

Analysis of chloride-binding domains of the extrinsic 12-kDa protein by proteolysis and directed mutagenesis

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Introduction

Photosynthetic oxygen evolution is catalyzed by the photosystem II (PS II) complex which contains a number of intrinsic membrane-spanning protein components and several extrinsic proteins. The extrinsic proteins are different among cyanobacteria, red algae and higher plants: Higher plant PS II contains the 33-kDa, 23-kDa and 17-kDa proteins, whereas cyanobacterial and red algal PS II contains the 33-kDa, 12-kDa proteins and cytochrome *c*-550 (cyt *c*-550) (red algal PS II contains a fourth protein, a unique 20-kDa protein) (Enami et al. 2000). The 12-kDa protein and cyt *c*-550 in red algal PS II from *Cyanidium caldarium* play a role in minimizing the chloride and calcium requirement of oxygen-evolving activity, which resembles the function of the 23- and 17-kDa proteins in higher plant PS II (Enami et al. 1998). It was also reported that in *Synechocystis* PCC 6803, a deletion mutant of the *psbU* gene encoding the 12-kDa protein grew slower than the wild type under the growth medium lacking Ca²⁺ or Cl⁻ (Shen et al. 1997). These suggested that the 12-kDa protein of cyanobacterial and red algal PS II functions in minimizing the chloride and calcium requirement for oxygen-evolving activity.

The *psbU* gene coding for the red algal (*C. caldarium*) 12-kDa protein shows that the mature protein consists of 93 amino acids with a molecular mass of 10,513 Da (Ohta et al. 1999). In order to analyze the functional domains in the 12 kDa protein, we prepared the red algal 12-kDa protein partially lacking its N- and/or C-terminal peptides by limited proteolysis and directed mutagenesis, and examined their binding and reactivating abilities with PS II by means of reconstitution experiments.

Materials and Methods

Expression and purification of full-length and various mutants of the 12-kDa protein

The *psbU* gene encoding the 12-kDa protein of the red alga, *C. caldarium*, was cloned into the LIC site of plasmid pET-32Xa/LIC (Novagen, WI), resulting in a fusion protein with a His-tag (Ohta et al. 1999). The mutant sequences of the 12-kDa protein lacking 10 residues of the C-terminus and 9 residues of the N-terminus were generated by PCR method. The

recombinant plasmids were transformed into *E. coli* BL21 (DE3) and expressed efficiently by induction with 1 mM IPTG. The fusion proteins were purified with nickel affinity column, treated with factor Xa, and then the target proteins were purified by applying again to the nickel affinity column.

Limited proteolysis of the 12-kDa protein

The full length, recombinant 12-kDa protein was digested by bovine chymotrypsin (Sigma) or V8 protease (Sigma) in 10 mM MES-NaOH (pH 6.5) at 21°C for 1 hr, at an enzyme to 12-kDa ratio of 1 to 47 or 1 to 23, respectively. The reaction was stopped by addition of 1.3 mM phenylmethylsulfonyl fluoride for chymotrypsin or 2 mM diisopropyl fluorophosphate for V8 protease. For determination of cleavage sites, molecular masses and N-terminal sequences of the digested peptides were analyzed by MALDI-TOF MS (KOMPACT MALDI, Shimadzu/KRATOS) and an amino acid sequencer (model 477A, Applied Biosystems) after Edman auto-degradation.

Reconstitution experiments

Rebinding and functional properties of the digested fragments and mutants of the 12-kDa protein were examined by reconstitution experiments with CaCl_2 -washed PS II, as described previously (Enami et al. 1998). Oxygen evolution was measured with a Clark-type oxygen electrode under saturation light in 50 mM MES-NaOH (pH6.5) and 25% glycerol at 25°C in the absence or presence of 10 mM NaCl or 5 mM CaCl_2 , with 0.4 mM phenyl-*p*-benzoquinone as the electron acceptor.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was carried out with a gradient gel of 16-22% acrylamide containing 7.5 M urea. Samples were solubilized with 5% lithium dodecyl sulfate and 75 mM dithiothreitol for 30 min. After electrophoresis, gels were stained with Coomassie Brilliant Blue R-250.

Results and Discussion

Determination of cleavage sites of the 12-kDa protein by protease and reconstitution of the digested proteins with CaCl_2 -washed PS II

The digested peptide fragments of the 12-kDa protein by chymotrypsin and V8 protease were separated by SDS-PAGE, blotted to PVDF membranes, and their N-terminal sequences were determined by Edman auto-degradation. Molecular masses of the digested peptide fragments were also determined by MALDI-TOF MS. The results showed that chymotrypsin cleaved the protein at Tyr-9 and Phe-83, resulting in a peptide fragment from Leu-10 to Phe-83, and the V8 protease cleaved at Glu-5, resulting in a peptide fragment from Gly-6 to C-terminus (see Fig. 1).

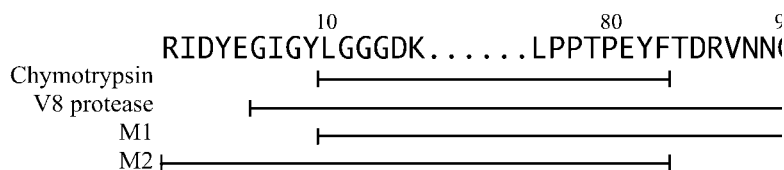


Fig. 1. Amino acid sequences of peptide fragments from limited proteolysis and mutants of the 12-kDa protein.

Table 1 shows the recovery of oxygen-evolution of the CaCl_2 -washed PS II reconstituted with the digested fragments of the 12-kDa protein, together with the 33-kDa, 20-kDa proteins

and cyt *c*-550. Reconstitution with the 33-kDa, 20-kDa proteins and cyt *c*-550 in the absence of the 12-kDa protein resulted in no restoration of oxygen evolution in the absence of Cl^- and Ca^{2+} ions; a partial recovery in the presence of Cl^- and further recovery in the presence of Ca^{2+} was observed. In contrast, reconstitution with the 12-kDa protein in addition to the three extrinsic proteins showed a high restoration even in the absence of Cl^- and Ca^{2+} ions. These indicate that the 12-kDa protein functions in minimizing the chloride and calcium requirement for oxygen evolution. The peptide fragment from Leu-10 to Phe-83 (Chymotrypsin-treated 12-kDa protein) was able to bind to PS II completely (data not shown), but no restoration of oxygen evolution was observed by its rebinding. On the other hand, the peptide fragment from Gly-6 to C-terminus (V8 protease-treated 12-kDa protein) also bound completely to PS II, but this binding resulted in the reactivation of oxygen evolution partially in the absence of ions and significantly in the presence of only the Cl^- ion. These indicate that either the C-terminus or N-terminus or both are functionally important domains to activate oxygen evolution in the absence of Cl^- and Ca^{2+} ions.

Reconstitution of mutants of the 12-kDa protein

To determine the functional domains of the 12-kDa protein on the basis of the above results,

Table. 1. Oxygen evolution of the CaCl_2 -washed PS II reconstituted with various peptide fragments of the 12-kDa protein, together with the other three native extrinsic proteins. Oxygen-evolving activities of control PS II (100) were 3230, 3521, 3680 $\mu\text{mol O}_2/\text{mg chl/h}$, respectively, in the absence or presence of 10 mM NaCl or 5 mM CaCl_2 .

	Oxygen evolution (Relative value %)		
	- ion	+ 10 mM NaCl	+ 5 mM CaCl_2
Untreated PS II	100	100	100
CaCl_2 -washed PS II	0	4	7
+33-kDa + 20-kDa + cyt <i>c</i> -550	4	36	52
+Full length 12-kDa protein	58	67	72
+Chymotrypsin-treated 12-kDa	5	35	53
+V8 protease-treated 12-kDa	30	59	64
+M1 (Leu-10 to C-terminus)	29	55	63
+M2 (N-terminus to Phe-83)	4	33	51

we constructed two mutants of the 12-kDa protein; one lacks 9 residues at the N-terminus (M1) and the other one lacks 10 residues at the C-terminus (M2) (Fig. 1). Both of the M1 and M2 mutants completely bound to PS II (not shown). The recovery of oxygen evolution after reconstitution with M1 or M2 were almost the same as those reconstituted with V8 protease-treated or chymotrypsin-treated 12-kDa protein, respectively. These results indicate that the C-terminus of the 12-kDa protein, at least 10 residues, plays an essential role in minimizing the Cl^- and Ca^{2+} requirements of oxygen evolution, and its N-terminus (at least 5 residues) also has a function in minimizing the Cl^- requirement.

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