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# PARTIAL DISINTEGRATION AND RECONSTITUTION OF THE PHOTOSYNTHETIC OXYGEN EVOLUTION SYSTEM

## BINDING OF 24 KILODALTON AND 18 KILODALTON POLYPEPTIDES

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**Treatment with 1 M NaCl almost totally removed two polypeptides of 24 and 18 kDa from the Photosystem II particles of spinach chloroplasts and reduced the oxygen-evolution activity by about half. Both polypeptides were able to rebind to the NaCl-treated particles in a low-salt medium. The rebinding of the 24 kDa polypeptide showed a saturation curve whose maximum level was close to that naturally occurring in the untreated particles. In parallel with the amount of rebound 24 kDa polypeptide, the oxygen-evolution activity was recovered. The 18 kDa polypeptide bound to the NaCl-treated particles without saturation. When the 18 kDa polypeptide was added to the particles previously treated with NaCl and then supplemented with a saturating amount of 24 kDa polypeptide, there appeared, in addition to the binding without saturation, another binding of the 18 kDa polypeptide with saturation to a maximum level close to that naturally occurring in the untreated particles. The 18 kDa polypeptide did not restore the oxygen-evolution activity. These findings suggest that there are specific binding sites; one for the 24 kDa polypeptide located on the Photosystem II particles, and the other for the 18 kDa polypeptide on the 24 kDa polypeptide.**

## Introduction

The photosynthetic oxygen-evolution system is likely to involve three polypeptides having molecular masses of 33, 24 and 18 kDa [1–6]. These polypeptides seem to be localized on the inner side of the native thylakoid membranes [3,4] and can be removed from some thylakoid membrane preparations by treatment with concentrated Tris buffer [1,3,4] or alkaline pH [1,2].

Treatment of PS II particles [2] or inside-out thylakoid membranes [6] with highly concentrated NaCl released two polypeptides, of 24 and 18 kDa, and partially inactivated the oxygen evolution. Åkerlund et al. [6] reactivated the oxygen-evolution system in the NaCl-treated inside-out thylakoid vesicles by adding back the 24 kDa polypeptide.

In the present study, we used PS II particles prepared from spinach chloroplasts with Triton X-100 [1] to analyze quantitatively the dissociation and reassociation of the 24 and 18 kDa polypeptides in relation to the oxygen-evolution activity of the particles.

## Materials and Methods

PS II particles were prepared from spinach chloroplasts with Triton X-100 according to the method described previously [1]. They were suspended in 30% (v/v) ethylene glycol containing

Abbreviations: Chl, chlorophyll; PS, photosystem; Mes, 2-(*N*-morpholino)ethanesulfonic acid.

300 mM sucrose, 10 mM NaCl and 25 mM Mes-NaOH (pH 6.5) and stored in liquid nitrogen. Before use, the material was thawed at 20°C and the particles collected by centrifugation at 35000 $\times g$ 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) (designated hereinafter as medium A). The pellet was finally suspended in medium A for use in experiments. After storage for 2 months in liquid nitrogen, the particles retained 80–100% of their original level of oxygen-evolution activity with phenyl-*p*-benzoquinone as an electron acceptor of the Hill reaction.

For the 1.0 M NaCl treatment of PS II particles, 1.2 M NaCl containing 300 mM sucrose and 25 mM Mes-NaOH (pH 6.5) was added to 1/6 vol. of the particle suspension (3 mg Chl/ml) in medium A, giving a final chlorophyll concentration of 0.5 mg/ml. After standing for 30 min at 0°C under room light except as otherwise stated, the suspension was centrifuged at 100000 $\times g$ for 30 min. The resulting pellet, consisting of NaCl-treated PS II particles, was washed once with medium A by resuspension and recentrifugation before being used for experiments. Particles kept suspended in medium A for 30 min were used as a control.

The supernatant of the centrifugation following the NaCl treatment was concentrated by ultrafiltration with an Amicon YM 5 Diaflo membrane and then dialyzed against medium A for 4 h. This solution was then either directly used for binding experiments or subjected to column chromatography on DEAE-Sepharose CL-6B (Pharmacia Fine Chemicals). In the latter case, the column was equilibrated with 10 mM NaCl and 20 mM sodium phosphate buffer (pH 6.9). The 18 kDa polypeptide was not adsorbed; it was eluted with 10 mM NaCl and 20 mM sodium phosphate buffer (pH 6.9). The 24 kDa polypeptide, which was adsorbed, was eluted with 50 mM NaCl and 20 mM sodium phosphate buffer (pH 6.9). Purity of the 18 and 24 kDa polypeptide preparations was examined by SDS-polyacrylamide gel electrophoresis in the manner described below. Only one band was detected in each preparation, suggesting that practically no impurities were present in the preparations.

In order to investigate the possibility of the rebinding of the polypeptides to the NaCl-treated particles and of its effect on restoring oxygen-evolution activity, various combinations were tested: a suspension of NaCl-treated particles alone, or of those supplemented with the 24 kDa polypeptide, was mixed with either the 18 or 24 kDa polypeptide or a combination of the two (in all cases to a final chlorophyll concentration of 1.0 mg/ml in medium A). For measurement of oxygen evolution, each suspension was diluted with 80 vol. medium A. To analyze the binding of the polypeptides to the particles, the suspension was diluted with 5 vol. medium A and centrifuged at 100000 $\times g$ for 30 min. This pellet was suspended in medium A and recentrifuged at 100000 $\times g$ for 30 min, and the final pellet was subjected to polypeptide analysis. All the above procedures were performed at 0–4°C.

Previous research [2] showed that the 33, 24 and 18 kDa polypeptides bound to PS II particles could be totally removed with a highly concentrated Tris-HCl buffer of pH 9.3. On the basis of this finding, the particles which were treated in the various ways described above were incubated for 15 min at 0°C in 1 M Tris-HCl (pH 9.3 at 4°C) and 300 mM sucrose at a chlorophyll concentration of 0.5 mg/ml and then the suspension was centrifuged at 100000 $\times g$ for 30 min. The pellet was discarded, and the supernatant analyzed by SDS-polyacrylamide gel electrophoresis in the buffer system of Laemmli [7], omitting 2-mercaptoethanol from all solutions. The stacking gel contained 5% polyacrylamide, and the separation gel consisted of a linear gradient of polyacrylamide concentration from 8 to 15% with a gradient of sucrose from 5 to 20%. The gel was stained with Coomassie brilliant blue R-250 [8]; the electrophoretic pattern was recorded with a dual-wavelength TLC scanner (Shimadzu, CS-910). The relative contents of the polypeptides were estimated according to the peak heights of the stained bands in the densitogram.

The oxygen-evolution activity was measured with phenyl-*p*-benzoquinone as an electron acceptor at 25°C with a Clark-type oxygen electrode in medium A with a supplement of 0.3 mM phenyl-*p*-benzoquinone and 0.05% bovine serum albumin as described previously [1]. The chlorophyll con-

centration was determined according to the method of Arnon [9], and the protein concentration according to that of Lowry et al. [10], with a modification described by Bensadoun et al. [11], using bovine serum albumin as a standard.

## Results

Treatment of the PS II particles with 1 M NaCl released two polypeptides having molecular masses of 24 and 18 kDa and reduced the oxygen-evolution activity as described previously [2]. As shown in Table I, illumination enhanced the effects of NaCl treatment: under illumination with white fluorescent light of 1000 lx, almost all the 18 and 24 kDa polypeptides were released from the particles, whereas in the dark three-quarters of each polypeptide was removed. The oxygen-evolution activity was reduced to half in the light and to two-thirds in the dark. When the particles were treated twice with 1 M NaCl in the light, the two polypeptides were totally removed (Table I). Under ambient room light conditions, the effectiveness of NaCl in inactivating the oxygen evolution and releasing the polypeptides was the same as under the 1000 lx illumination. Therefore, the treatment hereinafter described was performed under room light conditions.

When the released protein fraction containing both the 24 and 18 kDa polypeptides was added back to the NaCl-treated PS II particles at low ionic strength, the oxygen-evolution activity doubled to regain the control level (Table II). Addition of the released protein fraction to the control increased the activity by about 10%. When the released protein fraction was treated at 95°C for 5 min and added to the NaCl-treated particles, the oxygen-evolution activity of the particles was restored to 70% of the control level.

The change with the various treatments in polypeptide composition of the PS II particles is indicated in Fig. 1 by the densitograms from the SDS gel electrophoresis. The dissociation and reassociation of the 33, 24 and 18 kDa polypeptides are seen in the electrophoresis of components released from the particles by 1 M Tris-HCl (pH 9.3). Clearly, the NaCl-treated particles contained the 33 kDa polypeptide but only trace amounts of the

TABLE I

INACTIVATION OF OXYGEN EVOLUTION AND RELEASE OF POLYPEPTIDES UPON TREATMENT OF PS II PARTICLES WITH NaCl

PS II particles were treated with 1 M NaCl for 30 min under various conditions after being kept in the dark in medium A for 2 h. Then, the particle suspension was centrifuged at 100000 × *g* for 30 min. After the pellet (NaCl-treated particles) was washed with, and resuspended in, medium A, the oxygen-evolution activity was measured with a supplement of 0.3 mM phenyl-*p*-benzoquinone and 0.05% bovine serum albumin. The values in parentheses are percentages of the control activity. The supernatant was subjected to SDS-polyacrylamide gel electrophoresis for determination of the released polypeptides. The PS II particles were treated also with 1 M Tris-HCl (pH 9.3 at 4°C) and 300 mM sucrose, in order to remove totally the three polypeptides from the PS II particles [2]. The amounts of polypeptides released by the NaCl are presented as a percentage of those released by Tris-HCl by comparing peak heights on densitograms from the electrophoresis.

| Treatment | $O_2$ evolution (μmol/mg Chl per h) | Release of polypeptide (%) | | |
|---|---|---|---|---|
| | | 18 kDa | 24 kDa | 33 kDa |
| Control, light | 380 (100) | 0 | 0 | 0 |
| 1 M NaCl, dark | 250 (66) | 76 | 75 | 0 |
| 1 M NaCl, light | 200 (53) | 97 | 92 | 0 |
| 1 M NaCl, light (two treatments) | 200 (53) | 100 | 100 | 2 |

TABLE II

REACTIVATION OF OXYGEN EVOLUTION BY ADDING BACK POLYPEPTIDES TO NaCl-TREATED PS II PARTICLES

The 24 and 18 kDa polypeptides released from the PS II particles by 1 M NaCl treatment were added back to untreated or NaCl-treated particles either directly or after heat treatment at 95°C for 5 min. The proportion of polypeptides to particles (0.2 mg polypeptides added per mg Chl) was equivalent to the level originally occurring in untreated particles. The values in parentheses indicate percentages of the oxygen-evolution activity of the control without the polypeptide addition.

| Type of particles | Addition | $O_2$ evolution (μmol/mg Chl per h) |
|---|---|---|
| Control | None | 250 (100) |
| | Polypeptides | 280 (112) |
| NaCl-treated | None | 120 (48) |
| | Polypeptides | 250 (100) |
| | Heat-treated polypeptides | 170 (68) |

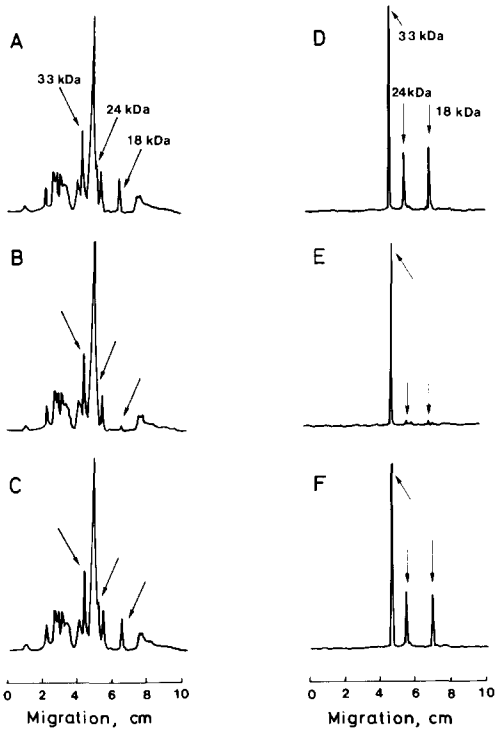


Fig. 1. SDS gel electrophoresis of polypeptides in the untreated, NaCl-treated and reconstituted PS II particles and of polypeptides released from these particles with 1 M Tris-HCl (pH 9.3). (A) Control (untreated) particles; (B) NaCl-treated particles; (C) particles reconstituted with a 24 kDa plus 18 kDa polypeptide mixture so that the ratio of added polypeptides to chlorophyll in the particles was 1 : 5 (w/w); (D) polypeptides released from the control particles with 1 M Tris-HCl (pH 9.3) and 300 mM sucrose; (E) polypeptides released from the NaCl-treated particles with 1 M Tris-HCl (pH 9.3) and 300 mM sucrose; (F) polypeptides released from the reconstituted particles with Tris-HCl (pH 9.3) and 300 mM sucrose.

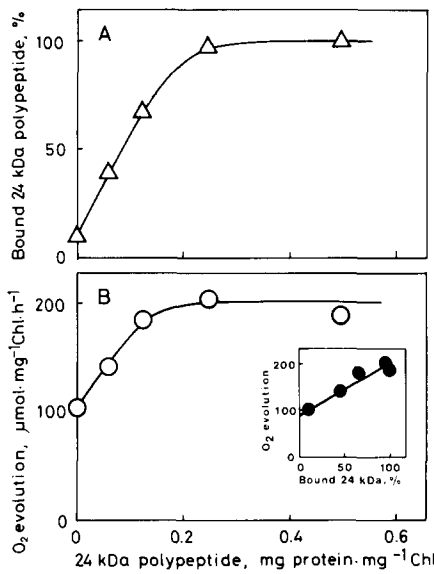


Fig. 2. Changes in the amount of bound 24 kDa polypeptide and the activity of oxygen evolution when 24 kDa polypeptide was added to the NaCl-treated PS II particles. (A) Amount of 24 kDa polypeptide bound to the particles, 100% corresponding to the level in the untreated particles; (B) activity of oxygen evolution; (inset) relationship between the amount of bound 24 kDa polypeptide and the activity of oxygen evolution.

24 and 18 kDa polypeptides. When the 24 and 18 kDa polypeptides were added back to the NaCl-treated particles in a low-salt medium, both polypeptides rebound to the particles.

Fig. 2 shows the amounts of bound 24 kDa polypeptide and oxygen-evolution activity versus the amount of 24 kDa polypeptide added to the particles. The binding saturated with a maximum level close to that naturally occurring in the untreated particles (Fig. 2A). The oxygen-evolution activity also followed a saturation curve (Fig. 2B). The inset in Fig. 2B shows that the activity of oxygen evolution correlated well with the amount of bound 24 kDa polypeptide.

The 18 kDa polypeptide could bind again to the NaCl-treated particles, but without saturation (Fig. 3A). However, the oxygen-evolution activity was not stimulated at all by this polypeptide (Fig. 3B). In order to investigate the effect of the 24 kDa polypeptide on the rebinding of the 18 kDa polypeptide to the particles, the PS II particles were first treated with 1 M NaCl to remove the 24 and 18 kDa polypeptides and then suspended in medium A containing 200 μg/ml of 24 kDa polypeptide, thus producing particles containing the original level of 24 kDa polypeptide but no 18 kDa polypeptide. When the 18 kDa polypeptide was added to these particles in medium A, a greater amount of 18 kDa polypeptide bound to the particles in the presence of 24 kDa polypeptide than in its absence (Fig. 3A). This extra binding of 18 kDa polypeptide followed a saturation curve similar to that of the 24 kDa polypeptide with the NaCl-treated particles; its maximum level, which was estimated by extrapolating the straight line to the ordinate, was the same as the original level in the untreated PS II particles. Again, the rebinding

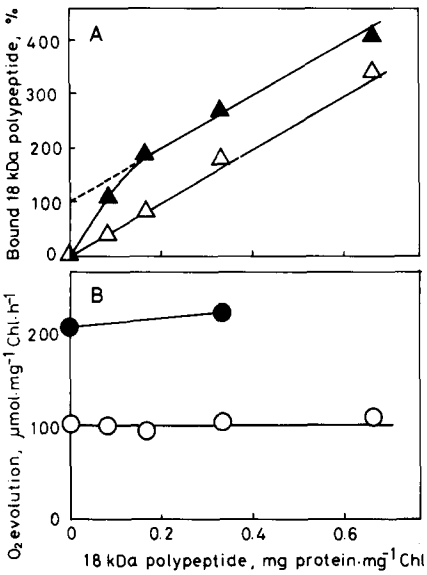


Fig. 3. Changes in the amount of bound 18 kDa polypeptide and the activity of oxygen evolution when 18 kDa polypeptide was added to either the NaCl-treated, or the NaCl-treated and 24 kDa polypeptide-supplemented, PS II particles. (A) Amount of 18 kDa polypeptide bound to the particles, 100% corresponding to the level in the untreated particles. (△———△) NaCl-treated particles, (▲———▲) NaCl-treated and 24 kDa polypeptide-supplemented particles. (B) Activity of oxygen evolution. (○———○) NaCl-treated particles, (●———●) NaCl-treated and 24 kDa polypeptide-supplemented particles.

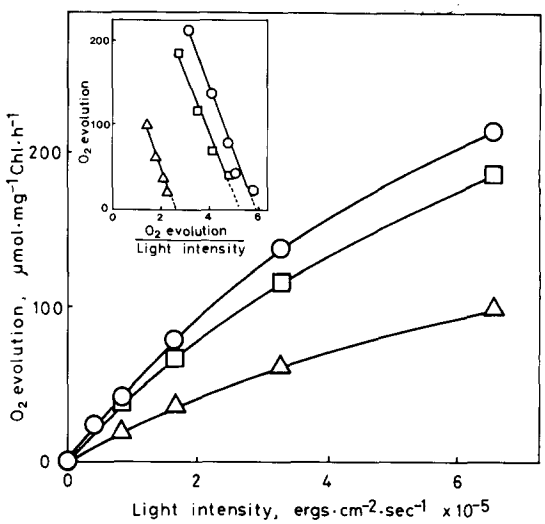


Fig. 4. Plot of the oxygen-evolution activity versus light intensity in the untreated, NaCl-treated and reconstituted PS II particles. The oxygen-evolution activity was measured as in Table I, and the light intensity varied with neutral density filters. (○———○) Untreated particles. (△———△) NaCl-treated particles. (□———□) NaCl-treated particles reconstituted with a 24 kDa plus 18 kDa polypeptide mixture at a protein-to-chlorophyll ratio of 1:4 (w/w). (Inset) Plot of the oxygen-evolution activity versus activity divided by light intensity. Units of the ordinate and abscissa are μmol $O_2$/mg Chl per h and $10^4 \times$(μmol $O_2$/mg Chl per h)/(erg/cm$^2$ per s), respectively.

of the 18 kDa polypeptide to the particles did not stimulate oxygen evolution (Fig. 3B).

The oxygen-evolution activity of the untreated, NaCl-treated and reconstituted PS II particles was measured at various light intensities (Fig. 4). The reaction was not saturated at the highest light intensity employed in this experiment. The NaCl treatment decreased the oxygen-evolution activity to about half and the reconstitution treatment almost restored it at the high and low light intensities. The activity was also plotted against the activity divided by light intensity (Fig. 4, inset). Each plot was approximated by a straight line having a slope similar to those of the others. If the plots can be analyzed in a way similar to the enzyme kinetics, the slope, abscissa intercept and ordinate intercept of the line correspond to the apparent Michaelis constant ($K'_m$), relative quantum yield ($\phi_{rel}$) and maximum rate ($V_{max}$) and are presented in Table III. $K'_m$ was relatively constant throughout the NaCl and reconstitution treatments. Both $\phi_{rel}$ and $V_{max}$ were reduced to about half by the NaCl treatment and almost restored by the rebinding of the polypeptides.

TABLE III

PARAMETERS FOR THE OXYGEN EVOLUTION OF UNTREATED, NaCl-TREATED AND RECONSTITUTED PS II PARTICLES

Values were determined from the plot in Fig. 4 (inset). The apparent Michaelis constant ($K'_m$), the relative quantum yield ($\phi_{rel}$) and the maximum rate ($V_{max}$) correspond to the slope, abscissa intercept and ordinate intercept of the line, respectively. The relative quantum yield is normalized to the value of the untreated PS II particles.

| Type of particles | $K'_m$ (erg/cm$^2$ per s) ($\times 10^5$) | $\phi_{rel}$ | $V_{max}$ (μmol/mg Chl per h) |
|---|---|---|---|
| Untreated | 7.8 | 1.00 | 460 |
| NaCl-treated | 7.7 | 0.47 | 210 |
| Reconstituted | 7.3 | 0.93 | 380 |

## Discussion

It was shown in our previous study [2] that treatment with concentrated NaCl inactivated the photosynthetic oxygen evolution by removing the 24 and 18 kDa polypeptides. A similar result was obtained by Åkerlund et al. [6] with their inside-out thylakoids. The data in Table I in the present study confirm this finding and, in addition, reveal the effect of illumination on the NaCl treatment. In intact thylakoid membranes, illmination is necessary for inactivation of the photosynthetic oxygen evolution by treatment with either concentrated Tris-HCl buffer [12] or alkaline pH [13]. In the PS II particles used in the present study, the inactivation of oxygen evolution and the release of the 24 and 18 kDa polypeptides with 1 M NaCl took place in the dark, and illumination enhanced the effectiveness of NaCl treatment. This difference between the intact thylakoids and the PS II particles may have resulted from an alteration in membrane integrity during the preparation of the particles with Triton X-100. In Table I it may also be seen that the activity dropped to only half the control level even when both 24 and 18 kDa polypeptides were completely removed by a double washing of the particles with 1 M NaCl. This observation suggests that the 24 kDa polypeptide is supplementary rather than essential to the oxygen-evolution system. As already shown [2], the 18 kDa polypeptide is apparently not essential either.

The 24 and 18 kDa polypeptides were able to bind to the NaCl-treated particles in a low-salt medium. The observation that the binding of the 24 kDa polypeptide was saturated at the level originally occurring in untreated particles indicates that there is a specific binding site for the 24 kDa polypeptide on the surface of the particles. The parallel between the binding of the 24 kDa polypeptide and the recovery of oxygen-evolution activity clearly suggests the involvement of the 24 kDa polypeptide in the oxygen evolution of the particles.

The 18 kDa polypeptide bound to the NaCl-treated particles without saturation. An extra binding site with a high affinity for the 18 kDa polypeptide appeared when the NaCl-treated particles were supplemented with the 24 kDa polypeptide. This binding of the 18 kDa polypeptide showed a saturation phenomenon with a maximum level of the same value as that naturally occurring in untreated particles. These findings suggest that there is a specific binding site for the 18 kDa polypeptide on the 24 kDa polypeptide. The 18 kDa polypeptide can bind to the particles with a low nonspecific affinity. However, the 18 kDa polypeptide rebound to the particles with either high or low affinity did not restore the oxygen-evolution activity.

Åkerlund et al. [6], using the inside-out vesicles of granal thylakoids, observed that the 24 kDa, but not the 18 kDa, polypeptide restored the oxygen-evolution activity of the NaCl-treated vesicles. The present study indicates that their observation is in agreement with ours also in the case of the PS II particles prepared with Triton X-100. Toyoshima and Fukutaka [14], on the other hand, claimed that the 18 kDa, but not the 24 kDa, polypeptide was effective in restoring the oxygen-evolution activity in their cholate-treated subchloroplast particles. The reason for the discrepancy between the NaCl-treated particles and the cholate-treated particles in the role of the 24 and 18 kDa polypeptides in the oxygen-evolution system is still in question.

Dissociation of the 24 kDa polypeptide from the particles reduced the quantum yield and the maximum rate of the Hill reaction with phenyl-*p*-benzoquinone, while its reassociation almost restored them. The apparent Michaelis constant, however, was not affected by the presence or absence of the 24 kDa polypeptide. We are not yet able to propose a model that can account for the fact that the total removel of the 24 kDa polypeptide partially suppressed the quantum yield and the maximum rate but did not affect the apparent Michaelis constant.

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