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PROTON-INDUCED GRANA FORMATION IN CHLOROPLASTS

DISTRIBUTION OF CHLOROPHYLL-PROTEIN COMPLEXES AND PHOTOSYSTEM II PHOTOCHEMISTRY

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Lowering the pH of the incubation medium to pH 5.4 leads to grana formation morphologically similar to that induced by metal cations. The same phenomenon is observed in EDTA-washed chloroplasts, indicating that it is not due in part to electrostatic 'masking' by residual cations associated with the membranes. Digitonin fractionation studies have indicated that the distribution of the major chlorophyll-protein complexes between granal and stromal membrane regions is similar at pH 5.4 in the absence of Mg^{2+}, and at pH 7.4 in the presence of Mg^{2+}. Chlorophyll fluorescence induction studies have indicated that the primary photochemistry of Photosystem II (PS II) is stimulated by lowering the pH to 5.4, just as it is upon metal cation addition at higher pH values. The failure to observe such an increase at pH 5.4 by measuring electron transport to ferricyanide is attributed to a combination of an inhibition by this pH of electron transport at a site after Q reduction and an increase in the number of PS II centres detached from the plastoquinone pool. We conclude that the stacked configuration of chloroplast membranes leads to increased PS II primary photochemistry, which is most simply explained in terms of a redistribution of excitation energy towards PS II.

Introduction

The organization of chloroplast membranes into grana stacks and non-stacked intergranal regions has excited considerable interest in recent years. Substantial progress has been achieved in understanding the mechanism by which stacking is brought about, which has led to a realization of the involvement of electrostatic screening of the negative surface membrane charge by cations [1–6]. This conclusion is supported by our demonstration that chloroplast membranes can be induced to form grana stacks even in the absence of metal cations by lowering the pH of the suspension medium to pH 5.4 [7,8]. Such an observation is perhaps surprising inasmuch as this pH is well above the isoelectric point of chloroplast membranes, which is usually measured at about pH 4.5 [9–12].

In this paper we present data which demonstrate that proton-induced grana stacks resemble those induced by metal cations not only morphologically, but also biochemically.

The idea has been frequently put forward that membrane stacking may function to increase the photochemical efficiency of PS II [13–18]. Data have been presented both for [18–21] and against [8,14,22–24] this hypothesis. Recently, it has been

Abbreviations: Chl, chlorophyll; PS II, Photosystem II; CP_1, CP_{1a} and CP_a, chlorophyll-protein complexes 1, 1a and a, respectively; LHCP, light-harvesting chlorophyll-protein complex; DCMU, 3′-(3′,4′-dichlorophenyl)-1,1-dimethylurea; Tricine, *N*-tris(hydroxymethyl)methylglycine; Mes, 4-morpholineethanesulphonic acid; SDS, sodium dodecyl sulphate.

suggested that relatively small increases in PS II photochemistry may be achieved by increased PS II-PS II energy transfer upon membrane stacking [25,26]. Owing to the importance of this aspect for an understanding of the biological significance of membrane stacking, in the present work we reinvestigate the problem. We have persevered with the approach introduced recently by ourselves [8,25] of comparing chloroplasts in which membranes are induced to form grana either by divalent cation addition at pH 6.4, or by lowering the pH to 5.4 without cation addition. The main advantage of this method is that direct effects of metal cations on PS II can be distinguished from membrane stacking effects. Previous data have demonstrated that when energy transfer between PS II units is largely eliminated by maintaining the PS II traps oxidized, no stimulation of the reduction of ferricyanide can be observed in chloroplasts in which grana are induced to form by protons [8]. Thus, we concluded that the divalent cation-induced stimulation of PS II photochemistry under similar conditions may not have been a result of grana formation. In the present work we compare the effect of proton- and metal cation-induced granal stacking on PS II photochemistry using chlorophyll fluorescence induction techniques. We conclude that membrane stacking can indeed lead to an increase in PS II primary photochemistry distinct from that caused by increased PS II-PS II energy transfer.

Materials and Methods

Chloroplasts were prepared from freshly harvested spinach leaves by homogenization in Tricine buffer (30 mM, pH 8.0) also containing sucrose (0.4 M) and NaCl (10 mM). The suspension was centrifuged for 5 min at $1500 \times g$, and the pellet was subsequently resuspended and washed once in the same buffer. Chloroplasts were stored in the same buffer until required.

The pH 5.4 and 6.4 buffers used for measurement of reactions were Mes (25 mM) brought to the required pH with NaOH. NaCl was added to give a final Na^+ concentration of 25 mM.

Digitonin fractionation was performed essentially as described previously [27] with the chlorophyll concentration at about 600–800 μg/ml. Subsequent to the digitonin treatment and prior to the centrifugation at $10\,000 \times g$, all samples were diluted five times with Tricine (50 mM, pH 7.4). The $10\,000 \times g$ supernatant was diluted a further three times with the same buffer and centrifuged at about $200\,000 \times g$ for 1.5 h.

Electrophoresis of chlorophyll-protein complexes was performed according to the method of Anderson [28]. Absorption spectra, Chl *a*/*b* ratios and relative electrophoretic mobilities of the six complexes were similar to those reported [28,29].

The preparation of samples for electron microscopy was performed as described previously [24].

Fluorescence induction kinetics were measured at 90° to the excitation beam through a Balzers (691 nm) interference filter. The signal was processed by an 8-bit analogue-to-digital converter and then memorized by a Rockwell R65000 microcomputer. The signal was displayed on a chart recorder after reconversion to the analogue form.

Results

In Fig. 1 data are presented from two experiments in which distribution of the major chlorophyll-protein complexes between the light and heavy digitonin fractions was examined. Fig. 1a shows that very little fractionation occurred at pH 7.4 in the absence of divalent cations. On lowering the pH to 5.4, however, the light fraction became enriched in CP_1 and CP_{1a} and depleted in the three LHCP complexes and CP_a. The heavy fraction at pH 5.4 was, on the other hand, enriched in the LHCP complexes and CP_a.

Fig. 1b shows the effect of incubation at pH 5.4 and 7.4 in the presence of Mg^{2+} prior to and during digitonin fractionation. In both cases, the light fractions were enriched in CP_1 and CP_{1a} and depleted in the other complexes. It should be mentioned that the presence of EDTA (0.1 mM) during the washing stage to remove traces of Mg^{2+}, which may have remained bound to the membrane surfaces [30], did not qualitatively influence the distribution of the complexes between the digitonin fractions, though fractionation seemed to be less pronounced in both pH 5.4- and Mg^{2+}-treated chloroplasts (data not presented).

Our previous studies on proton-induced membrane stacking [7,8] were performed in the presence of 20–30 mN Na^+. Thus, our results could be interpreted as being due to a combination of charge neutralization

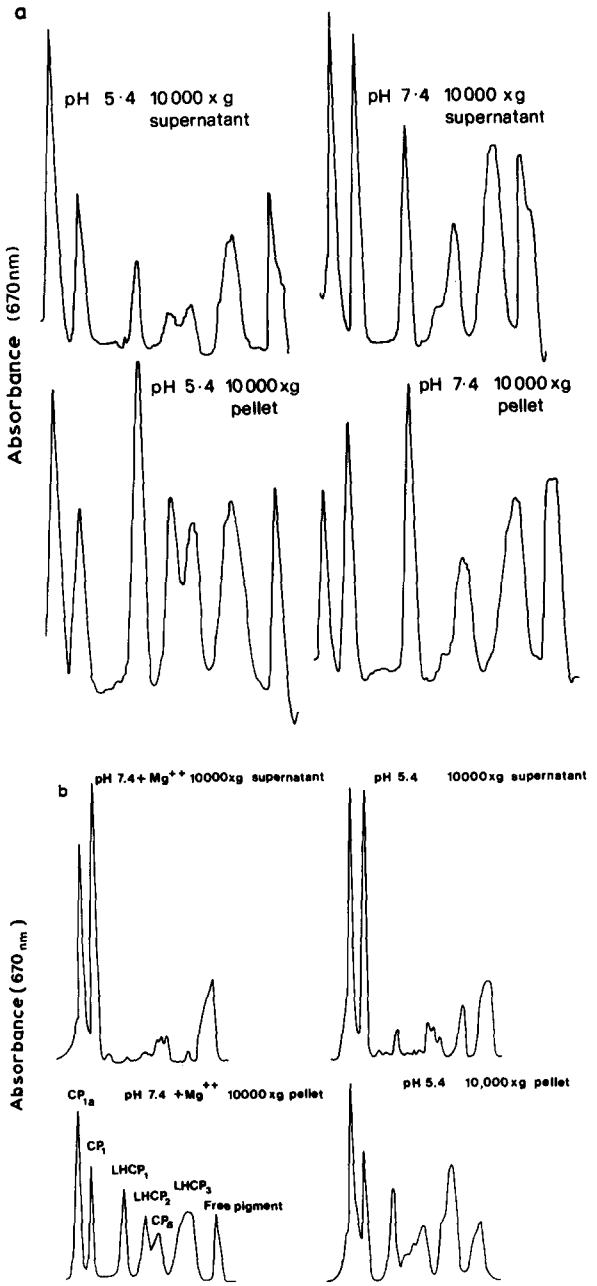


Fig. 1. SDS-polyacrylamide-gel electrophoresis profiles of chlorophyll-protein complexes from light and heavy digitonin fractions of spinach chloroplasts. (a) After the initial centrifugation, chloroplasts were resuspended for about 1 min in water before addition of an equal volume of Tricine-sucrose buffer (see Materials and Methods). (b) Chloroplasts were prepared as described in Materials and Methods. In all cases, after being washed, chloroplasts were resuspended at room temperature at the indicated pH values ±$MgCl_2$ (5 mM) for 4 min. They were subsequently incubated for a further 3–4 min in ice prior to digitonin addition. For other details see Materials and Methods. The terminology used to identify the bands is that of Anderson et al. [29].

of negative groups on the membrane surfaces and charge screening by Na^+. To investigate this further, we exhaustively washed chloroplast membranes in salt-free media and also with EDTA. They were then resuspended at pH 5.4 in the absence of cations or with 25 mN Na^+, or at pH 8 in the presence or absence of $MgCl_2$ (5 mM). The electron micrographs presented in Fig. 2 show that the grana formed at pH 5.4 in the absence of metal cations are similar to those formed at pH 8 in the presence of Mg^{2+}. We therefore conclude that membrane stacking at pH 5.4 is exclusively due to charge neutralization effects.

The photochemistry of PS II has been investigated by measuring fluorescence induction curves both in the presence and absence of DCMU. Fig. 3 and 4 show typical examples of the type of induction curve obtained under the various conditions used. In the absence of DCMU (Fig. 3) one sees the typical biphasic rise, the first phase (the minor one) of which is thought to be due to the presence of centres which are detached from the plastoquinone pool [31] and hence do not participate in electron transport to most artificial electron acceptors or to PS I. The second and major phase is indicative of the accumulation of reduced Q, subsequent to the reduction of all electron carriers up to and including the PS I centres [31, 32]. In the present work we will not deal with the kinetics of the first phase which will be the subject of a subsequent work. From Table I it can be seen that the half-time of the fluorescence rise of the second phase is greatly reduced by Mg^{2+} addition at pH 6.4, while lowering the pH to 5.4 brought about only a very small reduction of this parameter. Also presented in Table I are data for the fluorescence rise in the presence of DCMU. Here both Mg^{2+} addition and incubation at pH 5.4 brought about large decreases in the half-time. We would point out that the percent effect of Mg^{2+} either in the presence or absence of DCMU is very nearly the same, while that at pH 5.4 is clearly very different.

While both phases 1 and 2 of the induction curves measured in the absence of DCMU are increased by Mg^{2+}, the second phase is considerably more sensitive.

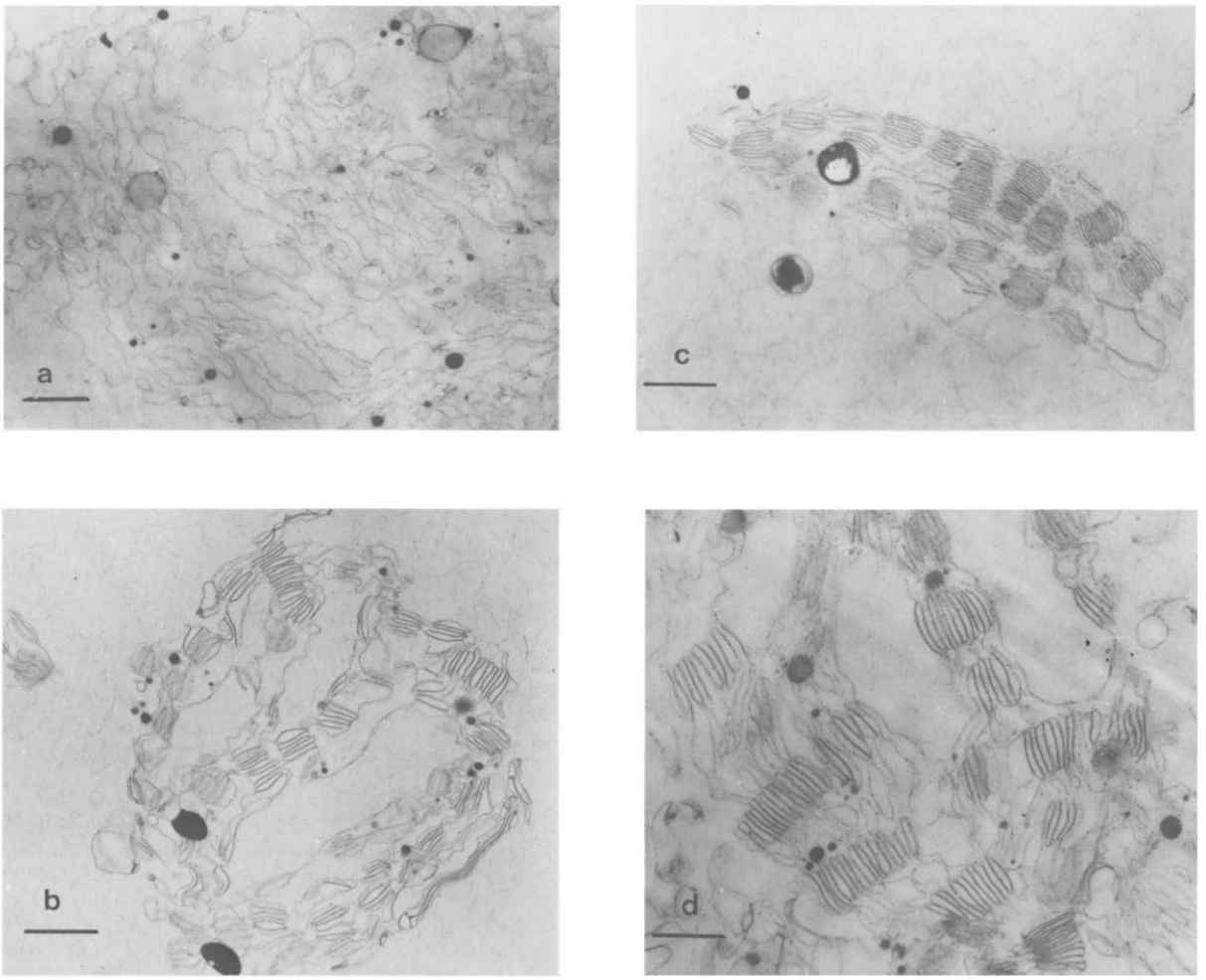


Fig. 2. Electron microscope pictures of chloroplasts suspended at pH 5.4 in the presence and absence of NaCl (25 mM), and at pH 8 in the presence and absence of $MgCl_2$ (5 mM). Chloroplasts were extracted as usual, resuspended in a small volume of the same buffer, and washed in NaCl (10 mM) and subsequently in sucrose (0.1 M) with EDTA (4 mM) at pH 7.7. They were then washed a further two times with unbuffered sucrose (0.1 M). The final resuspension was at a chlorophyll concentration of 30 μg/ml in sucrose (0.1 M), and the pH of the different samples was adjusted with either succinic acid or Tris. Sucrose was deionized to remove traces of metal cations. (a) pH 8, NaCl 0, $MgCl_2$ 0; (b) pH 8, NaCl 0, $MgCl_2$ 5 mM; (c) pH 5.4, NaCl 0, $MgCl_2$ 0; (d) pH 5.4, NaCl 25 mM, $MgCl_2$ 0. Bar, 1 μm.

Interestingly, pH 5.4-treated chloroplasts display an extremely large increase in the first phase with a much smaller stimulation of the second phase.

The data presented above lend themselves to a purely qualitative interpretation (see Discussion). In an attempt to obtain more quantitative information we adopted the method of Melis and Homann [17, 33]. Thus, the areas above the induction curves, measured in the presence of DCMU, were treated according to first-order reaction kinetic theory, as indicated in Fig. 4. As reported by Melis and Homann [17,33], two distinct phases were observed, indicating two types of PS II centre; the slow β-centres and the fast α-centres. Also in agreement with these authors, we noted both an increase in the 'rate constant' of the fast centres, k_α, on Mg^{2+} addition and also an increase

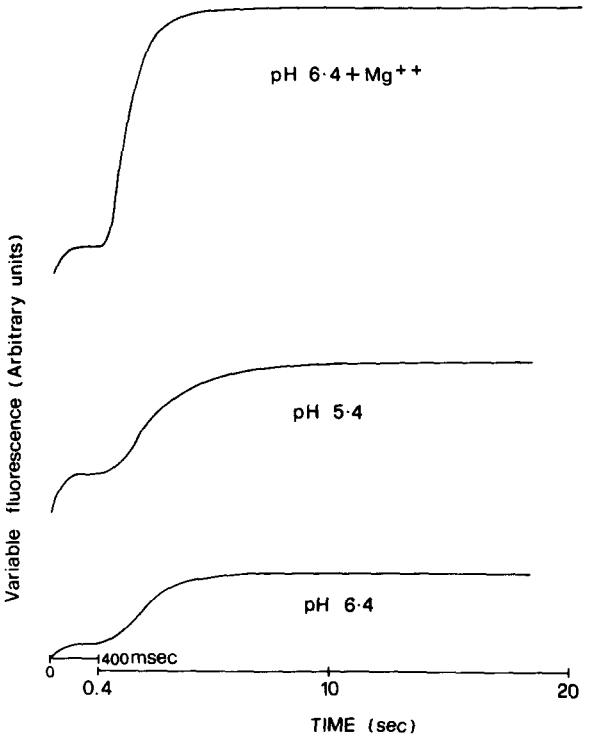


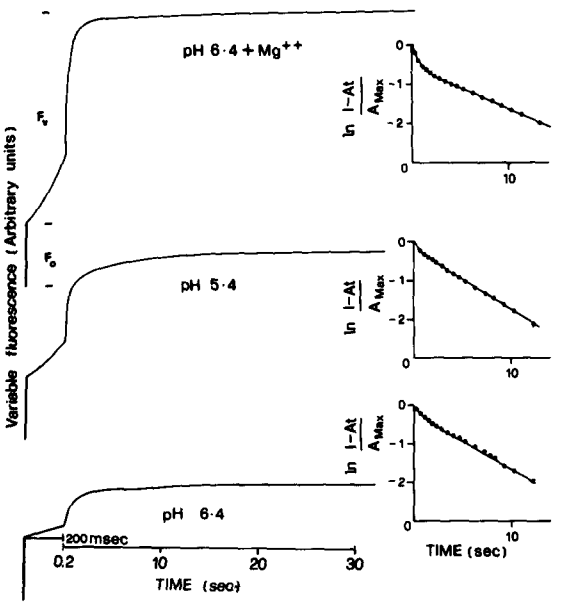
Fig. 3. Fluorescence induction curves for chloroplasts suspended at pH 5.4 and at pH 6.4 ± $MgCl_2$ (5 mM) in the absence of DCMU. For experimental details see the legend to Table I. The sampling frequencies used were 5 kHz for the first 400 ms and subsequently 0.05 kHz.

TABLE I

THE EFFECT OF INCUBATION AT pH 5.4 and pH 6.4 IN THE PRESENCE AND ABSENCE OF Mg^{2+} ON SOME FLUORESCENCE INDUCTION PARAMETERS WITH OR WITHOUT DCMU (25 μM)

Chloroplasts were prepared as described in Materials and Methods and then incubated in the reaction medium for 6 min at 18–20°C before illumination. Chloroplasts not treated with DCMU were illuminated with light of approx. 3 000 erg/cm^2 per s while those treated with DCMU were illuminated with approx. 800 erg/cm^2 per s. The light was filtered through a Corning 4-96 filter. Gramicidin (1 μM) and bicarbonate (1 mM) were also included in the reaction medium. The chlorophyll concentration was 4 μg/ml. F_o is the non-variable fluorescence, F_v the variable fluorescence and F_{v1} and F_{v2} the variable fluorescence of phase 1 and 2 respectively. Data are the average of 12 experiments.

	pH 6.4	pH 5.4	pH 6.4 + Mg^{2+}
−DCMU			
$T_{1/2}$ fluorescence rise (phase 2) (s)	2.5	2.2	1.3
F_o	15.7	15.0	14.2
F_{v1}	2.7	9.1	6.1
F_{v2}	14.3	21.3	46.9
+DCMU			
$T_{1/2}$ fluorescence rise (ms)	498	292	285
F_o	15.9	16.8	16.4
F_v	14.0	25.3	46.0



in the total amount of these centres (Table II). It should be pointed out that k_α is not a true first-order rate constant, due to energy transfer between PS II units. Here it has been estimated simply as the initial velocity of Q reduction after subtracting the contribution due to the β-centres. Lowering the pH to 5.4 leads to an increase in the k_α value. No change in the amount of the fast and slow centres was observed on incubating chloroplasts at pH 5.4.

Lowering the pH to 5.4 brings about an increase in

Fig. 4. Fluorescence induction curves and the relative first-order kinetic plots for chloroplasts suspended at pH 5.4 and pH 6.4 ± $MgCl_2$ (5 mm) in the presence of DCMU. For experimental details see the legend to Table I. A_t is the area above the induction curve at any time, while A_{Max} is the total area above the induction curve upon attainment of the maximal fluorescence. The sampling frequencies used were 5 kHz for the first 200 ms and subsequently 0.05 kHz.

TABLE II

THE EFFECT OF INCUBATION OF CHLOROPLASTS AT pH 5.4 and pH 6.4 IN THE PRESENCE AND ABSENCE OF Mg^{2+} ON THE FLUORESCENCE INDUCTION PARAMETERS OF PS II IN THE PRESENCE OF DCMU

See the legend to Table I for experimental details. k_β was estimated as the first-order rate constant while k_α was determined from the initial velocity of Q reduction during the first 50 ms after subtracting the contribution due to the slow β-centres. Data are the average of 14 measurements.

	pH 6.4	pH 5.4	pH 6.4 + Mg^{2+}
k_α (s^{-1})	1.6	2.1	2.2
k_β (s^{-1})	0.13	0.14	0.13
α-centres (% total)	31	31	51
F_O	16.9	20.7	19.7
F_V	15.4	30.6	50.5

the fluorescence yield, in accordance with the results of Briantais et al. [34]. This resembles the effect of metal cations on the fluorescence yield in mainly involving a stimulated variable fluorescence component. At both pH 5.4 and 6.4 in the presence of Mg^{2+}, the fluorescence rise curve displays an initial sigmoidicity in the presence of DCMU (Fig. 4). This was previously noted [25] and was attributed to membrane stacking leading to increased energy transfer between PS II units.

Discussion

We have previously demonstrated [7,8] that at pH 5.4, spinach chloroplasts form structures which seem morphologically identical to the grana induced by metal cations at higher pH values. In this paper we extend this observation to include a partial biochemical characterization of the pH 5.4-induced grana. These were separated from stromal membranes by digitonin fractionation, and the SDS-chlorophyll-protein complexes were examined. It is demonstrated that the heavy (granal) fraction is enriched in the three LHCP complexes and the CP_a complex, which according to Anderson et al. [29] and Anderson [28] may correspond to the PS II complex. The light (intergranal) fraction is correspondingly enriched in the CP_1 and CP_{1a} complexes, which are associated with PS I [28,29]. Thus, protein-induced grana seem to be biochemically similar to Mg^{2+}-induced grana.

The possibility existed that the presence of grana at pH 5.4 may be due to a proton-induced inhibition of destacking, rather than the formation of grana in previously unstacked membranes. In this paper we demonstrated the presence of grana in hypotonically washed chloroplasts, pretreated with EDTA. Thus, this possibility would not seem tenable, in agreement with recent work from Barber's group (Barber, J; personal communication).

The most likely explanation of our data would seem to be that upon lowering the pH to 5.4 a number of high-p*K* surface-exposed carboxyl groups are titrated, thus reducing the membrane surface charge density near certain complexes. This would then be envisaged to permit the rearrangement of charge complexes within the membranes as suggested by Barber and Chow [1], leading subsequently to regions of low charge density which then stack and regions of high charge density which remain unstacked. Alternatively, the reduction of charge density may be sufficient in itself to permit the membranes to approach each other and stack, with the movement of the chlorophyll-protein complexes into and out of the stacked zones occurring after. These two possibilities cannot be distinguished at the moment.

Nakatani et al. [12] could see little or no change in the electrophoretic mobility of pea chloroplast membranes when the pH was lowered to 5.4. On the other hand, Schapendonk et al. [35] noticed quite major decreases in electrophoretic mobility on lowering the pH from 7 to values in the region of 5.4. These differences are presumably due to differences in chloroplast preparation techniques. Thus, at present it is not possible to comment on the charge density reduction required to induce proton-mediated stacking.

We have previously demonstrated that ferricyanide reduction is not increased by grana formation at pH 5.4 [8,25]. Thus, we concluded that while metal cations stimulate PS II photochemistry under these conditions, this effect is not a consequence of grana formation. This conclusion is not supported by the fluorescence induction data presented here. Firstly, let us consider the significance of the measurements of half-time of the fluorescence rise. It is well known that in the presence of enery transfer between PS II units the fluorescence rise is not directly proportional to the area above the induction curve [36,37]. As it

is the area above the curve which is directly proportional to the reduction of the electron acceptor pool [32], the fluorescence yield cannot be used as a quantitative indicator of photochemical rate. Here we have measured the half-times of the fluorescence rise under the three experimental treatment conditions of pH 6.4 ± Mg^{2+} and pH 5.4, both in the presence and the absence of DCMU. As DCMU should not change the relationship between fluorescence quenching and the redox state of Q, we feel that a qualitative comparison can be made between the induction curves measured with or without the inhibitor. We noted that Mg^{2+} decreased the half-time in both the presence and absence of DCMU by the same extent. According to the above reasoning, this result is expected and indicates the well known Mg^{2+} stimulation of PS II primary photochemistry. Applying the same reasoning to the pH 5.4-incubated chloroplasts, we can conclude that while in the presence of DCMU this treatment leads to an increased primary photochemistry, this effect seems to be greatly reduced in the absence of DCMU.

When the area changes above the induction curves, measured in the presence of DCMU, were analyzed according to first-order reaction kinetic theory [17, 33], we noted that the rate constant for the fast α component was increased significantly by both Mg^{2+} and protons. These data are in agreement with the half-time fluorescence data, measured in the presence of DCMU. The failure to observe this stimulation on lowering the pH to 5.4 in the absence of DCMU would seem then to be due to this pH inhibiting electron transport at some site after Q reduction. Such a conclusion is in accord with the fact that ferricyanide reduction is not increased at pH 5.4 when the PS II centres are substantially open [8,25].

Lowering the pH to 5.4 leads to a large increase in the ratio of the phase 1 variable fluorescence component, observed in the absence of DCMU, to the total fluorescence. This may indicate the detachment of some PS II centres from the plastoquinone pool [31]. The failure to observe increased ferricyanide reduction rates at low light intensities on lowering the pH to 5.4 may also be due to this fact.

It has been suggested [17,18] that membrane stacking could be the major reason for the large increase in the number of fast α-centres observed on Mg^{2+} addition to chloroplasts. The data presented here do not support such an idea as no such change was observed in proton-stacked membranes. Recently, Homann and Theg [38] have reached a similar conclusion based on proteolytic digestion studies of metal cation-stacked membranes.

In conclusion, the present work demonstrates that proton-induced grana are associated with a distribution of the major chlorophyll-protein complexes similar to that of metal cation-stacked membranes. Furthermore, when only the primary photochemistry is observed, in the presence of DCMU, it can be shown that both treatments lead to increased PS II activity. Thus, we conclude that membrane stacking functions to increase PS II primary photochemistry as well as PS II-PS II energy transfer [25,26]. We have previously shown that both proton- and metal cation-induced grana are accompanied by a decrease in PS I photochemistry [8]. Clearly, these three effects can be most simply explained on the basis of a decreased probability of energy transfer from PS II to PS I in the stacked configuration.

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