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Photobleaching of photosynthetic pigments in spinach thylakoid membranes. Effect of temperature, oxygen and DCMU

M.Y. Velitchkova*, R. Picorel

Estacion Experimental de Aula Dei, CSIC, Apdo. 202, 50059 Zaragoza, Spain

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Abstract

The time dependence of photobleaching of photosynthetic pigments under high light illumination of isolated spinach thylakoid membranes at 22 and 4 °C was investigated. At 22 °C, the bleaching at 678, 472 and 436 nm was prominent but lowering the temperature up to 4 °C during illumination prevented the pigments from bleaching almost completely. The accelerating effect on pigment photobleaching by the presence of 3-(3,4 dichlorophenyl)-1,1-dimethyl-urea)—(DCMU), a well-known inhibitor of the electron transport and known to prevent photosystem I (PSI) and photosystem II (PSII) against photoinhibitory damage, was also suppressed at low temperature. At 22 °C in the presence and absence of DCMU, the decrease of the absorption at 678 and 472 nm was accompanied by a shift to the shorter wavelengths. To check the involvement of reactive oxygen species in the process, pigment photobleaching was followed in anaerobiosis. The effects of the three different environmental factors—light, temperature and DCMU—on the dynamics of photobleaching are discussed in terms of different susceptibility of the main pigment–protein complexes to photoinhibition.

Keywords: Environmental factors; Photobleaching; Pigments; Spectroscopy; Thylakoids

1. Introduction

During prolonged exposure of leaves or choroplasts to high light intensity two processes were observed—photoinhibition and photobleaching. Light-induced damage targets mainly to PSII, causing inactivation of electron transport and subsequent oxidative damage of the reaction center [1]. In recent years, it has been demonstrated that PSI is also photoinhibited, particularly at low temperature in chilling sensitive plants [2]. During the photoinhibition of isolated PSI complex the light-harvesting complex (LHC) of PSI was more affected than the core complex [3]. These findings raise the question about the involvement and importance of photobleaching of photosynthetic pigments in the process of photoinhibition.

The photobleaching involves a loss of bulk photosynthetic pigments. The holochromes located at the end of the energy migration pathways receive a greater proportion of the excess energy and should undergo faster photobleaching. The

*Corresponding author. *Address*: Institute of Biophysics, Bulgarian Academy of Sciences, Acad. G. Bonchev str. bl. 21, 1113 Sofia, Bulgaria. Tel.: +359-2-979-2621; fax: +359-2-971-2493.

E-mail address: mayav@obzor.bio21.bas.bg (M.Y. Velitchkova).

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chlorophyll aggregates absorbing at longer wavelengths bleach before the holochromes absorbing at shorter wavelengths [4,5]. Miller and Carpentier [6] demonstrated that the pigments of PSI submembrane fractions bleached faster than those of PSII complex, the latter being more sensitive towards photoinhibition.

The photobleaching of photosynthetic pigments is accelerated in the presence of inhibitors of photosynthesis, including the herbicides affecting the acceptor side of PSII. The degradation of pigments is considered to result from photooxidation induced by its inability to dissipate absorbed excitation energy when the electron transport is blocked. During illumination in vitro, Ridley (1977) observed rapid destruction of chlorophylls from light harvesting complex II (LHCII) [7]. However, in the presence of DCMU the chlorophyll *a* associated with the complex of PSI is also destroyed approximately to the same extent. Later, Horton and Ridley proposed that the DCMU-induced enhancement of the pigment destruction would only be produced when the herbicide dose was sufficiently high to inhibit not only the intersystem electron transport but also to block the natural cyclic flow associated with PSI as well [8]. A similar effect on photobleaching was also observed when the inhibition of PSII electron transport was achieved by different manners—exposure to high and low pH, pretreatments with phospholipase A_2 and linolenic acid, short time heat stress [9,10]. It is supposed that the most affected pigments are those associated with PSI [10]. The chlorophyll photobleaching was enhanced also in the early stages of greening of cucumber cotyledons [11].

The involvement of active oxygen radicals in photobleaching of chloroplast pigments has been demonstrated by the protective role of some oxygen species scavengers, like superoxide dismutase [12], flavonols [13], carotenoids [14], α-tocopherol [15] and some reductants [16]. It was supposed that the damages of chlorophylls were primarily caused by singlet oxygen. The production of singlet oxygen occurs via interaction between the ground state electrons of triplet oxygen with the triplet state of chlorophyll [17] or by participation of Fe–S centers in thylakoid membranes [18].

The present work showed that the bleaching of the pigments could be considerably suppressed by lowering the temperature up to 4 °C during the photoinhibitory treatment. The involvement of oxygen reactive species on pigment photobleaching was proved experimentally in anaerobiosis. The temperature and the oxygen dependence of the stimulating effect of DCMU on pigment destruction were also studied. The data obtained demonstrated the different susceptibility of different pigment pools to photobleaching.

2. Experimental

2.1. Thylakoid membrane isolation

The thylakoid membranes were isolated from market spinach as described in [19]. The final pellet was resuspended in 20 mM Tricine (pH 7.6), 0.33 M sucrose, 10 mM NaCl, and 5 mM $MgCl_2$. Chl concentration was determined by the method of Lichtenthaler [20].

2.2. High light treatment

The illumination of isolated membranes was carried out in temperature-controlled vessel under continuous stirring. For both sets of measurements the temperature was 22 and 4 °C. The Chl concentration during illumination was 150 μg Chl/ml and 20 μM DCMU was present where indicated. The thylakoid suspension was illuminated with a project lamp giving intensity on the vessel surface of 1800 $\mu E\ m^{-2}\ s^{-1}$. Anaerobiosis during photoinhibition was achieved by bubbling of the medium for 30 min before resuspending the thylakoid membranes and maintaining N_2 flow during illumination. Control samples were kept in the dark under identical conditions to the illuminated samples in order to monitor dark inactivation processes.

2.3. Absorption spectra measurements and analysis

Absorption spectra of control and treated thylakoid membranes were performed with a Beckman

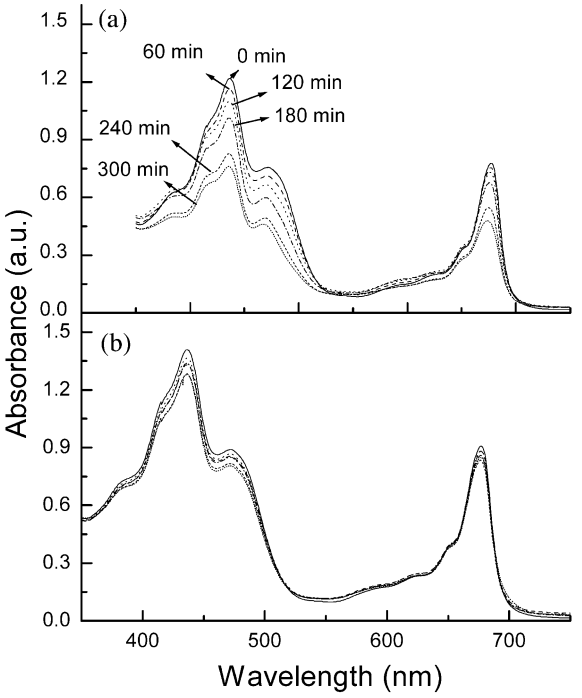

Fig. 1. Absorption spectra of isolated thylakoid membranes illuminated for various periods of time with strong white light (1800 μE m^{-2} s^{-1}) at 22 °C (a) and 4 °C (b) in the absence of DCMU. Thylakoid membranes were resuspended in 20 mM Tricine (pH 7.6), 0.33 M sucrose, 10 mM NaCl, and 5 mM $MgCl_2$ at a Chl concentration of 15 μg Chl/ml. Spectra were recorded at room temperature.

DU-640 spectrophotometer at room temperature in 1 cm pathlength cuvettes. The chlorophyll concentration during measurements was 15 μg Chl/ml. The spectra were analyzed using an Origin 5.0 (Microcal Software Inc, USA).

3. Results

3.1. Changes of absorption spectra during illumination in aerobiosis

The dependence of absorption spectra changes of thylakoid membranes with the time of high light intensity illumination was followed at 4 °C and compared with those obtained at 22 °C (Fig. 1). In Fig. 1a the absorption spectra of thylakoid membranes, illuminated at 22 °C in the presence of oxygen are presented. With the increase of the time of illumination, the absorption decreased equally (by approx. 30–36%) in the blue and in the red region. However, the absorption at 650 nm was less affected. A concomitant blue shift of the bands at 678 and 472 nm was also observed. Illumination of thylakoid membranes for the same time period but at 4 °C did not lead to a significant decrease of absorption and no changes of the peak wavelengths were observed (Fig. 1b).

In the presence of DCMU the bleaching of photosynthetic pigments proceeded more intensively. Fig. 2a shows the absorption spectra of thylakoid membranes treated with high light intensity in the presence of 20 μM DCMU at 22 °C. The intensities of the absorption bands decrease considerably. Lowering the temperature up to 4 °C during light treatment reduced the bleaching even in the presence of DCMU (Fig. 2b).

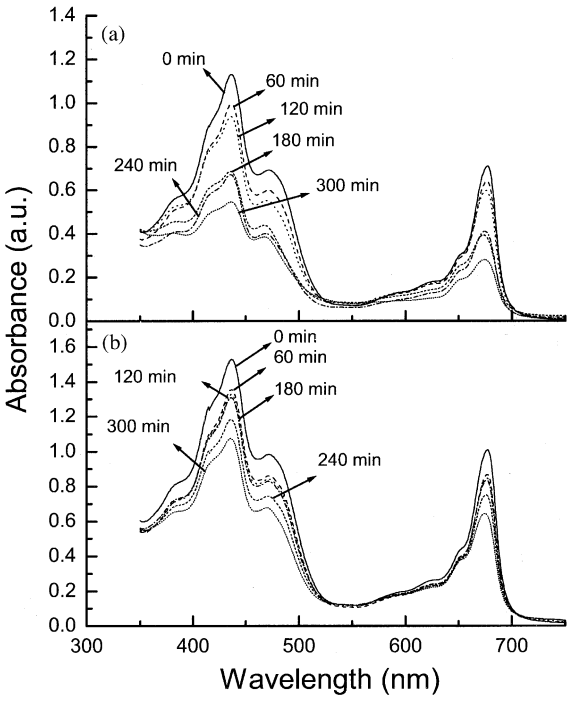

Fig. 2. Absorption spectra of isolated thylakoid membranes illuminated for various periods of time with strong white light (1800 μE m^{-2} s^{-1}) at 22 °C (a) and 4 °C (b) in the presence of 20 μM DCMU. Experimental conditions were as in Fig. 1.

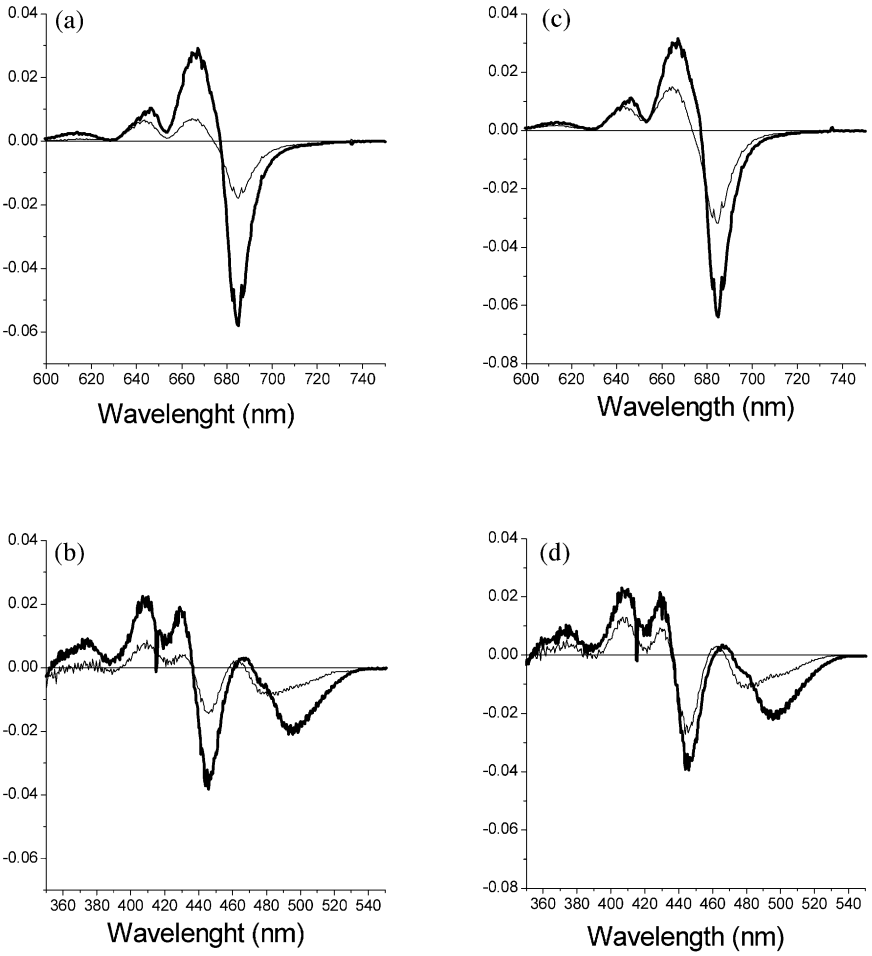

Fig. 3. First derivatives of the absorption spectra of control and illuminated for 5 h thylakoid membranes in the presence of 20 μM DCMU at 22 °C (a and b) and at 4 °C (c and d). Red spectral region (a and c); blue spectral region (b and d).

The positions of the main peaks were verified by analysis of the first derivatives of the absorption spectra. In Fig. 3 the first derivatives of the absorption spectra in the red (a, c) and blue (b, d) regions of control and illuminated with high light intensity for 5 h thylakoid membranes are presented. The spectra were recorded in the presence of 20 μM DCMU at 22 °C (a, b) and at 4 °C (c, d). In the red region, the blue shift of the maximum at 678 nm was approximately 4 nm. In the blue region, no shift of the peak at 436 nm was observed, but the peak at approximately 472 nm became shifted to shorter wavelengths. At 4 °C in the presence of DCMU a blue shift of the red maximum was also observed. In the presence of DCMU the change of amplitude of maximum at 472 nm was approximately the same at both studied temperatures (Fig. 3b,d).

The values for the decrease of absorption and blue shifts of the main maxima for control thylakoid membranes and illuminated for 5 h at indicated conditions are summarized in Table 1. No blue shift is observed at 436 nm at any condition investigated. The shift of the maxima at 472 and 678 nm are more pronounced in the presence of DCMU and do not depend on temperature when

Table 1
Values for the intensity decrease of the main absorption maxima (I; in % from non-illuminated samples) and values for blue shift $\Delta\lambda$ (in nm) for thylakoid membranes illuminated for 5 h in aerobiosis in the absence and in the presence of 20 μM DCMU at 22 and 4 °C

	−DCMU		+ DCMU	
	22 °C	4 °C	22 °C	4 °C
I_{436} (%)	30	9	51	39
$\Delta\lambda_{436}$ (nm)	0	0	0	0
I_{472}(%)	31	9	37	39
$\Delta\lambda_{472}$(nm)	2	0	4	4
I_{678}(%)	36	6	61	38
$\Delta\lambda_{678}$(nm)	2	0	4	4

20 μM DCMU are present in the reaction medium during illumination. In the absence of DCMU, the shift is observed only at 22 °C. The intensity of the shoulder at 650 nm, attributed to absorption of Chl *b* is much less affected, especially in the absence of DCMU—no shift and no significant change of intensity was observed at 4 °C, and only a small change of amplitude at 22 °C was detected (data not shown). In the presence of DCMU, the absorption at 650 nm seems to be reduced. However, the absorption at 650 nm is dominated by the main red band peaking at 678 nm. Therefore, the decrease at 650 nm is most probably due to the decrease of the main band but not to a specific change at 650 nm. This suggestion is supported by the observation that after 5 h high light treatment the ratio of the intensities at 678 and 650 nm (I678/I650) decreased to the same extent as the intensity at 678 nm. Lowering the temperature up to 4 °C reduces the bleaching at 678 and 436 nm. In the presence of DCMU the absorption at 472 nm decreases to the same extent independently of the temperature.

Taking in mind the contribution of Chl *b* and carotenoids in the absorption at 472 nm, we analyzed the second derivatives of the absorption spectra in this region for untreated thylakoid membranes and illuminated for 5 h in the absence and in the presence of DCMU at room temperature (Fig. 4). It is evident that the long wavelength carotenoid band (indicated with a arrow) is completely diminished and as result, the resulting maximum is reduced and blue shifted.

3.2. Time dependence of band absorption intensities

The data on the time dependence of absorption maxima in different spectral regions at both temperatures are summarized in Fig. 5. At 22 °C, the photobleaching of bands at 436, 472 and 678 nm proceeds by a similar manner. At this temperature and in the presence of DCMU two phases of absorption bleaching at 436 and 472 nm could be distinguished—a faster one within the first 3 h and a slower one between 3 and 5 h. The shape of the intensity time dependence of the absorption maxima for all bands tested at 22 °C without DCMU are very similar to those observed at 4 °C in the presence of the inhibitor.

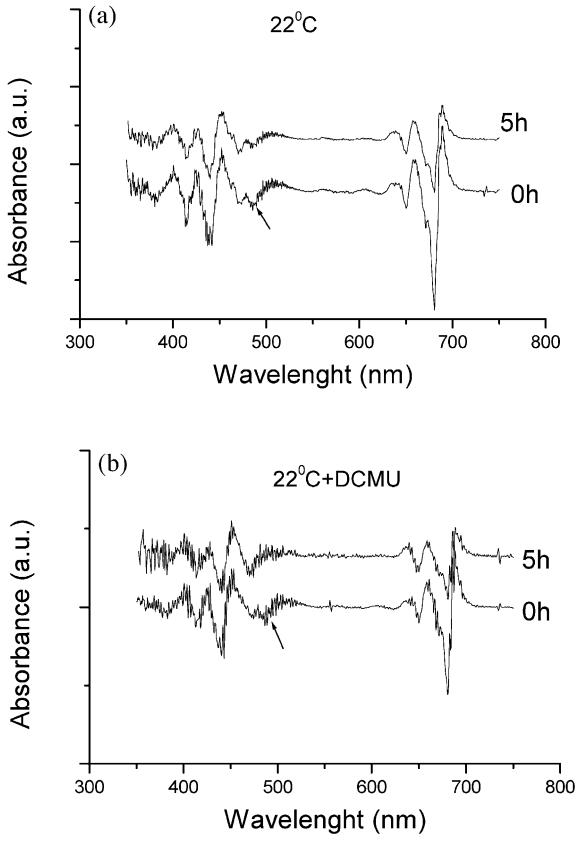

Fig. 4. Second derivative of absorption spectra of control and illuminated for 5 h thylakoid membranes at room temperature in the absence (a) and in the presence (b) of 20 μM DCMU. The arrows indicate the absorption attributed to carotenoids.

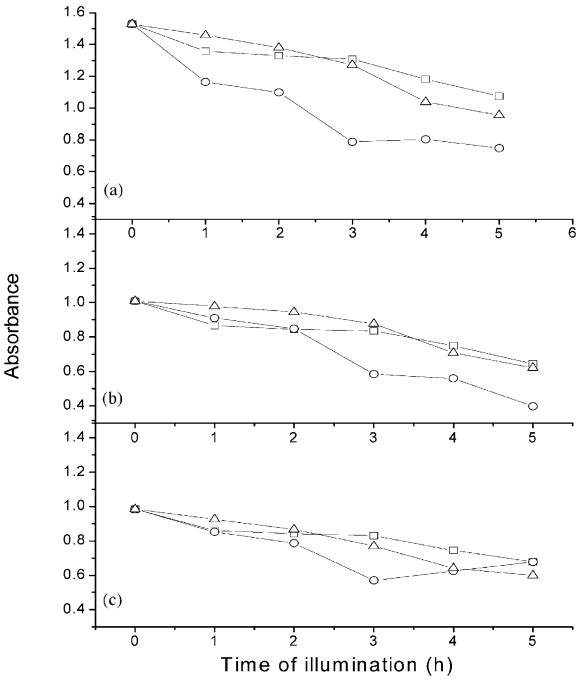

Fig. 5. Time dependence of the intensities of absorption maxima at 436 nm (a), 678 nm (b) and 472 nm (c) under different conditions. At 22 °C—squares; 22 °C + 20 μM DCMU—circles; 4 °C + 20 μM DCMU—triangles.

3.3. Photobleaching under anaerobiosis

In order to check to what extent the oxygen radicals are responsible for pigment photobleaching, the same set of experiments is carried out under anaerobiosis**.** In these conditions no significant changes of the thylakoid absorption spectra are observed under illumination. At 22 °C the decrease of intensity of absorption maxima at 436 and 678 nm is appoximately 2–5% and a slight enhance is observed in the presence of 20 μM DCMU, but the change is not significant. No other spectral changes are observed. The light-induced decrease of absorption at 4 °C is as at 22 °C and is not affected by the presence of DCMU. No blue shift is observed at any wavelength (data not shown).

4. Discussion

Different kinetics of pigment photobleaching were observed during illumination of isolated thylakoid membranes under different sets of environmental conditions- room and low temperatures, presence and absence of oxygen, and presence and absence of DCMU.

Under anaerobiosis no bleaching or blue shift of the main bands were observed, independently of the applied temperature. The lack of pigment bleaching in anaerobiosis was also shown for PSII core complex [21]. It was also shown that under anaerobiosis the increased susceptibility to pigments destruction of heat stressed membranes was not observed [10]. In this work, we show that under anaerobiosis no significant pigment bleaching is detected even in the presence of DCMU. Thus, the enhance effect of DCMU on the destruction of photosynthetic pigments under high light illumination was manifested only in the presence of oxygen.

In aerobiosis the extent of pigment bleaching depends on the temperature and the presence of DCMU. However, the different absorbance bands do not exhibit the same illumination time dependence. The most intriguing finding is that the photobleaching of pigments can be suppressed almost completely at 4 °C—approximately only 2–9% of absorption band reduction takes place at 678 and 436 nm during illumination at low temperature. No blue shifts of the bands at 472 and 678 nm are observed when the illumination is carried out at 4 °C. The protective effect of low temperature is reduced in the presence of DCMU—when the linear and cyclic electron flows are inhibited. The nature of that low temperature protection against pigment destruction even in the presence of oxygen is not clear. Several hypotheses can be consider: (i) the production of oxygen radicals is suppressed at low temperature; (ii) the conformational state of the pigment–protein complexes at this temperature provides better pigment protection; (iii) low temperature facilitates a better dissipation of excess light energy; (iv) oxygen species mobility is reduced considerably at this temperature. Between all these hypotheses, the latter seems to be the more plausible one. At temperatures as low as 4 °C the mobility of the chemical reactants would be drastically reduced. This slower mobility could affect in two ways: (i) the oxygen would have more difficulties to reach

and interact with the electron transport chains reducing, therefore, the efficiency of light-induced oxygen radical formation, and (ii) the oxygen radicals formed would move slower at low temperature than at 22 °C reducing the probability to reach their sensitive targets. This effect would even be more pronounced when the targets (i.e. the pigment–protein complexes) are embedded in the thylakoid membranes, as the permeability of biological membranes for oxygen would be very much reduced at low temperatures due to the strong temperature dependence of membrane fluidity.

In the absence of DCMU, the blue shift of the maxima at 678 and 472 nm is observed at room temperature only. It must be noted that no shift is observed for the band at 436 nm independently of the temperature and the presence of DCMU. The blue shift of the maximum at 678 nm is due most probably to a preferential bleaching of Chl molecules absorbing at longer wavelengths. Earlier it has been reported, that this shift was observed in PSI preparations but not in PSII preparation [6]. Therefore, we can suppose that the observed shift is related to the long wavelength absorbing pigments, associated mainly with PSI. The blue shift of the absorption band at 472 nm is due to the bleaching of carotenoids, absorbing in this spectral region, although the partial destruction of Chl *b*, contributing to the absorption at 472 nm cannot be excluded (Fig. 3). However, the absorption at 650 nm, attributed to Chl *b* does not change. Thus, the bleaching of carotenoids seems to be the main reason for the blue shift at 472 nm.

The enhance effect of DCMU on photobleaching of photosynthetic pigments at room temperature has widely been discussed in the literature. Our data demonstrate that DCMU does not affect in the same manner the bleaching of different absorption bands. In the absence of DCMU at 22 °C, the bleaching of pigments absorbing at 436, 472 and 678 nm proceeds almost homogeneously. The addition of DCMU in the reaction medium during light treatment results in different kinetics of pigments bleaching. The bleaching of absorbance bands attributed to Chl *a* (436 and 678 nm) is enhanced up to 50–60%. However, at 22 °C the extent of bleaching at 472 nm is slightly affected by the presence of DCMU. On the other hand, in the presence of DCMU the protective effect of low temperature on the bleaching is manifested only for bands at 678 and 436 nm, while it is not observed for the band at 472 nm associated with carotenoids destruction. Therefore, it could be assumed that different mechanisms of Chl and carotenoids destruction take place in isolated thylakoid membranes, especially when the electron transport is inhibited. Both mechanisms are oxygen mediated but they exhibit different temperature sensitivity depending on the presence of electron transport inhibitors.

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