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# BACTERIORHODOPSIN - THE SIMPLEST BIOENERGETIC SYSTEM FOR ATP PRODUCTION

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**Abstract:** Bacteriorhodopsin (BRh), which is present in the cell membranes of the archaeon *Halobacterium salinarum*, harvests light wave energy which causes conformational changes in this retinal protein leading to the release of adenosine triphosphate (ATP) by ATP synthase. Since some protons are released during light-induced excitation of BRh molecules, the chemiosmotic hypothesis advanced by Peter Mitchell was used to explain the ATP formation by translocation of protons across the simple membranes of *H. salinarum*. However, protons can be released as a result of photoexcitation of some molecules, and these protons are only responsible for increasing the acidity ($pK_a$) of their solutions; therefore, the protons released by BRh probably have no role in ATP production. In contrast, the 138 nm spectral shift between the two photointermediates, L550 and M412, corresponds to an energy amount of 17.41 kcal/mole, which can be successfully absorbed by ATP synthase as near-infrared radiation (NIR). The conformational changes of F0 ATP-synthase can induce the rotation of F1 ATP-synthase, which is associated with the removal of ATP molecules already formed by F1 ATP-synthase from ADP and inorganic phosphate. The NIR radiant energy can thus be confusingly associated with proton release and not with direct light-induced ATP formation. The already proposed mechanism of ATP production by BRh-ATP-synthase system is supported by a large body of literature data. However, such data that contradict old theories can be interpreted in the light of new understandings of living organisms. Therefore, a new perspective on ATP production in biological systems from the simplest to higher organisms is required.

***Keywords*:** Bioenergetics; Bacteriorhodopsin; ATP production; NIR, Chemiosmosis

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## Introduction

The single-celled microorganism *Halobacterium salinarum* is one of the members of an ancient kingdom that lived in salted lakes even before the Earth had an oxygen atmosphere.[1,2] However, the detailed molecular mechanisms that allow these single-celled microorganisms to survive and produce ATP have remained an open question for decades.[3] It is still thought that the retinal protein bacteriorhodopsin (BRh) found in the purple membrane of *H. salinarum* acts as a light-driven proton pump.[4] Thus, the proton gradient generated upon light illumination of BRh was proposed to explain ATP synthesis by membrane bound $H^+$ ATPase.[5] Since protons are released during light-induced excitation of BRh molecules, the chemiosmotic hypothesis advanced by Peter Mitchell has been applied to explain the formation of ATP molecules.[6,7] Reconstituted vesicles containing ATP synthase and bacteriorhodopsin were then used by Stoeckenius and Racker to confirm the Mitchell chemiosmotic hypothesis.[8] They thought that, upon illumination, bacteriorhodopsin pumps protons into these vesicles, and the resulting proton gradient is sufficient to cause ATP synthesis by ATP synthase.[9] Recently, based on quantum-mechanical calculations, it was found that the mechanism of proton translocation across the cell membrane cannot fully explain ATP formation by *H. salinarum* cells.[10,11] However, BRh is currently still believed to act as a light-driven proton pump, which captures light energy and uses it to transfer protons vectorially across the membrane from the cytoplasmic side to the outside of the cell against a concentration gradient.[12] The resulting proton gradient would subsequently be converted into chemical energy.

In this paper, we provide some evidence that may shed new lights on the mechanism of proton liberation by BRh. In addition, we discuss a

vibrational mechanism of ATP formation by the BRh-ATP synthase biosystem, as well as the limitations of old hypotheses, theories and experiments based on the assumption that translocation of protons across biological membranes might be able to release ATP molecules.

**Results and Discussion**

*Acidity of excited state of bacteriorhodopsin*. Based on the Förster cycle it is possible to calculate the change in the equilibrium constant, ΔK, upon light excitation of molecules in singlet or triplet state.[13-15] Changes in the electron distribution on excitation of BRh are also connected with changes in its acidity (Figure 1). There, ΔE and ΔE' correspond to the excitation energies and ΔH and $\Delta H^*$ to the reaction enthalpies associated with the equilibrium in the ground and excited state (Equation 1), respectively. Therefore, if one considers the equilibrium:

$$BRh^+\text{-}H \rightleftharpoons BRh + H^+ \qquad \text{Equation 1,}$$

then the following equation can be written:

$$K = \frac{[BRh] \cdot [H^+]}{[BRh^+ - H]} \qquad \text{Equation 2,}$$

where the concentrations of molecular species are in brackets.

From the ground state $S_0$ the excited state $BRh^*+H^+$ can be reached in two different ways, and the following equation can be written:

$$N_L h\nu_{bRH}{}^+ + \Delta H^* = N_L h\nu_{bR} + \Delta H \qquad \text{Equation 3}$$

or

$$\Delta H^* - \Delta H = N_L(h\nu_{bR} - h\nu_{HbR}{}^+),$$

where ΔH and $\Delta H^*$ are the enthalpy changes in the ground and excited states, and $N_L$ is the Avogadro number. If ΔH can be approximated in dilute

solutions by the standard value $\Delta H^0$, and entropy effects may be neglected, then considering the well-known relationship $\Delta G^0 = -RT\ln K = -2.303\ RTpK$, it follows:

$$N_L(h\nu_{BR} - h\nu_{BRH^+}) \cong 2.303\ RT\Delta pK \qquad \text{Equation 4}$$

Upon inserting the numerical values of the constants, the following relationship between the change in the pK value and the $\Delta\bar{\upsilon}$ shift of the absorption maximum for the lowest excited state due to protonation is obtained for T = 298 K:

$$\Delta pK = 0.00209\ (\bar{\upsilon}_{BRh} - \bar{\upsilon}_{BRhH^+}) \qquad \text{Equation 5}$$

where $\bar{\upsilon}$ is the wavenumber expressed as $cm^{-1}$.

Therefore, a spectral shift of 138 nm from 550 nm to 412 nm and back to 550 nm, when bacteriorhodopsin passes from the photointermediate $L_{550}$ to $M_{412}$ and then to $N_{550}$ corresponds to a change in wavenumber of $\Delta\bar{\upsilon} = 6{,}090.02\ cm^{-1}$, which results in a pK value change of 12.73 units:

$$\Delta pK = 0.00209 \cdot \left(\frac{10^7}{412} - \frac{10^7}{550}\right) = 12.728 \qquad \text{Equation 6}$$

Thus, BRh-$H^+$ will become a very strong acid under illumination, because the proton concentration can theoretically increase $10^{12.728} = 5{,}345{,}643{,}593{,}969.7$ times!

We need to be aware that the liberated protons spread both inside and outside bacterial cells. Otherwise we should take into account that the protons are penetrating the membrane of the unicellular microorganism only in one direction, outwards, they are not diluted in salt water and that they return to the cell due to a strange energy they release through ATP-synthase.

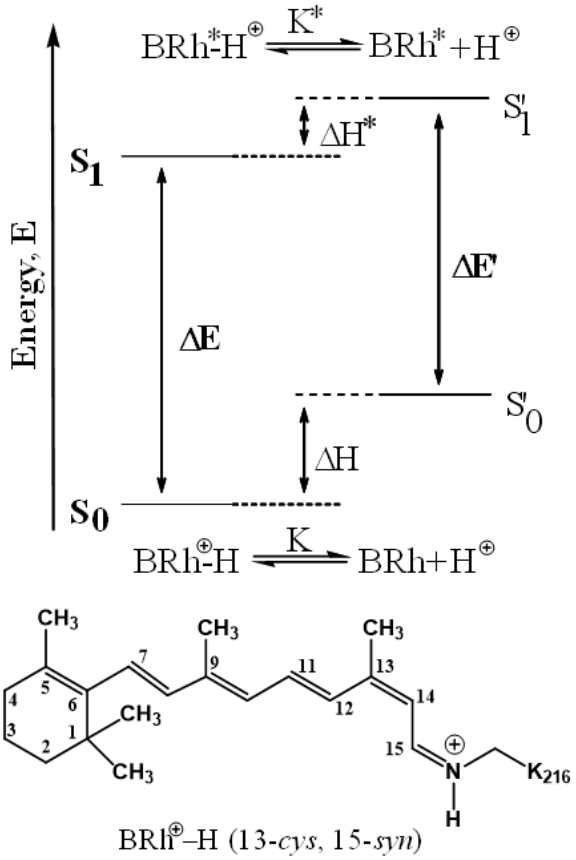



**Figure 1.** Förster cycle for BRh, explaining the increased acidity of this compound under blue light illumination (412 nm).

However, because protons are very small and positively charged, they bind to at least one water molecule to form $H_3O^+$ ions. In fact, they surround themselves with more water molecules. We have also to assume that protons may pass freely through the microbial membrane on both sides. Indeed, there are no experiments, only assumptions for outward vectorial transport of protons.

The released protons under illumination may have therefore no role in ATP production. Instead, the spectral shift of 138 nm (550 nm – 412 nm), between $M_{412}$ and $N_{550}$, which is associated with proton releasing, is associated with enough energy to release a mole of ATP from ATP-synthase.

We calculated an amount of energy of 17.41 kcal/mole as the difference between the two absorbencies:

$$\Delta E = \frac{28595}{412} - \frac{28595}{550} = 17.414\ \text{kcal/mole} \qquad \text{Equation 7}$$

The same amount of energy is provided by the NADH molecule for each of the three ATP molecules it releases. It is well-known that ATP-synthase binds inorganic phosphate and adenosine diphosphate (ADP) to produce ATP, which is bound to this enzyme. Thus, Paul Boyer demonstrated that ATP synthesis does not require any energy input when substrates are bound to the F1 subunit of ATP-synthase.[16] Some energy is only required to induce conformational changes in the F0 subunit capable of rotating the F1 subunit, which leads to the release of ATP from the binding site.

*Shortcomings of the chemiosmotic hypothesis*. Peter Mitchell (Nobel Laureate, 1967) tried to explain the whole complexity of the living organisms by proton translocation through the biological membranes.[6] According to his hypothesis, the function of the respiratory chain is to translocate protons thereby establishing a proton motive force, which consists of a ΔpH and a trans-membrane potential. That time, the chemiosmotic hypothesis has been accepted as one of the great unifying principles of twentieth century biology. Although the proposed mechanism has stimulated further biochemical research, it has so far lacked direct and convincing experimental support. For example, once released outside *H. salinarum* cells, protons would have to return back into the cells via the F0 subunit of ATP synthase to induce its conformational changes capable of removing the ATP molecule from F1. First, functional BRh produces ATP at a pH between 4.0 and 9.0, while it only works efficiently at a pH above 5.0.[17] In addition, it is not possible for protons released from *H. salinarum* cells and diluted in the externally saline

medium to return to the cell at pH > 7.0, such as pH 9.5. In addition, if the extracellular medium is buffered to pH 7.0, it is not possible for protons to enter the cell via ATP synthase. Second, there is no explanation for the mechanism by which protons make such conformational changes to the F0 subunit.

According to Mitchell's hypothesis, uncouplers, including 2,4-dinitrophenol, operate by chemically driving protons across the lipid membrane, thereby short-circuiting the proton flow.[18] However, such agents which induce permeability of membranes to protons (2,4-dinitrophenol, carbonylcyanide-*m*-chlorophenylhydrazone) have no influence on the formation of the 412 nm intermediate and in recovery of the 568 nm chromophore.[19] The chemiosmotic hypothesis appears to need updating, as some of its assumptions have proven incorrect in the light of the latest structural data on respiratory chain complexes, bacteriorhodopsin and proton pumps.[20,21] Proton translocation is lateral rather than transversal with respect to the coupling membrane.

*Discussion*. The liberation of protons is therefore the result of BRh photoexcitation, which is responsible for the increase in acidity, but has no role in ATP production. In contrast, the 138 nm spectral shift from the L550 photointermediate to the M412 photointermediate corresponds to an amount of energy of 17.41 kcal/mole, which can be successfully absorbed by ATP synthase as near-infrared (NIR) radiation.[10,11] Conformational changes in ATP synthase F0 can induce ATP synthase F1 turnover, which is associated with the removal of ATP molecules already formed by ATP synthase F1 from ADP and inorganic phosphate. NIR radiant energy can thus be confusingly associated with proton release and not direct light-induced ATP formation. The already proposed mechanism of ATP production by the BRh-ATP-

synthase system is suggested by a large number of observations in the literature and by the fact that there was not find proton channels through the membrane protein.[22-24] In fact, there is no electrogenic transfer of protons.[25] Moreover, protons are stored in $H_5O_2^+$ rather than on any of the glutamic acids, thus establishing the theoretical plausibility of storing the release proton in bacteriorhodopsin in a hydrogen-bonded water network.[26]

Bacteriorhodopsin, when illuminated, shows a net proton release at neutral and alkaline pH; however, it shows an uptake of protons in acidic pH. Acetylation of bacteriorhodopsin caused no alteration in the absorption spectrum of purple complex ($bR_{570}$) and $M_{412}$-intermediate. Light-induced release of protons was not observed even in neutral pH values, and only the proton uptake was noticed by acetylated purple membrane fragments. These fragments have no ability for light-induced proton transport.[27]

During the BRh photocycle, proton is transferred from the retinal Schiff base to aspartate-85.[28] When aspartate-85 is replaced with threonine, the mutated bacteriorhodopsin became a chloride ion pump. In addition, the original physical arguments and calculations made in support of steady state electrogenic ion transfer and thus chemiosmosis violate Gauss's law.[29,30] These findings are among many arguments against applying the chemiosmotic hypothesis to describe light-induced ATP formation in *H. salinarum* cells.

Working with chloroplasts, maximum ΔpH values of about 3.5 were found, which is only equivalent to 200 mV of protonmotive force.[31] However, the light-induced phosphate potential is about 17 kcal/mole in chloroplasts, which is equivalent to 700 mV; therefore, the minimum $H^+$/ATP ratio consistent with these values must be greater than 3.5, which is not consistent

with the stoichiometry proposed by Peter Mitchell.[32] This result suggests that the changes of pH are not dependent on ATP formation.

The present paper can be classified under biophysics and computational biology and is very similar to other biophysical papers. For example, some authors used mathematical computation to explain previously experiments reported in other papers.[33] Thus, previous experiments on similar fluorescent protein at the single-molecule level provide evidence of previously unknown short- and long-lived states and of related excited-state decay channels. A quantum chemical calculation on cis-trans photoisomerization paths of chromophores in their ground and first singlet excited states was done to explain the observed behaviors from a common perspective.[33]

According to literature, BRh can be used in the light, biological, chemical, optical, gas, and electrical sensors, immunosensors, nanosensors, and various other sensors.[34] Thus, a thin film of purple membranes containing BRh was coated on a two-dimensional array of electrode pixels and was made into a junction with an electrolyte gel layer having a counter electrode to form an artificial photoreceptor.[35] Photocurrent signals from each pixel showed a differential responsiveness to light intensity intrinsic to this liquid junction photocell. Molecules with ionizable protons show excited state prototropism. In condensed environments, the immediate environment surrounding the excited molecule state prototropism can significantly influence the emission spectral parameters of different prototropic species.[36] The fluorescence of different prototropic forms shows a sensitive response to changes in the local environment surrounding the organized microheterogeneous medium in terms of physical properties, local structure and dynamics. Consequently, excited fluorescent molecules can easily liberate protons under illumination.

In conclusion, we have shown for the first time that light energy can be used directly to obtain ATP in the BRh photocycle, while the release of protons from the Schiff base is a secondary phenomenon.

**Experimental**

We investigated several experimental data described extensively in the literature, but which were conducted in the light of older theories and hypotheses. Thus, in recent decades a huge amount of information has accumulated that needs to be interpreted in the light of new theories and views. From these findings, we have carefully explored the following data:

1) *The oxidation of one mole of NADH* by the electron transport system generates a significant amount of free energy (ΔG°' = -52.38 kcal/mole).[37] The energy produced is sufficient to release seven moles of ATP, if it is incorrectly assumed that only 7.3 kilocalories are needed to form one mole of ATP. In fact, one mole of NADH produces three moles of ATP, while one mole of $FADH_2$ releases only two moles (although many researchers claim that the actual number of moles of ATP is 2.5 and 1.5 respectively). However, we consider that 0.5 moles represent the energy that cannot correctly be quantified due to the complexity of cellular metabolism.

2) *The intracellular pH* (pHi) of living cells is only slightly sensitive even to large changes of extracellular pH values.[38] In fact, pHi is maintained within relatively narrow limits and does not respond quickly to external pH changes as proton translocation-based hypotheses claim.

3) *Calculating the energy of photons.* The amount of energy of one mole of protons (1 einstein) can be determined by the following equation:

$$E = \frac{N \cdot h \cdot c}{\lambda} = \frac{(6.023 \cdot 10^{23}) \cdot (6.626 \cdot 10^{-34}\ \mathrm{J \cdot s}) \cdot (2.9979 \cdot 10^{8}\ \mathrm{m \cdot s^{-1}})}{\lambda} =$$

$$= \frac{119.641 \cdot 10^{-3}\ \mathrm{J \cdot m}}{\lambda} = \frac{119.641 \cdot 10^{-3}\ \mathrm{J \cdot m}}{\lambda_{(nm)} \cdot 10^{-9}\ \mathrm{m}} = \frac{119.641 \cdot 10^{6}\ \mathrm{cal}}{4.184 \cdot \lambda_{(nm)}} = \frac{28595\ \mathrm{kcal/mole}}{\lambda_{(nm)}},$$

where N = Avogadro's number; h = Plank constant; and *c* = speed of light; λ = wavelength.[39]

4) Some uncoupling agents and metabolic inhibitors absorb strongly in IR in the range around 6000 $cm^{-1}$, which suggests that they may capture the quantum of electromagnetic vibration corresponding to 14-17 kcal/mole, emitted by cytochromes or bacteriorhodopsin.[40]

5) *Bacteriorhodopsin photocycle*. The sequence of conformational changes of BRh following photoisomerization of its all-*trans* retinal chromophore into a 13-*cis* configuration has been extensively described.[41] Light-induced conformational changes in the membrane protein BRh have also been studied (Figure 1a).[42,43] BRh photointermediates have been defined in kinetic and spectroscopic terms as BR570, K590, L550, M412 (69.50 kcal/mole), N560 (51.13 kcal/mole), O640.[44] During the photocycle, the retinal changes its conformation between the all-*trans*, 15-antiprotonated Schiff base and 13-*cis*, 15-antiprotonated Schiff base found in its metastable M-state. Excitation of the retinal protein around the ground state absorption wavelength of 568 nm drives BRh into the M-state. In exchange, illumination of BRh around the M state absorption peak of 412 nm directly recovers the ground state (Figure 1b).[45]

The broad absorption band of BRh extends from the near ultraviolet to the maximum in the visible at 570 nm. The fluorescence band shows a maximum at 730 nm, but extends into the near-infrared.[46] The amount of energy involved in the L550-M412 transitions is $\Delta E = E_{412} - E_{550} = 17.414$ kcal/mole!

6) Upon illumination, BRh retinal is isomerized. The protonated Schiff base then releases its proton into the extracellular environment and is subsequently reprotonated from the cytoplasm.[47] Most scientists believe this,

although experimental data are being sought to confirm a possible mechanism by which protons return to the cell via ATP synthase. This process has not yet been clarified.

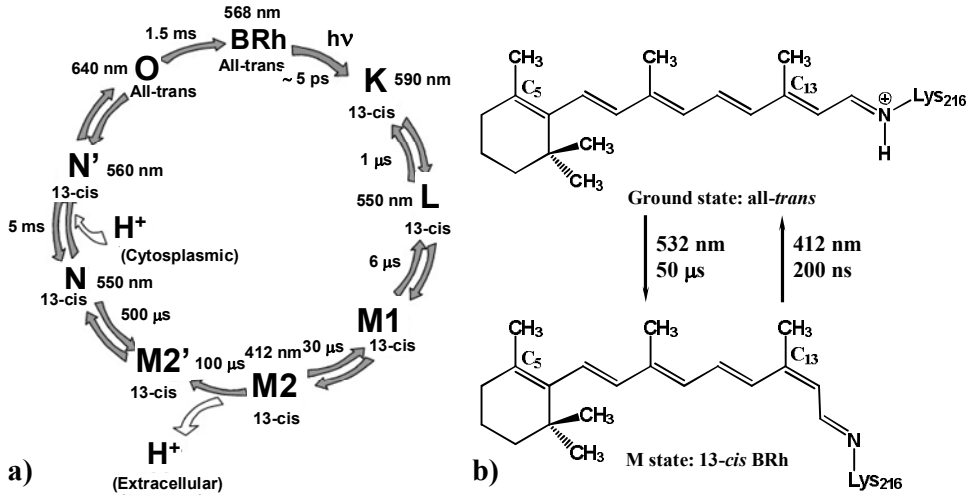
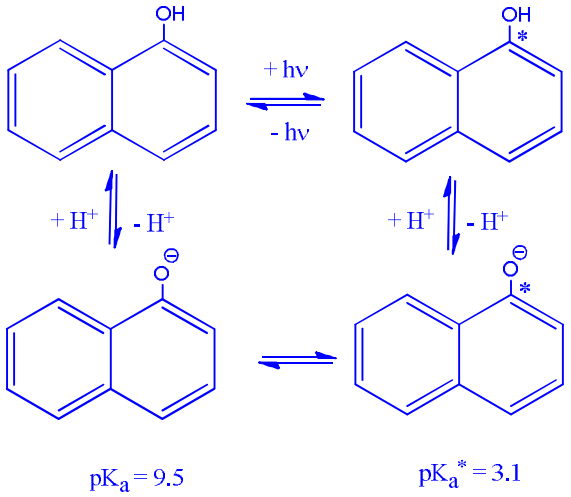



**Figure 2.** a) Photocycle of bacteriorhodopsin;[44] b) Photoinduced switching between ground state and intermediate state M of BRh.[45]

*Acidity of excited molecules*. Kaupp reported a close relationship between singlet excitation of the model compound α-naphtol and its acidity (pKa).[14] Förster cycle can be used to calculate variation of pKa on illumination of fluorescent molecules. Thus, the proton concentration of α-naphthol can increase 2,511,886.4-fold, from pKa 9.5 to pKa 3.1, as shown by both experimental data and theoretical calculations.

*Racker's experiment.* Stoeckenius and Racker's vesicles, which contain both ATP synthase and BRh, increase the acidity of their suspensions upon illumination.[8] In reconstituted systems, the measurements at pH 6.8 yielded an extrapolated stoichiometry for the proton pumping of 1.1 $H^+/e^-$ for beef heart and 0.6 $H^+/e^-$ for *Paracoccus* cytochrome *c* oxidase, respectively, whereas at pH 7.4 and 7.7, the stoichiometry was reduced by about 0.2 $H^+/e^-$.

When cytochrome *c* oxidase, reconstituted into phospholipid vesicles, was supplied with a pulse of reduced cytochrome *c,*




**Scheme 1**. The relationship between singlet excitation of α-naphtol and its acidity (p$K_a$).[14]

acidification of the extravesicular space was observed. This prompted us to consider that NADH + $H^+$, produced in Krebs cycle, serves as a reducer for ubiquinone as well as cytochromes *b*, $c_1$, *c*, *a* and $a_3$, and thus the electrons are transferred to oxygen. The oxygen molecule is not only an oxidizer but also an excitatory agent which transform the potential energy of the reduced molecule-oxygen system into an excitation form of energy.

7) The fluorescence of the M412 intermediate with a 13-cis retinal Schiff base was measured.[46] Singlet excitation of BRh is observed via its fluorescence and absorption properties.[48,49] Therefore, because BRh can be excited in the singlet or triplet state, its acidity differs in the ground state from the excited state.[50]

Both the acid and its conjugate base must be fluorescent or phosphorescent.[51] Each excited state exists long enough for equilibrium with the medium to be established prior to emission. BRh meets all these requirements. Therefore, the method of obtaining $pK_a^*$ is based on a simple

thermodynamic apparatus known as the Förster cycle. The $pK_a^*$ value can only be determined indirectly because the lifetime of excited states is usually shorter than the time required to establish acid-base equilibrium.[51]

**Conclusions**

In this work, we have demonstrated the mechanism by which protons are released from the Schiff base of bacteriorhodopsin. In addition, we have shown that an amount of energy of 17.41 kcal/mole is required to remove the ATP molecule from the enzyme ATP synthase and that this energy is equal to the energy difference between intermediates L550 and M412 or between M412 and N550. Consequently, the chemiosmotic hypothesis of proton translocation across biological membranes advanced by Peter Mitchell cannot fully explain ATP formation in *Halobacterium salinarum*.

Bacteriorhodopsin (BRh) from *H. salinarum* is therefore not a light-activated proton pump that transports protons across the plasma membrane, but a source of infrared radiation. Electromagnetic coupling between the excited states of BRh and the F0 subunit of ATP synthase may better explain the ATP production. Our analysis of experimental and theoretical data shows that the vectorial proton pumping mechanism is not correct. Protons are released due to BRh excitation only as a secondary process and spread both inside and outside. Most probable, the protons do not play an essential role in ATP production in *H. salinarum*. Instead, the 138 nm spectral shift between the L550 and M412 photointermediates, which is also associated with proton release, corresponds to an energy quantum of 17.41 kcal/mole.

A conformational change in the F0F1 system is required to remove ATP from the ATP synthase molecule. The amount of energy of 17.41 kcal/mole as a near-infrared beam (6091 $cm^{-1}$) can indeed induce a

conformational change in F0 capable of releasing one mole of ATP from F1-ATP synthase.

Overall, our investigation suggests that light energy transfer is the crucial event leading to ATP formation during acute light exposure, while $H^+$ increase is a secondary, in fact less important, phenomenon. To clarify obscure aspects of ATP production in *H. salinarum*, further investigations should be undertaken using new experimental and theoretical tools.

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