Biochimica et Biophysica Acta, 501 (1978) 94—102

BBA 47420

DUAL ACTION OF IONOPHORE A23187 ON INTACT CHLOROPLASTS

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(Received August 29th, 1977)

Summary

1. Ionophore A23187 induces uncoupling of potassium ferricyanide-dependent O_2 evolution by envelope-free chloroplasts and oxaloacetate-dependent O_2 evolution by intact chloroplasts. The half maximal concentration ($C_{½}$) for stimulation of oxygen evolution in both cases is approximately 4 μM · 100 μg chlorophyll · ml^{-1}.

2. Ionophore A23187 also induces inhibition of CO_2 and 3-phosphoglycerate-dependent O_2 evolution by intact chloroplasts in the presence of 3 mM $MgCl_2$. The half maximal concentrations ($C_{½}$) for inhibition of O_2 evolution are 3 μM and 5 μM respectively · 100 μg^{-1} chlorophyll · ml^{-1}.

3. A very high concentration of ionophore A23187 (10 μM · 20 μg^{-1} chlorophyll · ml^{-1}) plus 0.1 mM EDTA lowers the fluorescence yield of intact chloroplasts suspended in a cation-free medium in the presence of 3-(3,4-dichlorophenyl)-1,1-dimethylurea, indicating loss of divalent cation from the diffuse double layers of the thylakoid membranes.

4. These results are discussed in relation to ionophore A23187-induced divalent cation/proton exchange at both the thylakoid and the envelope membranes of intact chloroplasts.

Introduction

A23187, an acidic ionophore which facilitates electrically neutral divalent cation/proton exchange across lipid membranes [1], uncouples electron transport by broken chloroplasts [2,3]. Telfer et al. [4] also reported that ionophore A23187 uncouples electron transport in intact chloroplasts. Oxaloacetate-dependent oxygen evolution, which does not require ATP, was stimulated while 3-phosphoglycerate-dependent oxygen evolution, which requires both ATP and NADPH, was inhibited. However, it was also suggested by these authors that ionophore A23187 brings about Mg^{2+} exchange across the chloroplast outer membranes and that this process might prevent its uncoupling

action at the thylakoid membrane. Recently, Portis and Heldt [5] have demonstrated that a low concentration of ionophore A23187 (2 μM) in the presence of 0.1 mM EDTA lowers the stromal concentration of Mg^{2+} and, as a consequence, CO_2 fixation is inhibited. However, they found that under these conditions externally-added Mg^{2+} restored CO_2-dependent oxygen evolution. They also found that 3-phosphoglycerate reduction was far less sensitive to ionophore A23187 than CO_2 reduction and was not completely inhibited unless the concentration was 10 μM and 1 mM EDTA was present. These observations lead Portis and Heldt [5] to conclude that ionophore A23187 does not bring about significant uncoupling in intact chloroplasts and that the main action of the ionophore is to reduce the stromal Mg^{2+} level sufficiently to inhibit Mg^{2+}-dependent enzymic activities of the Calvin cycle.

In this paper we have extended our work on the effect of ionophore A23187 with the view of clarifying its possible multiple action on intact chloroplast metabolism.

Materials and Methods

Intact chloroplasts were isolated from peas as described previously [6]. The chloroplasts were suspended in a divalent cation-free media consisting of 0.33 M sorbitol and 0.05 M *N*-2-hydroxyethylpiperazine *N'*-2-ethanesulphonic acid (HEPES), brought to pH 7.6 with KOH. The final K^+ concentration was between 20 and 30 mM. Chlorophyll was determined by the method of Arnon [7]. The chloroplasts were shown to be 70—80% intact as described previously [4]. Envelope-free chloroplasts were obtained by subjecting isolated intact chloroplasts to an osmotic shock in distilled water before addition of double strength assay medium. Oxygen evolution was measured using a Clark-type oxygen electrode (Hansatech Ltd., Norfolk), based on the design of Delieu and Walker [8]. Illumination was by a 150 W projector via a heat filter and an orange filter transmitting 540—740 nm light (Cinemoid No. 5a, Strand Electrics Ltd.). Temperature was maintained at 22°C. Oxygen evolution was assayed in the divalent cation-free suspending medium at a chlorophyll concentration of 100 $\mu g \cdot ml^{-1}$. Chlorophyll fluorescence was measured as described previously [9], at a chlorophyll concentration of 20 $\mu g \cdot ml^{-1}$.

Ionophore A23187 was obtained as a gift from Dr. R.L. Hamill of Eli Lilley and Co., Indianapolis, and stock solutions were made up in ethanol and protected from the light.

Results

CO_2-dependent O_2 evolution by intact chloroplasts is usually studied using a relatively high chlorophyll concentration (100 μg chlorophyll $\cdot ml^{-1}$). In order to investigate the ability of ionophore A23187 to bring about uncoupling in intact chloroplasts, we first determined the half maximal concentration ($C_{1/2}$) requirement for ionophore A23187 stimulation of ferricyanide-dependent O_2 evolution by envelope-free chloroplasts under non-phosphorylating conditions at a chlorophyll concentration of 100 $\mu g \cdot ml^{-1}$. The $C_{1/2}$ was found to be approximately 4 μM.

We have confirmed the data of Portis and Heldt [5] that 2 μM ionophore A23187 completely inhibits CO_2-dependent O_2 evolution by intact chloroplasts suspended in a Mg^{2+}-free medium. We also confirmed their data that subsequent addition of Mg^{2+} restores O_2 evolution, that the time required to bring about complete inhibition is dependent on the ionophore A23187 concentration and that EDTA increases the effectiveness of ionophore A23187. Therefore, we agree with their conclusion that ionophore A23187 at this concentration lowers the Mg^{2+} level of the stroma to below the critical level required to activate certain enzymes of the Calvin cycle. As the concentration required is well below that required to bring about uncoupling in broken chloroplasts, it is not surprising that there is no apparent uncoupling effect of ionophore A23187 in these experiments, and that this concentration of ionophore A23187 (2 μM) has virtually no effect on 3-phosphoglycerate- and oxaloacetate-dependent O_2 evolution, which do not involve Mg^{2+}-activated enzymes.

We have reinvestigated the effect of a higher concentration of ionophore A23187 on intact chloroplast activities in the presence and absence of Mg^{2+}. Fig. 1 shows the effect of 10 μM ionophore A23187 on O_2 evolution with three different electron acceptors. Oxaloacetate-dependent O_2 evolution in the absence of EDTA (trace a) was stimulated by ionophore A23187 and this rate was not affected by the addition of Mg^{2+}. However, if Mg^{2+} was added before ionophore A23187 it prevented the stimulation. The apparent temporary increase in rate on addition of ionophore A23187 is due to the O_2 dissolved in the ethanolic solution of ionophore A23187. 3-Phosphoglycerate-dependent O_2 evolution in the presence of 0.1 mM EDTA (trace b), after a brief but significant stimulation, was partially inhibited by ionophore A23187 and subsequent addition of Mg^{2+} brought about complete inhibition. However, there was very little inhibition if ionophore A23187 was added after Mg^{2+}. CO_2-dependent O_2 evolution, also in the presence of 0.1 mM EDTA (trace c), was simi-

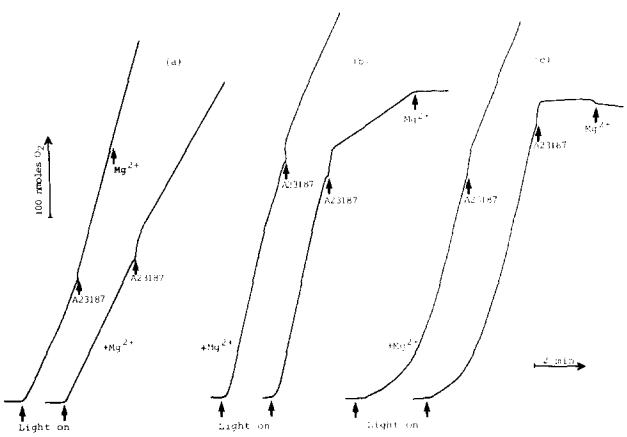

Fig. 1. Oxaloacetate (a), 3-phosphoglycerate- (b) and CO_2- (c) dependent O_2 evolution by intact chloroplasts. The reaction medium was as described in Materials and Methods. In addition, a contains 0.6 mM oxaloacetate and 3 mM $MgCl_2$ (where indicated), b contains 2.5 mM 3-phosphoglycerate, 0.1 mM EDTA and 3 mM $MgCl_2$ (where indicated) and c contains 10 mM $NaHCO_3$, 0.1 mM 3-phosphoglycerate, 0.2 mM ADP, 0.1 mM EDTA and 3 mM $MgCl_2$ (where indicated). 10 μM ionophore A23187 and 3 mM $MgCl_2$ were injected as indicated.

larly affected except that 10 μM ionophore A23187, again after a brief stimulation, completely inhibited O_2 evolution before the addition of Mg^{2+}.

Fig. 2 shows the concentration curve for stimulation of oxaloacetate-dependent O_2 evolution by ionophore A23187 in the presence and absence of added Mg^{2+}. In this experiment Mg^{2+} was routinely added 15 s after ionophore A23187 but before the onset of illumination. The approximate $C_{1/2}$ for stimulation was 4.5 μM and was more or less independent of the presence of Mg^{2+}. This concentration is similar to the concentration required for uncoupling ferricyanide reduction by broken chloroplasts. In this experiment, if Mg^{2+} was added before ionophore A23187 we found no stimulation of O_2 evolution.

We also investigated the effect of ionophore A23187 on both 3-phosphoglycerate- and CO_2-dependent O_2 evolution in the presence of Mg^{2+} (added after ionophore A23187). Fig. 3 shows that complete inhibition of 3-phosphoglycerate-dependent O_2 evolution occurred with a $C_{1/2}$ of approximately 5 μM which suggests that ionophore A23187 is uncoupling, and hence inhibiting, an ATP-dependent reaction. CO_2-dependent O_2 evolution was also inhibited by ionophore A23187 (see Fig. 3) but was found to be somewhat more sensitive than 3-phosphoglycerate-dependent O_2 evolution ($C_{1/2}$ approximately 3 μM). In the absence of added Mg^{2+}, CO_2-dependent O_2 evolution was inhibited by very low concentrations of ionophore A23187 (<2 μM, see ref. 5) and as the inhibition was time dependent it cannot be displayed as a concentration curve. On the other, hand 3-phosphoglycerate-dependent O_2 evolution, in the absence of added Mg^{2+}, was extremely resistant to ionophore A23187. Even 15 μM ionophore A23187 did not completely inhibit O_2 evolution unless either Mg^{2+} or EDTA was added.

As shown in Fig. 1, ionophore A23187 was an effective inhibitor of CO_2- and 3-phosphoglycerate-dependent O_2 evolution and also an effective uncoupler of oxaloacetate-dependent O_2 evolution in the presence of Mg^{2+} only if

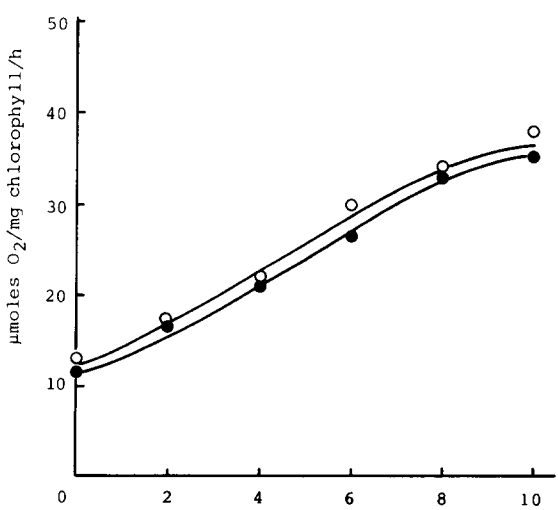

Fig. 2. Effect of ionophore A23187 on oxaloacetate-dependent O_2 evolution by intact chloroplasts in the presence (closed circles) and absence (open circles) of $MgCl_2$. The medium was as described in Materials and Methods. In addition it contained 2 mM oxaloacetate. Ionophore A23187 was added to the chloroplast suspension 15 s before 3 mM $MgCl_2$.

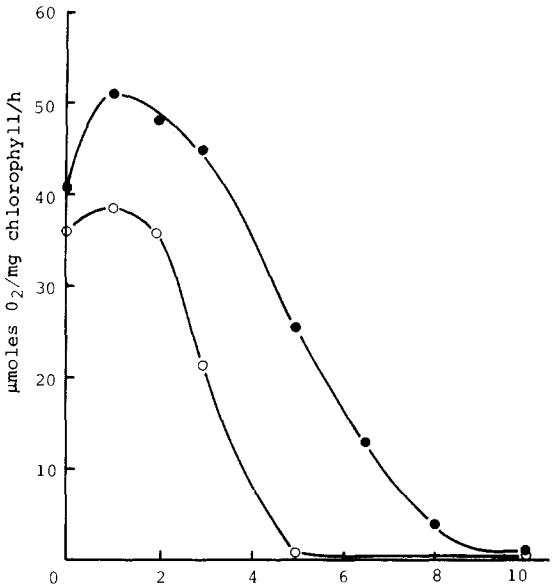

Fig. 3. Effect of ionophore A23187 on CO_2- (open circles) and 3-phosphoglycerate- (closed circles) dependent O_2 evolution by intact chloroplasts in the presence of $MgCl_2$. The media for CO_2-dependent O_2 evolution and 3-phosphoglycerate-dependent O_2 evolution were as described in Figs. 1c and Fig. 1b, respectively, except that EDTA was omitted. Ionophore A23187 was added to the chloroplast suspension 15 s before 3 mM $MgCl_2$.

ionophore A23187 was added to the reaction vessel before Mg^{2+}. Fig. 4 shows the effect of 10 μM ionophore A23187 and 5 mM Mg^{2+} on 3-phosphoglycerate-dependent O_2 evolution and the importance of the order of addition of the ionophore and Mg^{2+}. Trace a shows the rate of O_2 evolution by chloroplasts alone. Mg^{2+} had little effect on this rate (not shown). Traces b and c show that O_2 evolution is completely inhibited by 10 μM ionophore A23187 and Mg^{2+}, provided that the ionophore is added before the Mg^{2+}. If the Mg^{2+} is added first there is no effect on the rate of O_2 evolution. When ionophore A23187 is added to the reaction vessel before the chloroplasts (trace d) O_2 evolution is very severely inhibited and addition of Mg^{2+} brings about complete inhibition. However, if Mg^{2+} is added to the reaction vessel with ionophore A23187 before the chloroplasts (trace e) there is no significant effect on the rate of O_2 evolution. Traces b and e suggest that ionophore A23187 complexes rapidly with divalent cations and that this complex has a reduced ability to enter the chloroplast membranes. As ionophore A23187 alone is a fairly effective inhibitor if added before the chloroplasts, its lack of effect in trace e cannot be due to precipitation of ionophore A23187 in the absence of a hydrophobic environment.

In order to investigate the effect of ionophore A23187 and EDTA on the divalent cation content of intact chloroplasts we have utilized the well known divalent cation effect on the chlorophyll fluorescence yield of thylakoid membranes originally reported by Homann [10] and Murata [11]. At concentrations of monovalent cations below 20—30 mM the fluorescence yield is low, but on addition of divalent cations (approx. 5 mM) or on increasing the monovalent cations to 100 mM there is an increase in fluorescence yield. This has

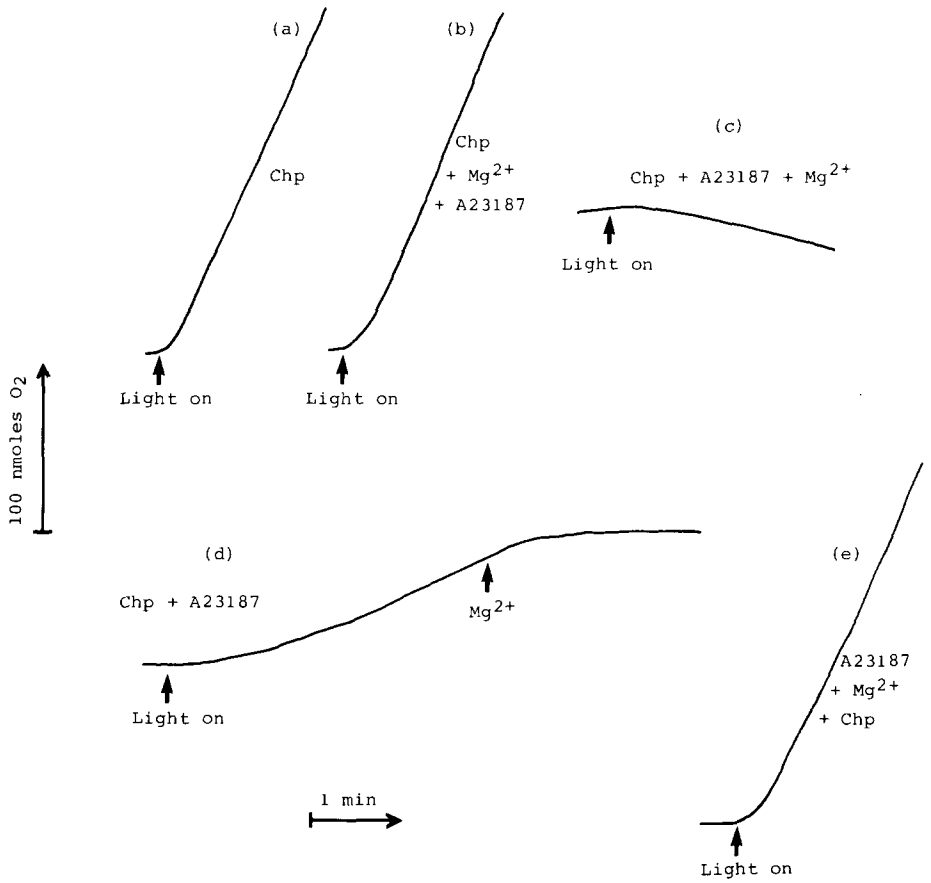

Fig. 4. 3-Phosphoglycerate-dependent O_2 evolution by intact chloroplasts. The medium was as described in Materials and Methods, except that 1.5 mM 3-phosphoglycerate was also present. The order of addition of the chloroplasts, 10 μM ionophore A23187 and 3 mM $MgCl_2$ (before the onset of illumination) is indicated for each trace. The concentration of $MgCl_2$ injected in trace d was also 3 mM. Chp, chlorophyll.

been attributed to changes in the cation composition of the diffuse electrical layer at the surface of the thylakoid membrane [12,13]. Fig. 5 shows that intact chloroplasts in the presence of 3-(3,4-dichlorophenyl)-1,1-dimethyl urea (DCMU) and suspended in a divalent cation-free medium have a high fluorescence yield (trace a) while chloroplasts from which the envelope has been removed by an osmotic shock suspended in the same medium have a low fluorescence yield (trace c). The slow decrease in the fluorescence yield of intact chloroplasts (traces a and b) was induced by the relatively high light intensity of the exciting light and has been studied by Sokolove and Marsho [14]. Neither ionophore A23187 nor EDTA (up to 1 mM) caused further significant quenching of fluorescence yield when added alone. However, traces a and b show that the combination of 10 μM ionophore A23187 and 0.1 mM EDTA rapidly lowers the fluorescence yield to that seen with the shocked chloroplasts (trace c) and trace a shows that subsequent addition of Mg^{2+} brings about complete reversal of the rapid quenching induced by ionophore A23187 plus EDTA. The lowering of fluorescence yield brought about by the ionophore A23187

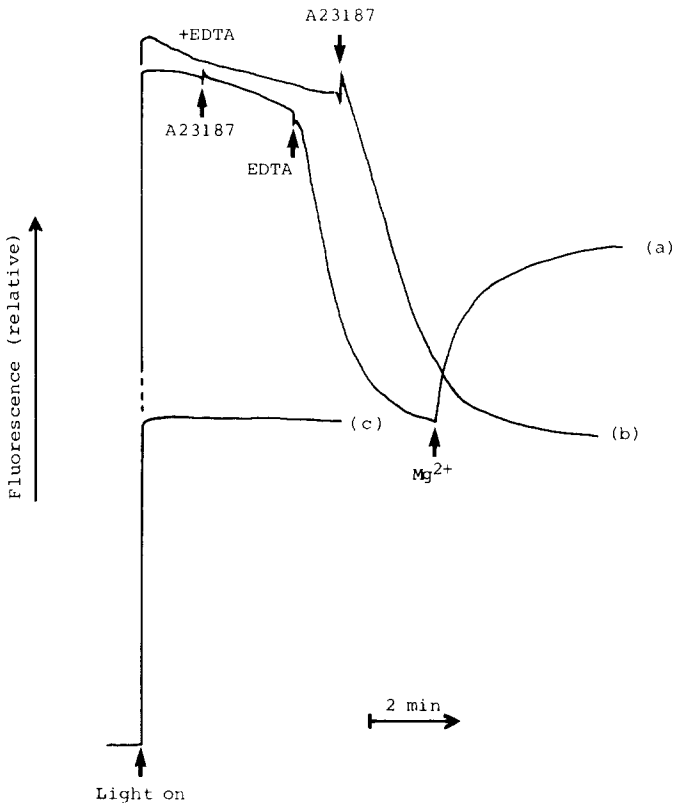

Fig. 5. Relative chlorophyll fluorescence yield of intact chloroplasts alone (a), intact chloroplast plus 0.1 mM EDTA (b) and chloroplasts subjected to an osmotic shock in the reaction vessel as described in Materials and Methods (c). The reaction medium was as described in Materials and Methods. It also contained $2 \cdot 10^{-5}$ M DCMU. 10 μM Ionophore A23187, 0.1 mM EDTA and 10 mM $MgCl_2$ were added as indicated.

and EDTA combination was independent of illumination and was completely inhibited if divalent cations were added immediately after EDTA and ionophore A23187. For fluorescence measurements the chlorophyll concentration had to be lowered to a fifth of that used in the O_2 evolution measurements; thus the ionophore A23187 concentration required to lower the fluorescence yield relative to chlorophyll concentration was very high.

Discussion

In this paper we have shown that the action of ionophore A23187 on intact chloroplast metabolism must be interpreted in relation to its action both on the thylakoid membrane and envelope. Under some conditions there is preferential action at the envelope, as shown by the fact that CO_2 fixation is inhibited by a low concentration (2 μM) of ionophore A23187, even in the absence of EDTA, and is restored by addition of Mg^{2+} to the external medium [5]. We have shown that uncoupling of electron transport by broken chloroplasts (100 μg chlorophyll $\cdot$ ml^{-1}) requires a relatively high concentration ($C_{1/2} \sim 4$ μM) of ionophore

A23187. The difference in concentration of ionophore A23187 required to release sufficient divalent cations to inhibit CO_2 fixation compared to that required to bring about uncoupling may be related to the much greater area of thylakoid membrane in the intact chloroplast as compared with the area of the envelope membrane.

We have shown that if the ionophore A23187-induced loss of Mg^{2+} from the stroma is prevented by the presence of externally added Mg^{2+}, higher levels of ionophore A23187 can act as an effective uncoupler of CO_2-, 3-phosphoglycerate- and oxaloacetate-dependent O_2 evolution. The slight differences in the concentration requirement for this uncoupling can probably be related to the difference in the ATP/NADPH ratio required for each electron acceptor.

If intact chloroplasts are suspended in a divalent cation-free medium the effect of ionophore A23187 is more dependent on the nature of the electron acceptor. In the case of oxaloacetate-dependent O_2 evolution electron transport is uncoupled by the same concentration range of ionophore A23187 as in the presence of Mg^{2+}. Thus there must be sufficient divalent cation remaining in the stroma to act as the counterion for a decrease in the size of the proton gradient across the thylakoid membrane and, hence, release of control of the rate of electron flow. In a previous paper we showed that ionophore A23187-uncoupled electron flow to oxaloacetate was inhibited on addition of EDTA and restored by excess Mg^{2+} [4]. Therefore, in the presence of EDTA and ionophore A23187 the divalent cation level required for the ionophore to act as an uncoupler must be too low. However, CO_2 fixation is very sensitive to the Mg^{2+} concentration [5,15] and, hence, is inhibited by very low concentrations of ionophore A23187. 3-Phosphoglycerate reduction, on the other hand, using a divalent cation-free medium is more resistent to ionophore A23187 (Fig. 1 and ref. 5). With 10 μM ionophore A23187 electron transport was not completely inhibited unless Mg^{2+} or EDTA was present. We interpret this resistance to a balance between the effect of ionophore A23187 at the two membranes; i.e. in a cation-free medium ionophore A23187 lowers the divalent cation level of the stroma by catalysing Mg^{2+}/H^+ exchange across the envelope, thus allowing only partial uncoupling by Mg^{2+}/H^+ exchange across the thylakoid membrane. Although in principle this would result in a reduction in the stromal ATP level there is apparently sufficient to drive some 3-phosphoglycerate reduction.

Further evidence that a high level of ionophore A23187 inhibits electron flow to 3-phosphoglycerate or CO_2 by uncoupling is shown in Fig. 1 where it can be seen that on addition of the ionophore there is a brief stimulation in the rate of O_2 evolution before inhibition occurs. This seems to suggest that the ATP/NADPH ratio in the stroma is high and that a temporary stimulation in the rate of NADPH formation occurs until the ATP supply becomes limiting.

We also found that the ionophore A23187 · Mg^{2+} complex, which would be in the closed conformation according to Pfeiffer et al. [16], has a very reduced affinity for the lipid membrane of chloroplasts compared to the free acid which would be in the open conformational state. Care must therefore be taken in the order of addition of ionophore A23187 and divalent cations to chloroplasts when studying its uncoupling action.

Fig. 5 shows that the combination of EDTA and ionophore A23187 will lower the fluorescence yield of intact chloroplasts and that this is due to loss of

divalent cation from the stroma. This is the first direct evidence that it is divalent and not monovalent cations which are the in vivo co-ions for the fixed membrane charges of the thylakoid membrane [13]. Although monovalent cations are present in the chloroplasts [17] they cannot be present at a high enough concentration to induce the high fluorescing state.

Acknowledgements

The authors wish to thank the Science Research Council and the European Commission for Solar Energy Research and Development Programme for financial support.

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