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OXIDATION-REDUCTION COUPLED PHOSPHORYLATION IN THE DARK WITH ISOLATED SPINACH CHLOROPLASTS

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SUMMARY

1. Spinach chloroplasts, pre-incubated with ferricyanide, acquire the ability to make ATP in the dark provided they are supplied with a reductant and a lipophilic mediator that can penetrate the membrane. The mediator must be of the type that, upon oxidation, releases protons into the surrounding medium such as 2,3,5,6-tetramethyl-*p*-phenylenediamine (DAD).

2. Dark phosphorylation is not affected by the electron transport inhibitor, 3(3,4-dichlorophenyl)-1,1-dimethyl urea (DCMU) or 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (DBMIB), but is inhibited by uncouplers of photophosphorylation (e.g. NH_4Cl and carbonylcyanide-*m*-chlorophenylhydrazone (CCCP)) and high concentrations of the energy transfer inhibitor, Dio-9.

3. Because only catalytic amounts of the mediator DAD are required to saturate dark phosphorylation, it is concluded that DAD shuttles reducing equivalents across the membrane from the reductant, ascorbate, on the outside, to ferricyanide, the oxidant, trapped on the inside.

4. The results are interpreted within the framework of the chemiosmotic hypothesis for the coupling of electron transport to phosphorylation.

INTRODUCTION

Although the chemical mechanism for the formation of ATP in coupled electron transport systems still remains to be resolved, within the past 15 to 20 years

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Abbreviations: CCCP, carbonylcyanide-*m*-chlorophenylhydrazone; Cl_2Ind, 2,6-dichlorophenol indophenol; DAD, 2,3,5,6-tetramethyl-*p*-phenylenediamine (diaminodurene); DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (dibromothymoquinone); DCCD, dicyclohexyl carbodiimide; DCMU, 3(3,4-dichlorophenyl)-1,1-dimethyl urea; DEPT, *N,N*-diethyl-*p*-toluidine; DMPD, *N,N*-dimethyl-*p*-phenylenediamine; EDTA, ethylenediamine tetraacetic acid; PD, *p*-phenylenediamine; PMS, *N*-methylphenazonium methylsulphate; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine.

an enormous amount of information concerning the conservation of energy during mitochondrial and photosynthetic electron transport has been accumulated. Early major contributions were made by Shen and Shen [1] and Hind and Jagendorf [2] who measured the ability of chloroplasts to phosphorylate in the dark following a period of illumination. They showed that the processes of photosynthetic electron transport and phosphorylation are coupled to each other through some form of "high energy intermediate" which they termed X_e. Jagendorf and co-workers [3, 4] later demonstrated that chloroplasts can drive the formation of ATP completely in the dark if a pH gradient is impressed across the membrane. These results, combined with the observation, again from Jagendorf and co-workers [5], that illuminated chloroplasts take up protons from the outer media, were the earliest findings that tended to corroborate the predictions of Mitchell's chemiosmotic hypothesis for the coupling of electron transport to phosphorylation [6].

In more recent years, the question of how chloroplasts generate a protonmotive force and, in particular, a proton gradient has been addressed by many laboratories (for review see ref. 7). It is now recognized that both photosystems I and II are coupled to phosphorylation [8–11]. Energy conservation by photosystem II can be attributed to the location of the water splitting reaction, or at least the site where the protons from water are deposited, as being on the inside of the thylakoid membrane [12–15]. Energy conservation by photosystem I is believed to be due to the action of plastoquinone pumping protons across the membrane during concerted oxidation-reduction reactions [14–16].

There are, however, several partial reactions utilizing photosystems I and II that are coupled to phosphorylation but that cannot be explained either by the internal release of protons from water or the proton pumping action of plastoquinone [11, 17, 18]. In addition, there are partial reactions with donors for both photosystems that apparently bypass one or both coupling sites [17, 19]. Hauska et al. [19, 20] have attempted to explain this apparent anomaly in terms of the chemistry of the various donor compounds used. Those compounds which are coupled to ATP formation are thought to release protons on the inside of the thylakoid membrane upon oxidation and, thus, generate a proton gradient capable of driving phosphorylation. In contrast, compounds which are good donors but are not coupled to phosphorylation only undergo redox reactions involving electrons and the neccessary pH gradient is not formed. This hypothesis has been supported by Hauska and Prince [21] who measured the ability of various donor compounds to generate a pH gradient in ferricyanide loaded liposomes.

Hinkle and co-workers [22] and Racker and co-workers [23] have been able to incorporate electron transport carriers and energy coupling proteins isolated from mitochondria into artificial vesicular membranes. Under certain conditions these systems are capable of various energy conserving reactions including active ion transport [22] and "oxidative" phosphorylation [24, 25]. Apparently a requirement for energy conservation in these vesicles is the production of a pH gradient generated by electron transport.

In this paper we show that a biological membrane system, spinach chloroplasts, can be induced to conserve energy from oxidation-reduction reactions that occur completely in the dark. In these experiments the oxidant, ferricyanide, is spacially separated from the reductant, ascorbate, by being trapped on the inside of

the thylakoid membrane. Electron transport is then catalyzed by a lipophilic mediator. We show that only those mediators that undergo redox reactions involving both protons and electrons can catalyze the formation of ATP. Our results are consistent with and interpreted within the framework of the Mitchell chemiosmotic hypothesis [6]. A preliminary report of these experiments has already been published [26].

METHODS AND MATERIALS

Spinach chloroplasts were isolated as previously described [27] in buffer containing 50 mM tricine/NaOH (pH 8.0), 10 mM KCl, 5 mM $MgCl_2$, 0.4 M sucrose, and 2.0 mg/ml bovine serum albumin and resuspendend at 2–3 mg chlorophyll/ml (2–3 ml total volume) in the same buffer containing, however, 0.1 M sucrose and, in addition, 0.1 M potassium ferricyanide and 20 μM 3(3,4-dichlorophenyl)-1,1-dimethyl urea (DCMU). The chloroplast suspension was then diluted to 100 ml in a solution containing 50 μg chlorophyll/ml, 50 mM tricine/NaOH (pH 8.0), 10 mM KCl, 5 mM $MgCl_2$, 2.5 mM sucrose (carried over with the chloroplast suspension), 2.0 mg albumin/ml, 0.1 M potassium ferricyanide, and 20 μM DCMU. The suspension was stored in the dark at 0–4 °C for 30 min and was then centrifuged at $12000 \times g$ for 5 min. The pellet was resuspended in a small volume of the supernatant.

Unless otherwise stated, reaction mixtures contained in a total volume of 1.5 ml: chloroplasts, equivalent to 0.2–0.25 mg chlorophyll/ml, 6.0 mM potassium ferricyanide (carried over with the addition of the chloroplasts), 50 mM tricine/NaOH buffer (pH 8.0), 10 mM KCl, 5.0 mM $MgCl_2$, 2.0 mg albumin/ml, 1.3 μM DCMU, 10 mM sodium ascorbate, 5.0 mM ADP, and 3.3 mM [^{32}P]phosphate (approximately 2.5×10^6 CPM/ml). All additions, unless indicated, were made before the addition of the chloroplasts to the reaction mixture. Reactions were allowed to proceed in the dark at 15 °C for 2 min after which an 0.8 ml aliquot was removed, added to 0.2 ml of 1.0 M $HClO_4$, and analyzed for [^{32}P]ATP. Experiments (data not presented) have shown that the yield of ATP does not increase after 30 s of incubation. A 2-min incubation was chosen to assure the highest possible yield of ATP under all conditions. All samples were measured in triplicate and the error reported is the standard derivation.

Esterified phosphate was determined by measuring the Čerenkov radiation in the aqueous phase after the extraction of unesterified phosphate, essentially as described by Gould et al. [28]. In order to avoid possible quenching of the radiation by the intense blue color of the samples, 0.1 ml of a solution of 0.4 M Na_2HPO_4 was added to each sample and the mixtures were extracted a second time. This procedure removes essentially all of the excess molybdate from the aqueous phase and the solutions are, and remain, colorless. 5 ml of the aqueous phase were counted in a Packard Tricarb Liquid Scintillation Spectrometer model 3385 using the programmed ^{3}H channel.

RESULTS

(*I*) *Diaminodurene catalyzed ATP formation in the dark*

In a previous paper it was reported that chloroplasts, sonicated in the presence

of ferricyanide, are capable of making ATP in the dark when they are added to a reaction mixture containing ADP, phosphate, ascorbate, and a permeable electron transport mediator such as 2,3,5,6-tetramethyl-*p*-phenylenediamine (DAD) [26]. A major disadvantage of that procedure is, however, the inherent irreproducibility associated with sonication. (The sonication procedure, when carried out longer than 10–15 s, often yields vesicles incapable of phosphorylation.) An alternative procedure was therefore sought. The following simpler technique has proven to be more reproducible and not nearly as detrimental to the integrity of the thylakoid membrane. Chloroplasts, suspended in 0.1 M sucrose, are diluted approximately 50-fold in a medium containing ferricyanide (and DCMU) and are incubated for one half hour in the dark before being collected by centrifugation. In order to insure that the ferricyanide which has penetrated the membrane does not leak out upon resuspension of the chloroplasts due to a large ferricyanide gradient across the membrane, the chloroplasts are resuspended in a small volume of the ferricyanide preincubation medium. Chloroplasts membranes treated in this manner with ferricyanide have been used for all of the following experiments.

Table I shows the results of an experiment in which ferricyanide pre-treated chloroplasts were added to a solution containing ascorbate, ADP, phosphate, and DAD ("complete"). After correction for the background (usually equivalent to 8–12 nmol ATP/mg chlorophyll), it is seen that these membranes generated approximately 34 nmol ATP/mg chlorophyll in the dark. Table I also shows that the formation of ATP in the dark is absolutely dependent upon the inclusion of both ADP and, in this experiment, DAD in the reaction mixture. The maximal yield of ATP generated in the dark by ferricyanide pre-treated chloroplasts varied among different chloroplast preparations between 30 and 70 nmol per mg chlorophyll although within any given experiment the standard deviation was only about $\pm 5\,\%$. The cause for this wide variation is not entirely clear, however, it probably depends on the amount of ferricyanide that penetrates into the chloroplast membrane and the coupling state of the chloroplasts after the pre-treatment.

The yield of ATP generated in the dark by pre-treated chloroplast membranes in complete reaction mixtures is a rather critical function of the ferricyanide concentration in the pre-incubation mixture, as shown in Fig. 1. The inhibition seen

TABLE I

DIAMINODURENE CATALYZED PHOSPHORYLATION IN THE DARK WITH FERRICYANIDE PRE-TREATED CHLOROPLASTS

Experimental conditions are as described in Methods and Materials. Chloroplasts were added to a final concentration of 0.23 mg chlorophyll/ml and the concentration of DAD was 5 μM. In reaction mixtures in which DAD or ADP has been omitted, the radioactivity has been demonstrated to be due to non-nucleotide contaminants (see Section 1 of Results).

Reaction mixture	nmol ATP / mg chlorophyll
Complete	47 ± 2
Minus DAD	13 ± 3
Minus ADP	12 ± 2

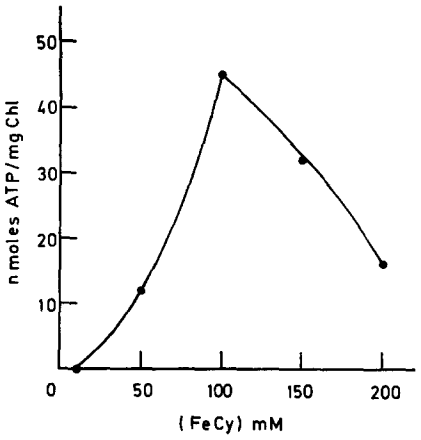

Fig. 1. Yield of ATP synthesized in the dark as a function of the ferricyanide concentration during pre-incubation. Chloroplasts were pre-incubated in solutions containing various amounts of ferricyanide and tested for their capacity to make ATP in the dark as described in Materials and Methods. Reactions mixtures contained chloroplasts, equivalent to 0.20 mg chlorophyll/ml and 5 μM DAD.

above 100 mM ferricyanide may be due to (i) an inactivation of the coupling factor on the membrane by the severe oxidizing conditions of the incubation medium and/or (ii) a severe shrinkage of the thylakoid membrane decreasing the internal volume and hence the amount of ferricyanide that is accumulated internally. If ferrocyanide is substituted for ferricyanide during the preincubation no ATP is made in complete reaction mixture (see Table III). Furthermore, other experiments have shown that the amount of ATP generated in the reaction mixture is linearly proportional to the amount of pre-treated chloroplasts added to complete reaction mixtures.

It should be added that the technique used to measure [^{32}P]ATP is based on the extraction of all unesterified orthophosphate and what is measured is either ^{32}P-labeled organic or polyphosphates (see Methods and Materials and ref. 28). In order to identify the radioactive products of the reaction, samples were prepared as usual (containing ascorbate [^{32}P]phosphate, and DAD) with and without ADP. The reactions were stopped by the addition of $HClO_4$, the aggregated chloroplasts centrifuged and the adenine nucleotides in the clear supernatant separated on a DEAE-formate column as described by Adams et al. [29]. Analysis of the eluent showed that the only product of the reaction labeled with [^{32}P]phosphate is ATP and only in samples to which ADP was added (data not shown).

(II) Sensitivity of dark phosphorylation to uncouplers, electron transport, and energy transfer inhibitors

Table II shows that dark phosphorylation catalyzed by ferricyanide pretreated chloroplasts can be inhibited by the uncouplers NH_4Cl and carbonylcyanide-*m*-chlorophenylhydrozone (CCCP). Other uncouplers, including methylamine, 1,1,3,3-tetramethylbutamine,valinomycin plus nigericin and arsenate, have also been tested and behave similary (data not shown). It should be pointed out that the concentration of most uncouplers tested inhibit dark phosphorylation with ferricyanide

TABLE II

SENSITIVITY OF DAD CATALYZED DARK PHOSPHORYLATION TO UNCOUPLERS, ELECTRON TRANSPORT AND ENERGY TRANSFER INHIBITORS

Experimental conditions are as described in Methods and Material. Chloroplasts were added to a final concentration of 0.22 mg chlorophyll/ml (Expt. No. 1), 0.25 mg chlorophyll/ml (Expt. No. 2) and 0.24 mg chlorophyll/ml (Expt. No. 3). The concentration of DAD was in all experiments 5 μM. Note that in Expt. No. 3 the addition of DAD was delayed for 30 s.

Additions to complete reaction mixtures	nmol ATP / mg chlorophyll		
	Expt. No. 1	Expt. No. 2	Expt. No. 3
None	43±5	56±6	36±0
Minus ADP	2±1	5±1	6±0
NH_4Cl (5 mM)	3±0	—	—
CCCP (10 μM)	4±2	—	—
DCMU (10 μM)	43±3	—	—
DCMU (50 μM)	47±5	—	—
DBMIB (10 μM)	54±3	—	—
DBMIB (50 μM)	56±4	—	—
Dio-9 (10 μg/ml)	—	43±5	33±1
Dio-9 (50 μg/ml)	—	31±5	15±2
Dio-9 (100 μg/ml)	—	15±1	11±1
DCCD (50 μM)	50±4	—	—
DCCD (100 μM)	51±2	49±9	42±1
DCCD (500 μM)	—	66±2	51±2
DCCD (1.0 mM)	—	61±3	39±1

pre-treated chloroplasts in the same concentration range needed to inhibit photophosphorylation. With chloroplasts membranes prepared by sonication, it was noted that the concentration of uncouplers needed to inhibit dark phosphorylation was higher than that required to inhibit photophosphorylation [26]. The cause for this difference in sensitivity is at present not clear.

Table II also shows that the electron transport inhibitors, DCMU, a compound which prevents the oxidation of the reduced primary acceptor of photosystem II [30], and 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (DBMIB), a compound which prevents the oxidation of plastohydroquinone [31] are ineffective in inhibiting dark phosphorylation. These results would be expected if the normal routes for the maintenance of a proton gradient (i.e. the deposition of the protons from water oxidation on the inside of the thylakoid membrane and the plastoquinone-plastohydroquinone "pumping" of protons across the membrane) are not involved in dark phosphorylation.

Finally, several so-called energy transfer inhibitors, compounds which inhibit phosphorylation at the level of the coupling factor, have also been tested. The results for two, Dio-9 and dicyclohexyl carbodimide (DCCD), are shown in Table II. At relatively low concentrations, concentrations generally required to inhibit photophosphorylation, no influence is seen on dark phosphorylation. Dio-9, however, inhibits dark phosphorylation approximately 50% and 80% at 50 and 100 μg/ml, respectively, whereas DCCD shows a marked stimulation (approx. 20–30%) at

500 μM. Concentrations of DCCD higher than 1.0 mM DCCD do inhibit dark phosphorylation (data not shown). Column three in Table II (Expt. No. 3) shows the results of an experiment in which the addition of DAD to the "complete" reaction mixture was delayed 30 s in order to give DCCD time to react with the chloroplast membrane (see Section V). Even under these conditions, 1.0 mM DCCD does not inhibit dark phosphorylation.

(III) Nature of the mediator catalyzing dark phosphorylation

Table I clearly shows that in the absence of DAD, ferricyanide pre-treated chloroplasts are not capable of generating ATP in the dark even in the presence of a large excess of ascorbate (the ascorbate concentration in the reaction mixture was 10 mM). The requirement for DAD suggested the need for a compound capable of penetrating the membrane and that can be oxidized by ferricyanide. Several compounds have been tested for their ability to catalyze phosphorylation in the dark and the results for a few are shown in Table III. The correlation between those compounds which are capable of catalyzing dark phosphorylation and photophosphorylation (either cyclic or in photosystem I partial reactions) is striking. One feature that these compounds have in common is that, upon oxidation, they all release protons into the medium [32]. (Note that the H^+/e^- ratio for DAD, Cl_2Ind (Cl_2Ind, 2,6-dichlorophenol indophenol), and *p*-phenylenediamine (PD) is one, whereas for *N*-methylphenazonium methylsulphate (PMS) it is only 0.5.) On the other hand, it is known that *N*-substituted-*p*-phenylenediamines, upon oxidation, do not release protons into the medium but form, instead, radical cations [32]. Although they have been used as donors for photosystem I partial reactions, they are not coupled to phosphorylation [19]. Table III shows that three such examples, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD), *N,N*-dimethyl-*p*-phenylenediamine (DMPD), and *N,N*-diethyl-*p*-toluidine (DEPT), are also completely incapable of catalyzing dark phosphorylation with ferricyanide pre-treated chloroplasts.

(IV) DAD shuttle across the membrane

We have previously proposed an electron-proton shuttle mechanism for

TABLE III

COMPARISON OF DIFFERENT ELECTRON TRANSPORT MEDIATORS ON THE YIELD OF DARK PHOSPHORYLATION

Experimental conditions are as described in Materials and Methods. Chloroplasts were added to a final concentration of 0.21 mg chlorophyll/ml. The concentration of all mediators tested was 50 μM. The yields of phosphorylation are expressed as nmol ATP/mg chlorophyll.

Mediator	Chloroplasts pre-incubated with potassium ferricyanide	Chloroplasts pre-incubated with potassium ferrocyanide
None	6±1	4±0
DAD	35±1	4±1
Cl_2Ind	24±2	–
PD	17±1	5±1
PMS	11±0	–
TMPD	7±1	4±1
DMPD	6±1	–
DEPT	7±1	–

TABLE IV

THE YIELD OF DARK PHOSPHORYLATION AS A FUNCTION OF DAD CONCENTRATION

Experimental conditions are as described in Materials and Methods. Chloroplasts were added to a final concentration of 0.24 mg chlorophyll/ml.

Concentration of DAD (μM)	nmol ATP / mg chlorophyll	DAD / ATP
0	10 ± 0	—
1	72 ± 1	0.065
2	83 ± 2	0.115
5	88 ± 3	0.265
10	85 ± 13	0.555
20	71 ± 2	1.365

DAD (and other related mediators) in order to explain dark phosphorylation with sonicated chloroplasts vesicles [26]. DAD was pictured as traversing the membrane-bridging ascorbate, on the outside, and ferricyanide, on the inside; the reaction occurring until all of the entrapped ferricyanide has been reduced. However, such a proposed shuttle was not necessary because the amount of DAD in the reaction mixture was in extreme excess compared to the amount of ATP generated by the chloroplasts (see Table I, ref. 26) and probably the total amount of ferricyanide entrapped by the chloroplasts. (Assuming an internal chloroplast volume of 10 μl/mg chlorophyll [33] and that the ferricyanide concentration inside the vesicles was the same as the concentration outside after brief sonication, i.e. 0.1 M, the concentration of entrapped ferricyanide would have been equivalent to 0.2 mM after the addition of the chloroplasts to the reaction mixture. The concentration of DAD was 0.5 mM. In terms of reducing equivalents, that would have been a 5-fold excess of DAD with respect to ferricyanide.)

This question has been re-examined using catalytic rather than substrate concentrations of DAD. The results are shown in Table IV. The data in the third column are expressed as the DAD concentration in the reaction mixture divided by the concentration of ATP generated in the dark. If it is assumed that DAD only penetrates the membrane once and does not shuttle back and forth between ascorbate and ferricyanide, it would be possible to estimate an H^+/ATP ratio for dark phosphorylation. Upon oxidation, DAD loses two protons and two electrons. Thus, the hypothetical H^+/ATP ratio would simply be one-half the DAD/ATP ratio. At 1.0 μM DAD, this calculated H^+/ATP ratio is 0.033 and would represent a very high efficiency if the assumption were correct that DAD does not shuttle. This value, however, must be compared with the value of 2–3 usually found for thylakoid membranes [7]. Using this more realistic ratio, in order for 1.0 μM DAD to generate enough protons on the inside of the thylakoid membrane to make 62 nmol ATP/mg chlorophyll, each DAD molecule must shuttle reducing equivalents across the membrane a minimum of 16–24 times.

(V) The kinetics of DAD catalyzed dark phosphorylation

The kinetics of DAD catalyzed phosphorylation are of interest, particularly

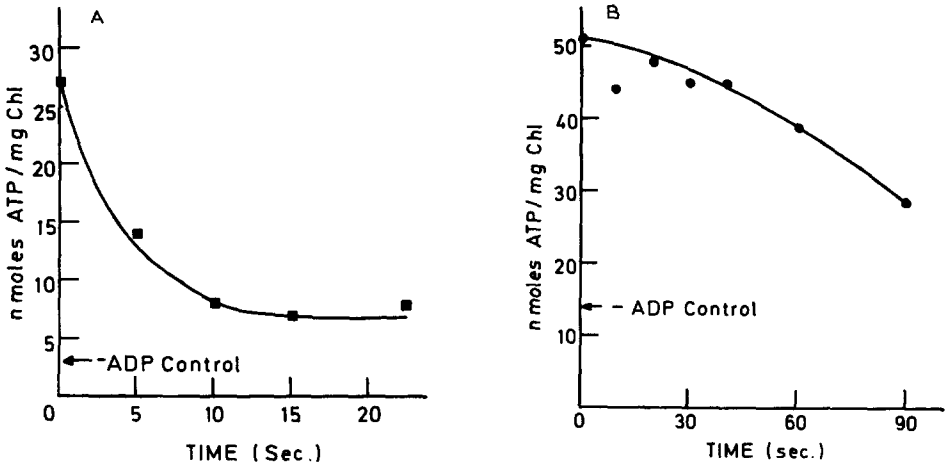

Fig. 2. (A) Decay kinetics for dark phosphorylation when the addition of phosphate to the reaction mixture is delayed. Experimental conditions are as described in Methods and Materials. Chloroplasts, equivalent to 0.20 mg chlorophyll/ml, were added to reaction mixtures containing 50 μM DAD. Phosphate was added at various times thereafter, as indicated. (B) Decay kinetics for dark phosphorylation when the addition of DAD to the reaction mixture is delayed. Experimental conditions as in (A) except that the addition of 50 μM DAD was delayed as indicated.

with respect to the half time for the overall reaction (i.e. the rapidity with which the internal pool of ferricyanide is consumed and the stability of the "high energy intermediate" (proton gradient)) and with respect to the stability of the chloroplasts after addition to the reaction mixture but in the absence of a mediator (i.e. the rapidity with which ferricyanide diffuses out of the thylakoid membrane). Preliminary experiments in which the reaction was terminated by the addition of $HClO_4$ to the reaction mixture indicated that the half time for the overall reaction is less than 15 s. However, due to technical difficulties, it was not possible to accurately determine the half time in this manner.

In order to investigate the kinetics of the overall reaction, a decay of the ability of chloroplasts to make ATP was determined by delaying the addition of phosphate to otherwise complete reaction mixtures. The results, as seen in Fig. 2A, show that the half time for the decay of the complete process is on the order of 5 to 6 s. This decay could be due to either a rapid decay of the high energy state (i.e. diffusion of internally accumulated protons through the membrane resulting in the loss of a transmembrane pH gradient) or to the rapid diffusion of ferricyanide out of the internal space of the thylakoid membrane.

In order to distinguish between these two possibilities, the addition of the mediator, DAD, to the reaction mixture was delayed. These results are shown in Fig. 2B. It is clear that, although ferricyanide pre-treated chloroplasts do lose their ability to synthesize ATP in the dark under these conditions as well, the half time for the decay is about 80 s or more than one order of magnitude greater than that seen when the addition of phosphate is delayed.

DISCUSSION

Chloroplast membranes, pre-incubated with ferricyanide, have the ability to make ATP in the dark when they are supplied with a reductant, such as ascorbate, and a lipophilic mediator. We believe that the role of the mediator is to shuttle reducing equivalents from ascorbate, located on the outside of the membrane, to ferricyanide, trapped on the inside, as shown in Fig. 3. The requirement for a lipophilic mediator clearly indicates that ascorbate, although in great excess, cannot react with ferricyanide either directly or indirectly through plastoquinone. This result is not surprising and is consistent with the observation that partial reactions driven by photosystem I require a mediator to bridge ascorbate and plastocyanin, the donor to photosystem I [34].

It is clear, however, that not all mediators that can be used to support photosystem I driven electron flow are capable of catalyzing dark phosphorylation. Those mediators that are functional are of the type that, upon oxidation, lose protons as well as electrons. This correlation between the chemical nature of the mediator and the ability of chloroplasts to drive photophosphorylation in partial reactions of photosystem I was first observed by Hauska et al. [19, 20]. In terms of this hypothesis, we believe that, upon penetration of the chloroplast membrane and oxidation by ferricyanide, protons must be released into the "inner space" of the thylakoid membrane in order to develop a pH gradient across the membrane. The pH gradient then provides the energy required for chloroplasts to drive the synthesis of ATP in the dark via the coupling factor protein in accordance with the Mitchell hypothesis [6]. Thus, mediators such as TMPD which form stable radical cations, would be capable of reducing ferricyanide but would be incapable of generating a pH gradient and hence cannot function as catalysts for dark phosphorylation. Experiments measuring

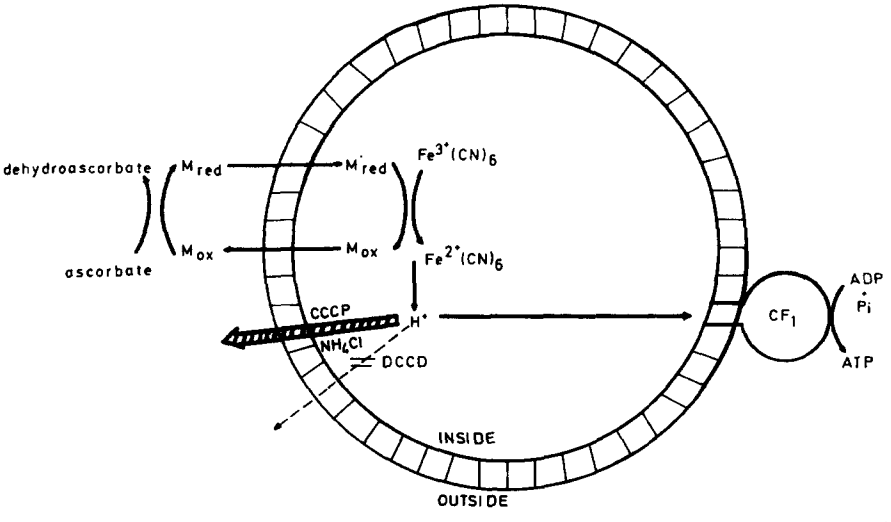

Fig. 3. Model to explain the ability of ferricyanide pre-treated chloroplasts to make ATP in the dark. Abbreviations: M, mediator; red and ox, the reduced and oxidized forms of the mediator, M; CF_1, chloroplast coupling factor 1; broken arrow indicates the postulated non-specific leak of protons through the membrane.

9-amino acridine fluorescence quenching, have indeed shown that, under these conditions, the addition of compounds such as DAD lead to a large transient pH gradient, whereas compounds such as TMPD do not [35].

In terms of the mechanism involved, the system that we have developed using artificial reduction-oxidation reactions across the thylakoid membrane is principally very similar to the pH shift experiments of Jagendorf and co-workers [3]. In our experiments, the oxidation of a compound that releases protons on the inside of the membrane develops the pH gradient, whereas in pH shift experiments the pH gradient is developed by loading the chloroplasts with a permeable acid at relatively low pH followed by an increase in the external pH.

We have shown that ATP formation with ferricyanide pre-treated chloroplasts is completely sensitive to inhibition by uncouplers of photophosphorylation and insensitive to compounds, such as DBMIB and DCMU, which inhibit photosynthetic electron transport. With respect to the model shown in Fig. 3, these results are consistent with the predictions that compounds which inhibit the formation of a pH gradient inhibit dark phosphorylation, while compounds which have no influence on the mechanism of phosphorylation do not interfere with ATP formation.

The effect of energy transfer inhibitors on dark phosphorylation is, however, more difficult to interpret. At concentrations routinely used to inhibit photophosphorylation, we have observed no inhibition by either Dio-9 or DCCD. Both Dio-9 and DCCD do, however, inhibit dark phosphorylation at relatively high concentrations, and a stimulation of the ATP yield is seen with moderately high concentrations of DCCD. McCarty and Racker [36] have shown that DCCD can stimulate ATP synthesis and completely restore the proton pumping ability of EDTA-washed chloroplasts. We have also observed a stimulation of photophosphorylation by very low concentrations of DCCD in untreated chloroplasts (unpublished data). Thus, it may be that the effect of DCCD, under conditions in which dark phosphorylation is measured, is to decrease the non-specific permeability of protons through the membrane. This would increase the magnitude of the pH gradient developed by the redox reactions and the result would be an enhancement in the yield of ATP. The observation that very high concentrations of energy transfer inhibitors must be present before they effect dark phosphorylation can be explained by the relatively high chlorophyll concentrations (0.20–0.25 mg/ml) that we generally used.

The phosphorylation system that we have developed using ferricyanide pretreated chloroplasts is an extension of the work done with artificial membrane systems capable of energy conservation [22–24]. We believe that our system may provide a useful tool in the study of the mechanism of phosphorylation.

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