ELECTRIC EVIDENCE FOR THE ISOLATION OF INSIDE-OUT VESICLES FROM SPINACH CHLOROPLASTS

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1. Introduction

Subchloroplast particles obtained by Yeda-press treatment have been shown [1] to contain different types of vesicles which can be separated in an aqueous two phase system on the basis of differences in surface properties. The vesicles partitioned to the bottom phase (B-vesicles) were enriched in photosystem II and showed a reversible proton extrusion when illuminated [1,2]. Vesicles from the top phase (T-vesicles) were slightly enriched in photosystem I and showed in continuous light a reversible proton uptake like normal chloroplasts [1,2]. On the basis of these results the B-vesicles were proposed to contain mainly 'inside-out' thylakoids although other explanations have been discussed [2].

If these B-vesicles are indeed 'inside-out' thylakoids it is expected that the primary process of charge separation occurs in the opposite direction as in normal chloroplasts, i.e., electrons should be transported from the outside to the inside.

The polarity of the light-induced charge separation process in membrane vesicles can be determined directly with macroscopic electrodes by measuring the polarity of the dipole field generated by a non-saturating flash in a suspension of vesicles [3–6].

It is shown here that the light-induced vectorial electron transport in B-vesicles is directed from the outside to the inside of the vesicle, i.e., in the opposite direction as compared to normal class II chloroplasts and T-vesicles. Therefore, B-vesicles are considered to consist of 'inside-out' thylakoids.

Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; MES, 2-(*N*-morpholino)ethanesulfonic acid; tricine, *N*-tris(hydroxymethyl)methylglycine

2. Materials and methods

Washed class II chloroplasts were prepared from spinach as in [1,2,7]. The chloroplasts were pressed twice through a Yeda press then fractionated by phase partition as in [1,2]. The phase partition was repeated 3 times. After the third partition the material from the top phase (T-vesicles) and the bottom phase (B-vesicles) were collected by centrifugation (100 000 g for 1 h) and resuspended in 40 mM KCl containing 5% dimethylsulfoxide. The chloroplasts as well as the T- and the B-vesicles were frozen and stored under liquid nitrogen until use.

The principle for the determination of the polarity of membrane vesicles is illustrated in fig.1 and described below (see also [3–6]). The membrane vesicles were suspended in the reaction medium then 1 ml carefully filtered onto a membrane filter (Sartorius SM 11307, pore size 0.2 μm) so that no air was sucked through the filter. The filter was rinsed with reaction medium to remove excess chloroplasts then mounted in a reaction cell so that it separated the two compartments of the plastic cell (fig.1C). Both compartments contain always the same reaction medium. The cell can be illuminated through a glass window and the potential in both compartments was monitored by two platinum ring electrodes. The electrodes were shielded from direct illumination.

Excitation of photosynthesis was carried out by short flashes (30 W, 16 kV, Verre et Quartz, duration 1 μs, wavelength >610 nm). The electric signals were amplified (amplifier: Tektronix type 1 A 7A) and fed into a transient recorder (Biomation 8100, time resolution 10 ns/point). The electrical bandwidth of the measuring apparatus was set to 100 Hz–1 MHz. To increase the signal-to-noise ratio 100 measurements were accumulated in an averager (NIC 80). The repetition frequency was always 1 Hz. In order to correct for the electric artifacts which result from the discharge of the flash lamp a second set of the same number of signals was accumulated but with the reaction cell optically shielded from the excitation flashes. The resulting signals reflecting only the electric artifact were subtracted from the first set of signals.

The reaction medium for the electric measurements contained 1 mM KCl, 0.1 mM benzylviologen, 0.1 mM MES (pH 6) and vesicles giving 0.2 mM chlorophyll. In experiments with DCMU it was 0.1 mM. The membrane vesicles were always incubated for at least 1 min in the reaction medium before filtration.

The electrochromic ΔA_{515} was measured with a repetitive pulse spectrophotometer as in [8]. The vesicles were suspended in 5 ml reaction medium (20 mM MES (pH 6.5), 40 mM KCl, 0.1 mM benzylviologen) giving 5 μM chlorophyll. Optical pathlength was 2 cm. Photosynthesis was excited with saturating single turnover flashes (duration 20 μs, wavelength >610 nm).

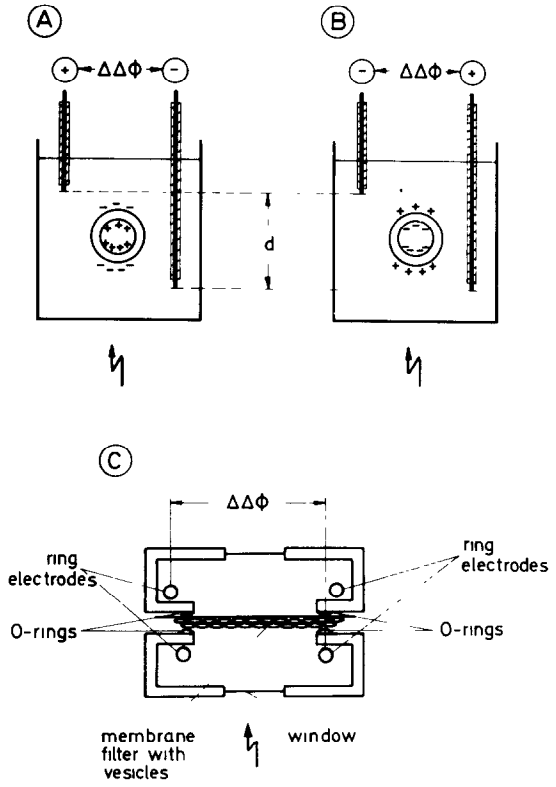

Fig.1. Principle of the electrostatic induction method: (A) Negative charges generated at the outside of the vesicle, (B) Positive charges generated at the outside of the vesicle; (C) Experimental realisation using a cell with a membrane filter. Details see section 2 and text.

3. Results

The determination of the polarity of the membrane vesicles was based on the method developed in [3–6]. Figure 1A,B schematically show the principle of this method. Chloroplasts were suspended between two electrodes which have the distance d. For clarity only one membrane vesicle is shown. If a non-saturating flash is fired from the bottom of the cuvette the lower membrane of the vesicle absorbs more light than the upper one.

The related transmembrane electric potential which is proportional to the number of charge separations is larger across the lower membrane $\Delta\phi_l$ as compared to the upper membrane $\Delta\phi_u$. Summation over all vesicles in the cuvette results in a macroscopically measurable electric potential difference $\Delta\Delta\phi$ which is proportional to the difference of the transmembrane potentials $\Delta\phi_l - \Delta\phi_u$ [10].

Depending on the direction of the charge separation at the vesicle membrane either more negative (fig.1A) or more positive (fig.1B) charges are generated at the outside of the lower membrane than at the outside of the upper one. This results in either a negative or a positive amplitude of $\Delta\Delta\phi$. Therefore, a negative value of $\Delta\Delta\phi$ indicates that negative charges are generated at the outside, a positive value of $\Delta\Delta\phi$ indicates that positive charges are generated at the outside.

The same principle can be realized with the membrane filter technique (see section 2). In fig.2 the

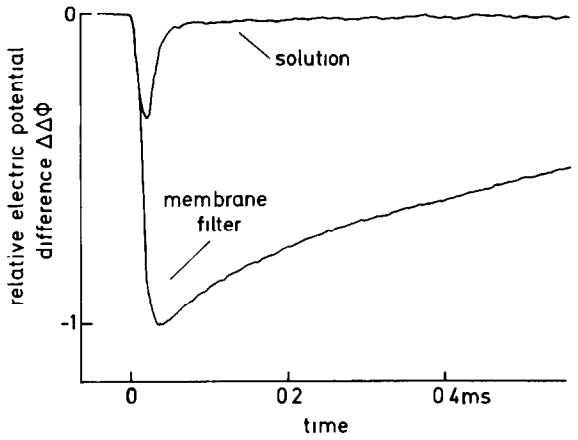

Fig.2. Electric potential difference $\Delta\Delta\phi$ measured by macroscopic electrodes from class-II chloroplasts on a membrane filter and from the same amount of chloroplasts suspended in solution. Reaction medium: 0.5 mM tricine (pH 7.5), 10 mM KCl, 0.1 mM benzylviologen. Flash duration was 20 μs. Further details see section 2 and text.

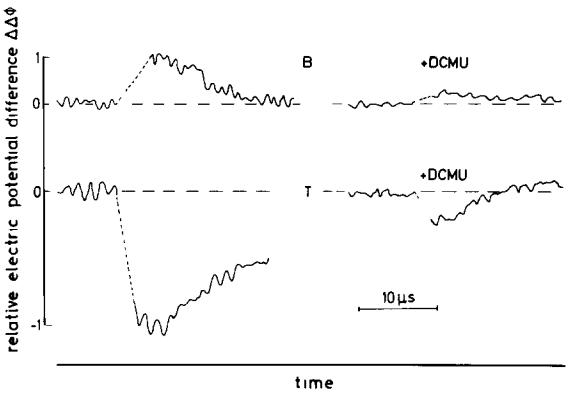

Fig.3. Electric potential difference, $\Delta\Delta\phi$, measured by macroscopic electrodes. Dashed lines indicate the time region of the flash. Top: B-vesicles. Bottom: T-vesicles. Details see section 2 and text.

electric signals are compared from class-II chloroplasts in solution and from class-II chloroplasts filtered onto a membrane filter. The amplitude of the signal obtained from chloroplasts on the filter is increased (in this experiment about a factor 3) and the relaxation time is also increased (in this experiment about a factor 30). Generally, this cell improves the sensitivity and slows down the relaxation of the charge asymmetry (see section 4). In view of these advantages this cell was used for the following experiments.

For the T-vesicles a flash-induced negative potential, $\Delta\Delta\phi$, is found (fig.3 bottom) i.e., negative charges are generated at the outside of the vesicle. This is the same polarity as is found for class-II chloroplasts (fig.2) [4,5]. The B-vesicles showed under identical experimental conditions a positive signal amplitude, $\Delta\Delta\phi$ (fig.3 top) i.e., at the membrane of B-vesicles positive charges are generated at the outside. Addition of DCMU reduces the observed signals about 70–80% for both T- and B-vesicles. This shows that the signals are due to the photochemical activity of the electron-transport chains in the vesicles membranes. Similar results have been obtained with freshly-prepared B- and T-vesicles.

It is known that the electrochromic absorption changes indicate linearly the transmembrane electric field [9,11] and the sign of the absorption changes depends on the orientation of the pigments in respect to the field. Figure 4 shows the field indicating absorption change for the T- and B-vesicles measured at 515 nm. In both cases the same sign of the absorption change is observed. This shows that the orienta-

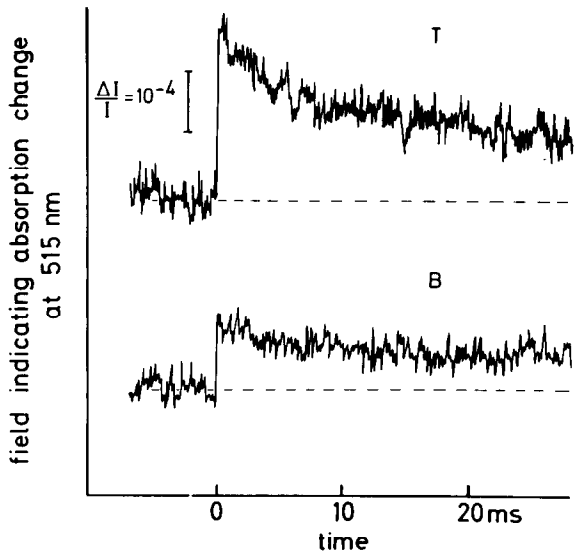

Fig.4. Field indicating ΔA_{515}. Top: T-vesicles. Bottom: B-vesicles. Details see section2 and text.

tion of the bulk pigments (chl-*a*, chl-*b*, carotenoids) with respect to the orientation of the field generating reaction centers is unchanged in both types of vesicles. The electrochromic nature of both signals was demonstrated by the acceleration of the decay on addition of the ionophore valinomycin (not shown). The different amplitudes of the absorption changes reflect the different amount of active photosystems in the T- and B-vesicles.

4. Discussion

It is demonstrated by the result depicted in fig.3 that positive charges are generated at the outside of the membrane of B-vesicles and negative charges at the outside of the membrane of T-vesicles and class-II chloroplasts. This indicates that the primary process of charge separation across the membrane in the B-vesicle occurs in the opposite direction as compared with class-II chloroplasts and with T-vesicles. As the electric method detects only a transmembrane charge separation the experiments show that in T- as well as in B-vesicles a vectorial electron transport exists, however, the sidedness of the reaction centers within the membrane is reversed.

As expected, the electrochromic absorption change has the same sign in the T and in the B-vesicles indicating that the orientation of the bulk pigments is not changed with respect to the reaction centers.

It can be concluded from these results that the preparation procedure leaves the structural organisation quite unchanged besides the effect of turning the sidedness of the whole membrane is some vesicles. Therefore, the B-vesicles can be regarded to consist predominantly of 'inside-out' thylakoids.

For these conclusions only the signs of the electric and electrochromic signals are necessary. The interpretation of the kinetics of the electric signals obtained by the membrane filter technique is more complicated:

1. The observed amplitude $\Delta\Delta\phi$, is increased by a factor 2–4 as compared with the corresponding signal in solution under identical experimental conditions (fig.2). This may result from the arrangement of all vesicles in a thin layer and a small distance of the electrodes from the generated charges [10].
2. The relaxation time found for chloroplasts on the membrane filter is increased by a factor 10–100 as compared with that found in solution under identical experimental conditions. It is known that the relaxation time (and the observed amplitude) of the signal depends on the ionic conductivity of the reaction medium (ion concentration, viscosity) [4,5,10].

A high conductivity facilitates the ion flux around the membrane vesicle and thereby accelerates the equilibration of the charge asymmetry. Obviously, the filtration onto a membrane filter considerably slows down the ion flux around the vesicle (fig.2). This can be understood by a decrease of the free space outside the vesicle so that the ion flux around the vesicle is strongly restricted.

If the ion flux around the B- and the T-vesicles is also reduced by a factor 10–100 (as found for the class-II chloroplasts) a relaxation time of 0.2–2 μs and 0.1–1 μs, respectively, is calculated for the vesicles in solution using the experimental relaxation times found for the T- (20 μs) and B-vesicles (10 μs) on the membrane filter (fig.3). This time is shorter than the time resolution of our apparatus and correspondingly no signals have been detected from these vesicles in solution.

An explanation for the difference of the relaxation times found in chloroplasts and in T- and B-vesicles cannot be given at this time. If the relaxation of the charge asymmetry would result from the ion flux around the vesicles a difference of about a factor 2 is expected because the diameter of a grana thylakoid is about twice of that of the T- and a B-vesicle [1]. Two possible reasons may be suggested tentatively for this discrepancy:

1. The electric signals measured in chloroplasts result mainly from large stroma thylakoids which would be a factor of 10 larger than T- and B-vesicles.
2. The surface conductivity is considerable lower in chloroplasts than in T- and B-vesicles. This may be due either to the unstacking and/or due to an alteration of the surface properties during preparation.

The experiments reported here are not appropriate to draw quantitative conclusions neither from the kinetics nor from the magnitude of the signals. Besides the geometric effect discussed above the magnitude depends also on the amount of chloroplasts remaining on the filter after washing. Furthermore the kinetics depend on the actual geometry of the space between

vesicle membrane and pore wall of the filter and on the conductivity of this space.

As the electric method measures directly the direction of the primary vectorial charge separation at the membrane vesicles the qualitative results reported here are sufficient to conclude that the sidedness of the B-vesicles is opposite to that of T-vesicles and class-II chloroplasts in accordance with earlier conclusions based on the direction of proton translocation [2].

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