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Biochimica et Biophysica Acta 1460 (2000) 220–229

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

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Review

Analogies between halorhodopsin and bacteriorhodopsin

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Received 24 March 2000; accepted 24 March 2000

Abstract

The light-activated proton-pumping bacteriorhodopsin and chloride ion-pumping halorhodopsin are compared. They belong to the family of retinal proteins, with 25% amino acid sequence homology. Both proteins have seven α helices across the membrane, surrounding the retinal binding pocket. Photoexcitation of all-*trans* retinal leads to ion transporting photocycles, which exhibit great similarities in the two proteins, despite the differences in the ion transported. The spectra of the K, L, N and O intermediates, calculated using time-resolved spectroscopic measurements, are very similar in both proteins. The absorption kinetic measurements reveal that the chloride ion transporting photocycle of halorhodopsin does not have intermediate M characteristic for deprotonated Schiff base, and intermediate L dominates the process. Energetically the photocycle of bacteriorhodopsin is driven mostly by the decrease of the entropic energy, while the photocycle of halorhodopsin is enthalpy-driven. The ion transporting steps were characterized by the electrogenicity of the intermediates, calculated from the photoinduced transient electric signal measurements. The function of both proteins could be described with the 'local access' model developed for bacteriorhodopsin. In the framework of this model it is easy to understand how bacteriorhodopsin can be converted into a chloride pump, and halorhodopsin into a proton pump, by changing the ion specificity with added ions or site-directed mutagenesis. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Retinal protein; Bacteriorhodopsin; Halorhodopsin; Chloride pump; Proton pump; Photocycle

1. Introduction

Living cells preserve their functions, particularly in very harsh environments, by building up and maintaining ion gradients across their cell and organelle membranes. The ions are driven across these membranes by active pumps that use chemical or light energy. Many of these pumps are complex multi-subunit proteins. The ion pumps, which make use of the light-driven bond rotation in retinal, and the conformational changes in the protein that follow, in order to change the accessibility of the transported ion between the two sides of a membrane, are the simplest of such membrane-spanning proteins.

About 30 years ago, a light-driven proton pump, bacteriorhodopsin (BR), was discovered in *Halobacterium salinarum* [1]. In this organism, the purple membrane, a part of the cell membrane, contains only BR in a highly ordered two-dimensional hexagonal crystalline form [2]. Several years later, another ion pump, salinarum halorhodopsin (sHR or HR in general) was discovered in the same organism, though present in a much lower concentration [3]. HR was initially considered to be a light-activated sodium pump [4–6], but was later shown to transport chloride ion through the membrane [7]. *Natronobacterium pharaonis* was found to contain a very similar light-activated chloride pump, pharaonis halorhodopsin (pHR) [8,9].

BR and both HRs are integral membrane proteins,

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PII: S0005-2728(00)00141-9

with seven transmembrane helices enclosing a binding pocket of the chromophore [10,11]. The light-sensitive chromophore in all rhodopsin-type proteins, i.e., the two kinds of ion pumps, the sensory rhodopsins and the visual rhodopsins of higher organisms, is a retinal bound to a lysine via a protonated Schiff base. In the ion-pumping rhodopsins, the retinal in the protein binding pocket is in a thermal equilibrium between the all-*trans*,15-*anti* and 13-*cis*,15-*syn* configurations [12–14], when the protein is dark-adapted.

The natural two-dimensional crystalline arrangement of BR in the purple membrane had great advantages for determination of the structure of the protein [2], but only recently was a structure with resolution as high as 1.55 Å published [15]. The overall three-dimensional structure of sHR exhibits great similarity to that of BR, although the details are not yet known because at the time of this writing the best resolution achieved is only 6 Å, and there is only a projection map [11].

Illumination of the ion-pumping rhodopsins initiates a multistep reaction cycle in both retinal isomers. The 13-*cis* photocycle has two intermediates [16,17], and under physiological conditions is without ion transport activity. The all-*trans* retinal-containing protein proceeds through a series of thermal steps, and this is the process that transports the ion across the membrane [17–21].

Through the use of a large variety of spectroscopic methods, such as fast and ultrafast laser spectroscopy, resonance Raman, kinetic and static Fourier transform infrared (FTIR), nuclear magnetic resonance, etc. on wild-type and mutant BR, the conformational changes in the retinal and protein have been described in detail (for reviews see [22–25]). The time course of proton transport through the membrane and its release and uptake steps have also been thoroughly studied [26–29]. The same methods have also been applied to HR, but less information has accumulated [19,30–34]. The purpose of this review is to compare the available information, and to point out the similarities and differences between the proton-pumping BR and the chloride ion-pumping HR. For additional information on these pump rhodopsins, the reader is referred to the articles in this issue and to other recent reviews [23–25,35–37].

2. Structure

2.1. Amino acid sequence

The amino acid sequence of BR, determined in 1979, was the first primary structure for an integral membrane protein [38,39]. The protein contains 248 residues (molecular weight about 26 kDa) and a retinal covalently bound to Lys-216. The predominance of α helices in the secondary structure of the protein was deduced from the hydrophobicity of the sequence and confirmed by low-resolution electron diffraction [2]. The protein consists, in fact, mainly of seven α helices, each spanning the membrane, and only short extramembrane segments [38–40].

The primary structures of sHR [41] and pHR [42] were determined from their gene sequence. The sequence identities reveal a homology of about 25% between the two HRs and BR, with a greater conservation in the transmembrane part (36%) and less in the loops (19%) [41]. On the same basis, the extent of sequence identity between the two HR molecules is much higher (66%). In BR, helix C holds the proton acceptor group (Asp-85) and the donor group (Asp-96); both are replaced in HR with neutral residues (Fig. 1). On the extracellular side, there is a proton release complex in BR (Arg-82, Glu-194, Glu-204 and several bound water molecules); this complex is perturbed in HR because of the replacement of Glu-204 by Thr.

In sHR and pHR, helices C and F display the highest similarity; but while in helix F there are several conservative replacements, in helix C apart from two surface amino acids, the sequence is identical (Fig. 1). There are several highly conserved amino acids, such as Asp-212 in BR (position 238 in sHR and position 252 in pHR) and Arg-82 (position 108 in sHR and position 123 in pHR). They form a counterion complex and stabilize the protonated Schiff base [43–46]. Some other Arg, Thr and Ser are also conserved [47].

The role of arginines in the chloride transporting process of HR was investigated, because it could have an important role in chloride ion binding [47,48]. Indeed, the Arg mentioned above (at position 108 in sHR and position 123 in pHR) plays an important role in the chloride-pumping activity. Its replacement by a neutral residue inactivates the chlo-

ride pump [47,49]. The single conserved histidine in HR, which is missing from BR, is also considered important in the chloride-pumping activity of these proteins [32,50].

2.2. Protein structure

The two-dimensional crystalline arrangement of BR in the purple membrane was an obvious and very promising object for electron diffraction study [51], but the lack of information on the third dimension was a strongly limiting factor, overcome partially by measurements with tilted samples. The introduction of cryo-microscopy improved the 7 Å resolution to 3.5 Å [10,52]. The location of the retinal was determined to be between the α helical segments [53]. Recently, a new crystallization method was introduced: the growth of highly ordered three-dimensional microcrystals in a cubic lipid phase [54]. The new developments have resulted in higher resolutions [54–56], leading most recently to a 1.55 Å resolution structure for BR [15]. In this structure the positions of the bound water molecules are identified and the H-bonded network in the extracellular half-channel revealed, which plays important roles in proton release to the extracellular surface during the photocycle, and in the later photocycle step that constitutes proton conduction from Asp-85 to the proton release group.

In wild-type *H. salinarum*, HR does not form purple membrane patches, but two-dimensional crystalline patches appear in the cell membrane of a mutated overproducing strain D2 [57]. This led to the determination of the structure at 6 Å resolution [11,57]. The projection map shows that the seven α helices have an arrangement similar to that in BR. The most important difference at this resolution is that the side chain volumes in the cytoplasmic half-channel are smaller than those for BR [11], and the intramolecular space between helices B, C, F, and G is larger. It was suggested that the first of these differences corresponds to the difference in function, namely that the transported chloride ions are larger than the protons.

Conformational changes in the protein during the photocycle can be followed by different methods, such as time-resolved linear dichroism [58], circular dichroism [59], optoacoustic [60], kinetic FTIR [61] or thermodynamic measurements [62], but none of these techniques provides specific molecular information about the location of the changes. The exact structural changes may be followed by low-temperature trapping and diffraction study of the intermediates. For BR these studies have already yielded promising results on intermediates K [63] and M [64,65]. No such studies have yet been reported with HR.

2.3. Retinal configuration

The retinal in visual rhodopsins is in the 11-*cis* form, but in the bacterial rhodopsins it is in the all-*trans*,15-*anti*, or 13-*cis*,15-*syn* configuration. Dark-adapted BR contains about 35% all-*trans* [14]. During sustained light adaptation, the all-*trans* retinal content increases to almost 100% [12,66]. Light adaptation has significant practical consequences for studying the photocycle, as the light-adapted sample becomes homogeneous in the retinal configuration.

The retinal in sHR in the dark-adapted state is 45% all-*trans* and 55% 13-*cis*. Sustained light adaptation shifts this equilibrium to a maximum of 75% all-*trans* content in sodium chloride, which means that a homogeneous sample can never be achieved, and only this 75% has ion-pumping activity [17]. In sodium sulfate there is no light adaptation; in both the dark- and the light-adapted forms, the all-*trans* content of the sample is about 67% [34]. In pHR the all-*trans* content of the preparation is constant at 85% under all conditions (different salts and illumi-

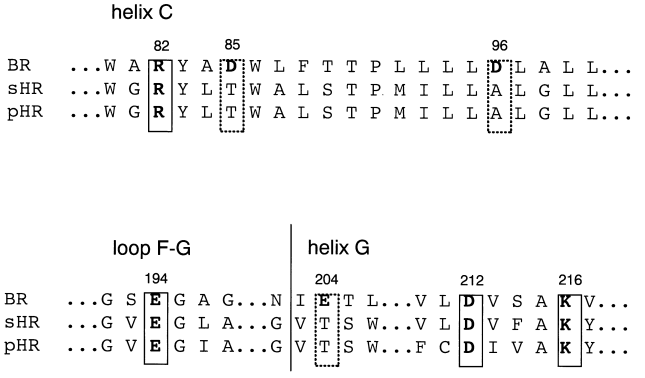

Fig. 1. Two segments from the aligned protein sequences for BR, sHR and pHR [42].

nation) [21,34]. Although the sample is not homogeneous, there is no evidence that the 13-*cis*-containing pHR is photoactive or takes part in the chloride-pumping process [21].

3. Ion binding

It has long been known that on titration with acid in the absence of chloride, the spectrum of BR shifts towards the red (the membranes change from purple to blue, the 'acid blue' form) through several intermediate states. On lowering of the pH in the presence of chloride, instead of the 'acid blue' form an 'acid purple' form is obtained [67–69]. Under physiological conditions, between pH 4 and 9, BR undergoes no spectral change and no important amino acid titrates. At above pH 9, a slight blue shift in the spectrum is observed. This complex pH titration of BR is explained by the fact that a complex of the proton acceptor Asp-85 and the proton release group that includes Glu-204 displays two pK_a values, a main transition at 2.6 and a secondary transition at 9.7 [70–74]. At low pH, the acid blue to acid purple transition suggests two chloride binding constants of 0.1 M and 1.3 M, each for one ion [68].

HR is more affected at high pH than BR. Under alkaline conditions, a yellow form appears, with a pK_a strongly dependent on the presence of sodium chloride or other salts in the solution [19,75]. The spectral data at lower pH gave a chloride binding constant of 10 mM for sHR; although the spectra are only slightly shifted, their amplitude changes appreciably [75–78]. The chloride-dependent spectral changes in pHR are greater. With increasing chloride concentration, the spectrum shifts by about 13 nm towards the blue and its amplitude increases [19,21], revealing a binding constant of 1 mM. This change in color is similar to that of the acid purple to acid blue transition in BR. FTIR and Raman spectroscopy indicate an interaction between the protonated Schiff base and the halide ion, predicting a chromophore–anion interaction [79,80]. A similar spectral shift was observed for the D85T BR mutant, which converts BR to a chloride pump [81]. Another interesting observation on pHR is its azide-dependent spectral shift. Increasing azide concentration in a chloride-free preparation causes the same spectral change as observed for chloride, but with a binding constant of 10 mM azide. Thus, azide is bound as an anion like chloride. Indeed these two ions compete for the same binding site [82].

4. Photocycle

Ion translocation during the photocycle involves a series of thermal reactions, initiated by the absorption of a photon. This cycle has been investigated by a wide variety of techniques; the most frequently used being real-time transient spectroscopy. Many photocycle models have been discussed, from the simplest, with a unidirectional reaction between the intermediates [83], to more complicated ones with reversible reactions, and parallel or branching photocycles [84–86]. The aim of this review is not to discuss all such models in detail. For additional information, the reader is referred to other papers in the present issue and to reviews [23–25,35–37].

4.1. The 13-cis photocycle

Although the absorption changes from the photocycle of the 13-*cis* retinal-containing protein are measured together with those from the all-*trans* photocycle, they can be separated mathematically by using two sets of measurements in which the all-*trans* to 13-*cis* ratio is changed. For both BR and sHR, this procedure led to a rather simple photocycle with one or two red-shifted intermediates, and the initial state recovers with multiexponential kinetics [16,17,87,88]. During the 13-*cis* photocycle, at neutral pH, charge motions may be measured inside the protein, but without a net charge transport across the membrane, proving that this cycle is not associated with a transport process [16]. On the other hand, at alkaline pH in the 13-*cis* photocycle the presence of intermediate M was observed and proton transport activity was measured [89,90].

In addition to these photocycles, which begin and end with the 13-*cis* state, both proteins undergo light adaptation. Light adaptation has been presumed to occur through an independent process, with very low quantum efficiency. In BR this has been estimated to be 0.035 [16,87]. In HR, even very long light adaptation cannot totally convert the 13-*cis* retinal con-

figuration to the all-*trans*: 25% always remains in the 13-*cis* configuration. Two possible explanations exist: either the thermal dark adaptation is fast, or a small portion of the all-*trans* retinal is also photoactively converted to 13-*cis*. After the light is off, the dark adaptation takes several hours, which makes the first explanation unlikely. There is one indirect feature that lends support to the second assumption: the wavelength dependence of the light adaptation [34].

4.2. The all-trans photocycle

4.2.1. Spectra of intermediates

The spectral characterization of the photocycle intermediates was initially performed by accumulating the intermediate states at low temperature [91–93]. The introduction of fast spectroscopic techniques, such as use of the optical multichannel analyzer, made it possible to determine the spectra by using real-time measurements at ambient temperature [94,95]. The development of the mathematical methods facilitated the accurate determination of the spectra of the BR intermediates (Fig. 2A), and they were demonstrated to be independent of pH in the pH range 4–9 [96].

Spectral calculations were carried out also for HR, by a similar technique as for BR (Fig. 2B) [17,21].

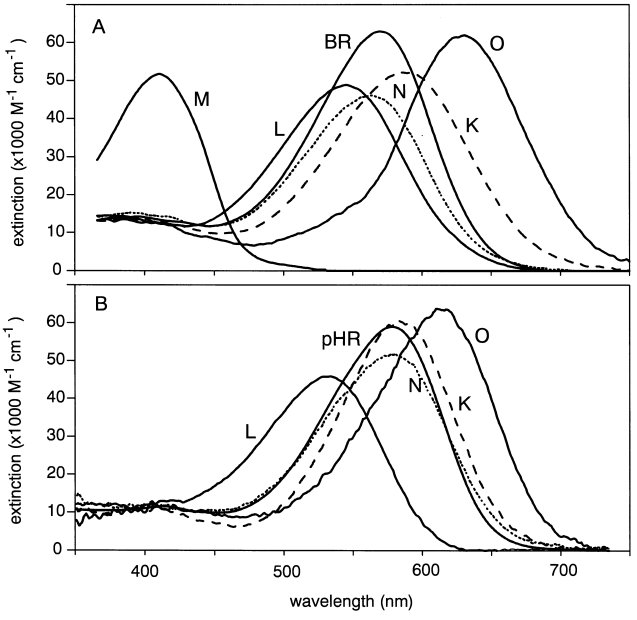

Fig. 2. Spectra of photocycle intermediates for BR and pHR.

Presumably for kinetic reasons, in the photocycle of sHR the O intermediate does not accumulate. A red-shifted intermediate, which was long considered to be O, was shown to belong to the 13-*cis* photocycle [17]. The two spectral sets for sHR and pHR halorhodopsin intermediates are almost identical, and they exhibit great similarity with the spectra of the intermediates calculated for BR (Fig. 2). The major difference is that the strongly blue-shifted M state is missing from the spectra of HR intermediates. The Schiff base cannot deprotonate, because the proton acceptor is missing [41,42]. As will be shown below, if the proton acceptor is replaced, the chloride pump can be converted to a proton pump.

4.2.2. Kinetics

From the large number of mathematically possible models, containing all the possible pathways between the intermediates, only those were considered which did not contradict other experimental facts. In this review only the most widely accepted model is presented, the sequential model with reversible reactions, which presents a number of common features in the studied ion pumps (Fig. 3). The reader interested in the different models is referred to appropriate reviews [20,22,24].

The red-shifted intermediate K with a 13-*cis*,15-*anti* retinal configuration appears in several picoseconds after the photoexcitation [97]. The all-*trans* to 13-*cis* isomerization that causes a conflict between the retinal and its binding site captures the photon energy necessary to transport the ion through the membrane. The stress induced by the isomerization is thermally relaxed as the protein converts to the blue-shifted intermediate L [98]. The next step in the BR is the L to M transition, i.e., the transfer of the proton from the Schiff base to the proton acceptor Asp-85 [99,100].

In BR, refinement of the sequential model has led to the introduction of 'silent' intermediates. These are spectrally indistinguishable substates that differ from one another in some properties of the protein. The most important 'silent transition' is the M_1 to M_2 transition (Fig. 3, BR cycle), when the protein switches between the extracellular and cytoplasmic conformations, after deprotonation of the Schiff base [101–103]. There are special conditions, such as solubilization or mutation, when the spectra of

M_1 and M_2 differ slightly [104,105]. The Schiff base is reprotonated by the proton donor Asp-96 on the cytoplasmic side [100], reflected by the appearance of intermediate N, followed by the uptake of the proton from the surrounding medium. When the retinal isomerizes back to all-*trans*, the red-shifted intermediate O appears and the protein relaxes back to the initial BR state.

The Schiff base in HR cannot deprotonate, because the proton acceptor Asp is replaced by a neutral Thr [41,42]. The intermediate L has a very long lifetime (Fig. 3, HR cycle), followed directly by N. In pHR the next intermediate is O, followed by a silent intermediate HR′ (evident only from an additional time constant), with the same spectrum as that of the unphotolysed HR [21].

The thermodynamic study of the two proteins yielded interesting details concerning the energetic features of the ion transport [33,62,106]. Apart from the last step, when the protein returns to the initial state, almost all the transitions proceed with little change in free energy, i.e., the reactions are close to equilibrium. In BR at pH above 6, the M_1 to M_2 transition becomes unidirectional. The free energy level of M_2 decreases relative to that of M_1, ensuring the unidirectionality of the proton transport even at very low proton concentration. Some transitions are strongly entropy-driven, with a rather large entropy increase [62]. For the M_1 to M_2 transition, the dependence on hydrostatic pressure suggested an approximate volume increase of 32 ml/mol [107]; this was attributed to the conformational change during the opening of the cytoplasmic side. In HR all reactions are enthalpy-driven, accompanied by a step by step entropy decrease, i.e., the intermediates are more highly ordered [33]. During the L to N transition in HR, a volume decrease of approximately 60 ml/mol was observed [33], suggesting that in this step HR closes the cytoplasmic space observed in the structure [11].

4.2.3. Electrogenicity

The ion translocation through the membrane is accompanied by an electric signal, which reflects charge rearrangements perpendicular to the membrane surface. The electric signals measured for different BR-containing oriented purple membrane systems demonstrated a remarkably good correlation with the photocycle [26,27,108]. In principle, the electric dipole moment of each intermediate will be a function of the charge configuration in the protein, which depends on the positions of the amino acid side chains and the transported ion. It was found that each intermediate had a well-determined electrogenicity, which expressed the change in its electric dipole moment relative to that of the unexcited protein [28,29]. A new sample preparation and measuring technique made it possible to solve the three-dimensional motion of the charges [109]. Apart from intermediate N, the calculated electrogenicities in BR were pH-independent, reflecting that in the pH range 4–9 no amino acid side chain that takes part in the proton transport was titrated, suggesting that the charge motions occur inside the protein.

Oriented samples of HR were also prepared and their electric signals were measured [30,110–112]. Kalaidzidis and co-workers could not correlate the slow part of the electric signals of pHR with the late pho-

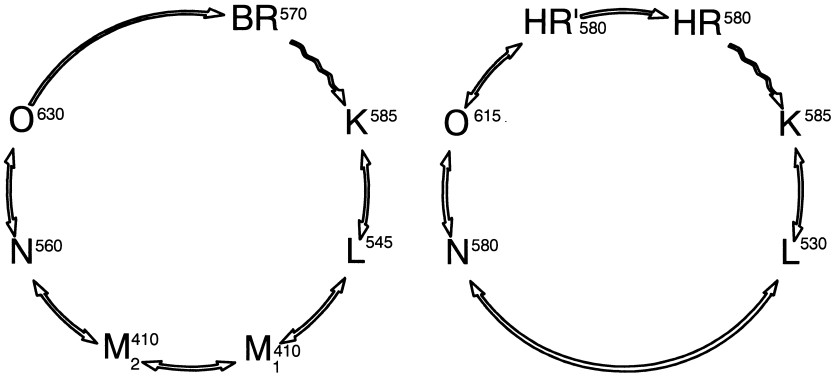

Fig. 3. Photocycle of BR and HR. The wavelengths of the absorption maxima are as indicated.

Table 1
Electrogenicity of BR and pHR, relative to the membrane thickness [29,112]

Intermediate	BR	pHR
K	−0.013	−0.022
L	−0.027	0.067
M_1	0.093	
M_2	0.25	
N	0.27	0.44
O	0.8	1
X'		1
X	1	1

tocycle intermediates [111]. A parallel measurement of electric signals and absorption kinetics proved the correlation and the electrogenicities of all intermediates were calculated [112]. In both forms of ion-pumping rhodopsins, the electrogenicity of intermediate K is negative, and reflects the isomerization of the retinal. Whereas in BR the intermediate L has also negative electrogenicity, in HR it is positive. All the other intermediates have positive electrogenicity, reflecting the general direction of charge transport (Table 1) [29,112].

5. Ion transport

The mechanism of ion transport could be deduced from the accumulated data, and a general transport model could be derived. The transport functions of retinal proteins have been described in terms of two different conceptual frameworks: the IST and the local access models.

The IST model simplifies the transport process to a combination of three basic events (isomerization/switch/transfer) [47,113]. The observed variety of transport modes in the D85N mutant of BR, in HR, and in sensory rhodopsins is explained by various sequences of these events. The fact that the sequence of the steps is not invariable leads to the interesting conclusion that their driving force is established in the first step of the cycle (photoisomerization), and they are therefore in kinetic competition with one another. It is indeed tempting to describe the transport in terms of three basic events, which are logical components of a light-activated transport. The IST model does not intend to describe the details of the mechanism of the ion transport steps.

The local access model concentrates more on the process and is designed to seek the basic rules that govern the light-activated ion transport [114,115]. It is meant, nevertheless, to account for the same data as the IST model, as well as more recent spectroscopic information [114]. In BR, the proton-transporting photocycle of the all-*trans*,15-*anti* isomeric form, after photoisomerization of the retinal in 13-*cis*,15-*anti*, in the L, M and N states the protein oscillates between extracellular (EC) and cytoplasmic (CP) configurations. In intermediate L, due to changes in the pK_a of the proton acceptor Asp-85, the proton is transferred toward the EC side, giving rise to M. M_1 is the EC, and M_2 the CP configuration of the protein. In M_2, the proton is transferred from the proton donor Asp-96 to the Schiff base, producing intermediate N. The change in accessibility from EC to CP is accompanied by a volume increase (measured by thermodynamic methods [107]), meaning that the CP conformation has the larger volume. In the final steps, going through O to BR, the retinal regains its all-*trans*,15-*anti* configuration and the protein once again has its original conformation. During the photocycle, the changing proton affinities of the proton acceptor and donor, modulated by the fluctuation of the local geometry and the accessibility of the entrance of EC and CP half-channel, determine the direction of proton transport [115]. The switch, therefore, is not associated with a single event of the photocycle. Also unlike the IST model is the fact that in the local access model the photocycle is a cascade, in which each step is the direct cause of the step that follows it.

Although data are not yet sufficient for a detailed mechanism of chloride transport in HR, a tentative model can be elaborated on the basis of the same local access model. The transported ion is negatively charged, and the transport in the cell is inward. In the unphotolysed state, the chloride binding site is accessible on the EC side, but with a protein structure with a larger unit cell volume as compared to BR [11]. After photoexcitation, during the K to L transition the retinal is driven to a relaxed 13-*cis* form, which results in the protein flickering between two conformations. The retinal configuration and the fluctuation of the protein lead to changes in the accessibility and the affinity of the binding site [79]. When the photocycle reaches intermediate N, in the

CP conformation the binding constant of the site becomes low, and the chloride is released. The reisomerization of the retinal to the all-*trans* form drives the protein through intermediate O, in the EC conformation. On the uptake of a chloride, intermediate HR′ appears, the excess energy is lost and the protein returns to its unexcited HR state. The CP conformation of the protein has a smaller volume compared to EC; this is reflected in a volume decrease during the L to N transition of the photocycle [33].

6. Interconversions of BR and HR

The great similarity between the two ion pumps raises the possibility of converting BR to a chloride pump or HR to a proton pump. There are two ways to change the ion specificity of the protein.

(a) With added ions. In the case of BR, the proton acceptor is neutralized at acid pH. Asp-85 has a very low pK_a of about 2.6 [70,71]. In this case, at pH about 2, but without chloride ion present, the spectrum of BR is red-shifted (acid blue form). When chloride is added, the spectrum shifts back (acid purple form). With quasi-continuous and continuous illumination it has been shown that the acid purple form, but not the acid blue form, performs transport through the membrane. Changing the halide ion, the transport activity can be changed. In contrast, when protons are replaced by deuterons, no change in transport activity is measured. These observations led to the conclusion that in the acid purple form BR transports chloride [116,117].

In the case of HR, where both the proton donor and acceptors are missing, it has been shown that chloride and azide compete for the same binding site. The addition of azide resulted in a BR-type photocycle. The accumulation of intermediate M is observed [19,82], and transport measurements with cell envelope vesicles revealed that proton is transported through the membrane, similarly as for BR [82]. It was suggested that the bound azide on the EC side replaces the proton acceptor, while another azide, shuttling between the Schiff base and the cytoplasmic surface, plays the role of proton donor.

(b) Mutants. The proton acceptor and donor sites can be modified by site-directed mutagenesis. In BR, when Thr replaced the proton acceptor Asp-85, the activity of the protein changed from proton to chloride translocation [81]. Although this BR mutant transports chloride in the same direction as HR, from the extracellular side to the cytoplasm, its efficiency is very low, in part as a consequence of the very low chloride affinity of the binding site. This is a reflection of the fact that for efficient chloride transport, not only the lack of the proton acceptor is important, but also the appropriate binding site configuration, to accommodate the ion. In the case of HR only very few mutants have been prepared and no information is available concerning its conversion to a proton pump in this way.

7. Conclusions

The comparison of different aspects of the two ion-pumping proteins, the proton-transporting BR and the chloride-transporting HR, has demonstrated interesting similarities and differences. Strikingly, these two ions, differing fundamentally in size, in charge and in chemical properties, can be transported through the membrane via similar steps during the photocycle. Unexpectedly, a real equivalence between the steps could be established by demonstrating that either protein can transport the ion specific for the other.

Acknowledgements

The author is grateful to J.K. Lanyi and L. Keszthelyi for critical reading of the manuscript. The author is supported by grants from the National Science Research Fund of Hungary (OTKA T022066) and the Research Fund of the Hungarian Academy of Sciences (AKP 97-71 3,3/52).

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