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Protons, the Thylakoid Membrane, and the Chloroplast ATP Synthase[a]

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Light-driven proton pumps and proton-translocating ATP synthases stand very close to the beginning of photoautotrophic life. Whereas nature found different solutions to proton pumping (e.g., retinal-based bacteriorhodopsin and (bacterio)chlorophyll-based reaction centers from eubacteria to green plants), the proton-translocating ATP synthases belong to one superfamily[1] of bipartite enzymes that are formed from a channel portion, which is embedded in the coupling membrane, and a peripheral, catalytic portion (reviewed in ref. 2). The mechanism of coupling between proton flow and ATP synthesis is usually discussed in the framework of the chemiosmotic theory.[3]

In the thylakoid membrane of chloroplasts a transmembrane protonmotive force is generated by light-driven electron transport[4] which involves three big protein-complexes, two photosystems, and the cytochrome b_6,f-complex (FIG. 1). Thylakoid membranes of shade plants are stacked (see upper insert in FIG. 1). While the ATP synthase CF_oCF_1 resides exclusively in exposed membrane regions, the proton pumping activity of photosystem II is exclusively located in appressed regions,[5] that is, up to 300 nm away from an ATP synthase. The lower insert in FIGURE 1 shows a simple equivalent circuit for the cyclic proton current, consisting of pumps, P, two line resistors, R_{L1} and R_{L2} (note the relatively large lateral distance between pumps and ATP synthases), an access resistor in the enzyme, R_A, and finally the largest resistor, R_C, representing the coupling site. Here protons are forced to do useful work, whereas proton flow over the other resistors only produces ohmic heat.

On the long way to understanding the coupling mechanism between proton flow and ATP synthesis we have studied the nature of the pathways for protons between pumps and ATP synthases, the magnitude of the losses of protonmotive force along these paths, the conductance of the channel portion of the ATP synthase, which is supposed to act as a proton well,[6] and, finally, elements of the protonic coupling site in the enzyme.

Thylakoids are a favorable object for two reasons: (1) Excitation of thylakoids with a short flash of light generates a voltage transient across the membrane (in nanoseconds) and a transient pH difference (microseconds to milliseconds). (2) The rise in the transient protonmotive force and its subsequent decay via leak conductances or via the ATP synthase can be followed at high time resolution by appropriate indicator dyes: intrinsic pigments respond to the transmembrane voltage by electrochromism,[7] the surface adsorbed neutral red (in the presence of a nonpermeant buffer) reports small pH transients at the lumen side of the thylakoid membrane,[8–11] and any hydrophilic pH indicator is practically selective for pH transients in the medium.[12] Taken together, the spectrophotometric techniques have allowed "complete tracking of proton flow."[13,14]

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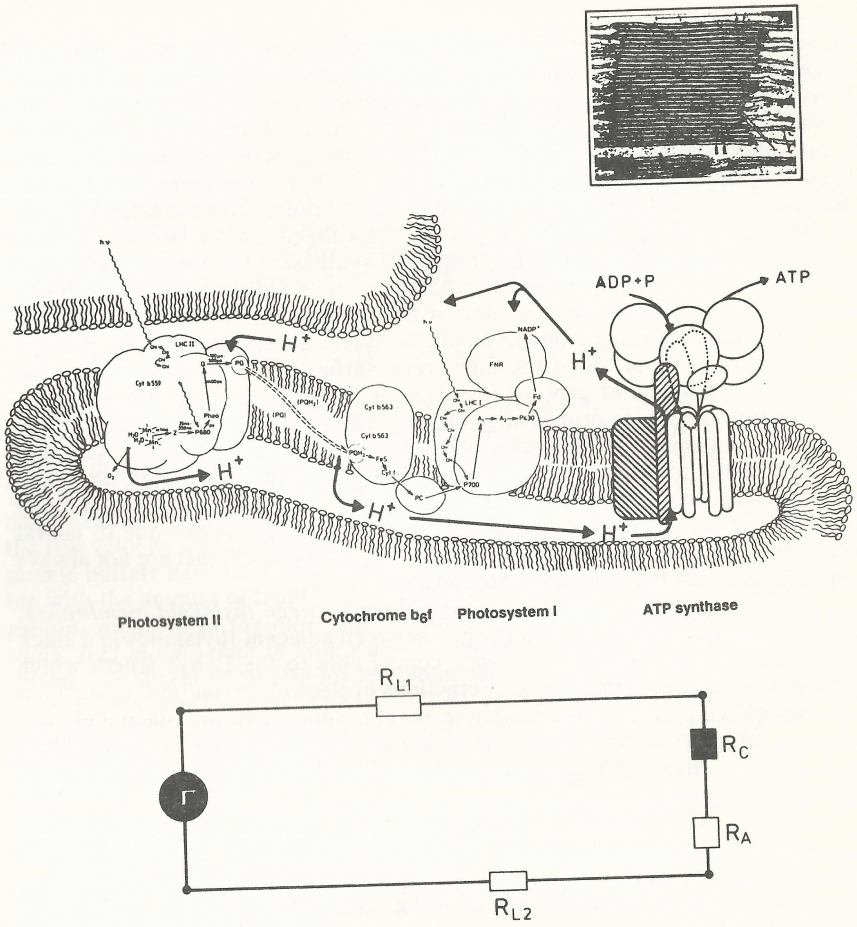


FIGURE 1. Schematic drawing of the thylakoid membrane with the three large electron transfer complexes and the ATP synthase. Apposed membrane portions with photosystem II and exposed portions with photosystem I and the synthase are apparent. Sites of light driven proton binding from the outer medium (the stroma) and of proton release into the lumen, lateral proton flow, and transmembrane proton backflow over the ATP synthase are indicated. The insert in the upper right shows an electron micrograph of stacked thylakoid membranes. Each of the disk-like structures contains at least 100 pieces of any protein complex, but the true function unit is larger as disks are interconnected with each other. The lower portion shows a simplistic equivalent circuit for cyclic proton flow between pumps and the ATP synthase.

ON THE PATHWAY OF PROTONS BETWEEN PUMPS AND ATP SYNTHASES AND THE DICHOTOMY BETWEEN LOCALIZED AND DELOCALIZED COUPLING

There is a long-standing debate on whether very many proton pumps are coupled with very many ATP synthases simply by proton flow through aqueous

compartments, as postulated by Mitchell[3] (delocalized coupling), or whether there is a preferential interaction with pumps feeding protons directly into neighboring ATP synthases (localized coupling). Detailed theories for the latter case (e.g., ref. 15) have been put forward mainly to account for certain observations in mitochondria and photosynthetic bacteria.[16] The dichotomy between localized and delocalized coupling has two different aspects, one related to the dimension in plane of the membrane and the other normal to the membrane. Over the lateral dimension and in thylakoids coupling is *per se* delocalized simply because a large fraction of proton pumps can be far from ATP synthases. For the normal dimension, however, it is still under debate whether protons *obligatorily* move through the aqueous volumes that are separated by the coupling membrane, thereby interacting with many buffering groups,[3] or whether there are special ducts for protons in the membrane[17,18] or along the membrane surface.[19,20] It is evident that the nature of the pathway for protons into F_0F_1 ATP synthases [e.g., through nonaqueous environment, as proposed[17]] strongly bears on the energetics and on the molecular mechanism of photophosphorylation. Four facets of this issue are dealt with in the following: (a) The properties of the very narrow (5 nm wide) spaces between membranes. (b) The possibility of enhanced proton diffusion at the surface of the membrane. (c) The magnitude of ohmic losses due to lateral proton flow. (d) The existence of intramembrane proton ducts that are not always in equilibrium with the adjacent water spaces.

(*a*) *Properties of the narrow aqueous phases between thylakoid membranes.* Both the lumen of thylakoids and the gap between adjacent thylakoids in a stack are only about 5 nm wide,[21] a distance comparable to the Debye length which describes the range of electrostatic interactions in electrolyte solutions (e.g., 3 nm at 10 mM of a 1 : 1 electrolyte[22]). These narrow spaces are not adequately described as an aqueous bulk phase but rather as charged surface or Donnan-matrix.[23] They are filled with charged groups on lipid heads and on the surface of membrane proteins.

Thylakoids, which appear as stacked disks in the electron micrograph inserted in FIGURE 1 (radius about 300 nm, repetition period about 20 nm), are truly interconnected and able to form large spherical blebs (radius about 1 μm) when suspended in distilled water. In the following discussion we neglect this complicated connectivity, and we use the term thylakoid in a loose way, denoting stacked, disk-shaped membranes. The internal volume of a thylakoid disk is so small ($1.4 \cdot 10^6$ nm^3) that one might wonder whether the concept of pH is still valid. At pH 7 this volume contains an average of less than 0.1 free protons. In other words, a snapshot reveals only one free proton in one disk and no free proton in nine others. That the concept of pH is valid is owed to many buffering groups (ensemble average) and to the rapidity of protonation/deprotonation reactions (time average). The specific buffering capacity of the thylakoid lumen, approximately 100 mmol/mol chlorophyll around neutral pH, has been determined by optical[9] and by spin probe techniques.[24] The number of buffering groups in the aforementioned small volume can be calculated from the definition of the buffering capacity; see Eq. 1a:[9]

$$\beta = -\Delta[H^+_{total}]/\Delta pH \qquad \textbf{(1a)}$$

wherein β denotes the buffering capacity, which is the ratio between the change in total concentration of protons (bound plus free) and the resulting pH change. If only one type of buffering group is present (dissociation constant K and concentration c_{buffer}), the buffering capacity depends on the concentration of free protons, $[H^+]$, as given in Eq. 1b.

$$\beta = 2.3 \cdot c_{buffer} \cdot [H^+] \cdot K/([H^+] + K)^2 \qquad \textbf{(1b)}$$

If the measured buffering capacity of the thylakoid lumen[9,24] was not attributable to a host of groups with different pK (as in reality), but to a homogeneous set with uniform pK 7, the number of such buffering groups in this small volume was about $3 \cdot 10^4$.

According to Eigen,[25] and for buffering groups at the surface of lipid structures and proteins experimentally established by Gutman,[26] each of these groups undergoes rapid protonation/deprotonation with a relaxation time in the order of 100 μs. As the diffusion of protons over aqueous distances of 300 nm occurs in a few microseconds, these buffering groups can be conceived of as one common pool. Then, the concentration of free protons in this small volume is the time average over protolytic events in the pool. On a time scale of 100 μs there will be more than 10^4 events, that is, the concentration of free protons will be sharply defined with less than 1% standard deviation.

The observation[10] that certain hydrophilic buffers, added perhaps at 10 mM, do not affect the magnitude of flash-induced pH transients in the lumen of unswollen thylakoids is at least in part a consequence of the tiny internal volume (3 l/mol chlorophyll). A concentration of 10 mM of a hydrophilic but neutral compound implies less than 10^4 molecules in the aforementioned small volume. This is much less than the amount of intrinsic buffers. The same buffers, however, are effective in swollen thylakoids[9] with the specific internal volume expanded more than 10-fold higher from the original 3 l/mol chlorophyll,[21,24] which means that now more than 10^5 buffer molecules are present, more than intrinsically. Supporting this argument, lipid soluble or amphiphilic buffers like imidazole also act on unswollen thylakoids, probably because they are enriched by adsorption to the membrane surface and not restricted to the small aqueous volume. On the same line, they are still more effective than hydrophilic buffers even in swollen thylakoids.[9]

In summary, although the behavior of the narrow spaces between thylakoid membranes in some respects differs from one of an extended aqueous bulk phase, there is no reason to doubt that the pH is well defined and that these spaces can serve to couple proton pumps with ATP synthases.

(*b*) *Enhanced proton diffusion at the surface of the membrane?* One aspect of the old but ongoing debate over the validity of Mitchell's chemiosmotic theory[3] is the question of whether the diffusion of protons at the surface of the membrane is enhanced over the one in bulk water. Originally a matter of speculation,[19] it has gained thrust by the experiments of Prats, Tocanne, and Teissie[20] on proton diffusion along the surface of phospholipid monolayers deposited on an aqueous subphase. Their results have allowed them to claim 20-fold enhancement of the surface diffusion coefficient over the one in bulk water, and furthermore that this result is relevant for the protonic energy coupling in real biomembranes.

Experiments with stacked thylakoid membranes do not support this notion. The layout is illustrated in FIGURE 2. Excitation with one short flash of light causes one turnover of each photosystem II (FIG. 1) with comcomitant proton uptake from the outer phase (and proton release into the lumen). This generates an alkalinization jump in the narrow space between appressed membranes (hatched in FIG. 2). In the absence of ADP and P the membrane is rather proton

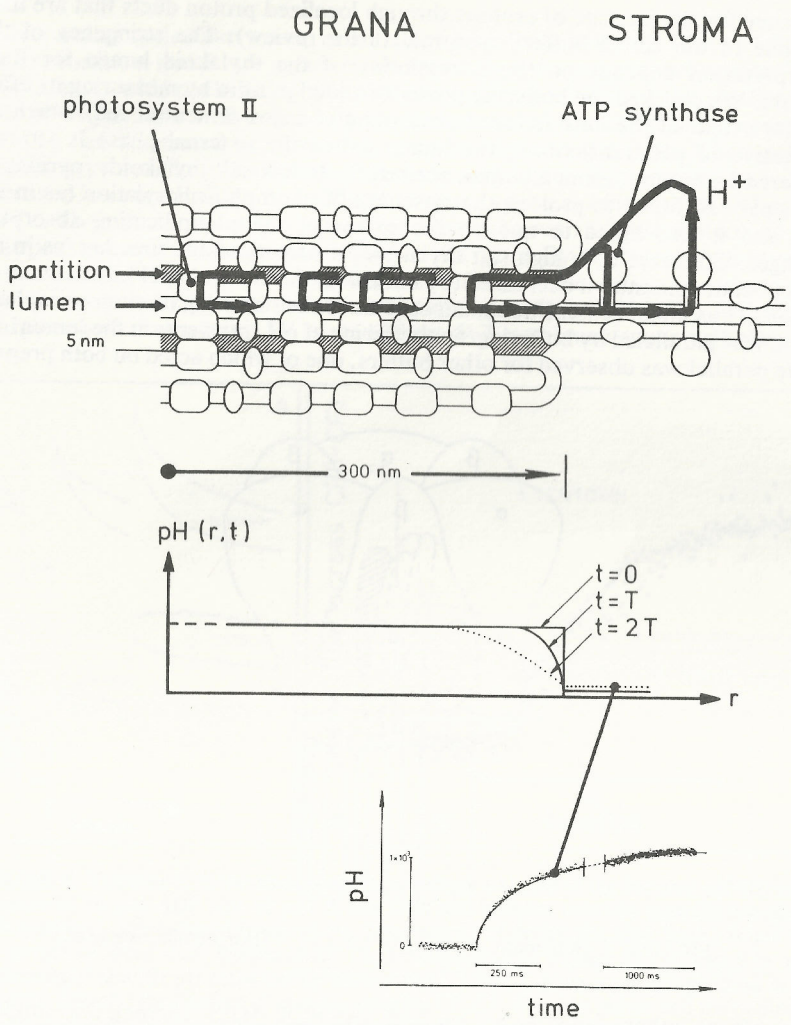


FIGURE 2. Schematic side view of stacked thylakoid membranes with appressed grana lamellae and interconnecting stroma lamellae. The narrow space between the outer surface of thylakoid membranes in a granum, called partition, is hatched. The *arrows* illustrate (a) light-driven proton pumping by photosystem II which is directed from the partition into the lumen, (b) opposite directed proton flow which is coupled to ATP synthesis, (c) lateral proton flow through the lumen, and (d) lateral backflow through partitions. The drawing in the middle illustrates the box shaped pH profile induced by excitation with a single flash of light at time zero and its evolution in time (t = T, t = 2T). The bottom trace is a reproduction of original data on the flash induced, and photosystem II-related pH rise in the stroma compartment and a theoretical curve (see text and refs. 27–29).

tight. (The relaxation time of a transmembrane pH difference then is 15 s.[11]) Thus, alkalinization relaxes by *lateral* diffusion of protons, hydroxyl anions, and mobile buffers along the surface to and from the medium. The concomitant rise of pH in the medium (data points and fit curve illustrated at the bottom of FIG. 2) is measured spectrophotometrically. At first glance the slowness of the observed slow rise is surprising. The half-rise time of about 100 ms[12,27] is 10^5 times larger than expected by solving Fick's equation and assuming the same diffusion coefficients for protons and hydroxyl ions as in water. But this can be understood by taking into account the presence of fixed buffers in this domain. The rise velocity of the medium pH is a function of the "effective diffusion coefficient," D^{eff} (see refs. 27 and 28):

$$D^{eff} = (2.3/\beta_{tot}) \cdot \{D_{H^+} \cdot [H^+] + D_{OH^-} \cdot [OH^-] + \Sigma(D_i \cdot \beta_i/2.3)\} \qquad (2)$$

depends on the buffering capacity, β, of the mobile buffers (suffix i) and of all buffers (fixed and mobile = *tot*al), respectively. D denotes the diffusion coefficient of protons, hydroxyl anions, and mobile buffers,[28] as specified by the suffix. The observed dependence of the relaxation on the medium pH and on added mobile buffers follows the expectation based on Eq. 1. The predicted minimum of the rise velocity around neutral pH and the enhancement of the velocity by added mobile buffers have been observed.[29] The 10^5-fold delay has been found compatible with the amount of fixed buffers present.[27] This has led to the conclusion *that protons adjacent to the outer side of stacked thylakoid membranes are, if anything, less mobile than in bulk water.*[29] It may be argued that enhanced surface diffusion is absent in protein-loaded biomembranes and only exists in membranes formed from pure lipid, as stated in ref. 20. This has become highly questionable because of recent experiments by Gutman *et al.*[30] and Menger *et al.*[31] The former find no evidence for enhanced proton diffusion in the ultrathin layer between the osmotically compressed bilayers in multilamellar lipid vesicles. The latter arrive at the same conclusion in their study on proton diffusion along the surface of a lipid monolayer spread on an aqueous subphase.

Does the narrowness of the aqueous phases and their granularity (by protruding proteins) invoke the critical diffusion phenomena described by percolation theory?[32] A tentative answer may be inferred from studies on proton conduction on lyophilized and lightly rehydrated purple membranes.[33] The threshold for the onset of conduction as a function of relative hydration is observed at a water-to-protein ratio (w/w) of 0.045 g/g, far below the point for the full water coverage, 0.25 g/g.[33] We calculated a water-to-protein ratio of 0.6 g/g for thylakoid membranes, which suggests that critical threshold phenomena that are predicted by percolation theory are not present in normally hydrated thylakoid membranes.

(c) *Losses of protonmotive force by lateral proton flow.* Comparing the efficiency of photosystem II to drive ATP synthesis with that of photosystem I, Haraux and de Kouchkowsky[34] found slightly higher figures for the latter. This might be understood in terms of the losses of the protonmotive force during lateral flow of protons and hydroxyl anions between photosystems II in the appressed membrane portions and the ATP synthases in the exposed ones (see FIG. 2). A total flux of protons, I (moles/s), over the boundary of a thylakoid disk requires a drop in proton concentration between the center (suffix c) and the fringe (suffix f) of the disk. For a disk that is homogeneously filled with pumps the concentration drop has been calculated:[27]

$$[H^+]_c - [H^+]_f = I/4\pi hD \qquad (3)$$

It depends on the thickness of the disk-shaped slab between membranes, h, and on the diffusion coefficient, D. A proton flux, which is equivalent to the highest rate of ATP synthesis in a model thylakoid (radius 300 nm, area per chlorophyll molecule 2.2 nm), namely, $1.3 \cdot 10^{-19}$ mol s^{-1}, and assuming a thickness of 5 nm and the diffusion coefficient as in bulk water, $D_{H^+} = 9.3 \cdot 10^{-9}$ m^2 s^{-1}, implies a drop in proton concentration of 0.23 μM.[27] The magnitude of the corresponding pH drop, $\Delta pH = -\Delta[H^+]/2.3 \cdot [H^+]$, depends on the pH in the medium. It decreases toward more acid pH. A similar relation holds in the alkaline pH domain, where the diffusion of OH^- dominates. Taking these results together and assuming the same diffusion coefficients as in bulk water the pH drop has been calculated.[27] At the outer surface of stacked thylakoids it amounts to 0.14 pH units, if the outer side is kept at pH 8. It is below 0.01 pH units at the internal side and at pH 4.[27] If the diffusion coefficients in these narrow spaces are lower than in bulk water, greater losses are expected. *It is probable that ohmic losses of protonmotive force are small, but they are not negligible in tightly stacked thylakoids.*

(*d*) *Are there localized proton ducts in the membrane?* This question is not easy to solve. It has been speculated that the respiratory chain and photosynthetic electron transport inject protons into the nonaqueous environment of the respective coupling membrane from where they are used for ATP synthesis.[17] For thylakoid membranes it has indeed been established by Dilley and coworkers (see ref. 18 for review) and also by others[35,36] that the membrane contains certain buffering groups that are not normally in equilibrium with the adjacent aqueous bulk phases. Amine groups with an unusually high pK (around 7.8) are involved.[18] These groups can transiently trap protons that are released, for example, by photosystem II as a consequence of water oxidation. The pool size is limited to about 6 protons per photosystem II, and proton liberation into the thylakoid lumen is again detectable after the pool is filled.[36] Under site-specific blocking of every second photosystem II by a herbicide (DCMU), the relative pool size per active reaction center II is doubled.[37] This has proved that the pool is delocalized and not, as one might expect, restricted to the particular protein molecule, such as photosystem II, from which protons are released. The question is whether or not these proton-trapping domains are relevant for photophosphorylation. There is ample evidence that a transmembrane and bulk-to-bulk pH difference[38] and an electric potential difference[39] (reviewed in ref. 40) can drive ATP synthesis in thylakoid membranes or in lipid vesicles with CF_oCF_1.[41] However, there is also evidence that the depletion from protons of the intramembrane buffering pool delays the onset of photophosphorylation in thylakoids that are excited by a series of light flashes.[42] This has been interpreted to indicate that the intramembranous groups may be on the pathway of protons into the ATP synthase. With the limited proton storage capacity of these groups,[35–37] this is relevant only for the abrupt onset of illumination from the dark but not for continuous illumination as experienced by plants in daytime. From the physiologic standpoint it is not much to worry about.

Another challenge to a chemiosmotic mechanism is also related to the onset lag of photophosphorylation. For thylakoids receiving a series of light flashes starting from the dark and with valinomycin added to minimize the electric component of the protonmotive force, the chemiosmotic theory predicts prolongation by added buffers of the onset lag of photophosphorylation. Buffers simply slow down the building up of a sufficiently large pH difference. On the contrary, in thylakoids that are prepared under low salt, added pyridin fails to enhance the onset lag of photophosphorylation[43] (and see Dilley, this volume). It behaves as expected only in high salt thylakoids. The former behavior has been interpreted as

evidence for the passage of protons through localized proton ducts that are inaccessible to the added buffer[43] (see ref. 18 for review). The stringency of this interpretation depends on the accessibility of the thylakoid lumen for these buffers. We checked the buffering power of added pyridin by measuring its effect on the extent of the flash-induced absorption changes of neutral red, which are indicative of pH transients in the lumen (when the external phase is strongly buffered by bovine serum albumin, see ref. 9). In low salt thylakoids, pyridin (10 mM, pH 7.8) failed to prolong the onset lag of photophosphorylation (as in ref. 43), and it also failed to quench the extent of the pH-indicating absorption changes. Conversely, in high salt thylakoids it prolonged the time lag (as in ref. 43) but concomitantly it decreased (by buffering) the extent of pH transients in the lumen. Thus enhancement/nonenhancement of the onset lag of photophosphorylation was paralleled by buffering/nonbuffering of pH transients in the lumen. The same parallel was observed for other buffers, one of which acted on both preparations (tris), others failed to act on both (HEPES, MES, tricine), and imidazole revealed a selective behavior as pyridin (A. Borchard & W. Junge, unpublished). These phenomena may be caused by different accessibility in different preparations of the thylakoid lumen to certain added buffers. *One should be careful to take the failure of pyridin to enhance the onset lag of photophosphorylation as proof of a role in photophosphorylation of intra-membrane proton ducts.*

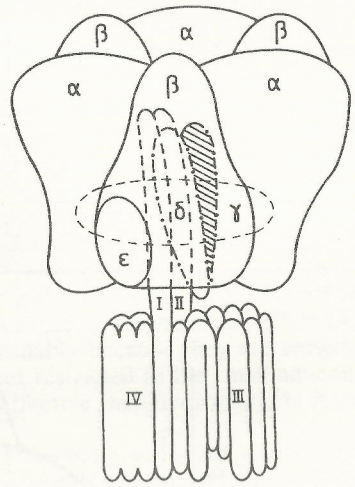


FIGURE 3. Schematic drawing of CF_oCF_1.[75] (For details, see text.)

PROTON FLOW THROUGH CFOCF1

The structure of the chloroplast ATP synthase is schematically illustrated in FIGURE 3. CF_1, the extrinsic portion, contains the catalytic sites of ATP synthesis and hydrolysis, and CF_o, a membrane-spanning complex, acts as a proton conductor.[2] CF_1 is composed of five different subunits, α, β, γ, δ, and ε, in the order

of decreasing molecular mass, in stoichiometric proportion 3 : 3 : 1 : 1 : 1.[44] The large subunits, α and β, are arranged alternatingly to form a pseudohexagon[45] and they interact with nucleotides and phosphate. The positions of the smaller subunits of CF_1 are less well defined; their arrangement at the interface between CF_o and CF_1, as illustrated in FIGURE 3, may be close to the truth. Their role in regulating proton flow through the synthase and in energy transduction will be discussed later on. In CF_o, four different subunits have been identified and named I to IV. Their stoichiometry is still under debate. Subunit III, the proteolipid, has a hairpin structure[46] and it is present in 6 to 12 copies. By sequence similarity with the subunits of the homologous enzyme of *Escherichia coli,* it is known that subunit I contains only one membrane-spanning helix with a bulky hydrophilic headpiece and that subunit IV is made from 5–7 membrane-spanning helices.[47]

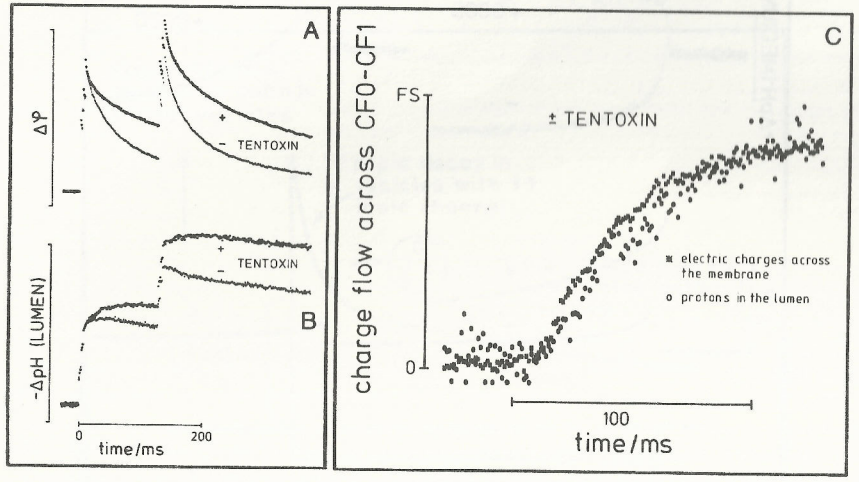


FIGURE 4. Transients of the transmembrane voltage **(A)** and of the lumenal pH **(B)** in the presence of 20 μM ADP and 60 μM P_i and with and without tentoxin (specific blocker of ATP synthase). Reproduced from ref. 14. Suspensions of thylakoids have been excited with two groups of three short flashes each. The transmembrane voltage was recorded by electrochromism[7] and the pH transient by neutral red.[8–11] Part **C** gives a superimposition of two data sets, namely, the number of protons entering CF_oCF_1 from the lumen and the number of charges crossing CF_oCF_1 in the time interval between the first and the second flash group (Normalization and procedure to extract these data from the traces in **A** and in **B** in ref. 14). Broadly speaking the two sets of data points in **C** coincided (see text).

Proton conduction by the integral enzyme is controlled by the presence of nucleotides which interact with CF_1. FIGURE 4 shows transients of the transmembrane voltage (FIG. 4A) and of the lumenal pH (FIG. 4B) under excitation of thylakoids with two groups of three short flashes each. The traces have been obtained with ADP and P_i! present without (−) and with added tentoxin (+) to block ATP synthesis.[14] If the ATP synthase is blocked, the voltage that is generated by two sets of three flashes decays more rapidly (some 100 ms) than does acidification of the lumen (some 10 s). This is indicative of an electric leak conductance which is higher for other ions than for the proton. It is responsible for the electric balance of the greater part of the inwardly directed proton pumping in thylakoids.[4] It is noteworthy that specific ion channels that might account for the

nonprotonic leak conductance of thylakoid membranes have not been characterized up to now, except for a voltage-dependent anion channel that has been detected by patch clamping of thylakoids from giant chloroplasts of *Peperomia metallica*.[48]

Only when the ATP synthase is active are the electric decay and the decay of the acidification accelerated concomitantly. This clearly shows the dominance of the protonic conductance through CF_oCF_1 over the leak conductances. FIGURE 4C shows a comparison of the extra charge displacement through active CF_oCF_1 (as extracted from FIG. 4A, see ref. 14) and of the extra proton displacement (from FIG. 4B) during the interval between the first and the second flash group. The two sets are coincident within noise limits. *Hence, every charge crossing the active ATP synthase is evident as a proton entering the enzyme from the lumen.*[14]

PROTON FLOW THROUGH CF_o, THE CHANNEL PORTION OF THE ATP SYNTHASE

Turnover numbers of CF_oCF_1 range up to more than 400 ATP molecules formed per second.[41] With a proton-to-ATP stoichiometry of 3 this implies a turnover number of 1,200 protons per second. In most experiments aiming at proton conduction by the channel portion, F_O or several of its subunits have been isolated and incorporated into lipid vesicles (see ref. 47 for review). Then proton leakage across the membrane has been monitored by pH electrodes. This approach has produced proton conductance that was sensitive to DCCD, as in F_O, but with turnover numbers of 10 s^{-1}, falling short by orders of magnitude from the required one. This was incommensurate with the proposed function as a low-impedance access for protons to the coupling site in the ATP synthase.[6] The shortcoming may have been due to the survival of only a small proportion of F_O channels in reconstitution experiments (safe guard mechanism?) and/or to insufficient time resolution of pH electrodes.

In an alternative approach to determine the time-averaged single channel conductance of CF_o, a fraction of CF_1 has been removed by EDTA treatment of thylakoid membranes. Relaxation of the flash light-induced transmembrane voltage and of the pH transients in the lumen and in the medium have been monitored.[13,49,50] FIGURE 5 illustrates the situation with CF_1 removed and with ferricyanide added as terminal electron acceptor, so that for any two protons released into the thylakoid lumen, only one proton is taken up from the suspending medium. FIGURE 6 shows the transients of the external pH (top), of the transmembrane voltage (middle), and of the lumenal pH (bottom) with both CF_o exposed (−DCCD) and CF_o blocked by DCCD. The two traces in the upper left show alkalinization of the suspending medium when the proton channel is blocked and a net acidification of same extent when the channel is open. (The efflux of two protons overcompensates the alkalinization by one proton.) Correlation of the electric and protonic decay processes (left column in FIG. 6) with the amount of *exposed CF_o* (determined by immunoelectrodiffusion) and application of the capacitor equation (assuming the usual 1 μF cm^{-2} for the thylakoid membrane) have revealed that: *Under the assumption that every exposed CF_o is actually conducting, the average proton conductance of exposed CF_o is about 10 fS.* This is equivalent to the translocation of 6,000 protons at 100 mV electric driving force.[13]

The traces in the right show the same set of experiments except that the electric potential difference has been shunted by the addition of gramicidin (with K^+ present). The short circuiting of the transmembrane voltage is immediately

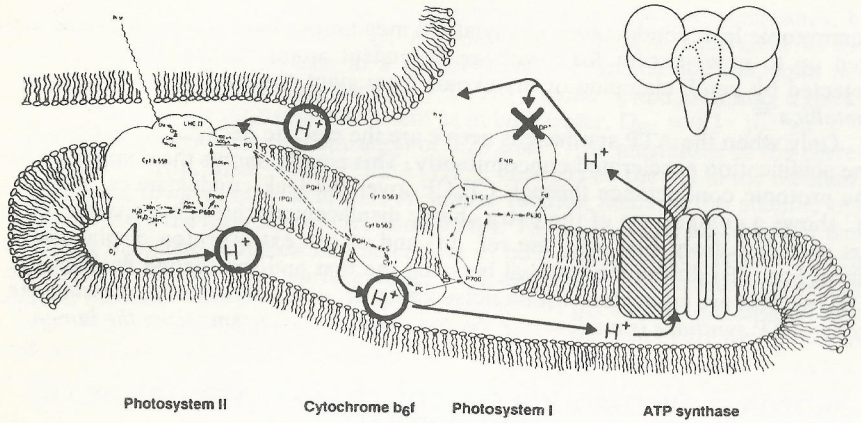


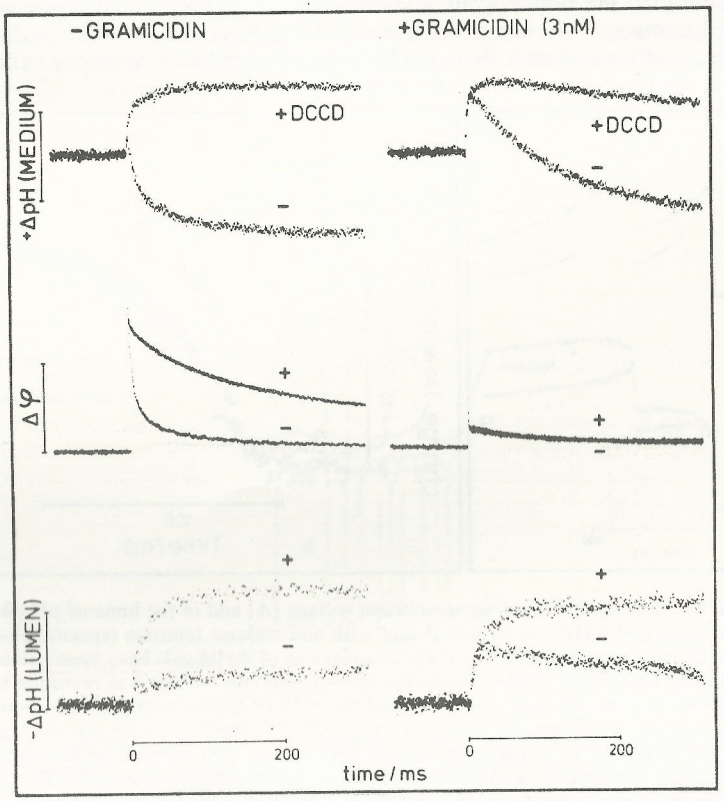
FIGURE 5. Schematic drawing of protolytic reaction sites when ferricyanide is added as terminal electron acceptor and with CF_1 removed by EDTA. This figure serves to illustrate the meaning of the original traces in Figure 6.

apparent from the middle traces. Under these conditions the pH difference across the membrane relaxes more slowly. It discharges the larger proton buffering capacity of the lumen rather than the electric capacitance of the membrane (as in the left column of FIG. 6). Around neutral pH the ratio between the specific electric capacitance and the equivalent term derived from the buffering capacity of the lumen is larger than 10, as determined in reference 9. This is the reason for the larger extent of transmembrane voltage after excitation of thylakoids with a single-turnover flash of light (namely, 30–50 mV[4]) as contrasted with the extent of transmembrane pH difference (namely, 0.06 units, only[9]). Conversely, comparison of the traces in the left and right columns of FIGURE 6 clearly demonstrates that in the absence of gramicidin there is no leak conductance to other cations, that is, CF_o is highly selective for protons (but see below).

CF_1 can be removed from thylakoid membranes without leaving behind proton conducting CF_o.[13,51] If the electric relaxation (see left column in FIG. 6) is attributable to only a few active channels out of many exposed ones, then the conductance of the open channels is higher than the average 10 fS. This has been subjected to a systematic study.[49,50] A lucky circumstance as well as a prerequisite for the experimental approach has been that EDTA treatment of thylakoid membranes, which is standard to remove the CF_1 counterpart of CF_o, also causes destacking of thylakoids and finally their fragmentation into smaller spherical vesicles containing a total of about 100 CF_oCF_1 or 10^5 chlorophyll molecules. Only the small size of these vesicles opens the way to a situation where among the about 10^{11} partially CF_1-depleted vesicles in the absorption cell of a typical experiment, some may have no conducting CF_o and others 1, 2, or more. Indeed, the relaxation of the flash light-induced voltage and of the pH transients has revealed a biphasic decay. This has been subjected to a statistical analysis based on Poisson's distribution of active channels over vesicles. Assuming ohmic behavior of CF_o (in the 50 mV range of these experiments), the decay of the voltage indicating electrochromic absorption changes is expected to follow Eq. 4:[49,52]

$$U_{app}(t) = U_0 \exp(-\tilde{n}) \exp(\tilde{n} \exp(-Gt/A \cdot \hat{c})). \qquad (4)$$

This equation has only two fit parameters, namely, $\tilde{n}$, the average number of open channels per vesicle, and G, the time-averaged conductance of one active CF_o. A, the area of one vesicle, can be inferred from similar experiments with the channel-forming antibiotic gramicidin (see below and ref. 49) and $\hat{c}$, the specific membrane capacitance, is taken as usual, namely, 1 μFcm^{-2}. The experimentally observed biphasic decay of the electrochromic absorption changes and its relation to the parameters $\tilde{n}$ and G is illustrated in FIGURE 7. Eq. 4 is a good approximation even if a vesicle area distribution with 30% standard deviation from the mean is



⇒ 10 fS per exposed CF_0
⇒ 6000 H^+ s^{-1} @ 100 mV
⇒ monospecific for protons

FIGURE 6. Complete tracking of proton flow through CF_o (adapted from ref. 13). Traces in the upper part show pH transients in the suspending medium of thylakoids, those in the middle transients of the transmembrane voltage, and traces at the bottom show pH transients in the lumen, all of them induced by one short flash of light. They are observed in partially CF_1-depleted thylakoids, with the covalent channel blocker DCCD present and absent, respectively. In the right column the transmembrane voltage is shunted by addition of the alkali cation pore former gramicidin. For details, see text.

taken into account.[49] Application of this analysis to CF_1-depleted vesicles has yielded a *time-averaged conductance of active CF_o of about 1 pS*. At 100 mV electric driving force this is equivalent to the passage of $6 \cdot 10^5$ protons per second. However, only a few percent of exposed CF_o have been highly conducting under these circumstances.

As a test for this statistical analysis of electrochromic absorption changes the conductance of gramicidin has been analyzed in the same vesicles as before. With CF_o blocked by DCCD, gramicidin has been added at pM concentration. The analysis has yielded a conductance of 2.5 pS, which is in the range of published figures for the open state of gramicidin under a given ionic milieu (see ref. 49 for

$$U_{app}(t) = U_0 \cdot exp(-\bar{n}) \cdot exp\left(\bar{n} \cdot exp\left(-\frac{G}{A \cdot \hat{c}} \cdot t\right)\right)$$

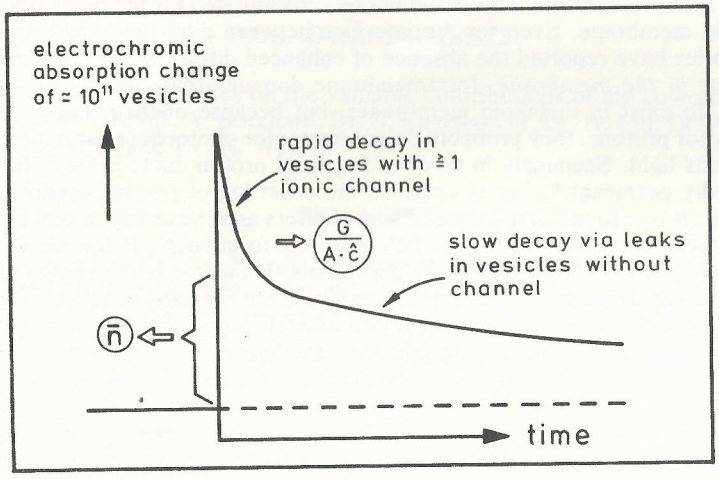


FIGURE 7. Schematic drawing of the flash-induced electrochromic absorption changes (ordinate) as function of time in a suspension with small thylakoid vesicles that are partially CF_1 depleted, so that each vesicle out of the 10^{11} vesicles that are contained in an optical absorption cell carried zero, one, two, . . . active CF_o channels. This has enabled a poisson statistical fit of measured decay curves with only two essential fit parameters, namely, the time-averaged single-channel conductance, G, and the average number of open channels, $\bar{n}$.[49,50]

details). Is the gramicidin channel always open in thylakoid membranes, though? This is indeed suggested by a linear concentration dependence of the electric relaxation rate in thylakoids for gramicidin concentrations above some 10 pS and a quadratic one for lower concentrations (Schönknecht *et al.*, unpublished). The rather high dimerization constant of gramicidin in thylakoids points to an open probability close to 1.

The conductance of CF_o has been investigated as a function of pH, pD (isotopic substitution), the concentration of other cations, addition of glycerol to alter the water structure, and under variations of temperature.[50] The technique has been the same as that already described. The results are as follows: (1) The proton

is the major charge carrier through CF_o even at pH 8 and against a background of 300 mM NaCl or KCl or 30 mM $MgCl_2$ in the medium. No other interpretation is possible, as time-resolved pH transients are one basis for this conclusion. The apparent discrimination by a factor of 10^7, say, against Na^+ is surprising, as the homologous F_oF_1 ATP synthase from *P. modestum* seems to be able to operate facultatively on H^+ or on Na^+.[53] (2) Between pH 5.6 and pH 8 the conductance of CF_o does not vary as a function of the medium pH, independent of the ionic strength. This shows that the conductance is not rate limited by the protonation/deprotonation of one particular group. (3) The conductance is lower in D_2O than in H_2O by a constant factor of 1.7. The isotope effect may be secondary. (4) Addition of glycerol decreases the conductance of CF_o and abolishes the hydrogen/deuterium isotope effect. This suggests that the conduction of the channel may then be governed by events related to the water structure in the channel mouth. (5) With 42 kJ/mol the Arrhenius activation energy of proton conduction by CF_o in the thylakoid membrane is intermediate between that of a pore (e.g., 30 kJ/mol for gramicidin) and that of a carrier (e.g., 65 kJ/mol for valinomycin) in the same membrane.

A protonic conductance of 1 pS exceeds by orders of magnitude the calculated convergence value that is limited by diffusive supply to a pore mouth of perhaps 1 nm diameter in a medium at neutral pH. I also greatly exceeds calculated rates of proton transfer through a short hydrogen bonded chain[55] and measured conductances (neutral pH) of gramicidin or of channels formed from leucine- and serine-containing synthetic polypeptides.[61] Protolytic reaction rates in excess of the diffusion limit have been reported for certain uncouplers in biomolecular lipid membranes.[54] Hydrolysis, the reaction of water with an acceptor group in the channel mouth, has been discussed to account for the seeming discrepancy.[54,55] This, however, is expected to reveal a pH dependence that has not been observed in the foregoing experiments with CF_o. At the moment, one can only speculate about the origin of the extremely high proton conductance of CF_o. Channel properties as large mouth and a short selectivity filter, which have been discussed in the context of K^+ maxi channels,[56] the drag force in the coulomb cage that surrounds the channel mouth,[57] the transient supply of protons from fixed buffers in its vicinity and/or mobile buffers may lift the supply rate of protons to the observed figure.

The foregoing experiments have established that *CF_o is a kinetically competent access channel for protons in the integral ATP synthase, CF_oCF_1, and the site of proton selectivity of CF_oCF_1 is the CF_o portion of the enzyme.*

CF_oCF_1 has recently been addressed by electrophysiologic techniques. In one approach, Wagner *et al.*[58] reconstituted purified CF_oCF_1 into azolectin vesicles, these were fused into a lipid monolayer on aqueous subphase, and a CF_oCF_1-doped bilayer was formed at the dip of a micropipette (dipstick technique). At voltages greater than 100 mV, there has been a steep rise in the open probability of channels with a conductance of 1–5 pS. Evidence has been presented for the protonic nature of the observed gated currents. That these are attributable to CF_oCF_1 and not to CF_o alone has become evident, as added ADP (3 μM) and P_i! (5 μM), both of which interact with the CF_1 portion, have decreased the open probability of conduction events.[58] These concentrations of ADP and P_i are known to affect tight-binding sites on CF_1, but they are too low for efficient ATP synthesis. Higher concentrations of ADP and P_i, however, lead to enhanced proton flow over CF_oCF_1 (see FIG. 4). This suggests that the aforementioned effects may represent a (over-voltage) valve reaction of CF_oCF_1, with CF_1 shifted in a voltage-dependent reaction to transiently expose the path of protons through

CF_o. This was compatible with a similar magnitude of conductance and with the same pH independence as in the previous studies on exposed CF_o (free of CF_1) in thylakoid membranes. It is noteworthy that the large turnover number of these channels ($>10^6 s^{-1}$ at 180 mV) in some circumstances has been sustained over several 100 ms (i.e., transport of more than 10^5 protons).

Another approach has been fusion of small CF_oCF_1-containing vesicles to form large liposomes (by dehydration/rehydration[59]). Patch clamping of these large liposomes has revealed cation channels with several conductance levels in the range of 10 pS (at 100 mM KCl or NaCl) but without a pronounced selectivity for protons.[60] Addition of venturicidin, an inhibitor of proton flow through CF_o, that is supposed to interact with the proteolipid, has decreased the open probability. Similar channels have been observed with subunit III of CF_o alone. This has been interpreted to indicate that some of the CF_oCF_1 molecules are deranged or disintegrated, as during dehydration/rehydration, and that the products, mainly subunit III, are capable of forming cation channels[60] as certain synthetic polypeptides.[61] The general cation conductance is obviously suppressed in intact CF_o. The sharp selectivity filter for protons may be brought about by interaction of subunit III with the other subunits of CF_o.

ON THE ROLE OF THE SMALLER SUBUNITS OF CF_oCF_1

With CF_o acting as a proton channel and the hexagon formed by the α and the β subunits interacting with the nucleotides, one may ask for the coupling site where protons are forced to do useful work. The γ subunit regulates proton flow through the enzyme. Its thiol groups[62] are involved in the diurnal redox modulation of the enzyme activity which is switched off at night to prevent the ATP produced by mitochondria to be dissipated by chloroplasts (see ref. 63). The ε subunit seems also involved in the regulation of proton conduction by the enzyme.[64] The δ subunit is necessary for efficient coupling between CF_o and CF_1,[65] but in contrast to the other two, which exert their respective roles only in conjunction with the other small subunits present, it seems to have some function of its own. After hints that δ may remain back on CF_o after removal of CF_1, where it keeps CF_o nonconducting,[66] we have found that *isolated* δ,[67] *when added back to CF_1-depleted thylakoids, blocks proton flow through open CF_o*[68] and thereby restores photophosphorylation[69] by those CF_oCF_1 that have remained on the CF_1-depleted membrane. The stopcock action of δ on CF_o has to be relieved in the intact, ATP-synthesizing enzyme. It is probable that δ *then either acts as (part of) the valve,* which admits protons from CF_o further up into the enzyme to the coupling site, *or as (part of) the conformational transducer* between protons and ATP. It is compatible with this role that δ, which is not necessary for the binding of CF_1 to CF_o,[65,70] can bind not only to CF_o[67–69] but also to CF_1 with one high (100 nm) and one or two low affinity sites.[71]

There is good evidence of a similarity between the quarternary structures of F_oF_1 ATP synthases from different sources. Yet there is only limited sequence homology, for example, between the δ subunits of *E. coli*[72] and spinach chloroplasts (36% including conservative replacements).[73] Nevertheless an attempt to construct functional hybrids between the *E. coli* EF_1 (minus δ) and chloroplast δ and vice versa has been successful. The hybrid constructs plugged proton conduction through CF_o or EF_o.[74] This is highly suggestive of a mechanical role of

subunit δ in a conformational coupling mechanism between protons and ATP formation,[75] perhaps by a rotating binding site mechanism as proposed.[76]

SUMMARY AND OUTLOOK

According to the chemiosmotic theory,[3] proton pumps and ATP synthases are coupled by lateral proton flow through aqueous phases. Three long-standing challenges to this concept, all of which have been loosely subsumed under 'localized coupling' in the literature, were examined in the light of experiments carried out with thylakoids: (1) *Nearest neighbor interaction between pumps and ATP synthases.* Considering the large distances between photosystem II and CF_oCF_1, in stacked thylakoids this is *a priori* absent. (2) *Enhanced proton diffusion along the surface of the membrane.* This could not be substantiated for the outer side of the thylakoid membrane. Even for the interface between pure lipid and water, two laboratories have reported the absence of enhanced diffusion. (3) *Localized proton ducts in the membrane.* Intramembrane domains that can transiently trap protons do exist in thylakoid membranes, but because of their limited storage capacity for protons, they probably do not matter for photophosphorylation under continuous light. Seemingly in favor of localized proton ducts is the failure of a supposedly permeant buffer to enhance the onset lag of photophosphorylation. However, it was found that failure of some buffers and the ability of others in this respect were correlated with their failure/ability to quench pH transients in the thylakoid lumen, as predicted by the chemiosmotic theory. It was shown that the chemiosmotic concept is a fair approximation, even for narrow aqueous phases, as in stacked thylakoids. These are approximately isopotential, and protons are taken in by the ATP synthase straight from the lumen.

The molecular mechanism by which F_oF_1 ATPases couple proton flow to ATP synthesis is still unknown. The threefold structural symmetry of the headpiece that, probably, finds a corollary in the channel portion of these enzymes appeals to the common wisdom that structural symmetry causes functional symmetry. "Rotation catalysis" has been proposed.[76] It is of heuristic value to visualize CF_oCF_1 as a mechanical coupling device. Its maximum turnover number ranges up to 400 s^{-1} for ATP and 1200 s^{-1} for protons. At about 200 mV electric driving force this implied a conductance of about 1 fS. Its channel portion (CF_o), however, has revealed a very large protonic conductance of 1 pS (three orders of magnitude greater than the protonic conductance of gramicidin around neutral pH). This was seemingly pH independent (between 5.8 and 8). The passage of other cations through CF_o is strictly suppressed (even at pH 8 and with 300 mM NaCl in the medium). Components of CF_o, on the other hand, mainly the proteolipid subunit, can form Na^+-permeable cation channels in lipid bilayers. The magnitude and specificity of the proton conductance of CF_o is not well understood physicochemically. In physiologic terms there is no need to supply protons at such high rate to a 10^3-fold slower enzyme.

The pathway of protons after their entry into CF_o is unknown, although several critical residues have been pinned down, for example, by site-directed mutagenesis (reviewed in refs. 3, 46, and 47). The entry of protons into the CF_1 portion with direct chemical action in the catalytic process has been proposed,[77] so far without any experimental evidence. If protons left the enzyme already at the interface between CF_o and CF_1, a mechanical mode of energy transduction to the

catalytic site had to be faced. Attempts have been made to identify the CF_1 subunits that throttle the very high rate of proton passage through CF_o to the slow coupled rate. These pointed to a role for subunit δ, an elongated protein (MW 20 k) that can block proton flow through CF_o. The nature of blocking is unknown, but again it is heuristically appealing to view δ as part of a conformational transducer.[75] The construction of hybrids from $F_1(-\delta)$, F_O, and δ taken from chloroplasts and from *E. coli* has revealed some functionality of the constructs. It is expected that further hybrids between even more remote enzymes of the F_0F_1 family may give a clue to a discrimination between mechanical and chemical coupling. Of course, the X-ray crystal structure analysis of this large enzyme (MW 550 k) is badly needed for any thorough understanding. Equally needed is a higher kinetic resolution of partial reactions and, if possible, of mechanical transients in the operating enzyme.

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