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PHOTOSYNTHETIC OXYGEN EVOLUTION DOES NOT REQUIRE THE PARTICIPATION OF POLYPEPTIDES OF 16 AND 24 KILODALTONS

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Oxygen evolution from Triton X-100 prepared photosystem II particles from spinach was inhibited 70-80% after treatment of the particles with high concentrations of salt (0.5 or 1 M NaCl). The salt-washed preparations were depleted of 16 and 24 kilodalton polypeptides. Nearly complete restoration of herbicide-sensitive electron transport activity was observed upon addition of Cl^-. Maintenance of the maximal rates required the presence of Ca^{2+}. Restoration of the activity did not require the readdition (reconstitution) of the extracted polypeptides indicating that the 16 and 24 kilodalton polypeptides do not participate directly in the water-oxidation process. They may, however, play a regulatory role, e.g., in the sequestering of Cl^- and/or Ca^{2+}.

Many studies have recently implicated functional importance of polypeptides having apparent molecular weights of 16, 24 and 33 kDa in photosynthetic oxygen evolution (1-3). Most of the importance attached to these polypeptides has resulted from observations that loss of (and readdition) of these polypeptides from oxygen-evolving PS II particles and inside-out chloroplast thylakoid vesicles correlated with the loss (and reconstitution) of oxygen-evolving activity (4-7). The 16 and 24 kDa polypeptides are apparently electrostatically bound to the inner thylakoid membrane surface since they can be easily removed from inverted vesicles by changes in ionic conditions (1-3,8,9). On the other hand, the 33 kDa polypeptide appears to be bound to the inner thylakoid membrane surface by hydrogen bonds since urea or changes in pH can lead to its removal (2,7,9).

ABBREVIATIONS: kDa, kilodaltons; PS II, photosystem II; MES, 2[N-morpholino]-ethane sulfonic acid; DPC, diphenylcarbazide; EPR, electron paramagnetic resonance; DPIP, dichlorophenolindophenol; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; Chl, chlorophyll; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethyl urea

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Since no exact functional roles have yet been assigned to any of these polypeptides, this study was undertaken to determine the effect of the removal of these polypeptides from oxygen-evolving PS II particles on the Cl^--cofactor requirement (11) on the oxidizing side of PS II and the effect of Ca^{2+} in these reactions.

MATERIALS AND METHODS

PS II oxygen-evolving particles were prepared from market spinach using a slight modification of the procedure reported by Berthold et al. (11,12). Leaves were homogenized in a medium containing 0.2 M sorbitol, 50 mM MES-NaOH, pH 6.5, 10 mM NaCl and 5 mM $MgCl_2$. The homogenate was filtered through ten layers of cheese cloth and the filtrate was centrifuged at 3,000 x g for 5 m. The pellet was resuspended in a wash buffer containing 20 mM MES-NaOH, pH 6.5, 10 mM NaCl and 5 mM $MgCl_2$ and again centrifuged at 3,000 x g for 5 m. The resultant stroma-free thylakoids (pellet) were resuspended in a small volume of the wash buffer and incubated with Triton X-100 at a Triton:Chl ratio of 25 at a final Chl concentration of 2 mg per ml. The incubation was carried out with stirring at 4°C for 30 m in the dark. The PS II particles were obtained as a pellet after centrifugation of the mixture at 40,000 x g for 30 m. The particles were used either directly after resuspension in a small aliquot of the wash buffer with no further Triton incubation or after washing once in the wash buffer to remove any residual chlorophyll and excess Triton. To treat the PS II particles with NaCl, the particles were resuspended in wash buffer to 2 mg Chl per ml; an equal volume of wash buffer containing the concentration of NaCl required (1 or 2 M NaCl) was added to dilute the Chl concentration to 1 mg per ml during the salt treatment. In addition, for the alkali-salt treatment, 50 mM Tris-HCl, pH 8.0 was added to the resuspension wash buffer in the presence of NaCl and small aliquots of Tris base was added to alter the pH to 9.2-9.3. After incubation of the PS II particles with salt or salt-alkali with stirring at 4°C in the dark, they were centrifuged at 40,000 x g for 15 m. The treated particles were resuspended in a medium simply containing 20 mM MES-NaOH, pH 6.5 to test for the effect of Cl^-; no sulfate was added as previously reported (10) since in these studies sulfate was found to lead to inhibition.

DPIP photoreduction was monitored at 580 nm with a Hitachi-110 spectrophotometer using a saturing red (> 600 nm) actinic light beam directed at the sample at 90° to the measuring beam. The phototube was protected from scattered light with a Corning 4-96 filter mounted in front of the phototube. Oxygen-electrode measurements were conducted with a Rank-type oxygen electrode thermostated at 20 °C.

SDS-PAGE was performed as previously described (13) with 12.5% acrylamide in the presence of 6 M urea. The molecular weight standards included: bovine serum albumin (68 kDa), ovalbumin 45 (kDa), carbonic anhydrase (29 kDa) and cytochrome c (12.4 kDa).

RESULTS

A comparison of the polypeptide profile of the PS II particle following salt and salt-alkali treatment is shown in Fig. 1. The results show a nearly complete loss of a 16 kDa polypeptide (16-18 kDa) and a loss of a large proportion of a 24 kDa polypeptide (22-24 kDa) after treatment of the particle with 0.5 M NaCl, see lane 3, Fig. 1. Both the 16 and 24 kDa polypeptides were lost upon treatment of

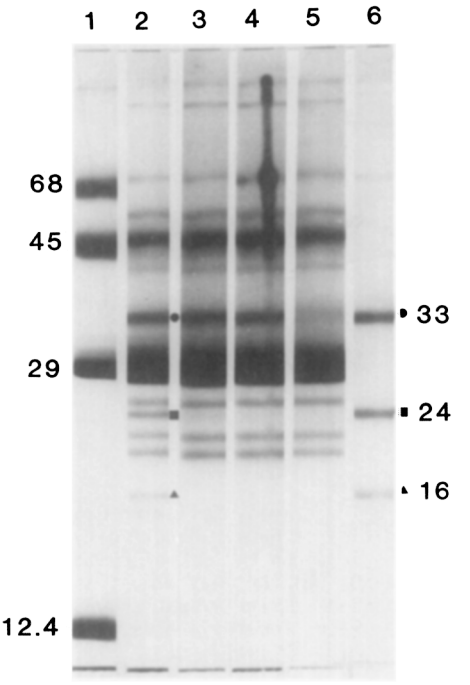

Figure 1. SDS-PAGE polypeptide profile for the PS II particle following various treatments: lane 1, molecular weight standards for bovine serum albumin, ovalbumin, carbonic anhydrase and cytochrome c in descending order (numbers adjacent to lane 1 correspond to the molecular weight of the standards in kilodaltons); lane 2, Triton-prepared PS II particle; lane 3, PS II particle treated with 0.5 M NaCl-showing major loss in 16 and 24 kDa polypeptides; lane 4, PS II particle treated with 1.0 M NaCl showing complete loss of the 16 and 24 kDa polypeptides; lane 5, PS II particle treated with 1 M NaCl at pH 9.2, showing loss of the 16, 24 and 33 kDa polypeptides (a diffuse band remains which has previously been attributed to the herbicide binding polypeptide); lane 6, the extract after salt-alkali-treatment of the PS II particle with the symbols and molecular weights of the extracted polypeptides. Symbols ▲, ■, ●, correspond to the 16, 24, and 33 kDa polypeptides, respectively.

the particle with 1 M NaCl; see lane 4, Fig. 1. If the particle was treated with 1 M NaCl at alkaline pH (9.2-9.3), in addition to the loss of the 16 and 24 kDa polypeptides, a 33 kDa polypeptide (32-34 kDa) was also extracted; see lane 5, Fig. 1. A diffusely staining polypeptide of a similar molecular weight size class (about 32 kDa) was not removed by this latter treatment and has been charac terized as the herbicide-binding protein of PS II (Q_B protein, refs. 13,14).

Loss of the 16, 24 and 33 kDa polypeptides has previously been correlated with loss in oxygen-evolving activity (1-3) and is confirmed in Fig. 2. The activity of the PS II particles which has been treated with either 0.5 or 1 M NaCl was decreased by approximately 70-80% as compared to untreated PS II

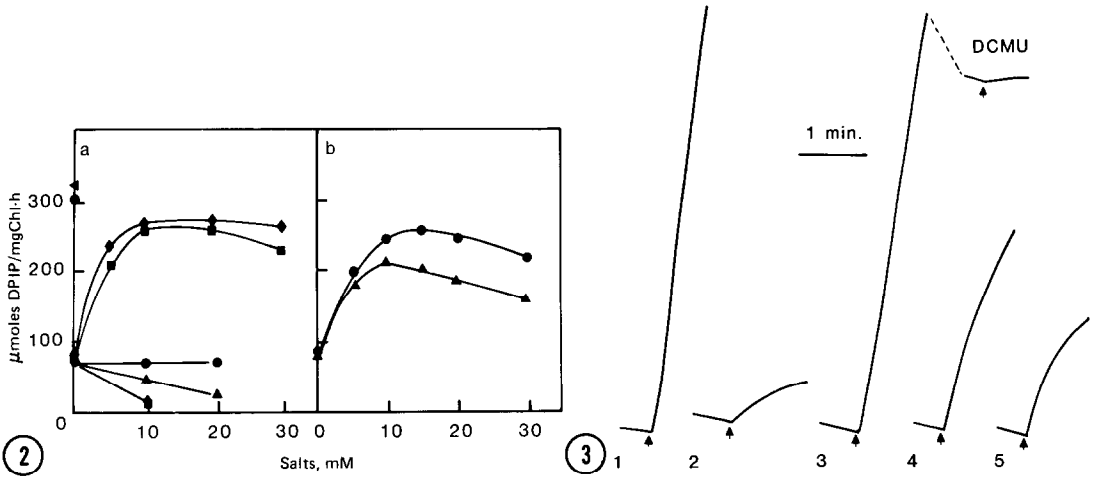

Figure 2a. Restoration of DPIP activity following 0.5 M NaCl treatment (◆) and 1 M NaCl treatment (■) by addition of NaCl. Specificity of Cl^- cofactor-requirement for reactivation is shown by lack of restoration upon addition of $NaNO_3$ (●) $NaHCO_3$ (▲) and Na_2SO_4 (◆) (Br^- was almost as active as Cl^-, not shown). K^+- salts responded similarly, not shown. Upper two symbols on x-axis correspond to control rates for thylakoids, uncoupled, (◀) and PS II particle ( ).
Figure 2b. Restoration of DPIP activity by the addition of $MgCl_2$ (▲) and $CaCl_2$ (●).

Figure 3. Restoration of O_2 evolution with 2,6-dichloro-p-benzoquinone as electron acceptor. Curve 1 is the control PS II sample; curve 2, 1 M NaCl-treated PS II particle in the absence of Cl^-; curve 3, 1 M NaCl-treated sample in the presence of 10 mM $CaCl_2$; curve 4, 1 M NaCl-treated sample in the presence of 10 mM NaCl and curve 5, 1 M NaCl-treated sample in the presence of 10 mM $MgCl_2$. Horizontal bar denotes the time scale. The arrows denote the onset of illumination; the dotted line in curve 3 denotes changes occurring during the addition in the dark of 10 μM DCMU. The maximal rate attained in curve 1 is 170 μmoles O_2 mg $Chl^{-1}h^{-1}$.

particles. Importantly, however, upon the addition of Cl^-, the rates of DPIP photoreduction in the salt-washed preparations were restored nearly to the control levels, Fig. 2a and 2b. The specific requirement of Cl^- is shown in Fig. 2a. Of the other anions tested, only Br^- was comparable in its ability to act as a cofactor, not shown.

The electron transport rates when measuring O_2-evolution directly (utilizing 2,6 dichloro-p-benzoquinone as electron acceptor) were restored to levels comparable to the control in the experiments shown in Fig. 3. The electron transport rates exhibit a time-dependent "photoinhibition" when Cl^- is supplied either as the Na^+ or Mg^{2+} salt (curves 4 and 5 in Fig. 3) whereas when Cl^- is added as the Ca^{2+} salt, long-term constant rates of O_2-

evolution were obtained. The "Cl-effect" defined as the ratio of the initial rate of O_2-evolution in the presence of Cl^- over the rate in its absence was observed to be 7.1 (for example, compare curves 2 and 3 in Fig. 3). Poor electron transport rates were encountered with other quinone acceptors in O_2 evolution assays indicating that some alteration had occurred to the quinone binding sites by the salt treatments. After removal of the 33 kDa polypeptide, following alkali-salt treatment, the oxygen evolution rates were almost negligible and could not be "reactivated" upon addition of any chloride salts.

The accessibility of the electron donor side of PS II was studied following the salt and salt-alkali treatments. Electron donation to PS II was observed with NH_2OH, DPC, Mn^{2+} and H_2O_2 in all preparations. Moreover, H_2O_2 donation was inhibited by azide and aminotriazole (see ref. 19), in all cases, this inhibition was overcome to different extents by DPC or NH_2OH (data not shown).

DISCUSSION

The results in this report indicate that the 16 and 24 kDa polypeptides, which have previously been implicated in the process of water-oxidation, are not mechanistically involved in this process. This has been shown by the removal of these polypeptides from oxygen-evolving PS II particles with the restoration to control levels of oxygen-evolution in the extracted preparations by the addition of Cl^-. A cofactor role for Cl^- has been known for the oxidizing side of PS II. This has recently been postulated to be associated with the S-states (15,16). In addition, Ca^{2+} was found to maintain maximal electron transport activity, i.e. to suppress photoinhibition. Ca^{2+} has previously been shown to be required for the "reactivation" of O_2-evolution in Tris-inhibited chloroplasts (17) as well as the "activation" of O_2-evolution from intact chloroplasts of intermittently-flashed wheat leaves (18). Furthermore, a Ca^{2+}-specific effect on EPR signal II has been reported (19). These combined results suggest a participatory role for Ca^{2+} in O_2-evolution.

Circumstantial evidence indicates that the 33 kDa polypeptide may be required to observe oxygen evolution and the ability of Cl^- to restore O_2-

evolving activity. However, it must be kept in mind that the loss of this activity may have been a consequence of damage to the "water-splitting" complex by the action of the salt and alkali conditions utilized to remove this protein (7). Hydrogen peroxide was found to be a donor on the oxidizing side of PS II following removal of this latter polypeptide (as well as the 16 and 24 kDa polypeptides); azide or aminotriazole inhibition of this activity was overcome by DPC and NH_2OH as shown previously (20) indicating the likelihood of a "cryptoperoxy" intermediate in oxygen evolution at a site removed from the donation site of NH_2OH and DPC.

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