Electrospray-ionization mass spectrometry of intact intrinsic membrane proteins

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Abstract
Membrane proteins drive and mediate many essential cellular processes making them a vital section of the proteome. However, the amphipathic nature of these molecules ensures their detailed structural analysis remains challenging. A versatile procedure for effective electrospray-ionization mass spectrometry (ESI-MS) of intact intrinsic membrane proteins purified using reverse-phase chromatography in aqueous formic acid/isopropanol is presented. The spectra of four examples, bacteriorhodopsin and its apoprotein from Halobacterium and the D1 and D2 reaction-center subunits from spinach thylakoids, achieve mass measurements that are within 0.01% of calculated theoretical values. All of the spectra reveal lesser quantities of other molecular species that can usually be equated with covalently modified subpopulations of these proteins. Our analysis of bovine rhodopsin, the first ESI-MS study of a G-protein coupled receptor, yielded a complex spectrum indicative of extensive molecular heterogeneity. The range of masses measured for the native molecule agrees well with the range calculated based upon variable glycosylation and reveals further heterogeneity arising from other covalent modifications. The technique described represents the most precise way to catalogue membrane proteins and their post-translational modifications. Resolution of the components of protein complexes provides insights into native protein/protein interactions. The apparent retention of structure by bacteriorhodopsin during the analysis raises the potential of obtaining tertiary structure information using more developed ESI-MS experiments.

Keywords: electrospray-ionization; G-protein coupled receptor; intrinsic membrane protein; mass spectrometry; proteome; reverse-phase HPLC; post-translational modifications

Mass spectrometry (MS) has a vital role to play in the structural analysis of the collection of proteins that constitutes an organism's proteome (Landers, 1996). The ability to monitor subtle covalent changes as a function of regulatory and developmental plasticity makes MS an indispensable analytical technique for both biochemist and physiologist (Chait & Kent, 1992; Burlingame et al., 1996; Roepstorff, 1997). Since the discovery of electrospray-ionization (ESI; Fenn et al., 1989) and matrix-assisted laser desorption ionization (MALDI; Karas & Hillenkamp, 1988), analysis of a variety of larger biological molecules, including globular proteins up to and exceeding 100 kDa has been possible. The intrinsic membrane proteins (IMPs) comprise a very significant portion of any proteome, and many are critical to the health and success of the organism. Recent Nobel Prize-winning studies on photosynthetic energy transduction (Deisenhofer & Michel, 1989), chemiosmotic ATP generation (Abrahams et al., 1994; Boyer, 1997) and transmembrane signal transduction (Gilman, 1987) all emphasize the importance of structural analysis of these proteins. The current investigation was undertaken with the goal of extending the benefits of ESI-MS to higher molecular weight IMPs, especially with respect to the covalent modifications not immediately revealed by knowledge of the gene sequence.

The ideal analysis of any protein includes a spectrum of the intact molecule to define the native covalent state and its heterogeneity. MALDI-MS can be used for such measurements of intact IMPs and spectra of both bacteriorhodopsin/bacteriopsin (BR/BO) and rhodopsin have been recorded (Schey et al., 1993; Rosinke et al., 1995). However, despite recent improvements in overall mass accuracy and measurement to measurement reproducibility, the ability to detect low abundance molecules with small mass differences compared to the main species (resolution) is superior when ESI-MS is used (Green, 1992); furthermore, the latter tech-
nique may be directly interfaced with liquid chromatography (LC/MS; Covey et al., 1988). Consequently, we are developing techniques for ESI-MS of IMPs. Electrospray-ionization of BO has been achieved by dissolving the protein in hexafluoroisopropanol and injecting it into chloroform/methanol/water (CMW; 2:5:2) as carrier solvent, although the mass reported (26,802) was not within 0.01% of the calculated value (26,783; Schindler et al., 1993). The CMW carrier solvent has proved promising and was used for analysis of IMPs up to 39 kDa from the inner mitochondrial membrane (Pearlman & Walker, 1996) as well as a more recent analysis of BO that did achieve 0.01% accuracy (Hufnagel et al., 1996). Mannose transporter subunits up to 35 kDa were analyzed after elution of a C8 reverse-phase column with undiluted formic acid, and 0.01% accuracy was achieved for BO when it was injected into undiluted formic acid as carrier solvent with the interface temperature raised to 100°C (Schaller et al., 1997). In another study, BO was eluted from an SDS gel and dissolved in formic acid/methanol/water prior to ESI-MS (Le Maire et al., 1993) achieving a mass accuracy close to 0.01%. Although these studies clearly demonstrate the feasibility of using electrospray ionization for IMPs, none have presented a reliable and versatile means of routinely recording data comparable in quality to that obtained for water-soluble proteins. A general method that copes with the typical problems associated with IMPs from a variety of sources, namely sample heterogeneity and small molecule contamination, is needed. Reverse-phase chromatography of membrane proteins (Heukeshoven & Dernick, 1982; Whitelegg et al., 1992) was adapted for the separation of simple and complex mixtures of IMPs to liquid chromatography–mass spectrometry (LC/MS) in an attempt to satisfy this requirement.

Previously, we separated hydrophobic, trypsin-generated IMP fragments derived from plant thylakoid PS2 by reverse-phase chromatography on a poly(styrene-divinylbenzene) support in aqueous formic acid/isopropanol, and obtained primary sequence data from the resolved fractions (Whitelegg et al., 1992). By applying this same chromatographic technique to intact IMPs, we have been able to ionize and record mass spectra of IMPs from the membranes of bacteria, plants, and animals with masses up to 56 kDa. In this paper we present as examples the spectra of four well-known IMPs with masses up to 42 kDa, whose native structures play a central role in light-driven transmembrane proton transport. The spectra we have five or seven transmembrane alpha helices. The spectra presented (Fig. 1B,D) although some batches of the reference IMPs to liquid chromatography–mass spectrometry (LC/MS) in an attempt to satisfy this requirement. The chromatographic separation of simple and complex mixtures of IMPs to liquid chromatography–mass spectrometry (LC/MS) in an attempt to satisfy this requirement.

Results and discussion

Bacteriorhodopsin (BR) is isolated from the purple membranes of *Halobacterium* carrying a Schiff-base linked retinal cofactor that plays a central role in light-driven transmembrane proton translocation by the protein (Oesterhelt & Stoeckenius, 1974). Our LC/MS analysis of an acetone precipitated commercial preparation of BR is shown in Figure 1. BR (47 min) was partially resolved from its apoprotein (BO; 45.1 min), and appears as a shoulder on the 280 nm elution profile (Fig. 1A). Integration of all scans from 44–52 min revealed a number of species of different mass (Fig. 1B) including both apo and holoprotein. The measured mass of BO (26,784.2; Fig. 1C) was within 0.01% of the calculated value (26,783.64) in agreement with the measurements of others (Schey et al., 1993; Hufnagel et al., 1996; Schaller et al., 1997). Since the experiment revealed a population that had apparently retained the chromophore (Fig. 1B; 27,051.2), the sample preparation procedure was modified to favor this species. The mass spectrum collected in a subsequent LC/MS experiment (Fig. 1D) revealed a species of measured mass 27,052.0, which falls within 0.01% of the value predicted for the intact BR molecule including a Schiff-base linked retinal molecule (27,050.06; see Fig. 1B also). Only the N-retinyl protein (produced by chemical reduction with borohydride, for example) could fall within the 0.01% window of mass accuracy, but is an unlikely structural candidate considering that our preparation was not reduced. Moreover, the absorption spectrum of the solubilized BR as it eluted from the column (λmax = 430 nm; unpubl. data) is consistent with a reversibly semi-denatured form of the holoprotein induced by organic solvent addition (Oesterhelt et al., 1973). Indeed, it was previously shown that dissolving BR in formic acid in the presence of organic solvent does not fully denature the protein (Huang et al., 1982) and other general studies (Heukeshoven & Dernick, 1982) indicated that a range of proteins apparently retained detectable higher order structure in formic acid–2-propanol provided they were not treated with SDS to induce more complete denaturation. Thus, it seems possible that, using the sample preparation procedure and LC/MS conditions described here, the protein retains sufficient structure to stabilize the Schiff linkage enabling retention of the chromophore, thereby allowing the ESI spectrum of the holoprotein to be recorded. The assertion that BR and other IMPs retain some elements of structure under these seemingly harsh conditions is being tested.

A number of minor components were revealed in the analysis of BR (Fig. 1B,D). Some species can be equated with incompletely processed forms of the protein (Hufnagel et al., 1996) but others, such as 26,590.4 and 27,655.8 for example (Fig. 1B), are hard to reconcile with the sequence of the BR precursor. It was noted that the 27,655.8 Da molecule was more highly retained than BR eluting at around 51 min. BR/BO has reported to be especially sensitive to formation of multiple Na+ adducts (Hufnagel et al., 1996). Clearly, only a minor proportion of the ions carry Na+ in the spectra presented (Fig. 1B,D) although some batches of the commercial preparation (84H68881) have been more susceptible to the formation of Na+ adducts.

To test the efficiency of the chromatographic separation, more heterogeneous samples were analyzed. The thylakoid-membrane pigment-protein complex, photosystem 2 (PS2), was chosen because earlier experiments had highlighted the amenability of this material to analysis by reverse-phase chromatography (Whitelegg et al., 1992). The complex is largely composed of a heterodimer of two large IMPs, D1 and D2, which constitute the herbicide-binding site of PS2 and, by homology to the published structure of the purple bacterial reaction center, each have five transmembrane α-helices (Trebst, 1987). As shown in Figure 2A, many of the PS2 proteins were effectively separated under our chromatographic conditions permitting successful LC/MS. The calculated and measured masses of the five reaction-center polypeptides are summarized in Table 1. D1 eluted as a major molecular species of measured mass 38,022.1 (Fig. 2B). This value is within 0.01% of the calculated value (38,020.6) for the 343 amino-acid D1 polypeptide that results from the spinach psbA gene product (Zurawski et al., 1982) after removal of initiating Met1 and N-acetylation (Michel et al., 1988), as well as C-terminal proteolytic processing after Ala344 (Takahashi et al., 1990). The D2 protein eluted later than D1; its mass was measured to be 39,418.8 (Fig. 2C) within 0.01% of the value calculated (39,418.3) after translation of spinach psbD,
ESI-MS analysis of bacteriorhodopsin (BR) and its apoprotein (BO). A: The UV elution profile (A280 nm) of BR/BO (200 µg) during reverse-phase HPLC in aqueous formic acid/isopropanol is shown. The holoprotein was more highly retained (47.0 min) than the apoprotein (45.1 min). After passing through the UV detector, column eluent was directed to the mass spectrometer (LC/MS). B: Molecular weight spectrum reconstructed from all scans in the range 44–52 min. Although M + nH⁺ ions were most abundant, Na⁺ occasionally replaced H⁺ leading to the observed shoulders (+Na). Where the original spectrum allowed estimates from five or more individual ions, the mass was given to six significant figures. C: Mass spectrum of bacterioopsin. The average of several scans recorded during elution of the 45.1 min peak (BO) is shown. D: Mass spectrum of bacteriorhodopsin. Based on the experiment described (A), the sample preparation procedure was modified (see Materials and methods) to maximize retention of the retinal chromophore on the protein. After LC/MS as in A, the apoprotein (BO) was barely detectable in the molecular weight reconstruction of the BR spectrum (inset). Measured mass was computed from the mass spectrum shown using as many different estimates as the spectrum allowed. Computer reconstructions of molecular weight spectra are shown inset for each spectrum (C and D).

N-terminal methionine removal and N-acetylation (Michel et al., 1988). The mass spectra reveal other molecular species, notably an entity of 38,100.4 (Fig. 2B), which is within 0.01% of the mass calculated for the phosphorylated D1 protein (38,100.6). Phosphorylation of D1 in vivo has been predicted from labeling studies and is apparently more prominent after photoinhibitory high-light stress (Whitelegge, 1989; Callahan et al., 1990; Whitelegge, 1996). We may now use ESI-MS to assess quantitatively the relationship
Fig. 2. ESI-MS analysis of polypeptides of the spinach photosystem 2 (PS2) membrane protein complex. A: The UV elution profile (A280 nm) of PS2 polypeptides (200 μg total protein) during reverse-phase HPLC in aqueous formic acid/isopropanol is shown. Reaction-center polypeptides are labeled along with their retention times (min). The peak (c) contains three polypeptides of the related light-harvesting complex (LHC) that were also revealed by this analysis. Mass spectra were recorded continuously after material eluting from the column had passed through the UV detector (LC/MS). B: Mass spectrum of D1. C: Mass spectrum of D2. Under the conditions used, the low molecular weight PS2-I subunit eluted with similar retention time to D2 contributing a strong ion (M + 3H⁺) at 1,399.5 (*), which is off-scale (100% relative intensity) in the figure. Molecular weight reconstructions of both D1 and D2 (11 and 9 strongest ions, respectively) are shown inset.
Table 1. Masses of spinach photosystem 2 reaction-center polypeptides

<table>
<thead>
<tr>
<th>Polypeptide&lt;sup&gt;a&lt;/sup&gt; (gene)</th>
<th>Molecular mass (measured)</th>
<th>Molecular mass (calculated)</th>
<th>Molecular mass (difference)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D1 (psbA)</td>
<td>38,022.1</td>
<td>38,020.6</td>
<td>1.5</td>
</tr>
<tr>
<td>D2 (psbD)</td>
<td>39,418.8</td>
<td>39,418.3</td>
<td>0.5</td>
</tr>
<tr>
<td>PS2-E (psbE)</td>
<td>9,254.5</td>
<td>9,255.4</td>
<td>-0.9</td>
</tr>
<tr>
<td>PS2-F (psbF)</td>
<td>4,409.8</td>
<td>4,409.2</td>
<td>0.6</td>
</tr>
<tr>
<td>PS2-I (psbI)</td>
<td>4,195.1</td>
<td>4,195.9</td>
<td>-0.8</td>
</tr>
</tbody>
</table>

*The masses measured using ESI-MS were compared to masses calculated for the five spinach reaction-center polypeptides.

<sup>a</sup>D1 was translated from spinach chloroplast psbA (Zurawski et al., 1982) and processed at the N-terminus by Met1 removal and N-acetylation (Michel et al., 1988) and at the C-terminus by cleavage after Ala344 (Takahashi et al., 1990). D2 was translated from spinach chloroplast psbD (Holschuh et al., 1984) and processed at the N-terminus by Met1 removal and N-acetylation (Michel et al., 1988). PS2-E was translated from spinach chloroplast psbE mRNA (Booker et al., 1993), and the N-terminus was processed by Met1 removal and N-acetylation. PS2-I mass was predicted based on translation of tobacco psbF (Shinozaki et al., 1986). The first 20 amino-acid residues of the spinach sequence have been confirmed by sequencing (Ikemura et al., 1985) as being identical to the tobacco product; at the N-terminus Met1 is intact and N-formylated (Sharma et al., 1997a). Average masses are based on natural isotopic abundance. Single representative measurements are presented.

between phosphorylation and the covalent photodamage to PS2 proteins that accompanies their function in vivo.<sup>5</sup>

Due to the intense interest currently being shown in membrane receptors, the ESI mass spectrum of a mammalian G-protein coupled receptor (GPCR) was recorded. The classical model of the GPCR is the photoreceptor rhodopsin (Khonara, 1993), an abundant component of retinal rod outer segment (ROS) membranes. An aliquot of these membranes isolated from bovine retinas was used to record the ESI spectrum of intact rhodopsin (Fig. 3). As shown in Figure 3A, the mass spectrum of rhodopsin is highly complex with each charge state containing an extensive family of molecular ions in contrast to the previous examples (Figs. 1, 2). The molecular weight profile (Fig. 3B) illustrates the extent of heterogeneity of the protein after reverse-phase chromatography. The observed distribution of masses agrees well with that calculated for the five common glycoforms of bovine rhodopsin (Fuikuda et al., 1979; Liang et al., 1979; see Fig. 3B). However, the extensive heterogeneity displayed in the mass spectrum reveals the presence of other modifications overlying the variable glycosylation; phosphorylation, oxidation, and formylation being probable can-

<sup>5</sup>Note added in proof. ESI mass spectra of pea D1 and D2 have recently been presented (Sharma et al., 1997b). The masses of D1 and D2 were measured to be 38,040.9 and 39,456.1 against theoretical values reported to be 38,033.6 and 39,463.5, respectively. Neither of these measurements achieve the 0.01% standard of mass accuracy. We have also measured the masses of pea D1 and D2 using the techniques described herein (Whitelegge, Gomez, Gundersen, Faulk, unpubl. data). Both of our measurements of the mass of pea D1 (38,036.4; 38,037.4) are within 0.01% of the value calculated after translation of pea psbA (Oishi et al., 1984) and processing, in agreement with Sharma et al. However, the masses we have measured for pea D2 (39,441.9; 39,445.3) are lower than that reported by Sharma et al. When the pea D2 sequence is calculated from the published pea psbD sequence (Bookjans et al., 1986) and processed as for the spinach polypeptide, the mass is calculated to be 39,479.5, or 39,437.5 if the N-terminus is not acetylated. Clearly the masses we have measured for pea D2 are closer to that calculated for the nonacylated form. Whether this discrepancy is due to nonacylation, some other covalent modification, or a sequencing error is under investigation.
dicates. The larger (>42,500) components in the molecular weight profile (Fig. 3B) are consistent with the presence of minor populations of rhodopsin with larger glycosyl constituents as others have reported for the bovine molecule (Barnidge et al., 1997), and was observed in the case of human rhodopsin (Fujita et al., 1994). ESI-MS is ideal for checking post-translational modifications of recombinant rhodopsin, and it is anticipated that other GPCRs and related polyhelix IMPs can be analyzed using the approach described here. We are currently attempting to enhance chromatographic resolution in order to improve our ability to define the range of native rhodopsin heterogeneity.

Our procedure yields significant improvements over previous studies. Through the development of appropriate solubilization conditions for intact IMPs, mass accuracy within 0.01% of the calculated values is routinely obtained and comparable to values obtained for “water-soluble” globular proteins on a similar instrument (Green & Oliver, 1991). Moreover, use of liquid chromatography provides the added advantage that samples need not be initially pure circumventing the usual contamination of membrane proteins with detergents and lipids. The combination of a poly(styrene-divinylbenzene) stationary phase with an aqueous formic acid/isopropanol mobile phase provided very robust separation of IMPs, from a variety of sources, that enabled resolution of the different components of a membrane protein complex (Fig. 2) and partial separation of BR holoprotein from its apoprotein (Fig. 1). The only disadvantage of this protocol involves chromatography in formic acid potentially leading to protein formylation during the separation procedure. We have yet to find a reliable method of preventing or reversing this modification, but by keeping run times brief (<1 h), formylation is minimal (Figs. 1, 2). Undoubtedly, some applications will require that formylation be avoided, and we are currently investigating solvent systems that are less reactive toward the sample but still capable of eluting IMPs efficiently.

ESI-MS analysis of intact IMPs can potentially provide numerous biological insights, which can be conveniently discussed in the context of the post-genome era (Landers, 1996; S.A. Carr, pers. comm.).

Posttranslational modification state of all proteins

Due to the difficulty in obtaining a complete peptide map, the spectrum of the intact parent is much more useful for viewing the overall covalent state of the molecule and its heterogeneity. The picture obtained may then be stored within the proteome database to provide a reference against which to compare spectra obtained under different biochemical or physiological conditions. It is unusual that the measured mass of the molecule agrees with that predicted from genome data due to post-translational or other modifications. Under these circumstances, further biochemical data, mass-based peptide mapping for example, would then be necessary to confirm the identity of the molecule and localize any modification sites. Since the eluent line between HPLC and mass spectrometer is routinely split, appropriate fractions are available for such analysis if required. Indeed, mass-based cyanogen bromide mapping (Whitelegge et al., 1997) was used to confirm the identities of all the proteins whose spectra we have presented here (unpubl. data).

Systematic catalogs of protein interactions

Mass spectrometry is well placed to take a central role in the mapping of protein/protein interactions. The analysis of the PS2 reaction center complex (Fig. 2) provides a good example. The complex was isolated by ion-exchange chromatography in the presence of detergents prior to our reverse-phase LC/MS analysis. The elution of the five reaction-center proteins as a complex during ion-exchange is indicative of the specific noncovalent associations that hold the complex together in vivo. The technique we describe will also potentially reveal nonstoichiometric relationships. The 27 kDa component that we detected in the commercial BR preparation is probably less than 1% by weight of the main component but was nevertheless resolved by both chromatographic behavior and mass.

Identification of all basic protein shapes

Although mass spectrometry does not directly provide high-resolution tertiary structure information, it is still possible to derive important insights from hydrogen-deuterium exchange (Zhang & Smith, 1993; Robinson et al., 1994) or limited proteolysis experiments (Seielstad et al., 1995), for example. Indeed, if the retention of structure eluded to in the case of BR (Fig. 1) is more generally observed, it may be possible to use ESI-MS measurements, such as hydrogen-deuterium exchange kinetics, to classify uncharacterized IMPs into existing structural categories without the need for classical high-resolution studies. Additionally, the heterogeneity read out provided by an ESI spectrum is essential to structural biologists on a day to day basis. For example, the molecular heterogeneity displayed by D1 (Fig. 2) provides a rational for the failure of PS2 to produce highly-ordered crystals. The ability to monitor covalent IMP structure by ESI-MS will surely accelerate the quest to solve more of their structures at atomic resolution.

Materials and methods

Sample preparation

BR (Halobacteria halobium) was obtained from Sigma (B-3636; batch 116H6834). Spinach (Spinacea oleracea) was obtained fresh from a local market (Stater Brothers) for photosystem 2 (PS2) reaction center preparation (Whitelegge et al., 1992). ROS membranes were prepared from bovine retinas (Pel Freeze) (Papamaster & Dreyer, 1974). All other chemicals were from Fischer unless otherwise stated. Samples were routinely prepared after acetone precipitation (Bouchon et al., 1993; Hufnagel et al., 1996). BR and ROS were suspended in 1 mM CHAPS, 10 mM Tris-HCl, pH 7.2. PS2 was suspended in 0.05% (w/v) Triton X100, 25 mM Tris-HCl, pH 7.2. Acetone (−20°C) was added to 80% (v/v) and the samples incubated at −20°C for 10 min prior to recovery of precipitated protein by centrifugation (1,500 × g; 5 min). Final resuspension of BR was in formic acid/2-propanol (1:1); PS2 polypeptides in 60% formic acid; ROS polypeptides in formic acid/water/isopropanol (50:25:25). Samples (100–200 μL) were cleared by centrifugation (10,000 × g) for 1 min prior to reverse-phase HPLC. To increase the yield of BR relative to BO the acetone precipitation step was eliminated; 50 μg BR was suspended in 30 μL 10 mM Tris, pH 7.2, 1 mM 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate (CHAPS) prior to addition of 60 μL undiluted formic acid (90%) and 40 μL isopropanol. Centrifugation of the sample (10,000 × g; 1 min) yielded a small pellet of insoluble material and a clear orange supernatant, which was subjected to HPLC.
ESI-MS of intact intrinsic membrane proteins

Reverse-phase chromatography

Reverse-phase chromatography was performed as previously described (Whitelegge et al., 1992) using a mobile phase modified from a previously published protocol (Gerber et al., 1979). The poly(styrene-divinylbenzene) copolymer (Polymer Labs. PLRP-S; 2.1 x 150 mm; 5 μm x 300 Å) stationary phase was eluted at a flow rate of 100 μL/min (40°C). Buffer A was 60% formic acid in water, buffer B was 2-propanol. The column was equilibrated in 95% A, 5% B, prior to linear gradient elution from 5% B, 5 min after injection to 100% B, 55 min after injection.

Electrospray-ionization mass spectrometry

Mass spectra were recorded on a Perkin Elmer Sciex (Thornhill, Canada) API III triple quadrupole mass spectrometer fitted with an Ionspray™ source. The mass spectrometer was tuned and calibrated before each LC/MS experiment by flow injection of 10 μL/min of a mixture of polypropylene glycol (PPG) 425, 1,000, and 2,000 (3.3 x 10⁻⁵, 1 x 10⁻⁴, and 2 x 10⁻⁴ M, respectively) in water/methanol (1/1, v/v) containing 2 mM ammonium formate and 0.1% acetic acid. Calibration across the m/z range 10–2,400 was effected by multiple ion monitoring of eight PPG solution signals (typically the singly charged ions at m/z 58.99, 326.25, 906.67, 1,254.92, 1,545.13, 1,863.34, and 2,010.47, and the doubly charged ion at m/z 520.4). The ion spray voltage was operated at 4.5 kV using hydrocarbon-depleted air for the spray nebulization (“zero” grade air, 40 psi, 0.6 L/min; Zero Air Generator, Peak Scientific, Chicago, IL), and spectra were produced with a curtain gas (0.6 L/min) produced from the vapors of liquid nitrogen. Spectra were obtained at instrument conditions sufficient to resolve the isotopes of the PPG/NH₄⁺ singly charged ion at m/z 906 with 40% valley. The instrument was scanned from m/z 600 to 2,200 with a step size of 0.3 and an orifice voltage (OR) of 55 V. The computations of protein molecular weight and the molecular weight reconstructions were performed using MacSpec 3.3 software (Sciex). Calculated molecular weights were generated using PeptideMap 2.1 (Sciex).

Acknowledgments

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