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INSIDE-OUT MEMBRANE VESICLES ISOLATED FROM SPINACH THYLAKOIDS

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Summary

Inside-out thylakoid vesicles have been separated from right-side-out material after press disruption of chloroplast lamellae. The separation was obtained by partition in an aqueous dextran-polyethylene glycol two-phase system, a method which utilizes differences in surface properties for separation of membrane particles. The isolated thylakoid vesicles showed the following inside-out properties: (1) light-induced reversible proton extrusion into the surrounding medium when supplied with the Photosystem II electron acceptor phenyl-*p*-benzoquinone; (2) a pH rise in the internal phase accompanying the external proton release, (3) sensitivity to trypsin treatment different from that of thylakoid membranes of normal orientation; (4) concave EF and convex PF freeze-fracture faces.

Introduction

The asymmetry of biological membranes is an important feature of membrane organization and function [1]. This has been studied extensively in membrane systems where isolation of inside-out vesicles has been possible, for example the red blood cell [2] and the inner mitochondrial membrane [3].

For the chloroplast thylakoid membrane system the lack of a preparation method of inside-out vesicles has made a study of the inner thylakoid surface difficult. Conclusions on the asymmetric arrangement of components across this membrane have therefore been drawn mainly from effects of antibodies and other non-penetrating reagents on the outer surface of the membrane [4].

In previous studies [5—7], partition in an aqueous dextran/polyethylene glycol two-phase system has been used for separation of press-disintegrated chloroplast lamellae. The phase partition method utilizes differences in surface

Abbreviation: DCMU, 3-(3,4-dichlorophenyl)1,1-dimethylurea; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone.

properties for separation of membrane particles rather than size and density [8,9]. By this method a grana-enriched fraction, further disintegrated under low salt conditions, was separated into two subpopulations on the basis of their different affinity for the two phases [5,6]. Proton translocation studies [5] showed that the material partitioning to the dextran-rich bottom phase showed light-induced reversible proton extrusion when supplied with phenyl-*p*-benzoquinone, while the material preferring the polyetheylene glycol-rich top phase showed normal proton uptake. These observations suggested that the bottom phase contained sealed vesicles with the inner thylakoid surface turned outside.

In this work the inside-out hypothesis is confirmed by characterization of the vesicles by the direction of light-induced pH changes both in their external and internal phases, by sensitivity to tryptic digestion and by freeze-fracture appearance.

Materials and Methods

Polyethylene glycol 4000 was obtained from Union Carbide, New York, N.Y., U.S.A. Dextran 500, batch No. 3447, was supplied by Pharmacia Fine Chemicals AB, Uppsala, Sweden. Trypsin, type XI, without chymotrypsin activity, was obtained from Sigma. Spinach (*Spinacia oleracea* L.) was grown in nutrient solution with a light period of 12 h.

Isolation procedure. Washed spinach class II chloroplasts, prepared as previously described [7], were suspended in 150 mM NaCl/50 mM sodium phosphate buffer, pH 7.4, and disintegrated by passage through a Yeda press [10] 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 gives further destacking and fragmentation of grana, producing mainly membrane vesicles as revealed by electron microscopy [6]. After removal of starch grains and residual unbroken lamellae by a low speed centrifugation (1000 × *g* for 10 min), this membrane population, designated 40 Kls (low salt), was fractionated by phase partition as illustrated in Fig. 1. 5 ml of the 40 Kls fraction (800 μg chlorophyll/ml) was added to 20 g of a polymer mixture yielding the following composition: 5.7% (w/w) dextran 500, 5.7% (w/w) polyethylene glycol 4000, 10 mmol sodium phosphate buffer (pH 7.4)/kg, 5 mmol NaCl/kg, 20 mmol sucrose/kg and chloroplast material corresponding to 4 mg chlorophyll. The concentration values are based on the total weigth of the phase system. The phase system was mixed carefully and allowed to separate. The mixture was usually centrifuged in a swing-out centrifuge (3 min at 1500 × *g*) to facilitate phase separation. The top phase (T1) and bottom phase (B1) were collected and repartitioned with pure bottom phase and top phase respectively, yielding fractions T2 and B2. The B2 fraction was purified by a further partition step giving a B3 fraction. The chloroplast material was removed from the viscous bottom phase by a final partition step with a top phase containing the positively charged trimethylaminopolyethylene glycol [11]. The temperature during mixing and separation of the two-phase systems is critical and should be 3—4°C. The polymer concentrations needed for successful separation

depend on the molecular weight distribution of dextran and polyethylene glycol and have to be tested for each new batch of the polymers. The concentrations needed vary between 5.2% and 6.2% (w/w).

Proton translocation studies. For these measurements the chloroplast membranes were removed from buffer and polymers by two consecutive centrifugations at 100 000 × *g* for 1 h. External pH changes and oxygen evolution were measured simultaneously in a vessel maintained at 20°C, supplied with a combined glass electrode and a Clark type oxygen electrode. Light was provided by two 375 W tungsten lamps placed on opposite sides of the reaction vessel. Both lamps were fitted with $CuSO_4$-filters. External pH changes were also measured, using the indicator Bromcresol purple. The absorbance change at 571 nm was followed after one saturating single turnover flash, in a medium containing 40 mM KCl, 0.3 mM phenyl-*p*-benzoquinone, 25 μM Bromcresol purple. The initial pH was set to 6.5.

Light-induced pH changes in the internal space of the vesicles were determined using the indicator Neutral Red and buffering the external medium with bovine serum albumin in a way similar to that described by Ausländer and Junge [12]. The light-induced absorbance change was followed by dual-wavelength measurements at 540 and 580 nm using an Aminco DW-2 spectrophotometer.

Trypsin treatment. The Photosystem I electron transport from ascorbate and 2,6-dichlorophenol-indophenol to methyl viologen and the Photosystem II reaction from water to phenyl-*p*-benzoquinone were studied after tryptic digestion of the top phase and bottom phase membranes. The incubations with trypsin were performed differently depending on the particular reaction studied. For the Photosystem I activity, trypsin was added to yield a trypsin/chlorophyll ratio of 0.6. The incubation was run at 20°C. For the Photosystem II activity, trypsin was added to yield a trypsin/chlorophyll ratio of 0.1. For these measurements the incubations were made at 4°C to preserve control activities. In both experiments the membranes were incubated without removal of the phase polymers. After increasing periods of tryptic digestion, samples were withdrawn and transferred to test tubes containing trypsin inhibitor (ratio of trypsin inhibitor/trypsin = 4). The control samples received a mixture of trypsin and trypsin inhibitor yielding the same final concentration as for the digested samples. Photochemical activities were measured as oxygen uptake or evolution using the same oxygen electrode and illumination source as for the proton translocation experiments. The reaction mixtures are given in the legends of Figs. 5 and 6.

Freeze-fracture studies. For freeze-fracturing the membranes were removed from the phase polymers by centrifugation at 100 000 × *g* for 60 min. The material was resuspended in 20% (w/v) glycerol/10 mM sodium phosphate buffer, pH 7.4/5 mM NaCl and sedimented once more. The samples were frozen using Freon 22 cooled in liquid nitrogen. Replicas were prepared according to standard procedures on a Balzers BAF 301 freeze-etching machine. Specimens were freeze-fractured at −110°C, and etching was carried out for 2 min at −100°C.

Results

The distribution of the 40 Kls material during the phase partition procedure is shown in Fig. 1. The chlorophyll composition, photochemical activities and ultrastructural appearance of such fractions have been reported previously [6], revealing an enrichment of Photosystem II in the bottom phase. In this study the two main subfractions (Fig. 1) were characterized with respect to membrane sidedness by proton translocation behaviour, resistance to tryptic digestion and freeze-fracture appearance.

Proton translocation studies

Glass electrode measurements revealed that when supplied with phenyl-*p*-benzoquinone the unpartitioned material (40 Kls) and the material partitioning to the top phase (T2) showed light-induced reversible proton uptake as is normally observed for thylakoid membranes (Fig. 2). Under identical conditions light-induced reversible proton extrusion was observed for the material preferring the bottom phase (B3). Similar results were obtained with the dye bromocresol purple as a pH indicator in the external medium (Fig. 3).

Addition of DCMU during the measurement severely inhibited both proton translocation and oxygen evolution in all fractions (Table I). In contrast dibromothymoquinone affected these reactions only slightly. The uncoupler NH_4Cl reduced the proton efflux of the B3 material by 85% without noticeably decreasing oxygen evolution. Similar effects of NH_4Cl were observed for the normal proton uptake of the 40 Kls and T2 fractions. These inhibition and uncoupling studies reveal that both the proton extrusion of the B3 material and the proton uptake of the 40 Kls and T2 fractions were mainly due to a pH gradient across the thylakoid membrane created by Photosystem II electron transport.

These observations strongly suggest that the B3 material contains sealed

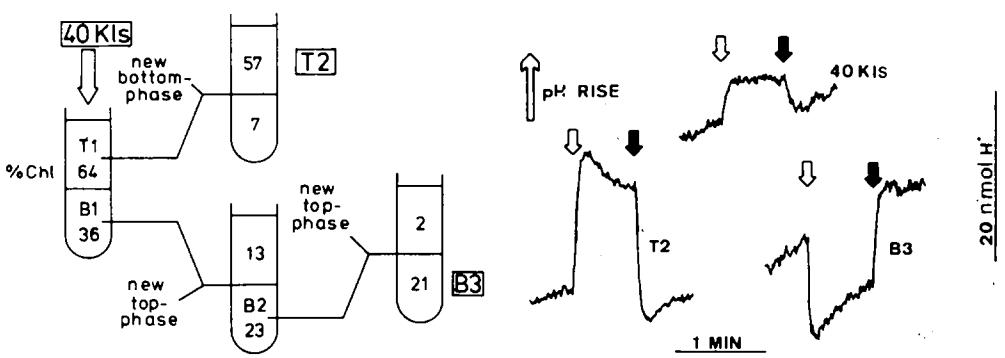

Fig. 1. Two-phase fractionation of the low salt disintegrated grana enriched fraction (40 Kls). The two main fractions, T2 and B3, were collected and characterized with respect to membrane sidedness.

Fig. 2. Light-induced proton transport associated with phenyl-*p*-benzoquinone reduction, measured with a glass electrode. The assay medium was composed of: 40 mM KCl, 0.37 mM phenyl-*p*-benzoquinone and chloroplast material corresponding to 100 μg chlorophyll/ml. Initial pH in the medium was 6.5 and the extent of proton uptake or extrusion was determined after each experiment by addition of 50 nmol NaOH.

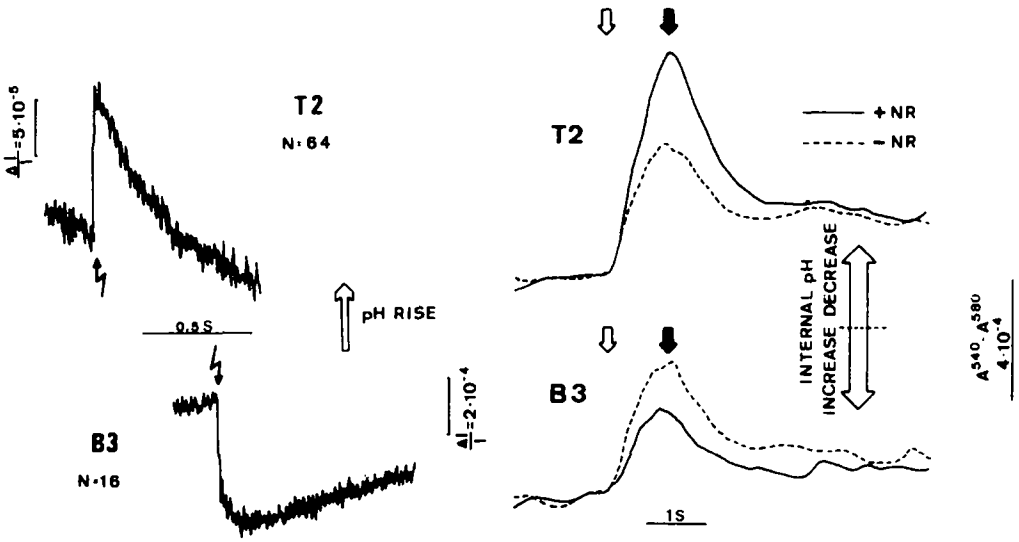

Fig. 3. pH changes after a single-turnover flash measured as the absorbance change of bromcresol purple in the T2 and B3 membrane fractions. The assay medium was composed of 40 mM KCl, 0.3 mM phenyl-*p*-benzoquinone, 25 μM bromcresol purple. Initial pH was set to 6.5. The signal-to-noise ratio was increased by averaging for N successive flashes.

Fig. 4. pH changes in the internal phase of the T2 and B3 membrane vesicles were measured as the light-induced absorbance change of Neutral Red (NR) in the presence of the external buffer bovine serum albumin. Assay medium was composed of: 37 mM KCl, 6.5 mg bovine serum albumin/ml (pH 6.8), 12.5 μM Neutral Red, 0.3 mM phenyl-*p*-benzoquinone and chloroplast material corresponding to 40 μg chlorophyll/ml. To increase the signal-to-noise ratio the signal was averaged for 16 repetetive illuminations.

thylakoid vesicles which are turned inside-out. A critical test of this suggestion would be provided by an investigation of whether the internal pH of the B3 material increases upon illumination, as would be expected for an inside-

TABLE I

EFFECT OF NH_4Cl, DBMIB AND DCMU ON STEADY STATE LEVEL OF LIGHT-INDUCED PROTON TRANSPORT AND OXYGEN EVOLUTION ASSOCIATED WITH PHENYL-*P*-BENZOQUINONE REDUCTION IN THE 40 KIs, T2 AND B3 MEMBRANE FRACTIONS

Experimental conditions as in Fig. 2 and when indicated NH_4Cl, DBMIB and DCMU was added to yield concentrations of 10 mM, 1.5 μM and 10 μM, respectively. Steady state level of proton transport (ΔH^+) and rate of oxygen evolution (O_2) are expressed as nmol H^+/mg chlorophyll and μmol O_2/mg chlorophyll per h, respectively.

Fraction	Control activities		Percent activity remaining					
	ΔH^+	O_2	+ NH_4Cl		+ DBMIB		+ DCMU	
			ΔH^+	O_2	ΔH^+	O_2	ΔH^+	O_2
48 KIs	42	25	10	98	99	98	21	21
T2	99	25	9	90	95	100	20	19
B3	—43	24	15	96	113	97	13	15

out vesicle. Such a study was performed using the pH indicator Neutral Red and buffering the external medium with bovine serum albumin (Fig. 4). The internal compartments of the T2 material showed an acidification upon illumination as is normally seen for thylakoid membranes. In contrast a rise in the internal pH of the B3 vesicles was registered, supporting the conclusions drawn from the measurements of the external pH changes. The absorbance change without addition of Neutral Red was probably due to light scattering changes.

Trypsin treatment

The effects on electron transport activities after tryptic digestion of chloroplast lamellae have been studied by several workers [13–15]. It is assumed that the resulting effects are caused by specific proteolysis of groups on the outer thylakoid surface, since trypsin should not be able to penetrate the lipid bilayer. Therefore, provided the T2 and B3 material are of opposite sidedness, different components should be digested by trypsin, leading subsequently to different inhibitory effects. Fig. 5 shows the effect of trypsin treatment on Photosystem I electron transport activity, in the T2 and B3 fractions. The

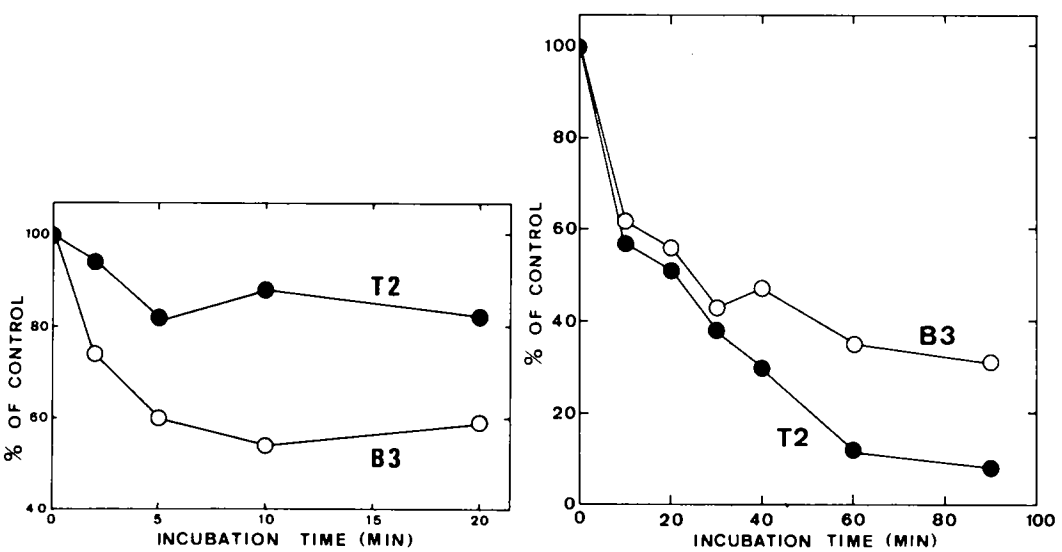

Fig. 5. Inhibition of the Photosystem I reduction of methyl viologen after trypsin incubation of the T2 and B3 membrane fractions. A trypsin/chlorophyll ratio of 0.6 was used. Oxygen consumption was measured with a Clark type oxygen electrode in 2.5 ml of a medium composed of: 40 mM sodium phosphate buffer (pH 7.4), 1 mM NaCl, 10 mM sucrose, 0.6 mM NaN_3, 0.12 mM methyl viologen, 0.288 mM 2,6-dichlorophenol-indophenol, 32 mM sodium ascorbate, 10 μM DCMU and chloroplast material corresponding to 100 μg of chlorophyll. The 100% rates were 117 and 54 μmol/mg chlorophyll per h for the T2 and B3 material, respectively.

Fig. 6. Inhibition of the reaction $H_2O \rightarrow$ Photosystem II $\rightarrow$ phenyl-*p*-benzoquinone after trypsin incubation of the T2 and B3 membrane fractions. A trypsin/chlorophyll ratio of 0.1 was used. The oxygen evolution was measured with a Clark type electrode in 2.5 ml of a medium composed of: 28 mM sodium phosphate buffer (pH 6.5), 4.8 mM NaCl, 0.24 mM phenyl-*p*-benzoquinone and chloroplast material corresponding to 50 μg chlorophyll. The 100% rates were 82 and 116 μmol O_2/mg chlorophyll per h for the T2 and B3 material respectively.

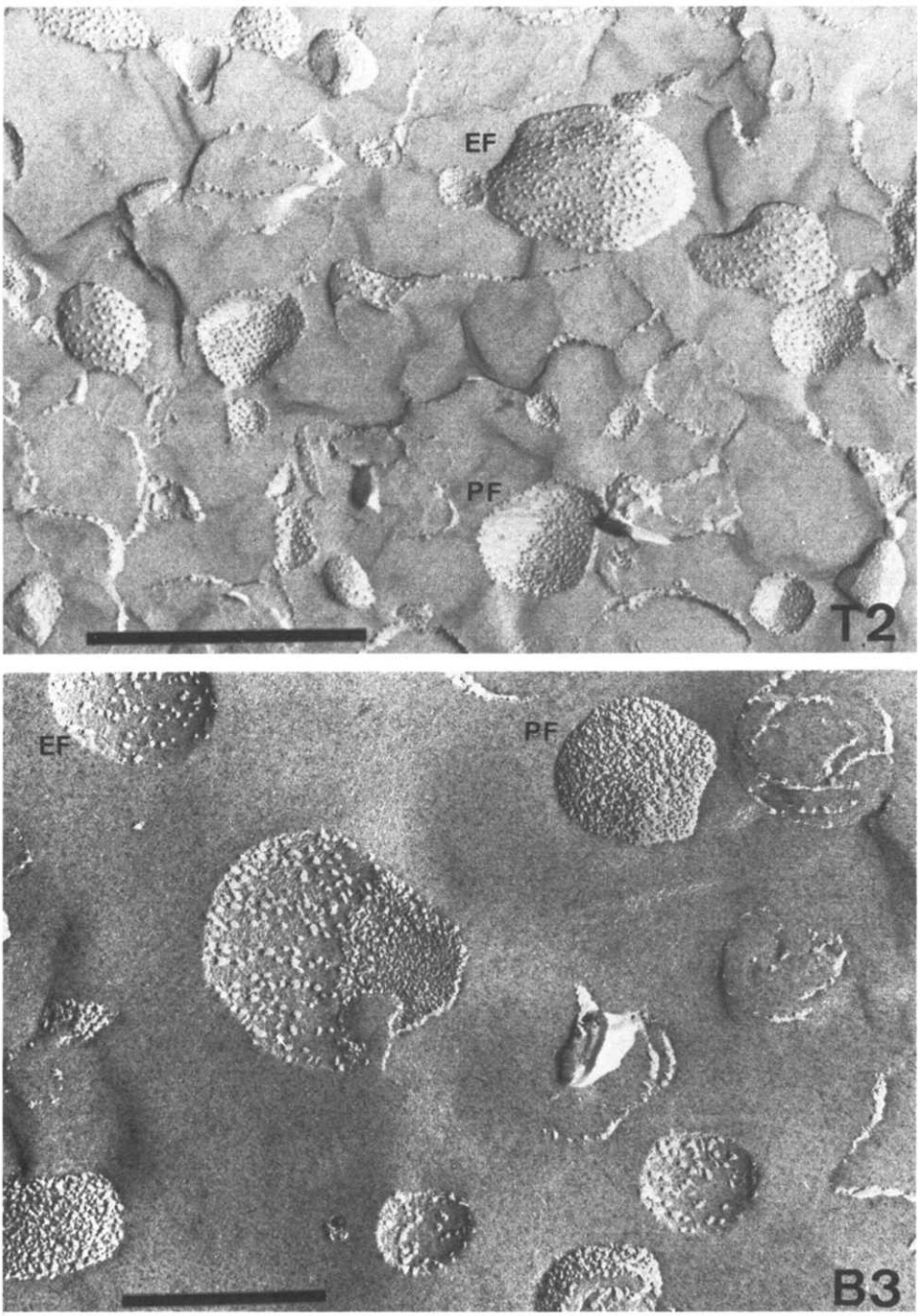

Fig. 7. Freeze-fracture appearance of the T2 and B3 membrane fractions. Bar represents 0.5 μm.

activity of the B3 fraction was inhibited 40—45% while under identical conditions the activity of the T2 fraction was inhibited by only approx. 15%. It is well established that this particular reaction is not affected by trypsin treatment of unbroken chloroplast lamellae [13,14]. Therefore the most likely

explanation for the relatively high inhibition of the B3 fraction is that it contains membranes exposing the inner thylakoid surface. Correspondingly the low inhibition of the T2 material confirms that it is a right-side-out fraction.

From Fig. 6 it is evident that the Photosystem II activity was also affected differently in the T2 and B3 fractions by trypsin treatment. The slope of the inhibition curve showed a biphasic appearance. First, a very rapid phase, resulting in about 40% decrease in activity of both fractions within 5 min of incubation. This rapid phase was followed by a slow phase in which there was a pronounced difference in activity decrease between the T2 and B3 materials. Between 5 and 90 min incubation the Photosystem II activity of the T2 fraction declined from 60% to below 10%. After the same incubation period, over 30% of the activity still remained in the B3 fraction. The results obtained from the trypsin inhibition experiments thus support the interpretation that the T2 and B3 material are of opposite sidedness, with the latter containing mainly inside-out thylakoids.

Freeze-fracture studies

Freeze-fracturing of thylakoid membranes results in the visualization of faces with characteristic topographies [16]. The fracture face next to the intra-thylakoid space (EF) shows relatively few, large distinct particles while the fracture face next to the stroma (PF) consists of mainly closely packed small particles. Simple vesicles derived from thylakoid membranes would show convex EF and concave PF faces provided they have retained their original sidedness (for nomenclature, see ref. 17). Consequently, inside-out thylakoid vesicles should have concave EF and convex PF faces. In fig. 7 are shown electron micrographs of representative replicas from the T2 and B3 fractions. A predominance of convex PF and concave EF faces was found for the B3 material as expected for inside-out thylakoid vesicles, while mainly right-side-out vesicles were observed for the T2 fraction.

Approx. 160 vesicular faces in each fraction were examined to estimate the relative contents of right-side-out and inside-out vesicles. Residual complex membrane structures were omitted from the calculations since a subdivision into concave and convex faces is not applicable for these. The results revealed that the B3 fraction contained 74% inside-out and 26% right-side-out vesicles while the T2 fraction contained 89% right-side-out and only 11% inside-out vesicles.

Discussion

Independently of each other, the different experiments presented in the previous section all provide evidence that phase partition of disintegrated chloroplast lamellae yields two subpopulations of chloroplast fragments with opposite sidedness.

Initially, the existence of inside-out thylakoid vesicles in the bottom-phase was suggested from observations on light-induced reversible proton extrusion associated with phenyl-*p*-benzoquinone reduction [5]. This interpretation is, however, correct only if the mechanism for proton translocation by the B3

material is the same as that normally observed for thylakoid membranes. In view of the present results this prerequisite is fulfilled since: (a) addition of the inhibitors dibromothymoquinone and DCMU affected both proton translocation and electron transport in a similar way (Tabel I); (b) approximately the same degree of uncoupling by NH_4Cl was observed for both T2 and B3 material (Table I); (c) the external proton release by the B3 vesicles was associated with a pH rise in the internal space of the vesicles while the opposite was observed for the T2 vesicles (Fig. 4).

Other investigators have reported light-induced proton release by chloroplasts where part of the coupling factors has been removed by EDTA or NaBr treatment [18,19]. It has been suggested that these types of proton release are the results of conformational changes [19]. Moreover, these types of proton release are insensitive to uncouplers but can be converted into proton uptake by treatment of the coupling factor depleted thylakoids with dicyclohexylcarbodiimide. The proton release by the B3 vesicles, however, has earlier been shown to be dicyclohexylcarbodiimide insensitive [5]. Obviously, the proton extrusion by the B3 vesicles does not resemble the proton extrusion observed for coupling factor depleted thylakoids.

The values for proton translocation in Table I are corrected for the weak "acidification"-response of the pH-electrode to light in the presence of phenyl-*p*-benzoquinone. In a previous report [5] we assumed that a minor uncoupler and DCMU insensitive part of the proton release by the bottom phase material might be due to some kind of conformational change. This residual proton release was, however, overestimated since no correction was made for the response of the electrode to light.

The proton gradient observed for thylakoids of normal sidedness, such as the T2 material, in the presence of phenyl-*p*-benzoquinone is suggested to be a result of internal release of protons from the water oxidation reaction and uptake of external protons by reduction of phenyl-*p*-benzoquinone. For the B3 material the only difference would be that the protons from the water oxidation reaction are released to the external medium and the protons for the reduction of phenyl-*p*-benzoquinone are taken from the internal space. At the present stage it is not known if the plastoquinone loop is involved in phenyl-*p*-benzoquinone reduction. The electron transport in intact thylakoids using phenyl-*p*-benzoquinone as acceptor was, however, insensitive to uncouplers. This observation corresponds to the results obtained for reduction of dibromothymoquinone and other class III acceptors [20], indicating that only coupling site II is involved in phenyl-*p*-benzoquinone reduction.

The two fractions T2 and B3 showed different resistance to tryptic digestion as revealed from electron transport inhibition studies. These observations can most easily be explained by assuming a different sidedness for the two subpopulations, thereby exposing different components sensitive for the membrane impermeable proteolytic enzyme.

The conclusions drawn from these trypsin treatment experiments demands that the electron acceptor used can penetrate the thylakoid membrane sufficiently. Methyl viologen is known to penetrate the chloroplast envelope freely [21] and is also likely to penetrate the thylakoid membrane in the small catalytic amounts needed for the Mehler reaction. For the highly hydrophobic

phenyl-*p*-benzoquinone no accessibility restriction would be expected.

At the present stage the effects of tryptic digestion on the T2 and B3 material should not be taken as evidence for the assymmetric localization of certain reaction sequences or components across the membrane. Such interpretations need extensive investigations, including comparative studies on inhibition of several partial electron transport reactions of the two different membrane populations. Such studies are in progress.

The replicas from freeze-fracturing of the B3 material demonstrated that this material was dominated by inside-out vesicles, since mainly concave EF and convex PF faces were observed. The replicas of the T2 and B3 fractions also gave a rough estimation of the relative amount of right-side-out and inside-out vesicles in each fraction. To avoid errors in the interpretation caused by residual unbroken thylakoids, only true vesicular shaped faces without surrounding crosssections were considered. A more extended study on freeze-fracture and freeze-etch appearance of the fractions will be published elsewhere [22].

Obviously the Yeda press treatment under the present conditions disintegrates the thylakoid membranes in a way which allows some membrane fragments to reseal into vesicles with the intra thylakoid surface turned outside and others to reseal into vesicles of original sidedness. Thus, the 40 Kls fraction contains a mixture of inside-out and right-side-out vesicles.

Due to asymmetric arrangement of the thylakoid membrane [4,23] the surface properties of the inside-out vesicles would differ from those of the right-side-out vesicles. This is the basis for their separation by phase partition, since this technique utilizes differences in membrane surface properties, such as charge and hydrophobicity, for separation [9]. Probably, varying amounts of inside-out vesicles are usually formed during different disintegration procedures described for the chloroplast lamellae. The reason why inside-out thylakoid vesicles have not been isolated previously would be that the separation methods used earlier depend predominantly on size and density differences of particles. The phase partition method has also been used successfully for isolation of inside-out erythrocyte ghosts [24].

The B3 fraction is apparently still contaminated with right-side-out material as judged by freeze-fracturing. Attempts are therefore in progress to improve the purity of the inside-out fraction using extensive centrifugation and phase partition procedures. Despite this contamination, the B3 material should be extremely suitable for exploring the inner thylakoid surface, to improve our knowledge of the asymmetric arrangement of components across this membrane.

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