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Review

# Protons @ interfaces: Implications for biological energy conversion

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## Abstract

The review focuses on the anisotropy of proton transfer at the surface of biological membranes. We consider (i) the data from “pulsed” experiments, where light-triggered enzymes capture or eject protons at the membrane surface, (ii) the electrostatic properties of water at charged interfaces, and (iii) the specific structural attributes of proton-translocating enzymes. The pulsed experiments revealed that proton exchange between the membrane surface and the bulk aqueous phase takes as much as about 1 ms, but could be accelerated by added mobile pH-buffers. Since the accelerating capacity of the latter decreased with the increase in their electric charge, it was concluded that the membrane surface is separated from the bulk aqueous phase by a barrier of electrostatic nature. The barrier could arise owing to the water polarization at the negatively charged membrane surface. The barrier height depends linearly on the charge of penetrating ions; for protons, it has been estimated as about 0.12 eV. While the proton exchange between the surface and the bulk aqueous phase is retarded by the interfacial barrier, the proton diffusion along the membrane, between neighboring enzymes, takes only microseconds. The proton spreading over the membrane is facilitated by the hydrogen-bonded networks at the surface. The membrane-buried layers of these networks can eventually serve as a storage/buffer for protons (proton sponges). As the proton equilibration between the surface and the bulk aqueous phase is slower than the lateral proton diffusion between the “sources” and “sinks”, the proton activity at the membrane surface, as sensed by the energy transducing enzymes at steady state, might deviate from that measured in the adjoining water phase. This trait should increase the driving force for ATP synthesis, especially in the case of alkaliphilic bacteria.

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...betrachtet man die wunderbaren Wirkungen der Elektrizität, die oft in geheimen tätig ist, obwohl sie überall verbreitet ist, so kann man nicht umhin, in ihr einen der wirksamsten Antriebe in den großen Verrichtungen der Natur zu sehen.

Theodor von Grotthuß, year 1805 [1]

## 1. Introduction

Two hundred years ago, the 20-year-old Theodor von Grotthuss, while experimenting in Italy with a voltaic pile,

has suggested a mechanism of charge transfer in water in the presence of electric field (see the review of S. Cukierman in this issue and Refs. [1–4]). The importance of the electric field-driven proton transfer for biological energy conversion has been realized much later; it appears that the Grotthuss’ prophecy on the universal significance of electricity, as quoted in the above epigraph, was regrettably overlooked by biochemists.

Forty-five years ago, P. Mitchell [5] and R.J.P. Williams [6,7] have put forward the idea that the ATP synthesis could be driven by the proton concentration gradient between two cellular compartments. They have noted that if proton release (upon substrate oxidation) and proton consumption (upon oxygen reduction) take place in separate sites, then the resulting difference in proton activity could be utilized for ATP synthesis.

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Mitchell has suggested that these separated compartments are the bulk aqueous phases on the opposite sides of a sealed, enzyme-carrying membrane. In the simplest case of a bacterial cell, he anticipated that the energy of substrate oxidation (and/or the energy of light in phototrophic bacteria) is utilized to pump protons out of the cell [5,8]. Thereby the interior of the bacterial cell becomes negatively charged, whereas the outer surface of the cell charges positively, so that one can speak about n- and p-sides of an energy-transducing membrane. The resulting transmembrane difference in the electrochemical potential of hydrogen ions ( $\Delta\tilde{\mu}_{\text{H}^+}$ ) was suggested to drive the energy-consuming enzymes, the ATP synthase in the first place. Mitchell [8] has defined the *protonmotive force* (*pmf*) as:

$$pmf = \Delta\tilde{\mu}_{\text{H}^+}/F = \Delta\psi - (2.3RT/F) \cdot \Delta\text{pH} \quad (1)$$

where  $\Delta\psi$  is the transmembrane difference in electric potential, and  $\Delta\text{pH}$  is the difference between the pH values in the two bulk water phases (*entirely delocalized coupling*).

Williams has considered the difference in the chemical activity of protons confined to different membrane loci (*localized coupling* [6,7,9,10]). In his opinion, unrestricted equilibration of the membrane acidic/alkaline domains with the bulk water would lead to the loss of the stored energy, so that no significant difference in proton concentration could build up. He wrote that “if charge is thrown out into medium, as in osmotic theories, then we face the problem of equilibration of the energy of a single cell on its outer side with the whole of the volume in which it is suspended, say the Pacific Ocean” [9]. Answering this criticism, Mitchell has noted that  $\Delta\text{pH}$  would be still formed because of the alkalization of the cellular interior, even when the external phase is indefinitely large, as in the case of a bacterial cell [11]. The intracellular pH, however, can increase only moderately because the cell enzymes cannot work at extremely high pH. Consider, e.g., the alkaliphilic bacteria, such as *Bacillus firmus*, which, because of the named constrain, have to keep their internal pH by about 3 pH units more acidic than the ambient one (see [12–14] for reviews). As  $|\Delta\psi|$  in these bacteria hardly increases above 200 mV [15], the straightforward application of Eq. (1) yields a *pmf* around zero. In fact, the energetics of alkaliphilic bacteria could be hardly explained by delocalized coupling.

Besides the clear-cut case of alkaliphilic bacteria, numerous other observations, as surveyed in Refs. [16–20], indicated deficiency of the delocalized coupling paradigm. In particular, several authors have reported poor correlation between the measured ATP yields and the *pmf* values, as estimated by applying Eq. (1) to the measured values of the bulk pH and  $\Delta\psi$  (see [16–21] and references therein).

To account for these observations, it has been repeatedly suggested that the measured “bulk” values of  $\Delta\text{pH}$  might not correspond to those sensed by the membrane-embedded “consumers” of *pmf*. One set of hypothetical models (for their surveys see [17–20]), implied a direct interaction between the producers and consumers of *pmf*, with protons directly shuttling between the “sources” and “sinks”. This rationale is referred to as *localized coupling* [9,10], *microchemiosmosis* [22] or *mosaic*

coupling [23]. The other proposal was that the ejected protons can spread freely over the membrane surface, while prevented from a prompt equilibration with the bulk aqueous phase [10,16,21,24,25]. In the latter case, one can speak of *anisotropically delocalized* or *surface-to-surface* coupling. Here, the steady-state pH value at the membrane *surface* ( $\text{pH}^{\text{S}}$ ) can differ from pH in the surrounding *bulk* phase ( $\text{pH}^{\text{B}}$ ). In particular, the  $\text{pH}^{\text{S}}$  value at the outer *p*-surface of metabolizing bacteria could then stay lower than  $\text{pH}^{\text{B}}$ . Hence, a retardation in the surface-to-bulk proton equilibration could result in a reasonable *pmf* even in respiring alkaliphilic bacteria [16,17,19,26].

It is noteworthy that the above noted experimental data on the deficiency of delocalized coupling have not been refuted. As well, the energetics of alkaliphilic bacteria has remained unclear. Still, the fierce discussions of 70-s and 80-s (see e.g., [11,27–29]) have gradually faded out and the mechanism of entirely delocalized coupling has found its place in the biochemistry textbooks. This development was due to (i) the poor understanding of events at the membrane/water interface and (ii) the absence of physically plausible mechanisms that could explain how a prompt proton equilibration between the membrane surface and the bulk aqueous phase can be hindered.

The last two decades have seen substantial progress in these two fields. The reactions of proton transfer were experimentally addressed in many membrane enzymes (see Ref. [30] for a comprehensive review). In particular, the reactions of proton ejection and/or proton binding were studied in bacteriorhodopsin (BR) [31–38], bacterial photosynthetic reaction center (RC) [39–43], photosystem II (PSII) [44–48], the cytochrome *bc*<sub>1</sub> complex [49–52], diverse oxidases [53–60], nitric oxide reductase [61], and ATP synthase [62–65]. Triggering of enzymes by short flashes of light made it possible to monitor proton displacements not only inside the membrane, but also across the membrane/water interface. Diverse studies have repeatedly shown that the spreading of protons along the membrane surface occurred much faster than their equilibration with the bulk aqueous phase (see Refs. [37,66,67] for surveys and Refs. [31,34–36,41,68–78] for experimental evidence). As well, the electrostatic nature of the interfacial kinetic barrier for ions could be elucidated [66,67,79–81].

In this review, we consider the proton dynamics at the surface of biological membranes. First, we survey the data from pulsed experiments, where light-triggered enzymes captured or ejected protons at the membrane surface. Then, we explicate the electrostatic properties of water at charged interfaces. Finally, we consider the structural attributes that facilitate the fast proton transfer along electrically charged membranes and, eventually, can serve for transient proton storage. Based on such a concurrent consideration, we suggest a following solution of the above outlined bioenergetic conundrum. Owing to the interfacial potential barrier of electrostatic origin, the equilibration of protons between the surface and the bulk water occurs slower than their spreading over the membrane. Then, at steady state, proton activity at the membrane surface might deviate from that in the bulk aqueous phase. Hence, in vivo, the pH at the outer surface of bacterial cells can be more acidic than that in

the surrounding medium. In that case, the surface-to-surface *pmf*, which drives the ATP synthase and other *pmf* consumers, can be larger than that estimated from the measured values of  $\Delta\psi$  and the “bulk” pH.

In relation to biological energy conversion, we focus here on the interplay between the retarded proton transfer across the membrane/water interface and the prompt proton spreading at the membrane surface. The physical properties of the surface water layers and the biological implications beyond bioenergetics are in the focus of another review [82], which is complementary to this one.

Last but not least, 45 years ago, when Mitchell and Williams published their seminal articles, the authors of this review were just born. So we missed, quite regrettably, the *Sturm und Drang* period in the history of bioenergetics. Because of this misfortune, our view is, perhaps, less biased than that of warriors who crossed their swords many years ago. This is the reason why we have dared, being guided by Theodor von Grothuss, to visit the historic battlefield and to take a new look on some of the artifacts abandoned there.

Wenn wir uns ein Atom Wasser so (+ -) versinnlichen, und mit - den Sauerstoff, mit + hingegen den Wasserstoff bezeichnen, so werden wahrscheinlich die Elemente eines zweiten, dem ersten beigefügten Atomes, sich nach polarelektrischen Gesetzen, nämlich so  $\pm \mp$ , ordnen. Dies ist aber gerade die Stellung, in welcher ein immerwährender wechselseitiger Austausch der Elementarteile des Wassers stattfinden kann...

Theodor von Grothuß, year 1819 [3]

## 2. Proton dynamics in bulk water

Protons move in water very fast. They are not carried by diffusing water ions but propagate according to a mechanism that has been first outlined by Grothuss in 1805. The insightful idea of Grothuss was that the water molecules instantly exchange their charged “parts” in a kind of relay (see Refs. [1,3] and the review of S. Cukierman in this issue). In current terms, the mechanism of proton diffusion in water can be described as follows [83–88]. The neutral water molecules are predominately tetrahedral and are involved in four hydrogen bonds (HBs) each (the perceptive Grothuss’ view on hydrogen bonding of water molecules is given in the epigraph). Unlike neutral water molecules, the solvated  $\text{H}_3\text{O}^+$  cation is a nearly plan-trigonal complex disallowing H-bonding to the oxygen atom. Together with the three water molecules in the first coordination shell this complex forms the ‘Eigen cation’  $\text{H}_9\text{O}_4^+$ , as shown in Fig. 1A. The H-bonds in this complex are rather strong (the O–O distances of 2.5 Å), so that the excess proton is highly stabilized. The proton transfer is controlled by the thermal cleavage of a HB between the first and second solvation shell. The cleavage reduces the coordination number of a water molecule in the first coordination shell. The breakage of one HB in the second solvation shell is accompanied by the shortening of the respective HB in the first solvation shell up to approximately 2.4 Å and by interconversion of  $\text{H}_9\text{O}_4^+$  into a

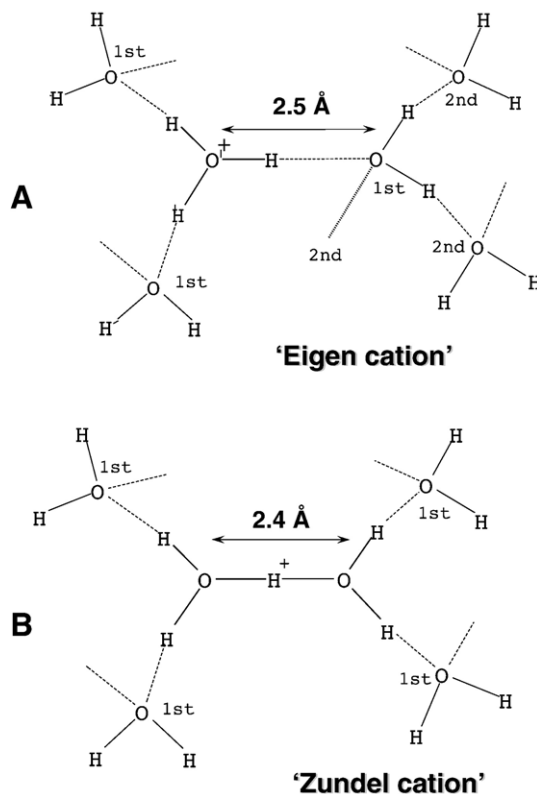


Fig. 1. Structures of the ‘Eigen cation’ (A) and of the ‘Zundel cation’ (B). See the text for further details and references.

strongly H-bonded, centrosymmetric  $\text{H}_5\text{O}_2^+$  complex (‘Zundel cation’ see Fig. 1B). The actual proton motion here is much faster than the solvent reorganization (in accordance with the insightful prediction of Grothuss), so that the mobility of the proton is determined by the rate of HB cleavage. The cleavage takes about 1 ps at room temperature and requires activation energy of 8–12 kJ/mol; the latter value corresponds precisely to the observable activation energy of proton motion in water (see Refs. [85–89] for further details).

Licht trennt die Bestandteile vieler ponderablen Verbindungen voneinander und zwingt sie neue Verbindungen mit seinen eigenen imponderablen Elementen (+E und -E) einzugehen, gerade wie es die Pole der Voltaschen Batterie, nur in einem höheren Grade, zu tun vermögen.

Theodor von Grothuß, year 1819 [3]

## 3. Retarded proton transfer across the membrane/water interface

As the proton mobility in water is high, the proton activity at the membrane surface can differ from that in the bulk water only if the proton equilibration is somehow impeded. The evidence of such retardation was obtained in “pulsed” experiments where short light flashes were used to trigger protolytic reactions in membrane photosynthetic enzymes. Here it seems appropriate to acknowledge that we owe this experimental setup to

Grotthuss. Besides coining the first law of photochemistry<sup>1</sup> in 1817 (see e.g., [3]), Grotthuss has provided us with the first description of a light-driven separation of electric charges, as documented by the epigraph to this section. Turning to protons at interface, the first experimental indications of their retardation were obtained when the flash-induced reduction and protonation of the secondary quinone acceptor ( $Q_B$ ) was monitored in diverse photosynthetic RCs (see Fig. 2A for the experiment scheme, and Ref. [90] for the survey of the earlier data). It was found that proton disappearance from the bulk aqueous phase, as reported by hydrophilic pH-indicators, was delayed as compared to the estimated time of  $Q_B$  reduction, both in the RCs of purple phototrophic bacteria [91–95] and in PSII of green plants [44]. These experiments, however, could not discriminate whether protons were impeded (i) on their way from the bulk water to the membrane surface or (ii) during their penetration through the protein towards the buried  $Q_B$  molecule.

This ambiguity has been clarified by Drachev and co-workers [31] who studied the flash-induced proton transfer by bacteriorhodopsin (BR) sheets (see Fig. 2B). These authors have followed not only the spectral changes (i) of BR proper and (ii) of the pH-indicator *p*-nitrophenol in the solution, but also have used capacitive electrometry to trace (iii) the movement of a proton from the buried retinal cofactor to the membrane surface. It was found that the proton delivery to the surface followed the formation of the M intermediate of the BR photocycle, whereas the protonation of the water-dissolved pH-indicator was distinctly retarded. The protonation of the pH-indicator could be accelerated by added hydrophilic pH-buffers. These observations showed that the kinetic barrier passes, quite paradoxically, not through the protein moiety, but through water, between the membrane surface and the bulk aqueous phase [31].

Heberle, Dencher and their co-authors have studied the same reaction of flash-induced proton release from BR by using two judiciously placed pH-indicators, namely fluorescein (Fluo), which was covalently bound to the surface, and pyranine (Pyr) that was dissolved in the solution (see Fig. 2B for the experimental setup). These authors have found that fluorescein was protonated at <0.1 ms, concomitant with the formation of the M-state, whereas pyranine was protonated much slower, at ~0.8 ms [33–35,71]. The delayed proton transfer from the BR surface into the bulk aqueous phase was thereafter confirmed in several other labs [36,75,78,96].

Proton transfer in the opposite direction, from the bulk water phase into the protein, was tracked with chromatophores—vesicular photosynthetic ensembles of purple phototrophic bacteria *Rhodobacter sphaeroides* and *Rhodobacter capsulatus*. It was found that proton transfer from the surface to  $Q_B$ , as traced by electrochromic absorption changes, followed the reduction of  $Q_B$  at ~0.1 ms, whereas the response of diverse pH-indicators in the solution was retarded up to 0.5–1 ms [41]. These data indicate that the kinetic barrier between the surface

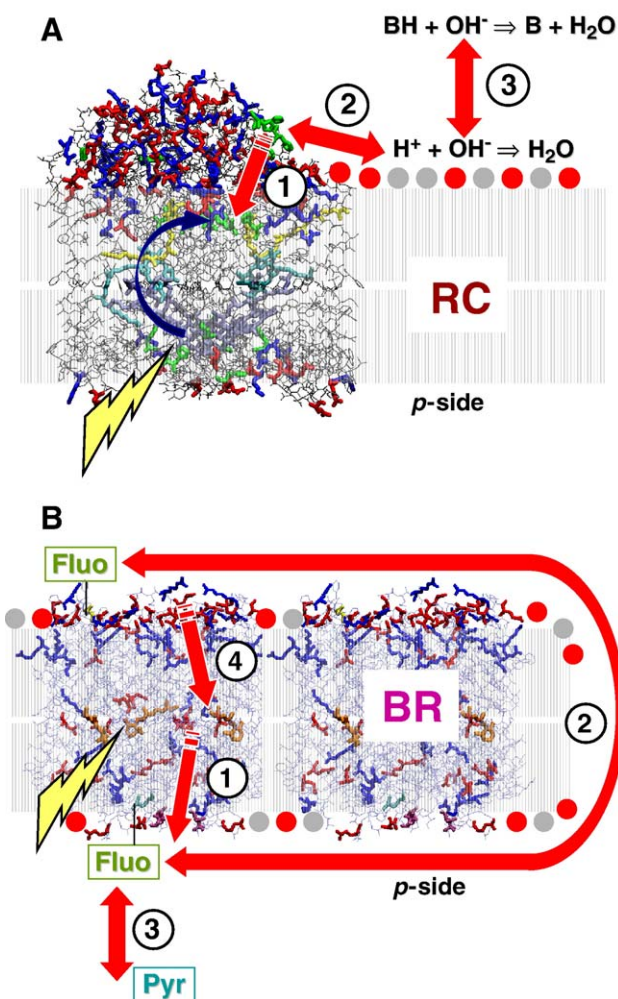


Fig. 2. Partial steps of proton exchange between the membrane enzymes and the bulk aqueous phase. The numbers indicate the sequence of proton transfer steps that are shown by thick red arrows. General color code: acidic residues (Asp and Glu) are shown by red, basic residues (Arg and Lys) by blue, histidine residues by green. The red and gray circles show schematically anionic and neutral lipid headgroups, respectively. (A) Proton trapping from the bulk aqueous phase by the RC of *Rb. sphaeroides* (the crystal structure from Ref. [160], PDB entry 1AIJ, is shown). Thin blue arrow, electron transfer to the  $Q_B$  ubiquinone. BH/B, protonated/deprotonated molecules of hydrophilic mobile pH-buffer, respectively. Color code: ubiquinone is shown by yellow, bacteriochlorophyll by ice-blue, bacteriopheophytin by cyan. (B) Proton transfer steps in BR (two identical crystal structures of BR trimers as described in Ref. [164], PDB entry 1BRR, are depicted). Fluo, fluorescein, Pyr, pyranine. Color code: retinal is shown by orange, the acidic groups of archaeobacterial glycolipids by mauve, Lys-129 is marked by cyan, Asp-36 is shown in yellow (see the text for further details). The figure was produced by using the VMD software package [213].

and the bulk aqueous phase is present also on the *n*-side of the coupling membrane.

The slow rate of proton equilibration has been initially attributed to the immobile pH buffers at the surface, i.e., to the ionizable lipid and protein groups that are able to retain protons [34,69,97–101]. Heberle and co-workers have shown that the replacement of particular amino acid residues at the surface of BR membranes affects the kinetics of proton uptake by BR [102]. These data indicated that the surface exposed amino acid residues were involved in the efficient collection of protons and,

<sup>1</sup> “Light must be absorbed by a chemical substance in order for a photochemical reaction to take place”.

correspondingly, could prevent proton escape into the bulk water. Numerous studies with diverse membrane preparations showed that by modifying the surface buffer groups it was possible to affect the proton dynamics, as monitored by pH-indicators, either covalently attached or nonspecifically bound/adsorbed at the surface (see Ref. [103] for a comprehensive review and Refs. [75,104–110] for representative data).

However, if the surface pH-buffers were *alone* responsible for the proton retardation, then the mobile, non-adsorbing pH-buffers or pH-indicators were expected to accelerate proton equilibration with the bulk aqueous phase if added at concentrations of  $>1\text{--}5\ \mu\text{M}$ , i.e. when they could kinetically compete with free protons at neutral pH (see discussion in Refs. [41,66,98]). As a rule, this was not the case. Only the monoanionic species accelerated the proton equilibration already at concentrations of  $\geq 25\ \mu\text{M}$  [31,78]. The di-anions such as phosphate and bromocresol purple were efficient only when added at  $>100\ \mu\text{M}$  [41,71,95,111]. Pyranine, which carries four negative charges, did not accelerate the proton exchange [71,78,99]. Apparently, the ability of mobile pH-buffers to accelerate proton equilibration depended on their electric charge. This finding has prompted a suggestion that the proton exchange between the surface and the bulk is retarded not just by the immobile pH-buffers but also by an interfacial potential barrier of electrostatic nature [66,67,79,80].

As argued in Refs. [67,80–82] and as discussed in more detail in Section 4 below, the interfacial barrier is just one of emanations of specific properties of water at electrically charged surfaces and might have complex physical nature. Still, some properties of the interfacial barrier could be inferred from experimental data. In particular, proton transfer across the barrier showed weak pH-dependence and high activation energy of 30–50 kJ/mol [41,71,76,95]. As argued in Ref. [41], both features point on participation of neutral water in proton transfer across the interface. Indeed, a direct collisional interaction of *mobile* pH-buffers, as coming from the bulk solution, with the *newborn* protons (or proton holes) at the surface should have low activation energy of  $\leq 10\ \text{kJ/mol}$ , typical for diffusion-controlled reactions. On the other hand, the observed high activation energy of 30–50 kJ/mol is characteristic for the protonation/deprotonation of neutral water. Apparently, the charged molecules of mobile pH-buffers fail, because of the interfacial potential barrier, to reach the newborn surface protons/proton vacancies before the latter interact with molecules of neutral water [41]. Hence, one gets a two-step mechanism: first a newborn charge at the surface interacts, in a reaction with high  $E_a$ , with neutral water molecules yielding either  $\text{H}_3\text{O}^+$  (upon proton release) or  $\text{OH}^-$  (upon proton binding), and only then these charged water species diffuse into the bulk (as depicted in Fig. 2A).

In a further attempt to reveal the properties of the interfacial barrier, it was analyzed, by solving a system of diffusion equations and by comparing the solution with the experimental data, which factors determine the rate of the pulsed protonic relaxation at the membrane/water interface of spherical vesicles in the presence of a potential barrier for ions. The modeling has shown that the rate of proton exchange with the bulk water is

determined by (i) the pH-buffering capacity of the surface, (ii) the height of the potential barrier, and (iii) the vesicle size [66]. The calculated dependence on the vesicle size corroborated quantitatively the experimental data. Several authors [68–70] have shown that protons were ejected by the cytochrome  $bc_1$  complex to the  $p$ -surface of cells and spheroplasts of purple phototrophic bacteria *Rb. sphaeroides* and *Rb. capsulatus* at  $\tau < 5\ \text{ms}$ , as followed by electrochromic shift of carotenoid pigments (which correlate with the absorbance changes of an amphiphilic, membrane-bound pH indicator neutral red [51]). These protons, however, were sensed by hydrophilic pH-indicators in the bulk water phase only at 30–70 ms [68–70]. In the case of right side-out vesicles with the diameter of about 100 nm, which were obtained by disruption of the spheroplasts, the same reaction proceeded at 4 ms [70]. In the latter case, the proton release rate was apparently limited by the turnover of the cytochrome  $bc_1$  complex, so that the genuine rate of protonic relaxation could be even faster. Hence, the proton retardation was stronger with whole cells or spheroplasts than with smaller vesicles. As well, the extent of proton retardation was larger with the BR sheets, as compared with smaller BR-containing phospholipid micelles [75].

As noted above, the mobile pH-buffers could accelerate the protonic equilibration between the membrane surface and the bulk aqueous phase. The kinetic modeling has revealed that acceleration is expected once the concentration of the pH-buffer exceeds a certain “threshold” [66]. The “threshold” value depends on the barrier height but is independent both of the vesicle size and of the surface buffering capacity. This feature helped to “extract”, from the experimental data, the height of the barrier, as “sensed” by different penetrating ions. As it is shown in Fig. 3, the barrier depends almost linearly on the electric charge of the penetrating ion and varies between 0.09 eV for  $p$ -nitrophenol and MES (with charge of  $-1$ ) and more than

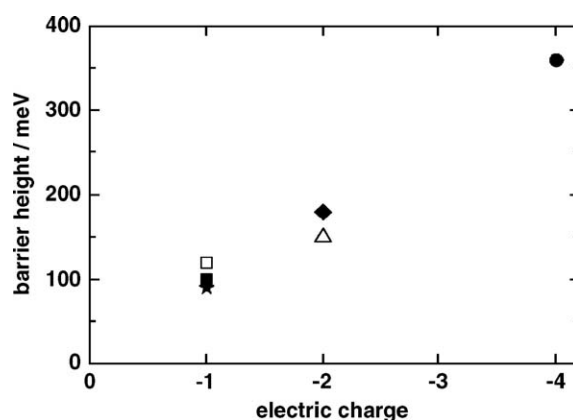


Fig. 3. The magnitudes of the interfacial barrier for five anionic pH buffers/indicators as function of their electric net charge in the deprotonated state. The values of barrier height were inferred from analysis of data on acceleration of protonic equilibration by added pH-buffers/pH-indicators in BR sheets and chromatophore membranes (see Ref. [66] for further details). Symbol code: stars,  $p$ -nitrophenol (BR membranes [31]); solid squares, MES (BR membranes [31]); open squares, MES (chromatophores of *Rb. sphaeroides* [41]); triangles, phosphate (BR membranes [32]); diamonds, bromocresol purple (chromatophores of *Rb. sphaeroides* [41]); solid circles, pyranine (BR membranes [71,78]). The figure corresponds to Fig. 6 of Ref. [66].

0.36 eV for pyranine (with charge of  $-4$ ). The barrier height for protons proper was found to be about 0.12 eV [66].

The steady state situation at the surface of a bacterial cell was modeled by solving the Smoluchowski equation for protons spreading away from proton “pumps” at the surface [79,80]. At typical pump turnover rates, a potential barrier of 0.12 eV could yield a steady-state surface  $\text{pH}^S$  of approximately 5.5–6.5, depending on the number of proton pumps involved (see Fig. 4). It was found that the contribution of ejected protons was essential at the surface but decayed promptly beyond the barrier, provided that the bulk water phase served as an infinite sink for the ejected protons (see Fig. 4). As a result, the total concentration of protons in the bulk remained close to their equilibrium concentration, whereas the concentration of protons at the surface might substantially differ from the equilibrium value, especially at alkaline and neutral conditions. In other words, the value of  $\text{pH}^S$  was independent of pH in the bulk aqueous phase [79,80]. This feature might help to understand the bioenergetics of alkaliphilic bacteria: the pH value at the surface of living cells, which is sensed by the membrane enzymes, could be much lower than in the surrounding medium. It is noteworthy that the surface buffering capacity does not matter at steady state [97], so that the surface proton activity (concentration) is determined by the height of the interfacial barrier and by the size of bacteria.

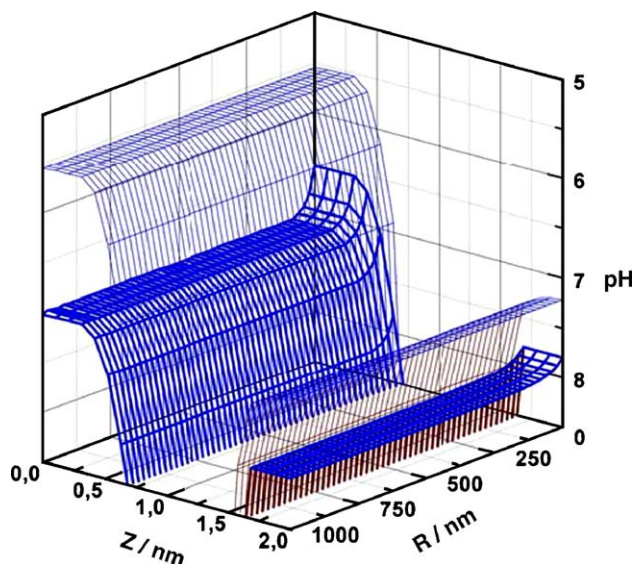


Fig. 4. The steady-state pH at a negatively charged membrane/water interface as resulting from the operation of a single proton pump (thick lines) or of a proton pump ensemble (thin lines). The figure is taken from a poster corresponding to Ref. [79]; the two-dimensional sections of same dependence are depicted as Fig. 2A and 2B in Ref. [80]; this article can be consulted for further details. The cylindrical axis  $z$  is perpendicular to the membrane plane, the axis  $r$  is directed along the membrane (note the different scales of the axes). The total pH generated by an ensemble of proton pumps with the surface density of  $2 \times 10^{11} \text{ cm}^{-2}$  (thin lines) was obtained by integration over a circle area with the radius of 1000 nm. The turnover rate of the pumps was  $5 \times 10^2 \text{ s}^{-1}$ , the height of the potential barrier was 0.12 eV, the surface potential was  $-0.06 \text{ V}$ , the diffusion coefficient of protons was  $10^{-5} \text{ cm}^2/\text{s}$  in the interfacial layer ( $z < 1.6 \text{ nm}$ ) and  $10^{-4} \text{ cm}^2/\text{s}$  in the bulk.

It is possible to say that in vivo (i) the interfacial potential barrier for protons and (ii) the relatively large size of bacterial cells do act synergetically in slowing the time constant of proton exchange between the surface and the bulk aqueous phase and keeping thus the proton concentration at the outer bacterial surface higher than in the surrounding medium.

Wenn wir z.B. das Kochsalz... im trockenem Zustande als Chlorinatronium betrachtet, und durch-die Chlorin, durch + Natronium bezeichnen, so wird ein Atom dieses Salzes mit einem Wasseratom sich auf folgende Weise zusammenstellen, dadurch aber entsteht wieder, wie schon erwähnt wurde, eine kreisförmige galvanische Molekularbewegung...

Theodor von Grothuß, year 1819 [3]

#### 4. Physical nature of the interfacial barrier

The negative charge of biological membranes imposes constraints on the mobility of the surface water molecules, so that one can say that the surface water is polarized. Then, however, the ability of the surface water to re-arrange in response to a probe charge should be diminished. In terms of ordinary electrostatics this means lower dielectric permittivity ( $\epsilon$ ) of the interfacial water [79,80]. It is well known that the  $\epsilon$  value of the first hydrating layer of water molecules at a charged surface is on the order of 4–6 [112], so that one can speak about dielectric saturation. The dielectric permittivity at the surface of lipid bilayers has been reported to be on the order of 10–30 [113,114]. Teschke and co-workers have determined the dielectric profile of water at the negatively charged mica surface by atomic force microscopy (AFM). They have measured the electrostatic immersion of highly polar silicon nitride and cobalt-coated tips and have calculated how the  $\epsilon$  value changed from 6 at the surface to 80 at the distance of 10 nm [115]. The importance of surface charge for water polarization follows from the observations of Ishino and co-workers that the negatively charged silicon nitride tips were attracted at small separations both to the positively and negatively charged Langmuir–Blodgett monolayers ( $-\text{NH}_2$  and  $-\text{COOH}$  functional groups), but not to the neutral stearyl amide ( $-\text{CONH}_2$ ) and stearyl alcohol ( $-\text{OH}$ ) monolayers [116]. The observed dependence on the surface charge is likely to be caused by the ordering of molecules in a nanoscopic water layer both at the positively and negatively charged surfaces. In the case of a neutral surface, the ordering is likely to be restricted to the first layer of water molecules.

The surface charge density of biological membranes is compatible to that of mica (both vary in a range of  $-0.001/-0.05 \text{ C m}^{-2}$ , see Ref. [80] and references cited therein). The AFM experiments [115] were, however, performed at low ionic strength, either in pure water or in the presence of 1 mM of various salts. The  $\epsilon$  profiles, as obtained in these experiments, could be still extrapolated to higher, biologically relevant ionic strengths [80]. At ionic strength corresponding to 0.1 M of monovalent salt, the calculated  $\epsilon$  value was diminished throughout first 1–2 nm of surface water (see Fig. 5). In this

stratum of pre-polarized water, the energy of an ion is higher than in the bulk aqueous phase. However, at the surface proper, because of numerous ion-binding groups (see the next section), the energy of ion should decrease yielding a potential minimum. The interplay between the decreased  $\epsilon$  of the surface water and the chemical affinity of the surface to ions results in a potential barrier some  $\leq 1$  nm from the membrane surface (see Fig. 5). The height of the barrier should depend on the charge of the probe ion. For monovalent cations the height of the barrier was estimated as 0.1–0.2 eV [80], compatible to the estimates as obtained from the analysis of experimental data (see the previous section and Ref. [66]).

More rigorously the electrostatics of surface water could be treated in the framework of nonlocal electrostatics [81]. The latter approach accounts for strong spatial and orientational correlations between water molecules (see e.g., [117]). The intermolecular correlations should cause extensive water structuring near the charged surface. In other words, the interfacial water tends to form layered structures, which were revealed by AFM [118,119] and by X-ray reflectivity [120]. Another emanation of the water layering, as first elegantly demonstrated by Israelachvili and Pashley, is the appearance of force oscillations in electrolyte solutions squeezed in nanoscopic films [121,122]. The origin of oscillations, which depended on salt concentration and which were taken as evidence for the high organization of interfacial water [123–

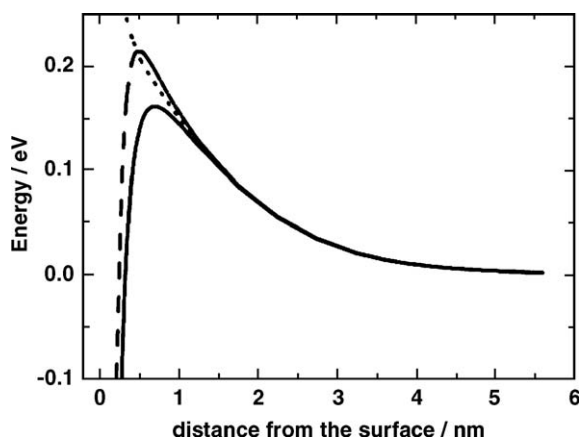


Fig. 5. Dielectric saturation at the membrane/water interface. The potential energy profiles of a monovalent cation (solid line) and of a monovalent anion (dashed line) at a charged membrane/water interface are plotted. The respective desolvation energy profile is shown by a dotted line. The electrostatic energy of a charged spherical particle with radius  $a$  near the membrane/water interface has two main contributions: (i) the electrostatic interaction with discrete charges at the membrane surface (they could be both negative and positive) and (ii) the Born desolvation penalty. Both contributions were calculated by numeric integration of the linearized Poisson–Boltzmann equation  $\nabla(\epsilon \nabla \phi) = -4\pi\rho + 4\pi \sum_i C_i q_i^2 \phi / k_B T$ . The discrete negative and positive charges at the membrane surface were represented by two periodic square lattices with the space intervals of 0.8 and 0.857 nm, respectively, the total charge density of  $-0.032 \text{ C m}^{-2}$  was assumed equal to the charge density at the surface of chromatophore vesicles from *Rb. sphaeroides* [214]. Discrete charges were approximated by spheres of radius of 0.25 nm with the uniformly distributed charge density  $\rho$ . The calculations were performed for the ionic strength of 0.1 M. Numeric integration was performed by the program MUDPACK [215]. The figure corresponds to Fig. 1B from Ref. [80].

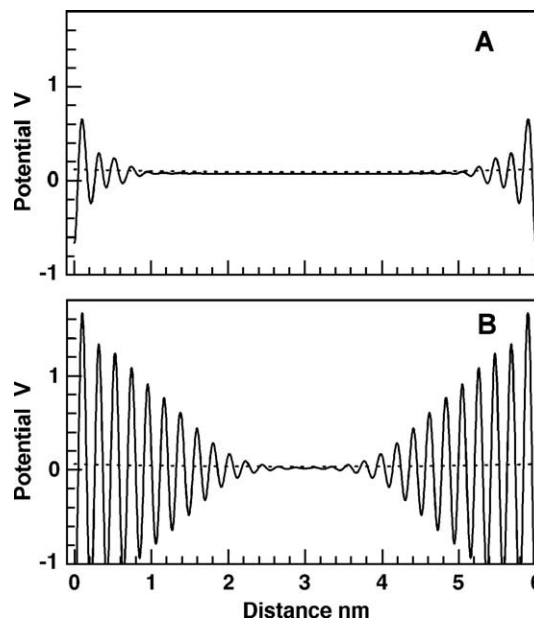


Fig. 6. The oscillations of electrostatic potential in an electrolyte solution confined between two charged parallel surfaces. The potential in a slab of thickness  $L=6$  nm was calculated by the nonlinear integrodifferential Poisson–Boltzmann equation either by using the nonlocal dielectric function as described in Ref. [81] (solid lines) or by using the static dielectric permittivity  $\epsilon=78$  (dotted lines). Curves in (A) and (B) were obtained at ionic strengths of  $10^{-3}$  and  $10^{-2}$  M, respectively. The picture corresponds to Fig. 3 from Ref. [81]. The latter article can be consulted for further details and the parameter values.

[28], has remained elusive so far. The consistent theoretical description of oscillations and of some other anomalies of interfacial water could be achieved by using nonlocal dielectric function of isotropic water [81]. The nonlocal electrostatics tackles the dielectric function with spatial dispersion. Namely, the theory accounts for the dependence of the dielectric displacement  $\vec{D}(\mathbf{r})$  at a given point  $\mathbf{r}$  on the electric field  $\vec{E}(\mathbf{r}')$  in the whole volume  $V$  of the dielectric medium via the nonlocal dielectric function  $\epsilon(\mathbf{r}, \mathbf{r}')$ :  $\vec{D}(\mathbf{r}) = \int_V \epsilon(\mathbf{r}, \mathbf{r}') \vec{E}(\mathbf{r}') d^3 r'$ . The Fourier transform of this function,  $\epsilon(k)$ , has a negative sign at some  $k$  values that means overscreening [129,130]. The molecular base of overscreening is the coupling between spatial and orientational correlations in liquid water. In an electrolyte solution the redistribution of mobile ions<sup>2</sup> exerts a positive feedback on the overscreening response of water that could cause potential oscillations in the vicinity of a charged surface [81]. This effect is analogous to the appearance of spin density waves in ferromagnetic systems and the charge density waves in plasma and high-temperature superconductors [81,131,132].

By using the nonlocal dielectric function of isotropic water, the force oscillations in a squeezed electrolyte were calculated in Ref. [81] for different electrolyte ionic strengths. This calculated behavior reproduced the experimentally measured

<sup>2</sup> The insightful guess of Grotthuss on the mechanism of electrostatic screening by salt ions, as documented by the epigraph to this chapter, deserves our acknowledgement.

dependence on ionic strength, with maximal oscillations appearing at a moderate ionic strength of  $10^{-3}$  M [121,122]. Relevantly to topic of this review, the model of surface water, as based on non-local electrostatics, predicts that a positive feedback on the overscreening response of water can cause resonant oscillations of the electrostatic potential in the vicinity of a charged surface (see Fig. 6). The height of the kinetic barrier, as caused by the potential oscillations, should depend linearly on the charge of penetrating ion [81]. At the non-ideal surface of biological membranes, the oscillations should dampen out. The linear dependence on the charge of penetrating ion is, however, still observed (see the previous section and Fig. 3).

Die Voltasche Säule besteht aus ... gehörig geordneten Wassermolekularsäulen, deren Wirkung durch die Anordnung an den Enden der Linien (den Polen) gesteigert wird.

Theodor von Grotthuß, year 1819 [3]

...verhält sich diese höchst dünne ... eingeengte Wasserschicht ganz wie ein fester Leiter oder wie ein edles Metall. Stimmt diese Tatsache... mit der... Hypothese überein, daß in dem flüssigen Wasser ein beständiger molekularpolarischer Austausch in sich selbst stattfindet, ... denn es ergibt sich daraus offenbar, daß wenn man die stete molekularpolarische Bewegung der Elemente des Wassers verhindert, wie in der eingeengten Wasserschicht, das Wasser alsdann aufhört, sich wie ein zersetzbarer Leiter... zu verhalten und die Eigenschaften eines unzersetzbares Leiters... annimmt.

Theodor von Grotthuß, year 1820 [4]

## 5. Lateral proton transfer along proton sponges and the feedback control of proton pumps

Beginning from the 60-s, the problem of lateral transfer of  $\Delta\tilde{\mu}_{\text{H}^+}$  along the biological membranes was continuously addressed (see [133] and references cited therein). As the rate of  $\Delta\psi$  transfer along the membrane should be fast, the overall rate of  $\Delta\tilde{\mu}_{\text{H}^+}$  propagation is determined by the rate of lateral proton spreading at the membrane surface. With various native and artificial surfaces, this rate has been estimated by diverse techniques yielding quite different values [134–142].

In the bioenergetic context, however, not the speed of the lateral proton spreading *per se* is important, but the ratio between the rates of proton transfer along the membrane and across the membrane/water interface. Here again, the pulsed experiments with BR-containing membranes were useful in providing quantitative information [35–37]. The experimental scenario from Ref. [35] is illustrated in Fig. 2B. The pH indicator fluorescein was covalently bound either to Lys-129 at the *p*-surface (shown by cyan in Fig. 2B) or to Cys-36 at the *n*-surface (marked by yellow in Fig. 2B). After a pulsed light excitation of BR, a proton was released to the *p*-surface at  $\sim 100$   $\mu\text{s}$ . Although the size of the BR-sheets was pretty large, on the order of 1  $\mu\text{M}$ , the fluorescein at the *n*-surface got this proton, after its lateral transfer around the edge of the purple membrane, at  $\sim 200$   $\mu\text{s}$ , i.e. faster than pyranine in the bulk

aqueous phase ( $\sim 800$   $\mu\text{s}$ , see Section 3). In analogous experiments of Alexiev and co-workers the rate of lateral proton transfer was even faster [36]. Serowy and co-workers “launched” light-triggered “caged” protons from lipid-soluble carriers used to imitate membrane enzymes. In this case as well, protons were promptly transferred along the surface with a diffusion coefficient of  $5.8 \times 10^{-5}$   $\text{cm}^2 \text{s}^{-1}$ , only two times smaller than in the bulk water [143].

The measurements of charge fluxes along surfaces of Langmuir monolayers and adsorbed films helped to clarify the mechanism of lateral proton transfer [137,139–142]. The Langmuir monolayers enable to control, in a quantitative way, several factors: the surface conductance, the monolayer composition, the surface charge density, the surface pressure, and the surface electrostatic potential. Upon studies of fatty acid films [139,142], assemblies of acidic DL- $\alpha$ -phosphatidyl-L-serine dipalmitoyl [140,141], and anionic polymers [144] it has been shown that the surface proton conductance increased sharply when the edge-to-edge distance between the neighboring anionic groups approached  $\sim 7$  Å. This effect was not observed with DL- $\alpha$ -phosphatidylcholine dipalmitoyl that lack a terminal acidic group [141]. Generally, the observations that the rates of lateral proton transfer, as observed in the same set-ups, differed depending on the nature of the lipid headgroup (see, e.g., [137,141]) are of key importance because they put limit on the contribution from proton transfer through the bulk water phase.

The increase in surface conductivity was accompanied by a sharp rise in surface electrostatic potential [139,142]. Apparently, at high density of surface anionic groups the effective dielectric constant ( $\epsilon_{\text{eff}}$ ) of water at the surface of monolayer decreased up to 6–7. Oliveira Jr. and co-workers [139,142] have suggested that at distances below the critical one of  $\sim 7$  Å, the water molecules get the opportunity to link up two neighboring acid groups, as shown in Fig. 7A. The authors have speculated that this structural feature leads to the increase in the strength of H-bonds and, as a consequence, to the more efficient/fast surface conductance.

To understand this rationale, it is useful to consider the energetics of proton transfer. Fig. 7B shows a double-well potential where the proton is transferred from a donor (left well in Fig. 5B) to the acceptor (right well in Fig. 7B). The efficiency of proton transfer is determined by the potential levels (free energy) of the donor and the acceptor, respectively (see [145,146] and references therein). The key for fast proton transfer lies, however, in the height of the intermittent barrier. For proton transfer in bulk water, as scrutinized in Section 2, the double-well potential is symmetrical and the barrier is low. In a heterogeneous proton-conducting chain, as at the surface of a membrane or a monolayer, the proton transfer barriers are expected to be higher because of intrinsic asymmetry of the system. As well, the experimentally demonstrated immobilization of water molecules in the first hydration layer (see e.g., Ref. [147]) might additionally increase the proton transfer barriers. Not surprisingly, in model systems the surface proton conductance was by orders of magnitude slower than in the bulk water—unless the distance between the acidic groups at



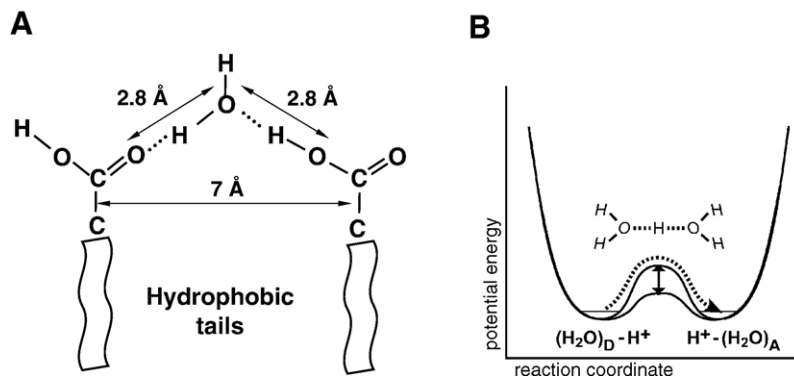


Fig. 7. Hydrogen bonds. (A) Geometrical arrangement of water and acidic groups, as needed to facilitate the proton transfer along the surface (as suggested by Leite et al. in Ref. [139], from which the figure has been redrawn). (B) Double-well potential showing the energy barrier to overcome for classical proton transfer (dashed line) from the donor molecule (left well) to the acceptor molecule (right well). Lowering the barrier (vertical arrow) accelerates proton transfer. The figure is taken from Ref. [146].

the surface reached the critical value of 7 Å [139–142]. The later observation testifies that proton transfer barriers can be lowered: bringing the donor and acceptor molecules in appropriate distance and orientation accelerates proton transfer. Apparently, the high density of charged groups results in formation of relatively inflexible networks of more or less symmetrical low-barrier hydrogen bonds. The formation of water-bridged hydrogen-bonded networks is supported by the pulsed field gradient NMR measurements of proton transfer at the surface of BR membranes. The measurements have revealed that the maximal proton transfer rate was observed with fully hydrated samples and that the translational proton jump distance at the surface was about 4 Å, three times larger than that observed in the bulk water [135]. The “rigidity” of such hydrogen-bonded networks follows not only from the neutron scattering data [147], but also from the exponential increase in surface potential, reflecting the decrease in the effective dielectric permittivity [139,142].

Symmetrical hydrogen bonds can be characterized by “large proton polarizability”: intense continua in the IR spectra in the range of 2000–3000  $\text{cm}^{-1}$  can arise upon protonation of such systems, as first shown by Zundel and co-workers [89,148,149]. In model systems, these broad IR bands appeared only at high concentration of amino acids [89]. The polarized water modes are involved in the intraprotein proton transfer in BR [38,150–152] and in the photosynthetic RCs of *Rb. sphaeroides* and *Rps. viridis* [153,154].

The recent simulations of proton transfer along a linear file of water molecules in a gramicidin channel, as done by using a fully microscopic empirical valence bond model, have revealed significant electrostatic barriers for a proton that had to cover long distances by moving along a chain of hydrogen bonds [155]. Apparently, the Grotthuss’ “hop-and-turn” mechanism is not quite efficient when proton has to go via *many* intermediate water molecules in a heterogeneous system. Therefore, when protons have to propagate promptly along surfaces or inside proteins, charged groups are needed as intermediates. The charged groups polarize the water molecules and, in the same time, appropriately orient the donor and acceptor groups (see e.g., [142,156]). It is worth to note that already Grotthuss has

realized the importance of *both* these factors for the efficient charge transfer, especially along thin water layers (see the two epigraphs to this section and Refs. [1–4]).

In search for structural elements that are common and characteristic for proton pumps, a statistical analysis of the available X-ray structures of membrane energy-converting proteins has been performed [157]. Thereby six proton pumps were compared with six energy-transducing enzymes that are not involved in the transmembrane proton translocation (see the caption to Fig. 8 for the chosen protein sets and the further details). The 3D structures of these enzymes have been analyzed and the distributions of shortest edge-to-edge distances between the ionizable groups have been calculated for the *p*-side of the membrane. It was found that the minimal distance between the ionizable groups at the proton releasing *p*-surfaces of proton translocators was, on average, about 7 Å (see the magenta profile in Fig. 8). This distance was distinctly shorter than in “not-pumps” where it was about 10 Å, on average (dark cyan profile in Fig. 8). The distance of 7 Å, as obtained for proton pumps, is in exact correspondence with that providing the fastest rates of proton transfer along acidic monolayers [139–142] and anionic polymers [144], as discussed above.

Fig. 9 shows the structures of the proton translocating *aa*<sub>3</sub>-type cytochrome *c* oxidase of *Rb. sphaeroides* [158] and of the cytochrome *bc*<sub>1</sub> complex of *Rb. capsulatus* [159], as well as the structure of the photosynthetic RC of *Rb. sphaeroides* [160], taken as a “not pump”; all three enzymes are embedded in a virtual membrane. This picture enables a visual comparison between densities of charged residues at the *p*-sides of two “proton emitters” and a “not-pump”, respectively, of the same/similar organism. As is evident from Fig. 9, the red, negatively charged Asp and Glu residues (pK in water ~4.0) densely cover the *p*-surfaces of proton pumps, while the blue, positively charged Arg and Lys residues (with pK in water of ~12.5 and ~10.0, respectively) are buried. One of preconditions of efficient proton transfer along a chain of ionizable groups is the matching of their pK values. The absence/scarcity of arginines and lysines from the *p*-surface should ensure fast proton propagation along the H-bonded networks. The proton transfer *between* the membrane proteins could be facilitated by acidic lipids,

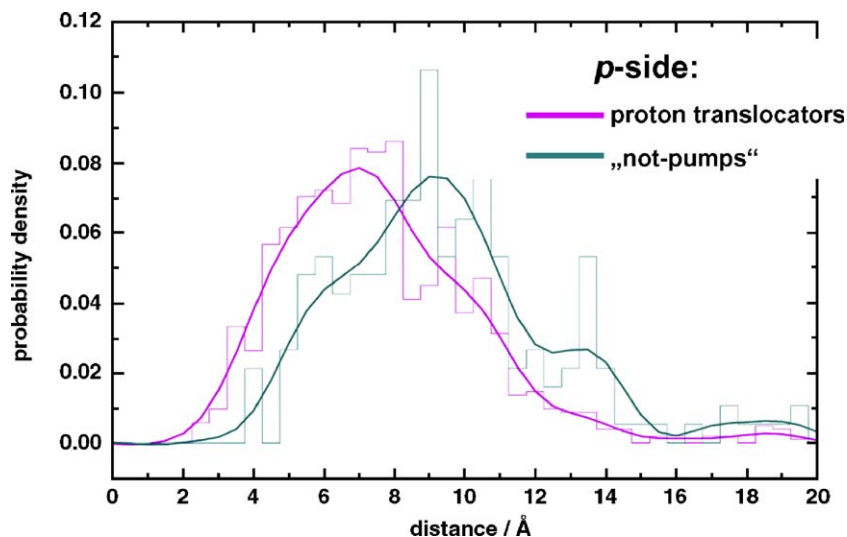


Fig. 8. Distribution of the shortest edge-to-edge distances between ionizable groups on the *p*-side of membrane proteins. Upon calculations, each membrane protein was “cut” in the middle and all ionizable residues (Asp, Glu, His, Arg, and Lys), as located between the section plane and the *p*-side of the protein, were considered. The distance distributions for proton translocators and “not-pumps” are shown by magenta and dark cyan, respectively. As (potential) proton transporters, the mitochondrial cytochrome *bc*<sub>1</sub> complexes from chicken (PDB entry 1BCC, [216]), and from beef (PDB entry 1BGY, [217]), fumarate-reductase from *Wolinella succinogenes*, PDB entry 1QLB [218], cytochrome *c* oxidase from *Paracoccus denitrificans* PDB entry 1AR1 [219], *ba*<sub>3</sub>-type cytochrome *c* oxidase from *Thermus thermophilus*, PDB entry 1EHK [220], and bacteriorhodopsin from *Halobacterium salinarum*, PDB entry 1C3W [221]) were taken. As “not-pumps”, the light-harvesting complex from *Rhodospseudomonas acidophila*, strain 10050, PDB entry 1KZU [222], the light-harvesting complex II from *Rhodospirillum molischianum*, PDB entry 1LGH [223], the B800–820 LH3 light-harvesting complex from the purple bacteria *Rhodospseudomonas acidophila* strain 7050, PDB entry 1IJD, [224], the RC from *Rhodobacter sphaeroides*, PDB entry 1AIJ, [160], the RC from *Rhodospseudomonas viridis*, PDB entry 1PRC [225], and the RC from *Thermochromatium tepidum*, PDB entry 1EYS [226], were analyzed.

phosphatidylserine ( $pK \sim 3.6$  [161]) and cardiolipin ( $pK_1 \sim 3.0$ ,  $pK_2 \sim 8.0$  [162,163]) in the first line. The interaction between acidic residues and acidic lipids is seen in the X-ray structure of a BR trimer that was co-crystallized with an internal patch of acidic glycolipids [164]. Fig. 2B documents that at the *p*-surface the head groups of acidic lipid (mauve colored) lie in a same plane with the peripheral acidic residues (red colored).

As the density of the acidic groups at the *p*-surfaces of different enzymes varies (see e.g., Fig. 9), protons would prefer the membrane patches where the density of acidic groups is higher. Thus, the patches with high density of acidic groups could channel the surface proton flows from sources to sinks. It

is necessary to appreciate that the picture of lateral proton transfer, as it is emerging from the structural analysis, corroborates in many points the ideas that R.J.P. Williams put forward before the structures of membrane enzymes were solved (cf. e.g., with Refs. [10,165]).

On a molecular level, it is possible to target protons into the sinks by tuning the  $pK$  values of the acids involved. Heines and Dencher have considered the possibility that cardiolipin, which has a second  $pK$  at about 8.0, might guide protons into the energy transducing enzymes [163]. Similarly, a surface histidine residue, with  $pK$  of about 7.0, if residing among many acidic groups, would serve as a trap that eventually can guide protons

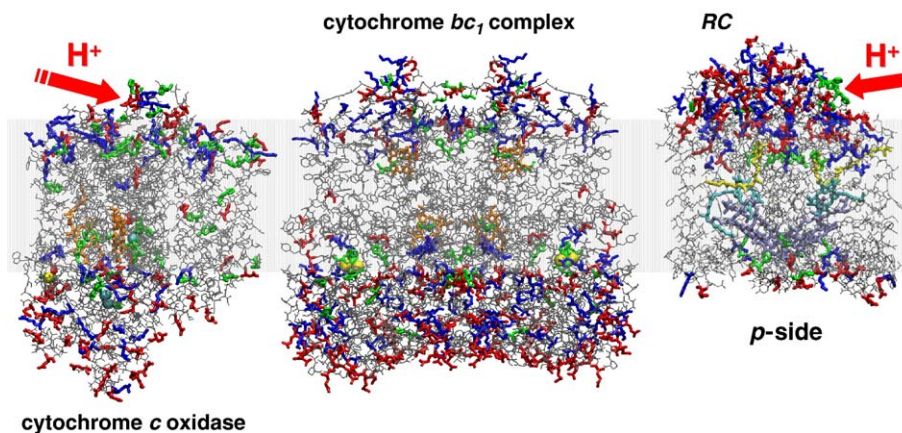


Fig. 9. Proton-conducting networks at the surfaces of energy-transducing enzymes of *Rhodobacter*. The 3D structures of the *aa*<sub>3</sub>-type cytochrome *c* oxidase of *Rb. sphaeroides*, PDB entry 1M56 [158] and of the cytochrome *bc*<sub>1</sub> complex of *Rb. capsulatus*, PDB entry 1ZRT [159], together with the structure of the photosynthetic RC of *Rb. sphaeroides*, PDB entry 1AIJ [160] are depicted. Color code as in Fig. 2; in addition, the hemes are shown by orange, copper by light-blue, and the iron-sulfur clusters as yellow-green balls. The figure was produced by using the VMD software package [213].

into a sink [10,41]. In line with this expectation, clusters of histidines and acidic residues seem to “mark” the proton gateways in membrane enzymes [103,166,167]. In many cases, such patches show affinity for  $Zn^{2+}$  ions and can be blocked by the latter [30,103]. The  $Zn^{2+}$ -sensitive proton *inlets*, as shown by arrows in Fig. 7, were identified in the RC of *Rb. sphaeroides* [42,166,168–173] and in the cytochrome *c* oxidase [174,175]. The indications of  $Zn^{2+}$ -sensitive proton *outlets* have been obtained for the cytochrome *c* oxidase [176] and the cytochrome *bc*<sub>1</sub> complex [52,167,177,178].

The rate of proton transfer along the surface is crucial not only for such exotic systems as large filamentous mitochondria [133,179] and cyanobacterial trichoms [180]. In Ref. [181] the proton-transporting  $F_O$  part of the  $H^+$ -ATP synthase of *Rb. capsulatus* was studied in a decoupled state when the time constant of its turnover was as small as  $\sim 3$  ms at neutral pH. At pH 10.0 the time constant slowed down only up to  $\sim 5$  ms. Hence, the proton delivery to this enzyme did not limit the turnover even at pH 10.0 [65]. From the kinetic analysis, the time of proton delivery to  $F_O$  could be estimated as  $\leq 1$   $\mu$ s at neutral pH [65,182]. As argued above, proton transfer across the interfacial barrier proceeds by three orders of magnitude slower. Hence the route along the surface is likely to dominate upon the proton transfer between the membrane sources and sinks. In other words, the lateral proton delivery at  $< 1$   $\mu$ s is a prerequisite of the enzyme ability to turn over at milliseconds under coupled conditions.

It is necessary to emphasize that the shortest distances between the ionizable groups, as plotted in Fig. 8, were determined not only at the surface, but for the whole 3D network of charged residues on the *p*-side of the membrane. We believe that these results are related to the asymmetric distribution of ionizable amino acids in the transmembrane parts of proton pumps, as noted by Wikström [183]. Concurrently, considering BR and the cytochrome *c* oxidase, which are quite different both evolutionary and functionally, Wikström has called attention to the similarities in distribution of charged residues in these two enzymes. On the *n*-side of the membrane they are scarce, while on the *p*-side one can see hydrogen-bonded networks of ionizable residues (hydrogen-bonded continua) in both cases (see Fig. 2B for the structure of BR and Fig. 9 for the structure of the cytochrome *c* oxidase). After the publication of Wikström's review, the crystal structure of one more primary proton pump, the cytochrome *bc*<sub>1</sub> complex, has been solved for several organisms (see e.g., [159,184]). Fig. 9 illustrates that an extensive H-bonded network is present on the *p*-side of cytochrome *bc*<sub>1</sub> complex as well.

Wikström has discussed the implications from his finding for the transmembrane proton transfer. This subject is beyond the scope of our review. In relation to the mechanism of the surface proton exchange, however, it is worth to note that the water-impregnated 3D networks of tightly packed ionizable residues can serve as a storage buffer for protons, as discussed earlier for thylakoid membranes by Dilley [185] and more recently for the cytochrome *c* oxidase by Ferguson-Miller and co-workers [186]. In this case, one can speak about membrane “proton capacitors” or “proton sponges”. It is worth to mention that the

term “proton sponge” has been already “booked” by organic chemists for aromatic diamines (e.g., diphenyl bis(octadecylamino)phosphonium bromide, see [187] and references therein). The ability of these compounds to scavenge and retain protons is due to the formation of strong symmetrical low-barrier hydrogen bonds, usually between two nitrogen atoms [187,188]. In these “chemical” proton sponges, as noted in Ref. [187], the deprotonation/protonation of symmetrical low-barrier hydrogen bonds is slow. It deserves notion that the “slow” proton-buffering groups at the *p*-surface of chloroplast thylakoids were earlier putatively identified as amines with unusually low *pK* values and attributed to lysine residues (see [189–191] and references therein). From the electrostatic viewpoint, *pK* values in the range of about 8.0 could be expected for the side chains of lysines or arginines if they are buried inside a membrane protein, so that their uncharged forms are selectively stabilized (see e.g., [192]). The crystal structures in Fig. 9 show arginine and lysine residues that are indeed “buried” beyond the layer of acidic residues at the *p*-surface. It is attractive to speculate that H-bonded networks that involve buried arginines and lysines can serve as membrane proton sponges. In case of physiological fluctuations in the activity of proton pumps, such sponges might eventually prevent the energy losses by “retaining” protons until their utilization by the ATP synthase becomes possible.

Protons, which are ejected by the pumps, can either move along the surface to the nearby “sink”, e.g., an ATP synthase, or escape over the interfacial barrier into the bulk phase (see Section 3 above). The rate of the former, productive reaction is determined by the protonic conductance of the “sink” (usually  $\leq 10^3$  protons per second under coupled conditions). The rate of the futile proton escape is just proportional to the proton concentration at the surface. In the simplest case, a gradual acidification of the surface would lead to the increase in the futile proton escape, so that the latter would dominate at pH  $< 5.5$  [80]. It seems more lucrative to impede the pumps *before* the futile proton efflux across the barrier reached remarkable values. In this relation it is noteworthy that the activity of the cytochrome *bc*<sub>1</sub> (*bf*) complexes, which serve as “hubs” in the vast majority of electron transfer chains [193–195], drop at pH  $< 6.5$  due to the back-pressure control from the generated *pmf* (see [196,197] and references therein). A similar feedback control is expected for the cytochrome *c* oxidase, as long as it can be driven backwards by *pmf* [198,199]. In BR, the *pK* value of the proton release complex, comprising glutamate residues and protonated water molecules, decreases from ca. 9.5 in the dark state to 5.8 in the M state [200]. At pH  $> 5.8$ , fast proton release ( $\tau = 80$   $\mu$ s) is observed [34] that is kinetically competent to drive ATP synthesis. At pH  $< 5.8$ , proton release is retarded and occurs in the late ms range [201]. Such a slow proton release may not be sufficiently potent to establish a large proton gradient across the membrane. It is noteworthy that the energy of a light quantum per se,  $\sim 200$  kJ/mol, is more than sufficient to overcome any imaginable back-pressure from membrane potential. Thus, the proton back-pressure controls the proton release in BR not thermodynamically, as in the cytochrome *bc*<sub>1</sub> complex and the cytochrome *c*

oxidase, but mechanistically, by shifting the  $pK$  value of the proton release complex. In all the considered cases,  $pH^S$  at the  $p$ -surface is unlikely to drop below approximately 6.0–6.5, which minimizes the futile proton escape into the bulk aqueous phase.

(i) The lateral proton transfer between the nearby proton sources and sinks at  $<1 \mu s$ , (ii) the presence of proton buffering capacitors (proton sponges), and (iii) the backpressure control over  $pH^S$ —all are instrumental in energy coupling. They help to avoid energy losses at steady state, in particular, by dampening down the physiological fluctuations in the activity of proton pumps.

Überhaupt ist jedes galvanische Phänomen nur eine Unterbrechung des zwischen den Elementarteilen des Wassers durch sich selbst und immer fort stattfindenden, oder (mit anderen Worten) eine Ausgleichung des Unendlichen kreisförmigen zu einem Endlichen linienförmigen.

Theodor von Grotthuß, year 1819 [3]

Je mehr man die Phänomene der galvanischen Elektrizität mit denen der Chemie vergleicht, desto mehr überzeugt man sich, daß letztere nur Resultate der ersteren, d.h. der elektrischen Spannungen sind...

Theodor von Grotthuß, year 1808 [2]

## 6. Outlook: implications for energy conversion

The surveyed experimental data let us to specify the mechanism of proton coupling in biological membranes as follows [35,37,41,66,67,80]:

- (1) The core mechanism, as invented by evolution and as realized in the present-day bacteria, consists in the ability

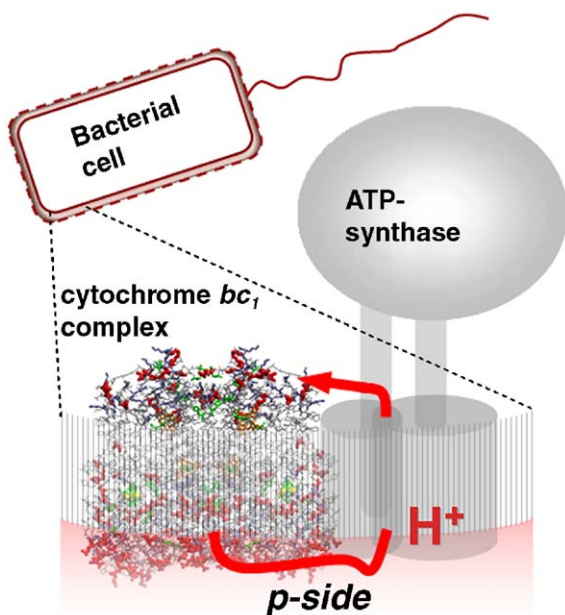


Fig. 10. Schematic presentation of a coupling membrane with protons moving from the cytochrome  $bc_1$  complex to the ATP synthase along the  $p$ -surface. The  $p$ -side of the coupling membrane is marked by a darker color. The figure is based on Fig. 3 of Ref. [80].

of redox- and light-driven proton pumps to charge the membrane by transferring protons from its  $n$ -side to the  $p$ -side (see Fig. 10). In the Grotthuss' terms, the external energy is used to turn the endless charge cycling between water molecules into a vectorial charge flow (see the first epigraph above). Because of the interfacial potential barrier, the proton equilibration between the surface and the bulk occurs slower (at  $\sim 0.1$ – $10$  ms) than the proton spreading along the surface (at  $\sim 0.1$ – $10 \mu s$ ). At steady state, the proton activity at the membrane surface might then deviate from the respective activity in the adjoining bulk aqueous phase.

Thus, in vivo the driving force beyond the ATP synthesis can be defined as surface-to-surface  $pmf$ :

$$pmf^S = \Delta\tilde{\mu}_{H^+}^S / F = \Delta\psi - (2.3RT/F) \cdot \Delta pH^S \quad (2)$$

As a rule,  $pmf^S$  is larger than the bulk-to-bulk  $pmf$ . At steady state, proton pumps operate close to dynamic equilibrium and are under back-pressure control from the generated  $pmf^S$ . Because of this dynamic feedback,  $pH^S$  at the  $p$ -surface is unlikely to drop below 6.0; this trait curbs the futile proton efflux into the bulk.

- (2) As the lateral proton propagation along the membrane is fast, the protonic connection between the sources and sinks goes predominantly along the surface (see Fig. 10).
- (3) Both the interfacial potential barrier and the ability of protons to diffuse promptly along the membrane are due to the high density of negatively charged acidic groups, especially at the  $p$ -surface of the membrane.
- (4) In the described simplest case, the energy coupling involves the bacterial cell proper plus the adjoining water layer with thickness of  $<2$  nm. The bulk solution beyond the interfacial barrier serves as a sink for the escaped protons.

As argued above, the futile proton escape at steady state is hindered, in the first line, (i) by the surface potential barrier, (ii) by the relatively large size of bacterial cells, and (iii) by the fast lateral proton transfer between sources and sinks. The development of the membrane proton sponges in the course of evolution can be considered as a further fine-tuning. The  $pH$ -buffering capacity of proton sponges could dampen down the physiological fluctuations in the activity of proton pumps and prevent undesirable over-acidification of the bacterial surface. In spite of all these “precautions”, the simple, bacterial machinery fails to entirely prevent the proton escape from the surface of bacterial cells and to avoid the losses of the entropic component of  $pmf$ .

On a next evolutionary step, some bacteria decreased the proton losses further by using membrane invaginations. These invaginations enable to “trap” the portions of the external  $p$ -phase inside the bacterial cell. The inventions of such intracellular structures happened, apparently, in different lineages [202] and, in particular, led to the formation of thylakoids in cyanobacteria and the intracellular vesicular

structures in purple bacteria. Because of the evolutionary relatedness of purple bacteria to mitochondria [203], the latter structures might have paved the way to the mitochondrial cristae. If invaginations are present, protons are ejected not into the external “Pacific Ocean” but into membraneous sacks; in this storage space protons dwell until being used (see e.g., Ref. [204]).

Throughout this review and quite on purpose, we have focused on a bacterial cell, where, at least, the external bulk aqueous phase can be unambiguously defined. We have not covered here the wealth of literature on proton dynamics in mitochondria and chloroplasts; their *p*-surfaces, because of the cell topology, are the *inner* surfaces of chloroplast thylakoids and of mitochondrial cristae. On one hand, fast proton equilibration in the limited volume of chloroplast thylakoids and mitochondrial cristae could be expected. On the other hand, in view of the complexity of water structure at charged interfaces, as here surveyed, it is difficult to make statements either on the water state inside these organelles or on the chemical nature of mobile pH-buffers involved. The events in the constrained inner volume of thylakoids and cristae are far from being clarified, in spite of remarkable efforts that have been invested in their investigation (see Refs. [17,189,191,197,205–211] and references therein). Perhaps, the conceptual framework, as outlined in this review, might be useful in further studies. Relevant to the topic of this review are the recent observations of Yaguzhinsky and co-workers on the possibility to decrease the efficiency of coupling in mitochondria by increasing the concentration of mobile pH-buffers [211]. As well, the possibility to switch from the “localized” coupling to the “delocalised” one by changing the salt concentration, as shown in earlier elegant studies of Ort and Dilley [205,206,210], might be related both to the sensitivity of the water structure at charged surfaces to ionic strength (see Section 4 above and Ref. [81]) and to the (expected) dependence of the buffering capacity of proton sponges on salt concentration.

The coupling mechanism, as outlined here, provides a coherent picture of electrochemical energy transduction. It reconciles Mitchell’s idea of *pmf* as the driving force beyond the ATP synthesis [8] with the existence of localized membrane acidic domains, as proposed by Williams [7,10]. In the suggested framework, the *pmf* corresponds to the *surface-to-surface*  $pmf^S = \Delta\tilde{\mu}_{H^+}^S/F$ , while the acidic domains might be attributed to the membrane *p*-surfaces (proton sponges) that stay locally acidified upon steady operation of proton pumps. We would like to emphasize that the suggested rationale is essentially based on the anisotropy of proton transfer at the membrane surface. The anisotropy was first foreseen [10,16,21,24] and then experimentally demonstrated [35,36].

Although apparently deviating from the best-known initial Mitchell’s concept of *delocalized* bulk-to-bulk coupling, the suggested mechanism is in full correspondence with the latest, less known notion of Peter Mitchell who wrote, in his last review, that the surfaces of the coupling membranes serve as “two proton conducting zones, *P* and *N*... in which the major part of the proton current that flows between the proticity producing and consuming modules is *localized*.” [212].

It seems appropriate to end this review by mentioning that as early as in 1808 Theodor von Grotthuss was already convinced that the *electric tension* (*die elektrische Spannung*) drives the chemical phenomena (see the second epigraph to this section and note that the current term *voltage* was not in use yet as Alessandro Volta was still alive and had just built his voltaic pile).

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