Interaction between the lumenal sides of the thylakoid membrane

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1. INTRODUCTION

The internal lamellae system of the chloroplast is composed of paired membranes, the thylakoids. These can either occur as single pairs, stroma lamellae, or packed into stacks, grana. In each granum one can distinguish appressed regions, where stacking occurs, and non-appressed regions of the end membranes and the margins.

A characteristic feature of the thylakoid is the extensive segregation in the lateral plane of several of its components. Thus, the ATP synthetase and ferredoxin-$NADP^+$ reductase is located on the stroma lamellae and on the non-appressed region of the grana [1–3]. By mechanical fractionation and separation by centrifugation it was shown that photosystem I is located in the stroma lamellae [4–6] while grana stacks have both photosystems I and II. Furthermore, mechanically disrupted grana could be separated by phase partition into vesicles rich in either photosystem I or II [7]. It was therefore concluded that grana photosystems I and II are separated into large domains (diam. 0.5 μm) of the thylakoid [7] and it was suggested in a model by Andersson [8] that the partition region is the site of photosystem II while photosystem I is restricted to the non-appressed regions. This model was strongly supported when it was shown that inside-out thylakoid vesicles [9,10] originate from the partition region [11] and that these were highly enriched in photosystem II as determined by the content of chlorophyll–protein complexes [12] and photochemical activities [13]. Also electron microscopical evidence supports this model [14].

In addition to the lateral segregation of the thylakoid components there is also a transverse asymmetry over the membrane. Oxygen evolution and concomitant production of protons occur at the inner, lumenal side of the membrane [15] while ATP synthesis and NADP reduction occur on the side facing the stroma [2]. Light-harvesting chlorophyll–protein complexes seem to span the membrane [16]. Thus, the asymmetry of the thylakoid membrane and its stacking lead to a spatial differentiation of photosynthesis both across the membrane and in the lateral plane.

The stacking of thylakoids and its function in photosynthesis have been extensively studied [17–20]. It has been shown on isolated chloroplasts that stacks are retained only in the presence of high salt concentrations (100 mM NaCl) or divalent cations (5 mM MgCl). If the salt concentration is reduced or the divalent cation removed the grana disappear due to unstacking of the thylakoid and the pre-segregated components of the thylakoid will randomize. This process is, however, reversible; if salt or divalent cations are added back the grana stacks will reform. From this it has been concluded that under stacking conditions forces act between components at the outer surface of the

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thylakoid. The major light-harvesting complex LHCP has been suggested to be such a stacking component. Very little is known, however, about the forces which stabilize this organization of the thylakoid.

Another characteristic feature of the thylakoids is their flat structure not only in the grana region and the stroma region but also in destacked chloroplasts. We suggested that this might be due to attractive forces between membrane pairs on the inner side facing the lumen [21]. Such lumenal attractive forces could be important in holding a pair of membranes in the thylakoid together in a parallel arrangement. They could also stabilize the structure of the inner surface of the thylakoid and the lateral segregation of its components and thereby allow effective thylakoid function and its regulation. Here, the interaction between the inner surfaces of the thylakoid has been studied by using inside-out thylakoid vesicles [9,10] which expose the lumenal side of the thylakoid. By monitoring the aggregation of such vesicles in different media the interaction betweeen the lumenal sides is demonstrated.

2. EXPERIMENTAL

Thylakoids were subfractionated into inside-out (B3), right-side-out (T2), and stroma vesicles (Y-100) using either NaCl [7,10] or $MgCl_2$ [22] as stacking agent. Also, vesicles (B4T) obtained by turning inside-out vesicles back to normal sidedness were prepared [21].

Aggregation of vesicles was studied by three independent methods: absorbance at 550 nm, centrifugation, and phase-contrast microscopy. For absorbance measurements buffer and salts were added to a vesicle suspension (700–900 μg Chl/ml) and after 5 min the absorbance at 550 nm was measured with a spectrophotometer. The same suspension was then centrifuged at $\sim 800 \times g$ for 10 min and the absorbance in the supernate was determined. The absorbance-percentage in the pellet was then calculated. The different samples were also inspected by phase-contrast microscopy.

3. RESULTS AND DISCUSSION

Fig. 1a shows absorbance at 550 nm of a suspension of inside-out vesicles (B3) at different pH and

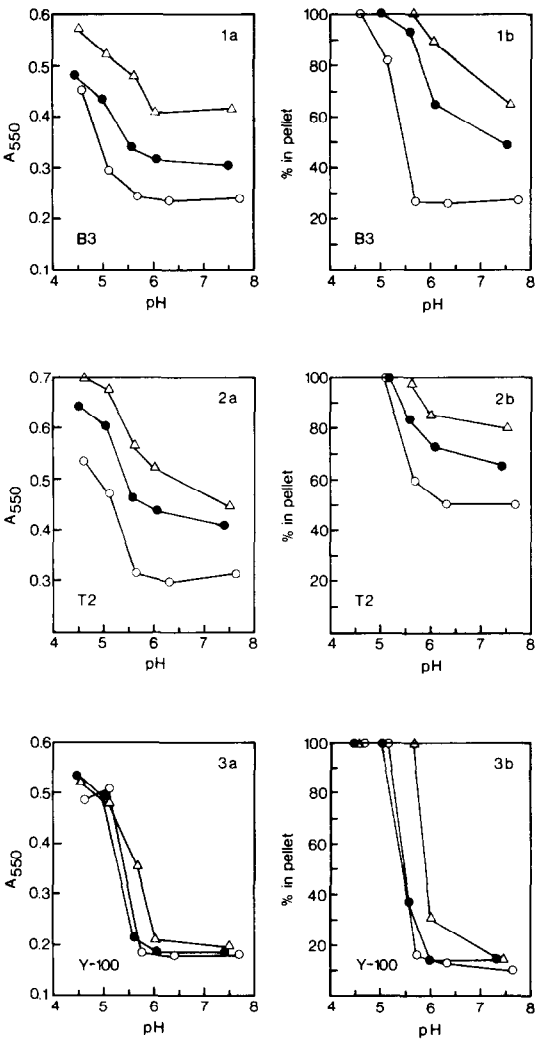

Fig. 1–3. Aggregation of different vesicles obtained by subfractionation of spinach thylakoids: (1) inside-out vesicles (B3); (2) right-side-out vesicles (T2); (3) stroma vesicles (Y-100); (a) absorbance at 550 nm at different pH and in different media, (○) buffer 10 mM (Na-phosphate, pH 6 and above; Na-acetate below pH 6), (●) buffer + 100 mM NaCl, (△) buffer + 5 mM $MgCl_2$. (b) Percentage of absorbance sedimented at $\sim 800 \times g$ for 10 min of the same samples as in (a).

in different ionic media. In buffer alone the absorbance is constant over pH 5.7–7.7. At lower pH there is an increase in absorbance with a steep increase between pH 5 and 4. In the presence of 100 mM NaCl or 5 mM $MgCl_2$ similarly shaped curves are obtained for buffer alone but at a given

pH the absorbance in NaCl is higher than for buffer alone, and $MgCl_2$ gives even higher absorbances. Absorbance of these vesicles at 550 mM is mainly due to light scattering and increase in absorbance at this wavelength is interpreted as an increase in aggregation. This was supported by centrifugation experiments on the same suspension. In fig. 1b the percentage of the absorbance which sedimented is plotted against pH. In buffer between pH 5.7 and 7.7 a constant amount (25%) of vesicles is pelleted; at pH 5 ~80%, and at pH 4.5, 100% of the material is pelleted demonstrating heavy aggregation at low pH. At neutral pH, NaCl and $MgCl_2$ promoted sedimentation indicating aggregation in these media (fig. 1b).

That the absorbance-increase was a result of aggregation was further supported by inspection with the phase contrast microscope. In buffer alone the inside-out vesicles were well suspended with the majority (>90%) of the vesicles in Brownian motion as separate single entities. In 100 mM NaCl small aggregates of different sizes were dominated. Each aggregate contained 5–10 vesicles. In 5 mM $MgCl_2$ much larger aggregates dominated. At the low pH the aggregation could be detected by the naked eye.

Similar experiments on suspension of right-side-out vesicles, originating partly from the grana (T2) and stroma vesicles (Y-100) are shown in fig. 2 and 3. The right-side-out grana vesicles show an aggregation rather similar to the inside-out vesicles. The vesicles exposing the right-side-out surface of the partition region (B4T) aggregated strongly in the presence of NaCl or $MgCl_2$ at neutral pH (not shown). The stroma vesicles differ in their aggregation behaviour in that between pH 6 and 7.5 there is no absorbance-increase upon addition of NaCl or $MgCl_2$ (fig. 3a) and no, or only a small, increase in the sediment after centrifugation (fig. 3b). Upon lowering the pH below 6 there is, however, a considerable aggregation of the stromal membranes. Intact thylakoids (i.e., class II chloroplasts) showed lack of aggregation at neutral pH upon addition of NaCl or $MgCl_2$ under the microscope, but they were aggregated at low pH.

In conclusion, all vesicles studied show aggregation upon lowering the pH below 5.5. At neutral pH aggregation is induced by NaCl (100 mM) or $MgCl_2$ (5 mM) for the vesicles originating from grana, both inside-out (B3) and right-side-out (T2 and B4T), but not for the vesicles originating from the stroma region (Y-100), nor for the intact thylakoids. It is of interest that stroma vesicles and intact thylakoids appear to have similar surface properties as judged from their counter-current distribution behaviour [23] and their isoelectric point [24].

The isoelectric point of the inside-out vesicle is 4.1; for the right-side-out vesicles (T2) it is 4.5 and for the stroma vesicles and intact thylakoids 4.7 [24]. Thus, all membrane vesicles carry negative charges at neutral pH. Whether aggregation occurs or not is governed by the balance between two opposing forces [20,25] acting between the vesicle surfaces:

(i) Attractive forces which can be van der Waal's forces, hydrophobic interaction, or electrostatic forces between negative and positive charges;
(ii) Repulsive forces which are mainly caused by negative charges on the surface.

In buffer, at neutral pH, the repulsive forces dominate and the vesicles are kept well apart. The repulsive forces can be reduced by removing the negative charges by acidification. Then the attractive forces dominate and aggregation occurs, as is the case for all membrane types in this study. Alternatively, the repulsive forces can be reduced by screening the negative charges by addition of either high concentration of NaCl or low concentration of $MgCl_2$ and the attractive forces will again dominate. This occurs only in the case of inside-out vesicles and right-side-out vesicles from the grana but not for the right-side-out stroma membranes. From this we can conclude that in addition to the attractive forces between two adjacent right-side-out surfaces of grana thylakoids, the so-called stacking forces, there are attractive forces between two adjacent inner surfaces of the thylakoids, the so-called lumenal attractive forces, which dominate over repulsive forces during conditions which are comparable to those in vivo. Thus, the Mg-concentration in vivo of the lumenal space is probably high enough [19] to promote attraction between the inside surfaces of the thylakoid. Upon illumination, protons are released on the inside of the thylakoid to such an extent that a large fraction of the negative charges are removed (pH is assumed to drop by about 3 units) just as in the low pH experiments of fig. 1a.

Thus, in vivo, both in the dark and in the light the lumenal attractive forces will keep the thylakoid internally stacked. The internal stacking might be tighter in the light since the acidification of the lumen removes the repulsive forces more effectively than is the case when screening by $MgCl_2$ in the dark. This interpretation agrees with electron microscopical observations [26] on the thylakoids and mobility studies on spin labels [27]. Thylakoids are essentially flat structures with a pair of membranes parallel to each other. Upon illumination the distance between opposing membranes over the lumenal space is reduced [26]. This shrinkage has been attributed to osmotic changes of the lumen and conformational changes of the membrane. The results of this study suggest, however, that the attractive forces and the reduction of the repulsive forces, acting between opposing lumenal sides of the thylakoid, are responsible for the reduced lumenal space.

Studies on the mobility of the spin label tempamine [27] while it resides in the lumen of spinach thylakoid have shown that upon illumination the mobility of tempamine is reduced. This was thought to be the result of reduced lumen volume and closer approach of the membranes. There are therefore several independent studies which indicate that the inner sides of two opposing membranes in a thylakoid are more tightly bound to each other in light. The approach between the inner faces is difficult to judge from electron microscopy. Conventional electron microscopy using thin sections shows an 'empty' space inside the thylakoid. However, by using mild embedding and fixation techniques [28] it has been shown that the lumen space contains electron-dense material. This may represent protruding proteins on the two lumenal sides which are in contact with each other.

The lumenal forces could have great significance for the structure and function of the thylakoid. They might hold various components of the inner side of the thylakoid in the correct position for their function in electron transport. For example, the lumenal forces could stabilize the lateral enrichment of photosystem II extrinsic proteins [22] in the grana region. Proton-induced appression of opposing inner sides of a thylakoid might also facilitate effective and controlled proton movement from the site of proton formation in the grana region to proton consumption in the ATP-synthesizing complexes of the stroma region. The lumenal forces together with the external stacking forces could also be important for light harvesting by organizing the light-harvesting chlorophyll–protein complexes in a three-dimensional lattice to maximize light utilization under low-light intensity conditions.

To study these problems one has to identify the components of the thylakoid inner surface which are responsible for the lumenal forces. Preliminary experiments indicate that trypsin reduces the aggregation at neutral pH which would mean that proteins are involved.

Inside-out thylakoids offer a unique possibility for studying the properties of the inner side of the thylakoid. Since they originate from the partition region the conclusions drawn here hold for the lumenal sides of the partition region only. It would therefore be of interest to be able to prepare inside-out stroma thylakoids too. At present such a preparation is not available however.

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