J. Membrane Biol. 98, 69–78 (1987)

The Journal of
Membrane Biology

Analysis of Ionic Channels by a Flash Spectrophotometric Technique Applicable to Thylakoid Membranes: CF_0, the Proton Channel of the Chloroplast ATP Synthase, and, for Comparison, Gramicidin

Holger Lill, Gerd Althoff, and Wolfgang Junge
Biophysik, Fachbereich Biologie/Chemie, Universität Osnabrück, 4500 Osnabrück, West Germany

Summary. We previously introduced a flash spectrophotometric method to analyze proton conduction by CF_0 in vesicles derived from thylakoid membranes (H. Lill, S. Engelbrecht, G. Schönknecht & W. Junge, 1986, *Eur. J. Biochem.* **160**:627–634). The unit conductance of CF_0, as revealed by this technique, was orders of magnitude higher than that theoretically expected for a hydrogen-bonded chain. We scrutinized the validity of this method. Small vesicles were derived from thylakoids by EDTA treatment. The intrinsic electric generators in the membrane were stimulated by short flashes of light and the relaxation of the voltage via ionic channels was measured through electrochromic absorption changes of intrinsic pigments. The voltage decay was stimulated by a statistical model. As the vesicle-size distribution had only a minor influence, the simulation required only two fit parameters, the first proportional to the unit conductance of an active channel G, and the second denoting the average number of active channels per vesicle $\bar{n}$. This technique was applied to CF_0, the proton channel of the chloroplast ATP synthase, and to gramicidin, serving as a standard. For both channels we found the above two fit parameters physically meaningful. They could be independently varied in predictable ways, i.e. $\bar{n}$ by addition of known inhibitors of F_0-type proton channels and G via the temperature. For gramicidin, the unit conductance (2.7 pS) was within the range described in the literature. This established the competence of this method for studies on the mechanism of proton conduction by CF_0, whose conductance so far has not been accessible to other, more conventional approaches. The time-averaged unit conductance of CF_0 was about 1 pS, equivalent to the turnover of 6×10^5 $H^+/(CF_0 \cdot sec)$ at 100 mV driving force.

Key Words flash spectrophotometry · unit conductance · gramicidin · proton conduction · CF_0 · ATP synthase

Introduction

Bioenergetic membranes utilize the electrochemical potential difference of the proton for ATP synthesis (Mitchell, 1961; 1977). Comparison of the "coupling factors," F_0F_1[1], from a wide range of organisms (e.g. MF_0MF_1 from mammalian mitochondria, CF_0 CF_1 from chloroplasts of higher plants, EF_0EF_1 from *Escherichia coli* and TF_0TF_1 from the thermophilic bacterium PS3) reveals clear homology. They are built from two distinct portions, an intrinsic membrane protein, F_0 (*see* Hoppe & Sebald, 1984, for review), which is regarded as a proton-conducting device, and the extrinsic F_1 which contains the nucleotide binding sites (*see* e.g. Vignais & Satre, 1984).

The nature and properties of the proton pathway through F_0 have been intensively studied. The genetic approach has substantiated speculation about a membrane-spanning "proton wire," constructed from various polar amino-acid residues interconnected by a chain of hydrogen bonds (Cain & Simoni, 1986; Kumamoto & Simoni, 1986). The conduction properties of hydrogen-bonded chains have been theoretically evaluated (Brünger, Schulten & Schulten, 1983; Nagle & Tristram-Nagle, 1983). On the other hand, it has been speculated that proton conduction is brought about by rotatory motion of subunits against each other (Mitchell, 1985; Cox et al., 1986). F_0 channels have been purified and functionally reconstituted into lipid vesicles (Schneider & Altendorf, 1982). The unit conductance of F_0, inferred from measurements of proton efflux from reconstituted vesicles seemed, however, orders of magnitude too low. Reported turnover numbers of 2 to 7 $H^+/(EF_0 \cdot sec)$ (Negrin, Foster & Fillingame, 1980; Friedl & Schairer, 1981; Schneider & Altendorf, 1982) or 47 $H^+/(TF_0 \cdot sec)$ (Sone, Hamamoto & Kagawa, 1981) are hardly sufficient for supporting rapid ATP synthesis. The highest turnover number of the ATP synthase in chloroplasts, for instance, 400 ATP/$(CF_0CF_1 \cdot sec)$ (Junesch & Gräber, 1985), required a turnover number of the proton channel of at least 1200 $H^+/(CF_0CF_1 \cdot sec)$ under the probable stoichiometry of 3 H^+/ATP (Junge, Rumberg & Schröder,

[1] *Abbreviations:* F_1: coupling factor 1; F_0: coupling factor 0; DCCD: *N,N'*-dicyclohexylcarbodiimide; TPT: triphenyltin-chloride.

1970; Davenport & McCarty, 1981). If the only function of the channel portion is to give protons unhindered access to the coupling site, located in F_1, the turnover number of the bare channel, F_0, would be expected to exceed that of the coupled enzyme by orders of magnitude. Seemingly, this was not the case.

We previously assessed the protonic unit conductance of CF_0 in thylakoid membranes using flash-spectrophotometric techniques. When assuming that all CF_0 which were exposed by extraction of their counterpart, CF_1, were conducting, and unit conductance of F_0, inferred from measure-thylakoids we obtained a unit conductance of 9 fS, equivalent to a turnover number of 6200 $H^+/(CF_0 \cdot$ sec) at 100 mV driving force (Schönknecht et al., 1986). This figure was more satisfying in the light of the supposed function. However, it represented only a lower limit. A closer look revealed that about half of the vesicles which had lost about 30 CF_1 molecules, in the average, remained proton tight. This implied that only a few percent of exposed CF_0 were actually conducting. By statistical analysis we determined the turnover number of the few active channels to be about 10^5 $H^+/(CF_0 \cdot$ sec) (Lill et al., 1986). This was amply sufficient for functioning as a proton well.

The advantage of thylakoids in studies aimed at determining the conductance of a F_0 channel is the possibility to generate protonic charge pulses by excitation with short flashes of light and to follow their decay by spectrophotometrical methods (reviewed in Junge, 1982). It is also favorable that proton flow can be completely tracked by independent measurements of voltage transients and of pH transients in both aqueous phases separated by the thylakoid membrane (Schönknecht et al., 1986). This gives access to the *proton-specific* conductance of thylakoids in addition to the overall conductance. In the above cited work we have analyzed the pulse decay in terms of a statistical model describing the electric relaxation in vesicles carrying very few active channels, distributed according to Poisson's distribution (Schmid & Junge, 1975; Lill et al., 1986). Here, we present a critical evaluation of this approach, which was extended to explicitly consider the vesicle-size distribution. It was applied to CF_0, as previously, and to gramicidin, for comparison with an ion channel with known properties. We demonstrated that the phenomenological parameters used for fitting the data, G and $\bar{n}$, had the assumed physical meaning, since they could be varied independently and in the expected way. They stood for the unit conductance of active channels and the average number of active channels per vesicle, respectively. Calculation of the unit conductance from the data required information on the vesicle area, which could be calculated from the experiments with gramicidin. It also required an estimate of the specific electric membrane capacitance. We assumed 1 $\mu F/cm^2$, as for many biological membranes. For gramicidin this produced a unit conductance within the limits of published figures. This, in turn, was taken as proof that the method was quantitative and that it could be used to determine the unit conductance of CF_0.

Materials and Methods

Broken pea chloroplasts were prepared according to the procedure for "stacked thylakoids" as in Polle and Junge (1986) except that Mg^{2+} was omitted in the final suspending medium. Thylakoids were stored as concentrated stock suspensions (2 to 3 mg chlorophyll/ml) on ice for up to 6 hr before use. They were stable during this interval. CF_1 depletion was carried out at room temperature in moderate light. Thylakoids were diluted to 10 μM chlorophyll in a solution containing 100 μM EDTA, 1 mM NaCl, 1 mM tricine/NaOH, pH 7.8 for variable incubation times. Release of CF_1 was stopped by adding 1 M NaCl to yield a final Na^+ concentration of 30 mM. The vesicles were spun down by a 20-min centrifugation at 30,000 × g (4°C) and resuspended in the same medium as used for the final resuspension after preparation: 10 mM NaCl, 100 mM sorbitol, 10 mM tricine/NaOH, pH 7.8.

Flash spectrophotometric measurements at room temperature were performed in the same setup as described previously (Junge, 1976; Förster, Hong & Junge, 1981). The measuring cuvette (2 cm optical pathlength, volume 15 ml) contained 10 μM chlorophyll, 10 μM methyl viologen, 10 mM NaCl, and 1 mM tricine/NaOH, pH 7.5. The suspension was excited with short (15 μsec) and saturating flashes of red light ($\lambda > 610$ nm, 1 mJ/cm^2) at 5-sec intervals. The decay of the electrical potential across the thylakoid membrane was measured via the electrochromic absorption changes of intrinsic pigments at 522 nm wavelength (Junge & Witt, 1968; Witt, 1979). Twenty signals were averaged at a digitalization time of 20 μsec per point. Measurements under variation of the temperature were performed in a 1-ml cuvette with an optical pathlength of 1 cm and with the chlorophyll concentration raised to 20 μM. Thermostating was precise within 0.5°C tolerance. The exciting flash was delivered by a ruby laser (Q switch, 2 mJ/cm^2).

Computer-aided curve fitting was performed on a DEC-PDP 11/34. Digitalized traces of electrochromic absorption transients were stored on disk. To facilitate data handling, the experimental information was contracted by averaging eight neighboring data points, spaced 200 μsec apart and starting from time zero when the exciting flash was fired. The averaged points were submitted to a fit routine based on the simplex algorithm (Caceci & Cacheris, 1984). The accuracy of the fits was controlled on-line on the display screen.

The inhibitors of F_0, N,N'-dicyclohexylcarbodiimide (DCCD) and triphenyltin chloride (TPT), as well as gramicidin were added from ethanolic stock solution. The ethanol concentration in the measuring cuvette was held below 0.5%. With DCCD, a 10-min incubation at room temperature in the dark was performed prior to measurement. No preincubation was necessary with TPT. DCCD and gramicidin were purchased from Sigma and TPT was from Fluka. According to the manufacturer

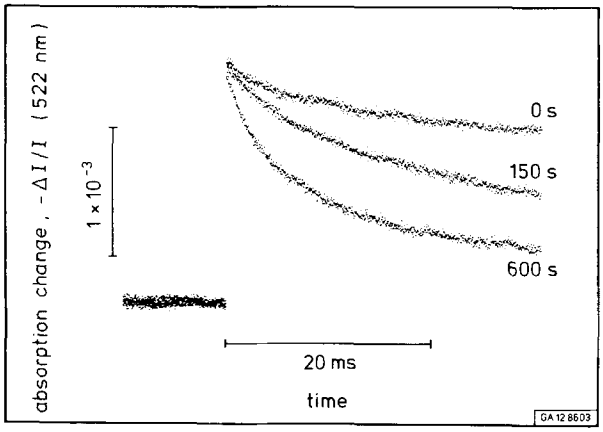

Fig. 1. Electrochromic absorption changes after single flash excitation. For CF_1 extraction, thylakoids were incubated in hypoosmolar EDTA solution. At indicated times, 1 M NaCl was added to yield a final Na^+ concentration of 30 mM. After centrifugation, thylakoids were resuspended and diluted to 10 μM chlorophyll. 20 signals were averaged; digitization time was 20 μsec per point

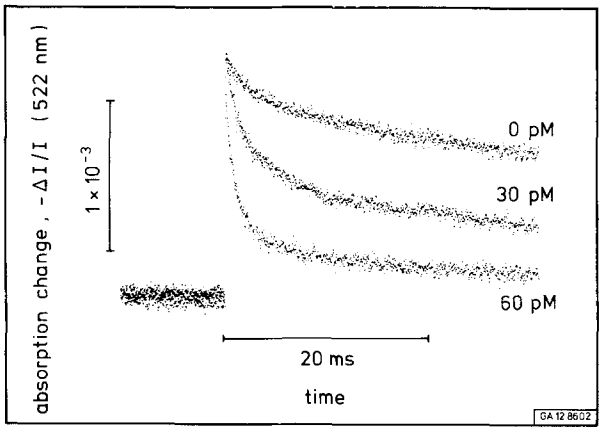

Fig. 2. Electrochromic absorption changes in the presence of gramicidin. Thylakoid vesicles (600-sec EDTA incubation, *see* trace marked "600 sec" in Fig. 1) were incubated for 10 min with 25 μM DCCD in the measuring solution. Indicated amounts of gramicidin were added prior to measurement and absorption changes at 522 nm after single flash excitation were recorded as in Fig. 1

gramicidin contained 87.5% gramicidin A, the channel-forming component with reference data available. Further components were 7.1% gramicidin B, 5.1% gramicidin C, and 0.3% gramicidin D.

Results

The Biphasic Decay of the Electrochromic Absorption Changes Caused by CF_0 or by Gramicidin

To expose the proton channel CF_0 by extraction of its counterpart CF_1, thylakoids were incubated in a medium containing EDTA as in Lill et al. (1986). A favorable side effect of the extraction procedure was the formation of smaller vesicles which contained about 10^5 to 10^6 molecules of chlorophyll. We previously found that these CF_1-depleted vesicles were divided into two classes; one still proton tight and competent in photophosphorylation, and the other leaky to protons and unable to synthesize ATP. Increasing degrees of CF_1 extraction induced by prolonged incubation time shifted the proportion between the two classes towards the leaky one. One possible interpretation was that out of a total of about 100 CF_0CF_1 per vesicle, and with 30 CF_0 exposed by removal of their CF_1 counterpart, in some vesicles only one CF_0 was active as proton channel. In other vesicles none of the exposed CF_0 was active (Lill et al., 1986).

Figure 1 shows the flash-induced generation of the transmembrane voltage and its decay in three samples differing in their incubation time in hypoosmolar EDTA solution (0, 150 and 600 sec). Extraction degrees ranged between 0 and 60% of original CF_1 as determined by electro-immunodiffusion as in Lill et al. (1986). The biphasic decay of the electrochromic absorption changes and the increased proportion of the rapid phase in response to longer incubation times was apparent. The dominating charge carrier of the rapid decay was in fact the proton as checked by parallel measurements of pH transients in both aqueous phases (*data not shown,* but *see* Schönknecht et al., 1986). Figure 2 demonstrates similar biphasic decay, but here it was induced by the addition of gramicidin. These traces were obtained with a sample which, by 600-sec incubation in EDTA solution, was leaky to protons (*see* respective trace in Fig. 1), but which was then further incubated for 10 min with 25 μM DCCD. This agent binds covalently to a single acidic residue in CF_0 (Sigrist-Nelson, Sigrist & Azzi, 1978). The upper trace in Fig. 2 revealed that the conductance of open CF_0 was blocked by DCCD (*compare* with lower trace in Fig. 1). The other two traces in Fig. 2 (bottom) show the reacceleration of the decay by added gramicidin. The concentration of gramicidin refers to the monomer.

Whereas the rapidly decaying component was appreciably faster than in Fig. 1, a similar biphasicity as in Fig. 1 was apparent. The increase of the gramicidin concentration increased the proportion of the rapid phase. At these very low concentrations the vesicles remained proton tight (checked via measurements of pH transients, *data not shown,* but *see* Schönknecht et al., 1986). This was

expected as the extra conductance induced by gramicidin was due to translocation of Na^+, the dominant alkaline cation in the medium. Broadly speaking the biphasic decay of the electrochromic absorption changes (Fig. 2) at low concentrations of gramicidin could be interpreted as follows: at 30 pM gramicidin about every second vesicle had received one gramicidin channel and at 60 pM it was about every vesicle. Further increase of the concentration left the decay approximately uniphasic [except for a minor nonelectrochromic background transient known from previous work (Junge & Witt, 1968)] with the decay rate proportional to the gramicidin concentration over several orders of magnitude (Schönknecht & Junge, *unpublished*). The transition from a biphasic to an approximately uniphasic decay at 60 pM served to obtain a semiquantitative estimate for the vesicle size: 60 pM gramicidin implied 30 pM gramicidin dimers. It is well documented that a pore is only formed by the dimer (Urry et al., 1971). With 10 μM chlorophyll in the sample and neglecting the statistical complications (channels distributed over vesicles, vesicles with different size) and assuming that each dimer was actually conducting (*see* Schmid & Junge, 1975) we estimated a vesicle-size equivalent to 3×10^5 chlorophyll molecules. With the accepted figure for the average membrane area per chlorophyll molecule, 2.2 nm^2 (Thomas, Minnaert & Elbers, 1956) the vesicle area became 0.66 μm^2, equivalent to a sphere with radius $\approx$230 nm. When the same experiment was performed with control thylakoids (no incubation in EDTA buffer) it yielded a value of 1 mol gramicidin dimer per $>10^7$ mol chlorophyll (G. Schönknecht, *personal communication*). Apparently, the incubation of thylakoids in hypoosmolar EDTA-containing buffer had caused their disintegration and resealing as smaller vesicles.

For the above gross estimate of the vesicle size we ignored the distribution of conducting channels over vesicles and we also ignored the distribution of vesicles over a size spectrum. This was explicitly mended in the following paragraph.

Theoretical Description of Optically Detected Charge-Pulse Relaxation in a Population of Vesicles Covering a Gaussian Size Spectrum and Doped with about One Active Ion Channel per Vesicle

Following previous considerations by Schmid and Junge (1975) and by Apell and Läuger (1986), we consider a population of vesicles covering a certain size spectrum. It is assumed that some vesicles contain one or a few ionic channels of ohmic behavior. At time zero a charge pulse induces the same starting voltage in each vesicle irrespective of the particular vesicle size. The vesicle capacitance is then discharged via the above ionic channel and the intrinsic leak conductance of the vesicle membrane. We assume that the leak conductance, i.e. the conductance which is due to any other conducting mechanism apart from the above ionic channel, is negligible. The voltage decay in a given vesicle with n channels is then monoexponential:

$$U(n, t) = U_0 \cdot \exp\left(-n \cdot \frac{G}{C} \cdot t\right) \tag{1a}$$

wherein G is the time-averaged unit conductance of a channel, C the electric capacitance of the vesicle and U_0 the starting voltage. With a vesicle having a surface area A and with $\hat{c}$ denoting the specific electric capacitance of the vesicle membrane:

$$U(n, A, t) = U_0 \cdot \exp\left(-n \cdot \frac{G}{A \cdot \hat{c}} \cdot t\right). \tag{1b}$$

The voltage decay can be optically detected by measurements of electrochromic absorption changes as e.g. in Junge and Witt (1968), Schmid and Junge (1975); reviewed in Junge (1982). In a typical experiment with vesicles derived from thylakoids the monitoring beam of the spectrophotometer probes a sample of 10 ml containing 10 μM chlorophyll. With an average vesicle size equivalent to about 3×10^5 chlorophyll molecules there are 2×10^{11} vesicles in the beam, a very large number, indeed. The observed decay of the electrochromic absorption changes reflects the exponential decay in each vesicle (according to Eq. 1) folded with the statistical properties of the population in two domains: 1) the distribution of channels over a subpopulation of given vesicle size, and 2) the size distribution of vesicles. To separate these two domains we classify the vesicles into subsets each of which contains vesicles of one size with surface area A. At first we consider only one of these subsets with an average number of channels per vesicle $\bar{n}$:

$$\bar{n} = A \cdot \chi \tag{2}$$

wherein χ denotes the average surface density of active channels and A is the surface area of a vesicle in the chosen subset. With many places per vesicle to accommodate channels but few active channels in the average the distribution follows Poisson's law:

$$P(n, A) = \frac{\bar{n}^n \cdot \exp(-\bar{n})}{n!} \tag{3}$$

wherein $P(n, A)$ denotes the probability to find n channels in a vesicle of surface area A and with $\bar{n}$ depending on the vesicle area as in Eq. (2).

Proceeding to the next level of complexity we consider several subsets of vesicles by assuming that their size distribution follows a Gaussian law in the domain of area:

$$W(A) = \frac{1}{\sigma \cdot \sqrt{2\pi}} \cdot \exp(-(A - \bar{A})^2/2\sigma^2) \quad (4)$$

wherein $W(A)$ denotes the probability to find vesicles with area A, $\bar{A}$ is the mean area and σ the standard deviation from the mean.

The apparent voltage decay as measured via electrochromic absorption changes is the average over contributions from all vesicles:

$$U_{app}(t) = \int_{A=0}^{\infty} W(A) \cdot \sum_{n=0}^{\infty} P(n, A) \cdot U(n, A, t) \cdot dA. \quad (5)$$

At first, we evaluate the sum while keeping A constant:

$$\begin{aligned} &\sum_{n=0}^{\infty} P(n, A) \cdot U(n, A, t) \\ &= \sum_{n=0}^{\infty} \frac{\bar{n}^n}{n!} \cdot \exp(-\bar{n}) \cdot U_0 \cdot \exp\left(-n \cdot \frac{G}{A \cdot \hat{c}} \cdot t\right) \\ &= U_0 \cdot \exp(-\bar{n}) \sum_{n=0}^{\infty} \frac{1}{n!} \cdot \left[\bar{n} \cdot \exp\left(-\frac{G}{A \cdot \hat{c}} \cdot t\right)\right]^n \\ &= U_0 \cdot \exp(-\bar{n}) \cdot \exp\left(\bar{n} \cdot \exp\left(\frac{G}{A \cdot \hat{c}} \cdot t\right)\right). \end{aligned} \quad (6)$$

Insertion of Eq. (2) into Eq. (6) and consideration of Eqs. (4) and (5) yields:

$$\begin{aligned} U_{app}(t) = U_0 \int_{A=0}^{\infty} dA \Bigg\{ &\frac{1}{\sigma \cdot \sqrt{2\pi}} \\ &\cdot \exp(-(A - \bar{A})^2/2\sigma^2) \Bigg\} \cdot \exp(-A \cdot \chi) \\ &\cdot \left\{\exp\left(A \cdot \chi \cdot \exp\left(\frac{G}{A \cdot \hat{c}} \cdot t\right)\right)\right\}. \end{aligned} \quad (7)$$

If the vesicle size is uniform, i.e. $W(A) = \delta(A - \bar{A})$ with δ being Dirac's function, Eq. (7) is simply

$$U_{app}(t) = U_0 \cdot \exp(-\bar{n}) \cdot \exp\left(\bar{n} \cdot \exp\left(-\frac{G}{A \cdot \hat{c}} \cdot t\right)\right). \quad (8)$$

This resembles the equation derived in Schmid and Junge (1975) and used in Lill et al. (1986). It contains only two fit parameters, namely $\bar{n}$, the average number of channels per vesicle, and k, the decay rate caused by a single channel:

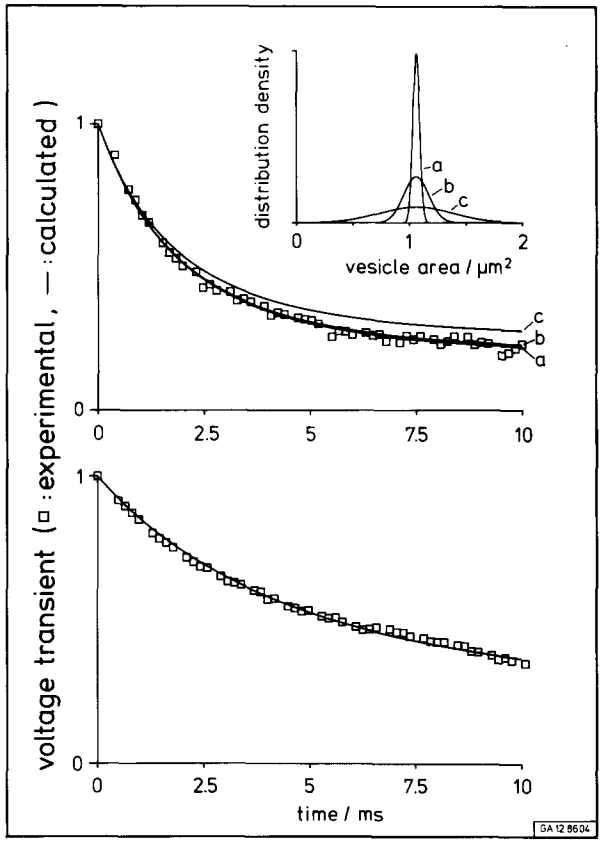

Fig. 3. Analysis of electrochromic decay by computer-aided fit routines. *Top:* Data from Fig. 2 (60 pM gramicidin). *Bottom:* Data from Fig. 1 (600-sec EDTA incubation). 50 points each were extracted at 200-μsec time intervals from the respective original traces. Each square represents eight points of the respective original curve, averaged by an extraction computer program in order to obtain one point as submitted to the fit program. *Insert:* Size distribution density $W(A)$ as function of the vesicle area under variation of the standard deviations from the mean (1.1×10^{-8} cm^2). Standard deviations assumed for simulation of the upper traces were: (*a*): 3%, (*b*): 10%, and (*c*): 30%

$$k = \frac{G}{A \cdot \hat{c}}. \quad (9)$$

If the vesicle area and the specific capacitance are known the time-averaged unit conductance of the ionic channel G can be calculated from the experimentally determined value of k.

Fitting the Biphasic Decay of the Electrochromic Absorption Changes with the Theoretical Model. The Influence of the Vesicle-Size Distribution

The decay of transient electrochromic absorption changes reflects the voltage decay averaged over all vesicles as modelled in Eqs. (7) or (8). In the upper part of Fig. 3, contracted data points as represented

Table. Absolute values for fits shown in Figure 3[a]

Trace	$\bar{n}_{corr}$	G (pS)	$\sigma \times 10^{-3}$
"60 pM"	1.46	2.5	2.6
"600 sec"	1.50	1.1	2.0

[a] σ in the last column denotes the standard deviation of the respective fit calculated from the sum of the least-squares roots. For further details, *see* text and legend to Fig. 3.

by squares were extracted from the measured decay curve in Fig. 2 with 60 pM gramicidin present. Neglecting the size distribution, forehand, and by applying Eq. (8) we obtained a best fit as given by the solid line (*a*). The parameter settings in the best fit to these particular data were as follows: $\bar{n}_{app} = 1.89$ and $k = 234$ sec^{-1}. We had to consider that, even without gramicidin added, some vesicles were already leaky despite the incubation with DCCD (*see* Fig. 2, upper curve). Submission of the upper curve in Fig. 2 to the same computer analysis gave $\bar{n}_0 = 0.43$. This had to be subtracted from the apparent $\bar{n}_{app}$ in order to obtain the corrected $\bar{n}_{corr} = 1.46$.

We assessed the influence of the vesicle-size spectrum on the quality of this fit. Simulation of the decay by Eq. (7a) required information on the vesicle area A and on the channel density χ. For gramicidin, χ could be calculated from the ratio of mol gramicidin dimer over mol chlorophyll, here 30 pM/10 μM divided by the area per chlorophyll molecule, 2.2 nm^2, as cited above. It was 1.36×10^8 channels per cm^2. According to Eq. (2) the average vesicle area was then $\bar{A} = 1.07 \times 10^{-8}$ cm^2, about twice the above semiquantitative estimate, where we neglected the statistical properties of the vesicle suspension. Inserting this and the fit value of k into Eq. (9) we calculated the ratio of the single-channel conductance over the capacitance: $G/\hat{c} = 2.5 \times 10^{-6}$ cm$^2 \cdot$ sec^{-1}. By inserting this into Eq. (7) we recalculated decay curves but now under variation of σ, the standard deviation from the mean vesicle area. The integration was carried out numerically. In the upper part of Fig. 3, the calculated decay curves were drawn as lines (*b*) and (*c*). The respective values of σ were: (*a*) 3%, (*b*) 10%, and (*c*) 30% of the mean vesicle area. In the insert of Fig. 3, the size distributions assumed in these simulations were visualized. The smaller two standard deviations yielded decay curves almost indistinguishable from each other and from the curve yielded by the less refined model according to Eq. (8). It was apparent that only the rather wide spread caused an appreciable deviation from the simpler fit curve which ignored any size distribution. The very small influence which was exerted by the width of the vesicle-size distribution justified its omission. Consequently, we used the simpler Eq. (8) instead of the more complete Eq. (7) throughout the following analysis.

The computer-assisted fit to the average voltage decay via CF_0 is represented in Fig. 3 (lower trace). The data were taken from a sample which was incubated for 600 sec in EDTA-containing buffer (*see* the respective trace in Fig. 1). The fit parameters used to calculate the traces in Fig. 3 were summarized in the Table. It turned out that the average number of channels was nearly the same. With A and $\hat{c}$ being the same in both experiments on gramicidin and on CF_0, the single-channel conductance of gramicidin was about three times greater than that of CF_0.

The Single-Channel Conductance of Gramicidin and of Open CF_0

The numerical fit of the observed transients of electrochromic absorption changes by Eq. (8) directly produced the parameters k and $\bar{n}$. The unit conductance G could be calculated from k, if the average vesicle area $\bar{A}$ and the specific capacitance of the membrane $\hat{c}$ were known (*see* Eq. (9). We tend to rely on the above estimate of $\bar{A}$, about 1.1×10^{-8} cm^2. The estimate for $\bar{A}$ was based on the action of gramicidin. We assumed that no significant amount of this agent was lost or adsorbed to sites where it could not contribute to ion conduction. This seemed warranted by the extremely low concentration at which it started to act on the voltage decay. If the concentration was further increased, the concentration dependence of the decay rate was proportional to the dimer concentration over more than six orders of magnitude (Schönknecht et al., *in preparation*). Where the specific capacitance of biological membranes has been determined it was close to 1 μF/cm^2 (Hille, 1984). Taking this figure for the thylakoid membrane and also $\bar{A}$ and k as above, according to Eq. (9) the unit conductance of gramicidin in the thylakoid membrane was 2.5 pS. Correspondingly, as the experiments with gramicidin and with CF_0 were carried out on the same vesicle population, which implied the same $\bar{A}$ and the same $\hat{c}$, the unit conductance of CF_0 was 1.1 pS (*see* Table).

The Effect of Operations which were Expected to Act Either on the Single-Channel Conductance or on the Number of Channels per Vesicle

Similar data for CF_0 and for gramicidin, as those exemplified in Figs. 1 and 2, were analyzed for G and $\bar{n}$. We increased the number of channels per

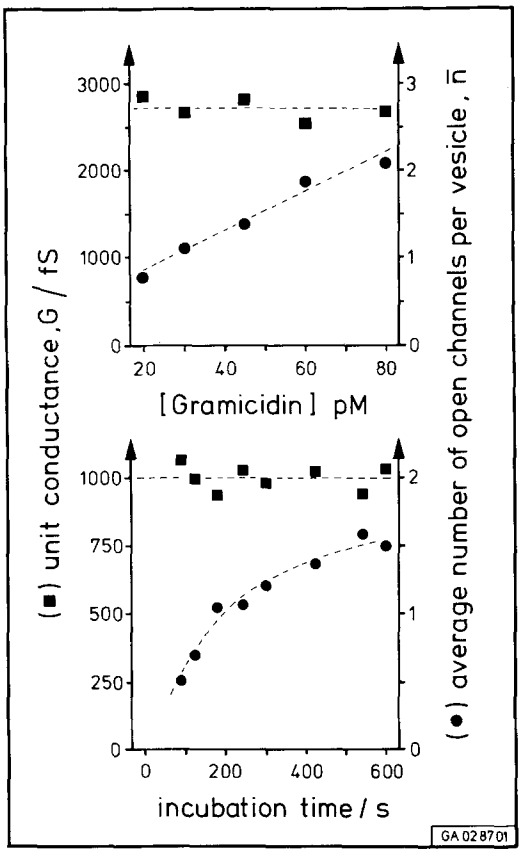

Fig. 4. Effects of increasing gramicidin concentration and prolonged incubation time of thylakoids in EDTA medium on G and $\bar{n}$. A set of traces obtained under identical conditions as in Fig. 2 has been subjected to the extraction and fit procedures (*cf.* Fig. 3). *Top:* Results obtained by increasing concentrations of gramicidin. The calculated unit conductance (rectangles), and the calculated average number of active channels per vesicle (circles) are shown as a function of gramicidin concentration. *Bottom:* Results obtained after various incubation times of thylakoids in EDTA medium. Symbols as in the upper part of the Figure

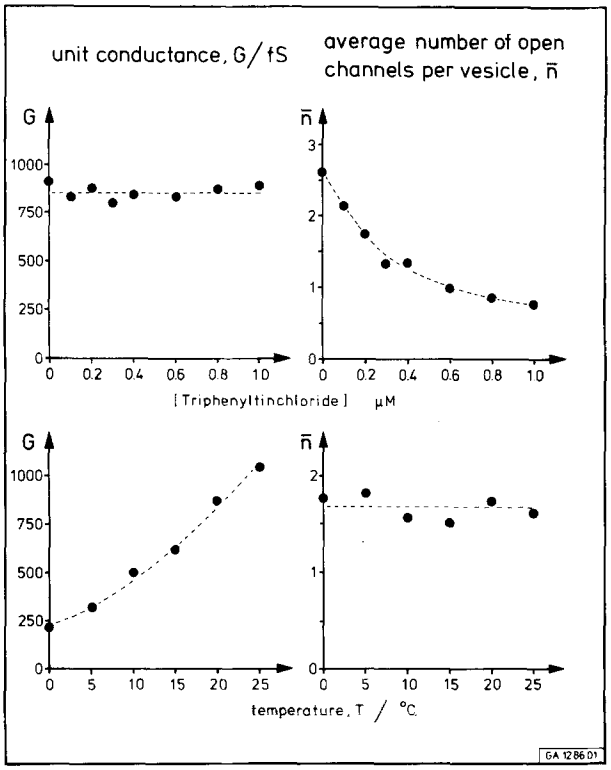

Fig. 5. Unit conductance and number of active CF_0 as functions of inhibitor concentration and temperature. In the upper part of the Figure, results obtained by fitting traces as recorded in the presence of various concentrations of TPT are shown. The lower part of the Figure depicts the respective parameters as a function of temperature. Thylakoids as in Fig. 1, "600 sec"

vesicle for CF_0 by increasing the incubation time in EDTA medium, and for gramicidin by increasing the concentration. The result of the analysis of original data as shown in Fig. 2 was plotted in Fig. 4. In both cases the average number of channels increased, as expected (*see* filled circles). But the single-channel conductance remained about constant, averaging 2.7 ± 0.15 pS for gramicidin and 1.0 ± 0.05 pS for CF_0.

In the reverse direction, we expected to decrease the number of conducting CF_0 channels by adding the CF_0 inhibitor, triphenyltinchloride (TPT). Although probably not binding covalently to F_0 channels (Cain, Partis & Griffiths, 1977), this reagent shows an even higher specificity for CF_0 than DCCD (Gould, 1976). The number of conducting channels was changed from 2.6 to 0.8 without change in the unit conductance (Fig. 5, upper part). We attempted to vary the unit conductance of CF_0 by lowering the temperature. The result, shown in Fig. 5 (lower part), was a change in the unit conductance from 1.1 to 0.2 pS without change in the average number of active channels per vesicle. It is obvious that $\bar{n}$ and G were parameters which were physically meaningful in the expected way.

Discussion

For many years studies on mechanisms of ion transport across biomembranes were focused on ion-transporting antibiotics as model compounds and mostly in model membranes. Technical progress in electrophysiology (*see* Hille, 1984) has opened the way to analyze the electrical behavior of various ion-conducting proteins. Patch-clamp techniques (Neher & Sakmann, 1976), in particular, yielded insight into the gating behavior of single channels. Limited sensitivity, about 500 fS, however, has not permitted their application to the proton channels, F_0, of ATP synthases for which a unit conductance in the order of 0.1 fS was reported in the literature

(Negrin *et al.*, 1980; Friedl & Schairer, 1981; Schneider & Altendorf, 1982; Sone et al., 1981). Recently we attempted to determine the unit conductance of CF_0, the respective channel of chloroplasts, by flash spectrophotometric methods. We measured the decay of the flash light-induced voltage in vesicles derived from thylakoids. Under the assumption that all CF_0 molecules which were deprived of their catalytic counterpart, CF_1, were proton conducting, a minimal conductance of 9 fS was calculated (Schönknecht et al., 1986). Re-examinating this assumption, we showed that only 3% of the exposed CF_0 were active (Lill et al., 1986). By statistical treatment of the data we calculated a unit conductance of 169 fS equivalent to the passage of 10^5 $H^+/(CF_0 \cdot sec)$ at 100-mV membrane potential. This result had two aspects: 1) It was satisfying that the *protonic unit conductance* was large enough for the supposed function as a *proton well.* 2) On the other hand, the unit conductance was much larger than theoretically expected. Under reasonable assumptions and for a hydrogen-bonded chain with only four amino-acid residues, Brünger et al. (1983) have calculated a turnover number in the order of some 10^3 $H^+ \cdot sec^{-1}$.

The discrepancy between the measured turnover number and the one expected for a hydrogen-bonded chain has to be resolved. It is worth studying the time-averaged unit conductance of CF_0 as a function of the pH, the temperature, the isotopic substitution of D^+ for H^+, and the viscosity of the membrane. The flash spectrophotometric method seemed appropriate both by sensitivity and because it is tolerant against variation of the environmental parameters in a wide range (e.g. measurements with CF_0 at −20°C have been carried out already). But was it valid and was it quantitative? The use in thylakoids (Junge & Witt, 1968) and in bacterial chromatophores (Jackson & Crofts, 1969) of electrochromic absorption changes of intrinsic pigments as linear indicators of the transmembrane voltage is accepted and the underlying physical mechanism has been reviewed (Witt, 1975; Junge & Jackson, 1982). Therefore, we concentrated on an assessment of the validity of our physical model and of the precision of the analysis of electrochromic absorption changes to yield the time-averaged unit conductance of ionic channels.

We treated the distribution of channels over vesicles of a given size class by Poisson's law and we assumed the distribution in the domain of vesicle area to be Gaussian. We found that the width of the vesicle-size spectrum had only a very small influence. This left us with a comparatively simple decay law, Eq. (8). It incorporated only two fit parameters, the mean number of active channels per vesicle $\bar{n}$, and the decay rate caused by a single channel k (*see* Schmid & Junge, 1975). We expected that k was equal to the unit conductance of this channel over the vesicle area A times the specific capacitance $\hat{c}$. This was established by experiments with operations expected to act either on $\bar{n}$ or on G. Indeed, the expected behavior was reproduced by the data analysis. To calculate the time-averaged unit conductance of a channel from k, however, one had to rely on estimates of A and of $\hat{c}$. Therefore, the unit conductance had, at best, the same degree of confidence as these estimates.

We checked the degree of confidence by applying this analysis to gramicidin. This antibiotic is perhaps the best-characterized molecular ion pore (Hille, 1984). We showed previously, that gramicidin binds practically irreversibly to thylakoid membranes (Schmid & Junge, 1975) where it acts as an ion pore at extremely low concentration. We calculated the mean vesicle area under the assumption that every gramicidin dimer in the vesicle suspension was acting as a pore. This figure, 1.1×10^{-8} cm^2, was a lower estimate. We assumed a specific capacitance of 1 μF for the thylakoid membrane. For gramicidin the analysis yielded a unit conductance of 2.5 pS. How does this compare with values published in the literature?

The time-averaged unit conductance of gramicidin A follows a Michaelis-Menten-type behavior as a function of the surface concentration of Na^+:

$$G = G_m \cdot \frac{[Na^+]_s}{[Na^+]_s + K_m}. \tag{10}$$

The unit conductance at saturating concentration of Na^+ varies in the literature (figures in pS, lipids given): 12 (Bamberg & Läuger, 1974—dioleoyllethicin); 14.6 (Finkelstein & Andersen, 1981—phosphatidylethanolamine/decane) and 24 (Hladky & Haydon, 1973—glycerolmonooleate). The K_m in a phosphatidylethanolamine/decane membrane is 310 mM (Finkelstein & Andersen, 1981). If this membrane is formed from pure material, it has virtually no surface potential (Szabo et al., 1972). This implies that the above K_m describes the half-saturation of the channel in terms of the surface concentration of Na^+. We calculated an expectation value of the unit conductance of gramicidin in the thylakoid membrane and for our experimental conditions according to the following considerations: 1) G_m is independent of the surface potential (Apell, Bamberg & Läuger, 1979). Irrespective of the question whether or not the factor-of-two discrepancy in the literature is caused by different lipids, we took a figure of 16 ± 8 pS as a reasonably wide estimate valid for thylakoid membranes. 2) The surface con-

centration of Na^+ at the lumenal side of the thylakoid membrane was calculated by taking the negative surface potential at the lumenal side of the membrane into account. [Note that it was the lumenal side of the membrane which was positively charged by flash excitation of thylakoids in our experiments (Junge, 1982).] The unit conductance was then given by Eq. (10) but with the surface concentration $[Na^+]_s$ related to the Na^+ concentration in the medium $[Na^+]_o$ as follows:

$$[Na^+]_s = [Na^+]_o \cdot \exp\left(-\frac{F \cdot \phi_s}{RT}\right) \quad (11)$$

wherein ϕ_s denotes the surface potential. The surface charge density at the lumenal side of the thylakoid membrane was taken from the literature. At about neutral pH it was $-0.9\ \mu C \cdot cm^{-2}$ at the oxidizing side of the photosystem I-complex and in sonicated chloroplasts (Itoh, 1979) and $-1.3\ \mu C \cdot cm^{-2}$ in the average as apparent from the redistribution of K^+ and Cl^- between lumen and medium in the dark (Rumberg & Muhle, 1976). We took the latter figure as a reasonable estimate. We calculated the expected surface potential according to the Gouy-Chapman equation (*see* McLaughlin, 1977, for review) and for 10 mM Na^+ in the medium. At 20°C a value of 46 mV was obtained. This agreed well with our previous measurement of the surface potential at the lumenal side of the membrane (Hong & Junge, 1983). It raised the surface concentration of Na^+ from 10 mM in the medium to 61 mM at the surface. Insertion of the above figures for G_m and $[Na^+]_s$ into Eq. (10) gave the expectation value for the unit conductance of gramicidin in the thylakoid membrane, $G_{expect} = 2.6 \pm 1.3$ pS. It was very satisfying that the experimentally determined value 2.7 ± 0.15 pS fell into this range. Conservatively speaking it may be safe to assume that the flash spectrophotometric analysis produced the unit conductance of gramicidin within ±50% error.

The experiments aimed at CF_0 were performed with the same thylakoid-derived vesicles as those aimed at gramicidin. Therefore, it was safe to assume that the vesicle capacitance $A \cdot \hat{c}$ was the same in both sets of experiments. This allowed the calibration of the unit conductance of CF_0 against that of gramicidin. The unit conductance of CF_0 was about 1 pS, again reliable within ±50%. In a previous manuscript we reported a unit conductance of 169 fS. In that work, a calibration against gramicidin was not performed. Moreover, the computer fit was extended over a wider time range, 30 msec instead of 10 msec in this manuscript. Thereby the fit also included an intermediate decay process, also attributable to CF_0, but with an about tenfold slower decay rate. This second conducting state of CF_0 will be treated elsewhere (Lill et al., *in preparation*).

It was satisfying that the turnover number which we determined was sufficient for the supposed function of CF_0 in photophosphorylation. However, it was disturbing that it was so much higher than calculated under reasonable molecular assumptions for a single hydrogen-bonded chain with only four amino-acid residues (Brünger et al., 1983). We expect a better understanding from ongoing studies on relative changes of the unit conductance as function of pH, temperature, isotopic substitution and viscosity, which found a firmer basis by this work.

We would like to thank Mrs. K. Schürmann for highly skilled and dedicated biochemical assistance, Ms. H. Kenneweg for photographs, Dr. N. Cook for critical comments on the manuscript, H. & W. for typing, and G. for drawing. Special thanks to Steven Theg. Financial support by the DFG (SFB 171/B3) is gratefully acknowledged.

References

Apell, H.J., Bamberg, E., Läuger, P. 1979. Effects of surface charge on the conductance of the gramicidin channel. *Biochim. Biophys. Acta* **552:**369–378

Apell, H.J., Läuger, P. 1986. Quantitative analysis of pump-mediated fluxes in reconstituted lipid vesicles. *Biochim. Biophys. Acta* **861:**302–310

Bamberg, E., Läuger, P. 1974. Temperature dependent properties of gramicidin A channels. *Biochim. Biophys. Acta* **367:**127–133

Brünger, A., Schulten, Z., Schulten, K. 1983. A network thermodynamic investigation of stationary and non-stationary proton transport through proteins. *Z. Phys. Chem.* **136:**1–63

Caceci, M.S., Cacheris, W.P. 1984. Fitting curves to data. The simplex algorithm is the answer. *Byte Mag.* **9:**340–362

Cain, K., Partis, M.D., & Griffiths, D.E. 1977. Dibutylchloromethyltin chloride, a covalent inhibitor of the adenosine triphosphate synthase complex. *Biochem. J.* **166:**593–602

Cain, B.D., Simoni, R.D. 1986. Impaired proton conductivity resulting from mutations in the *a* subunit of F_1F_0ATPase in *Escherichia coli*. *J. Biol. Chem.* **261:**10043–10050

Cox, G.B., Fimmel, A.L., Gibson, F., Hatch, L. 1986. The mechanism of ATP synthase: A reassessment of the functions of the *b* and *a* subunits. *Biochim. Biophys. Acta* **849:**62–69

Davenport, J.W., McCarty, R.E. 1981. Quantitative aspects of adenosine triphosphate-driven proton translocation in spinach chloroplast thylakoids. *J. Biol. Chem.* **256:**8947–8954

Finkelstein, A., Andersen, O.S. 1981. The gramicidin A channel: A review of its permeability characteristics with special reference to the single-file aspect of transport. *J. Membrane Biol.* **59:**155–171

Förster, V., Hong, Y.Q., Junge, W. 1981. Electron transfer and proton pumping under excitation of dark-adapted chloroplasts with flashes of light. *Biochim. Biophys. Acta* **638:**141–152

Friedl, P., Schairer, H.U. 1981. The isolated F_0 of *Escherichia*

coli ATP-synthase is reconstitutively active in H^+-conduction and ATP-dependent energy-transduction. *FEBS Lett.* **128:**261–264

Gould, J.M. 1976. Inhibition by triphenyltin chloride of a tightly-bound membrane component involved in photophosphorylation. *Eur. J. Biochem.* **62:**567–575

Hille, B. 1984. Ionic Channels of Excitable Membranes. Sinauer Associated, Inc., Sunderland, Mass.

Hladky, S.B., Haydon, D.A. 1973. Membrane conductance and surface potential. *Biochim. Biophys. Acta* **318:**464–468

Hong, Y.Q., Junge, W. 1983. Localized or delocalized protons in photophosphorylation? On the accessibility of the thylakoid lumen for ions and buffers. *Biochem. Biophys. Acta* **722:**197–208

Hoppe, J., Sebald, W. 1984. The proton conducting F_0-part of bacterial ATP synthases. *Biochem. Biophys. Acta* **768:**1–27

Itoh, S. 1979. Surface potential and reaction of the membrane-bound electron transfer components. II. Integrity of the chloroplast membrane and reaction of P-700. *Biochim. Biophys. Acta* **548:**596–607

Jackson, J.B., Crofts, A.R. 1969. High energy state in chromatophores of *Rhodopseudomonas spheroides*. *FEBS Lett.* **4:**185–189

Junesch, U., Gräber, P. 1985. The rate of ATP synthesis as a function of ΔpH in normal and dithiothreitol-modified chloroplasts. *Biochim. Biophys. Acta* **809:**429–434

Junge, W. 1976. Flash kinetic spectrophotometry in the study of plant pigments. *In:* Chemistry and Biochemistry of Plant Pigments. pp. 233–333. T.W. Goodwin, editor. Vol. II, Academic, New York

Junge, W. 1982. Electrogenic reactions and proton pumping in green plant photosynthesis. *Curr. Top. Membr. Transp.* **16:**431–465

Junge, W., Jackson, B. 1982. The development of electrochemical potential gradients across photosynthetic membranes. *In:* Photosynthesis: Energy Conversion by Plants and Bacteria. pp. 589–646. Govindjee, editor. Vol. II, Academic, New York

Junge, W., Rumberg, B., Schröder, H. 1970. The necessity of an electric potential difference and its use for photophosphorylation in short flash groups. *Eur. J. Biochem.* **14:**575–581

Junge, W., Witt, H. T. 1968. On the ion transport system of photosynthesis. Investigations on a molecular level. *Z. Naturforsch.* **38:**244–254

Kumamoto, C.A., Simoni, R.D. 1986. Genetic evidence for interaction between the *a* and *b* subunits of the F_0 portion of the *Escherichia coli* proton translocating ATPase. *J. Biol. Chem.* **261:**10037–10042

Lill, H., Engelbrecht, S., Schönknecht, G., Junge, W. 1986. The proton channel, CF_0, of thylakoid membranes. Only a low proportion of CF_1-lacking CF_0 is active with a high unit conductance (169 fS). *Eur. J. Biochem.* **160:**627–634

McLaughlin, S. 1977. Electrostatic potentials at membrane-solution interfaces. *Curr. Top. Membr. Transp.* **9:**71–144

Mitchell, P. 1961. Coupling of phosphorylation of electron and hydrogen transfer by a chemiosmotic type of mechanism. *Nature (London)* **191:**144–148

Mitchell, P. 1977. A commentary on alternative hypotheses of protonic coupling in the membrane systems catalysing oxidative and photosynthetic phosphorylation. *FEBS Lett.* **78:**1–20

Mitchell, P. 1985. Molecular mechanics of protonmotive F_0F_1ATPases. Rolling well and turnstile hypothesis. *FEBS Lett.* **182:**1–7

Nagle, J.F., Tristram-Nagle, S. 1983. Hydrogen bonded chain mechanisms for proton conduction and proton pumping. *J. Membrane Biol.* **74:**1–14

Negrin, R.S., Foster, D.L., Fillingame, R.H. 1980. Energy-transducing H^+-ATPase of *Escherichia coli*. Reconstitution of proton translocating activity of the intrinsic membrane sector. *J. Biol. Chem.* **255:**5643–5648

Neher, E., Sakmann, B. 1976. Single-channel currents recorded from membrane of denervated frog muscle fibers. *Nature (London)* **260:**779–802

Polle, A., Junge, W. 1986. The slow rise of the flash-induced alkalization by photosystem II of the suspending medium is reversibly related to thylakoid stacking. *Biochim. Biophys. Acta* **848:**257–264

Rumberg, B., Muhle, H. 1976. Investigation of the kinetics of proton translocation across the thylakoid membrane. *Bioelectrochem. Bioenerget.* **3:**393–403

Schmid, R., Junge, W. 1975. Current-voltage studies on the thylakoid membrane in the presence of ionophores. *Biochim. Biophys. Acta* **394:**76–92

Schneider E., Altendorf, K. 1982. ATP synthetase (F_1F_0) of *Escherichia coli* K-12. High-yield preparation of functional F_0 by hydrophobic affinity chromatography. *Eur. J. Biochem.* **126:**149–153

Schönknecht, G., Junge, W., Lill, H., Engelbrecht, S. 1986. Complete tracking of proton flow in thylakoids—The unit conductance of CF_0 is greater than 10 fS. *FEBS Lett.* **203:**289–294

Sigrist-Nelson, K., Sigrist, H., Azzi, A. 1978. Characterization of the dicyclohexylcarbodiimide binding protein isolated from chloroplast membranes. *Eur. J. Biochem.* **92:**9–14

Sone, N., Hamamoto, T., Kagawa, Y. 1981. pH dependence of H^+ conduction through the membrane moiety of the H^+-ATPase ($F_0 \cdot F_1$) and effects of tyrosyl residue modification. *J. Biol. Chem.* **256:**2873–2877

Szabo, G., Eisenman, G., McLaughlin, S.G.A., Krasne, S. 1972. Ionic probes of membrane structure. *Ann. N.Y. Acad. Sci.* **195:**273–290

Thomas, J.B., Minnaert, K., Elbers, P.D. 1956. Chlorophyll concentration in plastids of different groups of plants. *Acta Bot. Neerl.* **5:**314–321

Urry, D.W., Goodall, M.C., Glickson, J., Mayers, D.F. 1971. The gramicidin A transmembrane channel: Characteristics of head-to-head dimerized $\pi_{(L,D)}$ helices. *Proc. Natl. Acad. Sci. USA* **68:**1907–1911

Vignais, P.V., Satre, M. 1984. Recent developments on structural and functional aspects of the F_1 sector of H^+-linked ATPases. *Mol. Cell. Biochem.* **60:**33–70

Witt, H.T. 1975. Primary acts of energy conservation in the functional membrane of photosynthesis. *In:* Bioenergetics of Photosynthesis. Govindjee, editor. pp. 493–554. Academic, New York

Witt, H. T. 1979. Energy conversion in the functional membrane of photosynthesis. Analysis by light pulse and electric pulse methods. The central role of the electric field. *Biochim. Biophys. Acta* **505:**355–427

Received 17 February 1987