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Mass spectrometric analysis of integral membrane proteins: Application to complete mapping of bacteriorhodopsins and rhodopsin

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Abstract

Integral membrane proteins have not been readily amenable to the general methods developed for mass spectrometric (or internal Edman degradation) analysis of soluble proteins. We present here a sample preparation method and high performance liquid chromatography (HPLC) separation system which permits online HPLC–electrospray ionization mass spectrometry (ESI-MS) and –tandem mass spectrometry (MS/MS) analysis of cyanogen bromide cleavage fragments of integral membrane proteins. This method has been applied to wild type (WT) bacteriorhodopsin (bR), cysteine containing mutants of bR, and the prototypical G-protein coupled receptor, rhodopsin (Rh).

In the described method, the protein is reduced and the cysteine residues pyridylethylated prior to separating the protein from the membrane. Following delipidation, the pyridylethylated protein is cleaved with cyanogen bromide. The cleavage fragments are separated by reversed phase HPLC using an isopropanol/acetonitrile/aqueous TFA solvent system and the effluent peptides analyzed online with a Finnigan LCQ Ion Trap Mass Spectrometer. With the exception of single amino acid fragments and the glycosylated fragment of Rh, which is observable by matrix assisted laser desorption ionization (MALDI)-MS, this system permits analysis of the entire protein in a single HPLC run. This methodology will enable pursuit of chemical modification and crosslinking studies designed to probe the three dimensional structures and functional conformational changes in these proteins. The approach should also be generally applicable to analysis of other integral membrane proteins.

Keywords: bacteriorhodopsin; electrospray; G-protein coupled receptor; integral membrane proteins; HPLC; mass spectrometry; rhodopsin

Over the past several years, mass spectrometry has been increasingly applied to studies of protein structure (Karger & Hancock, 1996; Shively, 1994; McCloskey, 1990). Proteins to be examined by mass spectrometry (for information other than molecular weight of the intact protein) are usually fragmented, either enzymatically or chemically, and the resulting fragment mixture fractionated by HPLC prior to mass spectrometric analysis of the fragment peptides. These approaches have proven extremely useful for water soluble proteins. Difficulties in cleaving integral membrane proteins and difficulties in carrying out HPLC separations of the resulting cleavage fragments have severely limited the applicability of mass spectrometric methods (as well as conventional Edman degradation) to this biologically very important class of proteins. Integral membrane proteins, and their cleavage fragments, are amphiphilic molecules which tend to aggregate, often irreversibly, as well as bind irreversibly to commonly used HPLC column packings. Previously published methodology for HPLC separation of cleavage fragments of membrane proteins include reversed phase methods with alcohol containing solvents (Tarr & Crabb, 1983; Allmaier et al., 1986) as well as normal phase methods (Lerro et al., 1993), but we were unable to obtain consistent results with

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Abbreviations: HPLC, high performance liquid chromatography; ESI, electrospray ionization; MALDI, matrix assisted laser desorption ionization, TOF, time of flight; MS, mass spectrometry; MS/MS, tandem mass spectrometry; bR, bacteriorhodopsin; Rh, rhodopsin; TFA, trifluoroacetic acid; CNBr, cyanogen bromide; Fmoc, fluorenylmethyloxycarbonyl.

these methods. We present here a new sample preparation procedure, which includes reversed phase HPLC, and demonstrate its applicability to mass spectrometric mapping of some multiple transmembrane helix proteins: bacteriorhodopsin (bR), cysteine-containing mutants of bR, and rhodopsin (Rh).

The objective of this work was to develop an unbiased mass spectrometric mapping and structural analysis protocol which would allow observation of modifications anywhere on a membrane protein. Rh was studied as the prototypical G-protein coupled receptor, with bR and mutant bR's used for the initial method development. Mass spectrometric mapping of bR was reported over ten years ago (Allmaier et al., 1986), but Rh has proven much more difficult (Orlando et al., 1993; Barnidge et al., 1997). Initial work using bR as a model system yielded a cleavage and HPLC separation protocol which permitted complete analysis of the protein (Busman et al., 1996). In moving from wild type bR (which contains no cysteine) to cysteine containing mutants, it became apparent that the introduction of cysteine produced new problems which were not readily overcome using conventional reduction/alkylation approaches. We, therefore, developed a modified approach using organic soluble reagents (tributylphosphine and vinylpyridine) with reduction/alkylation done prior to removal of the protein from the membrane. The reduced, alkylated protein was then delipidated, cleaved with cyanogen bromide, and the fragments separated by HPLC with online ESI-MS and MS/MS analysis. We then extended the method to the typical multiple transmembrane helix integral membrane protein Rh which contains multiple cysteine residues, both as free thiols and as intramolecular disulfide bonds.

Rhodopsin, the light receptor protein of the retinal rod cells, is the most extensively studied of the family of G-protein coupled receptors which now includes well over a hundred members (Watson & Arkinstall, 1994). It was the first member of this protein family to be sequenced (Ovchinnikov, 1982) and is still the only one which has been directly sequenced in its entirety (the others having been derived primarily from DNA sequences). In spite of its central position in the study of integral membrane proteins in general and G-protein coupled receptors in particular, prior to this work, portions of the protein had continued to elude mass spectrometric observation. Using multiple experimental approaches, only 56% of the sequence had been observed in this laboratory (Papac, 1993). Another group subsequently observed an additional 10% of the previously unobserved sequence regions (of a total of only 15% of the sequence observed) as chymotryptic fragments (Orlando et al., 1993) using an HPLC separation developed for mapping bacteriorhodopsin (Lerro et al., 1993). Using matrix assisted laser desorption ionization (MALDI) MS, other workers observed a total of 55% of the protein as tryptic fragments which included 17% of the sequence which had previously been unobserved. The method reported here permits observation of all of the previously unobserved parts of the sequence allowing mass spectrometric mapping of the protein in its entirety. The results show no indication of any previously undescribed post-translational modifications on the protein. Further, this approach allows complete mapping of the functionally significant majority of the protein sequence in a single experiment. The doubly glycosylated N-terminal fragment not observed with this method is thought not to be critical to receptor function and is easily seen by conventional methods using tryptic digestion (Duffin et al., 1993). This new methodology removes the major obstacle to further study of higher order structure of rhodopsin and its interactions with other signal transduction proteins via chemical modification and cross-linking approaches.

Results

Figure 1 shows the amino acid sequences for WT bR as well as two examples (E194C and M145C) of the cysteine mutants which were examined. Mutant E194C is typical of the other mutants studied in that it yields the same number of CNBr cleavage fragments as WT; mutant M145C yields one fewer fragments since one of the methionine cleavage sites is removed. In the analysis of WT bR using the described protocol, all of the predicted cyanogen cleavage fragments were observed in the HPLC chromatogram, and the identities of the fragments were confirmed by both MS and MS/MS (data not shown). Analysis of the bR cysteine mutants following pyridylethylation likewise showed all of the predicted cleavage fragments, the structures of which were confirmed by MS and MS/MS. Figure 2 shows a chromatogram of the separation of the cleavage fragments of one of these mutants, E194C, on a C4 column. The tetrapeptide fragment #4 elutes near or in the solvent injection peak on the C4 column and is not observed as a distinct peak in this separation. All of the other cleavage fragments are observed as well shaped chromatographic peaks. Unlabeled peaks in Figure 2 did not appear to be peptide material. Figure 3 shows the MS/MS spectrum of fragment #9 of E194C which contains the pyridylethylated cysteine residue. The fragments are labeled by the conventional nomenclature (Biemann, 1990) where b type and y type ions result from cleavage of amide bonds between the carbonyl group and amide nitrogen with the charge retained on the N-terminal and C-terminal fragments respectively. Since ESI gen-

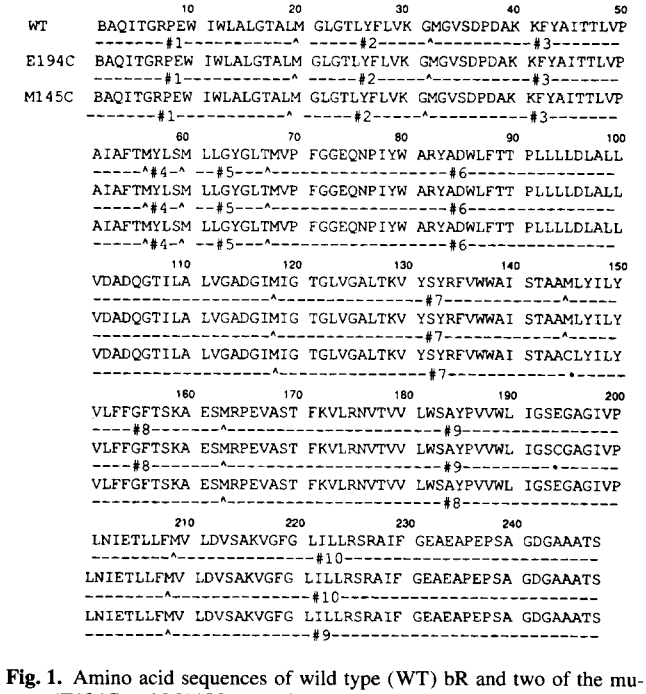

Fig. 1. Amino acid sequences of wild type (WT) bR and two of the mutants (E194C and M145C) examined (symbol B indicates pyroglutamate). The mutation sites are shown by closed circles. Cyanogen bromide cleavage sites are shown by the symbols (^). The cleavage fragments are numbered consecutively from the N-terminus (note that M145C has one less fragment due to mutation at a cleavage site).

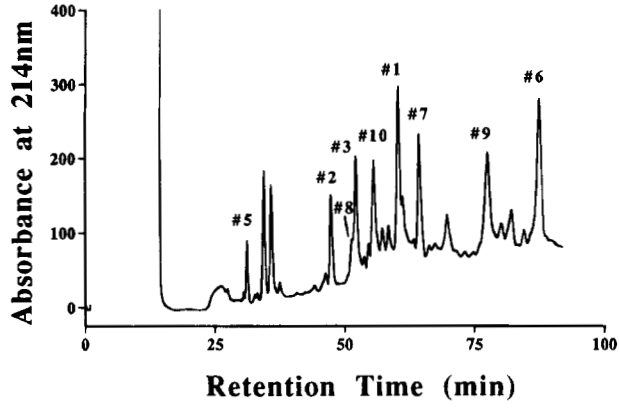

Fig. 2. Chromatogram of the HPLC separation of CNBr fragments of bacteriorhodopsin mutant E194C on a C4 column with detection at 214 nm. See text for column details and gradient conditions.

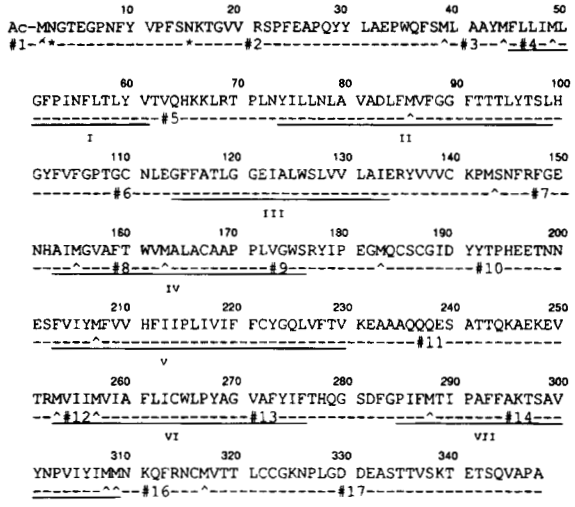

Fig. 4. Amino acid sequence of bovine rhodopsin. The symbols (^) indicate cyanogen bromide cleavage sites. The cleavage fragments are numbered consecutively from the N-terminus The asterisks (*) indicate sites of glycosylation. The solid lines with Roman numerals indicate the locations of the putative transmembrane helical regions of the protein (SWISS-PROT data bank (Bairoch & Apweiler, 1996), entry P02699).

erates ions of various charge states and the fragment ions of multiply charged precursor ions can have various charge states, the charges of the labeled fragment ions are also indicated.

Figure 4 shows the amino acid sequence of bovine rhodopsin and the predicted cyanogen bromide cleavage sites. Figure 5 shows chromatograms of the separation of the rhodopsin cleavage fragments on C4, C8, and C18 columns with the cleavage fragment peaks identified based upon MS data. Note again the well shaped chromatographic peaks suggesting little tendency toward aggregation or irreversible binding to the stationary phase. Table 1 shows the results of the mass spectrometric identifications of the separated peaks. These identities were further confirmed by MS/MS analysis (data not shown). Figure 6 shows the selected ion chromatograms for each of the cleavage fragments. These chromatograms were generated by plotting the signal for the indicated mass/charge ratios as a function of time. These mass/charge figures correspond to the most intense molecular ion charge state within the mass/charge range of the instrument (+1 for fragments 3, 4, 8, and 12; +2 for fragments 7, 9, 10, 13, 14, 16, and 17; +3 for fragment 5; and +4 for fragments 6 and 11). The retention times of the peaks on the selected ion chromatograms do not exactly coincide with those on the UV detector chromatogram (Fig. 5) because the two types of chromatograms were acquired with separate data systems. The most hydrophilic fragments (#3, #12, and #16) eluted in or near the injection solvent peak, and thus, were not observed on the UV detector trace using the C4 column. These peaks elute later using the alternative C8 or C18 columns, but with the disadvantage of longer retention of the larger peaks.

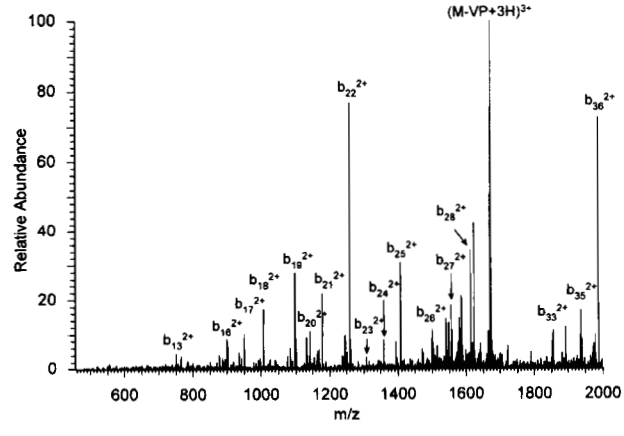

Fig. 3. MS/MS product ion spectrum from dissociation of the triply charged molecular ion of CNBr fragment #9 of bacteriorhodopsin mutant E194C. The fragment ion labeled $(M\text{-}VP + 3H)^{+3}$ is the product ion resulting from loss of vinylpyridine.

Discussion

Wild-type bacteriorhodopsin

The HPLC system described provided a reproducible separation of the peptide cleavage fragment mixtures. The quality of the separations was dependent upon use of the method for concentrating the samples on the column from a large injection volume, as well as prompt injection of the sample upon solubilization. The samples were loaded onto the 2.1 × 100 mm column in 5 mL of "weak" solvent identical to the initial mobile phase of the gradient. The HPLC of the separation of the wild-type bR cleavage peptides was monitored by UV absorption at 214 nm and by online ESI-MS and MS/MS. Peptides covering the entire protein sequence were identified by molecular weight and confirmed by MS/MS. Methionines were observed as homoserine lactone residues. Only 10% of the HPLC flow was directed to the mass spectrometer, allowing the remaining flow to be collected for other studies.

Bacteriorhodopsin mutants

Analysis of the cysteine mutants required alkylation of the cysteine residues. Conventional reduction/alkylation methods using dithio

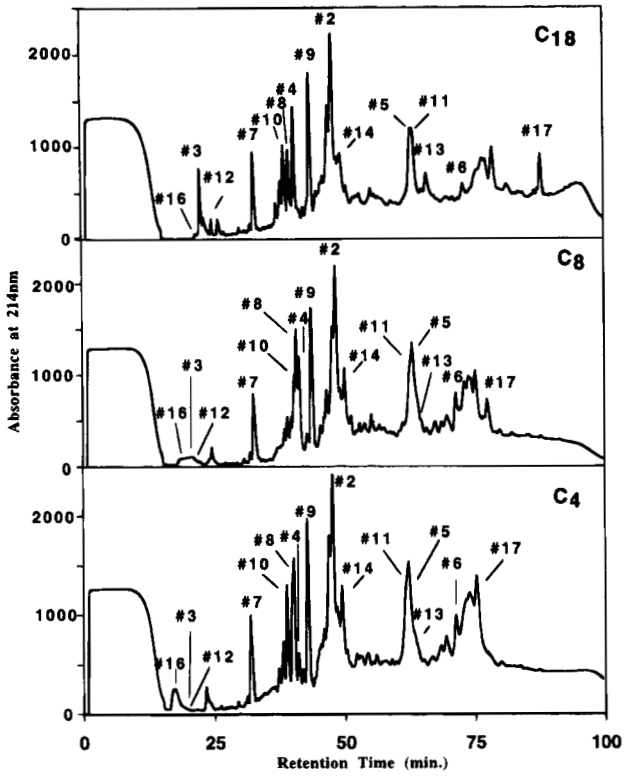

Fig. 5. Chromatograms of the HPLC separations of CNBr fragments of rhodopsin on C4, C8, and C18 columns with detection at 214 nm. See text for column details and gradient conditions.

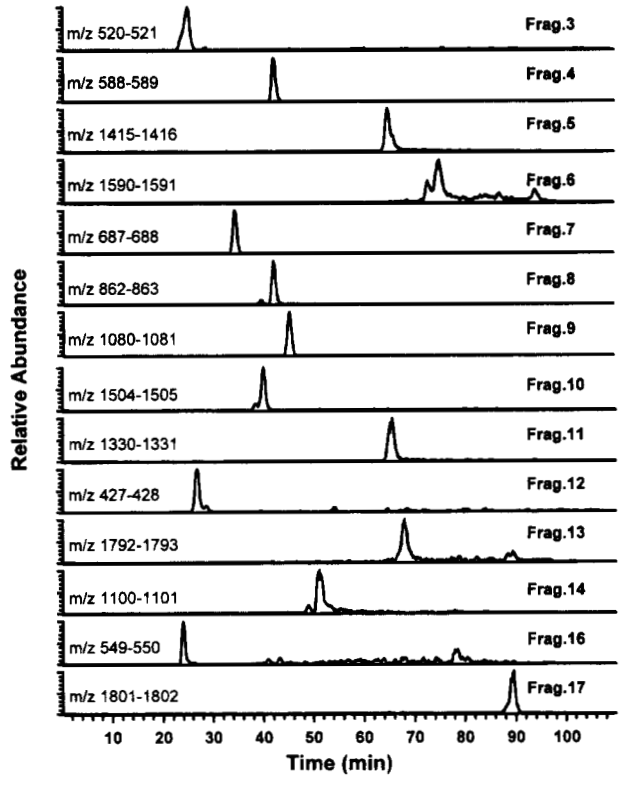

Fig. 6. Selected ion chromatograms from ESI-MS detection of peaks eluting from HPLC separation of CNBr fragments of rhodopsin on a C18 column.

Table 1. *Cyanogen bromide fragments of bovine rhodopsin*

Fragment	Residues	Mass expected as $(M + H)^{+1}$	Mass observed	Charge state observed	HPLC retention times (min) (C4)	(C8)	(C18)
1	1	144.1	N/D[a]	—	—	—	—
2[b]	2–39	6,501.8[c]	6,504.7	+1	47.6	48.3	47.8
3	40–44	520.3	520.1	+1	[d]	[d]	25.0
4	45–49	588.4	588.4	+1	42.0	42.1	42.2
5	50–86	4,244.1	4,243.9	+3	63.5	64.3	64.8
6	67–143	6,357.5	6,357.5	+4	72.2	72.6	75.0
7	144–155	1,374.7	1,374.7	+2	32.8	33.8	34.3
8	156–163	862.5	862.5	+1	41.0	41.8	42.1
9	164–183	2,160.6	2,160.4	+2	43.8	44.7	45.3
10[e]	184–207	3,007.3	3,007.0	+2	39.8	41.3	40.3
11	208–253	5,319.2	5,318.5	+3	62.5	63.5	65.6
12	254–257	427.3	427.1	+1	[d]	[d]	26.8
13	258–288	3,583.2	3,583.0	+2	64.3	65.1	68.0
14	289–308	2,199.6	2,199.6	+2	50.2	51.1	51.0
15	309	102.1	N/D	—	—	—	—
16	310–317	1,097.5	1,098.0	+1	18.8	21.3	24.0
17	318–348	3,602.3	3,602.2	+2	76.3	78.8	89.6

[a]N/D indicates fragments which were not detected.
[b]Observed by MALDI-TOF mass spectrometry.
[c]Most abundant glycoform.
[d]Not significantly retained; eluted in or near the injection solvent peak.
[e]Fragment 10 was also observed as +2 ion of the N-terminal pyroglutamate form in approximately equal abundance (retention times: C4, 40.8; C8, 42.6; C18, 41.0).

threitol and iodoacetic acid gave poor yields of the alkylated derivatives. We, therefore, turned to an alternative approach of performing the reduction and alkylation with organic soluble reagents prior to removal of the protein from the membrane. Tributylphosphine was chosen as the reductant because of its lipophilicity and because excess reductant would not react with the alkylating reagent, vinylpyridine, which was also selected because of its lipophilicity. Analysis of CNBr digests of the T121C, E194C, S35C, and M145C mutants allowed confirmation of the locations of the mutations. Each chromatogram includes a peak corresponding to the pyridylethylated cysteine peptide containing the mutation site with the molecular weight appropriate for the modified peptide. In the case of M145C, the replacement of the of the methionine residue removed a potential site for CNBr cleavage resulting in one fewer cleavage fragments. The MS/MS data for the pyridylethylated cysteine peptides further confirmed the identities of the mutated peptides. The MS/MS data for the pyridylethyated cysteine containing peptide (fragment #9) of E194C shown in Figure 3 are illustrative. The size of the peptide (5 kDa) is such that a complete series of sequence ions is not observed, however the identity of the peptide is clear from the series of thirteen contiguous b type ions (b_{16} – b_{28} as doubly charged ions) as well as additional individual and shorter sequences of b ions. The pair of b ions which specifically define the location of the pyridylethyl cysteine (b_{30} – b_{31}) are not prominent in this spectrum; a further cleavage of this large cyanogen bromide fragment would be required to locate this modified residue if the peptide were a complete unknown. For confirmation of a mutant of a known protein, however, the mapping data with confirmation of fragment identity from partial sequences would normally be sufficient to confirm the identity of the mutant.

Rhodopsin

Compared to bR and the cysteine mutants of bR, rhodopsin has the additional complications of ten cysteine residues present both as free sulfhydryls and as disulfide bonds, two glycosylated sites (asparagines 2 and 15), and two fatty acylation sites (palmitylations of cysteines 322 and 323 which are not cleaved by tributylphosphine). The analysis protocol developed using the single cysteine bR mutants proved equally applicable to Rh and allowed HPLC-ESI-MS observation of the entire sequence of the protein (except the two single amino acid fragments, methionine residues 1 and 309; and the glycosylated fragment #2 which was observed by MALDI-MS [data not shown]). The HPLC chromatograms of the Rh fragment separations shown in Figure 5 show well resolved peaks without the very broad envelope of absorption typically observed for membrane protein cleavage fragments. The two palmityl thioester substituents on cysteines 322 and 323 in fragment #17 survived the sample preparation, and the dipalmitylated peptide fragment gave a sharp chromatographic peak with successively longer retention going from C4 to C8 to C18 columns. The carbohydrate substituents at asparagines 2 and 15 also survived the sample preparation, and the doubly glycosylated fragment #2 was observed as a chromatographic peak which showed little difference in retention on the three columns.

The selected ion chromatograms for the molecular ions of the fragments shown in Figure 6 identify those peaks corresponding to cyanogen bromide fragments of Rh. The identities of the peaks were further confirmed by MS/MS data which showed at least partial sequences for each peptide peak. The retention of the two palmityl groups in fragment #17 was determined from the molecular weight and confirmation of the peak identity from partial MS/MS data, but sequence ions showing losses of the two palmitylcysteine residues were not observed. The glycosylated fragment #2 did not give definitive ESI data but was observed by MALDI analysis of the collected peak. In addition to the major species of mass corresponding to two hexasaccharide ($Man_3GlcNAc_3$) substituents, four additional minor peaks corresponding to successive additions of a hexose residue (+162 Da) were also found. These results are consistent with previous reports (Hargrave et al., 1984) identifying $Man_3GlcNAc_3$ as the major carbohydrate in bovine rhodopsin with minor components containing one and two additional mannose residues. The other peaks shown in the UV trace (Fig. 5) did not appear to be peptides; some of these peaks likely derive from residual lipids, but no effort was made to specifically identify them.

These results constitute the first complete sequence mapping of an integral membrane protein other than bR and confirm that there are no further posttranslational modifications in the previously unobserved regions of bovine Rh other than those previously reported (the possibility remains, however, that there could be heretofore undescribed acid-labile modifications which would be lost during the CNBr cleavage). The ability to observe the entire sequence of Rh in a single experiment now paves the way for application of chemical modification and crosslinking experiments to probe the higher order structures and protein-protein interactions involved in the function of this prototypical G-protein coupled receptor.

Conclusions

With the exception of some single amino acid fragments and the glycosylated N-terminal fragment of rhodopsin (which is observed by MALDI-MS), this approach permits analysis of the entire sequence of a membrane protein in a single HPLC-ESI-MS run. The observation of greater than 99% of the rhodopsin structure is more than by any other method reported to date and, to our knowledge, constitutes the first complete mapping of an integral membrane protein other than bR.

This methodology will enable pursuit of chemical modification and crosslinking studies designed to probe the three dimensional structure and functional conformational changes in the light transduction protein bR and the prototypical G-protein coupled receptor Rh. The approach should also be applicable to the analysis of other integral membrane proteins.

Materials and methods

Bacteriorhododpsins

Wild-type (WT) bR and mutant membranes were isolated and purified from cultured Halobacterium salinarium using standard procedures (Oesterhelt & Stoekenius, 1974). Site directed mutagenesis of bR T121C (cysteine replacement of the WT threonine at position 121), E194C, S35C, and M145C and the transformation of *Halobacterium salinarium* strain IV-8 was as previously described (Balashov et al., 1993; Balashov et al., 1997).

Harvested wild type or mutant bR membranes in 4 M NaCl (equivalent to 1 mg of protein as estimated by the absorbance at 570 nm for WT bR or the λ_{max} for the mutants) were centrifuged

at 88,800 g_{av} (50,000 rpm on a Beckman TL-100 ultracentrifuge using a TLA100.3 rotor) at 4 °C. The supernatant was removed by pipette and the pellet resuspended in 1 mL deionized water. The suspension was centrifuged at 88,800 g_{av} for 15 min, and the supernatant was removed. The wash was repeated to yield a purple pellet which was suspended in 300 μL 1.5 M TRIS pH 8.9 and combined with 300 μL 1-propanol. The vial was flushed with Ar, and 20 μL tributylphosphine and 20 μL 4-vinylpyridine were added by syringe. The reaction mixture was sonicated and vortexed intermittently for 3 h and then centrifuged at 88,800 g_{av} for 15 min. The pellet was washed twice with water by removing the supernatant resuspending the pellet in 1 mL water, and centrifuging at 88,800 g_{av} for 15 min.

Two methods were used for delipidating the pellet. In one method, the pellet was resuspended in 300 μL formic acid and 700 μL ethanol were added. This solution was loaded onto a Sephadex LH-20 column, and the derivatized protein was eluted with 70:30 (v/v) ethanol/formic acid. Collected fractions were screened for protein content by UV absorbance at 280 nm. Protein containing fractions were pooled and dried either by vacuum centrifugation or under a stream of nitrogen. In the second method, the pellet was delipidated by precipitation of the protein in 95% ethanol (2 mg pellet in 1 mL 95% ethanol at −18 °C overnight). The precipitated protein was separated by centrifugation as above then washed twice with 1 mL cold acetone and twice with 1 mL water. The ethanol precipitation method was more convenient than the column method and was found to be adequate for these analyses, but the column method would be expected to yield some additional purification of the protein (which would not necessarily be desirable in a chemical modification experiment).

The delipidated protein was dissolved in TFA and the solution adjusted to 50% TFA and 20% acetonitrile by dilution with acetonitrile and water. A 5M solution of CNBr in acetonitrile (ca. 2 mg per mg protein or about 400 moles per mole of methionine) was then added. The vial was flushed with nitrogen, sealed, and the contents allowed to react 18 h at room temp in the dark. The solution was then diluted fivefold with water and dried in a vacuum centrifuge.

The cleavage peptide mixture would not directly dissolve in the initial HPLC mobile phase. Solution was achieved by first dissolving the dried residue in 10 μL of TFA. Appropriate amounts of isopropanol, acetonitrile, and then water were added to yield a final solution equivalent to the initial gradient mobile phase (98% aqueous/2% organic). Samples (~0.25 mg total peptides in 5 mL of the initial mobile phase) were loaded onto a C4 (or C8) reversed phase HPLC column (Brownlee Aquapore, 2.1 × 100 mm) at 400 μL/min. The cleavage peptides were separated by HPLC using a gradient of 0.05% aqueous TFA (solvent A) and 2:1 (v/v) isopropanol/acetonitrile 0.05% TFA (solvent B) at a flow rate of 400 μL/min. The mobile phase composition was held at 2% B for 15 min (the injector loop was switched out of the solvent stream at 13 min) and then ramped from 2–15% B in 15 min, 15– 65% B in 75 min, and 65–98% B in 10 min.

Rhodopsin

Rod outer segments isolated from bovine retinae (McDowell & Kühn, 1977; equivalent to ca. 0.25 mg Rh) were reduced with tributylphosphine and alkylated with 4-vinylpyridine in 1:1 (v/v) 1.5 M Tris pH 8.9/1-propanol. The Rh was then delipidated by precipitation in 95% ethanol or by chromatography on Sephadex LH-20 as described above for bR. In the latter case, collected fractions were screened for protein content by UV absorbance at 280 nm and pooled. The intact derivatized protein in the fractions could also be detected by ESI-MS of the ethanol-formic acid fractions. The delipidated protein was cleaved with CNBr in 70% TFA under Ar in the dark for 18 h. After evaporating the cleavage solution to dryness, the fragment mixture was dissolved in TFA and diluted to yield a 5 mL solution equivalent to the initial gradient mobile phase (98% A, 2% B), where solvent A is 0.05% aqueous TFA and solvent B is 2:1 isopropanol:acetonitrile containing 0.05% TFA. The 5 mL sample was loaded onto a 2.1 mm × 100 mm C4, C8, or C18 Aquapore column (400 μL/min of 2% B for 14 min). The injector valve was returned to load position and the flow reduced to 200 μL/min. After 1 min at 2% B, the peptides were eluted with a gradient of 2–60% B in 60 min and 60–98% B in 20 min at a flow of 200 μL/min.

Mass spectrometry

The column effluent from the HPLC separation was split and 10% of the flow (40 μL/min for bR and mutants; 20 μL/min for Rh) directed into the ESI source of a Finnigan LCQ ion trap mass spectrometer. Data were acquired using LCQ version 1.1 beta 5 software. Instrument parameters were as follows: ESI needle voltage, 4.5 kV; ESI capillary temp, 200 °C; ion energy, 45%; isolation window, 2 amu; scan range 400–2,000 amu. MS data were acquired with repetitive scanning with MS/MS data automatically acquired on the most intense precursor ion in each MS spectrum.

The glycosylated fragment was observed on a Perseptive Voyager DE MALDI-TOF instrument with a 337 nm nitrogen laser using alpha-cyano-4-hydroxycinnamic acid as matrix. The dried HPLC fractions were each dissolved in 10 μL 85% acetic acid with vortex mixing. A 0.3 μL aliquot of the solution was spotted on the MALDI plate followed by 0.9 μL of 50 mM alpha-cyano-4-hydroxycinnamic acid in 70% acetonitrile. The solutions were mixed and allowed to dry. MALDI MS spectra were acquired using external standards.

Reference peptides

Reference cleavage fragments were synthesized (Knapp et al., 1993) using Fmoc chemistry as methionine peptides with one additional C-terminal residue and then cleaved with CNBr in 70% TFA.

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