

# Complete tracking of proton flow in thylakoids – the unit conductance of $CF_0$ is greater than 10 fS

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We investigated the proton conductance of the channel portion of chloroplast ATP synthase ( $CF_0$ ). Thylakoids were  $CF_1$ -depleted by EDTA treatment. Proton pumps were stimulated by short flashes of light. Proton flux through  $CF_0$  was measured spectrophotometrically in three different ways: as proton efflux from the lumen (via neutral red), charge flow across the membrane (via electrochromism) and proton influx into the medium (via phenol red). Hence we completely tracked the protons on their way from the lumen through  $CF_0$  into the medium. A first treatment with EDTA removed up to 12% of total  $CF_1$  without increasing the proton permeability of the membranes. A second treatment removed a further 20% of  $CF_1$  and increased the proton permeability of membranes by 3 orders of magnitude. The electric potential difference and the pH transients, in both the lumen and medium, decayed with a relaxation time of 7 ms indicating electrically driven proton flow through  $CF_0$ . If the electric driving force was shunted (e.g. by added gramicidin) both pH transients decayed at 85 ms compared with 20–60 s in control thylakoids. The longer relaxation time under chemical driving force was attributable to larger chemical than electrical capacitance of thylakoids. We calculated a lower limit of the unit conductance of  $CF_0$  under the assumption that all exposed  $CF_0$  were proton conducting. The value was 10 fS, corresponding to the passage of 6200 protons/s per  $CF_0$  (at 100 mV electric driving force) and by orders of magnitude higher than so far reported for any  $F_0$  channel.

*Photosynthesis     $H^+$ -ATPase     $CF_0$     Unit conductance*

## 1. INTRODUCTION

Proton-translocating ATP synthases 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 or hydrolysis [1,2]. The mechanism of proton conduction through  $F_0$  is still poorly understood despite an impressive body of genetical and biochemical information [3–5]. Published values of the proton con-

ductance of  $F_0$  are very low, some percent of a femto-Siemens. Turnover numbers (in protons/s per  $F_0$ ) were given for  $F_1$ -depleted submitochondrial vesicles,  $400 \text{ s}^{-1}$  [6], for  $F_1$ -depleted vesicles from *E. coli*,  $18 \text{ s}^{-1}$  [4], and for purified  $F_0$  from the thermophilic bacterium PS3, reconstituted in liposomes  $6$  [7] and  $47 \text{ s}^{-1}$  [8], and from *E. coli*,  $30$  [4] and  $25 \text{ s}^{-1}$  [9], all related to a driving force of the order of 100 mV. Compared to that, turnover numbers of more than  $900 \text{ s}^{-1}$  are required to support observed photophosphorylation rates as high as  $1100 \mu\text{mol ATP/mg chl per h}$  (see table 1) (calculated with  $1 \text{ CF}_0\text{-CF}_1/1000 \text{ chl}$  [10,38] and a stoichiometry of  $3\text{H}^+/\text{ATP}$  [11,12]).

We investigated the proton conductance of exposed  $CF_0$  in  $CF_1$ -depleted thylakoids. By flash excitation a voltage of the order of 50 mV (review [13]) and a pH difference of 0.06 units [14,15] were

*Abbreviations:*  $\text{Bu}_3\text{SnCl}$ , tributyltin chloride;  $\text{Ph}_3\text{SnCl}$ , triphenyltin chloride;  $(\text{C})F_1$ , (chloroplast) ATP synthase, soluble part;  $(\text{C})F_0$ , (chloroplast) ATP synthase, membrane part; chl, chlorophyll; DCCD,  $N,N'$ -dicyclohexylcarbodiimide; ETC, electron transport chain

generated across the thylakoid membrane. This caused a transient proton current from the lumen into the medium, which we completely tracked by three different spectrophotometric techniques.

## 2. MATERIALS AND METHODS

10–14-day-old pea seedlings were homogenized in 200 ml grinding medium (400 mM sorbitol, 10 mM Tricine-NaOH, pH 7.8). The homogenate was filtered through a nylon mesh (20  $\mu$ m) and centrifuged for 7 min at 1200  $\times$  g. The pellet was incubated with EDTA (1 mM), Tricine-NaOH (3 mM), pH 7.8, at 0.3–0.6 mM chl for 10 min on ice and subsequently centrifuged for 10 min at 20000  $\times$  g (1st EDTA wash). For other samples the same incubation procedure was repeated (2nd EDTA wash). Final dilution and preparation of stacked thylakoids (control) were as in [16].

The degree of extraction of CF<sub>1</sub> was determined by immunoelectrophoresis [22], for both the supernatant and pellet, as in [23]. It was checked via ATPase activity in the supernatant [19,20] (assuming a specific activity of 30  $\mu$ mol P<sub>i</sub>/mg chl per min as measured for purified pea CF<sub>1</sub> [23]). Standard procedures were used for determinations of protein concentration [18] and chlorophyll concentration [21]. ATP synthase activity with PMS was measured by the luciferin-luciferase system from LKB [24] and by the <sup>32</sup>P method [25]. Rates of O<sub>2</sub> evolution under continuous saturating illumination were determined by a Clark electrode.

Flash spectrophotometric experiments were car-

ried out in a setup as described in [26,41]. 15 ml sample in a cuvette of 2 cm path length contained 20  $\mu$ M chl, 10 mM NaCl and 200  $\mu$ M hexacyanoferrate(III) as electron acceptor (pH 7.3). Further additions are indicated in the figure legends. Samples were excited by saturating flashes of light typically at 5 s intervals and 20–40 transients were averaged. The electric potential difference across the membrane was measured via electrochromic absorption changes at 522 nm ([27], review [13]). pH transients in the suspending medium were determined by the absorption changes of phenol red (15  $\mu$ M) at 559 nm [28]. pH transients at the luminal surface of the thylakoid membranes were measured via neutral red (15  $\mu$ M plus 2.6 mg/ml BSA) at 548 nm [13,29,30]. In both cases the pH transients were obtained by subtraction of the signal recorded in the absence of the dye from that recorded in its presence. Recent criticism of the measuring technique with neutral red [43] was shown to be unwarranted in [31].

## 3. RESULTS AND DISCUSSION

### 3.1. 1st EDTA wash resulted in 12% extraction of CF<sub>1</sub> without generating proton leaks

After the 1st EDTA wash we found up to 12% of total CF<sub>1</sub> in the supernatant, while the O<sub>2</sub>-coupling ratio [ratio of O<sub>2</sub> evolution with and without nigericin (0.5  $\mu$ M)] was unaffected and the ATP synthesis rate was only slightly diminished (see table 1). Both indicated that the membrane remained proton-tight. This was confirmed by spec-

Table 1

CF<sub>1</sub> contents, coupling ratio of the rate of O<sub>2</sub> evolution and rates of cyclic photophosphorylation as functions of EDTA treatment

Preparation	CF <sub>1</sub> content (% of total)			O <sub>2</sub> coupling ratio	ATP synthesis rate ( $\mu$ mol ATP/mg chl per h)
	Immuno-electrophoresis	Pellet	Via ATPase supernatant		
Control	100	0	0	3.5	1110
1st EDTA wash	92	8	9	3.6	970
2nd EDTA wash	70	22	23	1.0	227

O<sub>2</sub> evolution was measured with 30  $\mu$ M chl and 2.2 mM hexacyanoferrate(III); ATP synthesis rates were determined with PMS (50  $\mu$ M)

trophotometric measurements. The diminution of the extent of spectrophotometric signals after EDTA treatments was caused by deactivation of the ETC, as evident from a proportional reduction of the rate of uncoupled  $O_2$  evolution. The acceleration of the rise of the pH transient (fig.1, middle) is related to unstacking of thylakoids by EDTA treatment as established in [16,17]. Hexacyanoferrate(III) ( $200 \mu\text{M}$ ) was used as specific electron acceptor for photosystem I. It did not compete with plastoquinone for photosystem II (see fig.1 in [16]) and, due to its low  $pK$ , did not bind protons upon reduction. So, flash excitation of both photosystems caused the uptake of only  $1 \text{ H}^+$

per ETC from the medium (at photosystem II) and the release of  $2 \text{ H}^+$  per ETC into the lumen ([16,28], review [13]). The net result is an acidification by  $1 \text{ H}^+$  per ETC. Therefore, with control chloroplasts we observed alkalization of the medium (see fig.1, upper trace) which relaxed in 20–60 s reflecting the passage of protons from the lumen into the medium to produce the expected net acidification (not visible in the time domain of fig.1). Thylakoids which had undergone one EDTA wash showed alkalization of the medium (fig.1, middle) with a relaxation time of approx. 14 s (measured on a longer time scale, not shown), proving that the membrane was almost as proton-tight as in the control. Incubation with DCCD had no influence (see fig.1).

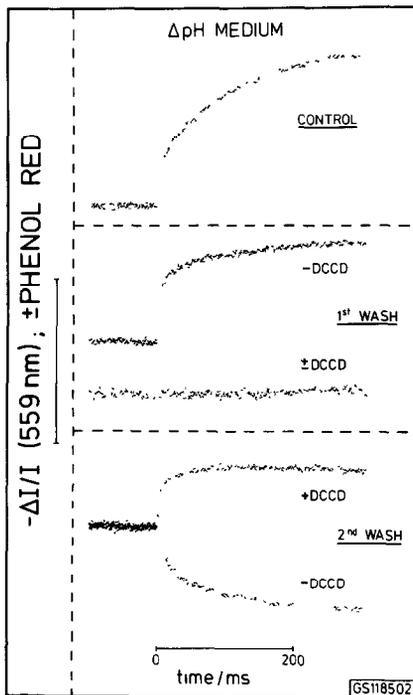


Fig.1. Time course of the absorption change of phenol red indicating transients of the pH in the suspending medium. (Top) Control thylakoids. (Middle, upper trace) Thylakoids after one EDTA wash. (Middle, lower trace) Difference of the upper trace and a trace measured after incubation with DCCD ( $25 \mu\text{M}$ , 10 min); the difference is zero. (Bottom) Thylakoids after second EDTA wash without DCCD (lower) and after incubation with DCCD (upper). Rising signals indicate alkalization of the medium. The bar indicates a relative change of transmitted intensity,  $\Delta I/I$ , of  $2 \times 10^{-3}$ .

### 3.2. 2nd EDTA wash led to a further 20% of $CF_1$ extraction and caused proton conductance through $CF_0$

After the 2nd EDTA wash no alkalization of the medium was observed but a rapid acidification (fig.1, bottom), accelerated by 3 orders of magnitude as compared to the control, indicating drastically increased proton permeability of the membrane. Correspondingly, the  $O_2$  evolution was uncoupled and the ATP synthesis rate dropped to 20% of controls (table 1). Incubation with DCCD ( $25 \mu\text{M}$ , 10 min) recoupled  $O_2$  evolution and blocked the high proton conductance, so that alkalization of the medium was again observed (fig.1, bottom, upper trace). Further established blocking agents of  $F_0$  channels [36], namely  $\text{Bu}_3\text{SnCl}$ ,  $\text{Ph}_3\text{SnCl}$  ( $5 \mu\text{M}$ ) [35] and venturicidin ( $50 \mu\text{g}/\text{mg}$  protein) [37] all acted similarly to DCCD, corroborating the view that the high proton permeability after 2 EDTA washes was caused by proton translocation through  $CF_0$ .

Fig.3 shows the time course of the pH transients in both aqueous phases – lumen and medium – for thylakoids washed twice with EDTA. Each of the four sections of fig.3 contains two traces. The lower traces were obtained with  $CF_0$  open, and the upper traces after blocking  $CF_0$  by DCCD. The difference between these two traces reflects the number of protons which passed the membrane via open  $CF_0$  channels. These differences are documented in fig.4. It is obvious that the time course of proton flow through  $CF_0$  was the same, whether viewed from the lumen or medium (see

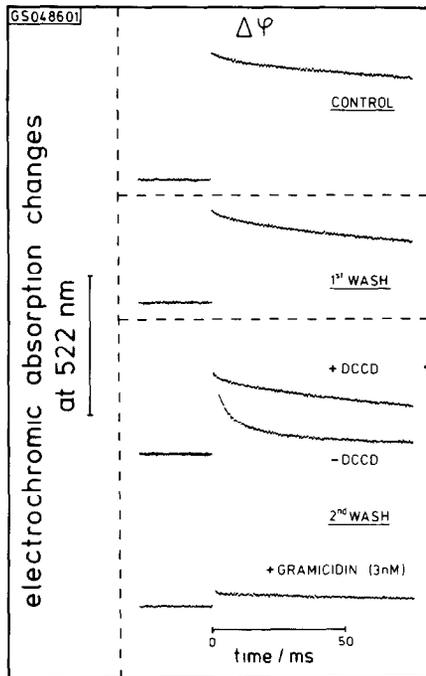


Fig.2. Time course of the field indicating electrochromic absorption changes at 522 nm. (Top) Control thylakoids. (Middle) Thylakoids after one EDTA wash. (Bottom, upper two traces) Thylakoids (washed with EDTA twice) before and after incubation with DCCD. (Bottom, lower trace) Thylakoids (twice EDTA washed) plus gramicidin (3 nM). The bar indicates  $\Delta I/I$  of  $5 \times 10^{-3}$ .

fig.4). The traces on the right in figs 3 and 4 were obtained with gramicidin (3 nM). The rationale for adding gramicidin was to eliminate the electric portion ( $F\Delta\phi$ ) of the electrochemical driving force ( $\Delta\mu\tilde{H}^+$ ) for protons and to leave over the chemical portion ( $-2.3RT\Delta\text{pH}$ ). Gramicidin (3 nM) shunted the electric potential difference in less than 1 ms (fig.2, bottom), while it only negligibly accelerated the relaxation of a pH difference (see fig.3, upper right). We attributed the obvious slowing down of the proton efflux by gramicidin (fig.4) to elimination of the electric force. Kinetic analysis for exponentials of the signals in fig.4 showed that the proton flux in the absence of gramicidin followed a biphasic decay, with a fast phase at 7.5 ms and a slow one at 60 ms, their extents being approximately equal. These relaxation times found a corollary in the following: (i) 7 ms was the decay time of the electric potential dif-

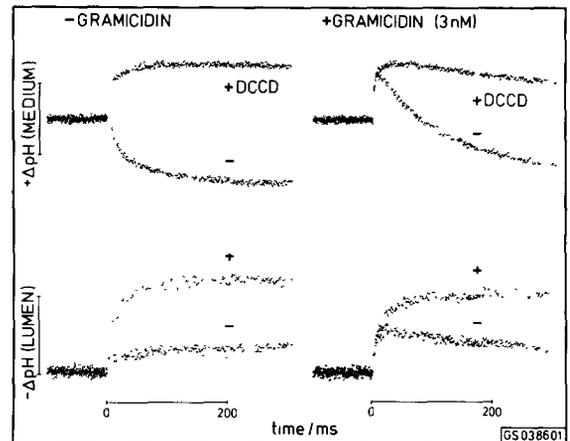


Fig.3. Time course of pH-indicating absorption changes in the medium ( $\pm 15 \mu\text{M}$  phenol red, upper) and in the lumen of thylakoids ( $\pm 15 \mu\text{M}$  neutral red plus 2.6 mg/ml BSA, lower) in the absence (left) and presence (right) of gramicidin (3 nM). Twice EDTA-washed thylakoids. Each of the four pairs of traces resulted from a measurement without DCCD (lower trace in each pair) and another measurement after incubation with DCCD (upper one). Positively directed signals indicate decreased absorption (see y-axis in fig.1) which represents alkalization of the medium (phenol red) and acidification of the lumen (neutral red), respectively. The bars indicate a  $\Delta I/I$  of  $10^{-3}$  (at wavelength 559 nm and for the medium) and  $\Delta I/I$  of  $4 \times 10^{-4}$  (at 548 nm and for the lumen).

ference of thylakoids washed twice with EDTA (fig.2). (ii) 85 ms was the relaxation time of the then monoexponential proton flow in the presence of gramicidin (fig.4, right). Hence we interpreted the biphasicity as follows: About one half of the protons passed through  $\text{CF}_0$  was mainly driven by electric force, while the other half was driven by chemical force ( $-2.3RT\Delta\text{pH}$ ) (see below).

### 3.3. The unit conductance of $\text{CF}_0$

Treating the thylakoid membrane as a capacitor we calculated the unit conductance. According to elementary physics the decay time of a transmembrane voltage is the ratio between the specific capacitance (usually assumed to be  $1 \mu\text{F}/\text{cm}^2$  for biological membranes) and the specific conductance. Thus, a decay time of 7 ms implied a specific conductance of  $1.43 \times 10^{-4} \text{ S}/\text{cm}^2$ . We took published values for the area per chlorophyll,

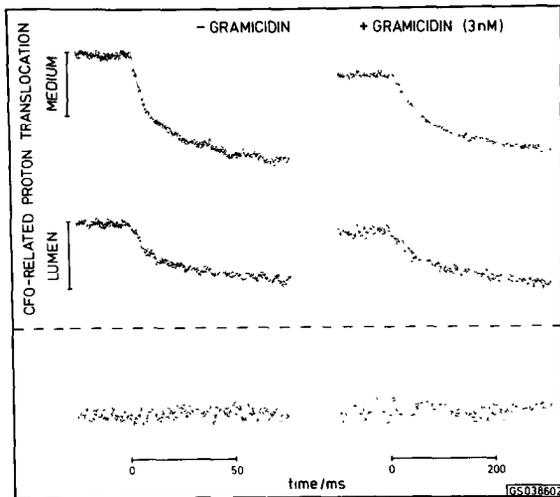


Fig.4. Time course of the proton flow through  $CF_0$ . The four traces in the upper part result from the four pairs of traces in fig.3 by subtracting (within each pair) the trace measured after incubation with DCCD from that without DCCD. Lower traces are the difference between the respective top and middle trace after scaling up of the middle traces by a factor 4.4. In the left part the time axis was spread by 4! The bars indicate the same as in fig.3.

$2.2 \text{ nm}^2$  [38], and for the frequency of  $CF_0$ - $CF_1$ ,  $1 CF_0$ - $CF_1/1000 \text{ chl}$  [10,39], and we assumed that the proton conductance was attributable to all  $CF_0$  which were exposed by  $CF_1$  extraction, approx. 30%. Then the density of exposed  $CF_0$  in the membrane was  $13.6 \times 10^9/\text{cm}^2$ . As a consequence the unit conductance was 10 fS. Almost certainly this underestimated the unit conductance since the tightness of the membrane after the first EDTA wash had already demonstrated that not all exposed  $CF_0$  were necessarily proton-conducting.

The ratio of 12 between the relaxation times under chemical driving force (85 ms) and under electrical force (7 ms) was attributable to the ratio between the electrical and the 'chemical' capacitance of thylakoids, as detailed in a forthcoming manuscript. We confirmed this ratio experimentally by measuring the protonic relaxation and electric relaxation for gramicidin concentrations between 3 and 300 nM with twice EDTA-washed thylakoids 'resealed' by DCCD. The ratio of the respective relaxation times was  $5 \times 10^4$  (protons/electric field) over the whole concentration range. As the pH was 7.3 and the  $Na^+$  concentra-

tion was 10 mM neither ion saturated gramicidin. Relating the ratio of the relaxation times to the ratio of the concentrations of both ions ( $2 \times 10^5$ ) and considering the intrinsic selectivity of gramicidin for protons over  $Na^+$ , approx. 50 according to [40], the corrected ratio of the protonic over the electric relaxation time became 12 ( $50 \times 5 \times 10^4/2 \times 10^5$ ). It was satisfying that this agreed with what we found for relaxation through  $CF_0$ .

#### 4. CONCLUSIONS

Proton flow across thylakoid membranes as mediated by exposed  $CF_0$ , the channel portion of the ATP synthase, was completely tracked. A lower limit for the unit conductance of  $CF_0$  was obtained under the assumption that all exposed  $CF_0$  were conducting. This value was 10 fS corresponding to the passage of 6200 protons per s at 100 mV electrical driving force. Even this lower limit exceeded published values for the conductance and the turnover numbers of  $F_0$  channels by orders of magnitude. It was very satisfying that it also exceeded the highest turnover numbers which were reported for the intact synthase, e.g. for  $CF_0$ - $CF_1$  (900 protons/s  $CF_0$ - $CF_1$ ). For the first time this was observed. The lack of increased proton conductance after the first EDTA wash was puzzling. It may indicate that the proton channel was plugged, e.g. by subunit  $\delta$  of  $CF_1$ , as previously proposed [43].

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