Unraveling Function of Biological switches by Mass Spectrometry

Response regulators and Histidine Kinases constitute a class of signaling proteins referred to as Two-component signaling proteins which are widely prevalent in bacteria, plants and lower eukaryotic animals. These proteins function as regulatory toggle switches that are turned on by phosphorylation. This switching mechanism is very important for cells to respond to environmental stimuli and for intracellular communication. One of the big questions in biology is how do these signaling proteins respond to environmental stimuli and how are these signals relayed? Our laboratory has been pioneering a powerful experimental method that has been used to probe how these biological switches work is Amide Deuterium Exchange Mass Spectrometry (HDXMS). This method relies on the ability of protein backbone hydrogen atoms to exchange with bulk water in solution, the rate of which is a function of how mobile a particular region of the protein is, and the ease at which solvent could access these sites. It has significant advantages over the conventional methods-X-ray crystallography and NMR. X-ray crystallography provides valuable high resolution structures of proteins but these are snapshot photographs that reveal little of how the switch operates in solution. NMR studies are limited to studying proteins in size smaller than ~ 40 kDa. RegA is a large, 190 kDa dimer protein that is beyond the scope of NMR. Our laboratory has been designated a Waters Center of Innovation, the first such center outside Europe or North America and only the second to be established worldwide for innovative research in Hydrogen/Deuterium Exchange Mass Spectrometry. (http://www.waters.com/waters/nav.htm?locale=en_US&cid=134644098, http://www.prnewswire.com/news-releases/waters-recognizes-the-dr-ganesh-anand-laboratory-at-the-national-university-of-singapore-as-center-of-innovation-132425043.html).

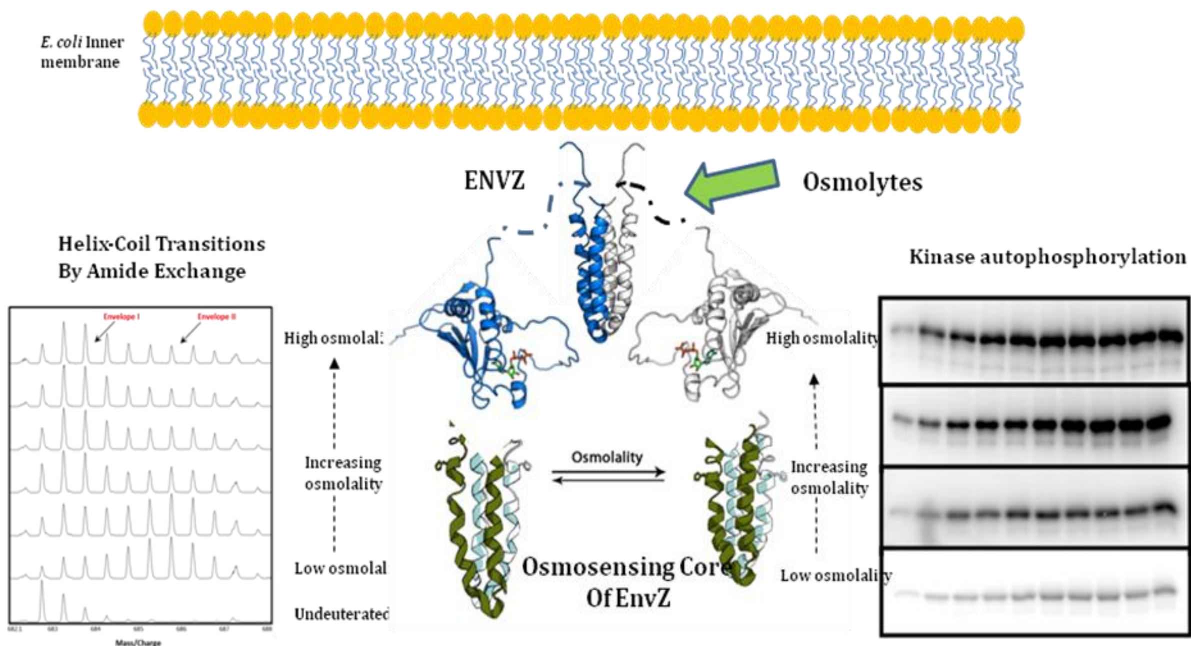
We have applied this technique to unravelling how signals are 'sensed' and how they are relayed across two signaling proteins. The first is RegA, an important response regulator in the protozoan, Dictyostelium discoideum.RegA contains two domains; an output hydrolase domain controlled by a switch domain. In the unphosphorylated state, the switch domain represses the activity of the hydrolase while phosphorylation of the switch domain at a single site turns on the hydrolase activity at a distal corner of the molecule. How phosphorylation at a single site in the switch domain is 'sensed' and propagated to the hydrolase domain has been a big question in the field of signaling proteins. Widely accepted models predict communication relays, analogous to electronic cables, coupling the site of phosphorylation with the output site. One model assumes a single relay in series while another model predicts multiple, alternate relays that operate as a parallel electrical circuit.

In one study, ((Moorthy, B.S. and Anand, G.S. (2012), we have compared the conformations of unphosphorylated, inactive RegA with phosphorylated, active RegA and a mutant that is active independent of phosphorylation, by HDXMS. Our results have allowed us to map the electric relays that radiate out from the site of phosphorylation from tracking of amides showing alterations in exchange in the phosphorylated state. This thus allows tracing of the circuits responsible for intramolecular communication. The site of the mutation is an important node in this relay and comparison of HDXMS of the mutant RegA reveals that communication relays from the phosphorylation switch converge on the node which is the mutation site. However after this node, the communication relays split up to form divergent parallel circuits to result in an output response. This finding challenges traditional assumptions of linearity in transmission of signals through the interiors of proteins and highlights that the large family of RegA-like signaling proteins work like parallel electric switches in sampling multiple conformational states. HDXMS has thus been uniquely powerful in unraveling the molecular equivalents of electronic cables, coupling switches and outputs. In addition to its relevance for a dynamic understanding of signaling processes, this study offers new important insights into inhibitor design and drug discovery and adaptive malleability inherent to signaling proteins. (See attached movie file).

In a second study (Wang Loo Chien, Morgan, L., Godakumbura, P., Kenney, LJ and Anand, GS (2012)) we have applied this technique to probe how changes in osmolality-are sensed by the bacterial histidine kinase, EnvZ, critical for enabling bacteria to sense and adapt to changes in their environments. Our results show that the cytosolic fragment (EnvZc) showed specific decreases in

deuterium exchange within the N-terminal domain of EnvZc alone under increased osmolality conditions with both NaCl and sucrose. Specifically, increasing osmolality resulted in decreased deuterium exchange that was localized to two helices of a four-helix bundle, one of which contains the autophosphorylation site (His^{243}). The second flanking helical segment showed characteristic bimodal distributions of deuterium exchange that provided key evidence for this subdomain existing as an ensemble of multiple conformations. Increases in osmolyte concentrations favored increased helicity in this region. The effects of osmolytes showed a clear concentration dependence with parallel shifts in deuterium exchange in both helices, indicating that the four-helix subdomain formed the osmosensing locus in EnvZ (Figure 2). Interestingly, decreased amide exchange showed a direct correlation to osmolality-dependent enhancement of EnvZ autophosphorylation at His^{243}. Mutation of the histidine (H243A) not only abolished autophosphorylation but also showed no differences in osmolality-dependent deuterium exchange. *In vivo* analysis further showed that the cytoplasmic domain of EnvZ was sufficient for osmosensing, i.e. no transmembrane domains were required. Our findings present a novel perspective on transmembrane signaling, in which a cytoplasmic sensor is capable of integrating and transducing distinct extracellular stimuli via a simple, yet effective molecular mechanism to elicit a common cellular response. Our results support a model where osmolytes promote intrahelical H-bonding that enhances helix stabilization, increasing autophosphorylation and downstream signaling, entirely within the cytoplasm. The model provides a conserved mechanism for signaling proteins that respond to diverse physical and mechanical stimuli. This also has important biopharmaceutical applications in identifying the effects of osmolytes in therapeutic protein formulation, storage and stability. We describe a novel application of HDXMS in monitoring osmolyte-induced helix-coil transitions in membrane receptor dynamics and signaling.

Figure 1.



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References:

1. Moorthy, B.S. and Anand, G.S. (2012) *Multi-State Allostery in Response Regulators: Phosphorylation and Mutagenesis Activate RegA via Alternate Modes. J. Mol. Biol***.** 417(5):468-8

2. Wang Loo Chien, Jeremy, Morgan, L., Godakumbura, P., Kenney, L.J. and Anand, G.S. (2012) *The inner membrane histidine kinase EnvZ senses osmolality via Helix-Coil Transitions in the cytoplasm. EMBO J.* Apr 27. doi: 10.1038/emboj.2012.99. [Epub ahead of print]