LIGHT-INDUCED REVERSIBLE PROTON EXTRUSION BY SPINACH-CHLOROPLAST PHOTOSYSTEM II VESICLES ISOLATED BY PHASE PARTITION

Bertil ANDERSSON, Hans-Erik ÅKERLUND and Per-Åke ALBERTSSON
Department of Biochemistry, University of Lund, Box 740, S-220 07 Lund; Sweden

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1. Introduction

In a recent communication [1] we showed that subchloroplast membrane vesicles could be fractionated, on the basis of differences in surface properties, by partition in an aqueous dextran–polyethylene glycol two-phase system. It was shown that the membrane vesicles which partitioned to the dextran-rich bottom phase were highly enriched in photosystem II, while the material preferring the polyethylene glycol-rich top phase was slightly enriched in photosystem I.

In the present paper we report on light-induced pH-changes associated with such subchloroplast membrane vesicles. When supplied with phenyl-*p*-benzoquinone, the photosystem II vesicles partitioning into the bottom phase showed light-induced reversible proton extrusion in contrast to the top phase material which showed normal proton uptake. Both the proton uptake and extrusion accompanying the phenyl-*p*-benzoquinone-reduction were associated with photosystem II electron-transport, since both could be inhibited by DCMU but were insensitive to DBMIB. Based upon these observations we suggest that the photosystem II material partitioning to the bottom phase contains mainly membrane vesicles which are turned inside-out. Other explanations for the light-induced proton extrusion will also be discussed.

Abbreviations: DCMU, 3-(3,4-dichlorophenyl) 1,1-dimethylurea; DBMIB, dibromothymoquinone; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DCCD, dicyclohexylcarbodiimide; PMS, *N*-methylphenazonium methosulfate

2. Materials and methods

Washed spinach class II-chloroplasts, prepared as previously described [1,2], were suspended in 150 mM NaCl/50 mM sodium phosphate buffer, pH 7.4, and disintegrated by passage through a Yeda press [3] at a nitrogen gas pressure of 10 MPa. The grana-enriched pellet obtained after centrifugation for 30 min at 40 000 × *g* was suspended in 10 mM sodium phosphate buffer, pH 7.4/5 mM NaCl/100 mM sucrose and passed twice through the Yeda press. This low-salt press treatment yields unstacking and further fragmentation of grana into membrane vesicles as revealed by electron microscopy [1]. This mixture of membrane vesicles, designated 40 K l.s. (low-salt) was fractionated by phase partition. 5 ml of the 40 K l.s.-fraction was added to 20 g of a polymer mixture to yield a two phase system of the following composition: 6.1% (w/w) dextran-500, 6.1% (w/w) polyethylene glycol 4000, 10 mmol sodium phosphate buffer (pH 7.4)/kg, 5 mmol NaCl/kg, and 20 mmol sucrose/kg.

The two phase system was mixed and allowed to separate. To facilitate phase-separation centrifugation for 3 min at 1500 × *g* was usually performed. The top phase and bottom phase were collected and repartitioned with pure bottom phase and top phase respectively yielding fractions T and B (fig.1). To remove the material from buffer and polymers each fraction was diluted with equal vol. 10 mM NaCl followed by two consecutive centrifugations at 100 000 × *g* for 3 h and 1 h. After each centrifugation the material was suspended in 10 mM NaCl. All preparation work was performed at 2–3°C. pH-

Changes and oxygen evolution were measured simultaneously in a thermostatted vessel (20°C), supplied with a combined glass electrode and a Clark type oxygen electrode. Light of saturating intensity was provided by a tungsten lamp, supplied with a $CuSO_4$-filter.

3. Results and discussion

Figure 1 summarizes the distribution of a low-salt press treated grana fraction (40 K l.s.) in the phase-partition procedure. The two main fractions (T and B) were collected and used, together with unpartitioned material, for light-induced proton translocation studies. The chlorophyll composition, photochemical activities and ultrastructural appearance of such fractions were recently reported [1]. The material preferring the bottom phase consisted of membrane vesicles highly enriched in photosystem II while the material which partitioned to the top phase was slightly enriched in photosystem I.

Figure 2 shows the proton movement by the fractions indicated in fig.1 (40 K l.s., T and B), when illuminated and supplied with phenyl-*p* benzoquinone. The unpartitioned 40 K l.s.-material and the T-material showed proton uptake from the surrounding medium. Under identical conditions light-induced reversible proton efflux was observed for the B-material. These results indicate that the 40 K l.s.-fraction contained a mixture of membrane vesicles which upon illumination translocated protons in opposite directions.

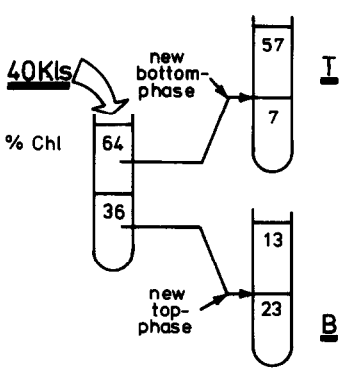

Fig.1. Chlorophyll distribution after two-phase fractionation of low-salt press treated grana-enriched material (40 K l.s.). The 40 K l.s., T- and B-fractions were used for proton translocation studies. Chl = chlorophyll.

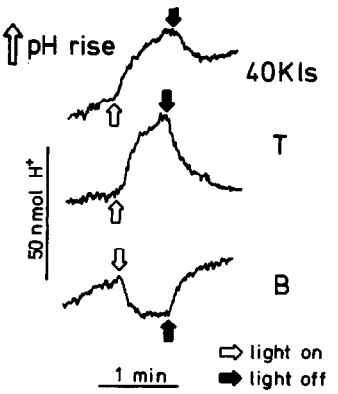

Fig.2. Light-induced reversible proton translocation accompanying phenyl-*p*-benzoquinone reduction. The assay medium (vol. 2.5 ml) consisted of: 10 mM NaCl, 0.3 mM phenyl-*p*-benzoquinone and chloroplast material corresponding to 175 μg chlorophyll/ml. The starting pH in the medium was 6.5–6.7. The extent of proton uptake and extrusion was determined by adding 50 nmol NaOH.

To study the mechanism of the reversed proton movement, inhibition and uncoupling studies were performed and compared with the normal proton uptake (table 1). The plastoquinone antagonist DBMIB [4] did not noticeably affect oxygen evolution nor proton translocation in any of the fractions. On the other hand, the photosystem II inhibitor DCMU caused severe inhibition of both proton transport and oxygen evolution in all three fractions. This shows that both proton uptake and efflux accompanying phenyl-*p*-benzoquinone reduction were mainly associated with photosystem II electron-transport from water to a site prior to the plastoquinone pool. There might be, however, some contribution of cyclic electron-transport, since some proton efflux by the B-material still occurred after nearly all oxygen evolution was abolished by DCMU.

The uncoupler NH_4Cl reduced the proton efflux of the B-material by 60% without decreasing oxygen evolution (table 2), indicating that this phenomenon was mainly due to a pH-gradient created across the thylakoid membrane. The proton uptake by the T-material was abolished by NH_4Cl and under identical conditions the proton uptake by the 40 K l.s.-

Table 1
Effect of DBMIB and DCMU on light-induced proton translocation and oxygen evolution in the 40 K l.s., T- and B- membrane-fractions

	(nmol H^+ × mg chl^{-1})		(μmol O_2 × mg chl^{-1} × h^{-1})	
	−DBMIB	+DBMIB	−DBMIB	+DBMIB
40 K l.s.	36	39	39	37
T	50	50	46	44
B	−35	−38	41	38
	−DCMU	+DCMU	−DCMU	+DCMU
40 K l.s.	17	− 2	26	2
T	37	0	30	1
B	−20	− 5	36	2

Experimental conditions as in fig.2 and when indicated DBMIB and DCMU was added to yield concentrations of 1.5 μM and 10 μM respectively

material was reversed into a weak proton efflux. This weak proton efflux by the unpartitioned 40 K l.s. material, in the presence of NH_4Cl, probably originated from B-fraction type particles possessing an uncoupler insensitive part of proton efflux. Similar results were obtained after addition of the uncoupler CCCP but the uncoupling effect was difficult to interprete since CCCP also inhibited oxygen evolution.

The mechanism of the proton uptake associated with the photosystem II electron-transport of the T-material can be explained by internal release of protons from water oxidation and uptake of external protons by reduction of phenyl-*p*-benzoquinone, a mechanism similar to that suggested for proton translocation associated with DBMIB reduction [5]. If the mechanism for the reversed proton translocation by the B-material is the same as that suggested for the proton uptake it would indicate that the B-material consisted of vesicles which were turned inside-out. Thus the unpartitioned 40 K l.s.-material would consist of a mixture of right-side-out (T) and inside-out (B) thylakoid vesicles which could be discriminated by the phase system.

The Yeda press treatment may disintegrate the thylakoid membrane system in a way which allows the membrane fragments to reseal into both right-side-out and inside out vesicles. The membrane surfaces of the inside out vesicles would differ from

Table 2
Effect of NH_4Cl

	(nmol H^+ × mg chl^{-1})		(μmol O_2 × mg chl^{-1} × h^{-1})	
	−NH_4Cl	+NH_4Cl	−NH_4Cl	+NH_4Cl
40 K l.s.	8	−3	31	31
T	30	0	28	29
B	−12	−5	33	32

Experimental conditions as in fig.2 and when indicated NH_4Cl was added to yield a concentration of 9 mM.

Table 3
Effect of DCCD

	nmol H^+ × mg chl^{-1}		μmol O_2 × mg chl^{-1} × h^{-1}	
	−DCCD	+DCCD	−DCCD	+DCCD
40 K l.s.	5	7	27	25
T	16	36	20	16
B	−14	−14	39	28

Experimental conditions as in fig.2 and when indicated DCCD was added to yield a concentration of 25 μM. The DCCD samples were incubated in the dark for 15 min.

those of the right-side-out vesicles due to the asymetric distribution of components across the thylakoid membrane [6,7]. This would be the basis for the separation of these vesicles by phase partition, since this technique utilizes differences in membrane surface properties for separation [8]. In fact, this method has already been used for the isolation of inside out vesicles from erythrocytes [9].

In addition to the inside out hypothesis alternative explanations for the proton efflux by the B-material must be considered. A light-induced, PMS-mediated, proton release by chloroplasts, where the coupling factor had been removed by NaBr treatment, was reported by Kamienietzky and Nelson [10]. That proton release could be reversed by addition of DCCD. In contrast, the proton efflux by our B-material was not effected by DCCD (table 3) indicating that a depletion of coupling factor was not the reason for the inversed proton movement. The proton uptake by the T-material was doubled by the same concentration of DCCD suggesting that these membranes had lost some of their coupling factor.

Kahn found [11] that *Euglena* chloroplasts excrete protons in the presence of NH_4Cl but take up protons in the presence of 2,4-dinitrophenol and suggested that these chloroplasts contained compartments translocating protons in opposite directions. The B-material could therefore originate from photosystem II compartments in the chloroplast, normally excreting protons upon illumination. But since the reported effects of the uncouplers on *Euglena* chloroplasts is not observed with spinach chloroplasts this explanation seems unlikely.

Conformational changes in the thylakoid membrane in the light, may cause a release of protons to the surrounding medium. We doubt that such a conformational change was responsible for the major part of the proton efflux by the B-material since it was sensitive to uncoupling and electron-transport inhibition. The residual – uncoupler and DCMU insensitive – part of the proton extrusion might however be associated with a conformational change.

In conclusion we therefore favour the interpretation that the B-fraction consists of thylakoid membrane vesicles turned inside-out. Such vesicles have so far not been isolated from the thylakoid lamellae. The B-fraction would therefore be useful for the characterization of the inner surface of the thylakoid membrane and its asymetric organisation.

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