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EVIDENCE FOR DIVALENT CATION MOVEMENT WITHIN ISOLATED WHOLE CHLOROPLASTS FROM STUDIES WITH IONOPHORE A23187

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Summary

Studies with osmotically shocked chloroplasts demonstrate that the slow chlorophyll fluorescence quenching observed with illuminated isolated whole chloroplasts is due to an energy dependent net movement of cations, other than protons, from granal to the stromal compartments. Ionophore A23187 inhibits this quenching and evidence is presented, both from fluorescence and electron transport studies, that this is due to a collapse of a light-induced divalent ion gradient created between the granal and stromal spaces. It seems that Ionophore A23187 can facilitate divalent cation—proton exchange across the thylakoid membranes.

There are indications that a net energy-dependent movement of Mg^{2+} from the granal to the stromal space may occur during illumination of intact chloroplasts [1,2]. If such a movement does occur then it could act both to regulate carbon fixation [1] and the distribution of light energy to the two photosystems [3,4]. Indirect evidence for Mg^{2+} movement within intact chloroplasts has come from recent chlorophyll fluorescence studies [4,5]. Isolated "whole" chloroplasts, like intact leaves, show dark-reversible slow fluorescence quenching when illuminated (see Fig.1) which is not observed after removal of their outer membranes by osmotic shock (see Fig.2). The rate of quenching is dependent on the ability of the chloroplasts to fix CO_2 and is speeded up by increasing the rate of electron transport and/or by reducing the lag period before O_2 evolution, for example by the addition of phosphoglycerate [4]. Inhibition of electron flow with 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) and uncoupling with compounds such as nigericin inhibit the formation of the low fluorescing state [4,5]. By varying the nature

of the electron acceptor it has been shown that this quenching is independent of the redox state of Photosystem II and is associated with the establishment of a high energy state [4]. Experiments with chloroplasts depleted of their outer membranes have demonstrated that the quenching is not directly brought about by the high energy state but results from an energy-dependent net efflux of cations, other than protons, from the thylakoid interiors [4,5]. In particular, increasing the Mg^{2+} concentration of the suspension raises the fluorescence yield to that observed before osmotic shock and regenerates the ability of the chloroplasts to show slow light-induced quenching (refs 4 and 5 and Fig.2). As shown in Fig.2, for Mg^{2+} addition to increase the fluorescence level with fully coupled chloroplasts there must be a dark pretreatment period [4,5]. Apparently only in the dark, when the proton gradient created by endogenous electron flow has collapsed, can the Mg^{2+} enter the thylakoid compartment and bring about the high-fluorescing state. Ca^{2+} will mimic Mg^{2+} with maximum effect at about 5 mM while for K^+ and Na^+, concentrations in excess of 100 mM are required to induce the same effect (refs 4 and 5 and Fig.2).

Although these fluorescence observations strongly suggest that net cation movements occur between the granal and stromal compartments of illuminated intact chloroplasts, they do not clearly indicate the significance of changes in Mg^{2+} distribution. In order to investigate the involvement of divalent cation movements in intact chloroplasts we have employed Ionophore A23187. This is a new carboxylic acid antibiotic which acts as a membrane carrier for Mg^{2+} and Ca^{2+} but not for K^+ [6]. The free acid has a molecular weight of 523 and an elemental analysis of $C_{29}H_{37}N_3O_6$ and is isolated by Eli Lilly and Company, Indianapolis, from whom we obtained our sample (Lot 361-066-275). Up to now its use has been mainly confined to mitochondrial work where it acts partly as an uncoupler, apparently by

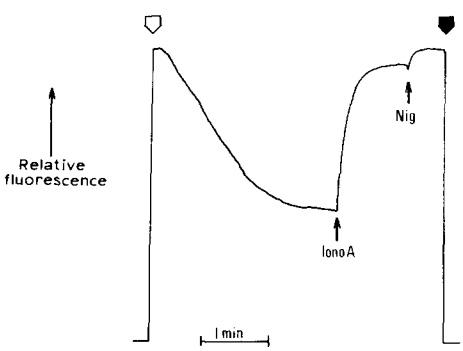

Fig.1. Slow fluorescence changes from a 3 ml suspension of isolated whole chloroplasts (approx. 65% whole as determined by the ferricyanide methods [7]). The chloroplasts were isolated from spinach by the method of Stokes and Walker [8] and were finally suspended in 0.33 M Sorbitol and 50 mM *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulphonic acid (HEPES) adjusted to pH 7.6 with NaOH (A.M.) at a chlorophyll concentration of 33 μg/ml. Illumination was via a Balzer Calflex C, 4 mm Schott BG18 filter combination at an intensity of 70 kergs·cm^{-2}·s^{-1} and its onset and termination are indicated by open and closed arrows respectively. Chlorophyll fluorescence was measured with an EMI 9558 photomultiplier protected by Balzer B40 695 and 6 mm Schott RG665 filters. Addition of Ionophore A23187 (Iono A) gave a final concentration of 1 μg/ml and nigericin (Nig) concentration was 10^{-7} M.

allowing proton—divalent ion exchange. As shown in Fig.1 we found that this antibiotic reversed the slow fluorescence quenching observed on illumination of isolated "whole" chloroplasts. Reversal was not always complete and there was usually a small additional nigericin-sensitive component. After subjecting the "whole" chloroplasts to an osmotic shock the fluorescence yield was lowered but as explained above the addition of 5 mM $MgCl_2$ or 100 mM KCl followed by a 2 min dark period resulted in suspensions which showed similar fluorescence properties to the "whole" preparations. As Fig.2 shows the addition of Ionophore A23187 to these reconstituted chloroplasts was only effective in reversing the quenching when Mg^{2+} was present. Similarly, pretreatment of the broken chloroplasts with Ionophore A23187 allowed the high-fluorescing state to occur only on the addition of Mg^{2+} and not K^+ (Fig.2).

Electron transport studies with broken chloroplasts treated with ferricyanide indicated that concentrations of Ionophore A23187 which relieve the low-fluorescing state uncouple electron flow. In the presence of sufficient Mg^{2+} (in excess of 3 mM) the Ionophore in the concentration range of 2—10 μg per 100 μg chlorophyll gave the same stimulated rate of electron flow as observed with an uncoupling concentration of NH_4Cl or nigericin (see Table I). In the absence of added Mg^{2+} the above concentrations of the antibiotic slightly stimulated electron flow while at higher concentrations and in the presence of Mg^{2+} there was an inhibition of the uncoupled rate. As

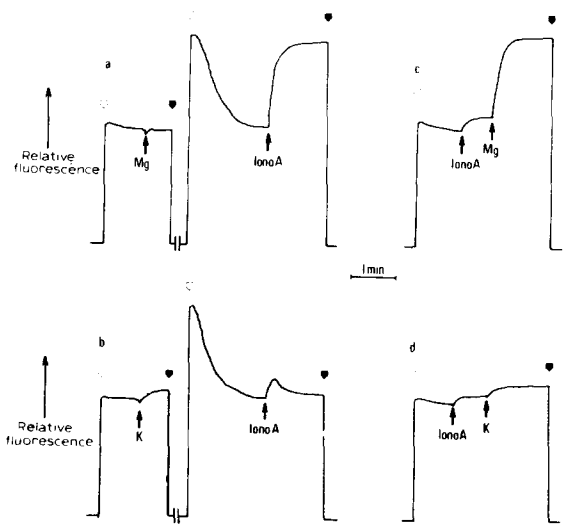

Fig. 2. Fluorescence time courses measured with spinach chloroplasts which had been subjected to osmotic shock by initial suspension in 1.5 ml distilled water followed by the addition of 1.5 ml double strength sorbitol/HEPES media. (a) Addition of 5 mM $MgCl_2$ followed by a 2 min dark period and then the injection of 1 μg/ml Ionophore A23187 (Iono A) as indicated during the second illumination period. (b) As (a) but carried out by injection of 100 mM KCl. (c) Addition of 5 mM $MgCl_2$ to illuminated chloroplasts treated with 1 μg/ml Ionophore A23187. (d) As (c) but addition of 100 mM KCl. Other conditions same as Fig.1.

TABLE I

EFFECT OF IONOPHORE A23187 ON ELECTRON TRANSPORT

The reaction mixture contained, in a volume of 2 ml, osmotically shocked chloroplasts equivalent to 98μg chlorophyll suspended in 0.33 M Sorbitol 50mM HEPES brought to pH 7.6 with NaOH(A.M.) after being initially shocked in 10% A.M. and subjected to two A.M. washes. Electron transport was induced by addition of 1.5 mM potassium ferricyanide. Measurements were made as described in the legend of Fig.3.

Ionophore Concentration (μg/ml)	Electron Transport Rate (μmoles O_2/mg chlorophyll per h)		
	−Mg	+10mM $MgCl_2$	+5mM NH_4Cl
0	23	27	86
0.15	22	31	85
0.5	26	42	85
1.5	25	64	83
2.5	31	82	83
5.0	35	85	85
15.0	31	46	46

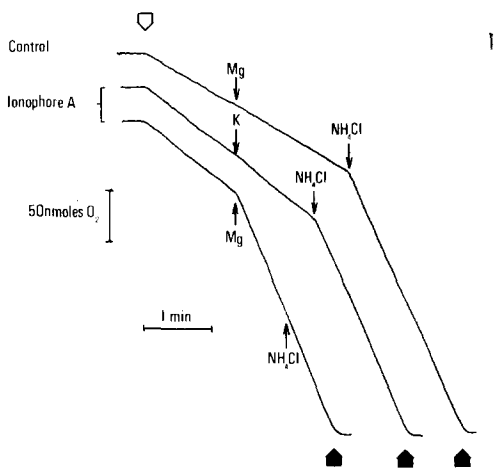

Fig.3. Electron transport to ferricyanide measured as O_2 evolution from H_2O with Rank O_2 electrode. Illumination was 200 kergs·cm^{-2}·s^{-1} white light through a heat filter and its onset and termination are indicated by open and closed arrows, respectively. The reaction mixture was the same as that given in Table I. Ionophore A23187 concentration was 2.5 μg/ml and $MgCl_2$ and KCl additions were 10 mM and 100 mM, respectively. NH_4Cl concentration was 5 mM.

Fig.3 shows, the stimulation of electron flow to the uncoupled rate by Ionophore A23187 was Mg^{2+} but not K^+ dependent.

These results suggest that Ionophore A23187 can act as an Mg^{2+}/H^+ exchanger across the thylakoid membranes. Such an exchange would account for the Mg^{2+}-dependent stimulation of electron flow and for the reversal of the Mg^{2+}-dependent quenching of chlorophyll fluorescence observed with the reconstituted chloroplast system. Therefore it seems likely that the reversal of the fluorescence quenching observed with isolated "whole" chloroplasts on addition of Ionophore A23187 is due to the collapse of a light-induced divalent ion gradient between the granal and stromal compartments. There

is, however, the alternative that the light-induced quenching is due entirely to the establishment of a monovalent cation gradient and that the ionophore relieves the quenching by "actively" driving cations into the thylakoids at the expense of the proton gradient. Although it is difficult to distinguish between these two possibilities it seems to us that the former is more likely since divalent cations are far more effective than monovalent cations at inducing the low fluorescing state with reconstituted systems. Finally, because the ionophore is capable of transporting both Ca^{2+} and Mg^{2+} it is impossible to decide at this stage which of these is involved in the facilitated cation exchange across the intact thylakoid membranes.

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