Visualizing Water Molecules in Transmembrane Proteins Using Radiolytic Labeling Methods[†]

Tivadar Orban,[‡] Sayan Gupta,[§,||] Krzysztof Palczewski,*,[‡] and Mark R. Chance*,[§,||,⊥]

[‡]*Department of Pharmacology*, [§]*Center for Proteomics and Bioinformatics*, [||]*Center for Synchrotron Biosciences, and* [⊥]*Department of Physiology and Biophysics, School of Medicine, Case Western Reserve University, Cleveland, Ohio 44106-4965*

ABSTRACT: Essential to cells and their organelles, water is both shuttled to where it is needed and trapped within cellular compartments and structures. Moreover, ordered waters within protein structures often colocalize with strategically placed polar or charged groups critical for protein function, yet it is unclear if these ordered water molecules provide structural stabilization, mediate conformational changes in signaling, neutralize charged residues, or carry out a combination of all these functions. Structures of many integral membrane proteins, including G protein-coupled receptors (GPCRs), reveal the presence of ordered water molecules that may act like prosthetic groups in a manner quite unlike bulk water. Identification of "ordered" waters within a crystalline protein structure requires sufficient occupancy of water to enable its detection in the protein's X-ray diffraction pattern, and thus, the observed waters likely represent a subset of tightly bound functional waters. In this review, we highlight recent studies that suggest the structures of ordered waters within GPCRs are as conserved (and thus as important) as conserved side chains. In addition, methods of radiolysis, coupled to structural mass spectrometry (protein footprinting), reveal dynamic changes in water structure that mediate transmembrane signaling. The idea of water as a prosthetic group mediating chemical reaction dynamics is not new in fields such as catalysis. However, the concept of water as a mediator of conformational dynamics in signaling is just emerging, because of advances in both crystallographic structure determination and new methods of protein footprinting. Although oil and water do not mix, understanding the roles of water is essential to understanding the function of membrane proteins.

WATER AND ITS IMPORTANCE IN BIOLOGICAL PROCESSES

A fundamental problem in modern structural biology is to understand the details of macromolecular structure and dynamics. Subtle structural changes induced by ligand binding or complex formation represent the quintessence of protein function in processes ranging from enzyme activity and signal transmission to nucleic acid binding. Many biological events are mediated to some extent by water. Water has been shown to mediate protein–protein interactions (*1*) and protein–ligand interactions (*2*) and was thought to facilitate protein folding (*3–5*). In the case of rhodopsin (*6*), a model for membrane-bound G protein-coupled receptors (GPCRs),[1] it has been shown that water has a significant role in forming the active site (*7*). Moreover, a comparison of known GPCR X-ray structures (*8–13*) revealed that water molecules in the hydrophobic core of these proteins interact with conserved residues, implying that these waters are probably as

important for GPCR function as the conserved residues themselves (*14*). Buried water molecules are critical for the overall function of rhodopsin (and bacteriorhodopsin as well), and these have been characterized not only by X-ray crystallography but also by Fourier transform IR spectroscopy (*15*). Recently, radiolytic protein footprinting has emerged as a promising tool for the analysis of side chain dynamics in the vicinity of bound water molecules. In this review, we present new developments and potential future directions for the study of transmembrane protein water molecules by radiolytic protein footprinting and the integration of these data with those from other biophysical techniques.

RADIOLYTIC PROTEIN FOOTPRINTING

Why Hydroxyl Radicals? X-ray crystallography and nuclear magnetic resonance (NMR) are currently the most valuable methods for revealing the structural details of macromolecules at atomic resolution. However, in many cases where proteins resist such analyses for certain conformational states of interest because of limitations of a specific system, structural mass spectrometry (MS) has become increasingly important as an alternative approach to addressing questions about structure and dynamics in relevant conformational states (*16, 17*). Structural changes that facilitate protein function are conveniently probed by covalent labeling methods like hydroxyl radical footprinting coupled to MS where radiolysis or other methods (see below) are used to generate radicals that can decorate solvent-accessible surfaces of macromolecules (*18–25*). These approaches allow

*To whom correspondence should be addressed. M.R.C.: Center for Proteomics and Bioinformatics, Case Western Reserve University, 10900 Euclid Ave., Cleveland, OH 44106; phone, (216) 368-4404; fax, (216) 368-3812; e-mail, mark.chance@case.edu. K.P.: Department of Pharmacology, Case Western Reserve University, 10900 Euclid Ave., Cleveland, OH 44106; phone, (216) 368-4631; fax, (216) 368-1300; e-mail, kxp65@case.edu.

[1]Abbreviations: FTIR, Fourier transform infrared; GPCRs, G protein-coupled receptors; MS, mass spectrometry; NMR, nuclear magnetic resonance; PDB, Protein Data Bank.

detailed examination of surface residues in conformational states of interest with resolution at the side chain level.

There are several unique benefits of structural MS. For example, MS approaches are not limited by the size of a biomolecular complex. Only limited amounts (in the range of nanomoles or picomoles) of a sample are required, and the technique provides the ability to test a wide range of solution conditions at physiologically relevant concentrations of protein and/or ligands in the nanomolar to micromolar range (26–28). For macromolecular footprinting with hydroxyl radicals, time-resolved studies that examine dynamic processes over periods ranging from microseconds to minutes are feasible (24, 29–31). In addition, radiolytic protein footprinting generates stable modified side chains, permitting a wide range of analytical approaches for the isolation of the protein and its peptides to yield a convenient readout of specific sites of modification that provide high-resolution structural information about the macromolecular species of interest (23, 32–34).

However, there are disadvantages in the application of structural MS methods as compared to more traditional procedures. For example, deuterium exchange and covalent labeling methods (including radiolytic footprinting) provide only local structural information without a tertiary or quaternary context. Cross-linking can provide distance information but has its own difficulties with respect to experimental design and data interpretation. Thus, the field has moved toward approaches that leverage structural information obtained at atomic resolution with examination of particular species of interest by MS to explore detailed mechanisms of conformational changes intimately associated with function (27, 28, 32, 33, 35–45).

Generation of Hydroxyl (OH•) Radicals. Hydroxyl radicals used for covalent labeling in structural mass spectrometry may be generated by a variety of methods such as radiolysis (18, 19, 44, 45), electric discharge of an electrospray ionization source (42, 46, 47), Fenton chemistry (48), and photochemistry of peroxide (49, 50). The primary focus for the remainder of this review is the use of radiolysis for generating OH•, specifically studies that rely on synchrotron X-ray radiation using beamline X28C of the National Synchrotron Light Source (51) because of its ability to implement dynamics studies by probing water molecules trapped inside the hydrophobic cores of proteins (52).

Mechanisms of Amino Acid−OH• Interactions. Mechanisms of amino acid modification by OH• radicals are very complex and depend largely on side chain chemistry. Extensive work in the field proposes that typical modifications of amino acid side chains include mass additions of 16 or 14 Da because of incorporation of hydroxyl or carbonyl groups. Residue-specific reaction mechanisms will produce characteristic mass changes subsequent to OH• radical exposure as follows: −43 Da for an Arg residue (53), −30 Da for Asp and Glu residues (54), +32 and +48 for Cys residues (55), and −22, −10, and + 5 for His residues (53) in addition to multiple incorporations of hydroxyl groups for aromatic residues ($+16n$, where $n = 1, 2,$ or 3). These modifications and the well-understood attenuations of retention time they induce in reverse-phase liquid chromatographic (LC) analyses (56) can be used either to set up rules to identify modifications on specific peptides on the basis of their LC−MS spectra as analyzed by software such as ProtMapMS (57) or to identify modifications of peptides in database search algorithms, such as Mascot (58).

Footprinting Experimental Setup and Data Analysis. The general steps involved in protein footprinting are illustrated in Figure 1. During exposure to ionizing radiation (Figure 1A), protein amino acid side chains react with OH• radicals generated from water molecules within the bulk solvent. Following radiation exposure, the remaining reaction products such as H_2O_2 or transient radical species are neutralized with methionine amides (59). Because of inherent covalent modifications produced by radiolytic labeling, a range of experimental conditions can be employed for this procedure such as different buffers and pHs and a wide range of proteases. One problem, however, is buffer components that scavenge generated radicals, such as glycerol, nucleotides, or other components used to stabilize proteins or conformations of interest. In such cases, the radiation dose (or the hydroxyl radical dose in general) must be carefully adjusted to provide sufficient energy for the desired peptide modifications while limiting secondary reactions resulting from overexposure. A number of studies have found that a rapidly delivered, high-flux dose is preferable to minimize unwanted overexposure and maximize the signal-to-noise ratio in LC−MS (21, 22, 25, 35, 51, 60–64).

Following exposure to X-rays, labeled proteins are digested with a protease (Figure 1B) and the protein proteolytic fragments are separated by reverse-phase high-performance liquid chromatography (Figure 1C) and analyzed by mass spectrometry. Unmodified peptides and their modified counterparts are identified by tandem mass spectrometry (MS/MS). To accelerate these time-consuming steps, recent efforts have concentrated on automating data analyses. These methods identify different modified species and also extract retention times (modified peptides elute before unmodified peptides) of modified and unmodified peptides (57). Following identification of the modified and unmodified species on the basis of MS/MS, the fraction unmodified is calculated from the change in the total area of the selected ion current (Figure 1D). Finally, the fraction unmodified is plotted as a function of dosage (exposure time) to determine the rate of modification by using a first-order function (Figure 1E).

Protein Dynamics Tested by Footprinting. Historically, hydroxyl radical-mediated protein footprinting was shown to be particularly well suited to testing solvent exposure of the modified residues. However, in recent experiments on the transmembrane protein rhodopsin, it was observed that several residues buried deep inside the hydrophobic core of the protein could exhibit modifications (52). Even more interesting, some of these modifications not only were extensive but also occurred after very short periods of exposure as compared to residues exposed to bulk solvent. Recent structural studies of bovine rhodopsin have shed some light on this peculiar reactivity of buried residues to hydroxyl radicals (14). These high-resolution studies identified water molecules in the proximity of many amino acid side chains that were efficiently modified. These findings are featured in this review to illustrate how radiolytic footprinting-mediated structural MS can be used as a unique tool to answer questions regarding the structure and function of membrane proteins and the dynamics of conformational changes involving internally bound water.

FOOTPRINTING OF A MODEL GPCR, RHODOPSIN

Rhodopsin is responsible for the initiation of signaling in visual transduction. Signaling starts upon absorption of a photon of light that causes a rapid trans isomerization of the bound 11-*cis*-retinal visual chromophore (65). After rhodopsin passes through several conformations corresponding to different bleached intermediates, the last one being Meta II, signal transduction is
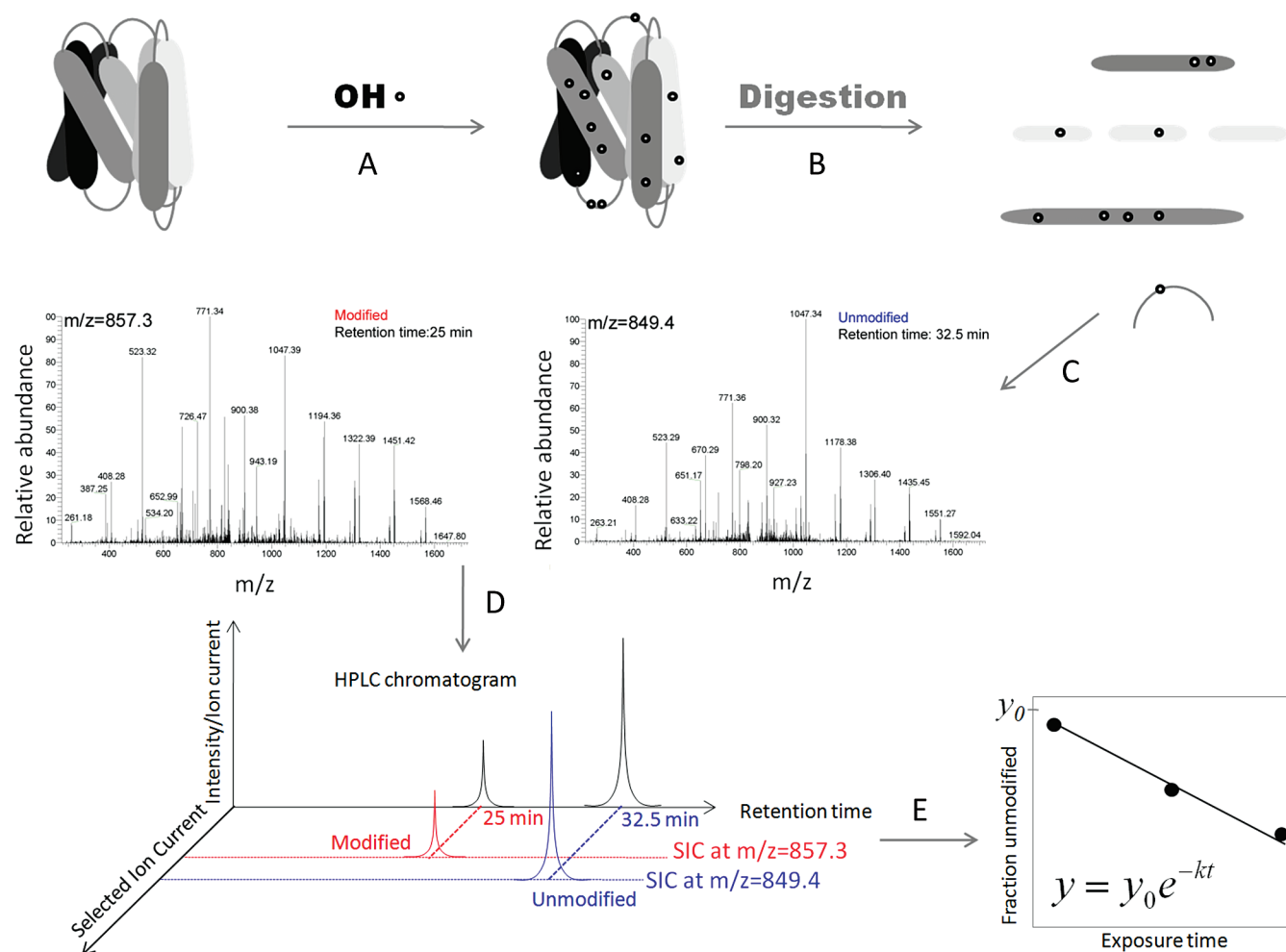
FIGURE 1: Radiolytic protein footprinting experiments and data analysis. (A) Proteins are exposed to X-rays for different time periods (milliseconds). (B) Following exposure, the protein is digested with a protease and its fragments are separated by using reverse-phase high-performance liquid chromatography coupled to a mass spectrometer. Searches for amino acid modifications such as +16 Da and other specific modifications (see Mechanisms of Amino Acid−OH• Interactions) are performed using programs such as Mascot (Matrix Science) and ProtMapMS (57). (C) Identification of the modified species on the basis of its MS/MS spectra. (D) The fraction unmodified is calculated by using the selected ion chromatogram peak area (modified colored red and unmodified colored blue). (E) The fraction unmodified is plotted as a function of exposure time, and the rate of modification is calculated by using nonlinear regression.

mediated by formation of the rhodopsin−transducin (G protein) complex. Contrary to its anticipated behavior, rhodopsin does not undergo large conformational changes following absorption of a photon of light, an event similar to binding of an agonist to other GPCRs (66). Superposed structures of bovine rhodopsin show little deviation (≤5 Å for a tip of helix VI and within 3 Å of normal protein dynamics for other parts); hence, the signal is probably transmitted by a different mechanism (67, 68). Such a mechanism was recently proposed following studies of radiolytic footprinting of this GPCR (52). The study confirmed that only small structural variations occur in the vicinity of the bound chromophore and furthermore offered a probable mechanism by which the absorption of a photon of light results in the conversion of GDP to GTP by the rhodopsin−transducin complex.

Figure 2 shows the residues of rhodopsin found to be modified following exposure to X-rays (52). Many modified residues are within the hydrophobic core; moreover, several amino acid residues exhibited unusually high rates of modification following exposure to X-rays. Phe116 had a particularly high modification rate, ~10-fold higher than that of any other residue in either rhodopsin or any soluble protein previously studied by radiolytic footprinting (18, 43, 53−55). Phe116 is located in the vicinity of

the Schiff base and close to a crystallographically identified water molecule (10) (see Figure 3A and also below). The high modification rate of Phe116 is attributed to its proximity to water molecule #2021 (10). In activated rhodopsin, changes in the modification rate of Phe116 result from re-positioning of the same internal water molecule. The residue with the largest change in relative rate constants upon rhodopsin activation was Met86. This residue is found in the vicinity of the highly conserved residue Asp83 previously determined to be critical for rhodopsin activation. A somewhat smaller change in modification rate (i.e., ~2-fold) was observed for residue Met288 upon activation. Although located in helix VII, Met288 is near the chromophore-binding pocket and crystallographically determined water molecules. Several water molecules were found to be conserved when X-ray structures of other GPCRs were compared (Figure 3B) (14). Moreover, these water molecules seem not to exchange with bulk solvent in any of the three rhodopsin states as demonstrated by rapid mixing with ^{18}O-labeled water (52). These results confirm previous predictions about the long-standing question of how rhodopsin transmits a visual signal in the absence of large conformational changes (69).
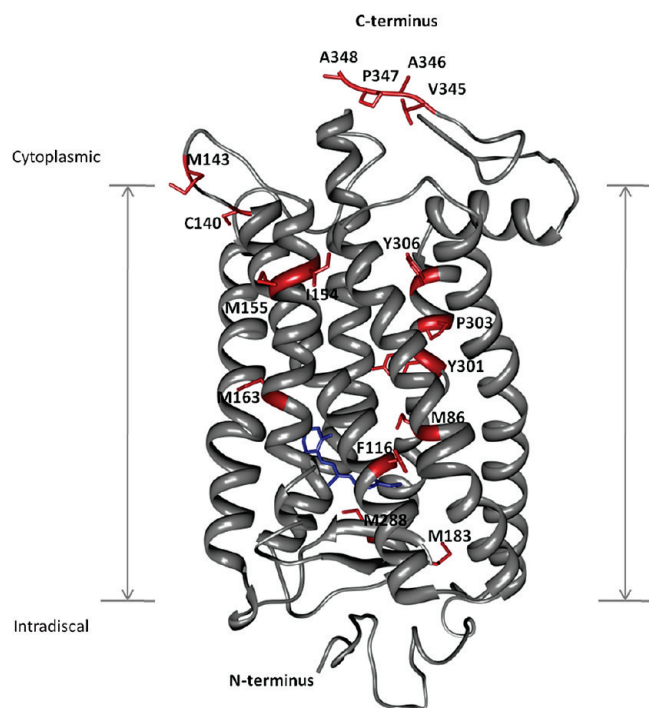
FIGURE 2: Footprinting of a model GPCR, rhodopsin. Rhodopsin is shown as gray ribbons, whereas amino acid residues found to be modified following exposure to X-rays are shown as red sticks. The N- and C-termini are indicated together with the transmembrane region. The rhodopsin chromophore, 11-*cis*-retinal, is colored blue near the intradiscal side of the membrane.

TRANSMEMBRANE WATERS DETECTED BY OTHER BIOPHYSICAL METHODS

Transmembrane Proteins and Waters Detected by Crystallography. GPCRs represent some of the most attractive drug targets because a high percentage of today's drugs act on these receptors. X-ray crystallography can elucidate the structure of proteins with atomic resolution that can ultimately be used as the foundation of structure-based rational drug design. Obtaining well ordered three-dimensional crystals is a critical step in determining protein structure by X-ray crystallography. Although a considerable number of soluble proteins have been determined to date, protein crystallization is still regarded an art. As one can imagine, additional challenges are presented in the case of membrane proteins attributable to their amphiphilic character. Unfortunately, because of the challenges posed by protein X-ray crystallography, the number of membrane proteins submitted to the Protein Data Bank (PDB) database is quite limited. Following the initial breakthrough of crystallization and subsequent determination of the structure of rhodopsin (*6*), several other GPCR structures such as bovine and squid rhodopsins (*10*, *11*), bovine opsin (*9*), β_1- and β_2-adrenergic receptors (*8*, *13*), and the A_{2A}-adenosine receptor (*12*) were determined. A recent study (*14*) performed in light of rhodopsin footprinting experiments (*52*) has shown that although the sequence similarity of these class A GPCRs is not high, several water molecules identified crystallographically within the hydrophobic region were conserved. Figure 3A shows the structure of rhodopsin, a seven-helix transmembrane protein (*70*), together with the
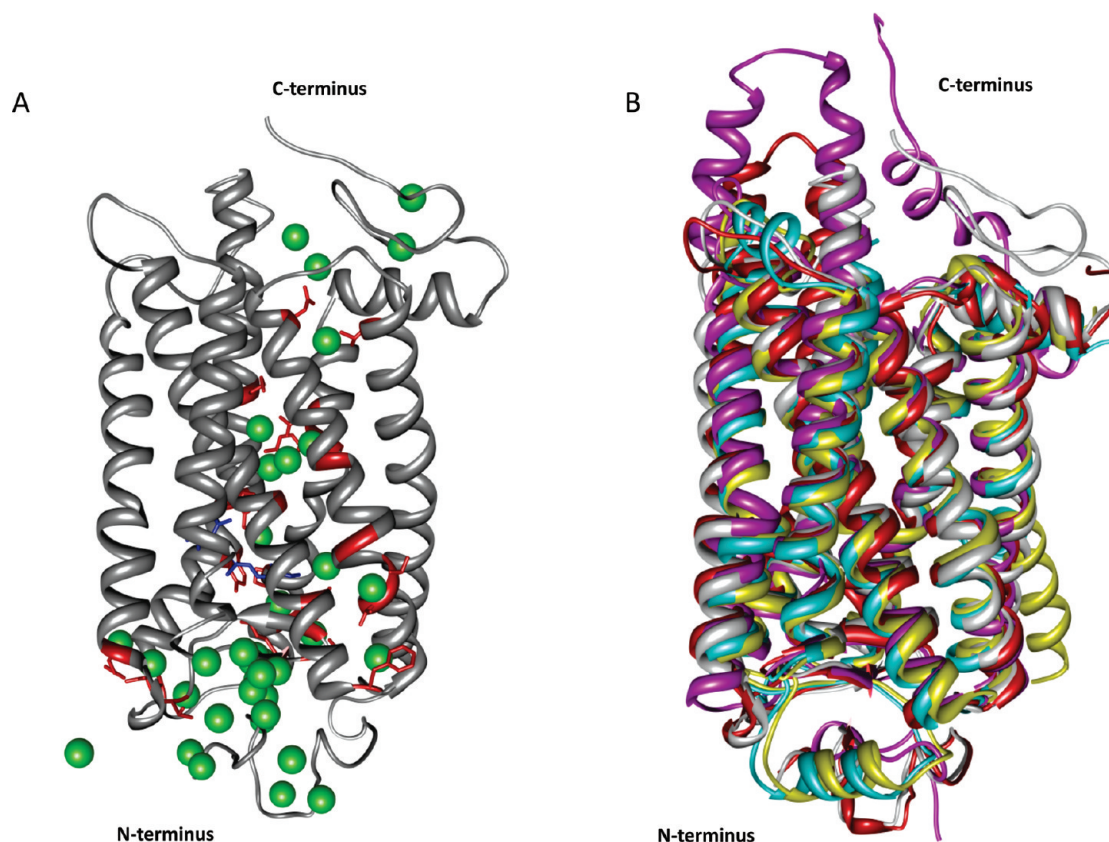

FIGURE 3: Transmembrane proteins and waters detected by crystallography. (A) Rhodopsin (PDB entry 1U19) is depicted with gray ribbons, and crystallographically determined water molecules are shown as green spheres. The rhodopsin chromophore, 11-*cis*-retinal, is shown as blue sticks, whereas amino acid residues from the transmembrane region in contact with water molecules are shown as red sticks. (B) Superposed structures of GPCRs such as squid rhodopsin (magenta), β_1-adrenergic receptor (cyan), β_2-adrenergic receptor (yellow), and bovine opsin (red).
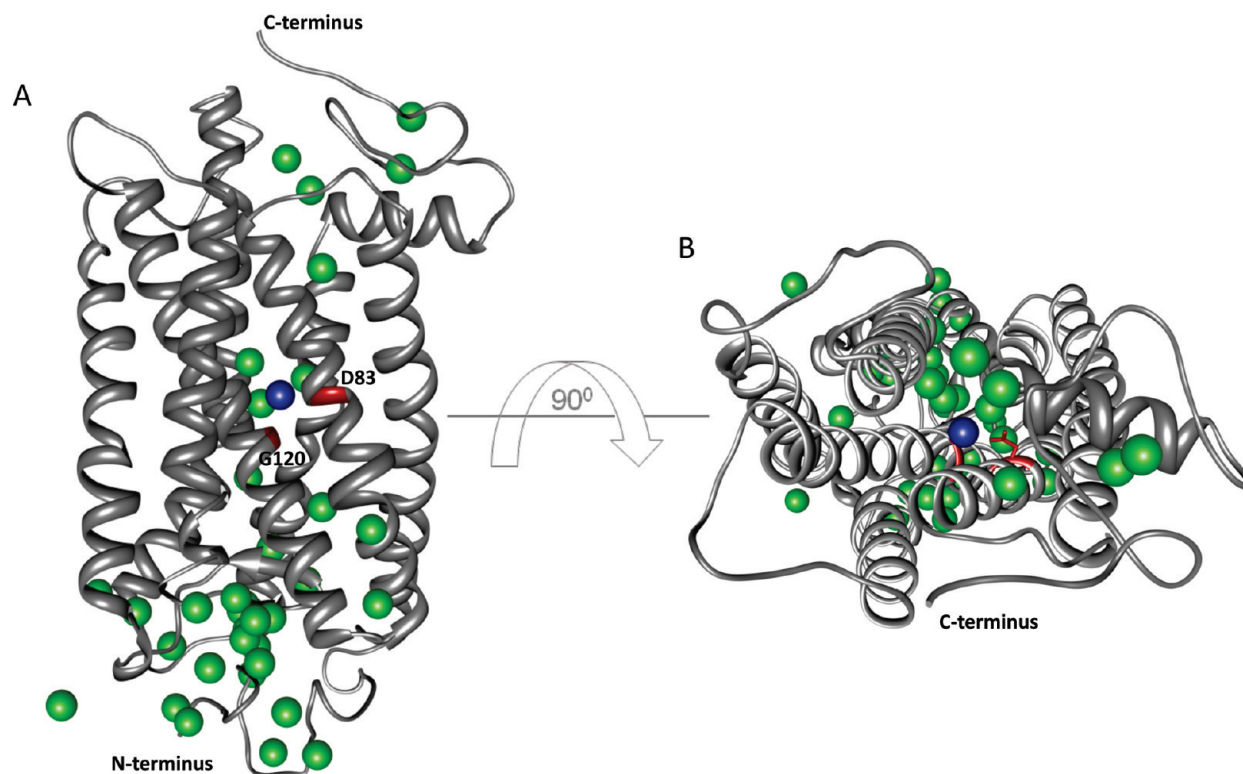
FIGURE 4: Transmembrane water molecules identified by FTIR. (A) Rhodopsin as gray ribbons, Asp83 and Gly120 colored red, and the interacting water molecule as a blue sphere. Water molecules determined by X-ray crystallography (PDB entry 1U19, chain A) are shown as green spheres. (B) Top view of rhodopsin with the same coloring scheme used in panel A.

crystallographically determined water molecules (PDB entry 1U19). A superposition of selected high-resolution GPCR structures is shown in Figure 3B. Although the alignment based on the α-carbons of these proteins is highly similar in the transmembrane region, regions found on either side of the membrane show considerable diversity as expected (*69*). The structure of several regions, such as the NPXXY motif (where X can be any amino acid) representing a highly conserved region among GPCRs (*71*), is greatly influenced by the presence of water molecules. Conserved water molecules were found to participate in hydrogen bonding networks that link transmembrane helices 2 and 6 (*12*) (Trp265···water #5772···water #5774···Asp83). These two residues, Asp83 and Trp265, are 94 and 71%, respectively, conserved among family A GPCRs. Another region linked by a hydrogen bonding network mediated by a water molecule is located between helices 6 and 7 in rhodopsin (*72*). In the case of the histamine H1 receptor (*73*), the hydrogen bonding network can be maintained with only one water molecule, but in the delta opioid receptor (*74*), the hydrogen bonding network is formed only by means of side chain interactions and the presence of water molecules seems not to be required.

Waters as an Integral Part of Proteins Detected by Fourier Transform IR Spectroscopy (FTIR). Fourier transform infrared spectroscopy (FTIR) was historically used to provide secondary structure information about biological molecules (frequency region being < 1800 cm^{-1}). It also has been successfully used to study water molecules within transmembrane proteins. FTIR spectroscopy can detect changes in hydrogen bonding strength based on the frequency of OH stretching vibrations (*75*). In the case of proteins, these vibrations are shifted from 2900 and 3200 cm^{-1} to 3200 and 3600 cm^{-1}, respectively, as a result of the strong absorption of bulk water. In the case of bacteriorhodopsin, water molecules were found

to be involved in the mechanism of proton pumping (*76, 77*). Specifically, several hydrogen bonding networks in which amino acid residues Asp85, Asp212, and Arg82 were implicated in the proton transfer mechanism were identified (*76*). Two water molecules were found to be altered in rhodopsin: the first in Meta I and the second at the Meta I−Meta II transition stage (*15*). Figure 4 shows rhodopsin with labeled amino acid residues Asp83 and Gly120 (red) and water molecule #2030 (blue sphere) (PDB entry 1U19) (*10*). Asp83, an important player in rhodopsin activation, is found near Met86, another residue shown to be important in rhodopsin activation by recent footprinting experiments (*52*). An important water molecule recently identified in the sensory rhodopsin (SRII)−transducer (HtrII) complex also was found to be located near residue Tyr199 (*78*). Thus, FTIR, crystallography, and footprinting all identify conserved and functionally important water molecules in membrane proteins, and these techniques can work in concert in improving our understanding of the role of water in mediating important functions such as ion transport and signaling.

FUTURE DIRECTIONS

Other Membrane Proteins and Ion Channels. The possibility of using footprinting to examine the details of water structure for a wide range of membrane proteins, both in vitro and in vivo, is discussed in this section. For example, structures of GPCRs other than rhodopsin have recently been published (*8, 12*); these structures, which are greatly similar to that of rhodopsin, provide interesting potential insights into GPCR signaling, but engineering of these proteins to enable the generation of diffraction quality crystals raises questions about their functionality. Radiolytic protein footprinting and FTIR studies, on both native forms and the engineered species, could provide novel insights into the presence and activity of bound waters in these proteins as well as their ligand-dependent signaling mechanisms.

Recently, radiolytic protein footprinting was used to understand the details of the conformational dynamics and the amino acids involved in K-channel gating (*79*). The closed state of bacterial inwardly rectifying K-channels is well understood (*80*), although high-resolution data for the open state are not yet available (*81, 82*). Footprinting data for detergent preparations of the inwardly rectifying (Kir) potassium channel of *Magnetospirillum magnetotacticum* (KirBac 3.1) in both open and closed states were collected, and the data were analyzed in the context of the known closed state structure. Differences in the extent of modification observed between the closed and open states were then used to reveal local conformational changes that occur during channel gating. The data suggest that specific side chain residues located in the inner helices of the transmembrane membrane pore as well as residues toward the extracellular side of the transmembrane region are involved in gating. These findings provide a basis for understanding the dynamics of gating at the side chain level for a wide variety of bacterial and eukaryotic channels in the presence of a range of ligands and other effectors that tune the gating process.

Because strongly bound waters can have long residence times, radiolytic labeling by short highly intense pulses of synchrotron X-rays or electron beams can generate local spurs of hydroxyl radicals that activate bound waters that rapidly modify adjacent side chain groups to provide information about the local dynamics of functionally important amino acid residues. In addition, recent advances in radiolytic labeling coupled to $H_2^{18}O$ exchange can probe the dynamics of membrane protein signaling and gating (*52*). In the latter experiments, $H_2^{18}O$ mixing of proteins followed by X-ray irradiation or pulse radiolysis generates ^{18}O-labeled covalent modifications primarily of aromatic or sulfur-containing groups that are accessible to bulk solvent. Time scales of these mixing experiments can be as short as 1−2 ms; thus, one can delay irradiation subsequent to mixing and monitor the time course of the movement of bulk water into crevices, channels, or pores for defined states of interest.

Prospects for developing new footprinting tools for in vivo probes of macromolecules are encouraging. In vivo footprinting of cells has a long history, and the use of X-rays to analyze nucleic acids structure in cells has been an important tool (*83*). Since a predominant constituent of a cell is water, ionizing radiation can generate hydroxyl radicals transiently inside and outside cells. However, nonradiolytic techniques of in vivo footprinting require harsh treatment of cells, whereas radiolytic approaches traditionally have required long exposures to γ-rays. Recently, in vivo X-ray footprinting was used to identify the folding pattern of a 16S rRNA structure in frozen *Escherichia coli* after subsecond exposures (*84*). The use high-flux X-rays from a synchrotron as the source of hydroxyl radicals reduced the exposure time considerably. With respect to protein footprinting, in situ generation of hydroxyl radicals by Fenton's reagents has shown different extents of oxidation for the open and close states of the porin, OmpF, in *E. coli* cells (*85*), thus providing a proof of principle for in vivo hydroxyl radical footprinting analyzed by MS. However, this requires a prolonged exposure to Fenton's reagent, so the approach is suitable only for analysis of extracellular motifs, unless cells are made porous. However, high-flux radiolysis sources coupled to MS analysis have the potential to provide very short time scales of exposure for the examination of structures and dynamics of living systems.

Generating Cross-Linked Side Chains. Additional applications of radiolysis chemistry yet to be explored may represent future directions for research. There are a myriad of chemical species, including hydrated electrons, which can be produced or consumed depending on experimental conditions. The history of radiolysis research includes not only aerobic radiolysis of bulk aqueous solutions (*86*) but also radiolysis: (1) under anaerobic conditions, (2) in the presence of scavengers, (3) after addition of N_2O to convert hydrated electrons to hydroxyl radicals, and (4) applied to gels and solids (*87, 88*). There are many biological structures and states, including membranes, which could be explored with this technology. In the presence of oxygen, radicals transferred from hydroxyl radicals to protein side chains are efficiently quenched, leading to the stable products observed. However, in the absence of oxygen when the observed yield of oxidized products is reduced (*23*), lifetimes of protein-derived radicals are extended. This raises the prospect of potentially effective zero-length cross-linking of radicals found on side chains in the proximity of each other. This could be explored as an efficient cross-linking strategy both in vitro and in vivo that would target multiple native residues without any engineering, compared to current methods that typically require use of native Cys or Lys residues, or their insertion in defined positions. The resulting data could provide distance constraints informative of tertiary structures as well as of quaternary structures within a protein complex.

In summary, visualizing water in transmembrane proteins using mass spectrometry is an important advance, and the use of radiolysis-related technologies to permit labeling of many types of macromolecules mediated by their interactions with water is developing rapidly. The coupling of hydroxyl radical labeling and MS is especially powerful for the study of membrane proteins, as femtomoles to picomoles of material are all that is required to provide very detailed examinations of structure. In addition, the study of more complex systems as well as the state of membrane proteins in vivo is increasingly possible. With further increases in the time of molecular dynamics simulations, it will be possible to merge experimental and theoretical approaches to gain a more realistic and mechanistic view of how membrane proteins work, including how they respond to the binding of ligands and transmit signals to partner proteins.

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