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POSSIBLE EFFECTS OF THE DETACHMENT OF STROMAL LAMELLAE FROM GRANAL STACKS ON SALT-INDUCED CHANGES IN SPILLOVER

A STUDY BY SONICATION OF CHLOROPLASTS

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Summary

Salt-induced chlorophyll fluorescence and spillover changes in control and briefly sonicated chloroplasts have been studied under conditions where Photosystem II traps are closed. In a low-salt medium containing 10 mM KCl, control envelope-free chloroplasts exhibited good spillover, as measured by low chlorophyll fluorescence yield at room temperature, a high ratio of the fluorescence peaks F_{735}/F_{685} at 77 K, and increased Photosystem I activity in the presence of 3-(3,4-dichlorophenyl)-1,1-dimethylurea and Photosystem II light. In contrast, when stacked chloroplasts were briefly sonicated and subsequently diluted into a low-salt medium, a high fluorescence yield at room temperature and a low ratio of F_{735}/F_{685} at 77 K persisted. When unstacked chloroplasts were sonicated and then diluted into a high-salt medium, the room temperature fluorescence yield remained low. The results are interpreted in terms of a model relating the changes in chlorophyll fluoresecence with the lateral diffusion of Photosystem I and Photosystem II chlorophyll-protein complexes in the plane of the thylakoid membrane creating randomized or segregated domains, depending on the degree of electrostatic screening of surface charges (Barber, J. (1980) FEBS Lett. 188, 1—10). It is argued that brief sonication of stacked chloroplasts separates stromal membranes from granal stacks, thus limiting the inter-mixing of the photosystems via lateral diffusion even when the ionic composition of the medium is varied. Consequently energy transfer from Photosystem II to Photosystem I is relatively poor and chlorophyll fluorescence from

Abbreviations: Chl, chlorophyll; $DCIPH_2$, reduced 2,6-dichlorophenolindophenol; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; EDTA, ethylenediaminetetraacetate; PS, Photosystem. Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid.

Photosystem II is enhanced. The loss of the salt effect on sonicated unstacked membranes can also be accomodated by the model. In this case it seems that the generation of small membrane fragments does not allow the normal salt-induced phase separation of the pigment-protein complexes to occur.

Introduction

A maximum efficiency of photosynthesis in red light approaches a value expected for the operation of the two photosystems in series [1]. When there is an unbalanced light input to the two photosystems, an optimal photosynthetic rate can be attained by a redistribution of the excitation energy between them. The possibility of energy transfer from PS II to PS I was originally recognized by Myers and Graham [2] who coined the term 'spillover' to describe the phenomenon. Murata [3] and Homann [4] discovered that cations control the excitation of the two photosytems in a way consistent with regulation of spillover. A number of studies on cation-induced chlorophyll fluorescence changes in 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU)-poisoned chloroplasts then followed (see Refs. 5—7), with the emerging picture that cation control of excitation energy distribution between the two photosystems could be both at the level of the initial quantal partitioning (characterized by α; Ref. 7) and that of spillover from PS II and PS I. A distinction between these two possibilities could be made, however, since a change in spillover, unlike that of α, would lead to a change in the lifetime of chlorophyll fluorescence accompanying a change in yield. Indeed Mg^{2+}-dependent changes in fluorescence lifetimes have been observed [8—10]giving support to the reality of spillover, although some effect on α may also be present [9,11,12].

The exact mechanism of cation control of spillover in broken chloroplasts remains to be elucidated. Suggestions have been put forward which invoke alterations of the separation (or mutual orientation) of PS II and PS I units (a) in adjacent membranes, interacting across the partition gap [13] or (b) in a bilayer membrane undergoing some conformation change, possibly leading to a change in membrane thickness [8]. Another suggestion is that cation-mediated associations between the PS II complex and the light-harvesting chlorophyll-protein complex are primary factors regulating spillover [14]. Extensive work from our laboratory has been done to test the idea that cations control the balance of forces between various protein complexes in the plane of the fluid thylakoid membrane, and between thylakoid membranes. Depending on the ionic composition of the bathing medium, the spatial distribution of PS II and PS I complexes, for example, may be random or non-random in the plane of a membrane, resulting in a corresponding statistical mean separation of the photosytems. This offers an explanation of cation effects on spillover, and associated changes in thylakoid membrane stacking [15—19].

Since the lateral displacement of protein complexes in the membrane is implicated in our proposed model, cleavage of thylakoid membranes into fragments is expected to alter the cation effects on spillover. The present study utilizes brief sonication of either stacked or unstacked chloroplasts to cleave thylakoid membranes in an attempt to test the current model further.

Materials and Methods

Envelope-free pea chloroplasts were prepared as described in Ref. 17 in the unstacked or stacked form by including 0.5 mM ethylenediaminetetraacetate (EDTA) or 1 mM $MgCl_2$, respectively, in the wash medium. The chloroplasts were stored on ice at 1 mg Chl/ml using the respective wash medium. An approx. 5 ml sample of the stock chloroplasts was taken for sonication by a 'Soniprobe' (Type 7530A, Dawe Instruments Ltd., U.K.) at low power (setting 3, approx. 1.5 A). An aliquot of the ice-cold sonicated stock was diluted 30- or 100-fold into the appropriate experimental medium when needed.

Spillover was monitored by (a) PS I activity using light absorbed preferentially by PS II; (b) steady-state chlorophyll fluorescence at room temperature, and (c) chlorophyll fluorescence peak heights at 77 K. Photosystem I activity was measured as the rate of O_2 consumption in the presence of methyl viologen. Chloroplasts were suspended in a room temperature basic medium containing 100 mM sorbitol, 10 mM KCl, 10 μM DCMU and 5 mM Hepes (pH 7.5, KOH) to which was added 20 μM methyl viologen, 1 mM sodium azide, 0.5 mM ascorbate, 50 μM 2,6-dichlorophenolindophenol (DCIP), 0.5 μM nigericin and 0.5 μM valinomycin (with or without 5 mM $MgCl_2$). Samples at a concentration of 30 μg Chl/ml were preincubated in the dark for 5—6 min. Actinic light intensity at 482 ± 5 nm was approx. 5 W/m^2 at maximum. Chlorophyll fluorescence was measured concurrently through the red filters Balzer B-40 693 plus Schott RG 695 and by means of a light guide inserted into the perspex O_2 electrode chamber. When chlorophyll fluorescence but not PS I activity was measured (Figs. 2A, 3A and 4), samples were diluted into the basic medium described above (with or without 5 mM $MgCl_2$) at 10 μg Chl/ml. All fluorescence values were normalized by setting that of a control chloroplast sample in the presence of 5 mM $MgCl_2$ equal to 100.

For fluorescence measurements at 77 K, chloroplast samples were sonicated for the durations indicated, diluted into the basic medium described above (with or without 5 mM $MgCl_2$, 10 μg Chl/ml), incubated for 5—10 min at room temperature and frozen in liquid nitrogen. Measurements of fluorescence were made using a Perkin-Elmer MPF 44A spectrofluorometer as described in Ref. 20. Samples were excited with 410 nm light (slit width 10 nm) and emission measured with slit width 4 nm.

Results

It is generally known (see Ref. 15) that isolated, broken chloroplasts suspended in a medium containing a few millimolar $MgCl_2$ exhibit a high chlorophyll fluorescence yield and extensive thylakoid stacking, and that a transition to a low fluorescence/unstacked state occurs on changing to a medium containing a low level of monovalent salt (e.g. 10 mM KCl). Basically the experiments reported below involve brief sonication of a concentrated stock of high fluorescence/stacked chloroplasts, followed by dilution 30- or 100-fold into a required salt medium. For a comparison, chloroplasts prewashed with EDTA to induce the low fluorescence/unstacked state were also sonicated prior to dilution.

Fig. 1 shows concurrent recordings of chlorophyll fluorescence and PS I

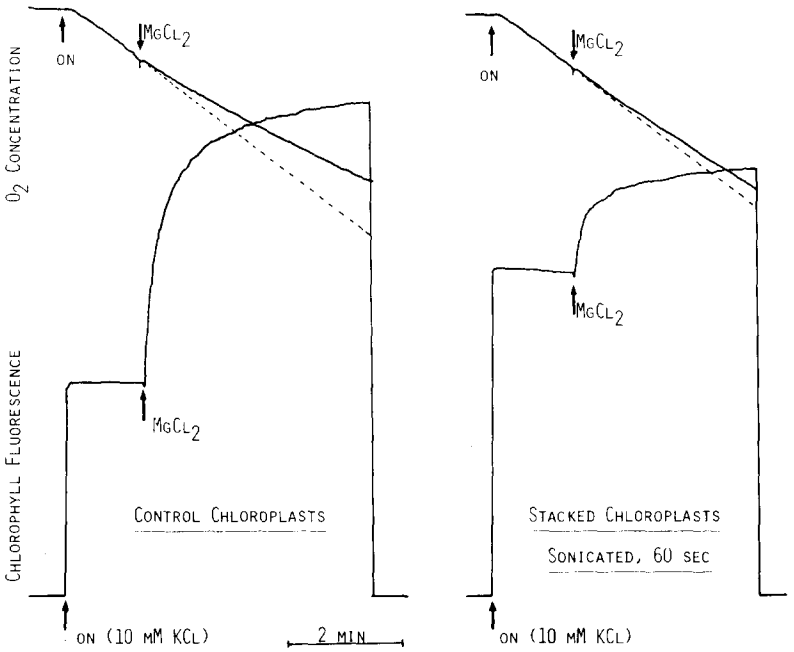

Fig. 1. Concurrent recording of O_2 consumption (PS I activity) and chlorophyll fluorescence before and after adding 5 mM $MgCl_2$. Excitation light intensity was 5 W/m^2 (482 nm). Control or sonicated stacked chloroplasts were diluted into the medium as described in Materials and Methods.

activity of chloroplasts diluted into a medium containing 10 mM KCl and DCMU. Prior to dilution, the chloroplasts were kept in 1 mM $MgCl_2$ and subjected to sonication for zero (control) or 60 s. It is noted that in the presence of 10 mM KCl, control chloroplasts attained a low fluorescence level, whereas the sonicated chloroplasts remained in a high fluorescence state. When 5 mM $MgCl_2$ was added to the suspensions, chlorophyll fluorescence increased by a smaller extent in the sonicated sample as compared with the control. PS I electron transport is given by the rate of O_2 consumption of DCMU-treated chloroplasts in the presence of methyl viologen and $DCIPH_2$ acting as the electron acceptor and donor, respectively. It is seen that the addition of $MgCl_2$ lowered PS I activity with a similar time course to that observed for the concomitant increase in fluorescence, and that the $MgCl_2$ effect on PS I activity was considerably reduced in the sonicated sample.

The effects of sonication time on chlorophyll fluorescence at room temperature and 77 K are shown in Fig. 2. Stock chloroplasts in 1 mM $MgCl_2$ were sonicated for the time durations indicated, and then diluted into a basic medium containing 10 mM KCl, with or without 5 mM $MgCl_2$. After measurement of the fluorescence at room temperature, the samples (with or without $MgCl_2$) were frozen in liquid nitrogen and the fluorescence peaks at 735 nm and 685 nm were recorded. As already indicated in Fig. 1, increasing the duration of sonication led to the maintenance of a high fluorescence state at room temperature, even in the absence of $MgCl_2$. As for the fluorescence peak ratios at 77 K, F_{735}/F_{685} was high in the absence, and low in the presence of $MgCl_2$

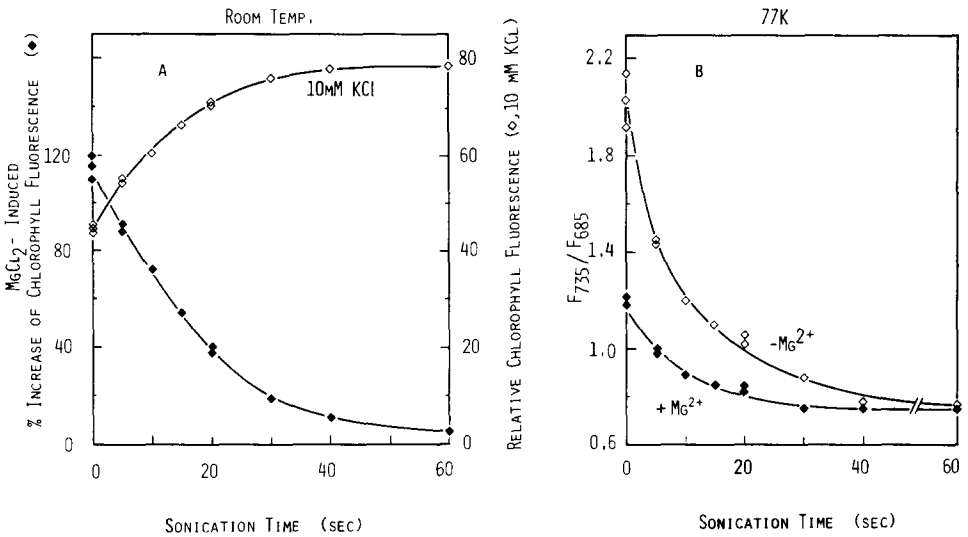

Fig. 2. Sonication of chloroplasts in the high fluorescence/stacked state. The sonicated mixture was diluted into a basic medium containing 10 mM KCl (with or without 5 mM $MgCl_2$). After incubation, chlorophyll fluorescence from samples (10 μg Chl/ml) was measured either at room temperature (A), or liquid nitrogen temperature (B). The cumulative sonication time is shown. In (A), the percent increase due to the addition of $MgCl_2$ is also shown (♦).

in control samples (zero sonication time), being attributable to high and low spillover, respectively. With increasing sonication time, the ratio F_{735}/F_{685} decreased in both sets of samples, but the change was greater in the absence of $MgCl_2$. Consequently, the two curves approach each other, so that the effect of $MgCl_2$ gradually disappears.

The above experiment on the effects of sonication time was also carried out with stock chloroplasts previously washed with 0.5 mM EDTA to induce the low fluorescence/unstacked state. Fig. 3A shows that upon dilution into a low-salt medium with 10 mM KCl, the suspensions remained in a low fluorescence state at room temperature for all durations of sonication, unlike the results of Fig. 2A. As in Fig. 2A it can be seen that the ability of 5 mM $MgCl_2$ to induce a rise in fluorescence declines with sonication time. A comparison of Fig. 3B and Fig. 2B reveals that at 77 K, there was a general decrease of F_{735}/F_{685} with sonication time in the absence of $MgCl_2$ in both types of chloroplasts fragments. A slight increase of the fluorescence peak ratio occurred in the $MgCl_2$-treated samples of Fig. 3B in contrast with the corresponding samples in Fig. 2B.

The biphasic variation of room temperature chlorophyll fluorescence with increasing monovalent cation concentrations in the presence of a low constant background of divalent cations was studied using control and sonicated chloroplasts. Fig. 4 shows that control chloroplasts exhibited the usual minimum in chlorophyll fluorescence levels with increasing monovalent salt. The level at very low or high monovalent salt concentrations was approx. 2.5-times as high as the minimum. Sonication of chloroplasts in high fluorescence/stacked state prior to dilution into the various salt media led to a more shallow minimum

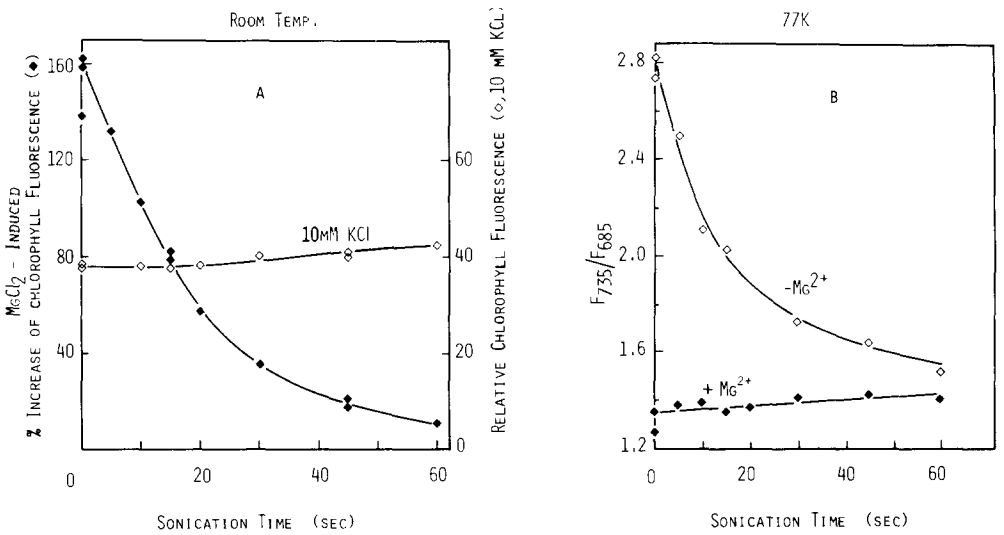

Fig. 3. Sonication of chloroplasts in the low fluorescence/unstacked state. Other conditions as in Fig. 2.

and retention of the high fluorescence state. On the other hand, sonication of chloroplasts already in the low fluorescence/unstacked state maintained a low and relatively constant fluorescence yield with increasing monovalent cation concentration.

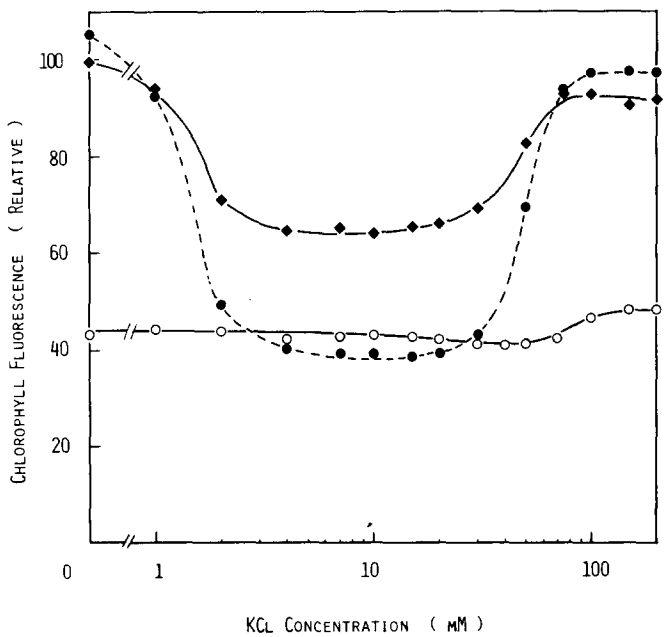

Fig. 4. The biphasic behaviour of chlorophyll fluorescence as a function of KCl concentration in the presence of 20 μM $MgCl_2$, studied in control chloroplasts (●), stacked chloroplasts sonicated for 20 s (◆) or unstacked chloroplasts sonicated for 30 s (○). Chlorophyll concentration was 10 μg/ml.

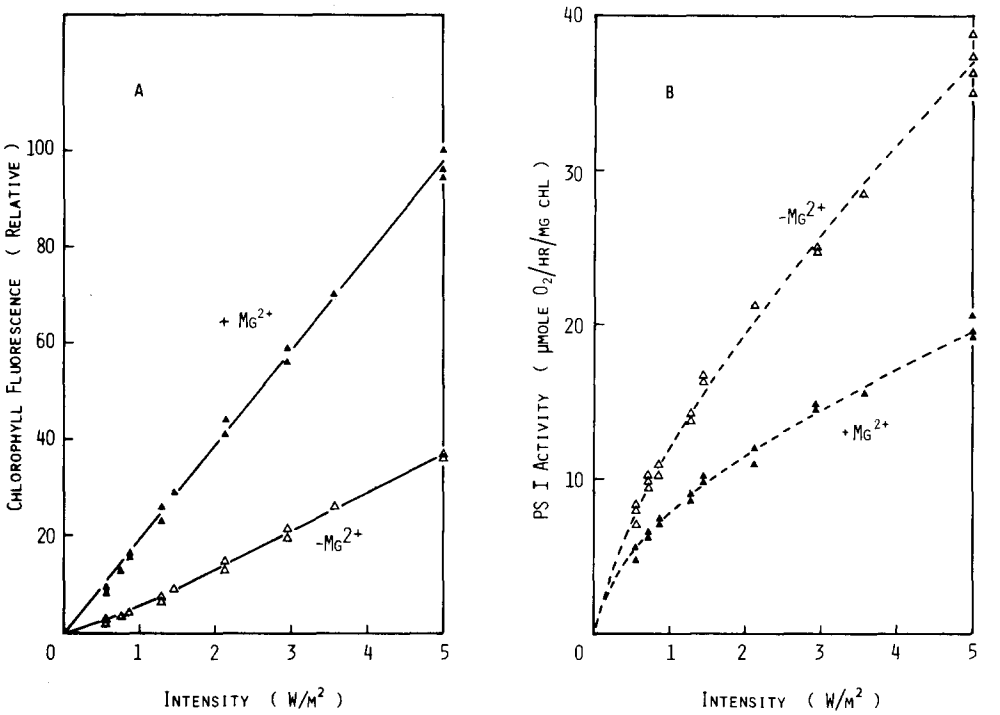

Fig. 5. Concurrent measurements of chlorophyll fluorescence (A) and PS I activity (B) for control chloroplasts. Chloroplast concentration after dilution was equivalent to 0.03 mg Chl/ml. The reaction medium (with or without 5 mM $MgCl_2$) is described in Materials and Methods. Light intensity was varied by neutral density filters.

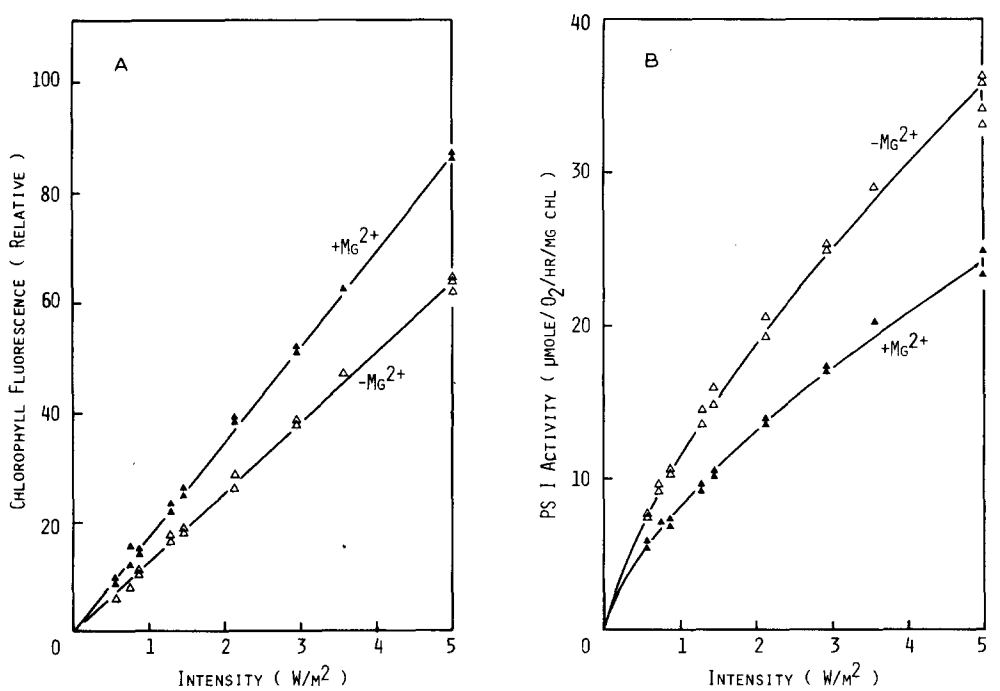

Fig. 6. Concurrent measurements of chlorophyll fluorescence (A) and PS I activity (B) for chloroplasts sonicated while in the high fluorescence/stacked state. Other conditions as in Fig. 5.

The intensity dependence of chlorophyll fluorescence and PS I electron transport rate, measured concurrently, is displayed in Fig. 5 for control chloroplasts and in Fig. 6 for chloroplasts sonicated for 60 s while in the high fluorescence/stacked state. A comparison of Fig. 5A and Fig. 6A reveals that the fluorescence intensity varies somewhat linearly with incident light internsity in all cases. The $MgCl_2$-induced fluorescence rise was greatly reduced in sonicated samples as compared with controls. In Figs. 5B and 6B the rate of PS I electron transport varied non-linearly with intensity. Sonication treatment did not alter the $MgCl_2$ effect on PS I activity as much as in the case of chlorophyll fluorescence.

Discussion

Electron micrographs have shown that brief sonication of stacked chloroplasts yielded granal stacks and vesicles, whereas similar treatment of mostly unstacked chloroplasts gave rise to a population of vesicles relatively devoid of grana [21,22]. In the former case, the technique represents a convenient means of detaching intergranal (stromal) lamellae from the basic structure of the granal stacks, without contamination from the use of a detergent. In the present study the resulting mixture of granal stacks and vesicles was found to be in a relatively high fluorescence state regardless of the presence or absence of $MgCl_2$ (Figs. 1 and 2A). Although not discussed in detail, a similar finding was reported by Murata [23] for French press chloroplasts. A simple interpretation is that, upon separation from the stacks, the stromal membrane vesicles with an enrichment of PS I can no longer effectively supply the fluorescence quenching centres ('secondary traps'; Ref. 24) to intermix with the PS II-enriched stacks by lateral protein diffusion. Such an interpretation is the basis of a model put forward to explain cation-induced changes of chlorophyll fluorescence, spillover and thylakoid stacking [15—19].

Vesicles derived from chloroplasts in the low fluorescence/unstacked state by sonication remained in the low fluorescence state regardless of the salt composition of the medium (Fig. 3A). Such vesicles have chlorophyll a/b ratios similar to that of the whole chloroplasts, and probably represent fragments of unstacked thylakoid membranes containing a random distribution of PS I and PS II complexes. Presumably these vesicles, though containing both PS I and PS II complexes, are subject to certain constraints such as size and curvature, so that domains of PS II-protein complexes cannot segregate from the PS I complexes when low levels of $MgCl_2$ or high levels of KCl are added as required by the lateral diffusion model [15]. This model requires the formation of heterogeneous domains of fluorescing and quenching complexes in order to account for the high fluorescence/low spillover state observed with normal thylakoid membranes.

A further distinction between control and sonicated chloroplasts can be found in the 'dip' behaviour of room temperature chlorophyll fluorescence as observed on increasing the KCl concentration in the presence of a low background concentration of $MgCl_2$ (Fig. 4). This dip behaviour [25—27] seems to be an expression of an electrostatic effect occurring immediately adjacent to the surfaces of the membranes and reflects changes in balance between

coulombic repulsive and Van der Waals attractive forces [19,27—30].

Fig. 4 clearly shows that sonication of stacked/high fluorescence chloroplasts helps to stabilize the high fluorescence state, in agreement with previous studies using digitonin-derived granal fragments [18]. In contrast, sonication of chloroplasts in the low fluorescence/unstacked condition maintained the low fluorescence state regardless of the salt regime. Although these results seem to agree with the lateral diffusion model they argue against the concept of local protein 'conformational changes' being responsible for changes in spillover, since such conformational changes should occur in both chloroplasts and their membrane fragments.

The ratio of the fluorescence peak heights F_{735}/F_{685} at 77 K is often taken as a measure of the degree of spillover [3,20,25,31], though artefacts are possible [32]. In Fig. 2B there is a general decline of F_{735}/F_{685} with sonication time, presumably due to the differences in the various sonicated samples altering the scattering and self-absorption properties of the solidified samples (see Ref. 32). Nevertheless, it is clear from Fig. 2B that with increasing sonication there is a loss in the ability of $MgCl_2$ to decrease spillover (as judged from the F_{735}/F_{685} ratio) since both curves (with or without Mg^{2+}) approach each other. A comparison between Figs. 2B and 3B reveals that, for the samples including $MgCl_2$, the fragments derived from chloroplasts in the low fluorescence state (Fig. 3B) maintained a higher F_{735}/F_{685} value or greater spillover (as compared with fragments obtained by sonication of chloroplasts already in the high fluorescence state, Fig. 2B).

When measured concurrently, PS I activity and chlorophyll fluorescence yield responded in opposite ways and with a similar time course to the addition of $MgCl_2$ (Fig. 1), as would be expected of the effect of this salt on spillover. However, in addition to affecting spillover, $MgCl_2$ may decrease PS I electron transport in another way, since with control chloroplasts in saturating light (broad band blue light, 250 W/m^2) the PS I rate was 200 and 278 μmol O_2/h per mg Chl in the presence and absence of $MgCl_2$ respectively. This difference may be attributed to an inhibition of PS I by $MgCl_2$, as has been reported by Bose et al. [33] for the same PS I reaction $DCIPH_2 \rightarrow$ methyl viologen. Such an inhibition would be superimposed on any decrease of spillover by $MgCl_2$. A dual effect of $MgCl_2$ on PS I activity could account for the seemingly smaller sensitivity of ΔV to sonication than ΔF, where ΔV and ΔF represent the $MgCl_2$-induced differences in PS I rates and chlorophyll fluorescence level, respectively (compare Figs. 5A with 6A, and 5B with 6B).

The mechanism of inhibition of the PS I reaction $DCIPH_2 \rightarrow$ methyl viologen is not clear. Bose et al. [33] found that another PS I reaction $DCIPH_2 \rightarrow$ NADP was stimulated by Mg^{2+} at all intensities. The opposite effects of Mg^{2+} on these two PS I reactions could be partly due to the control of the local concentration of charged substrates (positively charged methyl viologen, negatively charged ferredoxin and NADP) at the acceptor sites by the local negative surface potential the magnitude of which will be determined by the ionic composition of the medium [34].

In conclusion, the present experimental results suggest that cation-induced changes in spillover of excitation energy from PS II to PS I require the connection of stromal lamellae to granal stacks. It is suggested that once discon-

nected, the protein complexes from the two types of membranes cannot interact freely by lateral diffusion. Such a conclusion emphasizes the fluid and dynamic nature of the thylakoid membrane, and the possibility of the non-rigid nature of the Z-scheme itself.

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