Thylakoids from Pea Seedlings Grown under Intermittent Light: Biochemical and Flash-Spectrophotometric Properties†

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Received March 3, 1992

ABSTRACT: Thylakoid membranes were isolated from pea seedlings grown under intermittent light (2-min light/118-min dark cycles). These preparations differed from controls (thylakoids from plants grown under 16-h light/8-h dark cycles) in the following respects: 15 times smaller chlorophyll/protein ratio, 10 times greater chlorophyll *a*/*b* ratio, absence of light-harvesting chlorophyll *a*/*b* binding proteins, and 2–3-fold greater ratio of photosystem II over photosystem I. In addition we found the following: (1) Electrogenic electron transfer around cytochrome b_6/f under flashing light was greatly enhanced, probably as a consequence of the greater photosystem II/photosystem I ratio. (2) The rate of proton uptake from the medium at the acceptor side of photosystem II was enhanced, probably by unshielding of the quinone binding domain. (3) The *N*,*N*′-dicyclohexylcarbodiimide sensitivity of the proton-pumping activity of photosystem II was absent, which was consistent with the attribution of a *N*,*N*′-dicyclohexylcarbodiimide-induced protonic short circuit to chlorophyll *a*/*b* binding proteins. (4) The sensitivity of oxygen evolution under continuous light to variations of pH or the concentration of Ca^{2+} was altered. Chlorophyll *a*/*b* binding proteins serve as light-harvesting antennas. We found in addition that they modulated the activity of water oxidation and, in particular, the protolytic reactions around photosystem II.

In higher plants, photosynthetic electron transport from water to NADP is driven by three membrane-spanning protein complexes: photosystem II (PS II),[1] cytochrome b_6/f (cyt b_6/f), and photosystem I (PS I). Photosystem II is a highly complex entity that oxidizes water, reduces quinone, and thereby pumps protons across the thylakoid membrane. It is composed of a chlorophyll *a* (Chl *a*) containing reaction center core which is surrounded by light-harvesting chlorophyll *a*/*b* binding proteins (CAB-proteins).

CAB-proteins are a large class of proteins with molecular masses of 20–30 kDa serving as antennas to either photosystem [for reviews, see Peter and Thornber (1988), Green et al. (1991), and Kühlbrandt and Wang (1991)]. Chlorophyll *b* deficient mutants, which totally lack CAB-proteins, are capable of oxygenic photosynthesis (Ouija et al., 1988; Shen et al., 1988). Similarly, photosystem II complexes isolated from normal thylakoid membranes, but depleted of CAB-proteins by detergent treatment, evolve oxygen at high rates (Ghanotakis et al., 1987; Enami et al., 1989).

Is the antenna function then the only role of CAB-proteins? We have obtained circumstantial evidence for an involvement of certain proteins of this family in the channeling of protons from the catalytic center of water oxidation into the thylakoid lumen. The proton-pumping activity of PS II (i.e., proton release into the lumen during water oxidation and proton uptake from the stroma at quinone reduction) is short-circuited after treatment of stacked thylakoid membranes with *N*,*N*′-dicyclohexylcarbodiimide (DCCD) (Jahns et al., 1988; Jahns & Junge, 1989). Concomitantly, CAB-proteins are covalently labeled with DCCD at the lumen side of the membrane (Jahns & Junge, 1990). The involvement of *peripheral* proteins of PS II in proton channeling into the lumen is also suggested by variations in the pattern of proton release during water oxidation when thylakoids (Lavergne & Rappaport, 1990; Jahns et al., 1991), photosystem II enriched membrane fragments (Wacker et al., 1990; Rappaport & Lavergne, 1991), and photosystem II reaction center preparations (Lübbers & Junge, 1990) are compared.

One way to affect the CAB-protein content of thylakoid membranes is to grow plants under a regime of intermittent light (IML) (Akoyunoglou et al., 1966; Akoyunoglou & Argyroudi-Akoyunoglou, 1969), e.g., by a cycle with 2 min of light and 118 min of darkness. Thylakoids isolated from such plants, IML-thylakoids, differ greatly from those, named continuous light thylakoids (CL-thylakoids), isolated from plants grown under the standard long-day cycle (16-h light/8-h dark). Due to drastically diminished formation of Chl *b*, IML-thylakoids are characterized by a high Chl *a* to Chl *b* ratio (Akoyunoglou et al., 1966; Argyroudi-Akoyunoglou & Akoyunoglou, 1970; Tzinas et al., 1987), reduced amounts of CAB-proteins (Argyroudi-Akoyunoglou & Akoyunoglou, 1979; Day et al., 1984; White & Green, 1988), small antenna sizes in both photosystems (Tzinas et al., 1987; Armond et al., 1976; Dubertret & Lefort-Tran, 1981), the absence of membrane stacking and grana formation (Day et al., 1984; Armond et al., 1976; Dubertret & Lefort-Tran, 1981), and a higher PS II/PS I ratio (Tzinas et al., 1987). Both photosystems are active under continuous light (Tzinas et al., 1987; Armond et al., 1976; Ryrie & Young, 1990). IML-thylakoids seem to be ideal material to study the possible role of CAB-proteins in proton and electron transport.

We characterized a thylakoid membrane preparation from pea chloroplasts grown under intermittent light. Apart from corroborating and quantifying the above-cited properties of IML-thylakoids, we found alterations of the protolytic

† This work was financially supported by the Deutsche Forschungsgemeinschaft (Sonderforschungsbereich 171, Teilprojekt A2).

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[1] Abbreviations: PS II, I, photosystem II, I; cyt b_6/f, cytochrome b_6/f; Chl, chlorophyll; CAB, chlorophyll *a*/*b* binding; DCCD, *N*,*N*′-dicyclohexylcarbodiimide; IML, intermittent light; CL, continuous light; EDTA, ethylenediaminetetraacetic acid; BSA, bovine serum albumin; DNP-INT, dinitrophenyl ether of iodonitrothymol; Fecy, hexacyanoferrate III; MV, methyl viologen; NR, neutral red; PR, phenol red.

0006-2960/92/0431-7390$03.00/0

reactions of photosystem II and different Ca^{2+} and pH sensitivities of water oxidation.

MATERIALS AND METHODS

Pea seedlings (*Pisum sativum*, var. Kleine Rheinländerin) were grown either under intermittent illumination (2000 lux, 2-min light/118-min dark, variable, typically 10 days) or under continuous illumination (10 000 lux, 16-h light/8-h dark, 12–14 days).

Unstacked thylakoid membranes were prepared from seedlings of both growing conditions according to procedures described previously (Polle & Junge, 1986). Membranes from continuously illuminated plants were treated with EDTA for complete unstacking (Polle & Junge, 1986) and used as controls. Membranes from plants grown under intermittent light were intrinsically unstacked. The resulting pellets were resuspended in a medium containing 0.4 M sucrose, 10 mM NaCl, and 2 mM Tricine/NaOH, pH 7.8, to yield final chlorophyll concentrations between 1.7 and 2 mM for control CL-thylakoids and between 0.2 and 0.3 mM for IML-thylakoids. Stock suspensions were stored at −80 °C for up to 2 months. PS II membrane fragments were prepared according to Berthold et al. (1981), and oxygen-evolving reaction centers as described by Ghanotakis and Yocum (1986) with the modifications from Lübbers and Junge (1990).

For flash-spectrophotometric measurements [for methodological details see Junge (1982)], thylakoids equivalent to a final chlorophyll concentration of 10 μM for CL-thylakoids and 5 μM for IML-thylakoids were suspended in 10 mM NaCl. Electrochromic absorption changes (at 522 nm) (Junge & Witt, 1968) and absorption changes of P700 (at 819 nm) (Haehnel, 1984) were measured in the presence of 2 mM Tricine/NaOH, pH 7.8. pH transients in the thylakoid lumen were determined by absorption changes of neutral red (at 548 nm) (Junge et al., 1979) in the presence of 2.6 mg/mL BSA at pH 7.0–7.1, and pH transients in the medium by absorption changes of phenol red (at 559 nm) (Polle & Junge, 1986) without buffer at pH 7.0–7.1. As electron acceptor, either 20 μM methyl viologen (MV) or 2 mM hexacyanoferrate III (Fecy) was used, as indicated in the figure legends.

The rate of oxygen evolution under continuous illumination was determined by a Clark-type electrode with thylakoids suspended in 10 mM NaCl, 20 mM Tricine/NaOH, pH 7.8, 2 mM Fecy. The chlorophyll concentration was 30 μM for CL-thylakoids or 5 μM for IML-thylakoids. Other additions are indicated in the figure legends. For uncoupling, 1 μM of nigericin was added to each sample.

Chlorophyll concentrations and Chl *a*/Chl *b* ratios were calculated from absorption spectra of extracts in 80% acetone/water according to Porra et al. (1989). Protein content was determined by using the BCA protein assay reagent (Pierce).

The polypeptide composition was analyzed by SDS–PAGE according to Laemmli (1970) with the modifications described by Engelbrecht et al. (1986) using 5% acrylamide for the stacking gel and 13.5% acrylamide for the separating gels, with the separating gels containing 4 M urea. Protein bands were stained with coomassie brilliant blue.

RESULTS AND DISCUSSION

A. Biochemical Properties. Reduced chlorophyll synthesis and higher chlorophyll *a*/*b* ratios are characteristic for plants grown under intermittent light. The light-harvesting CAB-proteins of both photosystems are nearly absent in IML-thylakoids. As shown by Argyroudi-Akoyunoglou, Akoyonuglou, and co-workers (Akoyunoglou et al., 1966; Argyroudi-Akoyunoglou & Akoyunoglou, 1970; Tzinas et al., 1987), the Chl *a*/*b* ratio depends on the age of the plants and on the illumination conditions (light intensity, the number of light/dark cycles, and the duration of the dark intervals). We obtained IML-thylakoids with highly active photosystems by growing pea seedlings under 2-min light/118-min dark cycles. In contrast to protocols of the above authors we chose pea seedlings (instead of bean plants) and exposed them to intermittent light directly after sowing, without prior etiolation. Chlorophyll *a*/*b* ratios, rate of oxygen evolution, and polypeptide composition of 9–15-day-old pea seedlings grown under intermittent light were as follows:

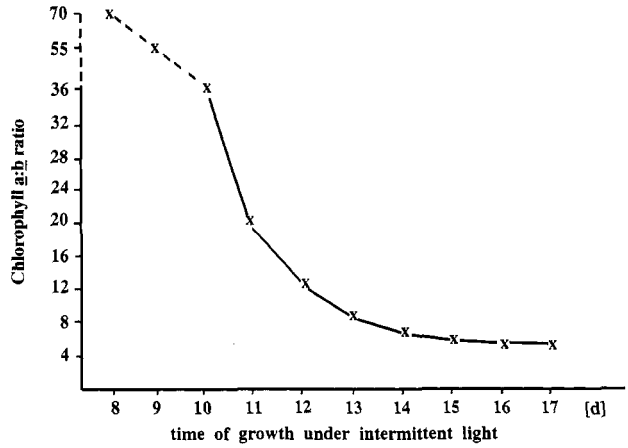

FIGURE 1: Chlorophyll *a*/*b* ratio from 8–17-day-old pea seedlings as a function of time after sowing. Growth under intermittent light as described in Materials and Methods. About 1 g of leaves was ground in the dark. The slurry was solved in 5 mL of 80% acetone/water. Chl *a*/*b* ratios of these solutions were determined photometrically according to Porra et al. (1989).

Chlorophyll a/b Ratio. With chlorophyll *a*/*b* ratios between >40 for 9–10-day-old seedlings and 6 for 13–15-day-old seedlings (see Figure 1), we obtained values similar to those measured for bean plants (Akoyunoglou et al., 1966; Argyroudi-Akoyunoglou & Akoyunoglou, 1970; Tzinas et al., 1987). After 9–10 days (i.e., about 120 light/dark cycles), we found the same Chl *a*/*b* ratio as described by Tzinas et al. (1987) for 10-day-old bean plants grown for 6 days in darkness followed by 4 days of illumination with 2-min light/98-min dark cycles (i.e., 56 light/dark cycles). This implied that the chlorophyll *a*/*b* ratio depended on the age of plants rather than on the number of light/dark cycles.

Rate of Oxygen Evolution. The specific rate of oxygen evolution was used as a control parameter for the activity of thylakoids isolated from this material. Highest rates (600–900 μmol of O_2/mg of Chl per h, see Table I) were obtained with 10-day-old plants. These rates were the same as those from isolated oxygen-evolving PS II reaction centers from control thylakoids [Table I; see also, Ghanotakis et al. (1987)]. IML-thylakoids revealed a similar Ca^{2+} requirement and the same pH optimum for the oxygen-evolving capacity under continuous illumination as isolated PS II reaction centers. Highest rates of 900 μmol of O_2/mg of Chl per h were obtained with 10 mM $CaCl_2$, compared to about 500 μmol of O_2/mg of Chl per h without any additional salt. Without Ca^{2+}, but with 10 mM Mg^{2+} or 100 mM Na^+, maximal rates dropped to only 600–650 μmol of O_2/mg of Chl per h. In contrast to the former, control thylakoids showed very little effect of any additional salt on the rate of oxygen evolution.

The pH dependence of the rate of oxygen evolution is shown in Figure 2. IML-thylakoids (circles) revealed an optimum around pH 6.5 and a sharp decrease at pH 8, very similar to

Table I: Biochemical Parameters of IML-Thylakoids, CL-Thylakoids, PS II Membrane Fragments (BBY-Particles), and PS II Reaction Center Core[a]

	μg of Chl/g of fresh weight	Chl *a*/ Chl *b*	protein/ Chl (g/g)	PS II/ PS I	μmol of O_2/mg of Chl per h
IML-thylakoids	350–400	30–40	45–60	2.5:1	600–900
CL-thylakoids	3000	4	3.5	1:1	135
BBY-particles		3	2.5	∞	300
rxn center core complexes		5.5	4.5	∞	800

[a] The PS II/PS I ratios were estimated from flash-spectrophotometric measurements (see Figure 5 and text). All other data were determined as described in Material and Methods and in Figure 1. For IML-thylakoids, the lower and upper limits are given for eight different preparations of 10-day-old seedlings. For the other preparations, a representative value is given. The data obtained with peas are in agreement with the literature on other species [see, for example, Murphy (1986), Tzinas et al. (1987), and Melis (1991)].

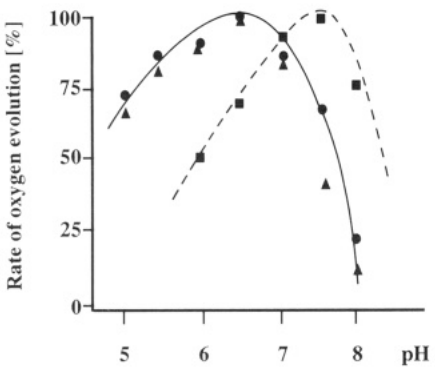

FIGURE 2: pH dependence of the rate of oxygen evolution under continuous illumination: IML-thylakoids (circles), PS II reaction centers (triangles), and control thylakoids (squares). The rates are given in percent of the respective highest value. For absolute rates see Table I. Assay media: 5 μM Chl, 10 mM $CaCl_2$ for IML-thylakoids and PS II reaction centers and 10 μM Chl, 10 mM NaCl for control thylakoids; 2 mM Fecy served as electron acceptor for both thylakoid types, 600 μM Fecy/150 μM 2,5-dichloro-*p*-benzoquinone for PS II reaction centers; 1 μM nigericin and 50 mM buffer were present at each sample; Tricine/NaOH was used for the range from pH 7.5 to 8.0, MOPS/NaOH from pH 6.5 to 7.5, and MES/NaOH from pH 5.0 to 6.5.

reaction centers (triangles). In control thylakoids (squares), however, the pH optimum was shifted to about 7.5. Was this difference caused by different surface potentials at the lumen side of IML- and CL-thylakoids (EDTA-treated!)? This was addressed by measuring the apparent p*K* of the membrane-adsorbed indicator dye neutral red (NR) [for details on the effect of surface potential on surface-adsorbed NR see Hong and Junge (1983)]. We found very similar apparent p*K*'s, i.e., similar surface potential, in both types of preparations.

An additional function of the CAB-proteins as concentrator of Ca^{2+} and for sustaining the optimal pH around the catalytic center was conceivable from these results. A function of several CAB-proteins in Ca^{2+} binding has been previously suggested by other authors (Shen et al., 1988; Webber & Gray, 1989; Irrgang et al., 1991). Webber and Gray (1989) speculated that the Ca^{2+} binding sites in two CAB-proteins with molecular masses of 26 and 24 kDa might be identical with DCCD-binding sites in these proteins. We have identified the 26- and 24-kDa Ca^{2+}-binding proteins as the DCCD-binding CAB-proteins of PS II (Jahns, Steinemann, and Junge, unpublished). The DCCD-binding sites (and thus probably the Ca^{2+}-binding sites) are localized at the lumenal side of the membrane within a segment between the first and second transmembrane helix of two CAB-proteins with molecular

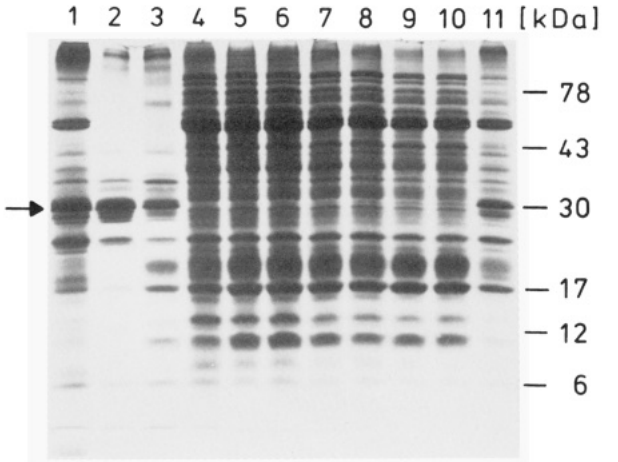

FIGURE 3: Coomassie-stained SDS–polyacrylamide gel showing the polypeptide pattern from the following: CL-thylakoids equivalent to 30 μg of Chl (lane 1), PS II membrane fragments (15 μg of Chl, lane 2), PS II reaction center core preparations (10 μg of Chl, lane 3), 9–15-day-old IML-thylakoids (3 μg of Chl, lanes 4–10), and 14-day-old IML-thylakoids, subsequently grown for 16 h under continuous illumination (8 μg of Chl, lane 11). Molecular masses are given on the right. The arrow indicates the position of the major LHC II protein (apparent molecular mass 27 kDa).

masses of 25 and 27 kDa (Jahns & Junge, 1990). Whether or not Ca^{2+} binding to CAB-proteins is responsible for the Ca^{2+} insensitivity of the oxygen-evolving capacity of normal thylakoids has to be further investigated.

Polypeptide Composition. Figure 3 shows a coomassie-stained acrylamide gel comparing the polypeptide composition of IML-thylakoids (from 9–15-day-old plants, lanes 4–10), CL-thylakoids (lane 1), PS II membrane fragments (lane 2), and PS II reaction center core preparations (lane 3). IML-thylakoids were strongly enriched in PS II reaction center core proteins; note the similar polypeptide patterns between lane 3 and lanes 4–10 with molecular masses lower than 30 kDa. On the other hand, IML-thylakoids showed drastically reduced amounts of all CAB-proteins. The major LHC II protein with an apparent molecular mass of about 27 kDa is indicated by an arrow on the left. After 13–15 days of exposure to intermittent light the Chl *b* content of IML-thylakoids rose (compare Figure 1). However, this was not paralleled by an increased level of the 27-kDa CAB-protein (see lanes 8–10 in Figure 3). Only after exposure of IML-thylakoids to 16 h of continuous light (lane 11) did increasing amounts of the major LHC II polypeptide appear. It was conceivable that the newly formed Chl *b* in 13-day-old plants was transiently adsorbed to the lipid moiety of the membrane. Alternatively, it could have been bound to other CAB-proteins which were already present. The presence of chlorophylls, which are "disconnected" from reaction centers, is held responsible for the dark level of chlorophyll fluorescence [the F_0 component; see, for example, Krause and Weis (1991)]. Indeed, preliminary experiments showed greatly enhanced F_0 levels in IML-thylakoids (Hecks and Trissl, personal communication). The problem of disconnected chlorophyll in these preparations should be kept in mind for all quantifications, which are based on chlorophyll content (e.g., rates of oxygen evolution or the amplitudes of absorption changes in the following flashphotometric experiments).

Table I summarizes our data on Chl content, Chl *a*/*b* ratios, protein conent, and the PS II/PS I ratios. IML-thylakoids (from 10-day-old pea seedlings) were compared with CL-thylakoids and PS II membrane preparations. The PS II/PS I ratio was estimated from flash-spectrophotometric mea-

surements to be 2.5:1 for IML-thylakoids (see also Figure 7). Tzinas et al. (1987) also found a PS II/PS I ratio of about 2–2.5:1 for 10-day-old IML-thylakoids from bean plants. The antenna size of one reaction center in IML-thylakoids can be estimated to be about 50 molecules of chlorophyll, based on the assumption that only Chl *a* binding proteins are present (D1/D2, CP 47, and CP 43 in PS II, subunits Ia/Ib in PS I). This is at variance with control thylakoids (CL-thylakoids) with about 250 molecules of chlorophyll for each photosynthetic reaction center [see, for example, Murphy (1986)]. When comparing specific, that is, chlorophyll-related, activities between IML- and CL-thylakoids, one expects 7-fold higher values for PS II and 3-fold higher values for PS I. In the presence of uncouplers and under saturating continuous light, we found 5–6-fold greater rates of uncoupled oxygen evolution in IML-thylakoids than in controls. If one takes the presence of disconnected chlorophyll into account, this is consistent with our estimates. Consequently, for further spectrophotometrical measurements, we expected about 5–6 times larger PS II dependent signals and 2–5 times larger PS I dependent signals than in controls (based on the same chlorophyll content).

According to Table I, the protein/Chl ratio in IML-thylakoids was about 15–20 times greater than in controls. This was reflected by greater turbidity of IML-thylakoid suspensions, which reduced the signal-to-noise ratio in spectrophotometric experiments and increased the buffering capacity for protons.

B. Flash-Spectrophotometrical Properties. For flash-spectrophotometric experiments (Figures 4–8) we used IML-thylakoids isolated from 10-day-old pea seedlings. The chlorophyll concentration for all experiments with IML-thylakoids was reduced to 5 μM (instead of 10 μM for control CL-thylakoids), in order to guarantee saturating flash excitation and to compensate for the greater turbidity.

Transmembrane Electric Potential. Figure 4 shows electrochromic absorption changes of IML-thylakoids at 522 nm which were induced by a short (5 μs) and saturating flash of light. The extent of these absorption changes is proportional to the transmembrane electric potential difference (Junge & Witt, 1968). It reflects the primary charge separation in both photosystems. The rate of the decay is proportional to the ionic current across the thylakoid membrane. The initial fast rise of the electrochromic absorption changes was even less in IML-thylakoids (bottom) than in controls (top). Only seemingly was this in contradiction to the expected 5–6- and 2–2.5-fold greater specific activity of PS II and PS I, respectively. It was probably caused by the lower specific Chl *b* content in IML-thylakoids, since the major electrochromic peak around 520 nm is due to carotenoids which are complexed to Chl *b* (Sewe & Reich, 1977). Chl *b* deficient pea mutants (Sewe & Reich, 1977) and tobacco mutants with low Chl content (Renger & Schmid, 1977) also reveal greatly reduced extents of electrochromic absorption changes.

When hexacyanoferrate III (Fecy) was used as electron acceptor, both the rapid rise and the slow decay of the transmembrane electric potential difference were similar in CL-thylakoids and IML-thylakoids (see Figure 4, lower curves in each pair). This indicated similar conductive properties of these membranes. With methyl viologen (MV) as electron acceptor, however, a pronounced slow rise (half-rise time about 5 ms) appeared in IML-thylakoids (Figure 4, below). It was absent in controls (Figure 4, top). A slow rise has also been reported for thylakoids from plants grown under continuous light (Joliot & Delosme, 1974; Jones & Whitmarsh, 1985).

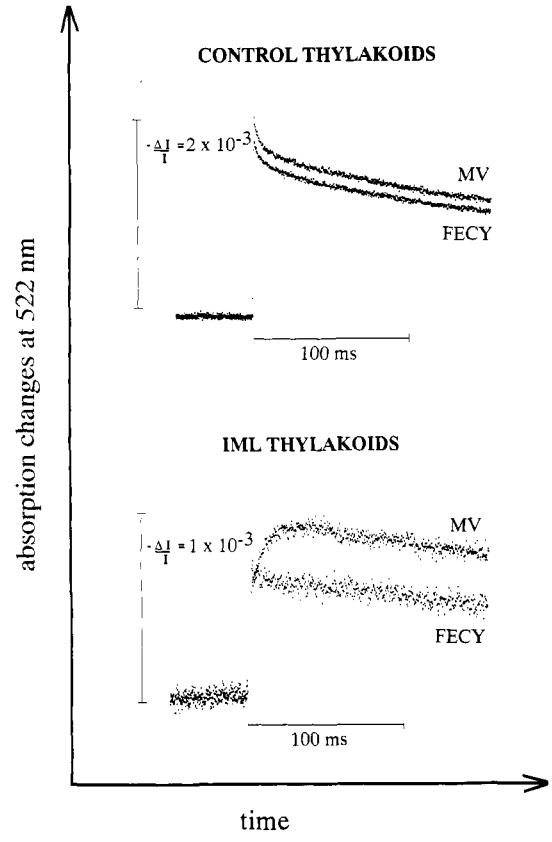

FIGURE 4: Transient electrochromic absorption changes at 522 nm. Excitation with a short flash of light. Twenty repetitive signals (0.1 Hz) were averaged. (Top) Control thylakoids (10 μM Chl); (bottom) IML-thylakoids (5 μM Chl). The respective upper traces in each pair were recorded in the presence of methyl viologen (20 μM, MV), the lower traces in the presence of hexacyanoferrate III (2 mM, Fecy) as artificial electron acceptor. Other conditions as given in Materials and Methods.

It requires an intermediate redox poise and is attributed to the slow electrogenic reaction in the cyt b_6/f complex. In EDTA-treated control thylakoids, this component was absent when MV served as terminal electron acceptor. Why did it appear in IML-thylakoids? As discussed above, there is a surplus of PS II over PS I. With MV as electron acceptor, but not with Fecy, a negative redox poise is generated by repetitive flashes and without chemically imposed redox poising.

Based on these results, the observed electrochromic absorption changes in IML-thylakoids conformed with the expectation for Chl *b* depleted thylakoid membranes. Additionally, IML-thylakoids seem to be an excellent system to investigate the cyclic cyt b_6/f activity in green plants.

Proton Uptake from the Medium. Figure 5 shows transient absorption changes of the hydrophilic dye phenol red (PR) which are indicative of pH transients in the suspending medium. The upward-directed transient reflects the alkalinization caused by proton uptake at the stroma side of the thylakoid membrane. Hexacyanoferrate III as electron acceptor left only proton uptake by bound quinones in PS II active (Polle & Junge, 1986; Junge & Ausländer, 1973). IML-thylakoids (Figure 5, bottom) differed from controls (top) in two respects: (1) The extent of the alkalinization was greatly diminished, and (2) the half-rise time was about 1 ms instead of 5 ms.

The first property was attributable to the much greater buffering capacity of IML-thylakoids. It was 180 μM per pH unit (at 5 μM chlorophyll) as compared to 30 μM per pH

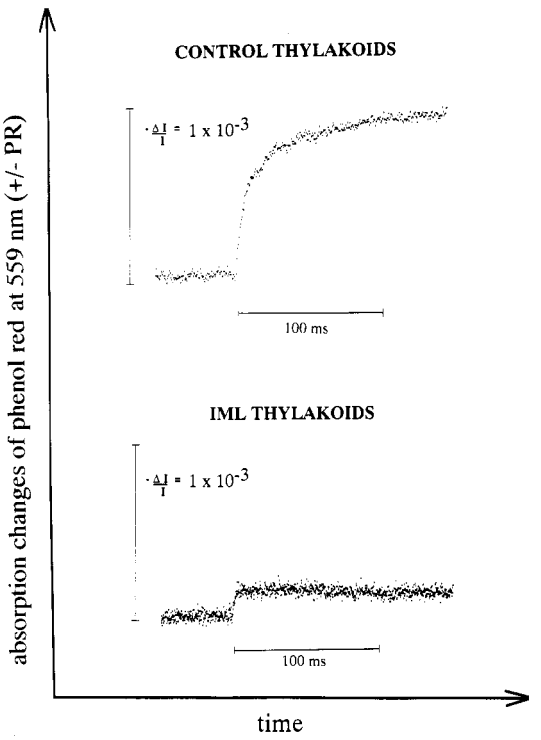

FIGURE 5: Transient absorption of phenol red (15 μM). Upward-directed signal indicates alkalinization of the suspending medium. Twenty signals were averaged. (Top) Control CL-thylakoids (10 μM Chl); (bottom) IML-thylakoids (5 μM Chl). With hexacyanoferrate III (2 mM, Fecy) as electron acceptor, only proton uptake at the reducing side of PS II was apparent. The drastically reduced extent for IML-thylakoids was mainly caused by the increased buffering capacity in comparison to controls (see text).

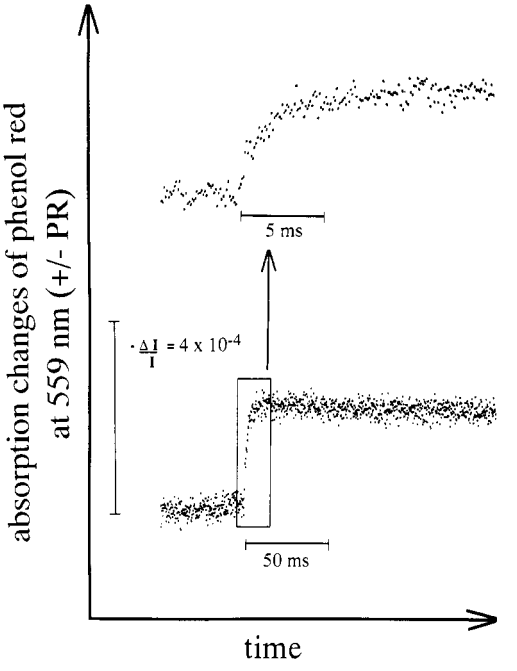

FIGURE 6: Transient absorption of phenol red (15 μM) indicating proton consumption at the reducing side of PS II. IML-thylakoids at pH 7.0; same conditions as in Figure 5. The signal-to-noise ratio was improved by averaging over 70 signals. The upper trace shows a cutout of the lower full trace as indicated by a rectangle in the figure (note the 10-times-expanded time scale in the upper part).

unit (at 10 μM chlorophyll) in controls. In relation to the same chlorophyll concentration, e.g., 10 μM, the buffering capacity was 12 times larger in IML-thylakoids. Taking the specific proportion of PS II into account, which was about 5–6-fold higher in IML-thylakoids, the expected reduction of alkalinization was a factor of about $^6/_{12}$ or 0.5. This was about 2 times larger than the observed one of 0.2–0.3 (compare Figure 5). The additional reduction was most probably caused by a subset of PS II reaction centers, which were directly accessible to Fecy as electron acceptor and therefore not protonated at the Q_B site. This subset may be identical with an enhanced proportion of PS II_β centers in IML-thylakoids, as has been reported by Melis and co-workers for developing and Chl *b* deficient chloroplasts [see, for review, Melis (1991)].

The half-rise time of alkalinization was much shorter (about 1 versus 5 ms) than in controls and also shorter than previously reported for unstacked thylakoids [2.7 ms, Polle and Junge (1986)]. A time-resolved measurement of the proton consumption at the PS II acceptor side under repetitive excitation and at pH 7.0 is shown in Figure 6. Analysis of the data revealed a monophasic increase of the curve with a half-rise time of 1.1 ms. It was shorter at more alkaline pH (about 600 μs at pH 8.0, not shown). These half-rise times are close to the time for the electron transfer from Q^-_A to Q^-_B [about 300–500 μs, (Crofts & Wraight, 1983; Andreasson & Vänngard, 1988)]. It is conceivable that CAB-proteins account for the slowing down of proton uptake from the medium at the reducing side of PS II (from about 1 ms to 2.7 ms or 5 ms).

Proton Release into the Thylakoid Lumen. Absorption changes of the amphiphillic indicator dye neutral red (NR) are shown in Figure 6. In the presence of high concentrations of bovine serum albumin (BSA) and with totally unstacked thylakoids, NR indicates exclusively pH transients at the lumenal surface of thylakoid membranes (Junge et al., 1979; Hong & Junge, 1983; Jahns et al., 1991). Absorption changes in IML-thylakoids (left side) and in controls (right side) are compared in Figure 7. Each pair of traces in the upper row shows pH transients in the presence of Fecy as electron acceptor with and without addition of DNP-INT. The traces in the lower row are the respective differences between the upper pairs. Upward-directed transients represent acidification of the lumen. Without DNP-INT (Figure 7, upper traces) both sites of proton release ino the lumen, water oxidation (fast) and plastoquinol oxidation (slow), were active. DNP-INT blocks the oxidation of plastoquinol by cyt b_6/f (Trebst et al., 1978). This leaves only proton release from water oxidation active [see, for example, Förster and Junge (1985)]. Therefore the smaller traces in the upper row (+DNP-INT) represent proton release by water oxidation only and the traces in the lower row (+/–DNP-INT) proton release by plastoquinol oxidation, which is powered by PS I. Thus, these traces allow an estimate of the PS II/PS I ratio in these two types of thylakoids. It was about 1:1 in controls (Figure 7, right) and about 2–2.5:1 in IML-thylakoids (Figure 7, left) in accordance with other results (see Table I and legend). In IML-thylakoids, the transient attributable to proton release by water oxidation was about 5-fold greater than in CL-thylakoids (when related to equal chlorophyll concentrations). This corresponds to the enrichment of the PS II/Chl proportion by a factor of about 5–6. IML-thylakoids are a favorable object for studies not only of the cyt b_6/f complex but also of PS II and water oxidation, especially with respect to a possible role for CAB-proteins in proton channeling into the lumen. The greater turbidity remains a problem.

We also investigated the stoichiometry of proton release during the four-step oxidation of water in dark-adapted IML-thylakoids. We found that the period-of-four pattern of proton release was absent in IML-thylakoids. These results are

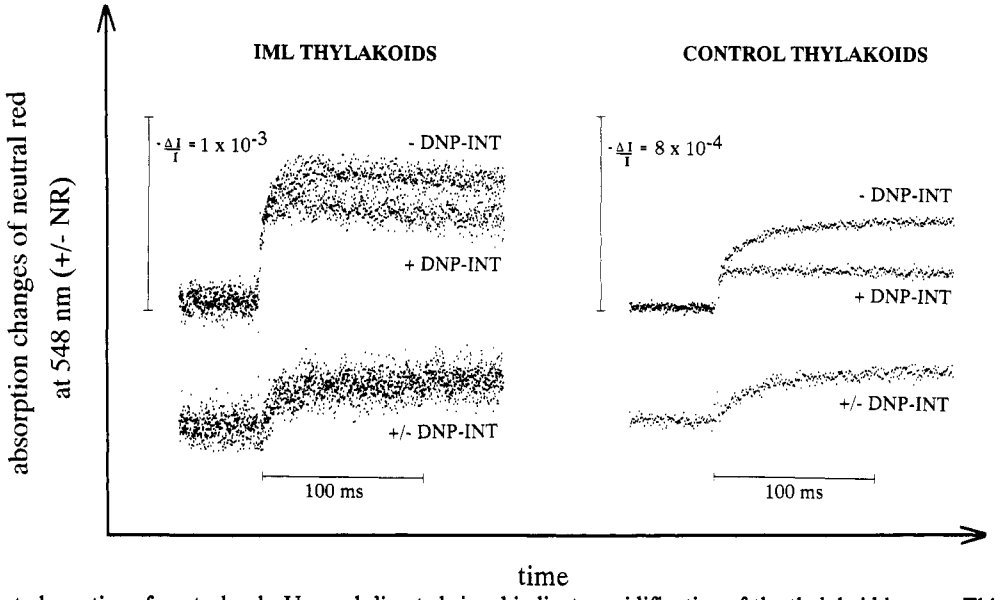

FIGURE 7: Transient absorption of neutral red. Upward-directed signal indicates acidification of the thylakoid lumen. Thirty samples were averaged under repetitive excitation. pH transients in the medium were selectively buffered by added bovine serum albumin (BSA, 2.6 mg/mL). For IML-thylakoids (left side, 5 μM Chl), 26 μM NR was used instead of 13 μM NR for controls (right side, 10 μM Chl). The upper part shows pH transients in the absence (–DNP-INT) and in the presence of 20 μM dinitrophenyl ether to iodonitrothymol (+DNP-INT). The lower part shows the respective differences (+/–DNP-INT) of the upper traces. The transients in the presence of DNP-INT indicate proton release from water oxidation (dependent on PS II activity), the differences (+/–DNP-INT) proton release from plastoquinol oxidation (dependent on PS I activity). From these traces we estimated the ratio of active PS II/PS I in both preparations.

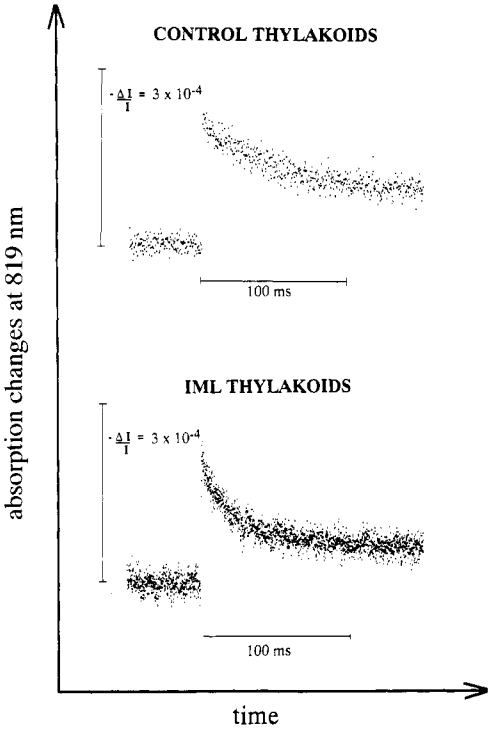

FIGURE 8: Transient absorption at 819 nm. Upward-directed signal indicates the oxidation of P700, the primary electron donor in PS I. (Top) Control CL-thylakoids (10 μM Chl); (bottom) IML-thylakoids (5 μM Chl). Hexacyanoferrate III (Fecy, 2 mM) served as electron acceptor.

presented in an accompanying article in this issue (Jahns & Junge, 1992).

Photosystem I Activity. Figure 8 shows absorption changes at 819 nm. They indicate the oxidation (rapid rise) and rereduction (slow decay) of P700, the primary electron donor of PS I. The oxidoreduction of P680 (PS II) was too fast to be resolved in these experiments (Schlodder et al., 1985). As expected, we found the specific extent of the absorption changes in IML-thylakoids to be about 2 times larger than in controls, again in accordance with other data (see Table I and Figure 7). Additionally, the reduction rate was faster in IML-thylakoids and biphasic. The dominating faster phase had a half-decay time of about 5 ms and a slower one of about 100 ms. The 5-ms phase was also apparent in the absorption changes of NR, indicative of proton release from plastoquinol oxidation (see Figure 7 for IML-thylakoids, left side, +/–DNP-INT). Electron transfer between PS II and PS I via the cyt b_6/f complex was faster in IML-thylakoids than in controls (5 ms compared to about 20 ms). This is at least partially understood by a greater PS II/PS I ratio (about 2.5:1) in IML-thylakoids, which produces a greater concentration of plastoquinol.

Action of DCCD on IML-Thylakoids. In previous studies (Jahns et al., 1988; Jahns & Junge, 1989) we have shown that incubation with DCCD short-circuits the proton-pumping activity of PS II by allowing a rapid transfer of protons from water oxidation to the site of quinone reduction. Interestingly, these effects occurred only in the presence of $MgCl_2$. Whether this $MgCl_2$ dependence was a direct salt effect (on the availability of the DCCD-binding sites) or a salt-mediated effect of thylakoid stacking could not be discriminated. Binding studies with [^{14}C]DCCD (Jahns & Junge, 1990) revealed CAB-proteins to be recipients of covalent modifications by DCCD. This suggested a role for these proteins in proton release from water oxidation into the lumen. Strictly speaking, however, a DCCD-induced intraprotein cross-link, perhaps in one of the reaction center proteins, could not be excluded as a cause for the observed short circuit.

With IML-thylakoid preparations, which lack most of the Chl a/b binding proteins, this question was addressed again. IML-thylakoids were incubated with DCCD under the same conditions as before (Jahns et al., 1988). The effects on the membrane potential and on pH transients were recorded. With IML-thylakoids, we found no influence of DCCD on proton release into the lumen, on proton uptake from the medium, or on the transient electric potential (not shown). Thus, there

was no indication for any short-circuiting by DCCD of the proton-pumping activity of PS II in IML-thylakoids.

This argues strongly against a Mg^{2+}-dependent intraprotein cross-link, say in the D1/D2 core of PS II, as the origin of the DCCD-induced protonic short circuit. It is, however, clearly compatible with our previous finding that CAB-proteins are susceptible to modification by DCCD and thus responsible for the short circuit.

CONCLUSIONS

We described biochemical and flash-spectrophotometric properties of thylakoid membranes which were isolated from pea seedlings grown under intermittent light. IML-thylakoids from other plant species have been biochemically characterized by other authors (see introduction). For IML-thylakoids from pea plants we found similar properties, namely, the low chlorophyll content, a high Chl *a*/*b* ratio, very much reduced amounts of CAB-proteins, and high specific rates of oxygen evolution (600–900 μmol of O_2/mg of Chl per h). The pH and Ca^{2+} dependencies of the rate of oxygen evolution of IML-thylakoids were similar to that in oxygen-evolving PS II reaction centers but differed from control thylakoids. One may speculate that CAB-proteins also serve as concentrators of Ca^{2+} and affect the electrostatic properties of the catalytic center of water oxidation.

We corroborated the intactness of the linear electron-transport chain and the photochemical activity of both photosystems in IML-thylakoids by flash-spectrophotometrical measurements. In agreement with Tzinas et al. (1987), we determined a PS II/PS I ratio of about 2.5:1 for IML-thylakoids. In addition to the known properties we found the following:

(1) IML-thylakoids revealed a greater activity of cyclic and electrogenic electron transport attributable to cyt b_6/f under excitation with repetitive single flashes and without chemically imposed redox poise. This was most probably due to the increased PS II/PS I ratio in comparison to control thylakoids. (2) The rates of proton uptake at the acceptor side of PS II were significantly faster in IML-thylakoids (1.1-ms half-rise time at pH 7) compared to the fastest one reported so far for control thylakoids [2.7 ms (Polle & Junge, 1986)]. It is conceivable that CAB-proteins shield the protonation site around Q_B. (3) Incubation of IML-thylakoids with DCCD did not induce the proton short ciruit in PS II in contrast to the situation in stacked control thylakoids (Jahns et al., 1988; Jahns & Junge, 1989). This supports our previous suggestion that DCCD binding to CAB-proteins is responsible for the short circuit (Jahns & Junge, 1990). (4) The known S-state-dependent periodicity of four in the pattern of proton release during water oxidation (Förster & Junge, 1985; Jahns et al., 1991; Rappaport & Lavergne, 1991) was absent in IML-thylakoids (see accompanying paper).

IML-thylakoids are a useful system for flash-spectrophotometrical measurements of the cyclic cyt b_6/f activity and protolytic reactions associated with PS II. The latter aspect is the subject of the accompanying paper.

ACKNOWLEDGMENT

We thank Hella Kenneweg for excellent technical assistance, drawings, and photographs and Karin Lübbers for advice on core preparations. Many thanks to the AG's "Pflanzenphysiologie" and "Spezielle Botanik" for leaving their growth chambers to us. Helpful discussions with Dr. R. Scheibe, Dr. E. Neuhaus, and M. Haumann are greatfully acknowledged.

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Registry No. Chl *a*, 479-61-8; chl *b*, 519-62-0; O_2, 7782-44-7.