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Heat inactivation of oxygen evolution in Photosystem II particles and its acceleration by chloride depletion and exogenous manganese

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Heat inactivation of oxygen evolution by isolated Photosystem II particles was accelerated by Cl^- depletion and exogenous Mn^{2+}. Weak red light also accelerated heat inactivation. Heat treatment released the 33, 24 and 18 kDa proteins and Mn from the Photosystem II particles. The protein release was stimulated by Cl^- depletion and exogenous Mn^{2+}, and the Mn release was also stimulated by Cl^- depletion. A 50% loss of Mn corresponded to full inactivation of oxygen evolution, whereas no direct correlation seemed to exist between the loss of any one protein and inactivation of oxygen evolution. Removal of the 24 and 18 kDa proteins from photosystem II particles only slightly decreased the heat stability of oxygen evolution.

Introduction

It has long been recognized that oxygen evolution is one of the most heat-sensitive processes in photosynthesis [1–3]. One direct result of heat inactivation is the release of functional Mn from PS II [4–6]. However, little is known about any heat-induced release of protein components. A major difficulty in such studies is that the oxygen-evolving site is located at the inner surface of the thylakoids, and hence any proteins released would not be exposed to the outside medium. With the advent of methods to prepare membrane particles enriched in PS II, where the oxygen-evolving complex is no longer retained within membrane vesicles, it is now possible to address this question more fully.

Here we describe the effect of Cl^- on the heat inactivation of oxygen evolution by isolated PS II particles, and the concomitant release of proteins and Mn from the particles. We also describe the acceleration of heat inactivation by exogenous Mn^{2+}.

Materials and Methods

PS II particles were prepared from spinach chloroplasts with Triton X-100 as described previously [7] and stored in liquid nitrogen in the presence of 30% (v/v) ethylene glycol [8]. In some cases, the particles were washed three times with 300 mM sucrose, 10 mM NaCl and 25 mM Mes-NaOH (pH 6.5) by centrifugation at 35 000 × *g* for 10 min and resuspension; these particles were designated Cl^--sufficient particles. In other cases the particles were washed three times with 300 mM sucrose and 25 mM Mes-NaOH (pH 6.5) in the same way as above; these particles were designated Cl^--deficient particles. Since no attempt was made to remove Cl^- from the reagents used, this medium contained about 0.1 mM Cl^-, which

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Abbreviations: Chl, chlorophyll; DCIP, 2,6-dichlorophenol indophenol; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; Mes, 4-morpholineethanesulphonic acid, PS, Photosystem

was detected by a Cl^- electrode. Treatment with 1.0 M NaCl to remove the 24 and 18 kDa proteins, and with Tris to remove the 33, 24 and 18 kDa proteins, was performed as described previously [8].

Heat treatment was carried out for 5 min by placing a sample (0.3–1.0 ml) of the particle suspension (1.0 mg Chl/ml) in a thin-walled glass tube, wrapped in aluminium foil, in a heated water bath. Oxygen evolution with 0.3 mM phenyl-*p*-benzoquinone as an electron acceptor, or reduction of DCIP at 0.06 mM in the presence of 10 mM NH_2OH, was assayed at 25°C in a medium of 300 mM sucrose, 10 mM NaCl, 25 mM Mes-NaOH (pH 6.5) and 0.05% bovine serum albumin [7] immediately after heat treatment. Each assay mixture for oxygen evolution contained 12 μg Chl/ml.

To measure the release of proteins and Mn, the particles and medium were separated after heat treatment by filtration within 40 s through a 0.22 μm filter (Millex-GV, Millipore Corp.) in the case of protein release, or by centrifugation at about 30°C for 6 min at $15000 \times g$ in the case of Mn release. The filtrate was subjected to SDS-urea gel electrophoresis as described previously [9], and gels were recorded with a dual-wavelength TLC scanner (Shimadzu CS-910). For the quantification of protein release, the relative area on the densitograms was measured and compared with those of standards (Tris extract of the same preparation) run on the same gel. The pellet from centrifugation was subjected to flameless atomic absorption spectrometry [10] for quantification of the Mn which remained bound to the PS II particles after heat treatment. Chl was determined according to MacKinney [11].

Results

Effects of chloride and manganese ions on heat inactivation

The profile of heat inactivation of oxygen evolution is shown in Fig. 1. Half-inactivation occurred at 47°C in the presence of 10 mM NaCl during heat treatment, and 42°C in its absence. This suggests that Cl^- partially protects the oxygen-evolving complex from heat inactivation. A similar protective effect of Cl^- on heat inactivation of the PS II reaction has been observed in

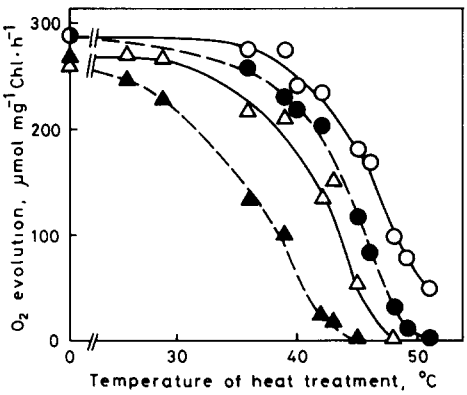

Fig. 1 Effects of Cl^--depletion and exogenous Mn^{2+} on heat inactivation of oxygen evolution of PS II particles. Cl^--sufficient particles (circles) or Cl^--deficient particles (triangles) were heated in the presence (closed symbols) or absence (open symbols) of 1 0 mM $Mn(CH_3CO_2)_2$. Oxygen evolution was assayed at 25°C in the presence of 10 mM NaCl

intact thylakoids [12]. The Cl^- requirement for maximum heat stability depended on pH of the medium; it was 2 mM at pH 6.5, and 20 mM at pH 7.6 (data not shown).

The heat inactivation of Cl^--sufficient particles was accelerated by 1.0 mM $Mn(CH_3CO_2)_2$: the temperature for half-inactivation was lowered by 2°C (Fig. 1). The half-inactivation temperature of Cl^--deficient particles in the presence of exogenous Mn^{2+} was lowered to 36°C (Fig. 1). This acceleration of heat inactivation was specific to Mn^{2+}, since the addition of 1.0 mM Mg^{2+} or Ca^{2+} during heat treatment displayed little or no effect (Table I). The addition of 1.0 mM $Mn(CH_3CO_2)_2$ to particles after heat treatment did not have any effect. Thus, the inhibition by exogenous Mn^{2+} depended on its presence during heat treatment. Hydroxylamine could almost completely restore the activity of DCIP reduction in the particles inactivated by heat treatment either in the presence or absence of Mn^{2+} (data not shown), indicating that the inhibition occurred solely at the water-oxidizing site.

Izawa and co-workers [13,14] reported that exogenous Mn^{2+} inhibited the Hill reaction of Cl^--deficient spinach thylakoids at the level of the water-oxidizing site, and that no inhibition was observed if 1.0 mM Cl^- was present. However, we found that the acceleration by exogenous Mn^{2+} of heat inactivation occurred both in the absence and

TABLE I

EFFECT OF CATIONS ON HEAT INACTIVATION

Cl^--sufficient and Cl^--deficient PS II particles were heated for 5 min at the temperatures shown in the presence of the salts indicated Oxygen evolution was measured in a medium containing 10 mM NaCl. The Cl^--sufficient and Cl^--deficient particles were obtained from separate PS II preparations

Type of particles	Heat treatment		Oxygen evolution	
	Temp. °C	Salt added (1.0 mM)	μmol/mg Chl per h	(Rel.)
Cl^--sufficient (10 mM NaCl)	4	–	222	(100)
	47	–	120	(54)
	47	$MgCl_2$	122	(55)
	47	$CaCl_2$	137	(62)
	47	$MnCl_2$	48	(22)
Cl^--deficient (no NaCl added)	4	–	328	(100)
	41	–	207	(63)
	41	$MgSO_4$	199	(61)
	41	$Ca(CH_3CO_2)_2$	189	(58)
	41	$Mn(CH_3CO_2)_2$	64	(20)

presence of Cl^- (Table I and Fig. 1).

We observed that light stimulated heat inactivation (Table II), with the effect being greater in the presence of Mn^{2+} than in its absence. A similar cooperative effect of light and Mn^{2+} was also observed in intact thylakoids by Muallem and Izawa [13]. The concentration of Mn^{2+} required for half-maximal effect was in the range 0.2–0.5 mM and did not appear to change greatly with the level of Cl^- present during heat treatment. Similarly, the Cl^- requirement for maximum heat stability did not vary much with the presence or absence of Mn^{2+} during heat treatment (data not shown).

Table III shows that there was some interaction between the effects of Cl^- deficiency and exogenous Mn^{2+}. When 10 mM NaCl was added to particles heated in the absence of Cl^- and Mn^{2+},

TABLE II

EFFECT OF WEAK RED LIGHT ON HEAT INACTIVATION

Cl^--deficient PS II particles were treated for 5 min at the temperatures shown in the presence of salts either in the dark, or light (645 nm, 5 W/m^2). Oxygen evolution was assayed in a medium containing 10 mM NaCl L and D represent light and dark conditions, respectively.

Heat treatment				Oxygen evolution	
Temp. (°C)	[NaCl] (mM)	[$Mn(CH_3CO_2)_2$] (mM)	L or D	μmol/mg Chl per h	(Rel.)
4	10	–	D	328	(100)
41	–	–	D	207	(63)
	–	–	L	175	(53)
	–	1 0	D	64	(19)
	–	1.0	L	21	(6)
45	10	–	D	223	(68)
	10	–	L	165	(50)
	10	1 0	D	126	(38)
	10	1.0	L	47	(14)

TABLE III

PARTIAL RECOVERY OF OXYGEN EVOLUTION BY ADDITION OF Cl^- TO Cl^--DEFICIENT HEAT-INACTIVATED PS II PARTICLES

Cl^--deficient PS II particles were treated for 5 min as shown, and oxygen evolution was assayed in the absence or presence of 10 mM NaCl (added during the assay). Numbers in parentheses represent relative activities of oxygen evolution.

Treatment		Oxygen evolution (μmol/mg Chl per h)	
Temp (°C)	Salt added during heat treatment	−NaCl	+10 mM NaCl
4	–	264 (82)	321 (100)
43	–	86 (27)	212 (66)
43	$Mn(CH_3CO_2)_2$ 1.0 mM	34 (11)	46 (14)
4	–	212 (74)	295 (100)
48	–	28 (10)	66 (22)

there was a considerable increase in the rate of oxygen evolution, well above that observed when 10 mM NaCl was added to unheated Cl^--deficient particles. That is, part of the heat inactivation of Cl^--deficient particles could be reversed by the addition of Cl^-. However, if the Cl^--deficient particles were heated in the presence of Mn^{2+}, this Cl^--induced recovery did not occur.

Release of proteins and Mn by heat treatment

The dependence of protein release on temperature of heat treatment is shown in Fig. 2. In particles heated without exogenous Mn^{2+}, the 33 and 24 kDa proteins were partially released, whereas the 18 kDa protein was not. Cl^- depletion stimulated heat-induced release of the 33 and 24 kDa proteins. In particles heated with exogenous Mn^{2+}, all three proteins were released. A protein of about 13 kDa was also released from the particles on heat treatment in the presence of Mn^{2+}, but the extent of this release appeared to be the same at all temperatures used. The release of the 24 and 18 kDa proteins, but not the 33 kDa protein, was stimulated by exogenous Mn^{2+}. In the presence of exogenous Mn^{2+}, the release of the three proteins was further stimulated by Cl^- depletion. Fig. 3 shows the relationship between oxygen-evolution activity and proteins remaining

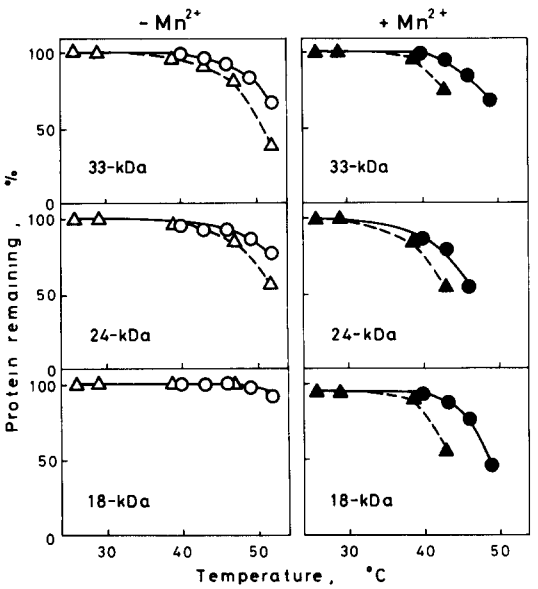

Fig. 2. Release of proteins after heat treatment of Cl^--sufficient and Cl^--deficient PS II particles. Symbols are the same as in Fig. 1 Other details are given in Materials and Methods

after heat treatments of Cl^--sufficient particles in the absence of exogenous Mn^{2+}. Similar plots were made for Cl^--sufficient particles with exogenous Mn^{2+} and for Cl^--deficient particles with and without exogenous Mn^{2+}. In all the cases, the loss of oxygen-evolution activity preceded the release of any one of the three proteins.

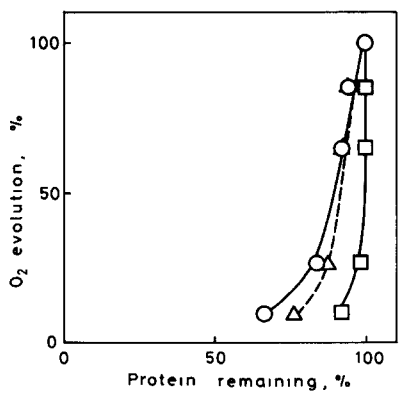

Fig 3. Relationship between oxygen-evolution activity and proteins remaining bound after heat treatment of Cl^--sufficient PS II particles without exogenous Mn^{2+} at various temperatures. Data are taken from Figs. 1 and 2 33 kDa protein (○———○); 24 kDa protein (△------△), 18 kDa protein (□———□).

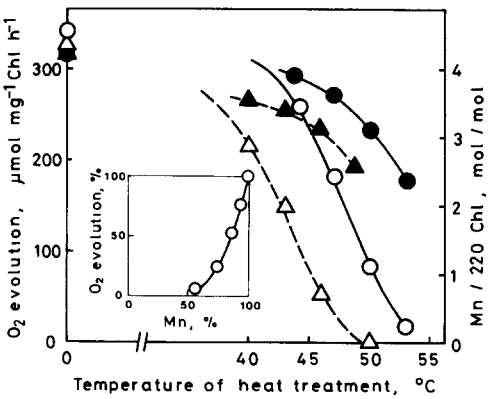

Fig. 4. Release of Mn after heat treatment of Cl^--sufficient and Cl^--deficient PS II particles. Mn remaining bound was assayed after heat treatment of Cl^--sufficient (●———●) and Cl^--deficient (▲---▲) PS II particles. Oxygen-evolution activity was assayed at 25°C in the presence of 10 mM NaCl after heat treatment of Cl^--sufficient (○———○) and Cl^--deficient (△---△) PS II particles. Inset, relationship between the oxygen-evolution activity and Mn remaining bound after heat treatment at various temperatures in Cl^--sufficient particles.

The dependence of Mn release on the temperature of the heat treatment is shown in Fig. 4. Cl^- depletion also stimulated the Mn release as the protein release. The relationship between the Mn remaining bound and the oxygen-evolution activity after heat treatment of Cl^--sufficient particles (inset of Fig. 4) indicates that the complete inactivation of oxygen evolution occurred with 50% loss of Mn. Since four Mn atoms exist in each oxygen-evolving complex accompanied by about 220 Chl molecules [15], this result suggests that loss of two of the four Mn atoms on heat treatment leads to complete inactivation of oxygen evolution.

Comparison of the present results on protein and Mn release with those previously reported shows some discrepancies. Yamamoto and Nishimura [16] reported that heat treatment at 50°C for 3 min of their PS II preparation caused the release of 98% of the 33 kDa protein and 80% of the Mn, whereas only 28% of the 24 and 22% of the 18 kDa proteins were lost under the same conditions. Franzén and Andréasson [17] reported that their preparation of deoxycholate-extracted thylakoid membranes lacking the 24 and 18 kDa proteins lost about 80% of the 33 kDa protein and about one-fourth of Mn when it was heated at 55°C for 3 min. The reason for these discrepancies between our results and those of other groups is not clear, but they may have originated from the different membrane structures of the PS II preparations.

TABLE IV

EFFECT OF REMOVAL OF 24 AND 18 kDa PROTEINS ON HEAT INACTIVATION

PS II particles depleted of the 24 and 18 kDa proteins (NaCl-treated particles) were prepared by treating Cl^--sufficient particles with 1.0 M NaCl at 0°C for 30 min [8]. The control consisted of Cl^--sufficient particles treated under the same conditions but in 10 mM NaCl instead of 1.0 M NaCl. Heat treatment was carried out at the given temperatures, and oxygen evolution was assayed in the presence of 10 mM NaCl. The numbers in parentheses represent the percentage of oxygen-evolution activity.

Type of particles	Oxygen evolution (μmol O_2/mg Chl per h)			
	Temp. (°C). 4	43	46	48
Control	262 (100)	193 (74)	175 (67)	120 (46)
NaCl-treated	134 (100)	80 (60)	72 (54)	48 (34)

Effect of removal of the 24 and 18 kDa proteins on heat inactivation

As heat treatment with exogenous Mn^{2+} caused a much greater loss of the 24 and 18 kDa proteins, we investigated whether the removal of these proteins would alter the heat stability of the particles or abolish the effect of exogenous Mn^{2+}. We treated PS II particles with 1.0 M NaCl to remove the 24 and 18 kDa proteins [8], and then compared their heat stability with that of an untreated sample (Table IV). Removal of the two proteins decreased, but not greatly, the heat stability of the particles. Exogenous Mn^{2+} still accelerated heat inactivation of the NaCl-treated particles (data not shown). Hence, exogenous Mn^{2+} does not exert its effect primarily by enhancing the removal of the 24 and 18 kDa proteins; this appears to be a secondary effect which may contribute partially to the decline in oxygen evolution.

Discussion

It has been recognized that Cl^- is a necessary factor for oxygen evolution in intact thylakoids [18–21] as well as in PS II particles [22,23]. The

Cl^- depletion in the thylakoids blocks the advancement of the S state of oxygen-evolving machinery from S_2 to S_3 [24,25]. This Cl^- effect may be related to the stimulation of heat inactivation of oxygen evolution by Cl^- depletion studied here. However, further studies are needed in order to understand the molecular mechanism for the effects of Cl^- depletion on the oxygen-evolving complex.

There are a number of parallels between our work with PS II particles and those of Izawa and co-workers [13,14] with Cl^--depleted thylakoids; the inhibition of oxygen evolution by treatment with exogenous Mn^{2+} and the exacerbation of this effect by weak red light. The major difference is that, in our results, exogenous Mn^{2+} stimulated the heat inactivation in Cl^--sufficient PS II particles, whereas unheated thylakoids required the absence of Cl^- for the effect to be observed [13]. This may suggest that heat treatment makes the particles appear functionally Cl^--deficient and hence renders the particles susceptible to attack by exogenous Mn^{2+}. In fact, early methods for Cl^- depletion of thylakoids sometimes required heating the thylakoids [18].

It has been reported that Mn^{2+} can act as a donor to PS II at concentrations as low as 12 μM [26]. Dilution of the heated samples for assay of oxygen evolution would leave this final concentration of Mn^{2+} in the assay medium. However, as there was no effect of adding Mn^{2+} after heat treatment, it is very unlikely that the released Mn^{2+} was acting as an electron donor in the heated particles. At present we can offer no explanation as to why exogenous Mn^{2+} should facilitate the release of the 24 and 18 kDa proteins. This may be partly an electrostatic effect, especially with the 18 kDa protein. Obviously this is an area worthy of further study.

Heat inactivation, and protein and Mn releases, were all stimulated by Cl^- depletion, suggesting that the protein and Mn releases are related to heat inactivation of oxygen evolution. Our previous study on the stoichiometry of components in PS II particles [15] showed that there are four Mn atoms and one molecule each of the 33, 24 and 18 kDa proteins per oxygen-evolving complex. In either urea-treated [10], or (urea + NaCl)-treated PS II particles [27], oxygen-evolution activity is lost when two of the four Mn atoms are released from the particles. These results are very similar to those of the heat treatment of PS II particles in the present study, which inactivated oxygen evolution with concomitant loss of the two Mn atoms. Therefore, we conclude that the loss of two Mn atoms from the oxygen-evolving complex is the major action of the heat inactivation of oxygen evolution in PS II particles. The proportion of protein release, on the other hand, was always smaller than that of the activity loss (Fig. 3). This suggests that the partial protein release may contribute, in part, to the heat inactivation and may have made the relationship between the Mn release and oxygen-evolution activity (Fig. 4, inset) a curved, rather than straight, line.

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