LIPOPHILICITY AND CATALYSIS OF PHOTOPHOSPHORYLATION

Artificial Proton Translocation by Lipophilic, Quinoid Hydrogen Carriers in Chloroplasts and Liposomes*

G.A. HAUSKA

Ruhr-Universität Bochum, Abteilung Biologie, Lehrstuhl für Biochemie der Pflanzen, 463 Bochum, Germany

and

R.C. PRINCE

University of Bristol, Medical School, Department of Biochemistry, Bristol BS8 1TD, England

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1. Introduction

The comparison of PMS‡ with PMS-S, and of DAD with TMPD suggests that artificial redox mediators in order to catalyse cyclic photophosphorylation in chloroplasts have to be lipid soluble and have to lose protons upon oxidation [1–3]. PMS-S is lipid insoluble, TMPD is lipid soluble but does not liberate protons upon oxidation, while PMS and DAD are lipophilic and lose protons. PMS-S and TMPD are inactive, PMS and DAD are active in cyclic phosphorylation. From this pattern it follows that artificial catalysts of cyclic photophosphorylation might act as artitificial proton translocators in a transmembrane redox cycle providing the energy for ATP synthesis via a chemiosmotic coupling mechanism (see [4] for a recent review).

In liposomes [5] transmembrane redox reactions can lead to electric potential- [6] and pH-differences [7].

In this paper we report on measurements of light-induced proton uptake [8] and transmembrane pH-differences [9], measured by the light induced quenching of 9-amino acridine fluorescence, in chloroplasts, comparing PMS with PMS-S, and DAD with TMPD. In addition the same redox compounds are studied as mediators of electron and proton flow in liposomes between external ascorbate and internal ferricyanide.

2. Methods

Chloroplasts were prepared as described [10]. Proton uptake in illuminated chloroplasts was measured as previously [11]. Details are given in table 1. Light induced fluorescence quench of 9-amino acridine in chloroplasts was observed in a Zeiss fluorimeter, model ZFM4, modified for cross illumination with red light using fiber glass optics. Excitation of fluorescence was obtained with the 365 nm line of an electronicly stabilised mercury lamp, isolated by the appropriate Zeiss glass filter and reduced to 10% intensity by a neutral density filter. Fluorescence was measured at 456 nm through the monochromator M4QIII at 2 mm slit width. The output of the photomultiplier was fed to a W/W Electronics recorder, model 3218. The detailed reaction mixture is given in table 1. The pH between the inner and outer space of chloroplasts was calculated according to Shuldiner

* Part 3. Previous parts [1, 2].

‡ Abbreviations: PMS, *N*-methylphenazonium methosulfate; PMS-S, *N*-methylphenazonium-3-sulphonate; DAD, 2,3,5,6-tetramethyl phenylenediamine; TMPD, *N,N'*-tetramethyl phenylenediamine; DCMU, dichlorophenyl-1,1-dimethylurea.

Table 1
Light-induced proton uptake and transmembrane pH-difference in chloroplasts during cyclic electron flow

	μEquivalents H^+ uptake per mg chlorophyll			Δ pH	% Quench
pH:	6.5	7.2	8.0	8.0	
PMS	0.49	0.31	0.08	3.7	55
PMS-S	< 0.02	< 0.02	< 0.02	< 1.6	< 1
DAD	0.58	1.14	0.39	3.6	47
TMPD	0.09	0.18	0.08	< 1.6	< 1

Proton uptake was measured at 12°C in 3 ml of the following reaction mixture: 50 mM NaCl; 5 mM $MgCl_2$; chloroplasts equivalent to 0.1 mg chlorophyll; 2×10^{-5} M DCMU and 5×10^{-5} M phenazines plus 1 mM ascorbate, or 10^{-4} M phenylenediamines. The reaction vessel was continuously flushed with argon. The pH was adjusted to the desired value by addition of dilute HCl or NaOH, the last small addition giving the buffer capacity. Illuminating white light had an intensity of 3×10^5 ergs/cm^2 per sec. The reaction mixture for light induced fluorescence quench of 9-amino acridine contained in a final volume of 3 ml, 50 mM NaCl, 5 mM $MgCl_2$, 50 mM Tricine–NaOH, pH 8.0, 2×10^{-5} M DCMU and either 2×10^{-5} M phenazines plus 1 mM ascorbate or 5×10^{-5} phenylenediamines. The base line of fluorescence was recorded before 9-amino acridine was added to 10^{-6} M. The illuminating red light had an intensity of 10^6 ergs/cm^2 per sec. The temperature was kept at 15°C. The values for the pH difference were calculated according to [9] assuming an internal volume of 40 μl/mg chlorophyll for the chloroplasts.

et al. [9] assuming an average internal volume for chloroplasts of 40 μl/mg chlorophyll.

Liposomes were prepared as previously described [6]. They were routinely made from aceton-washed soya bean phospholipid, obtained from Sigma, but were also prepared using egg lecithin, plus 5% dicetyl phosphate, to give negatively charged vesicles, or 5% cetyl pyridinium bromide, to give positively charged vesicles. All liposomes contained 2 mole % bacteriochlorophyll, to assist quenching of 9-amino acridine fluorescence, which was measured as described before [6]. The liposomes routinely contained 0.2 M potassium ferricyanide, or a potassium ferri–ferrocyanide mixture, and were suspended in sodium phosphate–pyrophosphate buffer, pH 7.0, of equivalent osmolarity. Sodium ascorbate was added to 100 μM and the reaction was started by addition of the mediator (figs. 3 and 5). The reduction of ferricyanide was

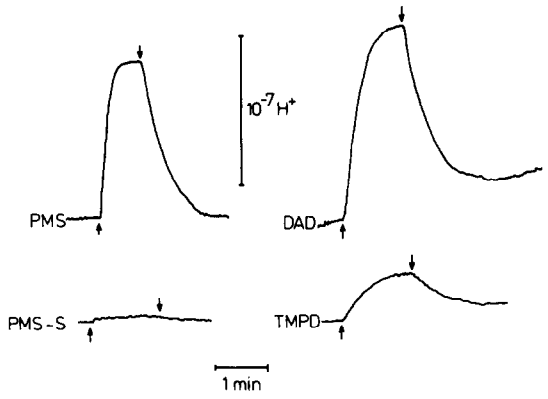

Fig. 1. Light-induced proton uptake in chloroplasts during cyclic electron flow. The measurement and the reaction conditions are described under Methods and in the legend for table 1.

monitored in a chopped double beam spectrophotometer [12] at 420–460 nm.

3. Results and discussion

Fig. 1 and table 1 clearly show that unlike PMS the sulphonated compound PMS-S does not catalyse light-induced proton uptake in chloroplasts in the presence of DCMU, corroborating the finding that it is inactive in cyclic photophosphorylation [1].

DAD also catalysed proton uptake very efficiently, the rise and the decay being slower than with PMS (fig. 1). This was even more pronounced at higher external pH, at which in addition the extent of proton uptake was much larger with DAD than with PMS. As a consequence with DAD the pH optimum was shifted towards higher pH values (table 1). This is reminiscent of the effects with amines of low pK, which increase the internal buffer capacity of chloroplasts without inhibiting proton uptake and photophosphorylation like amines with high pK's [13, 14]. DAD in the reduced form has its higher pK at around 6.

TMPD behaved similar to DAD but the extent of proton uptake was much less (fig. 1 and table 1). Since reduced TMPD efficiently donates electrons to photosystem I it is remarkable that it does not catalyse photophosphorylation [3] and is much poorer in mediating proton uptake.

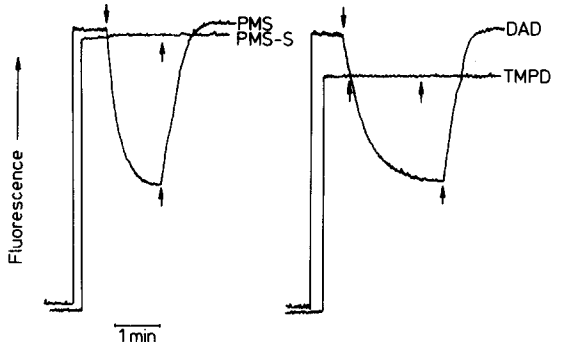

Fig. 2. Light-induced fluorescence quench of 9-amino acridine in chloroplasts during cyclic electron flow. The measurement and the reaction conditions are described under Methods and in the legend for table 1.

The lack of efficient proton translocation by TMPD and PMS-S as compared to DAD and PMS is also shown by measurements of fluorescence quenching (fig. 2). This method introduced for the measurement of the high energy state in chloroplasts by Kraayenhof [15] was shown to be useful for the determination of a proton gradient, i.e. for the determination of internal pH in chloroplasts [10]. Unfortunately this method is appropriate only for rather large pH differences. As seen in table 1, 1% quench would correspond to a pH difference of 1.6, which would be hidden in the noise of the recording. Therefore the question remains, whether the protons taken up in the presence of TMPD (fig. 1) do attive inside the thylakoids.

The results obtained with liposomes using the phenazines as mediators between internal ferricyanide and external ascrobate are summarised in fig. 3. As shown previously [7] at high oxidising potentials, PMS is a poor catalyst of electron and proton flow (figs. 1a and 1b, first additions of PMS). We attributed this to the production of a charged fully oxidised form (PMS^+) inside the liposome (fig. 5). When the redox potential had fallen below 480 mV, PMS acted as an excellent catalyst (figs. 1a and 1b, later additions of PMS). Rapid reduction of ferricyanide and internal proton release by PMS was observed if the liposomes contained a ferri-ferrocyanide mixture with a potential of about +480 mV at the start of the experiment (figs. 1c and 1d). Presumably this reflects the participation of an uncharged radical of oxidised

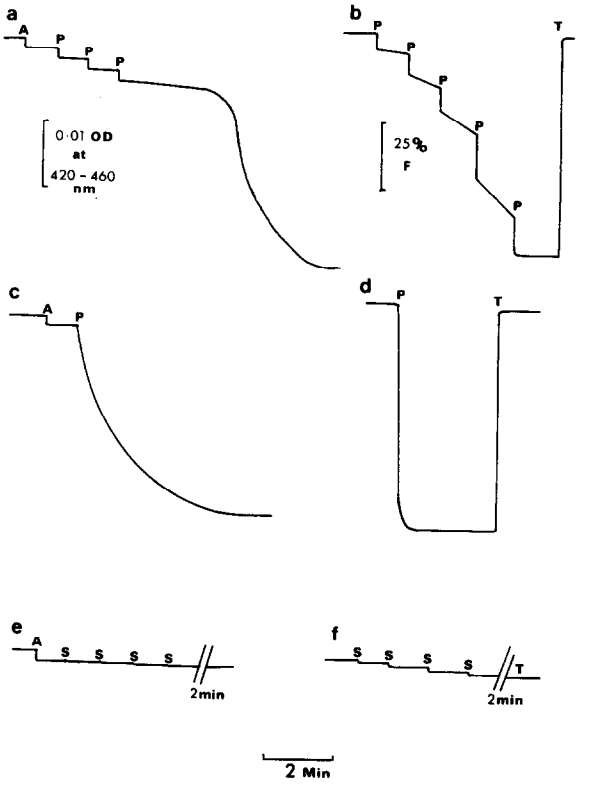

Fig. 3. Phenazines as redox mediators through the liposome membrane: a – Liposones (0.8 mg/ml) containing 0.2 M ferricyanide were suspended in 2.5 ml of buffer, and ascorbate (A) was added to 100 μM. PMS (P) was added in aliquots each an increase in final concentrations of 0.4 μM, and the reaction monitored in the double beam spectrophotometer at 420–460 nm; b – The conditions were identical to those in a, with the addition of 0.4 μM 9-amino acridine. The reaction was monitored in the fluorimeter previously described [6]. At the end of the experiment 0.1 μM Triton X-100 (T) was added to collapse the pH gradient; c – as a, but the liposomes contained a mixture of ferri–ferrocyanide with a redox potential of about + 450 mV; d – as c, but with the addition of 9-amino acridine as b; e – as c, but with PMS-S (S) instead of PMS. The sulphonated dye was added in 4 μM aliquots; f – as e, but with the addition of 9-amino acridine as in b.

PMS at lower redox potentials (fig. 5) which might be stabilised by the lipid phase.

Proposed reaction schemes for the mediation by $PMSH\text{-}PMS^+$, $PMS^{\cdot}$ are shown in fig. 5. Similar conclusions, concerning the different behaviour of PMS at different redox potentials have been drawn independently Hinkle (P. Hinkle, personal communication)

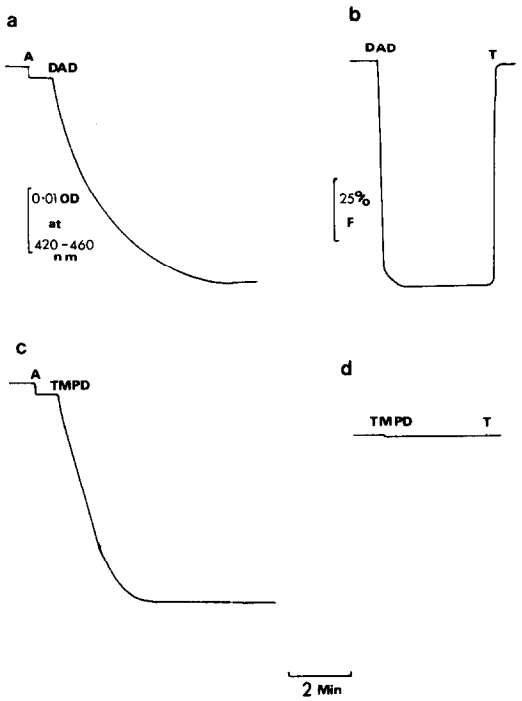

Fig. 4. *p*-Phenylenediamines as redox mediators through the liposome membrane: a – The experiment was identical to that of 1a, except DAD was added to 0.04 μM instead of PMS; b – as a, but with the addition of 9-amino acridine as in 1b; c – as a, but with 0.4μ M TMPD in place of 0.04 μM DAD; d – as b, but with 0.4μ M TMPD in place of 0.04 μM DAD. If an uncoupler such as carbonylcyanide-*p*-trifluoro-methoxy-phenylhydrazone was added during the experiment (not shown) in 1c or 4a the reduction of ferricyanide by PMS or DAD became similar to the reduction by TMPD and a pH gradient, as monitored by a decrease of 9-amino acridine fluorescence was not formed.

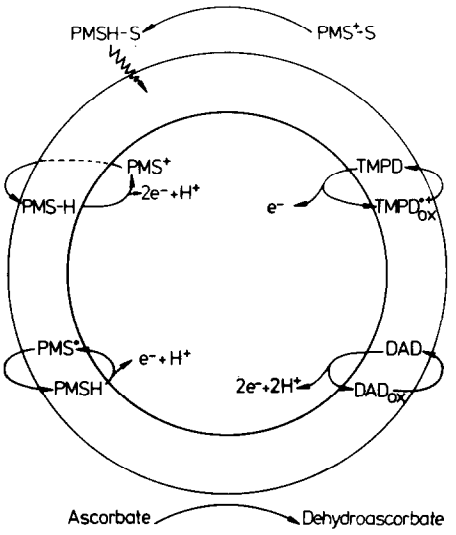

Fig. 5. Proposed schemes for redox mediation by phenazines and *p*-phenylenediamines across phospholipid membranes. See text for details.

using a redox state technique with liposomes containing trapped ascorbate and external ferricyanide.

The sulphonated derivative of PMS did not catalyse electron and proton flow through the phospholipid membrane at any redox potential (figs. 1e and 1f). Therefore PMS-S does not seem to penetrate into liposomes (fig. 5).

Fig. 4 shows the results obtained with the *p*-phenyldiamines. As seen in fig. 4a and 4c, both dyes (TMPD at 10-fold concentration of DAD) mediate electrons from ascorbate to ferricyanide through a lipid membrane. Both compounds, therefore, in the oxidised and reduced forms must be permeable. However, as seen in fig. 4b and 4d, while DAD also releases internal protons, TMPD does not. TMPD reduces internal ferricyanide steadier than does DAD (compare fig. 4a and 4c), presumably because no retardation by accumulating protons inside the vesicles takes place.

The oxidation–reduction cycles of DAD and TMPD in liposomes are depicted in fig. 5. TMPD can only be oxidised to a radical cation because of the two methyl groups on each of the amino nitrogens. DAD can be further oxidised to the diimine.

In summary the overall pattern of the reactions in liposomes corroborates the results with chloroplasts of this and the preceding papers [1, 2]. PMS-S does not catalyse transmembrane oxidation–reduction cycles like PMS, DAD and TMPD. Only PMS and DAD translocate protons during the cycle. Therefore, as anticipated [2], the ability to translocate hydrogen equivalents is associated with the ability to catalyse cyclic phosphorylation.

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