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Influence of the Membrane Potential on the Protonation of Bacteriorhodopsin: Insights from Electrostatic Calculations into the Regulation of Proton Pumping

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Abstract: Proton binding and release are elementary steps for the transfer of protons within proteins, which is a process that is crucial in biochemical catalysis and biological energy transduction. Local electric fields in proteins affect the proton binding energy compared to aqueous solution. In membrane proteins, also the membrane potential affects the local electrostatics and can thus be crucial for protein function. In this paper, we introduce a procedure to calculate the protonation probability of titratable sites of a membrane protein in the presence of a membrane potential. In the framework of continuum electrostatics, we use a modified Poisson−Boltzmann equation to include the influence of the membrane potential. Our method considers that in a transmembrane protein each titratable site is accessible for protons from only one side of the membrane depending on the hydrogen bond pattern of the protein. We show that the protonation of sites receiving their protons from different sides of the membrane is differently influenced by the membrane potential. In addition, the effect of the membrane potential is combined with the effect of the pH gradient resulting from proton pumping. Our method is applied to bacteriorhodopsin, a light-activated proton pump. We find that the proton pumping of this protein might be regulated by Asp115, a conserved residue for which no function has been identified yet. According to our calculations, the interaction of Asp115 with Asp85 leads to the protonation of the latter if the pH gradient or the membrane potential becomes too large. Since Asp85 is the primary proton acceptor in the photocycle, bacteriorhodopsin molecules in which Asp85 is protonated cannot pump protons. Furthermore, we estimate how the membrane potential affects the energetics of the individual proton-transfer reactions of the photocycle. Most reactions, except the initial proton transfer from the Schiff base to Asp85, are influenced. Our calculations give new insights into the mechanism with which bacteriorhodopsin senses the membrane potential and the pH gradient and how the proton pumping is regulated by these parameters.

Introduction

All living cells build up an electric potential difference across their membrane. This membrane potential originates from the unequal distribution of ions in the solution at the two sides of the membrane. The membrane potential is used in most cells for the transport of metabolites and proteins across the membrane. In neurons, the nerve signal is transduced along the axon of the neuron by changes of the membrane potential. Membrane potentials also play a prominent role in biological energy transduction where the electrochemical gradient is generated by proton pumps and redox loops.[1,2] This electrochemical gradient is consumed by the ATP-synthase to produce ATP from ADP and inorganic phosphate.

In many experiments, the membrane potential and concentration gradients across membranes are neglected, since it is often difficult or even impossible to set up the system in a proper way or to adjust these parameters under the given experimental conditions. However, the membrane potential and concentration gradients can be of great relevance to understand the function of membrane proteins in their physiological context. The function of membrane proteins in the presence of the membrane potential and concentration gradients can be investigated by electrophysiological methods using in vitro systems such as vesicles and black lipid membranes. Recently, *Xenopus* oocytes in which mRNA coding for membrane proteins is injected are often used to analyze the electrophysiology of membrane proteins under in vivo conditions.

The advances in structural biology give insights into the function of membrane proteins at a microscopic level. With this information, the interpretation of the structural origin of functional and spectroscopic properties of membrane proteins is possible. Also theoretical methods for investigating the

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(1) Cramer, W. A.; Knaff, D. B. *Energy Transduction in Biological Membranes.* Springer: New York, 1991.
(2) Nicholls, D. G.; Ferguson, S. J. *Bioenergetics 3*. Elsevier Academic Press: London, 2002.

influence of the membrane potential on the energetics of membrane proteins have been developed.[3] Recently, even all-atoms-simulations have been used to study the effect of an unequal ion distribution on a membrane.[4] The applicability of this approach is, however, limited since very large systems need to be simulated to get a biologically realistic membrane potential. Moreover, the membrane potential cannot be varied continuously in this kind of simulations. Up to now, only a pure lipid membrane has been simulated by this method.[4] Continuum electrostatic methods are an attractive alternative for studying membrane proteins theoretically in the membrane environment.[3,5−7] Continuum electrostatic models are also widely used for studying proton binding to proteins.[8−13] Calculating the protonation probability of titratable groups in proteins is particularly intricate because of the multiple binding equilibria that exist in proteins.[14,15] Although proton binding to membrane proteins has already been studied by continuum electrostatic methods,[16−24] membrane potentials have never been considered in these investigations.

In this work, we apply a continuum electrostatic method to study the effect of a membrane potential on the membrane protein bacteriorhodopsin (BR). This protein has been studied extensively structurally, thermodynamically, and kinetically.[25−29] It is today the best characterized membrane protein, and it has become a model system. BR is a proton pump which can generate an electrochemical proton gradient across the membrane.[30] The structure of BR and its orientation in the membrane are depicted in Figure 1. The sites involved in proton pumping are highlighted. The cofactor retinal is bound via a Schiff base to a lysinyl residue of BR. Light absorption by the retinal initializes a series of conformational changes and proton transfer reactions that finally lead to the transfer of a proton from the cytoplasm to the extracellular space. The first proton-transfer step of this process is the transfer of a proton from the retinal Schiff base to Asp85. Therefore, Asp85 is often called the initial proton acceptor. From this residue, the proton is transferred to the proton release group[31] from where it is eventually released to the extracellular space. The retinal Schiff base is reprotonated via Asp96 from the cytoplasm. The highly conserved residue Asp115, which lies close to the retinal, has a very high pK_a value under conditions where the pH is the same at the two sides of the membrane.[32−36] In previous calculations, we found that Asp115 deprotonates when the cytoplasmic pH becomes high while the extracellular pH remains about neutral.[18] According to our calculations, the deprotonation of Asp115 leads to the protonation of Asp85 due to a strong interaction between these two residues. The protonation of Asp85 inactivates the photocycle. This finding led us to suggest that Asp115 plays an important role in regulating the proton pumping of BR.

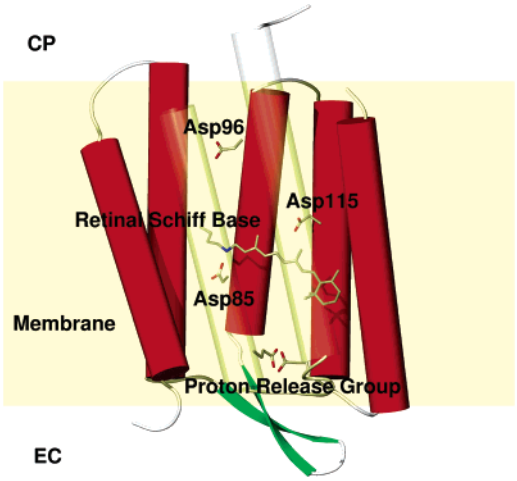

Figure 1. Structure of bacteriorhodopsin embedded in the membrane. The key sites that are involved in the proton pumping are highlighted. The extracellular (EC) and the cytoplasmic (CP) sides of the membrane are indicated.

Although BR has been studied extensively, only few studies investigated the influence of the electrochemical gradient on BR. Investigations of BR in vesicles are difficult and were done only rarely. Recent advances in expressing BR in *Xenopus* oocytes allows to study BR under biologically relevant conditions.[37]

Under physiological conditions, BR pumps protons against an electrochemical potential gradient which has two contributions: (i) the difference in the chemical potential of protons which is an entropic term related to the pH difference between the two sides of the membrane and (ii) the difference in the electric energy between the two sides of the membrane which is due to the presence of the membrane potential. In a previous investigation on the influence of the pH gradient on the protonation probabilities of BR, we accounted only for the

(3) Roux, B.; Allen, T. W.; Berneche, S.; Im, W. *Q. Rev. Biophys.* **2004**, *37*, 15−103.
(4) Sachs, J. N.; Crozier, P. S.; Woolf, T. B. *J. Chem. Phys.* **2004**, *121*, 10847−10851.
(5) Sengupta, D.; Meinhold, L.; Langosch, D.; Ullmann, G. M.; Smith, J. C. *Proteins* **2005**, *58*, 913−922.
(6) Roux, B. *Biophys. J.* **1997**, *73*, 2981−2989.
(7) Honig, B. H.; Hubbell, W. L.; Flewelling, R. F. *Annu. Rev. Biophys. Biophys. Chem.* **1986**, *15*, 163−193.
(8) Honig, B.; Nicholls, A. *Science* **1995**, *268*, 1144−1149.
(9) Ullmann, G. M.; Knapp, E. W. *Eur. Biophys. J.* **1999**, *28*, 533−551.
(10) Gunner, M.; Alexov, E. *Biochim. Biophys. Acta* **2000**, *1458*, 63−87.
(11) Bashford, D.; Karplus, M. *J. Phys. Chem.* **1991**, *95*, 9557−9561.
(12) Kloppmann, E.; Becker, T.; Ullmann, G. M. *Proteins* **2005**, *61*, 953−965.
(13) Li, H.; Hains, A. W.; Everts, J. E.; Robertson, A. D.; Jensen, J. H. *J. Phys. Chem. B* **2002**, *106*, 3486-3494.
(14) Ullmann, G. M. *J. Phys. Chem. B* **2003**, *107*, 6293−6301.
(15) Klingen, A. R.; Bombarda, E.; Ullmann, G. M. *Photochem. Photobiol. Sci.* **2006**, *5*, 588−596.
(16) Popovic, D. M.; Stuchebrukhov, A. A. *J. Am. Chem. Soc.* **2004**, *126*, 1858−1871.
(17) Rabenstein, B.; Ullmann, G. M.; Knapp, E. W. *Biochemistry* **2000**, *39*, 10487−10496.
(18) Calimet, N.; Ullmann, G. M. *J. Mol. Biol.* **2004**, *339*, 571−589.
(19) Olkhova, E.; Hunte, C.; Screpanti, E.; Padan, E.; Michel, H. *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103*, 2629−2634.
(20) Song, Y.; Mao, J.; Gunner, M. R. *Biochemistry* **2003**, *42*, 9875−9888.
(21) Spassov, V. Z.; Luecke, H.; Gerwert, K.; Bashford, D. *J. Mol. Biol.* **2001**, *312*, 203-219.
(22) Onufriev, A.; Smondyrev, A.; Bashford, D. *J. Mol. Biol.* **2003**, *332*, 1183−1193.
(23) Bashford, D.; Gerwert, K. *J. Mol. Biol.* **1992**, *224*, 473−486.
(24) Haas, A. H.; Lancaster, C. R. D. *Biophys. J.* **2004**, *87*, 4298−4315.
(25) Subramaniam, S.; Lindahl, M.; Bullough, P.; Faruqi, A. R.; Tittor, J.; Oesterhelt, D.; Brown, L.; Lanyi, J.; Henderson, R. *J. Mol. Biol.* **1999**, *287*, 145−161.
(26) Lanyi, J. K. *FEBS Lett.* **1999**, *464*, 103−107.
(27) Heberle, J. *Biochim. Biophys. Acta* **2000**, *1458*, 135−147.
(28) Haupts, U.; Tittor, J.; Oesterhelt, D. *Annu. Rev. Biophys. Struct. Biol.* **1999**, *28*, 367−399.
(29) Lanyi, J. K. *Annu. Rev. Physiol.* **2004**, 665−688.
(30) Michel, H.; Oesterhelt, D. *FEBS Lett.* **1976**, *65*, 175−178.
(31) Garczarek, F.; Brown, L. S.; Lanyi, J. K.; Gerwert, K. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 3633−3638.
(32) Gerwert, K.; Hess, B.; Soppa, J.; Oesterhelt, D. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 4943−4947.
(33) Engelhard, M.; Hess, B.; Emeis, D.; Metz, G.; Kreutz, W.; Siebert, F. *Biochemistry* **1989**, *28*, 3965−3975.
(34) Metz, G.; Siebert, F.; Engelhard, M. *Biochemistry* **1992**, *31*, 455−462.
(35) Száraz, S.; Oesterhelt, D.; Ormos, P. *Biophys. J.* **1994**, *67*, 1706−1712.
(36) Garczarek, F.; Gerwert, K. *J. Am. Chem. Soc.* **2006**, *128*, 28−29.
(37) Nagel, G.; Mockel, B.; Buldt, G.; Bamberg, E. *FEBS Lett.* **1995**, *377*, 263−266.

contribution due to the difference in the chemical potential.[18] We considered that the pH value at the two sides of the membrane can be different, but we assumed that the electric potential due to a proton imbalance is compensated by other ions. Experimentally this situation can be achieved, for instance, by the addition of H^+-Na^+ exchangers. Although experimentally these two electrochemical contributions can be adjusted independently, in living cells both components are present and they are interrelated. Therefore, it is of great importance to take both contributions into account.

In this paper, we introduce a method for calculating protonation probabilities of membrane proteins in the presence of a membrane potential. This method is applied to determine the protonation probabilities of BR in the ground state. We discuss how the titration behavior of BR is influenced by the membrane potential. Remarkably, this influence depends on whether the sites receive their proton from the cytoplasmic side or from the extracellular side. This behavior is caused by the noncontinuous hydrogen-bond network which characterizes proteins performing vectorial proton transfer, like bacteriorhodopsin.[38] Furthermore, we investigate how the presence of a membrane potential can influence the proton pumping activity, and we estimate the effect of the membrane potential on the proton-transfer reactions occurring in BR during the photocycle. The finding of our previous paper,[18] that Asp115 may be important for regulating the proton pumping activity of BR, is strengthened by our new results. Previously, we discovered that the interaction between Asp115 and Asp85 can explain why proton pumping stops, when the pH gradient becomes too large. Here, we show that a membrane potential even enhances this effect. Moreover, the interaction between Asp115 and Asp85 can also inhibit the proton pumping by BR when the membrane potential becomes too negative. Our new method can be applied also to other proteins and allows to study theoretically ion binding to membrane proteins in the presence of concentration gradients and membrane potential in general.

Theory

Membrane Potential within the Poisson−Boltzmann Theory. The influence of a membrane potential on the energetics of a membrane protein can be incorporated in the Poisson−Boltzmann theory.[6] The linearized Poisson−Boltzmann equation of a membrane system with a membrane potential Ψ present is given by

$$\nabla[\epsilon(\mathbf{r})\nabla\phi(\mathbf{r})] = -4\pi\rho_P(\mathbf{r}) + \epsilon(\mathbf{r})\bar{\kappa}(\mathbf{r})^2[\phi(\mathbf{r}) - \Psi\Theta(\mathbf{r})] \quad (1)$$

where $\phi(\mathbf{r})$ is the electrostatic potential of the system; $\Theta(\mathbf{r})$ is the Heaviside step function which is equal to zero on the extracellular side and equal to one on the cytoplasmic side; $\epsilon(\mathbf{r})$ defines the dielectric permittivity; $\rho_P(\mathbf{r})$ is the charge density inside the protein; and $\bar{\kappa}^2(\mathbf{r}) = (8\pi e_o^2 I(\mathbf{r})/RT)$ is a screening factor related to the ion distribution in the system, e_o is the elementary charge, $I(\mathbf{r})$ is the ionic strength which is zero inside the protein and the membrane and has a defined value in the solution, R is the gas constant, and T is the absolute temperature. For a detailed derivation of eq 1, see ref 6.

Assuming a discrete charge distribution, the solution of eq 1, i.e., the potential $\phi(\mathbf{r})$, can be expressed as[6,39]

$$\phi(\mathbf{r}) = \sum_{i=1}^{M} \frac{q_i}{\epsilon_p|\mathbf{r} - \mathbf{r}_i'|} + \phi_{rf}(\mathbf{r}) + \Psi\chi_{mp}(\mathbf{r}) \quad (2)$$

where the first term describes the Coulomb electrostatic potential at the position $\mathbf{r}$ caused by M point charges q_i at positions $\mathbf{r}_i'$ in a medium with a dielectric permittivity ϵ_p, the term $\phi_{rf}(\mathbf{r})$ describes the reaction field potential originating from the dielectric boundary between the protein and the solvent as well as from the distribution of ions in the solution, and the term $\Psi\chi_{mp}(\mathbf{r})$ describes the contribution due to the membrane potential. Here, $\chi_{mp}(\mathbf{r})$ is a dimensionless function with the property $0 \le \chi_{mp}(\mathbf{r}) \le 1$, which depends on the dielectric properties of the system and the ion distribution in the medium but not on the charge distribution within the protein.[6] The function $\chi_{mp}(\mathbf{r})$ describes how the membrane potential is modulated inside the membrane protein and it may deviate from a simple linear function.[6]

Protonation Probabilities in the Presence of a Transmembrane pH Gradient and a Membrane Potential. The calculation of protonation probabilities of proteins is an established method.[9,10,14,39,40] In a previous publication,[18] we extended the standard theory to take pH gradients into account. Here, we further extend the method to include also the effect of the membrane potential in the calculation of protonation probabilities.

In proton pumping proteins, like bacteriorhodopsin, protons are transferred via a hydrogen bond network. To avoid proton back-flow, the hydrogen bond network should not be continuous from the cytoplasm to the extracellular space. Thus in the ground state, proton pumping proteins should contain at least two separate hydrogen bond networks: one connected to the cytoplasmic side and the other connected to the extracellular side. The connectivity of the hydrogen bond networks can be searched by graph-theoretical methods.[18]

We consider a transmembrane protein that is in equilibrium with two different reservoirs of protons, i.e., the extracellular side, EC, and the cytoplasmic side, CP. The two reservoirs of protons have different proton electrochemical potentials, and they are not in contact with each other; i.e., protons cannot permeate through the membrane or through the protein. The protein has N sites connected to the extracellular side and K sites connected to the cytoplasmic side. None of the sites is connected to both reservoirs, because otherwise the reservoirs would be in contact with each other. All sites interact electrostatically. Such a system can adopt 2^{N+K} different protonation states, and each protonation state n can be represented by a protonation state vector $\vec{x}^n = \{x_1^n, x_2^n, ..., x_{N+K}^n\}$. The component x_i^n of this vector is either 1 or 0, depending whether site i is protonated or not.

In analogy to the chemical potential μ_c of a substance c, one can define the electrochemical potential $\bar{\mu}_c$ for the ionic species with activity a_c and charge z_c in a system with a membrane

(38) Haupts, U.; Tittor, J.; Bamberg, E.; Oesterhelt, D. *Biochemistry* **1997**, *36*, 2−7.
(39) You, T.; Bashford, D. *Biophys. J.* **1995**, *69*, 1721−1733.
(40) Onufriev, A.; Case, D. A.; Ullmann, G. M. *Biochemistry* **2001**, *40*, 3413−3419.

potential Ψ:

$$\bar{\mu}_c = \mu_c^\circ + RT \ln a_c + z_c F \Theta(\mathbf{r}) \Psi = \mu_c + z_c F \Theta(\mathbf{r}) \Psi \qquad (3)$$

where $\Theta(\mathbf{r})$ is the Heaviside step function which is equal to zero on the extracellular side and equal to one on the cytoplasmic side. The free energy for moving an ion across a membrane has thus two contributions: one arising from the work against the activity difference between the two sides of the membrane and a second arising from the work against the membrane potential Ψ.[1,2] The work required to transfer an ion from one side of the membrane to the other is given by the difference between the electrochemical potentials at the two sides of the membrane.

The energy G_n of the protonation state n of a protein is given by the following equation:

$$G_n(\bar{\mu}_{H+}^{EC}, \bar{\mu}_{H+}^{CP}) = \sum_{i=1}^{N} (x_i^n - x_i^\circ)(\bar{G}_{intr,i}^\circ - \bar{\mu}_{H+}^{EC}) + \sum_{j=1}^{K} (x_j^n - x_j^\circ)(\bar{G}_{intr,j}^\circ - \bar{\mu}_{H+}^{CP}) + \frac{1}{2}\sum_{i=1}^{N+K}\sum_{j=1}^{N+K} W_{ij}(x_i^n - x_i^\circ)(x_j^n - x_j^\circ) \qquad (4)$$

x_i° is the protonation of site i in its reference state. The term W_{ij} is the interaction energy between sites i and j when they are in their nonreference forms; $\bar{\mu}_{H+}^{EC}$ and $\bar{\mu}_{H+}^{CP}$ are the electrochemical potentials at the extracellular and the cytoplasmic side, respectively; the intrinsic proton binding energy ($\bar{G}_{intr,i}^\circ$) is the protonation energy that the site i would have in the presence of a membrane potential if all the other titratable sites are in their reference protonation form. The intrinsic proton binding energy $\bar{G}_{intr,i}^\circ$ has several contributions:

$$\bar{G}_{intr,i}^\circ = G_{model,i}^\circ + \Delta\Delta G_i^{solv} + \Delta\Delta G_i^{back} + \Delta\Delta G_i^{\Psi} \qquad (5)$$

It is calculated from the shift of the protonation energy of a model compound in aqueous solution $G_{model,i}^\circ$ compared to the protonation energy of this model compound in the protein environment. This shift is due to the changes in the solvation energy $\Delta\Delta G_i^{solv}$, the interaction with nontitratable background charges $\Delta\Delta G_i^{back}$, and the interaction with the membrane potential $\Delta\Delta G_i^{\Psi}$. The protonation energy of a model compound in aqueous solution $G_{model,i}^\circ$ is known from experiments or quantum chemical calculations.[9]

The different energy contributions can be calculated from the electrostatic potentials which are obtained from the numerical solution of the Poisson−Boltzmann equation. Explicit expressions for $\Delta\Delta G_i^{solv}$ and $\Delta\Delta G_i^{back}$ are given in a previous publication.[9] $\Delta\Delta G_i^{\Psi}$ can be obtained from the following equation

$$\Delta\Delta G_i^{\Psi} = F\Psi \sum_{k=1}^{M_i} \chi_{mp}(\boldsymbol{r}_k)(Q_{k,i}^h - Q_{k,i}^d) = F\Psi\,\Gamma_i \qquad (6)$$

where the sum named Γ_i describes the relative contribution of the membrane potential on the protonation energy of the site i; $\chi_{mp}(\mathbf{r})$ is the dimensionless function that modulates the membrane potential within the membrane defined in eq 2; M_i is the number of charges of site i that change during the protonation reaction; and $Q_{k,i}^h$ and $Q_{k,i}^d$ are the charges of atom k of site i in the protonated and deprotonated form, respectively. For protons, Γ_i has the property $0 \le \Gamma_i \le 1$.

If one wants to calculate the protonation probabilities for different pH values at the two sides of the membrane and for different membrane potentials, it is of advantage to rewrite eq 4. Using eqs 3, 5, and 6, the definition of the Heaviside function, and the fact that the charge of H^+ is +1, eq 4 becomes

$$G_n(\mu_{H+}^{EC},\mu_{H+}^{CP},\Psi) = \sum_{i=1}^{N} (x_i^n - x_i^\circ)(G_{intr,i}^\circ - \mu_{H+}^{EC} + F\Psi\Gamma_i) + \sum_{i=1}^{K} (x_i^n - x_i^\circ)(G_{intr,i}^\circ - \mu_{H+}^{CP} + F\Psi(\Gamma_i - 1)) + \frac{1}{2}\sum_{i=1}^{N+K}\sum_{j=1}^{N+K} W_{ij}(x_i^n - x_i^\circ)(x_j^n - x_j^\circ) \qquad (7)$$

where $G_{intr,i}^\circ = G_{model,i}^\circ + \Delta\Delta G_i^{solv} + \Delta\Delta G_i^{back}$, Γ_i is defined in eq 6. μ_{H+}^{EC} and μ_{H+}^{CP} are the proton chemical potentials at the extracellular and at the cytoplasmic side of the membrane without considering the effect of the membrane potential; i.e., they are equivalent to a pH value. In our system, no protons are present inside the membrane. It should be noted that due to the membrane potential, there is an important difference between the sites connected to the cytoplasm and those connected to the extracellular region. In eq 7, the energetic cost for protonating a given site amounts to $F\Psi\Gamma_i$ for sites connected to the extracellular side, while the cost is $F\Psi(\Gamma_i - 1)$ for sites connected to the cytoplasm. Thus, for a negative membrane potential (see Figure 2), protonation is favored on the extracellular side ($F\Psi\Gamma_i < 0$) since proton uptake is downhill with regard to the membrane potential. In contrast, protonation of sites connected to the cytoplasm is hindered ($F\Psi(\Gamma_i - 1) > 0$) since proton uptake is uphill with regard to the membrane potential.

Compared to eq 4, eq 7 has the advantage that membrane potential and pH are separated and can be changed independently from each other. Using eq 7, the protonation probability of site i at given environmental conditions (μ_{H+}^{EC}, μ_{H+}^{CP}, Ψ or equivalently pH_{EC}, pH_{CP}, Ψ) can be calculated by a thermodynamic average over all possible protonation states. Often, such an explicit averaging cannot be performed since too many protonation states are possible. In such cases, Monte Carlo simulations can be used to obtain the thermodynamic average.[41]

Methods

Preparation of Three Ground-State BR Structures. The high-resolution X-ray structure of ground-state BR with PDB code 1C3W[42] was used as the starting model for all calculations. The structure 1C3W lacks the intracellular EF loop (residues 155−162). We therefore took the EF loop coordinates from the 1QHJ structure[43] as described before.[18]

(41) Beroza, P.; Fredkin, D. R.; Okamura, M. Y.; Feher, G. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 5804−5808.
(42) Luecke, H.; Schobert, B.; Richter, H.-T.; Cartailler, J.-P.; Lanyi, J. K. *J. Mol. Biol.* **1999**, *291*, 899−911.
(43) Belrhali, H.; Nollert, P.; Royant, A.; Menzel, C.; Rosenbusch, J. P.; Landau, E. M.; Pebay-Peyroula, E. *Structure* **1999**, *7*, 909−917.

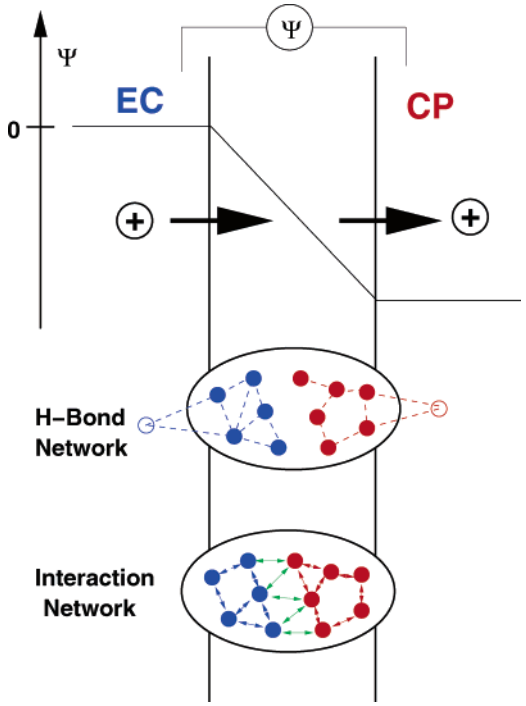

Figure 2. Schematic representation of the protonation of a membrane protein. In this scheme the membrane potential $\Psi = \psi_{CP} - \psi_{EC} < 0$, with ψ_{CP} and ψ_{EC} being the potentials in the cytoplasm (CP) and in the extracellular space (EC), respectively. The open circles symbolize the connections of titratable sites to the bulk solvent. The membrane protein is depicted as an ellipse. The hydrogen bond network relating the titratable sites to the EC region (blue) is not connected to the hydrogen bond network relating the titratable sites to the CP region (red); i.e., protons cannot flow through BR. Proton displacement along one hydrogen bond network is favored in the direction of decreasing potential (black arrows). Under these conditions, the membrane potential will enhance the protonation probability of a titratable site receiving a proton from the EC region (blue), and it will disfavor the protonation of a titratable site receiving a proton from the CP region (red). Due to the electrostatic interaction between sites connected to the extracellular space and sites connected to the cytoplasm (symbolized by the green arrows in the interaction network), the protonations of the sites that are connected to different sides of the membrane are not independent of each other. For clarity, only nearest-neighbor interactions are shown. In the calculations, all interactions were taken into account.

Lipid and water molecules were not considered explicitly. Neutral blocking groups were added to the termini, since the flanking residues are not resolved in any crystal structure of BR. Hydrogen positions were constructed with the HBUILD procedure[44] in CHARMM using the CHARMM22 parameter set[45] and subsequently optimized. To model the low dielectric region of the membrane, BR was embedded in a ring of dummy atoms (atoms with no charge) representing the hydrophobic part of the membrane lipids as described previously.[18] The membrane was assumed to range from −12 to 19 Å along the *z*-axis of the crystal structure.

Hydrogen Bond Network and Site Connectivity to the Bulk. The hydrogen bond network of the protein was analyzed by a graph theoretical method that has been described in detail in a previous publication.[18] This method was used to detect whether the titratable groups can receive a proton either from the extracellular side or from the cytoplasmic side. The two most important residues in this study are Asp85 and Asp115. Asp85 is connected to the EC side of the membrane via several water molecules, Arg82, and the proton release group. Asp115 is located at a distance of about 4 Å from a cavity which can connect this residue to the CP side of the membrane. This cavity is formed by the residues Leu94, Met118, Met145, Ile148, Leu149, Leu152, Thr178, and Trp182.

Continuum Electrostatic Calculations. The intrinsic proton binding energies and the interaction energies in eq 7 were obtained by continuum electrostatic calculations.[46] The Poisson−Boltzmann equation was solved by a finite-difference method[47] using the MEAD program suite.[48,49] All aspartate, glutamate, lysine, arginine, and tyrosine residues as well as the retinal Schiff base were considered as protonatable sites (BR has no histidines and cysteines). Atomic partial charges for amino acid groups were taken from the CHARMM22 parameter set.[45] The charges of the retinal Schiff base[18] were derived from a density functional calculation using the program ADF.[50] The pK_a values of the model compounds were taken from the literature.[9,21,51] The dielectric constant of the protein and membrane interiors was set to 4, and that of the solvent was set to 80. The ionic strength at both sides of the membrane was set to 0.15 M.

Continuum electrostatic calculations were performed using the focusing technique[47] in three steps. For the protein, the electrostatic potential was calculated by focusing using three cubic grids of 101^3, 81^3, and 101^3 grid points and grid spacings of 1.0, 0.5, and 0.2 Å, respectively. The largest spaced grid was centered on the protein, the other two grids were centered on the titratable group. For the model compounds, the electrostatic potential was calculated by focusing using three grids of 81^3, 81^3, and 101^3 grid points and grid spacings of 1.0, 0.5, and 0.2 Å, respectively. The largest spaced grid was centered on the model compound, and the other two grids were centered on the titratable group.

The influence of the membrane potential on the protonation energy of the titratable groups was calculated by the module PBEQ[52] of the program CHARMM.[53] The membrane was modeled as a slab. BR was embedded in a cylinder with a dielectric constant of 80 and with a radius of 26 Å. Additional dummy atoms were used to fill the parts of the cylinder in the membrane region that is not filled by the protein. Internal protein cavities were not filled with dummy atoms. The function $\chi_{mp}(\mathbf{r})$ was calculated from the solution of the Poisson−Boltzmann equation with and without membrane potential. From $\chi_{mp}(\mathbf{r})$, we calculated the quantity Γ_i using eq 6.

Since protons are charged particles, a pH gradient through the membrane can contribute to the membrane potential. This aspect was addressed in one of our calculations by modeling the membrane potential as a linear function of the transmembrane pH gradient $\Delta\text{pH} = \text{pH}_{EC} - \text{pH}_{CP}$, with a slope of $(RT/F \ln(10))$ since $\text{pH} = -\log a_{H+}$

$$\Psi(\text{pH}) = a \frac{RT}{F} \ln(10)\, \Delta\text{pH} \qquad (8)$$

where a is a factor which scales the increase of the membrane potential with increasing ΔpH.

In a living cell, the change of the membrane potential may deviate from linearity because of compensating ion gradients.[54] However, experiments on proton pumping BR expressed in *Xenopus* oocytes[55] showed that the total membrane potential can be well approximated

(44) Brünger, A. T.; Karplus, M. *Proteins* **1988**, *4*, 148−156.
(45) MacKerell, A. D. et al. *J. Phys. Chem. B* **1998**, *102*, 3586−3616.
(46) Bashford, D.; Karplus, M. *Biochemistry* **1990**, *29*, 10219−10225.
(47) Klapper, I.; Hagstrom, R.; Fine, R.; Sharp, K.; Honig, B. *Proteins* **1986**, *1*, 47−59.
(48) Bashford, D.; Gerwert, K. *J. Mol. Biol.* **1992**, *224*, 473−486.
(49) Bashford, D. In *Scientific Computing in Object-Oriented Parallel Environments*; Ishikawa, Y., Oldehoeft, R. R., Reynders, J. V. W., Tholburn, M., Eds.; Springer: Berlin, 1997; Vol. 1343, pp 233−240.
(50) te Velde, G.; Bickelhaupt, F. M.; Baerends, E. J.; Guerra, C. F.; van Gisbergen, S. J. A.; Snijders, J. G.; Ziegler, T. *J. Comput. Chem.* **2001**, *22*, 931−967.
(51) Bashford, D.; Case, D. A.; Dalvit, C.; Tennant, L.; Wright, P. E. *Biochemistry* **1993**, *32*, 8045−8056.
(52) Im, W.; Beglov, D.; Roux, B. *Comput. Phys. Commun.* **1998**, *111*, 59−75.
(53) Brooks, B. R.; Bruccoleri, R. E.; Olafson, B. D.; States, D. J.; Swaminathan, S.; Karplus, M. *J. Comput. Chem.* **1983**, *4*, 187−217.
(54) Schuster, S.; Ouhabi, R.; Rigoulet, M.; Mazat, J.-P. *Bioelectrochem. Bioenerg.* **1998**, *45*, 181−192.
(55) Geibel, S.; Friedrich, T.; Ormos, P.; Wood, P. G.; Nagel, G.; Bamberg, E. *Biophys. J.* **2001**, *81*, 2059−6.

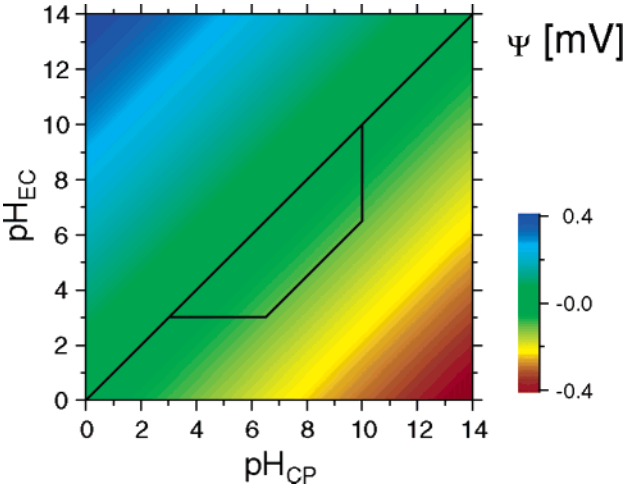

Figure 3. The proton pumping active region of BR and the membrane potential Ψ as a function of pH_{EC} and pH_{CP}. The region that is bordered by the black line indicates the region in which bacteriorhodopsin was found to pump protons.[58] The membrane potential is color-coded as indicated by the color scale. The potential is calculated as $\Psi(pH) = 0.5(RT/F)\ \ln(10)\ (pH_{EC} - pH_{CP})$.

by a linear function of the pH gradient over a large pH range with a factor *a* of about 0.5.

Calculation of the Titration Profile. We used a Monte Carlo (MC) approach[56] to calculate the protonation probabilities of all titratable sites of BR. The efficiency of the sampling is increased by a special treatment of pairs (double moves[56]) or triplets (triple moves[57]) of titratable sites that interact by more than a given energy threshold. Two-dimensional titration profiles were calculated for three different scenarios: (i) The pH and the membrane potential were varied with the constraint that the pH values at the two sides of the membrane (pH_{CP} and pH_{EC}) are equal. The membrane potential was varied in steps of 10 mV. (ii) pH_{CP} and pH_{EC} were varied independently. The membrane potential was set to zero. (iii) pH_{CP} and pH_{EC} were varied independently. The pH difference gives rise to a membrane potential according to eq 8 with $a = 0.5$.

In all the calculations, the pH was varied in steps of 0.2 pH units. The temperature was set to 300 K; the coupling threshold defining the pairs of titratable sites for double moves was set to 2 pK_a units; the coupling threshold for triple moves was set to 3 pK_a units. 20 000 MC scans were performed, where one MC scan comprises as many MC moves as titratable sites, pairs, and triplets are present in the molecule. At each pair of values (pH, Ψ or pH_{EC}, pH_{CP}), a randomly chosen initial state vector was equilibrated with 200 MC scans. All the MC calculations were done with our in-house program CMCT.

Results and Discussions

The pH region in which BR pumps protons was determined experimentally by Kouyama and co-workers with BR embedded in vesicles.[58] This region is shown by a solid line in Figure 3. Under illumination, BR pumps protons from the lumen of the vesicle to the exterior. Initially, the pH inside and outside of the vesicle are identical; i.e., the experimental starting conditions correspond to the diagonal of the diagram in Figure 3. Upon proton pumping, the pH inside the vesicle increases (the proton concentration decreases), and the pH in the exterior either decreases or is constant in case the external medium is buffered. Thus, under these conditions, the region on the left side of the diagonal in the diagram in Figure 3 is not explored. Our calculations fully reflect the experimental findings of Kouyama and co-workers; in fact the borders of the activity region indicated in Figure 3 coincide with the titration behavior of Asp85, a residue that is crucial for proton pumping. This titration behavior offers an explanation for the inability of BR to pump protons when the pH gradient and the membrane potential are too large.

Using the theory outlined above, we determine how the protonation probabilities of titratable sites in the membrane protein BR depend on the environmental conditions. We consider three different scenarios:

(i) The pH value of the solution and the membrane potential are varied independently. In these calculations, we assume that the pH value on the two sites of the membrane is the same. Experimentally, such a situation can be achieved by adding H^+-Na^+ exchangers to the system.

(ii) The pH values in the extracellular space and in the cytoplasm are varied independently. The membrane potential that could result from the pH gradient is compensated by an ion gradient in the opposite direction. This situation can be achieved experimentally when ionophores such as valinomycin are added to the system.

(iii) The pH values in the extracellular space and in the cytoplasm are varied independently. The pH gradient gives rise to a membrane potential which is partially compensated by other ion gradients. This situation most likely resembles physiological conditions. Experimentally, it was found that in *Xenopus* oocytes only about half of the pH gradient gives rise to a membrane potential.[55]

(i) Influence of the Membrane Potential on the Titration Behavior. The theory developed here clearly shows that the influence of the membrane potential on the protonation probability depends on the proton accessibility of a site, i.e., from which side of the membrane the titratable site receives its proton. Protonation against the membrane potential will be hindered, while protonation along the potential gradient will be favored. This idea is expressed in a quantitative way in eq 7. The membrane potential is therefore a critical parameter for investigating the titration behavior of a site in a protein embedded in a membrane.

Since the hydrogen bond network is not continuous from the extracellular side to the cytoplasmic side, protons do not freely flow through the protein, which is an indispensable property of vectorial proton pumps.[38] The effect of the membrane potential on the protonation can be best illustrated when the pH value is the same at the two sides of the membrane. In this scenario, no chemical potential gradient and only an electric potential gradient is present. In case the membrane potential Ψ is negative (i.e., the potential at the CP side is lower than that at the EC side), the proton displacement is favored from the extracellular region into the membrane and from the membrane into the cytoplasmic region. Under this condition, it is favorable to transfer a positively charged proton from the extracellular space into the membrane region since the membrane potential decreases in this direction. Therefore, the more the membrane potential becomes negative, the higher is the protonation probability of a site that receives the proton from the extracellular side of the membrane. Accordingly, the equilibrium

(56) Beroza, P.; Fredkin, D. R.; Okamura, M. Y.; Feher, G. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 5804−5808.
(57) Rabenstein, B.; Ullmann, G. M.; Knapp, E. W. *Eur. Biophys. J.* **1998**, *27*, 626−637.
(58) Kouyama, T.; Kinosita, K., Jr.; Ikegami, A. *Adv. Biophys.* **1988**, *24*, 123−175.

Table 1. Effect of the Membrane Potential on the Protonation Energy[a]

site	connection	Γ_i
Arg7	EC	0.24
Glu9	EC	0.24
Tyr26	CP	0.76
Lys30	CP	0.82
Asp36	CP	0.91
Asp38	CP	0.89
Lys40	CP	0.89
Lys41	CP	0.87
Tyr43	CP	0.81
Tyr57	EC	0.44
Tyr64	EC	0.30
Glu74	EC	0.12
Tyr79	EC	0.26
Arg82	EC	0.39
Tyr83	EC	0.39
Asp85	EC	0.50
Asp96	CP	0.73
Asp102	CP	0.90
Asp104	CP	0.89
Asp115	CP	0.58
Lys129	EC	0.15
Tyr131	EC	0.22
Tyr133	EC	0.18
Arg134	EC	0.26
Tyr147	CP	0.73
Tyr150	CP	0.79
Lys159	CP	0.90
Glu161	CP	0.90
Arg164	CP	0.92
Glu166	CP	0.93
Lys172	CP	0.88
Arg175	CP	0.80
Tyr185	EC	0.50
Glu194	EC	0.32
Glu204	EC	0.33
Asp212	EC	0.47
Retinal[b]	EC	0.52
Arg225	CP	0.80
Arg227	CP	0.91

[a] All the titratable sites in BR are listed in the column "site". The column "connection" indicates whether the site receives its proton from the extracellular (EC) or the cytoplasmic (CP) side of the membrane. Γ_i is defined in eq 6. Depending on whether the titratable site receives its proton from the extracellular or the cytoplasmic side of the membrane, Γ_i or $(\Gamma_i - 1)$ (see eq 7) has to be multiplied with the membrane potential and the Faraday constant in order to obtain the effect of the membrane potential on the protonation energy. [b] Retinal Schiff base covalently bound to Lys216.

protonation probability of a site that receives its proton from the cytoplasmic side of the membrane increases if the membrane potential becomes less negative or even positive. In the condition of increasing membrane potential, it is less and less favorable for the proton to stay in the cytoplasm, and thus the protonation probability within the membrane increases. On the contrary, the protonation probability of a site that receives its proton from the extracellular side of the membrane decreases with increasing membrane potential.

The titration behavior of the titratable sites of bacteriorhodopsin is analyzed according to the theoretical treatment developed here. In Table 1, all the titratable sites of BR are listed. Each of these sites is uniquely connected through a hydrogen bond network to either the cytoplasmic (CP) or the extracellular (EC) region. None of these sites is connected to both sides, as it is expected in a protein which performs vectorial proton pumping like BR. Table 1 lists also the quantity Γ_i, which describes the relative influence of the membrane potential on the protonation energy as described in eqs 6 and 7.

The protonation probabilities of all titratable sites of BR have been calculated. Two-dimensional titration curves ($\langle x_i \rangle$ vs (pH, Ψ)) of the functionally important sites are shown in Figure S1 of the Supporting Information. Particularly interesting is the titration behavior of the residues Asp85 and Asp115 in dependence on the membrane potential. In our previous work,[18] the electrostatic interaction among Asp85, Asp115, and the Schiff base together with the interplay of their protonation states has been suggested to provide the structural basis for the back-pressure effect in BR. We proposed a possible role for the highly conserved Asp115, whose function has not been understood yet. Under conditions when the pH is the same on both sides of the membrane or when no membrane potential is present, Asp115 was found to be always protonated in the physiological pH range.[28,29,36] This finding is in full agreement with our calculations as can be seen from the protonation probability of Asp115 at $\Psi = 0$ in Figure 4 where this residue only titrates at around pH = 12. From our calculations, we suggested that Asp115 senses the pH on the cytoplasmic side of the membrane and transmits this information to Asp85 and the Schiff base, two key sites in the proton-transfer mechanism of BR. In the present work, the role of Asp115 is further investigated. In the ground state of BR, Asp85 receives its proton from the extracellular side of the membrane, while Asp115 receives its proton from the cytoplasmic side. Thus, as outlined above, when the membrane potential increases (i.e., it becomes less negative), the protonation probability of Asp85 decreases, while the protonation probability of Asp115 increases. Asp85 is the first residue that receives the proton from the retinal Schiff base in the proton pumping cycle of BR. Thus, it is crucial for the proton transfer that Asp85 is deprotonated in the ground state of BR. Interestingly, at very negative membrane potential (Ψ between -250 to -450 mV), Asp85 and Asp115 titrate in the same pH range (between pH 6 and 8, Figure 4). Due to their strong interaction, the protonation of these residues is highly correlated: when Asp115 is deprotonated, Asp85 is protonated, and vice versa. Our calculations indicate that, at very negative membrane potential, the deprotonation of Asp115 leads to the protonation of Asp85 and thus to the inactivation of the proton pumping of BR. This finding underlines the important role of Asp115 in controlling the proton pumping activity of BR.

(ii) Influence of pH Gradient on the Titration Behavior. In the next calculations, we consider that BR is exposed to a transmembrane pH gradient. We assume that the electric potential gradient is compensated by other ions, and thus no membrane potential is present. This scenario has been discussed already in detail in a previous publication.[18] Here we only summarize the main results. Two-dimensional titration curves ($\langle x_i \rangle$ vs (pH_{EC}, pH_{CP}, $\Psi = 0$)) of the functionally important sites are shown in Figure S2 of the Supporting Information. The titration behavior of Asp85 and Asp115 in dependence on pH_{EC} and pH_{CP} is shown in Figure 5a.

Also in this scenario without the presence of a membrane potential, the interaction between Asp85 and Asp115 leads to interesting effects that may be relevant for the function of the protein. Asp115 is protonated up to a high pH under conditions where the pH is the same on the two sides of the membrane[28,29,36] as can be seen from the protonation probability along the diagonal of Figure 5a. However, Asp115 starts to deprotonate when the pH on the cytoplasmic side of the membrane

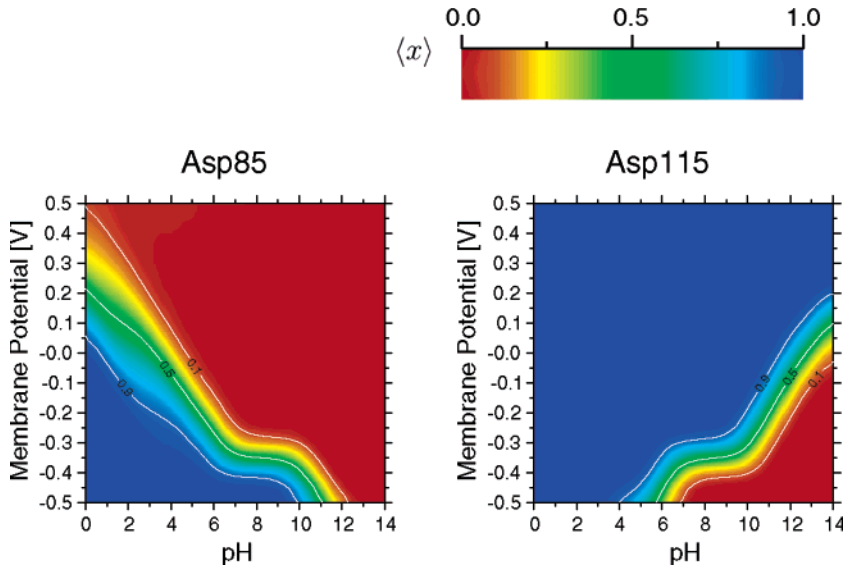

Figure 4. Protonation probabilities of Asp85 and Asp115 in dependence on the pH and the membrane potential Ψ. The protonation probability ⟨*x*⟩ is given by a color scale. It is assumed that the pH on the two sides of the membrane is the same and the membrane potential is adjusted by other ion gradients. Asp115 receives its proton from the cytoplasmic side of the membrane, and thus, at a given pH for the two sides of the membrane, its protonation probability decreases with decreasing membrane potential Ψ (i.e., more negative membrane potential). Instead, Asp85 receives its proton from the extracellular side and thus its protonation probability increases with decreasing membrane potential. At physiological pH, a membrane potential of about −400 mV leads to the protonation of Asp85 and the deprotonation of Asp115.

increases while the pH stays about constant in the extracellular region. The deprotonated Asp115 interacts strongly with Asp85 and causes the protonation of Asp85.[18] The protonation of Asp85 inactivates proton pumping of BR since the primary proton acceptor (Asp85) is already protonated. This effect is similar to the one discussed in the previous section. However, it is worth noting that the reason why Asp85 protonates and Asp115 deprotonates are different in these two scenarios. In the presence of a membrane potential (scenario i), the protonation energies of Asp85 and Asp115 are shifted due to the interaction of these residues with the membrane potential. Since the two residues receive their proton from different sides of the membrane, their titration behaviors are differently influenced by the membrane potential. In the presence of a pH gradient (scenario ii), the proton activities at the two sides of the membrane are different leading to altered titration behaviors of Asp85 and Asp115. However, in both situations, the interaction between Asp85 and Asp115 seems to be crucial for the regulation of the proton pumping activity.

(iii) Joint Influence of the Membrane Potential and the pH Gradient on the Titration Behavior. The scenarios discussed so far do most likely not correspond to the situation in the cell. The proton pumping activity of BR from the cytoplasmic side to the extracellular side of the membrane leads to a negative membrane potential. In the cell, this negative membrane potential due to the pH gradient is partially compensated by other ion gradients.[54,55,59] In vivo studies using BR expressed in *Xenopus* oocytes[55,59] have shown that in this system only about half of the proton gradient leads to a membrane potential. To mimic this situation in our calculations, we consider that only half of the pH gradient gives rise to a membrane potential, corresponding to using $a = 0.5$ in eq 8. Figure 3 shows how the membrane potential depends on the pH at the extracellular and cytoplasmic sides of the membrane.

Two-dimensional titration curves ($\langle x_i \rangle$ vs (pH_{EC}, pH_{CP}, $\Psi = 0.5 \ln(10) (RT/F)\Delta pH$)) of the functionally important sites are shown in Figure S3 of the Supporting Information. The titration curves for Asp85 and Asp115 are shown in Figure 5b. The titration curves are shifted toward the diagonal, due to the combination of two effects: the shift of the protonation energy caused by the membrane potential and the influence of the pH gradient between the two sides of the membrane. On one hand, Asp115 senses the negative membrane potential that is generated by the proton pumping activity of BR. This negative membrane potential lowers the proton affinity of Asp115 since this residue is connected to the cytoplasmic side of the membrane. On the other hand, Asp115 also senses the decreased proton concentration in the cytoplasm due to the proton pumping activity. Thus, both effects favor deprotonation of Asp115. The deprotonated Asp115 interacts strongly with Asp85 leading to an increased proton affinity of this residue and therefore its protonation. When Asp115 is deprotonated, Asp85 is protonated. Notably, the deprotonation of Asp115 does not lead to the creation of an additional negative charge in the retinal binding pocket. The negative charge is just shifted from Asp85 to Asp115. BR with a protonated Asp85 cannot pump protons anymore, since the primary proton acceptor is already protonated. The proton pumping active region determined by Kouyama and coworkers[58] shown in Figure 5 is clearly correlated with the deprotonation of Asp115 and the protonation of Asp85.

Influence of the Membrane Potential on the Photocycle Proton-Transfer Reactions in BR. The proton-transfer reactions occurring in BR during the photocycle can be represented as a sequence of protonation states. The energy involved during such reactions can be calculated using eq 7. The energetic contribution due to the membrane potential will correspond to $F\Psi\ \Delta\Gamma_{d\to a}$ where the term $\Delta\Gamma_{d\to a} = \Gamma_{i=a} - \Gamma_{i=d}$ describes the relative effect of the membrane potential on the energy required for the proton-transfer reaction between proton donor *d* and the proton acceptor *a*. The corresponding values of Γ_i can be found

(59) Nagel, G.; Kelety, B.; Mockel, B.; Buldt, G.; Bamberg, E. *Biophys. J.* **1998**, *74*, 403−412.

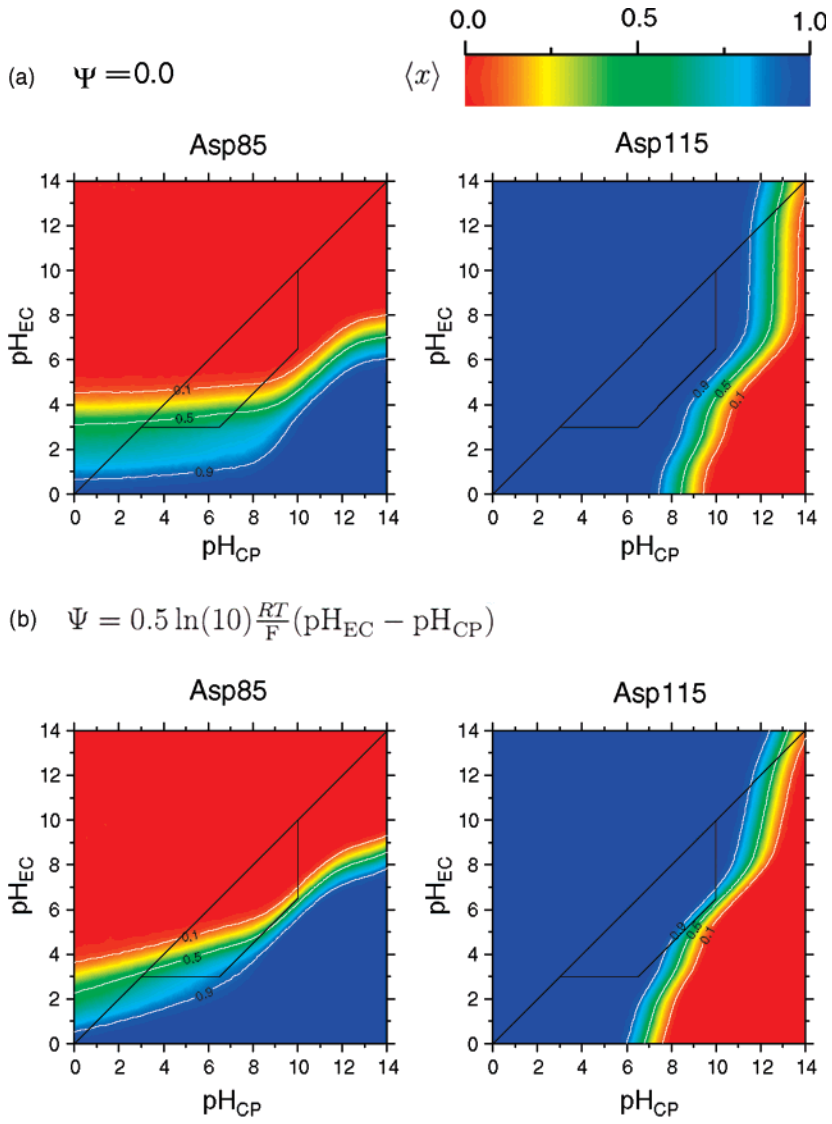

Figure 5. Protonation probability Asp85 and Asp115 as a function of pH_{EC} and pH_{CP}. The protonation probability is color-coded as indicated by the color scale on the top of the figure. The panels (a) and (b) differ in the definition of the membrane potential Ψ that arises from the pH gradient. In (a) the effect of the pH gradient on the membrane potential is compensated by other ions, and thus the membrane potential is zero. In (b) the pH gradient gives rise to a membrane potential which is partially compensated by other ions; the proportionality factor *a* is assumed to be 0.5. The situation depicted in (b) is the closest to experimental findings.

in Table 1. In addition, according to the definition of the membrane potential given in the Theory section (Figure 2), the value of Γ_i is 0 for the extracellular region and 1 for the cytoplasmic region. In Figure 6, the values of $\Delta\Gamma_{d\to a}$ are given for the proton-transfer reactions occurring during the photocycle of BR. The membrane potential does not affect the fast protonation of Asp85 in the L to M^1 transition, as the negligible value of $\Delta\Gamma_{d\to a}$ indicates. In contrast, the presence of a membrane potential affects significantly all the other proton-transfer reactions. In bacteriorhodopsin, the effect of the membrane potential is stronger during proton release and uptake than during proton-transfer reactions inside the proteins. Our findings are in agreement with experimental studies,[59] which have shown that the M^1 to M^2 transition is particularly voltage sensitive. Moreover, it has been shown[55,60] that the membrane potential affects also other charge-transfer steps, which are the reprotonation of Asp96 (N to O transition) and the slow proton transfer from Asp85 to the proton release group (O to bR transition) but not the fast protonation of Asp85 (L to M^1 transition). Also our calculations show that the energetics of these proton-transfer reactions are influenced by the membrane potential.

It should be kept in mind that the presence of a membrane potential will affect the equilibria between the photocycle intermediates implying a modification of their populations with consequences on the proton transfer rates. Moreover, the complex interaction network within bacteriorhodopsin may have other hardly predictable effects on the proton-transfer reactions. Also the protein conformational changes need to be taken into account. A rigorous evaluation of the effect of the membrane potential on the photocycle kinetics cannot be done with the current calculations, and it will be addressed in future work. Nevertheless, the presented estimation of the effect of the membrane potential on the proton-transfer reaction within BR provides a hint of how the membrane potential influences the proton pumping activity of BR.

(60) Hendler, R. W.; Drachev, L. A.; Bose, S.; Joshi, M. K. *Eur. J. Biochem.* **2000**, *267*, 5879−5890.

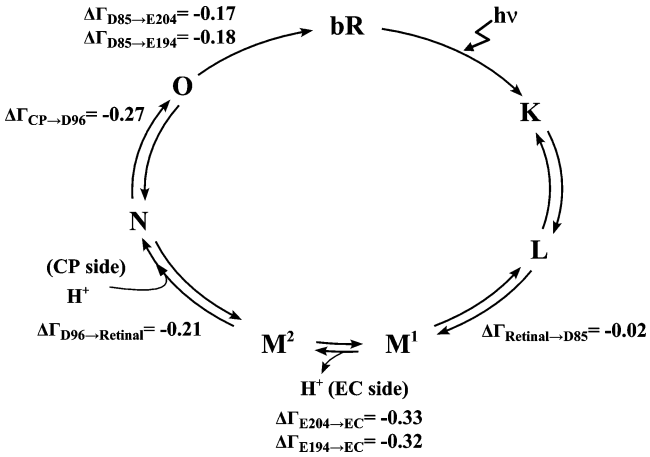

Figure 6. The photocycle of bacteriorhodopsin. The values of $\Delta\Gamma_{d\rightarrow a}$, where *d* is the proton donor and *a* the proton acceptor, are calculated for the proton-transfer reactions occurring during the photocycle. The membrane potential does not affect the fast protonation of Asp85 in the L to M^1 transition. In contrast, the presence of a membrane potential affects significantly all the other proton-transfer reactions. The strongest effect of the membrane potential is found during proton release and uptake, in the transitions M^1 to M^2 and N to O, respectively.

Consequences for the Regulation of BR by the Membrane Potential and by the pH Gradient. The initial absorption of a photon in the bR-state and the subsequent electronic relaxation lead to the first photocycle intermediate, the K-state, which is significantly higher in energy than the initial bR-state. An estimate for the enthalpy difference between the bR-state and the K-state lies at 11.8 kcal/mol,[61] but also higher values have been reported.[62−65] It has been shown experimentally that BR can generate a maximum electrochemical potential gradient of about 0.28 V[66] which corresponds to an energy of about 6.5 kcal/mol. This potential difference can be stored in the membrane potential, the pH gradient, or both. However considering the enthalpy that is stored in the K-state, BR could generate an electrochemical potential gradient of up to 0.5 V. Moreover, calorimetric measurements indicate that a substantial entropy increase is accompanied with the transition from the K-state to the bR-state along the photocycle,[65] which would lead to an even larger free energy available for proton pumping. Thus, the electrochemical potential that BR actually generates is smaller than the electrochemical potential that BR could generate theoretically, assuming that all the energy stored in the K-state can be used for proton pumping. Therefore, although it is difficult to estimate how much energy can be used for proton pumping, it seems likely that BR has a mechanism to regulate its pumping activity. From our titration calculations, we suggest that Asp115 provides the structural basis for such a regulation mechanism. Calculations on the Asp115Asn mutant of BR show a different titration behavior of Asp85 which would not be able to regulate the proton pumping of BR under conditions when the pH gradient or the membrane potential become too large (see Figure S4 in the Supporting Information).

There are several biological reasons why the regulation of the proton pumping might be required. The most obvious reason is the regulation of the cytoplasmic pH value. The proton pumping activity of BR leads to an increase of the cytoplasmic pH value (alkalinization). A too alkaline pH value within the cell could be harmful for the cell and could lead to the denaturation of cytoplasmic proteins and should thus be avoided. According to our calculations, a high cytoplasmic pH value leads to the deprotonation of Asp115 and consequently to the protonation of Asp85, which would stop the photocycle.

Since bacteria often live in large colonies, it can happen that the extracellular pH becomes too low which again would lead to too harsh conditions for survival of the bacteria. A too acidic extracellular pH would lead to the protonation of Asp85 which is stabilized by the deprotonation of Asp115. Thus, the interaction between Asp85 and Asp115 may be also important for regulating the extracellular medium.

Finally, the interaction between Asp85 and Asp115 could be involved in avoiding too negative membrane potentials as can be concluded from our calculations shown in Figure 4. A negative membrane potential stabilizes the protonation of Asp85 and the deprotonation of Asp115. This effect is enhanced at a very low membrane potential (lower than −300 mV) where the two residues titrate in the same pH range. A too negative membrane potential could for instance lead to unwanted conformational changes of membrane bound proteins. Moreover, a too negative membrane potential provides a large driving force for transporting positively charged substances into the cell and negatively charged substances out of the cell. Thus, too negative membrane potentials should be avoided by the cell. This protection can be achieved by the interaction between Asp85 and Asp115.

Conclusions

The membrane potential is of crucial importance in biology. Using the example of the proton pump bacteriorhodopsin, we show on a structural level how the membrane potential influences the energetics of the protonation of proteins. Moreover, we give a first estimation of the effect of the membrane potential on proton-transfer reactions. Besides the membrane potential, also the pH gradient is an important parameter in the cell especially for proton pumps. We have found that the membrane potential and the pH gradient influence significantly the protonation behavior of bacteriorhodopsin. Bacteriorhodopsin pumps protons from the cytoplasm to the extracellular space. Thus, proton pumping leads to an increase of the intracellular pH and to a negative membrane potential. A residue that is strongly influenced by the membrane potential is the highly conserved residue Asp115 to which no function has been attributed yet. It was found experimentally that Asp115 is always protonated under conditions where no membrane potential or no pH gradient are present. From our calculations, we see that the protonation of this residue depends strongly on the pH gradient and the membrane potential. A very negative membrane potential or a high pH in the cell lead to the deprotonation of Asp115. The negatively charged Asp115 interacts strongly with Asp85 and causes the protonation of Asp85. A protonated Asp85 cannot accept a proton from the retinal Schiff base, and thus the proton pumping activity of bacteriorhodopsin is inhibited. The membrane potential influences the photocycle in two

(61) Birge, R. R.; Cooper, T. M.; Lawrence, A. F.; Masthay, M. B.; Zhang, C. F.; Zidovetzki, R. *J. Am. Chem. Soc.* **1991**, *113*, 4327−4328.
(62) Birge, R. R.; Cooper, T. M. *Biophys. J.* **1983**, *42*, 61−69.
(63) Birge, R. R.; Cooper, T. M.; Lawrence, A. F.; Masthay, M. B.; Zhang, C. F.; Zidovetzki, R. *J. Am. Chem. Soc.* **1989**, *111*, 4063−4074.
(64) Ort, D. R.; Parson, W. W. *Biophys. J.* **1979**, *25*, 314−353.
(65) Ort, D. R.; Parson, W. W. *Biophys. J.* **1979**, *25*, 355−364.
(66) Michel, H.; Oesterhelt, D. *FEBS Lett.* **1976**, *65*, 175−178.

different ways which should be distinguished. On one hand, it affects the energetics of the individual proton transfer steps during the photocycle, since most of the proton transfer steps except the proton transfer between the Schiff base and Asp85 are influenced by the membrane potential. On the other hand, the membrane potential and the pH gradient affect the protonation of Asp115 which causes the protonation of Asp85. BR molecules in which Asp85 is protonated cannot undergo a normal photocycle and thus cannot contribute to the generation of a pH gradient. Our calculations show how bacteriorhodopsin can sense the membrane potential and the pH gradient in halobacteria and how the proton pumping is regulated by these parameters. The pivotal role of Asp115 in regulating the proton pumping activity, which we previously suggested,[18] is further underlined.

Our calculations demonstrate that it is important to analyze the function of membrane proteins in their natural environment. Experiments in which no membrane potentials and no pH gradients are considered may lead to results that differ from those that would have been obtained under physiological conditions.

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Supporting Information Available: Complete ref 45 and two-dimensional titration curves ($\langle x_i \rangle$ vs (pH, Ψ); $\langle x_i \rangle$ vs (pH_{EC}, pH_{CP}, $\Psi = 0$); $\langle x_i \rangle$ vs (pH_{EC}, pH_{CP}, $\Psi = 0.5 \ln(10) (RT/F)\Delta pH$)) of the functionally important sites are shown in Figures S1 to S3 and comparison of the titration behavior of Asp85 in the wild-type protein and in the mutant Asp115Asn under different conditions in Figure S4. This material is available free of charge via the Internet at http://pubs.acs.org.

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