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STIMULATION BY MANGANESE AND OTHER DIVALENT CATIONS OF THE ELECTRON DONATION REACTIONS OF PHOTOSYSTEM II

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Using inside-out thylakoid membranes, it has been shown that the oxidation of water and associated reduction of dichlorophenol indophenol is partially inhibited by low concentrations of cation chelators. This inhibition correlates with a removal of two manganese ions per Photosystem II reaction centre. The chelator-induced inhibition was completely reversed by the addition of low levels of Mn^{2+} ($C_{1/2} \approx 20$ μM) and higher levels of Mg^{2+} and Ca^{2+} ($C_{1/2} \approx 1$ mM). Other cations were not effective, indicating that the ability to overcome the inhibition did not involve a general electrostatic screening process. The degree of inhibition by chelators was greater at lower light intensities and after treatment with glutaraldehyde. In the presence of glutaraldehyde the stimulatory effect of Mn^{2+} was lost, while pretreatment with Mn^{2+} prevented the glutaraldehyde effect. These results are discussed in terms of conformational changes of the electron donation chains involving cation- (preferentially Mn-) dependent coupling between the oxygen evolving and reaction-centre complexes of Photosystem II.

Introduction

The water-plastoquinone oxidoreductase activity of Photosystem II seems to involve the cooperative interaction of three main electron-transfer protein complexes [1]: (i) A reaction centre associated with an antenna pigment system, in which absorbed light is used to drive a charge separation between the P-680 chlorophyll and a specialized plastoquinone designated Q_A [2]. (ii) An oxygen-evolving complex, catalysing the reduction of the photooxidised P-680 by oxidising water [3,4]. This complex, which is thought to contain manganese, is able to exist in several different oxidation states [5]. (iii) A complex which permits the oxidation of the photo-reduced Q_A by transferring reducing equivalents to the plastoquinone pool [6]. Associated with this complex is a 32 kDa polypeptide which binds herbicides such as DCMU [7,8].

Whether these three enzyme complexes are integrated into an individual macromolecular unit, or whether they exist as interactive but physically separate entities is unknown. Although it is often assumed that the oxygen-evolving complex forms a part of the reaction centre system [9], there is evidence that more than one reaction centre may cooperate with a single oxygen-evolving complex [10,11], thus indicating a less rigid organization.

The study of the electron donation reactions of PS II has gained impetus from the recent introduction of procedures enabling the isolation of insideout thylakoid vesicles in which the oxygen-evolving complex is exposed on the outer membrane surface [12]. This type of preparation has

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Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DCIP, dichlorophenol indophenol; EGTA, ethylenebis(oxyethylenenitrilo)tetra-acetic acid; PS II, Photosystem II; P680, reaction centre chlorophyll of Photosystem II.

been used in experiments where the inhibition of oxygen evolution has been correlated with the removal of proteins from the membrane [13,14]. Such work has been interpreted to indicate a functional role for extrinsic proteins, electrostatically attracted to membrane proteins associated with the donor side of PS II. Recently, we have presented evidence which suggests that the oxygen-evolving complexes may become dissociated from their reaction centres under conditions where the donor electron-transfer chains are partially inhibited [15].

In this paper we continue to use inside-out thylakoid vesicles to extend our investigation of factors which modify the electron donation reactions of PS II.

Materials and Methods

Preparation of chloroplasts and inside-out thylakoid vesicles

Chloroplasts were obtained from leaves of *Pisum sativum* (Feltham First), harvested after 14 days growth in vermiculite under greenhouse conditions with a 16 h daily photoperiod [16]. The isolated thylakoid membranes were washed once in 50 mM sucrose/10 mM NaCl/50 mM phosphate buffer (pH 7.4). Inside-out thylakoid vesicles were prepared using the procedure described by Andersson and Åkerlund [12], and were routinely suspended in a low-salt buffer medium comprising 150 mM sucrose/5 mM NaCl/10 mM phosphate buffer (pH 7.4) [17]. Chlorophyll concentrations were determined in 80% acetone [18]. For some experiments, the vesicles were suspended in the low-salt buffer supplemented with 2 mM EGTA. After a 10 min incubation at 19°C, the vesicles were pelleted by centrifugation and resuspended in the low-salt buffer medium devoid of chelator.

Assay of PS II photochemical activity by monitoring the rate of DCIP reduction

Dichlorophenol indophenol (DCIP) reduction was monitored spectrophotometrically [19] using a Perkin-Elmer (model 557) dual wavelength spectrophotometer. The cuvette routinely contained 1 ml of the low-salt buffer medium, and membranes corresponding to a chlorophyll concentration of between 5 and 40 μg with 0.05 mM DCIP. Actinic light was provided by an Intralux 150H light source fitted with a 668 nm interference filter (Balzer B40). The 100% light intensity was 5 $J \cdot s^{-1} \cdot m^{-2}$ and, where appropriate, the intensity was attenuated by Balzer neutral density filters. Diphenylcarbazide was added as a 50 mM stock solution in dimethylsulfoxide and was prepared just prior to use. The computed rates of DCIP reduction were corrected for any dark reaction rate.

Assay of manganese content of thylakoid membranes

Thylakoid membranes were assayed for their Mn content using a Perkin-Elmer atomic absorption spectrophotometer (Model 2280) equipped with a flameless graphite furnace [14]. Aliquots of the membranes, suspended in 0.2% nitric acid at a concentration of 50 μg/ml, were atomized at 2100°C for 5 s, and the absorption change at 279.5 nm was measured.

Results

Table I shows the light-dependent reduction rates of DCIP, registered as an absorption decrease at 560–520 nm, by inside-out thylakoid vesicles. An attenuated DCIP reduction rate supported by water oxidation was obtained when the membranes were treated with the cation chelator EDTA, while a stimulated reduction rate was obtained when $MnCl_2$ was present. The location of

TABLE I

THE LIGHT-DEPENDENT RATE OF DCIP REDUCTION CATALYSED BY INSIDE-OUT THYLAKOID VESICLES: EFFECT OF EDTA AND $MnCl_2$

Uncoupled rates of DCIP reduction were measured as described in Materials and Methods, using a Δe 560–520 nm of 6.8 $mM^{-1} \cdot cm^{-1}$. Each figure represents the averaged DCIP reduction rate from eight preparations (±S.D.), normalised to the H_2O to the DCIP rate obtained in the presence of 2 mM $MnCl_2$.

	μmol DCIP/mg Chl per h	
	H_2O to DCIP	DPC to DCIP
No addition	35±5	49±4
+2 mM EDTA	24±3	54±4
+2 mM $MnCl_2$	50	51±3

TABLE II

THE INHIBITION OF OXYGEN-EVOLUTION AND THE RELEASE OF Mn FROM INSIDE-OUT THYLAKOID VESICLES WASHED WITH 2 mM EDTA

The vesicles were pretreated by incubation for 10 min at 19°C in the absence or presence of 2 mM EDTA. A stoichiometry of 120 chlorophyll *b* molecules per PS II reaction centre [34] has been assumed. Results ± S.D. ($n = 4$).

	O_2 evolution (μmol O_2/ mg Chl per h)	Mn/reaction centre
Control membranes	44 ± 3	3.8 ± 0.28
2 mM EDTA -washed membranes	29 ± 3	2.1 ± 0.4

the apparent cation effect can be identified by the action of diphenylcarbazide. As can be seen in Table I, we have found that DCIP reduction by diphenylcarbazide is unaffected by the various treatments, indicating that it is the functional activity of the oxygen-evolving complex which is modified by cation depletion. Moreover, Table I shows that the accelerated H_2O to DCIP electron transfer rate in membranes treated with $MnCl_2$ is similar to the maximal reduction flux attained when diphenylcarbazide is used as a PS II donor. Similar results were obtained when EDTA was replaced by EGTA. Table II shows that the inhibition of oxygen-evolution caused by pre-washing

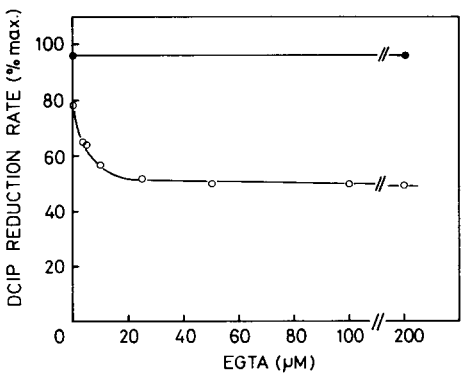

Fig. 1. The inhibition by EGTA of the DCIP reduction. The effect of EGTA on the H_2O to the DCIP rate is plotted as a percentage of the maximal rate measured in the presence of 0.5 mM diphenylcarbazide; (○——○) absence or (●——●) presence of 2 mM $MnCl_2$.

inside-out thylakoid vesicles with 2 mM EDTA is accompanied by the release of approximately 2 Mn/120 chlorophyll *b* from the membranes.

In Fig. 1 we give more information about the inhibitory effect of cation chelators. Addition of EGTA, at micromolar concentrations, leads to an increased inhibition; the half-maximal inhibition concentration is 5 μM. We observed, however, that the EGTA did not result in a complete inhibition, and at saturating concentrations of chelator approx. 50% of the DCIP reduction rate remained. The effect of micromolar concentrations of EGTA was completely reversed by the addition of 2 mM $MnCl_2$.

The partial inhibition of the H_2O to DCIP reduction rate in EDTA-treated inside-out thylakoid vesicles, shown in Table I, can be reversed by divalent cations other than Mn^{2+} (Table III). We found that mM concentrations of Mg^{2+} and Ca^{2+} ($C_{1/2}$ 1 mM) are effective but that other divalent cations, such as Cu^{2+} and Hg^{2+}, are potent inhibitors of oxygen evolution (see Table III). The addition of monovalent or trivalent cations, at concentrations sufficient to cause the screening of membrane negative charges, have little effect on the DCIP reduction rate. Moreover,

TABLE III

THE SELECTIVE ABILITY OF CATIONS TO STIMULATE THE DCIP REDUCTION RATE IN EDTA-WASHED INSIDE-OUT THYLAKOID VESICLES

Vesicles prewashed with 2 mM EDTA as described in Materials and Methods. $(DM)Br_2$ = decamethonium bromide; $(TEC)Cl_3$ = tris(ethylenediamine) cobalt(III) chloride.

	H_2O to DCIP (μmol DCIP/mg Chl per h)
No addition	17.1
+ 150 mM NaCl	18.7
+ 4 mM $(DM)Br_2$	18.7
+ 0.5 mM $(TEC)Cl_3$	17.1
+ 4 mM $MnCl_2$	33.6
+ 4 mM $MgCl_2$	30
+ 4 mM $CaCl_2$	31.4
+ 4 mM $SrCl_2$	21.8
+ 4 mM $BaCl_2$	18.9
+ 4 mM $CuCl_2$	6.2
+ 4 mM $HgCl_2$	2.6

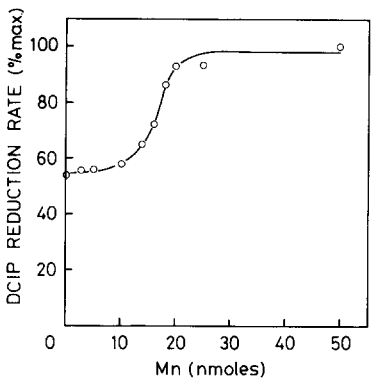

Fig. 2. The stimulation by $MnCl_2$ of the DCIP reduction role in EDTA-washed inside-out thylakoid vesicles. 4 mM decamethonium bromide was added to the suspension of vesicles to ensure that the membrane surface charges were screened. The effect of $MnCl_2$ on the percentage difference between the H_2O and DPC to DCIP reduction rates is shown.

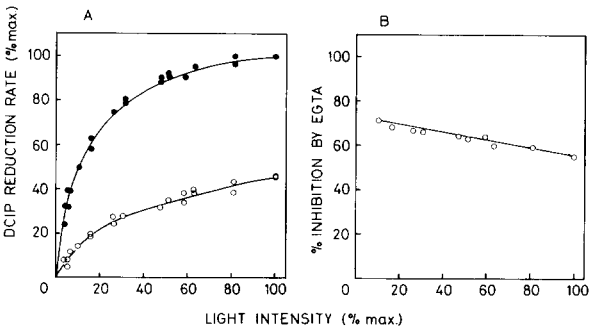

Fig. 3. Light intensity-dependent DCIP reduction rate. (A) The DCIP reduction rates obtained in the presence of either 2 mM EGTA (○———○) or 2 mM $MnCl_2$ (●———●). 100% maximal rate corresponded to 45 μmol DCIP/mg Chl per h at a light intensity of 5 $J \cdot m^{-2} \cdot s^{-1}$. (B) Plot of the extent of inhibition by EGTA. The line represents the percentage difference between the two light-saturation curves and the points, which represent the manipulated data, indicate the degree of error.

as Table III shows large divalent cations, such as the decamethonium cation, are also ineffective in promoting PS II photochemical activity.

In Fig. 2 we show that Mn^{2+} is effective in stimulating the H_2O to DCIP rate at 10–20 μM concentrations. As for Table III, the membranes were pre-washed with 2 mM EDTA, and 2 mM decamethonium bromide was added to the suspension to ensure that any effect due to changes in surface charge was negated. The main feature of the manganese titration shown in Fig. 2 is the sigmoidal nature of the curve, indicating a cooperativity of the Mn^{2+} binding sites. An analysis of the curve (data not shown) suggest that there are possibly two Mn^{2+} binding sites per oxygen-evolving complex.

The above data show that at high concentrations of EGTA, there remains a measurable rate of H_2O to DCIP electron transfer. We have investigated this feature further by monitoring the extent of inhibition of EGTA with decreasing intensity of the actinic illumination. From this experiment, we found that the degree of inhibition by EGTA increases as the light intensity is lowered. This is shown in Fig. 3A, where the rates of H_2O to DCIP electron transfer in the presence of either 2 mM Mn^{2+} or 2 mM EGTA at different light intensities are compared. As Fig. 3B shows, the degree of inhibition by EGTA varies from 55% at saturating light intensities to approx. 70% at a light intensity where the measured electron transfer rate for uninhibited membranes was half-maximal.

In Table IV we show the effect on the DCIP reduction rate of incubating the inverted thylakoid membranes with glutaraldehyde. Comparison of columns A and B show that glutaraldehyde had no

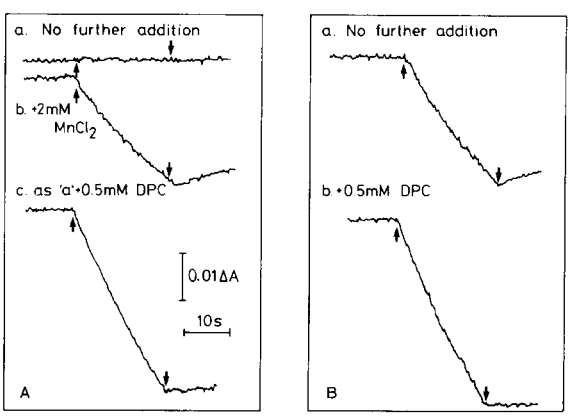

Fig. 4. The effect of trypsin on the DCIP reduction rate. Inside-out thylakoid vesicles were suspended in a low-salt buffer medium either comprising 2 mM decamethonium bromide and 0.5 mM EGTA (A) or 2 mM $MnCl_2$ (B). The digestion of the membranes was allowed to proceed for 5 min at 19°C with 50 μgs trypsin before the cessation of the reaction by the addition of 250 μgs trypsin inhibitor. Assay of PS II activity as for Table I with the additions as shown.

TABLE IV

THE EFFECT OF GLUTARALDEHYDE ON THE DCIP REDUCTION RATES

Inside-out thylakoid vesicles were incubated in the presence of either 1 mM EGTA or 2 mM $MnCl_2$ for 5 min before the addition of 0.25% glutaraldehyde. This amount of glutaraldehyde caused a 20% attenuation in the DPC to DCIP reduction rates. The rates shown in line 4 were obtained from vesicles supplemented with 2 mM $MnCl_2$ after glutaraldehyde and EGTA treatment.

	μmol DCIP/ mg chlorophyll per h	
	H_2O to DCIP	DPC to DCIP
A (no additions)	29.2	37.7
B (+2 mM $MnCl_2$)	39	39.8
C (+1 mM EGTA)	5.5	40
D (+1 mM EGTA, +2 mM $MnCl_2$)	7.5	40

effect on the ability of Mn^{2+} to stimulate the H_2O to DCIP rate to the maximal attained in the presence of diphenylcarbazide. However, membranes suspended in 1 mM EGTA and treated with glutaraldehyde results in an almost complete inhibition of the H_2O to DCIP electron transport without affecting the ability of diphenylcarbazide to donate to PS II. In contrast to Fig. 1, the inhibitory effect of EGTA cannot in this case be reversed by addition of $MnCl_2$.

Fig. 4A shows that in the presence of EGTA, trypsin digestion of the membranes (in the presence of 2 mM decamethonium bromide to ensure constant ionic strength) results in the complete abolition of the H_2O to DCIP electron transfer rate. The PS II photochemical activity under these conditions can, however, be partially restored by addition of $MnCl_2$ or the electron donor diphenylcarbazide. In contrast, membranes supplemented with $MnCl_2$ seem to be protected against trypsin attack (Fig. 4B). The action of $MnCl_2$ is not as an electron donor to the photo-oxidised P-680 chlorophyll, since we observe similar changes in light-induced oxygen release under the conditions of Fig. 4 (unpublished observations).

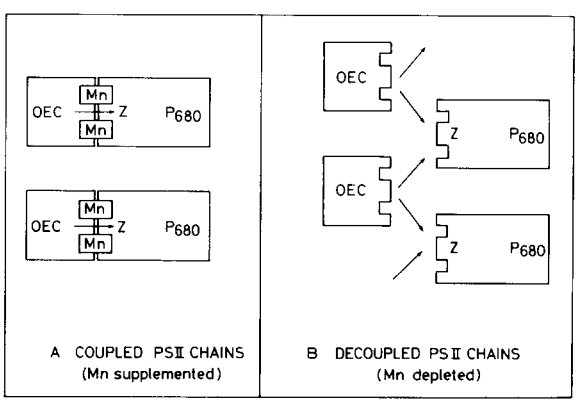

Fig. 5. Model of the donor electron-transfer pathway of PS II. The oxygen-evolving complexes (OEC) and the PS II reaction centres (containing the intermediary carrier Z and the P-680 chlorophyll) are envisaged either as being spatially inseparate (A) or spatially-distinct (B). It is presumed that the divalent cations, notably Mn^{2+}, act on the donor pathway by mediating the coupling of the two complexes. Diphenylcarbazide is believed to donate electrons to the intermediary carrier designated Z [33].

Discussion

A specific role for manganese in PS II photochemical activity is indicated from several lines of evidence [4,5,20]. However, there is as yet no convincing spectroscopic data to suggest that the oxygen-evolving complex can accommodate the oxidising equivalents of PS II turnovers by changes in the redox state of manganese [21]. In this paper, we report that the incubation of inside-out thylakoid vesicles with cation chelators, such as EDTA and EGTA, results in a partial inhibition of the oxygen-evolving process of PS II. That this inhibition is caused by the displacement of Mn is supported by the observed EGTA-induced loss of approx. 2 Mn/PS II reaction centre from the membrane and that the addition of μM concentration of $MnCl_2$ to the treated membranes fully restores the H_2O to DCIP electron transfer rate. The effect of the cation chelators is specific to inverted thylakoid membranes in which the electron donation pathway is exposed to the suspension buffer [14]. Under the conditions used in this report, unfractionated membranes catalyse a light-dependent DCPIP reduction rate which not only is insensitive to the cation chelators (but see Ref. 25) but which is also unaffected by the addition of diphenylcarbazide (unpublished observations).

As shown in Table III, both Mg^{2+} and Ca^{2+},

albeit at higher concentrations ($C_{1/2} \approx 1$ mM), can substitute for Mn^{2+} in stimulating the H_2O to DCIP electron-transfer rate in treated membranes. Since this stimulation was not observed with other cations, it is unlikely that a general electrostatic screening of membrane surface charge is involved. Moreover, the inhibitory action of Cu^{2+} and Hg^{2+}, which have similar ionic radii to Mn^{2+}, emphasises the cation specificity of the stimulation. From an analysis of the sigmoidal Mn^{2+} concentration curve (Fig. 2), it seems probably that there is a requirement for two Mn atoms to bind in order to achieve maximal H_2O to DCIP activity. These findings have some similarities to the recent studies of Klimov et al. [22] and Ono and Inoue [23], although in their work they did not measure the ability of diphenylcarbazide to restore PS II activity to determine the location of the inhibitory site.

We interpret the results of this paper as indicating that the action of cation chelators is to modify a site involved in optimising the structural organization of the electron donation pathway of PS II (Fig. 5). The placement of the electron donor, designated Z, on the PS II reaction centre complex [24] accounts for the resistance of the diphenylcarbazide donation reaction to the various treatments used in our work [15,17]. We envisage that the displacement of the divalent cations from their binding sites results in the decoupling of the oxygen-evolving complex from its reaction centre as depicted in Fig. 5A. The inability of the chelators to inhibit fully the H_2O oxidation rate indicates that, under these conditions, there are sufficient interactions between the complexes to maintain a reduced number of complete electron transport chains. Such an interaction is favoured at high light intensities where the postulated diffusion-limited step becomes less apparent as the density of photo-oxidised P-680 is increased [15]. This argument is supported by the observed ability of glutaraldehyde, not only to enhance the inhibition by EGTA of the H_2O to the DCIP rate but also to prevent the Mn^{2+}-induced re-organization which, in untreated membranes, can overcome the action of the chelator. In contrast, there is no effect of glutaraldehyde on the electron donation rate when Mn^{2+} was present prior to fixation, indicating that an optimal organization of the chains already existed (Fig. 5B). This concept that the donor electron-transport chains may become dissociated into individual complexes under cation-depleted conditions gains support from the observations of Bose et al. [25] who showed that the addition of 5 mM $MgCl_2$ to chloroplasts suspended in low-salt conditions caused a 2-fold increase in the flash-induced yield of oxygen evolution. Furthermore, the stimulation in the O_2 yield by Mg^{2+} was blocked by the pretreatment of the membranes with glutaraldehyde [25]. Several other reports have shown that the electron donation reactions of PS II can be inhibited by the chemical modification of chloroplasts. Some of the treatments used, such as iodination [26] and trypsin digestion [27], are specific to the outer membrane surface, indicating that the oxygen-evolving complex may span the thylakoid membrane. The results have been discussed in terms of a change in the structural organization of the membrane, and this interpretation is strengthened by the observation that pretreatment of the membranes with glutaraldehyde prevents the inhibition by lactoperoxidase [26]. In addition, Dilley and colleagues have shown, using dazoniumbenzenesulfonic acid, that light-dependent conformational changes occur at the level of the oxygen-evolving complex [28]. Fig. 4 shows that trypsin can inhibit the H_2O to DCIP electron-transfer rate in membranes treated with cation chelators without altering the PS II photochemical activity in the presence of diphenylcarbazide. Earlier work has already noted that trypsin digestion of inside-out thylakoid membranes results in the specific degradation of the 34 kDa polypeptide [29]. Mutant studies by Bishop and colleagues [30,31] have indicated a correlation between this polypeptide, Mn-binding and PS II activity. Our observations, therefore, that the addition of $MnCl_2$ to trypsin-digested membranes can partially restore the H_2O to DCIP electron transfer rate may question the role of the 34 kDa polypeptide in the PS II electron-donation pathway.

In this study we have discussed our results in terms of a diffusion-limited step involving the interaction of an oxygen-evolving complex with its reaction centre. The coupling of the two complexes is mediated by divalent cations, and is highly specific for Mn^{2+}. We are presently involved in determining whether such a diffusion-limited step

may have a functional role in untreated membrane preparations; for example, can the coupling between the oxygen-evolving complex and the PS II reaction centre change upon different S-state transitions?

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