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HEAVY METAL-INDUCED INHIBITION OF PHOTOSYNTHESIS: TARGETS OF *IN VIVO* HEAVY METAL CHLOROPHYLL FORMATION[1]

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The targets of heavy metal (here Cu^{2+} and Zn^{2+}) attack on the photosynthetic apparatus of algae belonging to different phyla were investigated. Experiments with the green alga *Scenedesmus quadricauda* confirmed previous findings that according to the irradiance level two different phenomena occur, which were further characterized by specific changes in several photosynthetic parameters. The reaction occurring under low irradiance (*shade reaction*) is characterized by heavy metal substitution of Mg^{2+} in chl molecules bound predominantly in the light harvesting complex II of Chlorophyta (LHC II). Under high irradiance (*sun reaction*) the LHC II chls are inaccessible to substitution and the damage occurs in the PSII reaction center instead. Algae with antenna proteins other than the LHC II did not show the two types of heavy metal attack at different irradiances. In red algae (*Antithamnion plumula*), low Cu^{2+} concentrations induced the sun reaction even at very low irradiance. In brown algae (*Ectocarpus siliculosus*) the shade reaction occurred even in saturating irradiance. These results also indicate that despite some similarity in their features, the primary step of the sun reaction and photoinhibition is different.

Key index words: **Chlorophyta; copper; heavy metal substituted chlorophyll; Phaeophyta; photosynthesis; Rhodophyta; zinc**

Abbreviations: **FCP, fucoxanthin chl protein; F_0, minimal fluorescence yield of a dark-adapted sample in nonactinic measuring light; F_m, maximum fluorescence yield of a dark-adapted sample; F_s, steady state fluorescence under the given actinic irradiance; F_v, variable fluorescence: $F_v = F_m - F_0$; hms-chl formation, formation of heavy metal substituted chl via substitution of the natural central ion of chl, Mg, by heavy metal ion or via incorporation of these ions into Pheo; LHCP, light harvesting chl protein; LHC II, light harvesting complex II of Chlorophyta; Pheo, pheophytin; Q_A, plastoquinone a in the PSII reaction center; RC, photosynthetic reaction center; TL, Thermoluminescence; total chl, the sum of chlorophyll(ide)s, pheophytins, pheophorbides, and transmetalated chl**

Heavy metals, such as Cu, Zn, Ni, and Mn, are essential trace elements for photosynthetic organisms. Thus, very low concentrations of their ions are indispensable components of nutrient media in which these organisms grow. In higher concentrations, however, these metals have severe toxic effects. Effects of heavy metals on plant metabolism have been subject to extensive research for several decades (reviewed e.g. in Barón et al. 1995, Fernandes and Henriques 1991, Krupa 1999, Maeda and Sakaguchi 1990). It is generally assumed that heavy metal resistance results

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in most cases either from the production of chelating substances that detoxify the heavy metals (e.g. Gledhill et al. 1999, Kneer and Zenk 1992) or from exclusion mechanisms preventing their accumulation inside the cell (Foster 1977). Toxicity may result in diverse effects, which depend on the type of plant, the nature and concentration of the metal, and the environmental conditions accompanying heavy metal stress. Among the mechanisms proposed to contribute to heavy metal damage are inhibition of enzymes (Stobart et al. 1985), inhibition at various sites of PSII reaction centers (RCs) (Barón et al. 1995, Clijsters and Van Assche 1985), enhancement of photoinhibition (Vavilin et al. 1995) and oxidative stress (Clijsters et al. 1999, Luna et al. 1994, Okamoto et al. 1996, Weckx and Clijsters 1996), the impediment of plastocyanin function (Kimimura and Katoh 1972), changes in lipid metabolism (Jones and Harwood 1993), and disturbances in the uptake of essential microelements (Zolotukhina 1995). However, many of the effects on enzymes and electron transport were examined only *in vitro* (discussed e.g. by Barón et al. 1995), and some of them either could not be observed to operate *in vivo* (Clijsters and Van Assche 1985, Sheoran et al. 1990) or could not be confirmed by later *in vitro* studies (Yruela et al. 1991). In several studies, heavy metals were applied at extremely high concentrations, which are far from being ecologically relevant (e.g. Weckx and Clijsters 1996).

One mechanism leading to inhibition of photosynthesis by heavy metals under environmentally relevant concentrations of the latter is their substitution for Mg^{2+} in the chl molecules. Küpper et al. (1996, 1998) described the consequences of this substitution for higher plants and green algae, and they discussed why heavy metal substituted chls (hms-chls) are unsuitable for photosynthesis. Other authors have published data that show *in vivo* formation of hms-chls in cyanobacteria (Kowalewska and Hoffmann 1989, Kowalewska et al. 1992), in unicellular algae such as *Chlorella* (De Filippis 1979, Gross et al. 1970), and in the photosymbionts of lichens (Puckett 1976). They did not study, however, the relationship between hms-chl formation and inhibition of photosynthesis.

In Chlorophyta, Küpper et al. (1996, 1998) found that the character of heavy metal attack strongly depends on the irradiation regime. Two types of processes were observed.

1) Under low irradiance (exact value depends on plant species, irradiance preadaptation, and length of the dark phase) interrupted by long dark intervals, massive substitution of Mg^{2+} in chl molecules by heavy metals (hms-chl formation) occurs. This leads to the type of damage that we call *shade reaction*. The large fraction of the total chl substituted in the shade reaction indicated that antenna chl was involved. The precise localization of hms-chl formation (light harvesting complex II of Chlorophyta [LHC II] or inner antenna) has not been specified. 2) In high irradiance, only a small fraction of total chl (<2%) was accessible to hms-chl formation, whereas the bulk of pigments became bleached in parallel with the destruction of the photosynthetic apparatus. This phenomenon has been named *sun reaction*. There were some indications that the small amount of hms-chl formation in sun reaction might take place in the PSII RC (Küpper et al. 1996, 1998).

In the present study we analyze the targets of the heavy metal chl formation during the shade and sun reactions using two approaches. (1) Cu^{2+} induced shade reaction and sun reaction in the green alga *Scenedesmus quadricauda* was examined by measuring chl fluorescence kinetics and photosynthetic oxygen evolution. Furthermore the pigment composition of the algae was analyzed *in vivo*, in extracts, and in pigment protein complexes prepared by native ("green") gel electrophoresis. (2) The effect of heavy metals (Cu^{2+}, Zn^{2+}) under low and high irradiance was investigated with algae having antennae that differ from the LHC II of the Chlorophyta: the red alga *Antithamnion plumula* and the brown alga *Ectocarpus siliculosus*.

MATERIALS AND METHODS

Plant material and culture conditions. The brown alga *Ectocarpus siliculosus* (Dillwyn) Lyngb. (strain Port Aransas) and the red alga *Antithamnion plumula* (Ellis) Thur. were kindly provided by D. G. Müller and I. Maier (Universität Konstanz). The green alga *Scenedesmus quadricauda* (Turpin) Bréb. (strain Greifswald 15) was obtained from the culture collection of the Botanical Institute (ASCR, Třeboň). Irradiance and temperature conditions for all experiments are given in Table 1.

Scenedesmus was grown in a modified medium of Šetlík et al. (1972): KNO_3 20 mM, KH_2PO_4 2.5 mM, $MgSO_4 \cdot 7H_2O$ 5 mM, $CaCl_2 \cdot 6H_2O$ 14 μM, H_3BO_3 50 μM, $MnSO_4 \cdot 4H_2O$ 7 μM, $ZnSO_4 \cdot 7H_2O$ 5 μM, $Na_2MoO_4 \cdot 2H_2O$ 1 μM, $CoSO_4 \cdot 7H_2O$ 5 μM, $(NH_4)_2Fe(SO_4)_2 \cdot 6H_2O$ 50 μM, pH 6.8. Cultures were aerated with air enriched with 2% CO_2. Cells incubated with or without copper were taken from a continuously maintained master culture, diluted to an optical density (at 750 nm) of 0.05 or 0.1, and incubated under the conditions listed in Table 1. Separate master cultures were grown for the low light and high light treatments, that is, cells were adapted to the respective irradiances before the experiments with copper were started.

Ectocarpus and *Antithamnion* were grown in artificial seawater medium1 (Maier and Calenberg 1994), with two modifications. Ascorbic acid (0.1 mM) was used instead of EDTA, because EDTA complexed copper so strongly that it drastically reduced its availability to the algae. The medium was adjusted to pH 6.8 instead of pH 7.5 to increase the solubility of heavy metals.

The algae were treated with $CuSO_4$ or $ZnSO_4$ (p.a., Merck, Darmstadt, Germany). Most experiments were carried out with Cu^{2+}, but important results were checked by additionally using Zn^{2+}. In experiments with *Scenedesmus*, metals were added only at the beginning of the experiment (batch culture), so that their uptake by the cells caused a gradual decline of their extracellular concentration during the treatment. In experiments with filamentous algae, the heavy metal concentration was maintained at 5×10^{-7} to 5×10^{-4} mol·L^{-1} by exchanging solutions continuously using peristaltic pumps (flow rate, 400 mL·d^{-1}; resident volume, approx. 90 mL). Algae were not washed out with the medium but were retained by the lid of the Petri dish (90 mm diameter) in which they were grown. A mixture of 97% air and 3% CO_2 aerated the medium in the storage bottles.

Altogether, 14 experiments with *S. quadricauda* were carried out, with a total of 22 Cu^{2+} and 4 Zn^{2+} treatments in low light and 16 Cu^{2+} treatments in high light. Four experiments with *A. plumula* were performed, consisting of 14 Cu^{2+} treatments in low light and 2 Cu^{2+} treatments in high light. In the case of *E. si-*

TABLE 1. Light and temperature conditions.

Organism	Type of experiment	Light intensity (μmol photons $\cdot m^{-2} \cdot s^{-1}$) (PAR)	Light period (h)	Dark period (h)	Average temperature (°C)
Scenedesmus	Shade[a]	20	12	12	30
	Sun[b]	400	14	10	30
Ectocarpus and *Antithamnion*	Shade[a]	8	14	10	18
	Sun[b]	140	16	8	18

All experiments were carried out under artificial radiation sources with a light:dark rhythm. In all experiments cool white Tesla® fluorescent tubes were used as light source.

[a] "Shade" conditions were set up in a way that average net photosynthesis was just above the compensation point to simulate shady conditions in nature.

[b] "Sun" means a (nearly) saturating but not inhibitory light intensity.

liculosus, eight experiments were carried out, with altogether 15 Cu^{2+} and 6 Zn^{2+} treatments in low light and 6 Cu^{2+} treatments in high light.

Irradiance-dependent differences in antenna accessibility were studied by growing the algae for several weeks either in low irradiance with a long dark phase or in high irradiance with a short dark phase (Table 1). In low irradiance treatments Cu^{2+} was added in the middle of the dark phase, whereas in high-irradiance treatments Cu^{2+} was added in the middle of the light phase.

Nondenaturing electrophoresis ("green gels"). The *Scenedesmus* cultures were first concentrated by filtration, and then the cells were pelleted by centrifugation (4000*g*, 10 min), washed with buffer A (25 mM Tris/HCl, 1 mM aminocaproic acid, 1 mM aminobenzamidine, pH 7.5), pelleted again by centrifugation, resuspended in a minimum amount of buffer A, frozen in liquid nitrogen, and stored in darkness at −70° C. Green gels were prepared according to Xiong et al. (1997), with the following modification. For solubilization of thylakoids in the stressed sample, the same amount of dodecylmaltoside was added as in the control. This yielded better solubilization and separation on the gel than if the concentration of the detergent in the stressed sample was lowered in accord with the lower concentration of chl. The gels consisted of 5%–10% polyacrylamide gradients containing 0.1% deriphat.

Hms-chl formation in isolated PSII RCs. For these analyses we used RC samples prepared by the procedure of Šetlíková et al. (1995). This procedure involves elution of RCs from a Cu-affinity column. To address the question of whether this method caused reduced activity due to hms-chl formation, we dried and extracted the samples as described below.

Extraction of pigments. Algal samples were washed in buffer A (see above), pelleted by centrifugation, lyophilized in the dark, and ground with sand. Green and red algal samples were then extracted for 2 days (in the dark) with cyclohexane (Uvasol® or LiChrosolv®, Merck) containing 0.5% of isopropanol (saturated/dried with $NaHCO_3$, $CaCl_2$, and K_2CO_3 [all p.a., Merck]). This method was chosen to prevent the formation of heavy metal ion-chl complexes during extraction and to minimize the degradation processes (Küpper et al. 1996). Because chl *c* is too polar to be extracted with this solvent mixture, brown algal pigments were extracted with 100% acetone.

For pigment extraction of bands from green gels, we used a method based on that described by Sofrova et al. (1992). Gel slices were ground, without adding solvent, for 1–5 min in a mortar with sand followed by the addition of a solubilization solution (55% acetone, 45% water, and 0.2% to 0.5% SDS). The suspension was incubated for about 12 h at 4° C. The extract was then diluted 1:1 with 10% NaCl solution and immediately with 1 mL of cyclohexane per 3 mL of diluted extract. After thorough mixing, the two phases were separated by centrifuging (30 min, 15,000*g*, 4° C). All extracts (both from plants and from gels) were dried with anhydrous $CaCl_2$, centrifuged (4000*g*, 10 min), and diluted to a maximum absorbance (550–700 nm) of 0.2 to 0.5.

UV/VIS spectroscopy and quantification of chls. Spectra of pigment extracts and *in vivo* spectra of intact algal cells and filaments were measured with the UV/VIS spectrophotometer Shimadzu UV3000, which is specially adapted to allow for accurate measurements of spectra from turbid samples. As previously described for Chlorophyta (Küpper et al. 1998), *in vivo* absorbance spectra reveal Cu-chl upon treatment with phosphoric acid, because Cu-chl is resistant to this treatment. In contrast, Mg-chl becomes converted to pheophytin (Pheo), which has a strongly red-shifted Q-band absorbance maximum compared with Mg-chl.

Chls and Pheo were quantified according to Küpper et al. (2000). Normalized spectra (550–750 nm) of pigment standards were mathematically described by a series of Gaussian peaks. These "Gauss-peak-spectra" were combined to simulate the contributions of possible constituents in the plant extracts, for example, Cu-chl *a*, Cu-chl *b*, Mg-chl *a*, Mg-chl *b*, Pheo a, Pheo b for a sample of Cu-stressed green algae. The combinations of Gauss-peak-spectra were then fitted to the sample spectrum to obtain the parameters representing the concentrations of the individual components. Additionally, baseline drift and wavelength inaccuracies of the spectrophotometers were automatically corrected.

HPLC was not used for quantification for the following reasons. First, artifacts of pigment degradation and transmetalation readily occur during the preparation of the hydrophilic samples for reversed-phase HPLC. Furthermore, and more importantly, samples from stressed organisms contain, in addition to the Mg-chls, heavy metal chls, and Pheos, compounds such as pheophorbides and chlides. Such chl derivatives have—in the relevant spectral region of 550–750 nm—the same absorbance as true chls with the same central ion. Hence, the percentage of Mg substitution is more accurately quantified by the photometric method. Furthermore, these compounds and the artifacts generated during preparation of samples for HPLC lead to a very large number of overlapping peaks in an HPLC analysis. These overlapping peaks with extinction coefficients that are not well defined in the HPLC solvent mixtures, and the risk of artifacts make the HPLC approach for quantification inaccurate.

In vivo *chl fluorescence measurements.* Kinetics of fluorescence induction were measured using a Walz PAM®-101 fluorimeter with white (tungsten) actinic light, white (tungsten) light for saturating pulses, and red (LED) measuring light. For some of the measurements (e.g. assessment of the effective cross-section of the PSII associated antenna according to Strasser et al. 2000), the double-modulated fast fluorimeter from Photon Systems Instruments (Brno) was used.

Oxygen measurements. Net photosynthetic oxygen release and respiratory oxygen uptake were measured using Clark-type electrodes and laboratory-designed chambers. For *Scenedesmus*, a chamber developed by Bartoš et al. (1975) was used and modified to allow simultaneous fluorescence measurements. The magnetic stirrer was driven by an electronically commutated motor, which reduced the noise of the electrode signal by nearly one order of magnitude. For filamentous algae, a new chamber was designed that also served for fluorescence measurements. The algae are kept in a fixed position by a platinum net on the window through which they are illuminated. The net separates the algal space from the electrode space, which is furnished by a magnetic stirrer. More details about the configuration and use of this system will be described elsewhere.

Thermoluminescence (TL) measurements. TL was measured with an instrument constructed by Prášil et al. (1996). *Ectocarpus* fila-

ments were cut into about 5 mm pieces, suspended in artificial seawater medium 1, and then homogeneously settled on a filter paper. *Antithamnion* filaments had to be distributed on the filter by the use of forceps to obtain acceptable homogeneity of the sample. *Scenedesmus* cells were suspended in *Scenedesmus* growth medium, which was subsequently removed by filtration through a Millipore HA filter (0.8 μm pore size, Millipore Corporation, Bedford, MA, USA).

Filters with homogeneously distributed filaments/cells was fixed in a sample holder and kept in the dark for 180 s at 25° C. The temperature was then lowered to 3° C and the S-state equilibrium was adjusted by one preflash. The final S2/S3 Q_B state of PSII was induced by two single turnover flashes. All flashes were provided by a xenon lamp. The TL curve recording began 2–3 s after illumination and was carried out using a 0.4° C·s^{-1} temperature gradient for warming up the sample to +70° C. The information on the activity of the PSII RCs contained in the TL glow curves was quantified by estimating the area between the TL curve and the baseline.

RESULTS

Light-dependent differences in Cu^{2+} stress in Scenedesmus. Cultures of *Scenedesmus* were preadapted for several weeks to specific irradiance conditions (Table 1). Fluorescence kinetics were measured *in vivo*, pigment content was assessed in extracts of whole cells, and the composition of pigment-protein complexes was analyzed by native ("green") gel electrophoresis.

Comparison of hms-chl formation and fluorescence. Hms-chl formation in shade reaction samples of *Scenedesmus* reached 45% of total chl *a* and 10% of total chl *b* within 5 h (Table 2). In contrast, in sun reaction samples a maximum of 2% of the chl *a* present at the start of copper stress were substituted. This low percentage of hms-chl formation during sun reaction was a matter of antenna chl accessibility. Under high irradiance, bleaching occurred within the first 5 h of exposure of the cells to Cu^{2+}. However, if hms-chl formation proceeded in the course of the sun reaction as fast as during the shade reaction, the time before bleaching occurred would have been sufficient to observe a much higher hms-chl formation than was actually observed.

In the course of the shade reaction, the maximal photochemical yield of PSII (measured as relative variable fluorescence, F_v/F_m) was hardly lowered, whereas the total fluorescence amplitude (F_m) decreased significantly ($P < 0.001$, Table 2). In contrast, the sun reaction led to a rapid decline in F_v/F_m due to a faster decrease of F_m compared with F_0. In some cases a rise of F_0 was observed additionally, leading to the same effect on F_v/F_m. Furthermore, the effective cross-section of the antenna associated with one active PSII RC did not change substantially under Cu-stress shade reaction conditions (data not shown). This was determined by the half-time of the fluorescence rise from F_0 to the P level in presence of 3-(3,4-dichlorophenyl)-1,1-dimethylurea.

In all experiments, the formation of Cu-chl in Cu-stressed algae was accompanied by the formation of Pheos, leading to a statistically significant ($P < 0.0001$) correlation between the two parameters (Fig. 1).

Nondenaturing gel electrophoresis. To identify the pigment-protein(s) in which hms-chl was formed under various irradiance conditions, the thylakoids of Cu-stressed *Scenedesmus* were solubilized and the pigment-protein complexes were separated by nondenaturing ("green") gel electrophoresis. Gels of *Scenedesmus* stressed with 20 μM Cu^{2+} for a long time under shade reaction conditions exhibited blue-green bands (Fig. 2A), which indicates Cu^{2+}-chl formation. The positions of the bands were altered relative to the control, making their identification impossible. The pigment-proteins of these Cu-poisoned cells appeared to be already highly degraded (forming a smear rather than clear bands on the gel), and hms-chl formation itself may have caused altered electrophoretic mobilities. In contrast, pigment-proteins from cells incubated with Cu^{2+} for only 5 h

TABLE 2. Fluorescence parameters, gross photosynthetic oxygen release (GPOR), and Mg substitution of *Scenedesmus quadricauda* stressed with Cu^{2+} in low and high irradiance.

Sample	Mg substitution (% of total chl *a*) (± SE)	Mg substitution (% of total chl *b*) (± SE)	GPOR (% of the control)	F_m (% of the control)	F_v/F_m
24 h 10 μM (exp. 88), ll	5 (± 1)	0	17	30	0.71
Control (exp. 88), ll	0	0	100	100	0.73
5 h 10 μM (exp. 101), ll	2.3 (± 0.5)	0	57	30	0.68
Control (exp. 101), ll	0	0	100	100	0.72
5 h 20 μM (exp. 89), ll	4 (± 0.5)	5 (± 2)	ca. 20	54	0.66
Control (exp. 89), ll	0	0	100	100	0.61
5 h 30 μM (exp. 101), ll	45 (± 1)	10 (± 4)	<1	<1	—
Control (exp. 101), ll	0	0	100	100	0.72
25 h 10 μM (exp. 88), hl	0.5 (± 0.5)	0	5	18	0.22
Control (exp. 88), hl	0	0	100	100	0.53
5 h 20 μM(exp. 89), hl	2 (± 0.5)	0	10	8	0.58
Control (ep. 89), hl	0	0	100	100	0.68
5 h 30 μM (exp. 93), hl	0.1 (±1)	0	< 1	—	—
Control (exp. 93), hl	0	0	100	—	—

hl, high light intensity (400 μmol photons·m^{-2}·s^{-1}); ll, low light intensity (20 μmol photons· m^{-2}·s^{-1}). Note that Cu^{2+} resistance of *S. quadricauda* is highly variable. The data show differences between control and treated plants and are from individual typical experiments to separate the differences in physiological status of algae used in replicate experiments (see Materials and Methods) from the effects of the metal treatments. SE therefore represents the measuring error within one sample (algal culture), not the deviation between replicate experiments.

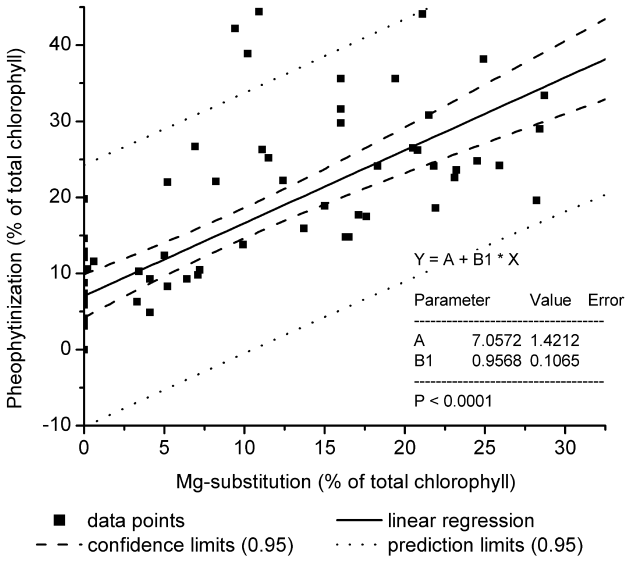

FIG. 1. Relation between pheophytinization and formation of Cu-chl in *Scenedesmus quadricauda* stressed with Cu^{2+} in low irradiance.

(Fig. 2B) had the same electrophoretic mobility as pigment-proteins isolated from control cells. However, solubilization of thylakoid membranes in Cu^{2+}-stressed samples was never complete. It is possible that only the most intact proteins have been solubilized from membranes of cells treated with Cu^{2+} for a short time. The pigment-proteins isolated from cells that had been stressed with Cu^{2+} in high irradiance were denatured under all experimental conditions (Fig. 2C).

Although pigment-containing bands were resolved on polyacrylamide gels, it was virtually impossible to get meaningful information by analyzing pigments extracted from these bands, for a number of reasons. First, extraction of the pigments from gel bands was always incomplete due to closure of gel pores by the organic solvents. This problem could be only partially solved by the choice of appropriate solvents and by extensive grinding of the gel before extraction. Furthermore, the relative content of Cu-chl in the extracts increased when extraction of the gel was less complete, making accurate quantification of Mg-substitution ratio in these complexes impossible. Second, in all bands of the control, Cu-chl formation was found to occur during electrophoresis. This is not surprising: The chemicals used to prepare the gel (highest standard analytical grade) may contain 0.0005% Cu, leading to 66 nmol Cu^{2+} in a typical lane of a preparative gel ($10 \times 100 \times 3$ mm), whereas usually <10 nmol (ca. 10 μg) chl are applied per lane. Because Cu^{2+} has a very high affinity for chl, the formation of Cu-chl during the approximately 5 h of electrophoresis (plus subsequent sample preparation) is not unexpected. This formation of transmetalated chl during electrophoresis, due to metal contaminations of the reagents used, is potentially a serious problem.

Hms-chl formation in isolated PSII RCs. The possibility that during the sun reaction Cu^{2+} could directly inhibit the PSII RCs was supported by the observation that PSII RCs eluted from a Cu-affinity column in the course of its purification (Šetlíková et al. 1995) contained Cu-chl. In diluted fractions of PSII RC, in which the relation of free Cu^{2+} to chl was high, the analyses showed that up to 8% of total chl was Cu-chl ($P < 0.001$).

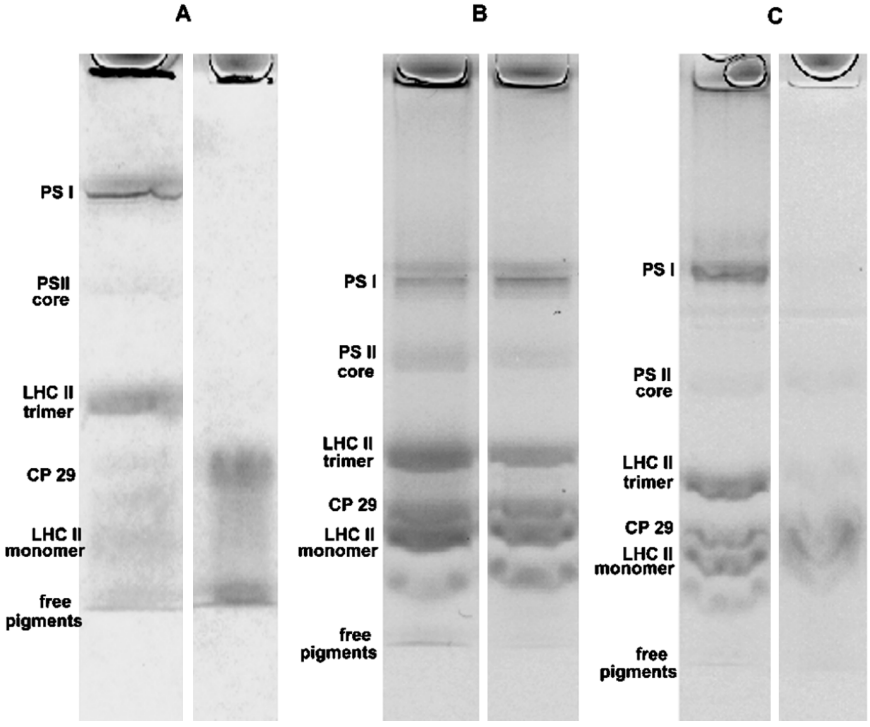

FIG. 2. Examples of "green gels" from *Scenedesmus quadricauda*. (A) Stressed for 24 h with 20 μM Cu^{2+} in low irradiance. (B) Stressed for 5 h with 20 μM Cu^{2+} in low irradiance. (C) Stressed for 5 h with 20 μM Cu^{2+} in high irradiance. Left side of each pair, control; right side of each pair, stressed with Cu^{2+}.

TABLE 3. Fluorescence parameters, gross photosynthetic oxygen release (GPOR), and Mg substitution of *Ectocarpus siliculosus* stressed for 1 week with Cu^{2+} and Zn^{2+} in low and high light intensity.

Light intensity	Sample	Mg substitution (% of total chl *a* in stressed sample ± SE)	GPOR (% of the control)	F_m (% of the control)	F_v/F_m
High	Control	0	100	100	0.65
	1 μM Cu^{2+}	0 (± 0.2)	90	79	0.71
	3 μM Cu^{2+}	0 (± 0.9)	80	71	0.65
	10 μM Cu^{2+}	1.5 (± 0.2)	45	31	0.66
	30 μM Cu^{2+}	15.5 (±3)	8	4	0.72
Low	Control	0 (± 0.2 for Cu, ± 0.5 for Zn)	100	100	0.64
	1 μM Cu^{2+}	0 (± 0.2)	80	90	0.71
	3 μM Cu^{2+}	1.1 (± 0.4)	70	89	0.67
	10 μM Cu^{2+}	2.5 (± 0.5)	40	45	0.61
	30 μM Cu^{2+}	16 (± 2)	9	24	0.44
	50 μM Zn^{2+}	0 (± 1)	85	96	0.61
	150 μM Zn^{2+}	4 (± 2)	75	86	0.59
	450 μM Zn^{2+}	19 (± 2)	55	88	0.44

High light intensity = 140 μmol photons·m^{-2}·s^{-1}; low light intensity = 8 μmol photons·m^{-2}·s^{-1}. Data were taken from typical experiments (for details of statistics see heading of Table 2 and Materials and Methods).

In the main fractions with a lower ratio of Cu^{2+}/PSII RC, the proportion of Cu-chl was <2%. The activity of the isolated RCs was tested by spectrophotometric measurements of the oxidation-reduction state of cytb559 and of P700. The results compared with standard values suggested that more than 70% of the centers were active in the separation of charges. Evidently, also other factors than Cu insertion contributed to the inactivation of a part of the complexes during isolation.

Comparison of antenna systems (structure-dependent differences in antenna accessibility). Brown algae (Ectocarpus): In *Ectocarpus* hms-chl formation corresponding to that observed in the shade reaction of green plants and algae occurred not only under low irradiance with a long dark phase but also occurred under high (saturating) irradiance with a short dark phase (Table 3). Bleaching of the algae was not observable even under high irradiance (Fig. 3A). Although the main focus of the study was on Cu^{2+}, a few experiments were done also with Zn^{2+} to check whether the same principles apply to this metal. Fluorescence microscopy clearly showed the formation of Zn-chl (Fig. 3B), as previously discussed in more detail in the case of Chlorophyta (Küpper et al. 1998): Although formation of nonfluorescent Cu-chl leads to an almost complete quenching of fluorescence, while formation of weakly fluorescent Zn-chl leads to a slight blue shift of the emission peak and a decreased but non-zero fluorescence quantum yield. The formation of Cu-chl was also followed *in situ* (Fig. 4) by means of acid treatment of samples combined with *in vivo* absorbance spectroscopy as described in Materials and Methods. However, analysis of extracts showed hms-chl formation only in chl *a*, whereas Cu-chl *c* was not detected even after 2 weeks of treatment.

Measurements of photosynthetic parameters also indicated that heavy metal-induced damage in brown algae is a typical shade reaction. Substitution of 10% ± 3% of the total chl *a* completely suppressed net photosynthetic oxygen release. Chl fluorescence induction ("Kautsky curves") in Cu-stressed *Ectocarpus* is only lowered in amplitude compared with the control; the relations between all parameters (F_0, F_m, F_v, F_s) are not changed significantly ($P = 0.05$, $n = 9$, *t*-test) until a very late stage of damage (Table 3). This is different with Zn^{2+} stress: Because Zn-chl exhibits fluorescence but is not functional (Küpper et al. 1998), it contributes to F_0 but not to F_v and thus lowers the relative variable fluorescence (F_v/F_m), as shown in Table 3. TL measurements of Cu- or Zn-stressed algae showed only a decrease in amplitude of the band, with no significant change in its shape or position (Fig. 5).

Red algae (Antithamnion). The red alga *Antithamnion* proved to be highly resistant toward Cu stress. To induce a measurable decrease in net photosynthetic oxygen release, the Cu^{2+} concentration had to be around 30 μM, which is about 10 times higher than in the case of *Ectocarpus* and sensitive *Scenedesmus* cultures (see results of this study) and about 100 times higher than in sensitive higher plants (e.g. *Stratiotes* and *Elodea*, Küpper et al. 1996).

Under inhibitory Cu^{2+} concentrations the most severe damage was a decrease in respiration, whereas photosynthetic oxygen production was affected much less (Fig. 6). The measurements of variable fluorescence indicated that during this first stage of Cu^{2+}-induced stress the photochemical yield of PSII (F_v/F_m) remained constant or even increased (Fig. 6, Table 4). These results are in sharp contrast to the usual damage pattern in green plants and brown algae, where respiration is much less affected than photosynthesis.

In later stages of Cu stress, photosynthesis in *Antithamnion* decreased and the algae bleached both in low and high irradiance. At 30 μM Cu^{2+}, the decline of F_v/F_m paralleled that of the rate of oxygen evolution (Fig. 6). It is noteworthy that both F_M and F_0 decrease but the former faster than the latter so that a drop of the ratio F_v/F_m results. *In vivo* absorbance spectra (Fig. 4) did not show any significant change in the shape of the peaks but only showed a decrease in their amplitude. The phycobiliproteins (peak around 550 nm) disappeared faster than the chls so that in the later stages the algae appeared green. An increased nonspecific absorbance in the blue region of spectra

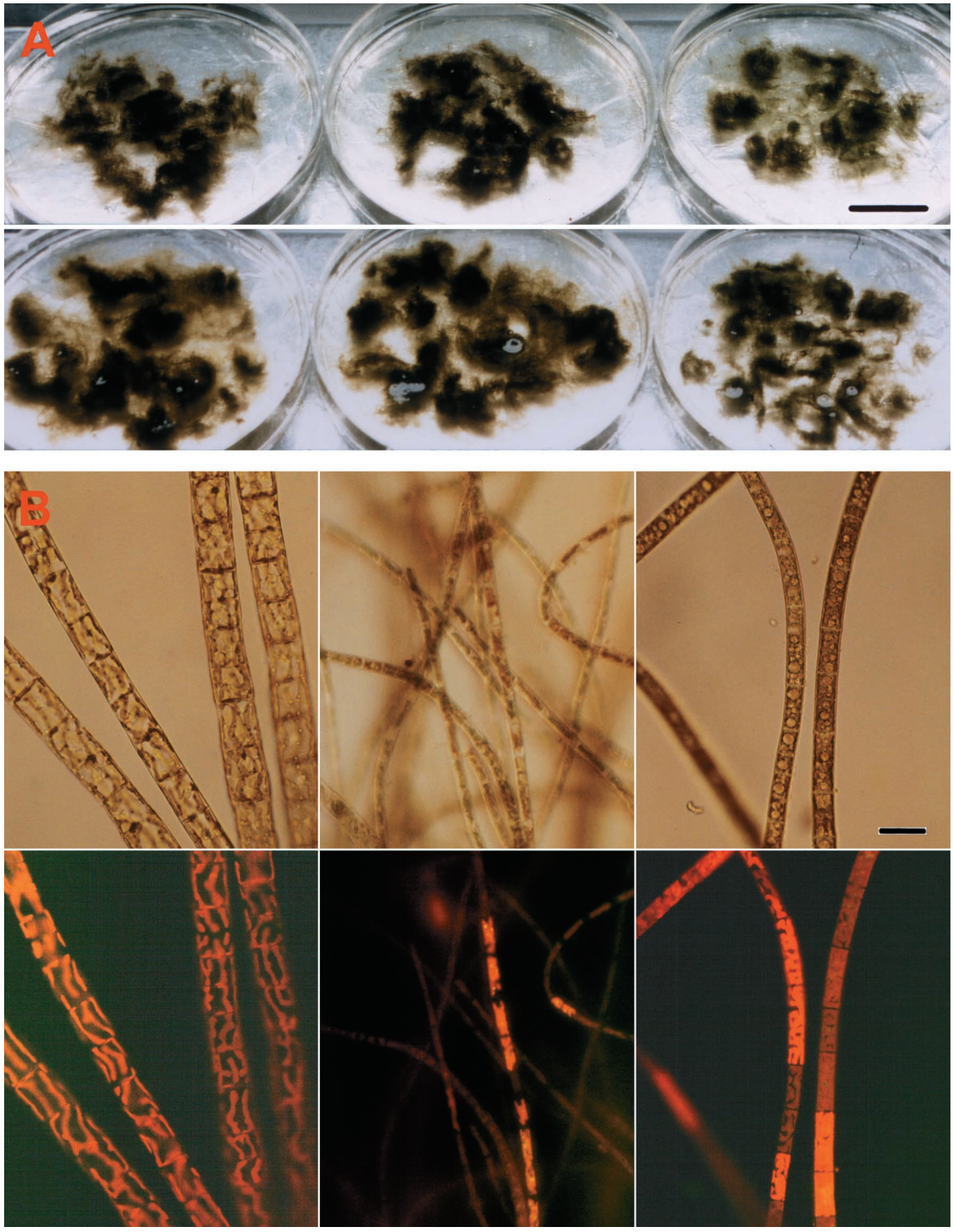

FIG. 3. Comparison of microscopic and macroscopic photographs of *Ectocarpus siliculosus* stressed with Cu^{2+} and Zn^{2+} for 1 week. (A) Comparison of macroscopic photographs of *E. siliculosus* stressed with Cu^{2+} in low and high irradiance, showing that even under high irradiance no bleaching of Cu-stressed algae occurs. The bar in the top row represents 2 cm. Top: high irradiance; bottom: low irradiance. Left: control; middle: 3 μM Cu^{2+}, right: 30 μM Cu^{2+}. (B) Comparison of transmission and epifluorescence microscopic photographs of *E. siliculosus* stressed with Cu^{2+} and Zn^{2+} in low irradiance. The bar in the top right image represents 50 μm. Top: transmission; bottom: fluorescence with green cut-off filter. Left: control; middle: stressed with 30 μM Cu^{2+} for 6 days; right: stressed with 450 μM Zn^{2+} for 7 days. The relatively high remaining fluorescence in Zn^{2+}-damaged cells is caused by the fluorescence of Zn-chl, whereas Cu-chl does not fluoresce leading to a stronger fluorescence decrease in cells damaged by Cu^{2+}.

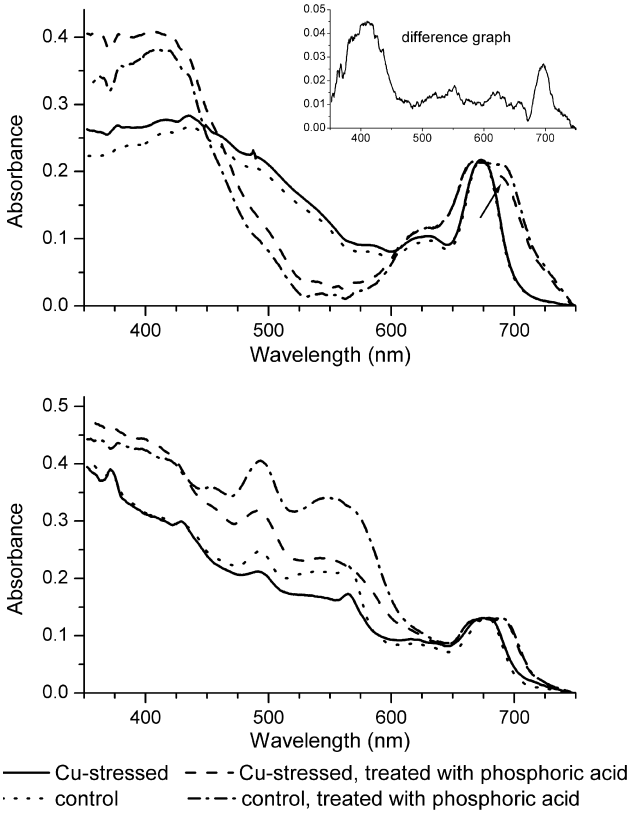

FIG. 4. *In vivo* absorbance spectra of *Ectocarpus siliculosus* and *Antithamnion plumula* stressed for 1 week with Cu^{2+} in low irradiance. Spectra are normalized to absorbance = 0 at 750 nm and equal absorbance at the red absorbance maximum to facilitate comparison of the shape of the chlorophyll/pheophytin peak. Top: *E. siliculosus*. Formation of Cu-chl in the sample stressed with 3 μM Cu^{2+} becomes visible by resistance of the chl toward pheophytinization (see region around 690 nm) upon treatment with phosphoric acid (Küpper et al. 1998). The small inset graph shows the above-described effects in pronounced form. It is a difference spectrum of the samples after treatment with phosphoric acid ([control]-[Cu^{2+} treated]) after removing the effect of increased scattering in the Cu-treated sample. Bottom: *A. plumula*, control and stressed with 90 μM Cu^{2+}.

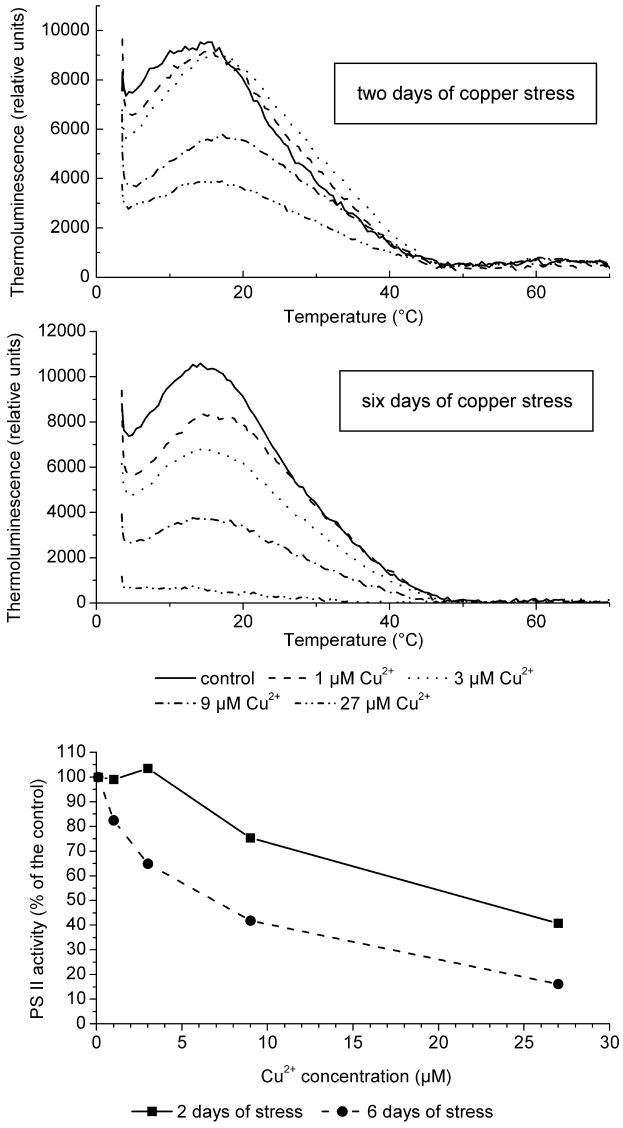

FIG. 5. Thermoluminescence of *Ectocarpus siliculosus* filaments stressed with Cu^{2+} in high irradiance. Top and middle: Thermoluminescence glow curves of samples that have been exposed to various concentrations of Cu^{2+} for 2 and 6 days. Bottom: PSII activity calculated from thermoluminescence vs. concentration of Cu^{2+} for cells exposed to various concentrations of Cu^{2+} for 2 and 6 days. PSII activity is plotted as percent of the control.

from stressed algae (Fig. 4) indicated an increase in light scattering. Quantification of pigments in cyclohexane extracts revealed an hms-chl formation lower than 1% of the pigment content that was present before the Cu^{2+} stress. At lethal Cu^{2+} concentrations (>100 μM) and after bleaching of most pigments, *in vivo* spectra of these algae showed effects similar to those described above. In addition, a slight decrease in pheophytinization upon the addition of phosphoric acid indicated the formation of stable Cu-chl (Küpper et al. 1998). Analysis of extracts yielded a Cu-chl content of up to 16% in the bleached sample, which corresponds to about 2% hms-chl formation of the original chl content.

TL measurements of *Antithamnion* with inhibited photosynthesis showed the same pattern as for *Ectocarpus* (i.e. a decrease in amplitude) but no significant change in position or form of the peaks.

DISCUSSION

The results described here prove that it is the LHC II complex that is differentially accessible to hms-chl formation in weak and strong light and leads to the two different types of Cu^{2+} attack in Chlorophyta described by Küpper et al. (1996, 1998). Evidence is further provided that algae with light harvesting antennae other than LHC II manifest one or the other type of Cu^{2+} attack regardless of the irradiance.

Hms-chl formation in red and brown algae. The red alga *Antithamnion* exhibited symptoms resembling the sun reaction even at very low irradiance. Under all circum-

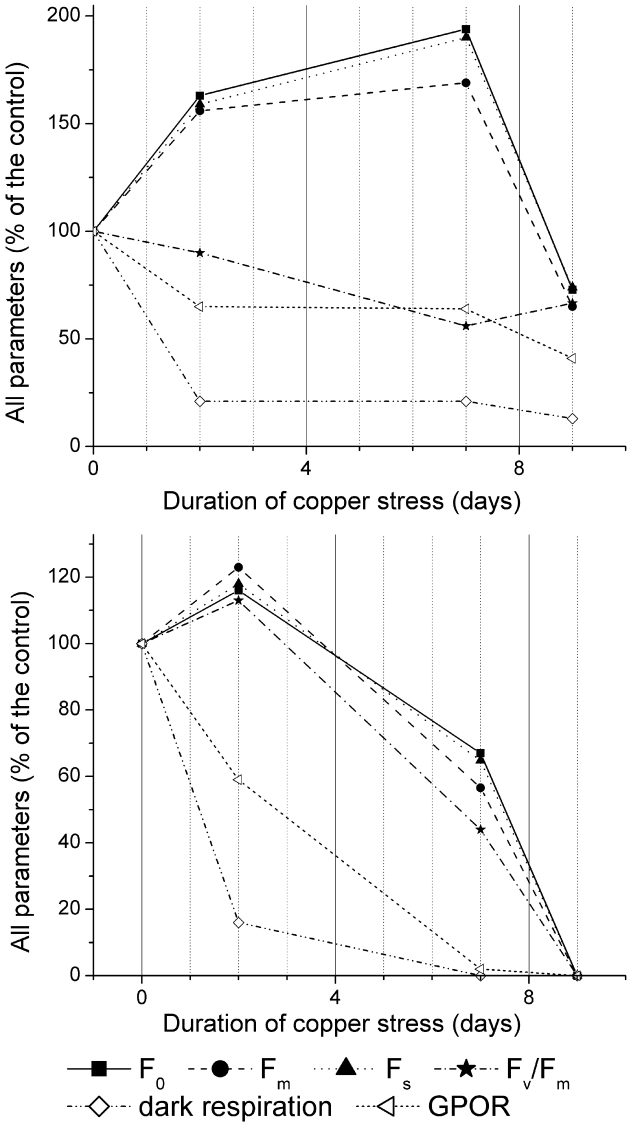

FIG. 6. Changes in parameters of fluorescence kinetics, respiration, and photosynthesis of *Antithamnion plumula* under copper stress in low irradiance. Top: 30 μM Cu^{2+}; bottom: 270 μM Cu^{2+}.

stances only a very small proportion of the total chl was accessible to hms-chl formation. Heavy metal attack caused first a decrease in F_v/F_m and ultimately bleaching of the stressed cells. Because the main difference in antenna systems between Rhodophyta and Chlorophyta is the absence of LHC II (the former contains phycobilisomes), the missing shade reaction in Rhodophyta suggests that the LHC II is the target of hms-chl formation during the shade reaction in Chlorophyta.

In contrast, the brown alga *Ectocarpus* showed symptoms of the shade reaction irrespective of irradiance. Even at high irradiance substantial hms-chl formation occurred that was accompanied by corresponding changes in fluorescence and thermoluminescence

TABLE 4. Fluorescence parameters and GPOR of *Antithamnion plumula* stressed with Cu^{2+} in low light intensity (8 μmol photons·m^{-2}·s^{-1}).

Sample	GPOR (% of the control)	F_m (% of the control)	F_v/F_m
Seven days stress			
Control	100	100	0.25
10 μM Cu^{2+}	107	166	0.24
30 μM Cu^{2+}	64	195	0.14
90 μM Cu^{2+}	44	102	0.23
270 μM Cu^{2+}	2	67	0.11
Nine days stress			
Control	100	100	0.27
10 μM Cu^{2+}	34	96	0.21
30 μM Cu^{2+}	41	73	0.18
90 μM Cu^{2+}	34	37	0.17
270 μM Cu^{2+}	0	<1	0

Data were taken from typical experiments (for details of statistics see heading of Table 2 and methods).

characteristics. This suggests that at least one of the light harvesting chl *a*/*c* proteins in brown algae was always accessible to hms-chl formation. Their affinity for hms-chl formation appears to be similarly high as that of the chl *a*/*b* LHC II of Chlorophyta in low irradiance. This similarity in affinity is most probably caused by the fact that the protein moiety of the chl *a*/*b* light harvesting chl proteins (LHCPs) displays a high homology with that of the chl *a*/*c*-xanthophyll LHCPs, both of the fucoxanthin chl protein (FCP) and of the intrinsic peridinin type (cf. Green and Durnford 1996, Durnford et al. 1999). The differences in structure of LHC II compared with the chl *a*/*c*-xanthophyll LHCPs that cause the different response to irradiance is a question that remains to be solved. The fact that the sun reaction in Chlorophyta takes place also if Cu^{2+} is added in the dark to cells grown previously in high light suggests that the sun reaction reflects a structural change in the LHC II that occurs during adaptation to high irradiance, rather than a change directly related to the light-dependent energization of thylakoid membranes. Susceptibility of LHC II to hms-chl formation in cells grown under low irradiance may occur during the assembly of LHC II trimers in low light. The FCPs have been shown to form large assemblies regardless of irradiance (Katoh and Ehara 1990, Mimuro et al. 1990), which might reflect their tendency to form hms-chl in both high and low irradiance.

In Cu^{2+}-treated *Ectocarpus* no Cu-chl *c* was found. This can be explained solely by the fact that chl *c* does not as readily loose its Mg^{2+} as chl *a* (Abele-Oeschger and Theede 1991). The inaccessibility of chl *c in vivo* can also reflect the specific protein environment of chl *c*. Finally, it cannot be ruled out that Cu-chl *c* has not been detected for technical reasons. Because the absorptivity of Cu-chl *c* in the red band is only about 30% of that of Cu-chl *a*, quantification of this pigment is less accurate. Because chl *c* amounts to 15%–20% of total chl (*a*+c) in *Ectocarpus*, a substitution of approximately 5% of the Mg^{2+} by Cu^{2+} in this pigment would mean the sensitivity of the assay would have to be high enough to detect

a change in 0.8% to 1% of the total chl content. This is not readily achieved using either spectroscopy or HPLC.

Shade reaction in Chlorophyta (Scenedesmus). Hms-chl formation from chl *a* occurred already at the lowest inhibitory heavy metal concentration. At higher Cu^{2+} concentrations, a significant ($P < 0.001$) amount of Cu-chl *b* was detected. Because chl *b* is known to be much more stable than chl *a* and less susceptible to substitution of the Mg^{2+} (cf. e.g. Berezin and Dobrysheva 1970, Schanderl et al. 1965), this indicates that chl *b* in the LHC II is also readily accessible to hms-chl formation.

During extraction of chl from cells, some pheophytinization of chl *a* always occurs. However, in Cu^{2+}-stressed *Scenedesmus* the amounts of Pheos were always much higher than in the controls, and their levels were proportional to that of Cu-chl, suggesting that their accumulation is a consequence of Cu-chl formation. The Cu-chl complexes do not bind axial ligands (see e.g. Boucher and Katz 1967), which is required for correct association of chl in the protein (e.g. Rebeiz and Belanger 1984) and for maintenance of the proper LHC II structure (Paulsen et al. 1993). Therefore, Cu^{2+}-chl formation in LHC II chls may induce a conformational change in the complex that results in exposing some Mg-chl to the acidic content of the thylakoid lumen. This would lead to pheophytinization. Krupa (1988) and Krupa et al. (1987) showed that Cd^{2+} decreased the ratio of trimeric/monomeric LHC II in radish. If this is due to disturbed pigment binding, a similar effect may occur during Zn^{2+} and Cu^{2+} stress. There are several reasons why all this Pheo may not be immediately converted to Cu-chl or Zn-chl. The environment around a pigment may change the selectivity such that transmetalation is favored over insertion of heavy metals into Pheo (Hartwich et al. 1998). Furthermore, once a cell is dying due to excess metal induced damage, pheophytinization might become faster than the transport of additional Cu^{2+} into the cell. Another question is whether hms-chl formation causes the conformational change in LHC II or whether another Cu^{2+}-conferred change in protein conformation renders the chl more accessible to substitution and pheophytinization. Micromolar Cu^{2+} concentrations can cause hms-chl formation, whereas usually millimolar concentrations trigger protein denaturation. Therefore it is possible to insert heavy metals into proteins (e.g. for x-ray crystallography) without causing protein denaturation. So it seems more likely that hms-chl formation is the primary step leading to pheophytinization. Results obtained with other heavy metals support this conclusion. Fourier transform infrared spectra published by Ahmed and Tajmir-Riahi (1993) showed that 10 μM of Hg^{2+} and Cd^{2+} modified chl absorption of LHC II, whereas denaturation of the latter became apparent only when the heavy metal concentration was above 1000 μM.

The shade reaction with Cu^{2+} is characterized by a parallel decrease of F_m and oxygen production without significant changes of F_v/F_m. The effective cross-section of the antenna serving one RC of PSII does not change significantly in Cu^{2+} affected cells in comparison with control cells. Oxygen production and variable fluorescence are completely suppressed when just about 10% of the total chl *a* is substituted. The same characteristics apply for the homologous reaction in brown algae. We propose the following explanation: The Cu^{2+} attack causes elimination of the excitation transfer from the LHC II antenna to the PSII RCs already by hms-chl formation in the first chl molecule(s) of the LHC II. Heavy metal chl molecules act as exciton quenchers as discussed by Küpper et al. (1998). Recently, such heavy metal quenching has been used as an analytical tool (Fiedor et al. 2001). The F_v/F_m ratio also decreases during the shade reaction with Cu^{2+} if the activity of the cells becomes strongly inhibited (below 10% of the control). At this stage the core of PSII might become deprived of the protection by the presence of a much larger number of potential ligands in LHC II. Thus, the RC becomes exposed to more direct Cu^{2+} attack, leading to processes that are characteristic of the sun reaction. Although Cu-chl is completely nonfluorescent, Zn^{2+} preserves some fluorescence and therefore the Zn^{2+}-induced shade reaction leads to a stronger decrease of F_m than of F_0.

Hms-chl formation during the sun reaction. In the sun reaction of Chlorophyta, most of the chl becomes inaccessible to hms-chl formation (Küpper et al. 1996, 1998). The reason for this is unknown and remains an important question for future investigations, as discussed above in the comparison of FCPs and LHC II. Nevertheless, small amounts of hms-chls are formed during the sun reaction, but in contrast to the shade reaction we do not know the exact target. The detection of Cu-chl in Cu^{2+}-treated red algae, which has been described here, demonstrates that hms-chl formation can also occur in complexes other than in LHC II. Because PSI resists attack of heavy metals even in high irradiance (e.g. Atal et al. 1991, Clijsters and Van Assche 1985, Gross et al. 1970), the core of PSII is the mostly likely target. Many authors have come to the most likely conclusion that the PSII RC is the target of heavy metal-induced inhibition. But there is less agreement about the exact site and mechanism of damage; literally all components participating in the energy conversion within PSII RCs have been proposed as targets (reviewed e.g. by Jegerschöld et al. 1995, Yruela et al. 1996). Most probably different effects occur when diverse materials are treated under various conditions (cf. Jegerschöld et al. 1995). In some cases, the high heavy metal concentrations applied will have led to reactions that are not relevant in nature. Most authors locate the site of heavy metal inhibition very close to the primary photochemical event in PSII (Cedeno-Maldonado et al. 1972, Wu and Lorenzen 1981, Samson et al. 1988, Yruela et al. 1993, Schröder et al. 1994, Jegerschöld et al. 1995, Boucher and Carpentier 1999), either on its oxidizing or on its reducing side.

We suggest that heavy metals inhibit charge separation in PSII. Hsu and Lee (1988) came to the same conclusion based on convincing experiments with copper treatment of spinach chloroplasts. Our arguments are

as follows. The sun reaction both in Chlorophyta and Rhodophyta is characterized by the gradual reduction of F_v/F_m. It is often argued that this copper-induced decrease of variable fluorescence is a consequence of inhibition of electron donation to the primary photochemical reaction. Cu^{2+} treatment might, however, induce a state analogous to photoinhibition (see Prášil et al. 1996) in which trapping of an exciton in the PSII RC is followed by its nonradiative dissipation. In the case of Cu^{2+}-induced inhibition, this state could most probably result from insertion of Cu^{2+} into the Pheo molecule of the PSII RC. We propose this as an explanation for the sun reaction (especially with Cu^{2+}). In this context, the extensive series of *in vitro* experiments performed by Yruela et al. (1991, 1992, 1993, 1996) are of particular importance. They provide convincing evidence that the inhibition of PSII activity by Cu^{2+} occurs at the Pheo-Q_A domain, which interferes with the stabilized charge separation ($P680^+ Q_A^-$). They concluded (Yruela et al. 1993), however, that the primary charge separation (i.e. the formation of the radical pair $P680^+$ $Pheo^-$) is not obstructed because they observed the accumulation of $Pheo^-$ in the presence of Cu^{2+} concentrations that inhibit the Q_A reduction. This observation cannot be extrapolated to conditions in living cells because the accumulation of $Pheo^-$ can be observed only under strongly reducing conditions, in the presence of dithionite. The latter may reduce cupric to cuprous ions, which are unlikely to be inserted into Pheo. On the other hand, the competition of Cu^{2+} with H^+ for the "inhibitory binding site" in PSII, found by Yruela et al. (1993), is in agreement with the idea proposed by us that heavy metals become bound to Pheo molecule.

The thermoluminescence data presented in this study are at variance with an inhibitory interaction of Cu^{2+} with Q_A or the protein environment in the Pheo/Q_A domain. Such interaction, proposed by Yruela et al. (1991, 1992, 1993, 1996), should result in a shift of the Q-band peak. We did not observe any change in the shape of the Q band but only its lowering; this indicates that entire PSII RCs are inactivated. Our observation of the Cu^{2+} content in the PSII RC complex provides an indirect indication that Cu^{2+} becomes inserted into Pheo because it remained bound in the PSII RC complex. This would probably not be the case if hms-chl formation took place in one of the RC chl molecules, because these are bound to the protein by the coordination valencies of Mg^{2+}. One might believe that the insertion of Cu^{2+} into the Pheo in PSII RC should lead to an observable decrease in Pheo. But it is practicably impossible to separate the small decrease of Pheo, which is caused by insertion of Cu into the Pheo a of PSII RCs, from the large decrease of total pigment due to bleaching, and the large amount of Pheos that are generated by the degradation of the pigment-proteins of dying cells.

CONCLUSIONS

From our results we can conclude that LHC II is indeed the main target of hms-chl formation that constitutes the typical shade reaction in Chlorophyta. In algae that do not possess LHC II there is no irradiance dependence of hms-chl formation. In contrast, at present it cannot be excluded that the small amount of Cu-chl occurring during Cu-induced sun reaction is formed unspecifically in different parts of the PSII core and that different mechanisms cause the inhibition of the PSII RC. But the data presented here and in earlier publications on hms-chl formation *in vivo* (Küpper et al. 1996, 1998) and the reports on PSII RC inhibition by authors quoted above suggest that the inhibition of the PSII RC during sun reaction is most likely the consequence of specific insertion of heavy metals in Pheo a.

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