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Preliminary structural characterisation of the 33 kDa protein (PsbO) in solution studied by site-directed mutagenesis and NMR spectroscopy†

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Site-directed mutagenesis experiments combined with 1D and 2D NMR spectra provide a preliminary insight into the structure and dynamics of the 33 kDa protein (PsbO) from *T. elongatus* free in solution. The NMR spectra suggest that PsbO rather than forming a 'molten globule' state or being 'natively unfolded', contains both a well folded core and highly flexible regions. This core shows remarkable stability over a broad range of temperatures and pH values. Additional experiments with Cys residues introduced at different positions indicate sites of increased accessibility/flexibility which may be important for the docking to the PS2 core complex.

Introduction

Photosystem 2 (PS2) is part of the thylakoid membrane of chloroplasts and cyanobacteria and performs one of the key reactions on our planet: the light-driven oxidation of water.[1,2] It consists of more than 20 polypeptides, most of them with no function identified yet. Especially, the role of the numerous small membrane spanning peptides is still unclear.[3] The primary electron transfer reaction occurs at the heterodimeric core of PS2 formed by the subunits D1 and D2, which bind most of the redox active cofactors. Water oxidation to molecular oxygen is catalysed *via* the sequence of four one-electron oxidation steps (S-states) by the manganese containing water-oxidising complex (WOC).[4] Recent advances in X-ray structure analysis of PS2 crystals[5,6] provide a detailed view of the structure and organisation of the WOC. At the lumen side of PS2, the WOC is shielded by several extrinsic proteins.[7] One of them, the 33 kDa protein (PsbO), is of special interest because it is ubiquitous in all photosynthetic organisms performing water-splitting and its extraction results in a strong decrease of the oxygen evolving activity. Therefore, it is often referred to as manganese stabilizing protein (MSP). However, it is obvious from the recently published PS2 X-ray structure[6] that the PsbO subunit is not directly involved in binding of manganese as part of the WOC.

Several lines of experiments using different kinds of spectroscopic techniques or biochemical and theoretical analyses have made suggestions about the PsbO structure and its behaviour free in solution. It was concluded from Fourier transform infrared (FTIR) spectroscopy[8–10] and circular dichroism (CD) spectroscopy[11,12] that the PsbO subunit in solution consists of a high content of β-sheet (30%) and random/loop structure (60%) whereas α-helical parts are only present in small amounts (10%). These results were consistent with secondary structure prediction methods and folding models of the 33 kDa protein.[13–15] Some authors suggested an unusual folding behaviour for PsbO in solution and therefore postulated an 'intrinsically disordered', 'natively unfolded' or 'molten globule' protein structure.[10,16] Experiments with focus on the dynamic properties of the PsbO subunit led to the observation of pH-dependent and cofactor-induced structural changes in solution.[11,17,18] Recently, it was reported that reconstitution of the PS2-complex with PsbO is accompanied by changes of its secondary structure.[19] Indeed, there are hints for structural changes of the bound PsbO subunit depending on the oxidation state of the manganese cluster.[20,21]

Here we report preliminary results from NMR-spectroscopy on PsbO from *Thermosynechococcus elongatus* (*T. elongatus*) describing its structural and dynamic properties. The 1D and 2D NMR spectra suggest that PsbO free in solution adopts a defined structured core. The spectra also indicate that the PsbO from *T. elongatus* in solution contains a considerable amount of flexible regions. The PsbO protein shows a remarkable temperature, pH, and long term stability beeing stable for at least four weeks under the conditions tested in this study. Due to this extraordinary stability of PsbO, we characterised four cysteine mutants serving as local probes for structural and dynamic properties of PsbO.

Materials and methods

Sample preparation

The recombinant 33 kDa protein was expressed in *E. coli* from an expression plasmid containing the full-length sequence of the *T. elongatus psbO* gene resulting in the export of PsbO into the periplasmic space.[22] Point mutations were constructed by oligonucleotide-directed mutagenesis.[23] The periplasmic cell-fraction highly enriched with 33 kDa protein was isolated,[24] dialysed against 20 mM Tris pH 8.0, 0.5 M mannitol, and loaded onto a ResourceQ column (Amersham-Pharmacia). The protein was eluted with a linear gradient from 0 to 0.5 M NaCl. By this method, we obtained highly purified PsbO (up to 20 mg L^{-1} culture). The ^{15}N-labeled samples were prepared from minimal medium with ^{15}N NH_4Cl as the nitrogen source.[25] Unless otherwise stated, PsbO protein samples for NMR experiments were prepared in 20 mM Na-phosphate buffer, pH 3.6, supplemented with 10% D_2O.

† Electronic supplementary information (ESI) available: colour version of Fig. 4. See http://www.rsc.org/suppdata/cp/b4/b407316a/

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To prove the integrity of the recombinant protein, reconstitution experiments were performed with PS2 particles prepared according to ref. 26. These particles were washed with 1 M $CaCl_2$ to remove the extrinsic proteins before adding the recombinant PsbO protein.[27]

NMR spectroscopy

NMR experiments were carried out at different pH and at different temperatures on a Bruker DRX600 spectrometer equipped with a 5 mm BBI probe and a *z*-axis gradient unit. Water suppression for 1D and 2D experiments was achieved by the watergate pulse scheme.[28]

The 1D ^{1}H NMR spectrum was recorded with a time domain of 32 k complex points and a sweep width of 12376.24 Hz. The 2D ^{1}H-^{15}N HSQC spectrum was recorded with a time domain of 2 k complex data points with 256 complex increments collected, and a sweep width of 12019.23 Hz in the ^{1}H and 2128.68 Hz in the ^{15}N dimension. Modified versions of the experiment proposed previously were used to determine ^{15}N{^{1}H}-NOE at 600.13 MHz proton frequency.[29–36] The ^{15}N{^{1}H}-NOE experiment was recorded using a 4.9 s saturation period and an additional recycle delay of 5 s. The ^{15}N{^{1}H}-NOE was recorded in an interleaved manner to reduce influences from possible instabilities under the chosen experimental conditions.

Data analysis

NMR spectra were processed and analysed with XWINNMR (Bruker, Rheinstetten, Germany), NMRPipe, NMRDraw, and NMRView.[37] NOE values were calculated from the ratio of the peak heights in the experiment with and without proton saturation. Uncertainties for NOE values were determined from triple recording of the whole relaxation experiment. Albeit no assignments are available, the NOE values were quantified based on the fraction of resolved peaks with NOE values less than 0.5 *versus* the total number of resolved resonances observed in the experiment.

Results and discussion

Recombinant wild-type PsbO (WT-PsbO)

In this report, we have used NMR-spectroscopy to elucidate preliminary structural and dynamic properties of the 33 kDa protein from the donor side of PS2. In order to investigate functional aspects of this protein, we have also carried out site-directed mutagenesis experiments. The PsbO protein from the thermophilic cyanobacterium *T. elongatus* was overexpressed in *E. coli*. Its ability to restore the water splitting activity of $CaCl_2$-washed PS2 was shown by reconstitution experiments. Routinely, about 50% of the original oxygen-evolving activity was obtained with the reconstituted PS2 (data not shown). Fig. 1 shows the 1D ^{1}H NMR spectrum of wild-type PsbO free in solution. The dispersion of aliphatic and amide proton resonances clearly indicates that the protein is, at least in part, comprised of a well folded core domain.

In order to test the influence of different experimental conditions on the stability of WT-PsbO we recorded several 1D ^{1}H NMR spectra at different pH values and at different temperatures. No significant changes in the 1D ^{1}H NMR spectra were observed for spectra recorded at pH 3.6, 6.5, and 8.0. Obviously, PsbO retains its fold and is not denatured by lowering the pH from 6.5 to 3.6. Therefore, we recorded several 1D NMR spectra at 298 K, 310 K, 313 K, and 318 K with a buffer pH adjusted to 3.6. The line width of these 1D spectra did not change dramatically suggesting that PsbO structure is stable at higher temperatures. In conclusion, the free thermophilic PsbO in solution is stable at pH 3.6 and 310 K as judged from the signal dispersion in the NMR

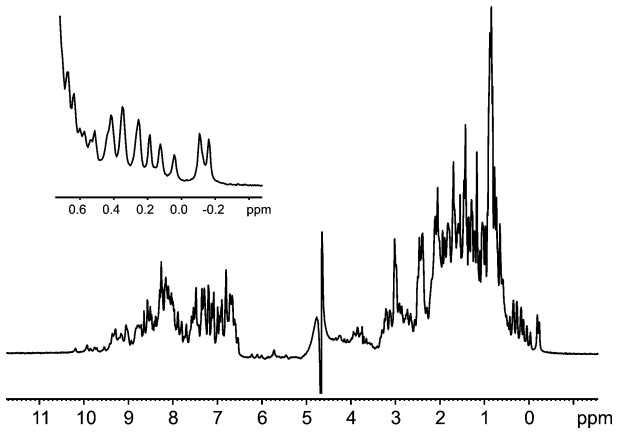

Fig. 1 600 MHz ^{1}H-NMR spectrum of WT-PsbO at pH 3.6 and 310 K. The inset shows the enlarged region of dispersed high field shifted resonances characteristic for a folded protein. For details refer to text.

spectra. No significant protein degradation was observed even after four weeks.

Fig. 2 shows a 2D ^{1}H-^{15}N HSQC spectrum of PsbO isotopically enriched with ^{15}N at pH 3.6 and 310 K. The 2D ^{1}H-^{15}N HSQC spectrum is characterised by a substantial amount of well dipersed resonances indicative for secondary structure elements (especially β-sheet) present in PsbO.[38–40]

However, the 1D ^{1}H NMR and the 2D ^{1}H-^{15}N HSQC spectra also show intense signals at 1–2 ppm and at 8.5 ppm characteristic for unstructured polypeptide chains. This is consistent with the X-ray structure of PsbO that contains 30% β-sheet and 5% α-helix with several loops involved in binding to the PS2-complex. Remarkably, 65% of PsbO do not show any secondary structure elements in the X-ray structure of the PS2 complex.[6]

This is further corroborated by the ^{15}N{^{1}H} NOE of wild-type PsbO. Out of 109 resolved and dispersed resonances in the ^{15}N{^{1}H} NOE experiment, approximately 29 show values less than 0.5 suggesting that these residues are part of unstructured elements of PsbO (data not shown). Peaks that cluster around 8.5 ppm have been omitted in this preliminary analysis due to spectral overlap. Based on the ^{15}N{^{1}H} NOE, at least an estimated 30% to 40% of all residues of WT-PsbO show increased flexibility on the ps to ns time scale (data not shown). Nevertheless, this is only in part consistent with the analysis of the B-factors obtained from the X-ray structure where the loops do not show elevated values compared to the core of PsbO. From this preliminary comparison, it appears that flexible regions of free PsbO are motional restricted when bound to the PS2 complex.

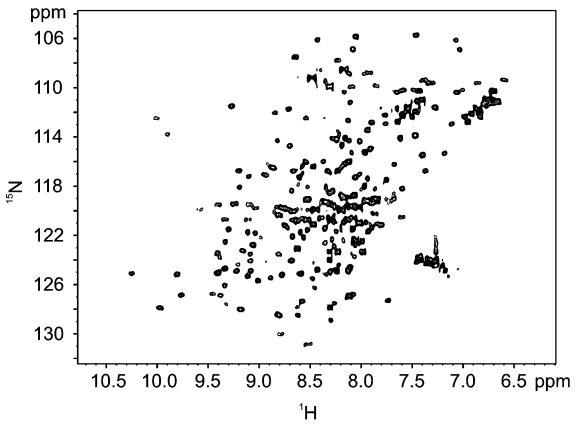

Fig. 2 600 MHz ^{1}H-^{15}N HSQC of WT-PsbO at pH 3.6 and 310 K. The signal dispersion shows that PsbO free in solution contains a well folded core in addition to flexible regions. For details refer to text.

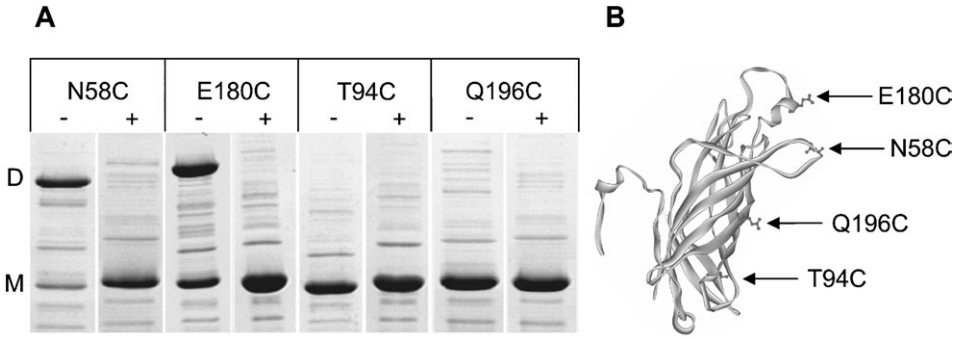

Fig. 3 (A) SDS-PAGE in the absence (−) and presence (+) of DTT performed with periplasmic fractions from *E. coli* cells expressing different PsbO mutants. Bands for monomers and dimers of PsbO are indicated by M and D, respectively. (B) Location of the additional cysteines, introduced by site-directed mutagenesis in PsbO of the PS2 complex from *T. elongatus* (PDB code: 1S5L, http://www.rcsb.org/).

Overall, the 2D ^{1}H-^{15}N HSQC spectrum of wild-type PsbO is characteristic for a folded protein which also contains a considerable amount of unstructured parts. However, the presence of a considerable amount of flexible regions in the free form of PsbO does not explain why PsbO from *T. elongatus* exhibits a higher thermostability than PsbO from spinach, especially as the content of Pro residues is virtually indentical in both organisms (5.6% for spinach compared to 5.3% for *T. elongatus*).[43] In conclusion, the structured core should be one of the major reasons for the thermostability of the thermophilic 33 kDa protein where a variety of factors may contribute to this stability as shown for other thermophilic proteins.[41]

Site-directed mutagenesis of PsbO

In order to investigate the accessibility and mobility of surface-exposed structural protein elements, we created four mutants containing an additional cystein each at a different position in the amino acid chain of PsbO. These mutants were analysed by SDS-PAGE in the presence and absence of DTT. Fig. 3 shows that two of them, N58C and E180C, form dimers whereas T94C and Q196C are monomeric under oxidizing conditions.

The mutations T94C and Q196C are located in the folded β-barrel core of PsbO while the mutations E180C and N58C are part of the extended loops. Obviously, the folded core region sterically prevents the formation of a dimer while the Cys residues in the loops are easily accessible for interaction. From these results we conclude, that the regions around residues Asn58 and Glu180 of PsbO are either flexible and/or solvent exposed to allow the formation of a disulfide bond between two PsbO molecules.

Fig. 4 shows 1D ^{1}H NMR spectra of the oxidised and the reduced form of mutant N58C and E180C. Interestingly, in our experiments the signal dispersion and the line width in the 1D NMR spectra of the reduced mutant proteins does not dramatically differ from the spectra recorded in the absence of DTT. Typically, the line width for several selected aliphatic resonances is approximately 24–27 Hz for WT-PsbO, 28–29 Hz for oxidised N58C and 21–22 Hz for reduced N58C, and approximately 24 Hz for oxidised E180C as well as for reduced E180C. These values indicate that the line width does not significantly change upon reduction of the oxidised mutants of PsbO as a more dramatic change would be expected from the decrease in molecular weight (52 kDa compared to 26 kDa).

This suggests that both PsbO domains of the disulfide linked dimer are folded and tumble, at least to a significant extent, independently free in solution. From this we conclude that the loops carrying the point mutation exhibit an increased degree of flexibility in the apo-form of PsbO. Especially, the reduced and the oxidised form of the PsbO E180C mutant show a signal dispersion and line widths in the 1D NMR spectrum that are virtually identical and comparable to the spectra of WT-PsbO.

It is interesting to note that especially the longer loop (carrying the mutation E180C) which is composed of 40 residues does not show any secondary structure elements in the X-ray structure of the PS2 complex from *T. elongatus*. This loop does not show elevated B-factors in comparison to the core of the protein which could be explained by its various contacts to other subunits of the PS2 complex, such as D2, CP47, and the 12 kDa-Protein (PsbU). Comparing the preliminary results presented in this study with the B-factors for loop regions and the core of PsbO from the X-ray structure, we propose that originally flexible loops of free PsbO, *e.g.* the longer loop, are structurally better defined after complexation with PS2. Furthermore, our analysis of the cysteine mutants indicates that the presence of the disulfide bond C19–C44 in PsbO from *T. elongatus* might not be critical for a stable fold. It is also in accordance with the observation that removal of the cysteins of PsbO by mutagenesis neither impaired its binding to PS2 nor influenced its water-splitting activity.[42] Further, Fig. 1 shows that the line width of the 1D ^{1}H NMR spectrum of

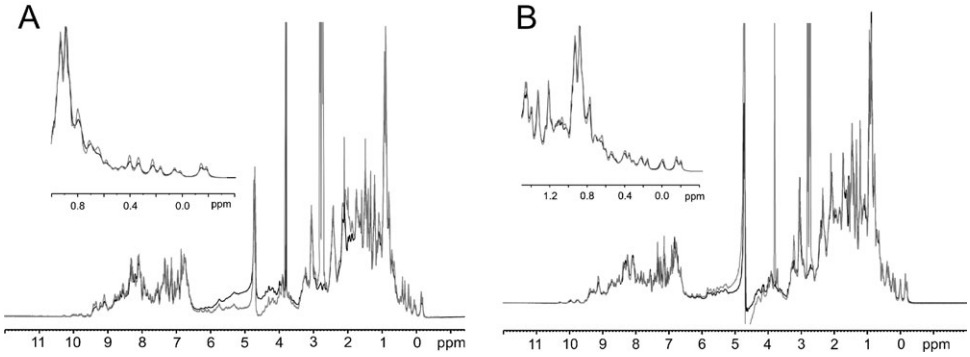

Fig. 4 600 MHz ^{1}H-NMR spectrum at pH 3.6 and 310 K of reduced (grey) and oxidised (black) PsbO N58C (A) and PsbO E180C (B). The inset shows the enlarged region of dispersed high field shifted resonances. For details see text.

WT-PsbO is characteristic for a monomeric state of the protein in solution. This is corroborated by the results from HPLC size exclusion experiments that show a monodispersed peak corresponding to an approximate molecular weight of 20 kDa for the recombinant PsbO (data not shown). This suggests that WT-PsbO is monomeric in solution.

In summary, we have shown by NMR-spectroscopy that apo-PsbO contains folded structural elements as concluded from the signal dispersion of 1D and 2D NMR spectra. Our data suggest that PsbO, rather than forming a 'molten globule' state or being 'natively unfolded', contains both a well folded core and highly flexible regions. In addition, the $^{15}N\{^{1}H\}$-NOE experiment provides evidence for a significant number of residues that show a higher degree of flexibility (approx. 30–40%). Spectra of the oxidised and reduced forms of PsbO N58C and PsbO E180C suggest that loops responsible for the interaction with the PS2 complex are flexible and/or solvent exposed when free in solution. Comparing our preliminary results with the B-factors for loop regions and the core of PsbO from the X-ray structure, there is evidence for the compelling hypothesis that originally flexible loops of free PsbO get motional restricted upon binding to PS2. This is further corroborated by the changes of apparent hyperfine splitting and line width of EPR spectra recorded with spin labeled PsbO E180C free in solution and after reconstitution of the PS2 complex (Nowaczyk, unpublished data). Based on these results we propose that changes in the flexibility between the free and bound form of PsbO, particularly of the loop regions, are responsible for an efficient binding of PsbO to the PS2 core and might play a crucial role in modulating the binding affinity.

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