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Biochimica et Biophysica Acta 1767 (2007) 583–588
Biochimica et Biophysica Acta
BBA
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Manganese binding to the 23 kDa extrinsic protein of Photosystem II

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Received 30 September 2006; received in revised form 19 December 2006; accepted 3 January 2007
Available online 10 January 2007

Abstract

The recombinant form of the extrinsic 23 kDa protein (psbP) of Photosystem II (PSII) was studied with respect to its capability to bind Mn. The stoichiometry was determined to be one manganese bound per protein. A very high binding constant, $K_A = 10^{-17}$ M^{-1}, was determined by dialysis of the Mn containing protein against increasing EDTA concentration. High Field EPR spectroscopy was used to distinguish between specific symmetrically ligated Mn(II) from those non-specifically Mn(II) attached to the protein surface. Upon Mn binding PsbP exhibited fluorescence emission with maxima at 415 and 435 nm when tryptophan residues were excited. The yield of this blue fluorescence was variable from sample to sample. It was likely that different conformational states of the protein were responsible for this variability. The importance of Mn binding to PsbP in the context of photoactivation of PSII is discussed.

Keywords: Photosynthesis; Photosystem II; 23 kDa protein; Manganese

1. Introduction

Photosystem II (PSII) is the protein complex which catalyzes the oxidation of water and the reduction of plastoquinone [1]. The oxidation of water takes place at a tetramanganese cluster bound to the reaction centre protein D1. In addition to the Mn cluster, the cofactors calcium and chloride are obligatorily required for photosynthetic water oxidation [2]. Three extrinsic proteins with the apparent molecular masses of 33, 23 and 17 kDa (PsbO, PsbP, PsbQ) are bound to the luminal side of PSII of green algae and higher plants [3,4]. In cyanobacteria, PsbP and PsbQ are functionally replaced by PsbU and PsbV (for review see [5]). It has been recently reported that in cyanobacteria homologues of PspP and PsbQ are expressed in addition to PsbU and PsbV [6]. PsbP was present in substoichiometric amounts while one copy of PsbQ was present per PSII [6]. Of all the extrinsic proteins, the PsbO protein is most strongly bound and stabilizes the Mn cluster. It has therefore also been termed the "manganese-stabilizing protein". This protein is absolutely required for photoautotropic growth [7]; while in the absence of PsbP and PsbQ plants can still grow photoautotrophically, although growth is retarded in the absence of PsbQ [8]. The structure of PsbP has been determined [9], but its function remains still a matter of debate. It has been proposed that PsbP is involved in increasing the binding affinities of both Ca^{2+} and Cl^-, in addition to their general function in stabilizing the Mn cluster (for review see [3,4]. The FUD mutant of *Chlamydomonas reinhardtii* lacks PsbP but possesses PsbO and PsbQ [10]. This mutant has a decreased photoactivation capacity and is more susceptible to photoinhibition than the wild type [11,12]. The same observations hold for higher plants as has been shown in studies of tobacco plants in which the level of PsbP was severely down-regulated by RNA interference technique [8].

Metal binding to the extrinsic proteins, especially to PsbO, has been studied in detail. An apparent calcium-binding site in this protein was identified in the recent structure of the PSII complex [13]. The binding of Ca^{2+} to the isolated PsbP was determined to be weak with a K_A on the order of 10^{-5} M^{-1} [14]. Ca^{2+} binding has been reported to induce changes in the secondary structure [15]. However in a second study using the same method, no such changes in the secondary structure were observed [16]. It has also been reported that PsbO is able to bind manganese [17,18] and it has been shown to have a carbonic anhydrase activity in the presence of manganese [19].

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doi:10.1016/j.bbabio.2007.01.001

It was also proposed that PsbP is also capable for binding calcium and manganese. It has been reported that the amino acid residues at the N-terminus, especially the aspartatic acid residue at codon 15, are involved in Ca^{2+} binding/retention [20]. Recently we have reported that PsbP can bind Mn and we have shown that isolated PsbP, which was preincubated with manganese, facilitated photoactivation [21]. Photoactivation was strongly accelerated and, in addition, occurred efficiently at low Mn(II) concentrations in the presence of PsbP.

In the present study we focus on characterising manganese binding to PsbP. The stoichiometry of Mn to protein and the binding constant were determined by dialysing the Mn containing protein against a Mn(II) free buffer or an EDTA-containing buffer. The Mn content was determined by atomic absorption spectroscopy. The Mn binding site was characterised by high field EPR spectroscopy. Furthermore the effect of Mn on the intrinsic protein fluorescence was studied.

2. Materials and methods

2.1. Recombinant PsbP

Expression and purification of PsbP were performed as described in [20]; the *E. coli* strain was kindly provided by K. Ifuku (Kyoto University, Japan). Protein quantification was done photometrically by measuring the absorption at 280 nm and using an extinction coefficient of 24924 M^{-1} cm^{-1} determined from the amino acid sequence by using an algorithm available at http://paris.chem.yale.edu/extinct.html.

2.2. Dialysis experiments

Protein was incubated with the given $MnCl_2$ concentration in 10 mM MES, pH 6.5, at room temperature and was shaken gently for 2 h. Small dialysis chambers with volumes between 150 and 500 µl were used and dialysed at 6 °C against 1000–6600 fold volume of 10 mM Mes, pH 6.5.

2.3. Atomic absorption spectroscopy

Mn content of the samples was determined at 279.5 nm using an atomic absorption spectrometer (Perkin Elmer 3030). Samples were diluted in 0.4% HNO_3. Aliquots (20 µl) of the final solution were pipetted automatically into the graphite furnace. Calibration was done by using a standard Mn solution (Sigma). The error of the measurements was less than 5% of the mean values.

2.4. Calculation of the binding constant K_A

The Mn–PsbP binding constant was calculated by using the equilibrium equations for MnEDTA (K*) and MnPsbP (K). K*=[MnEDTA]/([EDTA][Mn]), K=[MnPsbP]/([PsbP][Mn]), E=[EDTA]+[MnEDTA], F=[PsbP]+[MnPsbP], G=[Mn]+[MnEDTA]+[MnPsbP], H=[Mn]V_0+[MnEDTA]V_0+[MnPsbP]V_1. The binding constant is given by $K_A=(V_0G+V_0EK^*G)/(HF-HG-V_1GF+V_1G^2)$. The following values were used: $V_0=1$ l, $V_1=150\times10^{-6}$ l, K*=13.56 M^{-1}, E: variable, G: measured Mn content, $F=180$ nM, $H=180$ nM*150×10^{-6}l. The free Mn concentration was estimated to be close to 0.

2.5. High Field EPR

The spectrometer has been described in detail elsewhere [22]. Field calibration was based on a Mn(II) doped MgO standard ($g=2.000101$) sample and the absolute error in field measurements was 1G (0.1 mT) or 0.0001 in g. All spectra were taken with 2 Gauss modulation and under non-saturating conditions.

2.6. Fluorescence

Fluorescence measurements were performed using a Cary Eclipse Spectrofluorimeter (Varian) equipped with a cuvette holder which was connected to a water bath. The emission spectra were obtained by excitation of 1 µM protein at 297 nm at 25 °C in 10 mM Mes, pH 6.5.

3. Results

3.1. Determination of the specific binding capacity and binding constant of Mn to PsbP

We had shown previously that PsbP is capable of binding up to a 10-fold excess of Mn(II) [21]. However, we could not distinguish between the specific binding of manganese in a well defined binding site in contrast to loose attachment to the protein surface. Here we applied prolonged dialysis against a Mn(II)-free buffer to determine the amount of strongly bound manganese. The Mn content in the dialysis buffer was determined by atomic absorption spectroscopy. Before starting the dialysis, a 10-fold excess of $MnCl_2$ solution was added to a solution of recombinant PsbP protein. Fig. 1 shows the time course of Mn concentration in the dialysis buffer. From a control experiment without protein, it was found that after 2 h almost all manganese was detected in the dialysis buffer. In the presence of PsbP one Mn per protein was retained in the dialysis chamber even after a prolonged dialysis of 20 h. Therefore we concluded that the stoichiometry of Mn per protein is 1:1.

For the determination of the binding constant, samples containing PsbP protein and Mn in the stoichiometry 1:1 were dialysed for 2 h against different EDTA concentrations (Fig. 2). The Mn content in the dialysis chamber was measured and the Mn–PsbP binding constant was calculated by using the equilibrium equations and the value for the MnEDTA binding constant of 13.56 M^{-1}. The theoretical curve for Mn binding to PsbP as a function of the EDTA concentration is shown as a

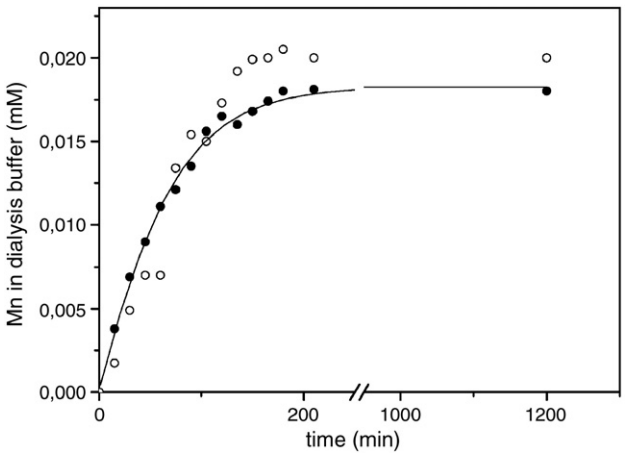

Fig. 1. Determination of Mn: protein stoichiometry. 20 µM protein was incubated with 200 µM$MnCl_2$ for 2 h. Mn release was measured during dialysis in Mn-free buffer as an increase of the Mn concentration in the dialysis buffer with time (closed symbols). As control 200 µM $MnCl_2$ solution without protein was dialysed (open symbols). The Mn:protein stoichiometry is 1:1±0.041 ($n=11$) after a minimum of 2 h dialysis.

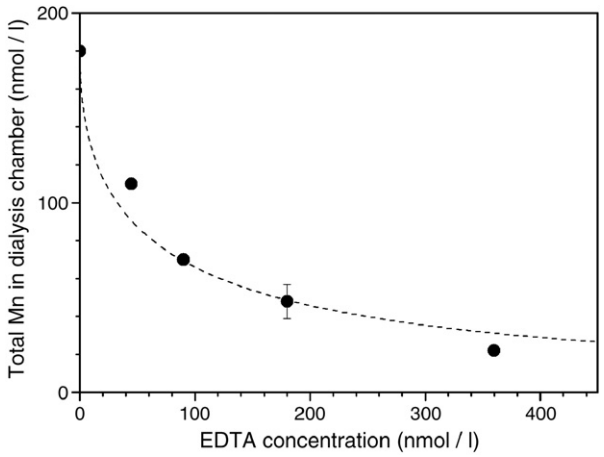

Fig. 2. Determination of the binding constant. 180 nM protein which contained 1 Mn per protein was dialysed for 2 h against dialysis buffer which contained the given EDTA concentrations. The Mn-content inside the dialysis chamber was measured. The dotted line shows the theoretical curve for binding equilibrium which was calculated using a $K_{A(EDTA)}=10^{-13.56}$ M^{-1}. The expected error of the measurements is as shown by one error bar ($n=2$).

dotted line in Fig. 2 and the binding constant MnPsbP was calculated to be $K_A=10^{-17.11\pm0.18}$ M^{-1}.

To study the exchange of manganese by other divalent cations, PsbP samples with one manganese per protein were dialysed against buffers containing either $CaCl_2$ or $ZnCl_2$. In one experiment, the cationic concentration in the dialysis buffer was equal to that of the protein in the dialysis chamber while in others a 5-fold to 10-fold higher concentration of metal ions was used. No exchange was observed when PsbP samples (1 Mn:1 protein) were dialysed for 2 h against $ZnCl_2$ solutions, while the manganese concentration was lowered by 50% when dialysed against a 10-fold excess of $CaCl_2$. Twenty-five percent of the Mn was replaced when dialysed against a 5-fold excess of $CaCl_2$. No loss of Mn was found when the $CaCl_2$ concentration of the dialysis buffer was the same as the Mn concentration inside the dialysis chamber. This indicated that Mn could be exchanged by Ca^{2+} but not by Zn^{2+}.

3.2. Detection of Mn bound to PsbP by High Field EPR spectroscopy

Mn bound to the protein could not be detected by X-band EPR spectroscopy even at liquid helium temperatures. Therefore we used High Field EPR spectroscopy using magnetic fields between 3 and 10 T. High field magnetic fields both enhance the detection of Mn(II) and also greatly simplify the spectra (for example see [23]). Fig. 3 shows the 285 GHz 10 T EPR spectra of PsbP protein which was incubated with a 10-fold excess of $MnCl_2$ and dialysed for 2 h or 18 h. The spectra show a relatively sharp six line signal which is characteristic for a six coordinated Mn(II) in an environment that is electronically symmetric. This does not necessarily imply that the ligands are identical or symmetrically placed, rather that the electronic structure is symmetric. An example of this is concanvalin-A where the Mn(II) is bound by three Glu, one His and 2 water molecules [23]. Even with such an arrangement the High Field EPR spectrum of Mn(II) yields six very sharp lines indicative of a small zero-field interaction and a very electronically symmetric binding site [24]. After 18 h dialysis of Mn containing PsbP the spectral lines were much narrower than after 2 h dialysis. This broadening of the spectra is an indication of Mn binding to different sites most likely at the surface of the protein. The narrow lines detected after removal of excess Mn indicate that Mn in its tight binding site has a very symmetrical magnetic coordination sphere. For purpose of comparison, a protein sample is shown prior to the addition of $MnCl_2$. In an attempt to enhance the difference in line width spectra were also recorded at 3 T. In general a reduction of the magnetic field leads to an increase of Mn(II) line width and this was indeed the case (Fig. 4). Spectra of samples were recorded which contained manganese in substoichiometric amounts (0.5 Mn:1 protein) and a twofold excess of manganese (2 Mn:1 protein) (Fig. 4). The spectra were recorded immediately without incubation and dialysis. The difference of the two spectra showed again six narrow lines indicative of manganese in its specific symmetrical binding site. This shows that Mn(II) is incorporated both very quickly and with high specificity.

3.3. Characterisation of Mn binding to PsbP by intrinsic fluorescence

Emission spectra of proteins are often used to characterise metal-induced alterations of the protein structure (see e.g. [14] for a study on Ca^{2+} and lanthanide-binding to PsbO). PsbP contains 7 Tyr and 2 Trp residues and emits a typical fluorescence emission with a maximum at 333 nm originating from Trp which originated either from direct excitation of the Trp residues or indirectly by excitation of the Tyr residues (Fig. 5). Addition of Mn(II) in stoichiometric amounts gave rise to new fluorescence bands with maxima at 415 and 435 nm with concomitant decrease in tryptophan fluorescence.

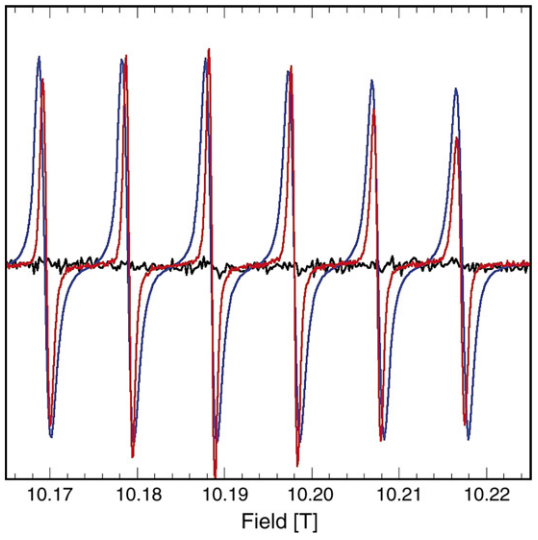

Fig. 3. High Field EPR spectra (10 T, 25 K) of psbP. 25 μM protein were incubated with 250 μM $MnCl_2$ for 2 h and subsequently dialysed for 2 h (blue line) and 18 h (red line). As a control psbP (25 μM) in the absence of Mn is shown (black line).

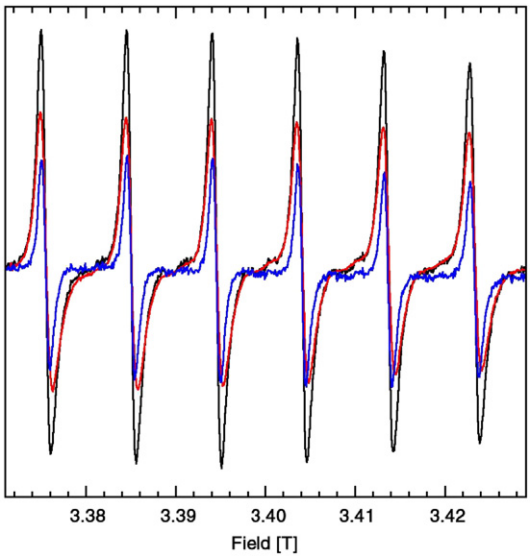

Fig. 4. High Field EPR spectra (3 T, 10 K) of psbP. 7.5 μM $MnCl_2$ (red line) and 30 μM $MnCl_2$ (black line), respectively, were added to 15 μM protein and immediately frozen (red line). The blue line shows the difference between the two signals.

The excitation spectra measured at 415 and 435 nm, respectively, showed a broad band between 320 and 380 nm (data not shown).

The intensity of the new fluorescence bands was very variable from sample to sample. In some samples, the fluorescence was as high as shown in Fig. 5. In samples exhibiting high fluorescence several cycles of freezing the samples resulted in about 30% loss of the fluorescence intensity. Different samples gave much lower fluorescence at 415 and 435 nm in the presence of Mn(II) as shown in Fig. 6. In these cases the fluorescence could be detected only shortly after the addition of $MnCl_2$ and disappeared after several minutes. The influence of NaCl concentrations, buffer compositions, pH and temperature was investigated but none of these conditions influenced the reproducibility of the fluorescence intensity. Denaturation of

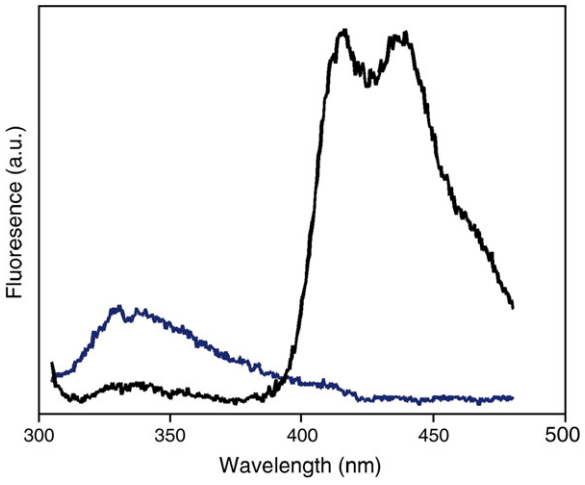

Fig. 5. Intrinsic fluorescence of psbP. Emission spectra of 2 μM psbP without Mn (blue line) and with 2 μM $MnCl_2$ (black line). Fluorescence was excited at 297 nm.

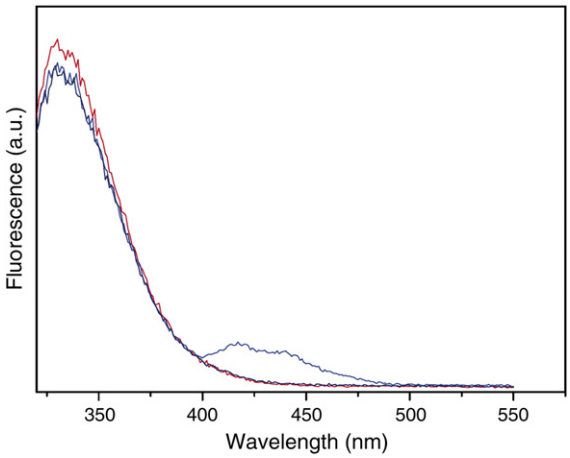

Fig. 6. Intrinsic fluorescence of psbP. Emission spectra of 2 μM psbP without Mn (red line) and with 2 μM $MnCl_2$ measured immediately after addition (blue line) and 5 min later (black line). Fluorescence was excited at 297 nm.

the protein by the addition of 6 M guanidinium hydrochloride restored the fluorescence, but only transiently (Fig. 7). At the same time the emission of the trpytophan fluorescence was shifted from 333 to 360 nm, a well known effect associated with denaturation. These data indicate that the conformational state of PsbP in the presence of Mn is important for both, the intensity and the stability of the new fluorescence bands.

4. Discussion

Addition of $MnCl_2$ to PsbP leads to a binding of manganese. The stoichiometry of Mn(II) uptake by PsbP was determined to be 1:1 when measured by atomic absorption spectroscopy (Fig. 1). This seems to represent a specific binding because the Mn content was not lowered by prolonged dialysis against Mn-free buffer. The binding constant was determined to be very high (Fig. 2). Similar high binding constants for Mn to protein has been determined for Mn(III) in MnSOD [25]. Proteins which bind Mn(II) have normally lower binding constants [26]. Previously we had determined a stoichiometry of 10 Mn:1 protein by room temperature X-band EPR spectroscopy [21]. In the previous experiments, samples had not been subjected to dialysis and non-specific loose binding to the surface occurred. High Field EPR clearly demonstrated these two modes of binding (Figs. 3 and 4). The X-ray structure of PsbP shows that nine glutamate and fourteen aspartate residues are located on the surface of the protein [9]. Mn prefers such hard ligands, therefore it seems very likely that many of these residues could be involved in loose attachment of Mn to the protein surface. Mn in the high affinity site of PsbP could be replaced by an excess of Ca^{2+} but not by Zn^{2+}. This cannot be explained by a difference in charge or in size, because the ionic radius of Mn^{2+} is about the same as Zn^{2+} and a bit smaller than that of Ca^{2+}. The difference may be caused by nature of the ligands which are involved in the metal coordination.

When Mn was added to PsbP fluorescence was emitted with maxima at 415 and 435 nm. The observed variability of the fluorescence yield (Figs. 5 and 6) was most likely caused by

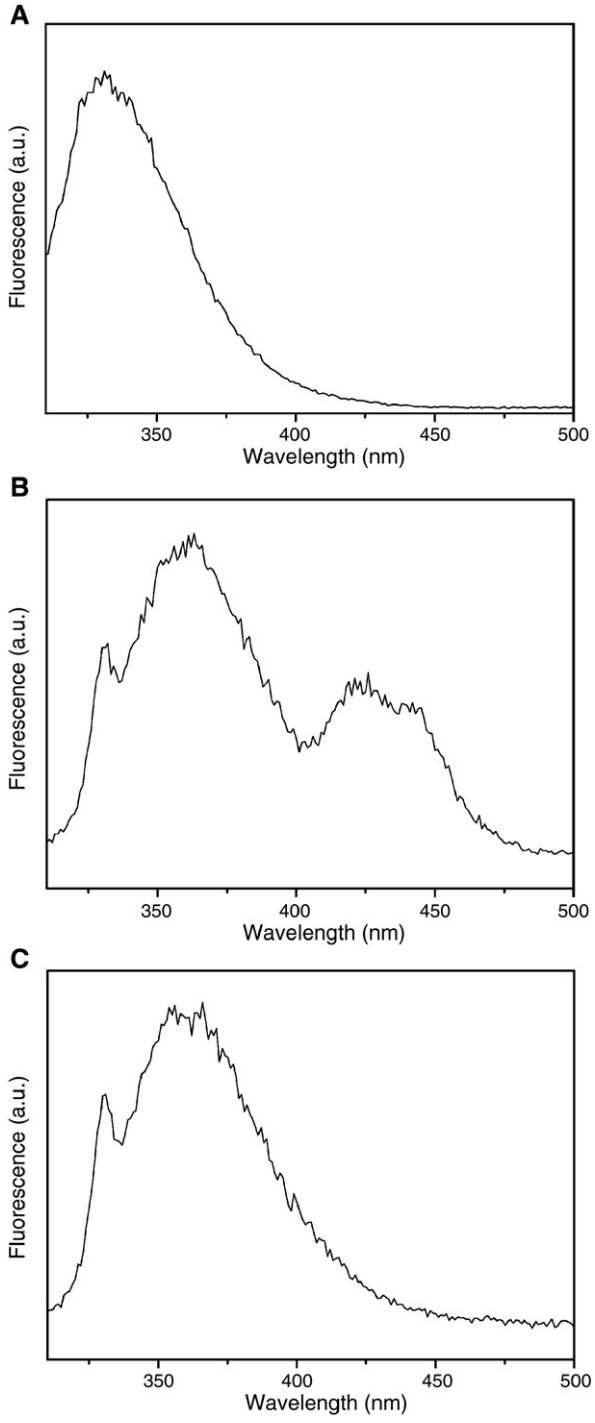

Fig. 7. Intrinsic fluorescence of psbP. Emission spectra of 2 μM psbP with 2 μM $MnCl_2$ (A), immediately (B) and 5 min later (C) after addition of 6 M GuHCl. Fluorescence was excited at 297 nm.

conformational changes. In some samples the fluorescent state relaxed to a non-fluorescent stable conformation. When a chaotropic agent was added (GuHCl in the experiment shown in Fig. 7), the unstable fluorescent state was populated transiently. Fluorescence disappeared when the completely unfolded state was reached.

To our knowledge such fluorescence bands have not been reported previously for Mn-containing proteins. The fluorescence bands were detected when either tyrosine (λ_{ex}=276 nm) or tryptophan residues (λ_{ex}=297 nm) were excited. The excitation spectra of the new fluorescence bands showed a broad band between 320 and 380 nm indicating that the excitation was given by excited tryptophan residues. The most likely explanation for the new fluorescence bands is that an excited tryptophan excites in turn by resonance energy transfer a Mn–ligand complex (MnLC). Upon excitation the Mn–ligand complex undergoes charge separation and a state $(Mn^{+}L^{-})^{*}$ is formed which emits fluorescence. The two emission bands might indicate that the state $(Mn^{+}L^{-})^{*}$ exists in different conformational states.

Metal–ligand complexes can fluoresce as has been shown for ruthenium, rhenium or osmium complexes with one or two diimine ligands [27]. These complexes show fluorescence upon a metal-to-ligand charge transfer (MLCT). Upon light absorption the metal becomes oxidised and the ligand reduced. Whether a metal fluoresces, depends on the energy levels of the binding d-orbitals relative to the energy levels of the MLCT complex. The fluorescence of known MLC-samples show a strong Stoke's shift, which is also found for the Mn-containing PsbP (excitation at 333 nm, emission at 415 and 435 nm, Figs. 5–7). The extinction coefficients for complexes which are known to undergo MLCT are high, 10000–30000 M^{-1} cm^{-1}. Although the emission from Mn-containing PsbP was variable, it was clear that it could reach such high yields (Fig. 5).

The crystal structure of PsbP was obtained in the absence of Mn [8]. The question arises at which site Mn is bound to PsbP. The binding site must be close enough to a Trp residue to explain the new fluorescence bands. In the protein there are two Trp residues (Trp 34 and Trp 168). If one looks at the structure [9] in the neighbourhood of Trp 34 there are three potential ligands for Mn in a close distance: Glu 50 (6.6 Å), Asp 51 (3.7 Å), Asp 54 (6.1 Å) and Glu 39 which is not too distant, which makes this site an attractive candidate for manganese binding. However, close to Trp 168, there are also three potential ligands: Glu 177 (5.8 Å), Asp 165 (6.2 Å) and His 144 (3.7 Å). In many known manganese binding proteins like oxalate dicarboxylase [28], oxalate oxidase [29], MnSOD [30] and concanvalin A [23,31] at least one histidine residue is involved in the coordination of manganese. Site-directed mutagenesis will help to answer the question which of these two putative sites is the tight binding site for Mn.

We have shown previously that Mn-containing PsbP accelerates photoactivation, that is the assembly of the Mn cluster in Mn-depleted PSII samples [21]. The question arises how this is achieved if the protein binds just one Mn with a very strong affinity. During photoactivation an instable intermediate is formed which contains two Mn [32]. Here we propose that PsbP delivers one of these Mn and helps to stabilize the intermediate state of assembly. One can imagine the following scenario: one Mn arrives at the binding site at the D1 protein, becomes oxidised and this is followed by the binding of PsbP to the reaction center of PSII. Upon binding the conformation of PsbP is changed, the binding of Mn to its binding site is weakened and the second Mn^{2+} is donated to the D1 protein. Different conformations of PsbP seem to exist

as shown by the instability of the fluorescence at 415 and 435 nm.

Acknowledgements

We would like to thank Kentaro Ifuku (Kyoto University) for the *E. coli* strain overexpressing PsbP, László Liszkay (CEA Saclay) for calculating the binding constant, Bill Rutherford (CEA Saclay) for stimulating discussions, Thanh-Lan Lai (CEA Saclay), Rainer Maier, Sascha Panahandeh and Salim Al-Babili (Universität Freiburg) for practical help. Financial support by the Deutsche Forschungsgemeinschaft (Li 883/6-3) is gratefully acknowledged.

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