

## Proton-Electrostatic Localization: Explaining the Bioenergetic Conundrum in Alkalophilic Bacteria

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### Abstract

The decades-longstanding energetic conundrum of alkalophilic bacteria as to how they are able to synthesize ATP has now, for the first time, been clearly solved using the proton-electrostatics localization hypothesis [Bioenergetics 1:104; doi:10.4172/2167-7662.1000104]. This is a major breakthrough advance in understanding proton-coupling bioenergetics over the Nobel-prize work of Peter Mitchell's chemiosmotic theory. The widespread textbook Mitchellian proton motive force (pmf) equation has now been significantly revised. Use of the newly derived equation results in an overall pmf value (215~233 mV) that is more than 4 times larger than that (44.3 mV) calculated from the Mitchellian equation for the alkalophilic bacteria growing at pH 10.5. This newly calculated value is sufficient to overcome the observed phosphorylation potential  $\Delta G_p$  of -478 mV to synthesize ATP in the bacteria, which can now explain the 30-year-longstanding bioenergetics conundrum. This finding may have fundamental implications not only in the science of bioenergetics but also in understanding the importance of water to life not only as a solvent and substrate but also as a proton conductor for proton coupling energy transduction.

**Keywords:** Excess protons; Excess hydroxyl anions; Localized proton coupling; Proton/cation capacitor; Bioenergetics in alkalophilic bacteria; Proton motive force; Chemiosmotic theory

### Introduction

By the early 1970s, the chemiosmotic hypothesis [1-3] of Peter Mitchell was widely accepted as the best conceptual scheme to explain how ATP is formed in oxidative or photosynthetic phosphorylation. According to Mitchell's chemiosmotic hypothesis, the ATP synthase is coupled to the redox proton pumps via bulk phase-to-bulk phase proton electrochemical potential gradients generated across the biological membrane; while the membrane is regarded as an insulator between the two bulk phases that plays no role in the lateral transduction of the protons to the ATP synthase. After Mitchell (1961), the proton motive force (pmf)  $\Delta p$  that drives the protons through the ATP synthase is commonly written as

$$\text{pmf } (\Delta p) = -\Delta\tilde{\mu}_H^+ / F = \Delta\psi - 2.3RT / F \times \Delta\text{pH} \quad (1)$$

Where  $\Delta\psi$  is the electrical potential difference across the membrane,  $\Delta\text{pH}$  is the pH difference between the two bulk aqueous phases separated by the membrane, R is the gas constant, T is the absolute temperature, and F denotes the Faraday's constant. The Gibbs energy change is denoted by  $\Delta\tilde{\mu}_H^+$ .

However, the question of whether the proton pathway is delocalized throughout the bulk aqueous volume or is localized at its membrane surface has remained open to discussion since it was first raised by Williams in 1961 [4-8]. The most clear-cut observations that cannot be explained by the Mitchellian delocalized view are in alkalophilic bacteria [9-11] such as *Bacillus pseudofirmus*, which, as illustrated in Figure 1, keep their internal pH about 2.3 pH units more acidic than the ambient bulk pH 10.5, while  $\Delta\psi$  is about 180 mV in the direction from outside across the cellular membrane to the cytoplasm [12-14]. The application of Equation 1 in this case yields a pmf value so small (44.3 mV at T=298K) that it has remained as an enigmatic problem for the last three decades as to how these organisms can synthesize ATP [15-17]. This long-standing unresolved energetic conundrum [18,19] can now be explained by the proton-electrostatics localization

hypothesis [20] as is reported here.

In the past, a number of hypotheses for sequestered proton transfer to the ATP synthase from primary proton pumps have been proposed. One of the earliest such hypotheses by Williams [21,22] speculated proton pathways with "anhydrous" protons in/along the membrane, which would require proximity and specific properties of participating respiratory proton pumps and the ATP synthase. Subsequent hypotheses along this line were the "localized proton microcircuits" models [23] which suggested direct coupling between the respiratory proton pumps and the proton motive ATP synthase in the membrane. Recently, by the use of differential scanning calorimetry and saturation transfer electron paramagnetic resonance in a reconstituted system, the physical interaction between the cytochrome *caa3* and  $F_1F_0$ -ATP synthase from *B. pseudofirmus* OF4 was demonstrated [24]. However, the protein-structure specific pathways which the "localized proton microcircuits" models would require for conduction of "anhydrous" protons in the membrane have never been observed.

The high cardiolipin content of *B. pseudofirmus* OF4 was once believed to play a role in the "localized proton microcircuits", such as the possibility of fostering formation of complexes and interaction among protein complexes in the membrane [25]. Cardiolipin is a type of diphosphatidylglycerol lipid in which two phosphatidylglycerols connect with a glycerol backbone in the center, forming a dimeric structure. If its hydrophilic head group, as part of the lipid bilayer,

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indeed serves as a proton “trap” or part of a conduction pathway along the membrane surface, as suggested by Haines and Dencher [26], then it would unavoidably expose the “anhydrous” protons to water molecules of the bulk aqueous phase. Therefore, it is also questionable whether such “localized proton microcircuits” via cardiolipin could really exist. It indeed has now experimentally confirmed as expected that cardiolipin is dispensable for oxidative phosphorylation and non-fermentative growth of alkaliphilic *Bacillus pseudofirmus* OF4 [27].

In an effort trying to explain localized proton-coupling phenomena, Cherepanov and Mulkidjanian et al. proposed an interfacial barrier model [28], which used the dielectric permittivity of interfacial water [29]. According to their model, there would be a potential barrier of about 0.12 eV for protons in the water phase some 0.5–1 nm away from the membrane surface. This potential barrier was thought to retard the proton exchange between the membrane surface and the bulk aqueous phase thus causing an elevation of the proton concentration at the interface. A similar study along this line has recently been reported by another group [30]. If that type of hypothesis were correct, one would expect a localized proton-coupling in nearly all bioenergetic systems including thylakoids, mitochondria and bacteria, regardless of whether the trans-membrane potential difference ( $\Delta\psi$ ) is involved or not. That is, for example, if the interfacial proton barrier really exists in thylakoids, one would expect a localized proton-coupling even when the trans-membrane potential difference is removed. However, the Davenport-McCarty experiment [31] demonstrated the opposite was true: in the presence of valinomycin and 50 mM KCl (to remove  $\Delta\psi$ ), the permeable buffer imidazole caused significant delay in the onset of photophosphorylation, indicating delocalized proton coupling. Therefore, it is questionable whether the interfacial barrier model could be applicable to real-world bioenergetics systems.

A more important question is whether the interfacial barrier (if it exists) would be sufficient to explain the bioenergetics problem in alkaliphilic bacteria, since, under the Cherepanov-Mulkidjanian model, protons could still be lost by “futile proton escape” into the bulk periplasmic p-phase. As Cherepanov and Mulkidjanian et al. discussed [28], their model might have an issue of “futile proton escape” at a speed possibly as fast as a microsecond/millisecond event. The cell culture growth of alkaliphilic bacteria operates at a time scale for hours and days. Therefore, the “futile proton escape” into the bulk periplasmic p-phase would be so significant that it would be hard to explain the commonly observed excellent growth of alkaliphilic bacteria culture.

Another hypothesis considered the possibility of variable contributions of a “high c-subunit stoichiometry” and speculated that the alkaliphilic bacteria would use a higher number of the c-subunits per ring for the ATP synthase rotor [32]. This is in regard to the chemiosmotic bioenergetic proton ( $H^+$ )/ATP ratio in relation to the number of the c-subunits per ring for the ATP synthase rotor. All known  $F_0F_1$ -ATPsynthases contain three  $\beta$ -subunits; the number of c-subunits seems to be species-dependent. It has been reported to be 10 in mitochondria [33], 14 in chloroplasts [34], 10 in *E. coli* [35], 10 in thermophilic *Bacillus* PS3 [36] and 13 in *Bacillus pseudofirmus* OF4 [37,38] and *Bacillus* sp. strain TA2A1 [39]. According to that hypothesis, the chemiosmotic  $H^+$ /ATP ratio should be equal to the number of c-subunits, divided by the  $\beta$ -subunits. Therefore, there would have to be an ATP synthase rotor with 32 ( $3 \times 478/44.3$ ) c-subunits per ring to account for the observed ratio of the phosphorylation potential  $\Delta G_p$  to the pmf at pH 10.5 ( $\Delta G_p = -478$  mV and pmf = 44.3 mV) in *Bacillus pseudofirmus* [40]. However, so far, no such ATP synthase with 32 c-subunits per rotor ring has ever been found.

The proton-electrostatics localization hypothesis, which we elaborate in this article, can naturally explain the bioenergetics conundrum in alkaliphilic bacteria without requiring any of the “localized proton microcircuits”, “high c-subunit stoichiometry”, or putative interfacial barrier models.

## Results and Discussions

The proton-electrostatics localization hypothesis is based on the idea that a microscopic water body, such as the water within a bacterium (Figure 1), acts as a quasi proton conductor. It is known that protons can quickly transfer among water molecules by the “hops and turns” mechanism [41-43]. Notice also that, from the negative charge point of view, hydroxyl anions are transferred in the opposite direction of proton conduction.

This understanding suggests that excess free protons in a microscopic water body behave like electrons in a perfect conductor. It is well known that for a charged electrical conductor at static equilibrium, all the (extra) electrons reside on the conducting body’s surface [44]. It is reasonable to expect this since electrons repel each other, and, being free to move, they will spread out to the surface. By the same token, it is reasonable to expect that free excess protons (or conversely the excess hydroxyl anions) in a microscopic water body will move to its surface. Adapting this view to excess free hydroxyl anions in the cytoplasm (created by pumping protons across the cytoplasm membrane through the proton-transfer-coupled respiratory electron transport into the liquid medium outside the cell), they will be electrostatically localized along the water-membrane interface at the cytoplasmic (n) side of the cell membrane. In addition, their negative charges ( $OH^-$ ) will attract the positively charged species, namely the protons ( $H^+$ ) outside the cell to the membrane-water interface at the periplasmic p-side, as illustrated in (Figure 2).

One can mathematically justify this argument by using the Gauss Law equation of electrostatics and the fact that there can be no electric field  $E$  inside a conductor. Gauss’s Law relates the net charge  $Q$  within a volume to the flux of electric field lines through the closed surface surrounding the volume in the following equation [44].

$$\epsilon_0 \oint \mathbf{E} \cdot d\mathbf{S} = Q \quad (2)$$

Where  $\epsilon_0$  is the electric permittivity constant and  $dS$  is a differential surface element. Here the small circle on the integral sign indicates that the integration is performed over the closed surface. Consider then a series of integration applications, where a small volume at the center of the cytoplasm is gradually increased until it is just inside the cytoplasmic surface, indicated by  $r$  in (Figure 2). By definition, the electric field  $E$  is zero everywhere in a conductive body. Thus, in each case the left side of Equation 2 vanishes and therefore the right side must also vanish, which means that no net charge ( $Q = 0$ ) is within the volume; the excess hydroxyl anions in this case must therefore be at the cytosol water-body surface, i.e. at the water-membrane interface along the cytoplasmic n side of the cell membrane surface.

Similarly, considering the proton-conductive water outside the bacterium, the electric field  $E=0$  holds true everywhere in the water body of the liquid culture medium. Applying Gauss’s Law to a series of volumes enclosing the entire bacterial system and decreasing them to be just outside the bacterium membrane surface (indicated by  $R$  in Figure 2), the surface integrals of Equation 2 vanish and so no net excess charge is found. Since the excess hydroxyl anions are inside the bacterium, the positive charges (excess protons) must be at the membrane-water interface along the periplasmic membrane surface,

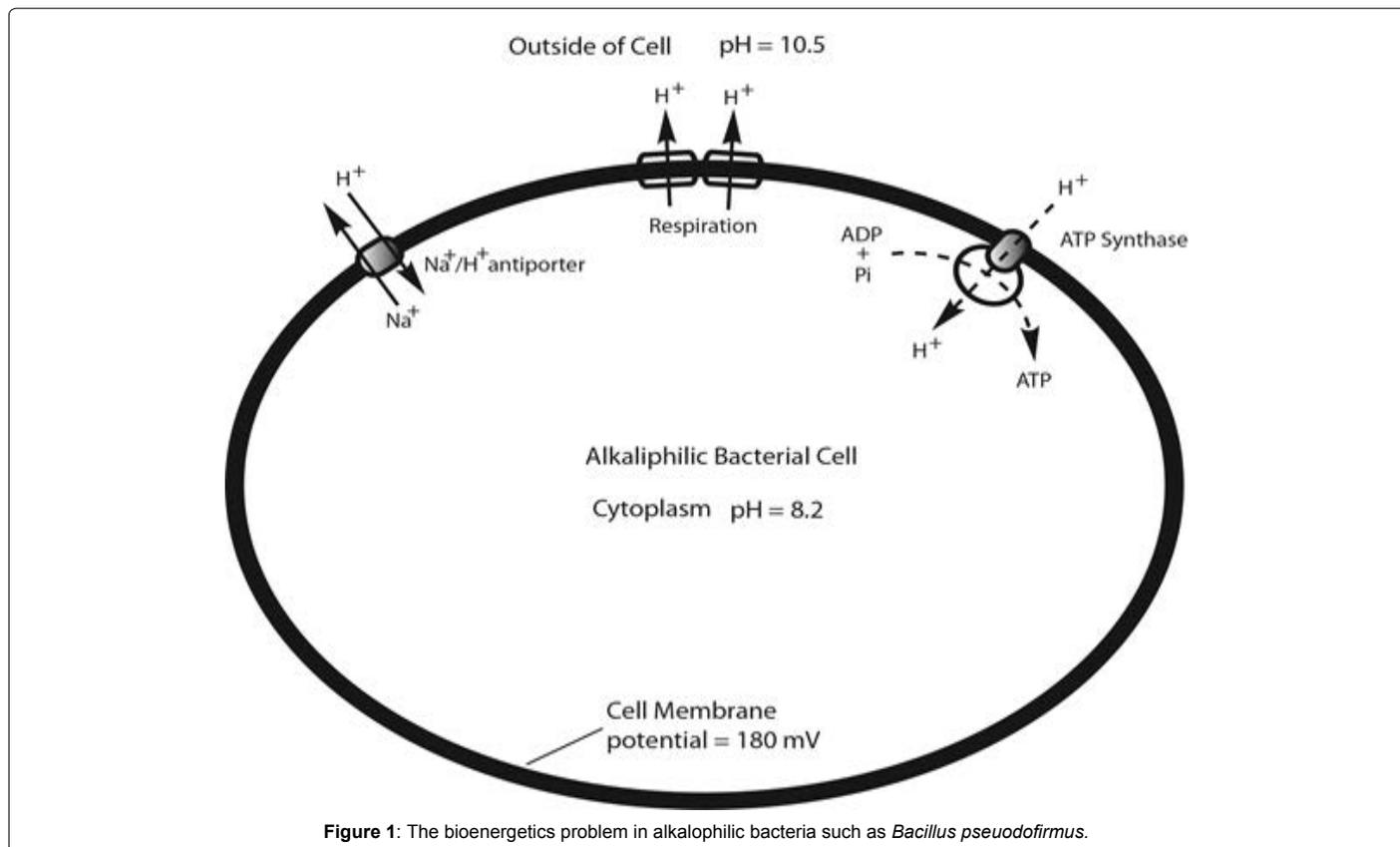


Figure 1: The bioenergetics problem in alkaliphilic bacteria such as *Bacillus pseudofirmus*.

precisely balancing the excess hydroxyl anions of the cytoplasmic side, making the total net charge of the entire system zero.

That is, when excess hydroxyl anions are created in the cytoplasm by the oxidative-driven proton pump across the membrane leaving excess protons outside the cell, the excess hydroxyl anions in the cytoplasm will not stay in the bulk water phase because of their mutual repulsion and consequently they go to the water-membrane interface at the cytoplasmic side of the membrane where they then attract the excess protons at the periplasmic side of the membrane, forming an “excess anions-membrane-excess protons” capacitor-like system (Figure 2).

It is noteworthy that a typical biological membrane contains negatively-charged surface groups, such as the phosphate groups of the membrane’s phospholipid molecules, at its two surface sides which can attract cations and/or protons and are believed to form “electrical double layers” along membrane surfaces [45]. Since these charges are fixed, their attracted protons (and/or cations) including the associated electrical double layers do not contribute to the pmf that drives proton flow through the ATP synthase [46]. One must not confuse the membrane fixed-charge-attached protons with the “excess free protons” that are electrostatically localized at the water-membrane interface. It is these “excess protons” generated by the respiratory electron transport systems that are relevant to the proton motive force, which can drive proton flow through the ATP synthase. Therefore, the surface-charges-attracted protons and/or cations including their associated electrical double layers are not shown in Figure 2, which focuses on illustrating the fundamental concept of the proton-electrostatics localization model that is relevant to the pmf.

According to the proton-electrostatics localization hypothesis, the Mitchellian proton motive force Equation 1, which has been in many

textbooks [47-53] for nearly a half century, must be revised. First of all, it is important to note that the proton-electrostatics localization (Figure 2) clearly indicates that the excess protons and hydroxyl ions may directly contribute to the trans-membrane potential difference  $\Delta\psi$ . In addition, the localized excess protons (their population density) will increase the probability for protons to be available at the ATP synthase, independently from that implied by the bulk pH value. To account for this effect, we now generalize the proton motive force equation for ATP synthesis as

$$\text{pmf}(\Delta p) = \Delta\psi + \frac{2.3RT}{F} \left( pH_{nb} + \log_{10} \left( [H_L^+] + [H_{pb}^+] \right) \right) \quad (3)$$

Where  $pH_{nb}$  is the cytoplasmic bulk phase pH;  $[H_L^+]$  is the effective concentration of the localized protons at the membrane-water interface; and  $[H_{pb}^+]$  is the proton concentration in the periplasmic bulk aqueous phase outside the cell.

How may we estimate the effective concentration of the localized protons ( $[H_L^+]$ )? According to Figure 2, excess protons in a theoretical pure water-membrane-water system are electrostatically localized at the membrane surface like a “proton capacitor”. Therefore, we may use the proton capacitor concept to analyze the localized proton density at the membrane-water interface from the associated trans-membrane potential  $\Delta\psi$ . The electrostatically localized protons (positive charges,  $Q$ ) per unit surface area ( $S$ ) at the membrane-water interface may be calculated as:

$$\frac{Q}{S} = \frac{C}{S} \cdot \frac{\Delta\psi}{F} = \frac{\Delta\psi \cdot \kappa \cdot \epsilon_0}{d \cdot F} \quad (4)$$

Where  $C/S$  is the membrane capacitance per unit surface area;  $F$  is

the Faraday constant;  $\kappa$  is the dielectric constant of the membrane;  $\epsilon_0$  is the electric permittivity; and  $d$  is the thickness of the membrane.

From the localized protons surface density (Equation 4), we can then calculate the effective concentration of the localized protons  $[H_L^+]^0$  at the membrane-water interface in a pure water-membrane-water system assuming a reasonable thickness ( $l$ ) for the localized proton layer using the following equation:

$$[H_L^+]^0 = \frac{C}{S} \cdot \frac{\Delta\psi}{l \cdot F} = \frac{\Delta\psi \cdot \kappa \cdot \epsilon_0}{d \cdot l \cdot F} \quad (5)$$

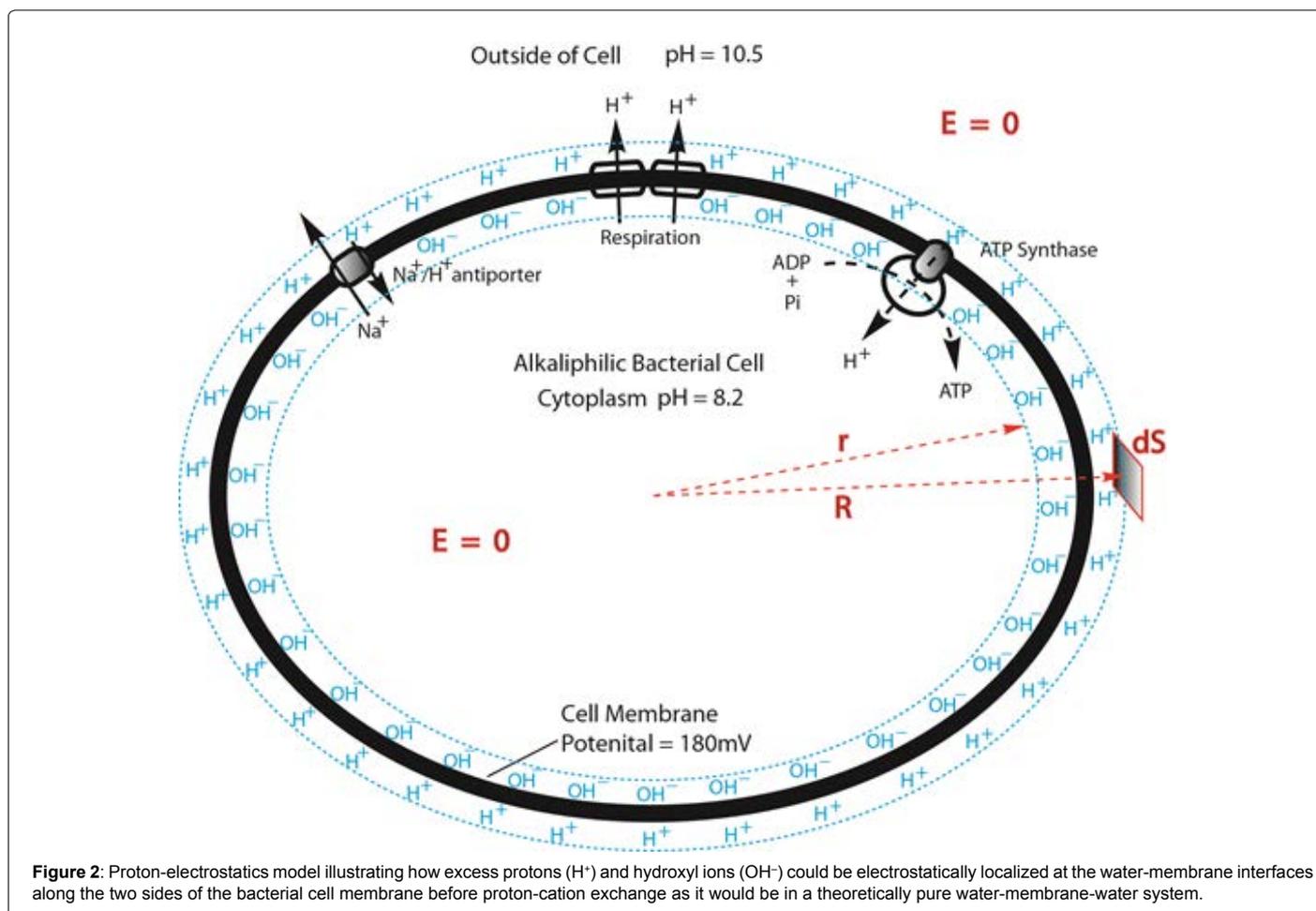
Recently, using nanoscale measurements with electrostatic force microscopy [54], the dielectric constant ( $\kappa$ ) of a lipid bilayer was determined to be about 3 units, which is in the expected range of 2~4 units [55]. Table 1 lists the calculation results for localized protons for a theoretical pure water-membrane-water system with Equations 4 and 5 using a lipid membrane dielectric constant  $\kappa$  of 3 units, membrane thickness  $d$  of 4 nm, trans-membrane potential difference  $\Delta\psi$  of 180 mV, and three assumed values for the proton layer thickness  $l$  of 0.5, 1.0, and 1.5 nm.

As shown in Table 1, the localized proton density per unit area was calculated to be  $1.238 \times 10^{-8}$  moles  $H^+ / m^2$ . The calculated effective concentration of localized proton ( $[H_L^+]^0$ ) was in a range from 8.25 mM to 24.76 mM if the localized proton layer is around  $1.0 \pm 0.5$  nm thick. The calculated effective pH of localized proton layer ( $pH_L^0$ ) was

1.61, 1.91, and 2.08 assuming that the localized proton layer is 0.5, 1.0, and 1.5-nm thick, respectively. It should be noted that this calculation assumed a pure-water membrane system or that the salt concentration in the culture medium is not too high, so that the effect of salt cation exchange with the localized protons may be neglected.

For a better analysis, these values calculated for a pure water-membrane-water system need to be corrected for the effect of exchange with other non-proton cations, such as  $Na^+$ , in the alkaliphilic bacteria liquid culture medium [19,40,56,57]. The liquid culture medium that was used in growing the alkaliphilic bacteria *Bacillus pseudofirmus* for the bioenergetics characterization at pH 10.5 contained about 300 mM  $Na^+$  (mainly 100 mM  $Na_2CO_3$  buffer plus 50 mM  $Na_2C_2H_2O(COO)_2$  as the carbon source) as its dominant non-proton cations. When non-proton cations in the bulk liquid phase exchange with the localized proton layer at the water-membrane interface, the effective concentration of the electrostatically localized protons may be decreased since some of the localized protons could be replaced by non-proton cations. The effective concentration of the electrostatically localized protons at the equilibrium of cation exchange can be calculated as:

$$[H_L^+] = \frac{[H_L^+]^0}{\prod_{i=1}^n \{K_{Pi} \left( \frac{[M_{pB}^{i+}]}{[H_{pB}^+]} \right) + 1\}} \quad (6)$$



**Figure 2:** Proton-electrostatics model illustrating how excess protons ( $H^+$ ) and hydroxyl ions ( $OH^-$ ) could be electrostatically localized at the water-membrane interfaces along the two sides of the bacterial cell membrane before proton-cation exchange as it would be in a theoretically pure water-membrane-water system.

Assumed thickness ( <i>l</i> ) of localized proton layer	0.5 nm	1.0 nm	1.5 nm
Localized proton density per unit area (moles H <sup>+</sup> /m <sup>2</sup> )	1.238 x 10 <sup>-8</sup>	1.238 x 10 <sup>-8</sup>	1.238 x 10 <sup>-8</sup>
Effective concentration of localized proton ([H <sub>L</sub> <sup>+</sup> ] <sup>0</sup> )	24.76 mM	12.38 mM	8.25 mM
Effective pH of localized proton layer (pH <sub>L</sub> <sup>0</sup> )	1.61	1.91	2.08

**Table 1:** Calculation of localized protons with equations 4 and 5 in a theoretical pure water-membrane-water system using a membrane dielectric constant  $\kappa$  of 3, membrane thickness  $d$  of 4 nm, and trans-membrane potential difference  $\Delta\psi$  of 180 mV.

Where  $[H_L^+]^0$  is the effective concentration of localized protons without cation exchange, as expressed in Equation 5. Here  $K_{p_i}$  is the equilibrium constant for non-proton cations ( $M^{i+}$ ) to exchange for the localized protons at the water-membrane interface;  $[M_{pB}^{i+}]$  is the concentration of the non-proton cations in the liquid culture medium; and  $[H_{pB}^+]$  is the proton concentration in the bulk phase of the liquid culture medium.

Consequently, when cation exchange with the localized protons is considered, we now have an updated new pmf ( $\Delta p$ ) equation, which is expanded from Equation 3 with Equation 6, and may be completely written as:

$$\text{pmf}(\Delta p) = \Delta\psi + \frac{2.3RT}{F} \left( pH_{nB} + \log_{10} \left( \frac{C}{S} \cdot \frac{\Delta\psi}{l \cdot F \left( \prod_{i=1}^n K_{p_i} \left( \frac{[M_{pB}^{i+}]}{[H_{pB}^+]} + 1 \right) \right)} + [H_{pB}^+] \right) \right) \quad (7)$$

Since protons have the smallest atomistic diameter and can exist as part of the water molecules, they can electrostatically distribute themselves to the water-membrane interface much more easily than any other cations, such as Na<sup>+</sup>, Mg<sup>2+</sup> or K<sup>+</sup>. Therefore, we expect that the equilibrium constant for protons to electrostatically occupy the cation sites at the water-membrane interface (in any possible competition with any other cations) should be much larger than one. Certain cation exchange experimental studies [58,59] have recently indicated that the equilibrium constant for protons to exchange with other cations for cation binding sites can be on the order of 4.7 x 10<sup>6</sup>. Conversely, the equilibrium constant  $K_{p_i}$  for non-proton cations such as Na<sup>+</sup> to delocalize the localized protons from the membrane-water interface is in the order of 2.1 x 10<sup>-7</sup>.

Table 2 presents bioenergetic properties calculated for the alkalophilic bacteria using Equation 7 with Equations 5 and 6. As listed in column 2 of Table 2, the effective concentration of localized proton  $[H_L^+]$ , after the cation exchange equilibrium, was calculated to be 2.439 x 10<sup>-8</sup> M, which translates to an effective pH (pH<sub>L</sub>) of 7.6 in the localized proton/cation layer at the membrane-water interface along the bacterial cell surface. The overall pmf ( $\Delta p$ ) of 215 mV was calculated at standard temperature of 298K with the following parameters:  $\Delta\psi$  of 180 mV; cytoplasmic  $pH_{nB}$  of 8.2; a membrane dielectric constant  $\kappa$  of 3 units; membrane thickness  $d$  of 4 nm; localized proton layer thickness  $l$  of 1 nm;  $K_{p_i}$  of 2.1 x 10<sup>-7</sup>; bulk phase non-proton cation concentration  $[M_{pB}^{i+}]$  of 300 mM Na<sup>+</sup>, 3.584 mM of K<sup>+</sup>, 0.1 mM of Mg<sup>2+</sup>, 0.4557 mM Ca<sup>2+</sup>, 38.08 μM Zn<sup>2+</sup>, 25.174 μM Fe<sup>2+</sup>, 5.557 μM Mn<sup>2+</sup>, 1.602 μM Cu<sup>2+</sup>, and 0.859 μM Co<sup>2+</sup>; and periplasmic bulk phase proton concentration  $[H_{pB}^+]$  of 10<sup>-10.5</sup> M outside the cell.

Using this calculated pmf (215 mV), which is over 4 times larger than the value of 44.3 mV calculated from the Mitchellian pmf of Equation 1, the ratio of the observed phosphorylation potential  $\Delta G_p$  (-478 mV/ATP) to the calculated pmf turned out to be about -2.2 protons/ATP. This means that the minimal thermodynamically

required number of protons to make an ATP could be around 2.2. Therefore, use of the newly calculated pmf through any of the known F<sub>0</sub>F<sub>1</sub>-ATP synthases with 13 or 10 c-subunits per ring (expecting the use of 3.3 protons per ATP) will be more than enough to overcome the observed phosphorylation potential  $\Delta G_p$  for ATP synthesis. That is, use of the proton-electrostatics localization hypothesis as numerically expressed in Equation 7 has now, for the first time, clearly solved the decades-longstanding bioenergetic conundrum in alkalophilic bacteria.

Recently, the specific membrane capacitance per unit surface area of single cells was measured to be 12.0 and 14.4 mf/m<sup>2</sup> for two model cell culture lines [60]. Using an averaged membrane capacitance of 13.2 mf/m<sup>2</sup> as shown in Table 2 (column 3), the effective concentration of localized proton  $[H_L^+]$  after the cation exchange equilibrium was calculated to be 4.853 x 10<sup>-8</sup> M, which translates to an effective pH (pH<sub>L</sub>) of 7.3 in the localized proton layer. The overall pmf in this calculation turned out to be 233 mV, which is again over 4 times larger than the value of 44.3 mV calculated from the Mitchellian pmf of Equation 1.

It is worthwhile to point out that significantly more research is needed on the proton-electrostatics localization hypothesis as well. For example, the thickness of the localized proton layer was assumed to be around 1 nm. Further research is needed to determine the exact effective thickness of the localized proton layer. It is also important to measure the effective concentration of localized proton  $[H_L^+]$  experimentally. We noticed that an independent study using the technique of pH-sensitive fluorescein-phosphatidyl-ethanolamine has recently detected a localized proton  $\Delta p H_L$  surface component in mitochondria [61]. Furthermore, as mentioned previously, the cation-proton exchange equilibrium constant  $K_{p_i}$  value (2.1 x 10<sup>-7</sup>) used in the pmf calculation above was estimated from the results of biochar surface cation exchange experimental studies. Further research is needed to more accurately determine the  $K_{p_i}$  value. Therefore, the newly calculated pmf ( $\Delta p$ ) (215~233 mV) as reported above represents the first, groundbreaking, numerical result from the proton-electrostatics localization model; although it may not be treated as a precise value, the numerical result nonetheless shows the usefulness of the revised pmf equation (Equation 7) in explaining the bioenergetic conundrum in alkalophilic bacteria.

## Conclusion

In conclusion, we have now, for the first time, explained the decades-longstanding bioenergetic conundrum in alkalophilic bacteria [62,63] numerically using the proton-electrostatics localization hypothesis. The widespread textbook Mitchellian proton motive force equation (Equation 1) has now been revised. Use of the newly derived pmf equation (Equation 7) resulted in an overall pmf value (215~233 mV) that is more than 4 times larger than that (44.3 mV) calculated from the Mitchellian equation for the alkalophilic bacteria growing at pH 10.5. This newly calculated pmf is sufficient to overcome the observed phosphorylation potential  $\Delta G_p$  of -478 mV to synthesize ATP through the known F<sub>0</sub>F<sub>1</sub>-ATP synthases with 13 or 10 c-subunits per ring. The conundrum is largely due to the deficiency of the Mitchellian equation (1) that underestimates the true pmf value by a factor of more than 4

	Using membrane dielectric constant $\kappa$ of 3 and thickness $d$ of 4 nm	Using averaged membrane capacitance of 13.2 mf /m <sup>2</sup>
Effective concentration of localized protons ( $[H_L^+]$ )	2.439 x10 <sup>-8</sup> M	4.853 x10 <sup>-8</sup> M
Effective pH of localized proton layer (pH <sub>L</sub> )	7.6	7.3
Cytoplasmic pH (pH <sub>nb</sub> )	8.2	8.2
Periplasmic bulk phase proton concentration ( $[H_{pb}^+]$ )	10 <sup>-10.5</sup> M	10 <sup>-10.5</sup> M
Trans-membrane potential difference ( $\Delta\psi$ )	180 mV	180 mV
Proton motive force pmf ( $\Delta p$ )	215 mV	233 mV

**Table 2:** Bioenergetic properties calculated for the alkaliphilic bacteria using equation 7 with equations 5 and 6 using  $\kappa_m$  of  $2.1 \times 10^{-7}$  in consideration of 300 mM Na<sup>+</sup>, 3.584 mM K<sup>+</sup>, 0.1 mM Mg<sup>2+</sup>, 0.4557 mM Ca<sup>2+</sup>, 38.08  $\mu$ M Zn<sup>2+</sup>, 25.174  $\mu$ M Fe<sup>2+</sup>, 5.557  $\mu$ M Mn<sup>2+</sup>, 1.602  $\mu$ M Cu<sup>2+</sup>, and 0.859  $\mu$ M Co<sup>2+</sup> in liquid culture medium in cation exchange with the localized proton layer (assumed to be 1 nm thick).

times in this case, since it fails to describe the contribution from the electrostatically localized proton cloud around the bacterial cell.

Finally, it is worthwhile to note: when the membrane potential  $\Delta\psi$  which is associated with the electrostatically localized proton cloud is zero, our newly derived pmf equation (7) naturally merges precisely with the Mitchellean equation (1), which can perfectly explain the Jagendorf acid-base jump experiment [64]. This also means that Peter Mitchell's chemiosmotic theory is not entirely wrong; as we acknowledged previously [20], the chemiosmotic theory is still a cornerstone in bioenergetics although it has its deficiency. Our work reported here may be regarded as a further development over it, which can now provide a unified framework for understanding the energetics in many biological systems. In addition to explaining the decades-longstanding bioenergetic conundrum in the alkaliphilic bacteria, the proton-electrostatics localization hypothesis may also have fundamental implications to better understand the bioenergetics in other biological systems [65-67] including (but not limited to) mitochondria [68-73] and chloroplasts [74-77]. It may also help to understand how life began on Earth in relation to the importance of water not only as a solvent and substrate but also as a proton conductor for proton coupling energy transduction. Through this study, it is now also quite clear that water serves not only as a solvent and substrate but also as a proton conductor for proton coupling energy transduction in living organisms.

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#### References

- Mitchell P (1961) Coupling of phosphorylation to electron and hydrogen transfer by a chemi-osmotic type of mechanism. *Nature* 191: 144-148.
- Mitchell P (1976) Possible molecular mechanisms of the protonmotive function of cytochrome systems. *J Theor Biol* 62: 327-367.
- Mitchell P (1978) David Keilin's respiratory chain concept and its chemiosmotic consequences. Nobel Lecture 295-330.
- Williams RJ (2011) Chemical advances in evolution by and changes in use of space during time. *J Theor Biol* 268: 146-159.

- Ferguson SJ (1985) Fully delocalized chemiosmotic or localized proton flow pathways in energy coupling? A scrutiny of experimental evidence. *Biochim Biophys Acta* 811: 47-95.
- Vinkler C, Avron M, Boyer PD (1978) Initial formation of ATP in photophosphorylation does not require a proton gradient. *FEBS Letters* 96: 129-134.
- Dilley RA, Theg SM, Beard WA (1987) Membrane-proton interaction in chloroplast bioenergetics: Localized proton domains. *Ann Rev Plant Physiol* 38: 347-387.
- Dilley RA (2004) On why thylakoids energize ATP formation using either delocalized or localized proton gradients - a Ca<sup>2+</sup> mediated role in thylakoid stress responses. *Photosynthesis Research* 80: 245-263.
- Krulwich TA, Guffanti (1989) Alkaliphilic bacteria. *Ann Rev Microbiol* 43: 435-463.
- Krulwich TA (1986) Bioenergetics of alkaliphilic bacteria. *J Membrane Biol* 89: 113-125.
- Olsson K, Keis S, Morgan HW, Dimroth P, Cook GM (2003) Bioenergetic properties of the thermo alkaliphilic *Bacillus* sp strain TA2.A1. *Journal of Bacteriology* 185: 461-465.
- Padan E, Bibi E, Ito M, Krulwich TA (2005) Alkaline pH homeostasis in bacteria: New insights. *Biochimica et Biophysica Acta* 1717: 67-88.
- Krulwich TA (1995) Alkaliphiles: 'basic' molecular problems of pH tolerance and bioenergetics. *Molecular Microbiology* 15: 403-410.
- Sturr MG, Guffanti AA, Krulwich TA (1994) Growth and bioenergetics of alkaliphilic *Bacillus firmus* OF4 in continuous culture at high pH. *Journal of Bacteriology* 176: 3111-3116.
- Guffanti AA, Krulwich TA (1984) Bioenergetic problems of alkaliphilic bacteria. *Biochem Soc Trans* 12: 411-414.
- Krulwich TA, Gilmour R, Hicks DB, Guffanti AA, Ito M (1998) Energetics of alkaliphilic *Bacillus* species: physiology and molecules. *Advances in Microbial Physiology* 40: 401-438.
- Krulwich TA, Liu J, Morino M, Fujisawa M, Hicks DB et al. (2011) Adaptive mechanisms of extreme alkaliphiles. *Extremophiles handbook* 119-139.
- Guffanti AA, Krulwich TA (1988) ATP synthesis is driven by an imposed  $\Delta p_H$  or  $\Delta \mu_H^+$  but not by a  $\Delta p_{Na^+}$  or  $\Delta \mu_{Na^+}$  in alkaliphilic *Bacillus firmus* OF4 at high pH. *The Journal of Biological Chemistry* 253: 14748-14752.
- Guffanti AA, Hicks DB (1991) Molar growth yields and bioenergetic parameters of extremely alkaliphilic *Bacillus* species in batch cultures, and growth in a chemostat at pH 10.5. *Journal of General Microbiology* 137: 2375-2379.
- Lee JW (2012) Proton-electrostatics hypothesis for localized proton coupling bioenergetics. *Bioenergetics* 1: 104.
- Williams RJP (1961) Possible function of chains of catalysts. *Journal of Theoretical Biology* 1: 1-17.
- Williams RJP (1975) Proton-driven phosphorylation reactions in mitochondrial and chloroplast membranes. *FEBS Letters* 53: 123-125.
- Branden M, Sanden T, Brzezinski P, Widengren J (2006) Localized proton microcircuits at the biological membrane-water interface. *Proc Natl Acad Sci* 103: 19766-19770.

24. Liu X, Gong X, Hicks DB, Krulwich TA, Yu L et al. (2007) Interaction between cytochrome *caa3* and  $F_1F_0$ -ATP synthase of alkaliphilic *Bacillus pseudofirmus* OF4 is demonstrated by saturation transfer electron paramagnetic resonance and differential scanning calorimetry assays. *Biochemistry* 46: 306-313.
25. Krulwich TA, Hicks DB, Swartz T, Ito M (2007) Bioenergetic adaptations that support alkaliphily, in *Physiology and Biochemistry of Extremophiles* edited by Gerday C and Glansdorf N, ASM Press, Washington, DC.
26. Hanes TH, Dencher NA (2002) Cardiolipin: a proton traps for oxidative phosphorylation. *FEBS Letters* 528: 35-39.
27. Liu J, Ryabichko S, Bogdanov M, Fackelmayer OJ, Dowhan W, et al. (2014) Cardiolipin Is Dispensable for Oxidative Phosphorylation and Non-fermentative Growth of Alkaliphilic *Bacillus pseudofirmus* OF4. *J Biol Chem* 289: 2960-2971.
28. Mulikidjanian AY, Heberle J, Cherepanov DA (2006) Protons @ interfaces: Implications for biological energy conversion. *Biochimica et Biophysica Acta* 1757: 913-930.
29. Cherepanov DA, Feniouk BA, Junge W, Mulikidjanian AY (2003) Low dielectric permittivity of water at the membrane interface: Effect on the energy coupling mechanism in biological membranes. *Biophysical Journal* 85: 1307-1316.
30. Zhang C, Knyazev DG, Vereshaga YA, Ippoliti E, Nguyen TH, et al. (2012) Water at hydrophobic interfaces delays proton surface-to-bulk transfer and provides a pathway for lateral proton diffusion. *Proc Natl Acad Sci* 109: 9744-9749.
31. Davenport JW, McCarty RE (1980) The onset of photophosphorylation correlates with the rise in transmembrane electrochemical proton gradients. *Biochimica et Biophysica Acta* 589: 353-357.
32. Hicks DB, Liu J, Fujisawa M, Krulwich TA (2010)  $F_1F_0$ -ATP synthase of alkaliphilic bacteria: lessons from their adaptations. *Biochim Biophys Acta* 1797: 1362-1377.
33. Stock D, Leslie A, Walker J (1999) Molecular architecture of the rotary motor in ATP Synthase. *Science* 286: 1700-1705.
34. Seelert H, Poetsch A, Dencher NA, Enge A, Stahlberg H, et al. (2000) Structural biology: Proton-powered turbine of a plant motor. *Nature* 405: 418-419.
35. Jiang W, Hermolin J, Fillingame RH (2001) The preferred stoichiometry of c subunits in the rotary motor sector of *Escherichia coli* ATP synthase is 10. *Proc Natl Acad Sci* 98: 4966-4971.
36. Mitome N, Suzuki T, Hayashi S, Yoshida M (2004) Thermophilic ATP synthase has a decamer c-ring: Indication of noninteger 10:3 H<sup>+</sup>/ATP ratio and permissive elastic coupling. *Proc Natl Acad Sci* 101: 12159-12164.
37. Preiss L, Klyszejko AL, Hicks DB, Liu J, Fackelmayer OJ, et al. (2013) The c-ring stoichiometry of ATP synthase is adapted to cell physiological requirements of alkaliphilic *Bacillus pseudofirmus* OF4. *Proc Natl Acad Sci* 110: 7874-7879.
38. Preiss L, Langer JD, Hicks DB, Liu J, Yildiz O, et al. (2014) The c-ring ion binding site of the ATP synthase from *Bacillus pseudofirmus* OF4 is adapted to alkaliphilic lifestyle. *Mol Micro biol* 92: 973-984.
39. Meier T, Morgner N, Matthies D, Pogoryelov D, Keis S, et al. (2007) A tridecameric c ring of the adenosine triphosphate (ATP) synthase from the thermoalkaliphilic *Bacillus sp.* strain TA2.A1 facilitates ATP synthesis at low electrochemical proton potential. *Mol Micro biol* 65: 1181-1192.
40. Sturr MG, Guffanti AA, Krulwich TA (1994) Growth and bioenergetics of alkaliphilic *Bacillus firmus* OF4 in continuous culture at high Ph. *Journal of Bacteriology* 176: 3111-3116.
41. Pomes R, Roux B (2002) Molecular mechanism of H<sup>+</sup> conduction in the single-file water chain of the gramicidin channel. *Biophysical Journal* 82: 2304-2316.
42. Mark D, Tuckerman ME, Hutter J, Parrinello M (1999) The nature of hydrated excess proton in water. *Nature* 397: 601-604.
43. Marx D (2006) Proton transfer 200 years after Von Grothuss: insights from ab initio simulation. *Chem Phys Chem* 7: 1848-1870.
44. Ohanian HC (1985) Gauss' law, in *Physics textbook* (ISBN 0-393-95401-3) W. W. Norton & Company. New York 24: 565-573.
45. McLaughlin S (1989) The electrostatic properties of membranes. *Ann Rev Biophys Biophys Chem* 18: 113-136.
46. Lee JW (2013) Membrane-surface-charges-attracted protons are not relevant to proton motive force. *Bioenergetics* 2: e114.
47. Nicholls DG, Ferguson SJ (1992) *Bioenergetics* 2. (2ndedn), Academic Press, Michigan, New York.
48. Nelson DN, Cox MC (2013) *Lehninger Principles of Biochemistry textbook*. W H Freeman and Company 731-748.
49. Garrett RH, Grisham CM (2013) *Biochemistry textbook* (5thedn), Brooks/Cole CENGAGE Learning, United States of America.
50. Voet D, Voet JG, Pratt CW (2013) *Fundamentals of Biochemistry textbook*. (4thedn), John Wiley & Sons, Inc, United States of America.
51. Berg JM, Tymoczko JL, Stryer L (2012) *Biochemistry textbook* (7thedn), W. H. Freeman, New York.
52. Mathews CK, Van Holde KE, Appling DR, Anthony-Cahill SJ (2013) *Biochemistry textbook*. (4thedn) Pearson Canada Inc., Ontario, Canada.
53. Devlin TM (2010) *Textbook of Biochemistry with Clinical Correlations*. (7thedn), John Wiley & Sons, United States of America.
54. Grames G, Dole-Perez A, Edwards MA, Fumagalli EL, Gomila G (2013) Nanoscale measurement of the dielectric constant of supported lipid bilayer in aqueous solutions with electrostatic force microscopy. *Biophysical Journal* 104: 1257-1262.
55. Heimburg T (2012) The capacitance and electromechanical coupling of lipid membranes close to transitions: the effect of electrostriction. *Biophysical Journal* 103: 918-929.
56. Guffanti AA, Susman P, Blanco R, Krulwich TA (1978) The protonmotive force and  $\alpha$ -aminoisobutyric acid transport in an obligately alkaliphilic bacterium. *The Journal of Biological Chemistry* 253: 708-715.
57. Krulwich TA (2014) The full recipe of the alkaliphilic bacteria liquid culture media, provided to Lee JW through their personal communications.
58. Lee JW, Kidder M, Evans BR, Paik S, Buchanan AC, et al. (2010) Characterization of biochars produced from cornstover for soil amendment. *Environ Sci Technol* 44: 7970-7974.
59. Skjemstad JO, Gillman GP, Massis A, Spouncer LR (2008) Measurement of cation exchange capacity of organic-matter fractions from soils using a modified compulsive exchange method. *Communications in Soil Science and Plant Analysis* 39: 926-937.
60. Zheng Y, Shojaei-Baghoni E, Wang C, Sun Y (2013) Microfluidic characterization of specific membrane capacitance and cytoplasm conductivity of single cells. *Biosensors and Bioelectronics* 42: 496-502.
61. Xiong JW, Zhu L, Jiao X, Liu SS (2010) Evidence for  $\Delta\text{pH}^{\text{S}}$  of proton motive force in ATP synthesis of mitochondria. *Biochimica et Biophysica Acta* 1800: 213-222.
62. Krulwich TA (2007) Bioenergetic adaptations that support alkaliphily. *Physiology and Biochemistry of Extremophiles* 24: 311-329.
63. Krulwich TA, Sachs G, Padan E (2011) Molecular aspects of bacterial pH sensing and homeostasis. *Nature Reviews: Microbiology* 9: 330-343.
64. Jagendorf AT, Uribe E (1966) ATP formation caused by acid-base transition of spinach chloroplasts. *Proc Natl Acad Sci* 55: 170-177.
65. Kell DB (1979) On the functional proton current pathway of electron transport phosphorylation. An electrodic view. *Biochimica et Biophysica Acta* 549: 55-99.
66. Westerhoff HV, Melandri BA, Venturoli G, Azzone GF, Bell DB (1984) A minimal hypothesis for membrane-linked free-energy transduction: the role of independent, small coupling units. *Biochimica et Biophysica Acta* 768: 257-292.
67. Van der Bend RL, Petersen J, Berden JA, Van Dam K, Westerhoff HV (1985) A critical appraisal of evidence for localized energy coupling. *Biochem J* 230: 543-549.
68. Hinkle PC (2005) P/O ratios of mitochondrial oxidative phosphorylation. *Biochimica et Biophysica Acta* 1706: 1-11.
69. Hinkle PC, Kumar MA, Resetar A, Harris DL (1991) Mechanistic stoichiometry of mitochondrial oxidative phosphorylation. *Biochemistry* 30: 3576-3582.
70. Krishnamoorthy G, Hinkle PC (1984) Non-Ohmic proton conductance of mitochondria and liposomes. *Biochemistry* 23: 1640-1645.
71. Scholes TA, Hinkle PC (1984) Energetics of ATP-driven reverse electron transfer from Cytochrome c to fumarate and from Succinate to NAD in submitochondrial particles. *Biochemistry* 23: 3341-3345.

72. Rottenberg H, Robertson DE, Rubin E (1985) The effect of temperature and chronic ethanol feeding on the proton electrochemical potential and phosphate potential in rat liver mitochondria. *Biochimica et Biophysica Acta* 809: 1-10.
73. Brand MD, Chien LF, Ainscow EK, Rolfe D, Porter RK (1994) The cause and functions of mitochondrial proton leak. *Biochimica et Biophysica Acta* 1187: 132-139.
74. Jagendorf AT (2002) Photophosphorylation and the chemiosmotic perspective. *Photosynth Res* 73: 233-241.
75. Junge W (1987) Complete tracking of transient proton flow through active chloroplast ATP synthase. *Proc Natl Acad Sci* 84: 7984-7088.
76. Junge W (2004) Protons, proteins and ATP. *Photosynth Res* 80: 197-221.
77. Barber J (1980) Membrane surface charges and potentials in relation to photosynthesis. *Biochimica et Biophysica Acta* 594: 253-308.