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Mass Spectrometry of Membrane Proteins: A Focus on Aquaporins

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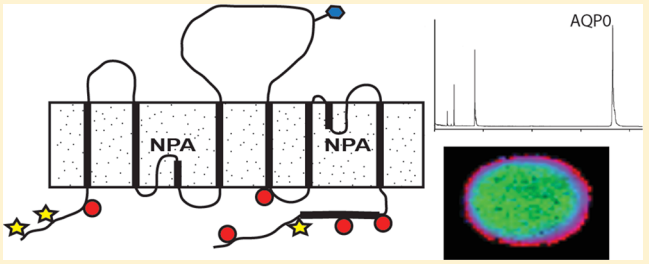
ABSTRACT: Membrane proteins are abundant, critically important biomolecules that conduct essential functions in all cells and are the targets of a significant number of therapeutic drugs. However, the analysis of their expression, modification, protein−protein interactions, and structure by mass spectrometry has lagged behind similar studies of soluble proteins. Here we review the limitations to analysis of integral membrane and membrane-associated proteins and highlight advances in sample preparation and mass spectrometry methods that have led to the successful analysis of this protein class. Advances in the analysis of membrane protein posttranslational modification, protein−protein interaction, protein structure, and tissue distributions by imaging mass spectrometry are discussed. Furthermore, we focus our discussion on the application of mass spectrometry for the analysis of aquaporins as a prototypical integral membrane protein and how advances in analytical methods have revealed new biological insights into the structure and function of this family of proteins.



Membrane proteins are encoded by 20−40% of the genome[1] and represent up to 70% of therapeutic targets for the most highly prescribed drugs.[2] This general class of proteins includes transporters, channels, receptors, recognition molecules, and adhesion molecules among others. Membrane proteins, including integral membrane and membrane-associated proteins, are critically important molecules for cell survival and maintenance of cell homeostasis because they conduct essential functions, including cell signaling, immune surveillance, molecular transport, and cell volume regulation. Furthermore, membrane proteins exist in a variety of specialized membrane domains such as lipid rafts or caveolae and in subcellular organelles such as the endoplasmic reticulum, Golgi network, and nuclear membranes. Lastly, secreted vesicles in the form of exosomes and microvesicles have recently been recognized as playing important roles in cancer metastases[3,4] and immune regulation,[5,6] with their membrane proteins likely to be involved in not only their biogenesis but also their targeting to distant destinations. Because of the essential functions of membrane proteins (some of which remain unknown) and their roles in disease, this class of proteins represents a highly validated analytical target. Hence, mass spectrometry, among other analytical techniques, is being applied at an unprecedented rate in the analysis of membrane proteins toward the development of new therapeutics, identification of potential disease biomarkers, and acquisition of a fundamental understanding of their function and regulation.

Although modern proteomics methods have yielded an extensive amount of new information about membrane protein expression levels, structure, modifications, and interactions, obstacles to obtaining a comprehensive view of membrane proteomes using mass spectrometric methods remain. Issues such as low abundance, limited solubility, and restricted enzyme accessibility are major factors in limiting the amount of information obtained in the study of membrane proteins. This review will discuss strategies and methods for the analysis of membrane proteins by mass spectrometry. Because global proteomic analysis of membrane proteomes has been the subject of multiple reviews,[2,7−11] this review focuses on targeted analyses of membrane proteins, with an emphasis on the analysis of aquaporin structure and function. The focus is on methods developed for the examination of posttranslational modifications (PTMs), protein−protein interactions, quantitative proteomics, and spatial localization through imaging mass spectrometry.

■ SAMPLE PREPARATION

The principal distinguishing feature between soluble protein and membrane protein analysis and the key to any successful proteomics analysis is sample preparation as summarized in Figure 1 for multiple mass spectrometry experiments. This point cannot be overemphasized in the analysis of membrane proteins. Because of the hydrophobic properties of membrane proteins as well as their lipid-rich environment and large range of abundances, developing sample preparation methods that are compatible with mass spectrometry has been a significant challenge. Initial studies of integral membrane proteins employed both matrix-assisted laser desorption ionization (MALDI) and electrospray ionization (ESI) for the successful ionization of intact membrane proteins. Key to the successes reported in these studies was the utilization of high

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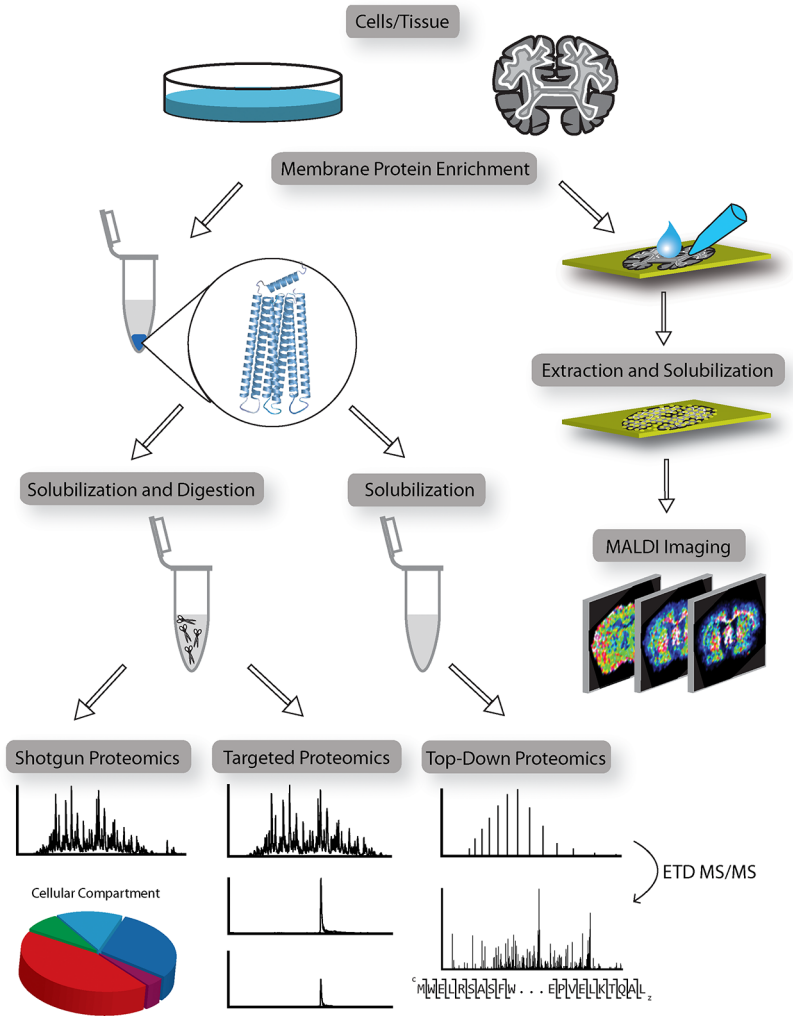

Figure 1. General overview of the sample preparation and analytical approaches in membrane protein analysis via mass spectrometry.

concentrations of organic solvents, formic acid for direct electrospray ionization[12] and acetonitrile, 2-propanol, and hexafluoro-2-propanol[13] or detergents[14] for direct MALDI analysis. Protein solubilization in these solvents combined with lipid removal was critically important for the separation of membrane proteins from abundant lipid contaminants that led to efficient protein desorption and ionization. Alternative approaches have also been successfully employed for membrane protein solubilization, including methanol solubilization,[15] and alternative detergent solubilization.[16,17] For intact membrane protein separations, early success was achieved when proteins were solubilized in formic acid and 2-propanol and separated over size exclusion and polystyrene/divinylbenzene columns with a solvent gradient from 60% formic acid to 100% 2-propanol.[18] More recently, size exclusion chromatography was performed using a chloroform/methanol/1% aqueous formic acid mixture (4/4/1), and reverse-phase chromatography was accomplished using an acetonitrile/2-propanol mixture (1/1) as the organic solvent.[19] Most initial studies were targeted to single proteins and were successful in identifying posttranslational modifications, including phosphorylation, palmitoylation, and glycosylation. Currently, top-down mass spectrometry methods for intact protein analysis are becoming more common in the analysis of intact integral membrane proteins (discussed below).

For global proteomics analyses, trypsin digestion is typically employed followed by one-dimensional or multidimensional high-performance liquid chromatography (HPLC)−tandem mass spectrometry (LC−MS/MS) analysis of resulting tryptic peptides. For integral membrane protein analysis, standard trypsin digestion typically provides sequence coverage limited to soluble loops and terminal tails; therefore, alternative enzymatic and/or chemical cleavage reagents, e.g., pepsin[20] and cyanogen bromide,[21] have been utilized to enhance sequence coverage. Cyanogen bromide cleaves at methionine residues and produces large hydrophobic fragments of proteins and, in the case of the prototypical G-protein-coupled receptor (GPCR) rhodopsin, greatly expands the sequence coverage observed.[21] HPLC parameters were also adjusted, including column type and solvent composition, to separate and elute the most hydrophobic portions of membrane proteins. Yates et al. used CNBr combined with trypsin to achieve more comprehensive proteome coverage in membrane proteomics studies.[22] This group also employed the nonspecific enzyme proteinase K in a membrane-disrupting high-pH buffer to provide information about the soluble loops and tails and membrane topology of brain membrane proteins.[7] Figure 1 shows the key steps in membrane protein sample preparation and analysis by mass spectrometry.

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MASS SPECTROMETRY TOOLBOX

A wide variety of mass spectrometric methods have been developed for protein analysis, some of which are generally applicable, e.g., shotgun proteomics for protein identification, and others of which are highly specific applications, e.g., hydrogen−deuterium exchange (HDX) for probing protein structure. These methods make up the protein mass spectrometrist's toolbox (Figure 2) that provides, when combined with appropriate sample preparation strategies (Figure 1), the necessary tools for answering key questions in proteomics. Many types of protein analyses by mass spectrometry rely on shotgun or "bottom-up" proteomics where proteins are digested prior to mass spectrometry analysis and the resulting peptides are analyzed. This basic approach has been used not only for protein identification but also for quantitative analysis of protein expression, for detection of posttranslational modifications, for structural studies when combined with chemical modification, or for protein−protein interaction studies when used with affinity purification. Alternatively, intact protein analysis can be employed in so-called "top-down" proteomics to determine primary amino acid sequence and posttranslational modifications or in "native mass spectrometry" to examine protein complexes. These mass spectrometry tools are briefly described below in the context of their application to membrane protein analysis.

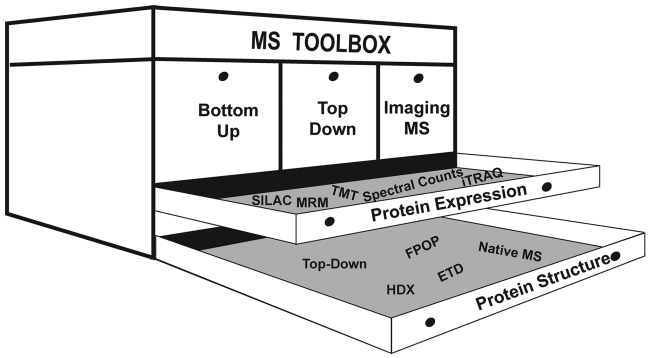

Figure 2. Mass spectrometry toolbox indicating a range of tools available for protein analysis.

TOP-DOWN VERSUS BOTTOM-UP

The vast majority of proteomics analyses rely on enzymatic or chemical cleavage of target proteins prior to analysis, termed bottom-up or shotgun analysis, because both liquid chromatography and mass spectrometry technologies perform optimally for peptide analysis. Bottom-up proteomics provides the highest sensitivity and therefore results in the largest number of proteins identified in a global proteomics experiment. Two or more dimensions of peptide separation, e.g., strong cation exchange and reverse-phase liquid chromatography in multidimensional protein identification technology (MudPIT)[22] or by isoelectric focusing and reverse-phase separations,[23] can be used to probe more deeply into a proteome. This strategy has recently provided near complete coverage of the yeast proteome.[24] Although not the focus of this review, global proteomics analysis of membrane proteomes has been applied to characterize the membrane proteome from many cell types and from subcellular membranes, including mitochondrial, Golgi, and nuclear membranes[25−27] and from specific plasma membrane domains such as lipid rafts.[28] One disadvantage of the bottom-up strategy is that information about the parent protein is lost upon digestion; therefore, without full sequence coverage, modifications can be missed and the stoichiometry of modification is difficult to determine. Importantly, for membrane protein analysis, transmembrane regions often go undetected. A useful tool from the MS toolbox for both bottom-up and top-down proteomics is electron transfer dissociation (ETD),[29,30] an alternative fragmentation method for tandem mass spectrometry that spares labile bonds from dissociation. This method is particularly useful for the detection of labile posttranslational modifications such as phosphorylation and glycosylation. Scott et al. characterized the glycoproteome from *Campylobacter jejuni* using ETD methods to identify peptides and sites of glycosylation.[31]

The top-down proteomics strategy relies on the separation and tandem mass spectrometry analysis of intact proteins. Although sample preparation methods for solubilization, delipidation, and enzyme compatibility are important for bottom-up strategies, membrane protein solubilization and delipidation are even more important for intact protein analysis because all regions of the protein, not solely the soluble portions, must be solubilized. Top-down methods allow protein isoforms to be interrogated and offer near complete sequence coverage that offers advantages for posttranslational modification mapping. Although top-down proteomics is rapidly developing and being applied to whole proteome analysis,[32] reports of top-down methods to whole membrane proteome analysis are limited.[33,34] Nevertheless, targeted membrane protein analysis by top-down methods has been useful in the analysis of posttranslational modifications[19,35] and is now being applied to the analysis of membrane protein complexes in native mass spectrometry as discussed below.

PROTEIN STRUCTURAL STUDIES

Multiple methodologies have been developed to obtain protein secondary, tertiary, and quaternary structural information using mass spectrometry. Chemical modification of "footprinting" and hydrogen−deuterium exchange followed by mass spectrometry detection are commonly used tools in this area of investigation. Chemical footprinting approaches typically employ oxidative chemistry using reagents such as hydroxyl radicals to covalently modify exposed regions of the protein.[36,37] Laser-induced oxidative labeling[38] and fast photochemical oxidation of proteins (FPOP)[39,40] have been used to rapidly (<1 ms) generate hydroxyl radicals from hydrogen peroxide to examine protein folding and unfolding. Experiments with the membrane protein bacteriorhodopsin (BR) indicated that the retinal chromophore plays an important role in the stability of the native protein structure.[38] In HDX experiments, exchangeable amide hydrogen atoms in the peptide backbone exchange with deuterium upon incubation of the protein in deuterated water. Through careful selection of post-exchange conditions (low pH and low temperature), back-exchange of hydrogen for deuterium during subsequent digestion and chromatography can be limited. The low-pH tolerant protease, pepsin, is typically employed under these experimental conditions, which has the added benefit of improving membrane sequence coverage.[20] To obtain structural information, the extent of modification of each amino acid is determined by mass spectrometry. HDX was first applied to membrane proteins to measure redox-dependent conformational changes in microsomal glutathione transferase, MGST1.[41] This work was followed by detection of redox

changes in cytochrome *c* oxidase,[42] light-induced changes in bacteriorhodopsin,[43] and the strengths of hydrogen bonding in transmembrane helices in BR.[44] In addition, both HDX and chemical footprinting have been used to map the structure of GPCR rhodopsin and its complexes with transducin.[45] Recently, phospholipid bilayer nanodisks have been used as a matrix for membrane protein conformation studies by HDX.[46]

Protein complexes can be studied as intact molecular species in native mass spectrometry,[47] a method pioneered by the Robinson group.[48] By careful selection of solution and instrumental conditions, membrane protein complexes have been ionized, detected, and structurally interrogated in a mass spectrometer.[49] The challenge, of course, is in keeping membrane protein complexes intact during sample preparation−solubilization and desorption−ionization steps. This is achieved, remarkably, through the use of detergent micelles that, upon removal in the gas phase, allow membrane protein complexes to remain intact.[50] A recent development in this regard, termed laser-induced liquid bead ion desorption (LILBID), involves infrared laser desorption of proteins from detergent micelles.[51] The energetics of desorption are such that intact membrane protein complexes as large as 750 kDa can be desorbed intact into the mass spectrometer for structural examination.[52]

QUANTITATIVE PROTEOMICS (REVIEWED IN CONNECTED CURRENT TOPIC MANUSCRIPT, DOI: 10.1021/BI400110B)

Two general approaches have been developed and applied to detect global protein expression differences: label-free and isotope labeling strategies. Label-free approaches rely on spectral counting or peak area comparisons to determine proteomic differences, and these approaches have the advantage of not requiring additional labeling chemistries in the sample preparation step. Spectral counts or peak areas can be calculated from standard bottom-up LC−MS/MS data sets where multiple replicates are used to improve the accuracy of protein expression measurements. The label-free approach can be problematic in membrane proteomics studies because of the aforementioned limited sequence coverage in the form of peptides from soluble loops and tails typically observed for integral membrane proteins. Thus, quantitative comparisons are made on very few peptide measurements per protein. Isotope labeling strategies such as SILAC,[53] iTRAQ,[54] or TMT[55] use chemical labeling of proteins or peptides in a multiplex format for comparing proteomes. In SILAC experiments, stable isotope-labeled amino acids are incorporated into each protein as cells replicate in cell culture media enriched in the labeled amino acids; therefore, the labeling occurs at the protein level. Extracted proteins from each sample are pooled and digested together prior to LC−MS/MS analysis. In iTRAQ or TMT tagging approaches, proteins from separate treatments are digested separately and resulting tryptic peptides are subsequently labeled with chemical tags unique to each sample that incorporate stable isotopes. After tagging chemistry occurs, peptides from each treatment are pooled and analyzed by LC−MS/MS. The different iTRAQ or TMT tags are designed to add the identical mass to each peptide and to fragment in the MS/MS experiment to produce a reporter ion unique to the sample of origin. The reporter ion intensities are therefore measures of the abundances of each peptide and, by inference, the parent proteins in each sample. The iTRAQ and TMT tags are engineered to allow up to 8-plex and 6-plex comparisons in a single experiment, respectively, and when those tags are combined with SILAC, an 18-plex experiment has been demonstrated.[56] Although limited sequence coverage for membrane proteins is also observed with these approaches, a direct comparison between multiple samples is made because of the nature of the multiplex format, thereby limiting sources of variance in sample handling and data acquisition.

The targeted quantitative method of multiple-reaction monitoring (MRM) has also been applied to measure changes in specific membrane proteins. In this approach, specific tryptic peptides are targeted for quantitative analysis, and their relative or absolute abundances are measured in an LC−MS/MS experiment. When only the targeted peptide ions and their sequence specific fragment ions are monitored, the sensitivity and specificity of the measurement are significantly enhanced. When these data are combined with stable isotope-labeled peptides of the same amino acid sequence, absolute quantitative information can be obtained in the so-called AQUA (absolute quantification) method.[57] Quantitative measurements can be fraught with difficulty because ionization efficiencies and peptide recoveries can differ widely for transmembrane-spanning helices and soluble tail/loop peptides. Therefore, the "gold standard" is to utilize stable isotope-labeled internal standards for quantitative accuracy either at the peptide level as in AQUA or at the whole protein level.[58−60]

Quantitative proteomics approaches have been widely applied to measure protein expression changes in membrane proteomes. Examples include measured increases in a membrane transporter in yeast grown in minimal medium,[61] changes in organellar distributions of membrane proteins,[62] changes in the pancreatic zymogen granule membrane proteome,[63] and changes in ocular lens membrane proteins and membrane-associated proteins with age.[64] In addition, combining quantitative methods with enrichment strategies, e.g., phosphopeptides, glycopeptides, or surface protein enrichment, has allowed more detailed changes in the membrane proteome to be detected.[65]

IMAGING MASS SPECTROMETRY (REVIEWED IN CONNECTED CURRENT TOPICS MANUSCRIPT, DOI: 10.1021/BI301519P)

Imaging mass spectrometry (IMS) as pioneered by the Caprioli group[66] involves the measurement of the spatial distribution of proteins and other analytes in tissues by direct sampling of the tissue surface. For protein imaging, MALDI ionization is the preferred method of ionization. IMS of integral membrane proteins represents a unique challenge with respect to protein solubility, mass range, and dynamic range. Because of these inherent limitations, alternative methods were required to both enrich and solubilize membrane proteins directly on tissue sections, without disturbing their spatial localization. As a result, novel methodologies were developed specifically for IMS to minimize the contribution of highly abundant, soluble proteins and maximize the detection of membrane proteins.[67,68] Specifically, the manner in which tissue is mounted onto the target plate has been demonstrated to be critical for membrane protein detection. Treating the target with methanol and mounting the tissue while it is still wet appear to be critical for membrane protein enrichment, most likely by solubilizing specific lipid classes and disrupting their hydrophobic interactions with proteins. Furthermore, multiple washes, with either water, formic acid, or acetonitrile, have been shown to aid in the removal of abundant, soluble proteins.[68] Lastly,

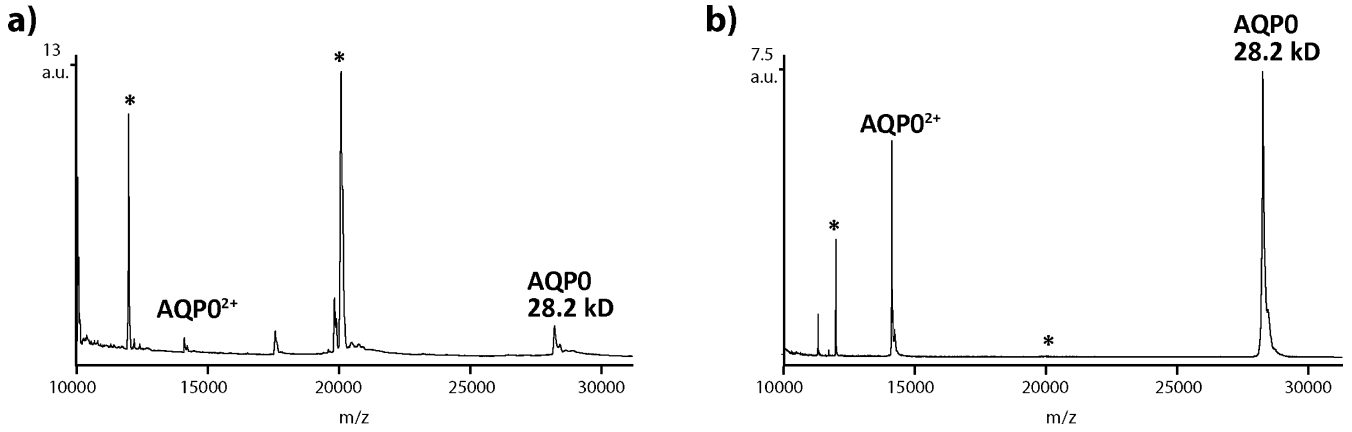

Figure 3. Comparison of averaged protein spectra from ocular lens tissue resulting from different tissue pretreatments for MALDI imaging. (a) MALDI mass spectrum acquired after thaw mounting of tissue, multiple water washes, a formic acid wash, and application of sinapinic acid in a high level of acetonitrile. (b) MALDI mass spectrum acquired after methanol mounting of tissue, multiple water washes, a formic acid wash, and application of sinapinic acid in 90% acetonitrile with 0.01% hexafluoro-2-propanol. In panel b, note the increased signal intensity for AQP0 and the significant reduction in the level of the highly abundant, soluble lens crystallin proteins (asterisks).

multiple studies have demonstrated the utility of spotting the MALDI matrix in solutions with a high organic percentage (90% acetonitrile) spiked with hexafluoro-2-propanol (0.1−1%) for enhanced membrane protein solubilization and detection via MALDI-MS.[69−71] The culmination of these sample preparation steps can be seen in Figure 3 where, without proper sample preparation, the ocular lens membrane protein aquaporin-0 (AQP0) is barely observed above the noise in MALDI mass spectra acquired from bovine lens tissue. Using membrane protein specific sample preparation protocols, AQP0 is sufficiently desorbed and ionized to produce an intense signal directly from lens tissue.

As mass spectrometry methods and instrumentation continue to be developed, examination of membrane proteins will become increasingly routine. Below we discuss the application of the aforementioned sample preparation methods and mass spectrometry tools in the analysis of the ubiquitous integral membrane protein family, the aquaporins.

A FOCUS ON AQUAPORINS

Aquaporins (AQPs) are a ubiquitous family of integral membrane proteins that span all domains of life;[72] their discovery was the subject of the 2003 Nobel Prize awarded to Dr. Peter Agre.[73] Their major function is to transport water through lipid membranes; however, one class of aquaporins, the aquaglyceroporins, also transports small molecules like glycerol, whereas other members have been reported to have cell adhesion functions. There are 13 members of the family found in humans with specific distributions throughout the body. Aquaporin mutations and dysfunction cause a number of human diseases,[74] yet their complete characterization, e.g., identification of regulatory posttranslational modifications and protein−protein interactions, remains to be accomplished. Their general topology in the lipid membrane bilayer and common sites of posttranslational modification are shown schematically in Figure 4, where six transmembrane helices can be seen along with the dual conserved NPA boxes that form the center of the water channel. AQPs exist as tetrameric complexes in the membrane, with each monomeric subunit possessing an active water channel. Mass spectrometry has played an important role in enhancing our understanding of AQP structure and function as reviewed below.

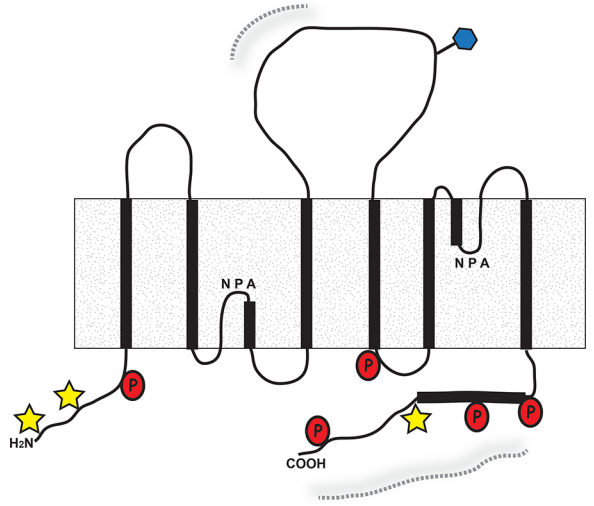

Figure 4. Cartoon diagram of aquaporin topology in the lipid bilayer showing regions of posttranslational modification, including phosphorylation (red circles), lipidation (yellow stars), and glycosylation (blue hexagon), as well as sites of protein−protein interaction (dashed lines).

POSTTRANSLATIONAL MODIFICATIONS

Significant progress has been made over the past 20 years regarding the characterization of the aquaporin family of transmembrane proteins via mass spectrometry. Much of this work has focused on aquaporin-0/major intrinsic protein (MIP) found in the ocular lens and aquaporin-2 (AQP2) found in the kidney. Both proteins function as regulators of water permeability in their respective organs; however, AQP0 has also been reported to possess cell adhesion properties.[75,76] Mass spectrometric studies have revealed that the cytoplasmically exposed C-terminal domains of these proteins are critically important in the regulation of membrane permeability to water. More specifically, these domains have been shown to be targets for numerous posttranslational modifications. Phosphorylation of AQP0 and AQP2 as well as glycation, fatty acid acylation, and nonenzymatic backbone truncation of AQP0 have all been detected by mass spectrometry, and their biological roles have been interrogated.

Phosphorylation. Phosphorylation, perhaps the most studied regulatory PTM, has been shown to play critical regulatory roles in AQP permeability and trafficking. In well-studied AQP0 and AQP2, regulation is achieved by C-terminal phosphorylation that alters protein−protein interactions that are important for trafficking or channel permeability. AQP0 C-terminal phosphorylation by protein kinase A (PKA) through interaction with AKAP2[77] alters calmodulin binding and channel permeability.[78−80] AQP2 C-terminal phosphorylation by PKA regulates trafficking to and from the apical plasma membrane via interactions with the "multiprotein motor complex".[81,82]

Mass spectrometry has been utilized to detect sites of phosphorylation on the C-terminal domains of both AQP0 and AQP2 and to assist in elucidating their biological roles. In early work, Schey et al. reacted purified AQP0 with cyanogen bromide to produce a peptide encompassing the entire C-terminus, residues 177−263, of the protein.[83] A slightly smaller C-terminal peptide was isolated by HPLC and subsequently analyzed by ESI-MS to reveal a multiply charged ion representing AQP0 N184−L263, as well as an 80 Da heavier ion corresponding to the singly phosphorylated peptide at roughly 25% abundance of the unmodified peptide. Additional confirmation of phosphorylation was achieved by digestion of the CNBr-produced peptide with trypsin. LC−MS/MS analysis of the peptide S_{229}VSERLSILK$_{238}$, containing one phosphorylation among three phosphorylatable serine residues, unambiguously assigned the major phosphorylation site as S235. This residue falls within a PKA motif and is highly conserved among cow, mouse, rat, frog, and human AQP0 sequences.

Additional work has focused on spatial differences in phosphorylation levels within the ocular lens and on defining the biological role of AQP0 phosphorylation. Both manual dissection and laser capture microdissection combined with LC−MS/MS analysis revealed spatial differences of pAQP0 levels within human and bovine lenses.[84,85] In both cases, label-free quantification was achieved by measuring the relative peak areas of selected peptides. In human lenses, the inner cortex and outer nuclear layer displayed the highest level of phosphorylation, at roughly 15% of total C-terminal peptide; the outer cortex and inner nuclear layers displayed the lowest levels of phosphorylation, at roughly 6 and 7% of total peptide, respectively. Additional phosphorylation sites were also discovered at S229 and S231. In bovine lenses, the level of phosphorylation of S235 was drastically higher in the nuclear region (62% relative abundance) than in any of the cortex layers (7% in the anterior cortex, 10% in the posterior cortex, and 22% in the equatorial cortex).[85] Phosphorylation of bovine AQP0 residues S243 and S245 did not display significant differences among the different lens regions. The authors concluded that S235 phosphorylation appeared to be spatially regulated and may be age-related given that the nucleus contains the oldest fiber cells within the entire lens.

The role of phosphorylation of AQP2 in water transport within vasopressin-sensitive inner medullary collecting duct (IMCD) cells of kidney has also been interrogated by mass spectrometry. Hoffert et al. combined trypsin digestion with phosphopeptide enrichment via immobilized metal affinity chromatography (IMAC) to identify phosphopeptides from rat kidney IMCD cells by LC−MS/MS.[86] Phosphorylation was detected on all four serines present on the C-terminus of AQP2, specifically on residues S256, S261, S264, and S269. Relative to a control sample, a 2.7-fold increase in the doubly phosphorylated peptide R_{254}QSVELHSPQSLPR$_{267}$, modified at S256 and S261, was detected in cells treated with vasopressin. Monophosphorylation at both S256 and S261 was also observed, and the changes in abundance for each form in the presence of vasopressin were determined to be a 7.2-fold increase and a 2.5-fold decrease, respectively, as compared to that of the untreated control. Given that peptides with different sequences have different ionization efficiencies, peptides monophosphorylated at S256 and S261 were synthesized and used to more accurately estimate phosphorylation levels. This study revealed that the peptide phosphorylated at S256 had greater ionization efficiency than the peptide phosphorylated at S261, indicating, after adjustment, that under control conditions, monophosphorylation at S261was roughly 24-fold more abundant than at S256 whereas upon vasopressin treatment, phosphorylation at S256 increased to roughly the same relative abundance. It was hypothesized, and later demonstrated, that this increase in phosphorylation levels of S256 in response to vasopressin regulates transport of AQP2 to the apical plasma membrane.

Global membrane proteomics studies have also revealed additional sites of aquaporin phosphorylation, for example, for aquaporin-4[7,86] and aquaporin-5.[87] These findings derived from mass spectrometry analysis can be used to inform future studies of biological relevance.

Fatty Acid Acylation. Many integral membrane and membrane-associated proteins are modified by the attachment of lipids, the role of which is to target or anchor the protein to the plasma membrane. The most common lipid modifications are S-palmitoylation on cysteine residues and N-terminal myristoylation. Mass spectrometry has been utilized to detect rare and unique fatty acid acylation on both the N- and C-termini of AQP0.[69] Direct, on-tissue MALDI profiling of intact AQP0 human and bovine lenses revealed a molecular ion roughly 264 Da higher in mass than intact, unmodified AQP0.[69,70] To determine the identity and site(s) of modification, bovine AQP0 was digested with trypsin and analyzed by LC−MS/MS. Analysis of late-eluting, hydrophobic peptides revealed multiple tryptic peptides that contained an addition of either 238 or 264 Da. Accurate mass measurements of the peptides identified these modifications as the addition of palmitic acid (238.2296 Da) and oleic acid (264.2453 Da). MS/MS analysis identified K238 and the N-terminal amino group as the two sites that were modified by either palmitic acid or oleic acid. Although fatty acid modifications to lysine residues have been reported for other membrane proteins, e.g., lung surfactant proteins,[88] this study was the first to identify lysine as a possible site of fatty acid acylation by oleic acid. Targeted mass spectrometric analysis of the lipidated and nonlipidated peptides revealed that the lipidated peptides were found exclusively in the detergent resistant membrane fraction, suggesting that the lipid modifications target AQP0 to lipid raft domains in the fiber cell membrane.[69] MALDI profiling and imaging (discussed below) were used to determine the spatial localization of lipidated AQP0.

The importance of AQP lipidation has also been demonstrated for aquaporin-4, where S-palmitoylation of C13 and C17 alters the ability of AQP4 to form square arrays;[89] however, this work was accomplished by site-directed mutagenesis, and lipid modifications of these sites have not been confirmed by mass spectrometry. Although other AQPs (AQP5, AQP8, and AQP9) have been shown to move between

plasma membrane microdomains,[90,91] lipidation of these AQPs has yet to be demonstrated.

Glycosylation. N-Glycosylation is a common PTM found on integral membrane proteins and is predicted to be present on multiple AQPs. AQP2 glycosylation on its extracellular loop is important for exiting the Golgi network and for proper trafficking to the plasma membrane.[92] The only mass spectrometry analysis of AQP glycosylation has been accomplished for AQP10,[93] an aquaglyceroporin found in enterocytes. Glycosylation at N133 of AQP10 was shown to be important for the thermal stability of the protein.

Truncation. The lens is an ideal tissue in which to study age-related modifications because of the loss of nuclei during fiber cell differentiation and concomitant loss of protein turnover with increasing age. Thus, molecules of AQP0 are retained for the entire life of an individual within the lens. Moreover, a number of age-related modifications have been shown to accumulate with lens age, particularly protein backbone truncation. It has been hypothesized that, in the absence of new protein synthesis, posttranslational modification is a mechanism used by lens fiber cells to alter protein function with age. Indeed, truncation of AQP0 has been shown to form a closed pore, junction-forming channel.[75,94,95]

Sites of protein backbone truncation were first mapped in human AQP0 as a function of lens age using the CNBr/trypsin cleavage strategy.[96] MALDI-MS analysis of the C-terminal AQP0 CNBr-generated peptides from lenses of different ages revealed identical patterns of truncation within each lens; however, the relative abundance of the various truncation products increased with age. The major truncation products observed were formed from cleavage at specific sites within the C-terminal region, including residues D243, N246, and N259. It was concluded that a variety of potentially nonspecific mechanisms, including nonenzymatic deamidation, could be responsible for the increase in the level of age-related backbone truncation along the C-terminus of AQP0. Additional studies of C-terminal truncation within different regions of human lens using both shotgun LC−MS/MS[84] and MALDI-MS of intact AQP0[70,71] approaches confirmed the correlation between fiber cell age and increased levels of truncation products, with the highest level of truncation products occurring in the older lens nucleus and the lowest level occurring in the younger, outer cortex regions. Interestingly, MALDI analysis of human fetal lenses showed no discernible AQP0 truncation, but 5-year-old lenses displayed significant truncation products, indicating that the pattern for truncation is established early in life[71] with an estimated half-life for intact AQP0 of 25 years. It has been proposed that an increased level of C-terminal truncation results in restriction of the AQP0 pore, resulting in reduced water permeability and potentially cataract formation.

Truncation has not been reported for other AQPs, but the methods developed for AQP0 truncation analysis may by useful for detecting AQP truncation in long-lived proteins, e.g., AQP4 in the brain.

■ PROTEIN−PROTEIN INTERACTIONS

Multiple approaches have been employed to identify proteins that interact with AQP0, including antibody pull-down,[97] use of "bait" peptides as affinity substrates,[98−100] and chemical cross-linking[101] followed by mass spectrometry analysis. An important aspect of the analysis of membrane protein interactions is the maintenance of native complexes during the solubilization step that is typically accomplished with detergents. Thus, for intact membrane protein affinity purifications and for cross-linking experiments, it is likely that only the strongest interactions are maintained.

The C-terminal tails of AQP0 and AQP2 have been shown to be critical regions of interactions with regulatory proteins. A standard bottom-up proteomic approach was used to identify proteins binding to affinity columns made with AQP0 C-terminal peptides corresponding to residues 251−263 and 240−263.[100] Multiple peptides from two lens specific intermediate filament proteins, filensin and CP49, were detected from both column enrichments relative to a control, no peptide, enrichment. Confirmation of these interactions was achieved by an AQP0 antibody pull-down experiment followed by digestion and MS analysis. Studies in knockout mice revealed that filensin and CP49, along with AQP0, are necessary for long-term maintenance of lens optical properties, fiber cell shape, and membrane organization.[102]

A chemical cross-linking approach was utilized to show a direct interaction between AQP0 and proteins from the ezrin/radixin/moesin (ERM) family of actin binding proteins.[101] While the specific sites of protein interaction can be determined using a cross-linking approach, identification of cross-linked peptides based on tandem mass spectra can be challenging. To identify specific sites of protein interaction between ezrin and AQP0, a lens membrane pellet was incubated with the zero-length 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC) cross-linking reagent that cross-links primary amines with carboxylic acid functional groups. Following the cross-linking reaction, proteins were digested with trypsin, and resulting cross-linked peptides were subjected to LC−MS/MS analysis. Cross-linked peptides from AQP0 and ezrin were detected; specifically, peptide 239−259 of AQP0 was shown to be cross-linked through its acidic residues to five different peptides that fall within the first 300 residues of ezrin/radixin. This confirmed the direct interaction between the C-terminal domain of AQP0 and ezrin/radixin. Pull-down experiments with a C-terminal AQP0 peptide and co-immunoprecipitation experiments with mass spectrometry detection confirmed the interaction. Given that the ERM family of proteins is involved in cell−cell adhesion and maintenance of cell shape, the age-related truncation of the AQP0 C-terminus could result in the loss of interaction between AQP0 and ERM proteins, leading to the loss of critically important cellular architecture and eventual cataract formation.

Chemical cross-linking with EDC was also used to determine the sites of interaction between AQP0 and calcium/calmodulin,[78] a known regulatory interaction in the lens.[78−80,103] In this case, four different forms of human AQP0 C-terminal peptide 224−241 were synthesized [(1) unmodified, (2) S231-phosphorylated, (3) S235-phosphorylated, and (4) S231- and S235-diphosphorylated] and were assessed for their ability to form cross-links with calmodulin. Using LC−MS/MS analysis, a direct interaction between K228 and K238 of unmodified AQP0 and E14, D78, D80, and E84 of calmodulin was identified. Cross-linking efficiency was ascertained by comparison of the peak area of cross-linked calmodulin peaks with the peak area of non-cross-linked calmodulin peaks. Monophosphorylation at either S231 or S235 of AQP0 resulted in a decreased cross-linking efficiency relative to that of the unmodified peptide, and diphosphorylation resulted in no observable cross-linked peptides. These results confirmed earlier studies that established weakened binding of calmodulin to pAQP0. This discovery, combined with the spatial

distribution of pAQP0 described above, is important in understanding fiber cell water permeability within different regions of the lens.

Antibody pull-down experiments with AQP2 complexes followed by in-gel digestion and MALDI-MS peptide mass fingerprinting revealed an interaction with tropomyosin as part of the multiprotein motor complex.[81,97] It is believed that this interaction is responsible for shuttling AQP2 to and from the apical membrane in collecting duct cells of the kidney. Additional experiments using phosphorylated and nonphosphorylated AQP2 C-terminal peptides for affinity purification experiments showed that the number of interactions with hsp70-5 (BiP) increased with phosphorylated AQP2 peptides, whereas the number of interactions with hsc70, hsp70-1, hsp70-2, and annexin II decreased with the phosphorylated AQP2 peptides.[98] C-Terminal peptides from AQP5 were used to affinity purify interacting proteins from lacrimal gland homogenates.[99] Sodium dodecyl sulfate−polyacrylamide gel electrophoresis separation followed by in-gel digestion and LC−MS/MS analysis identified prolactin-inducible protein (PiP) and major urinary protein (MUP4) as AQP5 interacting protein partners.

PROTEIN STRUCTURE

HDX and hydroxyl radical oxidative labeling analysis of an *Escherichia coli* member of the AQP family, the glycerol facilitator, revealed intriguing dynamic structural information.[104] As expected, N- and C-terminal tails showed rapid H−D exchange indicative of unstructured, highly solvent exposed regions of the protein and several transmembrane regions showed nearly complete protection from exchange. Interestingly, transmembrane region 7 (TM7) containing the second NPA motif that forms part of the solute selective channel was found to be highly flexible, similar to extracellular domains of the protein. The authors suggest that this flexibility acts to "lubricate" the channel, preventing prolonged binding of the solute within the channel.

TISSUE DISTRIBUTION

IMS has only recently been applied to measure spatial distributions of membrane proteins in tissues as demonstrated for lens AQP0.[68] Both unmodified and modified forms of AQP0 can be localized within a lens tissue section in a single imaging MS experiment as shown in Figure 5 for truncated AQP0. Truncation products identified in previous bottom-up experiments (residues 1−259 and 1−246) are present in the older inner cortical and nuclear fiber cells, whereas intact AQP0 is observed only in the outer cortical region. Lipidated forms of human AQP0 have also been profiled[70] and imaged[69] and shown to be highly abundant in the inner cortical region. As discussed above and shown in Figure 3, removal of substantial amounts of soluble proteins that suppress membrane protein signals is required for successful analysis.

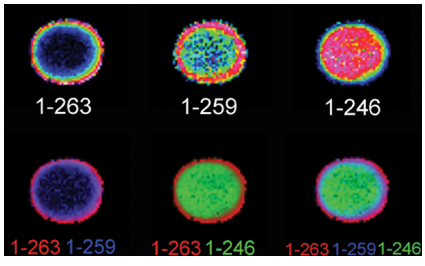

Figure 5. MALDI-MS images of full-length AQP0 (top left) and truncated forms of residues 1−259 (top middle) and 1−246 (top right) from a 51y human lens. The bottom row shows overlaid images of intact and truncated forms of AQP0.

CONCLUSIONS AND FUTURE DIRECTIONS

Mass spectrometry has developed into a powerful tool for the analysis of protein structure, modification, and localization, which all contribute greatly to our understanding of the underlying biology. As improvements in mass spectrometry technologies have occurred, applications to membrane protein analysis have developed in parallel with improvements in sample preparation protocols to enrich, solubilize, and digest this class of molecules. Future areas of application will depend on continued enhancements in both technology and sample preparation to provide greater sensitivity in membrane protein analysis. An area of rapid methodological development is in membrane protein structure studies using native mass spectrometry to examine the quaternary structure of large membrane protein complexes. In addition, as improvements in sensitivity and spatial resolution of imaging mass spectrometry occur, examination of membrane protein distributions in tissues at single-cell resolution will follow. An area of important biological relevance is the characterization of extracellular vesicles, exosomes, and microvesicles. These cell-derived microparticles are believed to play critical roles in cell−cell communication and are involved in cancer metastasis and immune system regulation.[3−6] Significant questions about the mechanisms regulating their biogenesis and targeting of these particles to distant sites remain. Characterization of exosomal membrane proteins will be a key step in understanding the regulation of their biology.

On the basis of the past decade and a half of investigation of membrane proteins by mass spectrometry, it is clear that improvements in mass spectrometry technology and sample preparation methods will continue to open the windows of discovery for membrane protein structure and function.

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Notes

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