

The electric unit size of thylakoid membranes

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The size of the function unit of electrical events in thylakoid membranes was estimated by the minimum amount of gramicidin needed to discharge the flash light generated electrical potential difference. Early flash spectroscopic measurements have indicated that a single gramicidin dimer operates on an electrical function unit containing at least 2×10^5 chlorophyll molecules [1]. In this study we present gramicidin titrations with more intact thylakoid preparations which revealed a more than hundred-fold greater lower limit for the electric unit size, namely 5×10^7 chlorophyll molecules. It is conceivable that the whole complicated thylakoid structure inside a chloroplast constitutes a single electric unit. It comprises more than 2×10^8 chlorophyll molecules in an area of more than $400 \mu\text{m}^2$.

Photosynthesis; Thylakoid; Gramicidin; Electrochromism; Size analysis

1. INTRODUCTION

Chloroplasts of higher plants contain a complex lamellar system, the thylakoid membrane, which carries the components of photosynthetic electron transport and ATP synthesis. Thylakoid membranes are differentiated into densely stacked membranes, grana lamellae, and a system of interconnecting membranes, stroma lamellae. Electronmicroscopic studies [2,3] suggest, that a stroma lamella connects a number of grana lamellae inside a single granum as well as different grana to each other. Each grana lamella is in contact with several stroma lamellae. When thylakoids are osmotically swollen in distilled water they form 'blebs' up to 10–20 μm in diameter [4,5] containing 2.5×10^8 chlorophyll molecules in the average [5]. This has yielded a specific area per chlorophyll molecule of 2 nm^2 [5] (in agreement with [6]). On the other hand, two of the, broadly speaking, disk shaped membranes in a granum (diameter about 500 nm) contain only 8×10^5 chlorophyll molecules. Light and electron microscopic studies support the notion that a bleb originates from the ensemble of thylakoid membranes inside one chloroplast [4,7]. The mechanism of 'unfolding' of the highly interlocked system of thylakoid membranes into a sphere is difficult to visualize.

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Abbreviations: DCCD, *N,N'*-dicyclohexylcarbodiimide; Mops, 3-(*N*-morpholino)propanesulfonic acid; MV, methylviologene; Tris, Tris(hydroxymethyl)aminomethane

How large is the size of the function unit of electrical events in thylakoid membranes compared to the geometrical size? Previously, this size has been determined by spectroscopic measurements of the minimum amount of gramicidin that accelerates the decay of the flash light induced transmembrane voltage. The minimum size of the electric unit has been equivalent to at least 2×10^5 chlorophyll molecules [1]. This corresponds to the area of a single grana lamella, 500 nm in diameter. It is smaller by orders of magnitude than the electron microscopic estimate. We reinvestigated the electric unit size of thylakoid membranes by the same technique as above, however, using more intact membranes.

2. MATERIALS AND METHODS

'Stacked thylakoids' and 'EDTA washed thylakoids' were prepared from pea seedlings as in [8], 'thylakoid vesicles' were prepared as in [9]. *EDTA washed thylakoids* were two times incubated in 1 mM EDTA at a chlorophyll concentration $>0.5 \text{ mM}$ in the dark and on ice, while *thylakoid vesicles* were incubated in 100 μM EDTA at 10 μM chlorophyll and at room temperature. Both EDTA treatments result in completely destacked thylakoids [8,9] and the peripheral proteins including CF_1 are largely detached [9,10]. *EDTA washed thylakoids* (chlorophyll concentration during EDTA incubation $>0.5 \text{ mM}$) and *thylakoid vesicles* (chlorophyll concentration 10 μM) differ in their size. While the latter are fragmented into small vesicles 500–600 nm in diameter [11] the former remain larger [10] (see below). To block proton conduction through CF_0 , EDTA treated thylakoids were incubated with 25 μM DCCD for 10 min before gramicidin was added.

The stock suspensions (1–4 mM chlorophyll) were stored on ice for up to 4 h before use. The decay of the electrical potential across the thylakoid membrane after a single-turnover flash was measured by electrochromic absorption changes of intrinsic pigments at 522 nm wavelength [1,12] under saturating excitation by a xenon flash lamp; duration 15 μs (FWHM), wavelength $>610 \text{ nm}$, 1 mJ/cm^2 .

Gramicidin was added to the thylakoid suspension from ethanolic stock solutions under vigorous stirring. The ethanol concentration in the measuring cuvette was held below 0.5%. Gramicidin was purchas-

ed from Sigma and contained 88% gramicidin A, 7% gramicidin B and 5% gramicidin C, according to the manufacturer.

3. RESULTS

Addition of gramicidin to thylakoid membranes accelerates the decay of the transmembrane electrical potential (after a single-turnover flash of light) [1]. For *stacked thylakoids* with rather low intrinsic conductance (half-decay time in the absence of gramicidin 510 ms) we determined the lowest gramicidin concentration resulting in a detectable acceleration of the decay of the electrochromic absorption change (Fig. 1). This minimal gramicidin concentration was about 10 pM at 20 μ M chlorophyll. If gramicidin was completely dimerized (see below) this was equivalent to one conducting gramicidin dimer per 4×10^6 chlorophyll molecules. A small portion of the electrochromic absorption change (about 10%) showed a fast decay, even in the absence of gramicidin. This was probably attributable to a fraction of thylakoids that had a much higher permeability. This part of the signal was omitted and the reading of the half-decay time was started at the point where the different transients diverged. In Fig. 1 (bottom) the reciprocal half-decay times were plotted against the gramicidin concentration. The half-decay times caused by gramicidin only ($t_{1/2,G}$, circles in Figs 1 and 2) were determined by correcting the measured half-decay times ($t_{1/2,M}$, crosses in Figs 1 and 2) by the half-decay time of the control ($t_{1/2,C}$, $t_{1/2,G}^{-1} = t_{1/2,M}^{-1} - t_{1/2,C}^{-1}$). As half-decay time of the control we took the mean from all electrochromic absorption changes without a detectable acceleration (indicated by the horizontal line). Gramicidin accelerated the decay over the whole extent of the electrochromic absorption change discernably, already at a concentration as low as 10 pM (Fig. 1, top). For an example of the contrary see below (Fig. 3, there the acceleration was restricted to one portion of the initial extent). This implied that each electric unit carried at least one, probably a few conducting gramicidin dimers. The data points for the inverse half-decay times caused by gramicidin (circles in Figs 1 and 2, bottom) were connected by linear regression, yielding a slope of 1.8 (in double logarithmic presentation). The approximately quadratic dependence was compatible with the notion that gramicidin dimers were the conducting species [13]. It also meant, that most added gramicidin was in the monomeric form [13]. Therefore the actual number of gramicidin dimers was lower than half of the monomer concentration and the number of chlorophyll molecules per electric unit was greater than 4×10^6 .

In studies on the dimerization constant of gramicidin in thylakoid membranes (to be published elsewhere), we found a great difference between *stacked thylakoids* and *EDTA washed thylakoids* (the dimerization constant was about ten times greater in the latter). Therefore

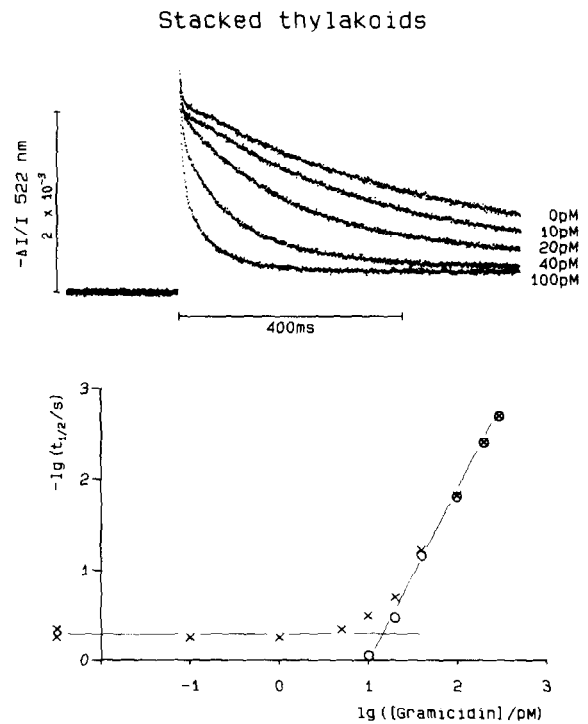


Fig. 1. Acceleration of the decay of the electrical potential across the thylakoid membrane by low concentrations of gramicidin (as indicated). *Stacked thylakoids*, 20 μ M chlorophyll, in 20 mM KCl, 10 mM $MgCl_2$, 10 μ M MV, 5 mM Mops/Tris, pH 7.1. Electrochromic absorption changes (top) and the corresponding double logarithmic presentation of the inverse half-decay times as a function of the gramicidin concentration (bottom). Crosses represent the measured half-decay times, data points left of the y-axis indicate no gramicidin added, the circles represent half-decay times caused by gramicidin only (details given in the text). The small slowly rising component in the upper two transients was probably caused by electrogenic events in cytochrome b_6/f [16].

the above gramicidin titration was repeated with *EDTA washed thylakoids* (the conductance of exposed CF_0 was blocked by DCCD). As expected, a detectable acceleration of the decay of the electrochromic absorption change occurred at even lower gramicidin concentrations (Fig. 2), namely at about 2 pM gramicidin at 20 μ M chlorophyll. This was at the most (all gramicidin dimerized, see below) one conducting gramicidin dimer per 2×10^7 chlorophyll molecules. Again the elevation of the gramicidin concentration resulted in a continuous increase of the acceleration, indicating that gramicidin acted on each electric unit. And again a nearly quadratic dependence on the gramicidin concentration was observed. So the electric unit of thylakoid membranes contained more than 2×10^7 chlorophyll molecules.

To characterise for illustrative purpose the different reaction to gramicidin of smaller vesicles we titrated with gramicidin fragmented *thylakoid vesicles* (containing only some 10^5 chlorophyll molecules [9,11]) (Fig. 3). Addition of small amounts of gramicidin resulted in

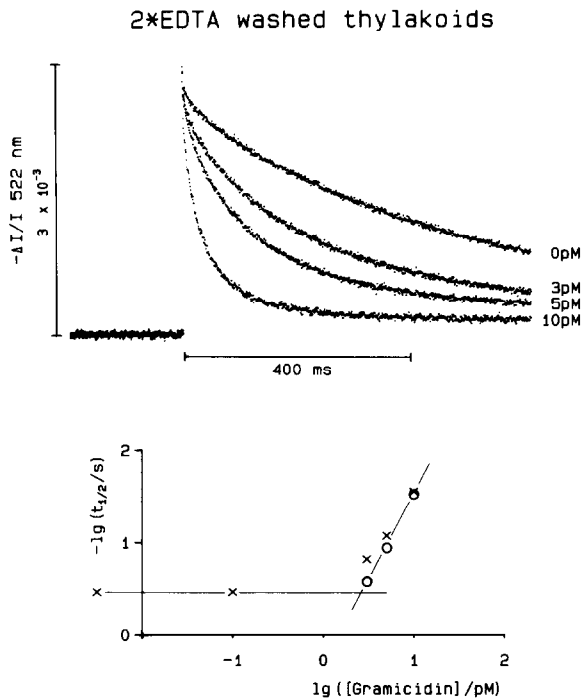


Fig. 2. Acceleration of the decay of the electrical potential across the thylakoid membrane by gramicidin (concentrations as indicated). EDTA washed thylakoids, 20 μM chlorophyll, in 10 mM NaCl, 200 μM K₃[Fe(CN)₆], pH 7.5, 10 min incubation in 25 μM DCCD. Electrochromic absorption changes (top) and the corresponding double logarithmic presentation of the inverse half decay time as a function of the gramicidin concentration (bottom). Crosses and circles as in Fig. 1 ($t_{1/2, Cr} = 350$ ms).

two populations of *thylakoid vesicles*, one carrying at least a single gramicidin dimer and another without any conducting dimer. This was reflected in a biphasic decay of the electrochromic absorption change (Fig. 3). As an example: at 20 pM gramicidin 40% of the extent showed an accelerated decay while the decay of the remaining 60% was as slow as in the control. This meant

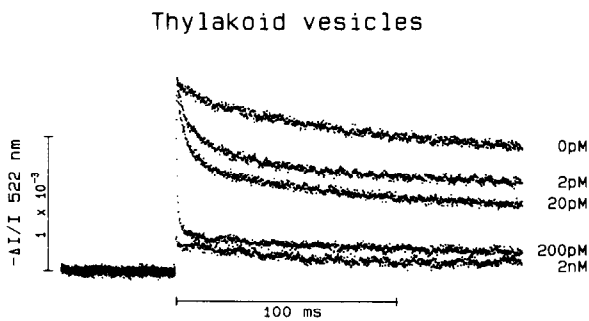


Fig. 3. Acceleration of the decay of the electrical potential across the thylakoid membrane by gramicidin (concentrations as indicated). Fragmented *thylakoid vesicles*, 10 μM chlorophyll, in 10 mM NaCl, 200 μM K₃[Fe(CN)₆], 2 mM Mops/NaOH, pH 7.2, 10 min incubation with 25 μM DCCD to block proton conduction through CF₀. The electrochromic absorption changes show a pronounced biphasic decay in contrast to Figs 1 and 2.

that 60% of the vesicles contained no gramicidin dimer ($P(0) = 0.6$), while the other 40% contained at least one conducting dimer. From this, with the aid of Poisson's statistics, an average number of channels per vesicle of $n = 0.5$ was calculated ($n = -\ln P(0)$; see [9,11] for its details). If gramicidin was completely dimerized, this was equivalent to 5×10^5 chlorophylls per vesicle (10 μM chlorophyll, 10 pM gramicidin dimers and $n = 0.5$). With 2 nm² per chlorophyll molecule [5,6], the area of one *thylakoid vesicle* containing 5×10^5 chlorophylls is 1 μm² corresponding to a sphere with 564 nm diameter. This agrees with earlier estimates [11] and recent results from quasi elastic light scattering (unpublished observations) for the size of fragmented *thylakoid vesicles*.

4. DISCUSSION

Flash spectroscopic measurements in the presence of gramicidin indicated that the electric unit of thylakoid membranes contained more than 4×10^6 chlorophyll molecules (*stacked thylakoids*) or more than 2×10^7 chlorophyll molecules (*EDTA washed thylakoids*). The apparent difference was probably caused by the higher dimerization constant of gramicidin in the latter. The number of 2×10^7 chlorophyll molecules was still at a lower limit.

There are two reasons why previous experiments resulted in a hundred-fold smaller lower limit for the electric unit size of thylakoid membranes [1]. First, the intrinsic conductance of the thylakoids (in the absence of gramicidin) was about ten-fold higher. Second, we found the dimerization constant of gramicidin in spinach thylakoids, as used earlier, to be one order of magnitude lower than in pea thylakoids, was used in this work (unpublished observation). So, much higher gramicidin concentrations had to be added for a detectable acceleration of the decay of the electrochromic absorption change.

The quadratic dependence of the accelerated decay of the transmembrane electrical potential on the gramicidin concentration indicated that much less than 50% of the gramicidin was dimerized. Accordingly, the number of chlorophyll molecules per electric unit rises from 2×10^7 to at least 5×10^7 . How does the conductance of one gramicidin dimer compare with the intrinsic leak conductance of the membrane area covered by 5×10^7 chlorophyll molecules? Take for the discharge time (τ) of a capacitor (capacitance C) via an ohmic resistor (conductance G): $\tau = C/G$. The specific capacitance of the thylakoid membrane is $\langle C \rangle = 1 \mu\text{F}/\text{cm}^2$ [14,15] and the observed half-decay time was 350 ms ($t_{1/2}/\ln 2 = \tau = 500$ ms) in the absence of gramicidin. This results in a specific conductance of $\langle G \rangle = 2 \mu\text{S}/\text{cm}^2$ (attributable to intrinsic leaks of the membrane). In an EDTA treated *thylakoid vesicle* at 10 mM NaCl one gramicidin dimer has a single conduc-

tance of 2.7 pS [11]. So 7.4×10^5 gramicidin dimers per cm^2 respectively one gramicidin dimer per $135 \mu\text{m}^2$ are needed to yield a specific conductance of $\langle C \rangle = 2 \mu\text{S}/\text{cm}^2$. With an area per chlorophyll of 2nm^2 [5,6] this corresponds to 6.8×10^7 chlorophyll molecules per gramicidin dimer. This comes close to the number of 5×10^7 chlorophyll molecules for the lower limit of the electric unit pointing out that the maximally detectable number is delimited by the conductance of a single gramicidin dimer (in relation to the intrinsic conductance of the very large membrane area) and not by the size of the electric unit of thylakoid membranes. Most probably the whole thylakoid membrane inside a single chloroplast (carrying about 2.5×10^8 chlorophyll molecules) forms a single electric unit and it takes at least four gramicidin dimers to get a detectable acceleration of the decay of the transmembrane electrical potential of this large electric unit. Assuming a vanishing low intrinsic membrane conductance, the discharge of this large electric unit by a single gramicidin dimer would take more than one second ($t_{1/2} = 4.350 \text{ms}$). As this process is solely limited by the conductance of gramicidin and not by lateral resistances inside the thylakoid lumen, one might expect the electric unit size of 2.5×10^8 chlorophyll molecules to be realized in a time range by far less than a second.

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