Time-Resolved Fluorescence Analysis of the Photosystem II Antenna Proteins in Detergent Micelles and Liposomes[†]

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Received February 19, 2001; Revised Manuscript Received July 19, 2001

ABSTRACT: We have studied the time-resolved fluorescence properties of the light-harvesting complexes (Lhc) of photosystem II (Lhcb) in order to obtain information on the mechanism of energy dissipation (non-photochemical quenching) which is correlated to the conversion of violaxanthin to zeaxanthin in excess light conditions. The chlorophyll fluorescence decay of Lhcb proteins LHCII, CP29, CP26, and CP24 in detergent solution is mostly determined by two lifetime components of 1.2−1.5 and 3.6−4 ns while the contribution of the faster component is higher in CP29, CP26, and CP24 with respect to LHCII. The xanthophyll composition of Lhc proteins affects the ratio of the lifetime components: when zeaxanthin is bound into the site L2 of LHCII, the relative amplitude of the faster component is increased and, consequently, the chlorophyll fluorescence quenching is enhanced. Analysis of quenching in mutants of *Arabidopsis thaliana*, which incorporate either violaxanthin or zeaxanthin in their Lhc proteins, shows that the extent of quenching is enhanced in the presence of zeaxanthin. The origin of the two fluorescence lifetimes was analyzed by their temperature dependence: since lifetime heterogeneity was not affected by cooling to 77 K, it is concluded that each lifetime component corresponds to a distinct conformation of the Lhc proteins. Upon incorporation of Lhc proteins into liposomes, a quenching of chlorophyll fluorescence was observed due to shortening of all their lifetime components: this indicates that the equilibrium between the two conformations of Lhcb proteins is displaced toward the quenched conformation in lipid membranes or thylakoids with respect to detergent solution. By increasing the protein density in the liposomes, and therefore the probability of protein−protein interactions, a further decrease of fluorescence lifetimes takes place down to values typical of quenched leaves. We conclude that at least two major factors determine the quenching of chlorophyll fluorescence in Lhcb proteins, i.e., intrasubunit conformational change and intersubunit interactions within the lipid membranes, and that these processes are both important in the photoprotection mechanism of nonphotochemical quenching in vivo.

The photosynthetic apparatus of higher plants is composed of two photosystems (PS),[1] PSI and PSII, each consisting of a core complex moiety, binding chlorophyll (Chl) *a* and *β*-carotene, and a peripheral antenna system, binding Chl *a*, Chl *b*, and xanthophylls (Cars). The major component of the PSII antenna system is LHCII, a heterotrimer of the Lhcb1, -2, and -3 gene products (also called LHCIIb) binding half of the thylakoid pigments. Minor components are CP29 (LHCIIa), CP26 (LHCIIc), and CP24 (LHCIId), respectively the products of the Lhcb4, -5, and -6 genes, which are monomeric and connect the PSII core complex to the peripheral LHCII trimers.

The large absorption cross-section of the antenna systems allows plants to grow in dim light. However, the light intensity experienced by a plant varies largely over relatively short time periods. When irradiance over-saturates the electron transport capacity, a decrease in the quantum efficiency of photosynthesis is observed, at the level of PSII, due to enhancement of the nonradiative deactivation. The down-regulation can be observed through its quenching effect

[†] This work was supported by the CNR (Target project in Biotechnology) and by Murst (COFIN 2000).

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[1] Abbreviations: Car, carotenoid; CCD, charge-coupled device; Chl, chlorophyll; CP, chlorophyll protein; DCCD, dicyclohexylcarbodiimide; DGDG, diacylgalactosylglycerol; DM, *n*-dodecyl-*β*-D-maltoside; F_M, maximum Chl fluorescence from dark-adapted leaves; F_M', maximum Chl fluorescence from leaves under actinic light; fwhm, full-width at half-maximum; GLC, gas−liquid chromatography; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; HPLC, high-performance liquid chromatography; IEF, isoelectric focusing; LED, light-emitting diode; Lhc, light-harvesting complex; Lhcb, light-harvesting complex of PSII; LHCII, the major light-harvesting complex of PSII; MGDG, diacylmonogalactosylglycerol; NPQ, nonphotochemical quenching; OGP, octyl glucopyranoside; PC, phosphatidylcholine; PL, total phospholipids; PMSF, phenylmethylsulfonyl fluoride; PS, photosystem; qE, first rapid phase (<1 min) of NPQ; RT, room temperature; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; S1, first singlet excited state; SQDG, sulfoquinovosyldiacylglycerol; TLC, thin-layer chromatography; *WT*, wild type.

10.1021/bi010342x CCC: $20.00
Published on Web 00/00/0000 PAGE EST: 9.7

on chlorophyll fluorescence, and the latter process, known as non-photochemical quenching (NPQ), protects PSII from irreversible photoinhibition (for a review, see ref *1*).

Photoprotection in higher plants is achieved by several different mechanisms. State 1−state 2 transition causes variations of antenna size leading to a redistribution of excitation energy between the two photosystems (*2*). This process is rather slow (half-time of dark-relaxation around 5 min) and correlates directly with the phosphorylation of LHCII proteins. Nonradiative dissipation is induced upon higher excess of excitation energy; it is catalyzed by faster mechanisms involving (i) the formation of a trans-thylakoid Δ*p*H and (ii) the de-epoxidation of violaxanthin to antheraxanthin and zeaxanthin. Although these processes have been shown to correlate with the rapid phase of NPQ (so-called qE) in vivo and in thylakoids (*3, 4*), the detailed mechanisms at a molecular level are still poorly understood.

Much evidence supports Lhc proteins as the principal site for NPQ, including: (i) the strong reduction of qE in Lhc protein-depleted plants (*5*); (ii) the binding of the xanthophyll cycle pigments to Lhc proteins rather than to core complex proteins (*6*); (iii) the strong decrease of NPQ in double mutants lacking zeaxanthin and lutein (*7*); (iv) the identification of binding sites for the NPQ inhibitor dicyclohexylcarbodiimide (DCCD) on CP26 and CP29 (*8*−*10*); (v) the strong decrease of NPQ amplitude when the psbS gene coding for the Lhc-like PSII subunit is deleted (*11*). Nevertheless, residual NPQ has been described in the *Chlorina f2* and *Chlorina 104* mutants lacking or reduced in Lhc proteins (*12, 13*). This suggests that the PSII core complex might contribute to NPQ.

Establishment of NPQ in vivo involves the building of quenching species within the pigment bed, leading to a transition in the overall average lifetime of fluorescence emission from 2.3 to 0.5 ns (*4, 14*−*16*); this is accomplished by an amplitude decrease of the 1.6−1.8 ns Chl *a* fluorescence component versus that of 0.4−0.5 ns (*17*). Formation of quenching molecular species has been proposed to involve either the direct de-excitation of ^{1}Chl* by Cars or the structural reorganization of Lhc proteins into a quenching state.

The direct quenching hypothesis required a reversible de-epoxidation of Cars with a change in their energy level of the S1 states, thus leading to an energy transfer from Chl to zeaxanthin, followed by rapid thermal decay (*18*).

Other models have been based on the modification of pigment−pigment and/or pigment−protein interactions within individual Lhc proteins triggered either by the protonation of lumen-exposed residues (*19*) or by changes in the aggregation state of Lhc proteins mediated by the presence of either violaxanthin or zeaxanthin in lipid membranes (*20*). The recent finding that violaxanthin and zeaxanthin have very similar energy levels in their S1 states (*21, 22*) disproved the direct quenching hypothesis, while the report that substitution of Car bound to the L2 site of recombinant Lhc proteins modulated fluorescence quenching (*10, 23*) supports the involvement of conformational changes, induced by the substitution of xanthophyll species in the allosteric site L2 of Lhcb proteins, in the formation of quenching states (*24*). The amplitude of zeaxanthin-induced quenching in isolated and nonaggregated Lhc proteins is within the range of 30−50% of the original fluorescence intensity (*10, 23*), thus making such a mechanism alone unlikely to account for the strong quenching that can be obtained in vivo. To clarify the role that both properties of individual Lhc proteins and their interactions within the thylakoid membrane have in the formation of quenching species, we have characterized the steady-state and time-resolved fluorescence behavior of the four components of the PSII antenna system (LHCII, CP29, CP26, and CP24) purified from maize grana membranes (*25*) in detergent solution and reconstituted into liposomes. Our results indicate a remarkable homogeneity in the fluorescence decay of Lhc proteins, which is dominated by two lifetimes of 1.1−1.5 and 3.6−4 ns, respectively. LHCII proteins binding zeaxanthin undergo static quenching by increasing the amplitude of the 1.1−1.5 ns versus the 3.6−4 ns components. Upon incorporation into liposomes, a further quenching of emission is observed due to the shortening of all the fluorescence lifetimes, suggesting that the conformation of Lhc proteins in detergent solution might be significantly different with respect to the native structure in thylakoid membranes. Increasing the protein density inside the liposomes, and thus protein−protein interactions, leads to a further reduction of the radiative lifetime down to values observed in quenched leaves. We conclude that protein−protein interactions within the thylakoid membrane affect the spectroscopic properties of Lhc proteins in vivo, and that their modulation may be an efficient mechanism for the NPQ process, in addition to the fluorescence quenching induced by de-epoxidation of violaxanthin to zeaxanthin.

MATERIALS AND METHODS

Isolation of Chl a/b Protein Complexes. LHCII, CP29, CP26, and CP24 were purified from maize thylakoids by nondenaturing isoelectric focusing (IEF) with 0.06% dodecylmaltoside (DM) or 1% octyl glucopyranoside (OGP) as detergents (*26*). Hereafter, we indicate as "LHCII" an IEF subpopulation of the whole antenna complex LHCII which displays the most complete polypeptide pattern in denaturing sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE), at least six polypeptides in the 26−30 kDa range (*27*), and is free from contamination by the minor antennae CP29, CP26, and CP24. The minor antenna proteins were purified by IEF followed by ultracentrifugation in a 0.1−1 M sucrose gradient including 10 mM *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (Hepes)−KOH, pH 7.6, and 0.06% DM. Analytical SDS−urea−PAGE (and nondenaturing SDS−glycerol−PAGE) were as in ref (*26*).

Isolation of Chloroplast Diacyl Lipids. Three thylakoid diacyl lipid classes, namely, diacylmonogalactosylglycerol (MGDG), diacylgalactosylglycerol (DGDG), sulfoquinovosyldiacylglycerol (SQDG), and total phospholipids (PL) were purified from spinach leaves by thin-layer chromatography (TLC) as in ref (*28*). The purity of the lipids was checked by TLC and their fatty acid composition analyzed by gas−liquid chromatography (GLC) as in ref (*29*).

Reconstitution of the Chl a/b Proteins into Liposomes. Purified thylakoid lipid classes in the physiological ratio MGDG/DGDG/PL = 45:40:15 (w/w/w) or crude soybean phosphatidylcholine (PC) (by Sigma) was taken up in chloroform and dried under nitrogen to a thin film. Then 15 mM Hepes (pH 7.2) was added to give a lipid concentration

of 0.5−1 mg/mL. Liposomes were formed by sonication under nitrogen at room temperature for 20 min in a bath sonicator. Ten milligram aliquots of the Lhcb proteins in 1% OGP, 15 mM Hepes (pH 7.2) were combined with liposomes by mixing at a lipid:Chl ratio of (6−36):1 (w/w), corresponding to a lipid:protein ratio of (30−300):1 (n/n), calculated on the basis of the chlorophyll/protein values of the antenna complexes obtained in ref (*30*). The detergent was removed by dialysis for about 20 h at 4 °C against 3 baths of 10 mM Hepes (pH 7.2) and 0.2 mM phenylmethylsulfonyl fluoride (PMSF) as protease inhibitor. Incorporation was monitored by a discontinuous Ficoll gradient ultracentrifugation (6, 10, 15, 20% Ficoll) in 10 mM Hepes (pH 7.2) (Beckmann SW41 rotor, 39 krpm, 16 h, 4 °C). All the samples, except for LHCII proteoliposomes with lipid:Chl ratio equal to 6 (w/w) which presented two bands, showed a single green band in the upper section of the gradient.

The gradient bands were concentrated by ultracentrifugation in a Kontron TST 60.4 rotor at 5.5 krpm for 1 h at 4 °C, after dilution of the bands in 10 mM Hepes (pH 7.2). The proteoliposome pellets were obtained only after a freeze−thaw step before the centrifugation. Aliquots were stored at −80 °C in 15 mM Hepes (pH 7.2). Before the measurements, the proteoliposomes were sonicated for 5 min in a bath sonicator to minimize the possible formation of multilamellar and heterogeneous-size liposomes due to the freezing.

Electron Microscopy. Liposome vesicles were pelleted by centrifugation and prepared for transmission electron microscopy and for freeze−fracture analysis as previously described in ref (*31*).

Spectroscopic Analysis. Steady-state absorption spectra were obtained at room temperature using a Perkin-Elmer Lambda 5 spectrophotometer.

Chlorophyll fluorescence lifetime measurements were carried out and analyzed as described in ref (*32*). The light source consisted of a mode-locked and cavity-dumped dye laser system (dye Rhodamine 6 G) synchronously pumped by a mode-locked Ar^+ laser. The system provided pulses of 10−15 ps duration and a repetition rate up to 4 MHz (actually 0.8 MHz in these measurements). The excitation wavelength was usually set to 630 nm. The fluorescence was measured at 90°, with respect to the excitation beam, in a 2 × 2 mm cuvette situated at the entrance slit of a monochromator (Jobin & Yvon M25). A bandwidth of 3 nm was used at the detection wavelength of 681 nm. To ensure the best rejection of the excitation light, a red filter was set after the monochromator (3 mm Schott RG 665), and removed when measuring the scattered light. The detector was a microchannel plate photomultiplier tube (Hamamatsu R1564U with S20 spectral response), kept at −30 °C by a Peltier cooler in order to reduce the dark counts to about 5−10 counts/s. As a consequence, no background was recorded in the analyzed time window (i.e., 20−40 ns). Such a wide time window is necessary to obtain a complete zeroing of the decay processes in the nanosecond range. Under these conditions, the instrument response function had approximately 60−80 ps of fwhm when the scattered light was examined instead of the fluorescence. The counting rate was set to 5000 counts/s for both flash and fluorescence. The decay spectra were accumulated over 1024 channels in a multichannel analyzer (Tracor Northern 1750) up to 10^4−3 × 10^4 counts at the peak channel. Each measurement was repeated several times until reproducibility of the results was reached.

Deconvolution of the decays into a sum of exponentials was carried out on-line by means of a least-squares code using the Marquardt's search algorithm and nonlinear parametrization. The quality of the fits was checked by a minimal reduced χ^2 and by the distribution of the weighted residuals. Time-resolved emission spectra, at steps of 2−3 nm, were obtained by analysis of the fluorescence signal followed by a global fit of all the decays using the same lifetime parameters as described in ref (*33*).

The single photon counting apparatus and deconvolution software have been checked by measuring and analyzing the time-resolved fluorescence of oxazine in methanol (*33*), which yielded a single-exponential decay with a lifetime of 785 ps and data fitting with a reduced χ^2, typically around unity.

Low-temperature measurements were carried out using a thin cylindrical cuvette of 0.1 mm path length (Hellma 124 QS 01) positioned in a variable-temperature cryostat (5−300 K) at 45° of the exciting beam.

Non-photochemical Quenching. The non-photochemical quenching of Chl fluorescence was measured on 3-week-old leaves of *Arabidopsis thaliana* wild type (*WT*), *aba-3* (*34*), and *npq1* mutants (*35*) under exposure to actinic light (halogen lamp) of intensity 1300 μE m^{-2} s^{-1}. Upon induction by 1.8 s pulses of 600 μE m^{-2} s^{-1} LED light at 590 nm, a video-imaging apparatus system by a CCD camera (*36*) detects the sample fluorescence above 690 nm and calculates the NPQ according to the equation (*37*): $NPQ = (F_m - F_m')/F_m'$, where F_m is the maximum Chl fluorescence from dark-adapted leaves and F_m' the maximum Chl fluorescence from leaves under actinic light exposition.

RESULTS

Purification and Biochemical Characterization of Lhcb Proteins. The Lhcb proteins were purified from maize PSII membranes by preparative IEF followed by sucrose gradient ultracentrifugation as previously reported (*25*), and then characterized for their polypeptide and pigment composition. The minor Lhcb proteins CP29, CP26, and CP24 migrated at 0.3 M sucrose after ultracentrifugation for 23 h at 39 000 rpm in an SW40 rotor and contained a single polypeptide in Tris−Tricine SDS−PAGE. The LHCII complex formed a single green band at 0.45 M sucrose and contained several polypeptides in the 26−28 kDa range according to their heterotrimeric state. Highly purified Lhcb proteins bound different complements of Chl and xanthophyll pigments (Table 1), in agreement with previous findings (*6*). LHCII purified from *A. thaliana WT* and from *aba-3* mutant had distinct pigment composition. In the complex from *WT* thylakoids, a Chl:Car molar ratio equal to 4 was found, equivalent to 3 Car and 12 Chl molecules per polypeptide (*25*). Due to inhibition of the zeaxanthin epoxidation, the antenna complex from the *aba-3* mutant was lacking violaxanthin and neoxanthin (*34*); zeaxanthin in the molar amount of 0.6 was bound to the complex up to a total, together with lutein, of two xanthophylls per polypeptide (*38*) as shown by the Chl:Car molar ratio of 6.

Table 1: Pigment Composition of Lhcb Proteins from Maize and *A. thaliana*[a]

	Chl *a*	Chl *b*	Lute	Neo	Viola	Zea
maize CP24	5	5	1	0	1	0
maize CP26	6	3	1	0.6	0.4	0
maize CP29	6	2	1	0.5	0.5	0
maize LHCII	7	5	1.8	1.0	0.2	0
A. thaliana WT LHCII	7	5	1.8	1.0	0.2	0
A. thaliana aba3 LHCII	7	5	1.4	0	0	0.6

[a] The data from HPLC analysis are expressed as moles per mole of Lhcb polypeptide.

Table 2

	*T*1 (ns) *A*1 (%)/ϕ1	*T*2 (ns) *A*2 (%)/ϕ2	*T*3 (ns) *A*3 (%)/ϕ3	$\langle T \rangle$ (ns)
(a) Chlorophyll Fluorescence Decay Analysis of Lhcb Proteins from Maize in Detergent Solution at 20 °C[a]				
CP29	<0.1	1.9	4.3	3.9
	<1	32/17	68/83	
CP26	<0.1	2.8	4.6	3.7
	<1	58/45	42/54	
CP24	<0.1	2.5	4.6	3.6
	<1	62/47	38/52	
LHCII	−	1.4	3.8	3.6
		19/8	81/92	
(b) Chlorophyll Fluorescence Decay Analysis of LHCII Preparations in Detergent Solution from *Arabidopsis thaliana WT* and *aba-3* Mutant (Binding Zeaxanthin) at 20 °C[b]				
A. thaliana WT LHCII	<0.1	1.1	3.7	3.1
	<1	10/3.2	90/96.5	
A. thaliana aba3 LHCII	<0.1	1.2	3.4	2.6
	<1	18/7	82/92	
(c) Fluorescence Decay Analysis of CP29 and LHCII Proteins from Maize in Detergent Solution at 77 K[c]				
CP29	<0.1	2.3	5.2	4.7
	<1	31/16	69/83	
LHCII	−	2.0	4.8	4.2
		41/22	59/78	

[a] The fitting lifetime (*T*) components are expressed in terms of their percentage amplitudes (*A*), and associated fluorescence yields (ϕ). $\langle T \rangle$ is the average chlorophyll fluorescence lifetime. [b] The fitting lifetime (*T*) components are expressed in terms of their percentage amplitudes (*A*), and associated fluorescence yields (ϕ). $\langle T \rangle$ is the average chlorophyll fluorescence lifetime. [c] The fitting lifetime (*T*) components are expressed in terms of their percentage amplitudes (*A*), and associated fluorescence yields (ϕ). $\langle T \rangle$ is the average chlorophyll fluorescence lifetime.

Steady-State Absorption Spectra. The steady-state absorption and fluorescence properties of the purified pigment proteins were in agreement with the data previously published (*39*, *40*). Incubation of Chl proteins in conditions typically used for measurements did not modify either the aggregation state or the optical properties of the complexes.

Time-Resolved Fluorescence Emission. The data obtained from time-resolved analysis of the Chl fluorescence emission from LHCII, CP24, CP26, and CP29 are reported in Table 2 , and, in view of the similarities among the various pigment−proteins, only some time-resolved emission spectra were selected and are shown in Figure 1. Two main lifetime components were required to best fit the fluorescence decays. In the case of LHCII, 92−95% of the total emission was due to a 3.8 ns lifetime component (T3) and the rest (a few percent) to a 1.4 ns component (T2). These values are in excellent agreement with the results of previous work with LHCII (*41*, *42*). The emission spectrum of the elementary components has been obtained by deconvolution of the decays measured at different wavelengths (see Materials and Methods). The time integrated emission spectra, as well as the resulting elementary bands, peak at 678 ± 1 nm in Figure 1B. In agreement, the averaged lifetime is independent of the emission wavelength.

The steady-state emission spectra of the minor Lhc proteins CP29 (Figure 1A), CP26, and CP24 (data not shown) do not reveal important differences in shape and peak wavelength, except for a small blue-shift of 1−2 nm in the case of CP24.

Time decay analysis revealed a slightly higher degree of heterogeneity for the minor antennae compared to LHCII since an extra fast component (A1 < 1% and T1 < 100 ps), in addition to the two major components in the nanosecond range, was required to best fit the fluorescence decays (Table 2a). In general, the main components of the minor antennae have longer lifetimes than LHCII, and their average lifetime is independent of the emission wavelength, as in the case of LHCII. Preexponential factors show that the different Lhc proteins diversify with respect to their molar ratio of chromophores emitting at the two major lifetimes: the T2-emitting population was the most represented in the case of CP24 and CP26 while CP29 and, even to a larger extent, LHCII emitted mainly by their longer (T3) component.

LHCII from WT and aba-3 Mutant. LHCII from *WT Arabidopsis* had fluorescence behavior very similar to the complex purified from *Zea mays* although trace amounts (A1 < 1%) of a rapid component (T1 < 100 ps) could be detected. The LHCII from *aba-3* mutant exhibited the same lifetime components; however, the average lifetime was shortened from 3.1 to 2.6 ns because of an increased contribution of the 1.2 ns component with respect to the 3.5 ns components (Table 2b). The molar ratio of chromophores emitting preferentially by the T2 versus the T3 component is increased with respect to the LHCII *WT* sample. Fluorescence yield, determined by steady-state fluorescence emission spectra upon excitation at 625 nm, showed that LHCII from *aba-3* has a 40% lower emission with respect to purified *WT* LHCII, although the overall shape of the spectra was identical (data not shown).

Low-Temperature Lifetime Analysis. To further characterize the origin of the two major fluorescence lifetime components, low-temperature measurements were also performed. Cryogenic temperatures induce both a narrowing of the absorption spectrum and, according to Paillotin (*43*), an increase of the absorption probability. The value of the intrinsic fluorescence lifetime (τ_0) depends only on the integral of the absorption spectra, which does not vary from room temperature (RT) down to 77 K: as a consequence, also τ_0 is not much affected in this temperature range. On the other hand, a strong effect is expected on chromophore−chromophore excitation exchange since, assuming the Boltzmann's equilibrium, uphill pathways are different and the energy transfer tends to become irreversible. Accordingly, the fluorescence emission of LHCII (and CP29) become narrower in going from RT to 77 K, their fwhm being reduced approximately from 20 to 12 nm (Figure 1), and the maximum peak is red-shifted by 3−5 nm.

Chl fluorescence decay analysis of CP29 at 77 K yielded two main lifetime components, both peaking at 680 nm (Figure 1C). Not surprisingly, lifetime values were somehow

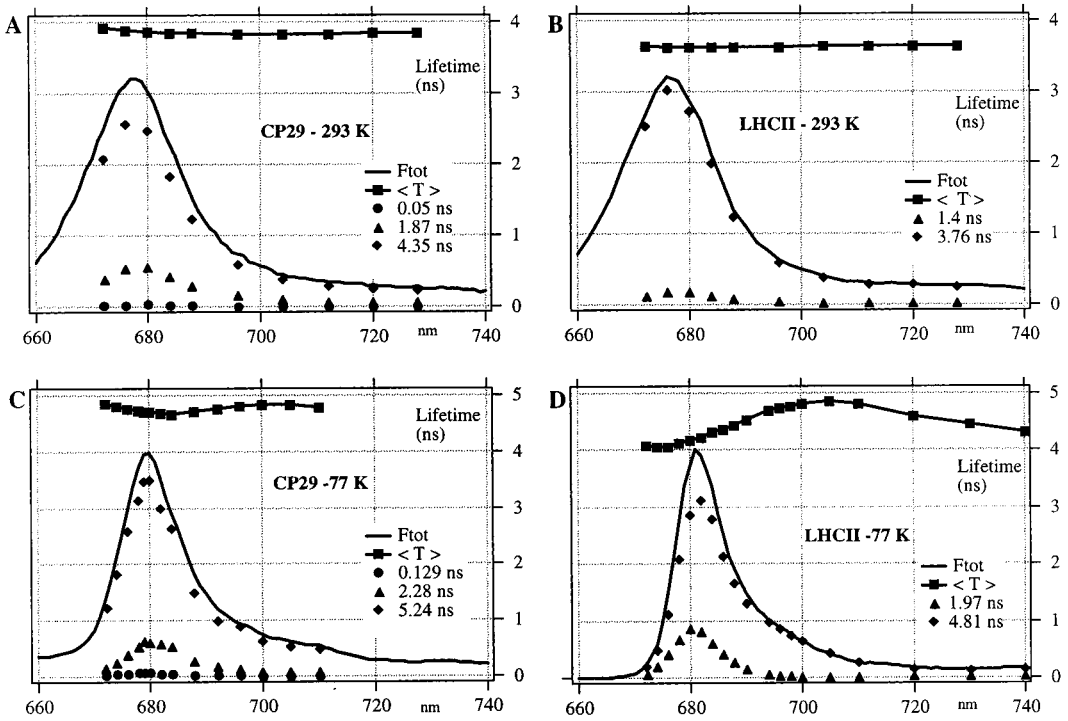

FIGURE 1: Time-resolved analysis of fluorescence decay from purified CP29 (A and C) and LHCII (B and D) complexes in 0.06% DM solution at 20 °C (A and B) and 77 K (C and D). The continuous curves are the emission spectra from the Q_y transitions in arbitrary units, while the right-hand scale refers to the lifetime components indicated by different symbols.

longer with respect to 20 °C (Table 2c); however, the relative contribution of the two lifetime components was essentially the same as that at RT, and, in practice, the averaged lifetime was still wavelength-insensitive.

The analysis of LHCII decay spectra at 77 K still showed two main components peaking, respectively, at 680 and 682 nm. As a consequence, the time-resolved spectra showed certain wavelength dependence in Figure 1D. Comparison with spectra of the same protein at RT still reveals longer lifetime values and also a stronger heterogeneity at 77 K, where the shorter lifetime contribution increased from 8 to 22%.

Lhc Proteins Reconstituted into Liposomes. It is known that the spectral properties and fluorescence decay behavior of pigment−protein complexes depend on interactions with the environment and, particularly, on their aggregation state (*41*, *42*). From this point of view, Chl−proteins isolated in detergent micelles represent an artificial system, which easily allows investigation of properties due to specific antenna subunits but in a situation rather far from physiological, i.e., where chlorophyll−protein interactions with each other and with thylakoid membrane lipids are not reproduced. However, useful information can be drawn from the comparison of the results observed under such conditions with those obtained in a more physiological environment like a lipid medium. On purpose, we reconstituted purified Lhc proteins into liposomes composed of a mixture of MGDG, DGDG, and PL, i.e., identical to the composition of the thylakoid membranes from which the Lhcb proteins were extracted (*28*).

Transmission electron microscopic analysis of liposomes showed that the procedure yielded single vesicles rather than multilayers. The size of the vesicles was slightly variable in different samples, apparently depending on the Lhc protein incorporated: on average, 460 ± 370 nm vesicle diameter was measured in the case of LHCII samples. Vesicles incorporating CP29 were slightly larger and very homogeneous in both size and shape. Vesicles with CP26 and CP24 were even larger. Unimportant changes in the general characteristics of vesicles were detected when changing the lipid-to-protein ratio.

Freeze−fracture analysis on LHCII-containing vesicles obtained with the highest lipid-to-protein ratio revealed isolated particles with a size of 9 nm, which is consistent with the size of trimeric LHCII particles described in thylakoid membranes and in liposomes (*44*). Small irregular aggregates of particles were randomly detected. Upon increasing the protein content, also the number of particles in patches increased; however, no regular arrays were detected by freeze−fracture analysis.

Similar situations were found in vesicles reconstituted with minor chlorophyll−proteins, although particles were smaller (sometime at the limit of detection), and showed areas with a rough appearance in contrast with smoother surfaces containing presumably only lipids. Again, no regular arrays of particles were detected.

One of the main spectroscopic features following incorporation of Lhc proteins into liposomes is the reduction of the amplitude of fluorescence emission by a factor of about 4.5, which can be estimated by the changes in the average lifetime (Figure 3A and Table 3). The time-resolved analysis of CP29 in lipid membranes (Figure 2A) showed a strong decrease of the lifetime values, thus leading to an average lifetime of 3−0.8 ns depending on the lipid-to-protein ratio: namely, the higher the protein content of liposomes, the shorter the lifetime values (Figure 3A). This effect is accompanied by a decrease of the relative contribution of the slowest lifetime component (T3) with respect to the other

Table 3: Chlorophyll Fluorescence Decay Analysis of Lhcb Proteins from Maize and Reconstituted in Liposomes[a]

	lipid/protein ratio (mol/mol)	*T*1 (ns) *A*1 (%)/ϕ1	*T*2 (ns) *A*2 (%)/ϕ2	*T*3 (ns) *A*3 (%)/ϕ3	$\langle T \rangle$ (ns)
CP29	2.9	0.2 31/5	1.0 51/5	2.3 18/43	1.5
CP26	2.9	0.3 30/8	1.1 56/61	2.3 14/31	1.4
CP24	2.9	0.2 43/13	0.9 49/63	2.3 8/24	1.2
LHCII	2.2	0.2 30/10	0.8 62/70	1.8 8/20	0.9
LHCII	6.6	0.2 22/3	1.0 49/40	2.3 29/57	1.7

[a] The lipid-to-protein ratio in the second column is expressed in terms of moles per mole. The fitting lifetime (*T*) components are expressed in terms of their percentage amplitudes (*A*), and associated fluorescence yields (ϕ). $\langle T \rangle$ is the average chlorophyll fluorescence lifetime.

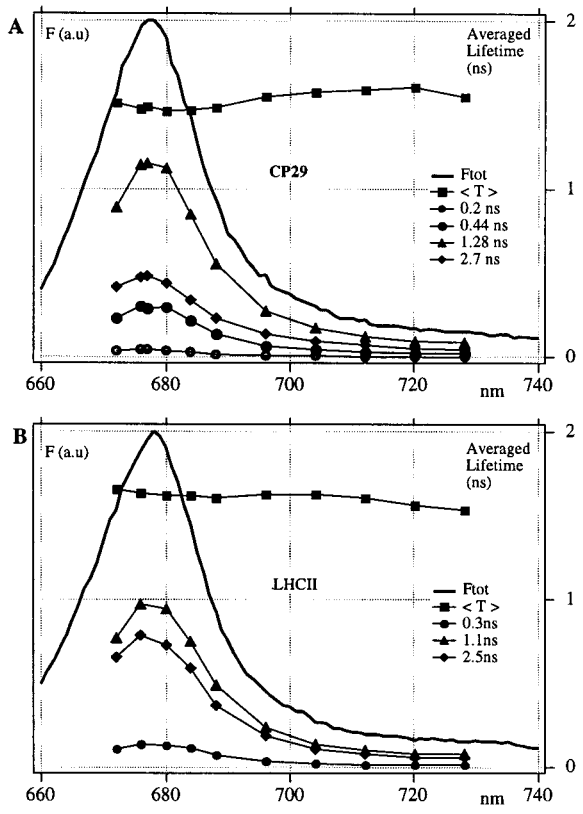

FIGURE 2: Time-resolved analysis of fluorescence decay from purified CP29 (A) and LHCII (B) complexes in liposomes at 20 °C. The continuous curves are the emission spectra from the Q_y transitions in arbitrary units, while the right-hand scale refers to the lifetime components indicated by different symbols.

ones T1 and T2 (Figure 3B). Similar effects in liposomes are also evident for LHCII *WT* (Figure 2B), and were observed in all the other Lhc proteins (Table 3).

Figure 3 shows the dependence of the fluorescence decay parameters on three lipid:protein ratios for CP29. The influence of the protein−protein interactions is expected to increase at lower lipid to protein (L/P) ratios. Essentially, comparison of the fluorescence decay parameters in detergent with those at L/P equal to 9 determines the effect of endogenous lipids on the protein structure; conversely, at lower L/P ratios, the influence of protein−protein interactions is mainly highlighted. The transition from detergent to lipid environment induces a decrease in the lifetime values of the

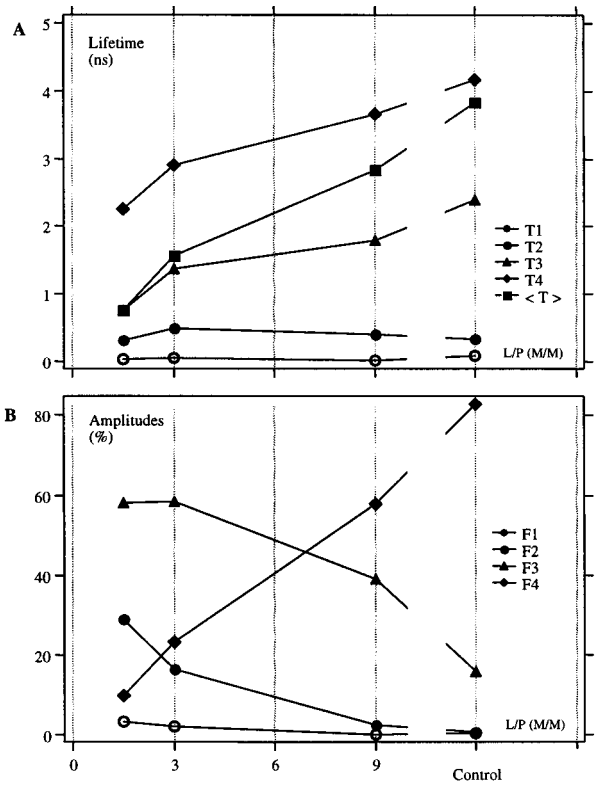

FIGURE 3: Dependence of the fluorescence lifetime parameters (A, lifetimes; and B, amplitudes) on the lipid-to-protein ratio for native CP29 at 20 °C.

individual decay components, as well as a much lower contribution of the longer lifetime component T3 with respect to T2. The contribution of the fastest component did not change with respect to the decay in detergent solution. An L/P ratio of 3 induces a further decrease in both the lifetime value and the relative amplitude of T3 accompanied by an increase of the T2 contribution. In this state, the complex also showed an increased relative contribution of T1, the fastest resolved component. At the lowest L/P ratio (1.5), the T3 contribution is further decreased, while T1 is increased. The T2 component did not show further changes of relative amplitude. Similar effects were observed in the case of LHCII CP26 and CP24 (data not shown).

Low-Temperature Analysis on Proteoliposomes. Further characterization of the decrease of fluorescence induced by the incorporation of Lhc proteins into liposomes was obtained by low-temperature analysis of lifetime fluorescence spectra. As an example, the time-resolved spectra of LHCII (L/P ratio of 3) at 77 K are shown in Figure 4. With respect to LHCII in detergent solution (Figure 1D), an increased heterogeneity of lifetime components was obtained. Four lifetimes are now resolved (Table 4) ranging from 0.32 to 5.3 ns. The lifetime values increase together with the peak wavelengths of their spectral components, distributed between 679 and 699 nm, showing that the low-energy forms act as sinks of the excitation energy. As a consequence, the average lifetime is strongly dependent on the emission wavelength.

Effect of Xanthophyll Composition on NPQ in Vivo. To evaluate whether the modified xanthophyll composition of Lhc proteins had any effect on the fluorescence quenching behavior of leaves, we performed in vivo measurements of NPQ on *WT* samples, whose xanthophyll composition changes upon exposure to strong actinic light due to the

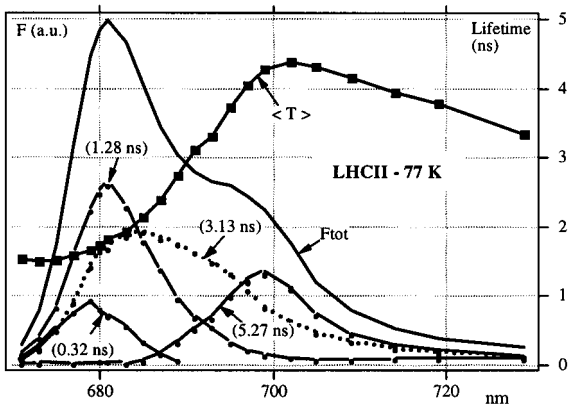

FIGURE 4: Analysis into time-resolved components of the fluorescence emission spectra from purified LHCII in liposomes at 77 K. The right-hand scales refer to the average lifetime shown by the solid squares.

Table 4: Characteristics of the Chlorophyll Fluorescence Lifetime Components at 77 K from LHCII Proteins Reconstituted into Liposomes with a Lipid/Protein Ratio Equal to 3 mol/mol[a]

	τ (ns)	λ_{peak} (nm)	A (%)
$T1$	0.3	679	13
$T2$	1.3	682	39
$T3$	3.1	685	28
$T4$	5.3	699	19

[a] Lifetimes (T) and wavelength peaks (λ_{peak}) associated with the components obtained from the spectral fitting are given together with their amplitudes (A).

activation of the violaxanthin de-epoxidase enzyme, which transforms violaxanthin to zeaxanthin (*45*), and on the two xanthophyll mutants *aba-3* (*34*) and *npq1* (*35*), which do not undergo de-epoxidation, being respectively blocked into either a full zeaxanthin or a full violaxanthin state. The NPQ kinetics in Figure 5 are remarkably different in the three genotypes. Following a rapid phase, developing within 20 s, *npq1* mutant rapidly reaches saturation with an NPQ value of about 1.0, while *aba-3* promptly responds with an NPQ value of ca. 2.0 and a half-time of 13 s. *WT Arabidopsis* develops a maximal NPQ value of 1.8 but with slower kinetics ($t_{1/2}$ = 24 s).

DISCUSSION

Fluorescence Lifetime Heterogeneity of Lhc Proteins in Detergent Solution. Lhc proteins in detergent solution show reduced heterogeneity since most of the Chl fluorescence is emitted with a slow-decaying component (3.7−4.6 ns). Nevertheless, alternative states of pigment−protein complexes were present in solution and characterized by shorter lifetimes, in the 1.4−2.8 ns range, depending on the individual Lhcb proteins. On considering the preexponential factors as representative of the molar fraction of the emitting species, different populations for short- and long-lived fluorescent systems were obtained. The major LHCII antenna showed the strongest preponderance of the long-living emitting form (3.8 ns), while in the cases of CP26 and CP24 the shorter lifetime (about 2.6 ns) component was more represented. CP29 exhibited an intermediate situation with 34% and 66% of total fluorophores emitting with lifetimes of 1.9 and 4.3 ns, respectively.

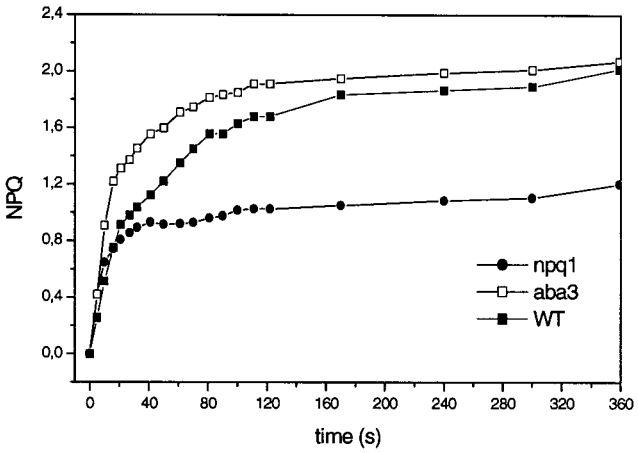

FIGURE 5: In vivo nonphotochemical quenching of *Arabidopsis thaliana WT*, *aba-3*, and *npq1* mutants. The kinetics were obtained by detecting the PSII fluorescence induced on leaves during exposure to intense actinic light (see text for details).

As for the origin of the two decay components, they may correspond either to different spectroscopic species of the same chlorophyll-protein complex or to different aggregation states. Aggregation may well generate short lifetime components (*41*, *42*, *46*), and yet it seems improbable here since the aggregation state of purified proteins, checked by sucrose gradient ultracentrifugation, was homogeneous both before and after the measurements. The presence of more than one component in the fluorescence decay of Lhc proteins is a well-known, although unexplained, observation which was previously attributed to the heterogeneity of the gene products or to the aggregation state in biochemical preparations from chloroplasts (*41*). These explanations are now made unlikely by the use of recombinant proteins—which are the product of a single gene, are monomeric in detergent solution, and have simple and reproducible pigment composition—and nevertheless maintain the lifetime heterogeneity described above (*10*, *23*). Two alternative hypotheses can be proposed to the presence of two fluorescence lifetimes in a preparation deriving from highly purified pigment−protein complexes with homogeneous aggregation state: (i) each pigment−protein molecule is endowed with two distinct fluorophores characterized by different τ_0; (ii) two alternative states of the protein are present in solution, each characterized by a different fluorescence lifetime.

To distinguish between the two cases, we must first have a closer look at the structure of Lhc proteins, and, for the sake of simplicity, we focus on CP29. This protein, binding eight Chl and two Car molecules per polypeptide, has been shown to exchange light excitation energy with transfer rates ranging from 30 fs^{-1} to 10 ps^{-1}, and it has been demonstrated to undergo complete Boltzmann's equilibration within less than 5 ps (*47*). Considering the difference by 3 orders of magnitude between this equilibration time and the fluorescence lifetime, the existence of two well-distinguishable lifetime emissions from two necessarily interconnected chromophores is impossible. Indeed, due to the extremely rapid energy interchange, both chromophores should decay with a unique lifetime. As a matter of fact, the elementary decay components of the emission spectra show the same peak values and bandwidths, implying that the energy levels associated with CP29 chlorophylls are also the same. Similar considerations can be drawn for the monomeric proteins

CP24 and CP26 (*30, 48*). The case of LHCII must be considered with caution due to its trimeric organization, implying slower intersubunit energy transfer processes. Nevertheless, in trimeric LHCII the intramonomeric energy transfer dynamic is known to dominate over intersubunit equilibration (*49*). Accordingly, the organization of the individual subunits is very similar to that of CP29 (*50, 51*), and the equilibration of Chl excitation is similarly accomplished in the few picoseconds time scale (*52, 53*).

The alternative explanation is that Lhcb proteins in solution may assume two different conformations each characterized by a distinct fluorescence lifetime. This hypothesis is consistent with the presence of well-resolved lifetimes in rapidly equilibrated multichromophore pigment−proteins.

Fluorescence Lifetime Distribution Can Be Modulated by Xanthophylls Bound to the L2 Site of Lhcb Proteins. In LHCII, Cars are bound to three sites, two of which (called L1 and L2) are localized in the central domain of the polypeptide formed by trans-membrane helices A and B (*54*), and the third site (N1) is localized nearby helix C (*55*). Neoxanthin can be exclusively bound into site N1 (*56, 57*), while lutein, violaxanthin, and zeaxanthin compete for sites L2 and L1 (the last one having the highest affinity to lutein). Thus, *WT* LHCII binds lutein in site L1 and neoxanthin in site N1, while site L2 is occupied by either lutein (80%) or violaxanthin (20%). LHCII from the *aba-3* mutant has the N1 site empty, the L1 site occupied by lutein, and the L2 site occupied by either zeaxanthin (60%) or lutein (40%). Biochemically, the major difference between LHCII from *WT* and *aba-3* mutant is the occupancy of site L2 by either violaxanthin or zeaxanthin. The consequence of this difference is a modification of the relative amplitude of the two fluorescence lifetimes and of the molar ratio of long- versus short-living chromophores. These results are consistent with the recent finding that recombinant CP26, reconstituted in vitro with zeaxanthin instead of violaxanthin, undergoes a reorganization of its fluorescence behavior by increasing the shorter (approximately 1.5 ns) emission lifetime, with respect to the long-living component (3.7 ns), and a decrease of the overall fluorescence yield of the complex (*23*). Moreover, thylakoid membranes undergoing ATP-induced NPQ show a redistribution of their fluorescence emission, thus decreasing the amplitude of long-living lifetimes in favor of short-living ones (*4, 17*).

Xanthophyll Composition of Lhc Proteins Affects NPQ in Vivo. Analysis of NPQ in *Arabidopsis WT*, and in two genotypes which incorporate either violaxanthin or zeaxanthin in their Lhc proteins, supports the hypothesis that a conformational change induced by the binding of zeaxanthin is a determinant of the NPQ mechanism. In fact, while the *npq1* mutant develops only the first rapid phase of quenching, it cannot reach full expression of NPQ. The *aba-3* mutant not only shows NPQ values even higher than *WT*, but also undergoes the transition from unquenched to quenched state much more rapidly. These results are consistent with the requirement of a zeaxanthin-dependent conformational change for the full expression of NPQ in vivo. In the *WT*, zeaxanthin is produced from violaxanthin upon high light exposure, and, consistently, its quenching kinetics are slower. In the *aba-3* mutant, this conformation is constitutively present; therefore, the NPQ can develop more promptly upon light exposure probably due to lumen acidification.

Temperature Independence of Fluorescence Lifetimes Supports the Hypothesis That Lhc Fluorescence Heterogeneity Is Due to the Existence of Two Protein Conformations. The previous hypothesis of two conformational states is indeed verified by the low-temperature analysis. The rationale of this experiment is that lifetime heterogeneity is expected to be strongly dependent on the temperature in the case of two interconnected chromophores (i.e., two chlorophyll emitters within the same pigment−protein complex), while no temperature dependence is expected if the two fluorophores do not exchange excitation energy (independent complexes). In the case of CP29, which is best suited for this analysis due to its monomeric nature, the experimental results clearly support the two-lifetime origin from independent chromophores; in fact, by comparing RT to cryogenic data, the relative ratio of the two main lifetime amplitudes and the molar ratios of the two components are not affected by temperature. The increased lifetime values at low temperature can be ascribed to the partial inhibition of the nonradiative decay channel of S1 Chl excited states, causing a longer ^{1}Chl* duration. Similar results were obtained in the case of CP26 and CP24 (data not shown). Analogous analysis of LHCII complex yields somehow different results, the relative weight of lifetime emission undergoing a change in favor of the shorter component. This difference with respect to the case of monomeric complexes might be due to changes in the protein−protein interactions within the trimer.

Lhc Proteins in Liposomes. The main effect of the reconstitution of the Lhcb proteins into liposomes is the appearance of Chl quenching. The highest lipid/protein ratio used in this investigation is much higher (about 10−100-fold) than what is found in the grana partition of thylakoid membrane, where a molar ratio of approximately 2.5 has been evaluated (*58*). Under these conditions, chlorophyll−protein complexes are likely to be in a rather homogeneous aggregation state surrounded by the lipid moiety. This situation is therefore similar to that in detergent, and the relatively small decrease of the slower lifetime is most probably due to an intramolecular quenching induced either by a conformational rearrangement or by direct interaction of the pigments with the lipid medium. Instead, the fluorescence intensity associated with the slowest component is more strongly affected (about 25% decrease) in going from detergent to the highest lipid/protein ratio (Figure 3B). Thus, the equilibrium between the two conformations of Lhc proteins is displaced toward the state with a short lifetime with respect to the situation in detergent solution. This might explain the results of fluorescence lifetime obtained from intact leaves (*16, 59*) or purified membranes (*43*), which never show the long-lifetime contribution typical of purified Lhc proteins although these are the major components of the plant photosystems.

At lower lipid/protein ratios, the protein−protein interactions within the lipid bilayer become more important as shown by the strong increase of the 0.3 ns component (T1), which was present in a trace amount both in detergent and at high lipid/protein ratios. This component is thus tentatively ascribed to Chl spectral forms due to new pigment−pigment or pigment−protein interactions. At intermediate lipid/protein ratios, between 3 and 9 (w/w), both the average lifetime and the main decay component observed in isolated antennae

reconstituted into liposomes are rather similar to those observed in the native membrane under closed reaction center conditions (*4, 33*). Although a coincidence cannot be excluded, this may reflect the obtaining of a state of pigment−proteins similar to that typical of interacting subunits forming supramolecular complexes in thylakoids (*60, 61*).

Low-temperature analysis of LHCII in lipid membrane shows that the protein−protein interactions produce low-energy spectral forms which are the best candidates of the overall quenching effect induced by incorporation of Lhc proteins into lipid membranes. In fact, these red forms are absent from the low-temperature spectra of Lhc proteins in detergent solution.

It has been proposed that reversible aggregation of Lhc proteins within the thylakoid membrane might be part of the mechanism of excitation energy dissipation (*1*). Nevertheless, no evidence for the aggregation of Lhc proteins within the thylakoid membranes during photoprotection has been so far reported. Our results show that quenching of Chl fluorescence can be produced in liposomes by rather weak protein−protein interactions, that can be dissolved by detergent treatment, and support the view that changes in protein−protein interactions might be part of the mechanism of the so-called non-photochemical quenching.

CONCLUSIONS

In this study we have shown that highly purified Lhc proteins, either monomeric or trimeric, exhibit a heterogeneity of Chl fluorescence which cannot be further reduced by purification, and which is maintained in recombinant Lhc proteins that are single gene products (*10, 23*). Low-temperature analysis confirms that distinct molecular conformations are responsible to the 1.2−1.5 and 3.6−4.0 ns fluorescent lifetimes. The relative amplitude of the two major lifetime components can be modulated by the xanthophyll species bound into site L2 through the conformational change induced to the protein. From these data, we conclude that Lhc proteins exist in two conformations, namely, a *conservative* (of excitation energy) conformation characterized by a 3.4−3.8 ns lifetime, and a *dissipative* conformation with a 1.0−1.4 ns lifetime. Such difference in fluorescence lifetime is probably due to variation in the Chl−Chl interactions within the complex elicited by a protein conformational change.

We conclude that the fluorescence yield of Lhc proteins can be modulated by at least two mechanisms: (i) transition between long-living and short-living molecular configurations mediated by the xanthophyll species bound to the L2 site of Lhc proteins (*23*); and (ii) modulation of protein−protein interactions which produce low-lying energy levels associated to red-most spectral forms. It is well-known that in thylakoid membranes violaxanthin is de-epoxidized to zeaxanthin during NPQ, and that both are incorporated into Lhc proteins (*6, 62*) and released into membrane lipids. Inhibition of violaxanthin de-epoxidation prevents the decrease of amplitude from the 1.8 ns lifetime emission and the increase of the 0.5 ns component (*4, 17*). Consistently, we found that xanthophyll biosynthesis mutants, endowed with a blocked xanthophyll cycle in either the violaxanthin or the zeaxanthin state, have NPQ amplitudes, respectively, limited to a reduced value of about 50% or increased ca. 20% with respect to the *WT*. We found that the *aba-3* mutant, which has the highest NPQ and fastest kinetics, actually shows the incorporation of zeaxanthin into Lhc proteins and a LHCII protein conformational equilibrium displaced toward short fluorescence lifetimes with respect to the *WT*. The two aspects of the fluorescence quenching reported, namely, intramolecular conformational change and establishment of protein−protein interactions, may represent successive steps of a unique mechanism of regulation of Chl excited states, i.e., of NPQ.

As for the physical mechanism of ^{1}Chl* quenching, this is still a matter of debate. It has been proposed that new Chl−Chl interactions, induced by conformational changes, may form low-lying energy states with enhanced dissipation through internal conversion (*62*). Carotenoids have also been involved in thermal de-excitation of ^{1}Chl* states. Early work suggested a direct quenching mechanism requiring a reversible de-epoxidation of Cars with a change in their S1 levels, thus leading to an energy transfer from Chl to zeaxanthin (*18*). However, violaxanthin and zeaxanthin have a very similar energy of their S1 states (*21, 22*). Still, the direct energy quenching cannot be excluded: if the rate of energy transfer from a Chl *a* Q_y transition to a zeaxanthin S1 state is slower than S1 internal conversion, then no S1 state will be populated to a spectroscopically detectable level. Alternatively, the catalyzed internal conversion (CIC) of ^{1}Chl*, namely, an indirect mechanism involving chlorophylls and carotenoids in van der Waals contact (not requiring excitation energy transfer from Chl to Car), has been reported to be active in aggregated LHCII (*63*) and could explain the present results.

ACKNOWLEDGMENT

We thank Antoine Tremolieres for help in the purification and analysis of thylakoid lipids, and Michel Recouvreur (Institute J. Monod, Paris) for technical assistance in electron microscopy. The *aba-3* and *npq1* mutants of *A. thaliana* were kindly provided by Jan Zeevaart (Plant Research Laboratory, Michigan State University) and Carlo Soave (Dipartimento di Biologia, Università di Milano).

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BI010342X