Proton-Electrostatics Hypothesis for Localized Proton Coupling Bioenergetics

James Weifu Lee[1,2]*

[1]Department of Chemistry and Biochemistry, Old Dominion University, Physical Sciences Building 3100, Norfolk, VA 23529, USA
[2]Whiting School of Engineering, Johns Hopkins University, 118 Latrobe Hall, Baltimore, MD 21218, USA

Abstract

According to the proton-electrostatics localization hypothesis presented in this work, protons injected into a thylakoid may be electrostatically localized at the water-membrane interface along the lumenal surface. This hypothesis provides a natural frame work to explain a wide range of experimental observations in the bioenergetics of chloroplast and other biological systems conducted since the 1960s, including the longstanding well-characterized energetic problems of alkalophilic bacteria such as *Bacillus firmus*. It can also help reconcile the elegant scientific observations of both the Dilley experiment and the Junge neutral-red thylakoid proton detection. Our analysis indicates that the Mitchellian view of delocalized proton coupling to ATP synthase could only be true under special circumstances; namely, when the membrane electrical potential difference is near zero and the bulk phase-to-bulk phase pH difference becomes the dominant factor. The proton coupling under most physiological conditions of photosynthesis is likely to occur in a mixed state of proton electrostatic localization of excess charge at the membrane surface and delocalization in the bulk media. The proton-electrostatics localization hypothesis leads to a new bioenergetics equation for the proton motive force which may provide a unified framework for understanding the energetics of many biological systems.

Introduction

Mitchellian view

By the early 1970s, the chemiosmotic hypothesis [1,2] of Peter Mitchell was widely accepted by bioenergetics researchers as the best conceptual scheme to explain how ATP is formed in oxidative or photosynthetic phosphorylation. It is well recognized that the driving force of ATP synthesis catalyzed by the thylakoid membranes in chloroplasts is linked to a flux of protons (H^+) between the membrane-bounded donors (redox H^+ pumps: photosystem II and cytochrome b/f complex) and acceptors (the ATP synthase enzymes: CF_oCF_1 complexes). According to Mitchell's chemiosmotic hypothesis, illustrated in Figure 1, the ATP synthase is coupled to the redox H^+ pumps via bulk phase-to-bulk phase proton electrochemical potential gradients generated across the thylakoid 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) that drives the protons through the ATP synthase is commonly written as

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

where $\Delta\psi$ is the electrical potential difference across the membrane (trans-membrane potential difference), ΔpH is the pH difference between the two bulk aqueous phases separated by the membrane, R is the gas constant, T is the temperature, and F denotes the Faraday constant. The Gibbs energy change is denoted by $\Delta \tilde{\mu}_{H+}$.

Contrary experiments

However, the question of whether the proton pathway is delocalized throughout the bulk interior aqueous volume or is localized at its membrane surface has remained open to discussion since it was first raised by Williams in 1961 [3,4]. There have been over 200 papers in the literature regarding this long-standing controversial issue. The most clear-cut observations that cannot be explained by the Mitchellian

delocalized view are in alkalophilic bacteria [5,6], such as *Bacillus firmus*, which keep their internal pH about 2-3 pH units more acidic than the ambient one, while $\Delta\psi$ is about 200 mV. The application of Equation 1 in this case yields a pmf value so small that it has remained as an enigmatic problem for decades as to how these organisms can synthesize ATP [7].

In the case of chloroplasts, the major conflicts with the classic chemiosmotic theory are from the following observations [8] by Dilley, Ort and others since 1976: (1) photophosphorylation occurs even in the presence of permeable buffers that abolish the pH difference between the two bulk phases; and (2) photophosphorylation begins before proton distribution into the bulk phase could occur.

In an effort to explain the apparent shortcomings of the delocalized bulk phase proton pathway, Dilley proposed a massive protein structure-based construction along the thylakoid membrane. That is, Dilley's model requires putative occluded domains to form a localized H^+ relay pathway along the membrane. However, such occluded protein domains have never been found. More recently, Cherepanov et al. [9] have proposed an interfacial proton barrier model to provide a proton pathway localized at the membrane surface. However, as discussed below, it is questionable whether the massive protein structure-based model and/or the putative interfacial proton barrier model could satisfactorily accommodate the observed complex proton-coupling phenomena. In the present paper, a proton-electrostatics localization hypothesis is introduced to provide a possibly unified framework to explain the observed phenomena.

Proton-electrostatics localization hypothesis

The proton-electrostatics localization hypothesis is based on the idea that a microscopic water body, such as the water within a thylakoid lumen, could be thought as a quasi proton conductor. As illustrated in Figure 2A, it is known that protons can quickly transfer among water molecules by the "hops and turns" mechanism [10]. Notice also, from the negative charge point of view, that hydroxyl anions are transferred in the opposite direction.

This understanding suggests that free excess 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 [11]. 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 in a microscopic water body will move to its surface. Adapting this view to protons injected into the thylakoid lumen, they will be electrostatically localized along the water-membrane interface. In addition, their positive charges will attract the negatively charged species, namely the hydroxyl anions (HO^-), to the membrane-water interface at the stromal side of the thylakoid membrane, as illustrated in Figure 2B.

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; namely shown in reference [11],

$$\varepsilon_o \oint E \bullet dS = Q \qquad (2)$$

where ε_o 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 applications, where a small volume at the center of the lumen is gradually increased until it is just inside the lumen surface, indicated by r in Figure 2B. By definition, the electric field E is zero everywhere in a conductive body. In each case, since E = 0 everywhere

inside the proton-conducting water body, 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 protons must therefore be on the lumen water-body surface, i.e. at the lumenal water-membrane interface.

Similarly, considering the conductive water outside the thylakoid, the electric field E = 0 holds true everywhere in the water body there too. Applying Gauss's Law to a series of volumes enclosing the entire thylakoid system and decreasing them to be just outside the stromal membrane surface (indicated by R in Figure 2B), the surface integrals of Equation (2) vanish and so no net excess charge is found. Since the excess protons are inside the thylakoid, the negative charges (such as hydroxyl anions) must be on the stromal membrane surface (i.e., at the stromal membrane-water interface), precisely balancing the excess positive (proton) charges of the lumen side, making the total net charge of the entire system zero.

It is worthwhile to note that typical biological membranes contain negatively-charged surface groups, such as the negatively-charged phosphate groups of the membrane's phospholipid molecules, at its two surface sides, which can attract cations, including protons, and are believed to form "electrical double layers" along membrane surfaces [12]. Since these membrane surface charges are fixed, their attracted protons (and/or cations) including the associated electrical double layers do not contribute to the proton motive force (pmf) that drives protons through the ATP synthase. Therefore, these surface-charges-attracted protons and/or cations including their associated electrical double layers are not shown in Figure 2B, which focuses on illustrating the fundamental concept of protons-electrostatics localization model that is relevant to the pmf.

It may also be noted that since protons can transfer among water molecules by the quick "hops and turns" mechanism (Figure 2A), their conduction is much faster than that of any other cations, such as Mg^{++} which in aqueous phase typically has about six bound water molecules that can drag its movement. Meanwhile, protons are the pointiest cations (with the smallest atomistic diameter) and can exist as part of
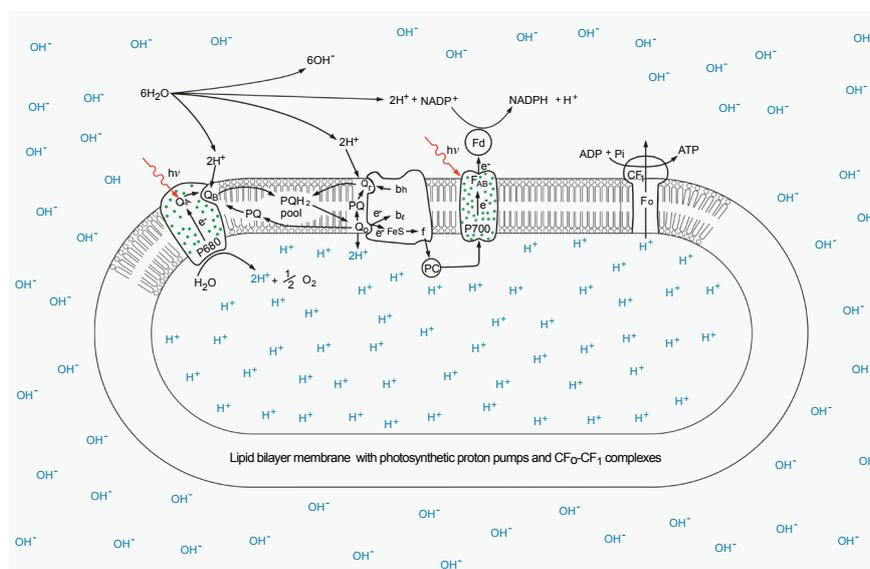

Figure 1: Mitchellian view: delocalized proton (H^+) distribution and coupling.
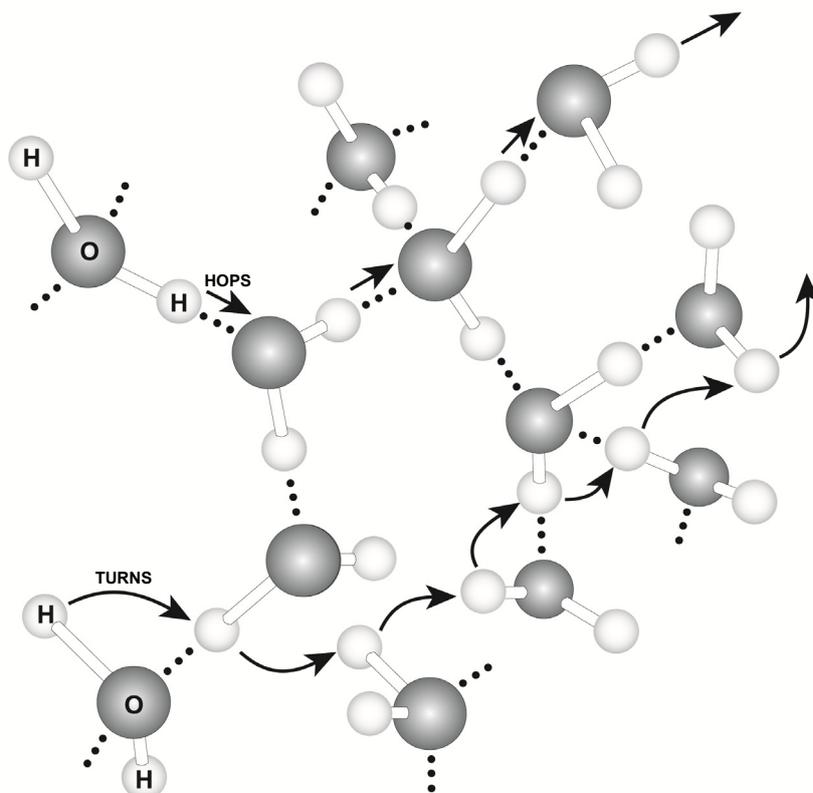
Figure 2A: Protons can quickly transfer among water molecules by the "hops and turns" mechanism so that a microscopic water body may be thought of as a quasi proton conductor.
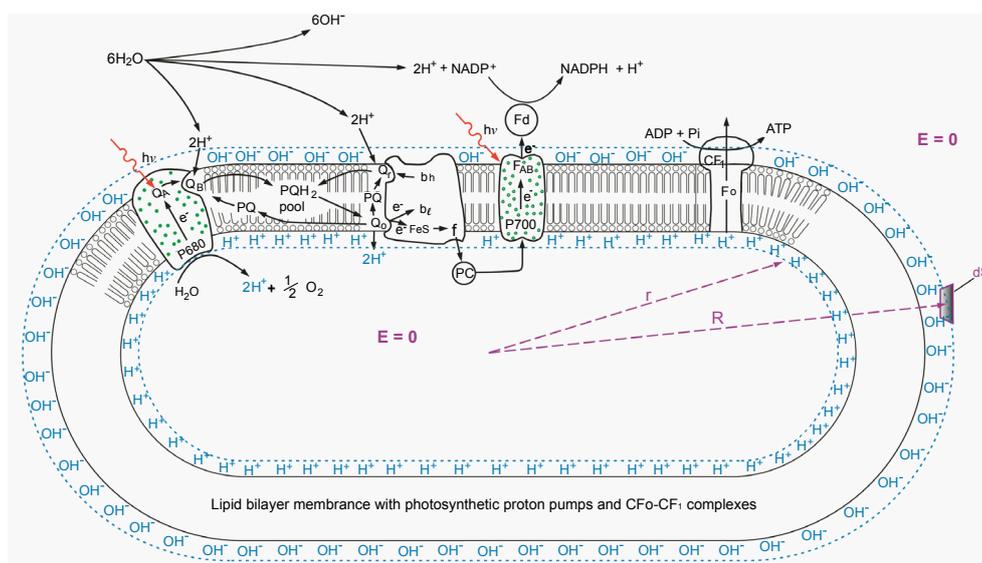

Figure 2B: Proton-electrostatics model illustrating how excess protons (H+) and hydroxyl ions (OH−) could be electrostatically localized at the water-membrane interfaces along the two sides of a thylakoid membrane system owing to the proton conductivity of the water under idealized low-salt conditions.

the water molecules. Consequently, they may electrostatically distribute themselves to the water-membrane interface much more easily than any other cations such as Mg^{++}, K^+, or Na^+. 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) is likely to be much larger than one. Certain cation

exchange experimental studies [13,14] have recently implicated that the equilibrium constant for protons to exchange with other cations for cation binding sites can be as large as around 4.7 x 10^{+6}. Furthermore, because of the unique "hops and turns" proton conduction mechanism, the proton-electrostatics localization coupling may likely represent a relatively fast acting dynamic process in many biological systems.

Revised proton motive force equation

According to the proton-electrostatics localization hypothesis, the proton motive force of Equation (1) must be revised. First of all, it is important to note that the proton-electrostatics localization point of view (Figure 2B) clearly indicates that the excess protons and hydroxyl ions can 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 generalize the proton motive force equation for ATP synthesis as

$$\text{pmf} = \Delta\psi - 2.3RT/F \times \Delta pH^L_{eff} - 2.3RT/F \times \Delta pH \qquad (3)$$

where ΔpH^L_{eff} in the second term is an effective change in pH due to the localized protons at the membrane-water interface causing a proton concentration gradient across the ATP synthase. By this construction, ΔpH^L_{eff} is always negative so that the first two terms of Equation (3) are positive. The following subsections discuss some significant implications of Equation (3).

Explanations from the proton-electrostatics localization model

The proton-electrostatics localization model can help to explain a wide range of experimental observations in chloroplast bioenergetics conducted since 1960s in relation to the proton localization and delocalization phenomena. This model may also have fundamental implications to further understand the proton-coupling phenomena in other biological systems [15-17] including (but not limited to) mitochondria [18] and bacteria [5,6].

The enigmatic energetics problem of alkalophilic bacteria

As mentioned previously, for alkalophilic bacteria the application of Mitchellian Equation (1) yields a pmf value so small that it has remained as an enigmatic problem for decades as to how these organisms can synthesize ATP [19]. According to the proton-electrostatics localization model illustrated in Figure 2B, the proton concentration density near the membrane-water interface, represented in Equation 3 by the term of $-2.3RT/F \times \Delta pH^L_{eff}$, could be significantly higher than the bulk phase-to-bulk phase pH difference, represented by $-2.3RT/F \times \Delta pH$. This could provide a natural explanation as to why the pmf in alkalophilic bacteria is large enough to synthesize ATP.

The effect of permeable amine in thylakoids

The proton-electrostatics localization model predicts that adding a permeable buffer, such as permeable amine (A), in the water body in a thylakoid lumen would have little effect on the electrostatically localized protons (Figure 3A). This is because the permeable amine molecules would most likely be located in the bulk water body in the lumen and not be able to significantly interact with the electrostatically localized protons coupling for ATP synthesis at the water-membrane interface. This predicted feature was demonstrated in Dilley's experiment [20] by the insensitivity to the numbers of single-turnover flashes needed to start ATP synthesis, and, more importantly, to the post-illumination ATP yield when adding 5 mM pyridine.

The effect of high salt solution

The proton-electrostatics localization model predicts that addition of a higher ionic strength (such as 100 mM KCl salt concentration) may partially delocalize protons via cation exchange with protons from the water-membrane interface (Figure 3B). In addition, a high salt concentration could enhance the migration of certain ions including Cl^- and K^+ across the thylakoid membrane, which could neutralize the electrostatic proton charges and thus cause proton delocalization as well (see more discussion in the next section). The delocalized protons in the bulky water phase could then interact with the added permeable
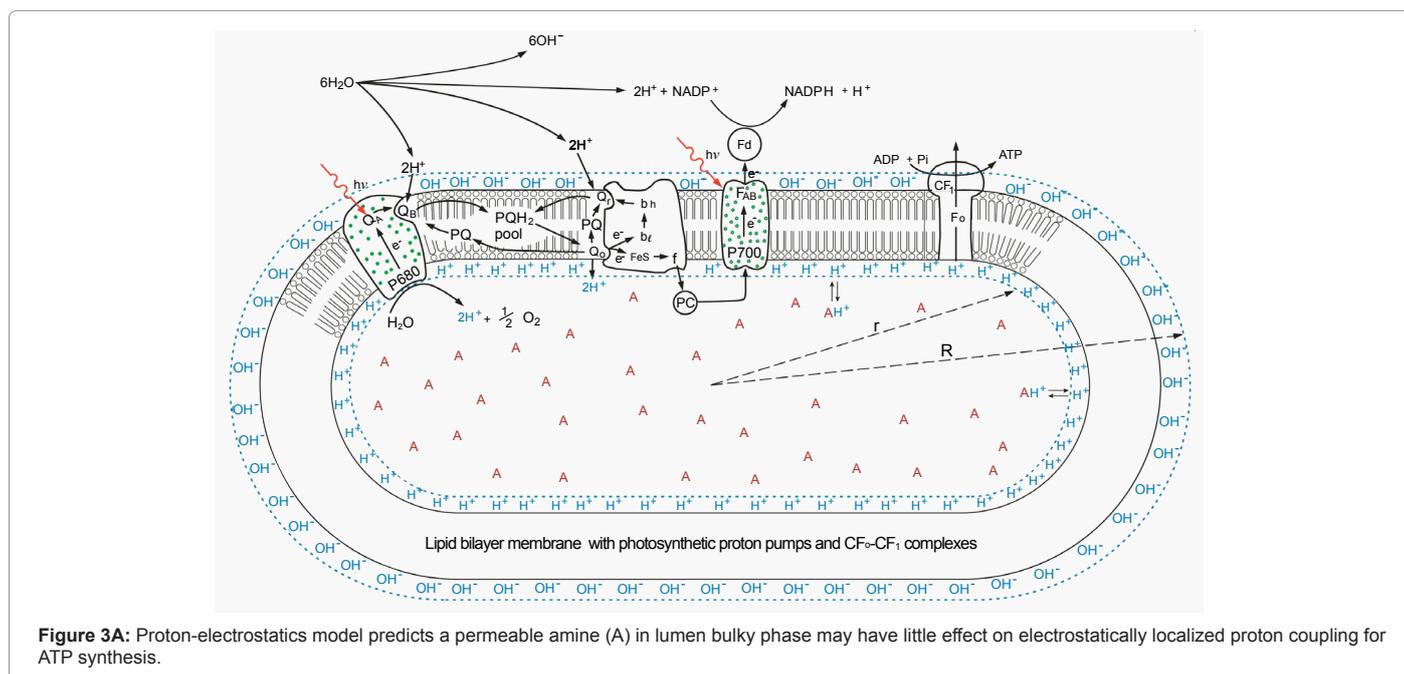

Figure 3A: Proton-electrostatics model predicts a permeable amine (A) in lumen bulky phase may have little effect on electrostatically localized proton coupling for ATP synthesis.

amine (A) to produce AH$^+$ by protonation. Therefore, this model would predict that an addition of permeable amine (A) such as pyridine in the presence of 100 mM KCl could result in a larger number of single turnover flashes required for the onset of ATP synthesis and a higher post-illumination ATP yield from the protons previously stored as AH$^+$ in the lumen. These predicted features of both a longer illumination time(requiring a larger number of single turnover flashes) for the onset of ATP synthesis and a higher post-illumination ATP yield were exactly what were observed in Dilley's experiment with an addition of 5 mM pyridine (permeable amine) in the presence of 100 mM KCl. The experimental results showed that it required 54 ± 2 and 42 ± 2 single turnover flashes for the onset of ATP synthesis in the presence and absence of pyridine, respectively. The post-illumination ATP yield was 7.2 ± 0.4 nmol ATP/mg chl in the presence of pyridine, about twice as much as 3.7 ± 0.5 nmol ATP/mg chl in the absence of the permeable buffer pyridine.

The effect of cation and anion migrations across the thylakoid membrane

The proton-electrostatics localization model predicts that the localized protons will be partially or fully delocalized when the electrical potential difference Δψ across the membrane becomes near zero.

In a chloroplast system, as protons are photosynthetically released at the lumenal surface, they can be quickly distributed along the lumenal water-membrane interface because the water body in the lumen is essentially a proton conductor. Consequently, photophosphorylation through a coupling factor CF$_o$CF$_1$ for ATP synthesis could begin with electrostatically localized protons before proton distribution into the bulk phase occurs.

As the photosynthesis process continues, in responding to the proton motive force, certain cations such as Mg^{2+} will move from the lumen into the stroma of the chloroplast [21] while Cl$^-$ anions will migrate from the stroma into the lumen because the thylakoid membrane contains certain natural ion channels [22,23] and/or transporters [24], as illustrated in Figure 3C. This type of cation and anion migrations could neutralize the electrostatic charges of both the protons in the lumen and the hydroxyl anions (OH$^-$) charges in the stroma. When charge neutralization occurs, the net electrostatic forces that hold the protons at the lumenal water-membrane interface will reduce. Consequently, the localized protons may now migrate from the water-membrane interface into the bulky water phase of the lumen, while the hydroxyl anions (OH$^-$) may move away from the surface of the thylakoid into the bulky water phase at the stroma. As a result, both the electrical potential difference Δψ and the localized effective pH difference ΔpH$^L_{eff}$ across the thylakoid membrane could gradually decrease and the bulk phase pH difference ΔpH may become a dominant coupling force for ATP synthesis. In addition, a high salt concentration in the chloroplast medium could also enhance the migration of certain ions, including K$^+$ and Cl$^-$, across the thylakoid membrane, which could also neutralize the electrostatic charges and thus cause proton delocalization as well.

The predicted feature of high-salt-enhanced K$^+$ and Cl$^-$ migration across the thylakoid membrane is another possible mechanism that could explain the longer illumination time required for onset of ATP synthesis and the higher post-illumination ATP yield observed in Dilley's experiment.

From the proton-electrostatics localization point of view, the Mitchellian view could be true only when both the trans-membrane potential difference (Δψ) and the localized proton coupling (ΔpH$^L_{eff}$) are near zero so that the bulk phase-to-bulk phase pH difference (ΔpH) becomes the dominant driving force for ATP synthesis. However, under most physiological conditions of photosynthesis, Δψ is non-zero even under the steady state of photosynthesis. Recently, it has been experimentally demonstrated that the light-driven Δψ can be stored *in vivo* over hours and that the steady-state Δψ can be between 20 and 90 mV, which represents about 60% of the total proton motive force [25]. The proton coupling at steady-state photosynthesis under most physiological conditions is likely to be in a mixed mode where all three terms of Equation (3) are significant.
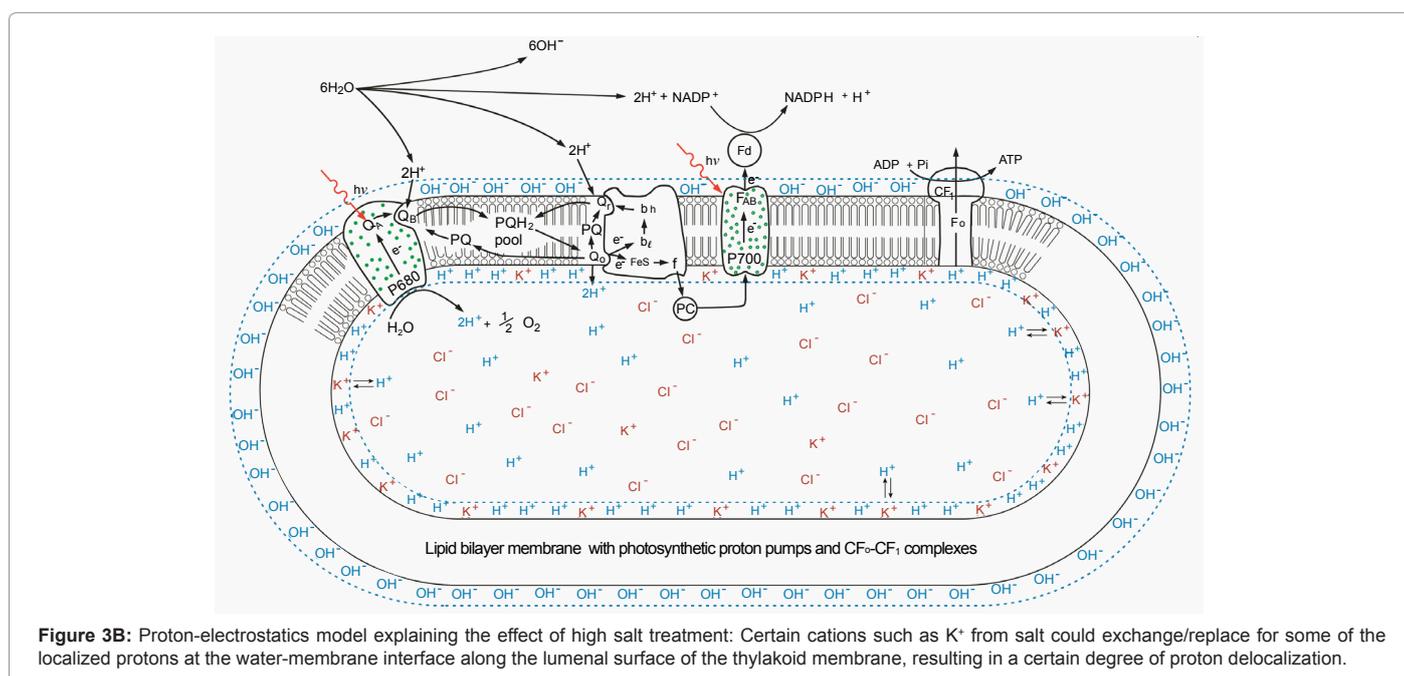

Figure 3B: Proton-electrostatics model explaining the effect of high salt treatment: Certain cations such as K$^+$ from salt could exchange/replace for some of the localized protons at the water-membrane interface along the lumenal surface of the thylakoid membrane, resulting in a certain degree of proton delocalization.

Interpretation of Junge's neutral-red thylakoid proton detection

The proton-electrostatics localization model predicts that a pH-indicting dye such as neutral red [26] that can interact with protons at the membrane-water interface in thylakoid lumen would be able to detect the electrostatically localized protons along the lumenal membrane-water interface. This predicted feature is exactly what was demonstrated by Junge's neutral-red proton-detection experiment in thylakoids, in which the transient proton flow through ATP synthase CF_oCF_1 was completely tracked from the lumen, across the membrane, and into the suspending medium by flash spectrophotometry in a very elegant manner [27]. However, Junge regarded the neutral-red thylakoid proton-detection result as an indication for delocalized protons and has held fast against Dilley's arguments of localized protons for more than 30 years [28].

With the proton-electrostatics localization hypothesis, it is now possible to help reconcile the scientific observations of both the Dilley experiment and the Junge neutral-red thylakoid proton detection. Since neutral red is a proton-indicating dye that can act at the membrane-water interface [29], Junge's neutral-red thylakoid proton measurements may now be explained as an evidence for localized protons that are predicted by the proton-electrostatics localization model at the lumenal membrane-water interface, but not necessarily for delocalized protons that Mitchell's chemiosmotic theory would expect to be in the lumen bulky water phase. Therefore, the proton-electrostatics localization model can now explain the results of not only the Dilley experiment, but also the Junge neutral-red thylakoid proton detection.
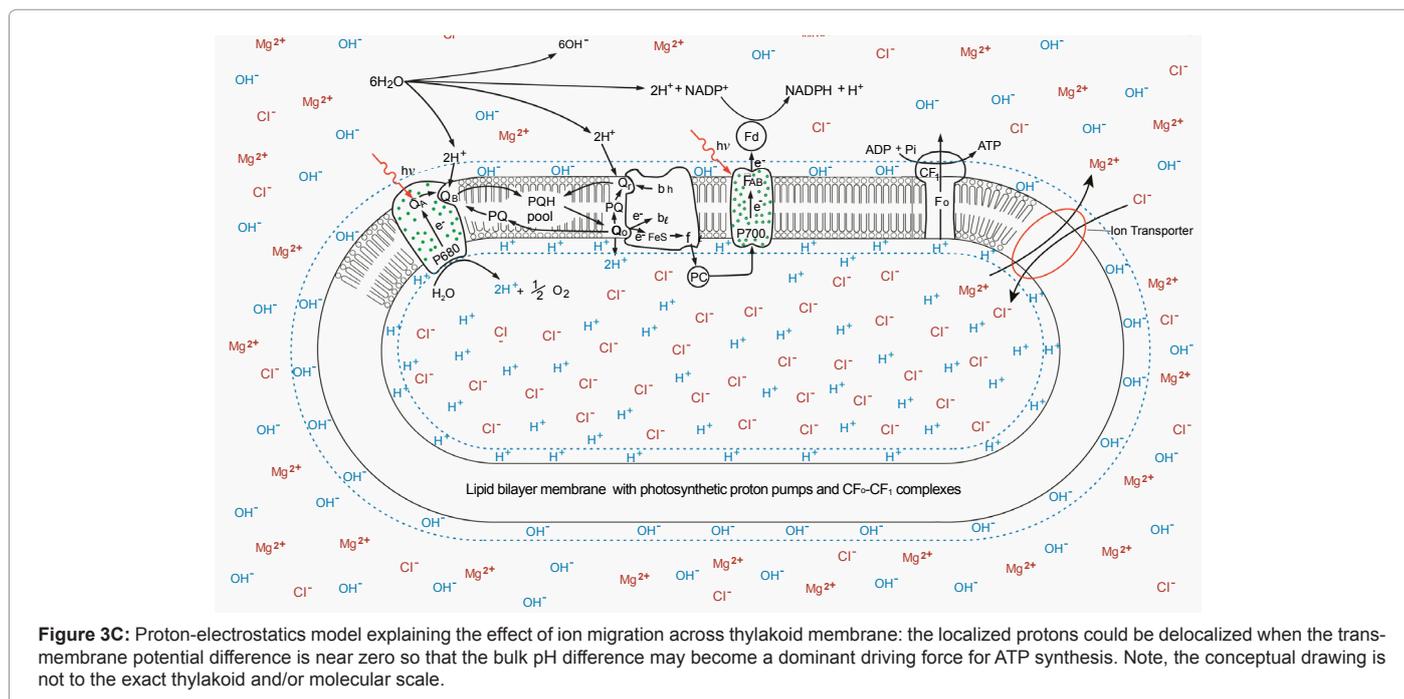
Davenport-McCarty experiment vs. an interfacial proton barrier model

Recently, in an effort trying to explain localized proton-coupling phenomena, Cherepanov and Mulkidjanian et al. [30] proposed an interfacial barrier model, which made use of the dielectric permittivity of interfacial water [31]. 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. If this understanding is correct, one would then 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, if the interfacial proton barrier really exists in thylakoids, one would expect a localized proton-coupling even when the trans-membrane potential difference ($\Delta\psi$) is removed. However, the Davenport-McCarty experiment [32] 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 thylakoid bioenergetics.

On the other hand, the proton-electrostatics hypothesis can well explain the Davenport-McCarty experiment since it predicts a delocalized proton coupling when $\Delta\psi$ is collapsed by the presence of K^+ and valinomycin. As explained previously with the effect of cation and anion migrations across thylakoid membrane (Figure 3C), the proton-electrostatics hypothesis predicts an association of localized proton coupling with $\Delta\psi$. That is, only in the presence of a significant $\Delta\psi$ (such as in the absence of K^+ and valinomycin), the proton-electrostatics model would predict that the permeable buffer (imidazole) in the lumen could not cause a significant delay in ATP synthesis. This predicted feature is also exactly what was observed in the Davenport-McCarty experiment using a thylakoid sample with 100 mM mannitol (without K^+ and valinomycin).

Another question is whether the interfacial barrier (if it exists) would be sufficient to explain the bioenergetics problem in alkalophilic bacteria [5,6,19], since, under the Cherepanov-Mulkidjanian model, protons could still be lost by "futile proton escape" into the bulk

Figure 3C: Proton-electrostatics model explaining the effect of ion migration across thylakoid membrane: the localized protons could be delocalized when the trans-membrane potential difference is near zero so that the bulk pH difference may become a dominant driving force for ATP synthesis. Note, the conceptual drawing is not to the exact thylakoid and/or molecular scale.

periplasmic p-phase. As Cherepanov and Mulkidjanian et al. [30] discussed, 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 (bioenergetics system) of alkalophilic bacteria operates at a time scale for hours and days. Therefore, if the "futile proton escape" is true, the loss of protons into the bulk periplasmic p-phase could be so significant that would be hard to explain the commonly observed growth of alkalophilic bacteria culture [5,6,19].

In contrast, the proton-electrostatics model does not require any putative interfacial potential barrier to explain the localized proton coupling. According to the proton-electrostatics hypothesis, when protons (H^+) and the hydroxyl anions (OH^-) are electrostatically localized along the two sides of the thylakoid membrane through the water body protons-conduction/protons-mutual repulsion effect, they are held together by their electric attraction forces across the membrane for energetic coupling in a dynamic manner as illustrated in Figure 2B.

Universal explanation of bioenergetics through the proton-electrostatics localization-delocalization proton motive force equation

As discussed above, the proton-electrostatics localization-delocalization proton motive force equation (Equation 3) can now readily explain the longstanding enigmatic bioenergetics problem in alkalophilic bacteria; it can also explain the bioenergetics in other biological systems such as the thylakoid systems. For example, during the initial onset of photosynthesis-driven proton translocation in Dilley's experiment, with a low salt medium where the cation exchange with the electrostatically localized protons is negligible, the pH difference between the two bulk aqueous phases across the thylakoid membrane initially is zero. The pmf in this special circumstance is expressed by the fully localized proton-coupling equation:

$$pmf = \Delta\psi - 2.3RT/F \times \Delta pH^L_{eff} \tag{4}$$

Note that the electrostatic localization of protons may occur quickly by their mutual positive-charge repulsion and their quick "hops and turns" conduction mechanism well before other cations such as K^+ and Mg^{++} which are dragged by their bound water molecules can possibly respond. The ΔpH^L_{eff} in Equation (4) represents the localized trans-membrane pH difference owing to the electrostatic localization of protons at the membrane-water interface, which could not be easily detected through the common bulky-phase pH-electrode measurements. This localized property of ΔpH^L_{eff} could give one the impression that the initial formation of ATP in photophosphorylation seems "not require a proton gradient". This phenomenon expected now by the proton-electrostatics hypothesis was well noticed by Boyer's group [33] in their experimental observation of initial photophosphorylation occurring within about a few milliseconds from when the actinic light was turned on.

As the photosynthesis-driven proton-translocation process continues building up the electrostatically localized proton gradient across the thylakoid membrane, the migrations of cations and anions across thylakoid membrane will progressively take place so that a significant portion of the electrical potential difference $\Delta\psi$ across the thylakoid membrane is gradually reduced while a significant portion of the electrostatically localized protons are released into the lumen bulky phase, which will result in an increase of the delocalized pH difference ΔpH between the two bulk aqueous phases across the thylakoid membrane. At this state, the general pmf expression of Equation 3 applies.

A significant insight obtained from the proton-electrostatics model analyses is that the electrostatically localized protons are closely associated with the transmembrane electrical potential difference $\Delta\psi$ because the localization results from the protons (net positive charges) electrostatic mutual repulsion in the thylakoid lumen water body; this makes sense since it is the net positive charges (mostly the excess protons) of the lumen water body in balance with the net negative charges of the stroma side that constitute the trans-membrane electrical potential difference $\Delta\psi$. In certain extreme cases such as the Davenport-McCarty experiment [32] where the trans-membrane potential difference $\Delta\psi$ is removed, all the $\Delta\psi$-associated electrostatically localized protons are released into the lumen bulky phase so that the delocalized proton gradient becomes the sole source of the pmf:

$$pmf = -2.3RT/F \times \Delta pH \tag{5}$$

This pmf expression (Equation 5) can also explain the Jagendorf-lab acid-base jump experiment [34,35], where the bulk pH difference ΔpH that was created by manipulating the proton concentrations in the two bulky aqueous phases across the thylakoid membrane was the sole driving force for ATP synthesis. Under most physiological conditions, however, the general proton-electrostatics localization-delocalization pmf expression of Equation 3 applies. Under the steady state of photosynthesis, as long as the salt concentration is not too high, localized proton coupling may account for at least about 60% of the total proton motive force. Therefore, the proton-electrostatics model can now provide a possible framework for understanding both the proton localization and delocalization phenomena in many bioenergetics systems.

Summary

1. Mitchell's chemiosmotic theory is a cornerstone in bioenergetics; however, the Mitchellian view of a single delocalized-proton coupling mechanism to drive the synthesis of ATP seems not to be entirely correct.

2. Localized proton coupling has been demonstrated experimentally beyond reasonable doubt by Dilley and others through the experimental observations in thylakoids and alkalophilic bacteria such as *Bacillus firmus*.

3. The proton-electrostatics localization hypothesis can now help provide a unified explanation for the proton localization and delocalization phenomena, without conjecturing any massive protein structure or any putative interfacial proton barrier.

4. According to the proton-electrostatics localization hypothesis, protons injected into a thylakoid may, owing to their mutual repulsion, be quickly localized electrostatically at the water-membrane interface along the lumenal surface of thylakoid membrane for energetic coupling in a fast-acting dynamic manner.

5. The electrostatically localized protons at the membrane-water interface are closely associated with the trans-membrane electrical potential difference because the electrostatic localization of protons is a result from the electrostatic mutual repulsion of the excess protons in thylakoid lumen water body.

6. The proton-electrostatics localization model indicates that the Mitchellian view could be true only in special cases when the trans-membrane potential difference is near zero and the bulk pH difference becomes the dominant driving force for ATP synthesis.

7. The proton-coupling under most physiological conditions of photosynthesis is likely to occur in a mixed mode of both electrostatic proton localization at the membrane surface and proton delocalization within the bulk phase.

8. The proton-electrostatics localization hypothesis suggests that the proton motive force (pmf) driving the synthesis of ATP should be written generally as $pmf = -\Delta\tilde{\mu}_{H^+}/F = \Delta\psi - 2.3RT/F \times \Delta pH^L_{eff} - 2.3RT/F \times \Delta pH$

9. The proton-electrostatics model-based pmf equation can now provide a possible unified explanation for both the proton localization and delocalization phenomena in many bioenergetics systems.

References

1. Mitchell P (1976) Possible molecular mechanisms of the proton motive function of cytochrome systems. J Theor Biol 62: 327-367.

2. Mitchell P (1979) Keilin's respiratory chain concept and its chemiosmotic consequences. Science 206: 1148-1159.

3. Ferguson SJ (1985) Fully delocalised chemiosmotic or localised proton flow pathways in energy coupling?: A scrutiny of experimental evidence. Biochim Biophys Acta 811: 47-95.

4. Dilley RA, Theg SM, Beard WA (1987) Membrane-proton interaction in chloroplast bioenergetics: Localized proton domains. Ann Rev Plant Physiol 38: 347-389.

5. Krulwich TA (1986) Bioenergetics of alkalophilic bacteria. J Membr Biol 89: 113-125.

6. Olsson K, Keis S, Morgan HW, Dimroth P, Cook GM (2003) Bioenergetic properties of the thermoalkaliphilic *Bacillus* sp. strain TA2.A1. J Bacteriol 185: 461-465.

7. Krulwich TA, Ito M, Gilmour R, Hicks DB, Guffanti AA (1998) Energetics of alkaliphilic *bacillus* species: physiology and molecules. Adv Microb Physiol 40: 401-438.

8. Dilley RA (2004) On why thylakoids energize ATP formation using either delocalized or localized proton gradients – a Ca(2+) mediated role in thylakoid stress responses. Photosynth Res 80: 245-263.

9. Cherepanov DA, Junge W, Mulkidjanian AY (2004) Proton transfer dynamics at the membrane/water interface: dependence on the fixed and mobile pH buffers, on the size and form of membrane particles, and on the interfacial potential barrier. Biophys J 86: 665-680.

10. Pomes R, Roux B (2002) Molecular mechanism of H+ conduction in the single-file water chain of the gramicidin channel. Biophys J 82: 2304-2316.

11. Ohanian HC (1985) Gauss' law, in Physics textbook (ISBN 0-393-95401-3). WW Norton & Company, New York, 565-573.

12. McLaughlin S (1989) The electrostatic properties of membranes. Annu Rev Biophys Biophys Chem 18: 113-136.

13. Lee JW, Kidder M, Evans BR, Paik S, Buchanan AC 3rd, et al. (2010) Characterization of biochars produced from cornstover for soil amendment. Environ Sci Technol 44: 7970-7974.

14. 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. Commun Soil Sci Plant Anal 39: 926-937.

15. Kell DB (1979) On the functional proton current pathway of electron transport phosphorylation. An electrodic view. Biochim Biophys Acta 549: 55-99.

16. Westerhoff HV, Melandri BA, Venturoli G, Azzone GF, Kell DB (1984) A minimal hypothesis for membrane-linked free-energy transduction: the role of independent, small coupling units. Biochim Biophys Acta 768: 257-292.

17. van der Bend RL, Petersen J, Berden JA, Van Dam K, Westerhoff HV (1985) A critical appraisal of evidence for localized energy coupling. Kinetic studies on liposomes containing bacteriorhodopsin and ATP synthase. Biochem J 230: 543-549.

18. 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. Biochim Biophys Acta 809: 1-10.

19. Krulwich TA, Ito M, Gilmour R, Sturr MG, Guffanti AA, et al. (1996) Energetic problems of extremely alkaliphilic aerobes. Biochim Biophys Acta 1275: 21-26.

20. Chiang GG, Dilley RA (1989) Intact chloroplasts show Ca-gated switching between localized and delocalized proton gradient energy coupling (ATP formation). Plant Physiol 90: 1513-1523.

21. Ishijima S, Uchibori A, Takagi H, Maki R, Ohnishi M (2003) Light-induced increase in free Mg2+ concentration in spinach chloroplasts: measurement of free Mg2+ by using a fluorescent probe and necessity of stromal alkalinization. Arch Biochem Biophys 412: 126-132.

22. Enz C, Steinkamp T, Wagner R (1993) Ion channels in the thylakoid membrane (a patch-clamp study). Biochim Biophysi Acta 1143: 67-76.

23. Tester M, Blatt MR (1989) Direct measurement of K channels in thylakoid membranes by incorporation of vesicles into planar lipid bilayers. Plant Physiol 91: 249-252.

24. Pavón LR, Lundh F, Lundin B, Mishra A, Persson BL, et al. (2008) Arabidopsis ANTR1 is a thylakoid Na+-dependent phosphate transporter: functional characterization in *Escherichia coli*. J Biol Chem 283: 13520-13527.

25. Cruz JA, Kanazawa A, Treff N, Kramer DM (2005) Storage of light-driven transthylakoid proton motive force as an electric field (Deltapsi) under steady-state conditions in intact cells of *Chlamydomonas reinhardtii*. Photosynth Res 85: 221-233.

26. Auslander W, Junge W (1975) Neutral red, a rapid indicator for pH-changes in the inner phase of thylakoids. FEBS Lett 59: 310-315.

27. Junge W (1987) Complete tracking of transient proton flow through active chloroplast ATP synthase. Proc Natl Acad Sci U S A 84: 7984-7088.

28. Junge W (2004) Protons, proteins and ATP. Photosynth Res 80: 197-221.

29. Hong YQ, Junge W (1983) Localized or delocalized protons in photophosphorylation? On the accessibility of the thylakoid lumen for ions and buffers. Biochim Biophys Acta 722: 197-208.

30. Mulkidjanian AY, Heberle J, Cherepanov DA (2006) Protons @ interfaces: Implications for biological energy conversion. Biochim Biophys Acta 1757: 913-930.

31. Cherepanov DA, Feniouk BA, Junge W, Mulkidjanian AY (2003) Low dielectric permittivity of water at the membrane interface: Effect on the energy coupling mechanism in biological membranes. Biophys J 85: 1307-1316.

32. Davenport JW, McCarty RE (1980) The onset of photophosphorylation correlates with the rise in transmembrane electrochemical proton gradients. Biochim Biophys Acta 589: 353-357.

33. Vinkler C, Avron M, Boyer PD (1978) Initial formation of ATP in photophosphorylation does not require a proton gradient. FEBS Lett 96: 129-134.

34. Jagendorf AT (2002) Photophosphorylation and the chemiosmotic perspective. Photosynth Res 73: 233-241.

35. Jagendorf AT, Uribe E (1966) ATP formation caused by acid-base transition of spinach chloroplasts. Proc Natl Acad Sci U S A 55: 170-177.