GENERATION OF LARGE AND ACTIVE MEMBRANE LAYERS FROM THYLAKOID VESICLES

Properties and possible applications

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1. Introduction

Biological membranes are interfaces which separate an inner from an outer space. Analysis of the vectorial molecular mechanisms at work in such 'in/out' systems are difficult because those reactions taking place at the inside of the membrane, or in the inner space, are not easily accessible. It would facilitate examinations, if these membranes could be transformed into planar interfaces of 'left/right' systems whose compartments are directly accessible. Such types of systems have been realized in black lipid membranes [1], bi-layers formed from association of two monolayers [2], and bi-layers formed from artificial vesicles or vesicular fragments of biological membranes [3].

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Layer formation from vesicles at air/water interfaces was first demonstrated in [5,6]. In all cases the layers were 'fixed' on one circular aperture sized <1 mm^2. The material of such small area is suitable for electric but not optical and chemical measurements. For the latter $\gtrsim 10^5$-times larger planes are necessary. This article will deal with the transformation of the membranes of thylakoid vesicles into such large, flat areas of 256 cm^2 (fig.1A) as (a) 'fluid thylakoid layers', and (b) 'rigid thylakoid layers'. It is shown that the layers have the same full photosynthetic activity as the corresponding amount of thylakoid vesicles in solutions. On top of this, it is shown that the lifetime of the activity of the rigid layers is 8-times longer than that of thylakoids in solutions. The possible advantage of optical and chemical analyses of large, planar systems is discussed with respect to the examination of the molecular mechanisms within membranes.

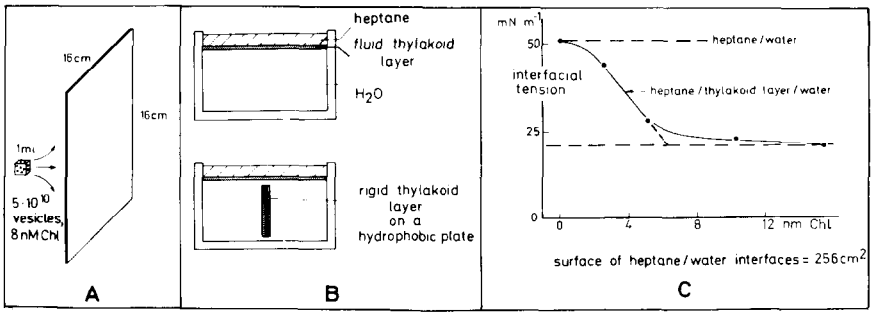

Fig.1. Schematic drawing. (A) Transformation of the membranes of $\sim 5 \times 10^{10}$ thylakoid vesicles and of 8×10^{-9} M chl, into flat membranes of ~ 250 cm^2. (B) Generation of a 'fluid' and a 'rigid' layer. (C) Interfacial tension in dependence of the amount of thylakoid membrane material and amount of chlorophyll, injected at a heptane/water interface of 256 cm^2.

2. Materials and methods

Chloroplast solutions of spinach were injected with a thin glass capillary into a heptane/water interface (area 250 cm^2) (fig.1B). Thereby, the chloroplast thylakoids are spread at the interface. This spreading can be monitored through measuring of the interfacial tension by Wilhelmy plate method (fig.1C). The tension between heptane/water decreases from 50–20 mN/m as the thylakoids are spreading. The decrease depends on the membrane amount being spread. The beginning of the lower limit suggests that the interface is saturated with membrane material (8 nM chl), either in form of closely packed thylakoid sacs or in form of opened plane membranes. Further material added (>8 nM chl) attaches to the interface or drops into the aqueous subphase. The minimum amount of membrane material or chlorophyll (chl) necessary in order to reach the lower limit of tension was 8 nM chl. Assuming within the thylakoid membrane an average area of 220–360 $Å^2$ for 1 chl molecule [4], the material could cover 220–360 cm^2 if it is spread as a flat plane monolayer. If a layer of closed thylakoid sacs is located at the interface, an area of only ~55–90 cm^2 could be covered. The experimentally given area of the heptane/water surface was 250 cm^2. A comparison of the data suggests that at ≤8 nM chl/250 cm^2 plane membranes, probably predominantly monolayers, are formed at the interface. This conclusion corresponds to results of spreading of artificial liposomes at an air/water interface [5,6]. In the following such layers are called 'fluid thylakoids layers'. These layers were used in our laboratory for electric measurements of the time course of charge separation within the layer [7]. So-called 'rigid thylakoid layers' are made by immersing hydrophobic glass plates (250 cm^2 divided into 10 plates of 25 cm^2 each) into the heptane phase all the way through the fluid layer to the aqueous phase (see fig.1B) which causes the 'fluid layer' to gather on the hydrophobic plate. During this process a constant interfacial tension has been maintained through continually adding material from the thylakoid reservoir of the syringe. Hydrophobic glass plates are made by incubating cleaned glass plates in a solution of 3% dimethyl dichlorosilane in chloroform for ~12 h. These hydrophobic glass plates are then rinsed first in chloroform, then in methanol. Before making the 'rigid thylakoid layer', the hydrophobic plates are stored for 1 h in distilled water.

A comparison was made of the photosynthetic activities of: (1) thylakoids in solutions; (2) 'fluid thylakoid layers'; (3) 'rigid thylakoid layers'; with amounts of membrane material and chlorophyll, of equal size (8 nM chl). Photosynthesis was brought about through flash excitation and continuous light. Activities of the following partial reactions were measured as a function of time:

(i) Light reaction of chlorophyll a_I (chl a_I) (P700) in photosystem I (PS I);
(ii) Transmembrane electric potential difference;
(iii) Electron transport determined by ferricyanide (FeCy) reduction;
(iv) Proton production; and
(v) Non-prompt fluorescence changes.

Measurements of these reactions by means of optical changes of the reactants, electrochromic absorption changes of intrinsic indicators, and fluorescence changes of pH-indicating probes with the repetitive pulse method [8] are described in [9] (see also legend of table 1).

Table 1

Activities (8 nM chl)	a	b	c	d	e
Thylakoids in solution (control)	1	1	1	1	1
'Fluid layer' (on 250 cm^2 heptane/water)	0	0	0	0	0
'Rigid layer' (on 256 cm^2 glass plate)	1	1	1	0.9	0.9

[a] Chl a_I oxidation (absorption change at 700 nm); [b] Electric field generation (electrochromic change at 515 nm as well as at 475 nm) (40 mM benzylviologen, 4 mM $MgCl_2$, 25 mM tricine, pH 8); [c] FeCy ($K_3[Fe(CN)_6]$) reduction (absorption change at 420 nm) (10 μM FeCy, 4 mM KCl, 1 mM NH_4Cl, 2 mM tricine, pH 8); [d] H^+ production (absorption change of cresol red at 574 nm) (20 μM FeCy, 4 mM $MgCl_2$, 1 μM cresol red); [e] Delayed fluorescence change at 684 nm; [a,b] Excited in flash light (10^{-5} s, average of 2000 repetitions); [c–e] Excited in continuous light (6 mW/cm^2, 25–100 s)

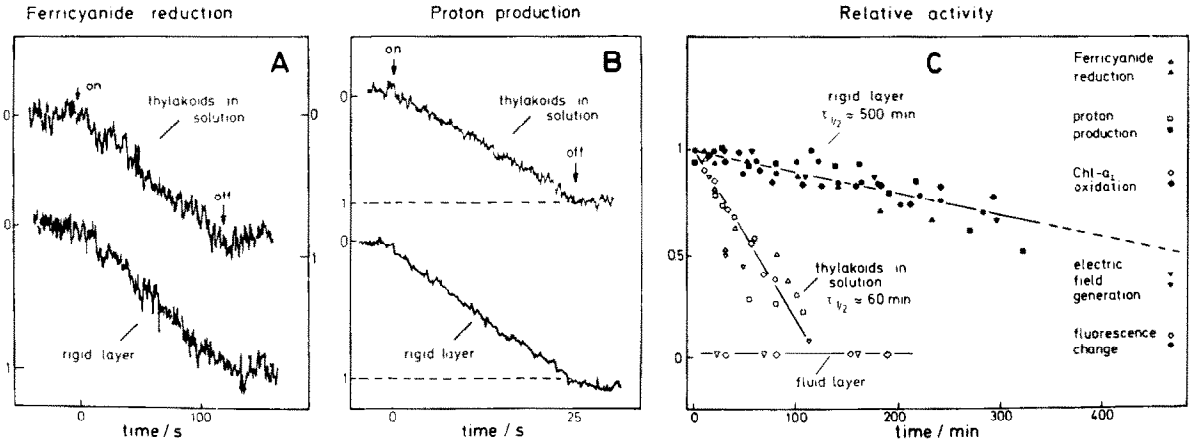

Fig.2. Activities of thylakoids in solution (8 nM chl) and rigid thylakoid layers (8 nM chl): details in table 1; (A) FeCy reduction measured with uncoupler at 420 nm (100 e^-/mM . chl . s); (B) H^+ production measured without uncoupler by cresol red at 574 nm (50 e^-/mM . chl . s); (C) lifetime of 5 different activities.

3. Results

3.1. *Rigid layers*

The activities mentioned in (i–v) and measured on the rigid layer are practically quantitatively the same as those of thylakoids in solutions (table 1, fig.2). Two examples are depicted in fig.2A,B. Moreover, it results that the activities of the rigid layers are maintained ~8-times longer (500 min in the dark) than those of thylakoids in solutions (60 min in the dark) (fig.2C). This may be due to an increased immobilization of the enzymes at the solid plate.

3.2. *Fluid layers*

Fig.3 shows that, after light excitation, the fluid layer is inactive in contrast to the rigid layer (fig.2C). This might be explained by the fact that complexes of the electron transport chain are separated from each other in the fluid layer. This is supported by the following 3 facts:

(1) After the transformation of the fluid layer into the rigid form, all activities appear in full (fig.2C, table 1). Obviously, the separated complexes are reintegrated when the fluid layer is attached to a hydrophobic plate (fig.2, table 1).

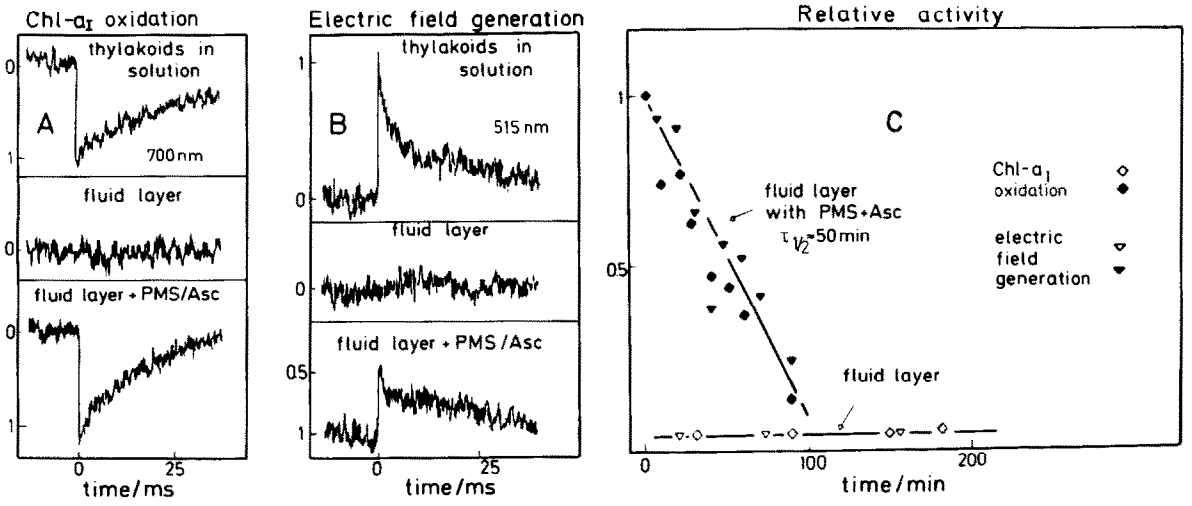

Fig.3. Activities of thylakoids in solution (8 nM chl) and fluid thylakoid layer (8 nM chl) with and without PMS 100 μM ascorbate 1.5 mM; details in table 1; (A) reaction of chl a_I (P700); (B) electric field generation; (C) life-time of the activities.

(2) After artificially separating the donor photosystem PS II from PS I on thylakoids in solutions, PS I can be reactivated if the isolated PS II is provided with artificial donors such as *N*-methylphenazonium-methodisulfate (PMS). Upon addition of PMS, the activities of chl a_I (700 nm) in PS I as well as the electrochromic absorption changes of the carotenoids at 515 nm, which indicate a transmembrane electric field, appear in the fluid layer (fig.3A,B). Since the electric field is generated in equal parts by PS I and II [8], it is expected that the PMS-regenerated photosystem I activity generates 50% of the total field and electrochromic changes, respectively. This has been observed in fig.3B.

(3) The electrochromic absorption changes are due to shifts of the absorption of all antennae pigments by the transmembrane electric field [9]. Since chl *b* is located mainly in the region of PS II, the change of chl *b* which is characteristical at 475 nm [10,11] can be observed only if the field generated by chl a_I has been delocalized along the integrated membrane. However, in contrast to the 515 nm change, we observed no 475 nm change at the reactivated fluid layer. This may support the conclusion that at least PS I is separated from PS II. The lifetime of the regenerated PS I activity of the fluid layer, $\tau_{1/2}$ ~50 min, is comparable to that of thylakoids in solutions (fig.3C).

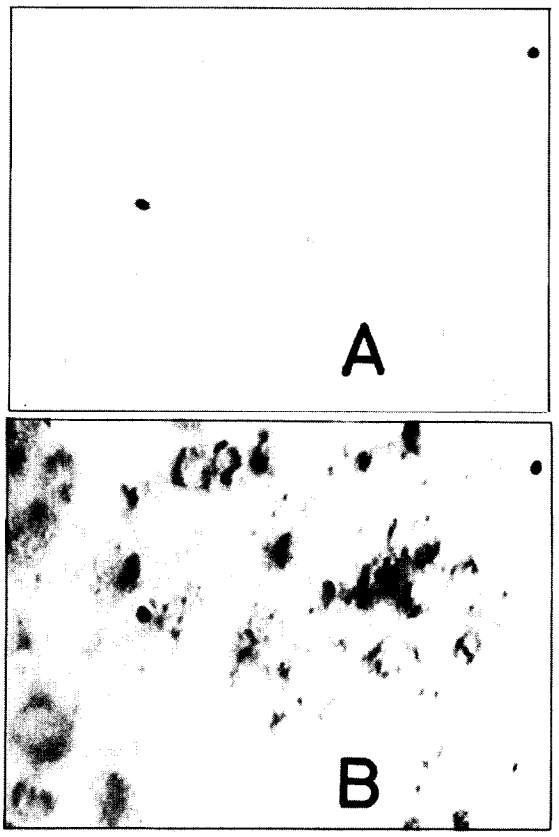

Fig.4. (A) Microscopic view of the fluid layer (enlarged 450×) taken after injection of thylakoids of 8 nM chl into a 250 cm² heptane/water interface; (B) after injection of 16 nM chl.

3.3. *State of the membrane layers*

The bi-layered membranes of the thylakoid sacs are obviously transformed predominantly into large, flat monolayers within the interface. This interpretation is suggested by the following 4 points:

(1) During the spreading, the interfacial tension between heptane/water decreases with increasing amounts of material and becomes constant at a lower level, at ~8 nM chl.

(2) Suggesting that this amount of material is spread predominantly in form of a planar monolayer, its area (220–360 cm²) is comparable to the available area of the heptane/water interface (250 cm²) (see fig.1 and section 2).

(3) A microscopic view of the fluid layer is shown, enlarged 450 times, under two different conditions. Fig.4A was taken when the amount of membrane injected reached the lower interfacial tension (8 nM chl). Fig.4B was taken when about double the amount of membrane was injected in the interface (16 nM chl). Under (fig.4A) no particle structure is noticeable, while under (fig.4B) the typical blebs of thylakoid membranes [12] ~10 μm diam. are visible. Obviously, first the material is transformed into large, flat membrane layers until the area of the interface is saturated at 8 nM chl/250 cm² (fig.4A). Further amounts of membrane material cannot be accepted into the interface, and this surplus remains in the original state partly absorbed at the membrane layer present (fig.4B). The same two pictures – unstructured and structure – have been obtained after the two kinds of arrangements (fig.4A,B) were translocated onto hydrophobic glass plates in order to form a rigid state (not shown).

(4) The fluid layer is, without PMS, inactive (see fig.3). A consequence of the result in fig.4 is that changing the condition of fig.4A (≤8 nM chl) to 4B (16 nM chl) the activity of the surplus material should appear even without PMS. Indeed, signals (i–v) are observable and proportional to the material injected at >8 nM chl/256 cm^2. Information from electron microscopy on the microstructure of the layer ≤8 nM chl/256 cm^2 is being gathered.

4. Discussion

The transformation of microscopic closed vesicles:

(i) Into opened, large macroscopic planar systems; and
(ii) With separated complexes in the fluid state; and
(iii) With full preservation of membrane activity in the rigid state; and
(iv) With drastic extension of lifetime can be of importance for the examination of open questions concerning the molecular mechanisms within membranes.

The layers could be used as follows:

1. Besides electric measurements the largeness of the layers allows optical and chemical measurements on planar membrane systems in contrast to the 25 000-times smaller area fixed on a circular aperture (see section 1).
2. The open state of the layers allows measurements and manipulations at enzymes located under normal conditions at the inner side of the closed system; for instance, the chl a_{II}-complex and the water-splitting enzyme complex located at the thylakoid inside are probably located in the fluid as well as in the rigid layer, at least in parts, at the open side towards the aqueous phase, a fact which improves the possibilities for direct measurements of the O_2 evolution and H^+ release, and the exchange of the complexes against artificial systems as organic dyes and metal oxides, respectively.
3. The separated complexes in the fluid layers (perhaps even separated protein complexes) could possibly be isolated in electrophorese set up in the plane of the fluid layer or vertically to it.
4. Injection of thylakoid material together with artificial lipids could result in a further separation. This may lead to an increased possibility for isolation.
5. Attacking the fluid layer with detergents may result in a better isolation of enzyme complexes than is normally the case when detergents are applied on closed vesicles for isolation.
6. Supported by the 8-times increased lifetime of the activities in the rigid layer, isolation of the immobilized enzymes through use of such detergents could be attempted which otherwise would deactivate the short-lived enzymes in thylakoid vesicles.
7. Spreading the membranes in form of rigid layers on hydrophobic metal plates, direct flash-induced potential measurements at the layer should be possible.
8. The qualities of the planar systems set up from material of thylakoids can be gained perhaps, too, if material is taken from membranes of bacteria, nerves and mitochondria.

Acknowledgement

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