Biochimica et Biophysica Acta, 688 (1982) 709–719
Elsevier Biomedical Press

BBA 71227

CONVERSION OF EVERTED THYLAKOIDS INTO VESICLES OF NORMAL SIDEDNESS EXPOSING THE OUTER GRANA PARTITION MEMBRANE SURFACE

CECILIA SUNDBY, BERTIL ANDERSSON and PER-ÅKE ALBERTSSON

Department of Biochemistry, University of Lund, P.O. Box 740, S-220 07 Lund (Sweden)

(Received December 31st, 1981)

Key words: Inside-out vesicle; Sidedness; Membrane refolding; Asymmetry; Phase partition, Thylakoid membrane; (Spinach chloroplast)

Inside-out spinach thylakoid vesicles can be isolated by aqueous polymer two-phase partition following mechanical disruption of spinach chloroplast lamellae (Andersson, B and Åkerlund, H.-E. (1978) Biochim. Biophys. Acta 503, 462–472) and a mechanism for their formation has been experimentally supported (Andersson B., Sundby, C. and Albertsson, P.-Å. (1980) Biochim. Biophys. Acta 599, 391–402). Upon disruption, inside-out vesicles may form under stacking conditions, e.g., in 5 mM $MgCl_2$ or 150 mM NaCl, while disruption under destacking conditions, i.e., low concentrations of monovalent cations, gives only right-side-out vesicles. This study deals with the sidedness stability of the isolated inside-out thylakoid vesicles when stored or disrupted by sonication in various ionic environments. The sidedness of thylakoid vesicles was determined by their partition behaviour in an aqueous polymer phase system, direction of proton translocation and aggregation response (stacking) upon addition of $MgCl_2$. The results show that no spontaneous change from everted to normal sidedness occurs upon storage of the inside-out thylakoids. In contrast, sonication of these vesicles under destacking conditions (5 mM NaCl) results in a nearly complete transformation to right-side-out orientation. Also, in the presence of 5 mM $MgCl_2$ or 150 mM NaCl, sonication induced a change in sidedness of the inside-out vesicles but to a lesser extent. The stabilizing effect on the everted sidedness by cations was shown to be a result of preventing vesicle fragmentation by maintaining internal thylakoid appressions rather than by influencing the membrane curvature during resealing. Once released from an appressed state by overcoming the stacking forces, an opened thylakoid membrane shows an absolute preference for turning right-side-out in all media tested. These results strongly support the proposed formation mechanism, in which pairs of neighbouring grana membranes after disruption reseal with each other promoted by their close proximity. Since the inside-out vesicles derive from the grana appressions, their transformation back to normal sidedness exposes the outer membrane surface of appressed thylakoids. This region of the thylakoid membrane is normally hidden in the grana appressions and removal of grana leads concomitantly to lateral intermixing with non-appressed thylakoid components. Thus the current isolation of right-sided vesicles derived from the grana appressions should be a new tool for studies on the molecular organization of the thylakoid membrane.

Introduction

Inside-out thylakoid vesicles can be isolated from spinach chloroplasts by aqueous polymer two-phase partition following mechanical fragmentation [1–3]. In several recent reports, such everted vesicles have been used for studies on the transverse and lateral organization of the thylakoid membrane and events taking place at the inner thylakoid surface (cf. Ref. 3).

Inside-out vesicles from several other types of biological membrane, such as the inner

0005-2736/82/0000–0000/$02.75

mitochondrial membrane [4], several plasma membranes [5–7], erythrocytes [8,9] and many different types of micro-organism [10,11] are available. The conditions under which optimal amounts of everted vesicles are obtained vary between different membranes. There are also membranes from which no inside-out vesicles have been obtained, such as the endoplasmic reticulum [12] or the intestinal brush border membrane [13].

In those cases where a formation mechanism for an everted vesicle has been put forward it is usually based upon the fact that asymmetric membranes behave as bilayer couples [14]. The expansion or contraction of one layer in response to variation of the ionic mileau relative to the other would induce a change in curvature.

Typical for thylakoids, compared with other membranes, is a special response to variations in ionic composition of the medium, namely the phenomenon of stacking. In a recent study [2] it was shown that stacking had a profound influence on the formation of inside-out vesicles. Such vesicles were formed only upon disruption from appressed regions induced by cations or protons (low pH). Unstacked thylakoid regions or thylakoids destacked in low ionic strength [15] give rise only to rightsided vesicles. This supports a formation mechanism [2,16] in which two neighbouring thylakoid grana membranes that remain appressed after being ruptured, reseal with each other. The thylakoid membrane in an inside-out vesicle may therefore be regarded as trapped in the everted orientation by the stacking forces. Thus, if an everted vesicle were opened up and brought into the transient metastable open state a refolding into right-sided orientation should be expected.

To test this hypothesis we have studied the sidedness stability of inside-out thylakoid vesicles under sonic treatment and storage in various media.

Materials and Methods

Spinach (*Spinach oleracea*) was grown hydroponically as previously described [1]. Leaves were harvested when 6 weeks old.

Dextran T-500, batch No 2386 was obtained from Pharmacia Fine Chemicals AB, Uppsala, Sweden and poly(ethylene glycol) 4000 (Carbowax PEG 3350) from Union Carbide, New York, NY, U.S.A.

Preparation of inside-out thylakoid vesicles. Washed spinach chloroplasts, prepared as previously described [17], were suspended in 150 mM NaCl/50 mM sodium phosphate buffer (pH 7.4) and fragmented by a Yeda press at a nitrogen gas pressure of 10 MPa. The grana-enriched pellet obtained after centrifugation for 30 min at 40000 $\times g$ was suspended in 5 mM NaCl/10 mM sodium phosphate (pH 7.4)/100 mM sucrose (low-salt buffer) and passed twice more through the press. Remaining unfragmented thylakoids and starch grains were removed by low-speed centrifugation (1000 $\times g$ for 10 min). The supernatant, containing a mixture of right-side-out and inside-out thylakoid vesicles, was fractionated by aqueous polymer two-phase partition [1]. 5 ml of this supernatant were added to 20 g of a polymer mixture yielding the following composition: 5.7% (w/w) dextran T-500/5.7% (w/w) poly(ethylene glycol) 4000/5 mM NaCl/10 mM sodium phosphate (pH 7.4)/20 mM sucrose. The system was mixed carefully and allowed to settle. (To facilitate phase settling, centrifugation was performed at 1500 $\times g$ for 3 min in a swing-out centrifuge.) The upper phase (T1) and the lower phase (B1) were collected and repartitioned with pure lower and upper phase, respectively, yielding fractions T2 and B2. The B2 fraction was purified by a further partition step giving a B3 fraction. All partition steps were performed at 3–4°C. The B3 fraction contains the inside-out vesicles, while the normal sided vesicles are recovered in the T2-fraction. The inside-out vesicles used for the subsequent studies were removed from the phase polymers by centrifugation at 100000 $\times g$ for 0.5–2 h.

Sonication and repartition of inside-out vesicles. Prior to sonication the inside-out vesicles were centrifuged and resuspended in either 5 mM NaCl (low salt), 5 mM $MgCl_2$ or 150 mM NaCl in addition to 10 mM sodium phosphate buffer (pH 7.4) and 100 mM sucrose. Ultrasonic treatment was performed with a tip sonicator (Model A 350 G, Ultrasonics Ltd, Shipley, U.K.) under continuous ice-bath cooling of the sample. The sample was subjected to ultrasound at optimal frequence with a microtip (power 7) at intervals of 30 s with 30 s breaks inbetween, resulting in up to 4 min of

sonic exposure. Time and strength for suitable sonication strongly depend on how the tuning adjustment is made and may vary between different types of sonicator. One aliquot of the vesicle suspension was withheld and used as control material. Since different cations influence the partition in various ways [18] the vesicles were spun down and resuspended in low salt medium prior to the repartition step.

The control and sonicated B3 vesicles were added to a 20 g polymer mixture of the same composition as before and repartitioned a fourth time, yielding B4 and B4T fractions. The distribution of membrane material in the phase system was estimated by determination of the chlorophyll content in withdrawn samples from upper and lower phases by measuring their absorbance at 680 nm.

Counter-current distribution. For an analytical investigation of the partition changes of the inside-out vesicles induced by sonication, counter-current distribution was performed using automatic thin-layer counter-current distribution apparatus [19,20]. The experiments were carried out with the same phase composition as in the tube experiments.

A sample (1 g) was added to 4 g of the polymer mixture to yield a 5 g sample system. The amount of material in a sample corresponded to about 800 μg chlorophyll. The volume ratio of the upper and lower phases was 3:2. For counter-current distribution a 1:1 volume ratio was desired; therefore 1 g pure lower phase (see below) was added. A 300 g bulk phase system (without chloroplast material) was made up in a funnel, mixed and allowed to settle overnight at 3–4°C. The upper and lower phase were collected and stored separately until use.

For running the experiment a rondell with 120 cavities (0–119) was used to run four samples simultaneously. Cavities 0–2, 30–32, 60–62 and 90–92 were loaded with sample material. From each sample system, which was shaken before use, 1.48 ml were added to each of three neighbouring cavities. The remaining 108 cavities were loaded with 0.74 ml lower phase and 0.74 ml upper phase collected from the bulk system using an automatic syringe. After each automatic transfer the rondell was shaken for 30 s followed by a settling time of 8 min before a new transfer was made. The interface was kept stationary during each partition cycle. After 27 transfers the fractions were diluted with 1.48 ml buffer in order to break the phase system. The diluted fractions were collected in tubes and the absorbance at 680 nm was measured and plotted against fraction number. For a detailed description of thin-layer counter-current distribution see Ref. 18 and 20.

Phase concentration. For proton translocation studies the vesicles are usually freed from buffer by consecutive centrifugations at $100000 \times g$ for 1 h and suspension in 40 mM KCl. An alternative and more rapid way to concentrate vesicles is to use a polymer two-phase system in which the volume of the lower phase is very small in volume compared to the upper phase [18]. This can be accomplished because the distribution of a certain membrane population between the two phases is independent of the phase volume ratio [18]. Thus, by choosing a phase system with small volume of the lower phase to which the membranes are distributed, a similar concentration effect is achieved after settling of the phases as when the membranes are sedimented by extensive ultracentrifugations.

Before phase concentration the material was first collected in the upper phase. Therefore, materials that partition to the lower phase in the phase system described above were transferred to a new upper phase by raising the temperature of the phase system from 3°C to 10°C before mixing. In that way one approaches the critical point of the phase system at which nearly all membrane particles partition entirely to the upper phase [20]. Then 14 g upper phase containing the diluted membrane vesicles were supplied with 0.3 g 40% (w/w) poly(ethylene glycol) 4000 and KCL to a final concentration of 9% and 10 mM, respectively. Since the original upper phase contains small amounts of dextran in addition to poly(ethylene glycol), this diminutive rise in poly(ethylene glycol) creates a new phase system with a lower phase of very small volume (upper/lower phase volume ratio 29:1). Partition of membranes depend on the ionic composition [18]. To ensure that the material partitions into the lower phase, in the concentration step KCl is added. After mixing and settling of the phase system by a 2 min centrifugation at $10000 \times g$, the upper phase was removed.

The small-volume lower phase was supplied with 14 ml fresh upper phase containing 9% poly(ethylene glycol) and 10 mM KCl and the partition cycle was repeated. After three consecutive repartition steps, the lower phase was collected and diluted several-fold with the desired volume of 40 mM KCl before use. It should be stressed that the centrifugations are performed only in order to facilitate phase settling, not to sediment the particles. A more thorough description of phase concentration of particles is given elsewhere [18].

Studies on proton translocation and electron transport. Light-induced pH changes associated with Photosystem II reduction of phenyl-*p*-benzoquinone were measured with a combined glass electrode in a vessel maintained at 20°C. For estimating the electron transport rate of the same reaction, the oxygen evolution was measured using a Hansatech oxygen electrode. For these measurments the thylakoid vesicles were suspended in 40 mM KCl after being concentrated by ultracentrifugation or phase concentration. Light was provided by a projector lamp placed behind a $CuSO_4$ filter.

Electron microscopy. Chloroplast membranes suspended in buffer were fixed in 3% glutaraldehyde. After 2 h they were pelleted, washed with buffer and post-fixed and stained with 2% OsO_4. The pellets were dehydrated stepwise in acetone with the final step in 100% acetone dried with $CuSO_4$. Embedding was done by gradually introducing Agar 100 in the acetone solution. Polymerization was done at 20°C for 12 h and 70 h at 60°C. Sections were cut and finally stained with aqueous solutions of uranyl acetate and lead citrate.

Results

Reorientation of inside-out thylakoid vesicles

In order to study whether an inside-out vesicle keeps its reversed sidedness if opened up in presence or absence of stacking forces, inside-out vesicles were subjected to sonic treatment under different ionic conditions. If this leads to a change in sidedness, one should expect a change in the vesicles with respect to their (a) partition behaviour in the polymer phase system, (b) direction of the light-induced reversible proton translocation, (c) specific aggregation response to added magnesium ions.

TABLE I

EFFECT OF SONIC TREATMENT ON INVERTED THYLAKOID VESICLES IN LOW IONIC STRENGTH: DISTRIBUTION OF MATERIAL IN AQUEOUS PHASE SYSTEM, PHOTOSYSTEM II ELECTRON TRANSPORT AND PROTON TRANSLOCATION

Sonic treatment was performed in a medium containing 5 mM NaCl/10 mM sodium phosphate (pH 7.4)/100 mM sucrose. Distribution of membrane material between the phases was estimated by chlorophyll absorption at 680 nm and expressed as percentage chlorophyll in each phase. Light-induced proton transport was measured with a glass electrode in 1 ml assay medium of the following composition: 40 mM KCl, 0.25 mM phenyl-*p*-benzoquinone and chloroplast material corresponding to 100 μg chlorophyll. Photosystem II was simultaneously recorded as oxygen evolution with a Clarke-type oxygen electrode and specific activity is expressed as μmol O_2 produced/mg chlorophyll per h.

	Unsonicated			Sonicated in 5 mM NaCl		
	%Chl	ΔH^+	O_2	%Chl	ΔH^+	O_2
B_4T	18	−0.8	–	79	+8.9	6.9
B_4	82	−23.2	17.3	21	−12.3	–

(a) By the phase-partition technique membrane particles are separated according to differences in surface properties [18,20]. This enables the discrimination of the outer and inner thylakoid surfaces in a way that inside-out vesicles partition to the lower dextran-rich phase (B3) while right-sided vesicles and unfragmented thylakoids partition to the upper, poly(ethylene glycol)-rich phase (T2) [1,2,17].

If the inside-out vesicles upon sonication change sidedness, an alteration in their partition behaviour towards that of right-sided thylakoids would be expected. This was investigated simply by performing a fourth partition step of the inside-out vesicles (B3) after sonication in the low-salt medium. As much as 80% of the sonicated vesicles now partition to the upper phase (B4 T) while 20% remain in the lower phase (B4) (Table I). This partition change is obviously caused by the sonication, since the control showed normal partition for B3 vesicles, i.e., 18% in top and 82% in bottom.

The structure of the material partitioning to the

lower and upper phases after sonication can be seen in the electron micrographs of Fig. 1a and b, respectively. The material that still prefers the lower phase (B4) shows the same size and appearance as previously shown for the original everted vesicles [16], while the upper phase material (B4 T) consists of much smaller vesicles. This implies that the material remaining in the lower phase consists of non-disrupted vesicles, while the material actually ruptured by sonication partitions to upper phase, as is the case for thylakoids of normal sidedness. This supports the conclusion that inside-out vesicles ruptured in a low-salt medium change their sidedness. However, to make such an interpretation unambiguous, better resolution is needed than that achieved after a single repartition step. Such an improved resolution can be obtained by using the thin-layer counter-current distribution apparatus [19] which allows a large number of partition steps to be performed automatically on an analytical scale. In that way the partition behaviour of different membrane populations can be accurately compared [20,21]. Fig. 2 shows the countercurrent diagrams obtained after a 30-transfer experiment of inside-out vesicles after increasing time of sonication. Membrane material that prefers the upper phase is seen to the right in the diagram, while material that prefers the lower phase is seen to the left. As expected, the unsonicated inside-out vesicles give a main peak to the left in the diagram with only small amounts of material to the right. 1 min of sonication of the inside-out vesicles produces a small peak to the right, showing the formation of vesicles with a high affinity for the upper phase. Longer sonication that induces more membrane breakage increases the amount of material under the peak to the right at the expense of the peak to the left. After 3 min of sonication nearly all material was found under the right peak. The position of this material in the counter-current distribution train very much resembles that of normal-sided thylakoids [17], which again demonstrates the transformation of inside-out vesicles into right-side-out vesicles upon rupture.

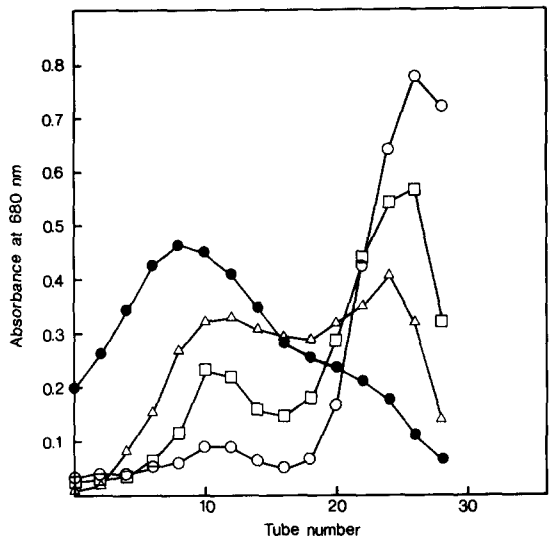

Fig. 2. Counter-current distribution after different times of sonic treatment of inverted thylakoid vesicles in a medium of low ionic strength (5 mM NaCl/10 mM sodium phosphate (pH 7.4)/100 mM sucrose). Duration of sonic treatment: ●, zero (control); △, 1 min; □, 2 min; ○, 3 min.

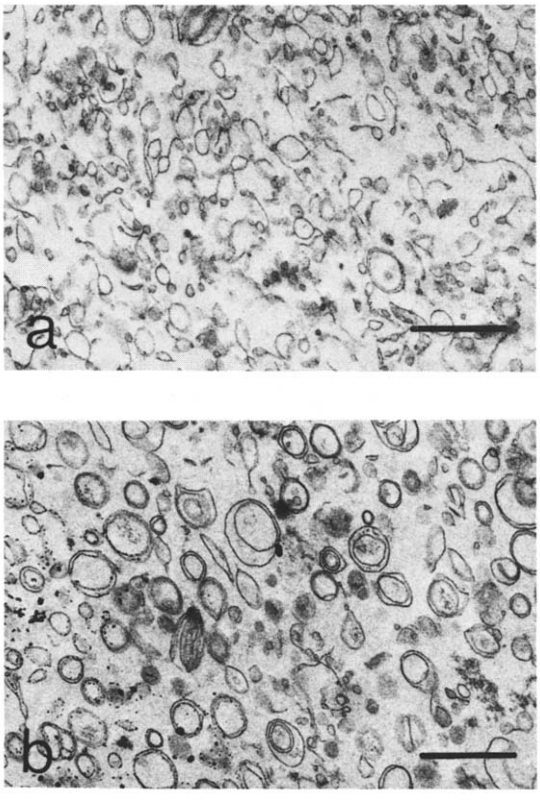

Fig. 1. Electron micrographs after sonic treatment of inverted vesicles in low ionic strength and subsequent repartition. (a) Upper phase; (b) lower phase (bar = 1 μm).

(b) The direction of the vectorial light-induced reversible proton translocation was also studied for the fractions from the one-step repartition experiment (Table I). In thylakoids of normal sidedness, protons are transferred from the outside to the inside during electron transport, leading to a pH rise in the surrounding medium [22]. In analogy, vesicles turned inside-out translocate protons in the opposite direction, leading to an external acidification [1]. Such a 'negative' proton transport was seen for the repartitioned control inside-out vesicles (B4 control) and vesicles remaining in the lower phase after sonication (B4 sonicated) (Table I). In contrast, sonicated vesicles partitioning to the upper phase (B4 T sonicated) show the normal proton uptake. This again provides very strong support that this material consists of right-sided vesicles formed from ruptured everted vesicles.

The numerical value of the pH changes of the B4 T sonicated material (+8.9 nmol H^+/mg chlorophyll) is smaller than that of the B4 control material (−23.2 nmol H^+/mg chlorophyll). As can be seen from oxygen measurements (Table I) this is accompanied with a decline in Photosystem II electron transport rate, necessary for the proton translocation. The reduced proton gradient can therefore be regarded mainly as a sonic damage to the electron transport chain rather than increased leakiness of the membranes or mixed sidedness of the vesicles.

(c) As a third sidedness assay, the aggregation response of the vesicles after addition of $MgCl_2$ up to 5 mM was tested. This can be used as a test, since the outer thylakoid surface is involved in the cation-induced stacking of chloroplasts [23]. Heavy aggregation was seen by light microscopy in the material partitioning into the upper phase following sonication (Fig. 3b), suggesting an exposure of the outer thylakoid partition surface. No aggregation was seen upon addition of magnesium in control inside-out vesicles (Fig. 3a), nor in the sonicated material remaining in the lower phase.

All observations described above independently support the conclusion that inside-out thylakoid vesicles fold back into right-sided orientation if ruptured at low ionic strength, i.e., under destacking conditions.

Formation of inside-out vesicles requires appressed thylakoids (stacking) prior to press disruption. Stacking can be maintained by low concentrations of divalent cations or high concentrations of monovalent cations [24]. In order to see whether inside-out vesicles changed their everted sidedness even if broken under the stacking conditions under which they were originally formed, sonication was performed of B3 vesicles suspended in media containing 5 mM $MgCl_2$ or 150 mM NaCl.

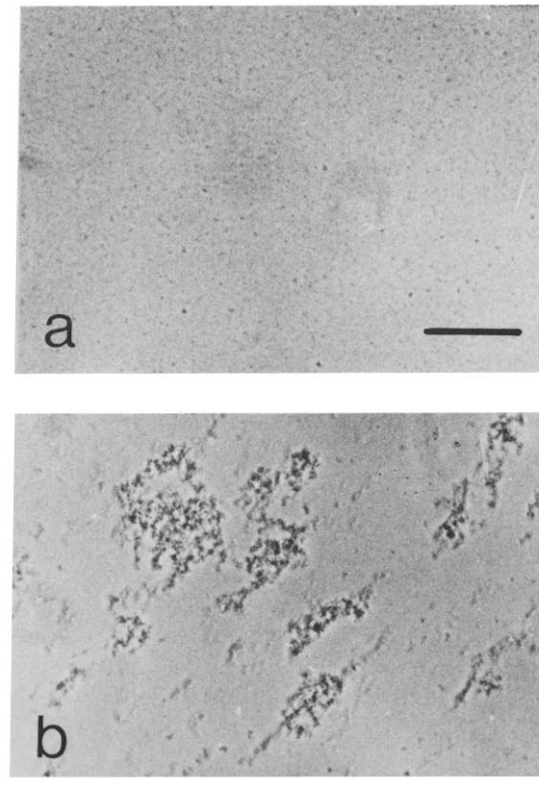

Fig. 3. Aggregation response upon addition of magnesium ions. (a) Inverted vesicles (B_3); (b) material found in upper phase after repartition of low-salt sonic-treated inverted vesicles (B_4T son). Photographs were taken under phase contrast in a Zeiss light microscope (bar = 0.5 mm).

The counter-current distribution diagram of inside-out vesicles sonicated in presence of 5 mM $MgCl_2$ is shown in Fig. 4. In spite of being subjected to 3 min of sonication, only a minor portion of the material changes its partition behaviour, since only a very small peak is found in the right-hand part of the diagram. As shown in Fig. 2,

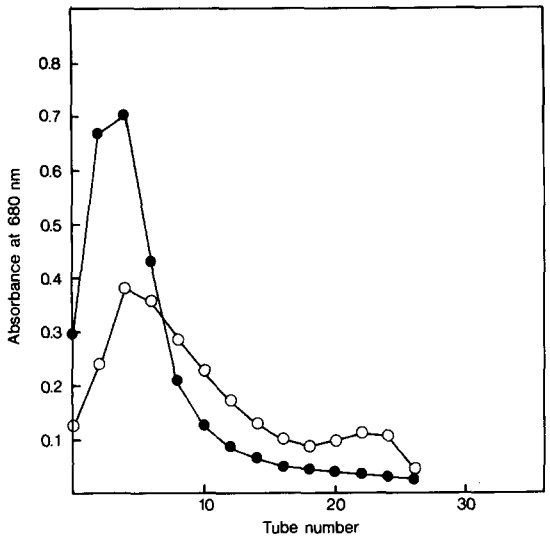

Fig. 4. Counter-current distribution after different times of sonic treatment of inverted thylakoid vesicles in the same medium as in Fig. 2, buth with 5 mM $MgCl_2$ instead of 5 mM NaCl. Duration of sonic treatment: ●, zero (control); ○, 3 min.

the same sonic treatment in the low salt caused all material to change its partition into the peak to the right. This could also be demonstrated by a one-step partition experiment (Table II), where 25% of the sonicated vesicles are found in the upper phase compared to 18% for the unsonicated control material. The material in the lower phase showed a negative pH gradient (Table II), and very weak aggregation in response to the addition of magnesium (not shown). In spite of a high electron-transport rate, the pH gradient is very small, suggesting an increased leakiness of protons of the sonicated vesicles. These results indicate very little folding (approx. 10%) into normal sidedness following sonic rupture in 5 mM $MgCl_2$ as judged from the small change in partition behaviour (Table II, Fig. 4).

Presence of 150 mM NaCl during sonication results in 55% of the material partitioning to the upper phase (Table II), indicating an intermediate

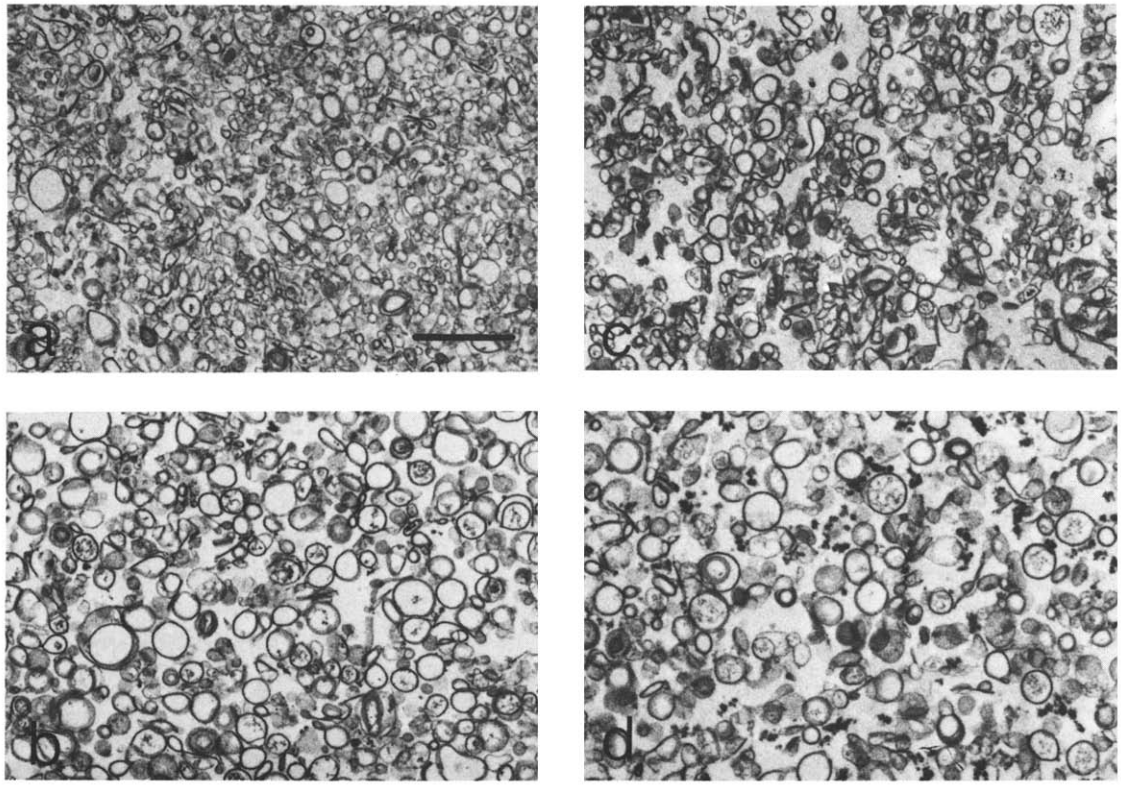

Fig. 5. Electron micrographs after sonic treatment of inverted vesicles in either 5 mM $MgCl_2$ or 150 mM NaCl and subsequent repartition. (a) sonication in $MgCl_2$; upper phase; (b) as a, but lower phase; (c) sonication in NaCl, upper phase; (d) as c, but lower phase (bar = 1 μm).

TABLE II

EFFECT OF SONIC TREATMENT ON INVERTED THYLAKOID VESICLES IN PRESENCE OF 5 mM $MgCl_2$ OR 150 mM NaCl: DISTRIBUTION OF MATERIAL IN AQUEOUS PHASE SYSTEM, PHOTOSYSTEM II ELECTRON TRANSPORT AND PROTON TRANSLOCATION

Sonic treatment was performed in a medium containing either 5 mM $MgCl_2$ or 150 mM NaCl in addition to 10 mM sodium phosphate (pH 7.4) and 100 mM sucrose. Assay conditions as in Table I.

	Sonicated in 5 mM $MgCl_2$			Sonicated in 150 mM NaCl		
	%Chl	ΔH^+	O_2	%Chl	ΔH^+	O_2
B_4T	25	–	–	55	+0.5	3
B_4	75	−4.3	16	45	−0.8	4

proportion of vesicles turning back to right-sided orientation. Only very low proton- and electron-transport activities remained for the vesicles ruptured in 150 mM NaCl (Table II). This is probably due to loss of water-splitting polypeptides from the inner thylakoid surface caused by the high NaCl concentration [25]. Still, a small positive pH change for the upper-phase vesicles and a negative for vesicles remaining in the lower phase give additional evidence for a reorientation of around half the population of everted vesicles. In addition, the upper phase vesicles showed a pronounced aggregation response to added Mg^{2+} compared with the vesicles in the lower phase (not shown).

The electron micrographs (Fig. 5) show that sonication of inside-out vesicles in presence of 5 mM $MgCl_2$ or 150 mM NaCl leads to disruption of vesicles into smaller fragments, which partition to the upper phase, while the unfragmented vesicles remain in the lower phase. In this respect, the results are the same as after the same treatment at low ionic strength (Fig. 1).

Stability of inside-out thylakoid vesicles during storage

The availability of inside-out thylakoid vesicles is of importance both for studies on the transverse membrane asymmetry and reactions taking place at its inner surface [3]. In all such studies the preservation of the reversed sidedness is crucial and the possibility of a sidedness change upon storage due to opening of the inside-out vesicles must be considered. The sidedness of the vesicles was therefore studied after storage in the low-salt media. No artificial stress such as ultrasonic treatment was applied. The vesicles (B3) were kept at 4°C for 27 h and samples were frequently collected and tested for sidedness, using phase partition and proton transport studies. Quite contrary to sonic treatment, storage does not change the low partition of the vesicles. Furthermore, the light-induced proton transport leads to proton extrusion throughout the experiment, i.e., is directed opposite to normal thylakoids. Thus both the phase partition and proton translocation show that the everted vesicles keep their everted sidedness. This was the case also after resuspension in distilled water. The vesicles also keep their sidedness and their photochemical activity after storage for 1 week at liquid nitrogen temperature in the presence of 5% dimethylsulfoxide.

Thus, when stored, the inside-out vesicles are quite stable with respect to sidedness.

Discussion

Studies on the transbilayer organization of biological membranes are favoured by the ability to manipulate the sidedness of membrane vesicles for selective probing of the outer and inner surfaces. Therefore, it is important to know the formation mechanism for inside-out vesicles and their ability to retain the everted sidedness. Usually the direction of folding for a disrupted membrane is explained in terms of expansion or contraction of one leaflet in a bilayer couple [14] in response to variations in the surrounding medium. This may affect the curvature preference and hence favour sealing into normal or everted sidedness. Such a model, dependent on medium-induced curvature changes like endocytosis, is usually applied to explain the formation of everted vesicles from several plasma membranes from different cells of higher organisms, including erythrocytes [5,8,9] and micro-organisms [10]. These curvature changes in response to the surrounding medium are likely to be an adaptation of the membrane to maintain an optimal packing in a bilayer leaflet [26]. Experi-

mentally, this has been demonstrated for erythrocytes, where different proportions of normal or everted vesicles were formed as a function of curvature changes induced by changing the cation composition during incubation [9]. These variations were interpreted as cation influence on the shape and hence the packing of anionic phospholipids. Also such curvature changes can be accompanied by lateral clustering of membrane components [27,28].

The present sonic rupture experiments of inside-out thylakoids strongly support our initial model [2,16] that such curvature changes in response to the cation content are of minor importance compared with the cation-dependent stacking forces in determining the sidedness of fragmented thylakoid membranes. Sonic opening of inside-out thylakoid vesicles results in membrane refolding forming right-side-out vesicles under low-salt conditions, as well as in the presence of high concentrations of monovalent ions or low concentrations of divalent cations (Tables I, II; Figs. 2, 4). However, the proportion of inside-out vesicles changing sidedness is strongly dependent on the medium in which sonic rupture is made. Sonication in a low-salt medium causes more vesicles to refold than in the presence of high concentrations of monovalent ions, which in turn gives more refolding than divalent cations (Table I and II). The stabilizing effect of cations on the everted sidedness is interpreted to be an effect of internal stacking. In an everted thylakoid vesicle the membrane surfaces normally involved in cation-induced grana thylakoid stacking [23] are facing the inner space of the vesicle. The inner space of these vesicles are marked with an asterisk in Fig. 6a. The vesicles have a cup-shaped structure and thin sectioning often gives rise to two concentric membrane profiles, with the space inbetween representing the internal space of the vesicle (cf. Ref. 16, Figs. 5 and 6). As demonstrated in the electron micrograph of Fig. 6a, the thickness of the internal space of the vesicles is around 240 Å in absence of stacking forces. The presence of magnesium induces the attractive stacking forces to keep the membranes so closely appressed that the internal space cannot be resolved except in certain areas (see arrow Fig. 6b). Two closely appressed membranes appear as a single unit. The size of the

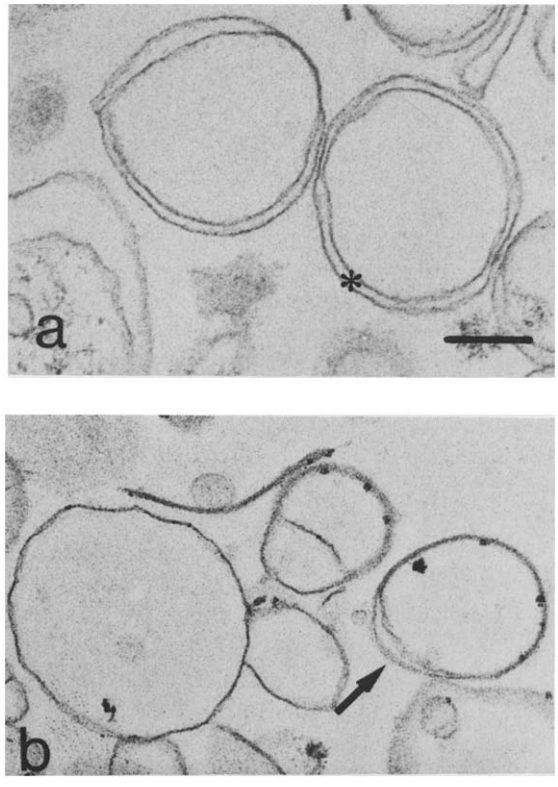

Fig. 6. Electron micrographs of everted vesicles suspended in: (a) low ionic strength. The vesicles are cup-shaped [16] and sections therefore show concentric profiles of two membranes. The asterisk marks the internal space of the vesicle. (b) 5 mM $MgCl_2$. Due to internal stacking the two membranes are tightly appressed and the internal space can be seen only in certain areas (see arrow). (bar = 0.2 μm.)

vesicles that have retained their everted sidedness after sonication is the same as for the original inside-out vesicles (B3), while refolded vesicles are considerably smaller (Fig. 5). These observations suggest that even if breakage across the bilayer has occurred in certain areas due to sonic shearing, vesicle fragmentation is limited because the attractive stacking forces keep the sheared membrane pieces attached to the parent vesicle. It is likely that right-side vesicles are formed only when the sonication in addition to breakage across the paired membranes has succeeded in pulling the appression apart by overcoming the stacking forces, thereby releasing a single open membrane fragment able to choose its sidedness. An essential conclusion is, therefore, that once a single open

membrane fragment is formed from an everted vesicle it reseals to right-sided orientation in the three media tested. Thus, the mechanism of the cation stabilization of the everted sidedness is a result of preventing the formation of such an unpaired open thylakoid membrane fragment rather than influencing the direction of sealing by contraction or expansion effect on the bilayer. In this respect thylakoids differ from other membranes studied so far. The reason for broken thylakoid membranes showing such an absolute preference to seal into right-sided orientation in the absence of appressions is not obvious. The membranes may have an intrinsic tendency to fold into right-side orientation due to the asymmetric organization of its components. Alternatively, there could be weak attractive forces between the lumenal sides of a thylakoid. Such 'lumenal forces' could also be responsible for the flat structure of thylakoids, with two membranes aligned in parallel, even under destacking conditions.

Our present observations that unpaired thylakoids upon disruption exclusively turn right-side-out is not in agreement with the results of Popov et al. [29]. They claim that everted thylakoids may be obtained from non-appressed stroma thylakoids upon digitonin treatment. However, the convex EF and concave PF fracture faces of their particles and the response to added ferredoxin, and ferredoxin-$NADP^+$ reductase do not support this claim.

Refolding of our preparation of everted vesicles exposes the outer surface of the appressed grana thylakoids (PS_s). This is of profound importance for studies on the molecular organization of the thylakoid membrane not only for studies on transverse asymmetry but also for studies on lateral heterogeneity. This is because non-appressed and appressed thylakoid regions [30] have been shown to be structurally and functionally different [31–38]. Therefore this lateral heterogeneity makes it insufficient to locate a certain component to the outer or inner surface only. As has been shown by previous freeze-fracture [16] and formation mechanism studies [2], the inside-out vesicles originate from the appressed regions of the grana thylakoid. Thus, the merit of turning the everted thylakoids back to normal sidedness also lies in exposing the outer surface of the appressed thylakoid region (PS_s). This surface cannot be studied simply in unbroken stacked thylakoids, since it is hidden in the appressions and destacking leads to mixing with components from the non-appressed regions [31].

Acknowledgements

We wish to thank professor Claes Weibull (Department of Microbiology, University of Lund) for providing facilities and advice concerning the electron microscopy. We are also indebted to Mrs. Adine Karlsson and Mrs. Ulla Wulf for preparing samples for electron microscopy. This work was supported by a grant from the Swedish Natural Science Research Council.

References

1 Andersson, B. and Åkerlund, H.-E. (1978) Biochim. Biophys. Acta 462–472
2 Andersson, B., Sundby, C. and Albertsson, P.-Å. (1980) Biochim. Biophys. Acta 599, 391–402
3 Andersson, B., Åkerlund, H.-E. and Albertsson, P.-Å. (1981) in Proceedings of the 5th International Congress of Photosynthesis (Akoyunoglou, G., ed.), vol. 3, pp. 55–66, Balaban International Science Services, Philadelphia, PA
4 Fleischer, S., Meissner, G., Snigel, M, and Wood, R. (1974) Methods Enzymol. 31, 292–299
5 Bennett, V. and Cuatrecasas, P. (1973) Biochim. Biophys. Acta 311, 362–380
6 Grover, A.K., Crankshaw, J., Garfield, R.E. and Daniel, E.E. (1980) Can J. Physiol. Pharmacol. 58, 1202–1211
7 Reinlin, L., Caroni, P. and Carafoli, E. (1981) FEBS Lett. 126, 74–76
8 Steck, T.L. (1974) in Methods in Membrane Biology (Korn, E.O., ed), vol. 2, pp. 245–281, Plenum, New York
9 Lin, G. and Macey, R. (1978) Biochim. Biophys. Acta 512, 270–283
10 Rosen, B.P. and Tsuchiya, T. (1979) Methods Enzymol. 56, 233–241
11 Barnes, E.M. Jr. and Bhattacharyya, P. (1977) J. Supramol. Struct. 6, 333–344
12 DePierre, J.W. and Ernster, L. (1977) Annu. Rev. Biochem. 46, 201–262
13 Haase, W., Schäfer, A., Murer, H. and Kinne, R. (1978) Biochem. J. 172, 57–62
14 Sheetz, M.P. and Singer, S.J. (1974) Proc. Natl. Acad. Sci. U.S.A. 71, 4457–4461
15 Izawa, S. and Good, N.E. (1966) Plant Physiol. 41, 544–552
16 Andersson, B., Simpson, D.J. and Høyer-Hansen, G. (1978) Carlsberg Res. Commun. 43, 77–89
17 Andersson, B., Åkerlund, H.-E. and Albertsson, P.-Å. (1976) Biochim. Biophys. Acta 423, 122–132
18 Albertsson, P.-Å. (1971) Partition of Cell Particles and

Macromolecules, 2nd edn., John Wiley and Sons, New York
19 Albertsson, P.-Å. (1965) Anal. Biochem. 11, 121–125
20 Albertsson, P.-Å., Andersson, B., Larsson, C. and Åkerlund, H.-E. (1982) in Methods in Biochemical Analysis (Glick, D., ed.), 28, pp. 115–150 Wiley Interscience, New York
21 Walter, H. (1977) in Methods of Cell Separation, vol. 1 (Catsimpoolas, N. ed.), pp 307–354, Plenum Publishing Corp., New York
22 Jagendorf, A.T. (1975) in Bioenergetics of Photosynthesis (Govindjee, ed.), pp 413–492, Academic Press, New York
23 Arntzen, C.J. (1978) in Current Topics in Bioenergetics (Sanadi, D.R. and Vernon, L.P., eds.), vol. 8B, pp 111–160, Academic Press, New York
24 Sculley, M.J., Duniec, J.T., Thorne, S.W., Chow, W.S. and Boardman, N.K. (1980) Arch. Biochem. Biophys. 201, 339–346
25 Åkerlund, H.-E. (1981) in Proceedings of 5th International Congres on Photosynthesis (Akoyunoglou, G., ed.), vol. 2, pp. 465–472, Balaban International Science Services, Philadelphia, PA
26 Israelachvilli, J.N., Marčelja, S. and Horn, R.G. (1980) Q. Rev. Biophys. 13, 121–200
27 Lutz, H., Barber, R. and McGuirre, R. (1976) J. Biol. Chem. 251, 3500–3510
28 Shukla, S.D., Berriman, J., Coleman, R., Finean, J.B. and Mitchell, R.H. (1978) FEBS Lett. 90, 289–292
29 Popov, V.I., Kaurov, B.S., Finakov, G.Z., Gulyaev, B.A., Kukarskikh, G.P., Allakhverdov, B.L. and Tageva, S.V. (1980) Photosynthetica 14, 343–354
30 Sane, P.V. (1977) in Encyclopedia of Plant Physiology (Trebst, A. and Avron, M., eds.), vol. 5, pp. 522–542, Springer Verlag, New York
31 Staehelin, L.A. (1976) J. Cell. Biol. 71, 136–158
32 Åkerlund, H.-E., Andersson, B. and Albertsson, P.-Å. (1976) Biochim. Biophys. Acta 449, 525–535
33 Armond, P.A. and Arntzen, C.J. (1977) Plant Physiol. 59, 398–404
34 Jennings, R.C., Garlashi, F.M., Gerola, P.D. and Forti, G. (1979) Biochim. Biophys. Acta 546, 207–219
35 Miller, K.R. (1980) Biochim. Biophys. Acta 592, 143–152
36 Andersson, B. and Anderson, J.M. (1980) Biochim. Biophys. Acta 593, 427–440
37 Henry, L.E.A. and Lindberg-Møller, B. (1981) Carlsberg Res. Commun. 46, 227–242
38 Sane, P.V., Goodchild, D.J. and Park, R.B. (1970) Biochim. Biophys. Acta 216, 162–178