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Heat-induced disassembly and degradation of chlorophyll-containing protein complexes in vivo

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ABSTRACT

Gradual heating of green leaves up to non-physiological temperatures is often used to estimate thermal stability of photosynthetic apparatus. However, a complete sequence of heat-induced disassembly and denaturation of chlorophyll-containing protein complexes (CPCs) has not been reported yet. In this work, we heated (1 °C·min^{-1}) barley leaves to temperatures selected according to the changes in the chlorophyll fluorescence temperature curve (FTC) and we analyzed CPC stability by two-dimensional native Deriphat/SDS–PAGE. The first distinct change in both structure and function of photosystem II (PSII) appeared at 40–50 °C. PSII core (CCII) dimers began to dissociate monomers, which was accompanied by a decrease in PSII photochemistry and reflected in FTC as the first fluorescence increase. Further changes in CPCs appeared at 57–60 °C, when FTC increases to its second maximum. Photosystem I (PSI) cores (CCI) partially dissociated from light-harvesting complexes of PSI (LHCI) and formed aggregates. The rest of CCI–LHCI complexes, as well as the CCI aggregates, degraded to the PSI-A/B heterodimer in leaves heated to 70 °C. Heating to these temperatures led to a complete degradation of CCII components and corresponding loss of PSII photochemistry. Trimeric light-harvesting complexes of PSII (LHCII) markedly dissociated to monomers and denatured, as evidenced by a release of large amount of free chlorophylls. Between 70 and 80 °C, a complete degradation of LHCII occurred, leaving the PSI-A/B heterodimer as the only detectable CPC in the membrane. This most thermostable CPC disappeared after heating to 90 °C, which corresponded to a loss of PSI photochemistry.

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1. Introduction

In the 1960s and the 1970s, investigations of photosynthetic activities in heated chloroplast preparations revealed that photosystem I (PSI) is more thermostable than photosystem II (PSII) (for review, see [1]). In the subsequent decade, research was focused on the correspondence between the inactivation and the denaturation of photosystems. Characteristic endothermic transitions of thylakoid membranes or photosystem preparations were obtained using differential scanning calorimetry (DSC).

The first DSC transition appearing at 42 to 48 °C was shown to reflect a disruption of the PSII donor side with corresponding loss of oxygen evolution [2], but no PSII protein was observed to denature during this transition [3]. A concomitant decrease in the amount of the high potential form of cytochrome b_{559} [2] and oxidized tyrosine D [3] implied that this transition involves structural changes in integral proteins of PSII. This rearrangement of PSII was supported by later findings that the heat-induced disruption of PSII donor side is associated with the release of components of oxygen evolving complex [4] and a change of the midpoint potential of the primary quinone acceptor Q_A [5].

The second endothermic transition in thylakoid membranes appears at about 60 °C (at neutral pH). It is accompanied by loss of electron transport through PSII [6] and it probably represents a denaturation of the PSII reaction centers. The next DSC transition at about 75 °C reflects a denaturation of light-harvesting complex of PSII (LHCII) [7]. The temperatures of these two DSC transitions were shown to be affected by particular sample preparation and the buffers used [3]. The nature of DSC transitions appearing in thylakoid membranes above 80 °C [2,7] has not been determined yet, but some of these transitions could reflect a denaturation of PSI.

It should be noted that the mentioned denaturation temperatures of individual chlorophyll-containing protein complexes (CPCs) determined by DSC were obtained at standard linear heating rate 1 °C·min^{-1}. A decrease of heating rate to 0.125 °C·min^{-1} can lower the DSC transitions by about 5 °C, as was recently demonstrated for isolated LHCII [8]. Thus, a thermal dose absorbed by the linearly heated CPCs can influence the denaturation temperatures.

From 1990s, conformation changes of CPCs during their heat denaturation have been studied using various techniques based on optical spectroscopy. Using the infrared absorption of amide I, De Las

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Rivas and Barber [9] documented that the reaction center of PSII changes its secondary structure at about 42 °C and that PSII core denatures around 60 °C [10], which confirmed the results of previous DSC studies. Using circular dichroism (CD) spectroscopy, Dobrikova et al. [11] showed that the heat denaturation of LHCII is preceded by a dissociation of trimeric LHCII to its monomeric form. This occurs at temperature between 60 and 74 °C, depending on the presence of surrounding lipids [12].

The heat-induced changes in the secondary structure of PSI have been reported only recently. Using infrared spectroscopy and 77K chlorophyll fluorescence spectra in isolated PSI complexes, Hu and coworkers [13] observed the major conformation transition at 60 to 70 °C, which was accompanied by the energetic disconnection of LHCI from PSI core. Denaturation of PSI was shown to take place in a wide temperature interval 70–90 °C [13].

All the abovementioned denaturation studies were performed with isolated thylakoid membranes or CPCs and therefore a question arises as to whether the heat transition temperatures are the same for CPCs *in vivo*. There are only a few studies which are focused on direct determination of the of heat stability of CPCs *in vivo* [14,15]. The authors employed native electrophoresis to study the heat-induced disassembly of LHCII and PSII after heating of leaves at temperatures not exceeding 50 °C. However, in these studies the leaves were heated at fixed temperatures (1 h and 5 min, respectively), and therefore the obtained results are hardly comparable with the *in vitro* denaturation studies using linear heating regime. There exist *in vivo* studies that use the linear heating regime, but they present only an indirect estimate of the CPC thermostability. These studies employ mainly the measurement of chlorophyll fluorescence (fluorescence temperature curve; FTC) [16–19] and thermo(chemi)luminescence (for reviews, see [20,21]) in continuously heated leaf samples. In order to understand these heat-induced changes in chlorophyll emission, it is important to directly analyze the disassembly and denaturation of CPCs during linear heating of leaves *in vivo*. At the same time, this analysis would enable the comparison of the data obtained *in vivo* with the *in vitro* denaturation studies.

In this work, we have analyzed the irreversible disassembly and denaturation of CPCs during linear heating of barley leaf segments. In order to obtain comparable results, we have used heating rate 1 $°C \cdot min^{-1}$ that was used in several previous DSC and FTC studies. In our approach, the changes of CPCs were determined using a two-dimensional native Deriphat/SDS–PAGE optimized for the separation of CPCs from barley [22,23]. Corresponding changes in the function of photosystems were detected directly in heated leaf segments by the simultaneous measurement of PSII fluorescence and PSI absorption changes. The results enabled us to determine a sequence of heat-induced structural/functional changes in CPCs *in vivo* within 25 to 90 °C and their relevance to the changes in FTC.

2. Materials and methods

2.1. Plant material

Barley (*Hordeum vulgare* L. cv. Akcent) seedlings were grown at 25 °C in a growth chamber with 16/8 hr photoperiod at 100 μmol photons$\cdot m^{-2} \cdot s^{-1}$ (photosynthetically active radiation, PAR). Middle parts of 8-day-old primary leaves were linearly heated (1 $°C \cdot min^{-1}$) from 25 °C to selected temperatures in the dark. After heating, the leaf segments were immediately cooled down in a water bath (25 °C, 30 s) and within 1 min used for the preparation of thylakoid membranes or for the measurement of photosystem function at room temperature.

2.2. Fluorescence temperature curve (FTC)

FTC is a dependence of chlorophyll fluorescence intensity on linearly increasing temperature. The FTC was measured with leaf segments using a fluorescence spectrophotometer F-4500 (Hitachi, Tokyo, Japan) extended by the laboratory setup with fiber optics allowing the measurement outside the sample chamber. Fluorescence was excited on the upper leaf side at 436 nm (10 μmol photons$\cdot m^{-2} \cdot s^{-1}$) and detected at 680 nm. The spectral slit-width of the excitation and emission monochromator was 10 and 5 nm, respectively. Leaf segments were placed on a sample holder, immersed in distilled water, and linearly heated from 25 to 90 °C at a rate of 1 $°C \cdot min^{-1}$ using a home-built computer-controlled heating device.

2.3. Preparation of thylakoid membranes

Thylakoid membranes for the subsequent electrophoretic separation of CPCs were isolated from unheated (25 °C) or preheated leaf segments according to reference [22]. The membranes were resuspended in the extraction buffer (11.3 mM Tris, 87 mM glycine, 10% glycerol (v/v)) to the final concentration of 1 mg chlorophyll/ml. The chlorophyll content was determined spectrophotometrically in 80% acetone according to Lichtenthaler [24].

2.4. Native Deriphat–PAGE, SDS–PAGE, and immunoblotting

Thylakoid membranes were solubilized with 20% decyl-β-D-maltoside to yield the final 25:1 (w/w) ratio of detergent to chlorophyll. Solubilized thylakoids were centrifuged at 7000×*g* for 135 s to remove the colorless insoluble material, and the green supernatant was immediately applied to polyacrylamide gel. The native Deriphat–PAGE of CPCs was performed according to published methods [22,23,25]. The solubilized thylakoids (7 μL) were loaded onto 6% (w/v) gel. Electrophoresis was performed at 2 °C in the dark at a constant voltage 50 V for 15 min and then at 90 V for 150 min. The gel containing green bands was scanned at 670 nm by a home-made 2-D gel densitometer [26]. Separated CPCs were analyzed and identified by denaturing SDS–PAGE and by immunoblotting according to reference [27]. The antibodies against D1 and CP47 were bought from Agrisera Company, and the others were gifts from others. The antibodies were detected using anti-rabbit (IgG)-peroxidase antibody (Sigma).

2.5. Deriphat purification

The commercial Deriphat (powder; Cognis, Cincinnati, OH) contained high amount of impurities disturbing CPCs; therefore, we purified it before use. We heated water solution of Deriphat at 70–80 °C and collected the crusts formed on the beaker surface as a purified Deriphat.

2.6. Determination of molecular mass of CPCs

Molecular mass of CPCs resolved by green native Deriphat–PAGE was determined using a method of Ferguson plot [28,29]. Solubilized CPCs and standard proteins of known molecular masses were electrophoresed in seven gels with different acrylamide concentration (4.5%, 5%, 5.5%, 6%, 7%, 8%, and 9% (w/v)).

2.7. Measurement of photosystem function

PSII and PSI functions in heat-treated leaf segments were monitored simultaneously using a Dual-PAM 100 with measuring heads Dual E and Dual DB (Walz, Effeltrich, Germany) at room temperature. PSII function was detected by the chlorophyll fluorescence ratio F_V/F_M (variable to maximum chlorophyll fluorescence level) that reflects the maximum quantum yield of PSII photochemistry. PSI function was monitored as the light-induced increase in a difference between the 830- and 875-nm leaf transmittance signals,

which reflect mainly the oxidation of P700, the donor of PSI. The preheated leaves were irradiated using the 1-s red light pulse (2000 μmol photons·m^{-2}·s^{-1}) to close the reaction centers of PSII and to induce the oxidation of P700 (see [30,31]). Near infrared LEDs emitting at 830 and 875 nm were both set at level 5. The chlorophyll fluorescence during the 1-s pulse was measured using a modulated measuring blue light.

3. Results

3.1. Fluorescence temperature curve (FTC)

Fig. 1 represents a typical FTC with two characteristic maxima (M1, M2) measured with the primary leaf of 8-day-old barley at a heating rate of 1 °C·min^{-1} within the temperature range of 25 to 90 °C. Similar FTCs have been already reported for leaves of different species (e.g., [32]), heating rates [18], and excitation wavelengths [33]. The whole course of FTC reflects changes in the fluorescence intensity of chlorophyll *a* [34].

The fluorescence level in FTC is rather constant up to 40 °C, but the subsequent heating to 50 °C is accompanied by a gradual increase in fluorescence intensity to the first maximum (M1) (Fig. 1). The latter phase reflects a gradual accumulation of Q_A^- at the acceptor side of PSII [35] due to the inhibition of electron transport from Q_A to Q_B [36–38], which is connected with the irreversible structural change of PSII (see Introduction). The initial part of this FTC phase is reversible [39,40], in particular at higher heating rates [41]. Although a detailed molecular mechanism of the heat-induced inhibition of electron transport from Q_A to Q_B is not known, a critical temperature of the fluorescence increase is often used as an indicator of PSII thermostability (see, e.g., [16–19]). The fluorescence decrease observed at 50 to 57 °C was shown to be partially reversible [39,41] and reflects probably a general temperature-dependent lowering of fluorescence quantum yield of chlorophyll *a* molecules [42]. The subsequent FTC phases are fully irreversible [18,43]. The steep increase of fluorescence to the M2 maximum (60 °C) has been ascribed to highly fluorescing chlorophyll *a* released from CPCs [44]. Finally, the fluorescence decrease above 60 °C has been explained by a progressive formation of low-fluorescing chlorophyll *a*-lipid aggregates [44]. A critical evaluation of the alternative causes of the individual phases in FTC, as well as a description of the heat-induced changes in chloroplasts responsible for the accumulation of Q_A^-, can be found in references [21,38,44].

Based on the FTC shape in Fig. 1, we selected eight distinct temperatures within the range 25 to 90 °C. The leaf segments heated to these temperatures were rapidly cooled down to 25 °C and used to monitor the irreversible disassembly and degradation of CPCs and photosystem function during linear heating.

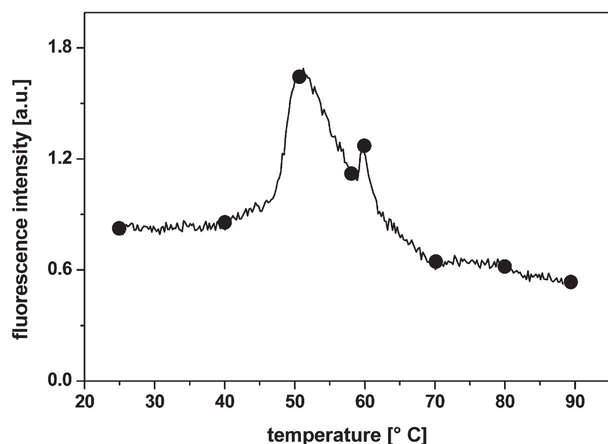

Fig. 1. A typical chlorophyll fluorescence temperature curve (FTC) of a barley leaf at a heating rate of 1 °C·min^{-1}. Eight selected temperatures in FTC (indicated) were chosen for the analysis of CPCs during heating. Excitation and emission wavelength were 436 and 680 nm, respectively.

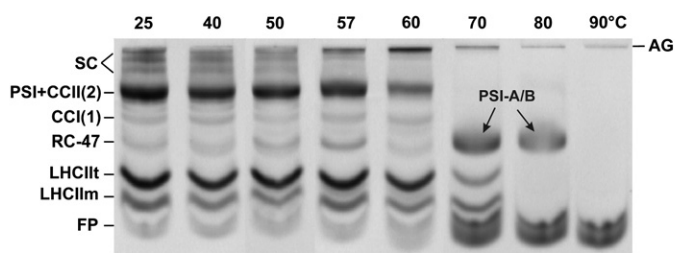

Fig. 2. Electrophoretograms of CPCs separated by native Deriphat–PAGE from thylakoid membranes of barley leaf segments linearly heated (1 °C·min^{-1}) to selected temperatures (Fig. 1). SC: supercomplexes; PSI + CCII(2) band: PSI core with bound LHCI and PSII core dimer (see also Fig. 3); CCI(1) band: monomeric PSI core; RC-47 band: reaction center of PS II (D1, D2, and CP47); LHCIIt(m): trimeric (monomeric) form of LHCII; FP: free pigments, AG: aggregates.

3.2. Pigment–protein complexes in control leaves

Native CPCs from thylakoid membranes of barley leaves were separated by a green native Deriphat–PAGE (Fig. 2). The Deriphat–PAGE has been developed as a method suitable for the separation of native CPCs from barley leaves with low amount of released free pigments [22]. The green bands in the gel were visualized by the monochromatic imaging at 670 nm. This monochromatic light, which coincides with the red absorption maximum of chlorophyll *a*, allows visualization of even faint-green bands [26].

The molecular masses of separated CPCs, determined by the method of Ferguson plot, were found to be about twice higher than those estimated previously using a simple semilog plot [23]. Our values are in agreement with present estimates of CPCs molecular masses (for details, see Appendix). The separated CPCs were identified using SDS–PAGE (Fig. 3) combined with immunodetection of selected proteins and 77K chlorophyll fluorescence spectra.

The separation of CPCs from control leaf thylakoids resulted in five main green bands with apparent molecular masses of approximately 435, 280, 215, 120, and 50 kDa (PSI + CCII(2), CCI(1), RC-47, LHCIIt, and LHCIIm; Fig. 2). Western blot analysis and SDS–PAGE revealed that the PSI + CCII(2) band contained dimeric core complex of PS II (CCII(2)) and PSI, the core complex of photosystem I (CCI) with LHCI. The CCI(1) band contained monomeric heterodimer of PSI-A/B and several small proteins. In the RC-47 band, D1, D2, and CP47 proteins were identified. LHCIIt and LHCIIm band contained trimeric and monomeric form of LHC II, respectively. Small amount of CP43, which was disconnected from CCII due to influence of Deriphat, co-migrated with the LHC IIt band.

The appearance of bands with supercomplexes (SC), containing both PSI and PSII proteins (Fig. 3), a faint band RC-47, and a very low content of free pigments (FP), confirmed that the separation of CPCs by Deriphat–PAGE was very mild. However, we note that a purification of Deriphat was necessary to obtain highly native CPCs. Without Deriphat purification, we observed much more distinct CCI and RC-47 bands and no supercomplexes in the electrophoretograms (not shown).

3.3. Pigment–protein complexes in heated leaves

The composition of CPCs was not affected by linear heating of barley leaves up to 40 °C (Figs. 2 and 3), which corresponded with the constant level of fluorescence detected in FTC (Fig. 1). The first change in CPCs appeared after heating to 50 °C when CCII dimers began to dissociate to monomers. This is documented by a relative decrease in the amount of PSII core proteins (D1, D2, CP47, and CP43) in the PSI + CCII(2) band in favor of the RC-47 band (Fig. 3).

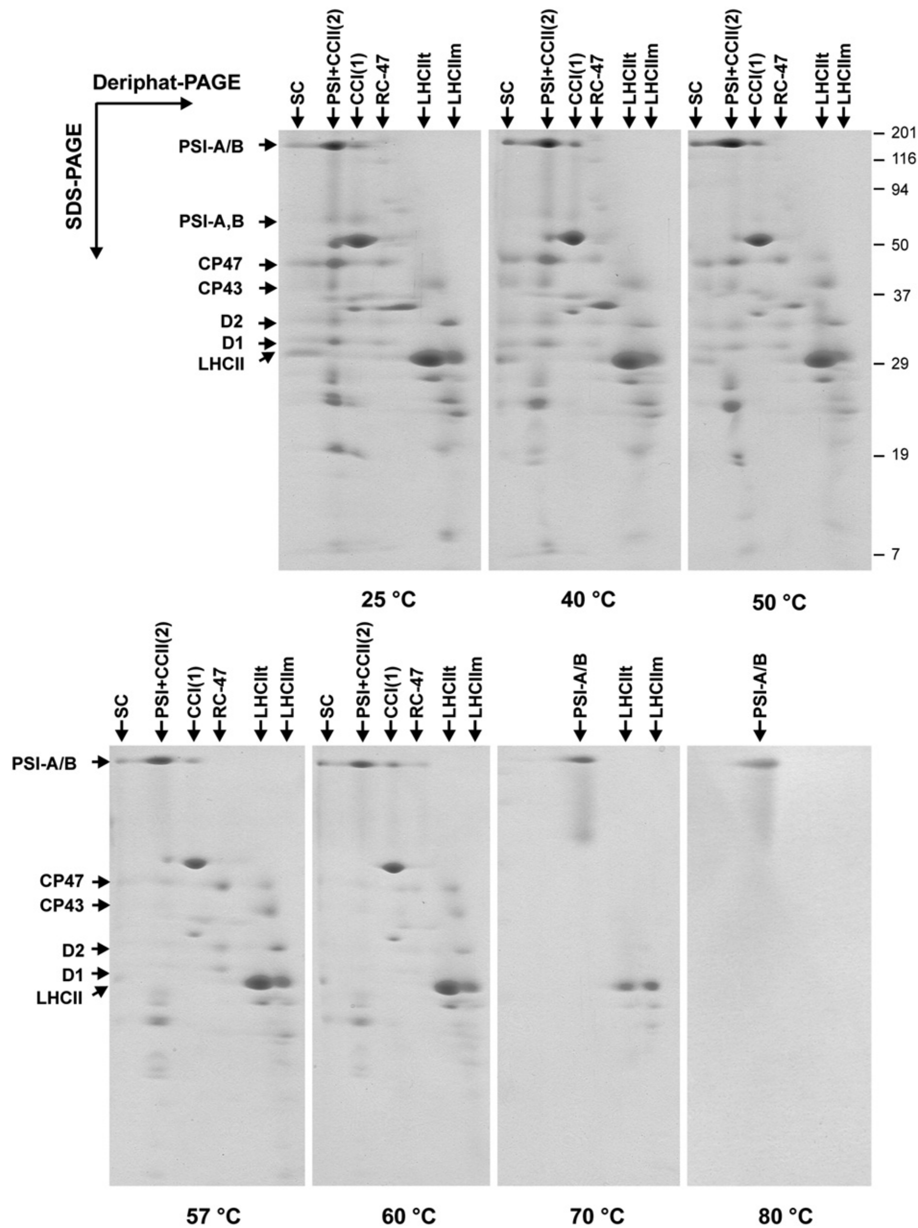

Fig. 3. Two-dimensional denaturing SDS–PAGE analysis of thylakoid protein complexes of barley. Excised lanes from Deriphat–PAGE (Fig. 2) were denatured, placed on the top of the SDS gel, electrophoresed on 12–20% (w/v) polyacrylamide gel containing 7 M urea and stained with Coomassie brilliant blue. Molecular mass markers (right): myosin (201 kDa), β-galactosidase (116 kDa), bovine serum albumin (94 kDa), ovalbumin (50 kDa), carbonic anhydrase (37 kDa), soybean trypsin inhibitor (29 kDa), lysozyme (19 kDa), and aprotinin (7 kDa).

This PSII structural change was related to the fluorescence increase to the M1 FTC maximum (Fig. 1), reflecting the inhibition of PSII electron transport. The dissociation of CCII dimers was almost complete after heating to 57 °C, which was the temperature just prior to the increase of fluorescence to the M2 maximum in the FTC (Fig. 1). After heating to 57 °C, a small amount of aggregates was detected on the top of the gel, and the supercomplexes above the PSI band almost completely disappeared (Figs. 2 and 3).

More pronounced changes in separated CPCs were detected after linear heating to 60 °C, the temperature of the M2 FTC maximum (Fig. 1). The PSI + CCII(2) band, involving just the PSI proteins, became fainter (Figs. 2 and 3). A part of PSI proteins remained on the top of the gel in the form of aggregates (Fig. 2), as evidenced by the 77K fluorescence emission spectrum (Fig. 4a). The spectrum of aggregates showed a distinct band at about 720 nm, which is attributed to chlorophyll *a* in CCI. This spectrum also revealed that CCI in the aggregates is not connected with LHCI because the CCI–LHCI complex emits above 720 nm [45]. In addition, the RC-47 band almost completely disappeared, however, we did not observe any increase in the amount of separated components D1, D2, and CP47 in the gel (Figs. 2 and 3). This indicates that these PSII components were either broken down or remained in the form of aggregates on the top of the gel.

The PSI + CCII(2), CCI(1), and RC-47 bands completely disappeared after heating to 70 °C, and a new green band appeared in the original position of RC-47 (Fig. 2). The molecular mass of this CPC was about 220 kDa as detected by using Ferguson plot (not shown). SDS–PAGE (Fig. 3) revealed that this new band contains only the PSI-A/B heterodimer. This assignment was confirmed by immunoblotting (not shown) and the measurement of 77K fluorescence emission spectrum, in which the 720-nm maximum was observed (Fig. 4b). In this emission spectrum, the second band with the maximum below 680 nm also appeared, indicating that some chlorophyll *a* molecules in the PSI-A/B complex became disconnected. A trace of the PSI-A/B

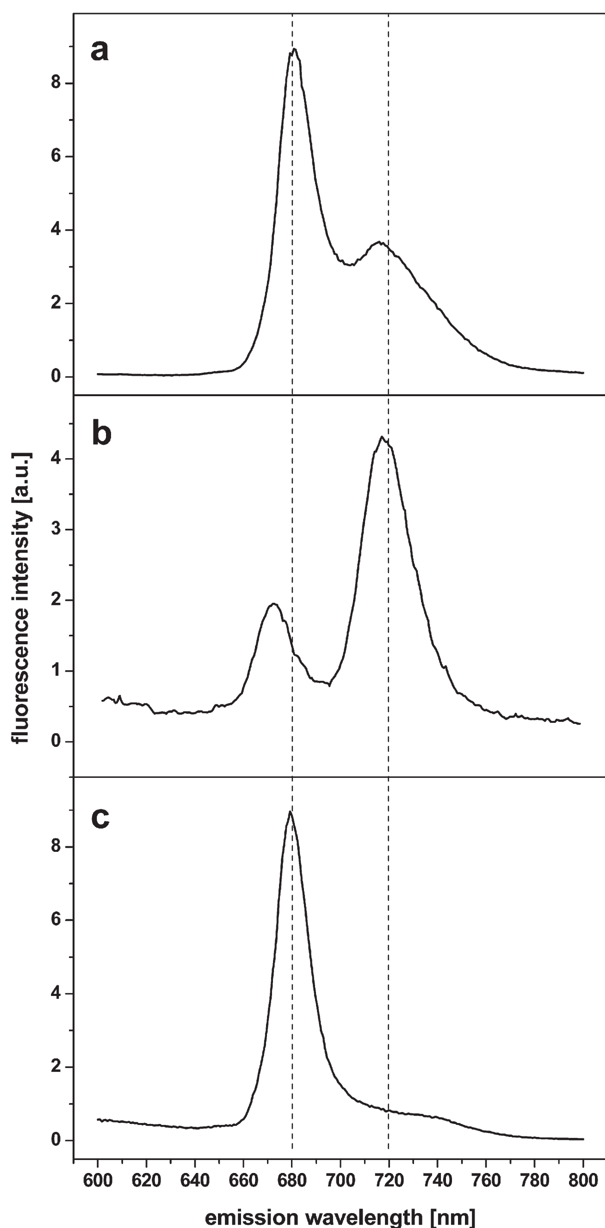

Fig. 4. 77K fluorescence emission spectra of aggregates remaining on the top of the gel (see Fig. 2) after linear heating of barley leaves to 60 °C (a) and 70 °C (c) and of PSI-A/B heterodimer separated by Deriphat–PAGE after heating to 70 °C (b). Excitation wavelength was 436 nm.

complex at the position of RC-47 band is also visible after heating to 60 °C (Fig. 3).

The other significant changes in CPCs after heating to 70 °C were a reduction of the level of LHCIIt and aggregates and a dramatic increase in the amount of free pigments (Fig. 2). The aggregates contained monodispersed LHCII complexes, as revealed by the 77K fluorescence emission spectrum (Fig. 4c). The observed narrow spectrum with the maximum at 680 nm is typical for LHCII [46].

After heating of leaves to 80 °C, only the PSI-A/B complex remained in the gel (Figs. 2 and 3). A small amount of chlorophyll-containing aggregates remaining on the top of the gel contained a part of weakly bound chlorophyll *a* and *b*, which was deduced from the low-wavelength emission maxima in 77K emission spectra under preferential excitation of chlorophyll *a* and *b* (not shown). The heating of leaves to 90 °C destroyed also the PSI-A/B (Figs. 2 and 3). A trace of chlorophyll-containing aggregates with weakly bound chlorophylls remained on the top of the gel.

3.4. PSII and PSI function

In order to reveal the connection between the heat-induced changes in the structure of CPCs and the function of photosystems, the photochemical activities of PSII and PSI were detected simultaneously in preheated leaves at room temperature (see Materials and methods). While the functionality of PSII was monitored via the measurement of the maximum quantum yield of PSII photochemistry (the parameter F_V/F_M), the photochemical function of PSI was estimated from the measurement of the amplitude of flash-induced oxidation of P700.

A significant decrease in PSII photochemistry was detected after preheating of leaves to 50 °C, when F_V/F_M ratio dropped from 0.8 to about 0.5 (Fig. 5a). This pronounced decrease coincided with the monomerization of CCII (Figs. 2 and 3) and with the increase of fluorescence intensity to the M1 FTC maximum (Fig. 1). The preheating of leaves to higher temperatures led to a gradual decrease in F_V/F_M ratio. PSII photochemistry completely disappeared in leaves preheated to 70 °C (Fig. 5a). Compared to PSII photochemistry, the PSI photochemistry was much more heat tolerant. The amplitude of P700 photooxidation was almost unchanged in leaves preheated up to 80 °C and dropped to zero after preheating to 90 °C (Fig. 5b).

4. Discussion

In this work, we studied a gradual disassembly and degradation of CPCs from thylakoid membranes during linear heating of barley leaves up to 90 °C and corresponding changes in the function of photosystems and FTC. A use of the native Deriphat–PAGE with purified Deriphat allowed us to separate PSI with large amount of CCII

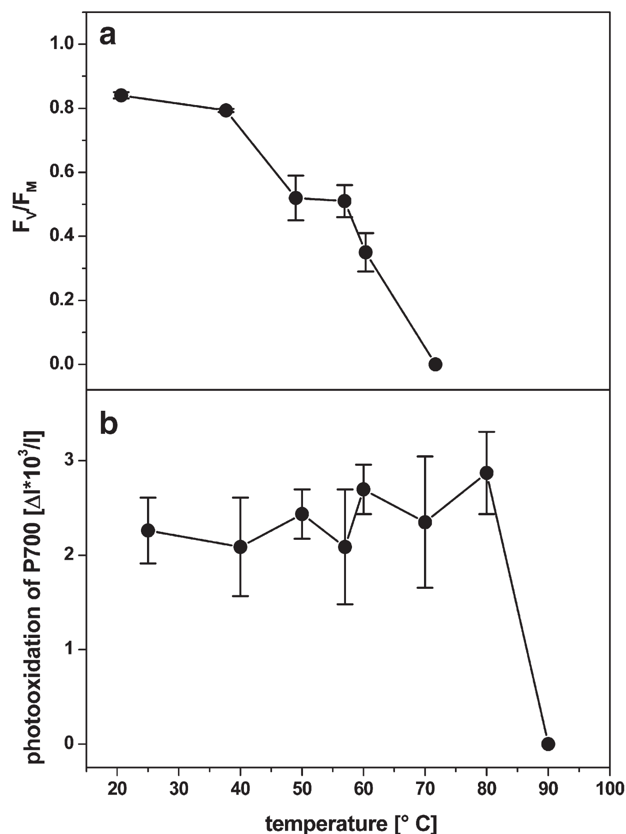

Fig. 5. The maximum quantum yield of PSII photochemistry estimated from the ratio of the variable (F_M–F_O) and maximal fluorescence intensity (F_V/F_M) (a) and the amplitude of light-induced oxidation of P700 (b) in barley leaves linearly heated (1 °C·min^{-1}) to various temperatures. For experimental details, see Materials and methods. Data represent means ± SD from four measurements.

Table 1
Molecular masses of CPCs from thylakoid membranes of barley leaves as determined by the method of Ferguson plot compared with those evaluated by semilog plot and those reported by Peter and Thornber [22,23] for the same separation method and plant material.

Pigment–protein complex	Molecular mass (kDa)		
	Ferguson plot	Semilog plot[a]	Peter and Thornber
PSI + CCII(2)	435	260	230
CCI(1)	280	120	120
RC-47	215	105	140[b]
LHCIIt	120	45	72
LHCIIm	50	24	25–35

[a] 7% (w/v) polyacrylamide gel was used.
[b] Molecular mass of the monomeric PSII reaction center that contained D1, D2, CP47, and CP43.

dimer and even some supercomplexes, making this technique similar to the blue-native PAGE (e.g., [47]).

4.1. Temperature range 25 to 50 °C

The first observed change in CPCs, the disassembly of CCII dimer (Figs. 2 and 3), took place between 40 and 50 °C. This temperature range corresponds with the temperature of the first DSC maximum detectable in isolated thylakoid membranes. This DSC transition does not involve any apparent degradation of PSII proteins, but it is associated with damage to the donor side of PSII (see Introduction). The relation between the disassembly of CCII and damage to the PSII donor side is not accidental. Hankamer et al. [48] and Danielsson et al. [49] have shown that the PSII activity measured as a rate of oxygen evolution is always higher in the samples having dimeric CCII. In line with these findings, in our experiments the heat-induced monomerization of CCII was accompanied by a decrease in PSII photochemistry (Fig. 5a) and by corresponding steep fluorescence increase to the M1 FTC maximum (Fig. 1). We note that the heat-induced disassembly of PSII dimer in this temperature range has also been observed in barley leaves preheated at fixed temperature (45 to 47 °C, 1 h) at low light [14].

4.2. Temperature range 50 to 60 °C

Marked changes in separated CPCs appeared after heating of the leaves to 60 °C. The pronounced disappearance of CCII from the gel indicates an extensive disassembly and degradation PSII complex. However, the degradation was not complete because the maximum yield of PSII photochemistry after heating did not drop to zero (Fig. 5a). The observed F_V/F_M value of 0.35 indicates that a part of CCII remained active. It seems that these active PSII centers did not enter into the gel and remained in the form of aggregates on the top of the gel. This relatively high thermostability of PSII photochemistry is supported by the previous DSC studies with thylakoid membranes, showing that CCII denatures at about 60 °C [6].

The heating of leaves to 57–60 °C induced the first changes in the organization of PSI. CCI markedly dissociated from LHCI and formed aggregates remaining on the top of the gel (Figs. 2–4). The heat-induced energetic disconnection of LHCI from CCI within 55 to 70 °C, interpreted from the measurement 77K fluorescence emission spectra, has been already documented in heated leaves [18], isolated chloroplasts [50], or isolated PSI complexes [13]. The formation of aggregates containing CCI components has been previously reported in detached *Arabidopsis* leaves senescing for several days [51].

The changes in the organization of CPCs after heating at 57 to 60 °C were accompanied by fluorescence increase to the M2 FTC maximum (Fig. 1), which reflects a weakening of chlorophyll *a*-protein interaction [34]. Ilík et al. [44] have suggested that this increase originates in the fluorescence of chlorophyll *a* released from some CPCs to the lipid fraction. Interestingly, we did not observe any significant increase in the amount of free chlorophylls in the gel after heating of leaves from 57 to 60 °C. This finding indicates that the fluorescence increase to the M2 FTC maximum may be connected with the observed structural changes in CCII or PSI rather then with a release of chlorophyll *a*. A suggestion that the M2 FTC maximum originates in PSI has already been mentioned [52,53].

4.3. Temperature range 60 to 90 °C

A marked disruption of trimeric LHCII, observed in leaves heated to 60 to 70 °C, was accompanied by a complete loss of PSII photochemical activity and by a pronounced release of chlorophyll molecules. The high amount of free chlorophyll indicates that the LHCII monomerization was followed by a significant denaturation of LHCII monomers (Figs. 2 and 3). The observed temperature range of LHCII denaturation agrees with that obtained in DSC studies *in vitro* [3,7,11].

After heating of leaves to 70 °C, the PSI CPC was detected only as the PSI-A/B heterodimer (Figs. 2 and 3). However, the activity of PSI, measured as the photooxidation of P700, remained unaffected. The most thermostable CPC, PSI-A/B complex, withstood also the heating to 80 °C; however, heating to 90 °C led to its destruction that corresponded with a disappearance of P700 photooxidation (Fig. 5b).

In summary, the main observed changes in the organization of PSII and PSI, i.e., the disassembly of CCII and the disconnection of LHCI from CCI with a separation of PSI-A/B heterodimer corresponded with the fluorescence increases in FTC. The observed heat-induced changes in PSI organization markedly resemble those that appeared in spinach leaves photoinhibited at low temperature in the presence of diethylthiocarbamate, the inhibitor of superoxide dismutase [54]. Hwang et al. [54] also observed the dissociation of LHCI from CCI and the separation of a new green band in native PAGE, which was mainly composed of PSI-A and PSI-B. These authors attributed these structural changes to the accumulation of superoxide, a reactive oxygen species (ROS), and proposed these steps to occur before the breakdown of CCI. These results indicate that our heat-induced changes in PSI can also be induced by the formation of ROS. This relationship is expected as heating of leaves or thylakoid membranes above 60 °C even in the dark leads to a production of ROS [55,56].

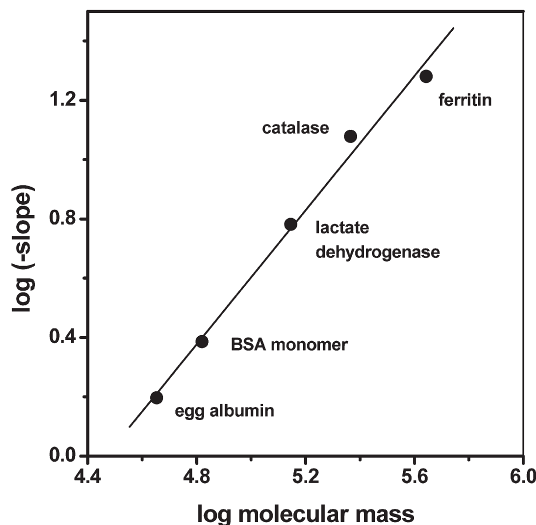

Fig. 6. Ferguson plot of Deriphat gels. Slopes were determined from linear fitting of the dependence of the migration distance of standard proteins on the percent gel concentration. Molecular mass markers: egg albumin (45 kDa), bovine serum albumin (66 kDa), lactate dehydrogenase (140 kDa), catalase (232 kDa), and ferritin (440 kDa).

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Appendix

There is a discrepancy between the values of molecular masses of CPCs reported in recent papers and the values that were estimated by Peter and Thornber [22,23] using Deriphat–PAGE. Markedly low molecular masses of CPCs separated by Deriphat–PAGE were also reported by Santini et al. [57] and Del Duca et al. [58]. In all these studies, authors used for the estimation of molecular masses a simple semilog plot, i.e., a direct comparison of mobilities of CPCs and protein standards in a polyacrylamide gel of one density. However, as noted by Poggese et al. [59], the use of a Ferguson plot is needed for a making proper estimate of molecular masses of CPCs separated by Deriphat–PAGE. The method of Ferguson plot, based on the measurement of mobility of protein complexes in gels of various density, can eliminate the effect of protein charges and shapes that influence the mobilities of protein complexes.

When a simple semilog plot was used for the estimation of CPC molecular masses, we obtained values that were very similar to those reported by Peter and Thornber [22,23]. However, when we used the Ferguson plot, the results were markedly different (Table 1). The CPC molecular masses were calculated using calibration plot obtained with protein standards (Fig. 6), and our results were very similar to those reported recently for CPCs separated by different methods (Table 1, e.g., [47]). The comparison presented in Table 1 clearly shows that the method of a Ferguson plot is essential for a proper estimation of molecular masses of CPCs separated by native PAGE.

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