THE ENERGIZATION OF CHLOROPLASTS IN THE DARK, INDUCED BY REDUCTION–OXIDATION REACTIONS ACROSS THE THYLAKOID MEMBRANE

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1. Introduction

We have recently shown that spinach chloroplasts which have trapped ferricyanide either by sonication [1] or by a prolonged incubation [2] in the presence of ferricyanide can make ATP in the dark when supplied with a reductant, such as ascorbate, and a lipophilic mediator. We have also demonstrated that the mediator must be of the type that, upon oxidation, loses protons as well as electrons. We have interpreted our results in terms of Mitchell's chemiosmotic hypothesis [3] and have suggested that the driving force for phosphorylation in our system is a pH gradient formed upon the internal oxidation of the lipophilic mediator by ferricyanide trapped on the inside of the thylakoid membrane [2].

In this communication, we show that trapped ferricyanide can be reduced by lipophilic phenylenediamines such as 2,3,5,6-tetramethyl-*p*-phenylenediamine (DAD), an electron-proton mediator, or *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD), an electron mediator. We have also measured 9-aminoacridine fluorescence after addition of the two phenylenediamines to ferricyanide pre-treated chloroplasts and have found that only the reduction of internally trapped ferricyanide by DAD results in transient fluorescence quenching.

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2. Methods

The methods for chloroplast isolation, treatment with ferricyanide, and the phosphorylation assay are described in another publication [2]. 9-Aminoacridine fluorescence was measured in a Zeiss fluorimeter model ZFM4 as previously described [4]. Ferricyanide reduction was measured at 420 nm in an American Instruments Co. dual wave length spectrophotometer (type DW-2 ultra violet/vis) using 470 nm as the reference wave length. A difference spectrum (data not shown) for the absorbance changes generated by the addition of either DAD or TMPD to ferricyanide pre-treated chloroplasts (as shown in fig.1) confirmed that the substance reduced was ferricyanide. The detailed reaction mixtures are described in the legends.

3. Results and discussion

3.1. *Phenylenediamine catalyzed dark phosphorylation*

Table 1 shows typical results for an experiment in which DAD and TMPD were tested for their ability to catalyze the formation of ATP in the dark upon addition to ferricyanide pre-treated spinach chloroplasts (in the presence of excess ascorbate). As previously shown [2], the addition of DAD, but not TMPD, leads to the generation of ATP. DAD catalyzed ATP formation in the dark is completely sensitive to the uncoupler combination valinomycin plus nigericin, confirming previous observations that dark phosphorylation can be completely inhibited by uncouplers of photosynthetic phosphorylation [2].

Two plausible explanations for the observation that

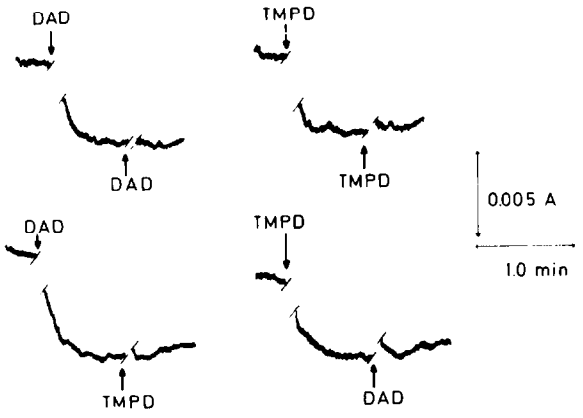

Fig.1. Phenylenediamine catalyzed ferricyanide reduction in ferricyanide pre-treated chloroplasts. Chloroplasts were incubated with 0.1 M ferricyanide for 30 min at 0°C. After the pre-treatment, the chloroplasts were collected by centrifugation and resuspended in tricine buffer containing 0.1 M potassium ferricyanide. Experiments were performed as follows: Ferricyanide pre-treated chloroplasts were added to reaction mixtures containing, in a total volume of 3.0 ml, chloroplasts, equivalent to 25 μg Chl/ml, 0.7 mM potassium ferricyanide, 1.3 mM sodium ascorbate, 50 mM Tricine–NaOH (pH 8.0), 10 mM KCl, and 5.0 mM $MgCl_2$. The ascorbate reduced the excess ferricyanide that was carried over with the chloroplast suspension (this absorbance change, equivalent to about 0.7 A, was not recorded). When the 420 minus 470 nm signal was stable, indicating that all of the external ferricyanide had been reduced (approx. 10–15 s), 5 μl of either DAD or TMPD (final concentrations of 1.0 and 5.0 μM respectively) were added and the signal recorded as shown in the traces. All reactions were measured at room temperature.

Table 1
Phosphorylation in the dark with ferricyanide pre-treated spinach chloroplasts

Additions	nmoles ATP/mg Chl
None	21 ± 5
(Minus ADP)	(18 ± 3)
DAD (5 μM)	63 ± 6
DAD (50 μM)	76 ± 3
DAD (5 μM)	
Valinomycin (1.7 μg/ml)	
Nigericin (1.7 μg/ml)	18 ± 2
TMPD (5 μM)	22 ± 1
TMPD (50 μM)	27 ± 2

Spinach chloroplasts were pre-incubated with 0.1 M ferricyanide for 30 min at 0°C. Complete reaction mixtures contained in a total volume of 1.5 ml, chloroplasts, equivalent to 0.26 mg Chl/ml, 6.0 mM potassium ferricyanide, 1.33 μM 3-(3,4-dichlorophenyl)-1,1-dimethylurea, 50 mM Tricine–NaOH (pH 8.0), 10 mM KCl, 5 mM $MgCl_2$, 2.0 mg/ml bovine serum albumin, 5 mM ADP, 3.33 mM [^{32}P]phosphate, 10 mM sodium ascorbate, and other additions as indicated. Reaction mixtures were incubated at 15°C for 1.0 min.

TMPD does not catalyze phosphorylation under these conditions are: (1) that the reduction of internally trapped ferricyanide by TMPD does not generate the 'high energy state' needed for phosphorylation (i.e. Δ pH), as previously suggested [2] or (2) that, under these experimental conditions, TMPD does not penetrate the membrane and hence cannot reduce internally trapped ferricyanide. These possibilities are examined in the following two sections.

3.2. *Phenylenediamine reduction of internally trapped ferricyanide*

Reduction of internal ferricyanide was measured at 420 nm in a dual wave length spectrophotometer after external ferricyanide (approx. 0.7 mM in the cuvette) had been reduced by the addition of ascorbate to the reaction mixture. Figure 1 shows some typical traces. Both DAD (1.0 μM) and TMPD (5.0 μM) are capable of reducing ascorbate-inaccessible ferricyanide. The subsequent addition of DAD or TMPD to samples that have already received DAD or TMPD does not result in any further decrease in absorbance indicating that there are not separate pools of ferricyanide available to DAD or TMPD.

Although no detailed studies on the kinetics of ferricyanide reduction have as yet been undertaken, it should be pointed out that, qualitatively, it is generally necessary to add about three times more TMPD than DAD in order to observe approximately the same reduction kinetics. The extent of reduction is, however, independent of the concentration of the mediator used. The slower kinetics observed with TMPD might very well be due to the generation of the TMPD radical cation inside the thylakoid membrane which then diffuses back outside slower than the uncharged diimine formed upon oxidation of DAD. In the case of TMPD, the diffusion process through the membrane may be rate limiting.

3.3. *9-Aminoacridine fluorescence quenching*

Schuldiner et al. [5] have studied the use of

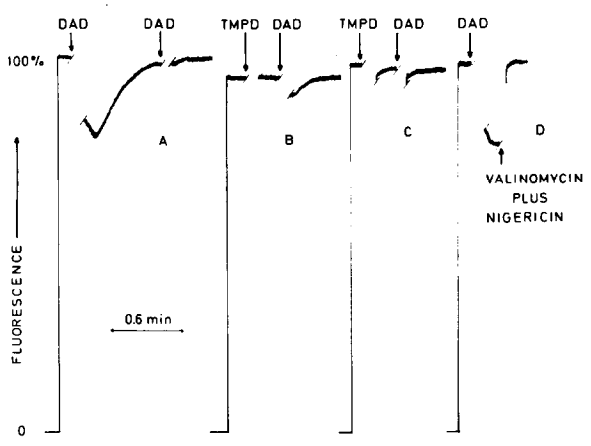

Fig.2. DAD induced transient 9-aminoacridine fluorescence quenching in ferricyanide pre-treated chloroplasts. Reaction mixtures contained in a total volume of 2.5 ml, ferricyanide pre-treated chloroplasts, equivalent to 7 μg Chl/ml, 2 mM potassium ferricyanide, 0.8 mM sodium ascorbate, 50 mM Tricine–NaOH buffer (pH 8.0), 10 mM KCl, 5 mM $MgCl_2$, and 5.0 μM 9-aminoacridine. Where indicated, DAD (3.0 μM), TMPD (trace B, 3.0 μM; trace C, 12.0 μM), and valinomycin plus nigericin (each 1.0 μg/ml) were added. Fluorescence was measured at room temperature.

9-aminoacridine as an indicator for the pH difference generated across the chloroplast membrane upon illumination. Although the quantitative reliability of this indicator has recently been seriously challenged by Fiolet et al. [6,7], it is clear that the fluorescence quenching properties of 9-aminoacridine reflect a general 'energization' of the thylakoid system. The ability of DAD and TMPD to induce 9-aminoacridine fluorescence quenching in ferricyanide pre-treated chloroplasts is shown in fig.2. As is the case with dark phosphorylation (table 1), DAD (trace 2A), but not TMPD (at either 3 or 12 μM, traces 2B and 2C respectively), is capable of inducing a fairly large transient quenching of 9-aminoacridine fluorescence. A second addition of DAD does not produce a second cycle of quenching. Furthermore, when TMPD is added first, a subsequent addition of DAD produces only a very small fluorescence quench at low TMPD concentration (3.0 μM) (probably reflecting the, as yet, incomplete reduction of internally trapped ferricyanide) or no fluorescence quench at high TMPD concentrations (12.0 μM). These results agree well with the ferricyanide reduction experiments shown in fig.1. The first addition of either phenylenediamine causes the exhaustion of the internal ferricyanide pool such that second addition of DAD is incapable of generating the 'high energy state' responsible for 9-aminoacridine fluorescence quenching.

Figure 2D shows a control experiment in which valinomycin plus nigericin were added at the peak of the DAD induced 9-aminoacridine fluorescence quench. The quenching of 9-aminoacridine fluorescence decayed faster under these conditions than we were able to record. When the uncoupler combination was added before the addition of DAD, no fluorescence quench was observed upon the subsequent addition of DAD (data not shown).

Selman and Ort [2] have previously shown that the energy transfer inhibitor dicyclohexylcarbodiimide (DCCD) unexpectedly stimulated the yield of dark phosphorylation. They postulated that this may be due to the ability of DCCD to inhibit the 'non-specific' leak of protons through the membrane causing an increase in the extent of the pH gradient developed upon internal oxidation of DAD and hence an increase in the yield of ATP. This hypothesis is supported by the experiment shown in fig.3 which depicts the effects of DCCD on 9-aminoacridine fluorescence quenching induced by DAD in the dark with ferricyanide pre-treated chloroplasts. Lower concentrations

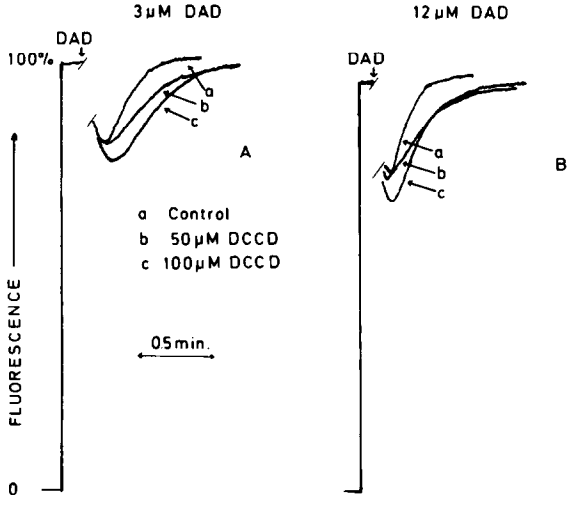

Fig.3. The effect of DCCD on the parameters of DAD induced 9-aminoacridine fluorescence quenching in ferricyanide pre-treated chloroplasts. Conditions as in fig.2.

of DCCD (approx. 50 μM) extend the half-time for the decay of the fluorescence quench about two fold, whereas higher concentrations increase both the apparent extent of the quenching as well as the half-time for the decay. The effects of DCCD on the kinetics of 9-aminoacridine fluorescence are qualitatively independent of the DAD concentration.

4. Conclusion

From these and previous experiments [1,2], we conclude that, during the ferricyanide pre-treatment, ferricyanide penetrates the thylakoid membrane where it is no longer reducible by the hydrophilic reductant ascorbate. This pool of ferricyanide can, however, be reduced by lipophilic phenylenediamines irrespective of the type, i.e. whether they lose both protons and electrons upon oxidation (e.g. DAD) or only electrons (e.g. TMPD). Only mediators of the former type are, however, capable of catalyzing the synthesis of ATP in the dark and, as experiments with 9-aminoacridine have shown, only those mediators lead to the generation of 'energized membranes'.

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