# THE ELECTROCHEMICAL RELAXATION AT THYLAKOID MEMBRANES

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SUMMARY. The primary processes of photosynthesis by green plants take place in thylakoids of chloroplasts. About one fourth of the useful work derived from light quanta is used for the generation of a transmembrane electrochemical potential difference of the proton, which, in turn, is used by a proton translocating ATP synthase to yield ATP. This article describes the electrochemical relaxation involving proton pumps, the ATP synthase, lateral and transversal conductances for protons and other ions. It relies mainly on flash spectrophotometry for the pulsed generation of the electrochemical potential difference and time-resolved detection of pH- and voltage-transients by appropriate dyes. The pore-forming antibiotic gramicidin serves as standard. The electrochemical analysis is complicated by the folding pattern of thylakoid membranes with tightly apposed and extended membrane domains which differ in their protein composition. In this article we discuss the following aspects: 1.) The single channel conductance of gramicidin in this protein-rich membrane compares well with figures reported for pure lipid bilayers. The area related dimerization constant is about ten-fold larger because of upconcentration in lipid domains. 2.) The whole contents of thylakoid membranes, a total area of several 100 $\mu m^2$ with more than $10^8$ molecules of chlorophyll and $10^5$ of ATP synthase, forms one contiguous entity. This capacitor can be discharged by a single gramicidin dimer. 3.) There is no evidence for surface-enhanced proton diffusion along this membrane. In a membrane patch with only $10^5$ molecules of chlorophyll and in stacked thylakoids the resistance to lateral proton flow between pumps and ATP synthases along tightly appressed membranes dissipates a few percent of the transmembrane protonmotive force. Thus the effective membrane unit for protonic coupling between pumps and ATP synthases is smaller than the electrically coupled one. 4.) The area specific electric capacitance of the membrane is by more than one order of magnitude smaller than the specific chemical (buffering) capacitance for protons. The greater damping power for pH-transients as compared to the one for electric transients may be why thylakoids (subject to fluctuating light intensity) use $\Delta pH$ rather than $\Delta\psi$ (like mitochondria) to drive ATP synthesis.

## 1. Introduction

Chloroplasts of higher plants carry an inner lamellar system, thylakoids, that is folded into densely stacked membranes (grana) and interconnecting membranes (stroma lamellae). The photosynthetic apparatus consists out of four large protein complexes: photosystem II oxidizes water, photosystem I reduces NADP, the cytochrome $b_6$,f-complex mediates electron transfer between the former, and $CF_OCF_1$ synthesizes ATP at the expense of the transmembrane electrochemical potential difference of the proton (Mitchell, 1961; Mitchell, 1966). Fig. 1 illustrates this structure. The protein complexes are seggregated according to the folding pattern



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of the membrane (Andersson & Anderson, 1980). Apposed membrane portions contain photosystem II, exposed membrane portions contain photosystem I and the ATP synthase. The upper inserts show an artist`s view of the complicated folding of thylakoids (left) and an electron micrograph of one granum. Each of the stacked disk-like structures (diameter about 0.5 μm) contains about 100 photosystem II molecules, but as the disks are interconnected by stroma lamellae the functional unit of the electrochemical events is much larger (see below). The inner lumen and the outer partitions between stacked thylakoid membranes are very narrow, only about 5 nm wide slabs.

The middle of Fig. 1 shows the electron transport chain. In reaction centres the primary event is a very rapid transmembrane transfer of electrons from the inner to the outer surface of the membrane (some 100 ps). This is followed by proton uptake from the outer phase (the chloroplast stroma) and proton release into the inner phase (thylakoid lumen), as indicated. The lower portion of Fig. 2 shows an equivalent circuit for cyclic proton flow between pumps and ATP synthases.

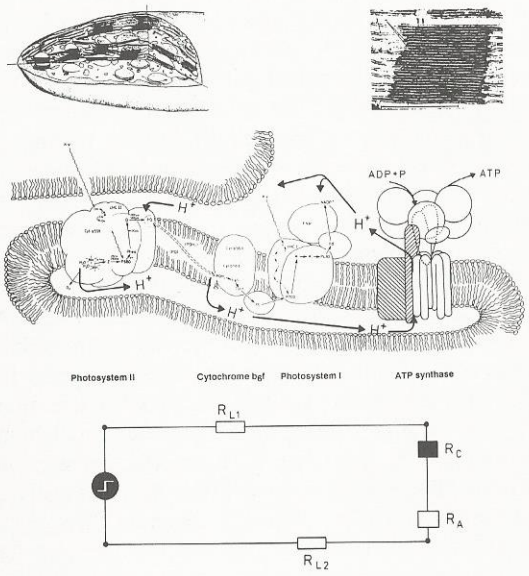


FIGURE 1. Schematic drawing of the thylakoid membrane with the three large electron transfer complexes and the ATP synthase. Apposed membrane proteins with photosystem II and exposed portions with photosystem I and the synthase are apparent. Sites of light driven proton binding from the outer medium (the stroma) and of proton release into the lumen, lateral proton flow, and transmembrane proton backflow over the ATP synthase are indicated. The insert in the upper right shows an electron micrograph of stacked thylakoid membranes. Each of the disk-like structures contains at least 100 pieces of any protein complex, but the true function unit is larger as disks are interconnected with each other. The lower portion shows a equivalent circuit for cyclic proton flow between pumps and the ATP synthase (adapted from (Junge, 1989)).

Photoinduced protolytic reactions have been studied at high time resolution by laser-flash spectrophotometry. Thylakoids are a favourable object for such studies. Photosystems are to be stimulated for a single turnover by short laser flash (<10 ns). Transients of the transmembrane voltage can be measured at ns time resolution by electrochromism of intrinsic pigments (Junge &



Witt, 1968; Witt, 1979; Junge & Jackson, 1982). Hydrophilic pH-indicating dyes are practically selective for pH-transients in the large external bulk phase (Polle & Junge, 1986) while membrane adsorbed indicators (mainly neutral red) react to pH transients at both surfaces of the membrane. In the presence of appropriate non-permeating buffers the latter can be selective for pH transients in the very narrow (only 5 nm wide) lumen (Auslaender & Junge, 1975; Junge et al.1979; Hong & Junge, 1983; Junge et al.1986). In stacked thylakoids, however, this selectivity is expressed only after the time interval necessary for the lateral relaxation of pH-differences along the narrow slabs between appressed membranes (Lavergne & Rappaport, 1990; Jahns et al.1991). While the response time of electrochromism so far has been instrument-limited and in the ns time range, pH indicating dyes respond in the time range of 100 μs (around neutral pH). With these three observables, namely pH(lumen), pH(bulk medium) and transmembrane voltage, the spectrophotometric techniques have allowed "complete tracking of proton flow" (Schoenknecht et al.1986; Junge, 1987).

## 2. The lateral relaxation of pH-transients between appressed thylakoid membranes

In stacked thylakoids certain sites of proton pumping, namely photosystem II, are laterally seggregated from ATP synthases. The center of a thylakoid disk may be 300 nm away from the nearest synthase. Cyclic proton flow between pumps and ATP synthases is illustrated in Fig.2 (top). A flash of light generates an electric potential difference (in some 100 ps) and a pH-difference (0.1-1ms) around each photosystem II. Initially the electric and the chemical portion of the protonmotive force are localized. The spreading of the electric potential difference is very fast. With the cable equation, the dimension of partitions and of lumen slabs and with the concentrations of ions present one arrives at an upper limit for the electric relaxation time of some 100 ns. The lateral relaxation of the chemical portion of the protonmotive force is much slower, indeed. Activation of photosystem II molecules by a flash of light generates an alkalinization jump in the *partition* region between opposing membranes in a stack. When the ATP synthase is inactive (e.g. too low concentrations of ADP present) the *transmembrane* relaxation of a pH-difference is much slower (20 s) than the *lateral* one (100 ms).

The lateral relaxation by diffusion of protons, of hydroxyl anions and of mobile buffers can be measured by hydrophilic dyes as pH-transients in the bulk phase. The expected pH-profile at different times is illustrated in the middle of Fig. 2, the observed transient alkalinization in the bulk (Polle & Junge, 1986) and a theoretical fit (Junge & Polle, 1986) are shown at the bottom. The rise of alkalinization in the external phase occurs in about 100 ms as contrasted with the expectation of about 1 μs by solving Fick`s equation under the assumption of the same diffusion coefficients as in bulk water.

The dramatic increase of the relaxation time is understood by theory of diffusion in domains with fixed and mobile buffers (Junge & Polle, 1986; Junge & McLaughlin, 1987). The relaxation is determined by an "effective diffusion coefficient", $D_{eff}$, made up of the "true" diffusion coefficients of $H^+$, $OH^-$ and of mobile buffers (index i):

(1) $$D_{eff} = (2.3/ß_{tot})\cdot(D_H\cdot[H^+]+D_{OH}\cdot[OH^-]+\Sigma(D_i\cdot ß_i/2.3))$$

wherein the ß denote the respective buffer capacities of mobile ($D \neq 0$) and immobile ($D = 0$) buffers. The buffering capacity is here defined for very small perturbations as $ß = -d[H^+]_{total}/dpH$, wherein the nominator denotes the total concentration change of protons, both, bound and free. It is important to note that the diffusion of protons between two captures by buffering groups is still governed by $D_H$.

In experiments with thylakoids the behaviour of the effective diffusion coefficient has followed the expectation (Equ.1). Both the predicted minimum around neutral pH and the acceleration by added mobile buffer have been observed (Polle & Junge, 1989). The delay over the expected relaxation in the absence of buffers by a factor of $10^5$ is compatible with the estimated amount of buffer intrinsically present in partitions (Junge & Polle, 1986). To account for this very large factor it has to be assumed that one half of the total buffering power of the thylakoid suspension (no extra buffers added) resides in partitions. This certainly overestimates the buffering capacity in this domain. Accordingly, the diffusion coefficients of hydrogen- and hydroxyl-ion are rather less than larger than in bulk water (Polle & Junge, 1989).

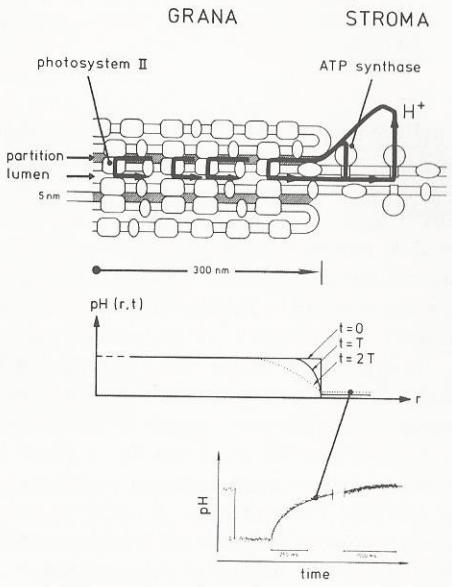


FIGURE 2. Schematic side view of stacked thylakoid membranes with appressed grana lamellae and interconnecting stroma lamellae. The narrow space between the outer surface of thylakoid membranes in a granum, called partition, is hatched. The *arrows* illustrate (a) light-driven proton pumping by photosystem II which is directed from the partition into the lumen, (b) opposite directed proton flow which is coupled to ATP synthesis, (c) lateral proton flow through the lumen, and (d) lateral backflow through partitions. The drawing in the middle illustrates the box shaped pH profile induced by excitation with a single flash of light at time zero and its evolution in time (t = T, t = 2T). The bottom trace is a reproduction of original data on the flash induced, and photosystem II-related pH rise in the stroma compartment and a theoretical curve (Junge & Polle, 1986; Polle & Junge, 1986).

It is worth noting that Equ.1 predicts that all buffers slow down the relaxation, since their diffusion coefficient is always smaller than the one of the proton (Eigen, 1963). This slowing down by buffering groups of a diffusive relaxation does not imply the diminuation of the steady proton flux. In the steady state the rates of proton binding and release by fixed buffers are equal. The steady flux is given by Fick`s first law and ∿ depends on the true diffusion coefficients (see 2.2).



## 2.1 THE CONTROVERSY OVER SURFACE ENHANCED DIFFUSION OF PROTONS

A controversy over the validity of Mitchell`s theory of a chemiosmotic mechanism of ATP synthesis (Mitchell, 1966; Mitchell, 1961) is focussed on whether or not the ATP synthase operates between two aqueous bulk phases separated by the coupling membrane or between more restricted domains, which are not in equilibrium with the respective bulk phase (see (Williams, 1988; Williams, 1961)). In this respect, surface-enhanced diffusion of protons at lipid monolayers has been hypothesized (Haines, 1983). Experiments with lipid monolayers bridging a barrier between two aqueous compartments led Prats and his collegues to the conclusion of 20-fold enhanced proton diffusion at the lipid/water interface (Prats et al.1986; Prats et al.1987). Moreover the authors claimed the absence of equilibration of the surface pH with the bulk. Their experimental layout has been criticized for stirring artifacts and also for neglect of equilibration with the bulk (Kasianowicz et al.1987). In an alternative approach the conductance between two electrodes immersed in ultrapure water (20 mm apart) has been measured, without a barrier and without stirring. The difference-conductance (plus/minus lipid monolayer) has revealed a sharp increase above a certain compression of the monolayer (Morgan et al.1991). This is not necessarily due to enhanced diffusion but it may be attributable to the upconcentration of protons in the diffuse double layer, which does matter, of course, in an electrophoretic experiment as this one. Experiments by laser-flash spectrophotometry on proton diffusion in the ultrathin layer between bilayers in osmotically compressed multi-lamellar lipid vesicles give no indication of enhanced diffusion (Gutman et al.1989). Our above experiments with thylakoids give no indication either (Polle & Junge, 1989). Thus it is very likely that there is no enhanced diffusion at the surface of *biological membranes* and there is certainly no inhibition of rapid equilibration with the surface and the bulk.

## 2.2 LOSSES OF PROTONMOTIVE FORCE BY LATERAL PROTON FLOW

In a comparison of the efficiencies of photosystem II and photosystem I to drive ATP synthesis slightly lower figures have been observed for the former (Haraux & de Kouchkowsky, 1982). This might be understood in terms of the losses of the protonmotive force during lateral flow of protons and hydroxyl anions between photosystem II in the appressed membrane portions and the ATP synthases in the exposed ones (see FIG.2). A total flux of protons, I (moles/s), over the boundary of a thylakoid disk requires a drop in proton concentration between the center (suffix c) and the fringe (suffix f) of the disk. For a disk that is homogeneously filled with pumps the concentration drop has been calculated (Junge & Polle, 1986):

(2) $$[H^+]_c - [H^+]_f = I/4\Pi hD$$

It depends on the thickness of the disk-shaped slap between membranes, h, and on the diffusion coefficient, D. A proton flux, which is equivalent to the highest rate of ATP synthesis in a model thylakoid (radius 300 nm, area per clorophyll molecule 2.2 nm), namely, $1.3 \times 10^{-19}$ mol $s^{-1}$, and assuming a thickness of 5 nm and the diffusion coefficient as in bulk water, $D_{H+} = 9.3 \times 10^{-9}$ $m^2$ $s^{-1}$, implies a drop proton concentration of 0.23 mM.[27] The magnitude of the corresponding pH drop, $DpH = -D[H^+]/2.3 \times [H^+]$, depends on the pH in the medium. It decreases toward more acid pH. A similar relation holds in the alkaline pH domain, where die diffusion of $OH^-$ dominates. Taking these results together and assuming the same diffusion coefficients as in bulk water the pH drop has been calculated. At the outer surface of stacked thylkoids it amounts to 0.14 pH units, if the outer side is kept at pH 8. It is below 0.01 pH units at the internal side and at pH 4 (Junge & Polle, 1986). If the diffusion coefficients in these narrow spaces are lower than in

bulk water, greater losses are expected. It is probable that ohmic losses of protonmotive force are small, but they are not negligible in tightly stacked thylakoids.

### 2.3 CONSEQUENCES FOR THE SPECIFICITY OF NEUTRAL RED FOR pH-TRANSIENTS IN THE THYLAKOID LUMEN

Neutral red is a hydrophobic pH-indicating dye which has a long history in microscopy as vital stain. It adsorbs to biomembranes. Because of this property it has been used as specific indicator of pH-transients in the lumen of thylakoids (Auslaender & Junge, 1975). The rationale of the experiments is illustrated in Fig. 3 (Jahns et al.1991). Since neutral red is adsorbed to both sides of the membrane it responds to pH-transients in both compartments. Its selectivity is elicited only by addition of a non-permeant buffer, typically bovine serum albumin, to eliminate pH-transients in the outer bulk phase. The well-behavedness of this indicator includes the following features: Although it is a redox-active compound (Prince et al.1981), its transient absorption changes in thylakoids are fully quenched by permeating buffers (Junge et al.1979). Its response to pH-transients and its binding to the membrane both vary as function of pH and salt composition in exactly the way expected for an indicator sensing the surface-pH, with $pH_s=pH_{bulk}-\psi_s/59mV$ (Hong & Junge, 1983). Although amphiphilic, the dye is only mildly protonophoric (Junge et al.1986). Hence the response to rapid pH-transients is first owed to dye molecules that are already in-place. A time resolution for pH-transients in the thylakoid lumen down to 100 μs is obtained (Foerster & Junge, 1985). A slow redistribution of the dye across the membrane and in response to a pH-difference occurs only in the range of several seconds (Junge et al.1986).

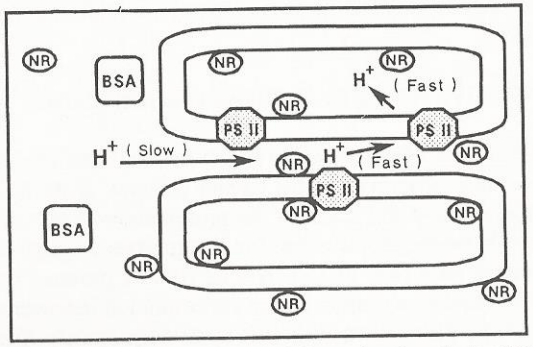


FIGURE 3. Scheme illustrating the interpretation of absorption transients of neutralred as indicator of pH-transients in the lumen of thylakoids (Lavergne & Rappaport, 1990; Jahns et al.1991). It is assumed that neutralred is adsorbed to the membrane surface at both sides of thylakoids in a grana stack. However, the non-permeating buffer, bovine serum albumin (BSA) is practically excluded from the narrow external partitions. Initially the dye responds rapidly to pH-transients at both sides of the membrane. The transient at the outer side is more slowly quenched by BSA (see above). Only thereafter is neutralred fully selective for the transient in the lumen phase.

In stacked thylakoids the well-behavedness of the indicator is blurred by the above described slow lateral relaxation of the alkalinization jump in partitions. This has been first pointed out by Lavergne and Rappaport (1990) and worked out in greater detail by Jahns et al. (Jahns et al.1991). Because of the tight appression of stacked thylakoid membranes the non-permeating buffer BSA is not present in the partition slab. Accordingly, the transient of neutral red also contains a smaller contribution from neutral red molecules at the outer side of the membrane.



Only after lateral relaxation in partitions, i.e. after some 100 ms, is it selective for pH-transients in the lumen. This is mended when thylakoids are unstacked. Then the added buffer has more direct access to the sites of proton uptake at the outer side of the membrane. These results have stimulated a reevalutation of the stoichiometry and kinetics of proton release during the four-stepped reaction of the water oxidase (photosystem II) with the result of a non-integer pattern of proton/electron ratio during the four successive transitions (Jahns et al.1991).

## 3 The transmembrane electric relaxation in the presence of the cation channel gramicidin

Gramicidin is perhaps the best characterized pore-forming antibiotic. It conducts alkali-cations across lipid bilayers as a head-to-head dimer (Veatch et al.1975; Durkin et al.1990). When the respective ATP synthase is inactive photosynthetic membranes are rather proton tight and only moderately pervious to other cations and to anions. The nature of the intrinsic *leak conductance* is a matter for itself and not further pursued here. In thylakoids the relaxation time of the electric potential difference (generated by one flash of light) ranges around 100 ms at 10 mM KCl and 3 mM $MgCl_2$. The capacitor equation links the relaxation time, τ, or the decay rate, k, to the specific conductance, G, and the specific electric capacitance, C, of the membrane:

(3) $\tau = C/G = 1/k$

where C comes in $F/m^2$ and G in $S/m^2$.

The area specific capacitance of thylakoids is about $10^{-2}$ $F/m^2$ (Arnold et al.1985). Thus a relaxation time of 100 ms gives rise to a specific leak conductance of $10^{-1}$ $S/m^2$. The total area of one thylakoid in a stack (It is not isolated from the rest, but see below.) is that of closed disk with radius 300 nm, namely $566 \cdot 10^{-15}$ $m^2$. The presence of one ion channel with time averaged conductance of only 57 fS accounts for the observed leak conductance and a powerful ion channel like gramicidin (several pS) causes a considerable acceleration of the electric relaxation. There are two limiting cases, in small vesicles (thylakoid fragments, chromatophores of photosynthetic bacteria) a single gramicidin dimer causes a drastic acceleration of the relaxation of the transmembrane voltage. In a typical spectrophotometric experiment the observed decay of the electrochromic absorption changes reflects the voltagedecay in more than $10^{10}$ vesicles. It is determined by the Poisson statistical ensemble properties (vesicles with 0 channels reveal a slow decay, others with 1, 2, ... channels a very fast one). On the other hand, with the large membrane area of interconnected, folded and stacked thylakoids one single dimer per large vesicle causes only little effect, the average number of channels is large and the ensemble behaves homogeneously. This has been used for a spectrophotometric determination of the unit conductance of gramicidin and its dimerization constant in photosynthetic membranes and furthermore to evaluate the electric unit size of chromatophores and of thylakoids. It has emerged that almost all thylakoid membranes within a chloroplast (see Fig. 1, top) are part of one electrically connected entity.

### 3.1 GRAMICIDIN IN SMALL VESICLES (BACTERIAL CHROMATOPHORES, FRAGMENTED THYLAKOIDS): POISSON`s DISTRIBUTION

It was claimed that the addition of gramicidin to chromatophores "inhibited" the generation by flash light of carotenoid absorption changes (Fleischmann & Clayton, 1968). These absorption transients are of electrochromic origin (Jackson & Crofts, 1969; Junge & Jackson, 1982). Seemingly, it did not accelerate their decay which was expected (Saphon et al.1975). Fig. 4 explains why (Althoff et al.1991). The transient traces in the upper half show the generation of transmembrane electric potential by flash light at time zero and a rather slow decay of the voltage by low leak conductance of the membrane. In the presence of gramicidin at increasing amounts the extent of the voltage is seemingly diminished while the decay rate is less affected. The same experiment but now at thousand-fold higher time resolution (lower half of Fig. 4) reveals a very rapid decay, which has excaped detection at lower time resolution. The slow decay is attributed to vesicles without any gramicidin dimer and the fast one to those with 1, 2, ... dimers.

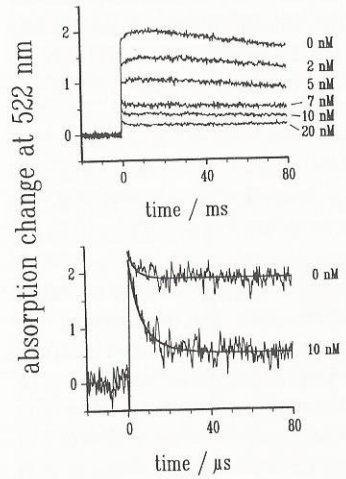


FIGURE 4. Electrochromic absorption changes after single flash-excitation. Chromatophores were incubated for 30 min in the presence of different gramicidin (monomer) concentrations. The ordinate indicates -ΔI/I and was scaled up by a factor of 1000. Top: 40 transients with 200 µs time resolution were averaged. Gramicidin concentration as indicated. Bottom: 120 transients with 200 ns time resolution were averaged. Gramicidin concentration as indicated. The transients were fitted according to Eq. (6) with the following parameters: n=0.27, k= 0.24 $\mu s^{-1}$ for 0 nM and n = 1.35, k = 0.11 $\mu s^{-1}$ for 10 nM (Althoff et al.1991).

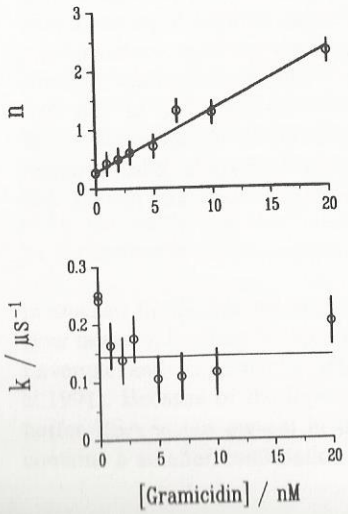


FIGURE 5. Results obtained by fitting traces as shown in Fig. 4, lower part. Top: Average number of conducting gramicidin dimers per chromatophore, n, as function of gramicidin (monomer) concentration in the chromatophore suspension. Solid line by linear regression of datapoints (see text). The error bars indicate the standard deviation from the mean of the fitted n values. Bottom: Decay rate caused by a single gramicidin dimer, k, as function of the gramicidin (monomer) concentration. The solid line indicates the mean k value of the data points in the range from 1 nM to 20 nM gramicidin.



The theory for this situation was developed previously ((Schmid & Junge, 1975), see also (Apell & Läuger, 1986)). In a vesicle with m ohmic channels the voltage decays exponentially.

(4) $U(t) = U_0 \cdot \exp(-m \cdot k \cdot t)$

wherein k denotes the decay rate caused by one channel and m the number of channels in this vesicle. In a population of many identical vesicles doped with n channels, in the average, the probablity to find m channels is given by Poisson`s distribution:

(5) $P(m) = (n^m \cdot \exp(-n))/m!$

Merging these equations yields the double-exponential decay law for the average behaviour of a very large ensemble:

(6) $U`(t) = U`_0 \cdot \exp(-n) \cdot \exp(n \cdot \exp(-kt))$

It has been shown elsewhere (Lill et al.1987) that the incorporation of the vesicle size-distribution into this equation does not lead to much different behaviour in a wide range of sizes. Equation 5 has only two essential fit parameters, n, the average number of open channels per vesicle, and k, the decay rate caused by one channel per vesicle, which is related to the specific conductance by Equation 2.

The decay of the electrochromic absorption changes as documented in Fig. 4 has been analyzed in terms of Equation 5 and the fit parameters have been plotted as function the the gramicidin concentration (see Fig. 5). The detailed analysis is found elsewhere (Althoff et al.1991). Broadly speaking it has yielded the following results: 1.) The average number of channels (i.e. gramicidin dimers) per vesicle depends linearly on the concentration of monomers. This implies complete solvation and complete dimerization of the antibiotic in the chromatophore membrane. 2.) The average number of bacteriochlorophyll-molecules per vesicle is 770. They cover an area of $10^4$ $nm^2$. 3.) With 1.1 $\mu F/cm^2$ for the specific capacitance of the chromatophore-membrane (Casadio et al.1988) the single-channel conductance of one gramicidin-dimer is 15 pS. At the given $K^+$-concentration (115 mM) this compares well with its conductance in lipid bilayer membranes as determined by electrophysiological techniques (Hladky & Haydon, 1972; Neher et al.1978). A similar analysis has been carried out with small vesicles from thylakoid membranes, resealed fragments which were obtained by EDTA treatment. The membrane area of these vesicles is 1 $\mu m^2$ and they contain about $5 \cdot 10^5$ molecules of chlorophyll (Lill et al.1987)(Schoenknecht et al.1990).

### 3.2 GRAMICIDIN IN THYLAKOID MEMBRANES: THE VERY LARGE SIZE OF THE ELECTRIC UNIT IN CHLOROPLASTS

When the envelope of intact chloroplasts is broken by hypoosmolar shock and when broken thylakids are resuspended in salt containing medium (say at 3 mM MgCl) the complicated folding pattern with grana of stacked membranes, interconnected by stroma lamellae is conserved. In the absence of salts, however, they swell into spherical blebs with more than 10 µm wide diameter (DeGrooth et al.1980; Stolz & Walz, 1988). These blebs contain up to $2 \cdot 10^8$ molecules of chlorophyll covering a membrane area of 400 $\mu m^2$ (Stolz & Walz, 1988). Electron microscopic evidence suggests that these blebs represent the total contents of thylakoid membranes within one chloroplast. We have asked whether or not this large membrane area encloses one continuous internal space even when folded into the complicated topology with stacked and interconnecting membranes which is shown in the upper left of Fig.1. This has been approached by asking for the maximum size of the membrane area which is to be electrically discharged by a single dimer of gramicidin. Due to the larger membrane area and thereby the larger electric capacitance of integral thylakoids the effects of small concentrations of gramicidin

are less dramatic as for instance in chromatophores (Fig.4). Still the addition of 3 pM gramicidin on 20 μM chlorophyll has caused an appreciable acceleration of the electrochromic transient. Accordingly, the electric unit contains at least $10^7$ chlorophyll molecules. The detectability of the minimum action of a single dimer per large vesicle depends on the magnitude of the intrinsic leak conductances of the membrane. An analysis that takes these leak conductances into account has pushed the chlorophyll contents of the electric unit up to at least $7 \cdot 10^7$ molecules (Schoenknecht et al.1990). Thus it is probable that the whole contents of $2 \cdot 10^8$ molecules of chlorophyll of one chloroplast is organized in one coherent membrane enclosing one electrically coherent lumen space.

### 3.3 GRAMICIDIN IN THYLAKOID MEMBRANES: DIMERIZATION CONSTANT AND SINGLE-CHANNEL CONDUCTANCE IN A MEMBRANE RICH IN PROTEIN

In the above estimates on the electric unit size of photosynthetic membranes the (time averaged) single channel conductance of gramicidin dimers is read out straightforwardly from experiments with small vesicles where Poisson`s distribution dominates the decay of electrochromic transients. There is good coincidence with the unit conductance determined by patch clamp. What about the distribution between membrane and water and the dimerization constant in a membrane with high protein contents as in thylakoids? The equilibration of gramicidin between thylakoids, its distribution between membranes and water, its dimerization constant in the membrane and the single-channel conductance has been studied with thylakoids (Schönknecht et al., submitted). When two suspensions of thylakoids are mixed, one with gramicidin and one without, it takes more than 20 min until the initially bipartite distribution of the antibiotic over vesicles is homogeneous. When gramicidin is added to a suspension under vigorous stirring, its distribution is homogeneous, right from the beginning. In a suspension with 20 μM chlorophyll (or a total protein contents of 200 μg/ml) less than 1% of the antibiotic is free in the suspending medium. There is evidence for higher concentration in appressed membranes than in stroma lamellae. As the dimer is the conducting species one expected a quadratic dependence of the decay of the electric potential difference on the concentration of gramicidin. This has only been observed at very low concentrations. Above a few hundred pM further increase is linear. This is indicative of complete dimerization. The concentration range with quadratic-to-linear transition gives information on the dimerization constant. For thylakoids from spinach it is calculated to be $5 \cdot 10^{10}$ $m^2$/mol, ten times larger than reported for phosphatidyl choline bilayers (Veatch et al.1975). About 60 % of the total surface of thylakoids is covered by protein and one-half of the lipid is supposedly in protein annuli (Murphy, 1986). This leaves about 20 % of the area to free lipid. If there is further upconcentration of gramicidin in appressed membranes, the factor of 10 in favour of dimerization is plausible. Since the dimerization of gramicidin is complete above a certain concentration, and as the dimer represents the "open-state" of this channel, the single-channel conductance can be derived from the above experiments. When the negative surface potential is shielded the figures range around 0.5 pS at 10 mM NaCl, in very good agreement with published data obtained on bilayers made from neutral lipids (Finkelstein & Andersen, 1981). In the absence of screening cations, however, the conductance in thylakoid membranes is strongly modulated by the surface potential at the electro-positive side of the membrane (the lumen side), with differences between plant species (see Schönknecht, 1990).



## 4 The transmembrane relaxation of the protonmotive force

When thylakoids are excited with a short flash of light an electrochemical potential difference of the proton is generated by vectorial electron transport plus protolytic reactions. The main pathways of discharge are the proton-specific pathways through the ATP synthase plus leak conductances for other ions. The intact enzyme conducts protons in a reaction that is coupled to ATP synthesis (see (Junesch & Graeber, 1987) for a review, and (Junge, 1987) for an analysis of proton flow under flashing light). If the channel portion of this enzyme, CFO, is exposed by removal of the catalytic F1-portion, the channel acts as an extremely proton-selective pathway with a conductance of at least 10 fS (Junge et al.1986) and probably much higher (Althoff et al.1989; Lill et al.1987). It is extremely selective for protons even at pH 8 and against a background of 300 mM of other cations like $Na^+$ (Althoff et al.1989). Fig. 6 illustrates the relaxation behaviour of the membrane in the presence of the large and specific proton conductance through CFO, plus the comparatively small leak conductance to other ions (left column) or with added gramicidin to drastically increase the conductance for $K^+$ (right column). The traces in the upper row show pH-transients in the medium, the ones in the middle row show electrochromic signals and the ones at the bottom pH-transients in the lumen of destacked thylakoids (see also (Schoenknecht et al.1986)). The scheme at the top helps to read these traces. Pairs of traces are superimposed, with one obtained before and the other one after addition of a blocking agent to proton transfer through CFO. The pair in the upper left, for instance, shows the transient proton uptake from the medium (upward rising) when the proton channel is blocked, on one hand, and the rapid overcompensation of this uptake, by proton outflux through the open channel, on the other. Since the electron acceptor has been chosen as to eliminate proton uptake at one of the two sites at the outer surface of the membrane (see Fig. 6, top), there are two protons released in the lumen per proton taken up from the medium and per two electrons charging the membrane electrically. Therefore, the initial alkalinization was overcompensated through the open channel.

We consider now the initial events as viewed by all three observables: A short flash of light acidifies the thylakoid lumen (bottom, left, with DCCD present), it charges the membrane electrically (middle) and alkalinizes the medium (top). With the blocking agent to the proton channel present, the pH-difference across the membrane relaxes more slowly than the electric potential difference, in more than 10 s (Schoenknecht et al.1986). This reflects the dominance of the conductance for ions other than the proton. With the channel conducting, however, all three observables decay rapidly. Broadly speaking, the differences between the traces with and without the blocking agent reflect: proton intake by CFO from the lumen (bottom), charge passage across the membrane (middle) and proton arrival in the medium (top) (see (Lill et al.1987; Schoenknecht et al.1986) for details). The first two observables have the same relaxation time and they are coincident in their extent (number of charges versus protons, not documented here, but see (Althoff et al.1989)). This relaxation reflects the discharge of the specific capacitance of the membrane by the dominating proton conductance. From the relaxation time the specific conductance of CFO is obtained by Equ. 3.

An analysis of the density of CFO-molecules which were exposed by removal of their CF1-counterpart has revealed the time-averaged conductance per channel, 10 fS (Schoenknecht et al.1986). This is equivalent to about 6000 protons at a driving force of 100 mV. We just mention that furthergoing studies demonstrate that not all exposed channels are conducting, so that the conductance of the active channels is much greater (Lill et al.1987; Althoff et al.1989).

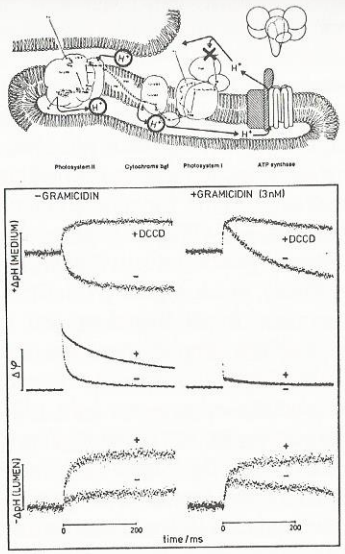


FIGURE 6. Flash-light induced transients of the pH in the outer medium (top traces), the transmembrane voltage (middle traces) and the pH in the lumen (bottom traces). Thylakoids were treated with EDTA to remove the catalytic portion of the ATP synthase in order to expose the channel portion CFO. The traces in the left column were obtained in the absence , those in the right column in the presence of the $K^+$-pore gramicidin (3 nM). Pairs of traces without and with DCCD (10 μM) to block proton conduction through CFO for details, see Schoenknecht, 1990).

The traces in the right column result from a similar experiment except for the addition of gramicidin to create a specific conductance for $K^+$ in great excess of the proton conductance. While the electric potential relaxes more rapidly by orders of magnitude the relaxation of the pH-transients is slowed down. In the framework of the linearized NernstPlanck Equation this can be understood as follows: With two dominating conduction pathways, for protons through CFO and for $K^+$ through gramicidin, the relaxation is governed by only five variables: the specific electric capacitance, C in $F \cdot m^{-2}$; the specific buffering capacitance (mainly of the lumen) for protons, $C_h$ in $F \cdot m^{-2}$; the specific storage capacitance for $K^+$, $C_k$ in $F \cdot m^{-2}$; the specific conductance for protons, $G_h$ in $S \cdot m^{-2}$; and the specific conductance for $K^+$, $G_k$ in $S \cdot m^{-2}$ (Junge and Schönknecht, in preparation). The definition of C, $G_h$ and $G_k$ is straightforward and the *chemical or storage capacitances* are:

(7) $C_h = ß \cdot F^2 \cdot V/(A \cdot 2.3RT)$ and

(8) $C_k = [K^+] \cdot F^2 \cdot V/(A \cdot RT)$

where ß denotes the differential buffering capacity as defined by:

(9) $ß = - dpH/d[H^+_{total}]$

$[K^+]$ is the average potassium ion concentration, V and A are volume and surface area of vesicles, *F* is the Faraday and RT are as usual. If the specific conductance for protons is larger than the one for any other ion (Fig. 6, left) one observes the discharge of the electric capacitance via the proton conductance and the relaxation time is given by Equ. 3. If the conductance for $K^+$ is much larger than the proton conductance (Fig. 6, right) the relaxation time of the pH-transient is governed by the chemical or storage capacitance for protons (Equ.s 7 and 9). If $C_k$ greatly exceeds $C_h$ the relaxation time is given simply by Equ. 10:

(10) $\tau = C_h/G_h$

If $C_k$ is of same order of magnitude as $C_h$ the relaxation is faster than given by Equ. 10.

Equ. 10 has been used to evaluate the specific buffering capacitance of thylakoid membranes around neutral pH (Schoenknecht, 1990). With about 7 $\mu F/cm^{-2}$, it is much larger than the specific electric capacitance, 1 $\mu F/cm^{-2}$ (Arnold et al.1985). The resulting figure for the buffering capacity ß compares well with published values (Walz, 1974; Haraux & de Kouchkowsky, 1979; Junge et al.1979) as determined by other techniques. The buffering capacitance increases from neutral to acid pH. It is obvious that the chemical storage capacitance of thylakoids for protons is larger than the electric capacitance of the membrane. Accordingly, transients of the chemical potential difference of the proton are more strongly damped than those of the electric potential difference. In this respect it is noteworthy that the major driving force for ATP synthesis in mitochondria is an electric potential difference while it is pH-difference in thylakoids. While mitochondria generate the electrochemical potential difference of the proton by substrate driven respiratory chain with only slow fluctuations of the substrate level, photosynthetic organisms (under water or under a cannopy of leaves) are subject to rapidly fluctuating supply of light. It is attractive to consider the greater damping of pH-transients as compared with electric transients as advantageous for the steady function of the ATP synthase.



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