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A study on PsbS and its role as a pH sensor

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Conclusion and Outlook

6.1 Conclusion

PsbS was discovered in 1986[1,2] followed by decades of research to understand its exact role in photoprotection and NPQ. NPQ is PsbS dependent[3] and many models have been proposed for the functional mechanism of PsbS[4,5]. There have been conflicting studies regarding its dimer-monomer transition[4,6,7], localization[8] and interaction with other photosynthetic components[9,10]. Although some insights into the role of PsbS as a photoprotector have been attained[11] from the X-ray crystal structure of Spinach-PsbS[7], the molecular response mechanism of PsbS during the pH lowering still remains unclear.

One of the technical challenges in the structural study of PsbS is the sample preparation for carrying out spectroscopic analyses. Native PsbS is very difficult to isolate and the yield is insufficient for structural characterization. Recombinant PsbS has been produced and refolded[9,12,13] but for spectroscopic techniques like NMR and FTIR, the yields of PsbS expression were low. For carrying out biochemical and spectroscopic studies on PsbS, its production, purification and refolding had to be optimized. As demonstrated in Chapter 3, with the new method, PsbS can be purified with 80 % efficiency as compared to 20 % efficiency by standard protocols that use nickel affinity columns. Particularly while using expensive isotopes for labelling, this purification steps helps in reducing the overall loss and labelling expenditure. In Chapter 3, PsbS was successfully refolded at neutral and low pH conditions using different types of detergents. For refolding of Patens-PsbS, OG and FC-12 were selected as the most suitable detergents for further experiments.

For understanding the molecular mechanism of PsbS, its oligomeric states are important[4]. PsbS has been suggested to be dimeric in alkaline conditions and monomeric in acidic conditions[10,11]. This model was challenged by the crystal structure of dimer PsbS at acidic pH, although the spinach used to isolate and make crystals of PsbS were not grown under high-light stress conditions[7]. In Chapter 4, the oligomeric states of PsbS at neutral and low pH conditions were studied. PsbS under pH 7.5 conditions forms predominantly dimers as compared to pH 5.0 conditions that forms a dimer-monomer equilibrium. Eventually, in both pH conditions, PsbS stabilizes as dimers.

Structural conformational changes in PsbS during pH lowering are hypothesized to be the mechanism of action due to protonation of certain acidic residues at the lumen site of PsbS[14]. HSQC NMR spectra show structural changes of several Gly signals with low pH. A peak of Arg residues appears to be present in the dimer sample and disappears in the monomer sample, suggesting the role of one Arg residue in dimer

formation and to serve as a marker peak in future experiments using NMR spectroscopy.

Mutation of two active Glu residues to Gln (for sake of simplicity, termed as E1 and E2) have shown *in vivo* reduction of NPQ by 60 - 70% [15,16]. To relate pH-dependent conformational changes to the functional mechanism of PsbS, in Chapter 5, three mutants of PsbS were analyzed. The single mutants M1 (E71Q), M2 (E176Q) and double mutant M3 (E71Q, E176Q) of Patens-PsbS were studied. The two Glu residues were proposed to be protonated during pH lowering[14]. Through 1D ^{13}C direct polarization NMR spectroscopy, the protonation of all the titratable Glu and Asp residues were observed. According to the crystal structure of PsbS[7], it is known that the E1 residue resides in the luminal loop that connects the two transmembrane helixes TM1 and TM2, while the E2 resides in an amphipathic short helix between TM3 and TM4 at the water-exposed side towards the lumen. The crystal structure of PsbS also shows that E2 forms hydrogen bonds between its carbonyl and carboxyl group with two backbone amides of amino acids in the adjacent monomer[7]. These hydrogen bonds stabilize the PsbS dimer. From the oligomeric analysis in Chapter 5, the M1 mutant is strongly disabled in dimer formation even though E1 is not residing close to the site of hydrogen bond formation. The highly reduced dimer content in M1, therefore, suggests that there is an allosteric effect of E1 on PsbS dimerization. Mutation of E1 to Gln might re-orient the luminal loop, thereby, disrupting the inter-monomer hydrogen bonds formed by E2.

The M2 mutant, on the other hand, is capable of dimer formation even though it lacks E2 that is involved in inter-monomer hydrogen bond formation. The substituted Gln might be able to form intermonomer hydrogen bonds via the backbone carbonyl and side chain amide. Since the mutant M2 does not show any structural response to pH, it is concluded that the lumen-facing amphipathic helix containing E2 is the site of plasticity that undergoes a pH-dependent structural change. As this glutamate residue is located in the hydrophobic membrane environment in the low-pH crystal structure of PsbS[7], while at neutral pH it resides in the aqueous region due to the negative charges on E2, it is possible that with protonation of E2 at low pH the amphipathic helix could move from water into the membrane phase. The structural conformation of M2 at both neutral and low pH resembles the low-pH conformation of WT PsbS, which is the active state. Reduced NPQ activity of the mutant M2 suggests that the ability to switch on and off during NPQ might be crucial for PsbS function.

The M3 mutant is still capable of forming dimers unlike the M1 mutant while having the same mutation at the E1 site. The FTIR data shows that the conformation of M1 and M3 neither resembles the WT at low pH nor at neutral pH, indicating that

mutation of E1 leads to a non-native fold of PsbS. Mutation of E1, thus, does more than neutralizing charges at the E1 site.

The roles of E1 and E2 in the PsbS response mechanism are non-equivalent. E1 is responsible for dimer formation and E2 brings about conformational changes in PsbS upon pH lowering presumably by a movement of the amphipathic short helix fragment where it is located. As the single mutation of E1 and E2 in PsbS have been reported to severely reduced NPQ activity[15], this implicates that both dimerization and flexibility of the amphipathic short helix stretch are important for the function of PsbS.

6.2 Outlook

The oligomeric state of the recombinant PsbS was explored extensively in Chapter 4 using techniques like SEC, DLS and DOSY NMR. However, the determination of molecular weight or size of membrane proteins like PsbS that are surrounded by detergent micelles is very challenging. SEC combined with multi-angle laser light scattering and a differential refractometer are required for accurate size estimation and analysis of membrane proteins[17]. In an SEC-UV/LS/RI system, the exact molecular weight and the dimerization constant can be determined[18,19]. With the use of these techniques, the pH-dependent oligomerization states of PsbS at neutral and low pH could be determined more accurately in future experiments.

With an NMR chemical shift assignment of the two active Glu residues, it would be possible to resolve their role in the pH-dependent function of PsbS. To assign the two specific peaks of Glu, selective labelling of Glu residues must be carried out. This would reduce the spectral crowding and assignment of the Glu residues would be possible. Alternatively, cell free expression system can be applied to minimize amino acid scrambling during selective labelling.

PsbS interacts with antenna proteins during NPQ and heat dissipation of excess energy takes place[20]. Protein interaction studies in liposomes containing PsbS and LHCII have shown to induce the quenched state of LHCII[12]. Understanding the exact interaction of LHCII and PsbS will help in comprehending the photoprotective function of PsbS in interactions with other membrane components. Reconstitution of isotopic labelled PsbS with unlabelled LHCII protein under varying pH conditions and analysis using NMR spectroscopy could provide a better insight into the interaction of the two proteins.

After characterization of PsbS refolded in detergents[21] (*this thesis*), characterization of PsbS reconstituted in liposomes will be useful to understand how it behaves in a

membrane-like environment. PsbS was reconstituted into liposomes and appeared to be in a monomeric state inside the liposomes, suggesting that the microenvironment is very important for the oligomeric state of PsbS (*Thesis of E. Crisafi*). The next step would be a reconstitution of isotopic labelled PsbS into native thylakoid membrane expressing only LHCII protein (and knocking out major photosynthetic proteins), to carry out NMR studies. This will help in understanding PsbS in its native environment and to understand its function as a photoprotector.

Site-selective mutagenesis studies on PsbS have been carried out to understand which residues are important for its function *in vivo*[15,16]. However, Chapter 5 shows that mutation of one of the active Glu effects the overall structural conformation of PsbS. Thus, it is important to realize that site-selective mutations might result in an improperly folded protein. *In vitro* structural studies as performed in this thesis can be used to test and validate whether PsbS mutants retain their overall fold.

Lastly, it will be important to validate the structural analyses of recombinant PsbS by comparing with analyses from the native PsbS. Sufficient amounts of native PsbS from plants is challenging to isolate. The yields might be difficult to achieve for NMR spectroscopy, but could be achieved for structural characterization by IR or CD spectroscopy.

6.3 References

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