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Review

Proton transfer reactions across bacteriorhodopsin and along the membrane

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Abstract

Bacteriorhodopsin is probably the best understood proton pump so far and is considered to be a model system for proton translocating membrane proteins. The basis of a molecular description of proton translocation is set by having the luxury of six highly resolved structural models at hand. Details of the mechanism and reaction dynamics were elucidated by a whole variety of biophysical techniques. The current molecular picture of catalysis by BR will be presented with examples from time-resolved spectroscopy. FT-IR spectroscopy monitors single proton transfer events within bacteriorhodopsin and judiciously positioned pH indicators detect proton migration at the membrane surface. Emerging properties are briefly outlined that underlie the efficient proton transfer across and along biological membranes.

Keywords: Retinal protein; Membrane; Electron crystallography; X-Ray structure; Infrared spectroscopy; pH probe

1. Introduction

The light-driven proton pump bacteriorhodopsin (BR) is synthesized by Haloarchaea when the oxygen concentration in the natural habitat, salt lakes and salterns, is low. BR aggregates within the plasma membrane in the form of a two-dimensional hexagonal lattice. From the color of these patches they are called purple membranes (PM). BR serves as a kind of emergency engine to drive phosphorylation when respiration ceases. The conversion of light energy into a proton gradient does not comprise electron transfer as an intermediate step as it is the case in the complex photosynthetic machinery of bacteria and plants. The much simpler, though less efficient, catalytic mechanism of BR involves a light-driven torsion of the chromophore retinal. This torsional strain is somehow transmitted to the surrounding protein by inducing a series of conformational changes. The resulting pK_a shifts of internal residues eventually lead to proton translocation across the membrane.

Plenty of reviews of BR have been published since its discovery three decades ago (see [1–12] for a personal choice). It is not the intention of this review to cover all aspects of research on BR, like, e.g. the role of BR in the physiology of Haloarchaea [13,14], folding of membrane proteins [15], the color regulation of the retinal chromophore [16], the appropriate model to describe the complex photocycle kinetics

Abbreviations: BR, bacteriorhodopsin; RSB, retinal Schiff base; PM, purple membrane; FT-IR, Fourier transform infrared; EC, extracellular; CP, cytoplasmic

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[17] or biotechnological applications of BR [18,19]. Rather, I will focus on the current knowledge about the task of BR, namely light-driven proton translocation across the membrane.

The enormous stability and the ease of preparation allows to use a whole variety of state-of-the-art biophysical instrumentation. Actually, many of these techniques have been developed with a sample of BR. Single proton transfer events can be monitored by time-resolved spectroscopy. Among the various spectroscopic techniques, FT-IR difference spectroscopy exerted probably the largest impact on the understanding of proton translocation across BR [20–24] and examples will be given therein. Studies on proton migration along the purple membrane and into the bulk water phase will be presented which have been performed with site-specific pH indicators. All of those proton transfer reactions will be discussed within the framework of the recently available highly resolved structural models of BR.

2. The structural basis

The pioneering electron crystallographic work of Henderson and Unwin [25] in the early days of bacteriorhodopsin marked a milestone in the description of biomembranes since it provided the first experimental evidence for α-helices protruding the membrane. The resolution of 7 Å in the membrane plane increased in the following years (PDB entry: 1BRD [26] and 2BRD [27]) to reach 3 Å in all three dimensions at present (1AT9 [28] and 2AT9 [29]). Beyond this resolution, X-ray crystallography is still unbeaten. Although BR was one of the first membrane proteins crystallized [30,31], it resisted for more than 25 years all efforts in obtaining well-ordered 3D crystals suitable for high resolution crystallography. The long period of desperation was terminated by Landau and Rosenbusch who used the cubic phase of a lysolipid as a matrix to crystallize BR [32,33]. BR is fully functional in the microcrystals arguing for a mild crystallization procedure [34]. The structure of BR could be resolved down to 2.5 Å (1AP9 [35]). The structure was further refined (2.3 Å) with crystals obtained with the same crystallization procedure (1BRX [36]). A major progress of these structural models was the detection of water molecules. Recently, two additional models were published based on different crystal forms of BR (1BRR [37] and 1BM1 [38]). However, there is still disagreement among the various structural models regarding the precise location and the spatial orientation of residues. A particular problem is the occurrence of merohedral twinning [39] in the crystals obtained from cubic phase crystallization [35,36]. It should be pointed out that most of the structural features of BR have already been elucidated by electron crystallography [27]. The overall accordance of the published structural models is not surprising since the phases obtained from electron microscopy have been used in the molecular replacement procedure for the X-ray data.

In the following, the model of Luecke et al. [36] is chosen to illustrate the basic structural and functional elements of BR (Fig. 1). Details of the other published structural models will be discussed where appropriate. The polypeptide comprises 248 amino acids and folds into the membrane as seven α-helices. The loop connecting helices B and C forms an antiparallel β-strand. The chromophore *all-trans* retinal (Ret in Fig. 1) is located approximately in the center of the membrane. It is bound to K216 via a protonated Schiff base (azomethine) linkage. The retinal Schiff base (RSB) divides BR into two parts: the extracellular pathway (EC) for proton release and the cytoplasmic (CP) pathway for proton uptake. The EC pathway comprises D85, R82, and the close lying E194 and E204 [37]. Other residues, like D212, T205, Y57, Y185, and W86, might also participate in the proton pathway but their involvement is not as definite as that of the former amino acids. The X-ray diffraction data with the highest resolution [36] allowed to detect three tightly bound water molecules which are exclusively located in the EC pathway (Fig. 1). The water molecules mark a putative proton pathway from the RSB to the EC surface. The presence of additional water molecules in the BR structure is expected from cavity analysis [27,40] and determined by neutron diffraction [41,42]. E9 is a further acidic residue along the extracellular surface of BR that was suggested to be member of the proton release chain [35].

Much less is known about the CP pathway. Important residues on the putative proton transfer chain from the CP surface to the RSB are D96,

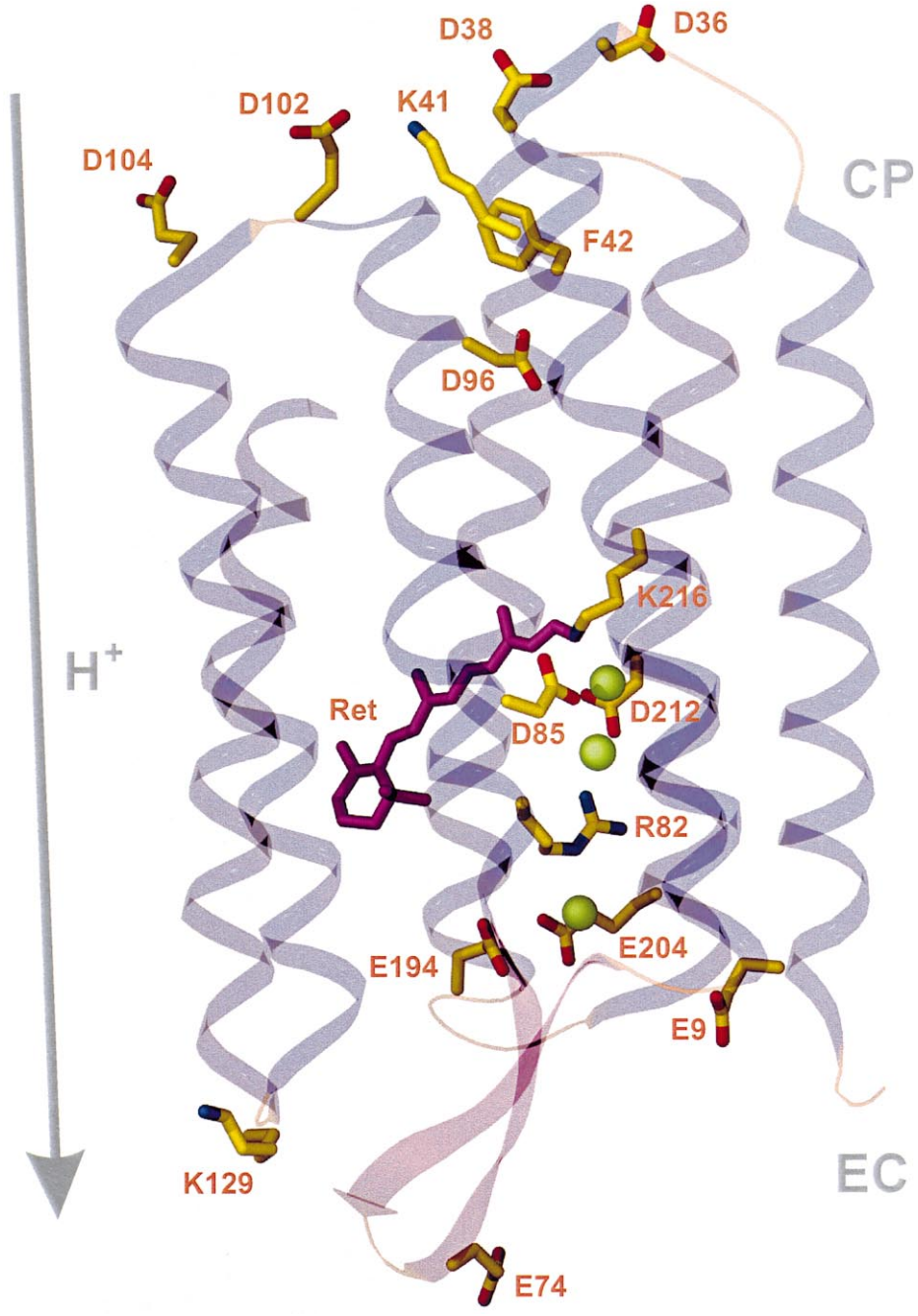

Fig. 1. Structural model of bacteriorhodopsin based on a resolution of 2.3 Å ([36]; data retrieved from the protein data bank, PDB entry: 1BRX). View is approximately parallel to the membrane plane. The thickness of the surrounding membrane is about 40 Å. The protein backbone is shown as ribbons. The chromophore retinal (Ret) and amino acids discussed in the text are represented as sticks. Three water molecules are shown as spheres. The arrow indicates the direction of proton translocation.

D38, T46, and F42 (Fig. 1). It is well established that D96 is the internal proton donor of the RSB. This residue is protonated in ground-state BR due to the hydrophobic environment of a surrounding leucine barrel (L95, L97, L100, and L223) with two phenylalanines (F42 and F219) covering the barrel. However, the distance of about 11 Å from D96 to the RSB is too long for direct proton transfer to occur.

Protonatable residues in this vicinity are threonins and serins but only the replacement of T46 exhibits a marked effect on proton transfer [43–45]. The simplest explanation for protons to cross the hydrophobic barrier between D96 and the RSB would be the presence of water molecules. However, the present structural models do not include water molecules in this area. The limited solvent accessibility of D96 is probably caused by F42 which shields D96 from the cytoplasmic surface. D38 that is located at the transition from the helical to the loop region, represents the first amino acid at the entrance of the CP pathway.

The loop region of the CP surface contains many charged residues (D36, D102, D104, K159, E161, R164, E166, R227)[1]. Such an antenna of proton binding sites represents a prerequisite for the efficient capture of protons from the alkaline cytoplasm of the cell [46]. The C-terminal tail contributes four negatively charged residues (E232, E234, E237 and D242) to the charge density of the CP surface. The EC surface is less charged (R7, E74, K129 and possibly E9) creating a large asymmetric charge distribution across the BR molecule. Determination of the net charge density at each of the membrane surfaces is difficult because the stoichiometry of cation binding [47–50] and the orientational distribution of the lipid molecules of the purple membrane is still unknown.

3. Proton transfer steps across bacteriorhodopsin

Proton translocation within BR is initiated by the light-induced isomerization of the chromophore retinal. Photon absorption leads to formation of a subpicosecond product, termed the J intermediate [51,52]. Though it is beyond the scope of this article to discuss the very early events of photoisomerization it should be stressed that the isomerization reaction of retinal is not a single step event (see [2,53,54] for reviews). Vibrational coherence effects and vibrational cooling lead to complex kinetics already in the very early photochemical reaction [55]. The transition of the vibrationally hot J intermediate [56] to the subsequent K state proceeds with a time constant of 3 ps [51]. The appearance of hydrogen-out-of-plane vibrations indicate a distorted 13-*cis* configuration of retinal [57,58]. Relaxation of this configurational strain leads via the KL intermediate to the L intermediate [59–61]. Thus, the initial photoinduced *trans*/*cis* isomerization of retinal is finally settled in the L intermediate. These structural motions of the retinal moiety set the scene for the following proton transfer reactions.

Isomerization causes an increase in acidity of the RSB because the Schiff base nitrogen is transferred into a less hydrogen-bonded environment [62]. This destabilizes the positive charge on the RSB and the high pK_a of the RSB ($pK_a^{(RSB)} = 13$ [63]) is decreased to approach the pK_a of D85. Consequently, the proton of the RSB is transferred to D85 and the M intermediate is established (step 1 in Fig. 2). This proton transfer reaction is monitored on the single vibrational level by FT-IR spectroscopy. Protonation of D85 leads to the corresponding aspartic acid whose C=O stretching vibration appears as a band at 1761 cm^{-1} [64,65]. The high frequency indicates the high pK_a of the carboxylic side chain (>10.5 [66]). The kinetics of this acid/base reaction are displayed in Fig. 2 (right, step 1). Although the distance of D212 to the RSB is about the same as that of D85, D212 does not actively participate in the proton release reaction [67].

Concurrent with protonation of D85, a proton is released to the extracellular membrane surface with a time constant of about 80 μs (step 2 in Fig. 2, left and right). This reaction is probed by transient absorption spectroscopy in the visible wavelength range by using the pH indicator fluorescein selectively bound to the ε-amino group of K129 (Fig. 1 and [68–72]). Two important consequences arise from the comparison of the two upper time traces in Fig. 2 (right). First, the released proton must originate from another yet unidentified group since D85 stays protonated until the latest stage of the photocycle. Second, the protonation of D85 induces imme-

[1] The orientation of amino acid side chains of the loop regions along both surfaces of BR can be obtained from the structural model derived from electron crystallography [28,29]. In contrast to the X-ray data, the electron diffraction data allowed to continuously trace the electron density of all residues in the helix-connecting loops. But still, most of the C- and N-termini could not be resolved due to the high mobility of this part of the polypeptide chain.

diate proton release to the EC surface. However, this correlation holds only at temperatures below 10°C. At temperatures above 10°C proton release is delayed with respect to the protonation reaction of D85. An activation energy of 35–40 kJ/mol [70,71,73,74] is determined for proton release above 10°C resulting in an increasing delay of proton release at elevated temperatures with respect to the deprotonation reaction of the RSB. This energy is, on the one hand, much lower than the activation energy of the photocycle intermediates (60–70 kJ/mol) but, on the other hand, much higher than proton diffusion in water (8–18 kJ/mol). The intermediate value might be explained by the torsional displacement of an amino acid side chain involved in the proton release reaction as it was suggested for R82 (see below). The kinetic isotope effect (KIE)[2] of the proton release reaction is >4 [71,75]. Such a large KIE indicates the involvement of a rate-limiting proton transfer reaction within the whole sequence of reactions that comprise proton release to the extracellular membrane surface. The large KIE for proton release compares to the small KIE of 1.6 for the proton uptake reaction which is governed by structural changes of the protein (see Section 4).

Many studies currently focus on the identity of the proton release group. In several scenarios E194 and/or E204 have/has been proposed to be the terminal proton release group in the EC proton transfer cascade because of severe alterations in the proton release kinetics when one of these residues was exchanged [76–79]. However, difference bands due to E194 and E204 have not been detected in time-resolved FT-IR experiments [72,80]. The most apparent interpretation of this finding is the postulation of an hydrogen-bonded network in the EC channel [72] comprising D85, R82, the dyad of E194 and E204 as well as intervening water molecules. It must be stressed, however, that the proton release reaction leaves a deprotonated group within the protein and that this group has not been identified yet! Though the spatial position might favor E9 as the proton release group (Fig. 1), time-resolved FT-IR and visible spectroscopy exclude E9 as well as E74 to be part of the proton release chain [80]. The functional role of water molecules in the proton release reaction was inferred from several studies [81–86] and direct evidence was provided by FT-IR spectroscopy [87–89].

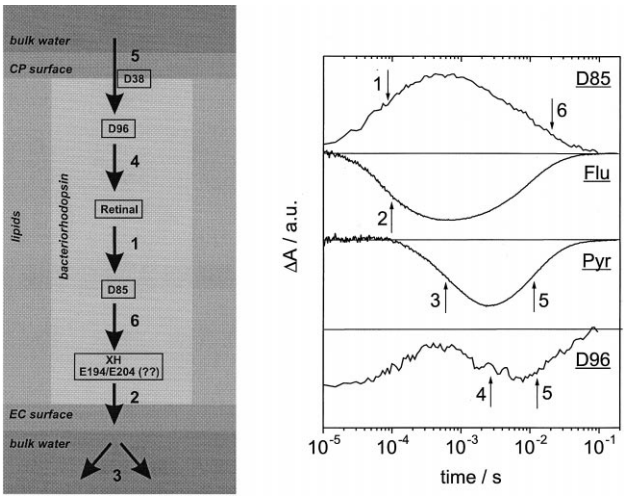

Fig. 2. (Left) Sketch of the proton transfer steps of bacteriorhodopsin. (Right) Time-resolved observation of the proton transfer steps of bacteriorhodopsin. Protonation changes of the internal amino acids D85 and D96 are detected by FT-IR spectroscopy. Protonation changes of the pH indicator fluorescein covalently bound to the extracellular surface of BR (Flu), and of pyranine dissolved in the aqueous bulk phase (Pyr), are detected by transient absorption spectroscopy in the visible range. Arrows denote proton transfer reactions discussed in the text.

R82 plays a critical role in proton release [90–93]. A combination of molecular dynamic, electrostatic and quantum chemical calculations suggested that R82 might flip from an 'upward' configuration with interaction to D85 to a 'downward' orientation [92,94] where it can interact with the E194/E204 dyad. This appealing mechanism explains how the information about the protonation state of D85 is transmitted to the proton releasing site. Unfortunately, the available structural models do not agree on the configuration of the side chain of R82. During structure determination the assignment of electron density to R82 is not unequivocal and might be confused with electron density from water molecules (see note added in proof, at the end of this paper).

D85 acts not only as the primary acceptor of the Schiff base proton, it is also involved in the actual proton release reaction. The interaction of D85 with

[2] The KIE represents the ratio of the time constants for transfer of a proton and of a deuteron. Experiments are performed in H_2O and in ^2H_2O, respectively.

the proton release group has been demonstrated for ground-state BR [95]. It has recently been shown by FT-IR spectroscopy that such an interaction is also valid in the M state [80]. Moreover, the conservative mutation D85E accelerated deprotonation of the RSB but decelerated proton release to the membrane surface [71,96].

After release to the extracellular membrane surface (arrow 2 in Fig. 2) protons dwell for about 1 ms along the surface of the purple membrane before they dissipate into the aqueous bulk phase (arrow 3 in Fig. 2). This step which is monitored by the highly water-soluble pH indicator pyranine (Pyr in Fig. 2, right), proceeds independently from the preceding internal proton transfer reactions and will be discussed in detail in Section 5. The maximum amplitude of the pyranine trace allows to determine the stoichiometry of proton pumping by BR [97]. Though quite controversial in the early days most investigators now agree on the fact that BR pumps one proton per photocycling molecule [70,73,98–101] and rule out higher stoichiometries [102–105].

Reprotonation of the RSB is accomplished by D96 (see step 4 in Fig. 2, left, and [106–108]). The change in protonation state of D96 can be followed at 1741 cm^{-1} (lowest time trace in Fig. 2, right) at which energy the C=O double bond vibrates [64,108]. The submillisecond kinetics have been attributed to environmental changes around D96 and D115 [109] that occur during the lifetime of the L intermediate. Arrow 4 in Fig. 1 indicates the time constant (2 ms) for the deprotonation reaction of D96. The unusually high pK_a of 11.4 [110] drops by more than 4 pH units to 7.1 [80] enabling D96 to reprotonate the RSB. This reaction defines the M-to-N transition. The increase in acidity of D96 might be caused by structural rearrangements observed with various techniques and discussed in Section 4.

D96 is reprotonated from the cytoplasm (arrow 5 in Fig. 2, left). Likewise to the proton release reaction, proton transfer within the CP channel does not limit the reprotonation rate of D96. That is evident from the comparison of the repopulation kinetics of protonated D96 with the rate of proton uptake from the bulk water phase (arrows 5 in Fig. 2, right). If F42 shields D96 in the ground state of BR then it must now move to let a proton pass from the cytoplasm to D96. K41 and D38 are further candidates for important residues lining the putative proton pathway from the cytoplasmic surface to D96. Mutations of D38 exerted drastic effects on the proton uptake reaction. Unexpectedly, however, it is the reprotonation reaction of the RSB that is affected and not the reprotonation of D96. This puzzling result suggests an involvement of D38 in the conformational changes of the protein. Indeed, changes in tertiary structure during the photocycle have been elucidated close to helix B where D38 is located (see Section 4). The non-appearance of conformational changes of the protein backbone in the D38R mutant supports this view [46,111,112]. It was concluded that the observed conformational changes during the BR photocycle are controlled by the charge of the amino acid at position 38 [113]. Mutations of K41, which is just one helix turn further into the membrane (Fig. 1), did neither affect the kinetics of the photo- nor of the proton cycle (Heberle and Tittor, unpublished). Though spatial position and presumed charge favors the interaction with D38, K41 is dispensable for proton pumping.

The final step in proton translocation by BR is the deprotonation of D85 (step 6 in Fig. 2, left). It can be deduced as the slow component in the biphasic decay of the C=O stretch of D85 (Fig. 2, right). The fast component corresponds to a band shift from 1761 to 1755 cm^{-1} during the M decay [114,115]. With proton transfer from D85 to the proton release group (XH) all proton donors and acceptors are reset to their initial states and the next sequence of proton transfer steps can be initiated by light.

It should be emphasized that the sequence of proton transfer reactions of BR was described for neutral pH. Proton transfer reactions are intrinsically pH-dependent and BR is an excellent example. At neutral pH the proton release reaction proceeds in the microsecond time domain (arrow 2 in Fig. 2). At pH < 5, however, proton release is delayed to the late millisecond range and the sequence of release and uptake is reversed [116–118]. This transition from 'early' to 'late' proton release [90] was ascribed to the protonation of the proton release group. The release group has a pK_a of 9.5 in the unphotolyzed state [91] that decreases to 5.2–5.8 (depending on the ionic strength of the medium [80,117,119]) during the lifetime of the M intermediate. At a pH below 3, a color change from purple to blue occurs [120] where

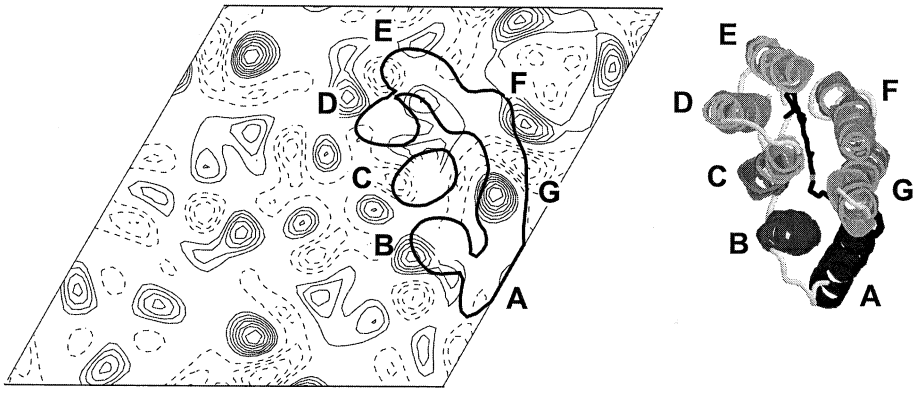

Fig. 3. Two-dimensional projection map at 7 Å resolution of the light-induced difference electron density of purple membrane where the D96N mutant of BR was trapped in the M_2 state (adapted from [144] by courtesy of H.-J. Sass). Continuous lines correspond to positive, dashed lines to negative electron density levels. The thick line indicates the position of the seven transmembrane α-helices (labeled by capital letters from A to G). Difference peaks are pronounced around helices B, F, G, and to a lesser extent also at helix D. For clarity, the protein backbone based on the high-resolution data [36] is displayed on the right side. The view is from the EC surface. Retinal is bound to K129 in the center of BR with the plane of the retinal molecule being perpendicular to the membrane.

D85 gets protonated [121] and the proton pumping activity of BR is lost [122,123]. At alkaline pH the deprotonation reaction of the RSB is accelerated [115,124,125]. The effect on the subsequent proton release to the extracellular side has not been studied yet because of experimental difficulties when working at high pH [90].

An interesting feature in the infrared difference spectra is the occurrence of a so-called 'continuum band'. An extremely broad absorption is observed across almost the entire mid-infrared range [126]. Zundel and coworkers elaborated the theoretical and experimental basis for this phenomenon which originates from the collective motion of protons within an hydrogen-bonded system (reviewed in [127,128]). A transient change in the continuum absorbance during proton pumping was reported from time-resolved FT-IR experiments [46,72,129,130]. This change is abolished in mutants of BR where residues crucial for proton translocation (D38, D96 and E204) had been exchanged. Evidence for such a continuum absorbance due to large proton polarizability was also provided in other proton translocating proteins (F_o complex of ATP synthase from *Escherichia coli* [131] and bacterial photosynthetic reaction center [132]). The relevance of these results for the detailed mechanism of proton pumping has to be elaborated in future studies to bring the rather general observation of changes in hydrogen bonding to a molecular level.

4. Changes in the tertiary structure of bacteriorhodopsin during catalysis

Several methods have been employed to study the conformational changes of the protein backbone of BR after photon excitation. Neutron diffraction [133] was first to convincingly demonstrate the occurrence of tertiary structural changes of the transmembrane part of BR during the transition from ground-state BR to the M intermediate. These results were confirmed and refined by numerous electron [112,134–140] and X-ray scattering experiments [69,113,141–146]. Fig. 3 displays a representative projection map that indicates the change in electron density when BR is illuminated and trapped in an intermediate state (here the M_2 state of the D96N mutant [113]). Prominent difference peaks evolve at helices B, F and G. These changes were attributed to an outward tilt of helix F and an ordering of helix G at the cytoplasmic end of both helices [134,138]. There seems to be a general consensus among crystallographers about the tertiary structural changes to occur during the M_1-M_2 transition[3] and to persist during the lifetime of the N intermediate. In light

[3] M_1 and M_2 are defined by the accessibility of the RSB. M_1 corresponds to a deprotonated 13-*cis* RSB that is accessible from the proton release side whereas in M_2 the RSB interacts with the proton uptake pathway.

of the recent results, earlier studies [147–150] who did not report large structural changes in the M state, may now be reinterpreted such that the M_1 intermediate was trapped.

Unfortunately, current crystallographic studies mostly suffer from poor time resolution. Therefore, samples have to be trapped in intermediate states by means of mutations, addition of chemicals or low temperature. These modifications bring along with changes in structure and function [112]. Thus, the correlation with the photoreaction of wild type BR in its natural environment is difficult [151]. Spectroscopic techniques offer high time resolution [152,153] but site-specific information can only be achieved with labels [70,154]. Time-resolved EPR spectroscopy is superior in this regard because relative distances can be determined [155,156]. Aside from the problem of using non-disturbing labels, spectroscopic studies mostly provide only a qualitative picture of the change in the environment of the respective label. Most studies agree that the structural change sensed by the spectroscopic probe is associated with the M decay. A distinction among substates of M has not been done because these are indistinguishable by visible spectroscopy. Infrared spectroscopy is sensitive to the secondary structure of proteins and the intensity of an amide I band (C=O stretch of the protein backbone) in the FT-IR difference experiment has been used to discriminate among the various M states [113,136,144,157–161].

It is obvious that knowledge of the three-dimensional structure of the photocycle intermediates is mandatory for a detailed description of catalysis by BR. Some of these structures are expected to be solved within the next years. However, since BR is a proton pump and protons are detectable by X-ray crystallography only at very high resolution, additional techniques must be applied to understand the complex dynamics of BR in its entity.

Determination of the tertiary structural changes is not only of importance for the structure-function relationship in BR. Insight into the underlying principles will certainly help to elucidate the mechanism of other membrane proteins, like e.g. seven-helical receptors. Among these, activation of the visual cascade by rhodopsin [162,163] and phototaxis in Haloarchaea by sensory rhodopsin [164,165] are prominent examples.

5. Proton transfer along the purple membrane

Proton release of BR leads to a transient acidification of the extracellular membrane surface. The subsequent proton transfer reactions along the membrane surface are of primary interest for coupling of the proton generator to a proton consumer, like e.g. an H^+-ATP synthase. Already in the classical reconstitution experiment by Racker and Stoeckenius [166], BR contributed substantially to the experimental verification of chemiosmosis [167]. The debate about 'localized' and 'delocalized' proton movements [168] was revived to a certain extent [169–175] by experiments on PM patches at which pH probes have been selectively positioned [73,74]. This well-defined system allowed for a detailed analysis of the proton transfer along the membrane surface and into the aqueous bulk phase.

The experimental scenario is illustrated in Fig. 4. The pH indicator fluorescein (Flu) was covalently bound either to K129 at the EC surface or to C36 at the CP surface[4]. The highly water soluble pyranine (Pyr) served as pH indicator in the water phase. After pulsed light excitation of BR a proton is released to the EC membrane surface with a time constant τ of about 80 μs (step 1 in Fig. 4). The released protons are detected in the water phase by more than one order of magnitude later (step 3 in Fig. 4; see Fig. 2 for the time trace). Lateral proton transfer around the edge of the PM to the CP surface proceeds with $\tau = 230$ μs (step 2). The fast equilibration of both membrane surfaces is surprising considering the large size of a PM patch (diameter of about 0.5 μm). These results were largely confirmed by Alexiev et al. [74] although they observed a faster lateral transfer rate in the PM system. However, in a study on monomeric BR micelles where the distance for protons to diffuse from one membrane face to the other is much smaller, these authors observed a delay between proton release and detection

[4] The cysteine residue was introduced by site-directed mutagenesis. Fluorescein was also bound to the engineered C161 at the CP surface. The response of fluorescein does not depend on the site of attachment at the CP surface. As a prerequisite for the experiments, neither the D36C nor the E161C mutation affected the proton release reaction by BR [46].

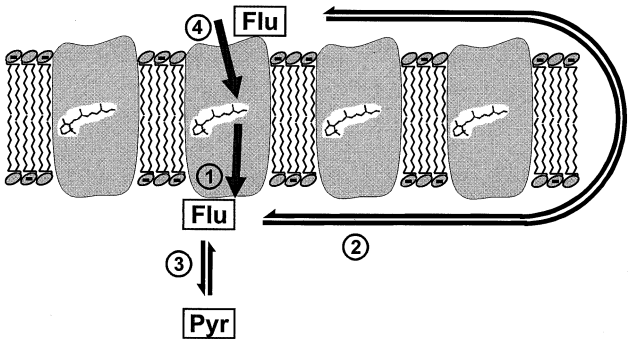

Fig. 4. Proton transfer reactions across and along the purple membrane. Proton release to the extracellular membrane surface is detected by fluorescein (Flu) covalently attached to K129 (bold arrow 1). Lateral proton transfer is monitored by fluorescein bound to C36 at the cytoplasmic surface (arrow 2). pH changes in the bulk water phase are recorded by the highly water soluble pyranine (Pyr) as pH indicator (arrow 3). Proton uptake by BR is monitored by either of the probes irrespective of location (bold arrow 4).

on the CP surface [176]. The reason for this discrepancy is not clear.

Lateral proton transfer is not only fast, it is also efficient. Determination of the proton pumping stoichiometry by pH titration of the fluorescein response reveals that the released protons are quantitatively transferred to the other membrane side. The experimentally determined stoichiometry of 4 H^+/BR [73] should be regarded as an apparent value since in the microsecond time domain the membrane surface is not in equilibrium with the bulk water phase. As stated in Section 2, the stoichiometry of proton pumping by BR is 1 as determined by the response of pyranine in the water phase.

Evidently, it is the long dwell time (approx. 1 ms) at the membrane surface for protons to migrate distances that are in the range of proton generators and consumers [177]. A faster proton diffusion than in bulk water [178] is not required to explain the data. It has been put forward that the dwell time of protons is due to the high buffering capacity of the (fixed) surface groups [179]. Mobile buffers are competitors to abstract protons from the membrane surface by collisional proton transfer [180,181]. Indeed, the addition of buffers to the purple membrane suspension accelerates the apparent surface-to-bulk proton transfer [70,73]. The retarded surface-to-bulk proton transfer is not a peculiar property of PM. It is also observed in BR reconstituted in vesicles of various lipid composition [75] and in detergent micelles [176].

The water structure and its dynamics [182] along the membrane surface also influence the surface-to-bulk proton transfer. Specifically, it was shown by neutron scattering that the first hydration layer strongly interacts with the PM surface. A higher rotational mobility accompanied by a reduced translational motion of the surface water molecules is observed with respect to bulk water [183,184]. This might facilitate two-dimensional proton diffusion parallel to the membrane surface by a 'hop-and-turn' mechanism [185]. It can be assumed that upon freezing of the bulk water phase, the structural discontinuity between surface water and bulk water is eliminated. In fact, PM in ice does not exhibit the delayed surface-to-bulk proton transfer reaction [68].

The CP surface of BR comprises many charged residues (Fig. 1). Point mutations of some of the acidic amino acids (D36, D102, D104, E161) exerted a slight deceleration of proton uptake by BR. The role of these amino acids was ascribed to efficiently collect protons from the aqueous bulk phase and funnel them to the entrance of the CP proton pathway where D38 is located. Time-resolved pH jump experiments [186] and structural studies by electron crystallography [28] and atomic force microscopy [187] put this phenomenological result on a quantitative basis. A proton collecting antenna was also found in cytochrome *c* oxidase [188] which points to a general property of proton pumps.

6. Conclusions

BR represents a prototype for proton translocating proteins. Examples have been presented that demonstrate how transmembrane proton translocation can be broken down into a sequence of single steps. Light excitation causes isomerization of retinal which along with structural changes of the surrounding protein, induces vectorial proton transfer. The released protons dwell along the membrane surface for about 1 ms allowing protons to migrate long distances. This result might have an impact on the mechanism of chemiosmotic coupling in general.

The model system BR allows to study basic principles of proton translocation. In comparison with proton pumping enzymes of the photosynthetic or respiratory machinery, there is no interference with electron transfer reactions which complicates the observation and interpretation of proton transfer reactions. Some of the basic properties elaborated with BR have already been confirmed by studies on other bioenergetic systems.

It should be stressed that the progress in understanding molecular details of proton transfer by BR is intimately connected to the development of experimental techniques. High resolution electron crystallography, femtosecond pump probe and step scan FT-IR spectroscopy are prominent examples. The biophysical techniques established in the BR field have already been successfully transferred to other systems.

Though a quite detailed picture of catalysis by BR has emerged, many questions remain to be answered, still. From a functional point of view, the most vital issue is the vectoriality of proton translocation. After proton transfer to D85 the accessibility of the RSB must change from EC to CP to be reprotonated from D96 (switch). If it is a matter of pK_a changes of donors and acceptors or a structural change in the hydrophobic part between the RSB and D96 or a combination of both has to be clarified in future experiments. Also, further studies are required to assess whether the observed changes in tertiary structure of BR are essential for proton translocation at all. Besides the unambiguously identified RSB, D85 and D96 the involvement of further members of the proton transfer chain across BR needs to be investigated. Determination of the position of water molecules and their role in proton translocation will certainly be a clue to the molecular understanding of proton transfer across BR.

7. Note added in proof

Indeed, the transient rearrangement of R82 has recently been confirmed by the structural models of the M state (PDB entry: 1C8S [189] and 1CWQ [190]).

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