Copper Ion Inhibition of Electron Transport Activity in Sodium Chloride Washed Photosystem II Particle Is Partially Prevented by Calcium Ion

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The inhibitory effects of copper ion (Cu^{2+}) on the photosynthetic electron transport function was investigated both in NaCl washed (depleted in 17 and 23 kDa polypeptides) and native (unwashed) photosystem II membrane preparations from spinach (*Beta vulgaris*) chloroplasts. Copper in the range of 2.0 to 15 μM strongly inhibited the electron flow from water to 2,6-dichlorobenzoquinone in NaCl washed particles in a concentration dependent manner. Complete inhibition was noticed at 15 μM Cu^{2+}. Oppositely in native membranes, 15 μM Cu^{2+} inhibited only 10–12% of control activity. It was found that calcium ion (Ca^{2+}) significantly reduced the Cu^{2+} inhibition of electron transport activity. The Ca^{2+} supported prevention of Cu^{2+} toxicity was specific to Ca^{2+}. Further analysis indicated that both Cu^{2+} and Ca^{2+} act competitively. Since Ca^{2+} is known to have stimulating/stabilizing effect at the donor side of photosystem II, it is therefore suggested that Cu^{2+} in NaCl washed particles exerts its inhibitory effect(s) at the oxidizing side of photosystem II; most possibly at the site where Ca^{2+} stimulates/stabilizes the oxygen evolution.

Introduction

Copper (Cu^{2+}) acts as a cofactor of plastocyanin; a redox component of photosynthetic electron transport chain. The cation also exhibits inhibitory effects on the electron transport function of higher plant (Mohanty *et al.,* 1989; Hsu and Lee, 1988), green algae (Samson *et al.,* 1988) and cyanobacterial (Singh and Singh, 1987) photosynthetic membranes. In photosystem (PS) II, the presence of Cu^{2+} inhibitory site at the oxidizing side of PS II; more precisely at Q_B has been proposed (Mohanty *et al.,* 1989). The ion has shown to impair the function of pheophytin – Q_A – Fe domain (Yruela *et al.,* 1991). Recently Cu^{2+} mediated impairment of electron flow form Tyr_z^- to P_{680}^+ has been more elaborately studied (Schroder *et al.,* 1994). From flash-induced absorption spectroscopic analysis it has been concluded (Schroder *et al.,* 1994) that Cu^{2+} does not effect the normal charge separation (P_{680}^+/Q_A^-) but specifically modifies the Tyr_z residue function such that the electron flow from Tyr_z^- to P_{680}^+ is blocked.

Calcium has been implicated as a co-factor for both advancement of S-state of manganese in the water oxidation complex and the reduction of P_{680}^+ by Tyr_z^- (Boussac *et al.,* 1992; Ono and Inoue, 1989). The ion (Ca^{2+}) facilitates electron flow from H_2O to Tyr_z^+ in higher plant chloroplasts (Ghanotakis *et al.,* 1984a) and from Tyr_z^- to P_{680}^+ in cyanobacterial electron transport system (Satoh and Katoh, 1985). Since both Cu^{2+} (Schroder *et al.,* 1994) and Ca^{2+} (Ono and Inoue, 1989; Ghanotakis *et al.,* 1984a) show their opposite [inhibitory (Cu^{2+}) and stimulatory (Ca^{2+})] effects on the oxidizing side of PS II, the effect of Ca^{2+} on the inhibitory action of Cu^{2+} in the electron transport activity has been studied in NaCl washed PS II particles in this present investigation. It should be mentioned that high salt (NaCl) washing renders the particles free of 17 and 23 kDa extrinsic polypeptides and the oxygen evolution activity attenuates (Kuwabara and Murata, 1983). These particles show a specific Ca^{2+} dependent restoration of sup-

Abbreviations: BIS-TRIS, (bis[2-hydroxyethyl]imino-tris [hydroxymethyl] methane); DCBQ, 2,6-dichlorobenzoquinone; DMSO, dimethylsulfoxide; MES, 2-(N-morpholino) ethanesulfonic acid; PD, *p*-phenylenediamine; P_{680}, photosystem II reaction center chlorophyll; Q_A/Q_B, primary and secondary quinone electron acceptors of photosystem II; TMPD, N,N,N′,N′, tetramethyl-*p*-phenylenediamine; TRIS, (tris[hydroxymethyl] aminomethane); Tyr_z, tyrosine residue acting as a donor to P_{680}^+.

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pressed oxygen evolution activity (Ghanotakis *et al.*, 1984 a). The results shown here indicate that in NaCl washed PS II particles the extent of Cu^{2+} mediated inhibition of electron flow from water to DCBQ is significantly reduced by Ca^{2+}. The results also suggest the presence of multiple sites of Cu^{2+} inhibition in the electron transport chain and one of these sites is Ca^{2+} sensitive. The Ca^{2+} sensitive Cu^{2+} inhibition become more prominent in NaCl washed PS II membranes.

Materials and Methods

Isolation of chloroplasts and preparation of PS II particles

The oxygen evolving PS II particles from spinach were prepared following the procedure as outlined earlier (van Leeuwen *et al.*, 1991) with slight modification. BIS-TRIS buffer used in the suspending medium of the original procedure was replaced by 20 mM MES-NaOH (pH 6.5). The particles were washed twice in the isolation buffer to remove triton-x 100 and untiluse they were kept frozen in the liquid nitrogen in presence of 5% DMSO. The chlorophyll (Chl) was estimated following Porra *et al.* (1989). The particles prepared following the above procedure had Chl *a/b* ratio of 2.79 as against 4.38 in chloroplasts. The low temperature (77 K) fluorescence emission peak at 735 (F_{735}) in chloroplasts was however, missing in PS II particles (data not shown).

Preparation of NaCl washed PS II particles for copper inhibition and calcium activation study

PS II particles were washed in medium containing 100 mM sucrose, 5 mM $MgCl_2$, 1.5 M NaCl and 5 mM MES (pH 6.5 adjusted with solid TRIS base). The washing was done twice by suspending the particles (500 μg Chl) in 10 ml of the above medium for 10 min at 4 °C in dark under continuous stirring and the washed particles were collected by 40,000 g centrifugation for 25 min. The 1.5 M NaCl washed (high salt washed) PS II particles were suspended in a medium containing 100 mM sucrose, 5 mM $MgCl_2$, 10 mM NaCl and 20 mM MES-NaOH (pH 6.5).

Inhibition with copper

Copper sulphate ($CuSO_4$, $5H_2O$) solution was used as the source of Cu^{2+}. Copper was added to the reaction mixture in dark. No significant difference in the extent of inhibiton was marked upon dark incubation (~ 10 min) of PS II particles with copper sulphate than when added in dark immediately before the assay.

Electron transport measurement

Electron transport activity was measured in terms of oxygen evolution in a Clark-type oxygen electrode. The reaction mixture in 1 ml contained; 100 mM sucrose, 10 mM NaCl, 5 mM $MgCl_2$ and 20 mM MES–NaOH (pH 6.5). Fresh ethanolic solution of DCBQ was used at rate saturating concentration (400 μM) as electron acceptor. Care was taken not to exceed the ethanol concentration more than 1% in the reaction mixture. All assays were carried out at 25 °C under rate saturating light intensity (1500 $\mu E\ m^{-2}\ s^{-1}$). The Chl concentration in the reaction mixture was maintained at 10 $\mu g\ ml^{-1}$. As when required, Ca^{2+} was added to the reaction mixture from 1 M stock solution of $CaCl_2$.

Result and Discussion

The PS II particles, prior to high salt washing, exhibited oxygen evolution activity of about 650 to 680 μmol oxygen $(mgChl)^{-1}\ h^{-1}$. Salt washing induced nearly 60–70% loss of this activity. The suppressed activity was restored nearly to 65–70% of control activity upon exogenous addition of $CaCl_2$ or $Ca(NO_3)_2$. Other salts like $MgCl_2$ or NaCl or KCl were not effective in this process (Table I). These observations provide evidence that the suppressed oxygen evolution activity due

Table I. Divalent and monovalent cation dependent restoration of oxygen evolution activity in NaCl washed PS II membranes. The PS II membranes were washed in high concentrations of NaCl. The ± values denote the SD obtained from three different observations.

Addition	Concentration [mM]	Electron transport rate μmol O_2 evolved $(mgChl)^{-1}\ h^{-1}$ $H_2O \rightarrow$ DCBQ
None	–	650 ± 15
NaCl washed (w)	–	260 ± 09
(w) + $CaCl_2$	20	490 ± 17
(w) + $Ca(NO_3)_2$	20	472 ± 19
(w) + $MgCl_2$	20	245 ± 20
(w) + NaCl	40	230 ± 21
(w) + KCl	40	237 ± 19

to salt washing can be specificially restored by Ca^{2+} (see also Ghanotakis *et al.*, 1984b).

In order to study the effect of Ca^{2+} on the inhibitory action of Cu^{2+}, the high salt washed particles were further examined for Cu^{2+} sensitivity in presence and absence of Ca^{2+}. The Cu^{2+} dependent inhibition of the fraction of oxygen evolution rate (E_{cu}) was calculated from the equation: [E_{cu} = (rate without copper) – (rate with copper) / (rate without copper)]. Fig. 1 depicts the inhibitory effects of varied micromolar concentrations of Cu^{2+} on the oxygen evolution activity of salt washed PS II particles in presence and absence of 20 mM $CaCl_2$. Copper addition severely inhibited the rate of oxygen evolution during the electron flow from water to DCBQ. Complete inhibition took place at about 15 μM $CuSO_4$ (Fig. 1 solid line). However, nearly 70–75% of Cu^{2+} mediated inhibition of PS II catalyzed electron transport activity in NaCl washed particles was restored on addition of $CaCl_2$ (Fig. 1 dotted line). However, Ca^{2+} found to protect significantly the high Cu^{2+} concentration (>5 μM) dependent impairment of electron flow as compared to low (<5 μM) concentration.

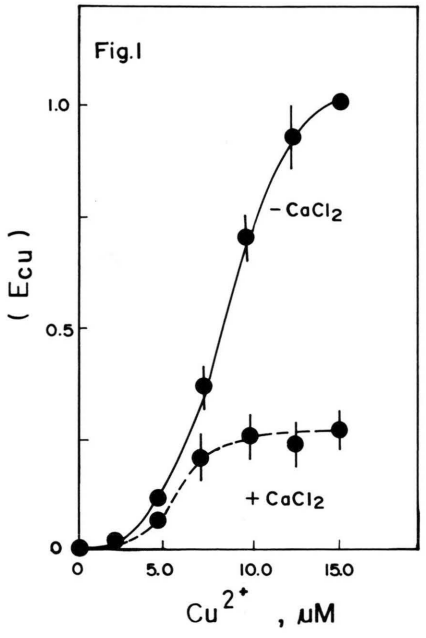

Fig. 1. Effect of different concentrations of Cu^{2+} on the fraction (E_{cu}) of oxygen (O_2) evolution activity during electron flow from water to DCBQ in NaCl washed PS II particles in absence (solid line) and in presence (dotted line) of 20 mM $CaCl_2$. The O_2 evolution activity of NaCl washed particles with and without $CaCl_2$ was normalized at 0 (zero) concentration of Cu^{2+}. The absolute electron transport activity of NaCl washed particles without and with added $CaCl_2$ were 270 and 500 (μmole O_2 evolved $(mgChl)^{-1}$ h^{-1}) respectively.

The concentration dependence of Ca^{2+} towards the attenuation of inhibition by Cu^{2+} in salt washed particles was evaluated (Fig. 2). In Fig. 2 it is shown that addition of Ca^{2+} in increasing millimolar concentrations stimulated the PS II activity in salt washed particles. Maximum stimulation was obtained with 5 mM $CaCl_2$ and the rate remained unchanged with further increase in Ca^{2+} concentration up to 20 mM (upper solid line). The electron transport activity of salt washed particles was reduced by about 90 to 95% on addition of 12.5 μM copper (Fig. 2 lower dotted line). However, the inhibition was gradually released on addition of increasing concentrations of $CaCl_2$. A maximum of 65 to 70% release of Cu^{2+} inhibited activity was noticed (Fig. 2, inset A). No other chloride salts of monovalent cations like Na^+ (20 mM) or K^+ (20 mM) or of divalent cations such as Mg^{2+} (20 mM) or Mn^{2+} (1 mM), but except Ca^{2+}, could able to prevent the Cu^{2+} inhibition (Table II).

The site of Ca^{2+} action in photosynthetic electron transport, as has been suggested, is largely confined to the water oxidation complex (Debus, 1992). Calcium ion has been shown to slow down the inhibitory action of NH_2OH (Mei and Yocum, 1991). The extent of inhibition by large reductants such as TMPD, hydroquinone, and PD on oxygen evolution activity specifically of 17 and 23 KDa polypeptide depleted (NaCl washed) particles has been shown to be attenuated both by Ca^{2+} and Cl^- (Tamura *et al.*, 1986; Ghanotakis *et al.*, 1984c; Mei and Yocum, 1992a and b). Inhibition of pho-

Table II. Extent of recovery of Cu^{2+} mediated inhibition of O_2 evolution activity in NaCl washed PS II particles with exogenous addition of different mono and divalent cations. The 100% activity for NaCl washed particle was about 260 μmol O_2 evolved $(mgChl)^{-1}$ h^{-1} which was about 40% of the control activity.

Addition	Concentration [mM]	Relative O_2 evolution
NaCl-washed (w)	–	100
(w) + Cu^{2+}	–	05
(w) + CaCl2	20	68
(w) + $Ca(NO_3)_2$	20	62
(w) + $MgCl_2$	20	07
(w) + $MnCl_2$	01	04
(w) + KCl	40	05

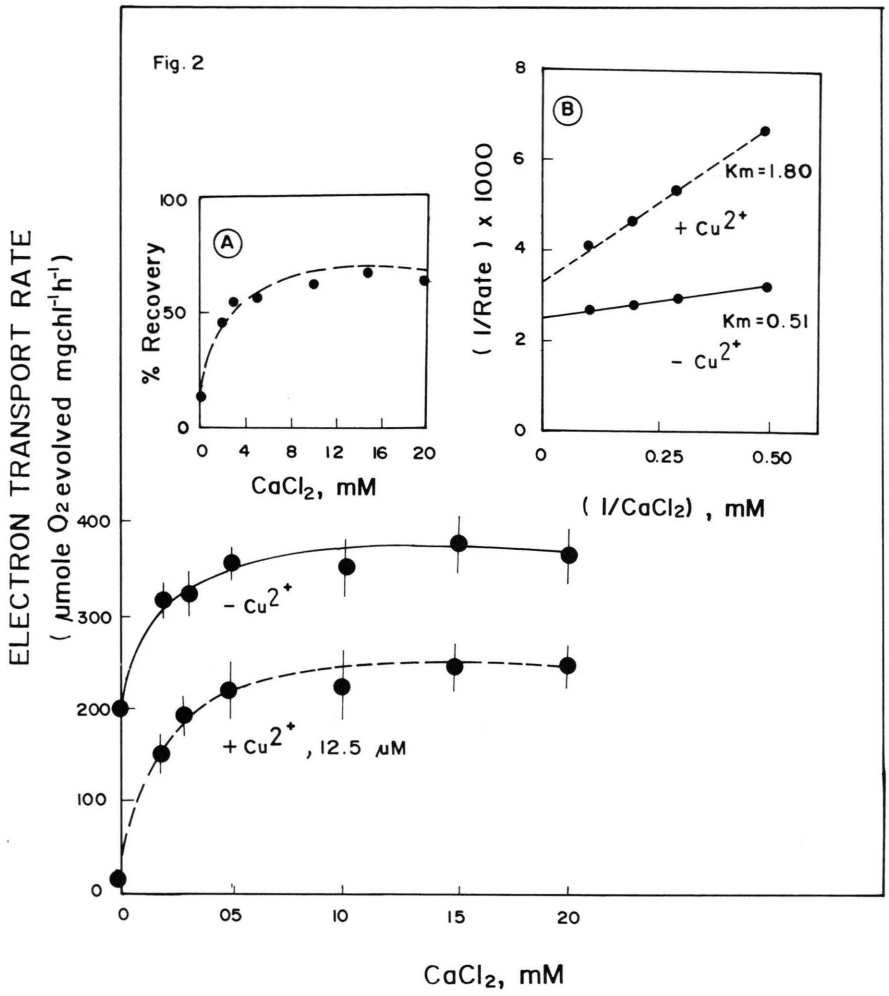

Fig. 2. DCBQ supported oxygen evolution activity of NaCl washed particles as a function of $CaCl_2$ concentration in presence (dotted line) and absence (solid line) of 12.5 µM Cu^{2+} the per cent recovery upon addition of Ca^{2+} has been shown in inset-A, and inset-B denotes the reciprocal plot of the data of Fig. 2.

tosynthetic electron transport by Zn^{2+} is also curtailed by Ca^{2+} (Rashid *et al.*, 1991). The severity of Cu^{2+} inhibition has also been claimed to be prevented by incubating the PS II membranes with increasing concentration of Fe^{3+} (as mentioned in Yruela *et al.*, 1991). Since, Fe^{3+} co-ordinates the electron flow between Q_A and Q_B it is believed that Cu^{2+} has a inhibitory site at the reducing side of PS II. In the present investigation it is seen that Ca^{2+} can also significantly prevent the Cu^{2+} mediated inhibition of electron flow from H_2O to DCBQ in salt washed PS II membranes. The reduced extent of Cu^{2+} mediated inhibition of electron transport activity in presence of Ca^{2+} suggests that Cu^{2+} possesses an inhibitory site on the oxidizing side of PS II. The site may be the site where Ca^{2+} acts as an activator/stabilizer of oxygen evolution in salt washed particles. Since Ca^{2+} could able to prevent only 65 to 70% inhibition caused by Cu^{2+} (Fig. 2), therefore it is assumed that the ion, besides its site of inhibition close to the Ca^{2+} sensitive site at the oxidizing side of PS II may have additional site(s) of inhibition in PS II linked electron transport chain.

That the Ca^{2+} and Cu^{2+} has a common binding site in the electron transport chain can be evaluated by examining the Km values of Ca^{2+} activation of oxygen evolution activity in presence and absence of Cu^{2+}. The data from Fig. 2 was replotted in form of a double – reciprocal plot. As can be seen from Fig. 2 (inset – B) the K_m values for Ca^{2+} activation of oxygen evolution activity in

Cu^{2+}-treated and -untreated samples were highly different. In Cu^{2+}-treated particles about 4 times higher concentration of Ca^{2+} requirement was noticed to obtain the half maximal velocity as compared to Cu^{2+} – nontreated samples. Hence the interaction of Cu^{2+} with Ca^{2+} is purely competitive. Furthermore it was marked that Ca^{2+} could able to relieve Cu^{2+} inhibition irrespective of its addition either after or before Cu^{2+} administration in the reaction mixture (data not shown).

Distinct stimulation by Ca^{2+} of oxygen evolution activity has been reported for $CaCl_2$ washed PS II membranes specifically depleted of 17 and 23 KDa polypeptides (Boussac and Rutherford, 1988; Ghanotakis *et al.*, 1984a). The similar situation may be expected in NaCl washed PS II particles used in this investigation. As Cu^{2+} inhibition was significantly and specifically curtailed by Ca^{2+} in NaCl washed PS II particles, it is reasonable to answer whether in native (i.e. unwashed) membranes where these extrinsic polypeptides are still attached show an altered sensitivity for Cu^{2+} inhibition as compared to NaCl washed particles. As shown in Table III, the Cu^{2+} inhibition which was significant in the range of 5 to 15 μM Cu^{2+} in NaCl washed particles, however, found to be ineffective in unwashed membranes. A small attenuation of activity (10–12%) was seen at highest concentration (15 μM) of Cu^{2+}; tested in this investigation.

The concentration dependent discrepancy in the extent of Cu^{2+} inhibition of electron transport activity in NaCl washed and native PS II membranes may have resulted due to the accessibility of Cu^{2+} to its site of action, i.e. in absence of the polypeptides the ion can have easy approach to the site of its action as compared to the membranes with intact extrinsic polypeptides. The Cu^{2+} mediated impairment of electron transport at the donor side of PS II in native membranes may be seen at high concentration (>15 μM) of the copper ion. This aspect although has not been examined in this report the experimental observations presented by Schroder *et al.* (1994) suggest that nearly 100 μM Cu^{2+} is required to induce the complete donor side impairment in native PS II particles.

Table III. Effect of $CuSO_4$ on the photosynthetic electron transport from H_2O to DCBQ in native and NaCl washed PS II particles. NaCl washed particles were prepared as mentioned in materials and methods. The ± values represent the SD calculated from three observations. – denotes no detectable O_2 evolution activity.

$CuSO_4$ addition [mM]	Electron transport rate μmol O_2 evolved $(mgChl)^{-1}$ h^{-1} $H_2O \rightarrow$ DCBQ	
	Native	NaCl washed
0.00	680 ± 15	250 ± 10
2.50	670 ± 20	220 ± 09
5.00	680 ± 17	200 ± 13
7.50	680 ± 14	150 ± 07
10.00	670 ± 20	70 ± 09
12.50	660 ± 22	20 ± 05
15.00	600 ± 13	–

These observations clearly suggest that Cu^{2+} mediated impairment of electron flow at the oxidizing side of PS II is highly sensitive to the presence or absence of 17 and 23 KDa polypeptides (the presence of these polypeptides and particularly 23 KDa, significantly reduce the Ca^{2+} requirement for activation of oxygen evolving activity). These findings are just opposite to the nature of Zn^{2+} mediated inhibition of electron transport activity at the oxidizing side of PS II (Rashid *et al.*, 1991). Zinc inhibition can be released by Ca^{2+} only in native PS II membranes. However, in $CaCl_2$ washed PS II membranes (membranes depleted of 17, 23 and 33 KDa polypeptides) Zn^{2+} inhibition can be prevented by Mn^{2+} in combination with H_2O_2 (Rashid *et al.*, 1991).

Hence it is inferred from the studies presented in this report that the inhibition of electron transport activity at the oxidizing side of PS II by Cu^{2+} depends on the presence or absence of extrinsic polypeptides (17 and 23 KDa) of water oxidation complex. When these polypeptides are removed by high salt washing, Cu^{2+} exerts its inhibitory effects and Ca^{2+} can partially prevent this inhibitory action.

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