Proton diffusion along the membrane surface of thylakoids is not enhanced over that in bulk water

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ABSTRACT In photosynthesis and respiration ATP synthesis is powered by a transmembrane protonmotive force. Membrane bound proton pumps and proton translocating ATPsynthases are coupled by lateral proton flow. Whether it leads through the aqueous bulk phases (chemiosmotic theory) or whether it is confined to the membrane or the membrane water interface, is still controversial. Another related controversy is whether or not proton diffusion along the interface between a phospholipid membrane and water is enhanced over the one in bulk water. Thylakoid membranes of plant chloroplasts are intrinsically closely apposed (≈5 nm). To study lateral proton diffusion along the narrow interfacial domain between adjacent thylakoid membranes, we stimulated the proton pumps by a flash of light. This generates an alkalinization jump. In the absence of ADP the membrane is relatively proton tight. Therefore, the alkalinization jump relaxes into the medium. The relaxation kinetics as function of pH and added buffers were studied by flash spectrophotometry. The results were compared with a theory dealing with the diffusion of protons, hydroxyl ions, and mobile buffers plus the action of fixed buffers. We came to the conclusion that the lateral diffusion coefficient both, for H^+ and for OH^- was less or of same magnitude as in bulk water.

INTRODUCTION

In his chemiosmotic theory of photo- and oxidative phosphorylation Mitchell (1) postulated the coupling of redox-driven proton pumps with proton translocating ATP synthases by lateral proton flow and backflow through the aqueous bulk phases that are separated by the coupling membrane. At the same time, Williams postulated (2) that protons follow special pathways within the coupling membrane. Whether or not intra-membrane proton conducting channels exist is still a matter of active research (see e.g., reference 20). This version of localized coupling schemes is not dealt with in this article. We focussed on another facet of the old but ongoing debate over the validity of the chemiosmotic theory, namely, on whether or not proton conduction along the surface of a coupling membrane is enhanced (3, 4). This debate has gained thrust by the interesting experiments of Prats et al. (5, 6) on proton diffusion along the surface of phospholipid monolayers deposited on an aqueous subphase.

Fig. 1 shows a schematic representation of stacked thylakoid membranes that form a contiguous system. Appressed grana lamellae are interconnected by nonappressed stroma lamellae. Three out of the four large membrane proteins act as proton pumps, namely photosystem II, photosystem I, and the cytochrome-bf-complex. The fourth complex, the ATP synthase, utilizes protons. The ATP synthase is exclusively located in nonappressed membranes and photosystem II predominantly in appressed ones (11). This necessitates lateral proton flow over distances of up to 300 nm between a center-placed photosystem II and the nearest ATP synthase at the periphery of a granum (see arrows in Fig. 1). Proton backflow from the ATP synthase to photosystem II follows the so-called partition (see hatched strips in Fig. 1). The halfwidth of partitions, ~2.5 nm (see reference 12), is comparable with the Debye length, which describes the range of electrostatic interactions in electrolyte solutions. Because of narrowness, partitions are sort of a surface region and therefore they are an adequate system to study proton diffusion along the membrane. Partitions are full of buffering groups, mostly on the large proteins which cover ~80% of the membrane area (12). The buffering capacity at the outer side of the membrane is practically constant in the pH-domain from 6 to 8 (19).

Upon excitation of thylakoids with a short flash of light (and with hexacyanoferrat (III) (200 μM) as electron acceptor) proton uptake by photosystem II generates a small, positive pH-jump in partitions ($\approx 10^{-3}$ pH units). This pH jump is relaxed by the lateral diffusion of H^+, OH^-, and of mobile buffers to and from the medium. The rise of the medium-pH can be kinetically resolved by hydrophilic pH-indicating dyes as detailed elsewhere (8). Its typical half-rise time is 100 ms, much shorter than the transmembrane relaxation time of the light induced pH-difference which takes ~10 s (in the absence of ADP, reference 22).

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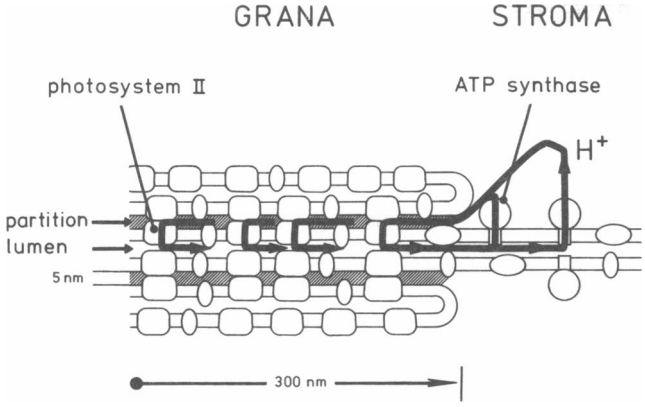

FIGURE 1 Schematic drawing of the side view on stacked thylakoids with appressed grana lamellae and interconnecting stroma lamellae. The narrow space between the outer surfaces of neighboring thylakoids in a granum, called partition, is hatched. The arrows illustrate (*a*) light driven proton pumping by photosystem II that is directed from the partition into the lumen, (*b*) opposite directed proton flow which is coupled to ATP synthesis by the ATP synthase, (*c*) lateral proton flow through the lumen, and (*d*) back-flow through the partition.

Treating the partitions as open cylinders (see reference 12 for their topology), the analytical solution to this diffusion problem is an infinite series of exponentials (see Eq. 13 in reference 9):

$$\mathrm{pH}(t)/\mathrm{pH}(\infty) = 1 - 4 \cdot \Sigma[(a_n^{-2} \cdot \exp(-a_n^2 \cdot D^{\mathrm{eff}} \cdot t/r^2)], \quad (1)$$

where pH(t) denotes the medium-pH at time t, r is the radius of a partition, the a_n are roots of the Bessel function (first kind, order zero) and D^{eff} is the effective lateral diffusion coefficient in the cylinder. The only free parameter, D^{eff}, depends on the concentrations of diffusing species (H^+, OH^-, and mobile buffers), on their respective diffusion coefficients, and on buffers present in the partition (see Eq. 19 in reference 10):

$$D^{\mathrm{eff}} \approx (2.3/\beta^{\mathrm{tot}}) \cdot \{D_{H^+} \cdot [H^+] + D_{OH^-} \cdot [OH] + \Sigma(D_i \cdot \beta_i/2.3)\}, \quad (2)$$

where β^{tot} is the total buffering capacity of all buffers (fixed and mobile), β_i and D_i are the buffering capacity and the diffusion coefficient of the mobile buffer (i) as defined in reference 9 and 10. In the physical model underlying Eqs. 1 and 2 it has been tacitly assumed that partitions are homogenous over the narrow (≈5 nm wide) dimension. For the pH this is justified as the titratable protein groups that protrude into this domain are expected (see reference 21) to undergo rapid protonation-deprotonation reactions at a time scale of 100 μs (~pH 7) and as the diffusion path perpendicular to the membrane is extremely narrow. Although a snapshot of any spatial element of a partition will mostly show no free proton, and only seldom one free proton, the rapid flickering of protons on and off the many buffering groups allows to define a pH as a time average.

Eq. 2 predicts that the effective diffusion coefficient, D^{eff}, equals the diffusion coefficient of the proton, D_{H^+}, if the contribution of OH^- to the diffusive relaxation of the pH-jump can be neglected (e.g., at acid pH) and if buffers are absent (i.e., $\beta^{\mathrm{tot}} = 2.3 \cdot [H^+]$). It is also apparent from Eq. 2 that fixed buffers diminish D^{eff} as compared with D_{H^+} or D_{OH^-}.

A previous study with stacked thylakoids (8) was restricted to alkaline pH and without mobile buffers added. In response to a light flash we have observed that the pH in the medium rose slowly, in ~100 ms (see also Figs. 2 and 4). Simulation of the pH-transient by Eq. 1 yielded an effective diffusion coefficient of $D^{\mathrm{eff}} \approx 5 \cdot 10^{-14}$ $m^2 \cdot s^{-1}$ (9). This was 10^5-times smaller than the diffusion coefficient of OH^- in bulk water (D_{OH^-}[bulk] = $5.3 \cdot 10^{-9}$ $m^2 s^{-1}$[13]). The 10^5-fold diminution of D^{eff} over D_{OH^-}(bulk) has been attributed to the factor $2.3 \cdot [OH^-]/\beta^{\mathrm{tot}}$ (see Eq. 2 and reference 9). An upper estimate for the buffering capacity in partitions, β^{tot}, has been obtained by assigning to these domains one half of the total buffering power of the thylakoid suspension. This was calculated from the experimentally determined buffering capacity of a thylakoid suspension, namely 15 μM/pH. With a calculated $\beta^{\mathrm{tot}} \leq 200$ mM/pH (see reference 9), and with $[OH^-] \leq 10^{-8}$ the factor of 10^{-5} between D^{eff} and D_{OH}(bulk) was obtained from Eq. 2. As pointed out in reference 9 the figure of $\beta^{\mathrm{tot}} = 200$ mM/pH was an upper limit of the buffering capacity of partition. As a consequence, the true diffusion coefficient of OH^- in the lateral direction could not have been much greater than in bulk water. If it had been greater there simply was not enough buffer for compensation as to yield the observed relaxation time of 100 ms. Here, we tested the validity of the physical model underlying this conclusion by measuring the dependence of D^{eff} on the medium-pH and on mobile buffers and comparing the results with the predictions of Eqs. 1 and 2.

MATERIALS AND METHODS

Stacked thylakoids were prepared from pea leaves according to standard procedures (detailed in reference 8). Concentrated stock (5 mM chlorophyll; 100 mM sorbitol; 10 mM NaCl; 10 mM Tricine-NaOH, pH 7.8) was stored on ice for up to 3 h. Aliquots were diluted to 10 μM chlorophyll in a medium with 5 mM $MgCl_2$; 10 mM NaCl, and with 200 μM Hexacyanoferrate as electron acceptor. The pH was adjusted by addition of HCl and of NaOH, and pH-indicating dyes were added when indicated in the legends to figures. The sample was filled

into an optical absorption cell (2 cm pathlength) mounted in a flash spectrophotometer (8). Photosynthetic electron transport and proton pumping was stimulated by excitation with short flashes of light (Xenon; 1 μs full width half maximum; wavelength > 610 nm), which saturated reaction centers. Transient absorption changes were recorded at a wavelength tuned to the absorption peak of the respective pH-indicator (Fig. 2 legend). 30 transients were averaged for signal-to-noise improvement. To discriminate the "pH_{out}-indicating absorption changes" of these dyes from a background of transients attributable to other events and to intrinsic pigments, a transient recorded in the absence of added dye was subtracted from transients recorded in its presence (8, 22). As the dyes are hydrophilic, they react specifically to pH-transients in the suspending medium (see reference 9).

RESULTS AND DISCUSSION

Records of flash-light induced pH-transients in the suspending medium were recorded at different pH values as shown in Fig. 2. The rise of the transmitted intensity was caused by proton uptake at the reducing end of photosystem II which is located at the partition side of the thylakoid membrane. As predicted by Eq. 2, the relaxation was slower at neutral than at acid and at alkaline medium-pH. We fitted the measured transients with the theoretically expected time course (according to Eq. 1 theoretical curves not shown, for an example see Fig. 2 of reference 9) and obtained the effective diffusion coefficient. Fig. 3 illustrates the dependence of the effective diffusion coefficient on the medium pH. For the expected behavior, which was calculated according to Eqs. 1 and 2 (see lines in Fig. 3) it was assumed that the diffusion coefficients of H^+ and OH^- were the same as in bulk water $D_{H^+}/D_{OH^-} = 1.76$ (13). It was also assumed that the

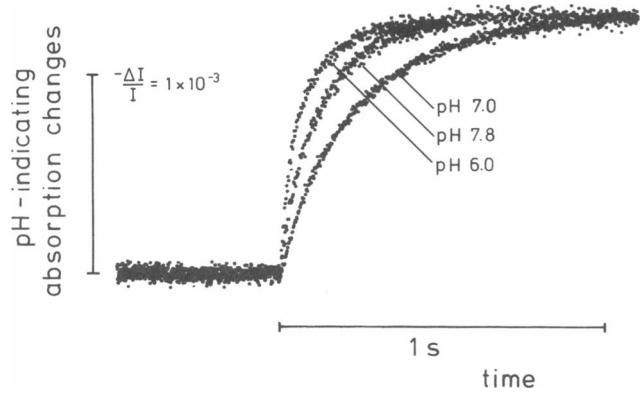

FIGURE 2 Transient alkalization in the suspension medium of stacked thylakoids at pH 6, 7, and 7.8 and induced by light flashes of 1 μs duration. Transient "pH_{out}-indicating absorption changes" of the following indicator dyes: pH 6(bromocresol purple, 20 μM, wavelength 590 nm); pH7 (phenol red, 15 μM, 554 nm), and pH7.8 (cresol red, 15 μM, 575 nm) by a procedure analogous to the one in reference 8. The ordinate scale gives relative changes of transmitted intensity (2 cm optical path).

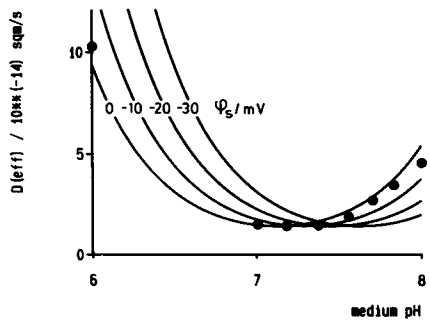

FIGURE 3 The dependence of the effective diffusion coefficient on the medium-pH and on the average surface potential in partitions. *Solid circles* show the experimental values. They resulted from a fit of measured pH-transients as shown in Fig. 2 by Eq. 1. An example for the fit-quality has been given in Fig. 2 of reference 9. Curves representing the theoretically expected behavior were calculated according to Eqs. 2 and 4.

total buffering capacity of the thylakoid suspension was practically constant between pH 6 and pH 8. Different curves were calculated for various values of the average electric surface potential in partitions, Φ_s, which affects the average equilibrium concentration of protons in the partition ($pH[part] = pH[bulk] + F \cdot \Phi_s/2.3 \cdot R \cdot T$).

The experimental results reproduced the predicted minimum near neutral pH. This showed the dominance of H^+-diffusion in the acid and of OH^- diffusion in the alkaline range. The observed curvature was compatible with a rather low average surface potential in the partition region, as suggested by theories on the stability of thylakoid stacking (14, 15).

Eq. 2 predicts that the addition of mobile buffers would increase D^{eff}. It is shown in Fig. 4 that the addition of 80 μM imidazole about doubled the rate of the pH-rise in the medium. At a medium-pH of 7.8 this concentration of imidazole (pK = 7.1) added an additional buffering capacity of 15 μM/pH to the suspension, which halved the extent of the pH-transient (see the two different ordinate-scales in Fig. 4). According to Eq. 2 a mobile buffer was expected to double the rise-rate if $D_i \cdot \beta_i \approx 2.3 \cdot D_{OH^-} \cdot [OH^-]$. Besides imidazole, glycylglycine, and cholamine chloride also accelerated the pH-rise when added at a concentration which doubled the total buffering capacity. With Tricine the same acceleration required much higher concentration. With other supposedly mobile buffers, namely phosphate, *N*-(Acetamido)-2-aminoethanesulfonic acid (ACES), Hepes, Tris, and *N*-Tris(hydroxymethyl)methyl-3-amino-propanesulfonic acid (TAPS) no acceleration was detectable. Whether they increased the rate of the pH-rise at very high concentration was not measurable because they quenched the extent of the pH-transient to below the noise level. At

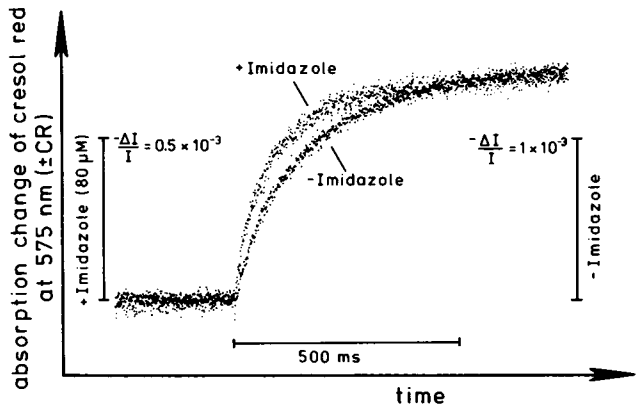

FIGURE 4 Transient alkalization of the suspension medium of stacked thylakoids at pH 7.8 in the absence and in the presence of imidazole, 80 μM. This contributed an additional buffering capacity of 15 μM/pH to the suspension, which decreased the extent of the pH-indicating absorption changes (note different scales). Other conditions as in Fig. 2.

present, we have no convincing explanation for the greater effect of e.g., imidazole over ACES. It should be mentioned, however, that similar differences were observed for the buffering power of various buffers of pH-transients in the very narrow lumen of freshly prepared thylakoids. As documented in Fig. 3 of reference 16 imidazole, glycylglycine and cholamine chloride quenched the extent of pH-transients more effectively than ten other buffers, ranging from pyrophosphate to standard zwitterionic amines.

The above results corroborated the validity of the proposed physical model for the extremely slow relaxation of a pH-jump induced in the partitions between stacked thylakoid membranes. The greater portion of the 10^5-fold diminution of the "effective diffusion coefficient," D^{eff} (operationally defined by Eq. 1), over the diffusion coefficients of H^+ and OH^- in bulk water, D_{H^+} and D_{OH^-}, was attributable to the fixed buffers present in partitions (upper limit of β^{tot}: 200 mM/pH, see reference 9). By inversion of the argument, it followed that the average lateral diffusion coefficients of H^+ and of OH^- in the extremely narrow partitions were smaller or comparable with those in bulk water. They could not be much greater, simply, because there was no titratable buffering capacity (in excess of 200 mM/pH) to compensate an eventual enhancement of proton diffusion.

One might argue that proteins protruding into the partitions narrow the volume for the diffusion of protons and that the diffusion coefficients along the narrow and winding lanes might still be greater than in bulk water. This is beyond the scope of our experimental approach and it does not affect the above conclusion which describes the average behavior of an only 2.5 nm wide layer at the surface of a biological membrane.

CONCLUSION

In the debate over the chemiosmotic theory and more localized concepts of energy coupling it has been proposed that the surface of coupling membranes facilitated the diffusion of protons between pumps and ATP synthases (e.g., 3, 4). This remained merely speculative until Prats et al. studied the diffusion of protons along the surface of a phospholipid monolayer on an aqueous subphase (5, 6). They measured the propagation of a pH-pulse between two aqueous compartments which were separated by a barrier which, in turn, was bridged by the phospholipid monolayer. The authors reported a 20-fold greater diffusion coefficient of the proton at the surface of the monolayer than in bulk water (6) (see exchange of critical arguments in references 7, 17, and 18). They speculated that "this observation strongly supports the semi-localized hypothesis" (6) of energy coupling in photosynthesis and respiration as opposed to Mitchell's delocalized chemiosmotic concept. Our results, however, show that generalization from a fluid phospholipid monolayer to a natural energy transducing membrane with a high protein content and, here, a low proportion of phospholipids, is not warranted. As protons adjacent to the outer side of the thylakoid membrane are, if anything, less mobile than in bulk water, there is no challenge to the original chemiosmotic concept that proton pumps are functionally linked with ATP synthases by aqueous phases.

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