Hg^{2+}-INDUCED TURNOVER OF THE CHLOROPLAST ATP SYNTHETASE COMPLEX IN THE ABSENCE OF ADP AND PHOSPHATE

J. Michael GOULD and Cheryl UNDERWOOD
Program in Biochemistry and Biophysics, Department of Chemistry, University of Notre Dame, Notre Dame, IN 46556, USA

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1. Introduction

The light-driven transfer of electrons along the chloroplast electron transfer chain is stoichiometrically coupled to the net movement of H^+ from the external aqueous phase into the thylakoid interior [1]. In the steady-state a sizable pH differential exists across the thylakoid membrane [2–5], and the rate of proton influx equals the rate of proton efflux. As a result, when all other requirements are saturated (e.g., light intensity, electron acceptors, etc.) the rate of proton-pumping electron transport is determined by those factors which limit the rate at which the accumulated H^+ can move back out of the chloroplasts.

Three principal routes of proton efflux from chloroplasts have been identified:

(1) Non-specific, passive diffusion of H^+ through the thylakoid membrane;

(2) Specific H^+ transport out of the chloroplast via a transmembrane protein complex (CF_0–CF_1) in a reaction which is stoichiometrically coupled to the synthesis of ATP [6,7]:

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

(3) Non-coupled 'leakage' of hydrogen ions through the CF_0–CF_1 complex [8–10].

Changes in the relative contribution of any of these three pathways can alter the total net proton efflux and result in altered electron transport rates. Thus, when phosphorylation is occurring, the magnitude of the steady-state transmembrane pH gradient is reduced [5,11,12] and the rate of electron transport is enhanced. The addition of an inhibitor which prevents phosphorylation by affecting directly either CF_0 or CF_1 (i.e., an energy transfer inhibitor) leads to an increase in the transmembrane pH gradient accompanied by a concomitant decrease in the rate of electron transport to the rate observed in the absence of phosphorylation substrates [13–15]. The rate of non-phosphorylating electron transport (measured in the absence of P_i) can also be lowered substantially by decreasing the non-coupled 'leakage' of protons through the CF_0–CF_1 complex with low levels of adenine nucleotides [8], or with certain CF_0 inhibitors [10]. When the formation of a transmembrane pH gradient is prevented altogether by greatly increasing the membrane proton permeability with an uncoupler such as methylamine, the highest rates of electron transport are observed [16].

The inhibition of phosphorylation and that portion of the electron transport dependent upon phosphorylation by energy transfer inhibitors is presumed to arise from an effect of the inhibitor directly upon CF_0 or CF_1 which prevents the enzyme from turning over catalytically. Hg^{2+} was reported [17] to act as a potent energy transfer inhibitor in chloroplasts. It was also noted [17] that, unlike other energy transfer inhibitors, in the absence of ADP and P_i Hg^{2+} actually increased the rate of electron transport to a rate equal to that observed (in the absence of Hg^{2+}) under normal phosphorylating conditions. We have re-examined this intriguing phenomenon and in this paper wish to report that the peculiar stimulation on non-phosphorylating (–ADP, –P_i) electron transport in Hg^{2+}-treated chloroplasts is due to the fact that, under these conditions, the CF_0–CF_1 ATP synthetase complex is apparently turning over at normal rates in

its proton-pumping (inside → outside) function, despite the complete absence of phosphorylation substrates, ADP and P_i.

2. Experimental

Chloroplasts were isolated from leaves of fresh market spinach essentially as in [13], with the exception that the buffer in the suspending medium was replaced with *N*-2-hydroxyethyl-piperazine-*N'*-propanesulfonic acid (HEPPS)/NaOH, pH 7.8. Electron transport was measured as the rate of oxygen uptake resulting from the reoxidation of reduced methylviologen using a membrane-covered Clark-type electrode. Reactions (1.5 ml final vol.) were run with continuous stirring at 18°C in a thermostatted glass chamber (Gilson Medical Electronics, Inc.). Broadband actinic illumination was supplied by a tungsten–halogen lamp equipped with a condensing lens and a Corning I-69 infrared filter.

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

3. Results

As with other energy transfer inhibitors, the addition of $HgCl_2$ to chloroplasts does not produce any significant effects upon either the rate of uncoupled electron transport (measured in the presence of methylamine), or upon the rate of non-phosphorylating electron transport (measured in the presence of ADP but in the absence of P_i). Unlike other energy transfer inhibitors, however, $HgCl_2$ does have a significant effect upon the rate of non-phosphorylating electron transport when it is measured in the absence of both ADP and P_i (fig.1). Under these conditions $HgCl_2$ causes a stimulation of the electron transport rate to a plateau equal to the electron transport rate obtained under phosphorylating conditions (i.e., +ADP, +P_i) in the absence of $HgCl_2$. Higher levels of $HgCl_2$ do not further stimulate the electron transport rate above the normal phosphorylating level. These increases in non-phosphorylating electron transport are not associated with a collapse of the transmembrane pH gradient, as with an uncoupler (not shown). The amount of $HgCl_2$ required to attain maximal stimulation of non-phosphorylating (–ADP, –P_i) electron transport is a function of the amount of chloroplast

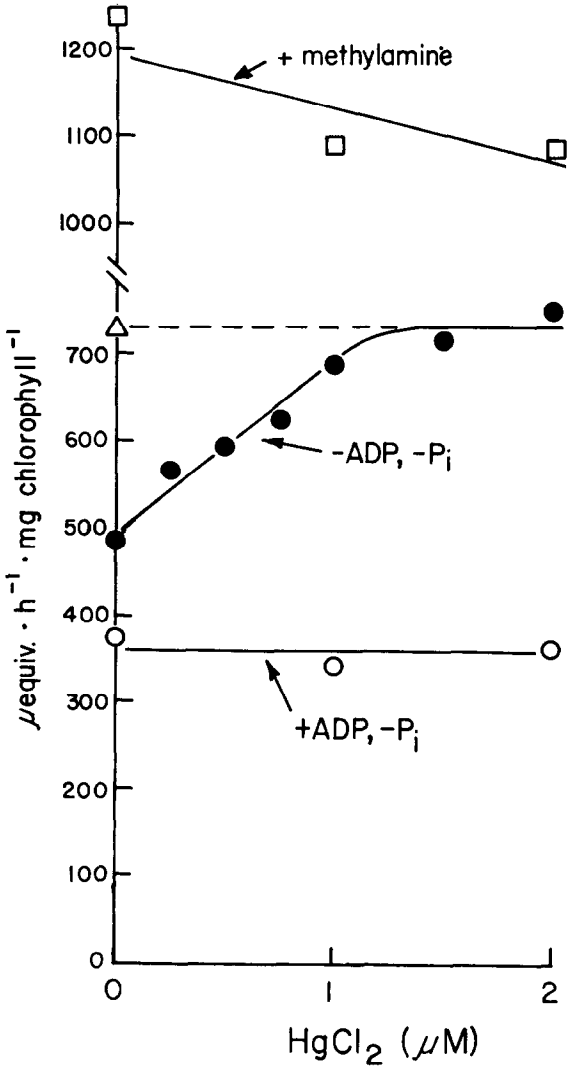

Fig.1. Stimulation of non-phosphorylating electron transport by Hg^{2+}. The reaction mixture (1.5 ml) contained 0.1 M sucrose, 2 mM $MgCl_2$, 20 mM HEPPS/NaOH (pH 8.2), 200 μM methylviologen, and chloroplasts equivalent to 25 μg chlorophyll. When added, ADP was 1.33 mM, methylamine was 10 mM, and inorganic phosphate (P_i) was 6.7 mM. Note that in the absence of ADP, $HgCl_2$ stimulates the rate of non-phosphorylating electron transport to a level equal to the rate of normal, phosphorylating electron transport (△, dashed line), but no further.

material present in the reaction mixture, with an Hg^{2+}/chlorophyll ratio of about 1/33 at 50% stimulation (not shown). A similar stoichiometry has been observed for Hg^{2+} inhibition of ATP formation [17].

In the initial observation of Hg^{2+}-stimulated non-phosphorylating electron transport, it was noted [17], and we have confirmed, that low levels (< 10 μM) of either ADP or ATP effectively reverse the stimulation by Hg^{2+}. Similar low levels of these nucleotides have been found to inhibit non-phosphorylating electron transport in the absence of Hg^{2+} [8–10,17], to increase the magnitude of the transmembrane pH gradient [8–10], and to increase the extent of delayed light emission [18]. These effects of ADP and ATP have been interpreted as arising from a direct effect of the nucleotide on CF_1 leading to a decrease in non-coupled proton 'leakage' through the CF_0–CF_1 complex [8]. In conjunction with the observation that the same levels of Hg^{2+} which stimulate non-phosphorylating electron transport in the absence of ADP cause an energy-transfer type inhibition in the presence of ADP plus P_i [17], these findings suggest that the effect of low levels of Hg^{2+} can probably be traced to an effect upon some component of the ATP synthetase, most likely CF_1. This in turn suggests the interesting possibility that the peculiar stimulation of non-phosphorylating electron transport induced by Hg^{2+} may be the result of Hg^{2+}-induced turnover of the ATP synthetase catalytic cycle leading to an increase in net proton efflux similar to that seen during normal ATP formation [5,11,12].

To test this idea we have examined the effects of other known energy transfer inhibitors of Hg^{2+}-stimulated non-phosphorylating electron transport. Both phlorizin (table 1), an energy transfer inhibitor [19] which specifically inhibits turnover of CF_1 [20], and triphenyltin chloride (fig.2), an energy transfer inhib-

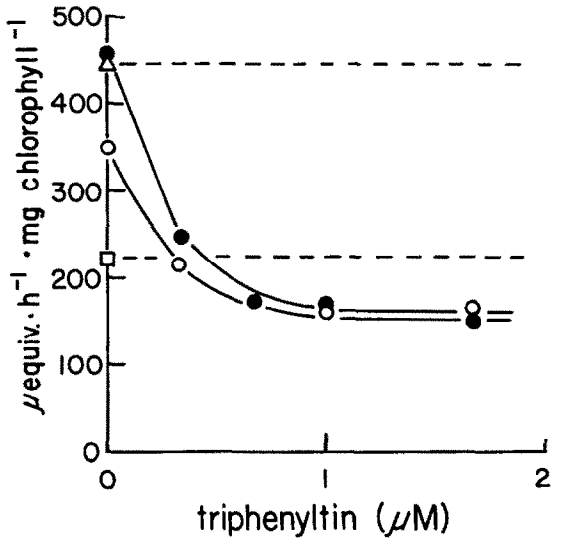

Fig.2. Inhibition of Hg^{2+}-stimulated non-phosphorylating electron transport by triphenyltin chloride. The reaction mixture (1.5 ml) contained 0.1 M sucrose, 2 mM $MgCl_2$, 20 mM HEPPS/NaOH (pH 8.2), 200 μM methylviologen and chloroplasts containing 25 μg chlorophyll. When added, $HgCl_2$ was 2 μM. Note that triphenyltin inhibits both normal, –ADP, $-P_i$ (○) and Hg^{2+}-stimulated (●) non-phosphorylating electron transport to the same rate. The reason that the triphenyltin-inhibited rate is lower than the rate seen with substrate levels of ADP (1.67 mM (□)) is discussed [10]. (△) Indicates the rate of phosphorylating electron transport (+1.67 mM ADP, 6.7 mM P_i) measured in the absence of Hg^{2+}.

Table 1
Inhibition of Hg^{2+}-stimulated non-phosphorylating electron transport by phlorizin

Reaction conditions	Electron transport rate (μequiv. · h^{-1} · mg chl.$^{-1}$)
–ADP, $-P_i$	400
+ADP, $-P_i$	154
+ADP, $+P_i$	528
–ADP, $-P_i$, $+Hg^{2+}$	511
–ADP, $-P_i$, $+Hg^{2+}$, + phlorizin (1.67 mM)	178
–ADP, $-P_i$, $+Hg^{2+}$, + phlorizin (3.3 mM)	156

Reactions were performed as described in the legend to fig.1. When added, $HgCl_2$ was 2 μM

itor which specifically blocks the flux of protons through CF_0 [13], completely inhibit the Hg^{2+}-dependent stimulation of non-phosphorylating electron transport. The findings, then, lend strong support to the conclusion that the mechanism of Hg^{2+} stimulation of non-phosphorylating electron transport requires the participation of the ATP synthetase complex.

4. Discussion

Several striking similarities between normal, phosphorylation-coupled electron transport and Hg^{2+}-stimulated, non-phosphorylating electron transport are now apparent:

(i) The maximum rate of Hg^{2+}-stimulated non-phosphorylating electron transport is equal to the maximum rate of electron transport stimulated by the addition of ADP and P_i;

(ii) Hg^{2+}-stimulated, non-phosphorylating electron transport is further stimulated when uncouplers are added, as is phosphorylating electron transport;

(iii) The stimulation of non-phosphorylating electron transport by either Hg^{2+} or ADP plus P_i can be effectively reversed by the CF_0 inhibitor triphenyltin or the CF_1 inhibitor phlorizin.

The stimulation of non-phosphorylating electron transport by ADP and P_i is clearly due to the function of these reagents as substrates of ATP synthesis. In the presence of ADP and P_i, the CF_0–CF_1 complex can catalyze the ATP formation reaction:

$$ADP + P_i \rightarrow ATP + H_2O \quad (2)$$

which, according to the chemiosmotic hypothesis [6], is obligatorily coupled to the efflux of internally accumulated protons:

$$nH^+_{in} \rightarrow nH^+_{out} \quad (3)$$

through the CF_0–CF_1 complex. The resultant increase in net proton efflux allows a corresponding increase in the rate of proton-pumping electron transport. The similarities between phosphorylating electron transport and Hg^{2+}-stimulated non-phosphorylating electron transport can most easily be understood if we postulate that in Hg^{2+}-treated chloroplasts the CF_0–CF_1 complex is in fact turning over, but in its proton-pumping function only (eq. (3)). The equivalence of the Hg^{2+}-stimulated non-phosphorylating electron transport rate and the normal, phosphorylating electron transport rate further suggests that the rate of turnover of the proton efflux reaction must be the very nearly same in both cases.

At the moment we have little information on the mechanism by which Hg^{2+} might allow the controlled flux of protons through the CF_0–CF_1 complex to occur at rates comparable to those occurring during ATP formation, despite the complete absence of phosphorylation substrates. Because these effects by Hg^{2+} can be prevented by the same low concentrations of adenine nucleotides [17] which have been shown to partially decrease the proton 'leakage' through CF_0–CF_1 [8], it is tempting to speculate that Hg^{2+} may be modifying some component of the ATP synthetase involved in the regulation of a gated proton channel through the enzyme complex. In any event, it seems most likely that the site of the Hg^{2+} effect is a sulfhydryl residue(s) within CF_1. This conclusion is further supported by the observation (C.U. and J.M.G., in preparation; see also [21]) that chloroplasts treated with *N*-ethylmaleimide, which has been shown to specifically derivatize a sulfhydryl residue on the γ-subunit of CF_1 [21,22], exhibit an ATP, ADP and energy transfer inhibitor-sensitive stimulated non-phosphorylating electron transport rate similar to that described here for Hg^{2+}-treated chloroplasts.

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