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The Kinetics of P515 in Relation to the Lipid Composition of the Thylakoid Membrane

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Abstract

Flash-induced P515 absorbance changes have been studied in dark-adapted chloroplasts isolated from spinach plants grown under two different light intensities. The slow component (reaction 2), normally present in the P515 response of chloroplasts isolated from plants grown at an intensity of 60 $W \cdot m^{-2}$, was largely reduced in chloroplasts isolated from plants grown at an intensity of 6 $W \cdot m^{-2}$. This reduction of the slow component in the P515 response appeared to be coincident with an alteration in the lipid composition of the thylakoid membrane. Mainly the ratio monogalactosyldiacylglycerol to digalactosyldiacylglycerol appeared to be altered. In thylakoids from plants grown at 6 $W \cdot m^{-2}$, the ratio was approximately 35% lower than that of plants grown at 60 $W \cdot m^{-2}$. The amount of both cytochrome b_{563} and cytochrome *f* was largely reduced in chloroplasts isolated from plants grown at low light intensity. These results may indicate a possible correlation between structural organization of the thylakoid membrane and the kinetics of the flash-induced P515 response.

Key Words: P515; electrochromic bandshift; thylakoid membrane; lipid composition; cyt b_{563}; cyt *f*.

Introduction

The P515[2] electrochromic bandshift (for a review see Witt, 1979) following a saturating single turnover light flash in dark-adapted chloroplasts shows multiphasic rise and decay kinetics (Schapendonk *et al.*, 1979; Joliot and

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[2]Abbreviations: P515, absorbance change at 518 nm; R1, reaction 1; R2, reaction 2; P_{700}, photosystem 1 reaction center; Fe–S, Rieske iron–sulfur protein; cyt *b*, cytochrome b_{563}; cyt *f* cytochrome *f*; PQ, plastoquinone; ATP, adenosine-5′-triphosphate; Tricine, *N*-tris(hydroxymethyl)methylglycine; Hepes, *N*-2-Hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; DCMU, 3-(3′,4′-dichlorophenyl)-1-1′-dimethylurea; MGDG, monogalactosyldiacylglycerol; DGDG, digalactosyldiacylglycerol; PL, total phospholipids; chl, chlorophyll; Σ, molar extinction coefficient ($mM \cdot cm^{-1}$).

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Delosme, 1974; Horváth *et al.*, 1978). According to Schapendonk *et al.* (1979) this multiphasic kinetic pattern is the composite result of at least two different reactions, called reaction 1 and reaction 2. In their interpretation, reaction 1, characterized by a fast rise (<1 ns; Gräber and Trissl, 1981) and a subsequent single exponential dark decay with a rate constant of approximately 9.2 s^{-1}, is the reflection of the generation and decay, respectively, of a transmembrane electric field. Reaction 2, however, characterized by a relative slow increase in absorbance during the first 50–150 ms after the flash and a decay with a first-order rate constant of about 1.4 s^{-1} (i.e., the rate constant for the decay of reaction 1 is far greater), is thought to be related to intramembranal electrical phenomena (Schapendonk *et al.*, 1979; Schuurmans *et al.*, 1981; Schreiber and Rienits, 1982). It has been suggested by van Kooten (Westerhoff *et al.*, 1983) that reaction 2 is caused by a lateral and transverse delocalization of inner membrane electric fields associated with the liberation of protons in inner-membrane domains near the Fe–S cyt *b–f* protein complex. These domains might be connected via lateral H-conductive channels with other membrane domains which might act as proton sinks, i.e., ATP synthetase. Models depicting site-specific intramembranal proton processing in the thylakoid have been suggested by Dilley and by Kell (Dilley *et al.*, 1981; Kell and Morris, 1981). In this respect it is of interest to mention that, in agreement with the results of others (Schuurmans *et al.*, 1981; Schreiber and Rienits, 1982), we have shown that reaction 2 can also be induced in the dark toward its saturation level by ATP-driven reversed electron flow (cf. proton translocation) (Peters *et al.*, 1983b). However, the existence of the proposed intramembrane structures capable of stabilizing protons within a hydrophobic matrix remains to be demonstrated.

In contrast to reaction 1, the occurrence of reaction 2 appears to be dependent on the functional integrity of the membrane, i.e., it disappears upon aging, after a temperature shock and, as is shown in this paper, is largely reduced in plants grown at low light intensities. This paper deals with the question whether or not the occurrence and magnitude of reaction 2 in chloroplasts is associated with changes in the lipid composition of the thylakoid membrane. A comparative study has been made of light intensity effects during plant growth on the kinetics of the flash-induced P515 response in isolated chloroplasts and on the lipid composition of the thylakoid membrane. We find that a significant reduction of reaction 2 in the P515 response observed in chloroplasts isolated from plants grown at low light intensity (less than 10 $W \cdot m^{-2}$) is coincident with a 35% reduced ratio of the two major membrane lipids, MGDG to DGDG. The reduced ratio is mainly due to a reduced amount of MGDG in the low-light chloroplasts. In addition, the amount of both cytochrome b_{563} and cytochrome *f* in chloroplasts isolated from plants grown at 6 $W \cdot m^{-2}$ is approximately 40% of the amount found in

chloroplasts from plants grown at 60 W · m^{-2}. The concentration of other components of the electron-transport chain connecting photosystem 1 and photosystem 2 is approximately the same for both types of chloroplasts. This is qualitatively conclusive with results obtained with other plant species (Boardman *et al.*, 1974). The results presented in this paper may indicate a possible correlation between factors determining the structural organization of the thylakoid membrane and the kinetics of the flash-induced P515 response.

Materials and Methods

Plant Material

Spinacia oleracea plants were grown in a growth chamber at 18°C and at 80% relative humidity. Plants were exposed to a daily light period of 8 hr at two different light intensities, 6 and 60 W · m^{-2}, respectively. Plants were harvested at the age of 7 weeks.

Isolation and Characterization of Chloroplasts and P515 Measurements

Intact chloroplasts were isolated according to a modified method of Cockburn (Cockburn and Walker, 1968) as described by Schapendonk (1980). This procedure routinely yielded preparations with 90–95% intact chloroplasts as determined by ferricyanide reduction (Heber and Santarius, 1970). Broken chloroplasts were obtained by an osmotic shock on ice during 60 s, as described previously (Peters *et al.* 1983b). Determination of Hill reaction rate and absorbance changes at 518 nm was performed as described before (Peters *et al.*, 1983b). Determination of total chlorophyll content and quantitative analysis of chlorophylls *a* and *b* were performed as described by Bruinsma (1963). Spectral measurements were done with an Aminco DW-2A spectrophotometer. P_{700} was determined from light-induced absorbance changes at 700–740 nm, assuming an Σ of 64 mM · cm^{-1} (Hiyama and Ke, 1972). Cytochrome *f* was estimated from the hydroquinone-reduced minus ferricyanide-oxidized difference spectrum in a medium containing Hepes-KOH 100 mM (pH 7.0), Triton-X-100 (1% w/v), $MgCl_2$ 15 mM, and EDTA 4 mM, using an Σ of 22 mM · cm^{-1} (Bendall *et al.*, 1971). Cytochrome b_{563} was estimated from the dithionite-reduced minus ferricyanide-oxidized difference spectrum. An Σ of 20 mM · cm^{-1} was assumed according to Rich (Rich and Bendall, 1980). Reaction conditions were the same as for the determination of cyt *f*. Data analysis was performed as described by Peters (Peters *et al.*, 1983a). The size of the PQ pool was estimated by comparing the fluorescence induction curves in the presence and absence of DCMU as described by Malkin (Malkin and Kok, 1966).

For the determination of the lipid composition of the thylakoid membrane, envelope-free thylakoids (i.e., stripped thylakoids) were prepared by an osmotic shock during 60 s on ice in a medium containing Tricine-KOH 10 mM (pH 7.8), $MgCl_2$ 2 mM, and KCl 2.5 mM. Stripped thylakoids were separated from the outer envelope fraction and a remnant of intact chloroplasts by sucrose gradient centrifugation according to Douce (Douce and Joyard, 1982). Centrifugation was for 15 min at 2000 × *g*. Stripped thylakoids were recovered as a broad band at the interface of the 25 and 34% (w/w) sucrose layers. This band was aspirated, washed twice by centrifugation for 7 min at 4000 × *g* in a medium containing Hepes-KOH 50 mM (pH 7.5), sorbitol 330 mM, and $MgCl_2$ 2 mM, and stored at 0°C. Lipids were extracted with ice-cold chloroform–methanol (1:2 by vol.) according to the procedure of Allen (Allen *et al.*, 1966), except that the chloroform–methanol extract was washed with 0.1 M KCl instead of water. Lipids were separated on precoated TLC plates Silica Gel G-60 (20 × 20 cm, thickness 0.25 mm, 5 mg lipid applied) with acetone:benzene:water, 91:30:8 (v/v), as a developing agent (Pohl *et al.*, 1970). The plates were stained with rhodamine 6g (0.003% in a 4% NaOH solution) and immediately viewed under UV light at 366 nm. Glycolipids were stained on replicate plates with a solution of 0.5 g α-naphthol in methanol–water 1:1 (v/v), according to Siakotos (Siakotos and Rouser, 1965). Saponification of the lipids and methylation of the fatty acids with BF_3 in methanol were performed as described by Kuiper (1970). Separation of the fatty acid methyl esters by gas–liquid chromatography and their identification was done by using a Becker gas chromatograph model 421 equipped with a flame-ionization detector. A 160-cm glass column with a diameter of 2 mm was used, filled with Gaschrom Q (100–120 mesh) coated with 5% Silicar 10-C. Nitrogen was used as carrier gas (60 ml · min^{-1}). Operating temperature was 130–170°C. The gas chromatograph was connected with an Autolab integrator type 6300.

Results

A representative example of the time course of the absorbance change at 518 nm (ΔA518) upon a single turnover light flash in dark-adapted broken chloroplasts, isolated from plants grown at a light intensity of 60 W · m^{-2}, is illustrated in Fig. 1 (solid curve). From this figure it can be seen that ΔA518 under these conditions occurs with multiphasic rise and decay kinetics. By using double flashes (Schapendonk *et al.*, 1979; Peters *et al.*, 1983b) it has been shown that the single-flash response curve can be deconvoluted into three separate responses: (a) a fast response (reaction 1) with a fast rise (<0.5 ms) and a single exponential dark decay with a half-life of about 75 ms; (b) a slow

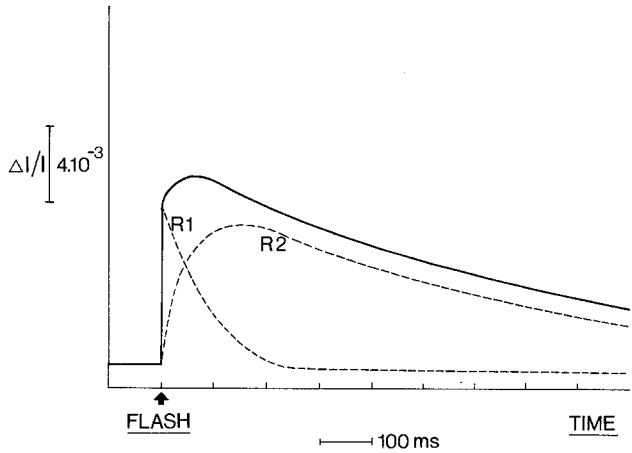

Fig. 1. Absorbance changes at 518 nm in dark-adapted broken chloroplasts induced by a single-turnover light flash (solid curve). Chloroplasts were isolated from spinach plants grown at a light intensity of 60 W · m^{-2}. The measurement was performed in a medium containing Hepes-KOH 50 mM (pH 7.5), sorbitol 330 mM, $MgCl_2$, 2 mM, $MnCl_2$ 1 mM, and EDTA 2 mM, at chl = 30 μg/ml. Average of 8 flashes fired at a rate of 0.10 Hz.

response (reaction 2) with a slow rise (within 150 ms after the flash) and a slow single exponential decay with a half-life of about 500 ms; and (c) a relatively small phase-d with an extremely slow decay (half-life of about 1500 ms). These responses (i.e., reactions 1 and 2), determined in our experiments according to the aforementioned procedure, are indicated in Fig. 1 by the dashed curves (phase-d not shown). From this figure, it can be seen that the decay of the overall P515 response is almost exclusively determined by the decay of reaction 2. Figure 2 shows the absorbance change at 518 nm upon a single turnover light flash in dark-adapted broken chloroplasts isolated from plants grown at a light intensity of 6 W · m^{-2} (solid curve). From this figure it can be seen that the contribution of reaction 2 to the overall kinetics of the P515 response in these chloroplasts is largely reduced. Whereas the rise and decay kinetics of reaction 2 itself are not altered significantly, the contribution of reaction 2 to the overall P515 response is reduced to about 25% in low-light chloroplasts as compared to the apparent high contribution in chloroplasts isolated from high-light grown plants. No significant differences between these two types of chloroplasts could be detected with respect to the kinetics of reaction 1.

Table I gives the results of a comparative analysis of these two types of chloroplasts with respect to some of the components involved in the functional and structural organization of the photosynthetic electron transport system.

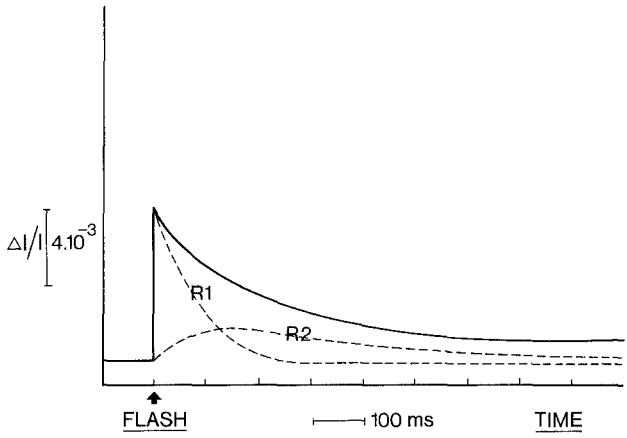

Fig. 2. Absorbance changes at 518 nm in dark-adapted broken chloroplasts induced by a single-turnover light flash (solid curve). Chloroplasts were isolated from spinach plants grown at a light intensity of 6 W · m^{-2}. Average of 9 flashes fired at a rate of 0.10 Hz. Conditions as in Fig. 1.

From the table it can be seen that there are no significant differences between these two types of chloroplasts with respect to the ratios of the molar concentrations of chlorophyll *a* and *b*, chlorophyll and P700, as well as for the size of the PQ pool. Figure 3 shows the fluorescence induction curves for the two different types of chloroplasts in the presence and absence of 2 times 10^{-6} M DCMU. Both types of chloroplasts show the same induction kinetics, and the amount of electrons that can be stored in the PQ pool, calculated by comparing the areas above the induction curves in the presence and absence of DCMU, appears to be about 12 for both samples. In contrast with this, the summed amount of cyt *f* and cyt b_{563} in chloroplasts isolated from plants grown at 6 W · m^{-2} appeared to be reduced to about 40% of the values measured in chloroplasts isolated from plants grown at 60 W · m^{-2} (see Fig. 4). From these experiments, the quantitative differences for each type of cytochrome could not be determined with sufficient precision. However, our data appear to be conclusive with a relative concentration ratio P700:cyt *f*:cyt b_{563} of 1:1:2.

Table I. Molar Ratios of Chlorophyll *a*/*b* Chlorophyll/P_{700}, and Chlorophyll/cyt b_{563} + cyt *f* (Relative Units), and the Values for the Hill Reaction Rate and the Size of the PQ Pool Relative to the Primary Acceptor Q_A, Determined in Chloroplasts Isolated from Plants Grown at a Light Intensity of 60 and 6 W · m^{-2}, Respectively

Light intensity	Chlorophyll *a*/*b*	Hill reaction (μmol O_2 · mg chl^{-1} · hr^{-1})	Chl/P_{700}	PQ/Q_A	Chl/cyt b_{563} + cyt *f* (relative units)
60 W · m^{-2}	2.8 ± 0.1	190 ± 10	490 ± 10	12 ± 1	100
6 W · m^{-2}	2.5 ± 0.1	155 ± 10	510 ± 10	12 ± 1	250

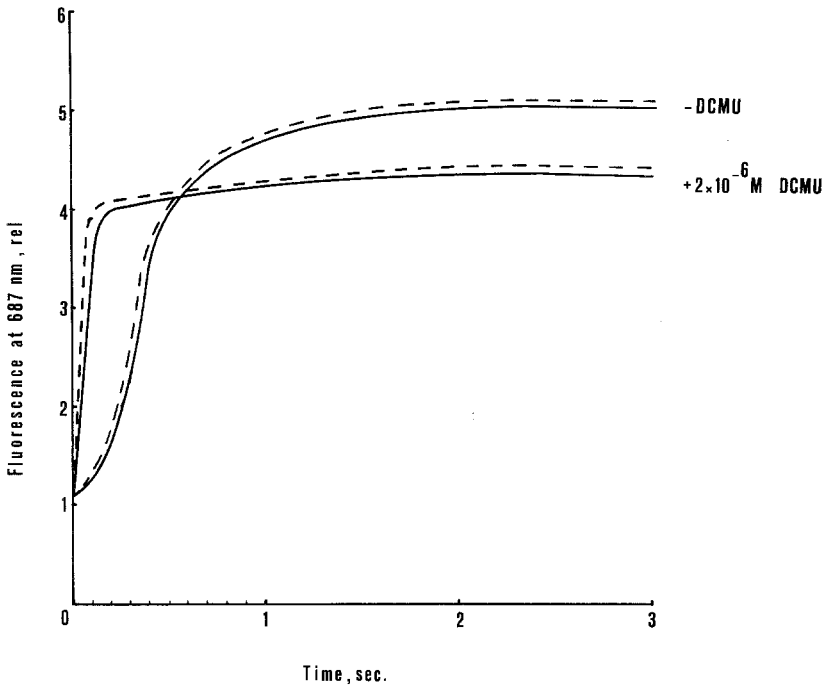

Fig. 3. Fluorescence kinetics at 20°C of chloroplasts isolated from spinach plants grown at 60 W · m^{-2} (dashed curve) and at 6 W · m^{-2} (solid curve). The induction curves were determined after a period of 90 min dark adaptation in the presence and absence of DCMU, in a medium containing sorbitol 330 mmol/liter, Hepes-KOH 50 mmol/liter, pH 7.4, $MgCl_2$ 1 mmol/liter, and 20 μg chl · ml^{-1}. Fluorescence (λ 687 nm) was excited with high-intensity light (λ 380–550 nm).

The reduction of the total amount of both cyt *f* and cyt b_{563} in low-light chloroplasts is coincident with a 20% reduced rate of the Hill reaction.

Table II reports on the lipid composition of stripped (i.e., envelope-free) thylakoids isolated from plants grown at 6 and 60 W · m^{-2}, respectively. From the table, an appreciable difference in the lipid composition between these two types of chloroplasts can be observed. In chloroplasts isolated from plants grown at a light intensity of 6 W · m^{-2}, the ratio of the two major membrane lipids, i.e., monogalactosyldiacylglycerol to digalactosyldiacylglycerol, is reduced to about 65% of the value found in high-light chloroplasts.

Discussion

On the basis of the kinetics of reaction 2 we suppose, in conformation with others (Schuurmans *et al.*, 1981; Schreiber and Rienits, 1982; Schapen-

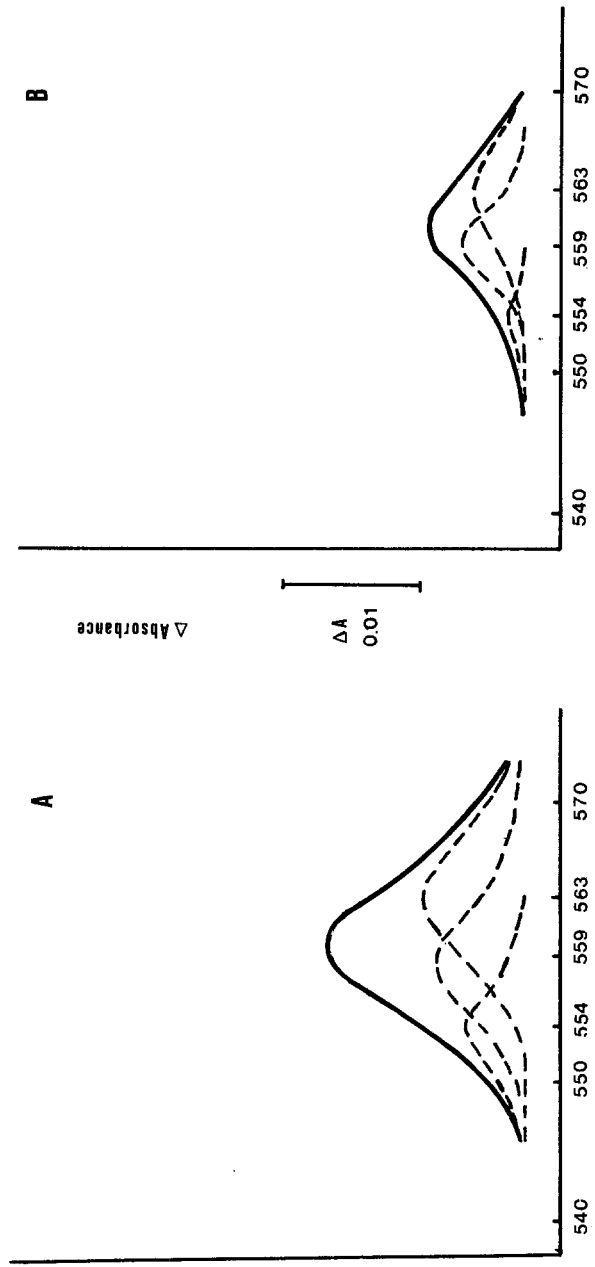

Fig. 4. Dithionite-reduced minus ferricyanide-oxidized difference spectrum of cytochromes in chloroplasts isolated from plants grown at 60 W · m^{-2} (A) and 6 W · m^{-2} (B), respectively. The dotted lines represent the calculated contribution of the different types of cytochromes to the overall spectrum.

Table II. Lipid Class Composition of Thylakoid Membranes Isolated from Plants Grown at Different Light Intensities

Light intensity	mol lipid/mol total chlorophyll	Lipid class composition (mol. %)			
		MGDG	DGDG	PL	MGDG/DGDG
60 W · m^{-2}	2.42	53.2	36.5	10.3	1.46
6 W · m^{-2}	1.46	47.5	46.5	6.0	1.02

donk *et al.*, 1979), that this reaction is not directly linked to a transmembranal potential but rather to an intramembranal local field. In particular, the slow decay associated with reaction 2 is an indication that this reaction is associated with an electrical event that takes place in an area, presumably a hydrophylic phase adjacent to or inside the membrane, which is not in direct equilibrium with the inner or the outer aqueous phase. In contrast to reaction 1, the occurrence of reaction 2 is strongly dependent on the state of activation of the thylakoid membrane. Reaction 2, most obvious in dark-adapted chloroplasts, is largely reduced or even absent from the P515 response under conditions at which the thylakoid membrane is energized preceding the actinic light flash. This suppression of reaction 2 can be brought about either by illumination (i.e., the second slow rise is largely absent in the P515 response of a single flash following two preceding light flashes separated in time for 100 ms) or by an activated ATPase (Peters *et al.*, 1983b). Obviously, the proposed inner-membranal electric fields associated with reaction 2 can be created by light-driven electron transport as well as by reversed electron flow caused by ATP hydrolysis in the dark. The energetic requirement to generate these fields apparently is low, because of the small number of single turnover light flashes required to saturate reaction 2, and the long periods at which the saturation can be sustained by an activated ATPase (Peters *et al.*, 1983b).

It has been proposed that reaction 2 is caused by lateral and transversel delocalization of inner-membrane electric fields associated with the liberation of protons in inner-membrane domains near the Fe–S cyt *b–f* protein complex (Westerhoff *et al.*, 1983). In this respect it is of interest that the suppression of reaction 2 in chloroplasts isolated from plants grown at a light intensity of 6 W · m^{-2} (Fig. 2) is associated with a 60% reduction of the amount of cyt *f* and cyt b_{563} (Table I). This reduction was found to have much less consequence for the rate of photosynthetic electron transport, as is indicated by the approximately 20% reduction of the Hill reaction in these chloroplasts (Table I). This indicates a low flux control of the electron transport rate by the cyt *b–f* complex, as was suggested by Kackser (Kackser and Burns, 1979). The existence of these proposed intramembrane structures capable of stabilizing protons within the hydrophobic matrix of the membrane remains to be demonstrated.

As can be seen from Table II, the apparent reduction of reaction 2 in the P515 response in chloroplasts isolated from plants grown at a light intensity of 6 W · m^{-2} is associated with an appreciable reduction of the ratio of the two major membrane lipids, monogalactosyldiacylglycerol to digalactosyldiacylglycerol. This reduced ratio was due mainly to a reduced amount of MGDG in the low-light chloroplasts. The difference in the ratio of MGDG to DGDG between chloroplasts isolated from plants grown at low and high light intensities, respectively, is probably associated with a difference in the stage of development between these two groups of plants at the moment of harvesting (7 weeks after sowing). Whereas the plants grown at a light intensity of 60 W · m^{-2} had, at the moment of harvesting, six fully expanded green leaves, the plants grown at a light intensity of 6 W · m^{-2} had only two. It has been reported that the MGDG/DGDG ratio is dependent on the stage of development of the plants, i.e., is low in the initial stages (Frey and Tevini, 1979). The apparent low amount of MGDG in thylakoid membranes isolated from plants grown at a low light intensity may affect the architecture and the structural organization of the membrane. As reported by Shipley (Shipley *et al.*, 1973), isolated MGDG dispersed in aqueous environments tends to form preferentially nonbilayer structures, described as a hexagonal type-II phase. Such nonbilayer structures adopted by the MGDG molecules could theoretically create hydrophilic microcompartments in the hydrophobic membrane that are able to stabilize protons or, in combination with proteins, protonated residues. This tendency of MGDG to form nonbilayer structures, at least *in vitro* (Sen *et al.*, 1981; Quinn and Williams, 1983), may have an impact on the heterogeneous organization of the thylakoid bilayer *in vivo*. According to Israelachvili (Israelachvili *et al.*, 1980) and Gounaris (Gounaris *et al.*, 1983), cone-shaped molecules like MGDG may serve conditions of a special packaging of large protein complexes in biological membranes. Thus, one might suggest that a reduced amount of MGDG in thylakoid membranes will alter the assembly of protein complexes in the membrane. This will, among others, affect the proton permeability of the membrane. If protons were stabilized within hydrophilic microcompartments adjacent to or inside the membrane, the stabilizing ability is reduced when the proton permeability is enhanced. This means that after proton loading, the microcompartments would equilibrate with the lumen at a higher rate and as a consequence, the extent of reaction 2 is smaller.

Our data are far from enabling us to reach firm conclusions about the nature and origin of the slow component of P515 (reaction 2). However, we feel that they are of relevance in view of current thoughts about the functional role of lipids and proteins in assembling the catalytic sites involved in the primary steps of the energy coupling mechanism.

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