CATION CONTROL OF CHLOROPLAST STRUCTURE AND CHLOROPHYLL *a* FLUORESCENCE YIELD AND ITS RELEVANCE TO THE INTACT CHLOROPLAST

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1. Introduction

Izawa and Good [1] and Ohki et al. [2] demonstrated that the stacking of thylakoid membranes to form grana is closely related to the cationic composition of the chloroplast suspending medium. Stacking is retained when 3–5 mM divalent cations e.g. Mg^{2+} or Ca^{2+} or 100–200 mM monovalent cations are present in the medium. If the cation concentration is lowered appreciably unstacking occurs but it can be restored by increasing the cation concentration again. Murakami and Packer [3] first showed that this cation control of stacking correlates with the Q independent cation control of fluorescence yield [4,5]. To date experiments with broken chloroplasts show that when stacking occurs there is a high yield of fluorescence and in the unstacked state the yield is low [3,6]. We have investigated cation control of chloroplast conformational changes and fluorescence yield and have found that there are conditions under which this correlation does not hold.

2. Methods

Intact chloroplasts were isolated from spinach or peas as described previously [7]. Experiments were carried out in a medium containing 0.33 M sorbitol, 50 mM Hepes (brought to pH 7.6 with KOH), 1 mM $MgCl_2$, 1 mM $MnCl_2$ and 2 mM EDTA. Where necessary the chloroplasts were shocked briefly in distilled water and medium, double strength of that described above, was added. The chlorophyll concentration was measured by the method of Arnon [8] and was approx. 30 μg/ml in all experiments.

Chlorophyll fluorescence measurements at room temperature were measured as described previously [9]. For low temperature fluorescence measurements 0.1 ml samples of diluted chloroplasts were frozen, after the stated pretreatment, in cylindrical silica quartz tubes in liquid nitrogen. The tubes were kept frozen in a clear dewar and the emission was detected at right angles to the excitation beam. The emission spectra were measured using a Perkin Elmer MPF4 spectrofluorimeter fitted with an R446F photomultiplier. The spectra were not corrected for the spectral sensitivity of the photomultiplier–monochrometer combination. The samples were excited with 435 nm light (slit width 20 nm). The intensity was sufficient to reduce the primary acceptor of photosystem two, Q, in all samples. The emission slit width was 5 nm and a 430 nm filter was used to eliminate second order components of the exciting beam. The motor driven monochrometer scanned the fluorescence emission spectrum at 60 nm/min.

Samples for electron microscopy were fixed by addition of an equal volume of 1% glutaraldehyde plus 0.23 M sorbitol and 0.01 M Hepes, pH 7.6, to the chloroplast suspension in assay medium, after the stated pretreatment. Where indicated Mg^{2+} was present in both the assay medium and the glutaraldehyde. Samples were incubated for 2 min in the presence of glutaraldehyde under the same pretreatment conditions as described in the figure legends and were then incubated on ice for 15 min. The samples were centrifuged in a Beckman microfuge for 2 min and the pellets were washed 3 times in distilled water over a period of 1 h. They were then postfixed with osmium tetroxide and chromate according to the method of Dalton [10], dehydrated with ethanol,

embedded in Epon 812 and stained with uranyl acetate and lead citrate. They were examined in an AEI 6B electron microscope.

The intensity of the pretreatment illumination of the samples for low temperature emission spectra and electron microscopy was 70 kergs cm^{-2} sec^{-1} of blue/green light.

3. Results

Fig.1a shows the light induced, dark reversible, fluorescence quenching pattern typically seen with intact chloroplasts which in this experiment are utilizing PGA as terminal electron acceptor. The quenching is inhibited by uncouplers, e.g. nigericin (see [11,12]). Fig.1b shows how chloroplasts from which the outer membrane has been removed and which are suspended in a medium containing a low level of monovalent cations, but no divalent cations, have lost these fluorescence characteristics. These chloroplasts retain a slow rate of electron transport and consequently generate a high energy state [13]. Fig.1b also shows that addition of divalent cations reinduces the high fluorescing state but that it is quenched under illuminated conditions.

Fig.2 shows electron micrographs of chloroplasts in the various fluorescence states seen in fig.1b and table 1 shows the actual fluorescence yield of the chloroplast samples just prior to fixation with glutaraldehyde. Fig.2a shows that thylakoid membranes suspended in a medium of low ionic strength exhibit the characteristic unstacking that has been reported by many others [1,2]. Illumination of these chloroplasts, which in this experiment caused a further slight quenching of fluorescence (table 1), does not bring about any change in the degree of thylakoid stacking (fig.2b). Addition of 5 mM $MgCl_2$ under these illuminated conditions does not increase the fluorescence yield significantly (table 1) but causes extensive restacking of the thylakoid membranes (fig.2c). The extent of stacking is not altered by a dark period (fig.2d) although this allows a two-fold increase in fluorescence yield (table 1). Further illumination brought about quenching of fluorescence to the initial low level but it did not alter the stacking (data not shown).

Next we investigated the effect of light on thylakoid stacking in intact chloroplasts. Figs.3a and 3b show that illumination does not affect the degree of thylakoid stacking although the fluorescence yield was quenched (table 1). Likewise figs.3c and 3d and table 1 show that illumination did not affect the stacking of chloroplasts shocked in a medium containing 5 mM $MgCl_2$ although fluorescence was quenched.

We have also investigated the effect of cations and illumination on the low temperature fluorescence emission spectra of intact and shocked chloroplasts.

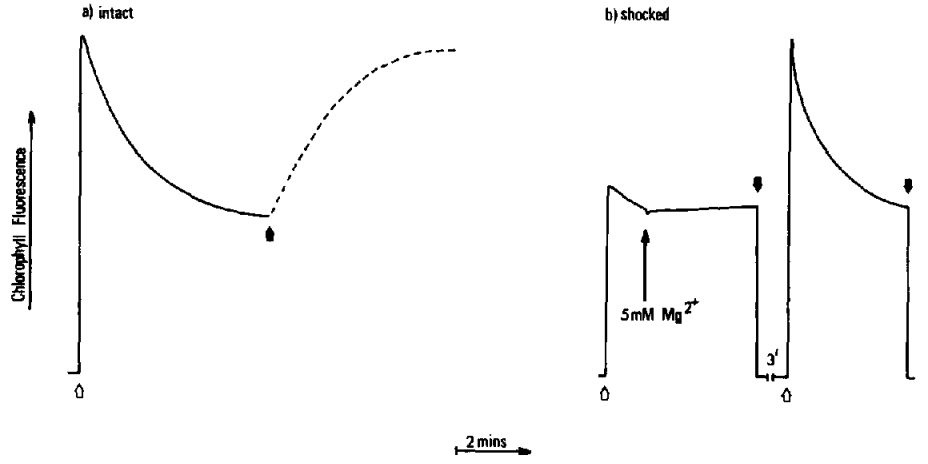

Fig.1. Comparison of fluorescence quenching characteristics of (a) intact chloroplasts and (b) shocked chloroplasts isolated from peas. Open and closed arrows indicate light on and off respectively. In curve (a) the dotted line represents the time course of the dark reversal of the fluorescence quenching measured by several brief periods of illumination.

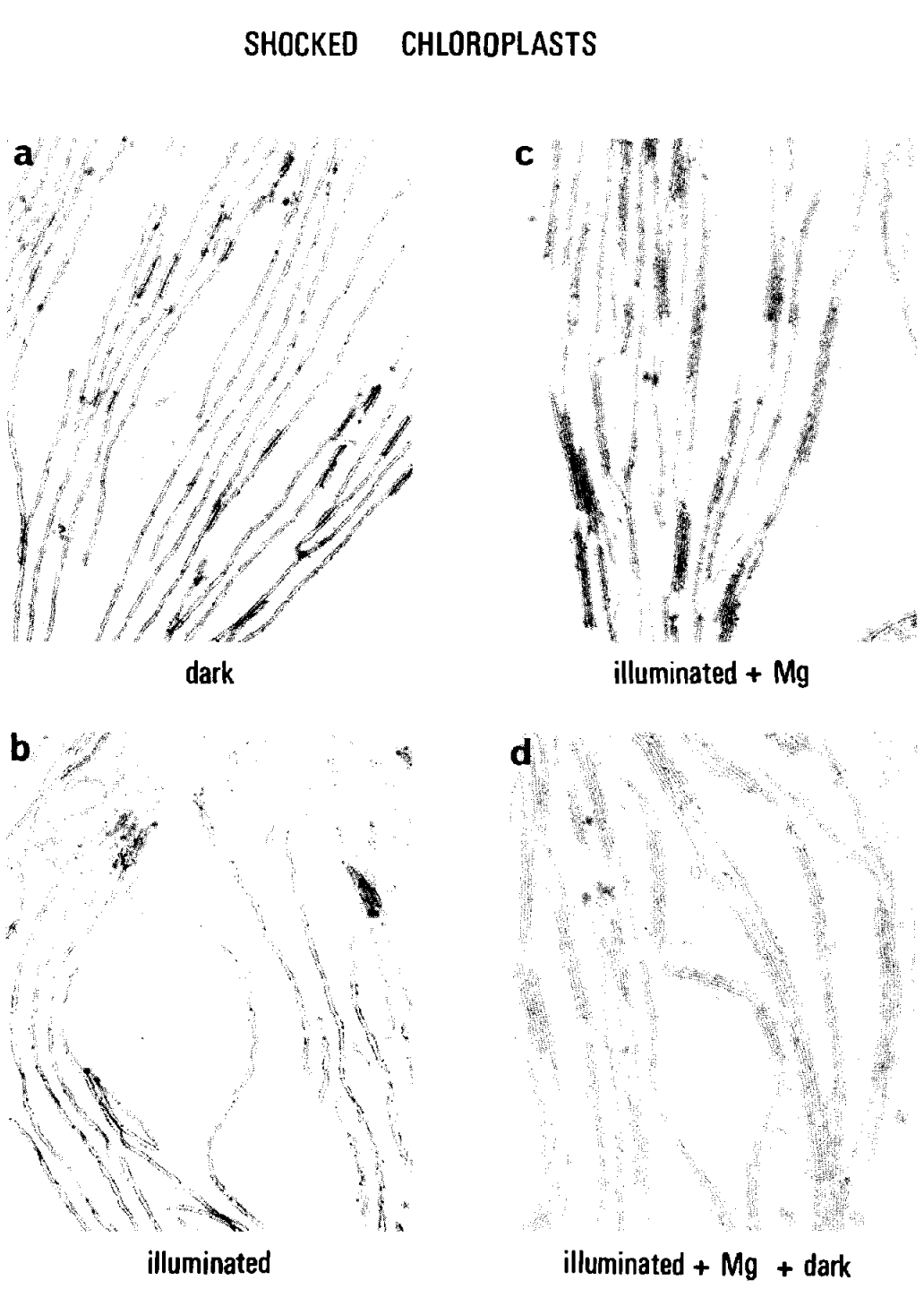

Fig.2. The effect of illumination and Mg^{2+} on the structure of shocked pea chloroplasts. 5 mM $MgCl_2$ was added where indicated during the period of illumination. The total incubation was 5 min in all cases (plus a 3 min dark reversal in (d) only). 0.3 μM methyl viologen was present in all reactions. Magnification × 27 000.

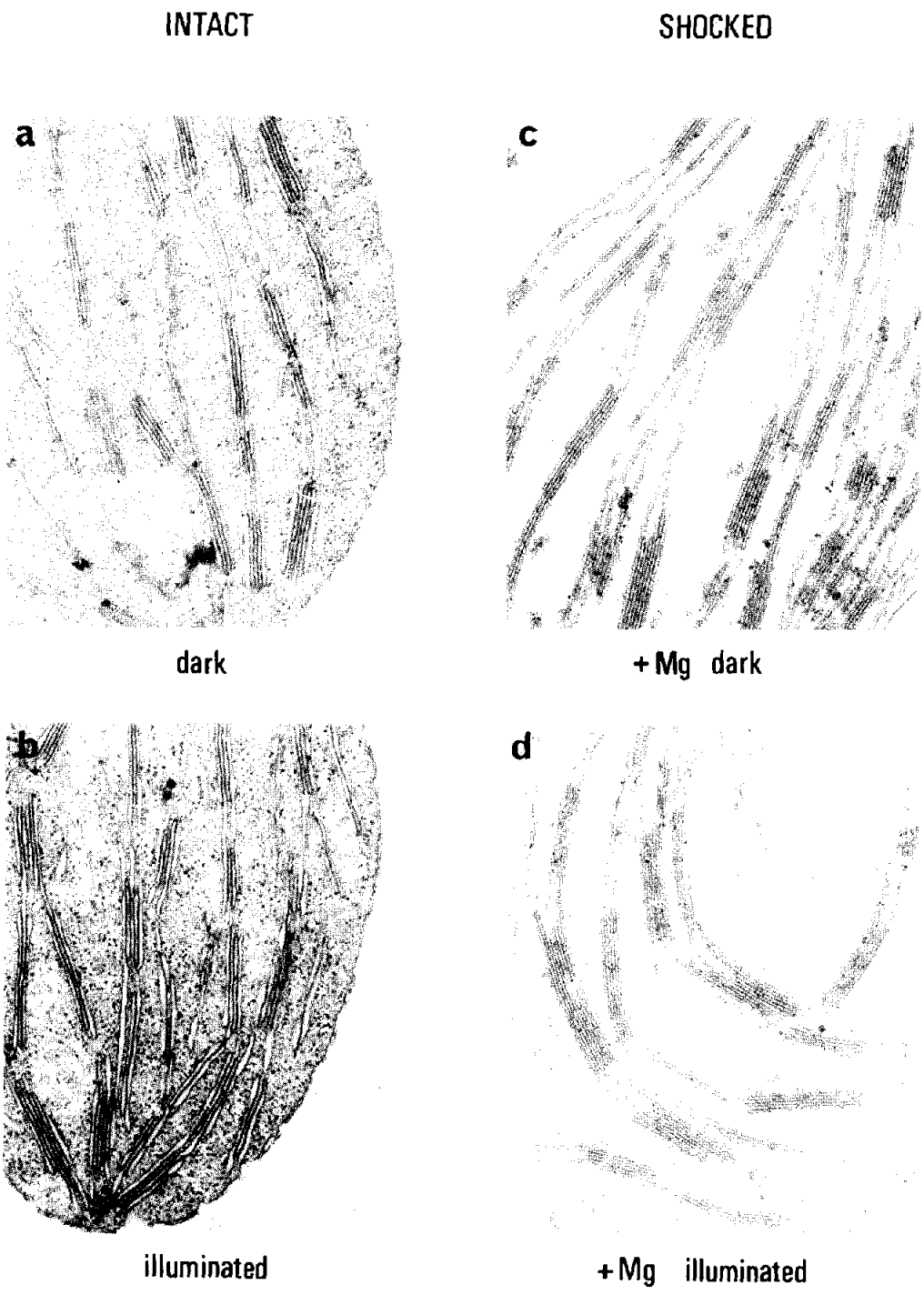

Fig. 3. The effect of illumination on the structure of pea chloroplasts. In (a) and (b) intact chloroplasts were incubated for 5 min under dark or light conditions in the presence of 1 mM PGA. In (c) and (d) chloroplasts were shocked in 5 mM $MgCl_2$ and incubated for 3 min under dark or light conditions. Magnification × 27 000.

Table 1
Fluorescence yield at room temperature of samples for electron microscopy just prior to fixation

Figure number	Experimental conditions	Fluorescence yield
	Shocked chloroplasts minus Mg^{2+}	
2a	Dark	33
2b	Illuminated	22
2c	Illuminated, Mg^{2+} added during incubation	26
2d	Illuminated, Mg^{2+} added during incubation, dark reversed	50
	Intact chloroplasts	
3a	Dark	54
3b	Illuminated	26
	Shocked chloroplasts plus Mg^{2+}	
3c	Dark	64
3d	Illuminated	41

Fig.4a shows the spectra obtained with samples of intact chloroplasts which were either pre-illuminated with bright light at room temperature and while being frozen or were incubated in the dark for the same length of time at room temperature and were subsequently frozen in the dark. The spectra were normalized at 685 nm and it can be seen that illumination caused an increase in the 735 nm peak height i.e. a decrease in the F685/F735 ratio. Fig.4b shows the difference in the spectra obtained if $MgCl_2$ was added to shocked chloroplasts in the dark at room temperature or if it was added in the light after a short pre-illumination period. These spectra were not normalised. It can be seen that when $MgCl_2$ is added after a high energy state has been established there is both a decrease in the 685 nm peak and an increase in the 735 nm peak. The absolute signal sizes cannot be compared too closely as there was a small variation in signal size due to slight differences in the size and shape of the silica quartz sample tubes. However it can be seen that in the illuminated sample the F685/F735 ratio was lower than it was in the dark sample.

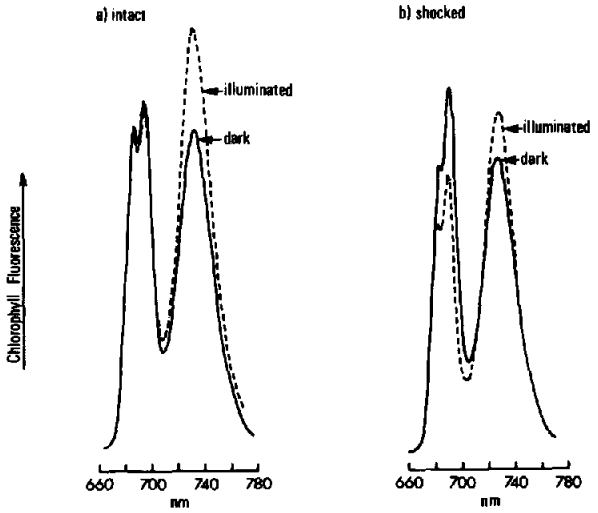

Fig.4. Low temperature fluorescence emission spectra of spinach chloroplasts. In (b) 5 mM $MgCl_2$ was added during the incubation in dark or light.

Table 2 shows the F685/F735 ratios of chloroplasts in the various fluorescence states illustrated in fig.1. Intact chloroplasts show a decrease in this ratio on illumination which is partially reversible in the dark. The room temperature fluorescence data on these particular samples showed a small irreversible quenching which also occurred in the presence of nigericin and which has frequently been shown to occur during the initial illumination period of intact chloroplasts. Shocked chloroplasts in $MgCl_2$ show a high F685/F735 ratio (similar to that seen with intact chloroplasts) while in the absence of $MgCl_2$ the ratio is much lower. The decrease, about 40%, is

Table 2
The effect of illumination on the 77°K fluorescence emission spectra of intact and shocked pea chloroplasts

Experimental conditions	Ratio of fluorescence intensity of 685 nm and 735 nm emission peaks (F685/F735)
Intact chloroplasts[a]	
Dark	0.97
Illuminated (11 min)	0.75
Illuminated (11 min), dark (5 min)	0.88
Shocked chloroplasts[b]	
+ Mg^{2+} dark	0.99
− Mg^{2+} dark	0.62
− Mg^{2+} illuminated (3 min)	0.59
− Mg^{2+} illuminated (1 min) Mg^{2+} added, illuminated (6 min)	0.69
− Mg^{2+} illuminated (1 min), Mg^{2+} added, illuminated (1 min), dark reversed (5 min)	0.87

[a] Chloroplasts suspended in assay medium as described in Methods.
[b] Chloroplasts shocked in distilled water and made up with double strength medium. Final reaction mixture: 0.33 M sorbitol, 20 mM Hepes, pH to 7.6 with KOH (final concentration of K^+, 30 mM). 5 mM $MgCl_2$ was added where indicated.

in the same order as that seen by others e.g. Murata [4] who found a 35% decrease. Illumination of shocked chloroplasts in the absence of Mg^{2+} caused a further slight decrease in the ratio and addition of Mg^{2+} in the light caused a slight increase. However the ratio was still low compared to the ratio found after a dark period. The latter treatment increased the ratio to the same level that was seen in the intact chloroplasts after a dark reversal period.

4. Discussion

We have found that the presence or absence of thylakoid stacking is directly correlated with the presence or absence of divalent cations but not always with high or low fluorescence yield. Fluorescence yield is controlled by the presence or absence of a high energy state as well as cation level. Addition of Mg^{2+} in the light to chloroplasts which have generated a high energy state did not increase the fluorescence yield although it did induce stacking.

This is similar to the situation seen with light scattering changes and fluorescence yield. In early experiments these changes appeared to be correlated [3,11] but Jennings and Forti [14] found that in *Euglena* chloroplasts the two processes could be distinguished kinetically and Krause [15] showed, using a similar experiment to that illustrated in fig.1b, that Mg^{2+} added in the light brings about the light scattering increase although the fluorescence increase only occurs after a dark period.

We have also shown that chloroplasts under conditions similar to those found in vivo (i.e. intact chloroplasts utilizing PGA as terminal electron acceptor for chloroplasts shocked in Mg^{2+} carrying out the Mehler reaction) do not show any light induced changes in thylakoid stacking although the high fluorescence yield is quenched to a low yield.

There have been other investigations of the effect

of light on intact chloroplasts to see whether there are any changes in thylakoid stacking that correlate with the change from State 1 (high fluorescence yield) to State 2 (low fluorescence yield) originally demonstrated by Bonaventura and Myers [16]. Bennoun and Jupin [17] showed that chloroplasts of *Chlamydomonas reinhardi* adapted to State 2 had a lower average number of thylakoids per grana stack than those adapted to State 1. However this change was not as marked as the change they induced by the absence of divalent cations. Vernotte et al. [18], using chloroplasts isolated with glutaraldehyde in the grinding medium from whole pea plants previously adapted to State 1 or State 2, did not find any change in thylakoid stacking.

It seems reasonable to suggest that cations have at least two different effects on membrane conformation one of which brings about 'large scale' alterations of the membrane's conformation (seen as changes in light scattering and thylakoid stacking) and the other 'small scale' alterations possibly similar to those proposed by Seely [19] to explain cation control of energy distribution between the two photosystems (seen as changes in fluorescence yield).

Evidence for changes in energy distribution between the two photosystems is provided by the low temperature emission spectra data presented here. Under conditions when fluorescence at room temperature, which comes mainly from photosystem 2, is quenched (absence of cations or presence of a high energy state) there is a corresponding increase at 77°K in the yield of the 735 nm fluorescence peak, which is thought to come from photosystem 1. This was found to be true for both the cation induced fluorescence yield changes (table 2 and fig.4), and the high energy state induced changes using physiological electron acceptors (table 2) which thus equates these fluorescence states with the State 1/State 2 adaption already studied in intact systems [16,18,20,21].

Overall our results support the concept that the slow fluorescence yield changes seen with intact chloroplasts or with well coupled broken chloroplasts treated with Mg^{2+} reflect changes in transfer of energy between photosystems 2 and 1. Our results do not, however, implicate cation induced thylakoid stacking with this energy transfer process.

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