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Studies on The Molecular Mechanism of 33 kDa extrinsic Protein in Photosystem II Oxygen-Evolving Complex

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33kDa extrinsic protein, an important protein in oxygenic photosynthesis, was known to have no fixed configuration in solution. At 20℃ and pH 6, 33kDa extrinsic protein showed changes of free energy of −14.6 kJ/mol^{-1} and of standard volume of −120mL/mol, respectively, with increase of hydrostatic pressure, comparatively lower than for most proteins. NBS modification of Trp241 in 33kDa extrinsic protein dramatically changes the secondary protein structure, its affinity to photosystem II as well as photosynthetic oxygen evolution. The relationship between structural change and transport of oxygen, water and proton is deserved a further study.

Key words: 33kDa extrinsic protein, structural change, NBS, Trp241, high hydrostatic pressure

INTRODUCTION

The oxygenic photosynthesis to oxidize water to molecular oxygen is catalyzed by photosystem II. In addition to the photosystem II reaction core, it includes a 33kDa extrinsic protein, the manganese stabilizing protein, that plays an important regulatory role in the process in plants and algae, although it is not involved in the electron transfer directly. 33kDa extrinsic protein is one of the three extrinsic proteins in photosystem II (PS II). The removal of 33kDa extrinsic protein from PS II particles by incubation with concentrated Tris/HCl buffer or high pH, results in loss of both Mn^{2+} ion and the oxygen-evolving activity. 33kDa extrinsic protein can also be released from the particles by washing with $CaCl_2$ or urea plus NaCl without loosing the Mn^{2+} ion. PS II particle can regain the oxygen-evolving activity mostly by rebinding of the protein. 33kDa extrinsic protein was isolated first from spinach by Kuwabara and Murata. Since then, its structure and function have been investigated. Two prominent structural features exist for 33kDa extrinsic protein. First, it is composed of 247 amino acid residues with only one tryptophan residue (Trp241), which can be specifically modified or monitored for studying protein structure. Secondly, 33kDa extrinsic protein was known to have no fixed configuration in solution. It is hard to obtain crystal structure up till now. If properly used, these two features can help understanding molecular mechanisms of 33kDa extrinsic protein. In this study, by using high hydrostatic pressure technique, NBS modification and analysis of fluorescence spectra, some molecular properties of 33kDa extrinsic protein was investigated.

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MATERIAL AND METHODS

PSII membranes were isolated from market spinach leaves with the method in Ref. [1] with minor modification [2]. 33kDa extrinsic protein was purified according to Ref. [3] with minor modifications. The fluorescence measurements were carried out using either an Aminco Bowman Series 2 (AB2) fluorospectrophotometer (SLM Co.) or a SLM 48000 fluorospectrophotometer (SLM Co.) in which the sample houses were modified to measure fluorescence under pressure from 0.1MPa to 600Mpa. Free energy change were estimated from the determination of standard volume change and $P_{1/2}$ (which is the pressure at which the degree of transition), according to the following expression: free energy change = standard volume change • $P_{1/2}$. For NBS modification, after each

sequential addition of 1μl 10mM NBS (Fluka AG), 33kDa extrinsic protein suspension (12μM, 1 ml) was stirred for 2 min. Then, ultraviolet (UV) absorption spectra were obtained with a Shimadzu UV-3000 spectrophotometer. The decrease of the absorption at 280nm was monitored until no further decrease took place. The number of the modified tryptophan in each protein was calculated by using the formula of Spande and Witkop. Before reconstitution, unmodified 33kDa extrinsic protein and NBS-modified 33kDa extrinsic protein were dialyzed separately against 50mM MES-NaOH, pH6.2 for 3 h. The precipitates, produced in the dialysis, were removed after centrifugation at 4000 g for 10 min. Then, the protein concentration was determined. 33kDa extrinsic protein reconstitution was made following the method in Ref. [4]. The concentrations of urea/NaCl treated PSII membranes were adjusted to 0.1 mg Chl ml^{-1}. 33kDa extrinsic protein was added into the reaction medium to obtain a desired protein-to-PSII membrane ratio. PSII membranes and 33kDa extrinsic protein were incubated at 4 °C for 30 min in the dark, and then centrifuged at 40000 g for 20 min. The pellets were washed twice with the SCN_{low} solution (0.4 M sucrose, 10 mM $CaCl_2$, 10 mM NaCl and 50 mM MES-NaOH, pH 6.2) to remove the loosely bound 33kDa extrinsic protein. The oxygen evolution activity was measured with a Clark-type oxygen electrode in the SCN_{low} solution at 25 °C. The chlorophyll concentration in the reaction medium was 10μg ml^{-1}. 0.8mM 2,6-dimethyl-p-benzoquinone (DMBQ) was used as the artificial electron acceptor. Protein content was analyzed with SDS-PAGE in the system of Laemmli [5] containing 6 M urea. A slab gel containing 5% (stacking gel) and 13.75% (resolving gel) acrylamide was used. The densitogram of the gel stained in Coomassie Brilliant blue R-250 was obtained with a Digital Imaging System (IS-1000), and the relative amounts of 33kDa extrinsic protein were determined by integrating the peak areas. The secondary structure of 33kDa extrinsic protein was examined with far-UV CD spectroscopy. pH of both the unmodified and the NBS-modified 33kDa extrinsic protein solutions were changed from 2.5 to 6.2 before measurement, through extensive dialysis in 10mM KH_2PO_4-K_2HPO_4 buffer (pH 6.2). After the 33kDa extrinsic protein solution was filtered through a polyethersulfone membrane (0.2nm), the CD spectra was measured by a Jasco J-715 spectropolarimeter at 22°C. The concentration of 33kDa extrinsic protein was adjusted to 10μM before each measurement. The cell length was 1 mm. Data were collected every 0.5nm with a 1.5nm bandwidth and 1 s time constant. Scan speed was 10nm min^{-1}. Four scanning spectra were averaged and the data were linearly smoothed ± 2 points.

RESULTS AND DISCUSSION

We noticed that study on 33kDa extrinsic protein folding-unfolding with guanidine hydrochloride (GdmCl) reported that 33kDa extrinsic protein has a very low free energy upon unfolding (-11.7kJ• mol^{-1} or -18.4 kJ• mol^{-1} obtained from two different calculation methods) [6]. To show folding-unfolding of 33kDa extrinsic protein much more directly, we selected high hydrostatic pressure technique and analysis of fluorescence spectra in this study (Fig.1). With an excitation at 295nm, the intrinsic fluorescence of the 33kDa extrinsic protein is mainly due to the tryptophan emission. The emission spectra in the region from 300 to 340nm have a gradual decrease in fluorescence intensity with pressure increasing to 180MPa, followed by an increase in the fluorescence intensity at wavelengths higher than around 340nm. Higher pressures up to 600MPa did not induce any further significant change in the fluorescence spectra, indicating that a stable transition state during unfolding is obtained at 180Mpa, much lower than that for most proteins. A sigmoid increase implies that the pressure-unfolding process can be described as a two-state transition. Changes in free energy and standard volume for the transition at pH 6.0 and 20°C are -14.6kJ/mol and -120mL/mol respectively, consistent with that obtained by chemical denature. The change of free energy is smaller by one magnitude order than that for most proteins. That for trypsin transiting from the native to the molten globule like state is -146.5kJ• mol^{-1} [7] with a corresponding pressure-transition of 650Mpa. This is consistent with the fact that 33kDa extrinsic protein has no fixed configuration in solution. Since a special function of 33kDa extrinsic protein is transferring water, oxygen and proton, it is reasonable to consider that the flexible configuration of 33kDa extrinsic protein favors the transfer. The fluorescence spectrum was almost totally recovered after pressure release to atmospheric pressure, indicating that the pressure-induced transition is reversible. The oxygen-evolving activity of the reconstituted system PS II with 33kDa extrinsic protein treated with a pressure of 200MPa is almost the same for that without treatment, indicating also that the unfolding transition of 33kDa extrinsic protein by pressure is reversible [8], and shows a strongly flexible configuration.

Table 1. Activity of oxygen evolution, measured after the reconstitution of the urea/NaCl-washed PSII membranes with the 33kDa extrinsic proteins.

Samples	Retained 33kDa extrinsic protein (%)	Oxygen-evolving activity (%)
1. Control PSII membranes	100	100
2. Urea/NaCl-washed PSII	0	19
3. 2 + 33kDa extrinsic protein (33kDa extrinsic protein: PSII=8mol:1 mol)	94	50
4. 2 + NBS-modified 33kDa extrinsic protein (33kDa extrinsic protein:PSII=20 mol:1 mol)	59	21

It is well know that heat shock, high salt concentration and high pH greatly affect the activity of photosynthetic oxygen evolution. All were studied IN folding-unfolding of 33kDa extrinsic protein with high hydrostatic pressure technique, and contradictory results were obtained. Free energy not standard volume changes from −14.6 to −8.0kJ/mol, when temperature was raised from 20 to 50℃, used for heat shock. But, changing suspension pH or salt concentration only induced change in standard volume (about 30%) not free energy, implying these treatment are much superficial than heat shock to affect the configuration change of 33kDa extrinsic protein. These information is very valuable for further functional study.

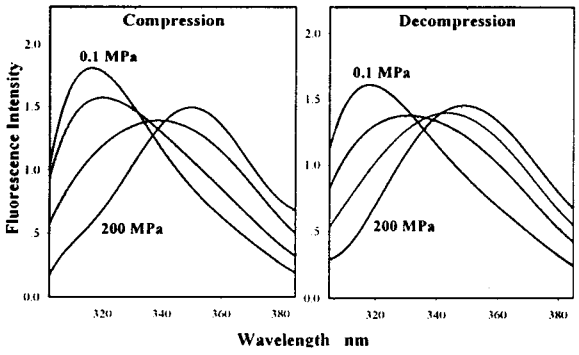

Figure 1. Fluorescence emission spectra of the 33kDa extrinsic protein upon excitation at 295nm (exciting only tryptophan) as a function of compressing pressure. Solution conditions: 33kDa extrinsic protein at 0.1 mg/mL in MES buffer, 0.05M, pH 6.0, T=20°C.

Trp241 of 33kDa extrinsic protein was specifically modified with N-bromosuccinimide (NBS), a frequently used chemical in the modification of tryptophan residues for its special reaction with tryptophan at acidic pH. CD results showed that NBS modification of Trp241 dramatically modified the secondary structure of the 33kDa extrinsic protein in solution (Fig. 2). β -sheets were greatly influenced because of the NBS modification of Trp241. More over, the significant difference in the region less than 215 nm in the CD spectrum reflects a large amount of random coil in modified protein. This means that the structure of 33kDa extrinsic protein became looser after NBS modification on this single tryptophan residue. Trp241 seems sensitive and important to configuration change. The affinity of the manganese stabilizing protein to photosystem II decreased greatly after the modification of Trp241, and no oxygen-evolving activity was recovered after its reconstitution (Table 1). The pH-dependence of the modification, the comparison of features of fluorescence spectra and hydropathy suggested that Trp241 is buried in the middle of the hydrophobic region at the C-terminus of the 33kDa extrinsic protein. It demonstrated that the C-terminus hydrophobic region of manganese stabilizing protein is also critical for maintaining its structure and function [9, 10].

Main conclusions could be draw from this study. First, the small change in free energy with low hydrostatic pressure support that 33kDa extrinsic protein is flexible to adjust its configuration to assistant transferring many contents involving in oxygen evolution. Secondly, Trp241 is sensitive to configuration change. It is crucial for protein binding and oxygen evolution. But we have no direct evidence to show that it is important for deliver water, oxygen and proton at this moment. Thirdly, realizing the

passing manner of contents is also important for the study of mechanism of photosynthetic oxygen evolution. Further related study is being designed and carried out in this Lab.

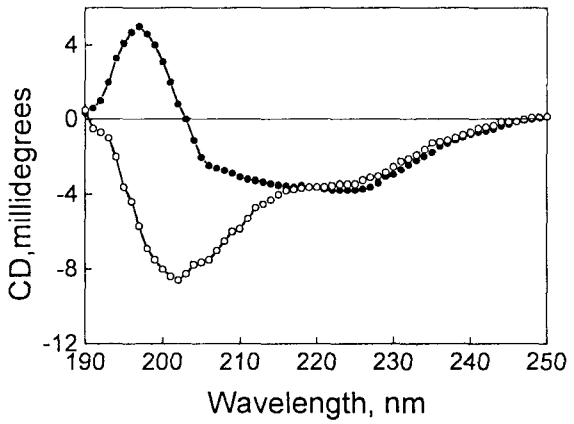

Figure 2. Far-UV CD spectra of 33kDa extrinsic protein. Solid circles, unmodified protein, Open circles, NBS-modified 33kDa extrinsic protein.

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