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Cause for Dark, Chilling-Induced Inactivation of Photosynthetic Oxygen-Evolving System in Cucumber Leaves[1]

Jian-Ren Shen*[2], Ichiro Terashima, and Sakae Katoh

Department of Biology, Faculty of Science, University of Tokyo, Hongo, Bunkyo-ku, Tokyo 113, Japan

ABSTRACT

Effects on oxygen evolution of the storage of detached cucumber (*Cucumis sativus*) leaves at 0°C in the dark were investigated with thylakoids and oxygen-evolving photosystem II membranes isolated from stored leaves. The cold and dark treatment of leaves selectively inactivated electron transport on the oxidizing side of photosystem II. Photosystem II membranes isolated from treated leaves were largely depleted of two proteins of 20 and 14 kilodaltons, which correspond to the extrinsic 23- and 17-kilodalton proteins of spinach functioning in oxygen evolution. The manganese content of photosystem II membranes was also markedly reduced by the treatment. Thus, the inactivation of oxygen evolution induced by the dark, chilling treatment is ascribed to solubilization of the 20- and 14-kilodalton proteins and extraction of manganese.

Photosynthetic oxygen evolution takes place in a discrete multiprotein complex of thylakoid membranes, which carries Chl *a*, carotenoids, and various reaction components or cofactors. Among others, four Mn atoms bound to the complex play a central role in cleavage of water molecules, which leads to the production of molecular oxygen. The Mn atoms are considered to be located on the D1 and D2 proteins of the PSII reaction center complex (13, 19). Three extrinsic proteins of 33, 23, and 17 kD, which are associated with the lumenal side of the thylakoid membranes, are also essential for oxygen evolution. The 33-kD protein is needed to maintain the functional conformation of the Mn cluster, whereas the extrinsic 23- and 17-kD proteins have regulatory roles in oxygen evolution (8). Removal of the two proteins from inside-out thylakoid vesicles or PSII membranes, with a high concentration of NaCl, strongly suppresses oxygen evolution (1), but the lost activity is largely restored by addition of Ca^{2+} and Cl^- (6, 14, 16). The two proteins seem to have a structural or protecting role because, on removal of the proteins, the Mn cluster becomes unstable in the presence of exogenous reductants (7).

Treatment of cucumber *(Cucumis sativus* L.) leaves at 0°C in the dark is known to inactivate selectively the oxygen-evolving system because thylakoid membranes isolated from treated leaves are capable of photoreducing DCIP[3] only in the presence of exogenous electron donors, such as DPC (4, 10, 20). Fluorescence induction determined at 77 K and 25°C also demonstrated that the chilling-sensitive site is not the PSII reaction center but electron transport on the oxidizing side of the reaction center (4, 20). The mechanism of the inactivation, however, still remains unclear. Kaniuga *et al.* (11) have shown that the Mn content of isolated chloroplasts decreased by 40 to 50% upon the cold and dark storage of tomato leaves, concomitant with an almost complete inactivation of the Hill reaction, and the changes were largely reversed by illumination of the treated leaves. The results suggest that loss of the Mn is the cause of inactivation. However, Mn atoms present in chloroplasts are heterogeneous in terms of both function and binding stability and there is a significant amount of Mn not related to photosynthetic oxygen evolution. Appearance of an EPR signal of free Mn^{2+} (11) indicates that the metal cations released from the functional sites are trapped in the lumen space of the thylakoids. Thus, it is difficult to relate quantitatively loss of Mn from chloroplasts to inactivation of oxygen evolution. Moreover, it is still unclear whether the release of Mn is the sole cause of the inactivation of oxygen evolution in leaves stored under cold and dark conditions.

In the present work, we used oxygen-evolving PSII membranes with an everted orientation to examine the effects of dark, chilling treatment on the oxygen-evolving machinery of cucumber leaves. Preferential inactivation of oxygen evolution was found to be related with release of two extrinsic proteins functioning in the water-oxidizing complex. In addition, Mn atoms were also solubilized upon the cold and dark storage of leaves. A part of the present results has been reported preliminarily in (21).

MATERIALS AND METHODS

Cucumber plants *(Cucumis sativus* L. cv Nanshin) were grown hydroponically in a controlled environment for about 20 d as described (21). For chilling treatment, leaves were detached and stored on ice at 0°C in the dark. Thylakoids were prepared as in Terashima *et al.* (20) and suspended in 20 mM Hepes/NaOH (pH 7.5), 0.3 M sorbitol, and 20 mM

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[2] Present address: Solar Energy Research Group, The Institute of Physical and Chemical Research (RIKEN), Wako-shi, Saitama 351-01, Japan.

[3] Abbreviations: DCIP, 2,6-dichloroindophenol; DPC, 1,5-diphenylcarbazide.

Table I. *Activities of DCIP Photoreduction and Oxygen Evolution in Thylakoid Membranes from Cucumber Leaves Stored at 0°C in the Dark*

Activities	Duration of Storage		
	0	24	48
		h	
Oxygen evolution (μmol O_2/mg Chl h)	267	58	36
DCIP photoreduction (μmol DCIP/mg Chl h)			
−DPC	129	65	22
+DPC	135	108	82

NaCl. For SDS-PAGE the thylakoids were washed twice with 40 mM Mes/NaOH (6.5), 0.4 M sucrose, 20 mM NaCl, and 2 mM $MgCl_2$ and resuspended in the same medium. PSII membranes were prepared by the method of Berthold *et al.* (3), with a Triton X-100 to Chl ratio of 20 (18). Photoreduction of DCIP was measured spectrophotometrically at 590 nm with a Hitachi 356 spectrophotometer. Concentration of DCIP was 200 μM and, where indicated, 1 mM DPC was supplemented. Oxygen evolution was determined at 27°C with a Clark-type oxygen electrode in the presence of 0.5 mM phenyl-*p*-benzoquinone as an electron acceptor. Basal reaction media contained 40 mM Hepes/NaOH (pH 7.0), 0.3 M sorbitol, and 20 mM NaCl and 20 mM methylamine for thylakoids or 40 mM Mes/NaOH (pH 6.0), 0.4 M sucrose, 10 mM NaCl, and 2 mM $MgCl_2$ for PSII membranes.

Polypeptide compositions were analyzed by SDS-PAGE using the buffer system of Laemmli (12). Samples were treated with 8 M urea, 2.5% SDS, and 5% β-mercaptoethanol and applied to a 10 to 15% acrylamide gradient gel containing 6 M urea. The gel was stained with Coomassie brilliant blue R-250 and scanned at 560 nm or photographed. Mn content of PSII membranes was determined with an atomic absorption spectrophotometer (Shimadzu AA640-01) equipped with a graphite furnace atomizer (GfA-2) (18). Chl concentration was determined according to Arnon (2).

RESULTS

Table I shows changes in the activities of DCIP photoreduction and oxygen evolution of the thylakoids isolated from detached cucumber leaves that had been kept at 0°C in the dark for various periods of time. In agreement with the results reported previously (4, 10, 20), the oxygen-evolving activity decreased markedly by the dark, chilling-treatment of leaves for 24 h and only 10 to 20% of the original activity remained after 48 h of the treatment. DCIP photoreduction determined in the presence of DPC, an artificial electron donor to PSII, was more resistant to the treatment than the photoreduction with water as electron donor. The preferential inactivation of electron transport on the oxidizing side of PSII was more clearly demonstrated with Triton-prepared PSII membranes. Table II shows that the dark, chilling traatment strongly suppressed electron transport from water to DCIP, while DPC-supported DCIP photoreduction was not at all affected by the treatment for 48 h. Oxygen evolution was also severely inactivated and addition of 5 mM $CaCl_2$ failed to restore the activity of PSII membranes isolated from treated leaves.

Polypeptide compositions of the thylakoids and PSII membranes were analyzed by SDS-PAGE (Fig. 1). Lanes 1 to 3 compare polypeptide compositions of the thylakoids from cucumber leaves which have been treated at 0°C in the dark for 0 to 48 h. Major polypeptides resolved from the thylakoid membranes were the large subunit of PSI (60 kD), the α and β subunits of H^+-ATPase (55–58 kD), the 47 and 43 kD Chl-carrying proteins of PSII reaction center complex, 27–29 kD apoproteins of light-harvesting Chl protein complexes and three extrinsic proteins of 31, 20, and 14 kD (see below), together with several other polypeptides in the small molecular mass region. Note that treatment of cucumber leaves at 0°C in the dark for 48 h did not alter the polypeptide composition of thylakoids at all (lane 3). In contrast, the dark, chilling-treatment of detached leaves caused notable changes in the polypeptide composition of PSII membranes. Compared with PSII membranes from untreated cucumber leaves (lane 4), the band intensity of the 20 and 14 kD proteins was significantly reduced after the treatment of 24 h (lane 5). The depletion of the two proteins was more marked in PSII membranes isolated from 48 h treated leaves (lane 6). Treatment of PSII membranes with alkaline Tris is known to solubilize extrinsic proteins of 33, 23, and 17 kD related to oxygen evolution (22). Lane 7 shows that the treatment solubilized the 20 and 14 kD proteins, together with the 31 kD protein, from PSII membranes isolated from untreated leaves. The released proteins were recovered in the supernatent after centrifugation (lane 8). The results indicate that the three proteins correspond to the extrinsic 33, 23, and 17 kD proteins of spinach (lane 9) which play important roles in oxygen evolution (8). Tris treatment also revealed the presence of a protein, which comigrates with the extrinsic 14 kD protein but remains bound after the treatment.

Table III summarizes the abundances of the extrinsic proteins in thylakoids and PSII membranes isolated after the dark, chilling treatment of leaves. There was no decrease in the relative abundance of the three extrinsic proteins in the thylakoids throughout the course of the treatment. Abundances of the three proteins, relative to the 47 kD Chl-carrying protein, in PSII membranes were comparable to those in the thylakoid membranes. In contrast to the thylakoids, about

Table II. *Activities of DCIP Photoreduction and Oxygen Evolution in PSII Membranes from Cucumber Leaves Stored at 0°C in the Dark*

Activities	Duration of Storage		
	0	24	48
		h	
DCIP photoreduction (μmol DCIP/mg Chl h)			
−DPC	167	55	28
+DPC	170	162	193
Oxygen evolution (μmol O_2/mg Chl h)			
−$CaCl_2$	390	101	21
+5 mM $CaCl_2$	440	109	20

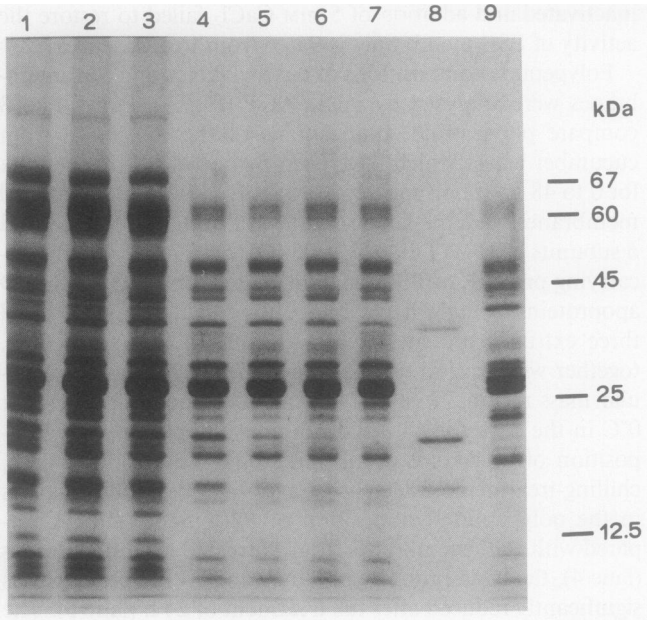

Figure 1. Effects of the cold and dark treatment of cucumber leaves on polypeptide compositions of the thylakoids and PSII membranes. Lane 1, thylakoid membranes from untreated cucumber leaves; lanes 2 and 3, thylakoid membranes from leaves treated at 0°C in the dark for 24 and 48 h, respectively; lane 4, PHII membranes from untreated cucumber leaves; lanes 5 and 6, PSII membranes from leaves treated at 0°C in the dark for 24 and 48 h, respectively; lane 7, 0.8 M Tris (pH 8.5)-treated cucumber PSII membranes; lane 8, extrinsic proteins extracted by the Tris treatment; lane 9, spinach PSII membranes. Molecular markers used were: bovine serum albumin (67 kD), catalase (60 kD), ovalbumin (45 kD), chymotrypsinogen (25 kD), and Cyt *c* (12.5 kD).

two-thirds of the 20 kD protein were solubilized during the treatment for 24 h and the subsequent 24 h treatment decreased the protein content to 15% of the original level. Amounts of the extrinsic 14 kD protein were determined by correcting for the comigrating protein that is not extracted by Tris wash. The 14 kD protein decreased more rapidly than the 20 kD protein and the treatment for 48 h resulted in a complete disappearance of the protein. In addition, there was a small but significant loss of the 31 kD extrinsic protein during the dark, chilling treatment. The results indicate that the extrinsic proteins are released from the lumenal surface of the thylakoid membranes in the dark-chilled leaves.

Removal of the 23 (20) and 17 (14) kD proteins from PSII membranes by NaCl treatment causes a strong inhibition of oxygen evolution (1). Thus, the inactivation of oxygen evolution observed here can be ascribed to the dissociation of the two proteins. In NaCl-washed membranes, the oxygen-evolving activity is largely restored by addition of Ca^{2+} (6, 14). As shown in Table II, however, oxygen evolution of PSII membranes from leaves stored under dark, chilling conditions was little affected by Ca^{2+}. This suggests that there may be another cause of inactivation. There was significant loss of Mn from PSII membranes during the treatment. Cucumber PSII membranes contained about 3.9 Mn per 200 Chl, in agreement with the stoichiometry of four Mn per PSII. The treatment of detached leaves at 0°C in the dark for 24 h resulted in solubilization of more than half the Mn atoms associated with the PSII membranes and there was only 0.8 Mn atom per PSII remained bound to PSII membranes after the treatment of 48 h.

DISCUSSION

Dark storage of cucumber leaves at 0°C has been shown to selectively inactivate the oxygen-evolving system, leaving other parts of the photosynthetic electron transport unaffected (4, 10, 20). Preferential inactivation of electron transport on the oxidizing side of PSII was also demonstrated in the present work, with PSII membranes prepared from the dark, chilling-treated leaves.

The previous experiments with chloroplasts showed that chilling treatment of tomato leaves in the dark caused solubilization or loss of Mn, concomitant with inactivation of oxygen evolution (11). PSII membranes employed in the present work have an advantage over chloroplasts or thylakoids in that PSII membranes have no extraneous Mn and all the four Mn atoms present per PSII function in water oxidation. In addition, due to the inside-out orientation of PSII membranes, amounts of Mn released from the functional binding sites could be directly determined. The results obtained show that release of Mn is indeed a cause of inactivation of oxygen evolution during the cold and dark storage of cucumber leaves. The degrees of the inactivation were always larger than those of the Mn extraction. This is consistent with the view that Mn atoms function as a cluster, so that partial release of the Mn atoms results in a total inactivation of oxygen evolution. Similar trends were reported with PSII membranes from which Mn had been extracted by various treatments (8). In most of the cases, a nearly complete inactivation occurs when the Mn/PSII ratio is reduced to 2.0 (8, 15). However, there was still significant activity in PSII membranes which lost more than half of the bound Mn atoms. This suggests that the cold and dark storage of leaves affect

Table III. *Relative Abundances of the Three Extrinsic Proteins in Thylakoids and PSII Membranes from Cucumber Leaves Treated at 0°C in the Dark*

Peak areas of the three proteins in the densitograms were determined with the 47 kD protein as a reference. Amounts of the 14 kD protein were corrected for the protein comigrated. Abundance of each protein in untreated samples was taken as 100%.

Proteins	Duration of Storage		
	0	24	48
kD		*h*	
Thylakoids			
31	100	116	113
20	100	119	112
14	100	108	100
PSII membranes			
31	100	91	84
20	100	33	15
14	100	16	0

the PSII electron transport not so homogeneously as the Mn-extracting treatments of isolated PSII membranes do.

Interestingly, PSII membranes from the dark, chilling-treated leaves were found to be largely depleted of the two proteins of 20 and 14 kD, which correspond to the extrinsic 23 and 17 kD proteins of spinach PSII. Depletion of the two extrinsic proteins, together with solubilization of Mn, have also been reported in PSII membranes from wheat leaves grown under intermittent flash illumination (17). The absence of the two proteins in PSII membranes from dark, chilling-treated cucumber leaves cannot be ascribed to proteolytic digestion of the proteins during the treatment because the two proteins were still present in the thylakoid membranes isolated from treated leaves. The results show that the proteins are dissociated from the inner surface of the thylakoid membranes but trapped in the lumen space of the thylakoid vesicles. Thus, the inactivation of oxygen evolution is at least partly ascribed to the dissociation of the two proteins. Removal of the two proteins has been shown to make the Mn cluster unstable in the presence of exogenous reductants (7). Thus, dissociation of the two proteins would promote solubilization of Mn, if the cold and dark storage of detached cucumber leaves accumulates endogenous reductants, such as ascorbic acid, in chloroplasts.

A question remains as to why the two extrinsic proteins are solubilized during the dark storage of cucumber leaves at 0°C. Degrees of the chilling sensitivity of different plant species are considered to depend on the temperature regions in which phase transition or phase separation of the membrane lipids occurs. Therefore, there is a possibility that the two proteins are dissociated as a result of changes in the physical state of membrane lipids during the prolonged treatment at 0°C. It has been suggested that the oxygen-evolving activity is inactivated by free fatty acids, in particular linolenic acid (5), which accumulates in leaves kept at a low temperature in the dark (9). An alternative, therefore, is that solubilization of the two proteins is a consequence of interactions of free fatty acids with the thylakoid membranes. However, the oxygen-evolving activity of chloroplasts isolated from chilling-sensitive tomato leaves were reported to be more resistant to the dark, chilling-treatment than were intact leaves (11). This seems to favor the view that damage at some other cellular site (such as vacuole) is responsible for the observed inhibition of oxygen evolution. Recently, we found that the two extrinsic proteins are released when PSII membranes are exposed to an acidic pH (our published data). Thus, the proteins may be solubilized if the internal space of the thylakoid membranes is acidified during the dark, chilling-treatment of leaves.

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