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Characterization of Cholesterol and Drug Ligand Interactions with Translocator Protein 18 KDA (TSPO) from *Rhodobacter Sphaeroides*

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The mitochondrial outer membrane protein Translocator Protein 18 kDa (TSPO), previously known as the peripheral benzodiazepine receptor (PBR), has been proposed to be a key component of the cholesterol transport system in mitochondria and a rate limiting step for steroidogenesis. Dysregulation of neurosteroids generation involving TSPO has been shown to be related to a variety of neurological and psychiatric diseases, including Alzheimer's disease, Parkinson's disease, as well as bipolar disorder. In addition, ligands of TSPO are widely used for imaging brain injury and inflammation, areas in which TSPO is highly expressed. A natural human single-nucleotide polymorphism (SNP) gives rise to a mutation, A147T, within the proposed cholesterol recognition site (CRAC). This mutation leads to altered ligand binding and has recently been reported to reduce pregnenolone production and to be associated with bipolar disorder. The same mutation is reported to inhibit a proposed TSPO catalyzed degradation of prototoporphrin IX. TSPO from *Rhodobacter sphaeroides (Rs*TSPO) shares high sequence similarity with the human protein but differs in the region of the SNP and shows significantly lower cholesterol binding. To better understand the interaction of TSPO with cholesterol, porphyrin and other ligands, we expressed and purified the recombinant *Rs*TSPO and several mutants in the cholesterol binding region. Binding properties were investigated with a sensitive tryptophan fluorescence quenching assay and the interactions were characterized by X-ray crystallography.(Supported by the MSU Center for Mitochondrial Science and Medicine, a Strategic Partnership Grant from Michigan State University Foundation and GM26916 to SFM).

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Monitoring Intramembrane Proteolytic Cleavage Reactions using Isotope-Assisted Vibrational Interrogation of Membrane Embedded (IVIBE) Proteins

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While several intramembrane cleaving proteases (iCliPs), enzymes that carry out proteolysis reactions within the membrane interior, have had their structures solved, basic biochemical questions such as what determines a substrate and how cleavage site is dictated remain. However, our inability to monitor enzyme-substrate interactions in a lipid environment is the dominant factor limiting our understanding of the central questions associated with these enzymes. Specifically, while there are many excellent techniques for the characterization of membrane proteins, many of them are only amenable to steady state measurements, require removal from the membrane, or are too low resolution to offer detailed information about changes in structure and environment. Here we offer a new method by which to observe structural fluctuations of the enzyme and the substrate during cleavage reactions within a membrane environment: isotope-assisted vibrational interrogation of bilayer-embedded systems (iVIBE). Deep UV resonance Raman (DUVRR) spectroscopy has previously been used to observe structural and environmental changes in both soluble and membrane proteins. Now, using an isotopically labeled protease and an unlabeled substrate we can resolve the spectral responses from each protein, allowing us to observe the cleavage reaction over time and determine the binding site, or structural fate of the substrate.

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Investigating Ligand-Modulation of GPCR Activation Pathways

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Molecular dynamics simulations can provide tremendous insight into atomistic details of biological mechanisms, but micro- to milliseconds timescales are historically only accessible on dedicated supercomputers. We demonstrate that cloud computing is a viable alternative, bringing long timescale processes within reach of a broader community. We used Google's Exacycle cloud computing platform to simulate an unprecedented 2 milliseconds of dynamics of the β2 adrenergic receptor (β2AR)_a major drug target G protein-coupled receptor (GPCR). Markov state models aggregating these independent simulations into a single statistical model are validated by previous computational and experimental results and provide the first atomistic description of multiple GPCR activation pathways. We show that agonists and inverse agonists interact differently with these pathways, creating an opportunity for developing drugs that interact more closely with diverse receptor states, for overall increased efficacy and specificity.

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Crystal Structure of MraY, an Essential Membrane Enzyme for Bacterial Cell Wall Synthesis

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The Phospho-MurNAc-pentapeptide translocase (MraY) is a prokaryotic membrane-spanning enzyme involved in an essential process of peptidoglycan synthesis: transfer the precursor phosphor-MurNAc-pentapeptide to carrier lipid undecaprenyl phosphate. MraY belongs to a subfamily of the polyprenyl-phosphate N-acetyl hexosamine 1-phosphate transferase (PNPT) superfamily whose members are involved in various biological processes including eukaryotic N-linked glycosylation. MraY has been a target for antibiotics development for its essentility and specificity in bacteria. Therefore atomic structure of MraY can provides valuable mechanistic information that can aid development of new antibiotics. We report the crystal structure of MraY from *Aquifex aeolicus* ($MraY_{AA}$), the first structure of the PNPT superfamily, at 3.3 Å resolution. The crystal structure, together with crystallographic and functional studies, reveals the architecture of $MraY_{AA}$, the location of Mg^{2+} at the active site and the putative binding sites of both substrates. Our crystallographic studies provide insights into the mechanism of how MraY attaches a building block of peptidoglycan to a carrier lipid.

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Combining Modelling and Site-Directed Mutagenesis to Explore Agonist Binding to Human Orexin Receptors

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Orexin-1 (OX1) and orexin-2 (OX2) are class A G-protein Coupled Receptors (GPCR)s located predominantly in the brain and are linked to a range of different physiological functions, including the control of energy metabolism and regulation of the sleep-wake cycle. The natural agonists for both receptors are two small peptides, Orexin A and Orexin B. Both peptides have activity at both receptors. Although no experimentally-derived structure has yet been published for the receptors in complex with either agonists or antagonists, a large amount of site-directed mutagenesis (SDM) has been reported and has provided important insight into the key determinants of agonist and antagonist activity. In drug-discovery, a working three-dimensional model can provide an intuitive way forward to explore new compounds. Thus we developed homology models that utilized existing SDM data, which we then explored further with MD simulation and ensemble-flexible docking to generate binding poses of the Orexin peptides in the OX receptors. We were then able to test the resulting poses with additional SDM experiments. As part of our modelling procedure we also developed a new method to analyze the structural data generated within an MD simulation to help distinguish between different GPCR substates. Our work demonstrates how this new method of structural assessment for GPCRs can be used to provide a working model for peptide-Orexin receptor interaction.

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Kinetic Exclusion Analysis (KinExa) of Avidity Enhancement of a Multi-Valent Adnectin Binding to Clustered Receptors on CHO Cells

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Multivalency is a strategy used in nature to gain avidity. A variety of cell surface receptors are known to cluster at the cell surface via protein or lipid (raft) interactions. Analytical methods to measure the effect of avidity as it exists at a cell surface are challenging. Kinetic Exclusion Analysis (KinExA) is a sensitive immunodetection analytical technique for measuring solution affinity. Adnectins™ are a proprietary type of targeted biologic derived from human fibronectin. Adnectin-A was selected with mRNA display (PROfusion™) to bind specifically to cell surface receptor X, and was formated as a multivalent fusion protein. To determine the affinity and avidity of Adnectin-A for receptor X clustered on cells, both the human and cynomolgus monkey homologues of