

ATP Synthase of Chloroplasts: Selective Action of Agents Binding to F₁ on Partial Reactions of Proton Transfer in F₀[†]

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ABSTRACT: We studied the basic steps of proton transfer through ATP synthase of chloroplasts, CF₀CF₁, under conditions of proton slip, a conducting state in the absence of added nucleotides. On the background of a steady transmembrane pH difference, voltage steps were induced by flashing light. Proton intake, transfer, and release by CF₀CF₁ were kinetically resolved by spectrophotometric probes. Kinetic disparities between these three steps were observed. Rapid but limited proton intake from the thylakoid lumen and proton release into the medium ($\tau_{1/2} \approx 5$ ms) preceded charge transfer across the dielectric barrier in the enzyme ($\tau_{1/2} \approx 30$ ms). The saturation behavior under multiple flashes suggested a sequential reaction mechanism. ADP and dequalinium, when bound to subunit β of the catalytic portion, CF₁, blocked different partial reactions involving protons. ADP in the catalytic cleft blocked the electrogenic transfer step, and dequalinium at the adjacent DELSEED sequence on the same subunit blocked the release of protons. Both effects prove a long-range conformational transmission between remote (≈ 10 nm) domains on F₁ and on F₀. For the normal sequence of events from protons to ATP they suggest a specific action of certain proton-transfer steps on different domains of the catalytic portion.

ATP synthase (F-ATPase)¹ is a key enzyme of photosynthesis and respiration in chloroplasts, mitochondria, and bacteria, where it produces ATP at the expense of proton motive force (Fillingame, 1990; Senior, 1990; Cross, 1992; Nelson, 1992). Its structure is bipartite with a peripheral portion, F₁, joined to the membrane intrinsic portion, F₀, by a slender stalk. The large subunits of F₁, $\alpha_3\beta_3$, are arranged approximately in C₃-symmetry providing a total of six nucleotide binding sites. One set of three binding sites is located mainly on subunit β with potentially catalytic function; the other set of three is located on α [for a structural characterization see Abrahams et al. (1994), and for a functional one, see Shapiro and McCarty (1988)]. When the membrane integral portion of the chloroplast enzyme, CF₀, is exposed by removal of its counterpart, CF₁, it acts as an extremely specific proton conductor (Althoff et al., 1989). The F₀ domain of certain bacteria, on the other hand, also mediates sodium cation transfer (Laubinger & Dimroth, 1987).

It has been proposed that the main part of the free energy provided by the translocation of protons (ions) across the F₀ domain is used for the extrusion of spontaneously formed but enzyme-bound ATP from catalytic sites into the aqueous environment (Penefsky, 1985b). These sites are located roughly at half-height in F₁, about 10 nm elevated (Mitra & Hammes, 1990; Abrahams et al., 1994) above Glu-61 (Asp-

61 in *Escherichia coli*), a fundamental amino acid of subunit III, which is found nearly in the middle of the membrane dielectric and which was proved to be essential for proton transfer coupled to ATP synthesis (Fillingame, 1992). The coupling mechanism may involve mechanical (conformational) transmission between the sites of proton transfer and the sites of catalysis [see Boyer (1993) for a recent review]. First evidence for long-range transmission was provided by Penefsky (1985a) demonstrating that chemical modification of subunit III by DCCD altered the affinity of the nucleotide binding sites in mitochondrial F₁. In this paper long-range transmissions in the opposite direction are described by demonstrating the effect of two agents interacting at different sites with F₁ on partial reactions of vectorial proton transfer in the membrane integral F₀-domain.

The most basic reactions involving the proton (for the chloroplast enzyme) are the following: (a) the intake by CF₀ from the lumen side, (b) the electrogenic transfer across the major dielectric barrier (which is probably attributable to CF₀), and (c) the release at the stroma side of the enzyme. We studied these reactions in pulse-relaxation experiments using spectrophotometric techniques. Thylakoids were exposed to a group of three short flashes of light to generate a step of the transmembrane voltage. It was superimposed on a background pH difference which was formed by a continuous illumination of the sample. Proton transfer across CF₀CF₁ was monitored at high time resolution by electrochromism of intrinsic pigments (b) and by appropriate pH-indicating dyes (a and c). This technique (coined as "complete tracking of proton flow") was used to investigate proton transfer by CF₀CF₁, both under conditions of ATP synthesis (Junge, 1987) and under *slip* (Groth & Junge, 1993), and also to analyze the proton conductance of the exposed CF₀ (Schönknecht et al., 1986; Althoff et al., 1989).

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¹ ACMA, 9-amino-6-chloro-2-methoxyaridine; Cyt *b_f*, cytochrome *b₆f* complex; DCCD, *N,N'*-dicyclocarbodiimide; DNP-INT, dinitrophenylether of iononitrothymol; EDTA, ethylenediaminetetraacetic acid; P_i, orthophosphate; PS, photosystem; F-ATPase, ATP synthase.

The integral ATP synthase of chloroplasts, CF_0CF_1 , shows two modes of proton conduction, namely, (1) the coupled translocation of protons to yield ATP in the presence of catalytic amounts of ADP, P_i , and Mg^{2+} and (2) a leak conductance, named *proton slip* in the absence of added nucleotides. Proton slip is blocked by subcatalytic concentrations of ADP ($K_i = 200$ nM at $[P_i] = 0.5$ mM), but also by GDP (2.5 μ M) and ATP (2 μ M). The nucleotide and phosphate dependence classifies the respective binding site as potentially catalytic (Groth & Junge, 1993). Proton slip is only apparent if the proton motive force exceeds a threshold of similar magnitude (about 3 pH units) as observed for ATP synthesis (Groth & Junge, 1993). It is reasonable to assume that the proton transfer under slip is closely related to the proton transfer which is coupled to ATP synthesis.

Under conditions of proton slip kinetic disparities between the three partial reactions became apparent. One portion of proton intake and release occurred much faster than charge transfer across the dielectric barrier of the enzyme. We found that dequalinium, a polycation binding to a highly conserved acid cluster in subunit β of mitochondrial and therefore probably also of chloroplast CF_1 , blocked proton release but not proton intake and charge transfer. ADP, which under the given conditions bound to one of the potentially catalytic sites on β , blocked two reactions, the release and the charge transfer, but not the transient intake of protons. This first clear demonstration of partial protolytic reactions also gave further evidence for a selective and long-range conformational action from CF_1 on CF_0 .

MATERIALS AND METHODS

Flash Spectroscopic Measurements. Stacked thylakoids were prepared from 10–12 day old pea seedlings as described in Polle and Junge (1986a). Thylakoids were suspended in 10 mM Tricine, pH 7.8, 100 mM sorbitol, 10 mM NaCl, and 5 mM $MgCl_2$ and stored on ice. Flash spectrophotometric measurements were done as outlined in Junge (1976). Thylakoids corresponding to an average chlorophyll concentration of 10 μ M were suspended in a reaction medium of 10 mM Tricine, 3 mM $MgCl_2$, 0.1 mM Na_2HPO_4 , and 10 μ M methylviologen at pH 8.0. For measurements of pH transients Tricine was omitted, or in experiments on the lumenal pH transients Tricine was replaced by bovine serum albumin (2.6 mg/mL) (Ausländer & Junge, 1975). Other additions are indicated in the figure captions.

Samples were placed in an optical cell (optical path length, 20 mm) and probed by a constant background of continuous, broad-band measuring light (band filter Schott DT-Grün, 495–595 nm, 1.5 and 2.5 mW/cm² when indicated), which caused a steady-state transmembrane pH difference across the thylakoid membrane. The spectral width of the measuring light was narrowed and tuned to the absorption of each of the three spectroscopic probes only after the cuvette, by placing interference filters on the cathode of the photomultiplier tube. This served to provide a constant intensity and quality of the background light through all three sets of data. The steady pH difference as caused by the background light was calibrated by ACMA fluorescence assay according to Casadio (1991). Proton slip occurs only if the proton motive force exceeds a certain threshold. The threshold of proton slip (Groth & Junge, 1993) is about the same as that of ATP

synthesis (about 3 pH units, see Junesch & Gräber, 1987). In the present experiments it was only surpassed by the superimposed voltage step. The applied groups of three flashes caused a voltage jump of about 100 mV and a much smaller equivalent of the chemical driving force (less than 0.2 pH-units (Junge et al., 1979)). Voltage and pH-transients were generated at a repetition rate of 0.1 Hz. The transmembrane voltage across the thylakoid membrane was measured by electrochromic absorption changes of intrinsic pigments at 522 nm (Junge & Witt, 1968). pH transients in the suspending medium were detected at a wavelength of 575 nm using the dye cresol red, 15 μ M (Polle & Junge, 1986a). Increased absorption after flash excitation corresponds to an alkalization of the suspending medium caused by the uptake of protons at the quinone side of photosystem II and by the terminal electron acceptor system. pH transients at the lumenal surface of the thylakoid membrane were recorded at a wavelength of 548 nm by the dye neutral red, 15 μ M (Ausländer & Junge, 1975; Junge et al., 1979). In this case increased absorption is related to an acidification of the lumenal phase as caused by proton release with kinetically distinct contributions from water oxidation (fast) and plastoquinone oxidation by cytochrome b_6f (slow). To extract the response of the dyes to pH transients from the kinetically complex pattern of superimposed transients of absorption, changes of absorption at the respective wavelength, 575 nm (CR) or 548 nm (NR), were recorded twice, in the presence and in the absence of the respective dye. Their difference was the pH-indicating signal. This has been presented elsewhere (Junge et al., 1979).

Flash excitation of thylakoid membranes generates a complex pattern of electrogenic and protolytic events. As a tag to those events which were attributable to CF_0CF_1 or to CF_0 we used the sensitivity to venturicidin, which specifically binds to the domain of the essential glutamic acid residue of the proteolipid subunit on the F_0 portion (Galanis et al., 1989). The difference between transient signals that were recorded with and without added venturicidin thus was attributed to the action of CF_0CF_1 . To check this procedure against possible side effects of venturicidin, we also used other inhibitors of proton transport at the level of the F_0 portion, namely, DCCD and organotin, with practically the same results.

Typically, 10–20 signals were averaged on a Tracor Northern TN 1500, to improve the signal-to-noise ratio.

Calibration of the Number of Charges Transported by CF_0CF_1 from Electrochromic Absorption Changes. The number of charges transported across CF_0CF_1 after excitation of the photosynthetic reaction centers by a series of three closely spaced flashes was calculated from the decay of the *electrochromic absorption changes* (see Figure 1). These transients are *voltage transients*, as the entire thylakoid system of one chloroplast behaves as an electrically contiguous capacitor (Schönknecht et al., 1990). The total current is obtained from the first derivative of the voltage. It is composed of the current through CF_0CF_1 and the current through all other conductors. To obtain the former, we calculated the difference of the currents obtained in the absence and in the presence of venturicidin. In terms of the capacitor equation the current attributable to CF_0CF_1 molecules is given by

$$I(\text{CF}_0\text{CF}_1) = C \left(\left(\frac{dU}{dt} \right)_{\text{total}} - \left(\frac{dU}{dt} \right)_{\text{vent}} \right) = C \left(\frac{dU}{dt} \right)_{\text{CF}_0\text{CF}_1} \quad (1)$$

where I denotes the current at a given voltage U , C is the electric capacitance of the membrane, and t is the time. The integration of the CF_0CF_1 -related current over time yields the number of charges, Q , which are transported over the dielectric barrier of the enzyme during the integration time interval.

$$Q(t) = C \int_{t=0}^t \left(\frac{dU}{dt} \right)_{\text{CF}_0\text{CF}_1} dt \quad (2)$$

The calculated figure $Q(t)$ as function of time was plotted in the figures by a line. The value of the proportionality constant C , which denotes the fractional membrane capacitance of the thylakoid membrane per molecule of CF_0CF_1 -total, follows from the extent of the transmembrane voltage, which is generated by three closely spaced flashes of red light. This extent results from the translocation of four elementary charges per electron transport chain (Junge 1982), or according to accepted figures of the $\text{CF}_0\text{CF}_1/\text{PS II}$ ratio of about 0.5 (Roos & Berzborn, 1983), eight charges per CF_0CF_1 complex. We corroborated the former stoichiometry by measurements of the reoxidation of P700, which showed that three closely spaced flashes (at 2-ms intervals) caused three turnover of PS II but only one turnover of PS I, giving rise to a total of 4 charge separations.

Calibration of Proton Intake By CF_0CF_1 . The acidification of the luminal phase of the thylakoids was monitored by the amphiphilic dye neutral red under strongly buffering pH transients in outer suspending medium as in previous work [e.g., Groth and Junge (1993) and Haumann and Junge (1994)]. Since the dye binds to the membrane surface, it is effectively upconcentrated in the thylakoid lumen by more than 2 orders of magnitude and responds to transients of the surface pH at the lumen side of the thylakoid membrane (Hong & Junge, 1982). Its response is very fast: in studies on proton release during the four reaction steps of water oxidation we found rise times ranging down to 10 μs (Haumann & Junge, 1994). The total extent of protons deposited into the lumen after excitation of thylakoids by three short flashes of light in close sequence (2-ms intervals) amounts to four per electron-transport chain or eight per CF_0CF_1 molecule (see above). It was detected as a quasistationary level in the presence of venturicidin to block proton slip. The thylakoid membrane is then rather impermeant to protons, and the relaxation of the flash-induced pH difference takes more than 10 s (Junge et al., 1986), which is very long compared with the time window of these experiments (200 ms). Proton intake into CF_0CF_1 was inferred from the difference between two transient signals obtained with and without added venturicidin. The extent of this CF_0CF_1 -related transient was calibrated by comparison with the maximum extent in the presence of venturicidin, namely, about eight protons per CF_0CF_1 -total.

Calibration of Proton Release by CF_0CF_1 was carried out by a procedure similar to that above. In the presence of venturicidin, the absorption transients of the hydrophilic dye cresol red that are linearly related to the alkalization of the external phase increase to a quasistationary level (with the same relaxation in about 10 s) which is caused by the uptake of four protons per electron-transport chain or about

eight protons per CF_0CF_1 -total at the stroma side of the thylakoid membrane (three at plastoquinone reduction and one by the electron acceptor, methylviologen/oxygen). Again the difference between transients recorded in the presence and in the absence of venturicidin presented the time course of proton release from CF_0CF_1 . It was calibrated against the maximum extent in the presence of venturicidin.

Biochemicals. Venturicidin was obtained from Sigma, Deisenhofen; ADP of high purity was from Boehringer Mannheim. Other chemicals were supplied by Merck, Darmstadt, and BioMol, Hamburg.

RESULTS

Figure 1 shows original transients of the transmembrane voltage (top), the pH in the lumen (middle), and the pH in the suspending medium (bottom) under excitation of thylakoids by a group of three closely spaced flashes on the background of an underlying transmembrane pH gradient. The generation of the electric potential difference is very rapid (sub-nanosecond), and the electrochromic response is also very fast. Thus three jumps following the three flashes were discernible. That the first jump had about twice the extent as the following ones was due to one additional turnover of photosystem I only during the first flash. Due to the kinetic competence of neutral red, the rapid proton release into the lumen by water oxidation was also apparent by three rapid steps. It was followed by 1 unit of slow proton release due to the oxidation of plastoquinone, a consequence of the single turnover of photosystem I after the first flash. In contrast to the former two sets of signals, the response of cresol red to the alkalization of the medium was rather slow. The delay was not due to this particular indicator dye but to the stacking of thylakoids under the given salt composition of the suspending medium. That the response of hydrophilic pH indicators to proton uptake from the partition region of stacked thylakoids is delayed by a mechanism of diffusion/reaction in a domain with fixed buffers has been demonstrated previously both experimentally (Polle & Junge, 1986a, 1989) and theoretically (Junge & Polle, 1986; Junge & McLaughlin, 1987). Proton uptake at the acceptor side of photosystem I was delayed by another mechanism. It was limited by the rate of superoxide dismutation (Polle & Junge, 1986b).

The control traces were obtained with venturicidin added to block any proton transfer by CF_0CF_1 . As mentioned, they revealed the known kinetic features of the light-driven, electrogenic electron transfer which generates the membrane potential and causes proton release into the lumen and proton uptake from the medium. This kinetic complexity was, however, without relevance for the present work, which was focused solely on those events which were related to CF_0CF_1 as evidenced by their sensitivity to venturicidin. The discharge of the membrane's electric capacitance by non-protonic leak conductance is faster ($\tau_{1/2} \approx 150$ ms, Figure 1, top) than that of the proton buffering capacity of the lumen [$\tau_{1/2} \approx 15$ s; see Junge et al. (1986) and Figure 1, middle and bottom].

Kinetics of Proton Intake, Transfer, and Release by CF_0CF_1 . In the absence of ADP and, of course, of venturicidin, the voltage decayed rapidly by proton slip through CF_0CF_1 ($\tau_{1/2} \approx 35$ ms, Figure 1, top). The extents of both the acidification of the luminal phase and the

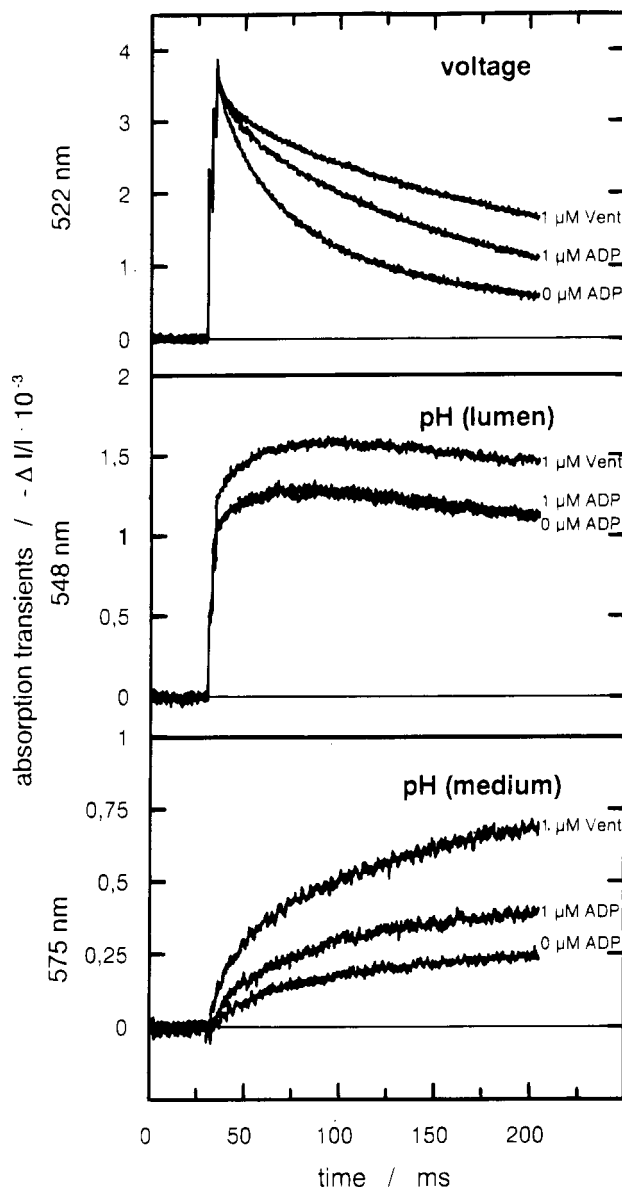


FIGURE 1: Transients of flash-induced transmembrane voltage and luminal and external pH. The transients were recorded under repetitive excitation of thylakoids with a flash group consisting of three xenon flashes spaced by 2 ms. The original traces represent the transmembrane voltage by electrochromism (top), the pH in the lumen by neutral red (middle), and the pH in the suspending medium by cresol red (bottom). They were obtained under a steady background pH difference of 1.5 pH units (for further chemical and spectroscopic details, see Material and Methods). The upward direction indicates a positive electrical charging of the thylakoid lumen (top), an acidification of the lumen (middle), or an alkalization of the medium (bottom). ADP and venturicidin were added to the reaction medium when indicated. The ordinate is scaled in terms of relative changes of transmitted intensity, $10^3 \times (\Delta I/I)$, at 522 (top), 548 (middle) and 575 nm (bottom).

alkalinization of the external medium were diminished accordingly (Figure 1, middle and bottom). By comparing these transients to the ones monitored in the presence of venturicidin, the ATP synthase related portion of the respective proton-transfer step was obtained. Broadly speaking, the respective differences between traces with and without venturicidin represented the transient charge translocation (top), proton intake from the lumen (middle), and proton release into the medium (bottom) by CF_0CF_1 (see Materials and Methods). For the charge transfer a finer analysis was

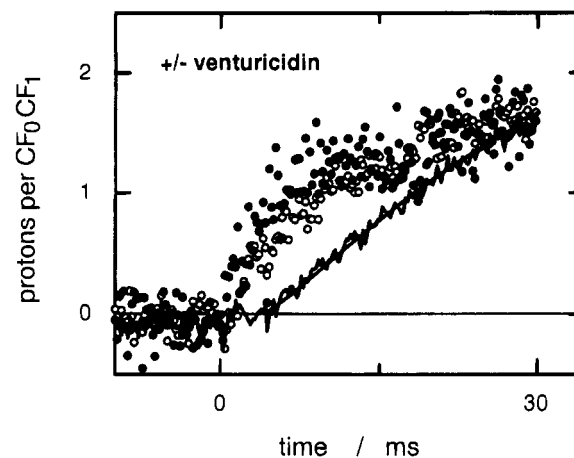


FIGURE 2: Partial protolytic reactions related to CF_0CF_1 under conditions of proton slip. Charge transfer (—), proton intake (●), and proton release (○) as attributable to CF_0CF_1 were calculated from original traces as shown in Figure 1 by comparison at each wavelength of one trace without and another one with venturicidin added. The ordinate was scaled in terms of moles of protons translocated per mole of CF_0CF_1 (total) as specified under Materials and Methods.

required as detailed in eqs 1 and 2. These three variables were plotted in Figure 2. It was apparent that one portion of proton intake and release was more rapid than the translocation of an equivalent number of charges. This was caused by neither the evaluation method for the latter (see original traces in Figure 1) nor an insufficient time resolution of the electrochromic indicator for the transmembrane voltage (see the rapid rise in Figure 1).

Saturation of Proton Intake. If the rapid component of proton intake and the slower proton transfer across the dielectric barrier were consecutive reactions, we expected the extent of proton intake to be saturated when the light flashes followed each other before the transmembrane relaxation was completed. This was indeed observed as documented in Figure 3A. The experiment was carried out at a medium pH of 8.0 to maximize the extent of rapid proton intake (see below). To avoid the additional contribution of PS I to the voltage, P700 was blocked by hexacyanoferrate(III) plus the Cyt *b₆f* inhibitor DNP-INT. The upper traces in Figure 3A show original pH transients in the lumen as induced by three flashes of light with narrow spacing (5 ms). The intensity of the continuous measuring light was increased from 1.5 mW/cm² (in Figure 1) to 2.5 mW/cm² (in Figure 3A) to augment the background proton motive force closer to the threshold of proton slip (Groth & Junge, 1993). Again two traces are depicted, with and without venturicidin added, both in the presence of 1 μM ADP. The bottom trace shows the difference between the upper two. Proton intake was diminished from the first to the second and the third flash. A repeat of this experiment with variation of the interval between consecutive flash series as given in Figure 3B showed the recovery of the extent of rapid proton intake with the same half-rise time as found for the recovery of the transmembrane charge transfer. It implies that the rapid intake of protons is a precursor of proton transfer across the dielectric barrier. These partial reactions are arranged in consecutive rather than parallel order.

Selective Blocking Agents. The selective blocking of proton release and charge transfer but not of proton intake was evident from the original traces in Figure 1. Proton

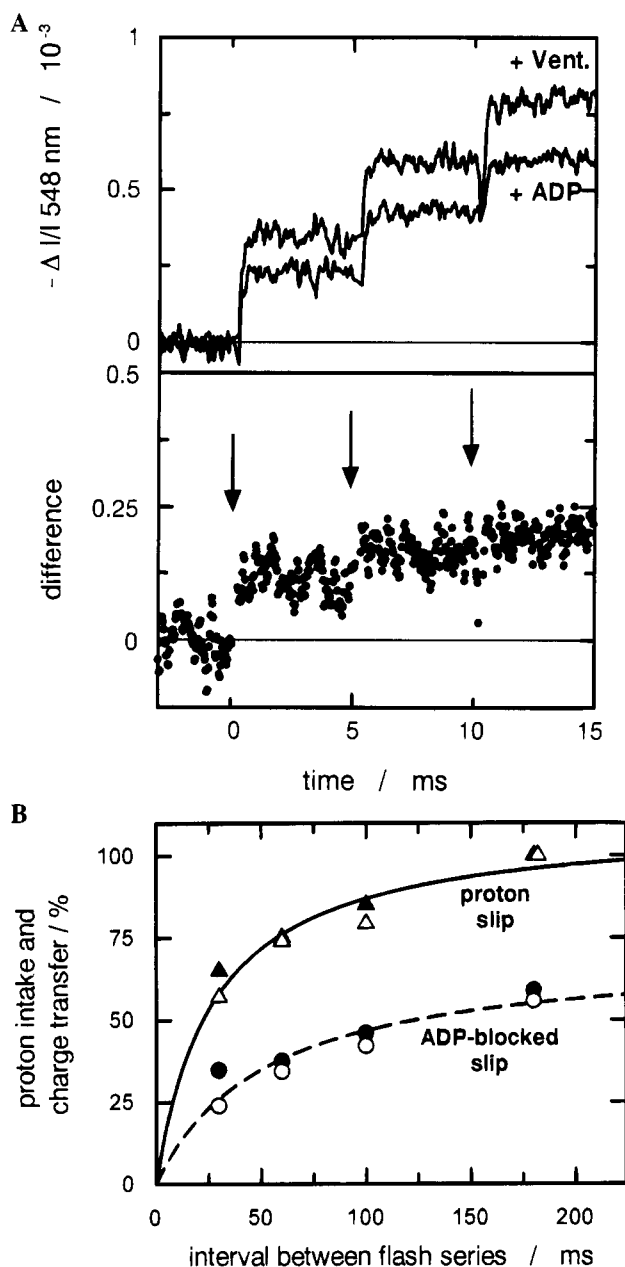


FIGURE 3: Saturation of flash-induced proton intake. (A) pH transients in the lumen of thylakoids as induced by a series of three xenon flashes spaced by 5 ms. In order to surpass the threshold of proton slip with a single flash, the background illumination of the sample was enhanced to 2.5 mW/cm², causing a transmembrane ΔX pH of nearly 2.5 units. Both original transients (top in Figure 3A) were obtained in the presence of 1 μ M ADP but only in the larger one was 1 μ M venturicidin additionally added. The lower part of Figure 3A represents the difference between the signals in the upper part. The timing of flash excitation is indicated by arrows. (B) Extent of transient proton intake (filled symbols) and charge transfer (open symbols) under repetitive excitation of thylakoids with groups of three closely spaced flashes (2 ms) at variable repetition intervals. Data points were calculated from original transients detected at 548 (proton intake) and 522 nm (charge transfer) as exemplified in Figure 1. In the presence of ADP (●, ○) the extent of both reactions was reduced. But with 1 μ M ADP or without (▲, △), the extents of the rapid intake of protons and of the transferred charges varied in parallel.

intake from the lumen (Figure 1, middle) was not affected by the addition of subcatalytic concentrations of ADP (e.g., 1 μ M, with 1 mM P_i always present), whereas the two other observables were changed in the same direction as by the

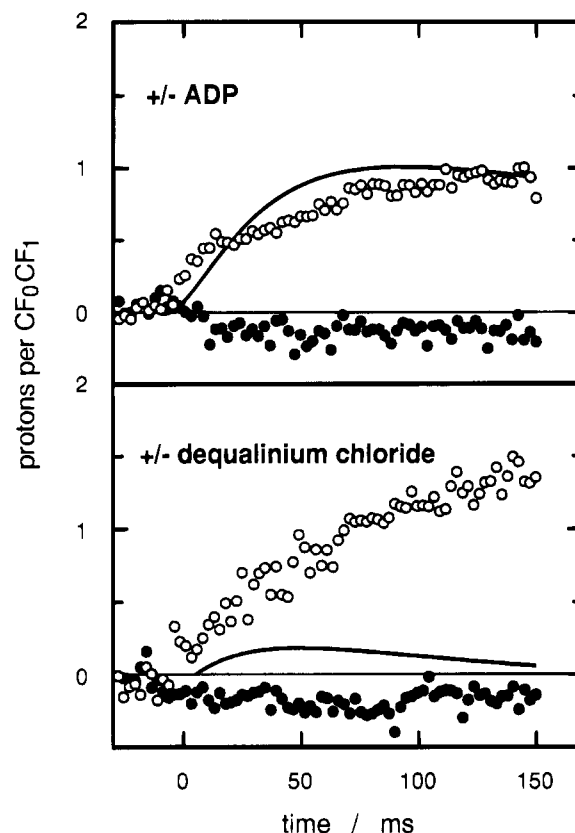


FIGURE 4: Selective blocking of partial protolytic reactions. Show are the portions of charge transfer (—), proton intake (●), and proton release (○) which are sensitive to ADP (top) and dequalinium (bottom). Traces were calculated from the original sets of traces, as exemplified in Figure 1, with and without ADP (1 μ M) and with and without dequalinium (1 μ M) added.

addition of venturicidin. In Figure 4 (top) we plotted the time course of this particular fraction of proton intake/transfer/release that was sensitive to the addition of ADP. These transients were calculated from the transients in Figure 1 that were obtained with and without the addition of 1 μ M ADP. Proton release and charge transfer were affected by ADP; proton intake was not. The lack of any effect of ADP on the rapid proton intake from the lumen was obviously no artifact caused by the added buffering capacity of ADP. Such effects are not even observed at 1000-fold higher concentration. Moreover, the addition of venturicidin on top of ADP produced the same signal extent as with venturicidin in the absence of ADP (not documented).

Figure 4 (bottom) shows a similar set of transients, those which were sensitive to dequalinium. This agent eliminated one portion of proton release, but not proton intake and charge transfer. The DELSEED cluster on subunit β has been identified as the binding site for dequalinium in mitochondrial ATP synthase (Bullough et al., 1986b, 1989a). We straightforwardly assumed that dequalinium interacted with the corresponding domain in chloroplast ATP synthase. Both effectors, ADP and dequalinium, bind to adjacent regions in the headpiece of the enzyme, CF₁. As evident from Figure 4, they blocked proton transfer before (ADP) and after the major dielectric barrier (dequalinium) in the enzyme.

The Apparent pK of the Proton Binding Groups. Figure 5 shows the extent of rapid proton intake as induced by a group of three short flashes as a function of the luminal pH.

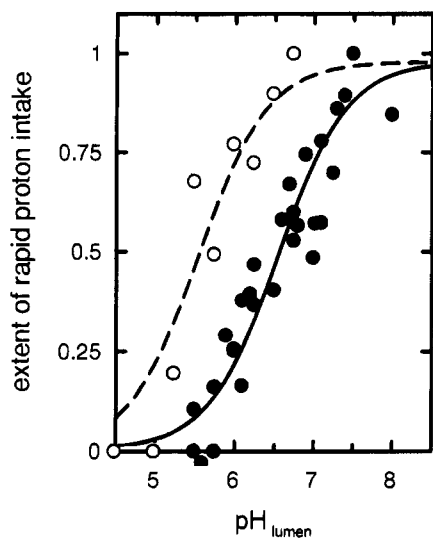


FIGURE 5: The apparent pK of the luminal proton binding sites of CF_0CF_1 . Shown is the extent of rapid proton intake from the lumen as a function of the luminal pH. The luminal pH was calculated from the external pH and the steady ΔpH values of 1.5 (closed circles) and 2.5 (open circles) units, respectively.

The luminal pH was calculated from the medium pH and the steady pH difference across the membrane, namely, 1.5 pH units (closed circles) and 2.5 pH units (open circles). The latter was calculated from the fluorescence quench of the acridine dye ACMA. Both data sets were fitted with a univalent Henderson–Hasselbalch titration curve (lines). Their apparent pK s differed depending on the pH difference; they were 6.2 and 5.7, respectively.

The Driving Force of Proton Intake. A flash of light which drives both photosystems through a single turnover generates a voltage rise of about 60 mV (Junge & Witt, 1968; Junge et al., 1979), but only a small acidification in the thylakoid lumen (Junge et al., 1979) by less than 0.1 pH unit (or 6 mV). Which was the driving force of proton intake? We monitored rapid proton intake in the absence of ADP and without or with 0.2 μM valinomycin added (and 2 mM KCl). The potassium cation carrier valinomycin collapsed the membrane voltage in 1.5 ms without affecting the underlying steady pH difference or the magnitude of the flash-induced pH step. In the presence of valinomycin rapid proton intake was abolished, as is evident from the transients of proton intake found without and with valinomycin, which are shown in Figure 6. This observation implies that the driving force of proton binding in flash spectrophotometric experiments is electrical rather than chemical.

DISCUSSION

It is unknown how and where CF_0CF_1 interacts with protons. A glutamic acid residue (aspartic acid in *E. coli*) on the multicopy proteolipid subunit of CF_0 is essential for proton conduction (Fillingame, 1990). This acid residue, which is functional only if positioned in the middle of either of the two membrane-spanning stretches of this subunit (Miller et al., 1990), is the target for the covalent binding of DCCD and for binding venturicidin (Galanis et al., 1989), both of which inhibit proton conduction. When exposed by removal of CF_1 , the membrane portion of chloroplast CF_0 is an extremely selective proton conductor [H^+/Na^+ selectivity $\approx 10^7$ (Althoff et al., 1989)]. But as the related F_0F_1 from

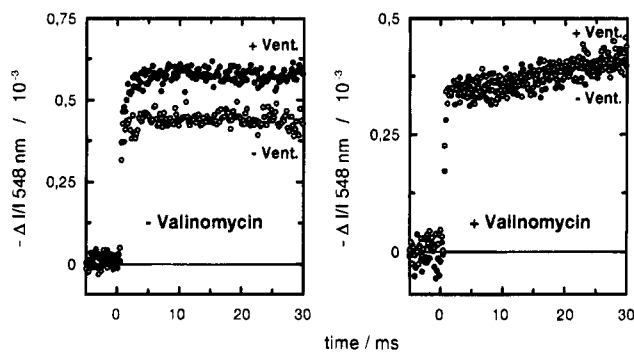


FIGURE 6: The driving force of rapid proton intake. Shown are flash-induced transients of the luminal pH in the absence and presence of 1 μM venturicidin under conditions of proton slip (no ADP) and of blocked proton flow across CF_0CF_1 (plus venturicidin). The transients were monitored without (left) or with (right) 200 nM valinomycin added in a reaction medium containing 2.6 mg/mL BSA, 2 mM KCl, 3 mM $MgCl_2$, 0.1 mM Na_2HPO_4 , 10 μM MV, and 15 μM neutral red. The reaction was induced by a single-turnover flash at a background illumination of 2.1 mW/cm^2 .

Propionigenium modestum can operate on Na^+ instead of H^+ (Laubinger & Dimroth, 1987), it has been proposed that the essential acid residue, instead of covalently binding protons, may act in a crown ether structure (Boyer, 1988). The structure of the F_1 portion has been disclosed at 0.28-nm resolution (Abrahams et al., 1994); the structure of the F_0 portion and particularly of the stalk which connects the channel and the catalytic portion is unknown in detail. With this limited information we discuss our experiments simply in terms of three types of sites: the site of proton intake (probably involving Glu-61 on subunit III of CF_0), the main dielectric barrier, and the exit site (probably located at the interface of CF_0/CF_1). A schematic illustration of these binding sites and of the agents acting on them is shown in Figure 7.

The proton conduction in the absence of ADP, named proton slip, is a phenomenon attributable to the holoenzyme, CF_0CF_1 , as evident from the selective blocking of certain reactions by ADP and dequalinium, both of which bind to CF_1 . We recorded proton intake, charge transfer across the main dielectric barrier, and proton release in response to a step of the transmembrane voltage (see Figures 1, 2, and 4). The extents were equal within the rather wide noise limits, but the rates differed. At pH 8.0 a large portion of proton intake, monitored by neutral red, was faster ($\tau_{1/2} \approx 5$ ms) than the charge translocation ($\tau_{1/2} \approx 30$ ms), detected by electrochromism (see Figure 2). The time resolution of electrochromism is better than nanoseconds, and that of the pH indicator neutral red at the given concentration, 15 μM , ranges down to 100 μs , as found in studies on proton release and proton uptake by photosystem II (Haumann & Junge, 1994). Thus the kinetic disparity was real and not caused by a limited time resolution of the spectroscopic probes. The rapid binding of protons from the lumen side and rapid release of protons into the medium preceded the transfer of charge (protons) across the main dielectric barrier. Still these partial reactions were arranged in sequential order and not in parallel. Evidence for this came from the saturation behavior of the rapid intake under a series of flashes fired at short intervals (see Figure 3A). In experiments with repetitive flashes with variation of the time interval the extent of rapid proton intake was proportional to the extent of the charge flow between the flash groups (see Figure 3B).

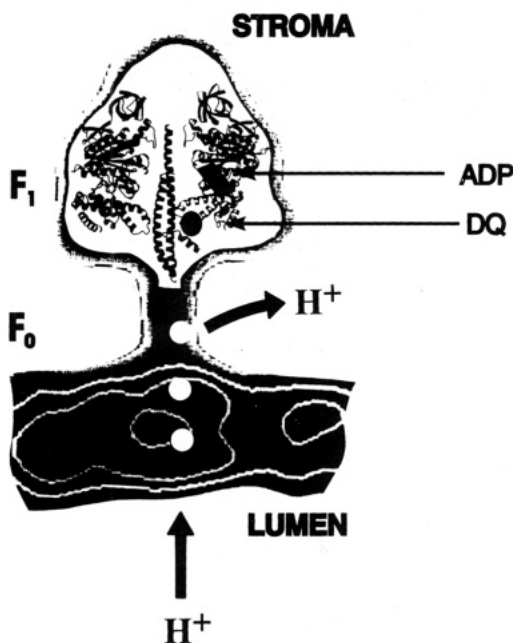


FIGURE 7: Schematic illustration of proton binding sites in CF_0 - CF_1 (white circles) and of the binding sites of ADP and dequalinium. The features are taken from cryoelectron microscopy (adapted from Capaldi et al. (1992)) and from X-ray crystallography (Abrahams et al., 1994). The stroma side and the lumen side of the chloroplast enzyme are indicated as well as the binding sites for ADP and dequalinium on subunit β . White circles spots stand for the sites of proton intake, the main dielectric barrier, and proton release. Their location is purely hypothetical.

A titration of the extent of proton intake from the lumen with variation of the medium pH but a constant pH difference between the medium and the lumen (1.5 and 2.5 units, respectively) yielded apparent luminal pKs of 6.2 and 5.7 (see Figure 5). More importantly, the titration curve was univalent, without any sign of cooperativity. This was in sharp contrast with the highly cooperative trapping of protons from the lumen which we previously reported under conditions where the ATP synthase was distorted by treatment with very low concentrations of EDTA (about 10 μ M) (Junge et al., 1984; Griwatz & Junge, 1992). Reinvestigating the seemingly cooperative proton trapping of the EDTA-treated enzyme but now with variation of both the medium pH and the lumen pH (by the intensity of the background light), we found that the high cooperativity of the pH profile was due to the *medium pH* (G. Groth and W. Junge, unpublished). The cooperative phenomenon which induced proton trapping was the distortion of the enzyme structure at alkaline pH at the stroma side of the membrane and not the behavior of the lumen-exposed, proton-accepting groups on CF_0 . It appeared as if divalent cations fixed CF_0 and CF_1 in their normal configuration. If these divalent cations were lacking, protons could mend this deficiency in a cooperative manner. This topic, which relates to the interfacing of CF_0 (probably via a stalk) and CF_1 , will be discussed elsewhere. There is no contradiction between the univalent titration behavior of proton intake under slip and the apparently highly cooperative one in the presence of EDTA.

Which was the driving force of rapid proton intake? The flash group induced a voltage jump of about 100 mV but a much smaller pH jump in the lumen (<0.2 unit), too small to explain the uptake of almost one proton per total CF_0CF_1 without cooperativity. When the flash-induced voltage jump

was rapidly collapsed by valinomycin (see Figure 6), rapid proton intake was eliminated. Thus the driving force for rapid proton intake was electric. The similar voltage dependence of proton trapping by the distorted enzyme [EDTA treatment; see Figure 3 in Griwatz and Junge (1992)] has been interpreted in the frame of Mitchell's proton well hypothesis (Mitchell, 1977) by the conversion of electric into chemical potential in a proton-selective matrix which ranges into the membrane dielectric.

The addition of venturicidin blocked all three partial protolytic reactions. This is plausible, as venturicidin interacts with the particular domain in the proteolipid (subunit III) (Galanis et al., 1989) containing Glu-61, which is essential for proton conduction by ATP synthase. ADP (1 μ M together with 100 μ M P_i) blocked proton transfer and proton release but not proton intake; i.e., it acted on the major electrogenic transfer step. On the other hand, it left the site of proton intake, plausibly around Glu-61 on subunit III, unaffected. According to our previous studies, ADP and P_i bind to one of the potentially catalytic (i.e., GDP receptive) sites (Groth & Junge, 1993), which according to the atomic structure (Abrahams et al., 1994) is located mainly on subunit β and roughly at half-height of the $\alpha_3\beta_3$ globule, about 10 nm from the middle of the membrane core and from the supposed sites of proton intake. Interactions between sites separated by this distance can only be explained by assuming long-range conformational transmission, long a matter of speculation regarding the functioning of this enzyme (Green & Harris, 1970; Boyer, 1975, 1977).

Dequalinium blocked proton release but much less proton intake and charge transfer. In the mitochondrial enzyme dequalinium interacts with the DELSEED sequence on β (Bullough et al., 1989a,b), which according to the X-ray structure establishes a catch contact with two lysines (around residue 90) on γ , but only in the particular β which is loaded with ATP (Abrahams et al., 1994). Whether or not the exit site of protons is identical with this domain is an open question, as noncompetitive effects of dequalinium on proton release could not be excluded on the basis of our experiments.

The selective and long-range action of dequalinium on proton release and of ADP on proton transfer may be turned around by the principle of microscopic reversibility. This then implies that different partial reactions of the proton act on different domains in the catalytic F_1 portion of the enzyme, where they may affect different partial reactions of catalysis. The results presented in this work are in line with, but extend, a previous report where DCCD binding to the proteolipid was shown to alter the affinity of nucleotide binding (Penefsky, 1985a). The differentiation between two types of action from CF_1 into CF_0 paves the way for studies aiming at the identification in the time domain of energy-transducing conformational changes which couple proton transfer to ATP extrusion.

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