DARK OXIDATION-REDUCTION COUPLED PHOSPHORYLATION IN SONICATED CHLOROPLAST VESICLES

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1. Introduction

The ability of isolated chloroplasts to phosphorylate in the dark has been well established by Jagendorf and co-workers [1]. These experiments have usually entailed either impressing an artificial pH gradient across the membrane by incubating the chloroplasts first in a permeable organic acid and then shifting the pH of the external medium to a higher value (acid – base phosphorylation) [2,3] or by illuminating the chloroplasts in the absence of the phosphorylation substrates (i.e. ADP and phosphate) followed by the addition of ADP and phosphate in the dark ('X_E' phosphorylation) [4]. In both of these experiments, phosphorylation is generally believed to be driven through a pH gradient across the membrane either directly applied, as in the case of the acid–base phosphorylation, or built up by the chloroplasts in the light and dissipated in the dark, as in the case of the 'X_E' experiments. The pH gradient across the membrane is thought to be either in equilibrium with a 'high energy intermediate' (the compound or 'state' of the membrane responsible for driving phosphorylation) [5] or to be the actual 'high energy intermediate' itself (see [1] for discussion).

In this paper we describe a new method for inducing chloroplast membranes to catalyze phosphorylation in the dark using only reductants and an oxidant. Chloroplast membranes are 'internally' loaded with a membrane non-permeable chemical oxidant and are then allowed to react with a membrane penetrable chemical reductant in the presence of ADP and phosphate. Under these conditions, ATP can be generated. The results are briefly discussed in terms of the Mitchell chemiosmotic hypothesis [6].

Abbreviations: DAD, 2,3,5,6-tetramethyl-*p*-phenylenediamine; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine; mCCP, carbonylcyanide -chlorphenylhydrazone

2. Materials and methods

Spinach chloroplasts were prepared as previously described [7] and suspended in a medium containing, in 2.0 ml final vol, chloroplasts equivalent to 3.0 mg Chl/ml, 50 mM Tricine–NaOH buffer (pH 8.0), 0.4 M sucrose, 10 mM KCl, 5.0 mM $MgCl_2$, 2.0 mg/ml bovine serum albumin (BSA), and 0.1 M potassium ferricyanide and stored in the dark at 0–4°C. The suspensions were sonicated for approx. 15 sec using a Branson Model B-12 sonifier equipped with a microtip at power setting 5. All precautions were taken to keep the chloroplast suspensions as dark as possible to avoid photosynthetic reduction of ferricyanide. 0.1 ml of the chloroplast suspension was then diluted to 1.5 ml final vol in a reaction mixture containing chloroplasts, equivalent to 0.2 mg Chl/ml, 6.0 mM potassium ferricyanide (carried over with the chloroplast suspension), 50 mM Tricine–NaOH (pH 8.0), 10 mM KCl, 5.0 mM $MgCl_2$, 0.4 M sucrose, 2.0 mg/ml BSA, 5.0 mM ADP, 3.3 mM [^{32}P]phosphate (equivalent to approx. 1.25×10^6 cpm/ml), 10 mM ascorbate, and 0.4 mM DAD, unless otherwise stated. After 2.0 min incubation at 20°C in the dark, 0.8 ml were withdrawn and added to 0.2 ml 1.0 N $HClO_4$. ATP was determined by liquid scintillation as previously described [7].

3. Results

Table 1 shows that chloroplasts, after being sonicated in the presence of ferricyanide, can incorporate phosphate into ATP in the dark when they are suspended in a reaction mixture containing ADP, phosphate, and reducing agents. As seen in table 1 (rows 1 and 2), the reductant ascorbate alone is not sufficient to catalyze ATP formation even though it is in substantial excess (10 mM ascorbate, 6.0 mM ferricyanide). However, when both ascorbate and DAD are added, the yield of ATP formation increases two- to three-fold*. In the absence of sonicated chloroplasts, only a little incorporated phosphate is detectable. The amount of ATP formed by the sonicated vesicles under these conditions ranges from 30 to 60 nmol per mg Chl (the amount obtained in the 'complete' sample corrected for the 'minus DAD' control), the total amount measured depending on the chlorophyll concentration tested. This is about one-half to one-fourth of the amount that can usually be obtained from an acid–base shift experiment [1].

Ferricyanide must be present during the sonication in order for the chloroplasts to be able to make ATP in the dark, as shown in table 2. In this experiment two batches of chloroplasts were sonicated under conditions that were as identical as possible with the one exception that the ferricyanide was left out of one batch. The chloroplasts sonicated in the absence of ferricyanide did not synthesize ATP upon suspension in the reaction mixture.

Table 3 shows that the ability of chloroplasts to incorporate phosphate into ATP in the dark can be inhibited by classical uncouplers of photosynthetic phosphorylation. The high concentration of uncouplers needed for inhibition most probably results from the high chlorophyll concentration needed to

* The control with DAD added in the absence of ascorbate also shows no increased incorporation over the 'minus ascorbate, minus DAD' control (data not shown). Due to the fact that the external ferricyanide concentration is 6.0 mM and only 0.4 mM DAD is added, all of the DAD would be oxidized on the outside of the membranes.

Table 1
Dependence of dark phosphorylation on sonicated chloroplasts and reducing agents

Reaction mixture	nmoles ATP / mg Chl
Complete	68
Minus DAD	23
Minus ascorbate minus DAD	7
Minus chloroplasts	28
Minus chloroplasts minus DAD	28
Minus chloroplasts minus ascorbate	16
Minus chloroplasts minus ascorbate minus DAD	11

Reaction conditions as described in Materials and methods.

Table 2
Dependence of dark phosphorylation on the presence of ferricyanide during sonication

Reaction mixture	Chloroplasts sonicated in the absence of ferricyanide	nmoles ATP / mg Chl	Chloroplasts sonicated in the presence of ferricyanide
Complete	26		77
Minus DAD	19		25
Minus ascorbate minus DAD	8		8

Chloroplasts were prepared and tested as described in Materials and methods with the exception that ferricyanide was omitted from one batch.

Table 3
Sensitivity of dark phosphorylation to uncouplers

Reaction mixture	nmoles ATP / mg Chl
Complete	64
Minus ascorbate minus DAD	12
Minus DAD	38
Plus NH_4Cl (50 mM)	37
Plus mCCP (50 μM)	33
Plus gramicidin (50 μM)	42

Reaction conditions as described in Materials and methods.

make the amount of ATP formed in the reaction mixture measureable.

In order to determine the chemical nature of the mediator necessary for the chloroplasts to make ATP in the dark, the amount of ATP formed with TMPD, a pure electron mediator [8], was compared to the amount formed with the C-substituted analog, DAD, a proton as well as electron mediator [8]. Upon oxidation, TMPD loses one electron and forms a stable radical cation (Wursters Blue) whereas DAD loses two electrons and two protons forming the uncharged diimine. From the results shown in table 4, it is clear that TMPD is unable to increase the yield of dark phosphorylation. Apparently the mediator bringing reducing equivalents from the outside of the membrane to ferricyanide, on the inside, must also bring along protons.

Table 4
Comparison of TMPD and DAD on the yield of dark phosphorylation

Complete reaction mixture	nmoles ATP / mg Chl
Minus ascorbate	9
Plus ascorbate	33
Plus ascorbate plus DAD	61
Plus ascorbate plus TMPD	32

Reaction conditions as described in Materials and methods. TMPD concentration was 0.4 mM.

4. Discussion

Since the pioneering experiments of Jagendorf and co-workers, it has long been known that, under certain conditions, chloroplasts have the ability to phosphorylate in the dark. Usually either an artificial pH gradient is impressed across the membrane by allowing the chloroplasts to incubate at low pH (in the presence of an organic acid) followed by a shift to high pH with the concomitant addition of ADP and phosphate [3], or the chloroplasts are illuminated in the presence of electron transfer cofactors and ADP and phosphate are immediately added in the dark [4].

We have now found new conditions under which the chloroplast membranes are able to catalyze phosphorylation in the dark, i.e. by a coupled oxidation-reduction reaction occurring across the membrane. The inclusion of a relatively non-penetrable oxidant on the 'inside' of the vesicles is achieved in these experiments by sonication of the chloroplasts in the presence of ferricyanide. The chloroplasts can then form a 'high energy state' in the dark in the presence of a reducing agent that can penetrate the membrane efficiently. The addition of ascorbate alone to these 'ferricyanide loaded' vesicles is insufficient to catalyze phosphorylation in the dark. A necessary requirement is the inclusion of a cofactor that can cross the membrane. Because of the dependence of the incorporation on a reductant, the ATP formed cannot be due to (i) a pH gradient induced by dilution of the chloroplasts in buffer (i.e. a pH shift of the type described by Jagendorf and Uribe [3]) or (ii) an electrical potential generated by a ferricyanide gradient induced by the dilution of the ferricyanide on the outside of the chloroplast membrane. A similar type of oxidative phosphorylation has been reported by Racker and Kandrach [9] who incorporated mitochondrial coupling factors into artificial membranes. In their experiments, reducing equivalents were transported from ascorbate, on the outside of the liposomes, to cytochrome *c*, on the inside, by phenazinemethosulfate.

In these experiments we have used DAD to increase the yield of phosphorylation in the dark, but similar results have also been obtained with dichlorophenolindophenol, phenazinemethosulfate, and phenylenediamine, always added together with ascorbate. Common to these mediators is that they are the type

that, upon oxidation, lose protons as well as electrons [8].

A second class of donor compounds yields only electrons upon oxidation. This group includes N-substituted *p*-phenylenediamines [8], ferrocyanide [10], and iodide [10] among others. TMPD forms, under the appropriate conditions, a stable radical cation [7]. It has long been known that when TMPD is used as a donor for photosystem I (in the presence of ascorbate and 3-(3,4 dichlorophenyl)-1,1-dimethylurea), electron transport is not coupled to phosphorylation [11]. Hauska et al. [12,13] have recently suggested that, in order for a donor to photosystem I to be coupled to phosphorylation, it must meet either of two conditions. (i) It must stimulate electron transport through the natural proton pump of the chloroplast electron transport chain (i.e. plastoquinone) or (ii) it must itself pump protons inside, that is, upon oxidation on the inside of the membrane it must yield protons contributing to the formation of a pH gradient. TMPD can neither reduce plastoquinone (because of the large difference in midpoint potentials) nor can it contribute protons to a pH gradient upon oxidation on the inside of the chloroplast membrane. TMPD is also unable to increase the yield of dark phosphorylation under conditions where other compounds, e.g. DAD, increase this yield by two to three fold (table 4).

Fig.1 shows a model for what we believe may be occurring in our system enabling the chloroplast membranes to generate ATP in the dark. Ferricyanide is trapped on the inside of the vesicles during sonication. It oxidizes the electron transport mediator, M, which is kept reduced on the outside of the membranes by the reductant, ascorbate (added in excess). Upon oxidation of M on the inside of the vesicles, protons must be released in order to develope a pH gradient across the membrane. The proton gradient so developed can then be used by the membranes, via the chloroplast coupling factor, CF_1, to drive the synthesis of ATP. The addition of uncouplers, known to dissipate the pH gradient in phosphorylating systems, leads to a marked inhibition of this dark phosphorylation.

We believe that this test system should be of value in determining whether or not artificial donor compounds routinely used as probes for coupling sites in electron transport can build a proton gradient across the thylakoid membrane upon oxidation when they are oxidized at the inside. We have now shown that TMPD, a photosystem I donor not coupled to phosphorylation, does not stimulate the yield of dark phosphorylation and are presently examining donors commonly used for photosystem II.

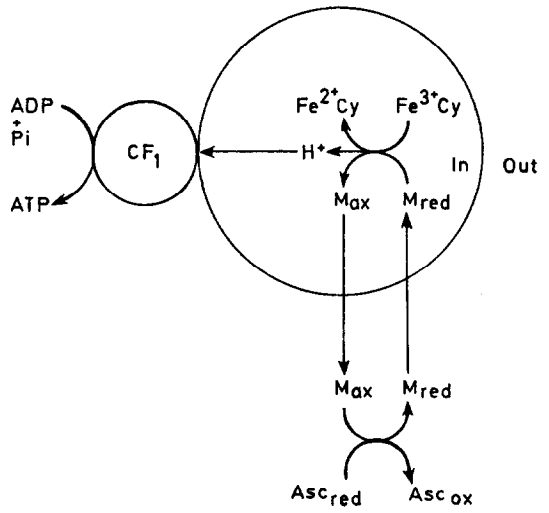

Fig.1. Model for oxidation–reduction coupled phosphorylation in sonicated chloroplast vesicles. Abbreviations: M_{red}, M_{ox}, reduced and oxidized forms of the membrane permeable mediator respectively; $Fe^{3+}Cy$, $Fe^{2+}Cy$, ferricyanide and ferrocyanide; Asc_{red}, Asc_{ox}, ascorbate and dehydroascorbate respectively; CF_1, chloroplast coupling factor 1.

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