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Review

Proton in the well and through the desolvation barrier

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Abstract

The concept of the membrane proton well was suggested by Peter Mitchell to account for the energetic equivalence of the chemical (ΔpH) and electrical ($\Delta\psi$) components of the proton-motive force. The proton well was defined as a proton-conducting crevice passing down into the membrane dielectric and able to accumulate protons in response to the generation either of $\Delta\psi$ or of ΔpH. In this review, the concept of proton well is contrasted to the desolvation penalty of >500 meV for transferring protons into the membrane core. The magnitude of the desolvation penalty argues against deep proton wells in the energy-transducing enzymes. The shallow ΔpH- and $\Delta\psi$-sensitive proton traps, mechanistically linked to the functional groups in the membrane interior, seem more realistic. In such constructs, the draw of a trapped proton into the membrane core can happen at the expense of some exergonic reaction, e.g., release of another proton from the membrane into the aqueous phase. It is argued that the proton transfer in the ATP synthase and the cytochrome *bc* complex could proceed in this way.

Keywords: Proton transfer; Solvation energy; Chemiosmotic theory; Membrane transport; Bacterial flagellum; F_OF_1-type ATP synthase; Cytochrome bc_1 complex; *Rhodobacter capsulatus*

Alice did not wish to offend the Dormouse again, so she began very cautiously: 'But I don't understand. Where did they draw the treacle from?'
'You can draw water out of a water-well,' said the Hatter; 'so I should think you could draw treacle out of a treacle-well-eh, stupid?'
'But they were in the well,' Alice said to the Dormouse, not choosing to notice this last remark.
'Of course they were', said the Dormouse;— 'well in.'

Lewis Carroll. Alice's Adventures in the Wonderland [1].[1]

1. Introduction

Proton cycling is the crux of biological energy conversion. Diverse redox- and light-driven enzymes generate the proton potential difference (the transmembrane difference in electrochemical potential of hydrogen ions, $\Delta\tilde{\mu}_{H^+}$), so that one side of the coupling membrane (*p*-side) becomes *p*ositively charged, whereas the other, *n*-side charges *n*egatively. The proton potential difference is then utilized by the "consumers", e.g., the ATP synthase [2–5]. In some bacteria, $\Delta\tilde{\mu}_{H^+}$ is functionally replaced/complemented by the sodium potential difference ($\Delta_{\tilde{N}a^+}$, see [6,7] for reviews). Still the majority of bacteria, the plant chloroplasts and the animal mitochondria use $\Delta\tilde{\mu}_{H^+}$. Mitchell has introduced the proton-motive force (*pmf*) as:

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

where $\Delta\psi$ is the transmembrane electrical potential difference, and ΔpH is the pH difference between the two bulk aqueous phases [3]. As argued elsewhere [8–11], the membrane surface is separated from the bulk aqueous phase by an electrostatic

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[1] Since alice had to overcome obstacles in Wonderland, some quotations from Carroll's books found to be useful in illustrating the energetic constraints on biological proton transfer. These quotations are used as epigraphs and highlighted in bold typeface throughout the text.

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barrier for ions; for proton, the barrier height is about 120 meV. Because of the barrier, the proton activity at the surface can deviate from that in the bulk aqueous phase at steady state, especially on the *p*-side of the membrane [8,10,11]. Thus, in vivo the *pmf* value can be defined as

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

where $\Delta\tilde{\mu}^{S}_{H^+}$ and ΔpH^{S} are *surface-to-surface* differences in electrochemical potential of proton and in pH, respectively. The surface-to-surface pmf, in general, is expected to be larger than the bulk-to-bulk *pmf*. The elevated proton activity at the *p*-surface should, in turn, favor the protonation of the functional groups.

In this review, the Mitchell's proton well concept is contrasted to the high desolvation penalty for transferring protons from water into the membrane core. It is suggested that the catalytic function of energy-transducing enzymes is dual: besides producing/consuming $\Delta\tilde{\mu}_{H^+}$, they decrease the desolvation penalty for ions, in particular, by coupling an unfavorable ion **draw out of water** with some energetically gainful reaction, e.g., with a release of another ion from the membrane into the aqueous phase.

…so after a minute Humpty Dumpty began again. 'They've a temper, some of them – particularly verbs, they're the proudest – adjectives you can do anything with, but not verbs — however, I can manage the whole lot of them! Impenetrability! That's what I say!
'Would you tell me, please,' said Alice, 'what that means?'

Lewis Carroll. Through the Looking Glass [12].

2. Proton well and the desolvation penalty

The concept of a membrane proton (ion) well is one of key mechanistic elements of the chemiosmotic paradigm. Initially the construct has been suggested by Mitchell [13] to account for the energetic equivalence of the $\Delta\psi$ and $-(2.3RT/F)\cdot\Delta pH$ terms in Eq. (1). Mitchell considered a proton-conducting crevice (half-channel) passing down into the membrane dielectric, as shown in Fig. 1A. He suggested that a proton in such a well would sense both the surface pH changes and the changes in $\Delta\psi$. Depending on their sign, the changes in $\Delta\psi$ and/or in surface pH would drive protons either inside the well (as shown in Fig. 1A) or out of it. The concept of a proton well, after being formulated by Mitchell, has been widely used upon describing the machinery of diverse generators and consumers of *pmf*. Many of these enzymes were considered as built up from proton half-channels connected by switching devices in the middle of the membrane, see, e.g., Refs. [5,13–18]. This general description is applicable, in particular, to the current working schemes of the cytochrome oxidase [19,20], bacteriorhodopsin [21], NADH dehydrogenase [22], and F_OF_1-ATP synthase [18,23]. The water chains, as revealed in the X-ray structures of some membrane enzymes [21,24–26], have added credit to this view.

In the given classical formulation, the concept of a proton well does not take into account the dielectric/desolvation penalty for transferring a proton from water into the hydrophobic membrane phase. The dielectric penalty of transferring an ion from a medium of dielectric constant ε_1 to ε_2 can be estimated, in meV, as [27,28]:

$$\Delta E_{desolvation} = \frac{14400q^2}{2a}\left(\frac{1}{\varepsilon_2} - \frac{1}{\varepsilon_1}\right) \quad (3)$$

where q is the electric charge of the ion (in units), and a is the size of a sphere (cavity) over which the charge is spread (in Å). In case of protons, $q=+1$, while the parameter a corresponds to the size of the protonated group, i.e., is about 2 Å for carboxylic or amino groups. The penalty for transferring a charged particle of 2 Å from water into a phospholipid membrane with ε of 2 has been estimated as about 1600 meV [28,29]. Generally, the membrane **impenetrability** for protons **means** high dielectric penalty and not the absence of proton carrying species—there is enough water in the phospholipid bilayer. Importantly, the penalty remains high even if a proton-conducting medium, as inside the Mitchell's proton well, is present. Only wide (>1 nm), water-filled pores can remarkably decrease the desolvation penalty [28,29]. Such wide pores, however, were not reported for the energy-transducing enzymes so far.

One can reasonably argue that the effective dielectric permittivity (ε_{eff}) inside a membrane protein might be higher than 2. Diverse estimates, as reviewed elsewhere [30], give values of $4<\varepsilon_{eff}<10$ for the core part of membrane proteins. Accordingly, the most recent, structure-based calculations of the proton energy profile in aquaporin yielded a barrier of about 600 meV [31]. Aquaporin, a water carrier, contains, however, a narrow water-filled pore [32]. In more hydrophobic energy-converting enzymes, the desolvation barrier is expected to be higher. Moreover, as argued by Krishtalik, the increase in ε_{eff} would decrease the equilibrium desolvation penalty but, in the same time, should increase the reorganization energy of proton transfer, so that the activation barrier of proton transfer would remain high [33].

The black curve in Fig. 1B shows the energy profile of a proton in the membrane for the case of equilibrium desolvation penalty of ~800 meV and of surface potential of −60 mV. The red curve shows the same profile after its superimposing over a $\Delta\psi$ energy profile of 200 mV (as shown by a dashed line, cf. with Fig. 1A). At first glance, the resulting energy profile leaves little space for membrane-protruding, proton-accessible wells, as the proton energy sharply rises immediately beyond the membrane/water boundary. The exact electrostatic calculations, as carried for aquaporin and ion channels [31,32,34–36], have shown, however, that the electrostatic profiles of membrane proteins are not smooth but contain local minima. The minima can be caused (i) by clustering of several protonatable groups and/or (ii) by constellation of (partial) negative charges. In the former case, the charge of a proton can be "spread" in space that would lead to a local decrease in desolvation penalty by increasing the value of term a in Eq. (3). The latter trick is used by ionophoric oligopeptides, such as valinomycin or nigericin, which form rings with the inside-pointing carbonyl oxygen of the backbone. Hence, amino acid residues can form a solvation

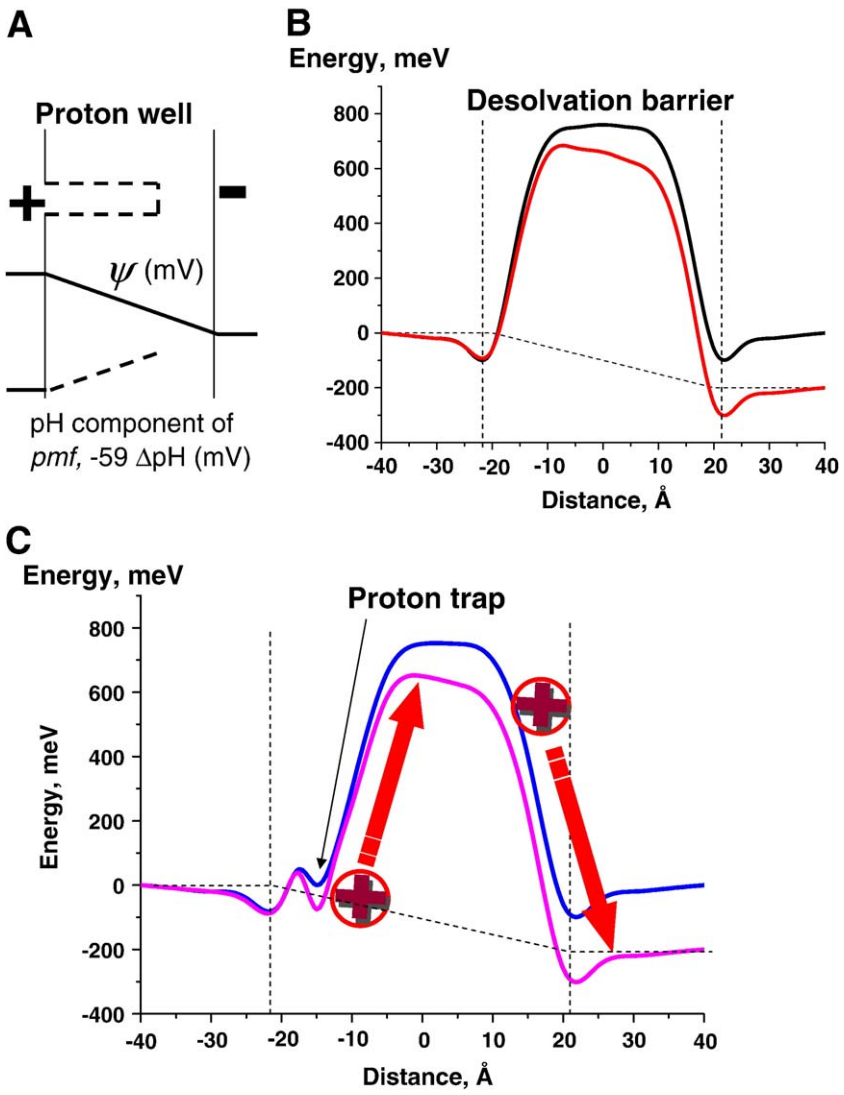

Fig. 1. Energy of a proton in the membrane. (A) Definition of a proton well; the picture is based upon Mitchell's illustration to Ref. [13]. (B) Dielectric penalty for proton in the membrane; the semi-quantitative plot is based on estimates from Ref. [28], see the text for the parameters. A *pmf* step of 200 meV is shown by a dashed line. (C) Proton trap. The desolvation penalty can be locally compensated by the protein yielding an energy minimum for proton. The respective site can trap a proton in response to a *pmf* step (dashed line). As shown schematically, the trapped proton can still leap over the desolvation barrier if driven by some exergonic reaction, e.g., as depicted, by release of another proton from the membrane.

shell around an ion; such a shell might energetically substitute for that provided by the bulk water. In this case, it is appropriate to speak about a substrate-induced fit [37], since ions are the substrates of ion transporters.

Thus, a protein can compensate the desolvation penalty by forming a proton-accepting cluster. If the cluster is membrane buried, its protonation state should be sensitive to the changes both in $\Delta\psi$ and in ΔpH (cf., the blue and magenta curves in Fig. 1C, as plotted in the absence and in the presence of *pmf* of 200 mV, respectively), so that the construct could operate as a Mitchell's proton well.

However, as it also follows from Fig. 1C, such proton well would be shallow. The rise of desolvation penalty is pretty steep. Therefore, to be able to change the protonation state in response both to $\Delta\psi$ and ΔpH, the proton-binding cluster has to be not far from the membrane boundary. Moreover, a proton, after hitting the bottom of a shallow proton well, is trapped and can hardly move further. The desolvation energy (>500 meV) is larger that the typical value of *pmf* (≤250 mV). Under coupled conditions, the energy-transducing systems operate close to equilibrium, so that even this small energy gain is usually not available. Hence, there is no evident source of energy to carry the proton over the desolvation barrier. A proton can, of course, wait for a thermal fluctuation. However, since proton equilibration along the membrane surface proceeds at microseconds [10,38,39], a thermally activated jump over a desolvation barrier of >500 meV would take minutes, at best.

A potential egress from this cul-de-sac is the linking of the proton draw into the membrane core to some exergonic reaction. Krishtalik has argued that the proton transfer into the membrane core could be facilitated if coupled with neutralization of a negative charge in the membrane [33]. At equilibrium, however, the electric charges in the protein interior are compensated by mobile ions and/or hydrogen bonds [39]. Therefore, only

removal/neutralization of *non-compensated* intra-membrane charges can help to pay the desolvation penalty. The protein charges that are not compensated (yet) are the just generated ones, i.e., the *newborn* charges. In particular, the expelling of a *newborn* proton out of the membrane, as shown in Fig. 1C, would be accompanied by its thermodynamically favorable solvation and, hence, can be used to draw another proton (ion) from water into the membrane. Below it is argued that the available data on proton transfer in the F_OF_1-ATP synthase and the cytochrome bc_1 complex can be described by mechanisms where the drawing of a proton into the membrane core is coupled with the neutralization/ejecting of non-compensated electrically charged species.

Alice remained looking thoughtfully at the mushroom for a minute, trying to make out which were the two sides of it; and as it was perfectly round, she found this a very difficult question.

Lewis Carroll. Alice's Adventures in the Wonderland [1].

3. Proton transfer by the rotary motor of the F_OF_1-ATP synthase

3.1. Rotary proton transporter: general scheme

F_OF_1-ATP synthase is a reversible molecular machine that couples the transfer of hydrogen or sodium ions across the membrane with the $ATP \leftrightarrow ADP + P_{inorganic}$ reaction (see [18,40–45] for reviews). As shown in Fig. 2A, this **mushroom**-like enzyme consists of two portions. The membrane F_O portion (a complex of $a_1b_2c_{10-15}$ subunits) translocates ions. The almost **perfectly round** hydrophilic F_1 portion (a complex of $\alpha_3\beta_3\gamma_1\delta_1\varepsilon_1$ subunits in bacteria) protrudes for about 100 Å from the membrane and catalyzes the synthesis/hydrolysis of ATP. The two parts of the enzyme are connected by two "stalks" formed by the centrally located γε-complex and by the off-center b_2 dimer, respectively (see Fig. 2A). F_OF_1 is a rotary machine: proton flow through F_O is believed to rotate the $\gamma_1\varepsilon_1c_{10-15}$-complex relative to the other subunits. The catalysis is achieved by sequential interaction of the rotating γ subunit with the three "catalytic" β subunits.

The paradigm of a rotary proton transporter has been formulated by Glagolev and Skulachev in relation to the $\Delta\tilde{\mu}_{H^+}$ driven flagellar motor of bacteria [15]. It has been suggested that such a transporter is built of a stator part and of a rotating disk covered by electrically neutral, potentially protonatable groups (NH_2), as shown in Fig. 2B. At any moment one of these groups is accessible from one side of the membrane via a water-filled half-channel in the stator. Another half-channel connects a negatively charged functional group of the stator (COO^-) with the other side of the membrane. The two half-channels are shifted relative to each other by a distance equal to that between the two neighbouring NH_2 groups. Then a protonation of a NH_2 group to NH_3^+ could promote the formation of a COO^- NH_3^+ ionic bond because of electrostatic attraction between two charges and, correspondingly, lead to the rotation of the ring by one step. As a result, the protonated NH_3^+ group gets the opportunity to release its proton via the output channel, whereas the next NH_2 group becomes accessible via the input channel and could be protonated to NH_3^+. A proton concentration bias from two sides of the membrane determines the direction of rotation and its rate. Later, after Mitchell has invoked the possibility of rotary catalysis for the ATP synthase [46], Skulachev has suggested that the same mechanism can describe the proton transfer through F_O [16]. After the site-specific mutagenesis has shown that only few amino acid residues are functionally irreplaceable in the F_O of *E. coli* [47–49], the rotary machine of the ATP synthase was specified by Junge in 1993 [18] and by Vik and Antonio [50]: namely, it was suggested that the ring of catalytic groups is formed by highly conserved carboxylic residues of multiple subunits *c* (Asp-61 in *E. coli*). The role of the functional group of the stator was attributed to the conserved arginine of helix 4 of the *a* subunit (Arg-210 in *E. coli*, see panels A and C in Fig. 2). Later this model was adapted to the Na-dependent F_OF_1-ATPase [51].

The scheme of a rotary proton transporter, as formulated by Glagolev and Skulachev and as adapted later for the ATP synthase, contains two distinct features: (1) separate groups are in protonic equilibrium with the opposite sides of the membrane and measure the proton concentration bias; (2) the protonation state of the groups changes in response to $\Delta\psi$ and ΔpH, since both $\Delta\psi$ and ΔpH can drive the bacterial flagella [15,52] and the ATP synthase [53,54]. To satisfy the latter condition, the protonatable groups were placed in the middle of the membrane, and two Mitchell's proton wells were used to connect them with the aqueous phases [15], as shown in Fig. 2B. This mechanistic solution, which was used later in the models of F_O [18,44,55,56], took, however, no notice of the desolvation penalty for proton entrance into the membrane dielectric (see the previous section). Moreover, the below considered data on proton transfer via F_O of purple phototrophic bacterium *Rhodobacter capsulatus* [54] do not support the existence of deep proton wells in F_O. Hence, this scaffold of a rotary ion transporter might ask for certain modification.

3.2. Proton transfer through F_O of Rhodobacter capsulatus

With photosynthetic membranes, it is possible to use light-triggered photosynthetic enzymes to generate a fast *pmf* step and then to trace the proton discharge through F_O. The accompanying voltage decay can be followed by electrochromic bandshift of native carotenoid pigments, while the reactions of proton binding and release can be tracked by appropriate pH indicators [57–60]. Native membrane vesicles (chromatophores), which serve as "energosomes" in some phototrophic bacteria, can be easily isolated after disruption of the cells. In the case of *R. capsulatus*, chromatophore vesicles contain, on average, only one ATP synthase each [61,62], so that the flash induced proton transfer through the ATP synthase can be tracked in a quasi-single-enzyme mode. The ATP synthases can be depleted from their F_1 parts by EDTA treatment. In this case, the proton transfer through F_O is not biased by load. Using this approach, it was found that the "bare" F_O of *R. capsulatus* showed following traits: the rate of proton transfer changed only slightly (by a factor of three) in the pH range between 5.0 and 10.0; the transfer rate was not affected by soluble pH buffers but revealed an H/D isotope effect of ~2.0 and a high activation energy (E_a) of ≥50 kJ/mol (~500 meV) [54]. The rate of proton

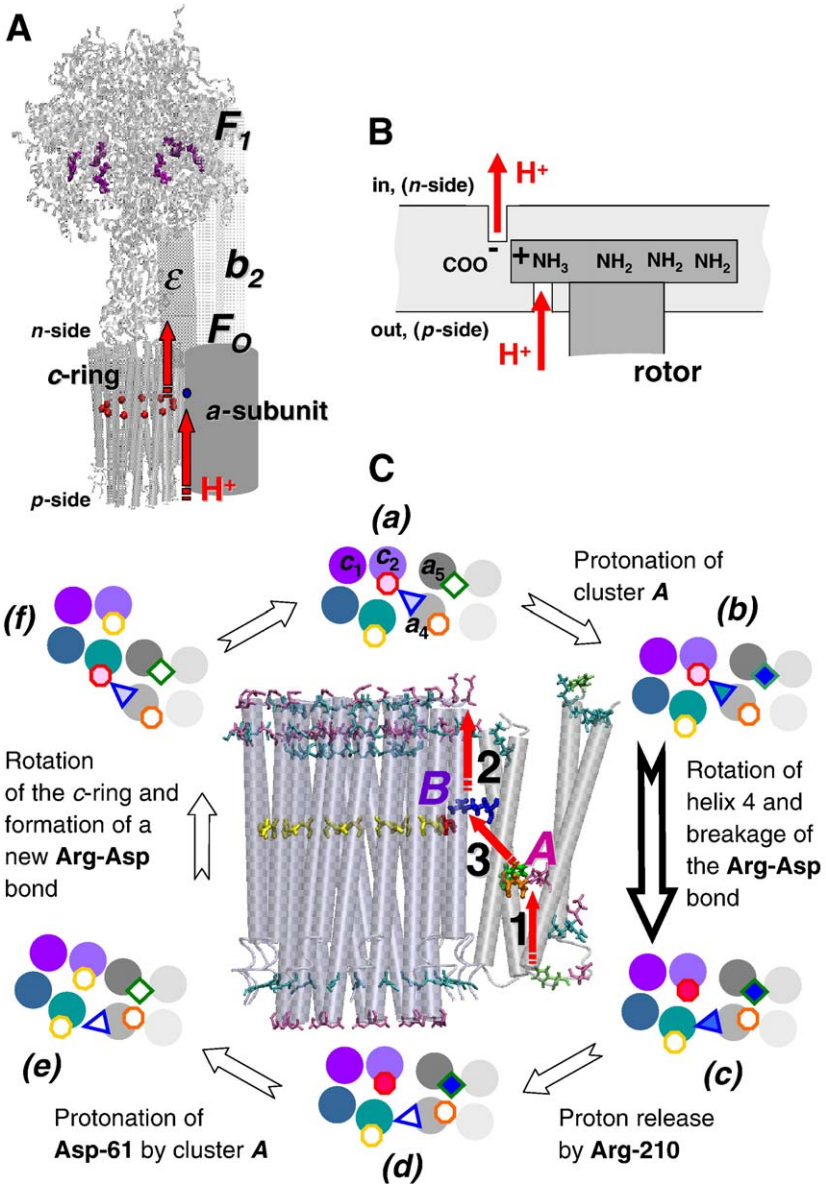

Fig. 2. Proton transfer by rotary transporters. (A) A scheme of proton transfer through F_O according to Refs. [18,56], as placed over a composite model of a F_OF_1-type ATP synthase (the X-ray structure of the F_1 part of the bovine H^+-ATP synthase (PDB entry 1H8E [42]) is combined with the crystal structure of the *c*-oligomer of the *Ilyobacter tartaricus* Na^+-ATP synthase (PDB entry 1YCE [68]). The subunits *a*, *b*, and ε are shown as cylinders. The conserved carboxylic residue in each *c* subunit is shown as a red ball, while the conserved arginine of the *a* subunit — as a blue one. The figure was produced with the help of the VMD software package [139]. (B) A hypothetical scheme of the rotary proton transport by bacterial flagella. The scheme is redrawn from Ref. [15]. (C) Tentative scheme of proton transfer by F_O of *E. coli*. In the middle of the panel the *c*-ring and the *a* subunit are shown from the side. The numbers indicate the sequence of proton transfer steps. In this composite structure, the crystal structure of the *c*-oligomer of the *I. tartaricus* Na^+-ATP synthase (PDB entry 1YCE [68]) is combined with the model of the four helices of the *E. coli a* subunit (PDB entry 1C17 [81]). Color code: yellow, neutral caroboxyls of the *c*-ring (Glu-65 of *I. tartaricus* corresponding to Asp-61 in *E. coli*), red, the only charged carboxylic residue in interaction with the blue Arg-210 of the *a* subunit, orange, Glu-219 of the *a* subunit, green, His-245 of the *a* subunit, cyan, other arginine and lysine residues, mauve, other aspartate and glutamate residues, lime, other histidine residues. The structures were produced with the help of the VMD software package [139]. The reaction steps of the catalytic cycle, which make a ring around the central picture, are traced in the synthesis direction and are shown from the membrane *n*-side. The α-helices of subunit *a* are shown in gray, the two adjoining subunits *c* are colored violet and turquoise, respectively; the blue triangle depicts Arg-210 of subunit *a*; octagons show Asp-61 of subunits *c* (yellow or red, cf., with the central picture) and Glu-219 of subunit *a* (orange); green diamond is for His-245 of subunit *a*; for the neutral residues the filling color is white; for the negatively charged residues – depending on the charge strength – from red to pink; for the positively charged residues – from blue to light-blue. On the panel (*a*) the α-helices are numbered. The states (*a*) and (*f*) are equivalent and differ only by the position of the subunit *a* relative to the *c*-ring. A thick arrow shows the rate-limiting step of the cycle. For other explanations, see text.

transfer by a bare F_O was compatible to the turnover rate of a coupled F_OF_1-ATP synthase. All these features match those reported previously for the bare chloroplast F_O [63]. The new finding was the independence of the proton transfer rate of $\Delta\psi$ [54].

The absence of slowing under the load and the weak pH dependence of proton transfer could be explained by a model where the protonatable group *A* in the equilibrium with the *p*-side of the membrane had an *acidic* p*K* of ~5–6, whereas the group *B*

in the equilibrium with the *n*-side had a *basic* p*K* of ~8–10 [54,56]. Then, if pH from two sides of the membrane is similar (a usual case for F_1-lacking membrane preparations), F_O operates away from its optimum at any pH. This feature prevents the futile proton escape through F_O under de-energized conditions and accounts for the weak pH dependence. The situation changes dramatically under conditions of ATP synthesis. Here, the proton activity differs from two sides of a coupling membrane and matches the p*K* values of groups *A* and *B*, so that proton transfer can proceed at a maximum rate.

Taken together, the weak pH dependence, high E_a, and H/D isotope effect of ~2.0 indicate that the rate limiting stage of proton transfer is a conformational transition coupled with a breakage of several hydrogen bonds, but not the proton transfer proper [54].

The revealed $\Delta\psi$ independence of the proton transfer rate [54] puts a major constraint on the position of groups A and B in the membrane. The kinetic data on proton transfer in *R. capsulatus* chromatophores could be described by the kinetic model only under assumption that the protonatable groups *A* and *B*, with p*K* values of ~6.0 and ~10.0, respectively, are located close to the membrane/water boundary. Otherwise the membrane potential would notably accelerate the proton transfer. The fraction of membrane dielectric between groups *A* and *B* was estimated as >50–80% [54].

The wealth of information on the structure and function of F_O [41,47–51,54,64–68] provides useful hints on the proton transfer mechanism. Fillingame and co-workers have identified two water-accessible crevices inside the F_O of *E. coli* [65,69,70]. As shown by the Ag^+ labeling after cysteine substitution, the *p*-side cleft is formed at the interface between helices 4, 5, 2 and 3 of the *a* subunit. In the case of *E. coli*, the key functional elements of this cleft are His245 of helix 5 and Glu219 of helix 4, which are close to the *p*-side of the membrane (see the structure in Fig. 2C). These residues apparently interact with each other, since their arrangement is inverted in mitochondria and some phototrophic bacteria (e.g., *R. capsulatus*): a histidine residue is found in helix 4, whereas a glutamate residue is found in helix 5. The tolerated replacements around these two positions were either amino acid residues capable of hydrogen bond formation, or small residues, such as glycine, which facilitated the accommodation of additional water molecules in this space [71–73]. It is likely that the crevice contains water molecules stabilized by hydrogen bonds with surrounding amino acid residues. This site might correspond to the "group *A*". The p*K* value of ~6.0, as obtained by modelling [54,56], is compatible with expected p*K* of a histidine residue, so that a proton can be taken by the conserved His-245 of helix 5 (His-173 of helix 4 in *R. capsulatus*). The involvement of glutamate as a proton acceptor is unlikely; the possibility to replace it by lysine without major consequences indicates that it is already protonated at neutral pH values (introduction of lysine instead of Glu-219 in *E. coli* [72,73] and instead of Glu-210 in *R. capsulatus* [74] did not block the enzyme function). Another thinkable possibility is that the proton might dwell in a hydrogen-bonded network. The studies of IR spectra of the bacterial RC [75] and of bacteriorhodopsin [76,77] have shown that protons tended to reside in a hydrogen-bonded water networks (presumably as $H_5O_2^+$ cations [78,79]). In the bacteriorhodopsin case, the functional p*K* of the $H_5O_2^+$ moiety was estimated as ~5.0 [79], close to the estimated p*K* value of group *A*. In fact, proton can be spread over several groups, so that one can speak not about group *A*, but about a proton-binding cluster *A*. As argued in the previous section, proton spreading, in combination with a specific affinity to proton, should decrease/compensate the desolvation penalty.

The *n*-side crevice is formed in *E. coli* by residues that, after being replaced by cysteines, were sensitive not just to Ag^+ but also to *N*-ethylmaleimide (NEM) [65,69,70]. This cleft is at the interface of helix 4 of the *a* subunit and the external helix 2 of the *c*-ring. The chain of residues consists of Ser-206, Asn-214, and, in all likelihood, the strictly conserved Arg-210 in-between. The accessibility of this space to the rather bulky NEM molecules implies a water cluster also in this cavity. The only irreplaceable residue with p*K* of ~10.0, as expected for group *B*, is the conserved Arg-210 proper (Arg-164 in *R. capsulatus*). The cross-linking data and the hydrophobicity plots [80] indicate that the distance between the *n*-surface and Arg-210 corresponds to the one-third of the membrane thickness (see Fig. 2C).

Fig. 2C shows a tentative scheme of F_O operation that takes into account the desolvation penalty and is based on the available functional and structural information [41,47–51,54,63–66,69–74,80–84] and the recent molecular dynamics simulations [67]. In the depicted scheme, the proton transfer through F_O is initiated by $\Delta\psi$ generation and/or proton activity elevation at the *p*-side of the membrane. The tentative catalytic cycle of F_O can be then described as follows (the *E. coli* numbering of amino acid residues is used):

(a) In the resting state the proton at Arg-210 is stabilized by interaction with the anionic Asp-61 [67,81].
(b) The $\Delta\psi$ generation and/or increase in proton activity from the *p*-side leads to protonation of the cluster *A* (with proton proper at His-245 or at $H_2O_5^+$).
(c) The amino acid residues of subunit *a* rearrange to form a solvation shell around the proton in cluster *A*. The rearrangement, because of involvement of Glu-219, causes a rotation of helix 4. This rotation should lead to a swinging movement of Arg-210 on the external side of helix 4 and to the breakage of a bond between Arg-210 and Asp-61, yielding two poorly compensated electrical charges almost in the center of membrane dielectric.
(d) The neutralization of the positive charge of Arg-210 could be achieved through its deprotonation via Ser-206. It is noteworthy, that the cross-linking experiments have shown that a cysteine residue, when introduced into position 62 of the *c* subunit next to the conserved carboxylic residue, formed a bond with a cysteine residue in the position 214 of the *a* subunit, one helix turn closer to the *p*-side than Arg-210 [83]. This might indicate that the helix 4 of the *a* subunit (or the whole *a* subunit) can slide along the *c*-oligomer, so that Arg-210, when not bound to Asp-61, can approach the aqueous phase. Generally, the electric field should favor the movement of the positively charged Arg-

210 towards the *n*-side of the membrane and of the negatively charged Asp-61 towards the *p*-side. This movement, so far hypothetical, can be considered as a recapitulation of the earlier "piston" model of F_O as suggested by Fillingame [84].

(e) The neutralization of the negative charge at Asp-61 can be then achieved by proton delivery from cluster *A*, perhaps, via Asn-214 [67]. It is thinkable that the protonation-induced rotation of helices in subunit *a* and, perhaps, the sliding of subunit *a* along the *c*-oligomer might establish a transient protonic connection between the cluster *A* and Asp-61.

(f) After all charges are neutralized, the system relaxes back, and Arg-210 restores the interaction with Asp-61. However, because of preceding rotation of helix 4 of the *a* subunit relative to the helix 2 of the *c* subunit, Arg-210 interacts now with the next Asp-61 of the *c*-ring.

In this scenario, the rate-limiting step of the turnover is the (b) → (c) transition, when the rotation of helices in subunit *a*, as driven by the need to accommodate a proton in the cluster *A*, leads to the breakage of the electrostatic interaction between Asp-61 and Arg-210 and to the appearance of uncompensated electric charges in the membrane. Apparently, this rotation is coupled with a rearrangement of several hydrogen bonds and might account for the isotopic factor of ~2.0 and the high E_a of ~50 kJ/mol [54].

According to this model, the proton, after getting into the "trap *A*" and being unable to pass further because of desolvation barrier, triggers a protein rearrangement accompanied by appearance of non-compensated electric charges in the membrane interior. The energetically favorable neutralization/removal of these charges is eventually coupled with the opening of an alternative exit for proton to the other side of the membrane.

The essence of the suggested scheme is that the helix 4 of the *a* subunit, via Arg-210 and Glu-219 (Arg-164 and His-173 in *R. capsulatus*), respectively, contributes to charge stabilization in two different proton traps that are located close to the opposite sides of the membrane (see Fig. 2C). Because of mechanistic constraints, the helix can stabilize only one proton-binding cluster at a time, so that the proton accommodation in one of the traps leads to the destabilization of the other one and proton release. This scheme is symmetric and hence can describe the proton transfer in both directions.

It is noteworthy, that the two proton-stabilizing moieties correspond functionally (and perhaps even structurally) to the ion-stabilizing rings of membrane ionophores (see above). The ionophore rings, however, stabilize both protons and metal ions. Therefore, the suggested scheme is applicable to the Na-dependent F_OF_1-ATP synthase. As already noted by Boyer, the high similarity between the Na-dependent and H^+-F_OF_1-ATP synthases implies a compatibility of their mechanisms [85]. The suggested scheme with two alternating ion-stabilizing traps fulfils this requirement.

The suggested scheme retains the key traits of a rotary transporter [15], namely (i), a rotor covered by a ring of identical ionizable groups interacts with a charged group of the stator; (ii) in the interaction site the rotor group gets an opportunity to give/take a proton; (iii) two different ionizable groups monitor the proton concentration bias and are sensitive both to $\Delta\psi$ and ΔpH. In deviation from the original scheme, (a) the two monitor groups are not identical to the functional groups of the rotor, (b) two deep proton wells are replaced by two shallow proton traps, and (c) the proton transfer rate is limited by a conformational change that enables a transient protonic connection between the two traps.

Turning to the bacterial flagella, it is worthy to note that according to the recent data (i) the flagellar rotor is not embedded in the membrane but just touches it from the *n*-side, and (ii) the only so far identified residue that might be involved in proton transfer, Asp-32 of the stator MotB subunit in *E. coli*, is located close to the *p*-side of the membrane [86]. It is thinkable, that a protonation of a trap close to the *p*-side (Asp-32?) triggers a conformational change of the MotB and MotA proteins. Such a change, on one hand, can move the flagellar rotor by one step via an interaction at the *n*-side of the membrane, and, on the other hand, can let proton to escape to the other side of the membrane.

The chief difficulty Alice found at first was in managing her flamingo: she succeeded in getting its body tucked away, comfortably enough, under her arm, …but generally, just as she had got its neck nicely straightened out, and was going to give the hedgehog a blow with its head…

Lewis Carroll. Alice's Adventures in the Wonderland [1].

4. Proton transfer by the cytochrome bc_1 complex of *R. capsulatus*

The cytochrome bc_1 complex (bc_1) is a dimeric membrane ubiquinol:cytochrome *c* oxidoreductase that serves as a "hub" in the vast majority of electron transfer (ET) chains [87–89]. The catalytic core of each bc_1 monomer is formed by the membrane-embedded cytochrome *b*, to which the iron-sulphur Rieske protein and cytochrome c_1 are anchored (see Fig. 3A). The catalytic reaction in the bc_1 proceeds according to the Mitchell's *Q*-cycle [90,91]. According to the current, structure-based models [88,92–98], ubiquinol is oxidized in the catalytic center *P* close to the *p*-side of the coupling membrane, at the interface between cytochrome *b* and the Rieske protein (see Fig. 3). The two released electrons go to different acceptors. One is taken by the [2Fe-2S] cluster of the Rieske protein to be transferred to cytochrome c_1. The other electron, after being accepted by the low-potential heme of cytochrome *b* (b_l), crosses the membrane on its way to the high-potential heme b_h and then reduces a quinone molecule in center *N* at the opposite *n*-side of the membrane, where a Q_NH_2 ubiquinol is ultimately formed.

Depending on the presence of inhibitors and the crystallization conditions, the redox active domain of the Rieske protein (FeS domain) was found in two different positions, reflecting its rotation by approximately 60° [25,89,92,93,99–103]. It was concluded that the **neck** of the Rieske protein is flexible and that its **head** – the FeS domain – moves upon shuttling an electron from center *P* to cytochrome c_1, as shown in Fig. 3B [92,93,100,104].

With chromatophores of purple phototrophic bacteria *Rhodobacter sphaeroides* and *R. capsulatus*, the generation of membrane voltage by bc_1 can be traced via spectral shifts of native carotenoid pigments and correlated with the electron and proton transfer reactions [57,58,105–107]. Taking the *Q*-cycle scheme at face value, one would expect that the proton release by bc_1 should follow the ubiquinol oxidation in center *P*, while proton binding should be coupled with the formation of another ubiquinol in center *N*. The partial steps of the flash-induced bc_1 turnover, as recently resolved in the presence of low concentrations of Zn^{2+} [106–108], indicate that the mechanism is less straightforward. It was found that the heme b_h was reduced by ubiquinol at 1–2 ms; the rate of this reaction was independent of Zn^{2+} concentration at <100 μM of the latter. Proton release from center *P*, $\Delta\psi$ generation, re-reduction of cytochrome c_1 and oxidation of heme b_h proceeded slower, even under single-turnover conditions. These reactions were sensitive to Zn^{2+} concentration; their time constants were in the range of 7–20 ms at 50 μM of Zn^{2+} [106–108]. It was concluded that the turnover of bc_1 proceeds in two steps, at least [98,106,107], as depicted in Fig. 3B. During the first step, the FeS domain takes the first electron and the first proton from ubiquinol, while the other electron is transferred to heme b_l. The second proton remains in center *P*, most probably at Glu-272 residue of cytochrome *b*. The electron moves then, in an electrically compensated way, from heme b_l to heme b_h at 1–2 ms. Upon the second, slower step (see Fig. 3B, right), the FeS domain relocates to re-reduce cytochrome c_1, protons are released both by the FeS domain and by Glu-272, $\Delta\psi$ is generated, and heme b_h is oxidized via center *N*. The bottom line is that the proton release from center *P* is much slower than the ubiquinol oxidation.

The interaction of Zn^{2+} with mitochondrial bc_1 is well studied [109,110], and the X-ray structure of the Zn^{2+}-containing enzyme is available [111]. As argued in more detail elsewhere [98,106,107,112], (a) the slowness of proton release and (b) the concurrent retardation of proton release, $\Delta\psi$ generation and the re-reduction of cytochrome c_1 by Zn^{2+} might indicate that the Zn^{2+}-binding histidine-rich cluster close to center *P*, as revealed in the crystal structure of mitochondrial bc_1 [111], serves as a proton outlet (see Fig. 3B). The comparison of different bc_1 structures shows that this proton exit is open when the FeS domain is docked to cytochrome c_1 (see the right panel in Fig. 3B and the caption to this figure). Hence, it is thinkable that protons get the opportunity to escape from center *P* only when the FeS domain relocates towards cytochrome c_1.

Relevant for our consideration is the kinetic correlation between the proton release from center *P*, $\Delta\psi$ generation and the re-reduction of cytochrome c_1, on one hand, and the oxidation of heme b_h via center *N*, on the other hand [106–108]. The elements

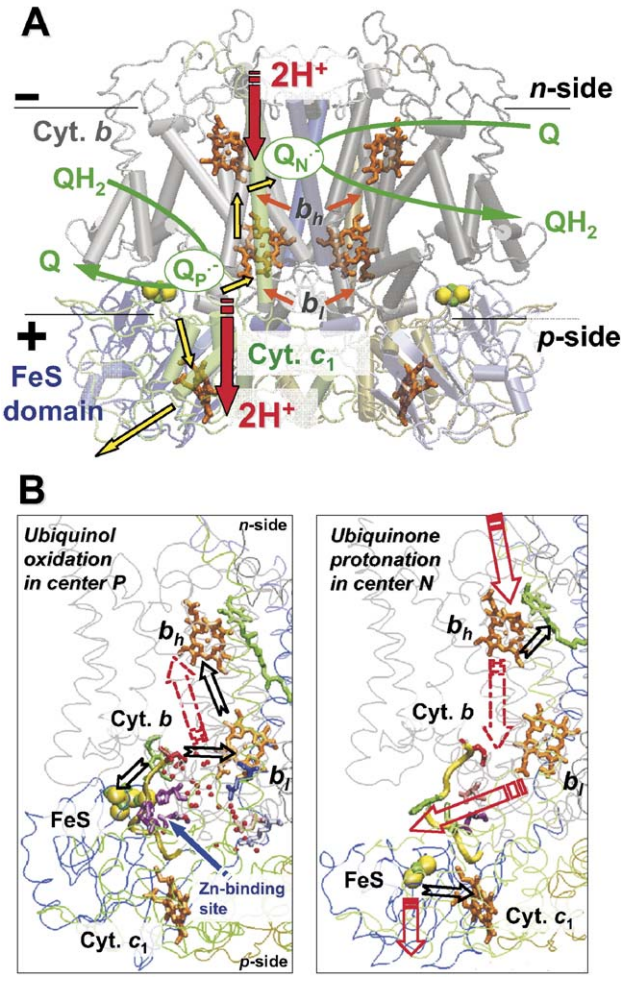

Fig. 3. Proton transfer by the cytochrome bc_1 complex. (A) Overview of structure and function of the cytochrome bc_1 complex. The scheme shows the Q-cycle scheme [90] placed over the X-ray structure of a dimeric bc_1 of *R. capsulatus* (PDB entry 1ZRT [89]) Color code: grey/black, cytochromes *b*; blue, Rieske protein; green, cytochromes c_1; orange, hemes; yellow/green, the FeS clusters. The figure was produced with the help of the VMD software package [139]. (B) Tentative scheme of electron and proton transfer during the initial steps of bc_1 turnover (as modified from Ref. [98]). Black arrows, ET events; red arrows, proton transfer events; dark red dotted arrows, dielectric relaxation of protein/water. The redox centers are colored as on panel A. Below the yeast numbering of amino acid residues is given by straight letters, while that of *R. capsulatus* is given in italic letters. The Glu-272 (*Glu-295*) of cytochrome *b* is colored red. The segment of the *ef* loop that interferes with the movement of the FeS domain (cytochrome *b* residues from 260 to 270 (*from 283 to 293 in R. capsulatus*) is shown as a thick yellow tube. As it follows from the figure, the Zn^{2+} binding can slow down both (i) the electrogenic proton release, and (ii) the movement of the FeS domain towards cytochrome c_1 – since one of the Zn^{2+} ligands – His-268 in the mitochondrial bc_1 and His-290 in the bc_1 of *R. capsulatus* – belongs to the cytochrome *b* loop that has to be "pushed aside" upon the movement of the FeS domain [140,141]. Left panel, fast step of ubiquinol oxidation in center *P*: the picture is a compilation of two crystal structures of the yeast bc_1: the water chains from the high resolution structure (PDB entry 1EZV [25]) are superimposed over the structure of a dimeric yeast bc_1 co-crystallized with cytochrome *c* (PDB entry 1KYO [142]). The bound stigmatellin in center *P* was replaced by ubiquinone. Water molecules, which are found in the vicinity of center *P*, are shown as red balls. The four amino acid residues, which correspond to the Zn^{2+}-binding ligands of the chicken bc_1, are depicted in violet. Thereby Ser-268 of yeast was replaced by histidine, as in *R. capsulatus* (*His-291*) and chicken; other residues are His-253 (*His-276*), Asp-255 (*Asp-278*) of cytochrome *b* and His-185 of cytochrome c_1 (no evident counterpart in *R. capsulatus*). Right panel, slower step of ubiquinone reduction in center *N*: the picture is based on the structure of the chicken bc_1 (PDB entry 1BCC [92]). Ubiquinol in center *N* is shown in the same position as it is found in the yeast bc_1 (PDB entry 1KYO [142]). The four amino acid residues, which bind Zn^{2+} in the chicken bc_1, are colored as follows: Asp-253 (*His-276*), pink, Glu-255 (*Asp-278*), violet, His-268 (*His-291*), green (all three are residues of cytochrome *b*), His-121 of cytochrome c_1 (no evident counterpart in *R. capsulatus*), light green. See text and Ref. [98] for further details.

of such kinetic correspondence were already observed upon studies of the mitochondrial bc_1 [113–117] and of the bc_1 of *Rhodobacter* [118–120]. These observations have prompted a suggestion that the ET from the FeS domain to cytochrome c_1 might be mechanistically coupled with a reaction in center *N* [121]. Daldal and co-workers have indeed found correlations between the occupancy of center *N* and the mobility of the FeS domain [122,123].

Here, it seems worthy to focus on the energetic rationale of the coupling between centers *N* and *P*. As argued elsewhere [94,112], the single-turnover data indicate that the relocation of the FeS domain towards cytochrome c_1 and the proton release from center *P* are coupled with the formation of the Q_NH_2 ubiquinol and/or its release from center *N*. In support of this suggestion, Cooley and co-workers have shown recently that the center *N* inhibitor antimycin A, on one hand, imitates either the presence of Q_NH_2 or the absence of quinone, and, on the other hand, increases the mobility of the FeS domain maximally as if it were to facilitate its movement away from center *P* [123].

The mechanism of ubiquinol formation in center *N* is not well understood. It is believed, however, that this reaction should resemble the well-studied flash-induced formation of the Q_BH_2 ubiquinol in the photosynthetic reaction center (RC) of *R. sphaeroides* [124–126]. The Q_B binding site is approximately at the same membrane depth as center *N*. In the case of the RC, it has been shown that the ubiquinol formation is limited by the second protonation step, i.e., by the $Q_BH^- + H^+ \leftrightarrow Q_BH_2$ reaction [127]. Its equilibrium constant drops to ~1 already at pH ~9.0 [128], so that the overall RC turnover is driven by the liberation of the ubiquinol from the binding site [127]. Apparently, the proton unwillingly gets into the protein, although the driving redox reaction is favorable: the midpoint potential at pH 7.0 (E_m^7) of the primary quinone Q_A^-, the electron donor to Q_B, has been estimated as $\sim -50 \div -200$ mV [129,130], notably lower than the E_m^7 of Q_B of $60 \div 80$ mV [130,131].

In the bc_1, the electron donor to Q_N is the b_h heme. Its E_m^7 is about +50 mV, much higher than that of Q_A [132]. Hence, the Q_NH_2 formation per se might be unfavorable and can require additional energy input. In the same time, the proton that stays in center *P* after ubiquinol oxidation, while initially "balancing" an electron at heme b_l, becomes electrostatically uncompensated after the electron relocates towards heme b_h and center *N*. The energetically favorable release of this proton from the membrane can, in principle, drive the ubiquinol formation in center *N*. However, to achieve this goal, the proton release from center *P* has to be held up until the proton binding in center *N* can take place. Exactly this kinetic behaviour has been observed [107,108]. It is attractive to think that the conformational transmission across the enzyme serves to drive the proton binding in center *N* by the energetically gainful release of an uncompensated proton from center *P* (see Fig. 3B).

It seems useful to consider some analogies with the cytochrome *c* oxidase. In this enzyme, the redox reactions that take place in the middle of the membrane are coupled with proton pumping. Initially, it has been believed that the "pumped" protons are "sucked" by the enzyme in response to the reduction of its redox centers. The most recent data indicate that the situation is more complex: the steps of proton binding and proton release are coupled not only with the redox changes of the redox groups, but also with each other, so that proton binding and release seem to proceed in a concerted manner [133–135].

Alice didn't dare to argue the point, but went on: '— and I thought I'd try and find my way to the top of that hill.
'When you say "hill,"' the Queen interrupted,' I could show you hills, in comparison with which you'd call that a valley.'

Lewis Carroll. Through the Looking Glass [12].

'…His name is Haigha, and he lives…'
'He lives on the Hill,' the King remarked simply, … 'The other Messenger's called Hatta. I must have two, you know — to come and go. One to come, and one to go.'
'… I didn't understand,' said Alice. 'Why one to come and one to go?'
'Didn't I tell you?' the King repeated impatiently. 'I must have two — to fetch and carry. One to fetch, and one to carry.'

Lewis Carroll. Through the Looking Glass [12].

5. Outlook: bootstrapping as a common strategy of transmembrane ion transfer

Any ion translocator has to find means to pay the desolvation penalty. If no external energy sources are available, enzymes are in the position of Baron Münchhausen, who had to lift himself out of a swamp by pulling his own ponytail. In later editions, Münchhausen was using his own boot straps [136], which gave rise to the term *bootstrapping*. In fact, many enzymes use bootstrapping to decrease the activation barrier of the reaction they catalyze. According to the common belief, enzymes "store" a part of the substrate binding energy and utilize it to decrease the activation barrier upon the rate-limiting step of the catalytic cycle [137]. Correspondingly, in the case of membrane translocators, the energy of initial ion binding might be used to generate non-compensated electric charge in the membrane interior. Its neutralization, e.g., by the translocated ion proper, could eventually result in the ion transfer across the membrane. This mechanism is based on the intrinsic ability of enzymes to go between isoenergetic conformations differing in chemical reactivity of the catalytic groups [138].

Hence, even hypothetical mechanistic schemes of membrane translocators have to give details on how the desolvation penalty is paid. This rather rigorous constraint, together with other constraints, as imposed by structural information and functional data, could essentially decrease the number of models that deserve experimental testing. And vice versa, enzymes that do not use any tricks to decrease the desolvation penalty may remain impermeable for ions even when containing a water pore, as, for example, aquaporin [31,32].

The desolvation penalty has been routinely taken into account when considering the membrane ion channels [31,32,34–36]. In this review, I have attempted to relate the desolvation penalty to the mechanisms of energy-converting enzymes. With these enzymes, certain confusion can arise from mixing up the desolvation penalty with the energy of proton translocation proper. It is important to emphasize the difference between these two energy terms. The energy required to pump a proton at steady state is on the order of 200–300 meV (it corresponds to *pmf*). This energy is irreversibly utilized to transfer a proton into a phase with a higher proton potential. The energy required for a proton leap over the desolvation barrier is likely to be higher, on the order of 600–1000 meV [33], so that **in comparison with this hill**, the moderate potential step of *pmf* **can be called a valley** (see Fig. 1B, C). This large energy quantum, however, is needed only transiently: the ultimate proton ejection to the other side of the membrane is coupled with the release of compatible amount of energy. Still for a particular translocated proton, the endergonic step always precedes the exergonic one, so that some energy-borrowing machinery, as considered in the previous sections, is almost unavoidable. In the simplest case, for **one** proton **to come, one has to go**, so that a translocator **must have** at least **two** different proton-binding sites, **one to fetch** the charge and **one to carry** it away.

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