

Orientation Dependence of Ire1 Receptor Dimer Activity

Accumulation of unfolded proteins in the endoplasmic reticulum beyond homeostatic levels is known as ER stress and triggers a transcriptional program called the unfolded protein response (UPR). In yeast, the protein responsible for detecting ER stress is a single-pass, transmembrane receptor called Ire1. It is thought that the binding of unfolded polypeptide by the luminal domain (LD) of Ire1 induces oligomerization of the receptor, activating its cytosolic Ser/Thr kinase and RNase domains. This activation results in trans-autophosphorylation of the kinase domains and splicing of the mRNA substrate, *HAC1* (1). In its spliced form, *HAC1* encodes the transcription factor Hac1p, which activates transcription of a number of genes that ultimately act to relieve ER stress.

It has been previously shown that replacing the LD with a leucine zipper motif creates obligate dimers that are partially and constitutively active (2). Very little is known, however, about the mechanism of activation. This proposal aims to elucidate the role of the transmembrane (TM) region in both dimerization and activation of signaling. Specifically, I will address the following questions.

1. How do the relative orientations of the TM helices relate to kinase and RNase activity?
2. Which orientations are energetically favored?

To determine the orientation dependence of Ire1 activity, the LD will be replaced with a leucine zipper dimerization motif from the transcription factor c-Jun. The junction between the leucine zipper region (JunLZ) and the transmembrane helix will be adjusted by the addition or subtraction of alanine residues such that all seven orientations of the helical wheel will be rigidly imposed. This strategy was used previously to search for active orientations of the *Escherichia Coli* aspartate receptor (3). The chimeric dimers will be expressed in *E. coli* and solubilized in detergent. The solubilized dimers will be assayed *in vitro* for both kinase and RNase activity in the methods presented by Nock, et al (4). Each activity will be scored as a function of the angle of rotation of the transmembrane helices with respect to the unmodified orientation. Because detergent micelles may not accurately mimic the environment of a biological membrane, the above assays will also be performed with the protein dimers inserted into synthetic vesicles. Detergent conditions will be optimized to yield results consistent with the liposome system.

Next, the contribution of the TM helix interactions to the overall free energy of dimerization will be determined by TROSY NMR. Perdeuterated versions of the above constructs will be expressed in *E. coli*. The methyl groups of Ile, Leu and Val (ILV) residues will be selectively protonated and ^{13}C labeled in the method of Goto, et al (5). The dimers will be solubilized in detergent and ^1H - ^{13}C HMQC spectra will be acquired. Because there is also a dimer interface in the TM region, certain orientations of the TM helix will compete with the JunLZ domain for the preferred interface. Since all resonances of c-Jun have been previously assigned (6), those associated with the zipper interface of JunLZ in the chimeric proteins may be compared with those of JunLZ alone. The fractions of monomeric and dimeric JunLZ domains will be determined by analyzing the dual peaks corresponding to each ILV residue. Thus, partition coefficients and dimerization free energies can be calculated for each construct.

To confirm that relative movement of the TM helices is transduced to the cytosolic domains, methyl-TROSY spectra will also be acquired for versions of the above constructs that have the RNase domains deleted, as well as constructs with both kinase and RNase domains deleted. Although individual assignments for Ire1 effector domains are not known, subtraction of each domain will account for the absence of a defined set of peaks from the spectra. Each set of peaks can then be analyzed to detect qualitative changes with the different TM orientations. We expect to see shifts in resonances associated with the kinase domains, since the transphosphorylation event necessitates an interaction. Kinase folds are sufficiently well-characterized that we can build a homology model of this domain. The proton and ^{13}C resonances can subsequently be assigned using methyl-TROSY in conjunction with alanine scanning mutagenesis. The ILV residues will then be identified in the model to help map the interaction surface.

The experiments I have outlined will identify the active orientations of the TM region and indicate whether dimerization guides monomers toward or away from a low energy state. Future experiments could probe the context of TM domain orientation *in vivo*. For example, one could query the oligomerization state of Ire1 prior to UPR with FRET and observe whether UPR induction causes monomers to associate, dimers to change conformation, or dimers to form higher order oligomers. Relating these observations to the energetics of the TM region would provide considerable insight to the mechanism of activation. Finally, when the structures of the cytosolic domains are solved, they can be docked with the known LD structure given the TM constraints we have determined. We would then have a picture of the full transmembrane protein in both active and inactive conformations.

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