Structural Rearrangements in Chloroplast Thylakoid Membranes Revealed by Differential Scanning Calorimetry and Circular Dichroism Spectroscopy. Thermo-optic Effect †

ARTICLE *in* BIOCHEMISTRY · OCTOBER 2003

Impact Factor: 3.02 · DOI: 10.1021/bi034899j · Source: PubMed

9 AUTHORS, INCLUDING:

Zsuzsanna Várkonyi
Hungarian Academy of Sciences
35 PUBLICATIONS **710** CITATIONS
SEE PROFILE

Sashka Krumova
Bulgarian Academy of Sciences
50 PUBLICATIONS **340** CITATIONS
SEE PROFILE

Stefka G Taneva
Bulgarian Academy of Sciences
96 PUBLICATIONS **859** CITATIONS
SEE PROFILE

Győző Garab
Hungarian Academy of Sciences
134 PUBLICATIONS **2,467** CITATIONS
SEE PROFILE

Structural Rearrangements in Chloroplast Thylakoid Membranes Revealed by Differential Scanning Calorimetry and Circular Dichroism Spectroscopy. Thermo-optic Effect†

Anelia G. Dobrikova,‡ Zsuzsanna Várkonyi,§ Sashka B. Krumova,‡,§ László Kovács,§ Georgi K. Kostov,‡ Svetla J. Todinova,‡ Mira C. Busheva,‡ Stefka G. Taneva,‡ and Győző Garab*,§

Institute of Biophysics, Bulgarian Academy of Sciences, Acad. G. Bonchev Bl. 21, Sofia 1113, Bulgaria, and Institute of Plant Biology, Biological Research Center, Hungarian Academy of Sciences, P.O. Box 521, H-6701 Szeged, Hungary

Received May 27, 2003; Revised Manuscript Received August 5, 2003

ABSTRACT: The thermo-optic mechanism in thylakoid membranes was earlier identified by measuring the thermal and light stabilities of pigment arrays with different levels of structural complexity [Cseh, Z., et al. (2000) *Biochemistry 39*, 15250−15257]. (According to the thermo-optic mechanism, fast local thermal transients, arising from the dissipation of excess, photosynthetically not used, excitation energy, induce elementary structural changes due to the "built-in" thermal instabilities of the given structural units.) The same mechanism was found to be responsible for the light-induced trimer-to-monomer transition in LHCII, the main chlorophyll *a*/*b* light-harvesting antenna of photosystem II (PSII) [Garab, G., et al. (2002) *Biochemistry 41*, 15121−15129]. In this paper, differential scanning calorimetry (DSC) and circular dichroism (CD) spectroscopy on thylakoid membranes of barley and pea are used to correlate the thermo-optically inducible structural changes with well-discernible calorimetric transitions. The thylakoid membranes exhibited six major DSC bands, with maxima between about 43 and 87 °C. The heat sorption curves were analyzed both by mathematical deconvolution of the overall endotherm and by a successive annealing procedure; these yielded similar thermodynamic parameters, transition temperature and calorimetric enthalpy. A systematic comparison of the DSC and CD data on samples with different levels of complexity revealed that the heat-induced disassembly of chirally organized macrodomains contributes profoundly to the first endothermic event, a weak and broad DSC band between 43 and 48 °C. Similarly to the main macrodomain-associated CD signals, this low enthalpy band could be diminished by prolonged photoinhibitory preillumination, the extent of which depended on the temperature of preillumination. By means of nondenaturing, "green" gel electrophoresis and CD fingerprinting, it is shown that the second main endotherm, around 60 °C, originates to a large extent from the monomerization of LHCII trimers. The main DSC band, around 70 °C, which exhibits the highest enthalpy change, and another band around 75−77 °C relate to the dismantling of LHCII and other pigment−protein complexes, which under physiologically relevant conditions cannot be induced by light. The currently available data suggest the following sequence of events of thermo-optically inducible changes: (i) unstacking of membranes, followed by (ii) lateral disassembly of the chiral macrodomains and (iii) monomerization of LHCII trimers. We propose that thermo-optical structural reorganizations provide a structural flexibility, which is proportional to the intensity of the excess excitation, while for their localized nature, the structural stability of the system can be retained.

The photosynthetic system of higher plants possesses the ability to be regulated by short-term variations in the external environmental conditions such as temperature and illumination (*1*, *2*). Most components of the multilevel regulatory mechanisms are known to involve structural changes in the thylakoid membranes. Vice versa, significant variations in the environmental conditions often induce structural changes, which in turn affect the photosynthetic functions.

The relative light-harvesting capability of the two photosystems can be regulated by redox-regulated reversible phosphorylation of LHCII;[1] during this process, part of LHCII is transferred from the stacked to the unstacked region of the thylakoid membranes (*3*−*5*). In strong light, i.e., when plants are exposed to excess radiation that cannot be used

† This work was supported by grants from the OTKA (Hungarian Research Fund, T34188 and T42696) to G.G., MY-K-1003/00 from the Bulgarian National Fund for Science Research to A.G.D., an exchange program between the Hungarian and Bulgarian Academy of Sciences, and the Center of Excellence Program of EU DGXII awarded to the Biological Research Center of the Hungarian Academy of Sciences (WP17).

* Corresponding author. E-mail: gyozo@nucleus.szbk.u-szeged.hu. Phone: +(36-62) 433131. Fax: +(36-62) 433434.

‡ Bulgarian Academy of Sciences.

§ Hungarian Academy of Sciences.

[1] Abbreviations: CD, circular dichroism; Chl, chlorophyll; DSC, differential scanning calorimetry; ΔH_{cal}, calorimetric enthalpy; LHCII, the main chlorophyll *a*/*b* light-harvesting pigment−protein complex of photosystem II; PSII, photosystem II; T_m, peak temperature of the endothermic transition.

10.1021/bi034899j CCC: $25.00
Published on Web 09/11/2003

in photosynthesis, different protective mechanisms are induced, which downregulate the energy utilization and thus prevent the excess excitation energy from causing sustained damage to the plant. For instance, the short-term (1−2 min) component of the regulated energy dissipation in the antenna system, qE, the energy-dependent nonphotochemical quenching, which depends on ΔpH, also involves conformational changes that modulate the aggregation state of LHCII (*6*). The nonphotochemical quenching of the excess excitation energy also depends on the presence of zeaxanthin and the PsbS protein of PSII (*7, 8*).

There is ample evidence for structural changes induced directly by environmental factors. Significant structural rearrangements, involving the dissociation and movement of LHCII, can be induced by the heat treatment of thylakoid membranes (*9*). It has been demonstrated that, upon illumination with moderate and high light intensities, thylakoid membranes undergo reversible structural changes, which, as revealed by CD spectroscopy, affect the long-range chiral order of the chromophores (*10*); prolonged illumination with intense light generally leads to similar, but irreversible changes (*11*). Although they display some similarities to the conformational changes accompanying qE (*12*), these structural rearrangements are largely independent of the photochemical activity of the membranes and of the transmembrane ΔpH. Further, lamellar aggregates of isolated LHCII have been shown to be capable of undergoing very similar reversible structural reorganizations (*13*, *14*), and light induces a significant degree of monomerization of LHCII in vivo and in vitro, which is in contrast with the preferentially trimeric organization of the complexes in the dark (*15*). These rearrangements are accompanied by Chl fluorescence quenching (*13*, *16*). Light-induced reorganizations, probably of a similar nature, have been found to be directly involved, at the substrate level, in the regulation of the phosphorylation of LHCII in vivo and in vitro (*17*−*19*).

Light-induced reorganizations in the isolated light-harvesting complexes and in the native membrane have been observed to be driven by a thermo-optic effect. In other terms, they are ascribed to fast, local thermal transients, *T*-jumps, due to the dissipation of the excess excitation energy in the antenna system, which in turn lead to elementary structural changes in the vicinity of the dissipating centers (*15*, *20*). This conclusion has been reached mainly from analyses of the thermal and light stabilities of macroarrays of different levels of structural complexity in thylakoid membranes and isolated complexes. The thermo-optic mechanism depends on the "built-in" thermal instability of molecular assemblies, which ensures the susceptibility of these structures to fast local thermal transients, the magnitude of which depends on the distance from the dissipating center, and the thermal transient (*T*-jump) can be significantly large within about a 1 nm radius around the dissipation center (*20*). (In the model, where the volume of the dissipation center was 1 nm^3 volume, the dissipation of a red photon energy led to a *T*-jump >65 °C and about a 10 °C jump in the neighboring 1 nm^3 "cell".) It is usually assumed, and verified experimentally, that the thermal instability of macroassemblies does not affect the molecular structure of the constituents. Hence, the arrays can undergo thermo-optically driven reorganizations without irreversible damage to their constituents. Indeed, the presence of such thermally unstable assemblies, LHCII-containing macrodomains and trimeric structures of LHCII, has been identified mainly via CD spectroscopy and biochemical tools (*15*, *20*). However, no calorimetric analysis has been performed, and accordingly, the thermodynamic parameters of the thermo-optically inducible changes remain unknown.

Earlier calorimetric analyses on thylakoids revealed the band structure of the thermograms and identified the origin of several bands with respect to the stroma and granum membranes and their membrane-associated enzymes and complexes (*21*−*29*). However, they have not been analyzed with respect to their sensitivity toward preillumination and other treatments affecting the long-range organization of the complexes. Furthermore, CD spectroscopy, which is a particularly sensitive tool for the detection of structural reorganizations both at the macroorganization level and in the individual complexes and particles (*30*, *31*), can be applied to establish correlations between the most prominent structural changes in the pigment system and some DSC bands. As summarized in ref *30*, CD can originate from asymmetric architectures of different complexity: (i) the so-called intrinsic CD arises from asymmetry in the electronic state of the individual molecules; this is a very weak signal and becomes clearly discernible only after the denaturation of the pigment−protein complexes; (ii) excitonic CD bands, typically "conservative" (+)/(−) band pairs, are given rise by short-range interactions between chromophores; since the excitonic CD signal is very sensitive to the distance and mutual orientation of the pigment dipoles (*31*), it can be used for fingerprinting of the complexes and testing their intactness; (iii) in chirally organized macroarrays, with sizes commensurate with the wavelength of the visible light, very intense and "anomalously shaped" so-called ψ-type CD bands are given rise; ψ-type bands are exhibited by intact granal thylakoid membranes, in particular by LHCII-containing macrodomains, as well as by lamellar aggregates of LHCII. Hence, the CD in chloroplasts is dominated by the ψ-type bands but also contains the contributions from excitonic and intrinsic bands and can be regarded as the superposition of these three types of signals. We shall take advantage of the fact, mainly our earlier observations (*10*−*15*, *20*), that the thermal and light sensitivity of these three types of CD signal are strikingly different and further analyze CD data on thylakoid membranes exposed to heat and light treatments. In the present work, by using DSC and CD spectroscopy, we have identified the broad but relatively weak DSC band around 43 °C as being associated with the LHCII-containing chiral macrodomains. The calorimetric data presented are fully consistent with the notion that the structural changes in the chiral macrodomains are of thermo-optic origin. Among the additional thermal transients, a band around 60 °C is found to be involved in the thermo-optically driven trimer-to-monomer transition of LHCII.

MATERIALS AND METHODS

Isolation of Thylakoid Membranes; Preillumination and Nondenaturing, "Green" Gel Electrophoresis. Thylakoid membranes were isolated from 10-day-old barley seedlings (*Hordeum vulgare* L.) or 2-week-old pea (*Pisum sativum* L.), grown in the greenhouse, by a slight modification of a procedure described earlier (*32*). No significant difference was observed between the two plant materials. The mem-

branes of 1−2 mg of Chl mL^{-1} were suspended in a medium containing 250 mM sorbitol, 5 mM $MgCl_2$, and 10 mM Tricine (pH 7.6) and stored on ice in the dark until use within 4−6 h after the isolation. Aliquots from this stock were suspended in the same medium or washed twice in Tricine buffer (10 mM, pH 7.6) in the presence of 50 μM EDTA or in 10 mM Tricine buffer supplemented with 250 mM sorbitol.

The chlorophyll concentration was determined spectrophotometrically (*33*).

Preillumination of the thylakoid membranes (in 10 mM Tricine, pH 7.6, 250 mM sorbitol), on ice or thermostated at 25 °C, was performed with heat-filtered white light (30 or 60 min, 1600 μE m^{-2} s^{-1}) at 2 mg of Chl mL^{-1}, in a thin layer, for DSC measurements and at 20 μg of Chl mL^{-1}, in a cell of 1 cm path length, for CD.

Nondenaturing, green gel electrophoresis on heat-treated and preilluminated thylakoid membranes (suspended in 10 mM Tricine buffer, pH 7.6, also containing 50 μM EDTA) was performed as in ref *9*. The bands associated with LHCII were excised for CD spectroscopy.

DSC Measurements. Calorimetric measurements were performed with a DASM4 (Biopribor, Pushchino, Russia) high-sensitivity scanning microcalorimeter (*34*). Runs were routinely made in the temperature range between 20 and 100 °C at a heating rate of 1 °C min^{-1}. A second heating of the sample in the calorimetric cell, immediately after cooling to 20 °C following the first run, checked the reversibility of the thermally induced transitions. The transition temperature (T_m) was defined as the temperature at the maximum of the excess heat capacity curve. The calorimetric enthalpy (ΔH_{cal}) of the transition was determined as the area under the excess heat capacity curve.

CD Measurements and Temperature Dependence. CD was measured between 400 and 800 nm in a Jobin-Yvon CD6 dichrograph equipped with a side-illumination attachment and a thermostatable sample holder. The Chl content of the samples was adjusted to 20 μg mL^{-1}, the optical path length of the cell was 1 cm, and the distance of the sample from the photomultiplier was 5 cm. The spectra were recorded in 0.5 nm steps with an integration time of 0.2 s and a bandpass of 2 nm.

The temperature dependence of the CD spectra of EDTA-washed thylakoid membranes was measured either in the thermostated sample holder after a 5 min incubation at the given temperature or at room temperature following the heat treatment of the sample in a block-heater unit. In the range of interest, between 40 and 80 °C, only minor differences were observed between the two types of treatments.

Each experiment was repeated on at least three independent batches, with identical tendencies and similar results. However, the thermal and light stabilities of the preparations from different batches of thylakoids differed somewhat from each other. In the paper, typical thermograms and CD spectra and temperature- and light-induced changes are presented.

RESULTS AND DISCUSSION

Mathematical Deconvolution and Experimental Dissection of Thylakoid Endotherms. Figure 1 presents the DSC profile of barley thylakoids, measured in medium of low ionic strength. The thermogram is characterized by six main

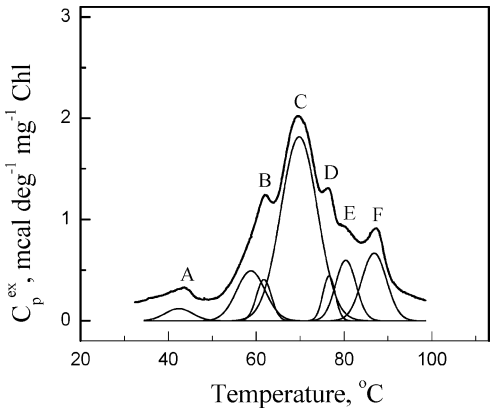

FIGURE 1: DSC thermogram of isolated thylakoid membranes (after baseline correction) suspended, at a concentration of 2 mg of Chl mL^{-1}, in a reaction medium containing 10 mM Tricine (pH 7.6) and 250 mM sorbitol and mathematical deconvolution of the DSC curve by Gaussian fitting. The scanning rate was 1 °C min^{-1}. The endothermic transitions are denoted as A−F following ref *21*. C_p^{ex} = excess heat capacity.

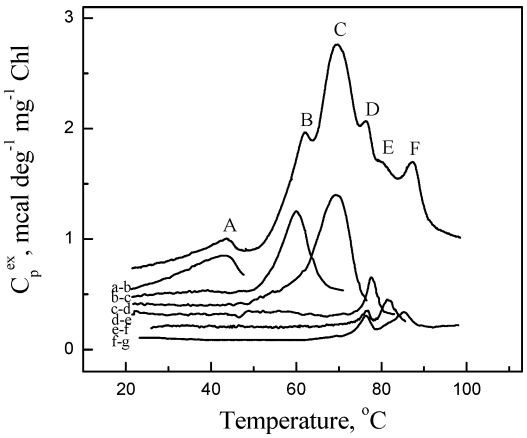

FIGURE 2: DSC profile of barley thylakoid membranes, from the same batch and under the same experimental conditions as in Figure 1, and dissection of the heat capacity curve by successive annealing procedures. Profiles of the difference curves of the successive annealings (a − b, b − c, c − d, d − e, e − f, and f − g) are obtained by subtracting each scan from the previous one. C_p^{ex} = excess heat capacity.

endothermic transitions, centered at 43, 62, 69.6, 76.5, 80, and 87 °C, denoted as bands A, B, C, D, E, and F, respectively (*21*). The overall endotherm was deconvoluted mathematically, using theoretical Gaussian fitting (Figure 1). With this, band B was split into bands B1 and B2, with peak positions at 59 and 61.8 °C, respectively. This was not analyzed further in this work. Also, in our deconvolution, the sharp and relatively weak band A1, which has been shown to be associated with the oxygen-evolving complex (*22*−*24*), remained unresolved in the broad and rather asymmetric and weak band A.

As shown in Figure 2, the heat sorption curves can be dissected into their constitutive bands by applying a successive annealing procedure (*35*, *36*). This experimental dissection procedure can be applied because the successive endothermic events are largely irreversible under our experimental conditions. This was confirmed by recording the second scans in barley and pea thylakoids (data not shown). Similarly to the endotherm, annealing yielded six DSC transitions. On the same thylakoid preparation, the peak positions were found at 43, 60, 69.2, 77.6, 81.5, and 85.3

Table 1: Thermodynamic Parameters of the Heat Sorption Curves of Barley Thylakoid Membranes in the Absence (Control) and Presence of 5 mM $MgCl_2$ and after 5 min Preincubation of the Sample at 48 °C, Followed by DSC Measurements between 20 and 100 °C[a]

		peak						
		A	B1	B2	C	D	E	F
control	T_m	43	59	61.8	69.8	76.6	80.4	86.6
deconvoluted	ΔH_{cal}	2 ± 0.4	3.56 ± 0.26	2.75 ± 0.68	18.9 ± 0.5	1.85 ± 0.16	3 ± 0.54	4.95 ± 0.67
control	T_m	43		60	69.2	77.6	81.5	85.3
annealing	ΔH_{cal}	4.56 ± 0.43		6.81 ± 0.62	14.8 ± 0.68	1.47 ± 0.12	0.76 ± 0.04	2.74 ± 0.21
$+Mg^{2+}$	T_m	46.6		62	69	76.3	80.5	85.3
deconvoluted	ΔH_{cal}	3.66 ± 0.53		25 ± 0.42	9.2 ± 0.89	2.25 ± 0.44	1.47 ± 0.29	2.3 ± 0.55
$+Mg^{2+}$	T_m	48		61	66.9	74.7	80.4	84.4
annealing	ΔH_{cal}	3.98 ± 0.32		17.7 ± 0.1	13.2 ± 0.38	4.32 ± 0.46	1.33 ± 0.33	0.37 ± 0.03
preincubated at 48 °C	T_m		57	62.2	69	76	81	86
($+Mg^{2+}$) deconvoluted	ΔH_{cal}		4.5	10.4	6.2	4.6	0.4	3.1

[a] The parameters were derived from the band parameters obtained either from mathematical deconvolution of the endotherm or from the experimentally dissected curves (using successive annealing). Thylakoid membranes were suspended, at a Chl concentration of 2 mg mL^{-1}, in 10 mM Tricine and 250 mM sorbitol (pH 7.6); the scanning rate was 1 °C min^{-1}. T_m (°C) is the temperature at maximum heat capacity, and ΔH_{cal} [mcal (mg of Chl)$^{-1}$] is the calorimetric enthalpy of the transitions. Mean values and standard errors were obtained from three to five independent experiments, except in the last row, where two essentially identical DSC curves were recorded. The standard error for T_m is less than 0.5 °C in all cases.

°C (Table 1), in good agreement with the fitted data. It is to be noted, however, that whereas the peak positions and bandwidths did not depend noticeably on the heating protocol, the amplitudes were somewhat sensitive to it. In particular, the amplitudes of the main bands, B and C, decreased upon application of the annealing procedure. It was also observed that the amplitudes of these bands were reduced when the thylakoids were preincubated for several minutes between 40 and 48 °C. This effect was largely independent of the peak position of band A, which could be shifted by varying the composition of the suspending medium, as shown in Table 1. This suggests that slower structural changes induced by heat or other processes, e.g., lipid peroxidation, phase transitions in the membrane, activation of proteases, are involved in the destabilization of the structure(s) associated with the high-temperature bands. The identification of these processes, which modify the relative intensities only slightly, is beyond the scope of the present work. However, comparison of these two protocols is important mainly because this permits us to apply variations in the experimental conditions. In particular, the fact that continuous heating, annealing, and preincubation at a given temperature followed by resumed heating yielded very similar endotherms, and hence similar thermodynamic parameters (Table 1), is important when the experimental protocol of DSC, i.e., continuous, slow heating, is difficult to apply, e.g., in CD measurements.

Thermal and Light Instability of LHCII-Containing Chiral Macrodomains: First Endothermic Event (Band A). Mainly through the use of circular dichroism spectroscopy, we have earlier shown that the thylakoid membranes of higher plant chloroplasts exhibit thermal instability, which originates from the disassembly of chirally organized LHCII-containing macrodomains (*20*). The fact that this transition occurs at relatively low temperatures (below 55 °C) suggests the involvement of macrodomains in the first DSC band. It has also been demonstrated that the transition temperature, determined from the temperature dependence of the main, ψ-type CD bands, depends on the suspending medium: the removal of sorbitol or Mg^{2+} (i.e., decreasing the osmolarity or the ionic strength, respectively) has been found to destabilize the macrodomains (*20*, *32*). It is therefore of interest that the position of the first endothermic event (band A) is very sensitive to the presence of millimolar concentrations of $MgCl_2$, which shift the peak position to higher temperatures (Figure 3, Table 1). This observation is in good agreement with the notion that the first DSC band involves a significant contribution from the heat-induced disassembly of the chiral macrodomains. Table 1 further reveals that the enthalpy changes associated with this disassembly are considerably smaller than those associated with the higher temperature bands. These latter bands evidently involve thermal transitions associated with further disassembly of the particles and the unfolding and denaturation of the main pigment−protein complexes (see also below).

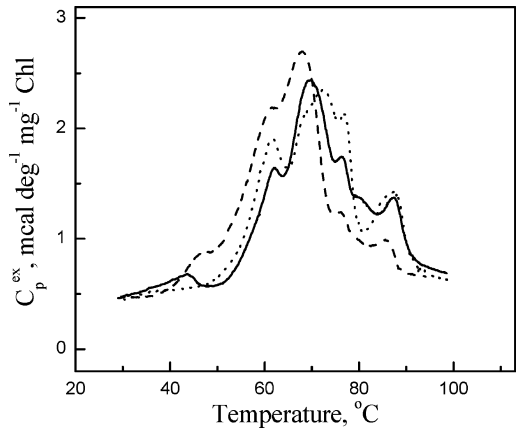

FIGURE 3: Effects of ionic strength and osmolarity on the calorimetric transitions of barley thylakoid membranes at a concentration of 2 mg of Chl mL^{-1}, suspended in 10 mM Tricine (pH 7.6) and supplemented with 250 mM sorbitol (as in Figure 1, solid line), 250 mM sorbitol and 5 mM $MgCl_2$ (dashed line), and 50 μM EDTA (dotted line). The scanning rate was 1 °C min^{-1}; for other conditions, see Materials and Methods. C_p^{ex} = excess heat capacity.

While DSC and CD data demonstrate that the correlation between band A and the disassembly of the macrodomains is satisfactory (and further support is given below), it must be noted that the two measurements are unlikely to yield exactly the same transition temperatures, even if the two measurements could be run under identical conditions. Analysis of the thermal instability of the main ψ-type bands in intact thylakoids revealed two components: the diminishment of the (−) 676 nm ψ-type band, associated with the

Table 2: Thermodynamic Parameters of the Heat Sorption Curves of Barley Thylakoid Membranes: Transition Temperatures (T_m) and Calorimetric Enthalpies (ΔH_{cal})[a]

		peak						
		A	B1	B2	C	D	E	F
control	T_m	43.0	59.0	61.8	69.8	76.6	80.4	86.6
	ΔH_{cal}	2.0 ± 0.4	3.56 ± 0.26	2.75 ± 0.68	18.9 ± 0.5	1.85 ± 0.16	3.0 ± 0.54	4.95 ± 0.67
EDTA-washed	T_m		58.8	61.3	69	73.2	76.8	85.5
	ΔH_{cal}		6.12 ± 0.46	5.32 ± 0.86	10 ± 0.88	4.62 ± 0.38	5.5 ± 0.51	7.35 ± 0.34
PI 30 min	T_m	35.0	59.0	61.0	70.9	76.7	81.4	87.4
at 0 °C	ΔH_{cal}	3.15 ± 0.5	4.47 ± 0.64	2.09 ± 0.24	22 ± 0.64	1.8 ± 0.48	2.7 ± 0.5	3.96 ± 0.44
PI 60 min	T_m	26.0	58.8	61.3	70.9	76.4	80.4	86.8
at 0 °C	ΔH_{cal}	4.15 ± 0.51	4.34 ± 0.36	1.98 ± 0.08	22.0 ± 0.23	1.08 ± 0.4	1.53 ± 0.17	5.67 ± 0.18
PI 30 min	T_m		57.9	60.9	69.8	76.8	81.5	87
at 25 °C	ΔH_{cal}		6.0 ± 0.6	1.67 ± 0.13	14.2 ± 0.2	2.53 ± 0.13	3.47 ± 0.23	3.59 ± 0.38

[a] Mean values and standard errors calculated using the band parameters from mathematical deconvolution (from three to five independent experiments). The standard error for T_m is less than 0.5 °C in all cases. The membranes, at 2 mg of Chl mL^{-1}, were suspended in 10 mM Tricine (pH 7.6) and 250 mM sorbitol (control) or washed with EDTA-containing media (see Materials and Methods). The control thylakoid samples were preilluminated (PI) with white light of 1600 μE m^{-2} s^{-1} at 0 °C for 30 or 60 min and at 25 °C for 30 min, as indicated. The scanning rate was 1 °C min^{-1}.

stacking, preceded that of the (+) 689 nm band (*20*). As pointed out earlier, the disassembly of macrodomains begins with the unstacking of membranes and then proceeds with a lateral disorganization of the chiral macrodomains (*37*). These two steps are not resolved in band A. Further, ψ-type CD is lost below a certain size of the domain, when the diameter falls below one-fourth of the wavelength of the corresponding band (*38*). On the other hand, the heat sorption can continue as long as the disassembly proceeds; albeit its magnitude may depend on the actual size of the domain. Additionally, DSC may contain other transitions, such as from the oxygen-evolving complex (*22*, *24*), which in part may be related to the macroorganization of LHCII (*29*, *39*); further independent or partly independent transitions may also be present, e.g., rearrangements in the lipid bilayer (*40*−*43*). Nonetheless, the thermodynamic parameters associated with the macrodomain organization of the complexes set the upper limits of the corresponding values and serve for comparison with other, more intense DSC transitions.

To support the above notion concerning the origin of band A and to reduce the contributions from independent factors, we performed experiments in the absence of chiral macrodomains. In hypotonic low-salt media in the presence of EDTA, thylakoid membranes do not contain sizable macrodomains (*11*, *32*). As can be seen in Figure 3 and Table 2, transition A is suppressed in ion-depleted thylakoids suspended in Tricine. Cations also affected transitions D and E and changed the relative intensities of the DSC peaks. This is in agreement with earlier data (*27*) and is not analyzed further in the present work.

It is known that the chiral macrodomains can be disassembled by preilluminating the thylakoid membranes with intense, photoinhibitory light (*11*). In accordance with the thermo-optic mechanism, this depends on the temperature of preillumination (*20*). In good agreement with expectations, band A could be eliminated by preillumination at 25 °C (Figure 4B), whereas the same light treatment at 0 °C led only to a shift toward lower temperatures (Figure 4A, Table 2). It is interesting to observe in the DSC curves that, fully consistent with the thermo-optic mechanism, preillumination at 25 °C exerted an effect very similar to that of preincubation of the sample at 48 °C; it also diminished the amplitudes of bands B and C (data not shown) without affecting the peak positions.

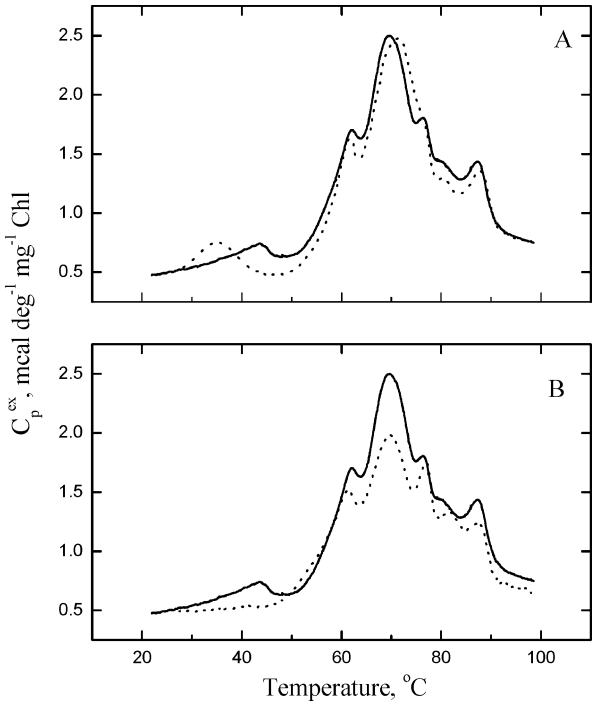

FIGURE 4: Effect of preillumination on the DSC profiles of barley thylakoid membranes suspended in 10 mM Tricine (pH 7.6) and 250 mM sorbitol at a concentration of 2 mg of Chl mL^{-1}. The thylakoids were illuminated at 0 °C (A) and 25 °C (B) for 30 min with white light of 1600 μE m^{-2} s^{-1} (dashed lines) or incubated in the dark (continuous lines). C_p^{ex} = excess heat capacity.

As expected, destabilization of the macrodomains by preillumination led to a shift in CD similar to that seen in DSC (Figure 5). In the dark, as indicated by the main, ψ-type CD bands, the macrodomains were quite stable up to 40 °C, and even at 50 °C the signal was strong. In contrast, upon preillumination at 0 and 25 °C, these bands were significantly reduced and almost fully eliminated, respectively (Figure 5). As shown in Figure 6, in the dark, the transition temperature (the temperature at which the intensity of the ψ-type CD bands decreases to 50% of the original value) was to be found around 45 °C. After a 30 min preillumination at 0 °C (Figure 6), the residual ψ-type bands shifted their transition from 47 to 39 °C, whereas longer preilluminations (≥60 min) at the same temperature (not shown) or the same preillumination at 25 °C led to essentially complete loss of the ψ-type

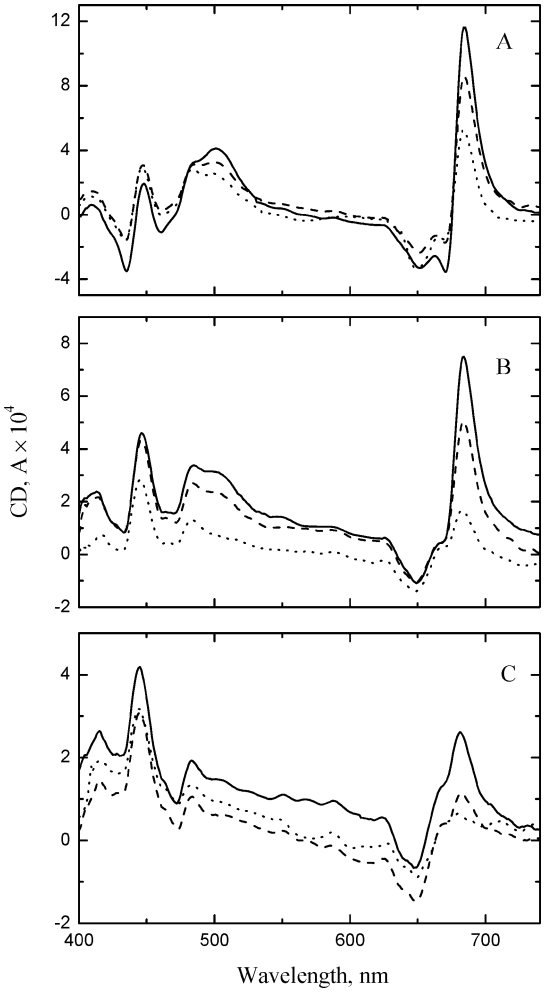

FIGURE 5: Thermal stability of barley thylakoid membranes suspended in 10 mM Tricine (pH 7.6) and 250 mM sorbitol at a concentration of 20 μg of Chl mL^{-1} in the dark (A) and following a preillumination (30 min, white light of 1600 μE m^{-2} s^{-1}) at 0 °C (B) and 25 °C (C). Spectra were recorded at 25 °C (continuous line), 40 °C (dashed line), and 50 °C (dotted line). The light treatment and the CD measurements were carried out as described in Materials and Methods.

bands (Figures 5C and 6). This finding is fully consistent with the thermo-optic origin of these changes. Moreover, it can be seen that, as observed earlier, photoinhibitory preillumination diminished mainly the array of complexes, i.e., the main, ψ-type bands (cf. Figure 5), without eliminating the excitonic band structure. In other terms, the preillumination did not destroy the structure of the individual complexes, as can be seen from the main excitonic bands, e.g., around 440 and 650 nm. It must be noted, however, that some of the excitonic bands also exhibited a heat sensitivity, as evident from a closer inspection of the CD spectra. For instance, the excitonic band centered around 476 nm displayed high susceptibility to heating (Figure 5). This will be further analyzed in the following section. At this point we conclude that the currently available DSC data and CD data indicate that the first DSC band includes a significant contribution from the disassembly of the chiral macrodomains. These data are consistent with the earlier conclusion that these macrodomains can be disassembled thermo-optically. Further, we demonstrate that this disassembly consumes relatively low thermal energies as compared with the main endothermic events in the thylakoid membranes.

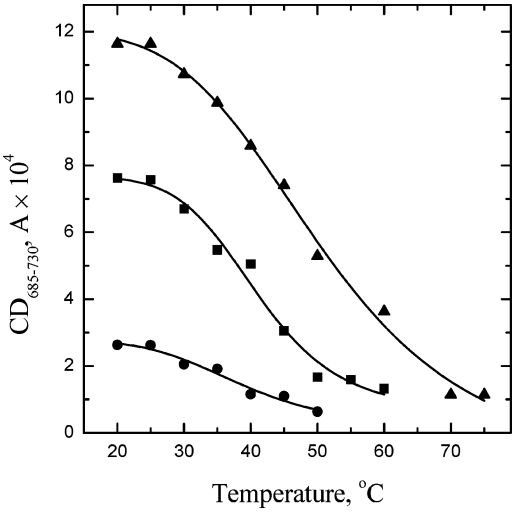

FIGURE 6: Temperature dependence of the intensity of the (+) 685 nm ψ-type CD band, with reference to 730 nm, in barley thylakoids in the dark (▲) and preilluminated with white light of 1600 μE m^{-2} s^{-1} for 30 min at 0 °C (■) and 25 °C (●). Other conditions as in Figure 5.

This explains why the thermal energy available locally and transiently from the dissipation of the unused excitation energy of the absorbed photons does not lead to the destruction of the complexes, at least under physiologically relevant conditions.

Trimer-to-Monomer Transition in LHCII: Second Endothermic Event (Band B). As pointed out above, besides causing the loss of the chiral macrodomains, the preillumination of the thylakoids with strong light resulted in weakening of the excitonic couplet centered around 476 nm. The same band pair, (+) 483 nm, (−) 470 nm, dominated the trimer-minus-monomer difference spectra, after both phospholipase A and light treatments (*15*). Further, monomerization of LHCII has also been ascribed to a thermo-optic effect. The (+) 483 nm, (−) 470 nm excitonic bands most likely originate from Chl *b* and/or neoxanthin molecules (*44*). The disruption of interactions between pigments on different monomeric subunits upon monomerization might be responsible for such an effect although the structural model of LHCII provides no likely candidates for such a change in interaction. Alternatively, monomerization might lead to a (small) structural change of monomeric subunits that causes a change in pigment−pigment interactions. Nevertheless, this band pair can be used as a fingerprint of the trimer-to-monomer transition (*15*). Hence, it is of interest to investigate whether the same "spectral transition" can be induced by heat in thylakoid membranes in the dark. Complementary data, relating to the heat-induced conversion of trimers into monomers in isolated LHCII around 55 °C (*15*) and the onset of monomerization, following heat treatment (45−47 and 60 °C, 1 h) of leaves were published earlier (*9*). Here, we inspected the thermal stability of the (+) 483 nm, (−) 470 nm excitonic CD band pair in the thylakoid membranes. In all samples, we found a clear indication of a thermal transition between 55 and 65 °C. However, in intact membranes the large CD variations associated with the gradual loss of the macrodomains hindered the resolution of this band pair (data not shown; see Figure 5A). This could be achieved more readily in samples that contained no macrodomains.

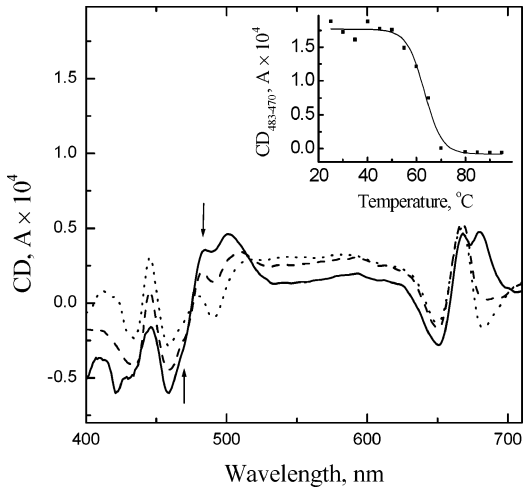

FIGURE 7: CD spectra of EDTA-washed barley thylakoid membranes incubated for 5 min at 45 °C (solid line), 53 °C (dashed line), and 62 °C (dotted line). Inset: Thermal stability of the (+) 483 nm/(−) 470 nm CD band pair, indicated by arrows. (For other conditions, see Materials and Methods.)

The spectra in Figure 7 clearly show that the intensity of this band pair, (+) 483 nm, (−) 470 nm, at 62 °C is largely diminished as compared with the control (at 45 °C) and the heat-treated sample (incubated for 5 min at 53 °C). At 45 °C, the residual macrodomain signals are eliminated in most samples, but minor contributions are often found, e.g., at 680 and 503 nm, even in EDTA-washed membranes (Figure 7).

To further substantiate the findings that elevated temperatures and light can induce monomerization of the trimeric LHCII in thylakoid membranes, we analyzed the oligomerization state of LHCII in the membrane by using a combination of green gel electrophoresis and CD spectroscopy. As shown in Figure 8, short (5 min) treatment of isolated thylakoid membranes at 65 °C leads to a substantial monomerization of LHCII; these data are in good agreement with those obtained after prolonged (1 h) heat stress at 45 and 60 °C on barley leaves (*9*). However, under our experimental conditions, i.e., following a much shorter (5 min) incubation on isolated thylakoids, no noticeable change occurred in the oligomerization (trimer/monomer) state of the complexes at 45 and 55 °C. This was also testified by CD spectroscopy, which revealed no substantial variation between the 45 °C (Figure 8B) and the 55 °C trimeric bands of the green gel, compared to the control also excised from the green gel, and only minor alterations at 65 °C (data not shown). On the other hand, the bands produced by heat treatment or preilluminations with strong light exhibited virtually identical CD spectra which were essentially also identical with those obtained in isolated trimeric LHCII upon monomerization by phospholipase treatment or by preillumination (*15*). Further, similarly to our earlier report on isolated LHCII (*15*), the difference spectra between the trimeric and monomeric spectra in Figure 8B were also dominated by the CD band pair (+) 483 nm, (−) 470 nm (data not shown).

It can be seen in both Figures 7 and 8B that the bands around (−) 650 nm and (+) 446 nm, which originate from excitonic interactions involving Chl *b* (of LHCII) and Chl *a*, respectively (*32*), retain their intensities even at 62 °C. (Their thermal destabilization between 65 and 70 °C will be addressed in the following section.) These data reveal that, as expected for the trimer-to-monomer transition, the changes around 60 °C do not lead to the disorganization of the main pigment−protein complex constituents of the membranes. Hence, it can be concluded that, as far as the main pigment−protein complexes are concerned, the second main endothermic event, band B, involves the monomerization of LHCII trimers but not the denaturation of the main complexes. These data are consistent with the assignment of this transition as of thermo-optic nature.

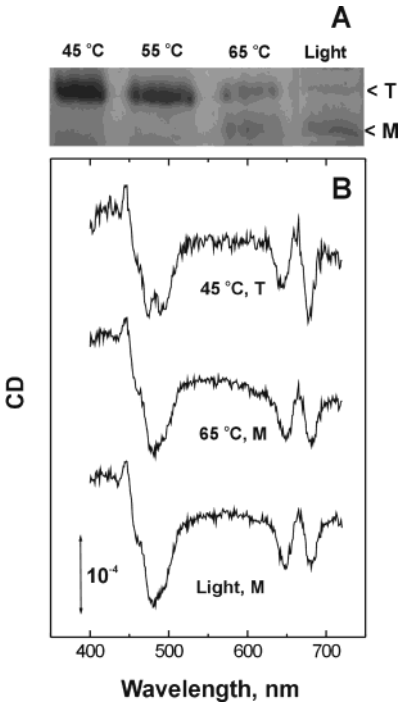

FIGURE 8: LHCII-containing bands from nondenaturing green gel electrophoresis (A) on EDTA-washed barley thylakoid membranes and CD spectra of excised bands (B). Thylakoid membranes were incubated for 5 min at 45, 55, and 65 °C, as indicated, or preillumanted at 25 °C for 1 h with white light of 1600 μE m^{-2} s^{-1}. The CD spectra, measured on the excised bands, were normalized for their (−) 650 nm band intensities, $CD_{650-625nm}$. T and M, respectively, denote bands containing predominantly trimeric and monomeric forms of LHCII.

Disassembly of LHCII: the Main DSC Band (Band C). As revealed by the loss of its characteristic CD fingerprint at 625−650 nm, the disorganization of LHCII occurs in a narrow temperature range, between 65 and 70 °C (Figure 9). This is in reasonable agreement with earlier published data, though these are at variance with each other as concerns the denaturation temperature (around 66 and 74 °C reported in refs *24* and *27*, respectively). This variation in the transition temperature is probably due to inherent thermal stability differences between different LHCII preparations (*15*, *45*). Variations are somewhat smaller in the thylakoid membranes, where the transition temperature was always found between 68 and 72 °C (Figure 9, inset). Similarly, the variations in the position of band C in different samples were confined to a relatively narrow range, between 67 and 71 °C. Hence, these data indicate that band C contains a contribution from the disassembly of the LHCII complexes, which can be inferred to lead to unfolding and denaturation of the polypeptide (*46*−*48*). Although contributions from other complexes cannot be ruled out, it seems very likely that LHCII furnishes the main contribution to band C: LHCII

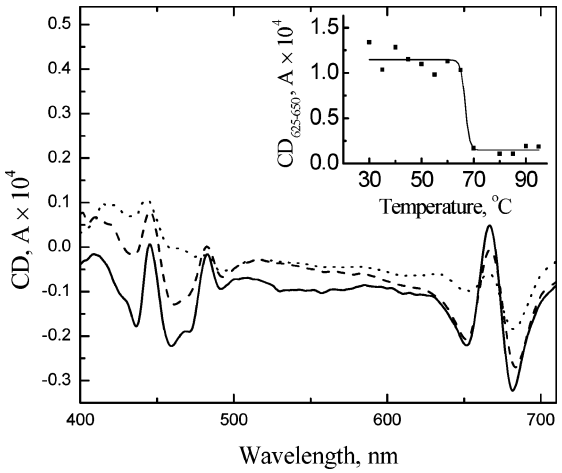

FIGURE 9: CD spectra of EDTA-washed barley thylakoids at 50 °C (solid line), 60 °C (dashed line), and 70 °C (dotted line). Inset: Thermal stability of the (+) 650 nm band, with reference to 625 nm, $CD_{625-650}$.

is the most abundant protein complex in the thylakoid membranes. Interestingly, however, even at 70 °C some CD bands are retained, albeit with reduced amplitudes, some of which, e.g., at 680 nm, are also present in LHCII. This suggests that the disassembly of LHCII is a multistep event, as revealed by recent light-sensitivity data suggesting the existence of internal pigment clusters in LHCII (*48*, *49*) and/or some other complexes which exhibit higher heat resistance than LHCII. These bands were eliminated between 72 and 80 °C, which suggests that this additional denaturing of the complexes contributes to band D.

At present, we have no indication as to whether these transitions, relating to bands C and D, are inducible thermo-optically under physiologically relevant conditions (i.e., in the temperature range below 45 °C and with moderate preillumination). A challenging question, far beyond the scope of the present paper, however, is that of what molecular organization ensures the high heat stability of these complexes and the safe "handling" of the heat packages from the dissipative processes.

CONCLUSIONS

Systematic comparison of CD data and the endothermic transitions in the thylakoid membranes revealed that characteristic structural transitions of the pigment−protein complexes, at different levels of structural complexity, contribute significantly to different calorimetric events, i.e., DSC bands. In particular, the first endothermic event, around 43 °C, could be assigned mainly to the LHCII-containing chiral macrodomains, i.e., a well-organized macroarray of complexes and/or PSII particles in the granum. The second endothermic reaction, band B, involves a trimer-to-monomer transition in LHCII in the native thylakoid membrane. These structural transitions can also be induced thermo-optically, i.e., by fast local thermal transients in the close vicinity of the dissipating centers (*15*, *20*). On the other hand, the thermodynamic parameters associated with the molecular disorganization of the main light-harvesting pigment−protein complexes do not appear to favor the thermo-optic mechanism. Hence, the available data suggest the sequence of events in thermo-optically driven transitions. The first target appears to be the disassembly of the macrodomains. As shown earlier and pointed out above, this begins with the unstacking of the membranes (*20*, *37*). This event is followed closely by a lateral disassembly of the macroarray; evidently, disassembly cannot occur without at least a partial unstacking. Following these steps, which might be essential in ensuring the mobility of certain protein complexes, including LHCII (*3*−*5*), the trimers of LHCII are transformed into monomers (*15*). This lends further structural flexibility to the membrane, which might be needed for different enzymatic activities (*17*, *50*). It is important to emphasize that when these changes are driven thermo-optically, i.e., the alterations are confined in space and time to the dissipation of the unused excitation energy, structural flexibility can be ensured, locally and temporarily, without attacking the overall organization of the membrane. In other terms, the main significance of the thermo-optical structural reorganizations might be that they ensure the dual requirement of structural stability, required to preserve the integrity of the membranes and the entire organelle, and flexibility to ensure the adaptability of the system, and evidently required in various steps of the multilevel regulatory system.

ACKNOWLEDGMENT

We are indebted to Prof. H. van Amerongen for advice concerning the origin of the (+) 483 nm, (−) 470 nm excitonic CD bands and for critically reading the manuscript.

REFERENCES

1. Anderson, J. M., and Andersson, B. (1988) *Trends Biochem. Sci. 13*, 351−355.
2. Horton, P., Ruban, A. V., and Walters, R. G. (1996) *Annu. Rev. Plant Physiol. Plant Mol. Biol. 47*, 655−684.
3. Allen, J. F., and Forsberg, J. (2001) *Trends Plant Sci. 6*, 317−326.
4. Barber, J. (1982) *Annu. Rev. Plant Physiol. 33*, 261−295.
5. Arntzen, C. J. (1978) *Curr. Top. Bioenerg. 8*, 111−160.
6. Ruban, A. V., Rees, D., Pascal, A. A., and Horton, P. (1992) *Biochim. Biophys. Acta 1102*, 39−44.
7. Li, X. P., Bjorkman, O., Shih, C., Grossman, A. R., Rosenquist, M., Jansson, S., and Niyogi, K. K. (2000) *Nature 403*, 391−395.
8. Aspinall-O'Dea, M., Wentworth, M., Pascal, A., Robert, B., Ruban, A., and Horton, P. (2002) *Proc. Natl. Acad. Sci. U.S.A. 99*, 16331−16335.
9. Takeuchi, T. S., and Thornber, J. P. (1994) *Aust. J. Plant Physiol. 21*, 759−770.
10. Garab, G., Faludi-Dániel, A., Sutherland, J. C., and Hind, G. (1988) *Biochemistry 27*, 2425−2430.
11. Gussakovsky, E. E., Barzda, V., Shahak, Y., and Garab, G. (1997) *Photosynth. Res. 51*, 119−126.
12. Istokovics, A., Simidjiev, I., Lajkó, F., and Garab, G. (1997) *Photosynth. Res. 54*, 45−53.
13. Barzda, V., Istokovics, A., Simidjiev, I., and Garab, G. (1996) *Biochemistry 35*, 8981−8985.
14. Simidjiev, I., Barzda, V., Mustárdy, L., and Garab, G. (1998) *Biochemistry 37*, 4169−4173.
15. Garab, G., Cseh, Z., Kovács, L., Rajagopal, S., Várkonyi, Zs., Wentworth, M., Mustárdy, L., Der, A., Ruban, A. V., Papp, E., Holzenburg, A., and Horton, P. (2002) *Biochemistry 41*, 15121−15129.
16. Barzda, V., Jennings, R. C., Zucchelli, G., and Garab, G. (1999) *Photochem. Photobiol. 70*, 751−759.
17. Zer, H., Vink, M., Shochat, S., Herrmann, R. G., Andersson, B., and Ohad, I. (2003) *Biochemistry 42*, 728−738.
18. Zer, H., Vink, M., Keren, N., Dilly-Hartwig, H. G., Paulsen, H., Herrmann, R. G., Andersson, B., and Ohad, I. (1999) *Proc. Natl. Acad. Sci. U.S.A. 96*, 8277−8282.
19. Aro, E. M., and Ohad, I. (2003) *Antioxid. Redox Signaling 5*, 55−67.
20. Cseh, Z., Rajagopal, S., Tsonev, T., Busheva, M., Papp, E., and Garab, G. (2000) *Biochemistry 39*, 15250−15257.

21. Cramer, W. A., Whitmarsh, J., and Low, P. S. (1981) *Biochemistry 20*, 157−162.
22. Thompson, L. K., Sturtevant, J. M., and Brudvig, G. W. (1986) *Biochemistry 25*, 6161−6169.
23. Thompson, L. K., Sturtevant, J. M., and Brudvig, G. W. (1987) in *Progress in Photosynthesis Research* (Biggins, J., Ed.) pp 609, Martinus Nijhoff Publishers, Dordrecht, The Netherlands.
24. Thompson, L. K., Blaylock, R., Sturtevant, J. M., and Brudvig, G. W. (1989) *Biochemistry 28*, 6686−6695.
25. Smith, K. A., Ardelt, B. K., and Low, P. S. (1986) *Biochemistry 25*, 7099−7105.
26. Smith, K. A., and Low, P. S. (1989) *Plant Physiol. 90*, 575−581.
27. Smith, K. A., Ardelt, B. K., Huner, N. P. A., Krol, M., Myscich, E., and Low, P. S. (1989) *Plant Physiol. 90*, 492−499.
28. Nolan, W. G., Hopkins, H. P., and Kalini, S. A. M. (1992) *Arch. Biochem. Biophys. 297*, 19−27.
29. Shutilova, N., Semenova, G., Klimov, V., and Shnyrov, V. (1995) *Biochem. Mol. Biol. Int. 35*, 1233−1243.
30. Garab, G. (1996) in *Biophysical Techniques in Photosynthesis* (Amesz, J., and Hoff, A. J., Eds.) pp 11−40, Kluwer Academic Publishers, Dordrecht, The Netherlands.
31. van Amerongen, H., Valkunas, L., and van Grondelle, R. (2000) *Photosynthetic Excitons*, World Scientific Publishing Co., Singapore.
32. Garab, G., Kieleczawa, J., Sutherland, J. C., Bustamante, C., and Hind, G. (1991) *Photochem. Photobiol. 54*, 273−281.
33. Lichtenthaler, H. K. (1987) *Methods Enzymol. 148*, 350−382.
34. Privalov, P. L., Plotnikov, V. V., and Filmonov, V. V. (1975) *J. Chem. Thermodyn. 7*, 41−47.
35. Lyubarev, A. E., and Kurganov, B. I. (2001) *Recent Res. Dev. Biophys. Chem. 2*, 141−165.
36. Shnyrov, V. L., and Mateo, P. L. (1993) *FEBS Lett. 324*, 237−240.
37. Garab, G., and Mustárdy, L. (1999) *Aust. J. Plant Physiol. 26*, 649−658.
38. Keller, D., and Bustamante, C. (1986) *J. Chem. Phys. 84*, 2972−2979.
39. Havaux, M., and Tardy, F. (1997) *Plant Physiol. 113*, 913−923.
40. Berry, J., and Bjornman, O. (1980) *Annu. Rev. Plant Physiol. 31*, 491−543.
41. Gounaris, K., Brain, A. R. R., Quinn, P. J., and Williams, W. P. (1984) *Biochim. Biophys. Acta 766*, 198−208.
42. Quinn, P., and Williams, W. P. (1985) in *Photosynthetic Mechanisms and the Environment* (Barber, J., and Baker, N. R., Eds.) pp 1−47, Elsevier, Amsterdam.
43. Kóta, Z., Horváth, L. I., Droppa, M., Horváth, G., Farkas, T., and Páli, T. (2002) *Proc. Natl. Acad. Sci. U.S.A. 99*, 12149−12154.
44. Gradinaru, C. C., van Grondelle, R., and van Amerongen, H. (2003) *J. Phys. Chem. B 107*, 3938−3943.
45. Taneva, S. G., Simidjiev, I., Garab, G., and Muga, A. (1998) in *Photosynthesis: Mechanisms and Effects* (Garab, G., Ed.) pp 325−328, Kluwer Academic Publishers, Dordrecht, The Netherlands.
46. Horn, R., and Paulsen, H. (2002) *J. Mol. Biol. 318*, 547−556.
47. Paulsen, H., Finkenzeller, B., and Kuhlein, N. (1993) *Eur. J. Biochem. 215*, 809−816.
48. Meyer, M., Wilhelm, C., and Garab, G. (1996) *Photosynth. Res. 49*, 71−81.
49. Olszowka, D., Maksymiec, W., Krupa, Z., and Krawczyk, S. (2003) *J. Photochem. Photobiol. B 70*, 21−30.
50. Yang, D. H., Paulsen, H., and Andersson, B. (2000) *FEBS Lett. 466*, 385−388.

BI034899J