Volume 258, number 2, 190–194 FEB 07864 December 1989

Cation channels by subunit III of the channel portion of the chloroplast H^+-ATPase*

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Received 14 September 1989

The chloroplast H^+-ATPase (CF_0CF_1) was isolated and reconstituted into lipid vesicles by dialysis technique Vesicles were fused by dehydration/rehydration to obtain cell-size liposomes, which were studied by patch-clamp techniques Single-channel activity was observed with several conductance levels in the range of some 10 pS (100 mM KCl) In contrast to intact CF_0, which conducts protons, only (even at pH 8), these channels were permeable for potassium and sodium Venturicidin, which blocks proton flow through intact CF_0, here greatly decreased the single-channel open probability Subunit III of CF_0, alone, yielded cation channels resembling the former Our tentative interpretation is, that dearrangement or fragmentation of CF_0 caused the potassium and sodium permeability, which, however, is suppressed in intact CF_0

ATPase, H^+, Reconstitution, Patch-clamp, Ion channel

1. INTRODUCTION

Proton-translocating ATPases of bacteria, chloroplasts and mitochondria are composed of two parts: the membrane-embedded F_0, acting as a proton channel, and the extrinsic F_1, containing the active site(s) for ATP synthesis and hydrolysis [1]. CF_0, the channel portion of the ATPase of chloroplasts is composed of four different subunits (I to IV) [2]. Subunit III is the only one existing in more than one copy per CF_0, namely, 6–12 [3–5]. With a molecular mass of about 8 kDa it forms a hairpin-like structure: two membrane spanning helices are connected by a short loop [6].

By flash spectrophotometry (electrochromism and pH-indicating dyes) the time-averaged conductance of CF_0 in thylakoid membranes has been determined, 1 pS. It is proton selective even at pH 8 without detectable conductivity for other cations [7,8]. With CF_0CF_1 incorporated into lipid bilayers (dip-stick technique) single-channel currents equivalent to about 2 pS have been observed, again specific for protons [9]. Direct patch-clamping of thylakoid membranes has proved difficult, except, so far, in giant chloroplasts of *Peperomia metallica*, where a voltage-dependent chloride channel has been detected [10].

For a further electrophysiological characterization of the chloroplast ATPase we used the strategy of Tank and Miller [11]: CF_0CF_1 and subunit III of CF_0 were isolated and reconstituted into lipid vesicles which were then fused to form large liposomes suitable for patch clamp measurements. Single-channel currents were observed which were still sensitive to Venturicidin (as is CF_0 [12]) yet conducting potassium and sodium (in contrast to intact CF_0).

2. MATERIALS AND METHODS

CF_0CF_1 was purified and reconstituted into azolectin vesicles using the dialysis technique [13] The integrity of the preparation was tested by measuring ATP synthesis driven by an artificial pH-gradient [13] From dialysis vesicles large liposomes were formed by a dehydration/rehydration procedure, as essentially described in [14] Subunit III of CF_0 was isolated by electroelution from SDS-gels [15], either from the 8 kDa band (monomer) or the 48 kDa band (hexamer [5]) and reconstituted into dialysis vesicles [15]. These were added to pure lipid vesicles prior to dehydration The protein concentration was about 1 mg/100 mg lipid Inside-out patches ($R \geq 5$ GΩ) were isolated and single-channel recordings performed as in [16] All potentials given refer to the pipette For further details see figure legends

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Abbreviations Tris, Tris(hydroxymethyl)aminomethane; Tricine, *N*-Tris(hydroxymethyl)methylglycine

* Part of this work was presented at the VIIIth International Congress on Photosynthesis (Stockholm, Sweden, 1989)

Published by Elsevier Science Publishers B V (Biomedical Division)
00145793/89/$3 50

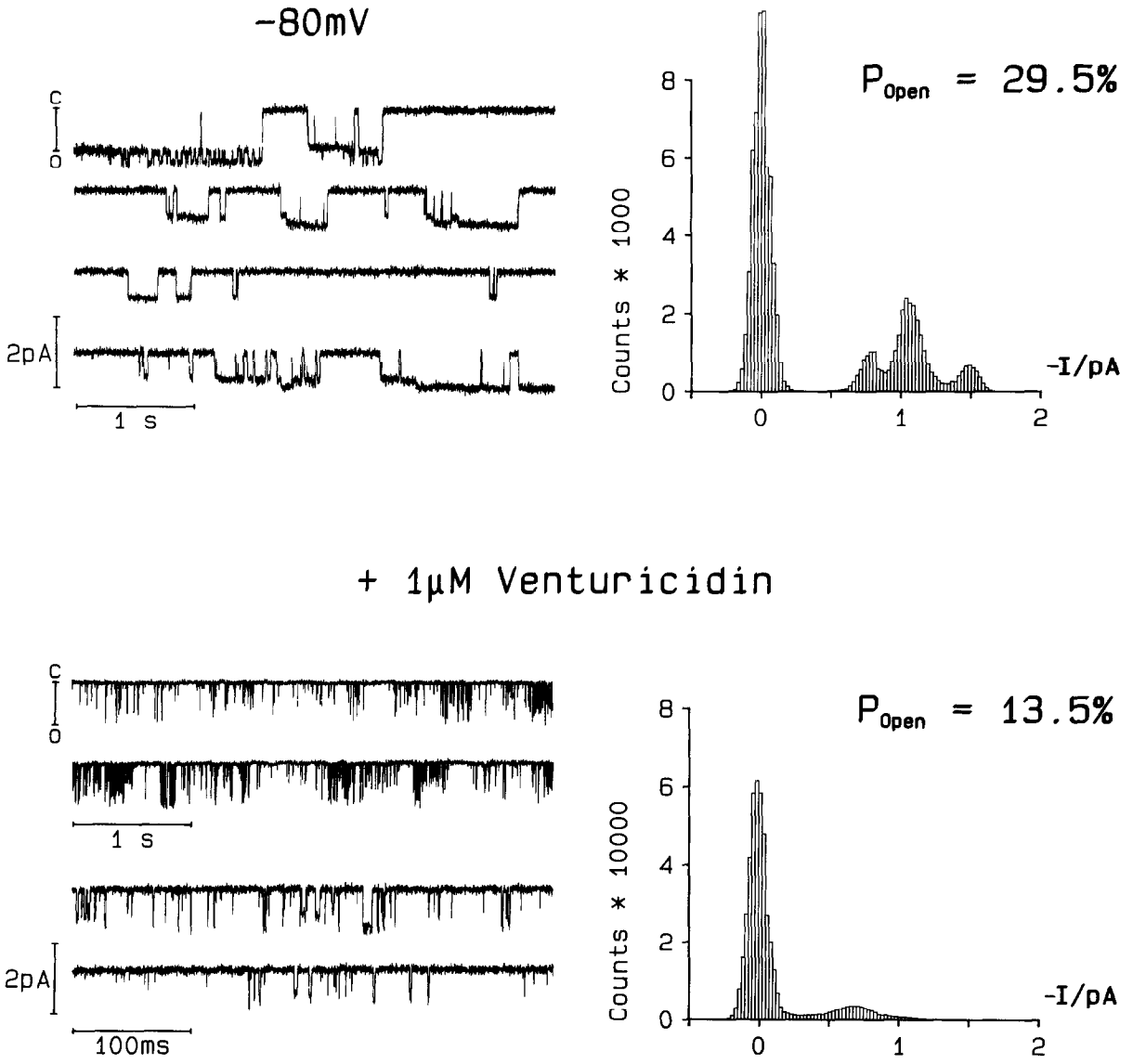

Fig.1 Single-channel recordings of liposomes with CF_0CF_1 incorporated. At −80 mV in 100 mM KCl, 5 mM $MgCl_2$, 10 mM Tricine/KOH pH 7.9. Single-channel traces (left) and corresponding total amplitude histograms (right, amplitude of each digitized point) before (top) and after (bottom) perfusion of 1 μM Venturicidin. Without Venturicidin current fluctuations showed 3 subconductance levels (direct transitions from all current levels to the closed state) (digitization rate 1 ms, cutoff-frequency 0 5 kHz, record length for total amplitude histogram 85 s) After perfusion of Venturicidin to the same patch channel gating was dramatically changed (note different time scales) (digitization rate 100 μs; cutoff-frequency 5 kHz, record length for total amplitude histogram 45 s)

3. RESULTS

After patch-formation a potential difference (−40 mV to −80 mV) was applied. Single-channel activity usually started 10–60 s after application of voltage. Large liposomes with CF_0CF_1 incorporated frequently showed one type of channel with 3 subconductance levels of 10 pS, 13 pS and 18.5 pS (in 100 mM KCl, see fig.1) with ohmic behaviour between +100 mV and −100 mV (data not shown). Under asymmetrical KCl or NaCl concentrations, the reversal potential of the single-channel currents shifted in direction of the Nernst potential of the cation, as expected for a cation channel. 1 μM Venturicidin (known to block proton flow through F_0 [12]) drastically decreased the mean open time of this cation channel and reduced the open probability (P_{open}) by more than a factor of 2 (fig.1). Occasionally further cation channels with higher conductances (about 30, 60 and 120 pS) were observed (not shown).

Incorporation of purified subunit III (of CF_0) into large liposomes also generated cation channels (fig.2). They were comparable to those observed with CF_0CF_1.

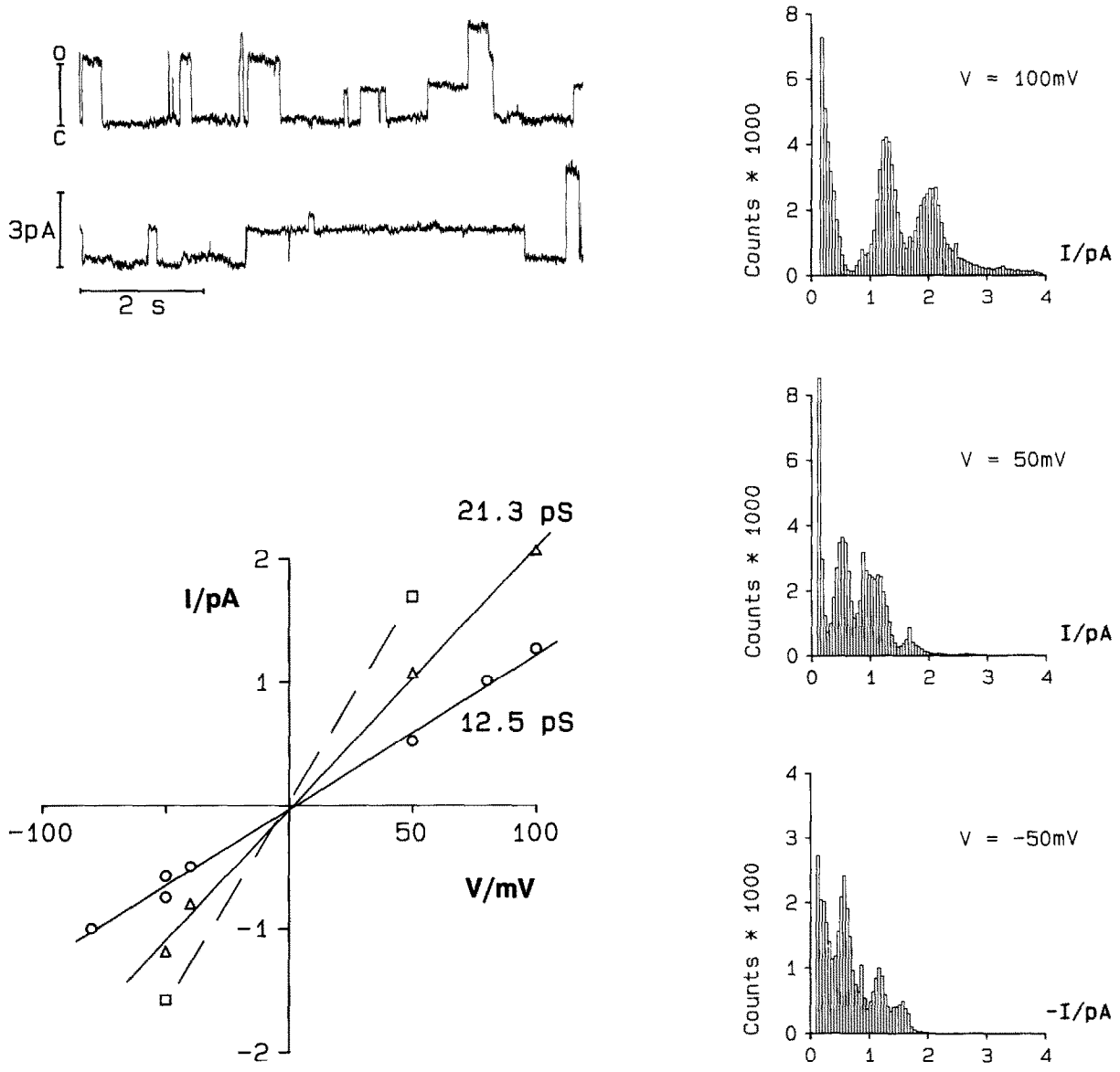

Fig.2 Single-channel recordings of liposomes with subunit III of CF_0 incorporated. 100 mM KCl, 5 mM $MgCl_2$, 20 mM Tris/Tricine pH 7 8 (Top) Single-channel records at +100 mV and the corresponding total amplitude histogram (digitization rate 2 ms, cutoff-frequency 0 3 kHz, record length for total amplitude histogram 8 min). (Right) Equivalent histograms for +50 mV (2 ms; 0 3 kHz, 8 min) and −50 mV (2 ms, 0 3 kHz; 6 min). (Lower left) Current-voltage relationship of open channels Data points were derived from total amplitude histograms

Aside from a conductance level of about 13 pS (in 100 mM KCl) at least two further conductance levels (of about 21 and 33 pS) were detected. These levels belonged to different channels, as they opened and closed independently (see upper trace in fig.2). All showed ohmic behaviour between +100 mV and −100 mV (fig.2).

In large liposomes, single-channel recordings obtained with CF_0CF_1 and with solely subunit III revealed some differences: the former frequently showed a single type of channel (also in patches which contained more than one channel) with subconductance levels (fig.1). The latter normally showed several independent channels with different conductances (fig.2). With subunit III we never detected subconductance levels as with CF_0CF_1.

To test whether the ability to form cation channels was restricted to CF_0CF_1, we examined EF_0, the channel portion of the homologous H^+-ATPase of *E. coli*. Purified EF_0 [17] was incorporated into large liposomes during dehydration/rehydration. In patch clamp measurements single-channel currents carried by potassium and corresponding to a conductance of 40 pS were observed (fig.3).

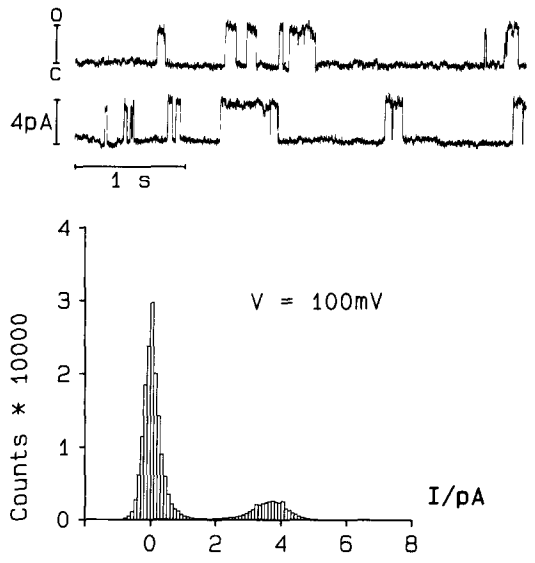

Fig 3 Single-channel recordings of liposomes with EF_0 incorporated 100 mM KCl, 5 mM $MgCl_2$, 20 mM Tris/Tricine pH 7 8 and total amplitude histogram (digitization rate 1 ms; cutoff-frequency 0 5 kHz, record length for total amplitude histogram 3 min)

4. DISCUSSION

Large liposomes formed by dehydration/rehydration of small dialysis vesicles which contained CF_0CF_1 revealed cation channels. This was unexpected, as it is known, that the intact enzyme conducts protons only [1,18]. Even in CF_1-depleted thylakoid membranes with CF_0 exposed, proton conductance is dominant during the first 100 ms after flash induced generation of an electric potential difference [7,8]. Dialysis vesicles containing CF_0CF_1 are competent to synthesize ATP in an acid-base jump and show single-channel currents carried by H^+ when incorporated into a lipid bilayer [9]. The lack of H^+ specificity in the present patch-clamp experiments with large liposomes might be explained by assuming, that CF_0CF_1-complexes had disintegrated during the dehydration/rehydration procedure, and some of the components gave rise to cation channels. The detection of only proton conductance in CF_1-depleted thylakoid membranes is then attributable to the still intact structure of at least a few CF_0-complexes. Even if deranged CF_0 existed in CF_1-depleted thylakoid membranes, one would not expect to detect cation channels during the first 100 ms after the onset of a membrane potential (by flash spectrophotometry) because channel activity usually started 10–60 s after voltage application (see above).

Venturicidin (1 μM), a highly specific blocker of F_0-type proton channels [12], changed the channel gating drastically and decreased the open probability of the cation channels significantly (fig.1). Therefore it is very unlikely, that these channels arose from contaminations in purified CF_0CF_1.

Venturicidin is assumed to bind to subunit III of the channel portion of the ATPase [19]. Purified subunit III of CF_0 incorporated into large liposomes (fig.2) gave rise to cation channels with similar conductances as those obtained with incorporated CF_0CF_1. Therefore it is likely that subunit III was also the major component forming the cation channels observed with CF_0CF_1. Yet it might be that in the presence of the other subunits of CF_0 channel formation by subunit III was modified.

So far, single-channel recordings were reported only for subunits of the channel portion of the mitochondrial H^+-ATPase: the synthetic transmembrane fragment of subunit 8 of the mitochondrial H^+-ATPase of *Saccharomyces cerevisiae* (not known to be homologous to any subunit of CF_0CF_1) was shown to have cation channel forming ability [20]. Subunit c (the homologous of subunit III of CF_0) from yeast mitochondria was reported to form proton channels in planar lipid bilayers [21], with no detectable sodium or potassium conductivity (at least at pH 2.2). However, linear extrapolation to pH 7.0 resulted in a turnover number for protons of 100 s^{-1} at 100 mV [21], which is one order of magnitude below turnover numbers calculated from maximum ATP synthesis rates in mitochondria [22] and chloroplasts [23] and several orders of magnitude below the reported value of 1 pS for CF_0 (corresponding to 6×10^5 H^+ s^{-1} at 100 mV) [7–9]. Recently, a synthetic amphiphilic model peptide (sequence: H_2N-(Leu-Ser-Ser-Leu-Leu-Ser-Leu)$_3$-$CONH_2$) has been shown to form cation channels with characteristics resembling those of large ion channel proteins and a dominant single-channel conductance of 14 pS in 100 mM KCl [24]. A slight modification (sequence: H_2N-(Leu-Ser-Leu-Leu-Leu-Ser-Leu)$_3$-$CONH_2$) generates a proton specific channel, with single-channel conductance of 120 pS in 0.5 M HCl and no detectable conductance for other cations [24]. Linear extrapolation of this conductance to pH 7.0, however, results in a value of 0.024 fS. Thus one should be careful to compare proton conductances observed with small peptides at very acid pH with the proton conductance of intact CF_0, which conducts protons with about 1 pS [7,9] even at pH 8.0 and without any detectable conductance for other ions (background of up to 100 mM other ions).

The present work showed the ability of subunit III of CF_0, alone, to form cation channels without, however, the extreme proton specificity of intact CF_0. So, the selectivity filter for protons of intact CF_0 should be caused by or with help of the other subunits (I, II, IV).

Acknowledgements Purified EF_0 was a friendly gift of Dr G Dekkers-Hebestreit, Dept Microbiology, Osnabruck. We thank Dr R. Hedrich and Dr W Hanke for introducing us into patch clamp electrophysiology and Dr H Luhring for helpful comments. Financial support by the Deutsche Forschungsgemeinschaft (SFB 171/B2 and B3)

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