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RECONSTITUTION OF PHOTOSYNTHETIC WATER SPLITTING AFTER SALT-WASHING OF OXYGEN-EVOLVING PHOTOSYSTEM-II PARTICLES

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Oxygen-evolving Photosystem-II particles were isolated after treatment of spinach chloroplasts with Triton X-100. Treatment of these particles with 2 M NaCl released polypeptides of 24 and 16 kDa concomitant with a loss of the water-splitting activity. Readdition of the concentrated 24–16 kDa protein fraction restored water splitting in the salt-washed particles, the extent of reconstitution being dependent upon the intensity of continuous light during the assay. Under flash illumination, the salt-washed particles transported the normal number of electrons from water to DCIP on the first two flashes but much less reduction occurred on all subsequent flashes; addition of reconstituting protein only slightly prevented this loss process. Absorbance difference spectroscopy revealed that the salt-washed particles were at least able to perform the normal S_1-S_2 transition. The results suggest that removal of the 24–16 kDa protein affects the efficiency of the higher S-state transitions. Additional components may be required for optimal reconstitution and interference with the secondary electron acceptor mechanism after salt washing was detected.

Introduction

The mechanism of photosynthetic oxygen evolution is still poorly understood. The kinetics of the process of charge accumulation are rather well known, but there is little information on the chemical nature of the components involved in this process [1,2]. The role of polypeptides operating in the water-splitting reactions, especially of presumed manganese-containing enzymes, has long been debated [3]. Various studies have recently yielded data which may shed some light on this problem.

Study of a mutant of *Scenedesmus* lacking water-splitting activity and having a low amount of manganese has implicated a role for a 34 kDa protein [4]. Tris or alkaline pH treatment of PS-II particles isolated by means of detergent fractionation of spinach chloroplasts results in the release of 33, 24 and 18 kDa polypeptides concomitant with a complete loss of the water-splitting activity [5,6]. Similarly, Tris treatment of so-called inside-out thylakoid vesicles results in the release of polypeptides with comparable M_r [7], whereas the 24 and 16 kDa polypeptides could also be released from inside-out vesicles by treatment with 0.25 M NaCl. Significantly, readdition of the 24 kDa polypeptide to salt-washed inside-out vesicles restored the water-splitting activity [8].

Such a protein-dependent, reversible inhibition of the water-splitting reactions opens new possibilities to study its mechanism. We, therefore, carried out some experiments in order to determine whether a similar reconstitution can be obtained in PS-II particles isolated after detergent fractiona-

Abbreviations: Chl, chlorophyll; DCIP, 2,6-dichlorophenolindophenol; DPC, 1,5-diphenylcarbazide; Mes, 4-morpholineethanesulphonic acid; PS, Photosystem; Tris, 2-amino-2-hydroxymethylpropane-1,3-diol.

tion and tried to localize the site of action of such reconstituting polypeptides.

Materials and Methods

Isolation of oxygen-evolving PS-II particles and inactivation by salt treatment

Oxygen-evolving PS-II particles were obtained after Triton X-100 treatment of spinach chloroplasts essentially as described in Ref. 9 with the exception that the second incubation with 1% Triton X-100 was omitted and instead the particles were washed with the extraction buffer without Triton X-100. Particles were suspended to a chlorophyll concentration of about 4 mg/ml in 20 mM Mes-NaOH (pH 6.0) containing 15 mM NaCl/5 mM $MgCl_2$/15% glycerol and stored under liquid nitrogen until use.

Salt-washed particles were always prepared on the day of the experiment. A sample of the stored PS-II particles was thawed and divided into two parts: one part was washed with 20 mM Mes-NaOH (pH 6.0)/2 M NaCl (hereafter referred to as salt-washed) and the other part was washed with 20 mM Mes-NaOH (pH 6.0) (referred to as control) at a concentration of about 1 mg Chl/ml. After centrifugation, the particles were suspended to 1 mg Chl/ml in 20 mM Mes-NAOH (pH 6.0) without further additions.

Isolation of protein fractions with reconstituting activity

A suspension of the above PS-II particles containing 50 mg Chl was centrifuged, resuspended in 20 ml 20 mM Mes-NaOH (pH 6.0)/2 M NaCl, incubated for 15 min, centrifuged again (15 min at $40\,000 \times g$) and to the supernatant 40 ml saturated $(NH_4)_2SO_4$ was added. The precipitate was collected by centrifugation, washed with saturated $(NH_4)_2SO_4$ and dissolved in 2 ml 20 mM Mes-NaOH (pH 6.0). Any remaining insoluble material was removed by centrifugation and the preparation was stored under liquid nitrogen.

Photochemical assays

Water-splitting activity was measured in terms of DCIP photoreduction by following the absorbance decrease at 570 nm using a double-beam spectrophotometer. A Corning CS4-96 – Schott AL 565 filter combination protected the photomultiplier from the actinic light, which was provided either by a continuous light source (maximum intensity 400 W/m^2) or by a xenon flash tube (2500 V, 2 μF), filtered by a Schott RG 645 – Calflex filter combination.

All measurements were carried out in 20 mM Mes (pH 6.0) at a chlorophyll concentration as indicated in the text, using 0.12 mM DCIP and 1.2 mM DPC as artificial donor (where indicated). The differential extinction coefficient of DCIP at 570 nm (pH 6.0) was determined to be 11.2 $mM^{-1} \cdot cm^{-1}$; flash-induced absorbance changes were averaged in a Nicolet model 527 signal averager.

PS-II reaction center concentrations were determined by measuring the amount of photoreducible Q using a differential extinction coefficient of 13 $mM^{-1} \cdot cm^{-1}$ at 325 nm [10]; the content of PS I, as determined from the absorbance change at 700 nm ($\epsilon = 64\ mM^{-1} \cdot cm^{-1}$), was generally less than one P-700 per 4000 chlorophyll molecules in the PS-II particles. Flash-induced oxygen yield measurements were performed with a polarographic device as described in Ref. 11.

Results

Protein composition of the PS-II particles and effect of treatments inactivating the water-splitting process

The protein composition of the isolated PS-II particles, as studied by SDS-polyacrylamide gel electrophoresis, closely resembles that of the PS-II preparation isolated by Kuwabara [6] by a slightly different procedure. Comparable protein profiles have also been published by others [12–14]. Washing the PS-II particles with Tris (0.8 M, pH 8.3) or at alkaline pH (pH 9.3) released proteins with $M_r = 33$, 24 and 16 kDa, whereas treatment with salt (2 M NaCl) released only the 24 and 16 kDa proteins. These results are comparable to those obtained by other authors using different PS-II preparations [5,6,8,15,16].

Rates of water splitting under conditions of continuous illumination

Under optimal conditions (0.12 mM DCIP), the absorbance decrease of DCIP caused by continuous illumination of control particles yielded a straight line; in the salt-washed particles, the ab-

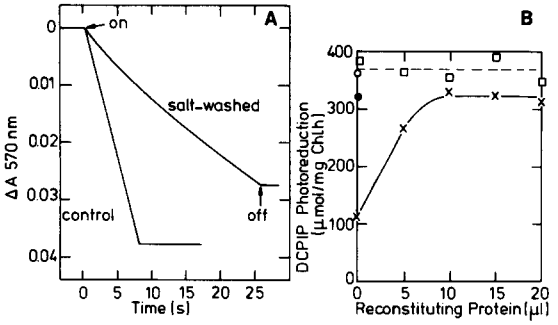


Fig. 1. (A) Absorbance changes of DCIP at 570 nm caused by continuous illumination (maximum intensity) of control and salt-washed PS-II particles. Chlorophyll concentration 10 μg/ml. (B) Effect of the addition of reconstituting protein upon the rate of DCIP reduction in salt-washed PS-II particles. Various amounts of reconstituting protein were added to 40 μg chlorophyll and subsequently assayed at 10 μg Chl/ml under maximum light intensity. × ——— × and □ ——— □, salt-washed PS-II particles without external donor and with 1.2 mM DPC as donor, respectively; ● and ○, control PS-II particles without and with DPC, respectively.

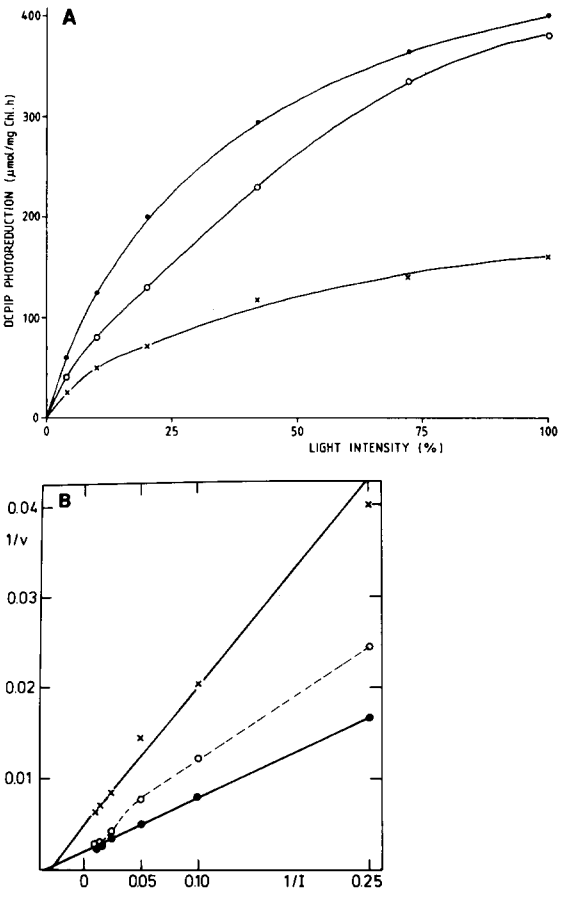


Fig. 2. (A) Rates of DCIP reduction as a function of the light intensity in control (● ——— ●), salt-washed (× ——— ×) and salt-washed/reconstituted (○ ——— ○) PS-II particles. Chlorophyll concentration, 25 μg/ml. (B) Double-reciprocal plots of the results depicted in Fig. 2a.

sorbance decrease always showed a slightly curved line, indicating that the rate of DCIP reduction slowly declines as illumination proceeds (Fig. 1a). Rates of DCIP reduction given below are based on the absorbance changes obtained after 5 s of illumination.

Fig. 1b shows the effect on the rate of DCIP reduction of the addition of reconstituting protein to the salt-washed particles. We observed a protein-dependent restoration of the rate of DCIP reduction back to control level whereas there was no effect on the rate of DCIP reduction with the artificial donor diphenylcarbazide, indicating that the reconstituting effect is specifically at the site of water splitting. This reconstituting effect was observed with protein fractions obtained after salt washing (24 and 16 kDa) or after alkaline-pH treatment (33, 24 and 16 kDa) of PS-II particles, but only when these fractions were added back to salt-washed PS-II particles; alkaline-pH- or Tris-treated PS-II particles could not be reconstituted by either protein fraction. All subsequent reconstitution experiments have been performed with an amount of protein sufficient to obtain maximum stimulation of the rate of DCIP reduction under maximum light intensity (10 μl/40 μg Chl). We have not attempted to separate and purify the individual 24 and 16 kDa proteins. Åkerlund et al. [8] have shown that the 24 kDa protein reconstitutes oxygen evolution in inside-out vesicles.

Fig. 2 shows the effects of light intensity on the rate of DCIP reduction in control, salt-washed and salt-washed/reconstituted PS-II particles. Analysis of these results by double-reciprocal plots should yield straight lines according to the theory of Lumry and Spikes [17]:

$$1/v = 1/k_D + 1/Ik_L$$

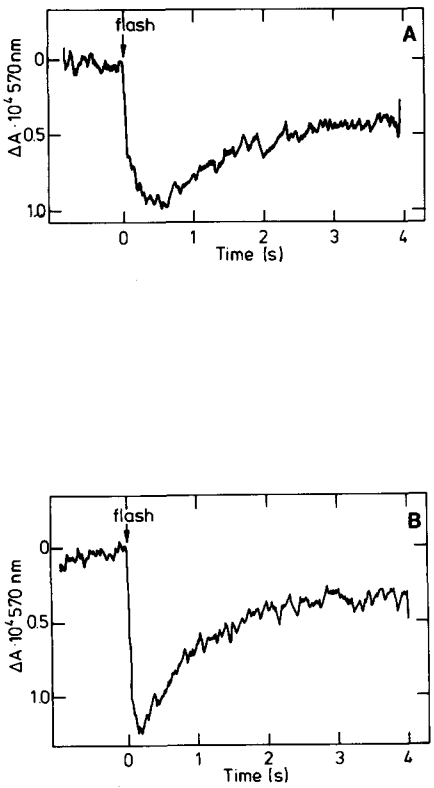
where v is the reaction rate, I the light intensity, k_D the rate constant of the rate limiting step in electron transport at infinite light intensity and k_L the quantum efficiency of the light reaction.

According to Fig. 2b this theory holds for the control and salt-washed particles which appear to differ only in their k_D, in agreement with the notion that the rate of water splitting is descreased after salt washing. The reconstituted particles, however, show a complex relationship: at intensities below 20%, there appears to be about 1.5 × stimulation of the rate in salt-washed particles; at higher intensities an additional stimulation occurs almost back to control level (about 2.5 ×). The intensity curves of DCIP reduction in the presence of the donor DPC were identical for control, salt-washed and reconstituted PS-II particles (not shown).

DCIP reduction under flash illumination

Fig. 3 shows the absorbance change of DCIP caused by a saturating flash of light in control and salt-washed PS-II particles. The absorbance change is slowly reversed in the dark, the half-time was about 2 s in control particles and was found to depend on the preparation. However, in all preparations it was about 2-times faster in salt-washed particles. In the presence of artificial donors like DPC or tetraphenylboron no reversibility was found, indicating that it is probably caused by a reaction of $DCIPH_2$ with an oxidized component associated with the water-splitting system.

The amplitude of the DCIP absorbance change in the salt-washed particles was identical to that in control particles. This is markedly in contrast to Tris- or alkaline-pH-treated particles which do not transport electrons from water to DCIP in a flash



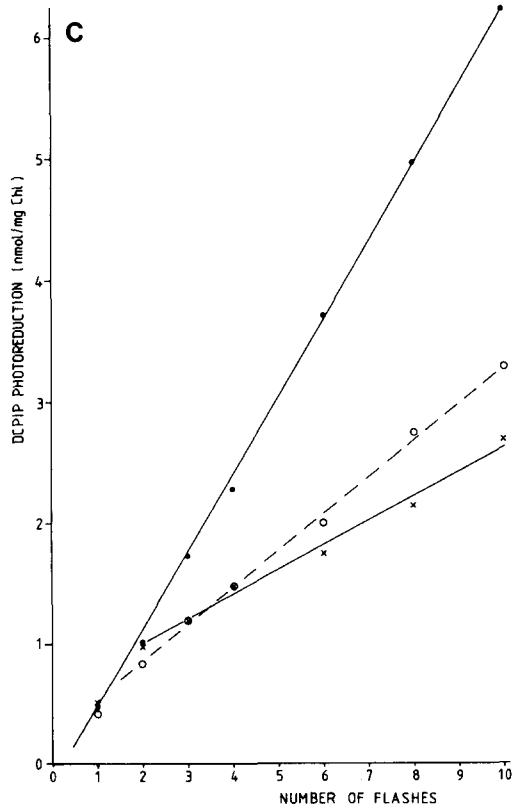


Fig. 3. Absorbance changes of DCIP at 570 nm caused by a saturating flash of light in control (A) and salt-washed (B) PS-II particles. Chlorophyll concentration 100 μg/ml, averaged over 32 flashes spaced 10 s apart. (C), DCIP reduction in a train of flashes (spaced 100 ms apart) in control (●———●), salt-washed (×———×) and salt-washed/reconstituted (○———○) PS-II particles. Chlorophyll concentration 100 μg/ml; each point represents the mean of two separate experiments, each flash train was averaged 16 times.

of light. Subsequently, we studied the effect of a train of flashes spaced at 100 ms. As shown in Fig. 3c, the amount of DCIP reduced in the first two flashes was identical in control and salt-washed particles. However, upon all subsequent flashes much less reduction of DCIP occurred. Addition of reconstituting protein only slightly increased the yield of DCIP reduction after a number of flashes; analysis of the straight lines gives about 1.5 × stimulation which is comparable to the stimulation of the rate of DCIP reduction at low intensities of continuous light (Fig. 2).

When we prolonged the flashing procedure – giving about 30 flashes – we observed the same result, that is, the stimulation of the amount of DCIP reduced is about 1.5 × in reconstituted particles; the total amount of DCIP reduced by such a train of flashes was identical to the amount reduced by continuous light of 2% intensity during the same period of time (3 s). This amount of DCIP reduced with 2% intensity of light falls in the linear part of the Lumry-Spikes plot (not shown in Fig. 2); at still lower intensities, deviations from linearity occur due to the reoxidation of $DCIPH_2$. The validity of this extrapolation of flashing light to continuous light indicates that the same number of reaction centers is involved in both types of experiment.

To determine whether light is required in order to obtain maximum efficiency of reconstitution, we subjected the same sample first to 3 s of flashing light, subsequently to 3 s of continuous light (100% intensity) and subsequently repeated the flash protocol; each time the DCIP absorbance change was recorded. The first flash series yielded the result already noted (1.5 × stimulation after reconstitution) whereas the extent of reconstitution was 85% under 100% continuous light. The second flash series, however, yielded identical results as the first one, indicating that light is not required to obtain the proper reinsertion of the proteins into the depleted particles (results not shown).

It should be noted that, in the experiments depicted in Fig. 3, the amount of DCIP reduced in a saturating flash was only about 1 electron equivalent per 1000 Chl molecules. This number is 4-times lower than the amount of photoreducible Q, which was determined to be 1 per 280 Chl molecules, in agreement with the estimate in Ref. 9. Depending upon the preparation used, we generally obtained values of 0.25–0.40 electron equivalents transported from water to DCIP per photoreducible Q. In the presence of DPC this number increased 1.5–2.0-times and in the presence of tetraphenylboron 3–4-times, indicating that, at least in case of water or DPC as electron donor, a substantial part of the reaction centers does not transfer electrons to DCIP.

Absorbance changes with periodicity four and two

The observation that after salt washing the PS-II particles are still able to transport the same number of electrons from water to DCIP on the first two flashes, prompted us to try to correlate this with transitions of the S-states. Measurement of the flash-induced oxygen yield pattern showed a pattern for control particles comparable to that of chloroplasts, indicating that the first flash elicits the S_1-S_2 transition. Our results (not shown) agreed with those obtained by Lavorel and Seibert [18,19] with the exception that we did not observe an anomalously large signal on the first flash. In salt-washed PS-II particles, however, we did observe a large signal on the first flash and only small signals on subsequent flashes which did not show reproducible oscillations. We, therefore, concluded that polarographic measurements were unsuitable to monitor S-state transitions in salt-washed PS-II particles.

It has been shown by Pulles et al. [20] that absorbance changes, oscillating with a periodicity of four and ascribed to the charge accumulating complex, can be observed around 320 nm. In control PS – II particles, we obtained a similar oscillation (Fig. 4a). That the flashes were spaced 1 s or 100 ms apart made no difference. The sample was supplied with 5 μM ferricyanide in order to oxidize any Q^- that may have been present in dark-adapted material; the dark-adaptation time was about 10 min before the measurement.

In chloroplasts, the oscillation with a periodicity of 4 is superimposed upon an oscillation with a period of two which is ascribed to the secondary acceptor R [20]. The latter oscillation can be detected by performing the measurements in the presence of hydroxylamine. The same method could be applied to the control PS-II particles,

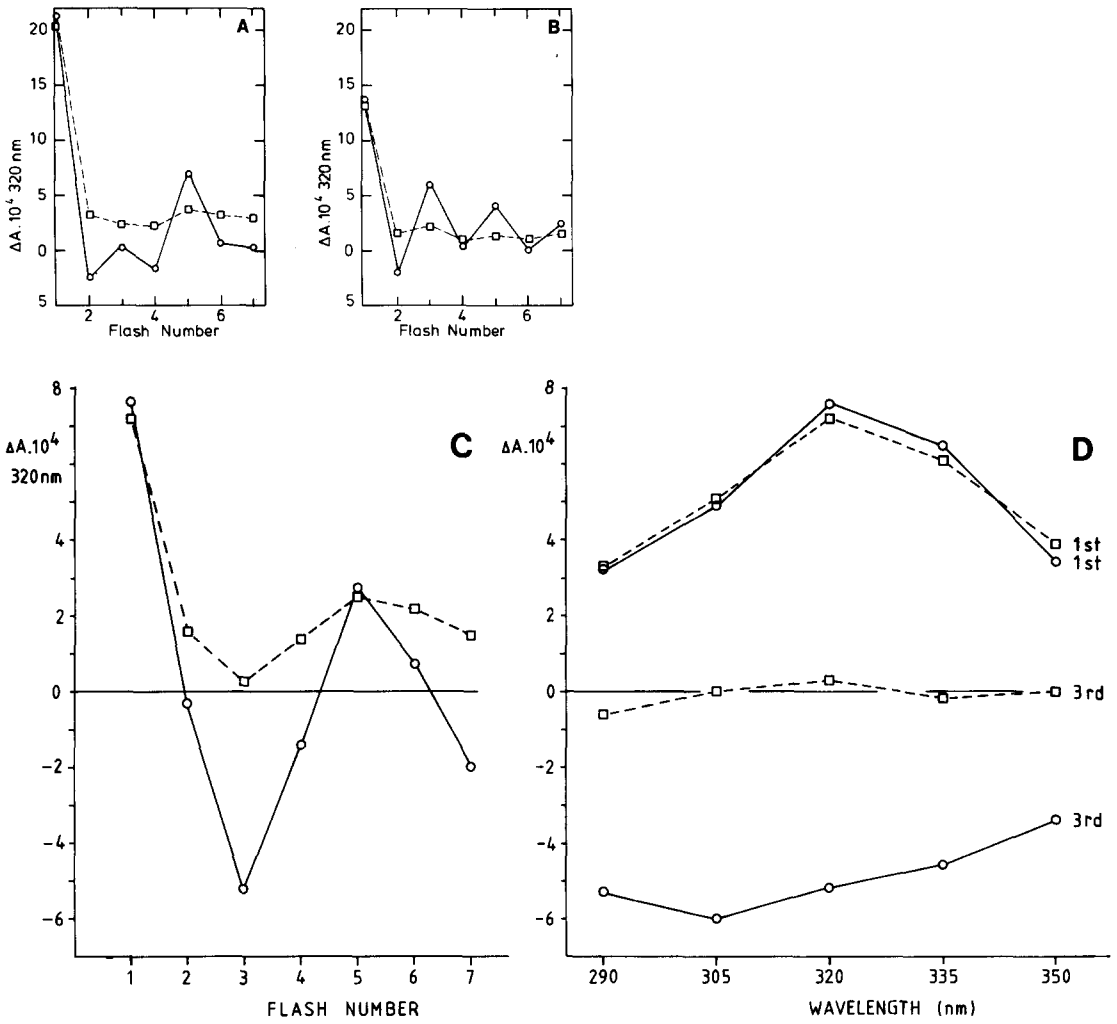


Fig. 4. Flash-induced absorbance changes in control (○———○) and salt-washed (□———□) PS-II particles. Chlorophyll concentration 200 μg/ml; flashes spaced 1 s apart, averaged 5 times. (A, B) Absorbance changes at 320 nm in the absence and presence of hydroxylamine (1 mM), respectively. (C) the results plotted in B were subtracted from those in A; (D) part of the difference spectrum as in C plotted for the first and third flash.

yielding the results depicted in Fig. 4b.

When the absorbance changes obtained in the presence of hydroxylamine are subtracted from those obtained in the absence of hydroxylamine, the resulting absorbance changes should reflect events related to the water-splitting donor site (Fig. 4c). The change upon the first flash can be ascribed to the oxidation of a component associated with the S_1-S_2 transition and the reversed change upon the third flash to the reduction of the same component associated with the S_3-(S_4)-S_0 transition, as originally proposed by Velthuys [2,21]. The latter proposition does not hold entirely for our measurements in PS-II particles, since the difference spectrum associated with the third flash is not just a mirror image of that associated with the first flash (Fig. 4d), indicating that more than one component is involved.

In salt-washed PS-II particles, the amplitudes of the absorbance changes upon the first flash, both in the absence and presence of hydroxylamine, were identical to those in control particles. This is

also the case for the difference spectrum associated with the first flash (Fig. 4d), indicating that the normal donor reactions associated with the S_1-S_2 transition take place in the salt-washed particles. On subsequent flashes, however, the periodicity of four is greatly diminished, indicating that the higher S-state transitions have an impaired efficiency. The complete lack of the spectrum associated with the S_3-(S_4)-S_0 transition would suggest that the inhibited site in the salt-washed particles is at the S_3-(S_4)-S_0 transition. However, a direct demonstration of a normal S_2-S_3 transition is not possible, since as yet no optical changes attributed to this transition have been established.

Moreover, the donor changes are complicated by the fact that the oscillation with a periodicity of two is not identical in control and salt-washed particles (Fig. 4b). A mathematical analysis of the absorbance changes with a periodicity of two indicates that, in control particles, 50–60% of the reaction centers transport electrons to the plastoquinone pool through the secondary acceptor mechanism. The remaining centers perform a reduction of the primary acceptor Q upon the first flash only. In salt-washed particles, less than 10% of the centers transport electrons through the secondary acceptor mechanism.

This observation was supported by fluorescence measurements, which indicate that the fluorescence level is at maximum after one flash, representing the accumulation of Q^- in nearly all of the centers. Moreover, the rate of re-reduction of the plastoquinone pool, measured as fluorescence induction, was not restored to control level by the addition of external electron donors like hydroxylamine, DPC or tetraphenylboron to salt-washed PS-II particles (not shown). The latter result differs from the situation in salt-washed inside-out vesicles, in which case the rate of plastoquinone pool reduction could be restored by the addition of hydroxylamine [8]. Possibly, the combination of salt with residual detergent may be deleterious to the secondary electron acceptor mechanism.

Absorbance change measurements at 320 nm performed in the presence of DCIP indicate that DCIP is able to accept electrons from part of the Q^- (or R^-) centers in control particles and from part of the Q^- centers in salt-washed particles, respectively, thereby relieving this inhibition at the acceptor site.

Due to this inhibition at the acceptor site, we were unable to demonstrate an effect of the addition of reconstituting protein upon in the absorbance changes in the salt-washed particles, despite the fact that we carefully checked – both before and after the absorbance change measurements – that the sample did indeed yield fully reconstituted rates of DCIP reduction at maximum intensity of continuous light. The explanation is that in the absence of DCIP, where these measurements were done, the reconstituting protein alone does not relieve the inhibition at the acceptor site.

Discussion

Oxygen-evolving PS-II preparations from spinach chloroplasts have recently been prepared by means of detergent fractionation using digitonin [5,22] or Triton X-100 [6,9]. We have used a slightly modified method of Berthold et al. [9] to obtain a similar preparation which closely resembles the other ones in regard to characteristics like protein composition, PS-I content and the effect of various treatments inactivating the oxygen-evolving complex.

High-salt treatment released the 24 and 16 kDa proteins, a result similar to that obtained with inside-out vesicles [8]. Readdition of the concentrated 24–16 kDa protein fraction restored the rate of water splitting in the salt-washed particles, the extent of reconstitution was found to depend on the light intensity at which the assay was performed. Low intensities, including flashing light with a spacing of 100 ms, caused a stimulation of 1.5 ×, increasing to almost control level when the light intensity increased. This result indicates that, in addition to the 24–16 kDa proteins, some other factor is necessary to achieve full reconstitution. This other factor must be related to a given rate of water splitting; from the results depicted in Fig. 2 we can estimate that the photoreactions have to occur within 10 ms in order to observe full reconstitution. Further confirmation of the involvement of a possible low-molecular-weight component in the process of reconstitution comes from the observation that, when salt-washed particles are washed a second time with buffer only, a further reduction occurs of the rate of DCIP reduction.

Such salt-washed/buffer-washed particles could not be reactivated to more than 50% of control level, even when saturating amounts of 24–16 kDa protein were added.

In contrast to Tris- or alkaline-pH-treated particles, the salt-washed particles were still able to transport electrons from water to DCIP under flash illumination. These measurements of DCIP reduction under flashing light indicated that a large part of the reaction centers do not transport electrons to DCIP with water or even DPC as donor. Only when tetraphenylboron was used as donor did all the centers transport electrons to DCIP. The reason for this behaviour is as yet unclear; it may be that DCIP causes a short cyclic reaction between acceptor and donor site in a majority of the reaction centers.

In the absence of an artificial acceptor, heterogeneous behaviour was also observed. About 40% of the centers can react only once by reducing the primary acceptor Q; this number was further increased by the salt treatment, complicating the interpretation of the absorbance changes. The identity of the absorbance changes attributed to both donor and acceptor reactions in control and salt-washed particles upon the first flash firmly established that the salt-washed particles perform a normal S_1-S_2 transition. The second flash also reduced the normal amount of DCIP, but a direct demonstration of an also normal S_2-S_3 transition was not possible. The subsequent transitions are clearly very inefficient, as demonstrated in the amount of DCIP reduced and the lack of the specific absorbance changes attributed to S_3-(S_4)-S_0.

It is interesting to note the similarities between our results and those obtained with Cl^--depleted chloroplasts, which are also able to store part of the positive charges [23]. The effect of Cl^--depletion has been related to a metastable H^+-pool which is derived from water oxidation [24,25]. It might be speculated that a common mechanism underlies these different observations: a difference between the S_1-S_2 transition on the one hand and the S_2-S_3 and S_3-(S_4)-S_0 transitions on the other hand is the absence of H^+-release on the first reaction [26]. A stabilizing factor for these higher S-state transitions may be the efficient removal of the released protons, in which the 24–16 kDA proteins may be involved. On the other hand, the 24–16 kDa proteins may simply be acting in a structural role whereby they shield the charge-accumulating complex against premature reduction by any unwanted internal or external reductants.

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