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Inhibition of primary photochemistry of Photosystem II by copper in isolated pea chloroplasts

M. Renganathan and Salil Bose

Centre for Biotechnology, Jawaharlal Nehru University, New Delhi (India)

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The light-saturated rates of electron transport of the photoreactions, $H_2O \rightarrow$ DCIP and $H_2O \rightarrow$ methyl viologen in isolated pea thylakoids are inhibited in the presence of cupric chloride with an I_{50} value of 7.5 μM. This inhibition is more or less equal for both the light-limited and the light-saturated rates suggesting a decrease in the concentration of active PS II reaction centres. Chlorophyll variable fluorescence (F_v) at room temperature in the presence and absence of DCMU is inhibited by copper; the apparent I_{50} value being 25 μM and 30% of F_v remains insensitive to copper at a concentration which inhibits electron transport completely. When the insensitive part of F_v is subtracted out, the I_{50} value becomes 7.5 μM. The rate of electron transport in $H_2O \rightarrow$ silicomolybdate (+DCMU) photoreaction is inhibited by copper in a manner similar to the inhibition of F_v. The inhibition of manganese $\rightarrow$ DCIP and manganese $\rightarrow$ silicomolybdate (+DCMU) rates of electron transport in heat-treated thylakoids is similar to that of $H_2O \rightarrow$ DCIP and $H_2O \rightarrow$ silicomolybdate (+DCMU), respectively. These results are interpreted as that copper inhibits the primary photochemistry of only one fraction of PS II centres (α centres/B-type centres) while the other fraction (β centres/non-B-type centres) is insensitive to copper. The strongest evidence of copper inhibition of the PS II reaction centre (RC) is that copper inhibits chlorophyll fluorescence in the isolated PS II RC which contains two polypeptides, D1 and D2, one cytochrome *b*-559 apoprotein, 4-5 Chl *a*, one P-680, two pheophytin and one β-carotene molecules but no Q_A or Q_B. Copper binds to the isolated RC in a stoichiometry of 0.8 copper per RC.

Introduction

Copper is one of the trace elements essential for the healthy life of plants, usually occurring as part of the prosthetic group of oxidizing enzymes. Contrary to its role as an essential element, copper at higher concentration (greater than 1 μM) can be toxic to bacilli, fungi and algae. In fact, copper has been extensively used as an algicide since the beginning of this century [1]. Copper has been shown to inhibit photosynthetic oxygen evolution in *Hydrilla* at micromolar concentration [2], photosynthesis in *Chlorella* [3], photosynthetic electron transport in isolated chloroplasts [4–8] and photosynthetic energy conversion [9] and facilitates thylakoid membrane lipid-peroxidation [10].

Copper inhibition of photosynthesis should be re-investigated for two reasons. First, copper binds certain organic buffers strongly [11]. We have indeed observed that depending on the buffer used in the assay medium copper inhibition of the Hill reaction ($H_2O \rightarrow$ DCIP) increases in the following order Tricine < Tris < Tes < phosphate < Hepes = Mes (the results will be published elsewhere). This observation is consistent with the fact that copper binding with buffer decreases in the same order. To investigate the effects of copper on photosyn-

Abbreviations: DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; DCIP, 2,6-dichlorophenolindophenol; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethyl urea; DPC, diphenylcarbazide; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; Mes, 4-morpholineethanesulphonic acid; Tes, 2-{[2-hydroxy-1,1-bis(hydroxymethyl)-ethyl]amino}ethanesulphonic acid; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; Tris, 2-amino-2-hydroxymethylpropone-1, 3-diol; I_{50}, inhibitor concentration causing 50% inhibition of electron transport; P-680, Reaction centre Chl *a* of PS II; Pheo, pheophytin, the primary electron acceptor of PS II; Q_A, first stable electron acceptor of PS II; Q_B, secondary stable electron acceptor of PS II; PS II, Photosystem II; PS I, Photosystem I; RC, reaction centre; Chl, chlorophyll; F_v, variable part of chlorophyll fluorescence; F_0, immediate non-variable fluorescence; F_i, initial variable fluorescence; F_{max}, maximal fluorescence.

Correspondence: S. Bose, Centre for Biotechnology, Jawaharlal Nehru University, New Delhi-110 067, India.

thetic electron transport, Shioi et al. [7] used Tris, Uribe and Stark [9] used Tricine, Habermann [4], Vierke and Struckmeier [6], Shioi et al. [12], Samuellsson and Öquist [8] and Sandmann and Böger [10] all used phosphate buffer in the assay medium. Consequently, the results obtained in their works are generally inconsistent.

The second reason is that electron donor DPC binds strongly with copper giving a strong purple colour; in fact, DPC has been used for quantitative estimation of copper spectrophotometrically [13]. Hydroxylamine cannot be used in the assay media for studies on copper inhibition because it reduces copper [14]. Cedono-Maldonado et al. [5], Vierke and Struckmeier [6] and Shioi et al. [7] have used DPC to find out the site of copper inhibition. Therefore, these results are not reliable. Furthermore, copper autooxidizes ascorbate [15], which is generally used to keep the donor (DCIP or TMPD) in reduced state. But Cedono-Maldonoado et al. [5], Shioi et al. [7], Samuellsson and Öquist [8] and Sandmann and Böger [10] have used DCIP/ascorbate system to examine the effect of copper on PS I electron transport. Consequently, these workers have obtained different results depending on the concentrations of the chemicals used and the buffer chosen for assay.

In addition, PS II-specific electron-transport reactions with substituted benzoquinones, with ferricyanide (in the presence of DCMU) or with DCIP (in the presence of DBMIB), could not be examined, because copper reacts chemically with quinone, ferricyanide and DBMIB in solution. PS II fluorescence in the presence of dithionite could not be examined because of chemical reduction of copper by dithionite [14].

We have therefore re-investigated the copper inhibition of photosynthetic electron transport with a view to elucidating the site(s) and mode of action of copper using Hepes buffer which does not bind to copper and is one of the best buffers for thylakoids at pH 7.5, and avoiding reagents that interact chemically with copper.

Materials and Methods

In all experiments 15-day-old pea (*Pisum sativum*) seedlings grown at 20 °C were used to isolate broken chloroplasts by grinding freshly isolated leaves in pre-chilled pestle and mortar, in ice-cold isolation medium containing 20 mM Hepes-NaOH (pH 7.8), 400 mM sucrose, 5 mM $MgCl_2$, 10 mM NaCl. The slurry was filtered through miracloth and chloroplasts were sedimented by centrifugation at $3000 \times g$ for 5 min at 4 °C. The pellet was re-suspended in a small volume of isolation buffer and kept on ice.

Oxygen evolution and uptake were measured with a Hansatech oxygen electrode, using the assay medium, 20 mM Hepes-NaOH (pH 7.5), 100 mM sucrose, 5 mM $MgCl_2$, 10 mM NaCl at 25 °C.

Reduction of DCIP and silicomolybdate was measured spectrophotometrically at 600 nm in a Hitachi-220 model double-beam spectrophotometer, modified for side illumination of the sample cuvette with red actinic light (greater than 620 nm) at a photon flux density of 650 $\mu E \cdot m^{-2} \cdot s^{-1}$, which was blocked from the photomultiplier tube by a CS 4-96 filter. The activities were calculated from the linear change in the absorbance during the initial 20 s of illumination.

Chlorophyll fluorescence transients were measured with a laboratory-built apparatus. Excitation light, provided by a d.c. power source, was passed through a corning CS 4-96 filter and the diode was protected by a corning red cut-off filter CS 2-64. The excitation light was controlled by an electromagnetic shutter (Uniblitz, U.S.A.) with a control unit (Uniblitz model 310B, Vincent Associates, U.S.A.). Fluorescence was detected by the Hansatech photodiode and the signal from the photodiode was stored in a storage oscilloscope, model 2090 explorer III (Nicolet Corporation, U.S.A.) and recorded on an $X-Y$ recorder (Hewlett Packard model No. 7015-B, U.S.A.).

The Photosystem II reaction centre complex was isolated in the laboratory of Prof. James Barber at the Imperial College of Science and Technology, London, following the method of J. Barber et al. [16]. The chlorophyll fluorescence emission from these reaction centres was measured using a Heinz Walz chlorophyll fluorimeter (model PAM 1). Copper binding to the reaction centre was monitored by a Perkin-Elmer atomic absorption spectrophotometer (model 2280). Chlorophyll concentration was estimated following Arnon [17].

Results

Copper inhibited the light-saturated rates of $H_2O \rightarrow$ DCIP and $H_2O \rightarrow$ methyl viologen photoreactions with an I_{50} value of 7.5 μM (Fig. 1). This inhibition is due to copper and not to anion, as different anionic salts like $CuCl_2$, $CuSO_4$, $CuNO_3$ and copper acetate inhibited the electron transport in a similar manner (data not shown). Complete inhibition of these reactions occurred at 30 μM of copper.

In the experiment of Fig. 2A 7.5 μM copper has been used for inhibition and the inhibited rate of electron transport was measured at various light intensities. The rate was calculated from the initial (0–20 s) linear part of the change in absorbance to make sure that during the measurement period the copper inhibition was not influenced by light. Fig. 2B is the Lineweaver-Burk plot of the data taken from Fig. 2A. The straight-line relationship plotted in Fig. 2B represents the following interpretation: $1/\text{rate} = 1/c \cdot k + 1/c \cdot a \cdot qy \cdot \text{intensity}$, where c is the concentration of the active reaction centres; k, the rate constant for return of phototransformed active centres to the photoactive state (via re-

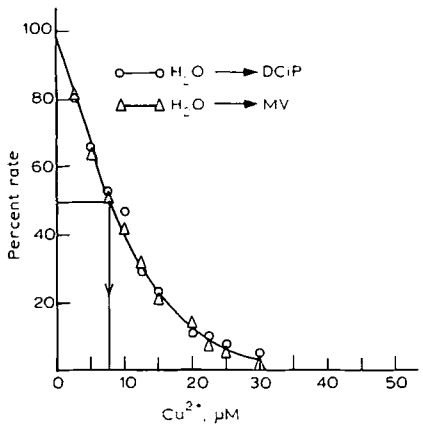

Fig. 1. Copper (cupric chloride) inhibition of the light-saturated rates of electron transport in $H_2O \rightarrow$ methyl viologen (MV) and $H_2O \rightarrow$ DCIP photoreactions. These photoreactions were measured using Hansatech oxygen electrode as oxygen uptake (with methyl viologen) or oxygen evolution (with DCIP). The reaction mixture contained 5 μg Chl per ml, 5 mM NH_4Cl, 1 mM methylviologen, or 100 μM DCIP, 10 mM NaCl, 5 mM $MgCl_2$ and 100 mM sucrose in 20 mM Hepes-NaOH (pH 7.5). Control rate for $H_2O \rightarrow$ methyl viologen photoreaction is 180 μmol O_2 consumed per mg Chl per h. Control rate for $H_2O \rightarrow$ DCIP is 150 μmol O_2 evolved per mg Chl per h. Inhibition was seen immediately after the addition of cupric chloride.

duction of e.g., DCIP); a, the effective size of the antenna of the centres; and qy, the quantum yield. The Y-axis intercept equals $1/c \cdot k$, the slope equals $1/c \cdot a \cdot qy$. Fig. 2B shows that the slope and the intercept values increase almost equally: the term $c \cdot k$ (intercept) decreases to 54% of that of the control, the term $c \cdot a \cdot qy$ (slope) to 42%. The simple interpretation of this result is that the inhibition is primarily due to the decreasing value of c.

Chlorophyll fluorescence at room temperature in the presence of DCMU is a good measure of PS II activity and no addition of an electron acceptor is required. Copper inhibited the variable part of chlorophyll fluorescence, F_v, both in the presence and absence of DCMU (Fig. 3, lower part). These results show that

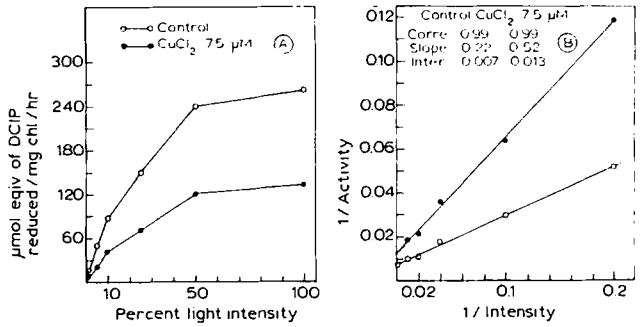

Fig. 2(A). Effect of 7.5 μM $CuCl_2$ on DCIP photoreduction measured spectrophotometrically at different light intensities. Assay conditions were the same as in Fig. 1, except that the concentration of DCIP was 30 μM. (b) Double reciprocal presentation of the data from Fig. 2A. Slope, intercept and correlation coefficient (Corre) are shown in the figure.

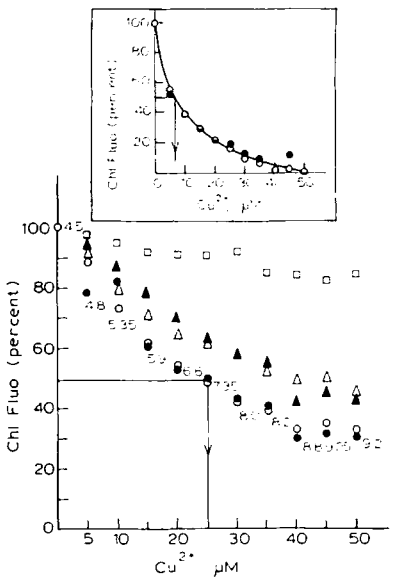

Fig. 3. Lower part: Copper ($CuCl_2$) inhibition of chlorophyll variable fluorescence (Fluo.) in the presence (closed symbols) and in the absence (open symbols) of 10 μM DCMU. The chlorophyll concentration of the sample was 5 μg Chl per ml; F_v (circle) F_m (triangle) F_0 (Square). The numbers given against experimental points (circles) represent half-rise time of F_v in ms in the presence of DCMU. Experimental details are given in Methods and Materials. Upper part: Inhibition of variable fluorescence by copper after subtracting the residual variable fluorescence at 50 μM copper concentration from the total variable fluorescence in the presence (closed circle) and absence of 10 μM DCMU (open circle).

inhibition by copper is not a DCMU-type inhibition and suggest that there is a site of inhibition before DCMU site. The apparent I_{50} value for the inhibition of F_v was 25 μM, which was higher than that observed for $H_2O \rightarrow$ DCIP or $H_2O \rightarrow$ methyl viologen photoreactions. It appears that there was a residual F_v which was not inhibited even at 50 μM, which inhibited $H_2O \rightarrow$ DCIP and $H_2O \rightarrow$ methyl viologen reactions completely. When this insensitive part of F_v was subtracted, then the I_{50} value became 7.5 μM (upper part of Fig. 3), which was similar to the I_{50} value of $H_2O \rightarrow$ DCIP reaction. The F_0 value was inhibited slightly. The reason for this is not clear, but this inhibition did not seem to be a consequence of non-specific quenching because copper did not quench chlorophyll fluorescence in acetone solution (data not shown). Moreover, a non-specific quenching of chlorophyll fluorescence cannot explain the inhibition of electron flow in saturating light.

We do not have a clear explanation why a part of F_v is insensitive to copper, but we propose that copper does not inhibit a fraction of PS II centres, which contribute to F_v but does not participate in DCIP reduction. It has been shown by other workers that the PS II β centres [18] or PS II non-B-type centres [19] or physiologically inactive PS II centres [20,21] do not participate in DCIP or methyl viologen reaction (be-

cause these centres are presumably not connected to the PQ pool) but these centres contribute to the chlorophyll variable fluorescence. Therefore, it is possible that these copper-insensitive centres are the PS II β centres, or non-B-type centres or inactive PS II centres. The PS II β centres, PS II non-B-type centres and physiologically inactive PS II centres are the different terminologies used for the same entity. A recent publication [22] has attempted to present a unifying terminology. It has been shown that the half-rise time of F_v for the PS II β centres is 3–5 times higher than that of α centres (Refs. 23 and 24; for a review on PS II heterogeneity, see Ref. 24). Our observation that the rise-time of F_v increased as the copper concentration was increased, (Fig. 3, numbers in ms represent half-rise time of F_v), supported the possibility that β centres are insensitive to copper inhibition.

Chlorophyll fluorescence induction at low light excitation is clearly resolved into three levels, the immediate non-variable fluorescence F_0, the initial variable fluorescence F_i, and the maximal fluorescence F_{max} (Fig. 4, control). Melis [18] identified the initial chlorophyll fluorescence rise from F_0 to F_i as the variable fluorescence controlled by the β centres of PS II and the fluorescence increase from initial level F_i to F_{max} as a manifestation of the reduction of the plastoquinone pool which is characteristic of the PS II_α centres and is involved in DCIP reduction. Earlier, Bose et al. [25] have shown that PS II fluorescence can be correlated to PS II electron-transport rate ($H_2O \rightarrow$ DCIP) only when F_i-to-F_{max} rise is taken into consideration. Fig. 4 shows that at 50 μM copper the fraction of F_v still present is predominantly the slow component of F_v. This is consistent with our suggestion that PS II β centres are not inhibited by copper.

It is generally known that, unlike DCIP, substituted benzoquinones accept electrons from PS II β centres because of their accessibility to the quinone binding site of PS II β centres [18]. Graan and Ort [20] found, using

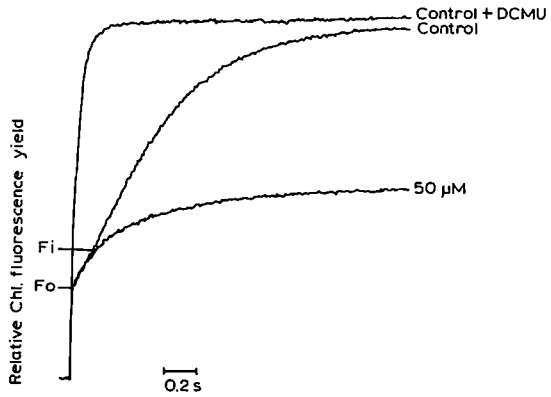

Fig. 4. Effect of $CuCl_2$ on the fluorescence induction kinetics of isolated pea thylakoids. Upper trace contained 10 μM DCMU. Lower trace contained 50 μM copper. The symbols are as mentioned in text.

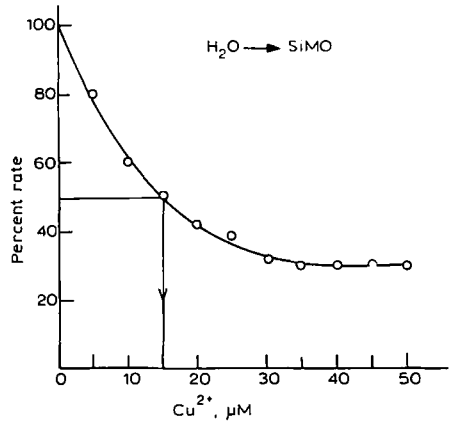

Fig. 5. Copper ($CuCl_2$) inhibition of $H_2O \rightarrow$ silicomolybdate photoreaction in the presence of DCMU. The reaction was monitored polarographically as described in Materials and Methods in the presence of 100 μg silicomolybdate per ml, 10 μM DCMU and 5 μg Chl per ml. Control rate for this photoreaction is 110 O_2 evolved per mg Chl per h.

certain halogenated benzoquinones, an increase in the number of oxygen-evolving PS II centres detected by flash yield measurements and concluded that approx. 40% of the PS II centres do not transfer electrons to plastoquinone at physiological rates in thylakoid membranes. We could not use substituted benzoquinones for our study because of their chemical reactions with copper. We, therefore, used silicomolybdate as an alternate electron acceptor as it is known to accept electrons in a DCMU-insensitive manner, presumably without requiring the PQ pool. $H_2O \rightarrow$ silicomolybdate rate in the presence of DCMU was also inhibited by copper, with a slightly higher I_{50} value (15 μM) than that of $H_2O \rightarrow$ DCIP or (methyl viologen) rate (Fig. 5). This inhibition was qualitatively similar to the inhibition of F_v in the sense that $H_2O \rightarrow$ silicomolybdate reduction contained a copper-insensitive part (30%) which when subtracted yielded an I_{50} value of 7.5 μM, which was similar to the I_{50} value of $H_2O \rightarrow$ DCIP and methyl viologen reactions. This again supports the possibility that copper-insensitive centres are the PS II β centres.

These results suggest that there is a copper inhibition site before DCMU inhibition site. As both chlorophyll fluorescence and electron transport, which are usually complementary to each other, are inhibited the inhibition could be either at the water-oxidation system or at the reaction centre of PS II. To examine this we have assayed manganese $\rightarrow$ DCIP and manganese $\rightarrow$ silicomolybdate(+DCMU) reactions using thylakoids, which were pretreated with heat just sufficiently to inactivate the water-oxidation system. The results in Fig. 6 show that copper inhibited a manganese $\rightarrow$ DCIP photoreaction in the same manner as that of $H_2O \rightarrow$ DCIP with the I_{50} value of 7.5 μM. In the manganese $\rightarrow$ silicomolybdate(+DCMU) photoreaction, as in the case of the $H_2O \rightarrow$ silicomolybdate(+DCMU) photoreac-

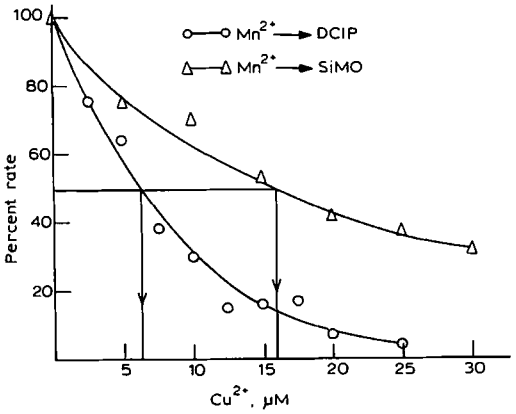

Fig. 6. Copper ($CuCl_2$) inhibition of manganese → DCIP and manganese → silicomolybdate(+DCMU) photoreactions. The reactions were monitored spectrophotometrically as described in Materials and Methods with 5 μg Chl per ml, 300 μM $MnCl_2$, 30 μM DCIP or 100 μg silicomolybdate per ml and 10 μM DCMU. Oxygen evolution was inactivated by treating thylakoids to 40 °C for 5 min. This heat-treated thylakoid suspension showed no reduction of DCIP or silicomolybdate before addition of manganese. Control rate for manganese → DCIP photoreaction is 230 μmol DCIP reduced per mg Chl per h. Control rate for manganese → silicomolybdate photoreaction is 400 μequiv. electrons per mg Chl per h.

tion, about 30% of the control rate was insensitive to copper inhibition. These results suggest that there is a site of copper inhibition between manganese donation and silicomolybdate acceptance. As manganese presumably donates electrons directly to P-680 [26], copper appears to act at the PS II reaction centre.

The above results prompted us to examine the effect of copper on isolated PS II reaction centre complex as prepared by Nanba and Satoh [27] and Barber et al. [16]. This complex consists of two 32 kDa polypeptides (D1 and D2), one cytochrome *b*-559 apoprotein, four or five Chl *a*, one P-680, two pheophytin *a* and one β-carotene molecules. There is no Q_A, Q_B, PQ pool or

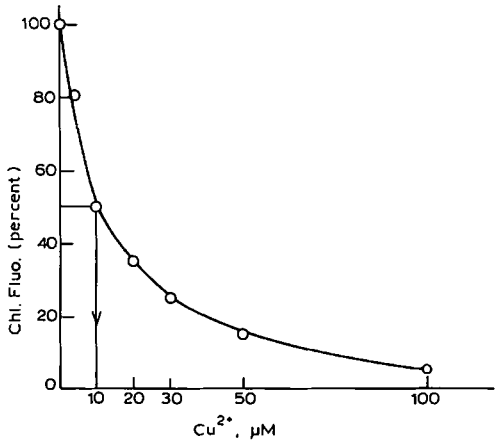

Fig. 7. Copper ($CuCl_2$) inhibition of chlorophyll (Chl.) fluorescence (Fluo.) in isolated PS II reaction centre complex. The concentration of PS II reaction centre was equivalent to 5 μg Chl per ml. Reaction centre complex was prepared and chlorophyll fluorescence was measured at 4 °C as described in Ref. 16.

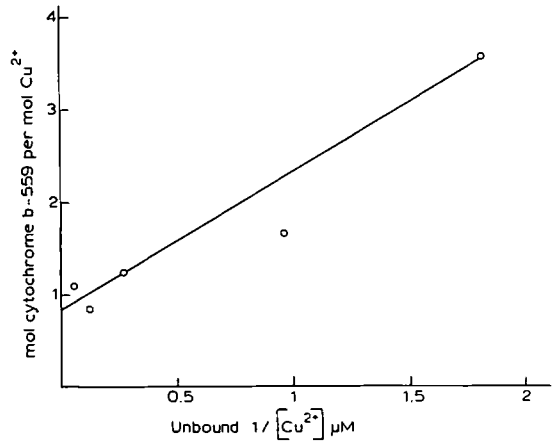

Fig. 8. Copper ($CuCl_2$) binding to PS II reaction centre complex. The amount of bound copper was estimated by subtracting the free copper from the total copper.

light-harvesting chlorophyll protein. In this preparation of PS II reaction centre complex the chlorophyll fluorescence emission was also inhibited by copper, further suggesting that the site of action of copper is at the reaction centre of PS II (Fig. 7). The I_{50} concentration for this inhibition was 10 μM which was close to that of H_2O → DCIP and H_2O → methyl viologen photoreactions, but not equal to that of the inhibition of F_v in thylakoids. This suggests, as is also expected, that the structural and functional heterogeneity in PS II is attributed to the level of organisation of PS II in the thylakoid membrane and not to the structure of PS II reaction centre as such.

Having recognized the action of copper on the isolated PS II reaction centre, an attempt has been made to detect copper binding to the reaction centre. The results (Fig. 8) show that copper binds to the PS II reaction centre complex with a stoichiometry of 0.8 mol to 1 mol of cytochrome *b*-559. As there is one cytochrome *b*-559 per PS II reaction centre in this preparation [16], we conclude that there is one copper binding site per PS II reaction centre. However, it should be noted that the I_{50} value for the inhibition of chlorophyll fluorescence of PS II reaction centre is more than the K_m value for copper binding to PS II reaction centre. This variation cannot be explained by the simple interpretation that the fluorescence quenching was due to only strongly bound copper ions. Fluorescence quenching is a complex process and copper can influence the fluorescence intensity not only by strong binding, but also through weak binding which could not be detected in direct binding experiments.

Discussion

The inhibition of manganese → silicomolybdate (+DCMU) photoreaction and chlorophyll variable fluorescence in the presence of DCMU by copper strongly

suggests that copper acts at PS II reaction centre. This suggestion is further strengthened by the inhibition of isolated PS II reaction centre chlorophyll fluorescence by copper. These observations do not exclude the possibility of copper action at other sites in the electron-transport chain, namely, on oxygen-evolving complex and on PS I. If there is a site on the oxygen-evolving complex, the sensitivity should be less, because of the similarity in the I_{50} values of $H_2O \rightarrow$ DCIP and manganese $\rightarrow$ DCIP photoreactions. We have not examined the typical Photosystem I electron-transport assay like DCIP/ascorbate or TMPD/ascorbate $\rightarrow$ methyl viologen, because of autooxidation of ascorbate by copper [15] resulting in non-photosynthetic oxygen uptake. However, work on the measurement of P-700 kinetics is in progress to find out the site(s) of copper inhibition, if any.

Although the PS II reaction centre appears to be a site of action of copper inhibition the mechanism of fluorescence quenching is not clear. According to Klimov's hypothesis [28] variable fluorescence arises from the charge recombination of $P\text{-}680^+$ and $Pheo^-$. Copper inhibition of both electron transport and chlorophyll variable fluorescence can occur provided either charge separation is inhibited in the presence of copper or the energy of the charge separated species ($P\text{-}680^+$ $Pheo^-$) is dissipated through an unknown way which competes favourably with fluorescence and electron transport. These two possibilities can be distinguished by examining the effects of copper on the amplitude and decay kinetics of $P\text{-}680^+$ generated by picosecond flashes [29].

There is recent evidence which does not support Klimov's hypothesis [30–33]. Copper inhibition of F_v and electron transport in the thylakoids can still be explained by classical Duysen's model [34], that Q_A in oxidized state is a quencher of variable fluorescence. If copper inhibits the reduction of Q_A, inhibition of both electron transport and variable fluorescence is expected. But this interpretation is not consistent with the observation that copper quenches variable fluorescence in the isolated reaction centre complex that does not contain Q_A. For that matter, the hypothesis of charge recombination as the source of variable fluorescence suits better in explaining the fluorescence quenching in both thylakoids and isolated reaction centre complex.

Copper-binding studies indicate that one copper binds to one PS II reaction centre. It is known that copper is coordinated to imidazole nitrogen atoms of histidines at the metal-binding region of copper proteins [35–37]. Michel and Deisenhofer [38] have suggested that the special-pair chlorophyll molecule P-680 and the non-heme iron atom make ligands with histidine amino acids of position 198, 215, in D1 and D2 proteins. Copper by interacting with any one of these histidines may disturb the environment of the prosthetic groups, thereby perturbing the normal function of the reaction centre.

Acknowledgments

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