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Minireview

A Unifying Concept for Ion Translocation by Retinal Proteins

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First, halorhodopsin is capable of pumping protons after illumination with green *and* blue light in the same direction as chloride. Second, mutated bacteriorhodopsin where the proton acceptor Asp85 and the proton donor Asp96 are replaced by Asn showed proton pump activity after illumination with blue light in the same direction as wildtype after green light illumination. These results can be explained by and are discussed in light of our new hypothesis: structural changes in either molecule lead to a change in ion affinity and accessibility for determining the vectoriality of the transport through the two proteins.

KEY WORDS: Isomerization; vectoriality; proton pump; chloride pump.

INTRODUCTION

This contribution deals specifically with a common mechanistic principle found in two different ion translocators: the proton pump bacteriorhodopsin (BR) and the anion pump halorhodopsin (HR). Both are retinal proteins occurring in halobacteria and have been repeatedly reviewed (Tittor, 1990; Oesterhelt *et al.*, 1992; Birge, 1990); Trissl, 1990; Kitagawa and Maeda, 1989; Lanyi, 1990; Mathies *et al.*, 1991). Here we intend to reduce an overwhelming body of biochemical and biophysical data into a simplified view of how the two ion pumps use the same principle of thermoreversible photoisomerization of retinal for the translocation of the same or different ions.

A considerable help for the interpretation of analytical data on the function of the molecules was the recent evaluation of a first atomic model for BR on the basis of electron crystallographic analysis (Henderson *et al.*, 1990). The molecule belongs to the 7-transmembrane helical family (Henderson and Schertler, 1990) and has dimensions of 21 by 41 Å spanning the lipid bilayer over a length of approximately 48 Å. The chromoprotein has a molecular weight of 26 kD with the retinal moiety bound to lysine residue 216 in the form of a protonated Schiff base. The seven transmembrane helices (A–G) are arranged in a circular manner and a transmembrane pore is formed mainly by helices B, C, F, and G. The Schiff base is located approximately halfway through the cross section of the total membrane span and separates an extracellular (EC) channel from a cytoplasmic (CP) channel. Ion translocation is believed to proceed through this pore *via* the Schiff base itself. Indeed at least four water molecules (at 15% relative humidity) could be located in this area of the molecule as deduced from neutron diffraction (Papadopoulos *et al.*, 1990). The retinal itself extends from its attachment point on helix G all the way across the interhelical space with a tilt of about 21° with respect to the plane of the membrane (Hauss *et al.*, 1990). The molecule in its binding pocket is closely packed with four tryptophan residues and the cyclohexene ring moiety touches helices D and E. The structural model specifically substantiated earlier analytical results suggesting the charged retinylidene moiety to be part of a proton donor–acceptor complex (Fischer and Oesterhelt, 1979, 1980; Oesterhelt, 1985). The model showed that aspartic acid 85 (D85) in the EC channel and aspartic acid 96 (D96) in the CP channel could fulfill donor and acceptor functions.

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R82 and R175 are also located within the membrane-spanning part on the two sides of the Schiff base, and these are conserved in the chloride pump halorhodopsin (HR). A first proposal for similar mechanisms of ion translocation in BR and HR was made on the basis of the retinylidene moiety being a *cis–trans* configurational switch between specific ion-binding sites (Oesterhelt *et al.*, 1986) and became more evident with the elucidation of the primary structure of HR (Blanck and Oesterhelt, 1987; Lanyi *et al.*, 1990a). The similarities have been described in detail in a recent article (Oesterhelt and Tittor, 1989).

A light-triggered switch between two ion-binding sites constitutes the simplest example of vectorial catalysis but also should be seen as a simplification of the actual passage of ions. Ions are translocated over a distance of 48 Å and more ion-binding steps might occur along the diffusive path in CP and EC. We define the role of the above-mentioned binding sites in a kinetic sense: Ion conduction in CP and EC is enhanced in the presence of such a particular amino acid side chain, and intermediate ion binding can be demonstrated by physical methods. Here we deal only with events which are decisive in vectorial ion translocation, and which can also be interpreted as molecular changes. These are (1) *cis–trans* isomerizations as configurational changes of the retinylidene moiety; (2) conformational changes of the retinylidene moiety; (3) changes of protonation states of the Schiff base; (4) changes in ion affinity of amino acid side chains for proton or chloride; (5) conformational changes of the protein as analyzed by kinetic X-ray analysis, electron diffraction, or Fourier transform infrared spectroscopy (FTIR). It will be shown that all available data on the two molecules can be coherently interpreted on the basis of a *cis–trans* switch function of the retinylidene moiety communicating alternatively with the extracellular and cytoplasmic channel in the molecules. This interpretation includes BR after removal of the specific proton donor and acceptor side chains and also manipulation of HR as a two-photon-driven proton pump with inversed translocation direction.

BACTERIORHODOPSIN AS A PARADIGM FOR HALOBACTERIAL RETINAL PROTEINS

Structure

Four different types of retinal proteins have been found so far in halobacteria. Besides the two pumps, two sensory rhodopsins, I and II, occur which mediate photoattractive and photophobic responses of cells to orange and to blue light (for reviews see Spudich and Bogomolni, 1988; Oesterhelt and Marwan, 1990). All the proteins bind *all-trans* retinal covalently, and the function of the pumps, possibly also the sensors, involves stereo-selective photoisomerization to the 13-*cis* configuration and its thermal reisomerization. Recently, more members of the halobacterial protein family have been identified and their genes sequenced so that a total of eight different primary structures is now available for comparison (Lanyi *et al.*, 1990a; Suyijama *et al.*, 1989; Uegaki *et al.*, 1991; Soppa *et al.*, 1992; Blanck *et al.*, 1989). With the structural model for BR at hand, the evaluation of homologies between proton pumps, chloride pumps, and one of the sensors (SR) (Fig. 1) suggests that the structural characteristics of bacteriorhodopsin very likely apply to the rest of the family. Secondary structure predictions clearly reveal for all the proteins seven transmembrane helices with conserved and precise boundaries (Soppa *et al.*, 1992). All the helices are amphipathic and, if arranged with the more hydrophilic faces toward the interhelical pore and the more hydrophobic part facing the lipid bilayer, most of the conserved amino acid positions are found in the interior. This is reasonable because the retinal is located here, as shown by neutron diffraction (Heyn *et al.*, 1988). Residues which are conserved in all known protein structures belong to three classes (Fig. 2): (i) amino acid residues in the retinal binding pocket. Obviously conserved groups are the retinal binding lysine residue 216 in helix G or aspartic acid 212 forming part of the counterion for the protonated Schiff base. A total of 21 residues has been identified in the binding pocket (Mathies *et al.*, 1991) and 13 of these are conserved in the pumps and the sensors. Part of these must participate in the stereo-selective photoisomerization to the 13-*cis* state which is accompanied by compensatory rotations around single bonds of retinal and the lysine 216 methylene groups. For this, the side chain of retinal and the cyclohexene ring seem to be held in place by four tryptophan residues in BR. Interaction with residues such as methionine 118, glycine 122, and proline 186 also serve a steric function. Other amino acids could be responsible for the catalytic function of thermal isomerization of 13-*cis* to *all-trans* retinal during the photocycle and the so-called "dark/light adaptation" reaction whereby an *all-trans*,15-*anti* configuration of retinal thermally equilibrates with a 13-*cis*,15-*syn* state (Oesterhelt *et al.*, 1973; Smith *et al.*, 1984). One example might be L93 (Subramaniam

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                              1     6          16         26         36       45
bop               MLELLPTA VEGVSQAQIT GRPEWIWLAL GTALMGLGTL YFLVKGMGVS DPDAKKFYAI
hop        MSITS VPGVVDAGVL GAQSAAAVRE NALLSSSLWV NVALAGIAIL VFVYMGRTIR PGRPRLIWGA
sop                                  MDAVATAYLG GAVALIVGVA FVWLLYRSLD GSPHQSALAP
     .......... .......... .......... .......... .......... .......... ..........

     46         56         66                                  80         90       99
bop  TTLVPAIAFT MYLSMLLGYG LTMVPFGG.. :......... ....EQNPIY WARYADWLFT TPLLLLDLAL
hop  TLMIPLVSIS SYLGLLSGLT VGMIEMPAGH ALAG...... ....EMVRSQ WGRYLTWALS TPMILLALGL
sop  LAIIPVFAGL SYVGMAYDIG TVIVNGNQ.. .......... ........IV GLRYIDWLVT TPILVGYVGY
     ....*..... .*........ .......... .......... .......... ..**  *... **........

     100        110        120        130        139        149        159    168
bop  LVDADQGTIL ALVGADGIMI GTGLVGALTK VY.SYRFVWW AISTAAMLYI LYVLFFGFTS KAESMRPEVA
hop  LADVDLGSLF TVIAADIGMC VTGLAAAMTT SALLFRWAFY AISCAFFVVV LSALVTDWAA SASSAGT..A
sop  AAGASRRSII GVMVADALMI AVGAGAVVTD GT..LKWALF GVSSIFHLSL FAYLYVIFP. RVVPDVPEQI
     .......... ....**..*. ..*.....*. .......... ..*....... ...*...... ..........

     169        179        189        198        208        218        228    237
bop  STFKVLRNVT VVLWSAYPVV WLIGSEGAG. IVPLNIETLL FMVLDVSAKV GFGLILLRSR AIFGEAEAPE
hop  EIFDTLRVLT VVLWLGYPIV WAVGVEGLAL VQSVGVTSWA YSVLDVFAKY VFAFILLRWV ANNERTVAVA
sop  GLFNLLKNHI GLLWLAYPLV WLFGPAGIG. EATAAGVALT YVFLDVLAKV PYVYFFYARR RVFMHSESPP
     ..*..*.... ..**..**.* *..*..*... .......... ...***.**. .......... ..........

     238        249
bop  PSAGDGAAAT SD*
hop  GQTLGTMSSD D*
sop  APEQATVEAT AAD*
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Fig. 1. Sequence comparison of bacterio-opsin, halo-opsin, and sensor-opsin. Residues of important roles for ion translocation are in bold; conserved residues in all three proteins are marked by an asterisk.

et al., 1991). (ii) The second group of amino acids is composed of side chains on the inner surface of the EC and CP channel and only two out of 30 residues are conserved. Considering the different functions of the three proteins, this is not surprising. (iii) The third group comprises 13 residues which are conserved but not located in the retinal-binding pocket or the ion-conducting pore. We attribute to these residues a structural determinant role and one example is proline 91 which causes a kink in helix C narrowing the CP channel.

An obvious pattern of characteristic residues dis-

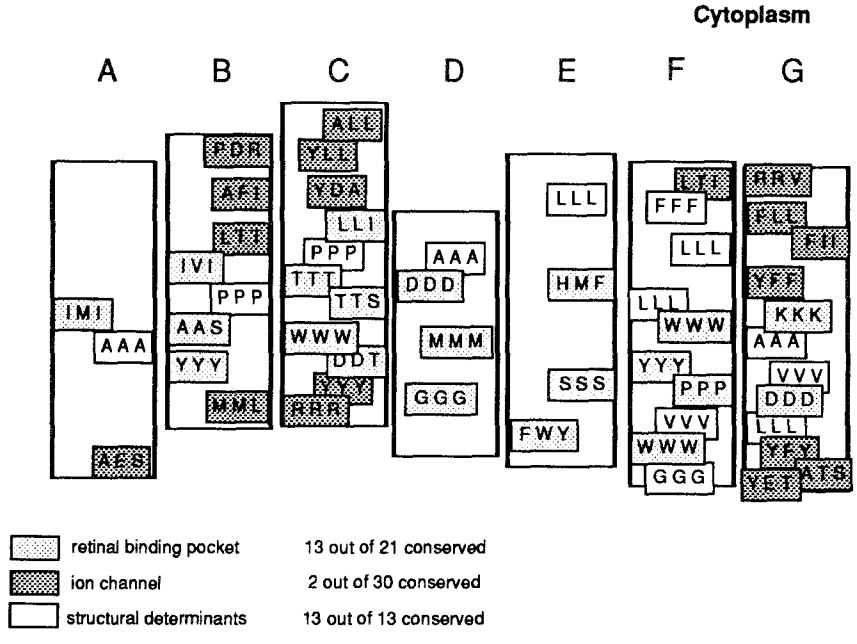

Fig. 2. Comparison of secondary structures of BR, HR, and SR.

criminates among the BR, HR, and SR type of molecules (Soppa *et al.*, 1992). The proton pumps always have aspartic acids 85 and 96. The sensors carry only a potential proton acceptor, and consequently their intermediate (deprotonated) state has a half-time of decay of about a second (not 10 ms as the pumps). The chloride pumps have none of these aspartic acids and no reversible deprotonation is linked to their catalytic cycle.

Color and Rotational Barriers

Protonated retinylidene Schiff bases of retinal absorb maximally at 440 nm in solution but around 570 nm in the proteins. This red shift in color ("opsin shift"; Nakanishi *et al.*, 1980) is mediated by specific retinal protein interaction and is of essential importance for color regulation not only in halobacterial retinal proteins but also in visual pigments. Color shifts are accompanied by changes of the energy barriers for rotation of double and single bonds in the retinylidene moiety. Both the color and the energy barriers are regulated by the average distance of the anionic charge compensating for the positively charged nitrogen of the Schiff base. Increasing the distance of the ionic charges causes an increasing red shift (Blatz *et al.*, 1972; de Groot *et al.*, 1989; Schulten *et al.*, 1980; Großjean and Tavan, 1988) and an increasing energy barrier for rotation of single bonds, especially the 14,15-bond (Tavan *et al.*, 1985). At the same time the energy barrier for double bond rotation is lowered to an extent which predicts values in the range of 14 kcal/mol for bacteriorhodopsin. Such values have been determined experimentally for 13-*cis* to *trans* isomerization in BR (Oesterhelt and Hess, 1973). The inverted height of barriers for single and double bond rotation is reversed upon deprotonation of the Schiff base or the approach of an anion (e.g., chloride in halorhodopsin). On this basis mechanistic models were suggested for the ion pumps which assumed isomerization around the 13,14 double bond accompanied by 14,15 single-bond rotations (Schulten and Tavan, 1978; Gerwert and Siebert, 1986; Oesterhelt *et al.*, 1986). In such mechanisms the step of single-bond rotation would be equivalent to a protein conformational change as discussed below.

Movement of Retinal

Any isomerization of the retinylidene moiety will cause a geometric change of atomic positions which can be large or small, depending on the extent of compensatory bond rotation in retinal and/or the oligomethylene chain of lysine 216. This question was addressed with the help of three-dimensional crystals of bacteriorhodopsin and measurement of the transition moment changes during the photochemical cycle of BR by measurements of linear dichroism. The result obtained (Schertler *et al.*, 1991) indicated that the Schiff base undergoes only a slight change of its transition moment, by $2.2 \pm 0.5°$, and this was interpreted as a displacement of one end of the chromophore by 0.6 Å. The movement of retinal might be limited to the Schiff base end of the chromophore if one takes into consideration that the side chain is sterically immobilized by contacts with tryptophan and other side chains. The structural model also favors rotation around the 14,15 single bond in addition to the 13-*cis* isomerization because only then does the proton of the Schiff base face the presumed proton acceptor D85 discussed in Schertler *et al.* (1991). Molecular dynamic calculation on BR also demonstrated that a rotation around both the 13,14 and the 14,15 bond is the most likely photoisomerization event (Nonella *et al.*, 1991).

It is worth mentioning that isomerization not only moves the charged nitrogen but also gives a different shape to the retinylidene moiety, and exerts a pull on the oligomethylene chain of the lysine. This could cause helix G to move. Indeed, it has been shown by several methods that during the photocycle of bacteriorhodopsin the protein moiety undergoes conformational changes (Dencher *et al.*, 1989; Gerwert *et al.*, 1990; Ormos, 1991; Koch *et al.*, 1991).

THE PRINCIPLE OF THE BACTERIORHODOPSIN MECHANISM OF PROTON TRANSLOCATION

When analysis of the chromophoric structure in BR showed that it is a proton donor–acceptor complex (Fischer and Oesterhelt, 1979; Fischer and Oesterhelt, 1980; Eisenstein *et al.*, 1987) this not only explained pH-dependent color changes but also suggested a *trans–cis* switch movement (Tsuda *et al.*, 1980; Pettei *et al.*, 1977) of retinal between two amino acid side chains of the appropriate protonation state as mechanism of proton translocation (Oesterhelt, 1982). As expected, the color changes of the chromophore at acidic pH to the blue and at the alkaline side to the red are both accompanied by inactivation of the

proton pump. Direct proof of different protonation states of important amino side chains was obtained by FTIR methods (Siebert *et al.*, 1982; Eisenstein *et al.*, 1987; Engelhard *et al.*, 1985; reviewed in Katagawa and Maeda, 1989). Aspartic acids 96 and 85 were shown to play a central role in the proton translocation mechanism of BR by analysis of proton currents in mutagenized BR molecules (Mogi *et al.*, 1988; Butt *et al.*, 1989; Marinetti *et al.*, 1989). Aspartic acid 85 in the EC channel is deprotonated over the entire physiological range (pK about 3.5) and acts as proton acceptor for the Schiff base, while aspartic acid 96 in the CP channel is protonated in the same range and acts as the proton donor to the Schiff base (Holz *et al.*, 1989; Miller and Oesterhelt, 1990; Otto *et al.*, 1989; Gerwert *et al.*, 1989). The protonation of D85 as acceptor prevents deprotonation of the Schiff base (formation of M) as exemplified by a D85E mutant with a pK higher than 7 and causes the characteristic blue color of the chromophore (Subramaniam *et al.*, 1990; Lanyi *et al.*, 1992). The lack of D96 (e.g., D96N mutant) slows the reprotonation of the Schiff base (decay of M) to seconds and renders the proton pump bulk pH-dependent (Miller and Oesterhelt, 1990).

The concept of the *cis–trans* movement of retinal between proton donor–acceptor groups, however, is not sufficient to explain vectorial catalysis (transport). As a general definition, this requires that a substrate (proton) enters a binding site (Schiff base) on a pathway (CP channel) different from the one it takes to leave (EC channel) this binding site. The substrate must be prevented from taking the same path by an irreversible step during the catalytic cycle. In other words, the vectorial catalyst must exist at least in three different states because A–B transitions are not enough for this type of reaction. For an ion pump such as BR this step could be combined with energy conservation, i.e., provision of the proton leaving via the D85 residue in the EC channel with a protonmotive force of 280 mV. This is approximately the proportion of the absorbed light energy stored across the halobacterial cell membrane as a result of bacteriorhodopsin's action (Michel and Oesterhelt, 1976). A plausible mechanism then implies that the Schiff base must undergo a change in pK during the catalytic cycle which is linked to *trans* to *cis* isomerization (Oesterhelt, 1976). Specifically, the Schiff base in the 13-*cis* state donates a proton to D85 with a pK lower than 3.5 and still in 13-*cis* accepts a proton from the D96 side chain with a pK higher than 10. These two states subdivided in B and C could be envisaged as a conformational change in retinal from a 14*s*-*cis* to the 14*s*-*trans* state after double photoisomerization (called M_I and M_{II} in Schulten and Tavan, 1978) or, alternatively, a conformational change of the protein itself as already discussed (Mathies *et al.*, 1991).

A recent spectroscopic analysis of the photocycle intermediates of BR has suggested that indeed an irreversible step occurs which subdivides the M state into M_1 and M_2 (Varo and Lanyi, 1991a, b). Thus we arrange the intermediates of the catalytic cycle of BR which can be spectroscopically detected (J to O, review in Mathies *et al.*, 1991) into the simple framework of an A–B–C–A transition in the following way:

$$A \longrightarrow B \longrightarrow C \longrightarrow A$$

$$BR \rightarrow J, K, L, M_1 \rightarrow M_2, N, O \rightarrow Br$$

It is not our aim to review details of the molecular changes so far elucidated by biophysical techniques (see Birge, 1990; Kitagawa and Maeda, 1989) nor to discuss the details of proton movements (Heberle and Dencher, 1990; Gerwert *et al.*, 1990) but rather to emphasize the general feature of vectorial ion transfer. The first product after absorption of a photon is J with a *cis* or pre-*cis* configuration which relaxes to K in about 5 ps. K is the first state which can be trapped thermally at low temperatures and further relaxes to the state L. The term relaxation reflects the fact that the initial photoisomerization is much faster than any of the molecular changes resulting from this event. The first chemically defined change after isomerization is the release of a proton to form the M state. The details of how the proton reaches aspartic acid 85 are not yet known, but it is clear that while this residue is protonated a proton appears on the surface of the protein and after a delay time due to diffusion through an unstirred layer in the bulk phase (Heberle and Dencher, 1990). It is not our aim to discuss the details of the second proton acceptor (a good candidate would be arginine 82). Once the proton is released from the Schiff base, a quick (Varo and Lanyi, 1990a) and irreversible transition from M_1 to M_2 can take place. If the proton acceptor D85 is missing or blocked by protonation (e.g., in HR, in D85E, or wild type at acidic pH), the molecule finally returns to the *trans* state without net proton transfer as a result. The M_2 state with its changed pK and accessibility starts the second half of the cycle, i.e., the C–A transition. The Schiff base accepts a proton from aspartic acid D96, forming the *N*-intermediate. After reprotonation of this residue a 13-*cis* to *trans* isomerization is

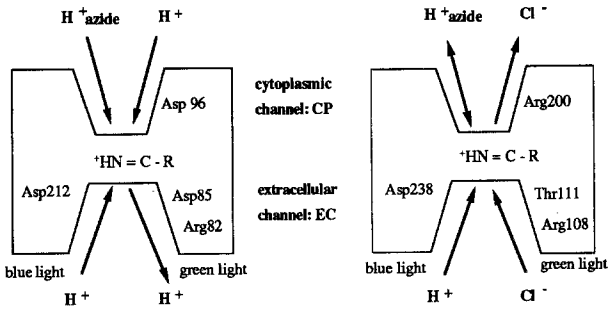

Fig. 3. Schematic presentation of ion translocation in BR and HR. Residues of important roles in the cytoplasmic and extracellular channel are shown.

possible and leads back to the A-, i.e., BR-state. Thus, the key events in the photocatalytic cycle of BR can be described as (1) *trans* to *cis* isomerization, (2) deprotonation of the Schiff base (protonation of D85), (3) transition of the Schiff base from a state of low pK, M_1, to a state of high pK, M_2, (4) reprotonation of the Schiff base by proton transfer from aspartic acid 96, and (5) reisomerization of the schiff base in a protein environment which may be alternatively specified by the protonation states of aspartic acid 85. These molecular events are clearly accompanied by conformational changes of the protein environment and current experimentation aims to define these conformational changes in terms of defined molecular events, e.g., environmental changes of specific amino acid residues. Another example is the alteration of continuum absorption of structured water or a hydrogen network within the protein as measured by the FTIR technique.

The M_2 state could live for seconds if D96 is converted to an asparagine (D96N), demonstrating that not only the pK is changed but accessibility through only the CP channel is strict. The *trans* state, i.e., the initial BR_{570} state, on the other hand, like the M_1 state, is able to exchange protons only by the EC channel, as has been shown by NMR and FTIR experiments (de Groot *et al.*, 1990; Ehrenberg *et al.*, 1980). Another channel-specific ion conduction is exhibited by inorganic anions such as azide. Addition of azide to mutant BR D96N, which is inactive as a proton pump in cells, allows a more efficient proton conduction specifically through the CP channel and thereby reconstitutes a fully active proton pump (Tittor *et al.*, 1989).

Figure 3 summarizes schematically the properties of the BR proton channel, and two more experimental results about proton conduction in bacteriorhodopsin deserve attention. The M intermediate receiving a photon of blue light is converted from the 13-*cis* to the *all-trans* state of its retinylidene moiety and regenerates the BR_{570} initial state in a fast reaction (Oesterhelt *et al.*, 1975; Ormos *et al.*, 1978). The proton uptake apparently is through the EC channel because electrical experiments showed that blue light inhibits the green light-driven proton pumping by BR (Ormos *et al.*, 1978). This corroborates with the fact that proton conduction in the dark is possible through the EC channel.

HALORHODOPSIN'S PROPERTIES

General

Halorhodopsin's function was discovered on the basis of light-dependent ATP synthesis and passive proton uptake in a BR-negative strain of *Halobacterium halobium*. Halorodopsin acts as an inwardly directed chloride pump and guarantees the osmotic balance of cells during growth (for history and review see Lanyi, 1990). Thus light energy contributes by a second pathway to the energy balance of cells growing under phototrophic conditions. It does not occur in two dimensional crystalline sheets within specialized domains of the cell membrane as typical for bacteriorhodopsin but was isolated in a detergent-solubilized state and reconstituted for functional studies in liposomes, black lipid membranes, or halobacterial lipid sheets (Lanyi, 1990).

Several key features demonstrate the high degree of similarity between the two ion pumps (see also Table I). The primary structure of halorhodopsin has been deduced from gene sequencing and protein chemistry of the isolated polypeptide chain (Blanck and Oesterhelt, 1987; Hegemann *et al.*, 1987). A very clear homology to bacteriorhodopsin was found and

Table I. Comparison of the Two Ion Pumps Demonstrating the High Degree of Similarity

Feature	Halorhodopsin	Bacteriorhodospin
Function	Chloride pump Cl^-, Br^-, I^-, not F^-	Proton pump (OH^-?)
Molecular weight	27 kD	26 kD
Prosthetic group	Retinal 1:1 as a Schiff base, protonated	Retinal 1:1 as a Schiff base, protonated
Absorption maximum	578 nm (light-adapted)	570 nm (light-adapted)
Secondary structure	7 transmembrane helices	7 transmembrane helices
Photochemical reaction	*trans* → 13-*cis*	*trans* → 13-*cis*
Primary photoproduct	H_{600} (5 ps)	J_{610} (450 fs)
Quantum yield	0.34	0.7
Longest living intermediate	H_{520}/H_{640}	M_{412}
Cycle time	~ 10 ms	~ 10 ms
Schiff's base	No deprotonation during active cycle	Reversible deprotonation during cycle
Ion-binding sites	Two chloride-specific sites	Two proton-specific sites
Ion affinity of protein	Change in chloride affinity	pK change (aspartate)

confirmed for structures of halorhodopsin from another halophilic species (Lanyi *et al.*, 1990a). Independent of the homology, hydrophobicity analysis revealed seven transmembrane domains of the molecule, and a helical wheel analysis suggested that these seven helices are amphipathic as those of bacteriorhodopsin. Most of the conserved residues are located on the inner surface of the ring formed by the seven helices. The suggested topography has been confirmed by several lines of experimentation: (i) proteolytic digestion experiments (Schobert *et al.*, 1988), (ii) labelling of the carboxy terminus on the cytoplasmic surface with specific antibodies (May *et al.*, 1988); (iii) 2-D crystallization of halorhodopsin in a halobacterial lipid bilayer (G. Rachel, W. Baumeister, and D. Oesterhelt, unpublished); the dimensions of the molecule are indistinguishable from bacteriorhodopsin (22 × 41 Å); and (iv) the retinal molecule is bound to the only lysine residue of the molecule in helix G like in bacteriorhodopsin (Blanck and Oesterhelt, 1987).

Spectroscopic Properties

Spectroscopic analysis of halorhodopsin, although not yet carried out in such detail as for bacteriorhodopsin, reveals complex behaviour. Here, only the functional cycle connected to ion transport and a side reaction leading to deprotonation of the molecule is regarded (Lanyi, 1990; Hegemann *et al.*, 1985b). The first identifiable intermediate on a picosecond time scale is H_{600}, which is converted into a species absorbing around 520 nm. This intermediate has been shown to be a 13-*cis* retinylidene moiety (Diller *et al.*, 1987; Fodor *et al.*, 1987) and was postulated to equilibrate in a chloride-dependent manner with a species absorbing at 640 nm (H_{640}). A chloride-dependent equilibrium exists for the initial state H_{578} also, with another form H_{565} in the dark. A photocycle accounting for the kinetics of the spectroscopic changes with a link between H_{640} and H_{565} was suggested (Oesterhelt *et al.*, 1985). Unfortunately no information on the isomerization state of H_{640} has been obtained, but the existence of two forms absorbing in that wavelength range has been demonstrated (Spencer and Dewey, 1991). The same has been postulated for H_{520} purely on the basis of theoretical considerations (Oesterhelt *et al.*, 1986). To form the analogy between HR and BR with respect to vectorial transport, the scheme of a three-step model can be applied:

$$A \longrightarrow B \longrightarrow C \longrightarrow A$$

$$H_{578}\,(H_{600}) \qquad H^{I}_{520} \qquad H^{II}_{520} \qquad H^{I,II}_{640},\ H_{565}H_{578}$$

This would attribute to the species H_{520} the function of having different affinity for chloride and different ion accessibility in analogy to the M_1, M_2 states in BR. It should be emphasized that in this working hypothesis the only experimental information available is the 13-*cis* state of H_{520} and therefore HR urgently needs more experimental attention.

Ion-Binding Sites

Ion-binding sites in HR have been characterized by optical spectroscopy, NMR, and resonance Raman

spectroscopic experiments and termed site I and site II (Schobert *et al.*, 1986; Falke *et al.*, 1984; Pande *et al.*, 1989). Without enumerating the properties of these two ion-binding sites in detail it should be mentioned that a possible assignment in the primary sequence has been made (Blanck and Oesterhelt, 1987; Lanyi, 1990). Site I could be represented by arginine 200 and is thought to be unoccupied in the initial state H_{578} but transiently occupied by chloride during the catalytic cycle (Lanyi *et al.*, 1990b). Site II, on the other hand, is thought to be permanently occupied by chloride (or nitrate) and is thought to be mainly constituted by arginine 108. It is site II which can lose a chloride ion to the extracellular side when forming H_{565} in the dark, and the affinity for chloride has been determined to be 10 mM. Both sites have distinct specificities for anions other than chloride, bromide, and iodide, but this will not be discussed in detail here. As a summary statement on halorhodopsin the molecule is similar to bacteriorhodopsin in all respects, structure and photochemistry, except the missing transient deprotonation durings its catalytic cycle. The Schiff base thereby stays permanently protonated (charged) and could move the anion through a *trans–cis* isomerization as a molecular switch. The functional analogy of the deprotonation of the Schiff base with salt formation with a chloride anion has been shown by theoretical calculations. Thus we hypothesize that like the formation of M^I in BR the formation of H^I_{520} is essential to change affinity and accessibility in a transition to H^{II}_{520} when Cl^- is entering the CP channel. The EC and CP half-channels in the protein structure conserve a similar function as in bacteriorhodopsin but the proton donor-accepting groups, D85 and D96, are replaced by two anion-binding sites, R108 and R200. Interestingly the amino acids are conserved in BR and upon blockade of the proton acceptor D85 by acidification anion-dependent stationary photocurrents were measured (Keszthelyi *et al.*, 1990; Der *et al.*, 1991).

Halorhodopsin's Deprotonation Reaction

The Schiff base in halorhodopsin has a pK of around 8, depending on the ionic conditions, which is in strong contrast to bacteriorhodopsin with a pK above 13. This leads to the formation of a deprotonated species (H^D_{410}) at the appropriate pH in the dark. Also in light a deprotonation can occur, forming a species H^L_{410} which has a lifetime in the minute time range. While the H^D_{410} form reflects the isomeric composition of the initial state H578 (upon preillumination mainly *all-trans*), H^L_{410} has been shown to be a 13-*cis* form by resonance Raman spectroscopy (Diller *et al.*, 1987). It is derived from one of the intermediates of the catalytic cycle, presumably H_{520}, but its rise from the form H640 has not been rigorously excluded. The fact that H^L_{410} can be produced by deprotonation during the photocycle indicates a change in pK of the Schiff base upon illumination, and this has been studied in detail (Lanyi, 1986). A most interesting property of halorhodopsin is the fact that addition of azide accelerates the formation and decay of the side product H^L_{410} dramatically until at saturating concentrations the formation of H^L_{410} effectively competes with the normal photocycle. As a result, chloride transport ceases in green light and H^L_{410} accumulates under stationary illumination to a large proportion (Hegemann *et al.*, 1985b). The effect of azide has been analyzed in detail with detergent-solubilized halorhodopsin and functionally studied in black lipid membrane experiments (Hegemann *et al.*, 1985b). Experiments with cell envelope vesicles show that the deprotonation reaction of the Schiff base catalyzed by azide occurs through the CP channel of the molecule (Lanyi, 1986), and this exactly is the half channel in which, also in bacteriorhodopsin azide, catalyzes the reprotonation of the Schiff base if D96 is removed with a mutation. Another property important for the general concept of ion translocation by HR and BR is worth mentioning. H^L_{410}, like M, can be isomerized to the *all-trans* configuration by absorption of blue light. Photoisomerization from *trans* to 13-*cis* in the deprotonated Schiff base (H^D_{410}) is also possible. Generalizing, the retinal in the protein's binding site will switch from *trans* to the 13-*cis* state or vice versa. Figure 3 summarizes and compares with BR the ion-conducting properties of HR.

VARIOUS MODES OF ION TRANSPORT

The properties of BR and HR make a distinct and common pattern of reactions obvious. First, retinal is switched by light between *trans* and *cis* and is able to reisomerize thermally only in the protonated state. Second, the *trans* state is accessible to protons in the dark through EC and has a high pK in BR and a lower pK in HR. Third, the 13-*cis* form (state B) has a low pK in BR and releases a proton through EC. This release is kinetically disfavored if D85 is protonated or absent. In this case BR eventually picks up a chloride like HR does in its standard photocatalytic cycle.

Fourth, the Schiff base nitrogen of the retinal in the 13-*cis* form (state C) is accessible only and exclusively through CP and accepts a proton from D96 or in its absence via azide. HR releases a chloride through CP, and BR under acidic condition might do so as well. HR further can lose a proton in this state through CP if azide is present. Leaving chloride transport and its mechanistic interpretation aside, proton translocation in BR and HR can be manipulated by two different approaches: mutagenesis of BR and change of physical conditions for HR.

The results with mutated bacteriorhodopsins can be summarized with respect to the proton donor (D96) and acceptor (D85) by the following statement: D85 must be a carboxylate group to guarantee fast deprotonation in the L- to -M_1 transition. D96 must be a protonated carboxyl group for efficient proton retransfer to the Schiff base in the M- to -N transition, guaranteeing the pH independence of the catalytic cycle and therefore the proton pump activity in the physiological pH range. If missing, azide can fully replace the proton donor function of aspartic acid 96 by catalyzing the proton exchange between the cytoplasm and the Schiff base. An interesting question concerns a situation where the proton donor and acceptor are both missing and such a molecule has been constructed by site-specific mutagenesis, transformation of halobacteria, and homologous expression of the mutant protein (D85, 96N, or D2N) (Tittor *et al.*, 1991). The molecule was shown to have two properties very similar to halorhodopsin. Its photocycle does not pass through a deprotonated state of the Schiff base, and the pK of the Schiff base in the dark is around 8.5. Deprotonation yields a yellow form which is the analogue of the HR species H^D_{410}. Surprisingly, upon blue light absorption the yellow form D2N showed proton transport activity in black lipid membrane experiments. Addition of azide increased this activity. The partial reactions of this blue light-driven translocation cycle are listed in Fig. 4. In the initial state this BR is a deprotonated Schiff base in *trans*. Upon blue light absorption it is isomerized to the 13-*cis* state which has become accessible to protons only from the cytoplasmic site and forms the intermediate 450 *cis*. Efficient proton uptake is made possible by added azide. The protonated Schiff base is now able to reisomerize to the *all-trans* state. Because of the lowered pK in the mutant protein D2N, the *trans* state has to release a proton from the Schiff base in the dark, which can occur only by release through EC. As a result, blue

system	educt	direction	result
BR	Initial state (trans)	from/to EC	rapid equilibration
	L cis M_2cis	to EC from CP	H^+ pump outward
	Mtrans	from EC	no net pumping
HR	$H_{520/640}$ H_{410} trans	to CP from EC	H^+ pump inward
D2N	410 cis 450 trans	from CP to EC	H^+ pump outward

Fig. 4. Synopsis of proton uptake and release and its vectoriality in bacteriorhodopsin, halorhodopsin, and mutated bacteriorhodopsin D2N. Educt designates the species undergoing the protonation change.

light in the presence of azide moves a proton as in wild-type bacteriorhodopsin from the cytoplasmic to the extracellular side, but with a somewhat reversed chemistry. *Trans*-to-*cis* isomerization leads to picking up a proton and *cis*-to-*trans* isomerization leads to release of the proton. Although the efficiency of this blue light-driven proton pump is not yet known, because quantitation of BLM experiments is difficult, in this mutant BR molecule the principle of vectorial catalysis is further and significantly reduced: without a specific proton donor and acceptor group, the minimal requirement for vectorial transport is a light-triggered switch between two differently accessible ion (proton) conducting half-channels of a transmembrane pore.

Another interesting case is found for halorhodopsin (Bamberg *et al.*, 1992). HR can act as a two-photon-driven device for the translocation of protons in the following way: Addition of azide and illumination with green light leads to the release of a proton from the Schiff base in the 13-*cis* state through the CP channel. The molecule, if subjected to thermal equilibration in the dark, would pick up the proton from that same side and no net transport would result. If, however, blue light is present in addition to green light, the deprotonated 13-*cis* Schiff base is photochemically switched to the *trans* state, and this exposes the Schiff base to the EC channel. Consequently, by reestablishing the initial state H_{578} and due to its intrinsic pK, a proton is taken up through the EC. The result is surprising again! Proton translocation through the halorhodopsin molecule is in the opposite direction compared to BR wild-type or mutant proteins. The important difference is that proton translocation

in HR starts with a 13-*cis* chromophore and photoisomerization leads to an *all-trans* state which is accessible for protons only from EC. This is clear evidence for the mechanistic description given above.

CONCLUSIONS

Although many details of the retinal proteins, halorhodopsin and bacteriorhodopsin, are still missing for the description of the individual steps of their catalytic cycles in a molecular sense, it seems obvious that a common principle of vectorial catalysis is verified in the two molecules. The most intriguing part of the catalytic cycle, not well understood in either of the cases, is the structural change in the molecule which leads to the *changed ion affinity* and accessibility. Any further biophysical experimentation will have to address this question. On the other hand, it seems obvious that the basic principle of a light-triggered switch between *cis* and *trans* or *trans* and *cis* is the key element for vectorial transport while ion-binding sites, at least in bacteriorhodopsin, seem to serve the main purpose of having made the proton pump optimally efficient and pH-independent during evolution. The hypothesis presented here for the mechanism of vectorial catalysis seems to be justified by the fact that not only the ion translocating activities of both wild-type molecules can mechanistically be understood but also the properties of single or double mutated bacteriorhodopsins and the manipulation of halorhodopsin from an anion to a proton pump.

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