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CATION CONTROL OF CHLOROPHYLL *a* FLUORESCENCE YIELD IN CHLOROPLASTS

LOCATION OF CATION SENSITIVE SITES

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SUMMARY

We have compared the effect of various cations on the fluorescence yield of chloroplasts under several different conditions in vitro.

1. In the absence of a high energy state but in the presence of low (10 mM) concentrations of monovalent cations, divalent metal cations increase steady state chlorophyll *a* fluorescence yield in a manner which does not involve transport of these cations across the thylakoid membranes.

2. The kinetics of this cation-induced fluorescence rise are relatively slow, and seem to reflect cation binding (or subsequent conformational changes) to sites on the outer surface of the thylakoid membrane.

3. In the absence of monovalent cations, the apparent binding constant for Mg^{2+} to sites on the outer side of the membrane is low. Addition of low concentrations of monovalent cations (10 mM) competitively inhibits divalent cation binding.

4. Control of fluorescence yield is also exerted by the high energy state, and seems to involve proton/metal cation exchange at sites on the inner side of the thylakoid.

5. When isolated chloroplasts are washed and resuspended in a medium containing no added cations, the initial fluorescence level is high, but is quenched on addition of monovalent cations, sodium EDTA being much more effective than sodium chloride. It is argued that when isolated under these conditions, chloroplasts retain sufficient divalent cations to saturate external negative sites, and that the fluorescence lowering is due to their removal.

6. Some other cations, such as poly(L-lysine), can displace divalent cations from their external sites in an irreversible manner, resulting in a fixed low fluorescence yield.

Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; HEPES, *N*-2-hydroxyethyl piperazine-*N*-2-ethanesulphonic acid; Tris, tris-(hydroxymethyl)-methylamine.

INTRODUCTION

Although the redox state of the primary electron acceptor, Q, of Photosystem II can control the yield of in vivo chlorophyll *a* fluorescence [1], there are other factors which affect the intensity of this emission. Several years ago it was shown that the addition of metal cations to broken chloroplast preparations increased the steady-state fluorescence yield [2–4]. Since the chloroplasts used had been treated with DCMU, the changes were not associated with changes in the redox state of the Photosystem II traps, and have in fact been implicated with a decrease in spillover of excitation energy from Photosystem II to Photosystem I (refs. 3, 4). More recently, Gross and Hess [5] have found that the cation sensitive fluorescence changes are more complex than originally thought. Using DCMU-treated broken chloroplasts suspended in a medium almost entirely free of organic and inorganic cations, they have shown that the chlorophyll fluorescence is initially high, and drops to a less intense level on addition of low levels of monovalent cations (2 to 10 mM). This lower fluorescence yield corresponds to the starting level of the experiments reported in the earlier papers [2–4], since in these experiments the chloroplasts were suspended in media already containing monovalent cations at sufficient concentrations to cause maximal quenching of the fluorescence yield. Addition of low concentrations of divalent cations (5 mM) or high concentrations of monovalent cations (100 mM), under these conditions [2–4], increases the yield back to the higher level.

It has also been found that fluorescence quenching can occur, in the presence of DCMU, when a high energy state is generated by coupled, cyclic electron transport through Photosystem I (refs. 6, 7).

In previous reports [8–11], we have found, as has Krause [12, 13], that under various conditions in which Q remains largely reduced, the high energy state and cation-dependent changes of chlorophyll fluorescence seem to be inter-related. Experiments with isolated, intact (class A, ref. 14) chloroplasts [8–13], broken chloroplasts treated with metal cations which have low rates of coupled non-cyclic electron flow [8, 9, 11, 12], and also with broken chloroplasts treated with DCMU and low levels of cofactors which promote cyclic electron flow (ref. 15 and Mills and Barber, unpublished), indicate that the high energy state induced fluorescence quenching can be explained by displacement of metal cations (thought to be Mg^{2+} in vivo, ref. 10) from sites on the inner side of the thylakoid membrane in response to electrogenic proton pumping. This interpretation suggests a "membrane sidedness" for the metal cation effects on fluorescence, and the experiments reported in this paper were designed to test this hypothesis.

MATERIALS AND METHODS

Intact chloroplasts were isolated by the method of Stokes and Walker [16] from spinach leaves, or from leaves of English Cos lettuce obtained from the local market. Intact chloroplasts were also isolated from 14 day old peas (Feltham First) grown in vermiculite without nutrient. Results were essentially identical whatever the source of material used. Broken chloroplasts were prepared in the measuring cuvette by subjecting intact chloroplasts to an osmotic shock in water, followed by immediate addition of double-strength suspending medium. Three different media

have been used: assay medium (0.33 M sorbitol, 2 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 50 mM HEPES/KOH, pH 7.6); low salt buffer (0.33 M sorbitol, 10 mM HEPES/Tris, pH 7.6); and Gross and Hess type medium (0.1 M sorbitol, Tris base to pH 7.2, which corresponds to about 0.15 mM Tris).

Chlorophyll fluorescence was induced by a broad band blue/green actinic beam transmitted by 2 mm Schott BG18, 2 mm Schott BG38 and Balzers Calflex C filters to give an intensity of 80 $J \cdot m^{-2} \cdot s^{-1}$ at the cuvette. The fluorescence emission was detected at right angles with an EMI 9558B photo-multiplier, screened by a Balzer 695 nm interference filter and appropriate Schott red cut-off filters to eliminate scattered actinic light. Measurements were made with chloroplasts suspended in a 10×10 mm glass cuvette at a chlorophyll concentration of 5–15 $\mu g \cdot ml^{-1}$.

Ionophore A23187 and Nigericin were obtained from Eli Lilly, Indianapolis, while Gramicidin D and poly(L-lysine) ($M_r \approx 2000$) were purchased from Sigma. All other reagents used were of Analar grade or equivalent.

RESULTS

When intact chloroplasts are subjected to an osmotic shock and resuspended without added electron acceptor in a medium containing low levels of monovalent cations (2–10 mM), they show sufficient endogenous Mehler reaction to maintain a substantial pH gradient across the membrane [12]. Under these conditions, a few seconds of illumination of high intensity (80 $J \cdot m^{-2} \cdot s^{-1}$) results in steady-state chlorophyll fluorescence corresponding to full reduction of Q [1].

The resulting increase in fluorescence on adding 100 mM KCl to such chloroplasts depends on the coupling state of the membranes as shown in Fig. 1. Two interesting features are evident, firstly that little increase in yield is observed until the uncoupler nigericin is added (Fig. 1a), and secondly that the kinetics of the fluorescence rise after addition of this ionophore are considerably faster than those observed when the uncoupler is added before injection of KCl (Fig. 1b).

In principle, the kinetics of the fluorescence increase could be limited by one of three processes; (i) transport of the cation across the membrane, if the sites of action are located on the inner side of the thylakoid; (ii) the binding process itself, or (iii) the membrane conformational changes which presumably alter the chlorophyll environment subsequent to cation binding. Thus, if the rise kinetics in Fig. 1 represent the action of cations at sites on the inner side of the membrane, one possible explanation for the difference in rise time would be different rates of cation transport across the membrane. Cation entry would normally be slow in uncoupled chloroplasts as seen in Fig. 1b, and very slow or absent in coupled chloroplasts where proton uptake would tend to drive cations out of the thylakoid interior (Fig. 1a). However, addition of uncoupler would then speed up cation entry (and hence the rise in fluorescence) over that normally seen, by promoting rapid H^+/cation exchange across the membrane as the pH gradient collapses.

In order to investigate whether the membrane permeability to cations is the overall rate-limiting step, we have studied the effect of specific ionophores on the rate of cation-induced increase in fluorescence in the absence of a proton gradient. It was found that in DCMU-treated chloroplasts, neither ionophore A23187 nor nigericin had any significant effect on the kinetics of the Mg^{2+} or K^+ induced rise.

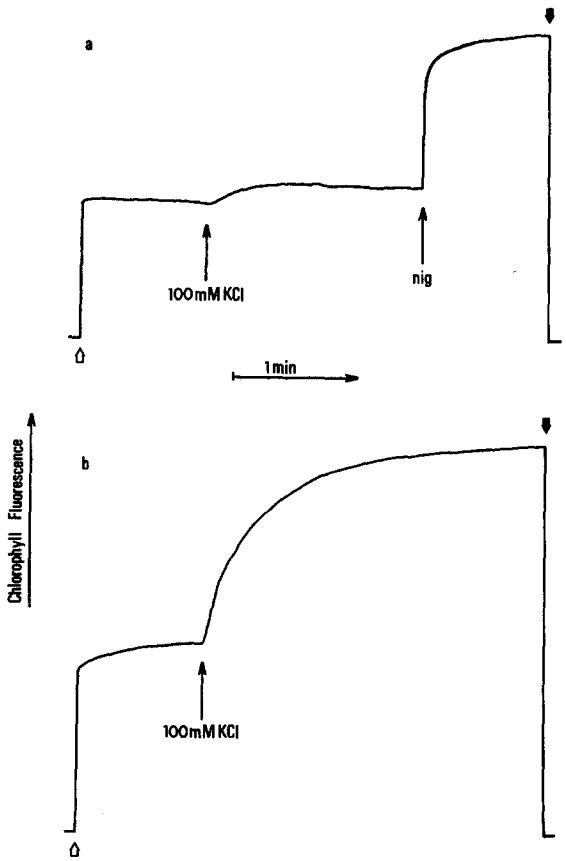

Fig. 1. The effect of nigericin on the rate of the K^+ induced rise in chlorophyll fluorescence yield. The shocked pea chloroplasts were suspended in assay medium (a) $1.8 \cdot 10^{-7}$ M nigericin (nig) was added 2 min after KCl (b) $1.8 \cdot 10^{-7}$ M nigericin was added before illumination. Other conditions as in Materials and Methods.

Similar negative results were obtained when gramicidin was used in place of, or together with nigericin.

These were surprising results and emphasise that in the absence of a proton gradient, the cation-induced rise in fluorescence is not controlled by the permeability of the membrane.

However, the results in Fig. 1 can be explained if fluorescence can be influenced by cations at more than one site, and we explore this possibility in the experiments reported subsequently in this paper.

If sites on the outer surface of the thylakoid membrane are involved in cation sensitive fluorescence yield changes these should be observed on the addition of impermeable, as well as permeable, cations provided that steric factors do not interfere with surface binding. Table I lists some cations which increase the fluorescence yield and it is relevant to note that the choline cation is almost as effective ($C_{\frac{1}{2}} \approx$ 95 mM) as K^+($C_{\frac{1}{2}} \approx$ 65 mM) but equiosmolar sucrose has no effect. We also found that the high fluorescence state created by choline addition to dark coupled chloro-

TABLE I

THE EFFECT OF VARIOUS ADDITIONS ON CHLOROPHYLL FLUORESCENCE OF SHOCKED PEA CHLOROPLASTS TREATED WITH DCMU

Isolated intact chloroplasts were shocked in water and double strength low salt buffer was then added. The fluorescence values were the constant level which was reached approximately 5 min after addition. $2 \cdot 10^{-5}$ M DCMU was present throughout.

Addition	Fluorescence yield (arbitrary units)	Concentration for half effect ($C_{\frac{1}{2}}$) (mM)
None	30	–
Sucrose (0.4 M)	29	–
KCl (0.2 M)	76	65
Choline Cl (0.2 M)	63	95
$MgCl_2$ (5 mM)	73	0.8

plasts showed the same light induced high energy state quenching as observed with metal cations in the medium [8–13]. This light-induced quenching observed in the presence of high concentrations of choline chloride was inhibited on addition of uncouplers.

These results suggest that external membrane binding sites can control the cation induced fluorescence rise, and we have considered this further by investigating the antagonistic effects of low concentrations of monovalent and divalent cations on fluorescence yield as reported originally by Gross and Hess [5]. In all the experiments discussed so far, the chloroplast suspending media contained sufficient monovalent cations to create the low fluorescence state. However when chloroplasts are suspended

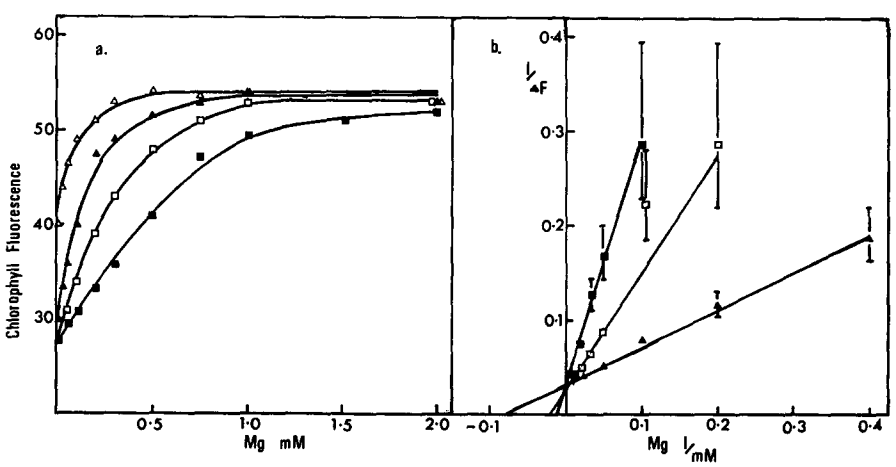

Fig. 2. Competitive inhibition by K^+ of the Mg^{2+} stimulated increase in chlorophyll fluorescence. In this case, (a), chloroplasts were washed in 0.5 mM EDTA, (pH 7.1) resuspended in a Gross and Hess type medium, and preincubated for 15 min with 10^{-5} DCMU and appropriate cations before noting the final fluorescence level. △, No KCl; ▲, 1 mM KCl; □, 3 mM KCl; ■, 10 mM KCl. (b) double reciprocal plot of the data in (a).

in a Gross and Hess type cation free medium the fluorescence is relatively high. As the vertical axis of Fig. 2a shows, the addition of low levels of K^+ under these conditions quenches fluorescence. This quenching is observed on addition of low concentrations of both inorganic and organic cations. When Mg^{2+} is then added, the fluorescence rises to the high level but the $C_{\frac{1}{2}}$ for this Mg^{2+} induced rise is increased as the K^+ level in the medium is raised from 0 to 10 mM. The reciprocal plot of the data shown in Fig. 2b seems to suggest that K^+ competitively inhibits the Mg^{2+} induced rise, (although there is some deviation from linearity near saturating Mg^{2+} concentrations).

Fig. 2 also shows that in the absence of both Mg^{2+} and K^+, the fluorescence yield was intermediate between the low and high fluorescence levels. This initial level was found to vary from preparation to preparation and tends to be reduced by subjecting the chloroplasts to washing in solutions which contain some monovalent cations. Under conditions when the initial fluorescence was low the addition of very low concentrations of Mg^{2+} created the high fluorescence state which could then be reduced to a low level on introducing monovalent cations to the medium (see Fig. 3 but also can be seen in Fig. 2). Additions of higher levels of Mg^{2+} then reversed this change and restored the chloroplasts to the high fluorescence state in the usual way (see Fig. 3).

The above results clearly demonstrate the competitive nature of low concentrations of divalent and monovalent cations on the fluorescence yield and further suggest that the high yield seen under low ionic conditions (i.e. in the Gross and Hess

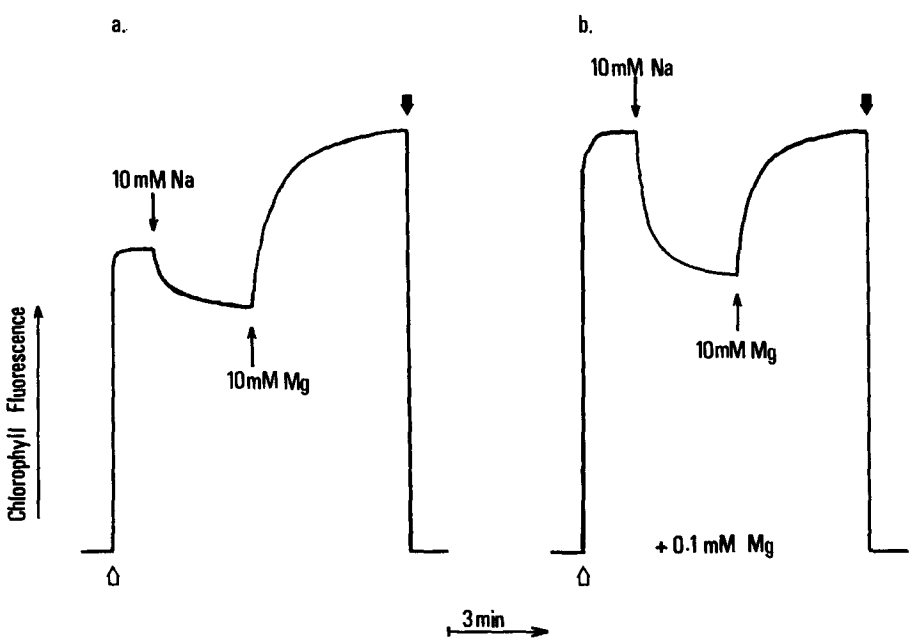

Fig. 3. Kinetics of the competitive effects of Na^+ and Mg^{2+} on chlorophyll fluorescence. Chloroplasts were washed in 2 mM KCl and resuspended in a Gross and Hess type medium containing 10^{-5} M DCMU. (a) No further additions, (b) preincubated for 5 min with 0.1 mM Mg^{2+}.

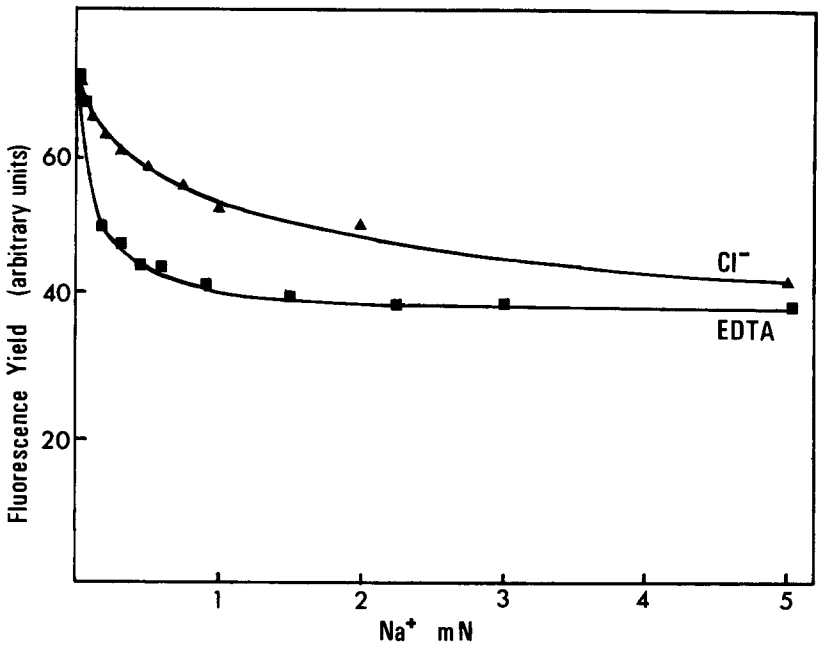

Fig. 4. Increased effectiveness of trisodium EDTA over NaCl on quenching of chlorophyll fluorescence observed at very low ionic strength. Chloroplasts were preincubated for 15 min in a Gross and Hess type medium containing 10^{-5} M DCMU and appropriate cations, before noting the final fluorescence level. Triangles, NaCl; squares, trisodium EDTA. Note that EDTA concentration (M) is one third the Na^+ normality shown on the horizontal axis.

TABLE II

THE EFFECT OF POLY(L-LYSINE) ON CHLOROPHYLL FLUORESCENCE AND ELECTRON TRANSPORT TO METHYL VIOLOGEN

Addition	Fluorescence yield* (arbitrary units)	Electron transport** (arbitrary units)
None	100	100
$MgCl_2$ (10 mM)	178	136
KCl (180 mM)	169	125
Polylysine (0.1 mg/ml)	90	32
Polylysine + $MgCl_2$ (10 mM)	90	32
Polylysine + KCl (180 mM)	126	21

* Fluorescence: as for Table I.

** Electron transport: 100 = 110 μatoms O_2 consumed · mg^{-1} chl · h. and was measured with a Rank O_2 electrode. Broken chloroplasts were suspended in 0.33 M sucrose, 15 mM Tricine/Tris pH 7.6 also containing 5 mM NH_4Cl, 0.75 mM sodium azide, 15 mM KCl and 60 μM methyl viologen. Illumination was by a 200 W projector source filtered through 2 mM Balzer K6 interference filter at an incident intensity of 15 W/m^2.

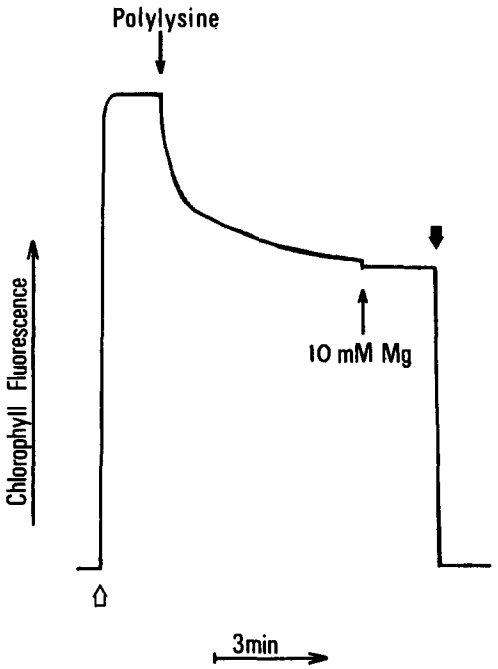

Fig. 5. Irreversible quenching by poly(L-lysine) of chlorophyll fluorescence observed at very low ionic strength. Conditions as for Fig. 3.

medium) may be due to residual divalent cation binding. The competition between monovalent and divalent cations may be due to binding to the same site or may involve allosteric interaction between two separate, but intimately related sites.

If the elevated fluorescence yield seen in the absence of added cations is due to residually bound divalent cations, then the addition of EDTA should remove these cations from their sites and lower the fluorescence yield. As shown in Fig. 4, sodium EDTA does reduce the fluorescence yield far more effectively ($C_{\frac{1}{2}} \approx 0.1$ mN) than does NaCl ($C_{\frac{1}{2}} \approx 0.9$ mN), when compared at equivalent sodium concentrations.

In order to study further the nature of the external binding sites we have used low molecular weight, poly(L-lysine). As shown in Table II, high concentrations of polylysine do not increase the fluorescence yield from the low level. If the chloroplasts are suspended in a Gross and Hess medium, polylysine will quench fluorescence in a manner analogous to monovalent cations (see Fig. 5) but then this nonpermeable compound blocks the fluorescence increase usually seen on adding low concentrations of Mg^{2+} or high concentrations of K^+. Electron transport was also inhibited by polylysine and this inhibition was not relieved by subsequent additions of cations, in agreement with others [17]. If high salt was present from the start some protection was afforded against the polylysine inhibition of both electron transport and quenching of the high fluorescence state.

DISCUSSION

In order to explain the slow quenching of chlorophyll *a* fluorescence observed on illuminating intact chloroplasts or broken chloroplasts pretreated with 5 mM Mg^{2+}, we proposed that Mg^{2+} was required on the inner side of the thylakoid membrane to induce the high fluorescing yield, and that protons taken up electrogenically on the commencement of coupled electron transport displaced Mg^{2+} from its site [10]. These cations then effluxed from the granal to stromal compartment in order to maintain an equal charge distribution across the membrane. We thus equated both the ΔpH induced quenching and the cation induced increase of fluorescence with the same internal sites [9, 10, 11].

However, experiments reported here suggest that the fluorescence changes induced by metal cations can also involve sites on the external surface of the thylakoid membrane. It seems that a high fluorescing conformational state exists when divalent cations are bound to negatively charged sites on the surface of the membrane. Since electroneutrality must be maintained, the thylakoids cannot be freed of divalent cations by simply washing in media containing no other exchangeable cations. Introduction of low levels of monovalent cations (10 mM) into the medium, however, allows the substitution of bound divalent cations to occur, and results in a conformational change which induces a decrease in fluorescence yield. Apparently, at higher concentrations (20–100 m-M), monovalent cations can interact with the sites controlling fluorescence in the same way as divalent cations, and the high fluorescence yield is restored.

Since the choline cation is almost as effective as K^+ or Na^+, it appears that at least one kind of cation-sensitive site influencing fluorescence is located on the outer side of the thylakoid membrane. Moreover, the lack of specificity between monovalent, and indeed divalent cations, suggests that the effects of these ions on fluorescence may not be a direct interaction at the binding sites per se, but predominantly an indirect action via the electrical double layer associated with the fixed negative charge density on the thylakoid surface [18]. The involvement of these phenomena in the physical mechanism of cation-induced chlorophyll fluorescence yield changes will be discussed at length in a subsequent paper.

The action of poly(L-lysine), which at pH 7.6 can be regarded as a "polymonovalent" cation [17] suggests that divalent cation-displacement from the external sites can be irreversible. This organic polycation quenches the relatively high fluorescing yield observed in the complete absence of monovalent cations and subsequently inhibits the fluorescence rise normally seen on addition of 100 mM K^+ or 5 mM Mg^{2+}. Presumably, polylysine screens the negative charges on the membrane by displacing divalent cations and blocking further binding by metal cations at the sites which control fluorescence. Similar effects have been observed with some other cations, including lanthanum (Mills & Barber, unpublished results).

It is worth noting here that according to Berg et al. [17] polylysine induces restacking of thylakoids suspended under "low-salt" conditions (in a medium containing sufficient monovalent cations to induce the low fluorescing state). Under similar conditions, polylysine does not increase the fluorescence yield, but rather stabilises the low fluorescing conformation in contrast to the magnesium ion [19]. It is clear that the conformational changes controlling fluorescence yield are rather

more subtle than the gross morphological changes concerned with stacking, evidence for which has been obtained from recent electron microscopy studies [20, 21].

From the results presented in this paper, we conclude that the effects of cations on chloroplast fluorescence in the absence of a pH gradient, are best explained by slow binding (or subsequent conformational changes) to sites on the outer side of the membrane. However, we need to extend this idea to explain the difference in fluorescence rise kinetics observed in the presence of a pH gradient across the thylakoid. It could be argued that the pH gradient induces some conformational change in the membrane causing displacement of divalent cations from sites on the outer side of the membrane. However, as discussed by Barber [15], it is likely that most of the protons taken up in the light are bound to fixed negative sites on the inner side of the thylakoid, thereby displacing an equivalent number of cations which efflux from the intra thylakoid space in order to maintain electroneutrality.

It thus appears that, in vitro, cation exchanges at sites on both the inner and outer surfaces of the thylakoid membrane can induce strikingly similar changes in the yield of fluorescence [8–13], but with inherently different kinetics. Proton/cation exchange on the inner side of the membrane can be fast, but is only observable when sufficient cations are present in the medium to satisfy the external sites(see for instance Fig. 1a). On the other hand, monovalent/divalent cation exchange at the external sites is relatively slow, and is only clearly seen when the pH gradient is abolished.

Finally, it would seem unlikely that in the intact chloroplast the external sites play any role in the control of in vivo fluorescence since when the outer envelope is intact the stromal cation level is sufficiently high to maintain a high fluorescence state [8–10, 12, 15]. Under these conditions the light induced fluorescence changes would reflect only cation/proton exchanges at sites on the inner surface of the intra-thylakoid compartment and the evidence to date favours a Mg^{2+}/H^{+} exchange [10, 11, 15].

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