J. exp. Biol. **172**, 461-474 (1992)
Printed in Great Britain © The Company of Biologists Limited 1992

THE CHLOROPLAST H^+-ATPase: PARTIAL REACTIONS OF THE PROTON

BY WOLFGANG JUNGE, SIEGFRIED ENGELBRECHT, CORD GRIWATZ AND GEORG GROTH

Biophysik, Fachbereich Biologie/Chemie, Universität Osnabrück, D-4500 Osnabrück, Bundesrepublik Deutschland

Summary

This article reviews proton intake, charge transfer and proton release by F-ATPases, based in part on flash spectrophotometric studies on the chloroplast ATPase in thylakoid membranes, CF_1F_o. The synthesis-coupled translocation of charges by CF_1F_o (maximum rate $<1500\,s^{-1}$) and the dissipative flow through its exposed channel portion, CF_o (rate $>10000\,s^{-1}$), are extremely proton-specific (selectivity $H^+:K^+>10^7:1$). The proton-specific filter is located in CF_o. Proton flow through exposed CF_o can be throttled by adding subunit δ or subunit β of CF_1. These subunits thus may provide energy-transducing contacts between CF_1 and CF_o. Recently, we characterized two conditions where, in contrast to the above situation, proton intake by CF_1F_o was decoupled from proton transfer across the main dielectric barrier: (a) CF_1F_o structurally distorted by low ionic strength transiently trapped protons in a highly cooperative manner, but remained proton tight. This result has been interpreted in terms of Mitchell's proton well. (b) In the absence of nucleotides there is a proton slip. Addition of nucleotides ($100\,nmol\,l^{-1}$ ADP) abolished proton conduction but not proton intake by CF_1F_o. These experiments *functionally* tag proton binding groups on CF_1F_o that are located before the main dielectric barrier.

Introduction

The proton side of F-ATPases appears to be even more difficult to assess than the nucleotide processing side. The proton-motive force not only drives ATP synthesis but also regulates enzyme activity (Junge *et al.* 1970; Bakker-Grunwald and van Dam, 1974; Junesch and Gräber, 1985, 1991). Three protons are translocated per molecule of ATP formed, whether concertedly or each one serving a particular function is unknown. The equivalence of the electrical portion of the proton-motive force, $\Delta\Psi$, and the chemical portion, ΔpH, as driving forces for ATP synthesis is plausible for near-equilibrium situations but not at all trivial far from equilibrium as in kinetic experiments, where it is also experimentally established (Junesch and Gräber, 1991). Specific amino acid residues which are essential for proton transfer have been identified by mutational analysis (Fraga and Fillingame, 1991; Miller *et al.* 1990) but evidence for their *direct* involvement is lacking.

Among F-ATPases those in photosynthetic membranes offer themselves for detailed

Key words: F-ATPase, proton, proton-motive force, photosynthesis, chloroplast.

kinetic studies on reactions involving the proton. The proton pumps are driven by light flashes, and transients of the transmembrane voltage and of the pH on both sides of the coupling membrane are detectable spectroscopically by intrinsic and extrinsic indicator dyes (Witt, 1979; Junge, 1982). When chloroplasts of green plants are excited by light the lumen of inner vesicles, thylakoids, is positively charged and acidified. The lumen is equivalent to the exoplasmatic side of unicellular organisms. The basic proton conductance of the thylakoid membrane is rather low. Proton conduction is enhanced when CF_1F_o synthesizes ATP or more dramatically when CF_o is exposed by removal of the catalytic portion CF_1. Under pulse stimulation by short flashes of light, proton intake from the lumen, charge transfer across the dielectric barrier and proton release into the suspending medium can be monitored, both for proton flow related to the ATP-synthesizing CF_1F_o (Junge, 1987) and for proton flow through the exposed channel portion CF_o (Schönknecht *et al.* 1986; Lill *et al.* 1987; Althoff *et al.* 1989). In both cases the intake of protons from the lumen and the transport of their charge equivalent across the membrane appear to be synchronized. A typical spectrophotometric experiment deals with more than 10^{13} molecules of CF_1F_o or CF_o which may operate independently and in an uncorrelated manner even when subjected to a sudden step of the electrochemical driving force. Thus, partial reactions of the proton could be blurred over. They may become apparent, however, if some reactions are frozen and completion of the transport cycle is blocked.

This article first reviews physicochemical properties of proton conduction by CF_1F_o and by CF_o and then reports new results on proton binding by CF_1F_o without concomitant proton transfer across the membrane.

The proton conductance of CF_1F_o and of CF_o

The turnover rate of CF_1F_o for protons has been inferred from measurements of the rate of ATP synthesis assuming a stoichiometry of $3H^+$/ATP. Values of up to 400 mol ATP mol^{-1} CF_1F_o s^{-1} have been found in CF_1F_o-reconstituted liposomes during the initial phase after a large pH jump (Schmidt and Gräber, 1987). Even higher figures ($500\,s^{-1}$) can be observed with spinach thylakoids under continuous light and with phenazine methosulfate as cofactor for cyclic electron transport. This result implies maximum turnover numbers for protons of up to $1500\,s^{-1}$. It is noteworthy, that reported rates for F_1F_o from all other sources are lower.

Fig. 1 documents proton flow through CF_1F_o under flashing light, where the turnover number is smaller than in the work cited above because of a smaller proton-motive force. The figure is taken from earlier work (Junge, 1987). Fig. 1A shows transients of the transmembrane voltage and Fig. 1B of the lumenal pH. They are elicited by excitation of thylakoids with two groups of three closely timed flashes of light. In the presence of a specific inhibitor of CF_1, tentoxin, the relaxation of both is slow. The transmembrane voltage (Fig. 1A) decays more rapidly than the acidification of the thylakoid lumen (Fig. 1B). This result reflects an electrical leak conductance by ions other than the proton. In the absence of tentoxin and with substrates (ADP and inorganic phosphate) present, ATP synthesis causes an accelerated decay of both the transmembrane voltage and the

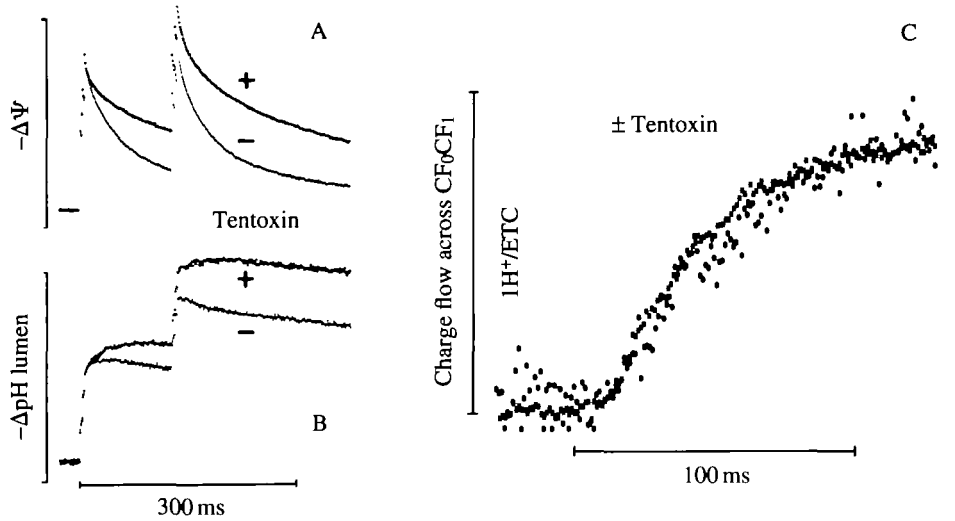

Fig. 1. Transients of the transmembrane voltage (A) and of the lumenal pH (B) in the presence of 20 μmol l^{-1} ADP and 60 μmol l^{-1} inorganic phosphate with and without tentoxin (a specific blocker of the ATP synthase). Reproduced from Junge (1989). A suspension of thylakoids was excited with two groups of three short flashes each. The transmembrane voltage was recorded by electrochromism and the pH transient by Neutral Red (details in Junge, 1987). C gives a superimposition of two data sets, namely the number of protons entering CF_1F_o from the lumen and the number of charges crossing CF_1F_o during the time interval between the first and the second group of three short flashes. These transients are the difference between each pair of original traces in A and B. For details, see Junge (1987). ETC, electron transport chain.

lumenal acidification. The difference between the two traces in Fig. 1A indicates the extra transmembrane charge flow that accompanies ATP synthesis. The difference between the two traces in Fig. 1B indicates extra proton intake from the lumen by CF_o. Both differences have been normalized and, for a short time segment, plotted on top of each other in Fig. 1C. Their coincidence within noise limits identifies the extra charges which pass the dielectric barrier in CF_1F_o as protons taken up from the lumen (Junge, 1987). Proton intake and charge transfer appear to be synchronized. The accelerated charge flow stops at a certain driving force and this is due to the gating of enzyme activity by the transmembrane voltage (Junge *et al.* 1970; Junge, 1970) or, more generally, by the transmembrane proton-motive force (Junesch and Gräber, 1985, 1991).

For a while, published figures of the proton conductance of the channel portion, F_o, have fallen short of the above turnover numbers. Values ranging from 10 to 100 s^{-1} have been obtained mainly with reconstituted archaebacterial and eubacterial channels in liposomes and by pH electrode (Negrin *et al.* 1980; Sone *et al.* 1981; Schneider and Altendorf, 1987). These low values are incompatible with the proposed role of F_o as a low-impedance access to the coupling site in the enzyme (Mitchell, 1966). One reason for these shortcomings might be an insufficient time resolution of the pH electrode (G. Althoff and G. Dekkers-Hebestreit, personal communication), another reason might be the survival of only a few active channels during reconstitution.

We investigated the protonic conduction of CF_o by flash spectrophotometry. The catalytic portion, CF_1, was removed from thylakoid membranes by treatment with EDTA. The number of CF_1 molecules removed from the membrane was determined by electro-immunodiffusion. Thylakoids were excited by short light flashes and the relaxation of the transmembrane voltage and of the pH transients in the lumen and in the suspending medium was monitored as shown in Fig. 1 (Althoff *et al.* 1989; Lill *et al.* 1987; Schönknecht *et al.* 1986). To label charge flow mediated by CF_o, signals were recorded pairwise, with and without blocking agent (DCCD, venturicidin or organotins; Linnett and Beechey, 1979). The area-specific electrical capacitance of thylakoid membranes and the specific number of CF_1 molecules removed were taken into account (Althoff *et al.* 1989). Assuming that any CF_1 which was removed leaves a conducting CF_o behind, the average protonic conductance per exposed CF_o was about 10 fS. The average was taken over two domains, the ensemble of those CF_o that had lost their CF_1 counterpart and the time (counting open and closed intervals of channels). At moderate driving force, 100 mV, this conductance is equivalent to a turnover number for protons of about $6000\,s^{-1}$. Under 200 mV driving force the turnover number is $12\,000\,s^{-1}$, ohmic behaviour assumed. This number is fully compatible with the proposed role of CF_o as the proton channel of the enzyme. As a time-averaged figure, it represents only a lower limit for the turnover number of active channels.

We attempted to determine the conductance of the subset of conducting CF_o channels. The rationale was to use vesicles so small as to contain 0, 1, 2 or very few active channels only and to determine the time-averaged conductance of single channels by a statistical analysis of electrochromic absorption transients. Thylakoids were fragmented into very small vesicles and the analysis technique was calibrated with gramicidin as a channel with known properties. Its conductance in thylakoids (Lill *et al.* 1987; Althoff *et al.* 1991; Schönknecht *et al.* 1992) was in the range of values published for its conductance in artificial bimolecular lipid membranes. The same type of analysis applied to CF_o (without gramicidin present) led to the conclusion that only one out of 100 exposed CF_o molecules per vesicle was conducting. Accordingly, the proton conductance of the few functional channels was much greater than the average conductance, namely 1 pS instead of 10 fS (Schönknecht *et al.* 1986). This result was in line with another report of a very large proton conductance of CF_o, namely of 3.5 pS, which was obtained for CF_1F_o by the (dip stick) patch-clamp technique (Wagner *et al.* 1989). We did not find any clear-cut mistake in our spectrophotometric analysis, particularly as it worked well with gramicidin. Still, the result is not compatible with theoretical and model studies. A proton conductance of 1 pS, particularly around neutral pH, exceeds by orders of magnitude (a) the calculated convergence conductance to a pore mouth of reasonable diameter (say 1 nm) (Peskoff and Bers, 1988), (b) calculated rates of net proton transfer through short hydrogen-bonded chains (Brünger *et al.* 1983), and (c) measured rates of proton transfer through other proton-specific channels (Veatch *et al.* 1975; Lear *et al.* 1988). Charge flow enhancing factors, a short selectivity filter and large channel mouth, as discussed in the context of K^+ maxi channels (Hille, 1984), the drag force in the coulomb cage (Peskoff and Bers, 1988) and mobile buffers (Decker and Levitt, 1988; Nunogaki and Kasai, 1988) do not really account for this discrepancy.

Other properties of this channel as determined by flash spectrophotometry (Althoff *et al.* 1989) are independent of the absolute value of the conductance. CF_o is proton-specific even at pH 8 against a background of 300 mmol l^{-1} NaCl or KCl, or 30 mmol l^{-1} $MgCl_2$ in the medium. The selectivity filter is therefore a property of CF_o and not of the rate-limiting (coupling) step in CF_1F_o. The proton specificity of over more than seven orders of magnitude is unparalleled by other known proton channels (e.g. gramicidin, selectivity for protons over other cations less than 100; Decker and Levitt, 1988). It is the more astounding, as a homologous enzyme to CF_1F_o has been described for *Propionigenium modestum* that can act both as a sodium- and as a proton-translocating ATPase (Dimroth, 1991). The conductance of CF_o is pH independent in the range between 5.6 and 8. In the same pH range there is a constant hydrogen/deuterium isotope effect of 1.7. Addition of glycerol decreases the conductance and abolishes the isotope effect. This finding suggests that the isotope effect may be caused by events in the channel and that the channel operates close to limitation by events in the water phase. The Arrhenius activation energy of proton conduction by CF_o is 42 kJ mol^{-1}, intermediate between the respective figures of a channel-type (30 kJ mol^{-1}, gramicidin) and a carrier-type antibiotic (65 kJ mol^{-1}, valinomycin) (see Althoff *et al.* 1989, for details).

In conclusion, CF_o is a very effective and selective proton channel. Its turnover number greatly exceeds that of the coupled enzyme. It can act as a low-impedance access channel to the coupling site. CF_o is endowed with an extremely specific selectivity filter for protons, unparalleled by other types of proton channels.

Subunits of CF_1 that can throttle proton conduction by CF_o

Three subunits of F_1, namely, γ, δ and ϵ, of the thermophylic bacterium PS3 were reported to block proton flow through the respective F_o channel (Kagawa, 1978). We asked whether *single* subunits of CF_1 can interact with exposed CF_o in such a way as to block proton flow. Triggered by the observation that δ may remain on CF_o after removal of CF_1, keeping CF_o non-conducting (Junge *et al.* 1984), we found that isolated δ, when added back to CF_1-depleted thylakoids, can throttle proton flow through exposed CF_o (Lill *et al.* 1988). Thereby, it restores photophosphorylation by those CF_1F_o that have remained intact on the membrane (Engelbrecht and Junge, 1988). Any 'stopcock' action of δ has to be overcome in the operating enzyme in favour of a controlled operation as valve, admitting protons from the channel further onto the coupling site, or as part of the conformational transducer between protons and ATP (Engelbrecht and Junge, 1990).

Following the same line of research we also found subunit β to be effective in throttling proton conduction by CF_o (monitored by electrochromic absorption transients), and restoring photophosphorylation in CF_1-depleted thylakoids (S. Engelbrecht and W. Junge, in preparation). In this series of experiments, subunit α could not be purified in sufficient amounts. The particular samples of γ, δ and ϵ were only marginally effective by themselves, but the combination of γ, δ and ϵ was, as if distorted δ was reshaped by its association with γ and ϵ (see also Engelbrecht and Junge, 1990). Interestingly, the combination of γ, δ and ϵ was equally effective on spinach CF_o, when subunit γ was taken either from spinach or from the thermophilic bacterium PS3. The finding that the

small subunits of CF_1 act in concerted manner and that subunit β by itself specifically interacted with the exposed proton channel, CF_o, qualifies the concept of δ just acting as a plug.

Instead, it seems as if there are several essential contacts between CF_o and CF_1. Following the hypothesis of a rotational mechanism of catalysis (Boyer, 1989) one may speculate that both portions of F_1, bearings (β?) and rotating shaft elements (at least γ and δ?), may interact with CF_o so as to block and, in CF_1F_o, so as to control, proton conduction.

Trapping of protons by CF_1F_o without concomitant conduction

Cooperative transient trapping of protons was first observed in thylakoids treated with very low concentrations of EDTA (Junge *et al.* 1984). This effect is documented in Fig. 2. Thylakoids isolated from spinach chloroplasts according to standard procedures, but without added magnesium in the medium, were incubated for 2 min in distilled water with very low concentrations of EDTA added (typically 10 μmol l^{-1} at 10 μmol l^{-1} chlorophyll, but see below). The exposure to low ionic strength was stopped by addition of NaCl to 10 mmol l^{-1}.

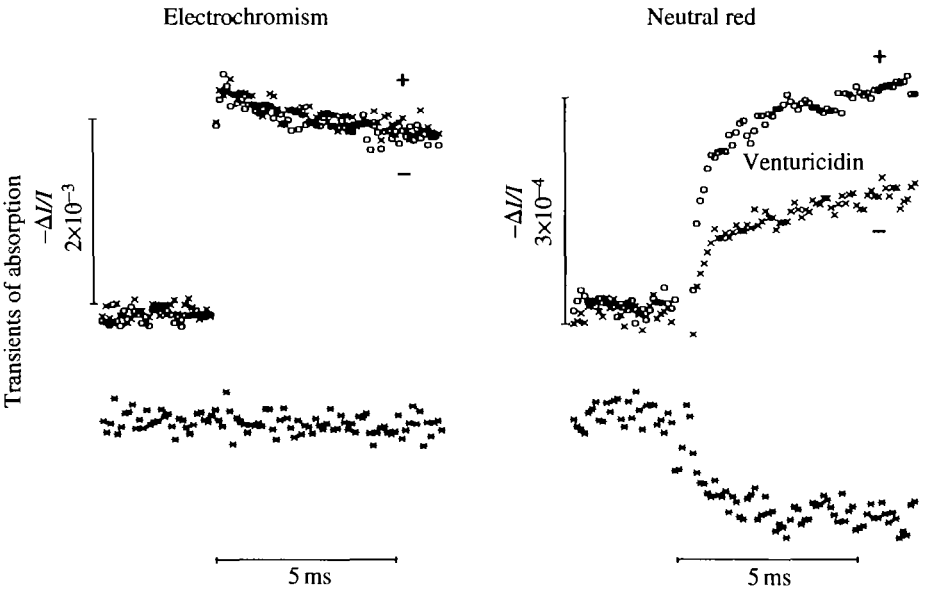

Fig. 2. Voltage transients (left) and pH transients (right) in the thylakoid lumen induced by a single flash of light. Spinach thylakoids prepared without addition of divalent cations were incubated for 2 min at low ionic strength (distilled water with 10 μmol l^{-1} EDTA). NaCl was then added (10 mmol l^{-1}). Voltage and pH transients were recorded twice without and with venturicidin (1 μmol l^{-1}) added. This procedure labelled events attributable to CF_o. EDTA treatment induced transient trapping of protons (see text) without equivalent charge transfer across the membrane. Each point represents a time interval of 68 μs. The lower traces are differences between each pair of top traces. The right-hand one shows the extent of proton intake by CF_o. For details, see Griwatz and Junge (1992).

Fig. 2 shows voltage transients (left) and pH transients (right) in the lumen for thylakoids that had undergone mild EDTA treatment. Although the extent and the decay of the voltage were unaffected by addition of venturicidin (blocker of F_o channels binding to the proteolipid, Galanis *et al.* 1989), the acidification of the lumen was seemingly increased. We attributed this increase to the abolition by venturicidin of a transient trapping of protons at the lumenal side of CF_1F_o or of CF_o. The extent of proton intake is shown in the lower part of Fig. 2 (right). It is simply the difference between the two traces at the top. On an enlarged time scale (Fig. 3) proton intake appeared biphasic, rapid trapping preceding a slow phase attributable to proton leakage through a few exposed CF_o channels. The pH dependence of proton trapping was remarkably steep (see Fig. 3). Whereas rapid trapping was absent at pH 7.2, it was fully expressed at pH 7.7. In contrast, the comparatively slow proton leakage was practically constant over this narrow pH range. The steep pH-dependence of proton trapping conformed with a hexacooperative binding isotherm (Hill coefficient around 6) (Griwatz and Junge, 1992; Junge *et al.* 1984). It is noteworthy that the proton-trapping capacity was saturated by the first flash of

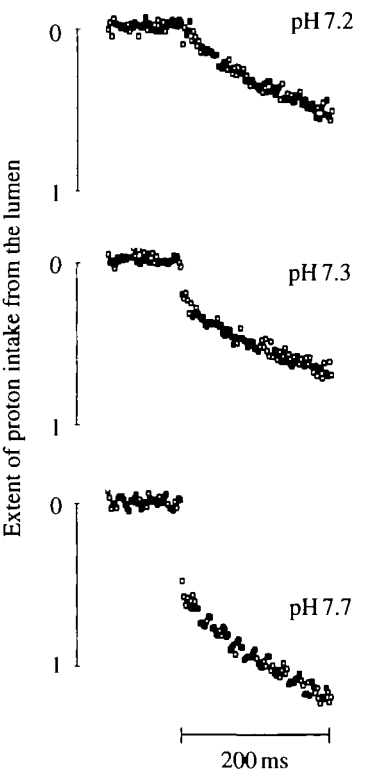

Fig. 3. Proton intake related to CF_o in EDTA-treated thylakoids as function of pH. The transients are equivalent to the one in the lower right of Fig. 2. The ordinate scale was normalized with respect to the pH-dependent sensitivity profile of the indicator dye Neutral Red. For details see Griwatz and Junge (1992).

light; further flashes did not produce further trapping. It was inhibited by various inhibitors of proton conduction through CF_o, namely, venturicidin, DCCD and organotins, and thus was clearly attributable to the ATPase. Trapping was also eliminated by adding very small concentrations of divalent cations (typically $10\,\mu mol\,l^{-1}$), concentrations too small to produce rebinding of possibly solubilized CF_1.

The pH difference which is generated by a single turnover of both photosystems is very small, about 0.05 units (Junge *et al.* 1979). What was the driving force for proton trapping? We found that rapid trapping could be eliminated if the flash-induced transmembrane voltage was even more rapidly shunted by an ionophore (e.g. valinomycin) (Griwatz and Junge, 1992). This finding led us to interpret these effects in the framework of Mitchell's concept of a proton well (Mitchell, 1977). Consider a proton-specific domain that reaches from the lumenal surface of the membrane into CF_1F_o. The lumenal surface still represents an *electrical* equipotential surface, but the *electrochemical* equipotential surface stretches out into this proton-conducting domain. Potentially buffering groups at its top sense a pH that is more acid than the one at the lumen surface. The pH decrease is equivalent to the voltage drop between the lumen and the location of these groups in the membrane. This concept for the transformation of electrical into chemical (entropic) force could account for the observed kinetic equivalence of $\Delta\Psi$ and ΔpH for ATP synthesis (Gräber *et al.* 1984; Junesch and Gräber, 1987). Without a proton well the kinetic equivalence is not at all trivial.

Proton trapping was highly cooperative. In spinach thylakoids the pK of the trapping groups was 7.3 and the Hill coefficient around 6. This behaviour suggests the presence of at least six identical and strongly interacting groups. The candidates are two essential residues, Glu61 and Arg41 (see Fraga and Fillingame, 1991; Miller *et al.* 1990, for *E. coli*) on subunit III. Only this subunit of CF_o is present in sufficient numbers (9–12 in *E. coli*). Derivatization of a single copy by DCCD is sufficient to block ATPase activity in *E. coli* (Hermolin and Fillingame, 1989).

Transient trapping of protons has only been observed with distorted CF_1F_o. Is the trapping state an intermediate of the operational cycle of this enzyme? In the same type of flash spectrophotometric experiments described above trapping has not been detected during ATP synthesis by CF_1F_o (Junge, 1987) (and see Fig. 1) or proton leakage through exposed CF_o (Althoff *et al.* 1989). In both cases the respective transporter has carried out multiple turnovers. Each experiment was an average over very many enzyme cycles. Time resolution of partial reaction steps was not expected.

Thus, it is conceivable that mild EDTA treatment stabilizes and thereby exposes to observation one particular intermediate of the normal operating cycle of the ATP synthase. The respective residues are placed on the lumenal side, beyond the selectivity filter, but before the main dielectric barrier in CF_1F_o. It is tempting to associate this barrier with the main coupling step in CF_1F_o.

Another situation where proton binding precedes conduction is related to proton slip through CF_1F_o. There are at least four mechanisms of proton conduction by thylakoid membranes: (1) coupled proton translocation by CF_1F_o during ATP synthesis; (2) proton channeling through exposed CF_o; (3) proton slip through CF_1F_o; and (4) any other proton leakage. While the first two are self-explanatory, proton slip differs from proton leakage

(through the bilayer or through proteins other than CF_IF_o) in its sensitivity to nucleotides and/or specific blockers of CF_I. In contrast to the proton leak in the cristae membrane of mitochondria, which is insensitive to blockers of F_IF_o (Brown and Brand, 1991), several authors have reported proton slip in thylakoid membranes (McCarty *et al.* 1971; Underwood and Gould, 1980; Gräber *et al.* 1981; Strotmann *et al.* 1986; Evron and Avron, 1990). It is particularly evident at very low nucleotide concentrations. Fig. 4 demonstrates this effect. Thylakoids were incubated in nucleotide-free medium and illuminated by a constant background intensity of green light. They were excited with a group of three short light flashes creating a voltage transient (Fig. 4A) and a pH transient (Fig. 4B) in the lumen on top of the steady acidification. The electrical leakage was greater in the absence of ADP than with ADP added (see Fig. 4A). The attribution of this slip to CF_IF_o was corroborated by its inhibition by venturicidin. Fig. 4B demonstrates

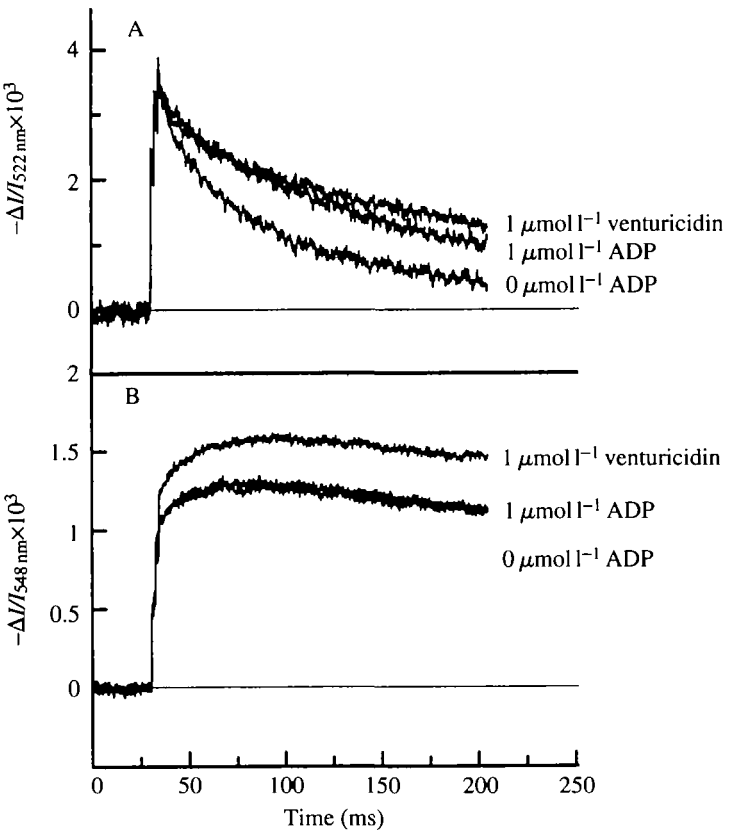

Fig. 4. Voltage transients (A) and pH transients (B) in the lumen of thylakoids in the absence of ADP, with 1 μmol l^{-1} ADP added and with 1 μmol l^{-1} venturicidin added. 0.1 mmol l^{-1} phosphate and 3 mmol l^{-1} $MgCl_2$ were present. A group of three short flashes was fired on a continuous background of green light (10 W m^{-2}). In the absence of ADP, electrical leakage and proton intake are evident. Sensitivity to venturicidin qualifies both as proton slip through CF_IF_o.

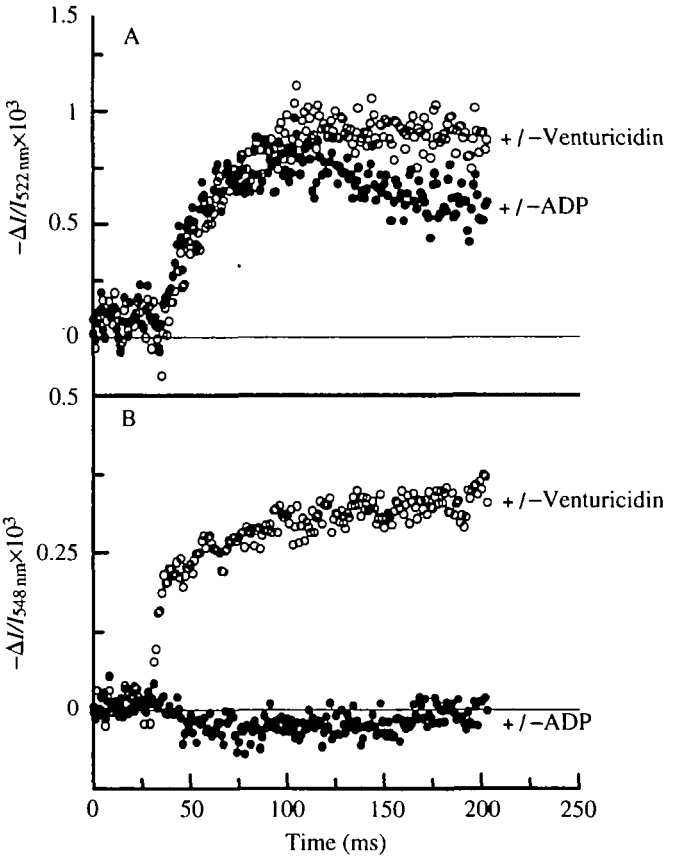

Fig. 5. Difference between pairs of traces in Fig. 4 representing charge slip (A) and proton slip (B). The addition of ADP eliminated charge slip but not proton intake, while venturicidin eliminated both. The intake of protons by CF_1F_o under slip conditions was more rapid than their transfer across the dielectric barrier.

that the slip was due to proton conduction. There was apparently less proton release into the lumen in the absence of venturicidin. As in the context of Fig. 1, this diminution was attributed to increased proton intake. Interestingly, the addition of ADP eliminated charge slip (Fig. 4A) but not proton intake (Fig. 4B). Still venturicidin eliminated both. Proton trapping was more rapid than proton conduction. This discrepancy is evident from a comparison of the respective differences between the traces shown in Fig. 4, which are plotted in Fig. 5. It has still to be determined how this example of proton intake by CF_1F_o without concomitant proton conduction relates to the result after EDTA treatment.

Other properties of the proton slip, as will be detailed elsewhere (G. Groth and W. Junge, in preparation), are as follows. The inhibition of the electrical slip by ADP occurred with an apparent K_i(ADP) of 200 nmol l^{-1}. It required inorganic phosphate; $K_i(P_i)$ of 45 μmol l^{-1}. GDP was also effective. There was an energetic threshold for the slip. More importantly, when a pH difference was created by continuous light, such that the electrical potential difference induced by a single additional flash of light was still below threshhold, there was no slip. The slip became evident, however, when the

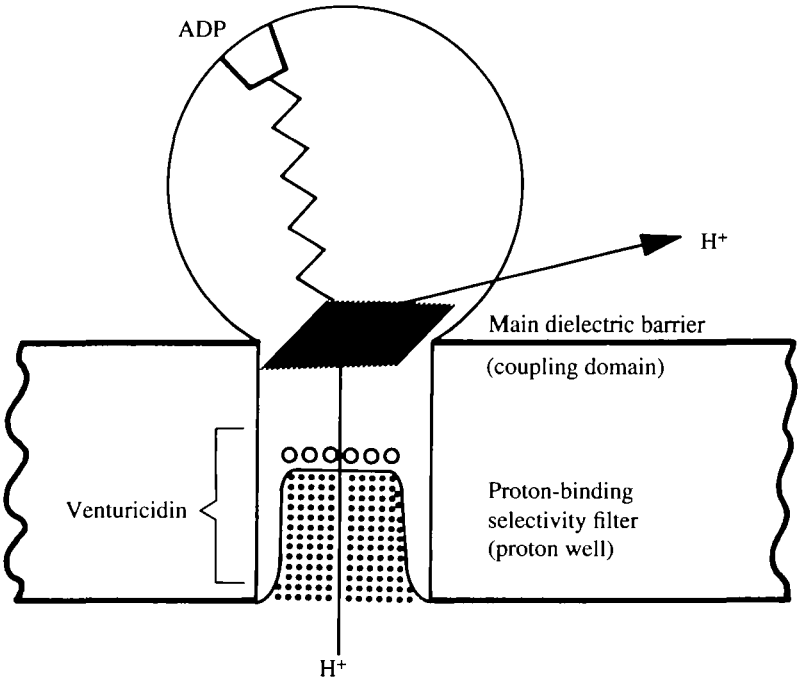

Fig. 6. Heuristic model for the location of proton-trapping groups and the main dielectric barrier in CF_1F_o.

electrical potential difference was increased (by firing three flashes instead on only one, see Fig. 4). This showed that the slip threshold was defined in terms of proton-motive force. The electrical component, $\Delta\Psi$, was additive to the chemical one, ΔpH.

A simplistic structural model based on the above kinetic studies on proteolytic reactions is given in Fig. 6. To avoid the impression that we can pin down any of the functional elements to certain protein domains, the enzyme is sketched as a ball and rod. For protons, the CF_1F_o entity represents a structure with three elements, an entry channel, a major dielectric barrier and an exit channel. Protons enter CF_1F_o through an extremely proton-selective filter, which is part of CF_o. On the *cis* side of the dielectric barrier, but after or within the filter, there are highly cooperative proton binding groups. They are freely accessible only in structurally distorted CF_1F_o (induced by EDTA treatment or, perhaps also, at low nucleotide concentration). Then a small portion of the total transmembrane electrical potential difference is converted into a pH difference at these groups, as postulated in Mitchell's (1977) concept of a proton well. It is tempting to identify the major dielectric barrier with the coupling step. Whether the proton trapping groups are involved in the reaction cycle of the undistorted ATP synthase remains to be seen. It is hoped that the above speculations are of heuristic value, at least until the atomic structure of CF_1F_o is available as a time-resolved movie.

This work was supported financially by the Deutsche Forschungsgemeinschaft (SFB171−TPB2) and by Fonds der Chemischen Industrie.

References

ALTHOFF, G., LILL, H. AND JUNGE, W. (1989). Proton channel of the chloroplast ATP synthase, CF_o: its time-averaged single-channel conductance as function of pH, temperature, isotopic and ionic medium composition. *J. Membr. Biol.* **108**, 263–271.

ALTHOFF, G., SCHÖNKNECHT, G. AND JUNGE, W. (1991). Gramicidin in chromatophores of *Rhodobacter sphaeroides*. *Eur. Biophys. J.* **19**, 213–216.

BAKKER-GRUNWALD, O. AND VAN DAM, K. (1974). On the mechanism of activation of the ATPase in chloroplasts. *Biochim. biophys. Acta* **347**, 290–298.

BOYER, P. D. (1989). A perspective of the binding change mechanism for ATP synthesis. *FASEB J.* **3**, 2164–2178.

BROWN, G. C. AND BRAND, M. D. (1991). On the nature of the mitochondrial proton leak. *Biochim. biophys. Acta* **1059**, 55–62.

BRÜNGER, A., SCHULTEN, Z. AND SCHULTEN, K. (1983). A network thermodynamic investigation of stationary and nonstationary proton transport through proteins. *Z. phys. Chem.* **136**, 1–63.

DECKER, E. R. AND LEVITT, D. G. (1988). Use of weak acids to determine the bulk diffusion limitation of hydrogen ion conductance through the gramicidin channel. *Biophys. J.* **53**, 25–32.

DIMROTH, P. (1991). Na^+-coupled alternative to H^+-coupled primary transport systems in bacteria. *BioEssays* **13**, 463–468.

ENGELBRECHT, S. AND JUNGE, W. (1988). Purified subunit δ of chloroplast coupling factor CF_1 reconstitutes photophosphorylation in partially CF_1-depleted membranes. *Eur. J. Biochem.* **172**, 213–218.

ENGELBRECHT, S. AND JUNGE, W. (1990). Subunit δ of H^+-ATPases: at the interface between proton flow and ATP synthesis. *Biochim. biophys. Acta* **1015**, 379–390.

EVRON, Y. AND AVRON, M. (1990). Characterization of an alkaline pH-dependent proton slip in the ATP synthase of lettuce thylakoids. *Biochim. biophys. Acta* **1019**, 115–120.

FRAGA, D. AND FILLINGAME, R. H. (1991). Essential residues in the polar loop region of subunit-C of *Escherichia coli* F_1F_o ATP synthase defined by random oligonucleotide-primed mutagenesis. *J. Bacteriol.* **173(8)**, 2639–2643.

GALANIS, M., MATTOON, J. R. AND NAGLEY, P. (1989). Amino acid substitutions in mitochondrial ATP synthase subunit 9 of *Saccharomyces cerevisiae* leading to venturicidin or ossamycin resistance. *FEBS Lett.* **249**, 333–336.

GRABER, P., BURMEISTER, M. AND HORTSCH, M. (1981). Regulation of the membrane permeability of spinach chloroplasts by binding of adenine nucleotides. *FEBS Lett.* **136**, 25–31.

GRABER, P., JUNESCH, U. AND SCHATZ, G. H. (1984). Kinetics of proton-transport-coupled ATP synthesis in chloroplasts. Activation of the ATPase by an artificially generated ΔpH and $\Delta\Psi$. *Ber. Bunsengeges. phys. Chem.* **88**, 599–608.

GRIWATZ, C. AND JUNGE, W. (1992). Cooperative transient trapping of protons by deranged chloroplast ATPase: Evidence for the proton well? *Biochim. biophys. Acta* (in press).

HERMOLIN, J. AND FILLINGAME, R. H. (1989). H^+-ATPase activity of *Escherichia coli* F_1F_o is blocked after reaction of dicyclohexylcarbodiimide with a single proteolipid (subunit c) of the F_o complex. *J. biol. Chem.* **264**, 3896–3903.

HILLE, B. (1984). *Ionic Channels of Excitable Membranes*. Sunderland, MA: Sinauer Associated.

JUNESCH, U. AND GRÄBER, P. (1985). The rate of ATP synthesis as a function of ΔpH in normal and dithiothreitol-modified chloroplasts. *Biochim. biophys. Acta* **809**, 429–434.

JUNESCH, U. AND GRÄBER, P. (1987). Influence of the redox state and the activation of the chloroplast ATP synthase on proton-transport-coupled ATP synthesis hydrolysis. *Biochim. biophys. Acta* **893**, 275–288.

JUNESCH, U. AND GRÄBER, P. (1991). The rate of ATP-Synthesis as a function of ΔpH and $\Delta\Psi$ catalyzed by the active, reduced H^+-ATPase from chloroplasts. *FEBS Lett.* **294**, 275–278.

JUNGE, W. (1970). Critical electric potential difference for photophosphorylation. Its relation to the chemiosomotic hypothesis and to the triggering requirements of the ATPase system. *Eur. J. Biochem.* **14**, 582–592.

JUNGE, W. (1982). Electrogenic reactions and proton pumping in green plant photosynthesis. *Curr. Topics Membr. Transport* **16**, 431–465.

JUNGE, W. (1987). Complete tracking of transient proton flow through active chloroplast ATP synthase. *Proc. natn. Acad. Sci. U.S.A.* **84**, 7084–7088.

JUNGE, W. (1989). Protons, the thylakoid membrane and the chloroplast ATP synthase. *Ann. N. Y. Acad. Sci.* **574**, 268–286.

JUNGE, W., AUSLAENDER, W., MCGEER, A. AND RUNGE, T. (1979). The buffering capacity of the internal phase of thylakoids and the magnitude of the pH changes inside under flashing light. *Biochim. biophys. Acta* **546**, 121–141.

JUNGE, W., HONG, Y. Q., QIAN, L. P. AND VIALE, A. (1984). Cooperative transient trapping of photosystem II protons by the integral membrane portion (CF_o) of chloroplast ATP-synthase after mild extraction of the four-subunit catalytic part (CF_1). *Proc. natn. Acad. Sci. U.S.A.* **81**, 3078–3082.

JUNGE, W., RUMBERG, B. AND SCHROEDER, H. (1970). Necessity of an electric potential difference and its use for photophosphorylation in short flash groups. *Eur. J. Biochem.* **14**, 575–581.

KAGAWA, Y. (1978). Reconstitution of the energy transformer, gate and channel subunit assembly, crystalline ATPase, and ATP synthesis. *Biochim. biophys. Acta* **505**, 45–93.

LEAR, J. D., WASSERMANN, Z. R. AND DEGRADO, W. F. (1988). Synthetic amphiphilic peptide models for protein ion channels. *Science* **240**, 1177–1181.

LILL, H., ALTHOFF, G. AND JUNGE, W. (1987). Analysis of ionic channels by a flash spectrophotometric technique applicable to thylakoid membranes: CF_o, the proton channel of the chloroplast ATP synthase, and, for comparison, gramicidin. *J. Membr. Biol.* **98**, 69–78.

LILL, H., ENGELBRECHT, S. AND JUNGE, W. (1988). δ subunit of chloroplast coupling factor 1 inhibits proton leakage through coupling factor O. *J. biol. Chem.* **263**, 14518–14522.

LINNETT, P. E. AND BEECHEY, R. B. (1979). Inhibitors of the ATP synthetase system. *Methods Enzymol.* **55**, 472–518.

MCCARTY, R. E., FUHRMAN, J. S. AND THUCHIYA, Y. (1971). Effects of adenine nucleotides on hydrogen-ion transport in chloroplasts. *Proc. natn. Acad. Sci. U.S.A.* **68**, 2522–2526.

MILLER, M. J., OLDENBURG, M. AND FILLINGAME, R. H. (1990). The essential carboxyl group in subunit c of the F_1F_o ATP synthase can be moved and H^+-translocating function retained. *Proc. natn. Acad. Sci. U.S.A.* **87**, 4900–4904.

MITCHELL, P. (1966). Chemiosmotic coupling in oxidative and photosynthetic phosphorylation. *Physiol. Rev.* **41**, 445–502.

MITCHELL, P. (1977). Epilogue: From energetic abstraction to biochemical mechanism. *Symp. Soc. gen. Microbiol.* **27**, 383–423.

NEGRIN, R. S., FOSTER, D. L. AND FILLINGAME, R. H. (1980). Energy-transducing H^+-ATPase of *Escherichia coli*. Reconstitution of proton translocating activity of the intrinsic membrane sector. *J. biol. Chem.* **255**, 5643–5648.

NUNOGAKI, K. AND KASAI, M. (1988). The proton hydroxide flux localizers around the channel mouth in buffered solution. *J. theor. Biol.* **134**, 403–415.

PESKOFF, A. AND BERS, D. M. (1988). Electrodiffusion of ions approaching the mouth of a conducting membrane channel. *Biophys. J.* **53**, 863–875.

SCHMIDT, G. AND GRÄBER, P. (1987). The rate of ATP hydrolysis catalyzed by reconstituted CF_oF_1-liposomes. *Z. Naturforsch.* C **42**, 231–236.

SCHNEIDER, E. AND ALTENDORF, K. (1987). Bacterial adenosine 5′-triphosphate synthase (F_1F_o): purification and reconstitution of F_o complexes and biochemical and functional characterization of their subunits. *Microbiol. Rev.* **51**, 477–497.

SCHÖNKNECHT, G., ALTHOFF, G. AND JUNGE, W. (1992). Dimerization constant and single-channel conductance of gramicidin in thylakoid membranes. *J. Membr. Biol.* **126**, 265–275.

SCHÖNKNECHT, G., JUNGE, W., LILL, H. AND ENGELBRECHT, S. (1986). Complete tracking of proton flow in thylakoids – the unit conductance of CF_o is greater than 10 fS. *FEBS Lett.* **203**, 289–294.

SONE, N., HAMAMOTO, T. AND KAGAWA, Y. (1981). pH dependence of H^+ conduction through the membrane moiety of the H^+-ATPase ($F_o \times F_1$) and effects of tyrosyl residue modification. *J. biol. Chem.* **256**, 2873–2877.

STROTMANN, H., KIEFER, K. AND ALTVATER-MACKENSEN, R. (1986). Equilibration of the ATPase reaction of chloroplasts at transition from strong light to weak light. *Biochim. biophys. Acta* **850**, 90–96.

UNDERWOOD, C. AND GOULD, J. M. (1980). Modulation of proton efflux from chloroplasts in the light by external pH. *Archs Biochem. Biophys.* **204**, 241–246.

VEATCH, W. R., MATHIES, R., EISENBERG, M. AND STRYER, L. (1975). Simultaneous fluorescence and conductance studies of planar bilayer membranes containing a highly active and fluorescent analog of gramicidin A. *J. molec. Biol.* **99**, 75–92.

WAGNER, R., APLEY, E. C. AND HANKE, W. (1989). Single channel hydrogen ion currents through reconstituted chloroplast ATP synthase CF_o-CF_1. *EMBO J.* **8**, 2827–2834.

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