Early stage toxicity of excess copper to photosystem II of Chlorella pyrenoidosa—OJIP chlorophyll a fluorescence analysis

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Abstract

Acute toxicity of excess Cu on the photosynthetic performance of Chlorella pyrenoidosa was examined by using chlorophyll a fluorescence transients and JIP-test after exposure to elevated Cu concentrations for a short time period. High Cu concentration resulted in a significant suppression in photosynthesis and respiration. The absorption flux (ABS/RC) per PSII reaction center increased with increasing Cu concentration, but the electron transport flux (ET/RC) decreased. Excess Cu had an insignificant effect on the trapping flux (TR/RC). The decline in the efficiency, with which a trapped exciton can move an electron into the electron transport chain further than QA- (ΨA), the maximal quantum yield of primary photochemistry (ΦP0), and the quantum yield of electron transport (ΦE0) were also observed. The amount of active PSII reaction centers per excited cross section (RC) was also in consistency with the change of photosynthesis when cells were exposed to excess Cu concentration. JIP-test parameters had a good linear relationship with the inactivation of PSII reaction centers and the inhibition of electron transport in the acceptor side.

Key words: Chlorella pyrenoidosa; chlorophyll a fluorescence; Cu; PSII
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Introduction

Copper is a widespread contaminant in natural freshwater system released mainly from mining and smelting activities (Gledhill et al., 1997). It is also considered to be one of the most toxic trace metals to plants although copper is required for electron transport in photosynthesis and by various enzyme systems (e.g., amine oxidase, cytochrome c oxidase) as an essential element for metabolic and physiological processes. 96h-EC50 of Cu in the green algae Closterium lunula and Chlorella pyrenoidosa was observed to be 199.5 and 67 µg/L, respectively (Yan et al., 2001; Zhang et al., 2001). Toxic effects of Cu on algae have also been well documented. For example, it has been reported that excess Cu could inhibit photosynthetic O2 evolution (Rijstenbil et al., 1994; Yu et al., 1994; Bhargava et al., 2008) and gene expression (Jamers et al., 2006), change the permeability of plasma membrane (De Filippis, 1979), inhibit nitrate uptake (Harrison et al., 1977), and reduce the levels of photosynthetic pigments, including chlorophyll a and b and accessory pigments such as carotenoids (Van and Clijsters, 1990). Photosystem II (PSII) has also been shown to be highly sensitive to Cu (Shioi et al., 1978). However, the toxic mechanisms of copper to PSII are still not fully understood and remain controversial (Barón et al., 1995).

Chlorophyll a (chl-a) fluorescence has been proved to be a very useful, non-invasive, and reliable tool for the study of the photosynthetic apparatus and more specifically the behavior of PSII (Krupa et al., 1993; Lu et al., 2000; Xia et al., 2004; Krüger et al., 1997). The fluorescence kinetics detected by means of direct, time-resolved fluorescence measurements with the resolution of 10 µs has been shown to be a polyphasic rise, including phases O, J, I and P. Moreover, those changes in PSII photochemistry can be quantified through the JIP test, which is derived from the O-J-I-P rise of chl-a fluorescence transients based on the theory of energy flux in biomembranes (Strasser, 1978, 1981). Environmental stresses such as high temperature (Strasser, 1997), light (Lu and Vonshek, 1999), salinity (Xia et al., 2004), and heavy metal (such as Cr) (Appenroth et al., 2001) induced the change of OJIP chl-a fluorescence transients. However, little information is available for the effects of excess Cu on the photosynthetic performance in algae by analyzing the so-called JIP-test, and very little is known about changes in PSII energy fluxes and yields during the initial stages of the responses to elevated Cu concentration in algae. These early responses may be important because they may determine whether cells are able to survive the fast transition and then undergo a longer metabolic stage of acclimation to elevated Cu.

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concentrations.

In the present study, we examined the change in the chl-α fluorescence transients (O-J-I-P) when *Chlorella pyrenoidosa* was cultured in different Cu concentrations for a short time period. The acute toxicity to PSII function of *C. pyrenoidosa* was also assessed by using JIP-test.

1 Materials and methods

1.1 Algal culture

*Chlorella pyrenoidosa* was obtained from the Institute of Hydrobiology, the Chinese Academy of Sciences, and cultivated in a Bristol culture medium at 25°C with aerated air. Light intensity was about 100 µmol/(m²·s) under a light:dark cycle of 12 h:12 h. Exponentially grown cells were harvested and resuspended in a fresh media containing elevated Cu concentrations of 0, 2, 5, 10, 20, 40 µmol/L and incubated in the condition as described above for 12 h. Copper as CuSO₄·5H₂O (analytical grade) was purchased from Guangzhou Chemical Reagent Factory, China.

1.2 Determination of oxygen evolution

Photosynthetic oxygen evolution and dark respiratory oxygen consumption were measured using an O₂ electrode connected to a biological oxygen monitor (YSI 5300, USA) under a constant temperature of 25°C. A halogen lamp was used as light source and photosynthetic photon flux was about 400 µmol/(m²·s). The cell was counted by a Z1-particle counter (Beckman Coulter, USA). Chlorophylls were extracted in 100% N,N-dimethylformamide (DMF) and determined according to the method described by Moran (1982).

1.3 Chl-α fluorescence parameters and JIP test

Chl-α fluorescence transients were measured at room temperature with a plant efficiency analyser (Hansatech Instruments Ltd., UK). The fluorescence signals were detected by a high performance PIN photodiode detector and recorded within a time span from 10 µs to 1 s with a data acquisition rate of 10⁷ readings/s for the first 2 ms and followed by 10³ readings/s. All samples were dark-adapted for 15 min prior to measurements. Figure 1 is a highly simplified model of energy fluxes (Strasser, 1978, 1981). Absorption flux (ABS) denotes the photons absorbed by the antenna pigments Chlα*. Part of this excitation energy is dissipated by fluorescence and heat. Another part is channelled as trapping flux (TR) to reaction center. There, the excitation energy is converted to redox energy by reducing an electron acceptor Qₐ to Qₐ⁻, thus creating an electron transport (ET) that maintains the metabolic reactions of the photosynthetic apparatus.

Chl-α fluorescence transients were analyzed according to JIP-test developed by Strasser and Strasser (1995). The energy fluxes for absorption (ABS), trapping (TR₀) and electron transport (ET₀) per reaction center (RC), the maximum quantum yield of primary photochemistry (Φₚ₀), the efficiency with which a trapped exciton can move an electron into the electron transport chain further than Qₐ⁻ (Ψ₀), the quantum yield of electron transport (ϕₑₑ) and the amount of active PSII reaction centers per excited cross section (RC/CS) were calculated by Eqs. (1)–(7) (Appenroth et al., 2001):

\[
\text{ABS/RC} = \frac{M₀}{V_j} \times \frac{F_m - F_o}{F_m} \quad (1)
\]

\[
\text{ET₀/RC} = \frac{M₀}{V_j} \times (1 - V_j) \quad (2)
\]

\[
\text{TR₀/RC} = \frac{M₀}{V_j} \quad (3)
\]

\[
\chi₀ = \frac{\text{TR₀/ABS}}{\text{ABS}} = \frac{F_m - F_o}{F_m} \quad (4)
\]

\[
\chi₀ = \frac{\text{ET₀/ABS}}{\text{ABS}} = \frac{F_m - F_o}{F_m \times (1 - V_j)} \quad (5)
\]

\[
\Psi₀ = \frac{\text{ET₀/TR₀}}{1 - V_j} \quad (6)
\]

\[
\text{RC/CS} = F_m \times \left( \frac{F_m - F_o}{F_m} \right) \times \frac{V_j}{M₀} \quad (7)
\]

where, \( F_o \) is the fluorescence intensity at 50 µs, \( F_m \) is the maximum fluorescence intensity, \( dV/dt₀ \) is the initial slope at the beginning of the variable fluorescence transients (theoretically at time 0), \( dV/dt₀ = \frac{F⁰₀ - F_m}{\Delta F_m - F_m} \times M₀ = 4 \times \frac{dV}{dt₀} \), \( V_j \) is the relative variable fluorescence at phase J.  

1.4 Statistical analysis

The data were analyzed according to one-way analysis of variance (ANOVA) followed by a least significant difference test or linear regression analysis. Minimum significance level was set as \( P < 0.05 \).

2 Results

Effects of excess Cu on the photosynthesis and respiration of *C. pyrenoidosa* in short period of incubation...
are shown in Fig. 2. Elevated Cu concentration from 2 to 40 µmol/L significantly decreased the photosynthetic 
O₂ evolution by 10.0% to 96.1% (P < 0.05), compared 
with the control. Dark respiration rate was significantly 
inhibited by Cu concentrations higher than 5 µmol/L (P < 0.05), for example, being 65.2% of the control value when cells were grown in the highest Cu concentration of 40 µmol/L.

Figure 3 shows the chl-a fluorescence transients in 
the cells exposed to different Cu concentrations in short 
time period, and it revealed a polyphasic rise of chl-a 
fluorescence, named by O, J, I and P. Steps J, I and P 
were observed to occur at approximately 2, 30 and 400 
ms, respectively. The chl-a fluorescence yield at phases 
O, J, I and P decreased sharply with the elevated Cu 
concentration.

To evaluate the effects of excess Cu on PSII, OJIP chl-a 
fluorescence parameters were calculated by JIP-test. The 
relative variable fluorescence (Vₐ) at J step had an obvious 
rise with increasing Cu concentration (P < 0.05), reached 
up to 126.8% of the control value when cells were exposed 
to the highest Cu concentration (Fig. 4). The energy fluxes 
for ABS, TR and ET per RC in response to elevated Cu 
concentrations are presented in Fig. 5a. Copper induced 
the changes of ABS/RC, ET₀/RC and TR₀/RC to varied 
degrees, significant increase in ABS/RC (P < 0.05), de- 
crease in ET₀/RC (P < 0.05) and constant in TR₀/RC (P > 0.05) on exposure to increased Cu concentrations 
were observed. In contrast, the quantum yield of primary pho-
tochemistry (qₚ₀) declined significantly when cells were 
exposed to increased Cu concentration (P < 0.05). Similar 
results were obtained in the yield of electron transport per 
trapped exciton (Ψ₀) as well as for the quantum yield of 
electron transport (qₑ₀) (P < 0.05) (Fig. 5b). qₚ₀, Ψ₀ and 
qₑ₀ significantly decreased by 30.9%, 75.8% and 64.0%, 
respectively, when cells were exposed to the highest Cu 
concentration.

Figure 6 presents the effects of excess Cu on the amount 
of active PSII reaction centers per excited cross section 
(RC/CS), and Cu exposure to 2, 5, 10, 20, and 40 µmol/L 
induced a significant decrease of RC/CS by 39.5%, 41.9%, 
59.8%, 55.1% and 61.2% compared with the control, 
respectively. Photosynthetic O₂ evolution increased with 
the increasing ϕₚ₀, Ψ₀ and ϕₑ₀, and exhibited a good linear 
relationship with them.

3 Discussion

Inhibition of photosynthesis by Cu had been observed in 
some higher plants and algae (Fernandes and Henriques, 
1991; Perale-Vela et al., 2007). In the present study, 
we have also demonstrated the Cu-induced inhibition of 
photosynthesis which occurs even in low Cu concentration 
(2 µmol/L). The reduction of photosynthesis could be well 
associated with the change of PSII function in excess Cu
**Fig. 5** Effects of excess Cu on the energy fluxes for absorption (ABS), trapping (TR) and electron transport (ET) per reaction center (RC) (a) and on the quantum yield of primary photochemistry ($\phi_P$), the efficiency that a trapped exciton by an active reaction center can be move an electron further than $Q_A^- (\Psi_0)$, and the quantum yield for electron transport ($\phi_E$) (b) in *C. pyrenoidosa* in short time (12 h). Data are means ± SD ($n = 5$).

**Fig. 6** Changes of the amount of active PSII reaction centers per excited cross section (RC/CS) when cells were exposed to increased Cu concentration in short time period (12 h). Data are means ± SD ($n = 5$).

Concentration (Shioi *et al.*, 1978). The role of Cu$^{2+}$ as an inhibitor of photosynthetic electron transport had been proposed that both the donor and acceptor of PSII can be the most sensitive sites for Cu (Schröder *et al.*, 1994; Yruela *et al.*, 1996). To further verify the site of action of Cu on PSII, the fast chl-a fluorescence signals were detected. In this study, *Chlorella* cells showed a typical polyphasic rise of chl-a fluorescence after the cells were dark-adapted, which was consistent with previous reports for higher plant, green algae (Appenroth *et al.*, 2001; Neubauer and Schreiber, 1987; Strasser and Strasser, 1995). The OJIP transients reflected the successive reduction of the electron acceptor pools of PSII (Govindjee, 1995). The J step considered to be associated with an accumulation of $Q_A^- Q_B^-$ form as demonstrated by experimental results and theoretical simulations. Accordingly, steps I and P were suggested to reflect an accumulation of the $Q_A^- Q_B^-$ and $Q_A^- Q_B^{2-}$-form (Lázár, 1999; Strasser *et al.*, 1995), respectively. Therefore, OJIP chl-a fluorescence transients can be used to determine the status of electron transport. Our study confirmed that electron transport at the acceptor side of PSII by JIP-test analysis was a Cu-induced inhibitory site when *Chlorella* cells exposed to excess Cu. The increase of $V_J$ have also indicated that the accumulation of $Q_A^-$ is strongly increased because $V_J$ can be taken as a measurement of the fraction of the electron acceptor QA being in its reduced state (Strasser *et al.*, 1995). Furthermore, a decreased value in the efficiency with which a trapped exciton can move an electron into the electron transport chain further than $Q_A^- (\Psi_0)$ and in the quantum yield of electron transport ($\phi_E$) in exposure to excess Cu also indicated an inhibition of the downstream of $Q_A^-$. Therefore, high concentration of Cu may inhibit the electron transport from $Q_A^- Q_B^-$. Copper had a regulatory role in photosynthetic electron transport as a part of polypeptides involved in electron transport (Droppa *et al.*, 1987). On the acceptor side, Cu$^{2+}$ interactions with the phloerythrin-$Q_A^- Q_B^- Q_B^{2-}$-domain or Cu$^{2+}$-induced modifications in the amino acid or lipid structure close to the $Q_A^-$ and $Q_B^-$-binding sites had been suggested to cause the inhibition of electron transport (Jegerschöld *et al.*, 1995; Yruela *et al.*, 1996). However, the present study can not determine whether the donor side of electron transport was affected by high concentration of Cu.

In this study, the decrease in the amount of active PSII reaction centers per excited cross section (RC/CS) indicated that excess Cu inactivated the reaction center, which was in agreement with the report by Kipper *et al.* (2002). Some authors had also suggested that the PSII reaction center was the target of heavy metal-induced inhibition, such as all components participating in the energy conversion within PSII RCs (Jegerschöld *et al.*, 1995; Yruela *et al.*, 1996). Hsu and Lee (1993) reported...
that Cu created a lesion close to the reaction center, which increased the probability of dissipation of the incoming excitation energy. It has been shown that PSII reaction centers have some heterogeneity and there are two different populations of PSII reactions centers, i.e., the active centers and the inactive centers, and ABS/RC and TR0/RC here refer only to the active (reduction of QA to QA−) centers (Lu and Vonshak, 1999). The change of ABS/RC, TR0/RC and ET0/RC in varied degrees also indicated that the PSII apparatus in *C. pyrenoidosa* maintained the equilibrium of energy fluxes for absorption, trapping and electron transport through a down-regulation of PSII reaction center in response to high Cu concentration. Photosynthetic O2 evolution had usually been regarded as an indicator of heavy metal stress. Good linear relationship between the parameter of JIP-test such as ϕp, ψ0 and ϕe0, and photosynthetic O2 evolution in this study indicated that the suppression of photosynthesis may be due to the change of the energy flux yield in high Cu concentration.

4 Conclusions

The present results show a sensitive and rapid Cu inhibition of photosynthesis and excess Cu induces a decrease in the efficiency with which a trapped exciton can move an electron into the electron transport chain further than QA− (ψ0), the maximal quantum yield of primary photochemistry (ϕp), the quantum yield of electron transport (ϕe), and the amount of active PSII reaction centers per excited cross section (RC/CS). Our study also suggest that the decrease in photosynthesis may be a result of the inactivation of PSII reaction centers and inhibition of electron transport in the acceptor side. In addition, it has also been demonstrated that the analysis of OJIP chlorophyll a fluorescence transients can be very useful in detecting early stage toxicity caused by excess Cu.

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