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CELL BIOPHYSICS

Assessment of the Effects of Methylmercury and Copper Ions on Primary Processes of Photosynthesis in Green Microalga *Chlamydomonas moewusii* by Analysis of the Kinetic Curves of Variable Chlorophyll Fluorescence

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Abstract—The effect of methylmercury and copper ions on the kinetics of light induction and dark relaxation of the variable fluorescence of chlorophyll *a* has been studied on cultures of unicellular alga *Chlamydomonas moewusii*. Methylmercury was effective at much lower levels. The toxicants at concentrations that did not decrease the photochemical activity of PS II (F_v/F_m) did affect the electron transport on the acceptor side of PS II, nonphotochemical quenching of excitation in the antenna, and reoxidation of the quinone pool. Our results indicate that this approach can be used for detecting the changes in plant cells at the early stages of toxicant action.

Key words: Chlamydomonas moewusii, copper sulfate, methylmercury, fluorescence induction curve, photosystem, biotesting

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INTRODUCTION

Technological pollution of water basins with heavy metals affects the phytoplankton, which is the primary producer determining the state of the whole aquatic ecosystem. For algae, the most toxic pollutants are mercury and copper compounds [1].

Heavy metal ions exert their effects by binding with organic acids or phosphate anions, blocking essential (e.g. sulfhydryl) groups, and substituting for other metals in proteins [2–4]. The sequelae of these events are lipid peroxidation, impairment of ion transport and homeostasis, upset ATP balance, enzyme inhibition, and DNA damage [5–7].

Mercury compounds are known to suppress the light [1, 8–12] and dark steps [13] of photosynthesis, mainly by hindering membrane processes through interaction with SH compounds and protein disulfide groups as well as by displacing enzyme cofactors [9]. Copper ions at higher concentrations inhibit electron transport in photosystem (PS) II at both donor and acceptor sides [14–15].

In studies of the primary reactions of photosynthesis and of the photosynthetic apparatus as a whole, widely used are fluorescence methods, which are highly sensitive and allow rapid assessment of a number of characteristics such as the concentration of photosynthetic pigments, the photochemical energy transduction capacity of PS II, the probability of electron transport from PS II to the plastoquinone (PQ) pool, and the ΔpH-dependent energy dissipation in the PS II antenna [16–19]. Estimation of the maximal quantum yield of photochemical energy transduction from the relative value of variable fluorescence F_v/F_m is a widespread means of assessing the functional state of PS II in various conditions, including the presence of toxicants [20–21]. Thus a decline in F_v/F_m is indicative of PS II degradation, i.e. appearance of centers incapable of Q_A reduction. Correct measurement of this parameter requires prior dark adaptation of the specimen, providing maximal Q_A oxidation and relaxation of the nonphotochemical quenching processes. However, in some cases, e.g. at early steps of toxic action or at low toxicant concentrations, F_v/F_m may change only slightly if at all. In this case it may be useful to analyze the kinetics of light induction and dark decay of variable fluorescence, which affords information on the changes in electron transport at both PS II sides and through the PQ pool as well as on the ΔpH-dependent energy dissipation [22–25]. The reactions of photosynthetic electron transport can be quite sensitive to external influences, and changes therein sometimes precede PS II destruction. Thus a marked decrease in the rate of electron transport from PS II to the PQ pool is not necessarily accompanied by a

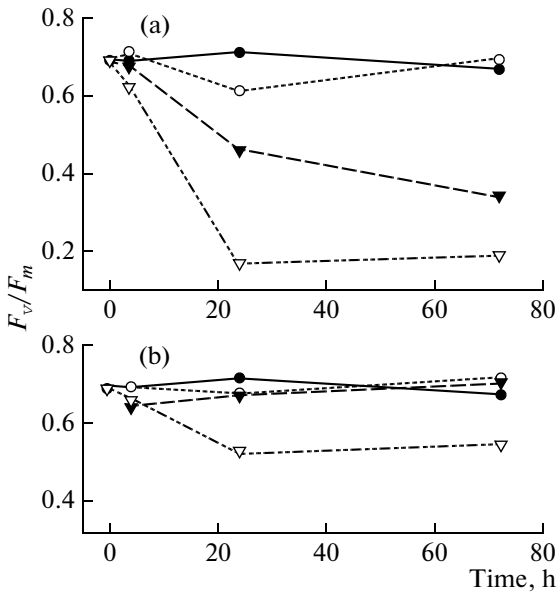

Fig. 1. Dynamics of the relative value of chlorophyll variable fluorescence (F_v/F_m) during incubation of *C. moewusii* under light (30 μE m^{-2} s^{-1}) in the control (filled circles) and in the presence of (a) MeHg (empty circles, 10^{-7} M; dark triangles, 5×10^{-7} M; empty triangles, 10^{-6} M) or (b) Cu^{2+} (empty circles, 10^{-6} M; dark triangles, 10^{-5} M; empty triangles, $5 \cdot 10^{-5}$ M). Prior to fluorescence assays, the specimens were kept in the dark for 5 min.

decline in F_v/F_m, e.g. upon the action of herbicides like diuron or atrazine. It should be noted that such kinetic measurements can be run with well-known commercially available instruments and take just a few seconds.

To see whether such analysis can be expedient in detecting the low-dose effects of heavy metals, here we have examined the characteristics of primary photosynthetic processes in microalgal (*Chlamydomonas moewusii*) cells exposed to methylmercury chloride and copper sulfate at concentrations that only slightly affect F_v/F_m.

EXPERIMENTAL

Algal culture and toxic exposure. An algologically pure culture of the green unicellular alga *C. moewusii* Gerloff (Lewin 1002, CALU 228 flagella-less mutant), obtained from the collection of the Chair of Microbiology, St. Petersburg State University, was grown phototrophically at 25°C in a Tris-acetate-phosphate medium (pH 7.0) under artificial daylight (30 μE m^{-2} s^{-1}, 14/10 h light/dark). Aliquots of stationary culture (25 mln cells in 100 mL) were transferred aseptically into 250-mL flasks, $CuSO_4 \cdot 5H_2O$ or MeHg chloride (Aldrich) were added at different concentrations, and the culture was further incubated under standard conditions up to 3 days.

Chlorophyll fluorescence assays. The F_v/F_m parameter in the algal suspension was determined using the pulse fluorimeter built at the Chair of Biophysics of the Biological Faculty, MSU for measurements on natural low-cell-density samples. The standard deviation calculated from data on four independent specimens did not exceed 8%.

The dark relaxation kinetics was recorded in a standard procedure using a PAM-2000 fluorimeter (Walz, Effelrich, Germany). Fluorescence was excited with modulated low-intensity light (0.1 μE m^{-2} s^{-1}, 655 nm, 600 Hz). To convert the PS II centers into the closed state with fully reduced Q_A, the specimen was illuminated for 2 s with intense white light (540 μE m^{-2} s^{-1}) from a halogen lamp (KL-1500, Schott, Germany). Four kinetic curves taken from four separate specimens were averaged, and the resulting curve was subjected to three-exponential fitting with SigmaPlot 9.0 (Systat Software).

The light induction kinetics was registered with Plant Efficiency Analyser (PEA) (Hansatech, King's Lynn, Norfolk, UK), which excites fluorescence with a powerful pulse at 650 nm; in our experiments, the pulse lasted 6 s at a flux density of 3000 μE m^{-2} s^{-1}.

RESULTS AND DISCUSSION

The F_v/F_m value characterizes the content of photochemically active PS II centers, which in its turn reflects the current balance between their light destruction and repair [14, 15]. These measurements were made during 72-h incubation of *C. moewusii* with different concentrations of MeHg or copper ions. As shown in Fig. 1, MeHg at 10^{-7} M slightly decreased the F_v/F_m (from 0.7 to 0.6 in 24 h) with further full recovery, indicating cell adaptation to the toxicant. At $5 \cdot 10^{-7}$ M, MeHg completely inactivated PS II, which is in line with its reported high toxicity for algae [1, 10]. Copper ions affected our object only at much higher concentrations; $5 \cdot 10^{-5}$ M Cu^{2+} decreased the F_v/F_m from 0.7 to 0.54 in 24 h, and the value did not further change through the observation period, suggesting equilibration of PS II destruction and repair.

It is known that the kinetics of induction of variable fluorescence measured in the millisecond range under saturating light comprises three phases of growth (*OJIP*) and subsequent decay (see Fig. 2a) [26]. The rise from the minimal (*O* or F_o) to the maximal level (*P* or F_m) mainly reflects the transition of PS II centers from the open state with oxidized Q_A to the closed state with reduced Q_A. The *OP* amplitude, just as F_v/F_m, corresponds to the content of photochemically active PS II center capable of Q_A reduction. The *OJ* phase reflects partial Q_A reduction while the PQ pool remains largely oxidized. The following *JIP* segment reflects the reduction of the remaining Q_A with complete reduction of the PQ pool. The relative amplitude

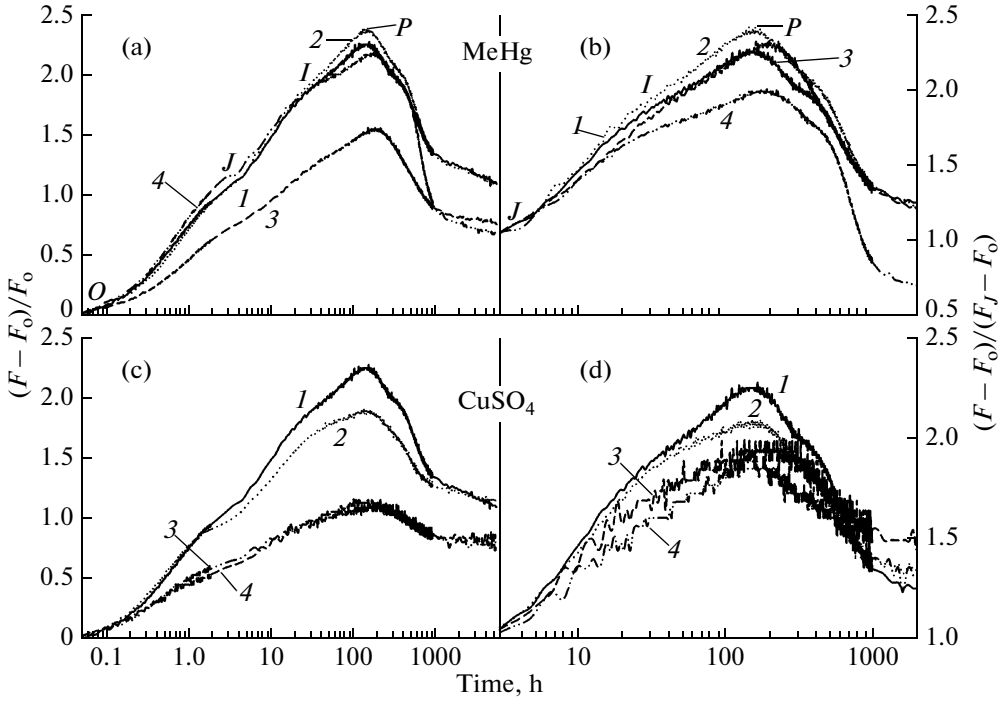

Fig. 2. Light-induced chlorophyll fluorescence transients (*OJIP*) measured in *C. moewusii* before (*1*) and after (*2*) 4, (*3*) 24, and (*4*) 72 h of incubation under light with (a, b) 10^{-7} M MeHg or (c, d) $5 \cdot 10^{-5}$ M Cu^{2+}. Left panels (a, c) show variable fluorescence ($F - F_o$) relative to F_o; right panels (b, d), relative to ($F_J - F_o$) where F_J is the fluorescence at 3 ms of illumination (inflection *J* on the curve). Prior to fluorescence assays, the specimens were kept in the dark for 5 min. Presented are typical curves chosen from measurements on four separate specimens using the PEA instrument.

of *JIP* characterizes the probability of electron transport from Q_A to the PQ pool [18], and its decrease testifies to impairment of electron transport. The time of reaching the maximal fluorescence yield $P(F_m)$ depends on the PQ pool reduction rate, and its increase testifies to impairment of electron transport [22]. The decline in fluorescence observed for several seconds after reaching the *P* level reflects nonphotochemical quenching caused by pH-dependent energy dissipation into heat in the PS II antenna [27]. The amplitude of this phase can indirectly characterize the ΔpH magnitude.

Figure 2 shows the *OJIP* transients recorded in *C. moewusii* cells before and after 4, 24, and 72 h of incubation with (a) 10^{-7} M MeHg or (c) $5 \cdot 10^{-5}$ M Cu^{2+} (the concentrations slightly affecting F_v/F_m, see above). One can see that the toxicants attenuated the *OP* amplitude (F_v/F_o) but the shape of the curves changed little. To estimate the *JIP* contribution, the curves were also normalized to the *OJ* amplitude (Fig. 2b,d). MeHg caused a small increase of *JIP* in 4 h, which could be due to hindered reoxidation of the PQ pool. Indeed, we have recently shown that dibromothymoquinone, an inhibitor of plastoquinone oxidation, slightly increases the maximal fluorescence yield (*P*) [28]. These results are in accord with the earlier data on the interruption of electron transport between PQ and PS I in the presence of MeHg [12]. The decrease in the *OP* amplitude observed in 24 h with MeHg indicates degradation of PS II centers. By 72 h the *OP* was restored to the initial level (a), but the *JIP* contribution decreased by 24% (b) and the time to $P(F_m)$ increased from 120 to 180 ms, indicating recovery of PS II photochemical activity and simultaneous appearance of centers with electron transport impaired in the acceptor part. In Fig. 2b one can also see that in 72 h with MeHg there was also an increase in the *qE* component of nonphotochemical quenching, which could reflect a greater transmembrane ΔpH. The latter can be caused by disturbed photophosphorylation in chloroplasts, which is also in line with published data [12]. Thus, prolonged incubation with 10^{-7} M MeHg did not cause PS II degradation but hindered the electron transport in PS II, decreased PQ reoxidation, and probably inhibited the ATPase.

As follows from Fig. 2c,d, $5 \cdot 10^{-5}$ M Cu^{2+} markedly lowered the maximal fluorescence yield (*OP* amplitude) while the *JIP* contribution and the curve shape changed less significantly. These results indicate a decrease of the number of functional PS II centers, possibly caused by suppressed repair [14].

The millisecond dark decay curves are resolved into three exponentials [23]. The fast and the medium components have been attributed to PS II reoxidation by the so-called "fast" and "slow" PQ pools [29], though these kinetic fractions probably reflect the het-

Lifetimes (τ) and amplitudes (*A*) of the fast (1), medium (2), and slow (3) components of the dark relaxation of variable fluorescence in *C. moewusii* cells incubated with MeHg and copper sulfate

Exposure	Incubation, h	τ_1, ms	τ_2, ms	τ_3, ms	A_1, %	A_2, %	A_3, %
Control	0	22	75	450	58	24	18
MeHg, 10^{-7} M	4	8	33	460	27	53	20
	24	9	37	500	24	56	20
	72	18	42	470	29	45	26
$CuSO_4$, $5 \cdot 10^{-5}$ M	4	4	62	832	24	42	34
	24	–	66	860	0	72	28
	72	–	52	700	0	69	31

Note: Decay measured with a PAM-2000 fluorimeter (see Experimental). Prior to measurements, the specimens were concentrated on a paper filter and kept in the dark for 5 min. Data averaged over four independently recorded kinetic curves were subjected to three-exponential fitting.

erogeneity of PS II in the affinity for PQ, and/or differences in PQ diffusion to and reoxidation at the cytochrome b_6/f complex. The slow relaxation component reflects recombination between Q_A^- and the S_2 state of the oxygen-evolving complex in the centers with impaired electron transport to PQ [30]. Accordingly, an increase in this component may indicate disturbances in the PS II acceptor part.

The table lists the results of three-exponential analysis of the dark relaxation curves measured in the same incubations as above. In the control, the fast component was dominant. Exposure to MeHg halved the amplitude of the fast component and correspondingly augmented the medium one, whereas the slow component increased somewhat only in 72 h. Thus, MeHg caused the PS II oxidation rate to decrease in a substantial fraction of the ensembles (adding to the "slow PQ pool"), and made some more centers incapable of PQ reduction. The latter result corresponds to the above analysis of the *OJIP* transients (Fig. 2). The marked shortening of the lifetimes calculated for the fast and medium components in the presence of MeHg may be associated with partial inhibition of PQ reoxidation.

Incubation with Cu^{2+} eliminated the fast component within 24 h, at the same time tripling the share of the medium component (i.e., the slowly oxidized PS II fraction) and almost doubling both the amplitude and the lifetime of the slow component. The latter effect indicates accumulation of PS II centers that cannot reduce PQ and have a lower rate of $Q_A^- - S_2$ recombination [30], probably because of hindered S-state transitions of the oxygen-evolving complex, which is consistent with the data on the inhibitory action of Cu^{2+} on the PS II donor part [14, 15].

CONCLUSIONS

The results presented here demonstrate the high sensitivity of PS II in the microalga *C. moewusii* to copper ions and MeHg, the latter being toxic at much lower concentrations. Both agents can suppress electron transport at the acceptor side of PS II and increase the fraction of PS II corresponding to the "slow PQ pool." Besides, MeHg hinders PQ reoxidation and increases the transmembrane pH gradient, which probably reflects impaired photophosphorylation in thylakoids.

Analysis of the light-induced transients and dark decay of chlorophyll fluorescence allows early detection of alterations caused by toxicants in algal cells, well before the onset of PS II destruction, and affords information on the possible mechanisms involved. This approach may prove useful in a variety of toxic and stress exposures.

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