Current place and perspectives of usage of X-ray radiolytic footprinting in study of structure and function of membrane proteins.

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Abstract: Membrane proteins, such as receptors, transporters and ion channels control the vast majority of cellular signaling and metabolite exchange processes and as such are increasingly becoming the key pharmacological targets. Difficulties of handling membrane proteins in high concentrations and requirements of membrane environments for their stabilization have made them difficult to study using traditional structural biology techniques, requiring the use of a hybrid, integrative approaches to study their dynamic properties and functional aspects in physiologically relevant conditions. In recent years significant progress has been made in the field of oxidative labeling techniques, and particularly X-radiolytic labeling in combination of mass spectroscopy (XF-MS) allowing these approaches to mature and provide valuable insights into structure and function of proteins, including membrane targets, which is difficult to obtain by other techniques, complementing available structural data. XF-MS has demonstrated unique capability for identification of structural waters and conformational changes in proteins at both high degree of spatial and temporal resolution. Here we provide a perspective of the place of XF-MS amongst other structural biology methods and showcase some of latest developments in usage of XF-MS in solving water mediated transmembrane signaling, ion transport, ion gating as well as ligand induced allosteric conformation changes in these membrane protein complexes.

Keywords: hydroxyl radical footprinting, mass spectrometry, ion channels, transporters,

Introduction.

Membrane proteins, representing around 30% of all proteins in the studied genomes, are crucial for cellular processes forming the core of the cellular sensory cascades and metabolite transport and hence are prime pharmacological targets1,2. Elucidation of their functional mechanisms which could be exploited to therapeutic means relies on our detailed understanding of their structural transitions. However, membrane proteins have earned a deserved reputation of being difficult targets for structural investigation. While classical structure determination approaches such as crystallography and NMR provide high resolution information, they are limited in terms of sample preparation requirements (i.e. the necessity for crystallization, high protein concentrations, and/or non-physiological buffer conditions)3-5. Other structural techniques such as electron microscopy6-8, small-angle scattering9,10 and EPR11,12 can provide novel structural information, but are limited in resolution, and are often conducted in non-natural environments. Recent advances in development of the oxidative labeling techniques coupled with mass-spectrometric analysis of the data, provides an alternative and highly complementary approach to these more common structural tools, with the advantage of providing amino-acid resolution under near-physiological conditions13. In this review we highlight the basic principle of oxidative labeling, its place relative to other mass-spectrometry based methods and its advantages in comparison to other available methods in application to membrane proteins and their complexes, using some recent examples of its successful applications. We also outline the latest developments and future promises of this exciting technology to study membrane protein structure and dynamics.

Part I: Comparison of different MS-based labeling approaches for study of protein structure and the place of oxidative labeling.

1.1 A brief history of footprinting approaches.

Footprinting as a technique has long been a staple of molecular biology. In a typical footprinting experiment, a control pattern of modification or degradation of a target macromolecule is being compared with the pattern obtained in the presence of a partner molecule or a ligand, revealing differences resulting from conformational changes of the target or suggestive of protection upon complex formation. Its usage for DNA both in vivo using dimethylsulfate
(DMS), a purine-methylating agent, or in vitro DNase I protection goes back to the 70es. A number of attempts to get a viable protein-protein interaction footprinting technology followed in the next couple of decades. It was not however until the mid-90es when the first demonstration of the feasibility of using modification strategies to footprint proteins have been developed. Using a combination of both reversible and irreversible Lys-targeting agents and a Lys-specific endopeptidase utilised such mapping for study of vaccinia virus topoisomerase. Nonetheless these approaches remained rather laborious and ultimately did not gain traction, so almost another decade had to pass so that the advances in mass-spectrometry (MS) and proteomics have finally allowed the development of several techniques capable of site-specific analysis of protein-protein interactions.

1.2. MS based approaches for studying protein-protein interactions.

When using native proteins, MS may be suitable for analysis of complexes, which are either very stable (e.g. immune complexes) or sampled using mild conditions – in what has become known as native MS. Indeed, epitope mapping by MS is possible in antibody-antigen complexes, which are robust enough to survive MALDI deposition and desorption. Individual epitopes could be identified by their respective ion's decreased signature in the antibody treated samples. Majority of the studied systems however are not stable enough for such approaches, and they are also not well adapted for study of conformational dynamics or solvent accessibility, however introduction of MS-identifiable labels into the studied proteins can overcome these issues.

As a result, over the recent years, the fast development of the mass-spectrometric methods has largely displaced the proteolytic-based footprinting approaches. There are a number of different flavours of methods but in general they rely on attachment of a probe to the surface of the protein, and/or crosslinking with subsequent identification of the location of the label by MS-based sequencing of the protein following a proteolytic digest. One particularly noteworthy MS approach is based on the usage of chemical cross-linkers to identify local proximity of residues, either in a protein in isolation or following a complex formation. Guided by available crystal structures of proteins in isolation, this is a powerful technique which can be used to map the interactions in large complexes. This approach, which has become known as CLMS (cross-linking/mass-spectrometry) has been successfully applied to the 180kDa Nde complex and a 15-subunit, 700kDa complex of RNA Pol II with TFIIF. Cross-linking efficiency may differ, and ultimately is restricted by the availability of cross-linkable residues, typically Lys or Cys, depending on the type of the cross-linker. It is rather difficult to quantify the effects and provide time-resolved studies, although some progress have recently been achieved using isotope-labels. An alternative method, also based on MS identification of the modification tag is the usage of isotope-coded affinity tag (ICAT) reagents, which can be used to sample cysteine accessibility. 13C labeled reactive tags (e.g. bromoacetamide) are first applied towards natively folded proteins (including in vivo), following which 12C tags are applied post-denaturation, allowing to identify accessible areas. Such ICAT footprinting was successfully used to map the surfaces of CheW that interact with the large multidomain histidine kinase CheA, as well as with the transmembrane chemoreceptor Tsr in native E. coli membranes.

Another powerful method based on MS usage is the H/D exchange (HD-X). This is a reversible modification method which relies on a MS detection of deuterium in a studied peptide sequence as a result of a exchange with amide hydrogen from the backbone of the target protein. Such exchange is only possible in solvent exposed regions, and the speed of exchange can be correlated with the relative accessibility. A significant drawback of the method is the continuous exchange of the H/D that often cannot survive the required handling of the sample and/or purification.

1.3. Oxidative footprinting or hydroxyl radical footprinting.

As its name suggests the oxidative footprinting approach relies on covalent modification (oxidation) of solvent accessible residues in the proteins, which are subsequently identified by means of quantitative mass spectrometry. There are several ways to generate these radicals. One approach is based on oxidative Fenton chemistry using Fe(II)-EDTA, which has been pioneered by Tuullius & Dombrovski for DNA footprinting. This oxidative cleavage approach was demonstrated to be usable for protein mapping as demonstrated early on. It has been commercialised under the name of FeBABE (referred to as "Iron Babe") (Pearce Biotechnology/Life Technologies), and uses Fe(III) (S)-1-(p-Bromoacetamido-benzyl)ethylenediamine tetracetic acid as a sulphydryl-reactive reagent that incorporates an Fe(III) EDTA moiety into a purified bait protein. The bromoacetyl group allows the reagent to attach covalently attached to sulphydryl groups. When activated by ascorbate and peroxide, the Fe(III) EDTA group cleaves peptide bonds, acting as an artificial protease using oxidative Fenton chemistry. This technique has been recently used with success in mapping the interactions within the large RNA Pol II pre-initiation complex. This approach has recently been successfully adapted for in vivo application in bacterial cells. Alternatively the active hydroxyl radicals used for the oxidative labelling can be generated using laser photolysis employing UV laser for dissociation of peroxide molecules. The method has become known as Fast photochemical oxidation of proteins (FPOP). Another method is actual generation of the oxidative species "on the
fly” using electrospray MS with high needle voltages and oxygen as a reactive nebulizer. However, the most popular currently employed approach is the usage of ionizing radiation for creating the active radicals from water, in a process that is known as radiolysis. Radiolysis could be achieved using gamma rays, or high energy electrons; however it is prevalently achieved using high-density X-rays from synchrotron sources, and it is hence commonly known as X-ray radiolytic footprinting or XRF, or when paired with mass-spectrometric analysis XF-MS. Both terms are used interchangeably from here on.

1.4. Basics on XRF.

Although it uses a different mechanism of generation of radicals, the principles of the XRF are essentially the same as the rest of oxidative labeling approaches. To generate reactive hydroxyl radicals in situ a high brilliance flux of white-light photons (~10^{12} per second) of varying energies in the 5-30 keV is usually applied, although a monochromated beam could also be used. The general reaction leading to generation of the reactive hydroxyl radicals in non-oxygenated solutions is the following:

\[ 2H_2O + e^- (hv \text{ ionising}) \rightarrow H_2O^+ + H_2O^+ + e_{aq}^- \]

Where H_2O* signifies excited water molecules. These excited molecules dissociate to hydroxyl radicals in less than 10^{-13} s, while the H_2O* reacts with another water molecule generating an additional hydroxyl radical

\[ H_2O^+ + H_2O \rightarrow H_2O^+ + HO^* \]

The general scheme of the typical XF-MS experiment is presented on Figure 1. In brief, a protein sample (in the micromolar range) is subjected to a short pulse of radiation using a microfluidic device or a fast kinetic setup such as KinTek, which causes oxidation events in the microsecond timeframe, following which the reaction is rapidly quenched using a reducing agent (e.g. methionine amide). The sample is then retrieved and is processed similar to an MS sample for proteomic analysis, with several steps that may include detergent removal, protease digestion and LC-MS/MS-MS identification of the fragments which have modifications. Experiments usually include a number of different exposure times, which are compared against a control, e.g. protein on its own vs protein in the presence of a partner. Dose-response (DR) curves are calculated for each peptide fragment analysed, and the effective dose received is calibrated using internal references, such as Alexa. Depending on the context, the detected differential labeling then can be attributed to conformational changes or interactions with partners.

Figure 1: General scheme for steady state and time resolved studies

Membrane proteins are surrounded by a number of extrinsic •OH scavengers (cofactors, detergents, phospholipids) that reduce the effective dose to the protein in comparison to soluble targets. An insufficient photon flux density requires longer time for delivery of the equivalent dose, which in turn increases secondary radiation damage resulting in poor data quality and artifacts due to protein unfolding. In fact, XF studies on several membrane protein were only made possible after the installation of a focusing mirror, which increased the useable flux density by 14 fold. A short duration of high flux dose provides better tolerance to the structural integrity of complex protein assembly and is a key factor in obtaining high quality data. The usage of pulsed beams allows to limit the radiation damage and the amount of hydroxyl radicals generated – (typically to between 0.2 to 3 μM range in deaerated buffers, thus also making sure that one-electron oxidized radical species and not two-electron oxidized forms are created). The new XF facility at the ALS and the upcoming XF beamline at NSLS II is designed to provide the higher flux density that is necessary to allow investigation of complex integral membrane proteins and extend current beamline capabilities to the sub-millisecond timescale.

Similar to the synchrotron radiation pulses laser photolysis and pulsed electron beams could operate on nanosecond scale, also allowing to sample protein conformational dynamics in real time. However, chemical or laser/H_2O_2 production of •OH relies on addition of reagents that can oxidize/damage and unfold proteins, as well as remove essential metal ions necessary for protein function. To the contrary, unlike laser photolysis however, XF-MS is a straightforward technique and the dose can simply be controlled by irradiation, permitting to quantify the reactivity rates of the modifiable sidechains. The most common variation utilizes a pulsed white synchrotron beam to which the sample is exposed on a millisecond and with the current advancements microsecond scale, however monochromated beams could also be used. One of the unique advantages of synchrotron-based XF-MS (5-10 keV) is that it has the ideal energy range to penetrate the sample solution and the cell membrane without severe perturbation to the protein. In addition, the high flux density provided by the latest generation of synchrotron sources has the potential to reduce the irradiation time to submillisecond and thus limits the secondary damage to the protein. In contrary, electron beam radiolysis (e.g. using a Van de Graaff generator), can generate modification submicrosecond, however, the energy deposition by 2 MeV electrons causes electron-induced protein damage. Further technical aspects of radiolysis have been covered extensively elsewhere and we will just highlight some of the advantages of the approach in the section below.
1.5. Advantages of XF in comparison to other methods.
In comparison to the other MS-based methods discussed above, the oxidative footprinting has a number of inherent advantages, e.g. it does not require any prior modification of the protein via mutagenesis or introduction of chemical labels, and unlike most of other label-based approaches relies on the smallest possible probe – the hydroxyl radical, which is usually generated in situ in aqueous solutions close to physiological conditions.

Another major advantage of the technique is that it uses a relatively non-specific oxidation reaction, which affects most amino acid residues, given their proximity to a ionisable water molecule. Thus, it is relatively independent of the protein sequence and it is also particularly effective in labeling hydrophobic and aromatic residues which are otherwise rather inert.\textsuperscript{13,48}

Importantly, it can be applied in microsecond timescales approaching the scale of conformational transitions in proteins, and in a time-resolved fashion allowing to sample conformational dynamics in proteins. As a covalent modification method it also doesn’t suffer from the issue of loss of signal and post-exposure artefactual labeling which could be an issue with H/D-X.

Oxidative labeling coupled to high-sensitivity MS also requires very little sample (nanogram amounts of protein) in comparison to many other structural biology techniques, which may be a major issue for difficult to obtain complexes and membrane targets.

Perhaps most importantly it provides direct information on the solvation state of a given residue and, as will be shown further below, on the role of bound water.

PART 2: Examples of usage of oxidative labeling in combination with MS for study of biological systems.

2.1 Complexes
The first successful application of the oxidative footprinting to study of protein complexes was in the case of the calcium dependent interactions of actin-gelsolin\textsuperscript{49}, and calmodulin-mellitin\textsuperscript{50}. In this case the radicals were generated in situ by coupling electrical discharges with electrospray MS. Soon after pulsed X-ray radiolysis was used to reveal for the first time the interaction of actin with coflin, highlighting the utility of the approach for studying difficult targets which are prone to polymerisation and hence difficult to handle by other structural approaches\textsuperscript{51,52}. The method has since successfully tackled ambitious complex targets such as the acto-myosin complex\textsuperscript{53}. Unlike most of the other structural biology methods XF-MS is not limited by the size of the studied complex, and as such it is very well suited for analysis of multiprotein complexes, including megadalton assemblies such as the Clp proteasome\textsuperscript{54} and even ribosome assemblies\textsuperscript{55,56}. While these ribosomal works are following the RNA-patterns, and are not strictly speaking dealing with protein-protein interactions, the latter is particularly significant as it describes an in vivo application of XRF.

Membrane protein complexes. The successes with soluble protein complexes, paved the way to the usage of XF-MS for more challenging membrane protein targets such as photosystem II (PSII), responsible for the light induced water splitting and production of oxygen\textsuperscript{57}. It functions as a dimeric supercomplex each of the parts of which contains 20 subunits. This study also validated the functional existence of the hypothesized water channels leading from the surface of the complex to the Mn\textsubscript{4}O\textsubscript{8}Ca cluster, that has been inferred from the high resolution structures of PS II\textsuperscript{58}. Another recent radiolytic footprinting study that combined XF-MS with crosslinking\textsuperscript{59} extended these findings providing new insights on the dynamic aspects of the interaction between the components of PSII and demonstrating the crucial importance of water molecules, which are in fact an integral part of the protein.

While these studies showcase the potential of the XF-MS to study of large membrane protein complexes, perhaps even more exciting than the ability of the technique to identify solvent channels and cavities within the membrane proteins however is its unique ability to detect bound water.

2.2. Bound water detection in by XF-MS
Bound or structural waters are required for folding, stability, enzymatic activity and protein-protein interactions\textsuperscript{60}. In particular, it has been shown that water has a significant role in forming the active site in a prototypical GPCR receptor, rhodopsin\textsuperscript{61}. Moreover, a comparison of known GPCR structures revealed that water molecules in the hydrophobic core of these proteins interact with conserved residues, implying that these waters are probably as important for function as the conserved residues themselves\textsuperscript{62}, forming an integral part of the signal transduction cascade. In potassium channels, bound water was also shown to play a major role in their slow inactivation while cavities inside transmembrane domain are formed by the interaction between bound water and amino acid residues\textsuperscript{63}.

Traditionally, water dynamics are explored using high-field NMR, such as nuclear Overhauser effect (NOE) and magnetic relaxation dispersion (MRD) methods. These techniques can indirectly calculate residence times of internal waters in proteins in the timescale from subnanoseconds to milliseconds\textsuperscript{64-70}. However, our understanding of site-specific structural and functional implications of bound water is limited due to lack of direct experimental evidence under near-physiological conditions and limitations of time-scale for many exchange-competent technologies.

As shown earlier, XF-MS has the unique ability to generate local spurs of hydroxyl radicals that activate bound waters, which rapidly modify adjacent side chain groups, providing a direct probe of the local dynamics of functionally important amino acid residues on millisecond timescales\textsuperscript{45}, even in cavities insulated from external solvent. **Temperature dependent XF**, allows to differentiate between bound and bulk water interactions and has been
successfully applied to small globular proteins such as cytochrome c and ubiquitin to identify bound water interactions on the surface and in cavities.

Recent development of time-resolved radiolytic labeling coupled to H\textsuperscript{18}O exchange demonstrates that footprinting can probe the dynamics of residues adjacent to bound waters. (see Figure 2). This is based on the fact, that the \textsuperscript{18}O from the attacking hydroxyl remains attached to the Met, Phe, Tyr, Trp, Cys residues, while in other side chains the covalently attached oxygen is derived from a secondary oxidation event.

Figure 2: General scheme for water exchange and labeling.

Unlike NMR approaches, this method is not limited by molecular size and sample concentration. However, the current time resolution is limited by the millisecond irradiation interval. Recently developed ultra-fast continuous flow mixing device, in combination with short pulse of intense X-rays from the focused bending magnet source at ALS or NSLSII will allow monitoring of the time course of the dynamics of the exchange of bound water in and out of crevices, channels, ion pumps or membrane protein pores on microsecond timescales, contributing significantly to our understanding of ion-transport and ion gating.

2.3. XF-MS in study of GPCRs

The conserved internal water network in the transmembrane domains of GPCRs participates in the transfer the signal from the chromophore or the agonist-binding site to the allosteric sites ultimately regulating the G-protein activation for signal transduction. In particular, in the photo-activation processes, which are well studied in the example of mammalian rhodopsin, demonstrated the involvement of transient intermediates leading to the so-called Meta II state that is competent of G-protein binding. The available X-ray crystal structures of rhodopsin and its several photo-intermediates have dramatically increased our understanding of structural rearrangements upon the activation of GPCRs. But it is also increasingly clear that static structures alone are not sufficient to provide a complete understanding of GPCRs function, especially given the prominent role that is played by structural waters, which are only visible in very high resolution crystal structures, that are hard to obtain for a number of membrane receptors. XF on the other hand has emerged as a novel approach to study GPCR by in situ labeling of transmembrane (TM) residues, located in proximity to bound water. The first molecular details of the photostimulation process came from the comparative XF studies of dark state, meta II and opsin from detergent-solubilised samples. These results are summarized in Figure 3 showing residues of bovine rhodopsin modified by XF. Figure 3: highlights of results on Rhodopsin and 5HT4

Results indicated an increase in labeling efficiency near the retinal-binding region, conserved TM domains and in a few residues of cytoplasmic and extracellular loops upon activation. The local conformational changes arising from the isomerization of the covalently bound retinal appear to be propagated to the cytoplasmic surface by means of water reorganization, and rearrangement of H-bonding network between bound water and amino-acid side chains in the TM domain as demonstrated by \textsuperscript{18}O-labeled water exchange studies. Combined XF and HDX experiments included the investigation of conformation changes due to the binding of heterotrimeric G-proteins. Monitoring the amount of HO\textsuperscript{−} radical-induced modification on rhodopsin, activated rhodopsin (meta II) and activated rhodopsin-G-protein allowed to elucidate the fine details of the dynamics of the internal water rearrangements that control the G-protein binding as well as location of the protein-protein interaction in both rhodopsin and G protein. This allowed to XF-MS to be used to structurally validate a homology model for 5-HT4 receptor, for which no high resolution structure was available, including predicted sites for internal water-side chain interaction, highlighting another important application of the XF-technology.

2.4. XF-MS to study K\textsuperscript{+} ion channels

Ion channels present exceptionally good targets for study by XF-MS, as their activation and the associated channel “gating” events usually result in dramatic changes in water accessibility of the central pore of the channel which is, in resting state, devoid of water, presenting a major energetic barrier to ion conduction. In the case of potassium channels, these gating events are connected to a variety of regulatory stimuli and include several critical conformational transitions that are suggested to propagate from the inner side of the membrane, including the so-called bundle crossing, which is thought to be the principle gating mechanism for the majority of the K+ channels, and it is absolutely required to be in an open conformation in order for the channel to be in a conductive state.

The study of the bundle crossing gating using crystallography proved to be challenging as the closed state appears to be energetically favourable resulting in channels preferentially crystallising in closed state, while their size precluded NMR analysis leaving the question of structural transitions during gating unanswered for a number of years. The first K-channel to be studied by XF-MS was KirBac 3.1, and the comparison of the closed state of the channel with a EDTA-induced opening revealed a dramatic increase of the solvent accessibility along the central cavity of the channel, as well as along the interface between transmembrane (gating) domain and the cytoplasmic regulatory domain of the channel, and conformational transitions in which were suggested to induce opening of the channel. In addition this study highlighted the possibility of the presence of a hydrophobic gate in this
inward rectifying potassium channel, namely the residue L124 which showed the highest level of change in accessibility between the two open and closed conformation of the channel. These prediction have been confirmed in full by the crystal structure of the open state of the channel. Notably, although the existence of such hydrophobic gating for K-channels has been suggested much earlier, the XF-MS directly visualized the role of the L124 during the transition. As hydrophobic gating is suggested to play a major role in a number of channels lacking traditional “bundle crossing gate”, such as K2P channels and a number of other channels including pentameric ligand gated and the bacterial mechanosensitive channels, XF-MS appears an ideally suited tool to interrogate the gating transitions in these channels in the future.

Similarly, during activation the pH-dependent bacterial KcsA channel, undergoes gating at the bundle crossing with several charged residues suggested to play the role of proton sensors. KcsA also possesses a unique C-terminal domain forming a cytoplasmic protrusion, which imposes steric limits on the bundle crossing gate and the contraction of which appears to control the opening of the channel and its inactivation. Using the comparison of water accessibility between the wild type and non-inactivatable mutant E71A of KcsA we were able not only to confirm the significant changes of the water accessibility of the C-terminal domain (Gupta, Chance, Tucker & Bavro in preparation) but also to demonstrate radical changes in the solvent profiles of residues behind the selectivity filter of the channel which harbours the E71A mutation. These findings are consistent with the recent suggestion of the important role played by structured/bound water in stabilizing the conductive selectivity filter conformation and highlight the high potential of XF-MS for study of these systems.

**Figure 4:** highlights of results on KirBac.

### 2.5. XF-MS to study ion transporter

Developing an experimental approach that can monitor proton movements at molecular level would have a major impact by providing valuable detailed understanding of structural and dynamic elements of the coupling mechanism by which the proton gradient is utilized to pump substrates against their concentration gradient by a number of secondary transporters. A recent study capitalized on the unique capabilities of XF to investigate the mechanism of a prototypical proton-coupled Zn$^{2+}$ transporter YiiP from the cytoplasmic membrane of *E. coli*. Intriguingly, the crystal structure of Zn-YiiP showed absence of any polar residue that can carry the proton to the Zn binding or transport site and identified a presence of hydrophobic barrier that divide the transport pathway between intra- and extra-cellular cavity. Comparative XF analysis on Zn$^{2+}$-bound and Apo-YiiP identified functionally important residues adjacent to these cavities and also within the hydrophobic barrier, and showed that they undergo large, reciprocal water accessibility changes. In particular, the active site Zn-binding residue D49 (TM helix 2) and residue L152 forming part of the hydrophobic gate, located on TM helix 5 (TM5), showed significant decrease in water accessibility upon zinc binding which is consistent with the available crystal structure of Zn-YiiP. Zinc access to the transport-site appeared to shut off water access to L152, suggesting that L152 may function as an inter-cavity gate that controls alternating access of zinc ion and water molecules to the transport-site. In contrast, increased water accessibility changes were observed in several methionine residues located at the N- and C-terminus of TM5, which are either located on the opposite TM5 face (L152) or packed against the opposite TM5 face (including D49). This reciprocal change in water accessibility on two opposite faces of the TM5 is consistent with its re-orientation in response to zinc binding, and provided a novel mechanism for zinc transport. In summary, XF-MS revealed that TM5 underwent a rigid-body re-orientation upon zinc binding and suggested that the release of zinc binding energy in the hydrophobic core might get transformed to mechanical energy to reorient TM5 and close the L152 gate.

This study highlights the potential of XF-MS for study of various membrane transporters, which would be of interest to the wider transporter community.

**Conclusion:**

Over the past decade, oxidative labeling, and more specifically it’s X-ray radiolytic footprinting (XF) variation has been developed into a nearly routine technique and has been applied to a diverse range of biological systems, yielding unique structural insights impossible to obtain by other standard high resolution structural methods. Synchrotron based XF has been used to elucidate structural changes within several membrane proteins, including a G-protein coupled receptors, potassium channel, photosystem proteins and ion transporters during their activated or functional states. Availability of high resolution X-ray crystal structures of these proteins in the their native or ground state has allowed to expand the use XF approach to determine plausible role of transmembrane structural water for regulation of their functional states. Moreover recent advancements in experimental strategies with the high flux density X-ray beam at the synchrotron facilities, together with significant improvement in mass spectrometry-based data analysis methodologies serve as an ideal platform for the development of a unique, integrative structural method universally suited to study any membrane protein system. XF is a future-proof method, which requires very low protein sample concentrations, yields residue-specific interaction, and can provide both structural and dynamics information. It is an ideal complement for both high and low-resolution structural studies, e.g. allowing the accurate placing of the components within low-to-medium
resolution maps form cryo-EM or solution scattering studies, while it can provide information on protein dynamics to static crystals structures. Given the above, there is little doubt that XF-MS has the potential to become one of the most important tools in solving structural problems related to large complexes and membrane proteins by a using hybrid approaches.

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