PHOTOREDUCTION OF CYTOCHROME *b*-559 AND PHOTOINHIBITION OF PHOTOSYSTEM II FROM HIGHER PLANTS: EFFECT OF Cu (II) INHIBITION.

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1. Introduction

The photosynthetic activity decreases when oxygenic organisms are exposed to prolonged illumination with high light intensities. This process, which includes the functional impairment of photosystem II (PSII) electron transport and the structural damage of one of the PSII reaction center (RC) proteins (D1), is known as photoinhibition [1]. It seems clear that the primary target of photoinhibition is the PSII RC. However there is a great deal of controversy whether the continuous strong illumination damages the electron transport at the acceptor or the donor side of the PSII. Many data on photoinhibition have been obtained using modified PSII preparations where the electron transport at the donor side or the acceptor side of PSII is impaired by treatment with some reagents [2-4], however the region close to Pheo has received less attention in these kind of studies. Our previous works [5-7] have demonstrated that Cu(II) inhibits electron transport at the acceptor side of PSII close to Pheo- Q_A domain and Cu(II)-inhibited PSII centers are photochemically inactive. The application of photoinhibitory conditions to Cu(II)-treated PSII samples and the comparison between these results and those of the intact systems can help to better understand the relationship between electron transport and photoinhibition .

2. Procedure

Oxygenic PSII-enriched membranes from sugar beet were isolated as described in ref. 5. Tris-treated PSII-enriched membranes were prepared by the method of Allakhverdiev et al. [8]. For cyt *b*-559 photoreduction measurements, samples (60 μg Chl. mL^{-1}) in buffer containing 0.3 mM sucrose, 10 mM NaCl mM Hepes-NaOH, pH 7.8 were incubated with 50 μM (I_{50}/RC) and 125 μM ($2I_{50}/\text{RC}$) of CuCl_2 for 10 min at 4 °C. For photoinhibitory experiments, samples (100 μg Chl. mL^{-1}) in 3 mL of buffer containing 0.3 mM sucrose, 10 mM NaCl and 25 mM Mes-NaOH, pH 6.5 were incubated with 250 μM (I_{50}/RC) and 500 μM ($2I_{50}/\text{RC}$)

of CuCl_2 and immediately exposed to heat filtered white light ($5,500 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$) under constant stirring. The temperature was maintained at 25°C or 4°C (see figure legend) depending of the experimental conditions. Anaerobic conditions were achieved by the addition of 10 mM glucose, 0.23 mg/mL glucose oxidase and 80 $\mu\text{g}/\text{mL}$ catalase before light treatment and maintaining N_2 bubling during illumination. The Cu(II) -inhibited samples were washed twice with 10 mM NaCl and 25 mM Mes-NaOH , pH 6.5 and resuspended in the same buffer plus 0.3 M sucrose after the illumination treatment and before oxygen evolution measurements. Oxygen evolution activity was measured with a Clark-type oxygen electrode [5]. The rate of DCIP photoreduction was spectrophotometrically monitored at 590 nm. The photoreduction kinetics of cyt *b*-559 were measured at 560 nm in the second range in both aerobic and anaerobic conditions with a Beckman DU 640 spectrophotometer. SDS-PAGE was performed in 15% acrylamide gels containing 6 M urea. After transfer to a nitrocellulose membrane, proteins were detected with a rabbit anti-D1 serum and goat anti-rabbit IgG coupled to horseradish peroxidase as a secondary antibody. The bands were quantified by densitometry.

3. Result and Discussion

We have studied the effect of Cu(II) -inhibition on the photoreduction of cytochrome *b*-559, loss of oxygen evolution activity and D1 degradation during aerobic and anaerobic photoinhibition. The effect of Cu(II) inhibition on the photoreduction of cyt *b*-559 is showed in Fig. 1.

Figure 1.- A) Light-induced absorbance change in the spectral range 540-585 nm after 25 s of continuous illumination of dark-adapted PSII membranes at pH 7.8 without addition (●) and pre-incubated with $50 \mu\text{M}$ CuCl_2 (I_{50}/RC) (■) for 10 min. B) Time course of the light-induced absorbance change at 560 nm in the absence (a), and the presence of $50 \mu\text{M}$ (I_{50}/RC) (b) and $125 \mu\text{M}$ ($2I_{50}/\text{RC}$) (c) of CuCl_2 .

The positive band centered at 560 nm, which correspond to a fraction of 40% of total cyt *b*-559 reduced, decreased in Cu(II)-inhibited samples (Fig 1A). The photoreduction kinetics of cyt *b*-559 in the 0-250 s range were also measured in the absence and presence of Cu(II). The PSII intact samples gave positive kinetics, however in the presence of increasing amounts of CuCl₂ (>50 μ M) negative kinetics were measured (Fig. 1B). This Cu(II) concentration corresponds to 75-80% inhibition of oxygen evolution activity [5]. The data suggest that Cu(II) inhibits photoreduction of cyt *b*-559. The possible chemical interaction between cyt *b*-559 and Cu(II) was discarded based on the Cu(II) has no effect on the reduced minus oxidized spectrum (data not shown). In intact systems it has been proposed that cyt *b*-559 mediates cyclic electron transport within PSII where it is able to accept electrons from the reduced plastoquinone pool or the bound quinone Q_B and then it can be oxidized directly *via* a monomeric chlorophyll. If we accept this function for cyt *b*-559 our results are consistent with the fact that the electron transport close to Q_A niche is impaired in Cu(II)-treated samples, as described in our previous works [5-7]. In addition to the Cu(II) effect on the photoreduction of cyt *b*-559, the data showed a second Cu(II) effect at higher Cu(II) concentrations (>50 μ M) which induces the photooxidation of cyt *b*-559.

Figure 2.- Inhibition of oxygen evolution activity during aerobic (closed symbols) and anaerobic (open symbols) photoinhibition in the absence (■, □) and presence (●, ○) of 250 μ M CuCl₂ (I_{50} /RC) at (A) 25 °C and (B) 4 °C. Samples were washed twice before measurements. The 100% oxygen evolution activity without addition was about 560 μ mol O₂.mg Chl⁻¹.h⁻¹ using DCBQ as electron acceptor.

The loss of oxygen evolution was markedly faster in Cu(II)-treated PSII samples when the photoinhibitory experiments were done in the presence of oxygen at 25 °C (Fig. 2A). However, similar kinetics were observed in anaerobic conditions. At 4 °C these differences were smaller and the loss of activity was slower than

that at 25 °C (Fig. 2B). It is known that photoinhibition is a temperature dependent phenomenon and it increases at higher temperatures [4]. A comparison of Fig. 1A and 1B indicates that the Cu(II) effect on photoinhibition is also temperature depending. After illumination, the presence of Cu(II) did not affect the D1-protein degradation rate. In contrast, a faster degradation was measured in Tris-washed PSII membranes (data not shown). These results indicate that the differences in the activity curves (Fig. 2) are unlikely to be due to the loss of the protein itself. D1 protein was degraded 20% after 10 min illumination while the loss of oxygen evolution activity in non-treated and Cu(II)-treated PSII preparations was 40% and 92%, respectively. The more pronounced impairment of the electron transport compared to D1-protein degradation has also been described by others [3]. It seems, therefore, that Cu(II)-inhibitory and Tris-treatment effects are the consequence of two different molecular mechanisms. Since the Tris-treatment removes the extrinsic proteins that stabilize the manganese complex of the PSII donor side, it is possible to suggest in these experimental conditions that the Cu(II)-inhibitory site is not located on the donor side of PSII. Moreover, the rate of DCIP-reduction in intact PSII samples decreased up to 50% and 68% after 15 min and 30 min of illumination, respectively. After these times, addition of 300 μ M DPC increased the rate of DCIP reduction, being then the loss of activity caused by photoinhibition of 9% and 13%, respectively. The increment in the control DCIP-reduction rate (non illuminated) was 28%. These findings provide the proportion that photoinhibition affects the electron transport on the donor side of PSII [2]. The addition of DPC to the photoinhibited Cu(II)-treated samples had no effect on the DCIP reduction indicating that photodamage in these Cu(II)-modified systems does not occur on the donor side of PSII.

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