Structural and Functional Characterization of the Preprotein Translocon Accessory Factor Ysy6p/RAMP4

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Abstract

The small integral membrane protein Ysy6p of *Saccharomyces cerevisiae* and its mammalian homolog Ribosome Associated Membrane Protein 4 (RAMP4) are accessory factors that interact stably with the Sec61 translocon. These proteins have been implicated in stabilizing integral membrane proteins in the ER under stress conditions. Ysy6p is a single-pass integral membrane protein of 65 amino acids in length and yet can stabilize a wide range of integral membrane proteins. In particular, Ysy6p can confer resistance to *Eschericia coli* strains bearing the temperature sensitive SecY24 mutation. Examination of the interaction between Ysy6p and SecYEG or Sec(Y24)EG should shed light on the nature of the interaction between Ysy6p and its targets. We are using NMR and other biophysical techniques to examine the mechanism by which Ysy6p stabilizes integral membrane proteins. Our studies begin with the characterization of Ysy6p and of the SecYEG/Sec(Y24)EG channel in isolation. Recombinant Ysy6p has been expressed and purified to homogeneity in 1,2-diheptanoyl-*sn*-glycero-3-phosphocholine (DHPC) or n-dodecyl-β-D-maltopyranoside (DDM). ^{1}H-^{15}N HSQC spectra have been collected on samples in either detergent. ^{13}C- and ^{15}N-labeled Ysy6 has been produced using the Single Protein Production expression system (Suzuki et al, 2005) for triple resonance multidimensional NMR experiments. SecYEG and Sec(Y24)EG complexes have also been expressed and purified. Here we present our findings on the structural characteristics of Ysy6p and our preliminary data on Sec(Y24)EG.

Introduction

The proteins of all organisms are synthesized on ribosomes in the cytoplasm. However, almost one third of the proteome encodes proteins that function in compartments other than the cytoplasm.[1] The movement of secretory proteins through and across membranes into extracytoplasmic compartments (protein translocation) is an essential process. At the core of this process is a complex of integral membrane proteins which form a channel or pore through which proteins pass. In bacteria, the major route is through the SecYEG translocon; eukaryotes use the homologous Sec61αγβ channel.[2] A number of additional associated proteins have been identified that facilitate translocation and folding of subsets of substrates or otherwise modulate translocon function[3,4].

One such protein is the small (65 amino acids, 7.3 kDa) integral membrane protein Ysy6p of *S. cerevisiae*. Ysy6p has been demonstrated to rescue the viability of a temperature sensitive mutant of *E. coli* SecY (SecY24), but its function in translocation and processing is not known.[5] The mammalian homolog of Ysy6p, RAMP4 (Ribosome Associated Membrane Protein 4) has been found associated with the Sec61αγβ channel. No bacterial homologs of YSY6/RAMP4 are known. RAMP4 is required for proper folding and glycosylation of a subset of membrane proteins under stress conditions.[6-9] We hypothesize that Ysy6p functions as a molecular chaperone by direct stabilization of transmembrane regions of translocation substrates. To test these hypotheses, we will use solution NMR and biophysical techniques to investigate the interaction of Ysy6p with the SecYEG and Sec(Y24)EG translocons in order probe Ysy6p function.

Methods

Recombinant protein expression: Fresh BL21(lDE3) transformants of Ysy6p in pET24A (Novagen) were grown in autoinduction media[10] for 6 hours at 37 ºC and grown for an additional 18 hours at room temperature. Uniform ^{15}N labelling was also achieved by growth in minimal autoinduction media. Cells were harvested and stored at -80 ºC. 13C/15N protein was produced in cells grown in M9 media supplemented with ^{15}N NH_4Cl and ^{13}C glucose and induced at OD_{600} of 0.6 for 16h at 23°C. SPP samples were generated according to published protocols.[12]

Protein purification: Cells were thawed at 25 ºC and resuspended in 20 mM TRIS-HCl pH 7.5, 500 mM NaCl, 10 mM EDTA, 10% glycerol, 1 mM PMSF. Cell suspension (~30%) was lysed by disruption (Avestin). Total membranes were solubilized in 0.5 g b-Dodecyl maltopyranoside (β–DDM, Anatrace), 50 mM TRIS pH 7.0, 500 mM NaCl, 10% glycerol, 5 mM EDTA for each 1.5 L worth of cell culture. Extraction was performed overnight at 4 ºC. Extracted Ysy6p was rapidly diluted 2-fold with 10mM Na Phosphate pH 7, 1 mM EDTA, 10% glycerol, 0.09% β-DDM (Buffer A) and loaded onto an SP sepharose column equilibrated into the same buffer. The column was washed in Buffer A + 200 mM NaCl, and eluted in a linear gradient to 1M NaCl. Pooled fractions were concentrated and loaded onto a Sephacryl S-300 gel filtration column (GE Healthcare). Eluted protein was concentrated and stored at -80 ºC in gel filtration buffer (10mM TRIS-HCl pH 7.5, 5% glycerol, 0.09% β-DDM, 300 mM NaCl). Protein purified in DHPC was subjected to the same protocol but using 2% DHPC for extraction and 4mM DHPC in chromatographic buffers.

Circular Dichroism: Experiments were performed in an Aviv spectropolarimeter at 25 ºC with a 1 nm bandwidth and 3 sec averaging time. Spectrum is an average of 3 scans.

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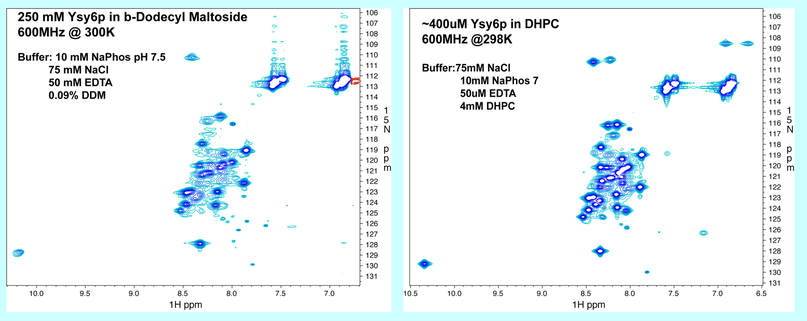
Results

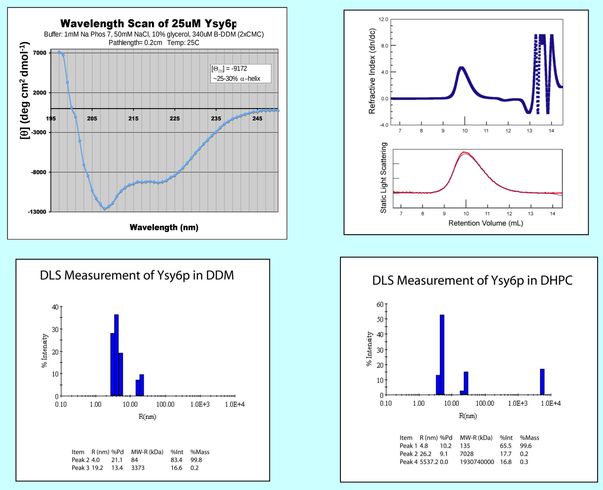
^{1}H-^{15}N HSQC spectra of detergent solubilized Ysy6p (conventional expression methods)



Standard ^{1}H-^{15}N HSQC spectra of Ysy6p were performed in different buffer and detergent conditions. A larger number of peaks, and in some cases better linewidths, is seen in the DHPC condition (right) than in β-DDM (left), even though the DHPC micelle is substantially larger by light scattering. In both cases, there appear to be regions of Ysy6p that are quite dynamic relative to the micelle.

Overall, the quality of the spectrum in DHPC is encouraging, as the SecYEG channel is functional in DHPC. Buffer conditions for protein produced using the SPP system are being explored.

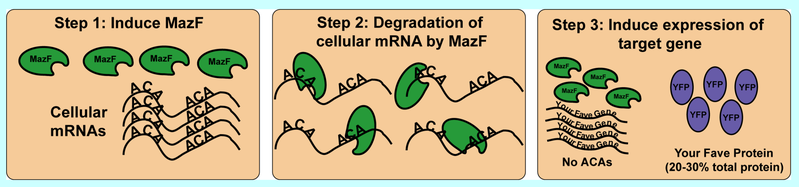
Characterization of Ysy6p-containing micelles by circular dichroism and light scattering

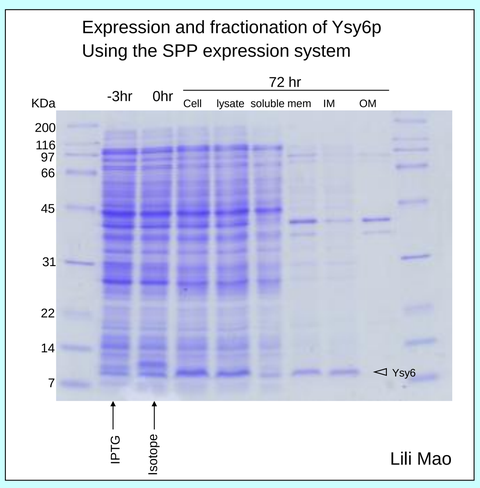


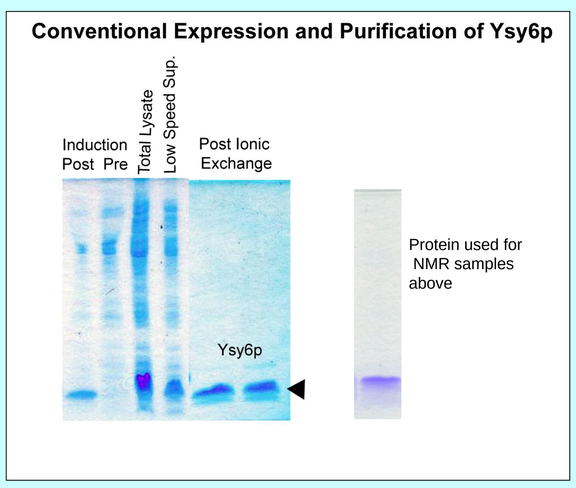
Features of circular dichroism spectra (top left) performed in β-DDM indicate that Ysy6p is at least partly folded and is approximately 30% helical.

Ysy6p micelles are primarily monodisperse in solution as judged by both static (top right) and dynamic (bottom panels) light scattering. Static light scattering experiments indicate that the Ysy6p particle in β-DDM is ~60kDa, indicating a single molecule of Ysy6p per micelle (top right and data not shown).

Condensed Single Protein Production Expression System







Ysy6 was expressed either using traditional growth and expression protocols (including autoinduction media)[10] or the the SPP protocol.[11,12]

In the SPP protocol, there are fewer contaminating proteins in the total membrane lane as compared to the equivalent low speed supernatant lane in the conventional gel and that Ysy6 is the most abundant protein in the inner membrane.

Conventionally produced Ysy6p typically requires additional ion exchange and gel filtration polishing steps.

The inset in the conventional purification panel is purified Ysy6p and typical of the protein used for the NMR studies above.

Conclusions and Future Directions

Detergent micelles containing Ysy6p are predominantly monodisperse and likely contain one monomer per micelle in the case of β–DDM. Micelle size is not a good predictor of NMR spectral quality.

Ysy6p is maximally structured in DHPC, a short chain phospholipid detergent in which the SecYEG channel is known to be stable and functional. Future experiments will be performed in DHPC.

Three-dimensional NMR experiments will be necessary to assign the backbone resonances of Ysy6p for chemical shift perturbation studies. TROSY-based experiments may not be necessary for studies of Ysy6p in isolation, although the difference in dynamic regimes sampled by different regions of the protein will need to be addressed.

Ysy6p can be expressed and purified using the SPP system. Optimal buffer conditions for solution NMR experiments still need to be determined. We intend to take advantage of the SPP system to examine the interaction of Ysy6p with SecYEG and with the less stable Sec(Y24)EG translocons.

We can express and purify SecYEG, however, conventional protocols need to be optimized to improve yields. Expression strain engineering may be necessary to allow use of the SPP system with SF100 cells.