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BRIEF COMMUNICATION

Secondary structure estimation of recombinant *psbH*, encoding a photosynthetic membrane protein of cyanobacterium *Synechocystis* sp. PCC 6803

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Abstract

The PsbH protein of cyanobacterium *Synechocystis* sp. PCC 6803 was expressed as a fusion protein with glutathione-S transferase (GST) in *E. coli* grown on a mineral medium enriched in ^{15}N isotope. After enzymatic cleavage of the fusion protein, the ^{1}H-^{15}N-HSQC spectrum of PsbH protein in presence of the detergent β-D-octyl-glucopyranoside (OG) was recorded on a *Bruker DRX* 500 MHz NMR spectrometer equipped with a 5 mm TXI cryoprobe to enhance the sensitivity and resolution. Non-labelled protein was used for secondary structure estimation by de-convolution from circular dichroism (CD) spectra. Experimental results were compared with our results from a structural model of PsbH using a restraint-based comparative modelling approach combined with molecular dynamics and energetic modelling. We found that PsbH shows 34–38 % α-helical structure (Thr36-Ser60), a maximum of around 15 % of β-sheet, and 12–19 % of β-turn.

Additional key words: CD spectroscopy; molecular dynamics calculations; NMR spectroscopy; photosystem 2; protein folding.

The PsbH protein is one of the small protein subunits of photosystem 2 (PS2), a membrane pigment-protein complex that catalyzes the light-induced electron transfer and water-splitting reactions. The PsbH protein was originally found in thylakoids of higher plants and detected as a 10 kDa phosphoprotein (Bennett 1977, Allen 1992). Genes encoding PsbH homologues have so far been identified in all oxygenic phototrophs studied (for review see Komenda *et al*. 2003) and its deduced amino acid sequence predicts a single transmembrane helix. In higher plants and green algae the PsbH protein undergoes reversible phosphorylation at two threonine residues close to the N-terminus (Michel and Bennett 1987, Vener *et al.* 2001). The cyanobacterial PsbH protein is truncated at the N-terminus and misses these phosphorylation sites (Koike *et al.* 1989). The function of PsbH in PS2 has been associated with control of the electron flow from Q_A to Q_B (Mayes *et al.* 1993), protection from photoinhibition (Komenda and Barber 1995), bicarbonate binding on its acceptor site (Komenda *et al.* 2002), or chaperon-like function in the assembly of the PS2 core and of small stress-induced chlorophyll (Chl) binding proteins (Komenda 2005). In *Chlamydomonas reinhardtii*, disruption of the PsbH subunit led to a rapid degradation of D2 protein and mutants lacking PsbH were unable to assemble any functional PS2 (Summer *et al.* 1997).

Three-dimensional structures of the PS2 core complex have been solved by electron microscopy (for review see

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Bumba and Vácha 2003) and X-ray crystallography (Zouni *et al.* 2001, Kamiya and Shen 2003, Ferreira *et al.* 2004). Apart from the major PS2 subunits (CP47, CP43, D1, and D2 protein) and components of cyt b_{559} (PsbE and PsbF proteins), identification and localization of the other small protein subunits has been contradictory concerning the crystal structures (see Shi and Schröder 2004). Recently, the location of PsbH subunit within the PS2 complex from electron microscopy and single particle analysis was identified (Bumba, unpublished), in good agreement with the crystal structure of the PS2 complex of cyanobacterium *Thermosynechococcus elongatus* (Ferreira *et al.* 2004). The location of the PsbH subunit close to the CP47 protein is also supported by the fact that the PsbH protein stabilizes the binding of CP47 to the D1-D2 heterodimer (Komenda *et al.* 2002) and thus supports the assembly of the PS2 core (Summer *et al.* 1997). PsbH is predicted to have a single transmembrane helix, a small C-terminal domain in the lumen, and a larger N-terminal domain in the stroma (Komenda *et al.* 2002). PsbH is one of the proteins expressed in etiolated and irradiated leaves in higher plants on the same level, which indicates that its function may be considered separately from the rest of the multiprotein complex.

The PsbH protein of cyanobacterium *Synechocystis* sp. PCC 6803 *psbH* gene (locuss12598) was over-expressed as a fusion protein with glutathione-S transferase (GST) in *E. coli* and the PsbH-GST fusion protein was enzymatically cleaved and purified according to the procedure given by Halbhuber *et al.* (2003).

We isolated non-labelled PsbH protein for circular dichroism (CD) spectrometry. CD spectra of the sample were recorded on a *Jasco*-instrument, controlled by PC-based *ISA OMA* software at 20 °C in 1-mm quartz cuvettes, after 15 min of temperature equilibration. Five scans were accumulated for each spectrum with a response time of 2 s, a bandwidth of 2 nm, and a scan speed of 10 nm min^{-1} from 190 to 250 nm. Background spectra without protein were subtracted. The protein concentration was 20 μM.

To study the structure of PsbH protein we performed uniform isotope labelling of the PsbH protein according the procedures using minimal media (M9) (Rhee *et al.* 1997, Halbhuber *et al.* 2003). We isolated and purified the ^{15}N labelled protein to a concentration of 1.1 kg m^{-3} in presence of non-ionic β-D-octyl-glucopyranoside. ^{1}H-^{15}N HSQC spectra were recorded on a *Bruker DRX* 500 MHz NMR spectrometer equipped with a 5 mm *TXI* cryoprobe to enhance sensitivity and resolution. The dialysed protein sample was mixed with D_2O (H_2O/D_2O 1 : 20) to perform the NMR experiments. The standard *Bruker* pulse sequence for these experiments was the *hsqc-etf3-gpsi2* pulse program.

A model of the the PsbH protein of cyanobacterium *Synechocystis* sp. PCC 6803 was generated by analogy to the crystal structure at 0.35 nm resolution of the PS2 complex of cyanobacterium *Thermosynechococcus elongatus* (Ferreira *et al.* 2004) (PDB code 1S5L). Bumba *et al.* (unpublished) recently showed the position of the PsbH in this structure to be correct, however, 22 amino acids at the N-terminal end of the protein are missing in the crystal structure. Therefore the suggested structure for these amino acids is not based on homology with the crystal but generated from a loop database. The three-dimensional model constituted by all non-hydrogen atoms was built and examined by the *MODELLER7* package (Sali and Blundell 1993). As the crystal structure has a very low resolution and large parts of the protein were not resolved, we decided to refine the homology model by running a 1.2 ns molecular dynamics simulation in aqueous solution using the *YAMBER2* force field (Krieger *et al.* 2004) to induce better folding of the protein. The protein structure was placed into a box of 7.3×8.2×9.6 nm size, which was 1 nm larger than the protein along all three axes. The box was filled with TIP3P water, sodium ions were iteratively placed at the coordinates with the lowest electrostatic potential until the cell was neutral. Molecular dynamics simulations

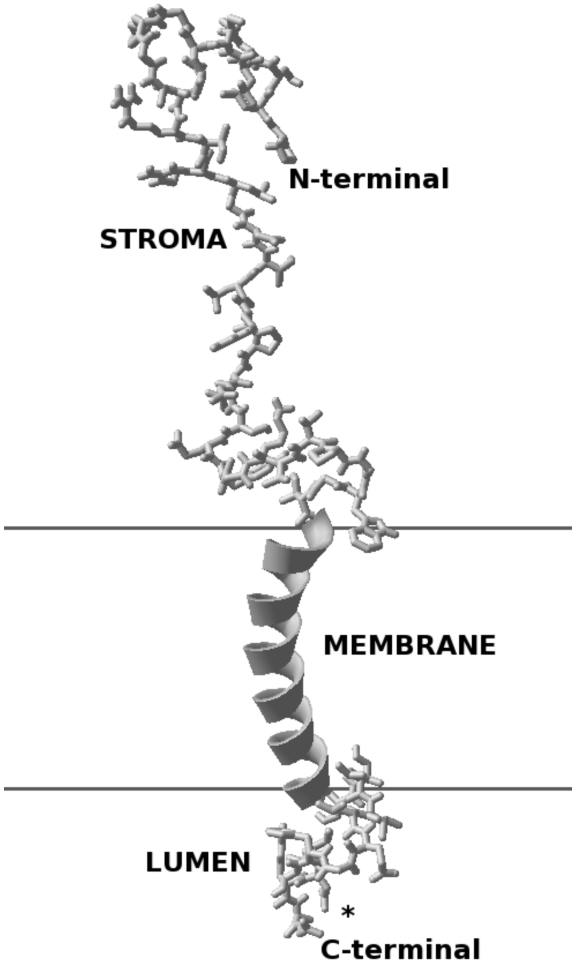

Fig. 1. Three-dimensional representation of the calculated structure of PsbH from *Synechocystis* sp. PCC 6803. The possible ion binding site in the lumen is marked *.

were run with *YASARA* (www.yasara.org), using a multiple time step of 1 fs for intra-molecular and 2 fs for intermolecular forces. A 1.2 nm cut-off was taken for Lennard Jones forces and the direct space portion of the electrostatic forces, which were calculated using the Particle Mesh Ewald method (Essman *et al.* 1995) with a grid spacing 0.1 nm, 4^{th} order B-splines, and a tolerance of 10^{-4} for the direct space sum. The simulation of the homology model was then run at 298 K and constant přessure (NPT ensemble) to account for volume changes due to fluctuations of homology models in solution. Residues 37–59 in the trans-membrane helix were restrained during the simulation to mimic the stabilization of this part by the membrane. Root mean square deviation for C_α and the total energy of the systems indicated that the protein structure arrive at an equilibrium state after 750 ps. The resulting structure was minimized to convergence of the energy gradient to less than 0.02 kJ mol^{-1} nm^{-1} using the *TRIPOS* force field included in the *MAXIMIN2* module of *SYBYL* (*TRIPOS Associates*). The minimization included electrostatic interactions based on Gasteiger Hückel partial charge distributions using a dielectric constant with a distance-dependent function $\varepsilon = 4\,r$ and a non-bonded interaction cut-off of 1.2 nm. The final model (Fig. 1) had 98.3 % of residues in the two most favoured regions of the Ramachandran plot and an acceptable overall geometry, both determined with the *ProCheck* program (Laskowski *et al.* 1993). No residues were found in disallowed regions. The overall *g*-factor of the structure obtained showed a value of –0.16. The *g*-factor should be above –0.5 and values below –1.0 may need investigation. Amino acids from Thr36-Ser60 adopted an α-helical fold, which corresponds to 34.2 % of the total secondary structure, and 12.3 % of the amino acids were found in a β-turn conformation. No stable β-sheet structure was found in the model. This has been confronted with the estimation of secondary structure from the experimental far-UV CD by deconvolution using the method of *VARSELEC* (variable selection method) included in the *Dicroprot V2.5* program (Deléage and Geourjon 1993) with 33 reference spectra. The calculation yielded a 38 % helical structure, 15–19 % sheet and 19 % β-turn. In our opinion, the deconvolution is reliable mostly for α-helix and to a lower extent for β-sheet and other structures. The distinction between anti-parallel and parallel β-sheets looks very difficult and is not very reliable, so that we summarized the overall β-sheet content.

Additionally, we used the *K2D* program based on neural network theory (Andrade *et al*. 1993). Calculations from CD spectra using the *K2d* software gave 37 % helical structure, 14 % sheet, and 49 % random coil. We can summarize the results of the quantitative analysis of the far-UV CD spectra in the following way: Both methods showed an α-helical content between 37 and 38 % and thus completely agreed with the model. Most critical and unreliable is the estimation of the β-structure from CD-spectra, which is an intrinsic limitation of the method.

Certainly, we can state that the CD spectra of the protein/detergent mixture indicate that the protein adopts a mixture of secondary structures, including helix, sheet, and random coil. As CD shows the average of all structures present in the sample, it is not possible to suggest from a CD spectrum if a protein is proper folded or if it is a mixture of different folds or aggregates. Therefore the 1H-15N-HSQC spectrum of PsbH protein in OG was recorded and, as shown in Fig. 2*B*, it has 70 resolved amino acid signals within the range of 7.0–9.0 mg kg^{-1}. Thus, we can clearly state that we have a properly folded protein and in future it will be promising to perform NMR structural assignments of doubly labelled ^{15}N-^{13}C protein/detergent system.

Pure unlabelled and ^{15}N labelled PsbH protein was prepared to study the secondary structure content of properly folded membrane protein PsbH. CD-spectra and two-dimensional ^{1}H-^{15}N-HSQC NMR experiments revealed that psbH protein in the detergent OG has a proper fold that fully corresponds to theoretical

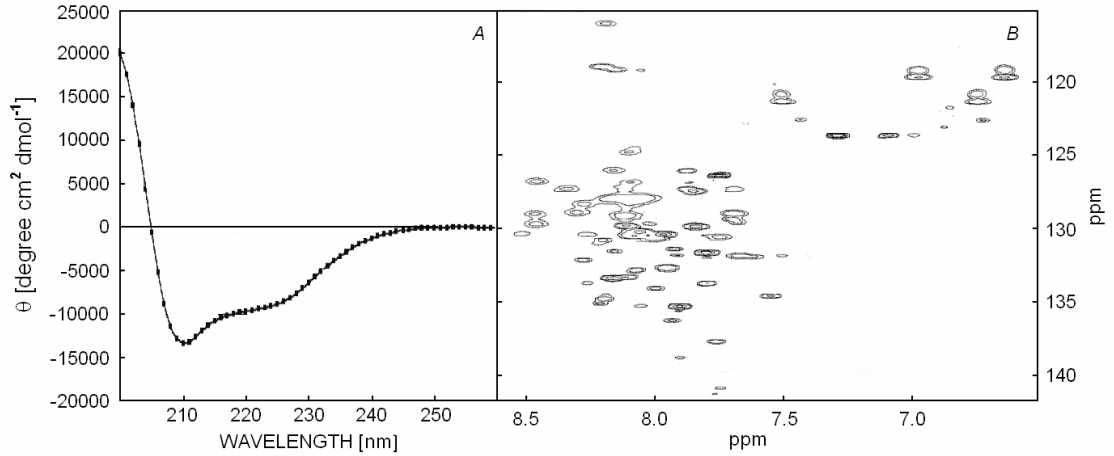

Fig. 2. *A*: Circular dichroism spectra of PsbH protein in OG. *B*: ^{1}H-^{15}N-HSQC spectrum of PsbH protein in OG.

calculations from molecular dynamics. The transmembrane helix from the structural model gives 34.2 % of the total secondary structure content, which corresponds to the helical structure measured by CD. This fact excludes the existence of additional helical structure in the stroma or lumen. Regarding the β-sheet content, the structural model does not show sheet structure, however, the β-turn from Glu7-Gly10 suggests a possible sheet structure at the N-terminus of the protein. Additionally, the fold adopted from Ser25-Gly35 shows Ψ-Φ values in the β-sheet region of the Ramachandran plot. In connection with the β-turn just before the helix this might lead to a β-structure under certain circumstances. The part of the protein in the lumen seems to adopt only a random coil structure. Interestingly, we find a high affinity for ions in the region formed by Glu65, Ser68, Asp70, and the C-terminal (Gly73) oxygen (marked * in Fig. 1). The possible ability of the C-terminal domain in the lumen to bind ions has to be explored further.

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