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Flexible membrane proteins: functional dynamics captured by mass spectrometry

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Membrane proteins are flexible molecular machines, responsible for the exchange of molecules in and out of the cell, which have evolved to perform specific and complex tasks with great efficiency. Obtaining accurate descriptions of their dynamics in the context of their function represents a major challenge for structural biology. Here we chart recent developments in mass spectrometry designed to characterize changes in the dynamics of membrane proteins in response to ligand binding or post-translational modifications. We focus on cooperative movements and structural changes across a range of timescales, from milliseconds to minutes, and highlight the contributions of mass spectrometry to our understanding of molecular mechanisms of diverse transmembrane processes.

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Introduction

Biological membranes delimitate the cellular entity and consist of phospholipid bilayers in which proteins are embedded [1]. Membrane proteins mediate much of the function of the cell, including energy conversion, transport, signaling and recognition [2]. As such it is not surprising that membrane proteins comprise more than half of all known drug targets [3]. Uncovering the molecular mechanisms of these proteins in action therefore represents a prominent frontier in modern structural biology.

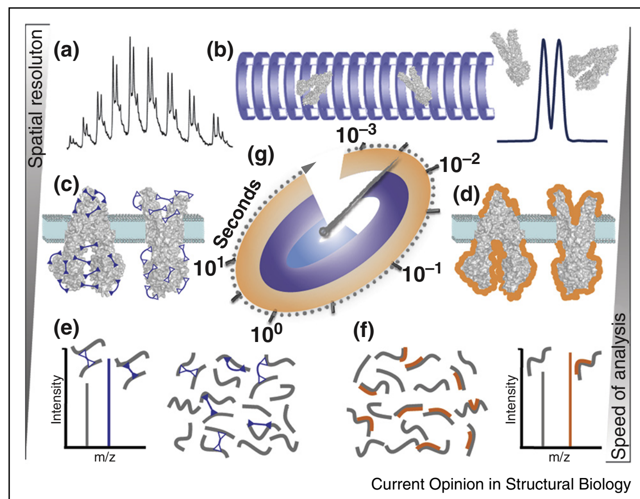
Over the past decades considerable insight has been garnered from high-resolution structural methods that are indispensable to the mechanistic studies of membrane-associated events [4–7]. When applied to membrane proteins these methods are often hampered due to the low solubility and stability of these proteins once extracted from the natural lipid environment. Equally challenging is the characterization of their flexibility and unique motions. These movements often underlie the function and regulation of transmembrane processes and span a range of timescales from milliseconds to minutes [8–10]. Moreover, membrane proteins frequently undergo dynamic interactions and constant reorganization within the fluidic biological membrane [11,12], forming heterogeneous macromolecular assemblies that are often beyond the scope of traditional structural biology approaches such as X-ray diffraction and NMR.

The gap between the biomedical significance and existing structural knowledge of membrane protein dynamics has motivated new experimental approaches [13–17]. Among them is mass spectrometry. In recent years mass spectrometry based methods have emerged as alternative means to characterize biological heterogeneity and dynamics with temporal resolution down to the millisecond scale [18–23]. Moreover the breakthrough in MS for studying membrane protein complexes, delivered directly from micellar solutions, is now paving the way for the elucidation of composition, stoichiometry and interactions of integral and peripheral membrane protein assemblies [24–28]. Here we discuss recent advances in mass spectrometric approaches pinpointing membrane protein motions that span over six orders of magnitude, from submillisecond to seconds or longer (Figure 1), and summarize novel insights that have been gained on the functional dynamics of membrane-embedded assemblies.

Capturing rapid structural fluctuations by chemical labeling mass spectrometry

The marriage of chemical labeling techniques and mass spectrometry some years previously has enabled the elucidation of conformational change and dynamics of soluble proteins [15,29–31]. Its applicability to membrane proteins however has only been demonstrated recently, primarily due to the practical difficulties associated with labeling reagents penetrating the detergent micelle or membrane bilayer. Chemical labeling experiments fall into two broad categories: labile modification, for example, isotopic exchange, and permanent modification introduced by covalent labeling such as hydroxyl radical footprinting. Both are capable of exploring rapid structural fluctuations with labeling reactions taking place on a millisecond timescale [32,33]. The former, represented by hydrogen-deuterium exchange mass spectrometry

Figure 1



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Mass spectrometry approaches designed to study membrane protein dynamics. Membrane protein complexes can be transferred intact into the gas phase of the mass spectrometer allowing direct interrogation of small molecule binding **(a)** and simultaneous monitoring of the resultant conformational changes by ion mobility **(b)**. Alternatively, comparative cross linking **(c)** or labeling techniques **(d)** can be employed to capture the dynamic behavior of membrane proteins before enzymatic cleavage and subsequent MS analysis **(e)** and **(f)**. In (c) and (e) light and heavy cross linkers are denoted with empty and filled arrows respectively. **(g)** Temporal resolution of different MS methods is indicated for the labeling techniques (orange), ion mobility mass spectrometry (blue) and crosslinking (light blue). Sidebars represent trends toward increased spatial resolution and speed of analysis.

(HDX-MS), has been employed in several studies for the investigation of the mechanism of activation of G protein-coupled receptors (GPCR) and transmembrane signaling. Application of HDX-MS to these targets has been demonstrated in revealing conformational changes that occur on a millisecond timescale [34–37].

Heterotrimeric G proteins are composed of a nucleotide binding α subunit (Gα) and an obligate dimer of the β and γ subunits (Gβγ) [38,39]. In their inactive form, Gα subunits are bound to GDP and tightly associated with Gβγ. Binding with the receptor activates heterotrimeric G proteins (Gs, the stimulatory G protein for adenylyl cyclase) through promoting the release of GDP, thus initiating a cascade of transmembrane signaling events. Results obtained from HDX-MS experiments complement the high-resolution snapshot provided by the crystal structure and uncover the mechanistic details of receptor-mediated activation. In the case of the β_2 adrenergic receptor (β_2AR) HDX-MS reveals a dramatic increase in exchange rate in the β1 strand. This strand links the second intracellular helix ICL2 of the agonist-bound receptor to the P-loop that coordinates the β-phosphate of GDP in Gαs [35]. Increased flexibility at the receptor-binding surface (β1 strand) destabilizes the local hydrogen-bonding network, and is coupled to conformational changes in the highly conserved P-loop at the nucleotide-binding pocket. Overall therefore the study uncovers an important link between structural dynamics of the two regions and relates receptor binding to GDP dissociation.

Continuing our focus on β_2AR activation but this time with respect to changes in dynamics associated with ligand binding, a marked decrease in flexibility of the intracellular helix ICL2 was revealed through HDX-MS [34]. A range of functionally distinct ligands of β_2AR were screened including a full agonist isoproterenol, a partial agonist clenbuterol, an antagonist alprenolol, and two inverse agonists carazolol and timolol [40]. Results showed that binding of agonists yielded greater protection from exchange than antagonists and inverse agonists. Conformational modulation was observed in regions spanning extracellular to intracellular regions of the receptor. Notably, binding of all five ligands yielded a significant reduction in dynamics in the ICL2 region. In the *apo*β_2AR structure ICL2 is proposed to be highly dynamic adopting either an extended/unstructured [41] or helical form [42]. The HDX data therefore suggest a shift in the conformational equilibrium of ICL2 toward a more rigid helical state of ICL2, priming it for the recruitment of G proteins or other effectors at the intracellular side.

Interestingly for both of the two HDX studies outlined above the dynamic motions involved in β_2AR-Gs activation were found to be on a comparable timescale to the fast amide exchange rate; under these conditions a maximum time resolution of millisecond is therefore achievable. When combined these investigations uncover important insight into the activation mechanism of GPCR-mediated signaling, its inhibition and the structural elements that are responsible for performing and controlling function.

Dynamics play a fundamental role in gating of membrane-embedded channels that are thought to operate via domain movements, again on a millisecond timescale. The dynamic gating mechanism of the potassium channel KirBac3.1 regulates the selective flow of K^+ in response to a variety of stimuli [43]. To probe the molecular mechanism, and to compare the open and closed states of KirBac3.1, hydroxyl radicals were employed to induce oxidation of amino acids located on the surface of protein. In this case the exposure period for oxidative labeling can be as brief as just a few milliseconds in order to capture rapid transitions between the two states. Results were consistent with considerable flexibility and large allosteric changes, including a rotational movement of the pore-lining transmembrane helices, which combine to open the gate. The study thus provides direct evidence for ion channel gating dynamics on a millisecond timescale and pinpoints the helices that move apart at the 'helix-bundle crossing' [44].

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