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PROTON EFFLUX THROUGH THE CHLOROPLAST ATP SYNTHASE ($CF_0 \cdot CF_1$) IN THE PRESENCE OF SULFHYDRYL-MODIFYING AGENTS

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Summary

The rate of photosynthetic electron transport measured in the absence of ADP and P_i is stimulated by low levels of Hg^{2+} or Ag^+ (50% stimulation $\approx$ 3 Hg^{2+} or 6 Ag^+/100 chlorophyll) to a plateau equal to the transport rate under normal phosphorylating conditions (i.e. +ADP, +P_i). Chloroplasts pretreated in the light under energizing conditions with *N*-ethylmaleimide show a similar stimulation of non-phosphorylating electron transport. The stimulations of non-phosphorylating electron transport by Hg^{2+}, Ag^+ and *N*-ethylmaleimide are reversed by the CF_1 inhibitor phlorizin, the CF_0 inhibitor triphenyltin chloride, and can be further stimulated by uncouplers such as methylamine. The Hg^{2+} and *N*-ethylmaleimide stimulations, but not the Ag^+ stimulation, are completely reversed by low levels of ADP (2 μM), ATP (2 μM), and P_i (400 μM). Ag^+, which is a potent inhibitor of ATP synthesis, has little or no effect upon phosphorylating electron transport (+ADP, +P_i). Concomitant with the stimulations of non-phosphorylating electron transport by Hg^{2+}, Ag^+ and ADP + P_i, there is a decrease in the level of membrane energization (as measured by atebrin fluorescence quenching) which is reversed when the CF_0 channel is blocked by triphenyltin. These results suggest that modification of critical CF_1 sulfhydryl residues by Hg^{2+}, Ag^+ or *N*-ethylmaleimide leads to the loss of intra-enzyme coupling between the transmembrane proton-transferring and the ATP synthesis activities of the CF_0-CF_1 ATP synthase complex.

Abbreviations: DCCD, *N,N'*-dicyclohexylcarbodiimide; Tricine, *N*-tris(hydroxymethyl)methylglycine; Hepps, *N*-2-hydroxyethylpiperazine-*N'*-propanesulfonic acid. CF_0, chloroplast coupling factor 0; CF_1, chloroplast coupling factor 1.

Introduction

In the steady state, when factors such as light intensity, electron acceptors, etc., are saturated, the rate of proton efflux from the interior of the chloroplast thylakoid vesicle must be equal to the rate of proton influx, and, as a result, the rate of proton-pumping electron transport is determined by the overall rate of proton efflux [1,2]. In chloroplasts, this proton efflux may occur via three main pathways:

(i) nonspecific, passive diffusion through the membrane; (ii) efflux through the CF_0-CF_1 complex in a reaction coupled to ATP synthesis:

$$ADP + P_i + nH^+_{in} \rightarrow ATP + H_2O + nH^+_{out}$$

and (iii) efflux through the CF_0-CF_1 complex via a pathway not coupled to ATP synthesis (i.e. non-coupled 'leakage' of protons through the ATP synthase complex) [3,4]. A change in the contribution of any one of these pathways leading to a change in total H^+ efflux will necessarily cause a parallel change in the steady-state rate of electron transport.

Experimentally, the relationship between rate of proton efflux and rate of electron transport has been shown under a variety of conditions. Non-phosphorylating electron transport (in the absence of ADP and P_i) is much slower than phosphorylating electron transport, since proton efflux under non-phosphorylating conditions is limited to passive diffusion through the membrane and to non-coupled 'leakage' through CF_0-CF_1 [3,4].

The stimulation of electron transport and proton efflux by phosphorylation can be reversed by inhibitors of CF_0 or CF_1 functions (energy transfer inhibitors). CF_1 inhibitors, such as phlorizin [5] and Dio-9 [6], characteristically inhibit the rate of phosphorylation-coupled electron transport to the non-phosphorylating rate (+ADP, $-P_i$), while inhibiting ATP formation completely and increasing the magnitude of the transmembrane pH gradient [7,8]. CF_0 inhibitors such as *N,N'*-dicyclohexylcarbodiimide (DCCD) [9] and triphenyltin chloride [10] have very similar effects, although they may inhibit electron transport to a level somewhat below the non-phosphorylating rate, since these inhibitors block both non-coupled leakage and coupled proton efflux through the CF_0-CF_1 complex [4].

A number of sulfhydryl-modifying agents have been shown to act as energy transfer inhibitors, including Hg^{2+} [11], Cd^{2+} [12], *p*-chloromercuribenzoate [11], 2,2'-dithiobis(5-nitropyridine) [13], *N*-ethylmaleimide [14], and *o*-iodosobenzoate [15]. In an effort to further elucidate the role of sulfhydryl residues in the H^+ transfer and ATP synthesis reactions of CF_0-CF_1, we have recently reinvestigated the earlier finding of Izawa and Good [11] that, in the absence of ADP and P_i, Hg^{2+} stimulates the rate of non-phosphorylating electron transport to a rate equal to the rate of phosphorylating electron transport (minus Hg^{2+}), but no further. In a preliminary study [16] we found that Hg^{2+}-stimulated, non-phosphorylating electron transport exhibited properties very similar to normal phosphorylating electron transport: it could be further stimulated by uncouplers, and was inhibited by energy transfer inhibitors such as phlorizin and triphenyltin. These findings led to the postulation that in Hg^{2+}-treated chloroplasts, in the absence of ADP and P_i, CF_0-CF_1 is modified such

that it can turn over normally in its H^+-transferring function, and that this H^+ transfer reaction is no longer obligatorily coupled to ATP synthesis. This unique form of intra-enzyme uncoupling has now been found to occur not only in Hg^{2+}-treated chloroplasts, but in Ag^+ and *N*-ethylmaleimide-treated chloroplasts as well.

Materials and Methods

Chloroplasts (intact, naked lamellae) were isolated from market spinach, at 4° C, by the following procedure: washed leaves were ground for about 10 s in a Waring blender in 0.3 M NaCl, 3 mM $MgCl_2$, 0.5 mM EDTA and 0.03 M Tricine/NaOH (pH 7.8). The homogenate was filtered through several layers of cheesecloth, centrifuged at $3000 \times g$ for 2 min, and the pellet was resuspended in 0.2 M sucrose, 2 mM $MgCl_2$, and 5 mM Hepps/NaOH (pH 7.8). This suspension was briefly centrifuged (15 s) at $760 \times g$ to remove intact cells, and the supernatant centrifuged again for 4 min at $3000 \times g$. The resulting pellet was taken up in a small amount of the sucrose/$MgCl_2$/Hepps medium. The chlorophyll concentration of this suspension was estimated by the method of Arnon [17].

Electron transport was measured with a membrane-covered Clark electrode as the rate of oxygen uptake resulting from the aerobic oxidation of reduced methyl viologen. Reactions (1.5 ml final volume) were carried out with continuous stirring at 18° C in a thermostatted glass chamber. Additions of $HgCl_2$, $AgNO_3$ and triphenyltin chloride were made in microlitre amounts to the required concentration.

Broad band saturating actinic illumination was supplied by a tungsten-halogen lamp equipped with a condensing lens and a Corning 1-69 infrared filter.

Photophosphorylation was measured as the irreversible light-induced proton consumption according to the equation

$$MgADP^{1-} + P_i^{2-} + nH^+ \rightarrow MgATP^{2-} + H_2O$$

where $n = 0.96$ in the presence of Mg^{2+} at pH 8 [18]. Reactions were run in a thermostatted 2 ml reaction vessel at 18° C with continuous stirring. The pH of the final reaction mixture (containing chloroplasts) was adjusted to pH 8.2 with dilute NaOH immediately before each experiment. Saturating actinic illumination (greater than 560 nm) was supplied by a 500 W projector lamp focussed through a 250 ml round-bottomed flask containing approximately 2% $CuSO_4$ solution as an infrared filter.

The light-dependent incorporation of *N*-ethylmaleimide into the chloroplast membrane-bound CF_1 was performed essentially as described by McCarty et al. [14]. Chloroplasts were illuminated with continuous stirring for 90 s in the presence of *N*-ethylmaleimide. Control chloroplasts were treated similarly but in the absence of *N*-ethylmaleimide. The incubation mixture (6.0 ml final volume) contained chloroplasts equivalent to 360 μg chlorophyll, 0.1 M sucrose, 2 mM $MgCl_2$, 20 mM Hepps/NaOH (pH 8.2), 200 μM methyl viologen and 5 mM *N*-ethylmaleimide. The illumination was carried out in a thermostatted reaction vessel at 18° C. Illumination was provided by two 500-W pro-

jector lamps, each focused through a 250 ml round-bottomed flask containing 2% $CuSO_4$ solution as an infrared filter.

Energy-dependent changes in the fluorescence of 3-chloro-*p*-(4-dimethylamino-1-methylbutyl)-7-methoxyacridine (atebrin) were detected in an Aminco-Bowman spectrofluorimeter. The exciting light (390 nm) was defined by a monochromator and a Corning 7-54 cut-off filter. The fluorescence emission passed through a monochromator (520 nm) and a Corning 3-72 filter. Saturating actinic illumination, provided by a 500 W projector lamp, passed through a Corning 2-60 cut-off filter and was channelled through a 12 inch long, 1/4 inch diameter light pipe into the top of the cuvette. The reaction mixture was stirred continuously. Experiments were conducted at room temperature (23°C).

Triphenyltin chloride (Alpha Inorganics) was recrystallized twice from ethanol before use. All other reagents were of the highest purity commercially available.

Results

$HgCl_2$

Izawa and Good [11] first reported that low concentrations of mercuric salts behave as energy transfer inhibitors in chloroplasts. The subsequent observation that the stimulation of non-phosphorylating electron transport by Hg^{2+} could be reversed by inhibitors affecting either CF_0 (triphenyltin) or CF_1 (phlorizin), suggests that this effect can also be ascribed to an effect upon the ATP synthase complex [16]. This notion is reinforced by the observation, first made by Izawa and Good [11], that very low levels of ATP (2 μM) completely reverse the Hg^{2+} stimulation of non-phosphorylating electron transport (Fig. 1). Similar results were also obtained with low levels of ADP (not shown).

There is a rather precise stoichiometric relationship between the amount of Hg^{2+} required to maximally stimulate electron transport and the amount of chloroplast material present in the reaction mixture (Fig. 2), with a ratio of about 3 Hg^{2+}/100 chlorophyll required for a 50% stimulation of electron transport. A similar stoichiometry was found by Izawa and Good [11] for the inhibition of photophosphorylation. The involvement of CF_1 is also consistent with the finding that inorganic phosphate reverses the Hg^{2+}-dependent stimulation of non-phosphorylating electron transport (Fig. 3). Interestingly, the concentration of inorganic phosphate required to reverse the Hg^{2+} stimulation (50% reversal = 200 μM P_i) was significantly lower than the concentration of inorganic phosphate required to stimulate basal (+ADP) electron transport and support phosphorylation (50% stimulation = 500 μM).

N-Ethylmaleimide

When chloroplasts are incubated with *N*-ethylmaleimide in the light under conditions which promote the formation of a large transmembrane pH gradient, CF_1 undergoes a conformational transition in which previously inaccessible SH group(s) on the γ-subunit of the enzyme [19] become susceptible to attack by *N*-ethylmaleimide [14]. The *N*-ethylmaleimide-modified enzyme is no longer capable of catalyzing ATP synthesis, although the capacity

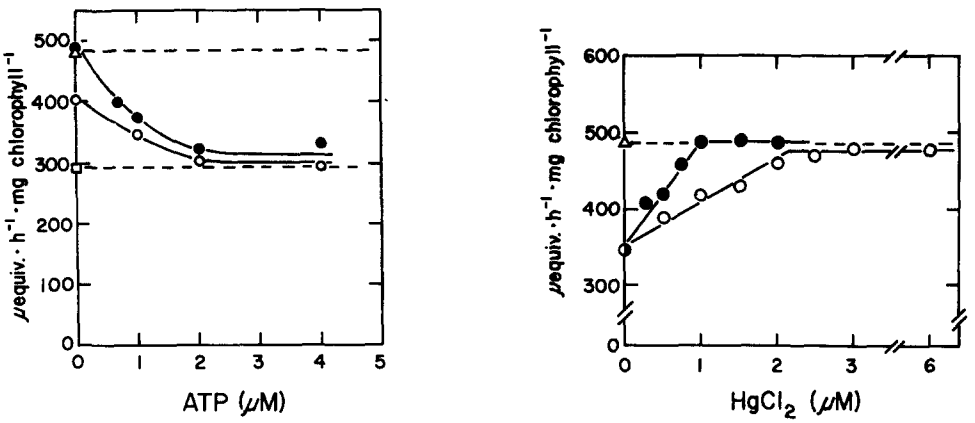

Fig. 1. Inhibition of Hg^{2+}-stimulated non-phosphorylating electron transport by ATP. The basic reaction mixture (1.5 ml) contained 0.1 M sucrose, 2 mM $MgCl_2$, 20 mM Hepps/NaOH (pH 8.2), 200 μM methyl viologen and chloroplasts equivalent to 25 μg chlorophyll. Note that in the presence of 2 μM $HgCl_2$ (●), the rate of electron transport is decreased by low levels of ATP to the rate seen in the absence of Hg^{2+} (○). □- - - - -□, the rate of non-phosphorylating electron transport (no Hg^{2+}) measured in the presence of 1.3 mM ADP. △- - - - -△, the rate of phosphorylating electron transport measured in the presence of 1.3 mM ADP plus 6.7 mM phosphate.

Fig. 2. Stimulation of non-phosphorylating electron transport by Hg^{2+} at different chloroplast concentrations. Reaction conditions were as described in the legend to Fig. 1, with no ATP, ADP or phosphate present. The reactions contained chloroplasts equivalent to 25 (●) or 50 (○) μg chlorophyll. △- - - - -△, the rate of phosphorylating electron transport measured (in the absence of Hg^{2+}) with 1.3 mM ADP plus 6.7 mM phosphate.

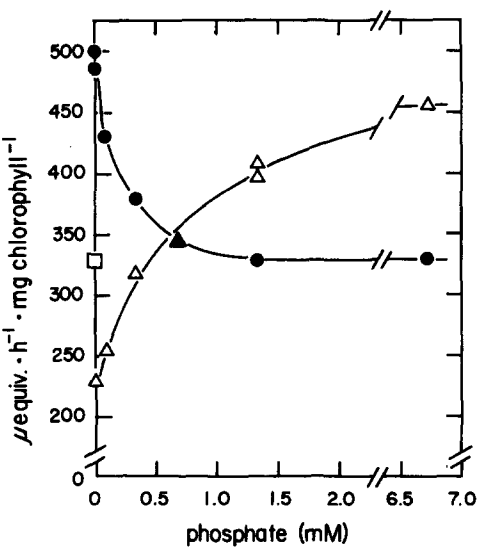

Fig. 3. Effect of inorganic phosphate concentration on phosphorylating and Hg^{2+}-stimulated non-phosphorylating electron transport. The reaction mixture (1.5 ml) contained 0.1 M sucrose, 2 mM $MgCl_2$, 20 mM Hepps/NaOH (pH 8.2), 200 μM methyl viologen, chloroplasts equivalent to 25 μg chlorophyll, and 2 μM $HgCl_2$ (●) or 1.3 mM ADP (△). Note that phosphate completely inhibits Hg^{2+}-stimulated electron transport to the rate of electron transport observed in the absence of Hg^{2+} when ADP and P_i are omitted (□).

of the chloroplasts to generate and maintain a transmembrane pH gradient is unaltered.

The effects of *N*-ethylmaleimide pretreatment of chloroplasts on light-driven electron transport are shown in Table I. As with Hg^{2+}, the rate of electron flow measured in the absence of both ADP and P_i was markedly stimulated in the *N*-ethylmaleimide-treated chloroplasts. The presence of as little as 5 μM ADP in the electron transport assay completely reversed this stimulation. The rate of *N*-ethylmaleimide-stimulated electron transport (—ADP, —P_i) could exceed the electron transport rate measured in *N*-ethylmaleimide-treated chloroplasts under phosphorylating conditions (+ADP, +P_i) and, in fact, approached the rate obtained under phosphorylating conditions in the absence of *N*-ethylmaleimide. Uncouplers further increased *N*-ethylmaleimide-stimulated electron transport, whereas energy transfer inhibitors such as phlorizin and triphenyltin reversed the stimulation. The striking similarity between the stimulation of non-phosphorylating electron transport (—ADP, —P_i) by Hg^{2+} [11,16] and by *N*-ethylmaleimide suggests a similar mode of action for these two inhibitors.

$AgNO_3$

Ag^+ binds strongly to thiol residues, although because of its monovalent character, Ag^+ probably exhibits a lesser propensity to form dimercaptide linkages (R-S-M-S-R) than does Hg^{2+}. Although previously reported to be an uncoupler [21], we have found that Ag^+ behaves as neither an uncoupler nor as an energy transfer inhibitor. As with Hg^{2+} and *N*-ethylmaleimide, the rate of non-phosphorylating electron transport was stimulated by Ag^+ to a plateau approximately equal to the phosphorylating rate measured in the absence of inhibitor (Fig. 4). Ag^+ stimulated non-phosphorylating electron transport was

TABLE I

STIMULATION OF NON-PHOSPHORYLATING ELECTRON TRANSPORT IN *N*-ETHYLMALEIMIDE-TREATED CHLOROPLASTS

Chloroplasts were pretreated in the light with or without *N*-ethylmaleimide (NEM) as described in Materials and Methods and in Ref. 14. The electron transport assay (1.5 ml) contained 0.1 M sucrose, 2 mM $MgCl_2$, 20 mM Hepps/NaOH (pH 8.2), 200 μM methyl viologen and chloroplasts equivalent to 25 μg chlorophyll. When added together, the concentrations of ADP and phosphate were 1.3 mM and 6.7 mM, respectively. The rates of electron transport are given in μequiv. $\cdot h^{-1} \cdot mg^{-1}$ chlorophyll.

Expt.	Additions	Electron transport rate	
		— NEM	+ NEM
I	None	384	453
	ADP (5 μM)	275	247
	ADP (1.3 mM)	264	261
	Phlorizin (3.3 mM)	250	267
	Triphenyltin (2 μM)	148	148
	ADP + P_i	575	448
	Methylamine (10 mM)	922	922
II	None	243	343
	ADP (1.3 mM)	157	164
	ADP + P_i	372	268

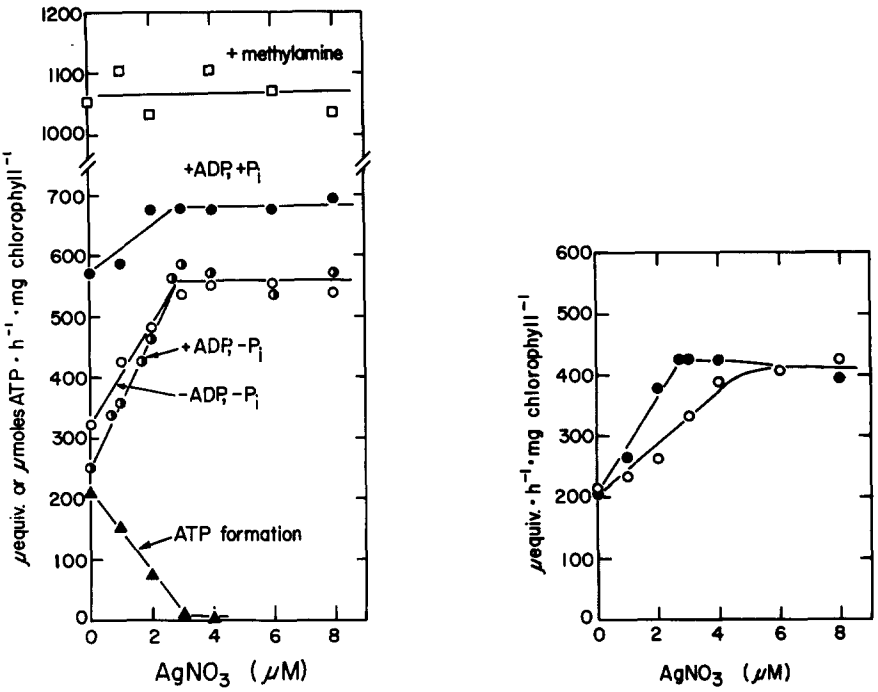

Fig. 4. Inhibition of photophosphorylation and stimulation of non-phosphorylating electron transport by Ag^+. The basic reaction mixture (1.5 ml) was as described in the legend to Fig. 1. When added, ADP was 1.3 mM, inorganic phosphate (P_i) was 6.7 mM, and methylamine was 10 mM.

Fig. 5. Stimulation of non-phosphorylating electron transport by Ag^+ at different chloroplast concentrations. The basic reaction mixture (1.5 ml) contained 0.1 sucrose, 2 mM $MgCl_2$, 20 mM Hepps/NaOH (pH 8.2), 200 μM methyl viologen, 1.3 mM ADP and chloroplasts equivalent to 25 (•) or 50 (○) μg chlorophyll.

also further stimulated by the addition of uncouplers. However, unlike the stimulations by Hg^{2+} and *N*-ethylmaleimide the rate of Ag^+-stimulated electron transport was not affected by even millimolar concentrations of either ADP or P_i. The same concentrations of Ag^+ which stimulated non-phosphorylating electron transport also inhibited ATP formation, although the rate of electron transport under phosphorylating conditions was not markedly affected (in some experiments a slight stimulation, such as that seen in Fig. 4, was observed).

The amount of Ag^+ required to cause maximal stimulation of electron transport (or inhibition of phosphorylation) was stoichiometrically related to the amount of chloroplast material present (Fig. 5). The amount of Ag^+ required to cause a 50% stimulation of non-phosphorylating electron transport (about 6 Ag^+/100 chlorophyll) was approximately twice the amount of Hg^{2+} required for a 50% effect.

The unusual effects of Ag^+ can most likely be traced to direct effects upon the ATP synthase since the energy transfer inhibitor triphenyltin (Fig. 6) reversed the Ag^+ stimulation of non-phosphorylating electron transport. This reversal is not the result of displacement of the Ag^+ from its binding site by

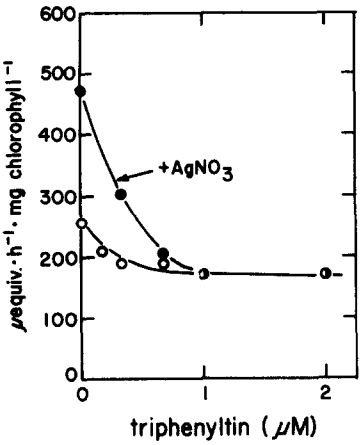

Fig. 6. Inhibition of Ag^+-stimulated non-phosphorylating electron transport by triphenyltin chloride. The basic reaction mixture (containing 25 μg chlorophyll) is described in Fig. 5. When added, $AgNO_3$ was 4 μM. Note that in the presence of 1 μM triphenyltin the stimulation by Ag^+ can no longer be observed. The partial inhibition by triphenyltin of electron transport in the absence of Ag^+ is discussed in Ref. 4.

triphenyltin, nor a direct interaction between the Ag^+ and triphenyltin, since the partial inhibition of Ag^+-stimulated non-phosphorylating electron transport by a suboptimal concentration of triphenyltin could not be reversed even at very much higher Ag^+ concentrations (not shown).

Effect of sulfhydryl-modifying agents on the extent of membrane energization

Atebrin and other acridine derivatives have been characterized and employed as fluorescent probes of the energized state of several biological membrane systems, including bacterial membranes [23], chromatophores [24], beef heart submitochondrial membranes [25] and chloroplasts [26,27]. Although the exact mechanism by which atebrin fluorescence responds to membrane energization is still questioned, an empirical relationship between the extent of fluorescence quenching and the degree of membrane energization has nevertheless been defined [26—29].

The observed effects of Hg^{2+}, Ag^+ and *N*-ethylmaleimide on electron transport and ATP formation can most likely be attributed to an increase in the overall rate of proton efflux from the thylakoid vesicles, which should in turn lead to a decrease in the steady-state level of membrane energization [7,8,22].

The effects of phosphorylation, Hg^{2+} treatment, and Ag^+ treatment on the magnitude of light-induced energy-dependent atebrin fluorescence quenching are shown in Fig. 7. As reported earlier [7], the addition of ADP plus P_i results in a significant decrease in the extent of membrane energization. When H^+ transfer through CF_0 is prevented by triphenyltin, the energy level of the membrane is restored to the same or higher [4,10] level. In the presence of the uncoupler gramicidin, little if any light-dependent decrease in atebrin fluorescence could be detected, even at very low atebrin concentrations (not shown).

Both Hg^{2+}-treated chloroplasts (—ADP, —P_i; Fig. 7b) and Ag^+-treated chloroplasts (±ADP, —P_i; Fig. 7c) exhibited decreases in the extent of energy-depen-

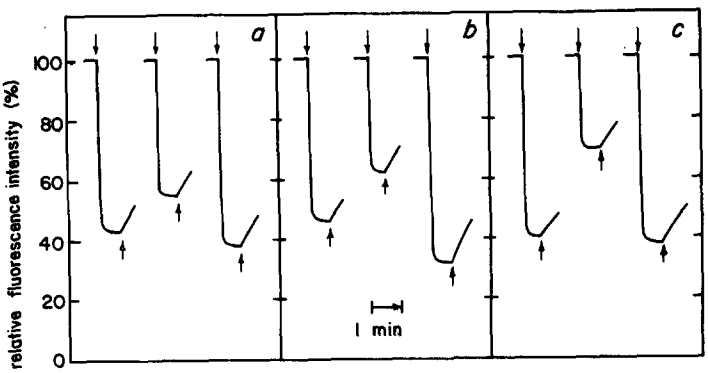

Fig. 7. Effects of ADP + P_i, Hg^{2+} and Ag^+ on energy-dependent quenching of atebrin fluorescence. The basic reaction mixture (2.0 ml) contained 0.1 M sucrose, 2 mM $MgCl_2$, 20 mM Hepps/NaOH (pH 8.2), 200 μM methyl viologen, 5 μM atebrin, and chloroplasts equivalent to 40 μg chlorophyll. The left hand trace in each panel shows the extent of atebrin fluorescence quenching under these non-phosphorulating conditions (downward arrow = light on; upward arrow = light off). The center trace in each panel shows the decrease in the extent of fluorescence quenching in the presence of 1 mM ADP plus 5 mM P_i (panel a), 2 μM $HgCl_2$ (panel b) or 5 μM $AgNO_3$ (panel c). Subsequent addition of 2 μM triphenyltin (right hand trace, each panel) restored the extent of fluorescence quenching to the original or higher level in each case. Triphenyltin added in the absence of ADP + P_i, Hg^{2+} or Ag^+ caused a 10—20% increase in the extent of fluorescence quenching under non-phosphorylating conditions (not shown).

dent atebrin fluorescence quenching similar to those observed under phosphorylating conditions, consistent with an additional H^+ efflux pathway operating in the treated chloroplasts. Triphenyltin effectively reversed the Hg^{2+} and Ag^+-dependent decrease in fluorescence quenching indicating that this additional H^+ efflux pathway includes CF_0. (Triphenyltin, Hg^{2+} and Ag^+ had no effect on the intensity of atebrin fluorescence in the absence of chloroplasts, or in the dark with chloroplasts.)

Discussion

Non-phosphorylating electron transport stimulated by Ag^+, Hg^{2+} or *N*-ethylmaleimide exhibits a number of striking similarities to electron transport coupled to ATP formation:

(i) The maximal rate of Hg^{2+}, Ag^+ or *N*-ethylmaleimide-stimulated non-phosphorylating electron transport is equal to the rate of phosphorylation-stimulated electron transport.

(ii) Hg^{2+}, Ag^+ or *N*-ethylmaleimide-stimulated electron transport is further stimulated by uncouplers.

(iii) Hg^{2+}, Ag^+, *N*-ethylmaleimide and ADP + P_i stimulations of non-phosphorylating electron transport are reversed by energy transfer inhibitors affecting either CF_1 or CF_0.

(iv) Hg^{2+}, Ag^+ and ADP + P_i diminish the steady-state membrane energy level in a triphenyltin-reversible manner.

These similarities suggest strongly that Hg^{2+} and Ag^+, like ADP + P_i, are eliciting their effects at the level of CF_1. This conclusion is further supported by the observation that, in the presence of ADP + P_i, Hg^{2+} exhibits the properties of an energy transfer inhibitor affecting CF_1 [11]. Furthermore, the effects

of Hg^{2+} are virtually identical to those of *N*-ethylmaleimide, which has been shown to react covalently with specific sulfhydryl residue(s) on the γ-subunit of CF_1 [19].

The net ATP synthesis reaction catalyzed by the CF_0-CF_1 complex,

$$ADP + P_i + nH^+_{in} \rightarrow ATP + H_2O + nH^+_{out} \quad (1)$$

may be considered to be the result of two separate, but tightly coupled reactions:

$$nH^+_{in} \rightarrow nH^+_{out} \quad (2)$$

and

$$ADP + P_i \rightarrow ATP + H_2O \quad (3)$$

In the presence of ADP and P_i, when Reaction 3 may proceed, the flux of protons out of the thylakoid (Reaction 2), and hence the rate of proton-pumping electron transport, is enhanced. The similarity between phosphorylating electron transport and non-phosphorylating Hg^{2+}, Ag^+ or *N*-ethylmaleimide-stimulated electron transport can most easily be understood if we conclude that, in the presence of the sulfhydryl modifiers, the ATP synthase (CF_0-CF_1) is turning over in its proton-pumping function (Reaction 2) but not in its ATP-synthesizing function (Reaction 3). Since the maximal rate of Hg^{2+}, Ag^+ or *N*-ethylmaleimide-stimulated electron transport is equal to that observed in normally phosphorylating chloroplasts, it appears that the rate-limiting step of normal phosphorylation is also rate limiting to proton transport in the treated chloroplasts. The phlorizin sensitivity of Hg^{2+} and *N*-ethylmaleimide-stimulated electron transport further supports this conclusion.

The effects of these sulfhydryl reagents appear to represent a unique type of intra-enzyme uncoupling between the proton-pumping and phosphorylation reactions of the CF_0-CF_1 enzyme complex. However, several significant differences among the effects of Hg^{2+}, Ag^+ and *N*-ethylmaleimide may also be noted. *N*-Ethylmaleimide does not inhibit ATP synthesis or stimulate electron transport unless the chloroplasts are preilluminated in the presence of the reagent for 90—120 s under conditions in which a large proton gradient is generated. This treatment results in a conformational change in CF_1, exposing the critical thiol residue(s) [14]. Preillumination is also necessary for the inhibition by bulky sulfhydryl-alkylating agents (e.g. *o*-iodosobenzoate, 2,2′-dithiobis(5-nitropyridine) [13,15]. However, preillumination is not required when Hg^{2+} or Ag^+ are used to modify CF_1 sulfhydryl residues, perhaps because the relatively small ions have access to the critical thiol group(s) in the absence of a conformational change.

Micromolar concentrations of ADP or ATP completely reverse the Hg^{2+} and *N*-ethylmaleimide-dependent stimulations of electron transport, while the Ag^+-dependent stimulation was unaffected by even millimolar concentrations of ADP. Although this could indicate different sites of action for Hg^{2+} and Ag^+, it is also possible that the difference arises from different modes of action at the same site. For instance, the formation of cross-linking dimercaptide linkages with Hg^{2+} (R-S-Hg-S-R) but not with Ag^+ (R-S-Ag Ag-S-R) might explain why rather precisely twice as much Ag^+ as Hg^{2+} is required to cause maximum

stimulation of electron transport. Measurements of the number of 2,2'-dithiobis(5-nitropyridine)-detectable SH residues indicate approximately 100—120 nmol SH/mg chlorophyll in chloroplasts in the dark [13]. The amount of heavy metal ion required for maximal stimulation of non-phosphorylating electron transport (Figs. 1 and 4) represents about 0.3 Hg^{2+}/SH as compared to about 0.6 Ag^{+}/SH. Triphenyltin chloride, which, like Hg^{2+} and Ag^{+} reacts stoichiometrically with chloroplasts, has been suggested to react with vicinal dithiols [30]. The concentration of triphenyltin required for maximal inhibition of CF_0 function represents about 0.3 triphenyltin/SH [10]. Unfortunately an unambiguous determination of the SH-metal adduct stoichiometries within the ATP synthase cannot be made in the absence of information on the number of accessible, non-CF_0-CF_1 sulfhydryl residues associated with the thylakoid membranes.

In any event, transmembrane proton transfer via the CF_0-CF_1 complex appears to be separated from the ATP synthesis reactions in chloroplasts in which certain critical sulfhydryl residue(s), most likely in CF_1, are blocked. The exact mechanism by which such an intra-enzyme uncoupling might occur is unknown, although the reversibility of Hg^{2+} and *N*-ethylmaleimide effects by low levels of adenine nucleotides suggests that the critical sulfhydryl residues are involved in a regulatory function [20]. The effects of Hg^{2+}, Ag^{+} and *N*-ethylmaleimide described here appear to be clearly distinct from the uncoupling effects of the bifunctional maleimide *o*-phenylenedimaleimide [31,32] which cross-links two sulfhydryl residues within the γ-subunit of CF_1. Weiss and McCarty [31] found that this cross-linking opened a proton channel in the ATP synthase (CF_0-CF_1) which was sensitive to DCCD but not to ATP. However, *o*-phenylenedimaleimide-treated chloroplasts showed an almost 2-fold stimulation in the rate of phosphorylating electron transport in conjunction with an 80% inhibition of ATP formation, indicating that the *o*-phenylenedimaleimide-dependent proton efflux through CF_0 was no longer controlled by CF_1. This is in contrast to the effects of Hg^{2+}, Ag^{+} and *N*-ethylmaleimide described in this paper, in which the ability of CF_1 to regulate (gate) proton efflux through the CF_0 channel is apparently preserved.

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