COPPER EFFECT ON CYTOCHROME b_{559} OF PHOTOSYSTEM II UNDER PHOTOINHIBITORY CONDITIONS

by

María Bernal[1], Mercedes Roncel[2], Jose María Ortega[2], Rafael Picorel[1] and Inmaculada Yruela[1,*]

[1]Estación Experimental de Aula Dei, Consejo Superior de Investigaciones Científicas (CSIC), Apdo. 202, E-50080 Zaragoza, Spain.
[2]Instituto de Bioquímica Vegetal y Fotosíntesis, Universidad de Sevilla-Consejo Superior de Investigaciones Científicas (CSIC), Americo Vespucio s/n, E-41092 Sevilla, Spain.

ABSTRACT

Toxic Cu(II) effect on Cytochrome b_{559} under aerobic photoinhibitory conditions was examined in two different PSII membrane preparations active in oxygen evolution. The preparations differ in the content of Cytochrome b_{559} redox potential forms. Difference absorption spectra showed that the presence of Cu(II) induced the oxidation of the high-potential form of Cytochrome b_{559} in the dark. Addition of hydroquinone reduced the total oxidised high-potential form of Cytochrome b_{559} present in Cu(II)-treated PSII membranes indicating that no conversion to the low-potential form took place. Spectroscopic determinations of Cytochrome b_{559} during photoinhibitory treatment showed slower kinetics of Cu(II) effect on Cytochrome b_{559} as compared to the rapid loss of oxygen evolution activity in the same conditions. This result indicates that Cytochrome b_{559} is affected after PSII centers are photoinhibited. The high-potential form was more

sensitive to toxic Cu(II) action than the low-potential form under illumination at pH 6.0. The content of the high-potential form of Cytochrome b_{559} was completely lost, however the low-potential content was unaffected in these conditions. This loss did not involve cytochrome protein degradation. Results are discussed in terms of different binding properties of the heme iron to the protonated or unprotonated histidine ligand in the high-potential and low-potential forms of Cytochrome b_{559}, respectively.

Keywords: cytochrome b_{559}, copper, photosystem II, photoinhibition, redox potential

Abbreviations: Cyt, cytochrome; DCBQ, dichlorobenzoquinone ; D1, polypeptide of the photosystem II reaction centre ; HP, high potential; LP, low potential; MES, 2-(N-morpholino) ethane-sulfonic acid; PAGE, polyacrilamide gel electrophoresis; PS, photosystem; RC, reaction center; SDS, sodium dodecyl sulphate ; Tris, Tris(hydroxymethyl)aminomethane.

INTRODUCTION

Several heavy metals are essential for plant growth and development, but their excess can easily lead to toxic effects. Among them Cu(II) is known to be toxic at high concentrations for photosynthetic organisms (Clijsters and van Asche, 1985; Maksymiec, 1997). Extensive *in vitro* studies have shown that photosystem II (PSII) is more susceptible to copper toxicity (for review see Droppa and Horváth, 1990; Barón et al. 1995) than photosystem I (PSI) (Ouzounidou et al. 1997). Both acceptor and donor sides of PSII have been proposed as copper-inhibitory sites. At the acceptor side, the Q_B binding site (Mohanty et al. 1989) and the Pheo-Fe-Q_A domain (Yruela et al. 1991, 1992, 1993, 1996a) have been reported. Evidences that copper ion impairs the function of the oxidising side has been reported (Cedeño-Maldonado et al. 1972; Vierke and Struckmeier, 1977; Shioi et al. 1978a,b; Bohner et al. 1980; Samuelsson and Öquist, 1980). It has been shown that the electron flow from Tyr_z to $P680^+$ is blocked by Cu(II) (Schröder et al. 1994; Arellano et al. 1995). Indeed, Králova et al. (1994) and Sersen et al. (1997) have proposed that Cu(II) interacts not only with Tyr_z but also with Tyr_D on D2 protein. A possible direct interaction between copper and calcium at the oxidising side of PSII was also shown both *in vitro* (Sabat, 1996) and *in vivo* (Maksymiec and Baszynski, 1999). Additional effects of Cu(II) toxicity on both donor and acceptor sides have been reported using higher copper concentrations. In such conditions the Mn-cluster and the extrinsic proteins on the donor side can be affected. Interaction of copper with the non-heme Fe^{2+} and cytochrome (Cyt) b_{559} on the acceptor side has been also reported. (Renger et al. 1993; Sersen et al. 1997; Jegerschöld et al. 1995, 1999).

The photosynthetic activity decreases when oxygenic organisms are exposed to prolonged illumination with high light intensities. This process which includes the functional impairment of PSII electron transport and the structural damage of the PSII reaction centre is known as photoinhibition (Aro el al. 1993). The influence of Cu(II) toxicity on photoinhibition has also been investigated (Yruela et al. 1996b; Pätsikkä et al. 1998) demonstrating that Cu(II) enhances the adverse effects of excess light on PSII. Over the past 10 years the participation of Cyt b_{559} in a redox mechanism to protect PSII against donor and acceptor side photoinhibition has been proposed (for review see Stewart and Brudvig, 1998). Evidence that Cyt b_{559} prevents the overreduction of PSII acceptor side have been reported (Nedbal et al. 1992; Poulson, 1995). On the other hand, it was postulated that the role of this heme protein is to act as an auxiliary electron donor delivering electrons to oxidised chlorophylls in the PSII reaction centre (Buser et al. 1992; Faller et al. 2001).

Cytochrome b_{559} can exhibit several redox potential forms, a labile high-potential (HP) form with a midpoint redox potential (E'_m) around +400 mV, a intermediate potential (IP) form ranging from +200 to +150 mV and a low-potential (LP) form with an E'_m value from +70 to +20 mV (Stewart and Brudvig, 1998; Kaminskaya et al. 1999; Mizusawa et al. 1999; Roncel et al. 2001). The IP form has been proposed to be a protonated LP form (Roncel et al. 2001). The HP form dominates in thylakoids and PSII membranes with an intact water-oxidizing complex. Several authors have reported that photoinhibition induces changes in the redox properties of Cyt b_{559} (Styring et al. 1990; Allakhverdiev et al. 1997; Ortega et al. 1999). The conversion of the HP to the LP form has been observed during photoinhibitory illumination in native PSII membranes (Ortega et al. 1999).

Cu(II) interaction with Cyt b_{559} has been reported earlier (Jegerschöld et al. 1995; Yruela et al. 1996b), however no data on its specific action during photoinhibition have been shown. The aim of this work is to investigate the changes of Cyt b_{559} caused by photoinhibition under low toxic Cu(II) conditions. The results show that HP Cyt b_{559} form is very sensitive to toxic Cu(II) action during photoinhibitory illumination whereas LP Cyt b_{559} form is not affected under the same experimental conditions.

MATERIALS AND METHODS

Biological material.- *Beta vulgaris* cv Monohill was grown hydroponically in a growth chamber in half-Hoagland nutrient solution under 325 μmol quanta.m^{-2}.s^{-1} from fluorescent and incandescent lamps at 25 ºC, 80% humidity and 16-h photoperiod. Spinach was obtained from local market.

Photosystem II membrane isolation.- Highly enriched PSII membranes with a rate of oxygen evolution activity of *c.a.* 500 μmol O_2 mg Chl^{-1} h^{-1}, using dichlorobenzoquinone (DCBQ) as artificial electron acceptor, were prepared from market spinach and sugar beet according to Berthold et al. (1981). Samples were suspended in 0.4 M sucrose, 15 mM NaCl, 5 mM $MgCl_2$ and 50 mM 2-(N-morpholino) ethanesulphonic acid (Mes-NaOH), pH 6.0, frozen in liquid nitrogen and stored at –80 ºC until use.

Photoinhibition.- Photoinhibitory treatment was done using intact and Cu(II)-treated oxygen-evolving PSII membranes preparations. PSII membranes at 200

μg Chl ml^{-1} resuspended in 3.5 ml buffer containing 0.4 M sucrose, 15 mM NaCl and 50 mM Mes-NaOH, pH 5.0-6.0 or 50 mM Tris(hydroxymethyl)aminomethane (Tris-HCl), pH 7.8, were incubated with 125 μM $CuCl_2$ (equivalent to a Cu(II)/PSII ratio of 137). To calculate the Cu(II) per PSII unit ratio in PSII membranes a content of 250 Chl per RC was assumed (Berthold et al. 1981). Incubations with Cu(II) were done at 23 ºC for 1 min under constant stirring in the dark before exposing the samples to heat-filtered white light (3,000 μmol quanta $m^{-2}s^{-1}$) to induce photoinhibitory conditions. The temperature was maintained at 23 ºC by circulating water from a temperature-controlled waterbath around the photoinhibition cell. The temperature increment inside of reaction cell was less than 1 ºC after 20 min illumination. Control samples were kept in the dark under identical conditions to the illuminated samples in order to monitor dark inactivation processes unrelated to photoinhibition. After treatment all of the samples were washed twice with 15 mM NaCl and 50 mM Mes-NaOH, pH 5.0-6.0 or 50 mM Tris-HCl, pH 7.8. The pellets obtained by centrifugation at 23,000 x g were resuspended in the same buffer plus 0.3 M sucrose and then, oxygen-evolution activity was measured.

Oxygen evolution activity.- Oxygen evolution activity was measured with a Clark-type oxygen electrode in the presence of 0.5 mM DCBQ as artificial electron acceptor. The light intensity on the surface of the cuvette was 3,000 μmol quanta $m^{-2}s^{-1}$. Samples were diluted in 3 ml buffer containing 25 mM Mes-NaOH, pH 6.5, 10 mM NaCl and 0.3 M sucrose. The Chl concentration was 10 μgml^{-1}.

Gel Electrophoresis and Immunoblotting.- The electrophoretic separation of PSII proteins was performed by SDS-PAGE on 12.5% (w/v) or 12-20% (w/v) acrylamide linear gradient gels containing 6 M urea according basically to Laemmli (1970). The gels were either stained with Coomassie B-Blue 250 or electroblotted to identify D1 and α-subunit of Cyt b_{559} proteins, respectively. After transferring to a nitrocellulose membrane, proteins were detected with a rabbit anti-D1 or anti-Cyt b_{559} α-subunit. To do that, the nitrocellulose membrane was cut across in two parts. Upper and lower parts of the membrane revealed the D1 and α-subunit of Cyt b_{559}, respectively, using goat anti-rabbit IgG coupled to horseradish peroxidase as a secondary antibody. To obtain extrinsic protein markers the spinach PSII membranes at 0.5 mg mL^{-1} were suspended in 0.8 M Tris-HCl (pH 9.1), 5 mM EDTA and incubated for two hours in darkness. Then, the membranes were treated with 1.5 M NaCl for 30 min in darkness, centrifuged at 35,000 x g for 20 min and washed twice with 20 mM NaCl, 0.4 M sucrose and 50 mM Mes-NaOH (pH 6.5). The supernatants containing the extrinsic proteins were concentrated with Centripep tubes (Amicon, cut-off 10,000 kDa) and used as marker in electrophoresis assays (Mizusawa et al. 1999).

Optical measurements.- Difference absorption spectra were recorded using 1-cm optical pathlength cuvettes at 10ºC with a Beckman DU 640 spectrophotometer. The content of HP and LP forms of Cyt b_{559} was determined from the difference absorption spectra in the 510-600 nm region. A differential extinction coefficient of 21 $mM^{-1}.cm^{-1}$ at the maximum at 559 nm *minus* the minimum at around 570 nm (Stewart and Brudvig, 1998) was used. Samples at 50 μg Chl ml^{-1} in 25 mM Mes-NaOH, pH 6.5, 10 mM NaCl and 0.3 M sucrose were

oxidised with 2 mM ferricyanide (HP_{red}) and then reduced with 4 mM hydroquinone (HP_{ox}) and 2 mM sodium dithionite (LP_{ox}). Sodium dithionite solution was freshly prepared in 0.5 M Tricine, pH 7.5.

Potentiometric redox titrations of PSII membrane suspensions were carried out at 20ºC under argon by following the absorbance changes at 559 nm *minus* 570 nm in the difference absorption spectra obtained by the sequential addition of aliquots of 0.1 M sodium dithionite. Spectra were recorded in an Aminco DW-2000 UV-Vis spectrophotometer. Samples were previously oxidized with 25 μM potassium ferricyanide. The redox potential in the reaction cell was simultaneously measured with a potentiometer (Methrom Herisau, Switzerland) provided with a combined Pt-Ag/AgCl microelectrode (Crison Instruments, Allela, Spain) previously calibrated against a saturated solution of quinhydrone (E'_m, pH7, +280 mV at 20ºC). Redox mediators used and midpoint redox potential determinations were as described in Roncel et al. (2001).

RESULTS

Effect of Cu(II) on Cytochrome b_{559} in the dark.

Firstly, Cyt b_{559} was examined in control PSII-enriched membranes active in oxygen evolution from spinach and sugar beet plants. The amounts of HP and LP forms of Cyt b_{559} under aerobic conditions were obtained from reduced *minus* oxidised difference absorption spectra in the 510-600 nm region (for details see Materials and Methods). Upon addition of ferricyanide to a dark incubated sample the initially reduced HP form becomes oxidised (Fig. 1). Then, it can be reduced by hydroquinone. A further addition of dithionite resulted in the reduction of the oxidised LP form present. Data show that PSII membranes from spinach and sugar beet differed in the content of Cyt b_{559} redox potential forms. Thus, spinach PSII membrane preparations contain around 20-50% reduced HP form and 80-50% oxidised LP form of Cyt b_{559} depending on the pH. It is worth mentioning that LP form assignment is given to all the Cyt b_{559} species that cannot be reduced by hydroquinone. The values are similar to that reported by others in these kinds of preparations (Ortega et al. 1999; Gadjieva et al. 1999). A marked difference is observed in PSII membranes from sugar beet where only the LP form of Cyt b_{559} was present at any pH value investigated. The absence of the HP form of Cyt b_{559} in oxygen-evolving PSII membranes was also found by others in preparations from pea (Roncel et al. 2001). Reductive potentiometric redox titrations at pH 6.0 showed clearly the existence of two different components with E'_m of +397 mV and +206 mV in spinach PSII membranes; the named HP and LP potential forms (Fig. 2). In the case of sugar beet preparations, only one redox component with an E'_m of +162 mV was found.

Cu(II)-treatment in the dark under aerobic conditions induced the oxidation of the reduced HP Cyt b_{559} form present in control PSII membranes at all the pH used (Table I). No significant modifications were observed in the Cyt b_{559} redox components of PSII preparations (Fig. 2). Only a slight shifted of E'_m values took place in spinach PSII membrane preparations. The HP and LP forms exhibited a E'_m of +352 mV and +172 mV, respectively. Thus, Cu(II) in the dark did not transform the HP form into the LP form under aerobic conditions. On the other hand, the LP Cyt b_{559} was not affected by Cu(II) in these conditions. It is worth mentioning that the oxidised HP present in the presence of Cu(II) can be reduced by hydroquinone indicating that the redox properties of HP Cyt b_{559} were not altered by this treatment. The Cu(II) concentrations used in the experiments correspond to a 40-50% inactivation of photosynthetic electron transport at pH 6.5 after 1 min incubation in the dark at 23 ºC (Yruela et al. 1991,1993).

Effect of Cu(II) on oxygen-evolution activity during illumination.

The effect of photoinhibitory illumination on the oxygen evolution activity was examined in both spinach and sugar beet PSII membrane preparations treated with toxic Cu(II) concentrations at different pH conditions. The Cu(II) concentrations used were the same as described above. The results were compared with those obtained in control preparations with no addition of copper. The time course of oxygen evolution activity was similar in both spinach and sugar beet PSII membranes during illumination treatment at pH 5.0-7.8 (Fig. 3). The loss of activity was markedly faster in Cu(II)-treated than in untreated samples consistent with previous results (Yruela et al. 1996b). The data also indicate that

damage caused by illumination is faster at slightly acid (pH 5.0) or basic (pH 7.8) conditions than at pH 6.0.

Effect of Cu(II) on redox potential forms of Cytochrome b_{559} during illumination.

The effect of Cu(II) on total Cyt b_{559} under photoinhibitory conditions was investigated by measuring the changes in the reduced (dithionite) *minus* oxidised (ferricyanide) difference absorption spectra after illumination treatment. At pH 5.0 the total content of Cyt b_{559} did not change in the presence of Cu(II) during illumination in the two kinds of PSII preparations investigated (Fig. 4A). The same result was observed at this pH in control conditions. At pH 6.0, however, differences were found between spinach and sugar beet PSII preparations. The total content of Cyt b_{559} decreased by 30% in spinach PSII membranes treated with Cu(II) (Fig. 4B) compared to the control. On the other hand, no Cu(II) effect on Cyt b_{559} was observed in sugar beet PSII preparations. At pH 7.8 the behaviour of Cyt b_{559} in both PSII membrane samples was independent of Cu(II) treatment (Fig. 4C). At this pH value the photoinhibitory effect on Cyt b_{559} was clearly more pronounced in spinach PSII membranes than in sugar beet ones where the total content of Cyt b_{559} decreases *c.a.* 60% and 20%, respectively. Therefore, it seems that Cyt b_{559} is more stable in PSII membranes from sugar beet where the HP form is absent (Table I). The data obtained at pH 6.0 suggest that the HP form of Cyt b_{559} gets lost after illumination in Cu(II)-treated samples. The results would also indicate that a pH 7.8 the HP form and a portion of the LP form is affected in these conditions.

In order to better investigate if the HP form is more sensitive to toxic Cu(II) than the LP Cyt b_{559} and considering that at pH 7.8 both HP and LP forms are

affected we measured the absorption spectral changes in the 510-600 nm region induced by the sequential addition of ferricyanide, hydroquinone and dithionite at pH 5.0 and 6.0. At pH 6.0, the initially reduced HP Cyt b_{559} in the control PSII membranes from spinach (Table I) decreased by 40% after 5 min of illumination being almost lost after 20 min (Fig. 5B). However, the total content of Cyt $b_{559,}$ inferred from the absorbance difference at 559 nm *minus* 570 nm between the fully oxidised and fully reduced state of Cyt $b_{559,}$ remained almost constant indicating that the HP Cyt b_{559} loss was not accompanied by a significant decrease of the total Cyt b_{559} content. This finding demonstrates that a conversion of the HP form to other Cyt b_{559} forms with lower redox potential occurs in the control PSII membranes under photoinhibitory conditions. A distinct feature was observed in Cu(II)-treated samples. The oxidised HP form of Cyt b_{559} present in the Cu(II)-treated PSII samples in darkness (Table I) was completely lost after 20 min illumination but the initial decrease was faster than in the control, i.e., the HP form diminished by 50% after 1-2 min illumination. The HP Cyt b_{559} lost was accompanied by a *c.a.* 30% decrease of the total Cyt b_{559} content during this time, remaining stable after that. The results indicate that contrary to what occurs in control PSII membranes an irreversible damage to the HP form takes place in the Cu(II)-treated preparations. At this point the question arises if the faster HP Cyt b_{559} decrease in the presence of Cu(II) is due to the fact that HP form is in the oxidised state in Cu(II)-treated PSII membranes or it is due to a direct Cu(II) interaction with the cytochrome. To elucidate that, photoinhibition experiments were done in pre-illuminated PSII membranes in order to photooxidise the HP_{red} form of Cyt b_{559} (Buser et al. 1992). The results were the same as those obtained in control conditions where HP was reduced (data not shown). This finding

indicates that HP form is highly sensitive to photoinhibition in the presence of Cu(II) independently of its redox state. The data also show that the LP Cyt b_{559} form is stable during illumination under toxic Cu(II) conditions at pH 6.0.

Similar results were obtained at pH 5.0 in the control PSII samples (Fig. 5A) but a different Cyt b_{559} behaviour was found in Cu(II)-treated preparations. The HP content decreased by approximately 30% after 5 min of illumination, much less value than at pH 6.0 where a 65% decrease was observed. At pH 5.0 the total Cyt b_{559} content did not vary during this time. The results would indicate that at this pH the Cu(II) interaction with Cyt b_{559} is prevented.

Effect of Cu(II) on polypeptide composition of PSII during illumination.

Taking into account that at pH 5.0-6.0 more intact PSII complexes exist than at pH 7.8, in terms of the donor side of PSII, and data at these pH values provide more selective information on the Cu(II) effect on Cyt b_{559}, we checked the integrity of the PSII complexes and Cyt b_{559} during photoinhibition under those pH values. To do that the polypeptide composition of control and Cu(II)-treated spinach PSII membranes in the dark and after illumination at pH 5.0 and 6.0 was assayed. In the dark both samples had the same electrophoretic profile at pH 6.0 (Fig. 6A) indicating that the Cu(II) concentrations used in our experiments did not affect the integrity of the PSII complexes in the dark. After illumination the major observed difference was that the 23 kDa protein was removed faster from the Cu(II)-treated samples than from the control. The 23 kDa protein is one of the extrinsic proteins of the oxidising side of PSII. It has been reported that this protein is very sensitive to high concentrations of Cu(II) and photoinhibitory treatment (Yruela et al. 2000; Pätsikkä et al. 2001). The 33 and 17 kDa extrinsic

proteins and the 47 and 43 kDa PSII intrinsic antenna complexes were unaffected after 20 min illumination. At pH 5.0 the results were similar (data not shown).

In order to know if the 30% decrease of total Cyt b_{559} content observed in the spectroscopic determinations at pH 6.0 (Fig. 4B,5B) corresponds to degradation of the heme-protein, immunoblot assays were done. Data show that α-subunit of Cyt b_{559} was not degraded under these conditions and Cu(II) does not stimulate the degradation of this heme protein (Fig. 6B). We also observed that D1 protein of the PSII reaction centre is not degraded during illumination indicating the extend of the photoinhibitory treatment applied.

DISCUSSION

In this work we report the photoinhibition effect on Cyt b_{559} of oxygen-evolving PSII membranes treated with toxic copper at pH within the 5.0-7.8 range under aerobic conditions. We have taken special care in controlling the Cu(II) concentrations used in our experiments in order to avoid secondary effects on the integrity of PSII complexes (Yruela et al. 2000). Two different types of oxygen-evolving PSII membrane preparations were investigated that differed in the content of the HP and LP forms of Cyt b_{559} (Fig. 2). This allowed the comparison of photoinhibition effects on both types of preparations in control conditions (absence of copper). The different content of Cyt b_{559} redox forms could be due to a different action in both biological materials of the detergent Triton X-100 used to isolate the PSII membranes (Roncel et al. 2001). Concerning the experiments performed in the presence of Cu(II) it is worth of noting that Cu(II) concentrations used in this work did not cause any apparent damage to the polypeptide integrity of the PSII complexes in the dark at pH 5.0-6.0 and in particular to the oxidising side of PSII as revealed by protein electrophoretic analysis of samples. In respect to Cyt $b_{559,}$ Cu(II) treatment induced the oxidation of the reduced HP form in the dark, but no conversion to the LP form was observed. The same observation was reported by Burda et al. (2003). Furthermore, total Cyt b_{559} content was maintained in the dark. Other authors have observed that toxic Cu(II) cause the complete conversion of the HP to the LP form (Jegerschöld et al. 1995). In fact, these authors reported the release of the 16 kDa extrinsic subunit during copper treatment. Such differences can be explained by the much lower Cu(II) concentrations used in our experiments. The mechanism of the Cyt b_{559} oxidation

by Cu(II) is difficult to explain precisely. It should be mentioned that a direct oxidation of the HP Cyt b_{559} form by Cu(II) is thermodynamically unfavourable because the midpoint redox potential of Cu(I)/Cu(II) ($E'_{m,7}$ = +167 mV) is markedly lower than that of HP Cyt b_{559} form ($E'_{m,7}$ = +350 - +400 mV). This phenomenon could only be possible if *i)* E_m of Cyt b_{559} is not any longer in HP form; *ii)* E_m of copper redox couple is increased; *iii*) a non-direct oxidation mechanism takes place. The former is discarded based on our results (Fig. 2); the second one in thermodynamic terms might be if the oxidizing agent were the redox couple Cu(I)/Cu ($E'_{m,7}$ = +521 mV). Cu(II) can be reduced *via* oxygen radical species (O_2^-) to Cu(I) under aerobic conditions. However, our results do not support this possibility since we do not observed that the redox potential of the medium to become more positive than +400 mV by addition of CuI(II). Thus, another oxidation mechanism should be considered. Recently, we have obtained information about the structure and the electronic distribution of Cyt b_{559} heme group in PSII reaction center preparations based on CW-EPR and HYSCORE experiments (Yruela et al. 2003; García-Rubio et al. 2003). Our results indicate that the exchangeable electron is localized in a confined iron orbital with a negligible mixture of nitrogen *p*-orbitals in the heme group of Cyt b_{559}. This makes very unlikely that any substrate or reactant can be located close enough to the active site for a direct one-step electron exchange. The transfer mechanism could be better understood if it were a multistage complex process where the iron acts as a final reservoir. Our investigations suggest that redox processes will depend in a complex way on the whole protein conformation.

Interestingly, we observed the rapid loss of oxygen evolution activity as compared to slower kinetics of Cu(II) effect on Cyt b_{559} suggesting the Cu(II)-

induced effects on Cyt b_{559} do not contribute to the fast rate of photoinhibition. It is also worth mentioning that at pH 5.0 the loss of oxygen evolution was faster than at pH 6.0 whereas the contrary was observed with respect to the Cyt b_{559} in the Cu(II)-treated samples during illumination. These findings indicate that Cyt b_{559} is affected after PSII centers are photoinhibited. The mechanisms underlying the Cu(II)-induced effect on oxygen evolution and Cyt b_{559} should be independent. It has been proposed that the protective role of Cyt b_{559} against photoinhibition is based on a conversion mechanism involving different redox components of this heme protein (Gadjieva et al. 1999; Ortega et al. 1999). In this sense our results show similar rate of oxygen evolution during photoinhibition in the two kinds of PSII preparations examined either in the absence or presence of Cu(II). Therefore the participation of Cyt b_{559} preventing photoinhibition process is not clear in our experiments. On the other hand, since any loss of Cyt b_{559} heme content was observed by addition of Cu(II) in the dark as well as in control conditions under illumination the results indicate that Cu(II) is probably acting directly on Cyt b_{559} in the light inducing the destabilisation of heme group.

The data show a pH-dependent behaviour of Cyt b_{559} during illumination in the presence of Cu(II). At pH 5.0, no Cu(II) effect on total Cyt b_{559} content was observed, however at pH 6.0-7.8 total Cyt b_{559} was rapidly affected (Fig. 4). This pH-dependence suggests that Cu(II) interaction with Cyt b_{559} takes place at a protonatable amino acid residue. It is well established that the His residue has a high affinity to bind copper, and evidence that Cu(II) inhibits photosynthetic complexes from purple bacteria by binding to His residues have been reported (Utschig et al. 2000, 2001; Rao et al. 2000). It has been suggested that in the HP form one of the two His ligands of heme group is protonated and forms a

hydrogen bond with a carbonyl group of the protein backbone providing a highly hydrophobic environment surrounding the heme group. Such ambience is considered essential for the maintenance of the unstable HP form of Cyt b_{559} (Berthomieu et al. 1992; Roncel et al. 2001). These authors also proposed that His residues in the LP form of Cyt b_{559} were unprotonated. Since the protonation of His weakens the bond between the heme iron and imidazole N^{δ} compared to the case of unprotonated His, the destabilisation of the heme group coordination by Cu(II) can be favoured in the HP form. Recently, Burda et al. (2003) have proposed that HP Cyt b_{559} oxidation in the dark probably occurs by deprotonation of His ligands. Such oxidation could make the HP form more sensitive to photoinhibition. However, we observed the HP Cyt b_{559} damage takes place independently of its redox state.

Interestingly, at pH 6.0-7.8 the HP form of Cyt b_{559} was more sensitive to toxic Cu(II) action than the LP form and irreversible damage to the HP form took place during photoinhibition contrary to what occurs in control PSII membranes where a conversion of the HP into the LP form was observed. Similar results were reported by others in fresh preparations (Styring et al. 1990; Mor et al. 1997), although the molecular mechanism involved in the conversion between the Cyt b_{559} forms is not clear at present. These findings indicate that Cu(II) interaction with Cyt b_{559} could impair the conversion process to the more stable LP form. The LP form was not affected (spinach and sugar beet preparations) in both control and Cu(II)-treated preparations during illumination at pH 5.0-6.0. Surprisingly, at pH 5.0 the extent of Cu(II) effect on the HP form was less than at pH 6.0 although this finding could be due to a protein conformational change at this low pH preventing the Cu(II)-induced damaging effect. In view of these considerations,

we suggest that the higher sensitivity of the HP form of Cyt b_{559} to photoinhibition in the presence of Cu(II) could be due to blocking of its conversion to the LP form. The destabilisation of the heme group did not involve cytochrome protein degradation.

ACKNOWLEDGEMENTS

The authors are grateful to Maria V. Ramiro for skilful technical assistance. We are indebted to Drs. A.K. Matto and R. Barbato for their kind gifts of antibody against the D1 and α-subunit of Cyt b_{559} polypeptides of PSII, respectively. M. Bernal was recipient of an I3P Programme fellowship from Consejo Superior de Investigaciones Científicas. This work was supported by the Dirección General de Investigación (Grant BMC2002-00031) to R.P. and Gobierno de Aragón (Grant P015/2001) to I.Y., and it has been done within GC DGA 2002 Program of Gobierno de Aragón.

REFERENCES

Allakhverdiev AI, Klimov VV, Carpentier R (1997) Evidence for the involvement of cyclic electron transport in the protection of photosystem II against photoinhibition: influence of a new phenolic compound. Biochemistry 36: 4149-4154

Arellano JB, Lázaro JJ, López-Gorgé J, Barón M (1995) The donor side of PSII as the copper-inhibitory binding site. Photosynth Res 45: 127-134

Aro EM, McCaffery S, Anderson JM (1993) Photoinhibition and D1 Protein Degradation in Peas Acclimated to Different Growth Irradiances. Plant Physiol 103: 835-843

Barón M, Arellano JB, López-Gorgé J (1995) Copper and photosystem II: A controversial relationship. Physiol Plant 94: 174-180

Berthold DA, Babcock GT, Yocum CF (1981) A highly resolved oxygen-evolving photosystem II preparation from spinach thylakoid membrane: EPR and electron transport properties. FEBS Lett 134: 231-234

Berthomieu C, Boussac A, Mäntele W, Breton J, Nabedryk E (1992) Molecular changes following oxidoreduction of cytochrome b_{559} characterized by Fourier transform infrarred different spectroscopy and electron paramagnetic resonance: Photooxidation in photosystem II and electrochemistry of isolated cytochrome b_{559} and iron protoporphyrin IX-bisimidazole model compounds. Biochemistry 31: 11460-11471

Bohner H, Böhme H, Böger P (1980) Reciprocal formation of plastocyanin and cytochrome c-553 and the influence of cupric ion on photosynthetic electron transport. Biochim Biophys Acta 592: 103-112

Burda K, Kruk J, Schmid GH, Strzalka K (2003) Inhibition of oxygen evolution in photosystem II by Cu(II) ions is associated with oxidation of cytochrome b_{559}. Biochem J 371: 597-601.

Buser CA, Diner BA, Brudvig GW (1992) Photooxidation of cytochrome b559 in oxygen-evolving photosystem II. Biochemistry 31: 11449-11459

Cedeño-Maldonado A, Swader JA (1972) The cupric ion as an inhibitor of photosynthetic electron transport in isolated chloroplasts. Plant Physiol 50: 698-701

Clijsters H, van Asche P (1985) Inhibition of photosysnthesis by heavy metals. Photosynth Res 7: 731-740

Droppa M, Horváth G (1990) The role of copper in photosynthesis. Crit Rev Plant Sci 9: 111-123

Faller P, Pascal A, Rutherford AW (2001) Beta-carotene redox reactions in photosystem II: electron transfer pathway. Biochemistry 40: 6431-6440

Gadjieva R, Mamedov F, Renger G, Styring S (1999) Interconversion of Low- and High- Potential forms of Cytochrome b_{559} in Tris-washed Photosystem II Membranes under aerobic and anerobic conditions. Biochemistry 38: 10578-10584

García-Rubio I, Martínez JI, Picorel R, Yruela I, Alonso PJ (2003) HYSCORE spectroscopy in the Cytochrome b_{559} of photosystem II reaction center. J. Am. Chem. Soc (in press).

Jegerschöld C, Arellano JB, Schröder WP, van Kan PJM, Barón M, Styring S (1995) Cu(II) inhibition of the electron transfer through Photosystem II studied by EPR spectroscopy. Biochemistry 34: 12747-12754

Jegerschöld C, MacMillan F, Lubitz W, Rutherford AW (1999) Effects of copper and zinc ions on photosystem II studied by EPR spectroscopy. Biochemistry 38: 12439-12445

Kaminskaya O, Kurreck J, Irrgang KD, Renger G, Shuvalov VA (1999) Redox and spectral properties of cytochrome b_{559} in different preparations of photosystem II. Biochemistry 38:16223-16235

Králová K, Sersen F, Blahová M (1994) Effects of Cu(II) complexes on photosynthesis in spinach chloroplasts. Aqua (aryloxiacetato)copper (II) complexes. Gen Physiol Biophys 13: 483-491

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage. Nature 227: 680-685

Maksymiec W (1997) Effect of copper on cellular processes in higher plants. Photosynthetica 34: 321-342

Maksymiec W, Bazynski T (1999) The role of Ca^{2+} ions in modulating changes induced in bean plants by an excess of Cu^{2+} ions. Clorophyll fluorescence measurements. Physiol Plant 105: 562-568

Mizusawa N, Yamashita T, Miyao M (1999) Restoration of the high-potential form of cytochrome b_{559} of photosystem II occurs via a two-step mechanism under illumination in the presence of manganese ions. Biochim Biophys Acta 1410: 273-286.

Mohanty N, Vass I, Demeter, S (1989) Copper toxicity affects photosystem II electron transport at the secondary quinone acceptor (Q_B). Plant Physiol 90: 175-179

Mor TS, Hundal T, Andersson B (1997) The fate of cytochrome b_{559} during anaerobic photoinhibition and its recovery processes. Photosynth Res 53: 205-213

Nedbal L, Samson G, Whitmarsh J (1992) Redox state of a one-electron component controls the rate of photoinhibition of photosystem II. Proc Natl Acad Sci USA 89: 7929-33

Ortega JM, Roncel M, Losada M (1999) Light-induced degradation of cytochrome *b*559 during photoinhibition of the photosystem II reaction center. FEBS Lett 458: 87-92

Ouzounidou G, Moustakas M, Strasser RJ (1997) Sites of action of copper in the photosynthetic apparatus of maize leaves: kinetic analysis of chlorophyll fluorecence, oxygen evolution, absorption changes and thermal dissipation as monitored by photoacoustic signals. Aust J Plant Physiol 24: 81-90

Pätsikkä E, Aro E-M, Tyystjärvi E (1998) Increase in the quantum yield of photoinhibition contributes to copper toxicity in vivo. Plant Physiol 117: 619-627

Pätsikkä E, Aro E-M, Tyystjärvi E (2001) Mechanism of copper-enhanced photoinibition in thylakoid membranes. Physiol Plant 113: 142-150

Poulson M, Samson G, Whitmarsh J (1995) Evidence that cytochrome b559 protects photosystem II against photoinhibition. Biochemistry. 34: 10932-10938

Rao SBK, Tyryshkin AM, Roberts AG, Bowman MK, Kramer DM (2000) Inhibitory copper binding site on the spinach cytochrome b_6f complex: Implications for Q_o site catalysis. Biochemistry 39: 3285-3296

Renger G, Gleiter HM, Haag E, Reifarth F (1993) Photosystem II: Thermodynamics and kinetics of electron transport from Q_A^- to Q_B (Q_B^-) and deleterious effects of copper (II). Z Naturforsch 48c: 234-240

Roncel M, Ortega JM, Losada M (2001) Factors determining the special redox properties of photosynthetic cytochrome b559. Eur J Biochem 268: 4961-4968.

Sabat SC (1996) Copper ion inhibition of electron transport activity in sodium chloride washed photosystem II particle is partially prevented by calcium ion. Z Naturforsch 51c: 179-184

Samuelsson G, Öquist G (1980) Effects of copper chloride on photosynthetic electron transport and chlorophyll-protein complexes of *Spinacea oleracea.* Plant Cell Physiol 21: 445-454

Sersen K, Králová K, Bumbálová A, Svajlenova O (1997) The effect of Cu(II) ions bound with tridentate Schiff base ligands upon the photosynthetic apparatus. J Plant Physiol 151: 299-305

Schröder WP, Arellano JB, Bittner T, Barón M, Eckert H-J, Renger G (1994) Flash-induced absorption spectroscopy studies of copper interaction with photosystem II in higher plants. J Biol Chem 269: 32865-32870

Shioi Y, Tamai H, Sasa T (1978a) Effects of copper on photosynthetic electron transport systems in spinach chloroplasts. Plant Cell Physiol 19: 203-209

Shioi Y, Tamai H, Sasa T (1978b) Inhibition of photosystem II in the green algae *Ankistrodesmus falcatus* by copper. Plant Physiol 44: 434-438

Stewart DH, Brudvig GW (1998) Cytochrome b_{559} of photosystem II. Biochim Biophys Acta 1367: 63-87

Styring SK, Virgin I, Ehrenberg A, Andersson B (1990) Strong light photoinhibition of electron transport in Photosystem II. Impairment of the function of the first quinone acceptor Q_A. Biochim Biophys Acta 1015: 269-278

Utschig LM, Poluektov O, Tiede DM, Thurnauer MC (2000) EPR investigation of Cu^{2+}-substituted photosynthetic bacterial reaction centers: evidence for histidine ligation at the surface metal site. Biochemistry 39: 2961-2969

Utschig LM, Poluektov O, Schlesselman SL, Thurnauer MC, Tiede DM (2001) Cu^{2+} site in photosynthetic bacterial reaction centers from *Rhodobacter sphaeroides, Rhodobacter capsulatus and Rhodopseudomonas viridis.* Biochemistry 40: 6132-6141.

Vierke G, Struckmeier P (1977) Binding of copper (II) to protein of the photosynthetic membrane and its correlation with inhibition of electron transport in class II chloroplasts of spinach. Z Naturforsch 32c: 605-610

Yruela I, Montoya G, Alonso PA, Picorel R (1991) Identification of the pheophytin-Q_A-Fe domain of the reducing side of the photosystem II as the Cu(II)-inhibitory binding site. J Biol Chem 266: 22847-22850

Yruela I, Montoya G, Picorel R (1992) The inhibitory mechanism of Cu on the photosystem II electron transport from higher plants. Photosynth Res 33: 227-233

Yruela I, Alfonso M, Ortiz de Zarate I, Montoya G, Picorel R (1993) Precise location of the Cu-inhibitory binding site in higher plant and bacterial photosynthetic reaction centers as probed by light-induced absorption changes. J Biol Chem 268: 1684-1689

Yruela I, Gatzen G, Picorel R, Holzwarth AR (1996a) Cu(II)-inhibitory effect on photosystem II from higher plants. A picosecond time-resolved fluorescence study. Biochemistry 35: 9469-9474

Yruela I, Pueyo JJ, Alonso PJ, Picorel R (1996b) Photoinhibition of photosystem II from higher plants: effect of copper inhibition. J Biol Chem 271: 27408-27415

Yruela I, Alfonso M, Barón M, Picorel R (2000) Copper effect on the protein composition of photosystem II. Physiol Plant 110: 551-557

Yruela I, García-Rubio I, Roncel M, Martínez JI, Ramiro MV, Ortega JM, Alonso PJ, Picorel R. (2003) Detergent effect on Cytochrome b_{559} electron paramagnetic

resonance signals in the photosystem II reaction centre. Photochem. Photobiol. Sci. 2: 437-442

TABLE I

Cytochrome b_{559} content in control and Cu(II)-treated PSII membranes in the dark

PSII membrane preparation [1]	Cyt b_{599} redox potential forms		
	HP_{red} (%)	HP_{ox} (%)	LP (%)
Spinach, pH 5.0			
Control	47 ± 3	–	53 ± 3
Cu(II)-treated	–	40 ± 4	60 ± 4
Spinach, pH 6.0			
Control	37 ± 5	–	63 ± 3
Cu(II)-treated	–	30 ± 3	70 ± 5
Spinach, pH 7.8			
Control	25 ± 3	–	72 ± 4
Cu(II)-treated	–	19 ± 5	83 ± 3
Sugar Beet, pH 5.0-7.8			
Control	–	–	100 ± 3
Cu(II)-treated	–	–	100 ± 3

[1] PSII membranes at 200 μg Chl ml^{-1} were incubated without or with 125 μM $CuCl_2$ for 1 min in the dark at 23ºC and subsequently washed twice with 15 mM NaCl and 50 mM Mes-NaOH, pH 5.0-6.0 or 50 mM Tris-HCl, pH 7.8.

FIGURE LEGENDS

Figure 1.- Chemically-induced difference absorption spectra in the α-band region of Cyt b_{559} in oxygen-evolving PSII membranes from spinach (A) and sugar beet (B) incubated for 20 min at 20 ºC in the dark at pH 6.0. Difference spectra are shown as follows: (a) control *minus* ferricyanide (2 mM); (b) hydroquinone (4 mM) *minus* ferricyanide (2 mM); (c) dithionite (2 mM) *minus* ferricyanide (2 mM).

Figure 2.- Reductive potentiometric redox titrations of Cyt b_{559} in oxygen-evolving PSII membranes from spinach (A) and sugar beet (B) at pH 6.0. Control (○), Cu(II)-treated samples (●). Solid curves through the points are the best fits of exponential data to the Nerst equation in accordance with one-electron processes (*n*=1). For details see Materials and Methods.

Figure 3.- Inhibition of oxygen evolution activity during aerobic illumination in the absence (○,△) and presence (●,▲) of 125 μM $CuCl_2$ [Cu(II) per PSII unit = 135] at 23 ºC. (A) pH 5.0; (B) pH 6.0; (C) pH 7.8. PSII membranes at 200 μg of Chl ml^{-1} from spinach (○,●) and sugar beet (△,▲) were illuminated with 3,000 μmol quanta.m^{-2}.s^{-1} in the absence and presence of $CuCl_2$ and then washed twice before measurements (for details see Materials and Methods). 100% activity corresponded to 320, 435 and 67 μmol O_2.mg Chl^{-1}.h^{-1} and 350, 390 and 59 at pH 5.0,

6.0 and 7.8 in control spinach and sugar beet PSII membranes, respectively. 100% activity corresponded to 223, 230 and 36 μmol O_2.mg Chl^{-1}.h^{-1} and 280, 187 and 23 at pH 5.0, 6.0 and 7.8 in Cu(II)-treated spinach and sugar beet PSII membranes, respectively.

Figure 4.- Time course of Cyt b_{559} content in oxygen-evolving PSII membranes from spinach (○,●) and sugar beet (△,▲) during photoinhibitory treatment in the absence (○,△) and presence (●,▲) of 125 μM $CuCl_2$ [Cu(II) per PSII unit = 135] at 23 ºC. (A) pH 5.0; (B) pH 6.0; (C) pH 7.8. Other experimental conditions as in Fig. 3.

Figure 5.- Time course of Cyt b_{559} content in oxygen-evolving PSII membranes from spinach during photoinhibitory illumination treatment in the absence (○,△) and presence (●,▲) of 125 μM $CuCl_2$ [Cu(II) per PSII unit = 135] at 23 ºC. (A) pH 5.0; (B) pH 6.0. ▲,△ content of HP form; ●,○ content of total Cyt b_{559}. Other experimental conditions as in Fig. 3.

Figure 6.- A) SDS-PAGE analysis of PSII membrane preparations from spinach incubated with 125 μM $CuCl_2$ [Cu(II) per PSII unit = 135] after photoinhibitory illumination treatment at pH 6.0. *Lanes* 1-4: control PSII membranes illuminated for 0, 5, 10, 20 min; *lanes* 5-8: Cu(II)-treated PSII membranes illuminated for 0, 5, 10, 20 min. B) Immunoblots with serum anti-D1 subunit and anti-α-subunit of Cyt b_{559} of PSII membrane

preparation from spinach incubated with 125 μM $CuCl_2$ [Cu(II) per PSII unit = 135] at 23 ºC after photoinhibitory illumination treatment at pH 6.0. *Lanes* 1-4: control PSII membranes illuminated for 0, 5, 10, 20 min; *lanes* 5-8: Cu(II)-treated PSII membranes illuminated for 0, 5, 10, 20 min.

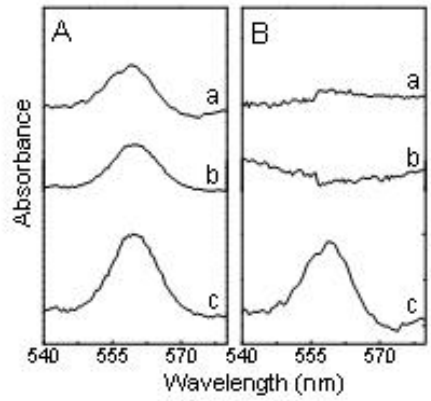

Fig.1

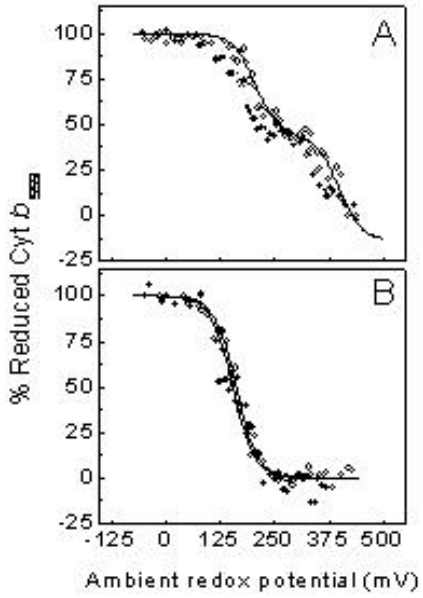
A
B
100
75
50
25
0
-25
% Reduced Cyt b_{559}
-125
0
125
250
375
500
Ambient redox potential (mV)

Fig. 2

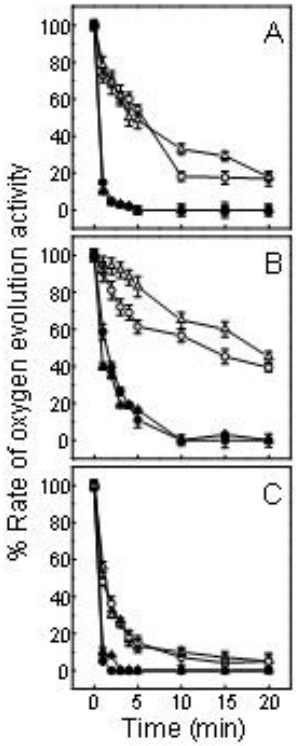
A
B
C
100
80
60
40
20
0
% Rate of oxygen evolution activity
0
5
10
15
20
Time (min)

Fig.3

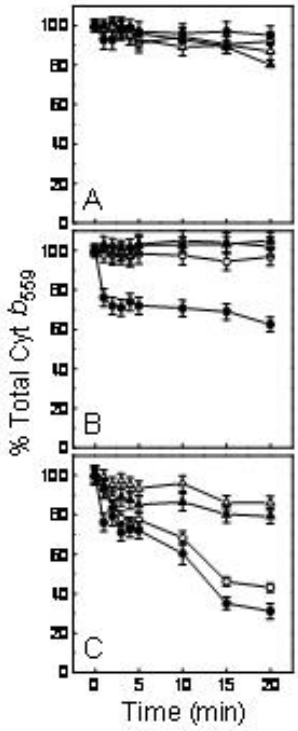

Fig. 4

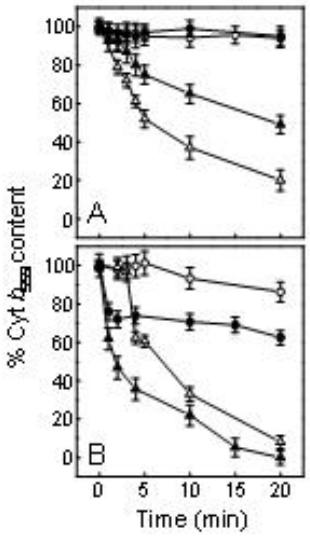

Fig. 5

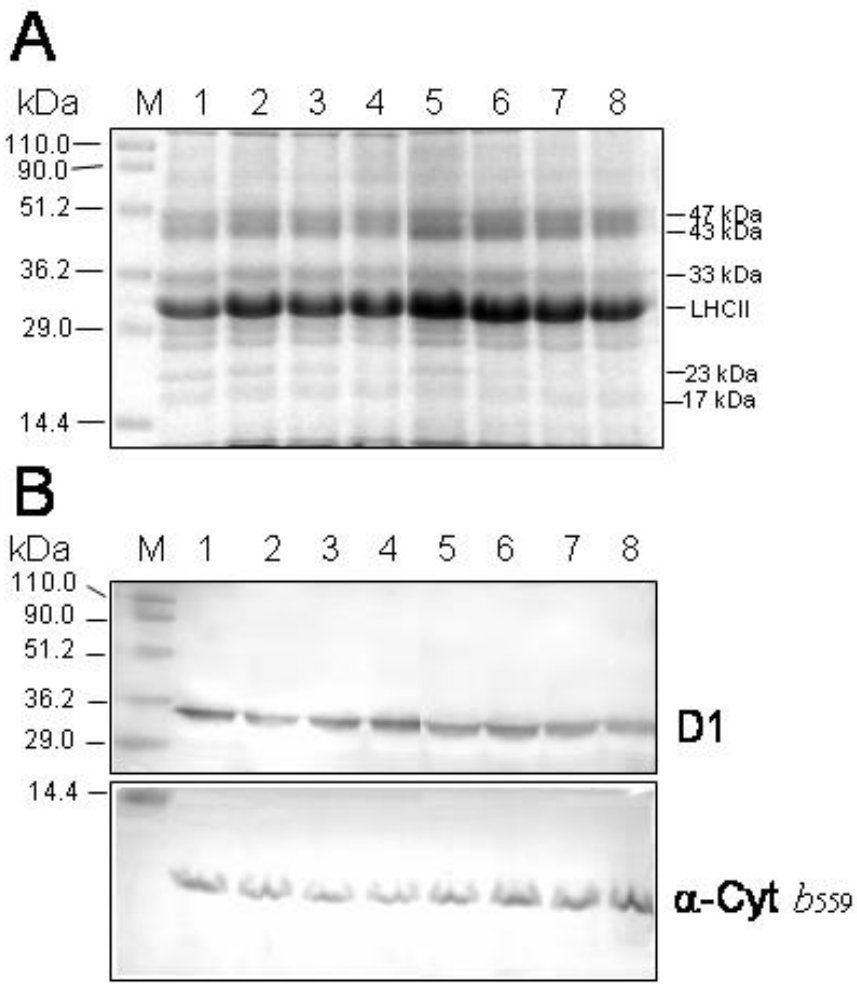
A
kDa
M
1
2
3
4
5
6
7
8
110.0
90.0
51.2
36.2
29.0
14.4
47 kDa
43 kDa
33 kDa
LHCII
23 kDa
17 kDa
B
kDa
M
1
2
3
4
5
6
7
8
110.0
90.0
51.2
36.2
29.0
14.4
D1
α-Cyt b559

Fig. 6