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Single channel H^+ currents through reconstituted chloroplast ATP synthase CF_0-CF_1

Richard Wagner, Enno C.Apley and Wolfgang Hanke

Biophysik, Universität Osnabrück, Fachbereich Biologie/Chemie Barbarastrasse 11, D-4500 Osnabrück, FRG

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The purified chloroplast ATP synthase (CF_0-CF_1) was reconstituted into azolectin liposomes from which bilayer membranes on the tip of a glass pipette ('dip stick technique') and planar bilayer membranes were formed. The CF_0-CF_1 facilitated ion conductance through the bilayer membranes. Our results clearly indicated that the observed single channel currents were carried by H^+ through the isolated and reconstituted chloroplast ATPase. We demonstrate that in proteoliposomes it is the whole enzyme complex CF_0-CF_1 and not the membrane sector CF_0 alone that constitutes a voltage-gated, proton-selective channel with a high conductance of 1–5 pS at pH 5.5–8.0. After removal of CF_1 from the liposomes by NaBr treatment the membrane sector CF_0 displayed various kinds of channels also permeable to monovalent cations. The open probability P_o of the CF_0-CF_1 channel increased considerably with increasing membrane voltage [from $P_o \leq 1\%$ ($V_m \leq$ 120 mV) to $P_o \leq 30\%$ (120 mV $\leq V_m$ 200 mV)]. In the presence of ADP (3 μM) and P_i (5 μM), which specifically bind to CF_1, the open probability decreased and venturicidin (1 μM), a specific inhibitor of H^+ flow through CF_0 in thylakoid membranes, blocked the channel almost completely. Our results, which reveal a high channel unit conductance, and at membrane voltages <100 mV low open probability with concomitant mean open times in the μs range, suggest a gated mechanism with channel openings in the μs timescale (<100 μs) for the energy coupling in the enzyme complex. At physiological membrane voltages for photophosphorylation (~30 mV) the enzyme complex would then display a time-averaged conductance of ~1 fS.

Key words: chloroplast ATPase/CF_0-CF_1 single channel proton conductance/reconstitution/energy coupling

Introduction

The proton translocating ATP synthases (ATPases) are the key enzymes of energy conversion in the membranes of bacteria, mitochondria and chloroplasts ('F_0-F_1 ATPases'). They are composed of two distinct moieties, the intrinsic membrane part F_0, involved in H^+ translocation across the membrane, and the water-soluble peripheral part F_1 containing the catalytic site(s).

In spite of a large number of studies the structure and molecular mechanism of the ATP synthases are still elusive. For reviews see Mitchell (1961, 1977), Maloney (1982) and Boyer (1987). In particular, it is not known by which mechanism the electrochemical energy of protons is coupled to ATP synthesis.

Here we report on measurements of single channel currents carried out in bilayer membranes that contained the reconstituted chloroplast ATP synthase. We demonstrate that it is the CF_0-CF_1 complex and not the membrane sector CF_0 alone that constitutes a voltage-gated H^+-specific channel with a high unit conductance and open probabilities <1% at membrane voltages <100 mV. The present data suggest that the channel part of the enzyme does not work at a constant turnover rate on the ms timescale but instead exhibits a gated mechanism with channel open times in the μs timescale.

Results

The purified chloroplast ATP synthase was reconstituted into azolectin liposomes using dialysis techniques. The ATP yield of the CF_0-CF_1 proteoliposomes after an acid/base transition was typically 40 ± 5 mol ATP/(s·mg proteins) which corresponds to ~50% of the maximum rate obtained from intact thylakoid membranes (Schmidt and Gräber, 1987).

CF_0-CF_1 was incorporated into bilayers that have been formed on the tip of a glass pipette by spreading proteoliposomes and exogenous lipids on an air/water interface and forming the bilayer from this monolayer ('dip stick technique', Wilmsen *et al.*, 1983; Hanke, 1985). In addition, macroscopic currents were measured in Mueller–Rudin type planar lipid bilayers that contained several copies of CF_0-CF_1 (Mueller *et al.*, 1962).

With the dip stick technique, employing hard-glass micropipettes of ~20 MΩ (~0.5 μm tip diameter), and symmetric solutions containing only 200 μM Tris–HCl (pH 6.8), almost no current fluctuations above the baseline noise could be observed when the applied voltage across the bilayer was ≤100 mV. Current fluctuations were observed at voltages >120 mV. Presumably, the absence of events at lower voltages may be attributed to a very low open probability. Current recordings at 180 mV exhibit a pronounced bursting behaviour (Figure 1A); however, short openings as shown from Figure 1B were mostly observed. The amplitude histogram of the traces in Figure 1A is shown in Figure 1C. The unit conductance of the open channel at 180 mV determined from this histogram is Λ = 4 ± 2 pS. Lifetime distributions of the open and the closed state of the channel at V_m = 180 mV were biphasic with mean open times of 2.39 ± 0.1 ms (72%) and 36 ± 3 ms (28%, Figure 1D), and mean closed times of 18.4 ± 5 ms (20%) and 177 ± 17 ms (80%, Figure 1E). These results indicate that the channel can adopt more than one open or closed state. This conclusion is supported by the 'on time' amplitude cross-correlation diagram shown in Figure 1F. This figure

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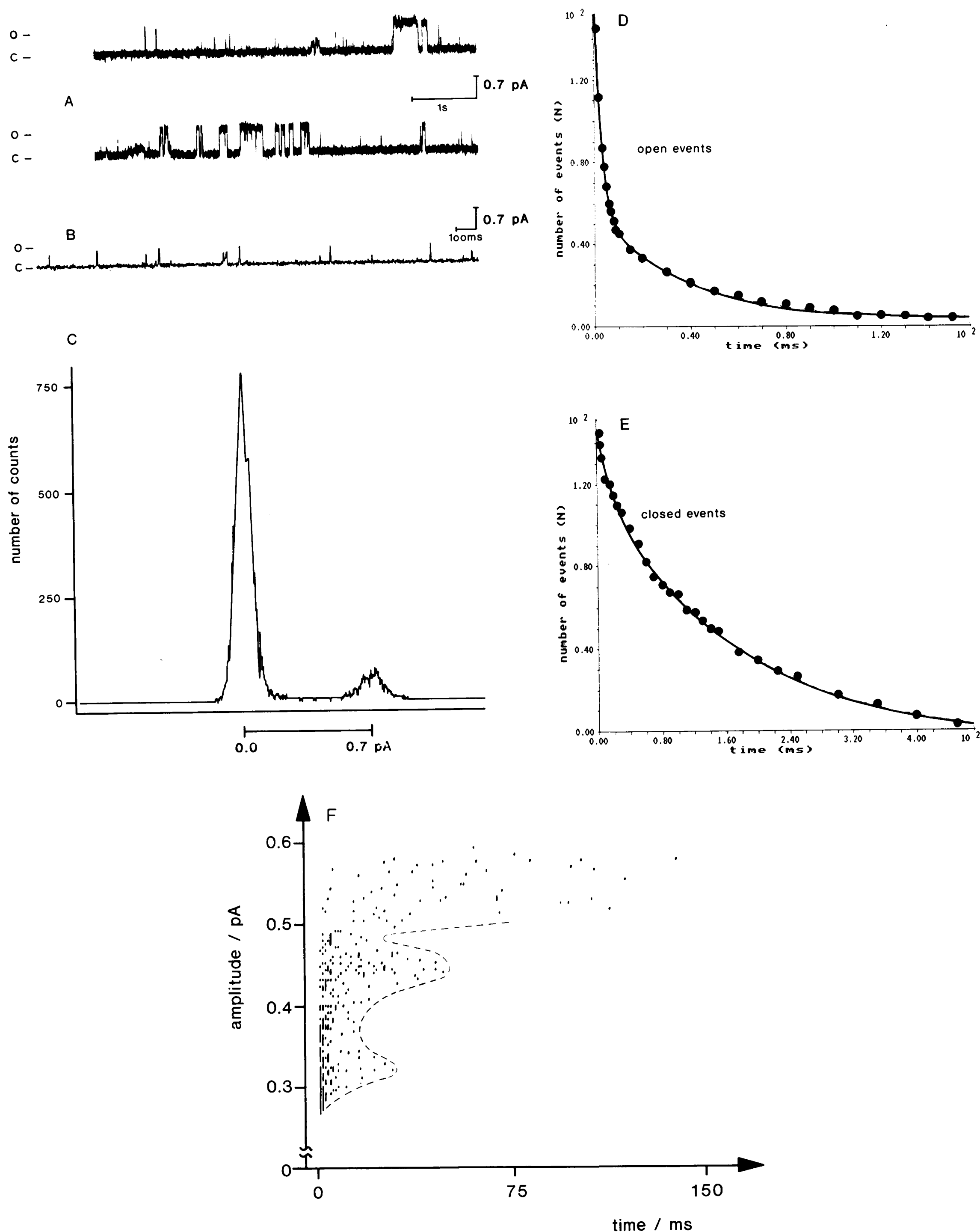

Fig. 1. Channel characteristics at V_m = 180 mV. (**A**) Voltage-activated current fluctuations of CF_0-CF_1 containing bilayers formed on the tip of a glass pipette at a transmembrane voltage of +180 mV. Measurements were performed at pH 6.8 with 200 μM Tris–HCl on both sides of the membrane. (**B**) Voltage-activated current fluctuations of CF_0-CF_1 containing bilayers. Experimental conditions as in (A) but with an expanded timescale. (**C**) Amplitude histogram of voltage-activated single channel fluctuations between the open (o) and the closed (c) state at V_m = 180 mV. (**D**) Lifetime distribution of the open state at V_m = +180 mV. The line shows a computer fit of the experimental points (+). Data were fitted with two exponentials to $N = N_1 \exp(-\tau/t_1) + N_2 \exp(-\tau/t_2)$. The line shows the computer fit of the exponential points (+). (**E**) Lifetime distribution of the closed state at V_m = 180 mV. (**F**) 'On-time' amplitude cross-correlation diagram at V_m 180 mV. The length of the analysed current traces (C–E) was 16 s, while here 64 s of current recordings have been analysed.

also shows that at least two types of channel openings occurred and that most open events revealed rather short open times (τ_{open} < 5 ms) with a conductivity of 1–5 pS. As shown in Figure 1A we did not observe multi-channel gating currents (simultaneous opening events of several channels). Gating of single channels was always observed, however, with slightly different current amplitudes. Concerning statistical considerations for having three different channels in a bilayer, one would expect to observe the simultaneous opening of at least two channels, which

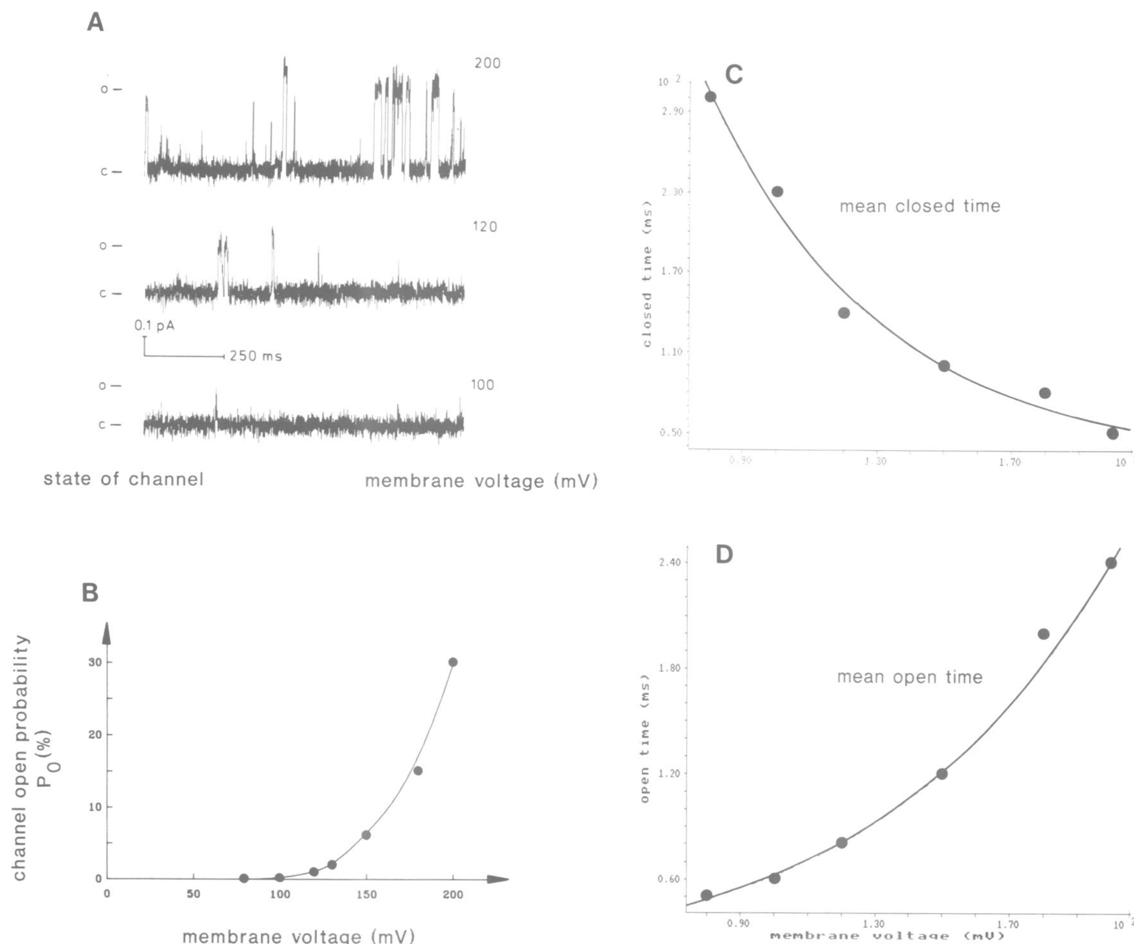

Fig. 2. Voltage dependence of channel gatings. (**A**) Voltage-activated single current fluctuations of CF_0-CF_1 in dip stick bilayers at the indicated membrane voltage. Measurements were performed at pH 6.8 with 200 μM Tris−HCl on both sides of the membrane. (**B**) Probability of the channel being in the open state as function of the transmembrane voltage (V_m). The open probability was obtained by integration of the areas under the respective peaks in the amplitude histogram (see Figure 1C). The trace length analysed for each voltage was 64 s ($n \geq 3$). (**C**) Dependent of the mean closed time on the membrane voltage. At the indicated V_m the lifetime distributions of the open and closed state was fitted by two exponentials as in Figure 1E and the time constant of the slower component, which in all cases covered at least 80% of the total events, was plotted versus V_m. The line shows the fit of the data by one exponential plus baseline. (**D**) Dependence of the mean open time on the membrane voltage. At the indicated V_m the lifetime distribution of the open states was fitted by two exponentials as in Figure 1D and the faster component of the fit, which in all cases covered at least 70% of the total events, was plotted versus V_m.

we in fact never observed. Therefore the most likely interpretation of Figure 1F is that we observed one type of channel with at least three slightly different open channel currents. At present we have performed measurements with seven different CF_0-CF_1 preparations using the dip stick technique. With solutions containing only 200 μM Tris−HCl (pH 6.8) on both sides of the membrane we estimated from the voltage dependence ($n = 5$, each preparation) of channel openings a single channel unit conductance of $\Lambda = 3 \pm 2$ pS in average.

The voltage dependence of the channel was investigated in more detail. Figure 2A shows single channel recordings from dip stick bilayers with symmetric solutions containing 200 μM Tris−HCl (pH 6.8) in the pipette and in the bath at three different V_m. The traces show two amplitude levels of channel openings, indicating that there may exist two conductance sublevels at least. In Figure 2B the channel open probability P_o calculated from the corresponding peaks in the amplitude histograms of current fluctuations (see Figure 1B) is shown as a function of V_m. At membrane potentials <100 mV the open probabilty is <1% but increases steeply with V_m. At the highest voltage applied (V_m = 200 mV) without destroying the bilayer, an open probability of 30 ± 5% was obtained. As shown in Figure 4 the open single channel displayed an ohmic current/voltage relationship. Since we were interested in estimating the time-averaged channel permeability, we further investigated the dependence of the mean channel open and closed times on V_m. At the measured membrane voltages the fits of the open time distributions were dominated by a fast decay component (half-time <3 ms) covering >70% of the opening events, while the fits of the closed time distributions were dominated by a slowly decaying component (half-time >50 ms) covering >80% of the closed events (see also Figure 1D,E). At higher V_m (>150 mV), long channel openings were accompanied by short-lived closed events (<20 ms), both apparently reflecting channel bursting. In the following section we therefore considered only the main components of the open and closed state distributions. The time constants of the fast decay component of the mean open time distribution and the time constants of the slow decay component of the closed time distribution are plotted as a function of

V_m (Figure 2C,D). These figures show that the mean open time of the channel increases exponentially with increasing membrane voltage (Figure 2C), while the mean closed time decreases exponentially with increasing V_m. For both curves in Figure 2C,D a good fit could only be obtained by including a voltage-independent component with values of 40 μs for the mean open and 33 ms for the mean closed time respectively. Considering a simple two-state model for the channel (open ⇋ closed), from the data shown in Figure 2C,D, the dependence of the channel open probability on V_m can be calculated [$P_o = \tau_{open}/(\tau_{open} + \tau_{closed})$]. It fits qualitatively well with the curve shown in Figure 2B. This indicates that in a first approximation the observed voltage dependence of the channel may well be described by this model.

One interesting feature of the observed voltage-dependent channel gating is the apparent low open probability at membrane voltages <100 mV. Although at present we do not have experimental values at <80 mV, approximate mean open and closed times at membrane voltages where the current amplitudes would be too small to be resolved may be obtained from Figure 2C,D. Extrapolation of the curves shown in this figure to $V_m = 30$ mV, for example, would yield an open time of 0.22 ms and a mean closed time of 730 ms. Considering the two state model for the channel, the calculated open probability would be $P_o \sim 3 \times 10^{-4}$ and the time averaged conductivity ~ 1 fS.

With 200 μM Tris−HCl (pH 6.8) facing the *cis* and *trans* side of the membrane, we measured the macroscopic current across a planar bilayer containing several CF_0-CF_1 copies. The current−voltage relationship exhibited a steep rectification (Figure 3A, control). This experiment demonstrates that only at high positive membrane voltage will channels open, and that they are incorporated into the bilayer with one preferable orientation. In order to see whether the voltage-activated current fluctuations require the whole CF_0-CF_1, we investigated the effect of low concentrations of ADP and P_i on the multi-channel currents in planar bilayers and also on voltage-activated current fluctuations in dip stick bilayers respectively. In the presence of ADP (3 μM) and P_i (5 μM) the macroscopic current is reduced (Figure 3A). Short open events in the range of detection limitation (<1 ms) could be observed mainly in the presence of APD and P_i, whereas the current amplitude was not affected (Figure 3D). In the absence of ADP and P_i, the mean open time increased and the channel displayed bursting behaviour. These results demonstrate that ADP and P_i, which bind to CF_1 specifically, affect the gating behaviour of the channel. Analysis of the single channel recordings up to $V_m = 180$ mV revealed that the open probability of the channel at a fixed V_m is decreased in the presence of 3 μM ADP + 5 μM P_i. Since the unit conductance was independent of V_m, at least over the range where open events could be detected, the smaller macroscopic currents in the presence of ADP and P_i (Figure 3A) should be due to a decrease in the open probability. The effect of low concentrations of ADP and P_i presumably on channel gating indicates that binding of the substrates to CF_1 increases the energy threshold of gating. Similar results have been obtained in thylakoid membranes, where low concentrations of ADP in the presence or absence of P_i decrease the time-averaged proton flux through the ATP synthase (McCarty *et al.*, 1971; Gräber *et al.*, 1981). In contrast to this, the nucleotide analogue (2′(3′)-*O*-(2,4,6-trinitrophenyl)-ADP (TNP-ADP) uncouples thylakoid membranes when it binds to CF_1 (Wagner *et al.*, 1986). In dip stick bilayers in the presence of 20 μM TNP-ADP, the number of opening events and also the open probability of the channel increased drastically (Figure 3E), while the current amplitude was not affected. Together, the above results suggest that the voltage-dependent channel gating requires not only the membrane part CF_0, but also CF_1.

Further, we investigated the effect of venturicidin (a specific blocker of proton flux through CF_0, Linnet and Beechy, 1979) on single channel fluctuations in dip stick bilayers and on multi-channel currents in planar bilayers. Figure 3B shows macroscopic current measurements supported by several copies of the CF_0-CF_1 complex in the presence of 50 nM and 1 μM venturicidin respectively. In the presence of 50 nM venturicidin the number of current fluctuations at $V_m = 180$ mV was slightly reduced (Figure 3D). The macroscopic current reduction by 50 nM venturicidin across the CF_0-CF_1-containing membrane is clearly enhanced by 1 μM venturicidin (Figure 3B). However, as shown in Figure 3D, venturicidin (50 nM and 1 μM, not shown) had no effect on the current amplitude in single channel recordings from dip stick bilayers. Therefore the macroscopic current reduction in the presence of venturicidin should be mainly due to a decrease in the channel open probability.

The selectivity of the channel towards H^+, Na^+ and Cl^- was tested at different V_m in dip stick bilayers with 200 μM Tris−HCl from pH 5.5 to pH 8.0 inside the pipette and using the same buffer with an additional 120 mM NaCl in the bathing solution. The results of these measurements are listed in Table I. As shown in Table I the channel conductance remained unchanged after the addition of 120 mM NaCl to the bath. Essentially the same result was obtained when KCl (120 mM, final concentration) was added instead of NaCl. Also, equal variations of pH between 5.5 and 8.0 on both sides of the membrane had no large effect on the channel unit conductance (see Table I). However, at present we can not exclude effects of pH on the channel open probability. Secondly, also with dip stick bilayers, the bath solution containing 200 μM Tris−HCl (pH 7.4) was titrated to pH 5.5, while the pH inside the pipette was kept at pH 7.4 with 200 μM Tris−HCl and the single channel currents were measured at different V_m. The results of these experiments as well as measurements at pH 7.4 on both sides of the membrane are shown in Figure 4. Because below $V_m =$ 80 mV and with a positive voltage inside the pipette (bath connected to ground) no current fluctuations could be observed, the obtained current−voltage relationships shown in Figure 4 were extrapolated to the voltage axis. With the applied transmembrane proton gradient of 1.9 units the I−V curve of the single channel was shifted parallel to that obtained from measurements with symmetrical pH 7.4. However, the extrapolated reversal potential was the actual equilibrium potential for protons. In a second titration experiment, also with dip stick bilayers, the bath solution containing 1 mM Tris−HCl, pH 7, was titrated to pH 8.5 by addition of 1 M Tris−base buffer, while the pH inside the pipette was kept at pH 7 with 1 mM Tris−HCl. At pH 8.5 in the bath single channel currents could only be resolved at membrane potentials >150 mV, where the bilayer tend to be rather unstable. The amplitude of the single

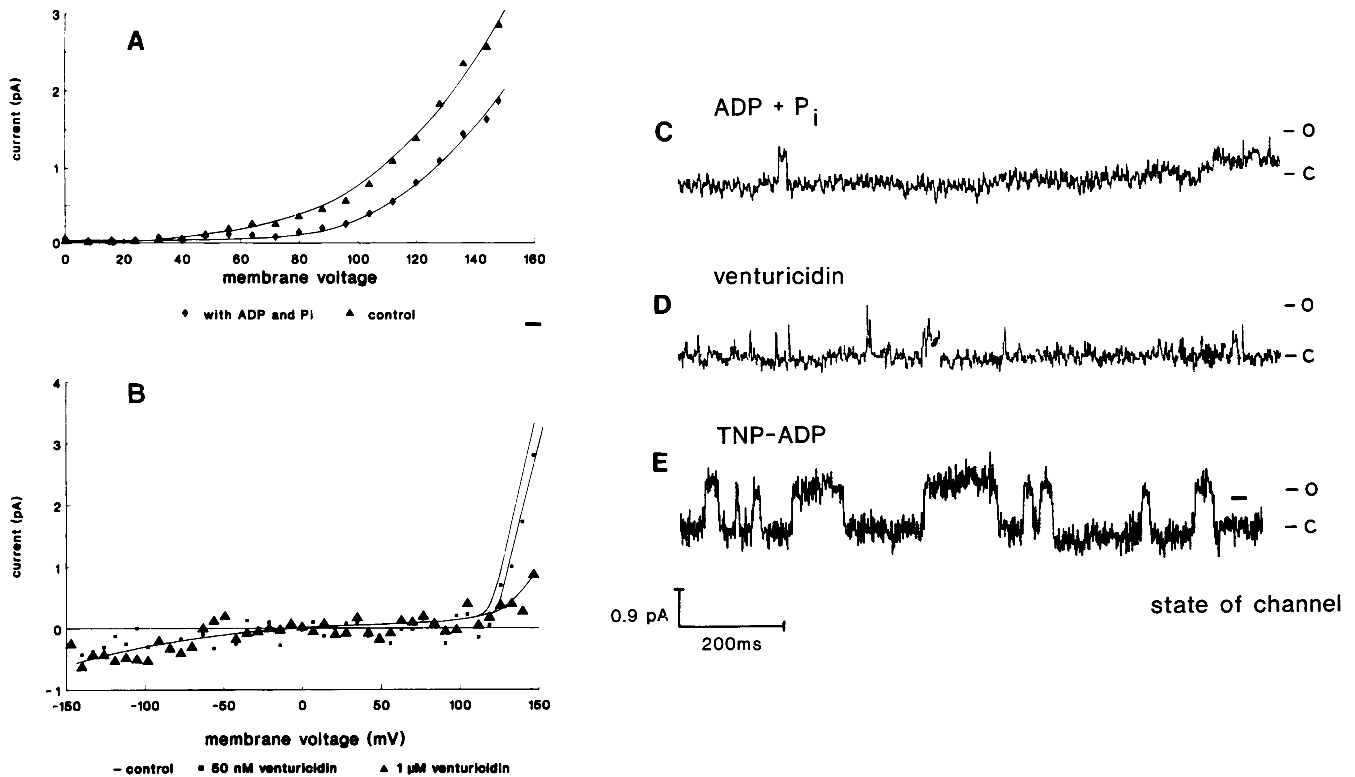

Fig. 3. Voltage dependence of macroscopic currents in planar bilayers and effect of substrates and venturicidin on channel currents. The data points in (A) and (B) are the average of three independent measurements each. (**A**) Voltage dependence of macroscopic currents in a planar bilayer containing several CF_0-CF_1 copies. Measurements with 200 μM Tris−HCl, pH 6.8, on both sides of the bilayer in the absence (▲) and presence (◆) of 5 μM ADP and 3 μM P_i were performed on the same bilayer. Since the $I-V$ curve at negative voltages was identical to the one shown in (B) it was omitted for clarity. (**B**) Voltage dependence of macroscopic currents in a planar bilayer in the absence (−) and presence of 50 nM (▲) and 1 μM (◆) venturicidin. The macroscopic $I-V$ relations were measured on planar bilayers containing several CF_0-CF_1 copies. Other experimental conditions as in (A). We estimated from the channel conductance that the planar bilayer contained ~50 copies of CF_0-CF_1.
(**C**) Voltage-activated current fluctuations of CF_0-CF_1 containing bilayers formed on the tip of a glass pipette with 200 μM Tris−HCl, pH 6.8, on both sides of the membrane in the presence of 5 μM ADP and 3 μM P_i at V_m = 180 mV. Current fluctuations were first recorded in the absence of ADP and P_i, both substrates were added to the bath side without destroying the seal, and after 5 min current fluctuations were recorded on the same bilayer. (**D**) Voltage-activated current fluctuations of CF_0-CF_1 containing dip stick bilayers in the presence of 50 nM venturicidin at V_m = 180 mV. Other experimental conditions as in (C). (**E**) Voltage-activated current fluctuations of CF_0-CF_1 containing bilayers formed by the dip stick technique in the presence of TNP-ADP (20 μM final concentration) at V_m = +180 mV. Other experimental conditions as in (C).

channel currents at 160, 180 and 200 mV are shown in Figure 4. Compared to the current amplitudes observed at symmetrical pH 7.4, the amplitudes are significantly lower, which might indicate that the apparent reversal potential was shifted by the applied ΔpH. With the same bilayer at pH 9.3 no single current fluctuations could be detected. However, when the bath side was titrated back to pH 6, current fluctuations were readily observed at membrane potentials >100 mV. These results clearly indicate that above pH 8 the amplitude of the channel currents decreases significantly. Considering the experimental protocol and purity of the buffer components used, the possible contamination of monovalent cations in the buffers used in dip stick experiments referred to as '200 μM/1 mM Tris−HCl' would be <50 μM. Therefore the above results strongly indicate that the voltage-activated CF_0-CF_1 channel is highly selective for H^+ (>100:1), and therefore also in buffers containing only 200 μM Tris−HCl on both sides of the membrane the measured single channel currents through the ATP synthase complex were carried exclusively by protons.

In order to see whether voltage-activated current fluctuations can also be observed when only CF_0 is incorporated into bilayer membranes we partially removed

Table I. Channel conductance of CF_0-CF_1 containing dipstick bilayers at different pH in the absence (−) and presence of (+) of 120 mM NaCl in the bathing solution

pH	NaCl 120 mM	Λ (pS)
5.5	±	3 ± 2
7.4	±	3 ± 2
8	±	3 ± 2

Measurements with symmetrical 200 μM Tris−HCl and identical pH on both sides of the membrane. Each value in the table represents the average of four measurements (V_m + 120, 140, 160, 180 mV). Single conductance values at a particular V_m were estimated by averaging the current amplitudes of a 16 s record. Single channel currents were recorded first in the absence and then, without destroying the bilayer, the salt was added to the bath side and after 5 min current recordings were performed on the same bilayer.

CF_1 from proteoliposomes by NaBr treatment. Upon NaBr incubation of CF_0-CF_1 proteoliposomes only 60 ± 10% of CF_1 was removed, as indicated by the remaining methanol-activated ATPase activity. Consequently, in SDS−gel electrophoresis of the NaBr-treated CF_0-CF_1 proteoliposomes the α, β and γ bands of CF_1 could still be

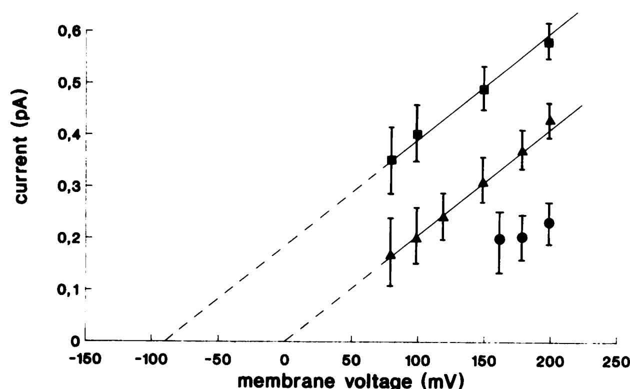

Fig. 4. Current−voltage relationship for open single channel. The shown data points are averages of three (■▲) and two (●) independent bilayers each. ▲ Measurements in dip stick bilayers with symmetrical 200 μM Tris−HCl, pH 7.4, on both sides of the membrane. ■ Measurements in dip stick bilayers with 200 μM Tris−HCl, pH 7.4, inside the pipette and 200 μM Tris−HCl, pH 5.5, in the bath. ● Measurements in dip stick bilayers with 1 mM Tris−HCl, pH 7.0, inside the pipette and 8 mM Tris−HCl, pH 8.5, in the bath.

detected. However, no ΔpH-driven ATP synthesis was observed in the NaBr-treated CF_0-CF_1 proteoliposomes. In dip stick bilayers with NaBr-treated proteoliposomes the conductance and gating behaviour was dramatically changed. Current fluctuations could already be observed at 60 mV, where P_o of the CF_0-CF_1 complex was far below 1% (see Figure 2B). Secondly, the H^+ selectivity and rather uniform size of the conductance of ~3 pS was lost. At least three different conductance levels between ~20 and ~60 pS (with 120 mM KCl, 200 μM Tris−HCl, pH 6.8, at both sites of the membrane) were observed (data not shown). A detailed description and analysis of these cation-specific channels also observed in patch-clamp studies with giant liposomes prepared from the smaller liposomes by dehydration/rehydration (Criado and Keller, 1987) containing either CF_0-CF_1, partially damaged CF_0-CF_1 or subunit III ('proteolipid') of the chloroplast ATP synthase is beyond the scope of this paper and will be the subject of a forthcoming report (H.Lühring and R.Wagner, unpublished).

Discussion

We have demonstrated voltage-gated single channel currents carried by protons through the isolated reconstituted ATP synthase CF_0-CF_1. In the artificial membranes proton-specific conductance could only be observed when the entire ATP synthase CF_0-CF_1 was used in reconstitution. The proton-selective channel revealed a unit conductance of ~3 pS between pH 5.5 and pH 8. The channel open probability P_o was strongly dependent on the applied membrane voltage and was <1% at V_m < 100mV.

The channel unit conductance of ~3 pS between pH 5.5 and pH 8 corresponds to a proton transport rate of ~10^6 H^+/s. This is by orders of magnitude larger than observed for other proton-conducting channels. The antibiotic gramicidin has a proton conductance of ~1 fS at pH 7 (Neher *et al.*, 1978). An oligopeptide containing only the amino acids leucin and serin (Leu-Ser-Leu-Leu-Leu-Ser-Leu)$_3$ was reported to conduct specifically protons (Lear *et al.*, 1988). These measurements with this oligopeptide were performed below pH 1 (in 0.5 M HCl). Extrapolation of the unit conductance to pH 7 yields a channel conductance of ~0.1 fS for the oligopeptide. However, a high channel conductance of 1 pS (time averaged) for the proton conductance through CF_0 in the thylakoid membrane has been obtained by flash spectrophotometric measurements in partially CF_1-depleted thylakoid membranes (Lill *et al.*, 1987). For this channel a selectivity of H^+/Na^+ > 10^7/1 has been reported (Althoff *et al.*, 1989). In the same study, it was reported, similar to our observations, that the conductance of CF_0 in the thylakoid membrane, between pH 5.6 and pH 8, seemingly was independent from the bulk pH. The applied spectrophotometric technique, however, did not allow to determine the CF_0 open probability and any voltage-dependent gating of the proton flux.

The theory of proton conduction across biological membranes along hydrogen-bonded chains would allow rates of 10^6 H^+/s (Schulten and Schulten, 1985). Also protonation of groups in the channel mouth can exceed rates of 10^5 H^+/s, provided the bulk pH and the pK of the groups are <5, or when both pH and pK are >9 (Brünger *et al.*, 1983, Kasianowicz *et al.*, 1987).

The supply of protons to the channel mouth, however, may be a problem. Recent calculations by Peskoff and Bers (1987, 1988) show that the supply of protons to the channel mouth can be ~10^4-10^5 H^+/s if it is limited by diffusion from the bulk. However, additional mechanisms may be involved in proton delivery to the channel mouth. In the context of the K^+-maxi-channels several possible mechanisms have been discussed in detail (Latorre and Miller, 1983; Bell and Miller, 1984; Jordan, 1987), which in principle may also be applied to CF_0-CF_1.

Due to negative surface charges the effective H^+ concentration at the membrane surface (channel mouth) is significantly higher than the bulk concentration. Likewise we determined the H^+ buffer capacity of our proteoliposomes between pH 5 and pH 8 (for experimental details see Junge *et al.*, 1979) to be of the order of 1 mM. This higher surface concentration of protons (we observed one channel in an area of ~1 μm^2) in combination with the possibly enhanced H^+ diffusion at the membrane surface (Prats *et al.*, 1987) would indeed be sufficient to allow up to ~1 s steady-state channel currents with a rate of 10^6 H^+/s. The observed independence (between pH 5.5 and pH 8) of the channel unit conductance from the bulk pH is consistent with the above mechanism. This result may also imply that a group with rather low pK is involved in conduction. In addition, a contribution from the Tris−HCl buffer to the on-rate of protons passing the channel is possible. As has been discussed before (Gutknecht and Tostenson, 1975; Ergasser and Horvath, 1974), mobile buffers can enhance proton flux through an aqueous unstirred larger adjacent to a membrane and, from a source, to a sink in an enzyme. However, if this effect was substantial it was already saturated at 200 μM Tris−HCl, since at pH 6.8 and up to 10 mM of the buffer no changes in the channel conductance were observed. Therefore, longer channel openings (~300 ms) with steady-state currents at a unit conductance of ~3 pS—as shown, for example, in Figure 1A—in principle may be explained by the current concepts, although the figures are strikingly high. We also have to point out that these longer channel openings (<50 ms) were rare events (see, for example, Figure 1F) which could be

observed only at membrane potentials >150 mV. The mean open time of the channel at 180 mV was 2.4 ms (72%) and 36 ms (28%). These values correspond to a time-averaged proton conductance of ~10 and ~140 fS. Moreover, at more physiological membrane potentials (<100 mV) the mean open time of the channels was even <1 ms (see Figure 2D) and therefore the time-averaged proton conductance was in the order of 1 fS. The time-averaged proton conductance obtained by Lill *et al.* (1897) of 1 pS for exposed CF_0 in the thyalkoid membrane is an order of magnitude higher. Nevertheless, the physiological significance of the longer channel openings as shown in Figure 1A is not clear at present and experiments with CF_0-CF_1 embedded in bilayers composed of neutral and positively charged phospholipids may be helpful to investigate the access of protons to the channel mouth.

How do our experimental findings compare with the known overall features of ATP synthesis under physiological conditions? Regarding the proton flux through the channel part of the ATP synthase, measured rates of ATP synthesis may be accounted for as the time and ensemble average of single channel conductance. Under physiological conditions ATP synthesis in chloroplasts is mainly driven by the pH gradient across the membrane at low membrane potential (30 mV), while in mitochondria the main driving force is the membrane voltage (Jung, 1977; Maloney, 1982). Neither the single channel conductance nor the open probability of the ATP synthase channel are known. Assuming a stoichiometry of $3H^+$/ATP (Junge *et al.*, 1970), steady-state rates of photophosphorylation require the passage of $\sim 10^3$ H^+/s through CF_0-CF_1 (Junesch and Gräber, 1985). With the measured single channel conductance of CF_0-CF_1 the transport rate of H^+ is $\sim 10^6$ H^+/s at 30 mV driving force, $\sim 10^3$ times faster than the turnover rate of protons required in steady-state photophosphorylation. Therefore, from the present data, the measured high unit conductance is not readily reconciled with the known features of the ATP synthesis. However, two feasible cases may be considered to explain the high unit conductance in the context of the known features of ATP synthesis. When gating of H^+ flow in the CF_0-CF_1 channel occurs without being coupled to ATP synthesis, this proton flow will be purely dissipative and the energy applied across the membrane will be dissipated very rapidly ($\sim 10^3$ H^+/ms). Thus, the ATP synthase would act as a type of 'safety valve' for the dissipation of excess energy (Strotmann *et al.*, 1986). On the other hand, it is tempting to speculate on a possible physiological relevance of the observed channel gating. The channel mean open times decreased with decreasing membrane potential. If the high unit conductance is also realized during photophosphorylation (~30 mV), this channel conductance may be paralleled by short channel open events (<100 μs) at low membrane voltage in order to yield a time-averaged rate of H^+ conductance of $\sim 10^3$ H^+/s (~1 fS) for the ATP synthase. In this model, the channel part of the enzyme would not work at a constant turnover rate on the ms timescale, but instead exhibits a gated mechanism with channel open times on the μs timescale.

Materials and methods

Preparation of CF_0-CF_1

CF_0-CF_1 was purified as described (Fromme *et al.*, 1987) and reconstituted into azolectin liposomes using the dialysis technique (Schmidt and Gräber, 1985). Azolectin (Sigma type IV-S) was purified as described by Cook *et al.*, (1986). SDS–gel electrophoresis (Laemmli, 1970) and ATP synthesis driven by an artificial pH gradient across the membrane (Schmidt and Gräber, 1985) was used to assess the purity and activity of the reconstituted enzyme.

Partial CF_1 depletion of CF_0-CF_1 proteoliposomes

To 1 ml of proteoliposomes in 10 mM Tris–Tricene, pH 8, 2.5 mM $MgCl_2$, 0.25 mM DTT (buffer A) a 5 M NaBr solution in the same buffer was added to yield a final NaBr concentration of 2 M. After 30 min at 4°C, 20 ml of buffer A were added. The proteolipids were collected by centrifugation, 30 min at 120 000 *g*, and resuspended in 0.5 ml buffer A (see above). ATP synthase activity (Schmidt and Gräber, 1985) and the methanol-activated ATPase activity of the NaBr-treated liposomes were measured (Anthon and Jaggendorf, 1986) and protein composition was analysed by SDS–gel electrophoresis (Laemmli, 1970). Protein was isolated from the proteoliposomes by incubation of 200 μl proteoliposomes with 800 μl cold (−20°C) acetone. The precipitate which predominantly contained protein was collected by centrifugation (20 min, 20 000 *g*) and resuspended in sample buffer for SDS–gel electrophoresis.

Incorporation of the protein into lipid bilayers formed on the tip of a glass pipette

Azolectin (Sigma type II-S) was dissolved in hexane (2 mg/ml) and 50 μl of this solution was spread on the air/water interface of a bath filled with 2.9 ml of a solution containing 0.2 mM Tris–HCl, pH 6.8, and 100 μl of a proteoliposome suspension. The liposomes containing 35 mg/ml azolectin and 0.1–0.3 mg/ml CF_0-CF_1 were dissolved in a buffer containing 1 mM Tris–Tricine, pH 8, 2.5 mM $MgCl_2$, 0.25 mM DTT and 0.2 mM EDTA. After 20 min of incubation, bilayers with a seal resistance between 10 and 30 GΩ were formed on the tip of a glass pipette (Wilmsen *et al.*, 1983). Following this protocol ~70% of the attempts were successful and the voltage-activated channels were observed. Voltage-activated current fluctuations were recorded and the single channel traces were analysed as described (Colquhoun and Sigworth, 1983; Hanke, 1985). The signal-to-noise ratio of the shown recordings was improved by filtering at 300 Hz (−3 dB, 48 dB/octave). Sampling rate on the computer (ISC-16) was 1 kHz and analysis of the data was performed with 1 kHz bandwidth. The bath solution was held at ground potential (V_0) and voltage is given according to the convention (V_0-V_i), where V_i is the voltage inside the pipette. For each set of experimental parameters $n > 2$ independent experiments were performed. The lifetime distributions of the open and closed state were fitted by a non-linear least-square analysis using the Marquardt algorithm (Bevington, 1969).

Incorporation of CF_0-CF_1 into planar bilayers

The macroscopic I–V relation was measured on a Mueller–Rudin type of planar bilayer containing several CF_0-CF_1 copies (Mueller *et al.*, 1962). Fusion of the CF_0-CF_1 proteoliposomes with the planar bilayers was performed as described elsewhere (Hanke, 1985) and the orientation of the CF_0-CF_1 complex in the bilayer membrane was determined from the voltage dependence of the macroscopic current *I*. The *I–V* curves were measured by sweeping the voltage between −150 mV and 150 mV at a rate of 30 mV/s. Single traces were averaged ($n > 2$) and the capacitive current drift as measured independently in a bilayer without protein was substracted.

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