Surface Charge Density Changes in Isolated Photosystem II Membranes Induced by Depletion of the Extrinsic Polypeptides of the Oxygen Evolving System

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Treatment of PS II particles with either 1 M NaCl or alkaline Tris (1 M, pH 8.4) caused a considerable decrease in the average net negative surface charge density, concomitant with depletion of the extrinsic 17, 24 and 33 kDa proteins of the oxygen evolving complex from the membranes. The partial recovery of the values for surface charge in both NaCl- and Tris-treated membranes was registered after reconstitution experiments with the three proteins. These results are compared with the data for the charge densities of the thylakoid membranes, to examine the role of the three extrinsic proteins in the formation of heterogeneous arrangement of surface charge across the appressed (granal) thylakoids.

Introduction

The involvement of the three extrinsic polypeptides with molecular masses of 17, 24 and 33 kDa, loosely bound to the inner site of thylakoid membranes in the photosynthetic water oxidation has been well documented and reviewed [1, 2]. The association of these proteins with thylakoid membranes and their nearest neighbour have been studied by various techniques [3–8]. However, despite intensive research activities concerning mainly the function of these proteins in the electron donor site of photosystem II, their role in the surface electrical properties of the thylakoid membranes still remain not fully elucidated. In the present study the specific role of the extrinsic 17, 24 and 33 kDa polypeptides in the surface charge density of thylakoid membranes is examined by selective, polypeptide depletion (NaCl- und Tris-treatment) from isolated PS II membranes.

Materials and Methods

Intact chloroplasts from pea leaves were isolated as described in [9] and the harvested material was resuspended in 0.33 M sucrose, 5 mM $MgCl_2$, 10 mM Tricine (pH 8.0). Envelope-free chloroplasts were obtained by osmotic rupture of intact chloroplasts in ice-cold 3 mM $MgCl_2$ for 30 s and subsequent addition of an equal volume of double strength buffer as above. The osmotically shocked material was centrifuged at 2500 × *g* for 12 min. Subchloroplast fraction consisting of appressed (granal) thylakoids was prepared by ultrasonic disintegration of envelope-free chloroplasts as described by Ford *et al.* [10].

Photosystem II-enriched subchloroplast particles were prepared from pea chloroplasts by Triton X-100 treatment essentially as described by Berthold *et al.* [11] and stored in liquid nitrogen. Before use the PS II (BBY) particles were washed twice in a medium containing 25 mM MES buffer (pH 6.5), 10 mM NaCl, 0.3 M sucrose and collected by centrifugation at 40000 × *g* for 20 min. The harvested material was resuspended in the same buffer as above at chlorophyll concentration of 3 mg/ml.

Chlorophyll concentration was estimated according to Wellburn and Lichtenthaler [12].

NaCl treatment of PS II membranes was performed in a medium containing 1 M NaCl, 25 mM MES buffer (pH 6.5), 0.3 M sucrose and final chlorophyll concentration 0.5 mg/ml following the procedure described in [5]. The depletion of the three extrinsic polypeptides from the PS II membranes was achieved by Tris (1 M, pH 8.4) treatment as in [4]. Before using for reconstitution experiments and assay of surface charge the NaCl- and Tris-treated preparations were washed three times in 25 mM (pH 6.5) and 0.3 M sucrose.

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The reconstitution experiments were performed by incubation of NaCl- and Tris-treated PS II membranes with a crude extract of extrinsic protein at a protein:chlorophyll ratio 15:1 as described by Akerlund *et al.* [3].

The surface charge density (σ) was determined by the method suggested in [13], following the salt-induced fluorescence changes of the positively charged probe 9-aminoacridine (9AA). The fluorescence measurements were carried out in medium containing 0.1 M sorbitol, 1 mM HEPES buffer (pH 7.5), 1 mM KOH, 10 μM DCMU, 50 μM EDTA, 20 μM 9AA and chlorophyll concentration of 10 μg per ml. 9AA fluorescence was excited at 390 nm and measured at 450 nm using a Jobin Yvon JY 3 spectrofluorimeter (slits width = 4 nm).

Results and Discussion

Since the introduction of salt-dependent fluorescence changes of 9-aminoacridine dye as a measure of charge density of artificial and native membrane surfaces [13, 14], this technique have been widely used for estimation of the electrostatic surface properties of a variety of cellular and subcellular preparations [9, 15–17]. In the present study the 9AA fluorescence measurements were applied to clarify the influence of polypeptide depletion of the extrinsic 17, 24 and 33 kDa proteins of water oxidizing system on the surface charge density of isolated photosystem II membranes. The removal of the three polypeptides was achieved by Tris-(1 M, pH 8.4) treatment [4], while the removal of only 17 kDa and 24 kDa polypeptides from the PS II particles was performed by NaCl-(1 M, pH 6.5) treatment [5] which did not affect the 33 kDa polypeptide. Gel electrophoresis analysis has indicated that NaCl-treatment resulted in release of 95% and 87% of the 17 kDa and 24 kDa proteins respectively without any remarkable effect on the 3 kDa protein, whilst Tris-treatment removed almost all the 17 kDa and 24 kDa polypeptides and about 83% of the 33 kDa protein from the PS II membranes (data not shown).

Fig. 1 represents the data of typical experiments where the effects of polypeptides depletion on the surface electrical properties of PS II membranes were evaluated. The resuspension of the PS II membranes in a low salt media (in the presence of EDTA) brought the fluorescence down to the minimum level, since the fluorescence of the positively charged 9AA molecules is quenched near the negatively charged membranes [13, 14, 18]. It is seen from Fig. 1 that the relative fluorescence quenching is less in the presence of both NaCl- and Tris-treated samples as compared to the control membranes, thus implying a decrease of the number of anionic sites along treated membranes. The flu-

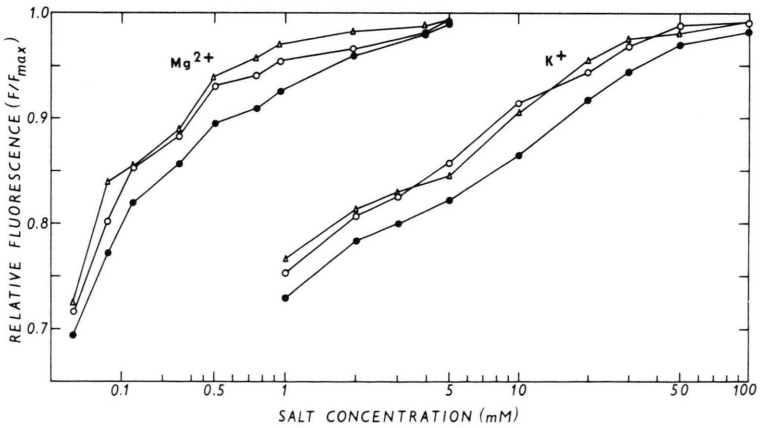

Fig. 1. The relative fluorescence intensity of 9-aminoacridine in the presence of Photosystem II (BBY) particles and different concentrations of K^+ and Mg^{2+}. The values of 9AA fluorescence *(F)* for a particular monovalent (K^+) and divalent (Mg^{2+}) salt concentration were normalized to the maximum fluorescence (F_{max}) reached by adding 20 mM Mg^{2+} at the end of experiments. The results in this figure were used in Table I to calculate C′ (K^+) and C″ (Mg^{2+}) and surface charge density of the PS II particles. (●)-control PS II particles; (○)-NaCl-treated PS II particles; (△)-Tris-treated PS II particles.

orescence quenching was released by titrating the suspensions with increased concentrations of either K^+ and Mg^{2+}, due to the cation-induced displacement of the dye molecules from the diffuse layer [13, 18]. Using the data for K^+ and Mg^{2+} concentrations obtained from S-shaped curves (Fig. 1), which give the same relative (F/F_{max}) fluorescence, the values of surface charge density were calculated essentially following the procedure described by Chow and Barber [13, 14].

It is shown (Table I) that the calculated values for the net negative surface charge density (σ) in control PS II membranes are markedly higher than in NaCl- and Tris-treated membranes at all F/F_{max} levels. It is also seen that the estimated values of σ increased with increasing of the salt concentrations. As mentioned earlier by Chow and Barber [14], this effect could be due to the inherent approximation of the analysis of fluorescence data and/or to the effect of lateral redistribution of charged protein complexes of PS II and PS I due to the increased charge screening in the presence of cations and concomitant separation of relatively less negatively charged complex of PS II in the appressed regions [32]. However, due to the fact that PS II particles are almost completely omitted from PS I complexes as proved by low temperature (77 K) fluorescence measurements (F_{735}/F_{685} ratio $= 0.14 \pm 0.02$, $n = 7$), the increased value of σ could be attributed mainly to the methodological reasons rather than to salt induced redistribution of protein complexes. Since the error in determining the salt concentrations at low and high F/F_{max} levels became significant [9, 16], the values for σ obtained at $F/F_{max} = 0.85$ are used for further comparisons. The σ value of $-0.032\ C \cdot m^2$ estimated for the control PS II (BBY) particles is markedly higher compared to the average surface charge density of envelope-free chloroplasts and appressed (granal) subchloroplast fraction (see Table II). The registered surface charge of the PS II membranes is quite close to the σ value reported previously for the "inside-out" PS II vesicles estimated by the same method [15]. This imply that the PS II particles (under our experimental conditions) could be related to the "inside-out" vesicles

Table I. Effects of NaCl- and Tris-treatments of the net negative surface charge density of PS II subchloroplast particles estimated from 9 AA fluorescence measurements. C′ (K^+) and C″ (Mg^{2+}) concentrations were calculated from Fig. 1 at the same F/F_{max} levels. The values for σ were calculated as in [13, 14]. Mean values ± s.e. were computed from 5 independent experiments.

F/F_{max}	C′ [mM]	C″ [mM]	σ [C·m^{-2}]
Control PS II particles			
0.70	1.375 ± 0.247	0.053 ± 0.003	−0.010 ± 0.001
0.75	1.930 ± 0.371	0.066 ± 0.004	−0.013 ± 0.002
0.80	3.587 ± 0.578	0.078 ± 0.011	−0.022 ± 0.002
0.85	7.250 ± 0.332	0.165 ± 0.018	−0.032 ± 0.001
0.90	15.000 ± 1.054	0.577 ± 0.078	−0.035 ± 0.001
0.95	29.660 ± 1.170	1.287 ± 0.071	−0.042 ± 0.001
NaCl-treated particles			
0.70	–	–	–
0.75	–	–	–
0.80	2.383 ± 0.232	0.077 ± 0.006	−0.015 ± 0.002
0.85	4.262 ± 0.305	0.137 ± 0.014	−0.021 ± 0.003
0.90	8.650 ± 0.430	0.312 ± 0.029	−0.027 ± 0.001
0.95	20.500 ± 1.914	1.460 ± 0.270	−0.029 ± 0.003
Tris-treated particles			
0.70	–	–	–
0.75	–	–	–
0.80	2.237 ± 0.400	0.073 ± 0.013	−0.014 ± 0.001
0.85	5.675 ± 0.299	0.166 ± 0.024	−0.025 ± 0.001
0.90	10.375 ± 1.073	0.408 ± 0.032	−0.029 ± 0.001
0.95	14.330 ± 2.753	0.650 ± 0.058	−0.030 ± 0.001

Table II. The calculated net negative surface charge density (σ) of PS II (BBY) particles, NaCl- and Tris-treated particles, envelope-free chloroplasts and appressed (granal) thylakoids. The values for σ are means ± s.e. (number of independent experiments) and are calculated at $F/F_{max} = 0.85$. Significance levels were determined from a t-test for the differences between control PS II particles and the other preparations. Statistically significant differences are marked by asterisks (* – $p < 0.05$; ** – $p < 0.001$).

Preparation	σ [C·m^{-2}]	
Envelope-free chloroplasts	−0.026 ± 0.001	(7)**
Appressed (granal) thylakoids	−0.017 ± 0.003	(3)**
Control PS II (BBY) particles	−0.032 ± 0.001	(5)
NaCl-treated PS II particles	−0.021 ± 0.003	(5)*
Tris-treated PS II particles	−0.025 ± 0.001	(5)**
NaCl-treated + P.E.	−0.030 ± 0	(2)
Tris-treated + P.E.	−0.028 ± 0	(2)

P.E. – crude protein extract of polypeptides included in the oxygen evolving complex used for the reconstitution experiments.

[3], rather than to stabilized flat sheets of granal membranes as proposed by Dunahay *et al.* [19]. Moreover, electron microscopic observations have indicated that the resuspension of isolated PS II particles in low salt (1 mM K^+, 0.1 M sorbitol) medium resulted in formation of single closed vesicles (data not shown). This morphological feature contradict to the freeze-fracture data indicating that PS II-enriched membrane fragments does not form closed vesicular structures [19]. The reason for this differences are not clear, but it must be noted that the final PS II preparations examined by freeze fracture are suspended in high salt (5 mM Mg^{2+}) and sucrose (0.4 M) medium. However, it has been also mentioned that some of the PS II preparations tend to vesiculate when the isolation procedure involve low salt treatment (Fig. 10 in ref. [19]). Assuming all above, it seems reasonable to suggest that the lumenal membrane surface which is exposed to the medium contribute to σ of PS II particles as distinct to the appressed (granal) fragments existing in "right-out" configuration where the value of σ could be attributed to the stromal membrane surface. These data are in a full agreement with earlier established significant charge asymmetry existing between the two leaflets of the granal thylakoids [15, 20]. However, the origin and molecular basis of this phenomenon still remain questionable.

It is found that polypeptide depletion of the PS II particles caused either by NaCl- or Tris-treatment leads to a remarkable decrease of the net negative charge of the PS II preparations as compared to the σ value of non-treated membranes, the difference being statistically significant. The lower values for NaCl- and Tris-treated membranes correspond to one electronic charge per 763 Å^2 and 641 Å^2 respectively as compared with 502 Å^2 for the non-treated PS II membranes. The decrease of the anionic sites along the PS II membranes as a result of polypeptide depletion could be completely understood within the framework of the data concerning the isoelectric points of the three extrinsic polypeptides. It has been demonstrated by isoelectrofocusing PAGE-SDS measurement that 24 kDa and 33 kDa polypeptides are negatively charged at neutral pH, while 17 kDa polypeptide is basic [21, 22].

The reconstitution of both NaCl- and Tris-washed BBY particles with a 15-fold excess of a crude protein extract (PE) containing the extrinsic polypeptides of the oxygen evolving complex results in a partial recovery of the net negative surface charge density, although the values remain lower than those in control PS II membranes (Table II, lines 6 and 7). Hence, it seems very likely that the removal of at least two of these polypeptides, *i.e.* 24 kDa and 33 kDa could be responsible for the observed decrease of the surface charge. These data confirm the earlier assumption that the binding of proteins of the oxygen evolving system to the inner site of the thylakoid membranes might be achieved through electrostatic interaction [1, 23, 24]. Moreover, similar decrease of the net surface charge has been reported recently for heat-treated PS II particles and the heat-induced partial release of the 17 kDa, 24 kDa and 33 kDa polypeptides was discussed as a possible reason for the observed effect [25]. Bearing in mind that the PS II (BBY) particles are "inside-out" membranes where the lumenal located proteins of the water splitting complex are exposed to the medium and that NaCl- and Tris-treatments lead to selective depletion of the negatively charged (24 kDa and 33 kDa) polypeptides it appears reasonable to assume that the charge asymmetry could be attributed (at least partially) to the presence of these polypeptides on the inner surface of the granal membranes. This assumption is in agreement with the freeze-frac-

ture electron microscopy observations of Simpson and Andersson [26], indicating that the ES_s tetrameric particles located on the outer surface of "inside-out" thylakoid vesicles are composed mainly of the extrinsic polypeptides of the oxygen evolving complex, although parts of the $LHC_{a/b}$ polypeptides has been also recognized on the inner thylakoid surface [27]. It has been demonstrated that when the extrinsic polypeptides were removed either by NaCl- or Tris-washing, the ES_s particles could no longer be resolved, and that the reappearance of particles on the membrane surface can be achieved by reconstitution with these polypeptides [26].

It is of interest to note that when the 33 kDa polypeptide was removed in addition to the 17 kDa and 24 kDa polypeptides by alkaline Tris, the value of σ is slightly higher as compared to that in NaCl-treated particles, although the differences are not statistically significant (Table II). This result is consistent with the model proposed by Itoh and Uwano [23] that the membrane binding site of 33 kDa polypeptide is negatively charged. The model is based on the assumption that 33 kDa protein is heterogeneous in respect to charge distribution and bears a positively charged domain probably originating from the lysin amino acid residues [21], although the protein carries a net negative charge above pH 5.2 [21, 22]. The effect of this positively charged domain would be to facilitate the binding of protein to the negatively charged membrane binding site [23]. Furthermore, the existence of negative point charges on the inner thylakoid surface in the vicinity of Z [28–30] and possible involvement of the 33 kDa-protein release in the inhibition of electron flow *via* the rise of the negative charge density in the region of Q [31] have been discussed earlier.

The particular role of the 17 kDa, 24 kDa and 33 kDa polypeptides of the oxygen evolving complex in the underlying molecular mechanism of the formation asymmetry across the thylakoid membranes remains to be clarified. Detailed rebinding experiments are required to solve this problem.

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