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STOICHIOMETRY OF COMPONENTS IN THE PHOTOSYNTHETIC OXYGEN EVOLUTION SYSTEM OF PHOTOSYSTEM II PARTICLES PREPARED WITH TRITON X-100 FROM SPINACH CHLOROPLASTS

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The stoichiometry of the proteins of the photosynthetic oxygen evolution system and of the electron transport components in Photosystem II particles prepared with Triton X-100 from spinach chloroplasts were determined. Per about 220 chlorophyll molecules, there were one reaction center II, one molecule each of the 33, 24 and 18 kDa proteins, four Mn atoms, two cytochromes *b*-559 (one high-potential, the other low-potential), and 3.5 plastoquinone-9 molecules, but practically no cytochrome *b*-563, cytochrome *f*, phylloquinone, α-tocopherol or α-tocopherylquinone.

Introduction

Photosystem II particles prepared from spinach chloroplasts with Triton X-100 are highly active in photosynthetic oxygen evolution [1,2]. When phenyl-*p*-benzoquinone is used as an electron acceptor, the rate of oxygen evolution ranges from 250 to 400 μmol O_2/mg Chl per h [1]. Oxygen evolution activity is very stable; after storage at 0°C for 1 week, 70% of the original activity still remains [1]. Electron microscopy of the PS II particles indicates that the particles are unsealed sheets of membranes [3].

Three protein components having molecular masses of 33, 24 and 18 kDa are released from PS II particles and similar preparations, when the particles are treated with a concentrated Tris buffer at alkaline pH or with NaSCN [1,5–7]. The fact that oxygen evolution activity is proportional to the amount of 33 kDa protein bound to the particles suggests that this protein is most essential among the three for oxygen evolution [5,8]. The 24 kDa protein seems supplementary, since a complete removal of this protein from the particles decreases oxygen evolution activity to about 40% of the original level [4,5,9]. There is, however, an opposing argument that the 24 kDa protein is essential to oxygen evolution [10]. The function of the 18 kDa protein in oxygen evolution is still obscure; PS II particles prepared with Triton X-100 having the 33 kDa and 24 kDa proteins but lacking the 18 kDa protein are fully active in oxygen evolution [9], whereas thylakoid membrane fragments prepared with cholate require the 18 kDa protein for oxygen evolution [11]. Manganese is known to be essential to the oxygen-evolution system, and its binding to the membranes seems to be related to the binding of 33 kDa protein [12].

In the present study, we determined the amounts of the 33, 24 and 18 kDa proteins, reaction center II, manganese, cytochromes and prenylquinones in PS II particles in order to establish the stoichiometry among them.

Abbreviations: Chl, chlorophyll; Mes, 4-morpholineethanesulphonic acid; PS, Photosystem; HPLC, high performance liquid chromatography.

Materials and Methods

Preparation of PS II particles, reaction center II and 33 kDa protein

PS II particles were prepared from spinach chloroplasts as described previously [1]. The particles were suspended in 30% (v/v) ethylene glycol containing 300 mM sucrose, 10 mM NaCl and 25 mM Mes/NaOH (pH 6.5) and stored in liquid nitrogen [9]. Before use, the sample was thawed at 20°C, and the particles were collected by centrifugation at 35 000 × *g* for 10 min. They were washed three times by resuspension and recentrifugation with a medium composed of 300 mM sucrose, 10 mM NaCl and 25 mM Mes/NaOH (pH 6.5).

A purified reaction center II preparation [13,14] was generously given by Professor Kimiyuki Satoh, Okayama University. The molar ratio of Chl to P-680 in this preparation was estimated to be about 50 : 1 by the light-induced absorbance change [15]. In a chemical analysis of pigments in the reaction center II preparation [47], a molar ratio of Chl to pheophytin *a*, the intermediate electron acceptor of the photochemical reaction II [16], was 24 : 1. Since one reaction center II is considered to contain two pheophytin molecules in analogy with the bacterial reaction center [17], this result confirms that the molar ratio of Chl to reaction center II in this preparation is about 50 : 1. In the following, we adopt this number for the ratio in the determination of the reaction center II content in PS II particles. The 33 kDa protein was prepared from an acetone powder extract of spinach chloroplasts as described previously [18].

Preparation of antiserum against 33 kDa protein

Antiserum against the 33 kDa protein was prepared as follows; 1 ml of 150 mM NaCl and 10 mM Na phosphate buffer (pH 7.2) (physiological saline) containing 2 mg of 33 kDa protein and 1 ml of Freund's complete adjuvant (Difco) were mixed and thoroughly emulsified. The paste was injected interdermally into the footpads and subcutaneously into several parts of the back of a rabbit. After 4 weeks, 1 mg protein dissolved in 2 ml of the physiological saline was injected intramuscularly into several parts of the back as a booster. The immunity of the serum was checked by double diffusion test according to Ouchterlony [19]. The rabbit was finally bled ten days after the booster injection.

Determination of 33 kDa, 24 kDa and 18 kDa proteins, reaction center II and manganese

The 33 kDa protein content in PS II particles was determined by a single radial immunodiffusion method in agarose gel, which was performed essentially according to Fahey and McKelvey [20]. The gel mixture contained 1.2% agarose, 1.0% Triton X-100, 150 mM NaCl, 0.1% NaN_3, 10 mM Na phosphate buffer (pH 7.2) and an anti-33-kDa protein-serum. The standard 33 kDa protein purified from an acetone powder extract of spinach chloroplasts was determined by its absorption coefficient at 276 nm of 20 $mM^{-1} \cdot cm^{-1}$ [21]. The purified protein, the PS II particles, and the thylakoid membranes were solubilized in 2.0% Triton X-100, and after standing for 30 min at room temperature, applied to the gel.

The 33 kDa protein and the reaction center II in PS II particles were determined by densitometry following SDS-urea polyacrylamide gel electrophoresis, which was performed as described previously [4]. The polyacrylamide concentrations were 5% in the stacking gel and 12% in the separation gel. The contents of 33 kDa protein and reaction center II in PS II particles were determined by comparing the peak heights in the densitogram with the purified 33 kDa protein and the reaction center II preparations as standards. In the case of the reaction center II determination, the peak heights of the 47 and 43 kDa polypeptides of the PS II particles were compared with those of the reaction center II preparation [22].

Relative contents of the 33, 24 and 18 kDa proteins in PS II particles were estimated by means of the amino acid contents of the proteins; PS II particles equivalent to about 1 mg Chl were treated with about 0.5 ml 1.0 M Tris/HCl (pH 9.3) and 0.3 M sucrose for 15 min to release all the 33, 24 and 18 kDa proteins [4]. The suspension was centrifuged at 13 000 × *g* for 7 min, and the supernatant was made up to 0.2% SDS by adding one-ninth volume of 2% SDS and then subjected to gel filtration HPLC with a series of two columns (TSK-GEL SW-3000, Toyo Soda). The effluent was 0.1% SDS in 70 mM Na phosphate buffer (pH 6.5). The three proteins were separated

by this method according to their molecular masses (see Fig. 2 in Results). PS II particles were also treated with 1.0 M NaCl, 0.3 M sucrose and 25 mM Mes/NaOH (pH 6.5) to release all the 24 and 18 kDa proteins [4], and these proteins were separated by gel filtration HPLC in the same way as above. The separated proteins were hydrolyzed in 6 M HCl at 110°C for 24 h. The amino acids were separated by column chromatography with a cation exchange resin (TSK-IEX-215, Toyo Soda), and detected by fluorescence after reaction with *o*-phthalaldehyde [23]. By this method, however, proline, tryptophan and cysteine could not be analyzed. By comparing the total amino acid contents in the fractions, the relative amounts of the three proteins were calculated.

The manganese content of PS II particles was determined by flameless atomic absorption spectrometry as described previously [4]. Chlorophyll was determined spectrophotometrically as described previously [9].

Determination of cytochromes and prenylquinones

Cytochromes *b*-559, *b*-563 and *f* were determined spectrophotometrically according to Bendall et al. [24]. The reduced-minus-oxidized millimolar difference absorption coefficients used were $E_{559-570} = 15\ \mathrm{mM}^{-1} \cdot \mathrm{cm}^{-1}$ for cytochrome *b*-559 [25], $E_{563-570} = 14\ \mathrm{mM}^{-1} \cdot \mathrm{cm}^{-1}$ for cytochrome *b*-563 [25], and $E_{554-565} = 23\ \mathrm{mM}^{-1} \cdot \mathrm{cm}^{-1}$ for cytochrome *f* [26].

Prenylquinones were extracted from PS II particles with chloroform/methanol (1 : 2, v/v) according to Bligh and Dyer [27], and determined by HPLC according to Lichtenthaler and Prenzel [28], using a high performance liquid chromatograph (Waters, 6000A) equipped with an ultraviolet detector (Shimadzu, SPD-2A). Elutions of the quinones were monitored at 260 nm for phylloquinone, plastoquinone-9 and α-tocopherylquinone, and at 290 nm for α-tocopherol. The quinones were determined according to the area under the elution bands, using absorption coefficients of $E_{cm}^{1\%} = 419$ at 248 nm for phylloquinone in isooctane [29], 210 at 255 nm for plastoquinone-9 in ethanol [30], 74.7 at 292 nm for α-tocopherol in ethanol [31], and 414 at 262 nm for α-tocopherylquinone in ethanol [30]. As chromatographic standards, phylloquinone was obtained from Tokyo Chemical Industry Co. (Tokyo), and α-tocopherol from Wako Pure Chemical Industries (Osaka). α-Tocopherylquinone was prepared from α-tocopherol with $FeCl_3$ according to Lichtenthaler et al. [32]. Plastoquinone-9 was prepared from spinach chloroplasts by TLC as described previously [46].

Results

Polypeptide compositions of preparations of reaction center II and PS II particles are compared in Fig. 1. The two prominent bands of 43 and 47 kDa in reaction center II [22], which are Chl-binding polypeptides, were also observed in PS II particles. The other polypeptides in the reaction center II preparation, such as the herbicide-binding protein at 30 kDa [22], cytochrome *b*-559 at 9 kDa and another polypeptide at 32 kDa [22], were all seen in the densitogram of the electrophoresis of the PS II particle preparation (Fig. 1). The PS II particles contained the 33, 24 and 18 kDa proteins and some other polypeptides in addition.

By using the peak heights of the 43 and 47 kDa polypeptides in the densitogram, the content of reaction center II in PS II particles could be calculated assuming that the molar ratio of Chl to P-680 is 50 : 1 [15] in the reaction center II preparation. The ratio of Chl to reaction center II in PS II particles was estimated, from three separate experiments, to be 212 ± 17 using the 47 kDa polypeptide band, and 215 ± 16 using the 43 kDa polypeptide band; thus we take 220 for the ratio of Chl to the reaction center II. A very similar number, 230, has been obtained by Lam et al. [33] for the ratio of Chl to Q, the primary electron acceptor of reaction center II, in their PS II particle preparation which was similar to that used in the present study.

Since the PS II particles in the present study had a Chl-*a*-to-*b* ratio of about 2.0 [1] and the Chl-to-reaction-center-II ratio of about 220, it contained, per reaction center II, about 90 Chl *a* and about 80 Chl *b* molecules in addition to 50 Chl *a* molecules which are directly bound to the reaction center II complex. These 170 Chl molecules are probably bound to the light-harvesting

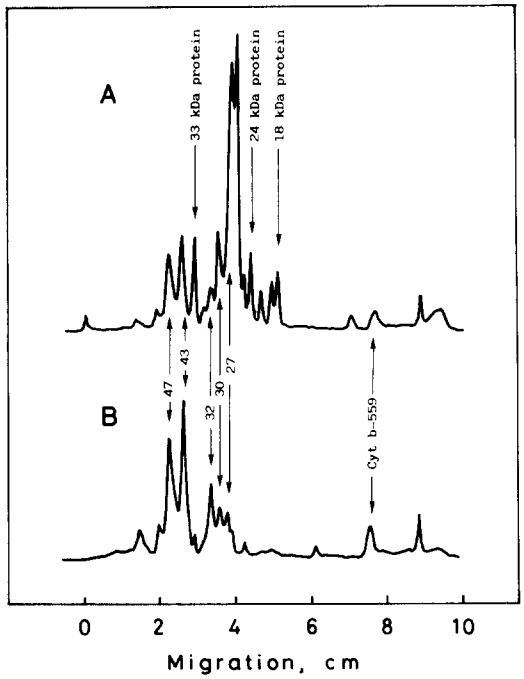

Fig. 1. SDS-urea polyacrylamide gel electrophoresis of PS II particles (A) and reaction center II (B).

Chl *a*/*b*-protein complex, which appeared as intensively stained bands at about 27 kDa after SDS-urea gel electrophoresis of PS II particles (Fig. 1A). Since the Chl-protein complex contains seven Chl molecules [34], the number of light-harvesting Chl-protein molecules per reaction center II in PS II particles can be estimated to be about 25.

The content of the 33 kDa protein was estimated by single radial immunodiffusion and also from its staining density after SDS-urea gel electrophoresis. The two methods produced similar results; the molar ratios of the 33 kDa protein to Chl molecules were 0.97 ($\pm$0.08):220 by immunodiffusion, and 1.03 ($\pm$0.07):220 by gel electrophoresis. The same methods were applied to the 33 kDa protein of intact thylakoid membranes of spinach chloroplasts. The molar ratio of Chl to 33 kDa protein was 270 $\pm$ 30 by immunodiffusion, and 300 $\pm$ 60 by densitometry after SDS-urea gel electrophoresis. Åkerlund [35] obtained similar numbers for the Chl-to-33-kDa-protein ratio in inside-out PS II vesicles and in intact thylakoid membranes.

The manganese content of PS II particles was determined by atomic absorption spectrometry to be 3.8 ($\pm$0.3) atoms per 220 Chl molecules. All these data suggest that there were one molecule each of reaction center II and 33 kDa protein, and four Mn atoms per about 220 Chl molecules. A very similar number for the Mn content in PS II particles has been reported by Sandusky et al. [36].

When PS II particles were treated with 1.0 M Tris/HCl (pH 9.3), the 33, 24 and 18 kDa proteins were all removed from the particles [4]. These three proteins could be separated by gel filtration HPLC in the presence of SDS (Fig. 2A). The 24 and 18 kDa proteins were also separated from the extract with 1.0 M NaCl (Fig. 2B). A large elution band with a retention time of 35–40 min was identified to be nucleic acids, since its absorption spectrum had a broad band at about 258 nm [21]. A benefit of this method is that all the proteins are recovered in eluted fractions, so that the amounts of the proteins can be estimated from the total amounts of amino acids in the respective protein fractions.

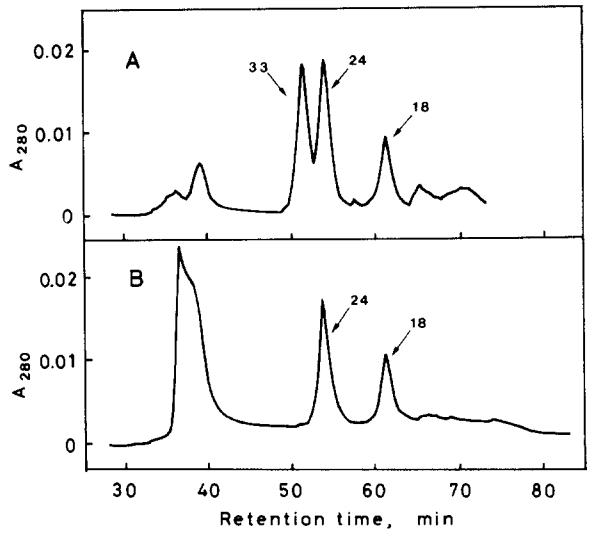

Fig. 2. Gel filtration HPLC of proteins extracted from PS II particles. The protein solution was made up to 0.2% SDS concentration bya dding one-ninth volume of 2% SDS solution and applied to the column which had been equilibrated and was developed with 0.1% SDS in 70 mM Na/phosphate buffer (pH 6.5). Elution of the proteins was monitored photometrically at 280 nm with an ultraviolet detector (Atto, SF-1205A). (A) Proteins released with 1.0 M Tris/HCl (pH 9.3) and 0.3 M sucrose. (B) Proteins released with 1.0 M NaCl, 0.3 M sucrose and 25 mM Mes/NaOH (pH 6.5).

TABLE I

AMINO ACID CONTENT OF THE 33, 24 AND 18 kDa PROTEINS, AND STOICHIOMETRY BETWEEN THE THREE PROTEINS IN PS II PARTICLES

The proteins were released by treating PS II particles with either 1.0 M NaCl, 0.3 M sucrose and 25 mM Mes/NaOH (pH 6.5), or 1.0 M Tris/HCl (pH 9.3) and 0.3 M sucrose. The particles and released proteins were separated by gel filtration HPLC as in Fig. 2. The fractionated proteins were hydrolyzed, and the resultant amino acids were quantitatively determined. Molar ratios of the proteins were calculated from the total amounts of amino acids in the protein fractions, divided by the molecular masses of the corresponding proteins.

Protein fraction	NaCl treatment		Tris treatment	
	Total amino acids [a]	Molar ratio [b]	Total amino acids [a]	Molar ratio [b]
33 kDa	–	–	119	0.99
24 kDa	105	1	87	1
18 kDa	75	0.95	60	0.92

[a] Measured in nmol in about 0.05 ml supernatant of the centrifugation.

[b] Normalized with the values for the 24 kDa protein taken as unity.

The three protein fractions were subjected to acidic hydrolysis in 6 M HCl. The amino acid compositions of the three proteins are similar to those reported previously [18,21,37,38], except that proline, tryptophan and cysteine are not listed, since these amino acids were not detected by the fluorescent method with *o*-phthaladehyde [23]. However, the relative amounts of the proteins could be estimated with errors less than 10% without determining these three amino acids, since proline does not exceed 10% and tryptophan and cysteine each amount to less than 1% in any of the three proteins [21]. This type of analysis (Table I) indicates that the molar ratio of the three proteins is 1 : 1 : 1.

The cytochromes in PS II particles were spectrophotometrically determined (Table II). Cytochrome *b*-559 amounted to about two molecules per 220 Chl molecules. This suggests that there are two molecules of this cytochrome per reaction center II. About half this cytochrome was reduced by hydroquinone and the other half by dithionite, suggesting that one of the two was of high potential and the other of low potential. The contents of

TABLE II

CYTOCHROME CONTENT OF PS II PARTICLES

Cytochrome	Molar ratio (molecules cytochrome per 220 Chl molecules)
b-559	1.94 ± 0.15
High-potential	1.03 ± 0.06
Low-potential	0.90 ± 0.10
f	0.05 ± 0.04
b-563	0.14 ± 0.04

cytochromes *f* and *b*-563 were very low. Lam et al. [33] and Sandusky et al. [36] have obtained similar results for the cytochrome content in their PS II particle preparations. The lack of cytochromes *f* and *b*-563 in PS II particles has also been pointed out by Bricker et al. [39].

The prenylquinone content of PS II particles was determined by HPLC. There were about 3.5 (± 0.2) molecules of plastoquinone-9 per 220 Chl molecules. This number is, however, about half of that in a similar PS II preparation of Lam et al. [33]. There were less than 0.20 molecules of phylloquinone and α-tocopherol per 220 Chl molecules, suggesting that there are practically none of these two quinones in PS II particles. α-Tocopherylquinone was not detected at all.

Discussion

The results in the present study suggest that there are one molecule each of the 33, 24 and 18 kDa proteins, four Mn atoms, two cytochrome *b*-559 molecules (one high-potential, one low-potential), 3.5 molecules of plastoquinone-9 and probably about 25 molecules of light-harvesting Chl-protein per reaction center II in PS II particles.

Two of the four Mn atoms seem to bind to the particles in cooperation with the 33 kDa protein, since, in most cases, they are released in parallel with the 33 kDa protein [12]. The other two Mn atoms seem to be more tightly bound to the particles.

Two of the 3.5 molecules of plastoquinone-9 are considered to be Q, the primary electron acceptor [40,41], and B, the secondary electron acceptor [42]. Nugent et al. [43] and Ghonotakis et al. [44]

suggest that the electron donor to reaction center II is also plastoquinone. These facts suggest that there is a very small pool of plastoquinone (0.5 molecules per reaction center II) in the PS II particles. Since Q is a one-electron carrier [41], and both B and pool plastoquinone are two-electron carriers [45], the total size of the electron acceptor pool of the PS II reaction can be calculated to be four-electron equivalents per reaction center II. This number is consistent with the results of Briantais (Briantais, J.-M., Laboratoire de Photosynthese, CNRS, Gif-sur-Yvette, personal communication), who found that the electron pool size of the acceptor side, estimated by fluorescence induction kinetics, is five-electron equivalents per reaction center II in the same PS II preparation.

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