Eur. J. Biochem. 179, 459–467 (1989)

CF_0, the proton channel of chloroplast ATP synthase

After removal of CF_1 it appears in two forms with highly different proton conductance

Holger LILL and Wolfgang JUNGE

Biophysik, Fachbereich Biologie/Chemie, Universität Osnabrück

(Received September 12, 1988) – EJB 881071

The discharge of the flash-induced transmembrane voltage through the exposed proton channel, CF_0, of the chloroplast ATP synthase, CF_0CF_1 was investigated. EDTA treatment of thylakoid membranes exposed approximately 50% of total CF_0 by removal of the CF_1 counterparts. This greatly accelerated the decay of the transmembrane voltage, as was apparent from electrochromic-absorption changes of intrinsic pigments and by pH-indicating-absorption changes of added dyes. Two decay processes were discernible, one rapid with a typical half-decay time of 2 ms, and a slower one with a half-decay time variable between 20–100 ms. Both were sensitive to CF_0 inhibitors, but only the rapid decay process was also inhibited by added CF_1. CF_1 was effective in surprisingly small amounts, which were significantly lower than those previously removed by EDTA treatment. This finding corroborated our previous conclusion that the rapid decay of the transmembrane voltage was attributable to only a few high-conductance channels among many CF_0 molecules, typically in the order of one channel/CF_1-depleted EDTA vesicle.

Inhibition of photophosphorylation in control thylakoids was measured as function of the concentration of CF_0 inhibitors. It was compared with the inhibition of proton conduction through exposed CF_0 in EDTA vesicles. Photophosphorylation and proton conduction by the high-conductance form of CF_0 were inhibited by the same low inhibitor concentrations. This suggested that the high-conducting form of CF_0 with a time-averaged single-channel conductance of 1 pS [Lill, H., Althoff, G. & Junge, W. (1987) *J. Membrane Biol. 98*, 69–78] represented the proton channel in the integral enzyme, which acted as a low-impedance access from the thylakoid lumen to the coupling site in CF_0CF_1.

The slow decay process was attributed to a majority of low-conductance CF_0 channels, i.e. about 50 molecules/vesicle. The conductance of these channels was more than 100-fold lower and they did not compete with the very few highly conducting channels for rebinding of added CF_1. The low proton conduction of the majority of exposed CF_0 molecules, possibly due to a structural rearrangement, may be protecting the thylakoid membrane against rapid energy dissipation caused by accidental loss of CF_1. It may also explain the low single-channel conductance of bacterial F_0 reported in the literature.

The ATP synthase of thylakoid membranes, CF_0CF_1, is closely related to F_0F_1-type ATPases in the energy-transducing membranes of other organisms (e.g. EF_0EF_1 in *Escherichia coli*, MF_0MF_1 in mitochondria, and TF_0TF_1 in the thermophilic bacterium, PS3). These complexes are composed of two distinct parts. F_1, the portion extrinsic to the membrane, contains the catalytic sites of ATP synthesis and hydrolysis (for review, see [1]). F_0, a membrane-spanning protein complex, is a proton conductor (for review, see [2]).

CF_1, the ATPase from thylakoid membranes, is composed of five different polypeptides, named α, β, γ, δ, and ε in order of decreasing molecular mass. The subunit stoichiometry probably is 3:3:1:1:1 [3] and the total mass in 410 kDa [4]. The large α and β subunits from nucleotide-binding sites; the catalytic sites are probably located on β or between two neighbouring α and β subunits [5]. The γ subunit might regulate proton flow through CF_0CF_1 [6, 7]. The ε subunit of CF_1 binds to γ [8], acts as an inhibitor of ATP hydrolysis and seems to be involved in the regulation of proton conduction by the whole enzyme [9]. The δ subunit appears to be necessary for efficient coupling of CF_1 to CF_0 [10]. Junge et al. [11] proposed that δ could remain bound to the thylakoid membrane upon extraction of CF_1 and act as a 'stopcock' to CF_0 rendering it inactive in proton translocation. In accordance with this, we showed recently that addition of δ to partially CF_1-depleted EDTA vesicles blocked open CF_0 [40], and thereby restored ATP synthesis [12].

In CF_0, four different subunits have been identified and named I–IV. Their stoichiometry is still under debate. In the integral enzyme, the proton permeability of CF_0 is controlled by CF_1. Proton conduction through the whole CF_0CF_1 complex normally occurs only under conditions which allow catalytic activity of CF_1 [9, 13]. In most experiments aiming at the proton conduction of the channel portion alone, F_0 [14], or one or more of its subunits [15], were isolated and incorporated into artificial lipid membranes. This approach has produced proton channels which were sensitive to *N,N*-dicyclohexylcarbodiimide $(cHxN)_2C$. However, the turnover numbers in the order of only 10 H^+/s (at 100 mV driving force) [14, 15] fell short by two orders of magnitude from the

Correspondence to W. Junge, Biophysik, Fachbereich Biologie/Chemie, Universität Osnabrück, Postfach 4469, D-4500 Osnabrück, Federal Republic of Germany

Abbreviations. CF_1, chloroplast ATP synthase (soluble portion); CF_0, chloroplast ATP synthase (membrane portion); $(cHxN)_2C$, *N,N*-dicyclohexylcarbodiimide; PMS, phenazine methosulphate.

Enzyme. ATP synthase (3.6.1.34).

proton turnover of the integral enzyme (at least 1200 H^+/s [16]) and 3 – 4 orders of magnitude from the expected turnover numbers of a 'proton well' which would serve as a low-impedance access to the coupling site in the ATP synthase [17]. This shortcoming might have been due to the fact that only a small fraction of intact channels survived in such reconstitution experiments [18]. In an alternative approach to determine the time-averaged single-channel conductance of CF_0, we removed CF_1 from some of the CF_0CF_1 complexes by EDTA treatment of thylakoids and studied proton flow through the exposed CF_0. A trans-membrane voltage was generated by excitation of vesicles with a short flash of light and the leakage to protons of CF_1-depleted thylakoid membranes was monitored spectrophotometrically by electrochromism of intrinsic pigments and by pH-indicating dyes. By statistical analysis, we found that only a low fraction, about 1% of total CF_0, which was exposed by extraction of CF_1, was highly active in proton translocation. For these rapid channels, we evaluated the high time-averaged single-channel conductance of 1 pS [19], corresponding to 6×10^5 H^+/s at 100 mV.

Schmid et al. have shown that the extra conductance in thylakoid membranes which is induced by EDTA treatment is only partially reversible by rebinding of extracted CF_1 [20]. The latter has been interpreted as being due to the additional opening of unspecific channels, which could not be closed by rebinding of CF_1. Here, we showed that these other channels were sensitive to several inhibitors of F_0-type channels [21] and therefore attributable to a low-conductance form of CF_0. This form of CF_0 was insensitive to added CF_1, presumably because it had lost the ability to rebind its CF_1 counterpart. In parallel experiments we measured the inhibition of photophosphorylation in control thylakoids and the inhibition of fast proton translocation in EDTA vesicles. Suppression of the catalytic activity was correlated with the inhibition of the high-conductance form of CF_0, suggesting that the high-conductance form of CF_0 was also present in active CF_0CF_1.

MATERIALS AND METHODS

Broken pea chloroplasts (thylakoids) were prepared from 10 – 14-day-old plants. They were CF_1-depleted by EDTA treatment as in [19]. The EDTA solution contained 100 μM EDTA, 1 mM NaCl, and 1 mM Tricine/NaOH, pH 7.8. CF_1 release was stopped by addition of 1 M NaCl to yield a final concentration of 30 mM.

The CF_1 content of EDTA vesicles was determined relative to untreated control samples by rocket immunoelectrophoresis by the method of Laurell [22] modified as in [23]. The buffer system was changed to Tris/borate instead of barbiturate. ATP synthesis was monitored via the luciferin/luciferase assay as in [16]. Methanol-activated Mg^{2+}-ATPase was measured as in [24]. Purification of spinach CF_1 and reconstitution of photophosphorylation was carried out as in [25].

Flash-spectrophotometric measurements were performed in the same setup as described [26, 27]. Thylakoids were excited with short flashes of light to drive the proton pumps through a single turnover. The measuring buffer contained 10 μM methyl viologen, 10 mM NaCl, 1 mM Tricine/NaOH, pH 7.5. The transient voltage across the thylakoid membrane after single-flash excitation was followed by the electrochromic-absorption changes of intrinsic pigments at 522-nm wavelength as previously described [19]. Computer-aided fits were performed either by means of DISCRETE routine [28] or by a self-written program based on the SIMPLEX algorithm [29].

Transient pH changes in the external suspending medium were monitored by the pH_{out}-indicating-absorption changes of phenol red (13 μM) at 559 nm wavelength [30]. The solution of measuring pH transients contained 10 μM methyl viologen and 10 mM NaCl. The pH was adjusted to 7.5 by titration with NaOH. The pH_{out}-indicating-absorption changes of the dye were obtained by subtraction of two transient signals, one of which was measured in 13 μM phenol red and the other without the indicator. 20 signals were averaged and subtrated in each case. The specificity of hydrophilic pH-indicating dyes for pH transients in the medium was established elsewhere [31]. In CF_1-depleted EDTA vesicles, with superoxide dismutase inactivated by EDTA treatment, the pH_{out}-indicating signals were composed of several events: the uptake of protons at the quinone-reducing site of photosystem II, which makes the suspending medium alkaline; and the liberation of protons into the thylakoid lumen, both at quinone and at water-oxidizing sites, which causes an acidification of the medium after passage of protons across the membrane (for a review of these reactions, refer to [32]). In control thylakoids, proton uptake from the outer side of the thylakoid membrane is apparent in the medium only after 100 ms (half-rise time, see [33]), whereas the efflux of protons which are liberated in the lumen is much slower (14 s, see [34]). In CF_1-depleted EDTA vesicles, with enhanced proton conductance via CF_0, this efflux occurs in the same time domain as proton uptake. The protonic events at both sides of the membrane are then superimposed on each other and mixed in the transients which are visualized using phenol red. In order to extract the CF_0-dependent proton displacement, CF_0 was blocked by covalent modification of subunit III with $(cHxN)_2C$ [33]. Subtraction of proton uptake from the medium, which is observed after resealing of CF_0 channels by $(cHxN)_2C$, from mixed transients which have been observed from the same batch before $(cHxN)_2C$-treatment then in turn yields a transient proportional to the proton displacement by CF_0 [34].

CF_0 inhibitors were added from ethanolic stock solution. The ethanol concentration was always held below 0.5% in order to avoid unspecific damage to thylakoid membranes. In the case of $(cHxN)_2C$, a 10-min incubation in the dark was performed prior to measurement. No preincubation was necessary with triphenyltin chloride or venturicidin. $(cHxN)_2C$ was purchased from Sigma, triphenyltin chloride was from Fluka, and venturicidin from BDH biochemicals. The luciferin/luciferase assay was obtained from LKB. All other chemicals were either from Merck or Sigma and of highest grade available.

RESULTS AND DISCUSSION

In [35] we examined the time dependence of CF_1 depletion upon incubation of thylakoids in hypo-osmolar EDTA solution. Two subpopulations of small EDTA vesicles were generated by this procedure, one of them contained highly conducting proton channels in their membranes and the other one lacked such channels [19, 35]. This was apparent by a biphasic decay of the electrochromic absorption changes after excitation of EDTA vesicles with a single short flash of light. Examples for this biphasic decay are shown in Fig. 1 for various incubation times of thylakoids in EDTA solution.

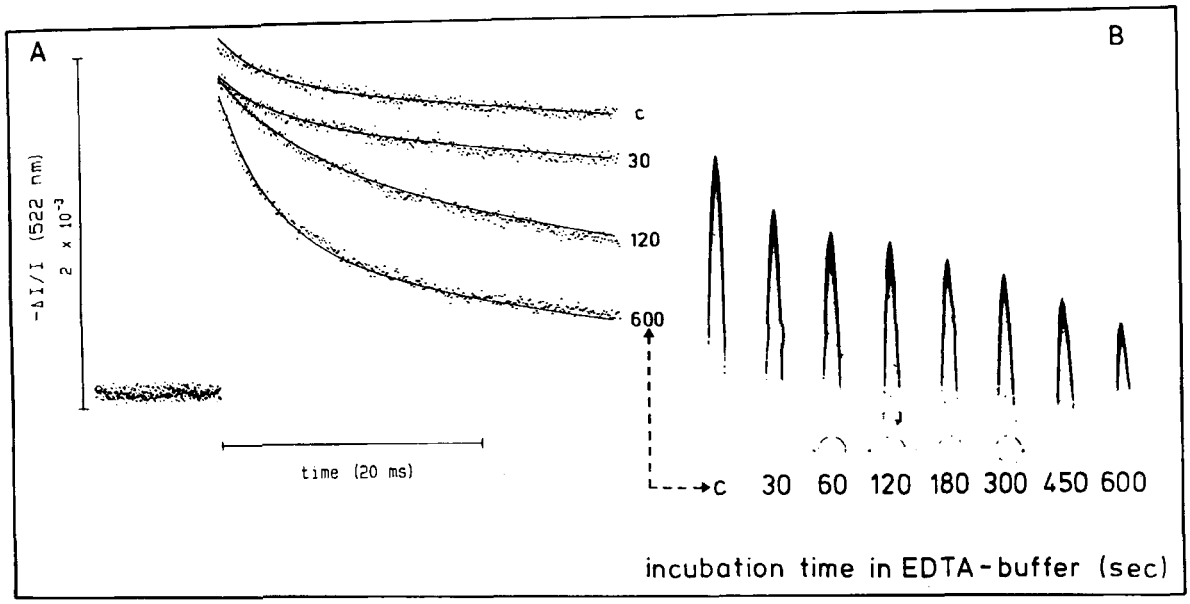

Fig. 1. *Flash-induced electrochromic-absorption changes after CF_1 extraction (A) and amount of CF_1 remaining on membranes as a function of the incubation time in EDTA solution (B).* (A) Transient electrochromic-absorption changes. After incubation in EDTA buffer (100 μM EDTA, 1 mM NaCl, 1 mM Tricine/NaOH, pH 7.5) for the indicated time period, CF_1 release was stopped by addition of 1 M NaCl to yield a final concentration of 30 mM (the numbers arrowed are in seconds; c, controls, i.e. untreated thylakoids). Solid lines represent numerical fits by Eqn (1). (B) Precipitation arcs obtained by rocket immunoelectrophoresis of thylakoid membranes. 4 μg chlorophyll was applied to every well, 150 μl of a rabbit antiserum raised against purified spinach CF_1 was added to the gel. Electrophoresis was carried out for 16 h at 2.5 mV/cm

The degree of CF_1 depletion of the respective membranes as detected by rocket immunoelectrophoresis is shown in the rigth part of the figure. In [19], the biphasic decay has been interpreted as follows: the incubation of thylakoids in hypo-osmolar EDTA solution, aside from solubilizing CF_1, generated smaller vesicles containing about 10^5 chlorophyll molecules or a total of 100 molecules of CF_0CF_1. Removal of, e.g. half of the 100 CF_1, molecules produced very few highly conducting CF_0 channels, in the order of 1 channel/EDTA vesicle, and in the order of 50 exposed CF_0 which were non-conducting or badly conducting. Thus the rapid-decay phase was attributable to those vesicles possessing one or more highly conducting channels. The slow-decay phase, on the other hand, was attributed to those vesicles without any high-conductance channel. Application of Poisson's statistics has led to a decay law of the type $\exp[\exp(-\alpha t)]$ which has been fitted to the data to evaluate the time-averaged unit of conductance of CF_0 (see [19]). This fit was applicable for the first 10 ms of the decay. For longer time domains however it could not be ignored that those vesicles without any high-conductance channel did not sustain the transmembrane voltage forever. Hence in this study we used a biexponential decay law with a slow component for those vesicles without any high-conductance channel. To simplify the matter, we approximated the rapid component by a single exponential, although one should note that the fast phase was composite with contributions from vesicles with one, two or more highly conducting channels, with decreasing probability. Following this simplifying mathematical approach, the decay of the electrochromic-absorption changes was simulated by:

$$\Delta A(t) = \Delta A_{(max)} \cdot [\alpha_1 \cdot \exp(-k_1 \cdot t) + \alpha_2 \cdot \exp(-k_2 \cdot t)] \quad (1)$$

where

$$\alpha_1 + \alpha_2 = 1 \quad (2)$$

$\Delta A(t)$ = extent of absorption change as a function of time t; $\Delta A_{(max)}$ = maximal extent of absorption change; a_1 = relative contribution of the first exponential to the overall signal; k_1 = rate constant of the first exponential; a_2 = relative contribution of the second exponential to the overall signal; k_2 = rat constant of the second exponential. The rate constants are related to the respective half-decay times as follow:

$$k = \frac{\ln 2}{1/2}. \quad (3)$$

In Fig. 1, the results of the computer-assisted fits have been plotted into the measured transients by solid lines.

Fig. 2 represents the effects of incubation of thylakoids in hypo-osmolar EDTA solution as a function of the incubation time. Fig. 2A shows the parameters resulting from fitting electrochromic-decay signals by Eqn (1) Fig. 2B shows the CF_1 content of the respective EDTA vesicles as determined by immunoelectrophoresis. The slowly decaying part of the voltage transients was accelerated during the first 2 min of EDTA incubation to a final half-decay time of approximately 20 ms. After reaching this value, further extraction of CF_1 did not further accelerate the decay of the slow phase. Instead, it shifted the relative contribution of the two phases to the overall decay from slow to fast. The half-decay time of the fast phase varied stochastically and therefore negligibly as a function of the incubation time (2.2 ± 0.7 ms).

We asked whether both the fast and the slow decay of the transmembrane voltage were due to CF_0-mediated ion translocation. EDTA vesicles which had been highly CF_1 depleted (10 min EDTA extraction, which removed typically 60% of the original CF_1, cf. Fig. 2B) were incubated with 25 μM $(cHxN)_2C$ or with 100 μg purified spinach CF_1/10 μg chlorophyll. The respective voltage transients are shown in Fig. 3. $(cHxN)_2C$ completely resealed the leaks introduced by removal of CF_1 (trace a), and the same hold true for two other CF_0-specific inhibitors [21], venturicidin and triphenyl chloride (not shown). Addition of CF_1 on the other hand, abolished the greater part of the rapid-decay phase but did

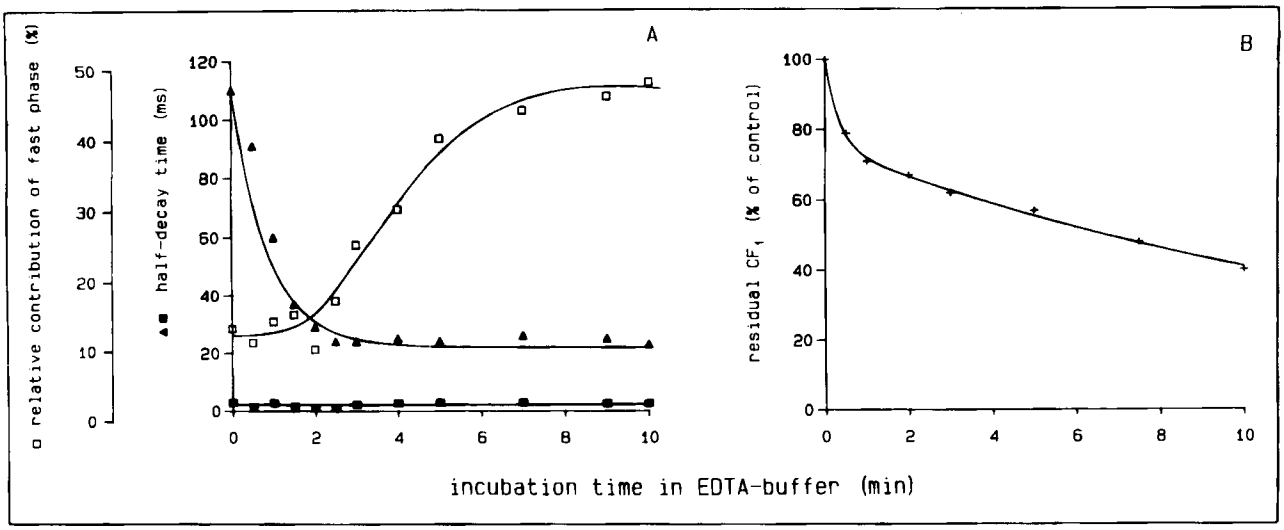

Fig. 2. *Quantification of effects introduced by EDTA incubation of thylakoids.* On the abscisae, incubation times of thylakoids in extraction buffer before adjustment of [NaCl] to 30 mM are indicated. (A) Fit parameters (according to Eqns 1–3) to the decay of electrochromic-absorption transients. The half-decay times of both phases (filled squares: fast phase; filled triangles: slow phase) and the relative contribution of the fast phase to the overall decay (open squares) are shown. (B) Relative amount of residual CF_1 still bound to the membranes as determined by rocket immunoelectrophoresis

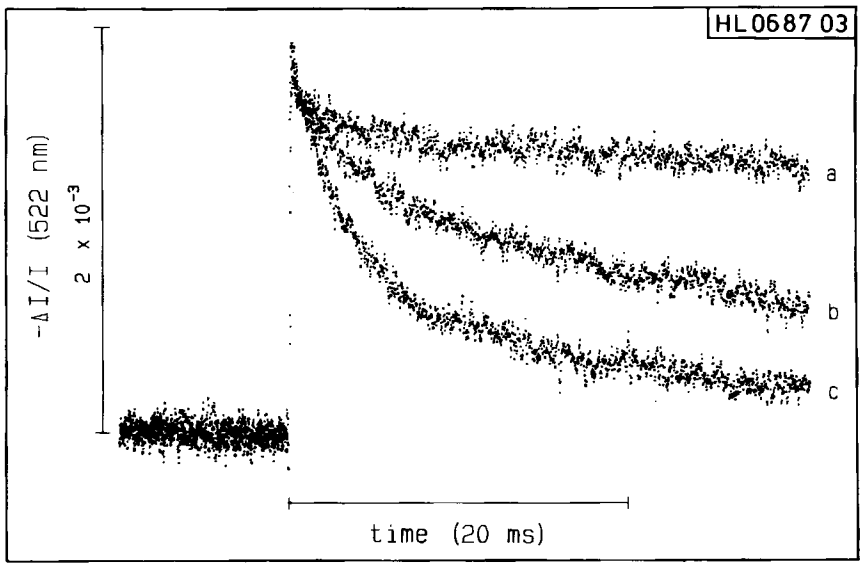

Fig. 3. *Electrochromic transients in EDTA vesicles with CF_1 and with $(CHxN)_2C$ added.* Thylakoids were incubated for 10 min in EDTA solution, resulting in 60% loss of CF_1 (cf. Fig. 2B). 20 µg chlorophyll was suspended in 500 µl measuring buffer (10 mM NaCl, 1 mM Tricine/NaOH, pH 7.5) without methyl viologen and incubated for 10 min. Additions were: (c) none; (b) 100 µg CF_1, the incubation was started by addition of 4 µl 1 M $MgCl_2$; (a) 40 µM $(cHxN)_2C$. Incubations were carried out at room temperature in the dark. Finally, 500 µl of the same buffer, but with an additional 40 µM methyl viologen was added. 1-ml microcuvettes with 1-cm optical pathlength were used for these measurements

not influence the slow phase. Electrochromic transients as shown in Fig. 3 were analyzed for the kinetic parameters introduced in Eqn (1) and also used in Fig. 2. The result is shown in Fig. 4A as a function of the amount of CF_1 which was added to highly CF_1-depleted EDTA vesicles. While very little CF_1 had to be added to suppress the rapid-decay phase, CF_1 had less influence on the half-decay time of the slow phase. A similar experiment with the addition of increasing amounts of $(cHxN)_2C$ instead of CF_1 produced a different result (Fig. 4B). With increasing concentrations of the inhibitor, the relative extent of the fast phase was reduced as already observed with CF_1. At further increased concentrations, however, the 20-ms phase was decelerated to control values without any further shift in the contributions of the two phases. This suggested that both kinetic phases were caused by CF_0-mediated ion translocation and led to the conclusion that CF_1-depleted CF_0 existed in two operating conditions, a high-conductance form which could be blocked by added CF_1 or $(cHxN)_2C$, and a low-conductance form which was sensitive to $(cHxN)_2C$.

The same highly CF_1-depleted EDTA-vesicles as used in the experiments described above were used for the measurement of transient pH changes after single-flash excitation of proton pumps. Fig. 5A and B show the pH transients without and with $(cHxN)_2C$ added. If the CF_0 channels were completely blocked by $(cHxN)_2C$ (25 µM, Fig. 5A) only proton

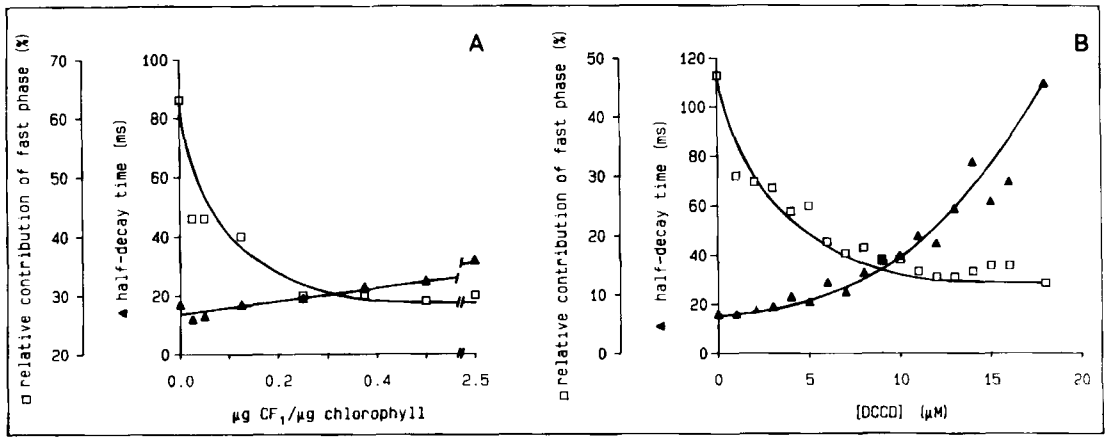

Fig. 4. *Fits of the decay of electrochromic-absorption changes after incubation of EDTA vesicles with CF_1 or $(cHxN)_2C$.* Potential decay traces as in Fig. 3 were fitted by Eqn (1) after incubation of highly extracted EDTA vesicles with various amounts of protein or $(cHxN)_2C$ (DCCD). Symbols stand for half-decay times of the slow phase (filled triangles) and relative contribution of the fast phase to the overall decay (open squares) as in Fig. 2. (A) Fits of traces derived from samples which were incubated with various amounts of CF_1. (B) Respective fits obtained from $(cHxN)_2C$-treated samples

uptake at the outer side of the membrane was apparent (resulting in an alkalinization of the medium, which is represented by a positive signal), while the backflow of protons from the lumen occurred over a much longer time scale (14 s [34]) and was therefore not recorded in the measurement shown in Fig. 5. The backflow, however, was apparent in the absence of $(cHxN)_2C$ (lower trace in Fig. 5B). In the other traces at 6 μM and 16 μM $(cHxN)_2C$ show an intermediate situation with some channels blocked and others not. Fig. 5C shows the difference between the traces in Fig. 5B and the one in Fig. 5A, representing proton displacement by CF_0 without the overlaid proton uptake.

Inspection of the traces describing proton displacement by CF_0, e.g. the trace denoted 0–25 in Fig. 5, again showed two decay processes, a fast one with a typical half-decay time of 5 ms, and a slow one with a typical half-decay time of 40 ms. This paralleled the biphasic decay of the transmembrane voltage (cf. Fig. 2). The traces were analyzed by a computer assisted fit to Eqn (1). The result was plotted as function of the $(cHxN)_2C$ concentration in Fig. 6A. The half-decay time of the fast phase (filled squares) was unaffected by $(cHxN)_2C$. But the relative contribution of the fast phase to the overall efflux was diminished in the concentration range 0–10 μM $(cHxN)_2C$. The half-decay time of the slow phase was decelerated from around 40 ms to 110 ms in this concentration range. At higher concentration of $(cHxN)_2C$, the decreased extent of the signal reduced the reliability of the fit, therefore the concentration range was restricted in Fig. 6A. This behaviour, suppression of the fast decay phase $(cHxN)_2C$ and conversion of vesicles with one or more of the high-conducting CF_0 into vesicles with many low-conductance channels, paralleled the one observed by monitoring the electric transients. It established that both forms of exposed CF_0 were specific for protons.

The inhibition by $(cHxN)_2C$ of fast proton displacement in CF_1-depleted EDTA vesicles was compared with the inhibition of ATP synthesis in control thylakoids. Fig. 6B shows that the inhibition of the fast phase, measured both by the pH-indicating dye or by electrochromism, revealed the same concentration dependence as the inhibition of ATP synthesis. The slow phase of proton translocation by CF_0, on the other hand, was much less sensitive to $(cHxN)_2C$. This was immediately obvious from inspection of the original pH transients in Fig. 5B and C. While the fast phase of proton displacement was practically abolished at 6 μM $(cHxN)_2C$, the slow phase survived this concentration, but was blocked at 25 μM $(cHxN)_2C$. That photophosphorylation and the high-conducting form of CF_0 showed the same sensitivity against $(cHxN)_2C$ suggested that the high-conductance form of CF_0 was like the form of the channel which was active in photophosphorylation.

The amounts of CF_1 necessary to achieve half-maximal inhibition of the fast channel were surprisingly low. The shift of the two phases was half completed at 0.06 μg CF_1 added/μg chlorophyll (Fig. 4A). With 60% losses of total CF_1 from the membranes used in these experiments and with an initial stoichiometry of 0.5 μg CF_1/μg chlorophyll [36], this amount comprised only 20% of the previously extracted CF_1. In a series of similar experiments, this fraction varied between 3–30%, indicating a number of CF_1-binding sites which was always lower than the number of exposed CF_0, even under the unlikely assumption that none of the added CF_1 molecules was lost and instead all were bound to a CF_0. This had two implications: (a) it corroborated by simple 'biochemical counting' our previous conclusion, that only a small fraction of CF_0, which was exposed by removal of its CF_1 counterpart, was proton conducting with a high conductance [19]; (b) the large majority of exposed CF_0 channels, which were in the low-conductance form, were much less able to rebind added CF_0 since they did not compete for the added CF_1 with the very few CF_0 channels which were in the high-conductance form. Both conclusions were corroborated by other lines of evidence.

a) The rebinding of CF_1 to the high-conductance form of CF_0 restored phenazinemethosulfate (PMS)-mediated-photophosphorylation activity in CF_1-depleted EDTA vesicles [25]. This is documented in Fig. 7A. Two types of electron acceptors were compared, methyl viologen acts as terminal electron acceptor for the linear electron-transport chain which starts from water oxidation. It allows a typical turnover time of proton pumping of 20 ms [32]. PMS, on the other hand, catalyzes a cyclic electron transport around photosystem I which gives rise to about tenfold higher rates of proton pumping under continuous light. Accordingly, proton

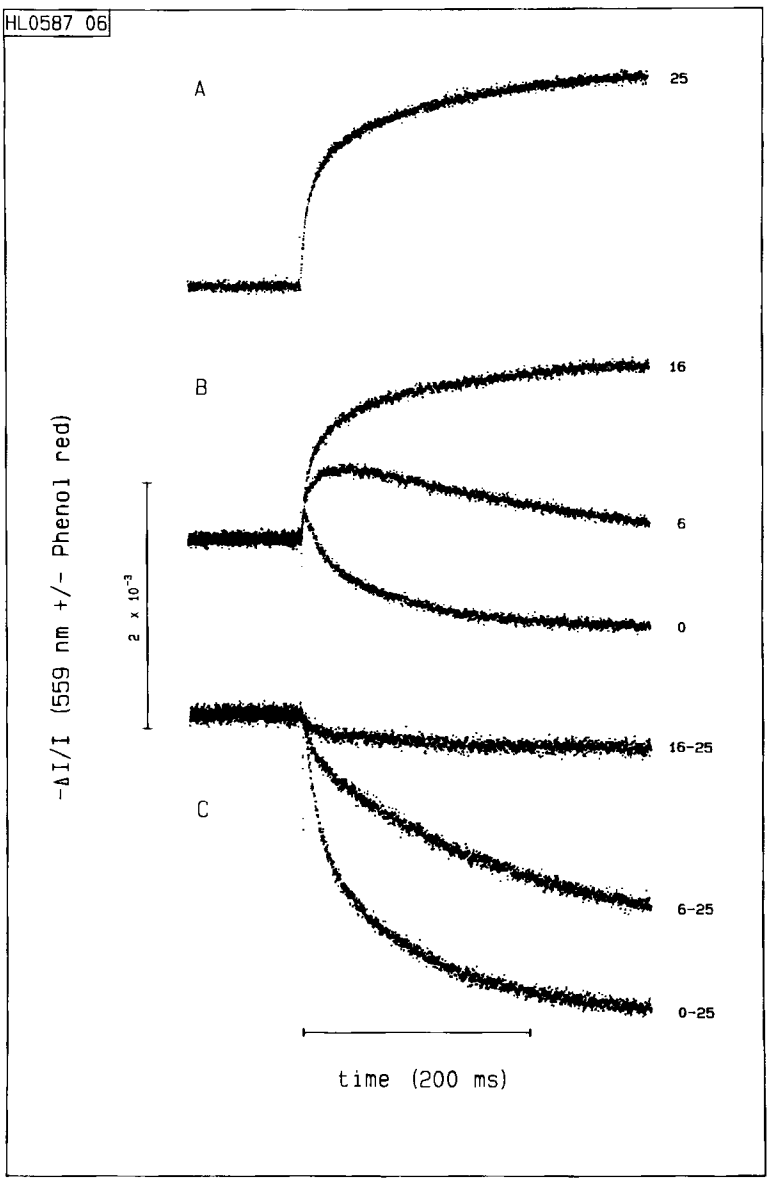

Fig. 5. *pH transients in the suspending medium of CF_1-depleted EDTA vesicles after incubation with various amounts of $(cHxN)_2C$, in μM.* Thylakoids had been incubated for 10 min in EDTA solution and thereby lost 60% of their CF_1, as determined by immunoelectrophoresis (cf. Fig. 2B). (A) pH transient in the outer suspending medium after 10 min incubation with 25 μM $(cHxN)_2C$. (B) pH transients after incubation without (0), with 6 μM and 16 μM $(cHxN)_2C$, respectively. (C) Proton displacement after incubation with various concentrations of $(cHxN)_2C$ as obtained by subtraction of the trace in (A) from traces in (B)

pumping mediated by PMS should be able to overrun a residual proton leakage of the membrane, while the same leakage still may drain out the proton-motive force generated by the slower linear electron-transport chain. The results in Fig. 7A suggested that the addition of CF_1 to depleted membranes resealed the high-conductance channels but still left a residual proton leak behind. This corroborated the conclusion derived from Fig. 4, the existence of two forms of CF_0, with the high-conductance form being the only one sensitive to added CF_1. Moreover, the saturation curve for the reconstitution of photophosphorylation showed nearly the same dependence on the amount of added CF_1 as did the respective curve for the inhibition of the fast charge translocation (cf. Fig. 4A).

b) We used the rate of CF_1-catalyzed ATP hydrolysis to assay the amount of CF_1 present on the thylakoid membrane. In Fig. 7B, the methanol activated Mg^{2+}-ATPase activity of EDTA vesicles incubated with a saturating amount of CF_1 (2.5 μg CF_1/μg chlorophyll) is shown as a function of the number of washes and centrifugations in CF_1-free buffer. Whereas in the absence of CF_1 the rate of ATP hydrolysis of CF_1-depleted EDTA vesicles did not change as a function of the number of washes, the ATPase activity which was regained after preincubation with saturating amounts of CF_1 was greatly reduced already after a first wash of the membranes. Concomitant with further centrifugations, the ATPase activity of the CF_1-incubated EDTA vesicles decreased towards the residual activity of the same vesicles without preincubation with CF_1. This finding could indicate that only very few CF_1 molecules were rebound firmly to CF_1-depleted membranes and that most of the ATPase activity regained after incubation with CF_1 and before washing was due to unspecific adsorption

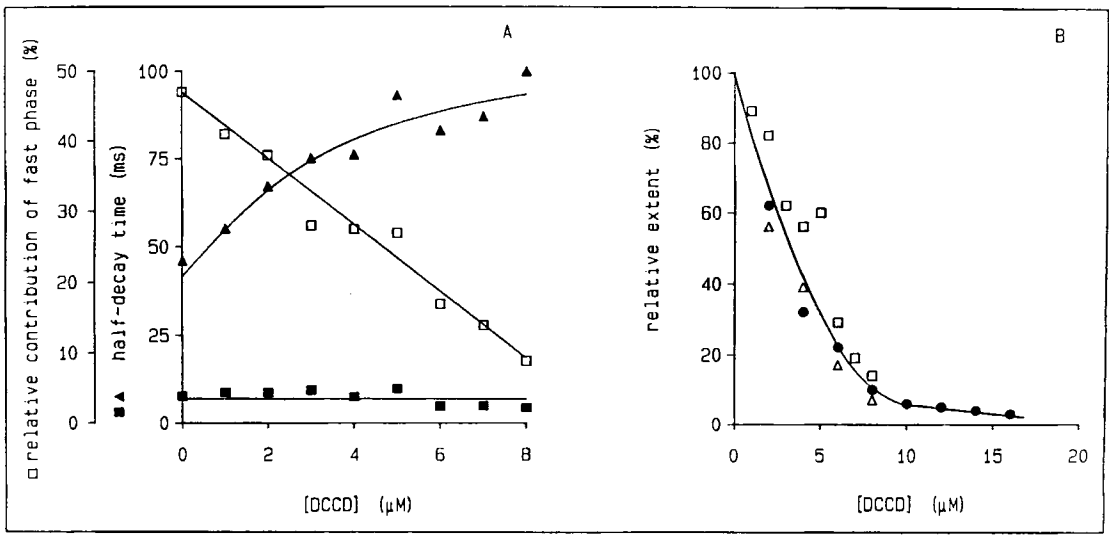

Fig. 6. *Inhibition of proton displacement through CF_0 by $(cHxN)_2C$.* (A) Parameters of a fit of transient proton displacement as documented in Fig. 5C by two exponentials (Eqn 1) as a function of the concentration of $(cHxN)_2C$ (DCCD). The half-decay times of the fast phase (filled squares) and the slow phase (filled triangles), and the relative contribution of the fast phase to the overall proton displacement (open squares) are shown. (B) Comparison of voltage transients and proton displacement by exposed CF_0 and ATP-synthesis rates in control thylakoids as a function of the concentration of $(cHxN)_2C$. The open squares show the contribution of fast proton displacement to the overall proton displacement as measured by the pH-indicating dye (see Fig. 5, traces A and C). Open triangles stand for the contribution of the fast phase to the overall decay of the voltage transients (cf. Fig. 4B). Filled circles show the rate of ATP synthesis. While the former two measurements were performed with CF_1-depleted EDTA vesicles as in (A), the rates of ATP synthesis were measured with control thylakoids

of enzymes to the thylakoid membranes. This in turn implied that the number of firm binding sites for CF_1, probably those on the few molecules of CF_0 in the high-conductance form, was very low in proportion to the number of exposed CF_0 molecules.

c) The above conclusions were further supported by another independent line of evidence. Fig. 7C shows the rate of photophosphorylation as a function of the incubation time of thylakoids. As in Fig. 1, the degree of CF_1 depletion of thylakoid membranes was varied by the incubation time in EDTA solution. The ATP-synthesis rates were determined with and without preincubation with saturating amounts of CF_1. Fig. 7C shows that the addition of CF_1 restored photophosphorylaton, but only to a degree which correlated with the amount of residual CF_1 associated with the thylakoid membranes (compare crosses and filled circles). It therefore followed that resumption of photophosphorylation was caused by the closing of very few high-conductance CF_0 channel. This reactivated those vesicles which were highly leaky, but as the number of highly conducting CF_0 molecules was very small in comparison with the number of exposed but badly conducting CF_0, which were unable to rebind CF_1, the maximal rate of photophosphorylation could not rise beyond that attributable to CF_1 which remained on the membrane after EDTA treatment.

Conclusion

Removal of CF_1 from chloroplast ATP synthase by EDTA treatment exposes the CF_0 counterpart and generates proton leaks. These leaks are apparent as an accelerated decay of the flash-induced transmembrane voltage. The decay of the electrochromic absorption changes was biphasic. It resulted from an ensemble of more than 10^{10} vesicles [37, 38]. We attributed the fast phase to the subset of vesicles which had one highly conducting channel, at least, and the slow phase to the complement without such channel. Both kinetic phases of this decay were sensitive to inhibitors of F_0-type proton channels, namely to $(cHxN)_2C$, venturicidin and triphenyltin. Only the fast phase however could be eliminated by addition of purified CF_1. The very high time-averaged single-channel conductance of highly conducting channels, 1 pS, reported previously [19] has resulted from a statistical analysis of this fast-decay process. It is a direct consequence of the fact that only a small percentage out of many exposed CF_0 molecules are highly proton conducting. This result was corroborated here by simple 'biochemical counting'. We found that the fast decay could be eliminated by adding back fewer molecules of purified CF_1 than had been removed from the membrane. The low-conductance CF_0 could not rebind CF_1. It is conceivable that the low conductance form of F_0 channels reported in the literature is attributable to the more frequent low-conductance form of these channels. There may therefore be a factor, as yet unknown, which causes a few out of many exposed channels to remain in the high-conductance form, whereas most of the CF_0 are converted into the low-conductance form upon removal of CF_1. The obvious physiological advantage of such conversion, which was already observed by Nelson and Eytan in 1979 [39], would be the protection of the thylakoid membrane against energy dissipation in case of accidental loss of CF_1.

The rate of PMS-mediated ATP synthesis in control thylakoids and the rapid phase of proton conduction in CF_1-depleted EDTA vesicles were inhibited in parallel at low concentrations of $(cHxN)_2C$. Both, the ability to rebind added CF_1 and the same sensitivity against the phosphorylation inhibitor make it probable that this highly conducting channel with a translocation rate of 6×10^5 H^+/s, at 100 mV driving force, resembles the form of CF_0 in the intact ATP synthase, CF_0CF_1. The proton-translocation rate of the ATP synthase, on the other hand, is much lower at high driving force, in the order of 10^3 H^+/s [6]. This dramatic slow down seems to be

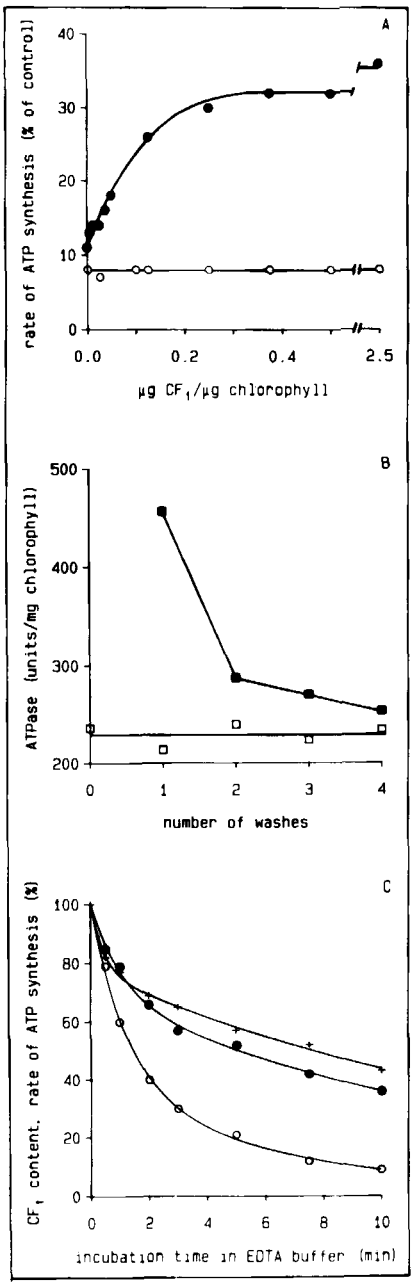

Fig. 7. *Rebinding of CF_1 to EDTA vesicles.* (A) Highly extracted EDTA vesicles (60% loss of CF_1) at 20 µg chlorophyll were incubated in a volume of 500 µl 10 mM NaCl, 40 mM Tricine/NaOH, pH 7.8, with the indicated amounts of CF_1. 4 µl 1 M $MgCl_2$ was added, followed by a 10-min incubation in the dark. ATP-synthesis rates were measured in a final volume of 1 ml of the same buffer with additional 50 µM PMS (filled circles) or 50 µM methyl viologen (open circles) as electron acceptor. (B) Highly extracted EDTA vesicles were incubated as in (A) with 2.5 µg CF_1 (filled squares) or bovine serum albumin (open squares)/µg chlorophyll. Samples were spun down for 2 min at 14000 × *g* and washed in 1 ml reconstitution buffer after each centrifugation as indicated on the abscissa. After the final resuspension in 50 µl 10 mM NaCl, 100 mM sucrose, 10 mM Tricine/NaOH, pH 7.8, MeOH-activated ATPase was measured as in [24]. (C) Thylakoids have been incubated in EDTA solutions for the times indicated on the abscissa. Their residual content of CF_1 was determined by rocket immunoelectrophoresis (crosses). ATP-synthesis rates have been measured with (filled circles) and without (open circles) preincubation of membranes with 2.5 µg CF_1/µg chlorophyll as in (A) and with 50 µM PMS as electron acceptor. (All values represent percentage of control, i.e. untreated thylakoids)

indicative of the coupling step between proton transfer through CF_0 and ATP liberation by CF_1, which is probably conformational and mediated by the δ-subunit of CF_1 [40]. Not unexpectedly, it appears that proton flow through CF_0 is not rate-limiting in ATP synthesis.

We thank Mrs K. Schürmann for highly skilled and dedicated assistance, and Ms H. Kenneweg for graphs and photographs. Dr S. Engelbrecht helped with many discussions and generously provided most of the purified CF_1 used in this work. Financial support by the *Deutsche Forschungsgemeinschaft* (SFB 171/B3) is gratefully acknowledged.

REFERENCES

1. Vignais, P. V. & Satre, M. (1984) *Mol. Cell. Biochem. 60*, 33–70.
2. Schneider, E. & Altendorf, K. (1987) *Microbiol. Rev. 51*, 477–497.
3. Binder, A., Jagendorf, A. & Ngo, E. (1978) *J. Biol. Chem. 253*, 3094–3100.
4. Moroney, J. V., Lopestri, L., McEwen, B. F., McCarty, R. E. & Hammes, G. (1983) *FEBS Lett. 158*, 58–62.
5. Williams, N. & Coleman, P. S. (1982) *J. Biol. Chem. 257*, 2834–2841.
6. Weiss, M. A. & McCarty, R. E. (1977) *J. Biol. Chem. 252*, 8007–8012.
7. Wagner, R. & Junge, W. (1977) *Biochim. Biophys. Acta 462*, 259–272.
8. Schumann, J., Richter, M. L. & McCarty, R. E. (1985) *J. Biol. Chem. 260*, 11817–11823.
9. Richter, M. L. & McCarty, R. E. (1987) *J. Biol. Chem. 262*, 15037–15040.
10. Andreo, C. S., Patrie, W. J. & McCarty, R. E. (1982) *J. Biol. Chem. 257*, 9968–9975.
11. Junge, W., Hong, Y. Q., Qian, L. P. & Viale, A. (1984) *Proc. Natl Acad. Sci. USA 81*, 3078–3082.
12. Engelbrecht, S. & Junge, W. (1987) *Eur. J. Biochem. 172*, 213–218.
13. Junge, W. (1987) *Proc. Natl Acad. Sci. USA 48*, 7084–7088.
14. Negrin, R. S., Foster, D. L. & Fillingame, R. H. (1980) *J. Biol. Chem. 255*, 5643–5648.
15. Schneider, E. & Altendorf, K. (1985) *EMBO J. 4*, 515–518.
16. Junesch, U. & Gräber, P. (1985) *Biochim. Biophys. Acta 809*, 429–434.
17. Mitchell, P. (1977) *FEBS Lett. 78*, 1–20.
18. Casey, R. P. (1984) *Biochim. Biophys. Acta 768*, 319–347.
19. Lill, H., Althoff, G. & Junge, W. (1987) *J. Membr. Biol. 98*, 69–78.
20. Schmid, R., Shavit, N. & Junge, W. (1976) *Biochim. Biophys. Acta 430*, 145–153.
21. Linnnett, P. E. & Beechey, R. B. (1979) *Methods Enzymol. 55*, 472–518.
22. Laurell, C. B. (1966) *Anal. Biochem. 15*, 45–52.
23. Roos, P. & Berzborn, R. J. (1983) *Z. Naturforsch. 38c*, 799–805.
24. Anthon, G. E. & Jagendorf, A. T. (1984) *Biochim. Biophys. Acta 766*, 354–362.
25. Engelbrecht, S., Lill, H. & Junge, W. (1986) *Eur. J. Biochem. 160*, 627–634.
26. Junge, W. (1976) in *Chemistry and biochemistry of plant pigments*, vol. II (Goodwin, T. D., ed.) pp. 233–333, Academic Press, New York.
27. Förster, V., Hong, Y. Q. & Junge, W. (1981) *Biochim. Biophys. Acta 638*, 141–152.
28. Provencher, S. W. (1976) *Biophys. J. 16*, 27–41.
29. Caceci, M. S. & Cacheris, W. P. (1984) *Byte Mag. 9*, 340–362.
30. Junge, W. & Ausländer, W. (1973) *Biochim. Biophys. Acta 333*, 59–70.
31. Junge, W. & Polle, A. (1986) *Biochim. Biophys. Acta 848*, 265–273.

32. Junge, W. & Jackson, J. B. (1982) in *Photosynthesis: energy conversion by plants and bacteria*, vol. 1 (Govindjee, ed.) pp. 589–646, Academic Press, New York.
33. Polle, A. & Junge, W. (1986) *Biochim. Biophys. Acta 848*, 257–264.
34. Schönknecht, G., Junge, W., Lill, H. & Engelbrecht, S. (1986) *FEBS Lett. 203*, 289–293.
35. Lill, H., Engelbrecht, S., Schönknecht, G. & Junge, W. (1986) *Eur. J. Biochem. 160*, 635–643.
36. Strotmann, H., Hesse, H. & Edelmann, K. (1973) *Biochim. Biophys. Acta 314*, 202–210.
37. Junge, W. & Witt, H. T. (1968) *Z. Naturforsch. 23b*, 244–254.
38. Emrich, H. M., Junge, W. & Witt, H. T. (1969) *Z. Naturforsch. 24b*, 1144–1146.
39. Nelson, N. & Eytan, E. (1979) in *Cation fluxes across biomembranes* (Mukohata et al., eds.) pp. 409–415, Academic Press, New York.
40. Lill, H., Engelbrecht, S. & Junge, W. (1988) *J. Biol. Chem. 263*, 14518–14523.