positively charged residues required for tryptic digestion. On the other hand, the membrane spanning regions are relatively abundant in the hydrophobic methionines, the residue for CNBr cleavage. The subpicomolar detection limit achieved in our experiments further expands the group of integral membrane proteins analyzable by mass spectrometry and approaches the detection limits previously obtainable only for soluble proteins.

2. Experimental

2.1. Generation of peptides for sequence analysis

Bovine rhodopsin was purified following a procedure published elsewhere [14]. In short, rod outer segment membranes (ROS) were separated from frozen bovine retina (WL Lawson Co., Lincoln, NE) by sucrose (J.T. Baker, Phillipsburg, NJ) flotation and purified by sucrose gradient ultracentrifugation (100,000 × *g* in a Beckman Optima TL ultracentrifuge, Beckman-Coulter, Fullerton, CA) followed by subsequent washes in 4 M and 7 M urea (Sigma Chem. Co., St. Louis, MO). The final ROS membranes were dissolved in 100 mM sodium phosphate buffer (pH 7.4; Sigma) at a rhodopsin concentration of 1 μg/μL and stored in the dark at −80 °C for later use. The rhodopsin concentration of the samples was determined by spectrophotometric measurements at 495 nm (HP 8452A, Agilent Technologies, Palo Alto, CA). For the purposes of mass spectrometric analysis, 100 μg rhodopsin in ROS membranes were centrifuged at 100,000 × *g* and the pellets solubilized in 100 μL 0.1% dodecyl maltoside (Calbiochem-Novabiochem, La Jolla, CA) in water and incubated at room temperature for 15 min. This sample was then centrifuged again at the same speed and the solubilized fraction used for the experiments.

The samples were reduced and alkylated by the addition 100 μL 1.5 M Tris buffer (pH 8.7), 100 μL *n*-propanol (Fisher Scientific, Fair Lawn, NJ), 5 μL tributylphosphine (Aldrich Chem. Co., Milwaukee, WI; >1000 molar excess over the cysteine content) and 5 μL 4-vinylpyridine (Sigma; >2400 molar excess over the cysteine content). The reaction was performed at room temperature for 1 h under argon with constant shaking on a rotator. The mixture was then precipitated with the addition of 1 mL of acetone (Sigma) at −20 °C for 15 min. The precipitant was centrifuged at 100,000 × *g* for 5 min at 4 °C, and the supernatant removed. The resulting pellet was washed two more times with 1 mL acetone, and centrifuged at 100,000 × *g* for 5 min at 4 °C. The pellet was dissolved in 400 μL TFA (Acros Organics, Fair Lawn, NJ), and 180 μL water was added to adjust the concentration to 70% TFA. The CNBr cleavage was performed by adding 10 μL, 5 mol/L CNBr solution in acetonitrile (Aldrich; more than 500 molar excess over the methionine content). The cleavage was carried out with shaking in the dark under argon overnight at room temperature. The reaction was quenched by the addition of 1 mL water and the solvents evaporated under vacuum (SpeedVac SC110, Savant Instruments Inc., Farmingdale, NY). The dried fragment mixture was redissolved in 5 μL TFA, 42 μL acetonitrile (Fisher), and 84 μL

isopropanol (J.T. Baker) and brought up to 5 mL with water. The resulting 5 mL sample [1 μL, 10 μL, and 50 μL (in different experiments)] was loaded onto a 75 μm × 150 mm, C18 Nano LC column (LC Packings, Sunnivale, CA) through a 300 μm × 1 mm C18 Nano-precolumn (LC Packings) at a 30 μL flow of 0.1% aqueous TFA, and the peptides eluted at a flow rate of 200 nL/min with a 75 min gradient from 1% to 99% organic phase with an Nano-HPLC system (LC Packings) followed by a 45 min wash with 99% organic phase. The aqueous phase was 0.05% TFA in water, the organic phase was 0.05% TFA in 2:1 isopropyl alcohol/acetonitrile. In similar experiments, the samples were loaded unto a 300 μm × 150 mm, C18 monomeric Vydac column (Bodman Industries, Aston, PA) and eluted with the same gradient as above at a flow rate of 10 μL/min with an Agilent 1100 capillary HPLC system (Agilent).

2.2. Mass spectrometry

The column effluent from the 75 μm column was directed into the nano electrospray source of a Finnigan LTQ ion-trap mass spectrometer (Thermo-Finnigan Instrument Systems Inc., San Jose, CA) fitted with a 360 μm o.d., 30 μm i.d., 10 μm tip Picotip emitter (New Objective, Woburn, MA). The 2.5 kV spray voltage was applied through the tubing connector of the source, no nebulization gas was utilized. The column effluent of the 300 μm column was directed into the IonMax source of the LTQ. The source was modified to contain a flat-end stainless steel emitter (HTX-27, Small Parts Inc., Miami Lakes, FL) to accommodate for low flow rates. In each cycle, one MS and one MS/MS spectra of the three most abundant molecular ions were automatically acquired using Xcalibur software (version 1.4 SR1) with repeatless dynamic exclusion for both setups.

3. Results and discussion

Rhodopsin is a unique integral membrane protein in the sense that it can easily be purified in high yields and relatively pure [9]. However, other integral membrane proteins are not so abundant or easily purified, and therefore, we continued to develop our mass spectrometric methodology to expand the scope of analyzable proteins. Combining immunoaffinity chromatography with mass spectrometry, recombinant rhodopsin (obtained in much lower quantities from a COS cell expression system) was also mapped, demonstrating the analysis of low abundance membrane proteins [8]. However, the detection limit for the analysis of the entire sequence of a membrane protein was still only demonstrated at the 0.1–1 nanomolar levels. In this paper, using rhodopsin as a model protein, we demonstrate that it is possible now to detect and entirely map less than 1 pmol of an integral membrane protein, which is comparable to the detection limit for soluble proteins.

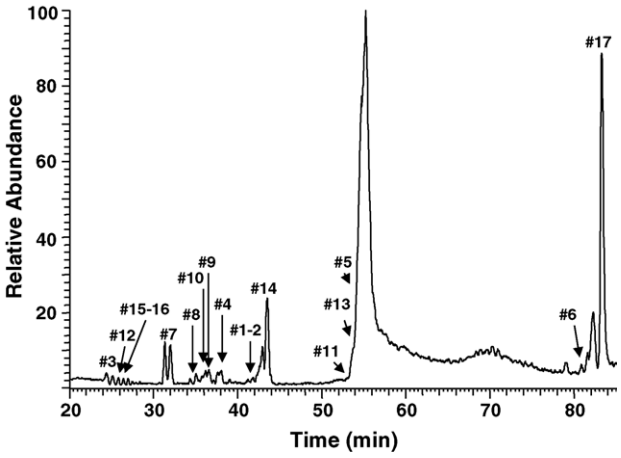

Fig. 1. Base peak chromatogram of 0.5 pmol bovine rhodopsin eluting from a 300 μm capillary column. The CNBr cleavage fragments are indicated. The entire rhodopsin sequence was covered by the peptides found in the chromatogram. The additional peaks are contaminants, the contaminant at 55 min originates from polyethylene glycol. Fragments 11, 13, and 5 coeluted with the contaminant.

We injected 20 pmol, 5 pmol and 0.5 pmol aliquots of a rhodopsin digest mixture onto two different capillary HPLC systems to test the detection limits obtainable for mapping rhodopsin with the LTQ mass spectrometer. Fig. 1 indicates the base peak chromatogram of a 0.5 pmol aliquot of bovine rhodopsin separated by the LC Packings nano HPLC system. The base peak chromatogram is produced by repetitive scanning of the HPLC effluent and indicating the signal intensity of the most abundant mass peak at each time points. It was possible to completely map rhodopsin from all three aliquots of the sample with both setups. The sensitivity obtained from the nano LC system surpasses that of the micro LC system, and as an additional advantage in the current trap-column setup the nano LC column cannot be overloaded. On the other hand, unlike the modified electrospray source, maintaining reliable constant spray throughout the gradient is a challenging task with the nanoelectrospray source. This counteracts the advantages of higher sensitivity arising from the lower chemical noise obtainable in the nano LC system in protein mapping experiments but causes fewer disadvantages for protein identification experiments, thus the two systems seem to be useful in different manners and in different experiments. Due to the lower flow rate, there is also an increase in the elution times with the nanoflow system compared to the microflow pump. It has to be emphasized that for easier processing of the sample, the injected amounts represent only aliquots of a larger scale sample preparation, the initial rhodopsin sample being around 2.5 nmol. Using essentially the same protein preparation methodology, we have shown previously that sample preparation is possible for 0.25 nmol initial sample. Therefore, with less initial sample, the final detection limit (0.5 pmol) might probably be lowered somewhat further (within one order of magnitude). The thorough analysis shows, however, that it is not possible to use the microflow HPLC setup orders of magnitude below the ∼0.5 pmol level

Table 1
Cyanogen bromide fragments of bovine rhodopsin

Fragment	Residues	Expected mass $[M+H]^{+1}$	Observed mass	Observed charge state	Retention time (min)[a]
1	1	144.1[b]	–	–	–
2	2–39	6497.8[c]	6498[d]	+4	42
1–2[e]	1–39	6670.9	6671	+4	42
3	40–44	520.3	520.9	+1	25
4	45–49	588.4	588.6[d]	+1	37
5	50–86	4241.4	4240[d]	+3	53
6	87–143	6353.3	6354[d]	+4	68
7[e]	144–155	1374.7	1374[d]	+2	32
8[e]	156–163	862.5	862.5[d]	+1	35
9	164–183	2159.1	2159[d]	+2	37
10	184–207	3005.3	3005[f]	+3	36
11	208–253	5315.8	5315[d]	+3	53
12	254–257	427.3	428.2	+1	27
13	258–288	3580.8	3580[d]	+3	54
14	289–308	2198.2	2198	+2	44
15	309	102.1	–	–	–
16	310–317	1097.5	1096	+2	27
15–16[e]	309–317	1228.6	1227	+2	27
17	318–348	3599.9[g]	3601	+3	72

[a] Capillary column (300 μm).
[b] With acetylation.
[c] The most abundant glycoform, multiple hexose additions observed.
[d] Also observed with homoserine C-terminal ending.
[e] Observed as incomplete cleavage.
[f] Also observed with pyroglutamic acid N-terminus.
[g] With palmitoylated cysteines.

for rhodopsin mapping, because some of the detected peptides were close to the detection limit. The higher sensitivity of the nanoflow HPLC setup might make it possible to lower the detection limit, but only if the current nanospray source is modified to provide more stable flow for extended time measurements. Therefore, using an order of magnitude lower initial quantity probably does not make it possible to cover the entire sequence of rhodopsin with the current setups. However, even with the current setups, it might be possible to detect or identify rhodopsin from a complex sample through one or two of its peptides at much lower levels, perhaps close to femtomoles. In the previous measurents with one order of magnitude less initial sample than now, the whole amount of final preparation had to be used, which resulted in ∼0.25 nmol of protein to be mapped. The new measurements demonstrate that 1/5000 of the final preparation of 2.5 nmol rhodopsin can be entirely mapped, which is a three orders of magnitude increase in the sensitivity of our measurements.

All the 17 CNBr fragments were observed in a single experiment providing 100% sequence coverage. Fragments 1 and 15 are single methionines and their masses are below the lower mass limit of the instrument, but they were detected through incomplete cleavages in combinations with their neighboring fragments (1–2 and 15–16 complexes). The fragments gave well-formed peaks, with different intensities. The most abundant fragments were 5, 7, 9, 14, 16, and 17; the rest had comparatively lower intensities. The CNBr fragments that contain tryptophan (fragments 6, 8, 9, and 13) were also observed with oxidized tryptophans as similarly observed in prior experiments [8,22,26,27]. This phenomenon may be attributed to contaminants in the CNBr and to sample aging. Table 1 shows the predicted and observed CNBr fragment masses and summarizes the data shown in Figs. 1 and 2.

Fig. 2 shows the selected ion chromatograms for the CNBr fragments. Two mass peaks (fragments 8 and 9) appear bimodal, the obtained MS/MS sequences are identical for the twin peaks (both in the case of fragments 8 and 9, data not shown). This was observed independent of sample concentration. The phenomenon is presumably due to two major conformations of these fragments in solution; alternatively a portion of the peptides might have non-covalently bound material lost only in the instrument source but not during reversed-phase chromatography. With the C18 capillary columns, even the smallest hydrophilic fragments (fragments 12 and 16) formed well-separated peaks, which had not been resolved this well previously [8,22,26] because they elute close to the injection solvent peak. The second peak in the selected ion plots for fragment 12 shows the presence of a contaminant with a similar molecular weight. Although the CNBr fragments could be identified from the selected ion chromatograms, their identity was further confirmed with MS/MS sequence data. In fact, the greatest improvement by upgrading the LCQ to an LTQ instrument was the quality of the MS/MS spectra. With the LTQ, it was possible to gather much more meaningful spectra even with the repeatless dynamic exclusion setting. As an example, the MS/MS spectrum of fragment 4 with a homoserine C-terminal ending is shown in Fig. 3. Homoserine is produced from

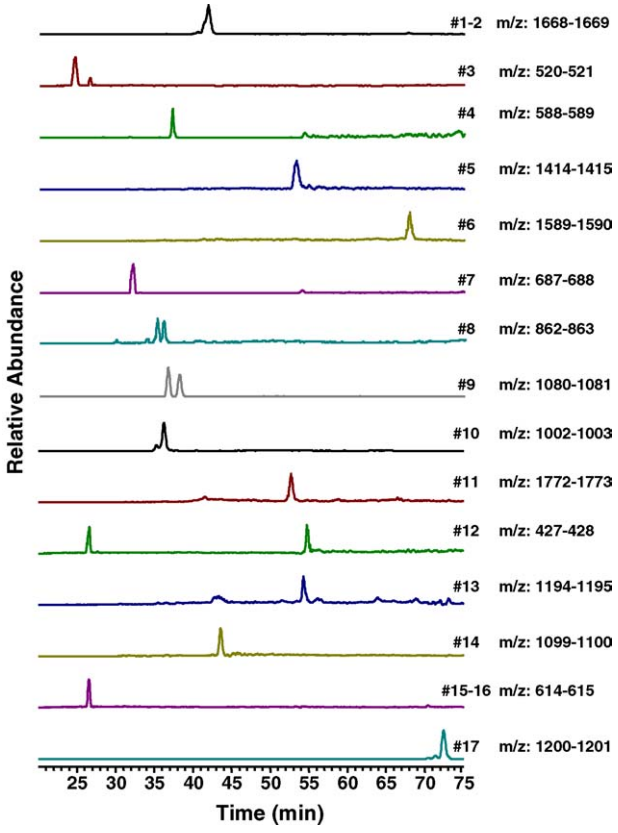

Fig. 2. Selected ion chromatograms of the CNBr fragments of bovine rhodopsin eluting from a 300 μm capillary column. The fragment numbers and the detected ions are indicated. The ions correspond to the most abundant charge states. The doublet peaks for fragments 8 and 9 reflect peptides with the same sequence, but apparently different steric conformations.

methionine during the CNBr cleavage process. The entire FLLIB peptide was covered by detected sequences.

Rhodopsin has six different types of posttranslational modification [34–43]. Two modifications are not detectable

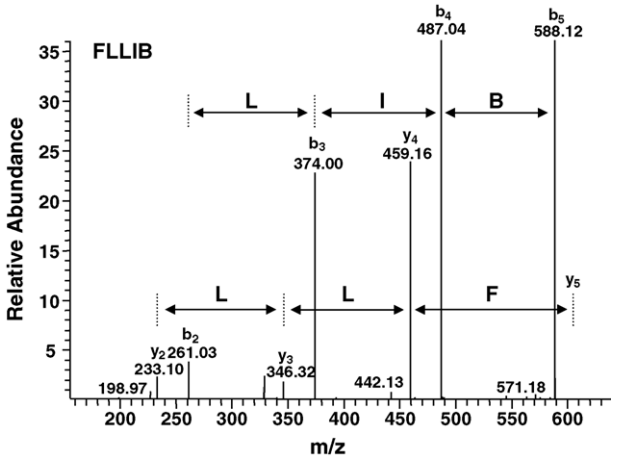

Fig. 3. Tandem mass spectrum of CNBr fragment 4 of bovine rhodopsin eluting from a 75 μm capillary column. The whole FLLIB sequence of the peptide was covered by b and y ions. B stands for homoserine produced from methionine during the CNBr cleavage process.

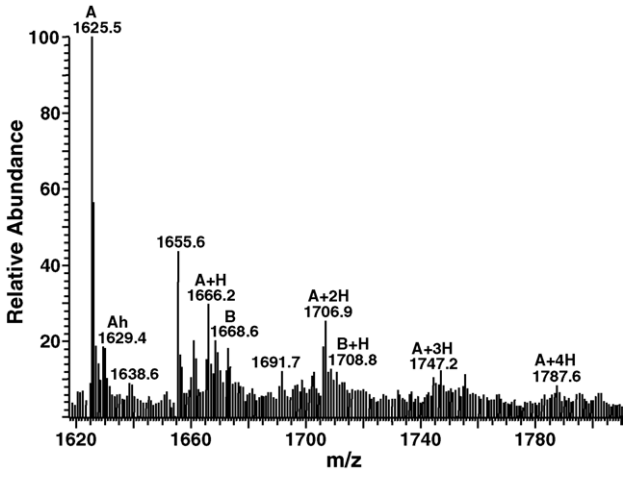

Fig. 4. Mass spectrum of the N-terminal rhodopsin glycopeptide. The sample contained 0.5 pmol bovine rhodopsin, measured with the 75 μm column. The rhodopsin glycopeptide consists of two core hexasaccharides with optional hexose additions. The spectrum indicates up to four hexose additions on the two glycans. Both fragment 2 (A) and the incompletely cleaved N terminally acetylated fragment 1–2 complex (B) are seen in the spectrum. H stands for hexose, Ah indicates homoserine ending for fragment 2.

with our procedures: the retinal chromophore, originally attached to lysine 296, and the disulfide bridge between cysteines 110 and 187, both of which are lost during sample preparation. The other four posttranslational modifications are retained during the processing steps. Two of these modifications are C-terminal: palmitoylation and phosphorylation. The presence of the two palmityl thioester substituents on cysteines 322 and 323 were confirmed both by MS and MS/MS data of CNBr fragment 17. Moreover, the lack of palmitates should coincide with the appearance of a new chromatographic peak at an earlier elution time, because the depalmitoylated fragment 17 has reduced hydrophobicity. In our measurements, no new early chromatographic peak was observed that is consistent with fragment 17 depalmitoylation. The other C-terminal modification is phosphorylation. As we used dark-adapted rhodopsin for our experiments, the amount of phosphorylation did not exceed the baseline levels [27].

In previous electrospray ionization measurements, the other two N-terminal modifications (glycosylation and acetylation) were not observed because CNBr fragments 1 and 2 were not observed in the HPLC-ESI-MS analysis. This is probably attributable to the higher flow rates and larger bore columns of those experiments [8,9,22,26,27]. However, both capillary systems were able to resolve this fragment and show the train of peaks characteristic for the rhodopsin glycopeptides previously seen only with MALDI in our analysis (Fig. 4). Therefore, we did not need separate MALDI confirmation of the detection of this fragment. The characteristic glycosylation pattern is a direct consequence of the two glycosylation sites present on the rhodopsin N-terminus (at asparagines 2 and 15) containing the core hexasaccharide and heterogenous hexose units at the end

of the glycan (one to three additional hexose molecules may be present on each glycan) [35]. Therefore, several mass peaks can be seen with regular 162 Da spacing with decreasing intensities. The rhodopsin modification of the acetylation of the N-terminal methionine was also observed. As CNBr cleaves at methionines, the N-terminal acetylated methionine can only be observed in the fragment 1–2 partially cleaved complexes. As seen in Fig. 4, an additional peak is present among the train of fragment 2 glycopeptide peaks whose mass is consistent with a methionine plus 42 Da (for acetylation) on the base glycopeptide and also on a glycopeptide with an additional hexose compared to the fragment 2 mass peaks. Although MS/MS data were collected for some of the most abundant peaks in Fig. 4, as CNBr fragment 2 is a long peptide (40 amino acids) and has two attached glycan structures made up of six to eight hexose units, the resulting MS/MS fragmentation is was too complex to analyze. Therefore, the peaks were identified by relative elution time (compared to the other peaks), molecular weight and peak pattern, all of which are similar to our previous measurements [26].

The results demonstrate that the improved methodology for the mass spectrometric analysis of integral membrane proteins permits the analysis of subpicomolar amounts of protein. The results obtained when using only 0.5 pmol of rhodopsin were comparable to the results previously obtained with more than three orders of magnitude larger amount of sample. As we used a relatively large, 2.5 nmol amount of initial sample, it is likely that the sample requirement can be further reduced, reinforcing our results that subpicomolar amounts of membrane proteins can be entirely mapped by mass spectrometry.

4. Conclusions

This observation of 100% of the structure of the bovine rhodopsin constitutes, to our knowledge, the first complete mass spectrometric mapping of an integral membrane protein at the subpicomolar level. Moreover, the determination of the complete sequence in a single experiment provided not only identification, but also the location of the posttranslational modifications. The improved methodology not only reduced detection limits, but it expanded the sequence coverage and the quality of the obtained sequences at the same time.

The ability to analyze subpicomolar amounts of proteins now opens a possibility for the application of this method to the analysis of very low abundance integral membrane proteins from either their native membranes or those produced by eukaryotic expression systems.

Acknowledgments

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