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Trans-cis isomerization of retinal and a mechanism for ion translocation in halorhodopsin

D. Oesterhelt[1], P. Hegemann[1], P. Tavan[2], and K. Schulten[2]

[1] Max-Planck-Institut für Biochemie, D-8033 Martinsried, Federal Republic of Germany
[2] Physik-Department, Technische Universität München, D-8046 Garching, Federal Republic of Germany

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Abstract. The chromophore in halorhodopsin (HR) which acts as a light-driven chloride pump in halobacteria shares many properties with its counterpart in bacteriorhodopsin (BR): *(i)* a similar retinal protein interaction, *(ii)* trans to cis isomerization and *(iii)* similar intermediates of its photocycle. One major difference between the two chromoproteins is that the HR chromophore does not become deprotonated during its photocycle. A mechanism for the photocycle of HR is presented, which, in close analogy to an earlier proposed mechanism for BR, involves the sequence of all-*trans* → 13-*cis*, 14*s*-*cis* → 13-*cis* → all-*trans* isomerizations of the chromophore, a Schiff base of retinal. In contrast to the situation in BR the 13-*cis*, 14*s*-*cis* → 13-*cis* isomerization is induced not by deprotonation of the retinal Schiff base chromophore but rather by the movement of an anion (Cl^-) towards the protonated nitrogen of the Schiff's base. The suggested mechanism involves the Schiff base directly in the chloride translocation in halorhodopsin.

Key words: Chloride pump, halorhodopsin, ion translocation, theoretical model, *cis-trans* isomerization

Introduction

Halorhodopsin (HR) is a retinal protein from the archaebacterial type of halophiles and acts in halobacteria as an inward-directed chloride pump (Schobert and Lanyi 1982; Bamberg et al. 1984a). Although its physiological function has not yet been established unequivocally, our hypothesis is that HR affects the net salt uptake of the halobacterial cell during volume increase by growth. HR pumps chloride and other halide ions as a single polypeptide chain of molecular weight 25 kD. This has been shown by isolation of the native chromoprotein and reconstitution of its function in a planar lipid membrane system (Steiner and Oesterhelt 1983; Bamberg et al. 1984b).

Halorhodopsin in the isolated state absorbs maximally at 578 nm, which is close to the absorption maximum of the proton pump bacteriorhodopsin (BR, λ_{max} 570 nm in the light-adapted state), the dominant retinal protein of halobacteria. Spectral as well as transport properties of HR are influenced by halide and other ions and, so far, at least three distinct anion binding sites have been identified on the molecule (Ogurusu et al. 1981; Hazemoto et al. 1984; Steiner et al. 1983; Falke et al. 1984; Schobert et al. 1986). Retinal is bound covalently to the protein and can be converted by reduction into a stable retinyl protein compound (Lanyi and Oesterhelt 1982). By means of resonance Raman spectra the chromophore has been identified as a protonated Schiff base (Smith et al. 1984; Alshuth et al. 1985) and detailed studies on the photocycle of halorhodopsin revealed further similarities with bacteriorhodopsin (Hegemann et al. 1985; Oesterhelt et al. 1985). It has also been suggested that the chromophore isomerizes during the photocycle (Lanyi 1984). In this paper experimental and theoretical evidence is presented for a model of the HR pump mechanism in which retinal undergoes steric transformations similar to those in BR and acts as a molecular switch for the transport of anions in HR.

Results and discussion

Evidence for trans to cis isomerization of retinal

The *trans* to 13-*cis* isomerization of retinal during the photocycle in BR has been well established by resonance Raman spectroscopy (Braiman and Mathies 1980) and extraction of retinal from intermediates (Aton et al. 1977). Another method to demonstrate light-induced *trans* to 13-*cis* isomerization

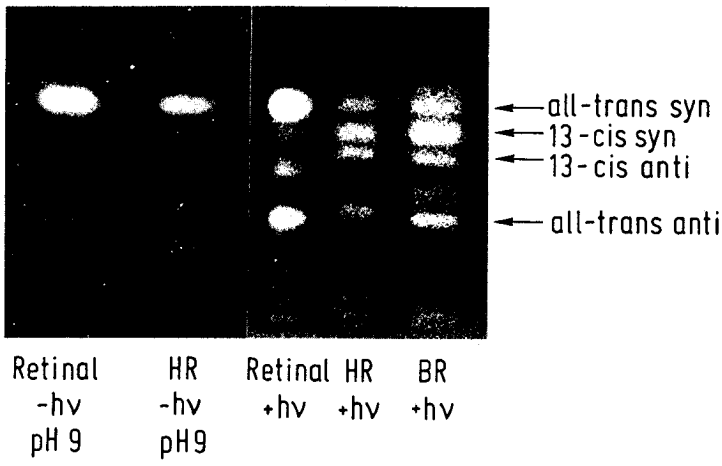

Fig. 1. Formation of 13-*cis* retinal in HR and BR upon illumination. To HR (10 nmol), isolated as described (Steiner and Oesterhelt 1983), and BR (10 nmol) in 2.5 ml 1 *M* NaCl containing 1% octylglucoside, 250 µl 2 *M* hydroxylamine solution pH 7 was added and the samples were illuminated for 30 min (HR) and 60 min (BR) at 10 °C with light from a 900 W Xenon lamp filtered through an OG495 cut off filter. As a control 10 nmol of all-*trans* retinal was treated under the same conditions. In a parallel experiment HR and all-*trans* retinal were reacted with hydroxylamine at pH 9 in the dark. After the reaction all samples were mixed with 700 µl ethanol and the retinal oximes extracted with hexane at 4 °C. Thin layer chromatography was carried out as described in Oesterhelt et al. (1973). The identification of the syn-/anti-isomers was done by NMR as described by Gödecke (1976)

is illumination of BR in the presence of hydroxylamine. As reaction products syn- and anti-oximes of 13-*cis* and all-*trans* retinal can be analyzed by thin layer chromatography. In the experiment presented in Fig. 1, BR served as the control for *trans* to 13-*cis* isomerization and the reaction products from HR obtained under identical conditions were analyzed on the same chromatogram. At neutral pH the reaction of HR with hydroxylamine is very slow in the dark. During illumination isolated HR reacts within minutes and the main products are 13-*cis* syn- and anti-oxime. Light-adapted HR, which was reacted as a control in the dark at pH 9 with hydroxylamine contains only all-*trans* retinal and traces of the 13-*cis* isomer. BR in light produces a very similar distribution of isomeric oximes as HR but, in addition to 13-*cis* and all-*trans* isomers, also some 11-*cis* oximes (between the anti-forms of 13-*cis* and all-*trans* and above all-*trans* syn-forms).

This evidence for a 13-*cis* form in the photocycle of HR is corroborated by two reports: *(i)* upon illumination of HR, the 13-*cis* retinal content increased while a species was formed absorbing around 400 nm (Ogurusu et al. 1981), presumably HR^{L}_{410} (see Hegemann et al. 1985); *(ii)* flash-induced absorption changes in the near UV in HR resembled the absorption changes seen in BR and by analogy it was concluded that the isomerization was a *trans* to 13-*cis* isomerization (Lany 1984).

The state of protonation of retinal during the photocycle

The resonance Raman spectrum of HR in H_2O and D_2O proved the existence of a protonated Schiff base in the chromophoric structure. Comparison of the spectrum with that of light-adapted BR (all-*trans*) reveals only minor differences in the Schiff base region, i.e. a smaller coupling of the N–H in plane bending motion with the C=N stretch mode (Smith et al. 1984; Alshuth et al. 1985). Otherwise the two chromophoric structures must be almost identical.

Light-absorption in HR produces HR_{600} within 5 ps and with a quantum yield of 0.34 (Polland 1984; Polland et al. 1985; Oesterhelt et al. 1985). HR_{600} is converted to HR_{520} and the molecule passes through the intermediates HR_{640} and HR_{565} before returning finally to the most stable state HR_{578}. This scheme of the HR photocycle has been confirmed by several lines of evidence (Oesterhelt et al. 1985; Lanyi and Vodyanoy 1986). The intermediate HR_{520} has the most blue-shifted absorption maximum (at 520 nm), the position of which indicates that the chromophore is not deprotonated as it is far to the red of the absorption of unprotonated retinal Schiff bases (Honig et al. 1976). The permanently protonated state of the retinal Schiff base in the HR photocycle is confirmed by the findings (Hegemann et al. 1985) that a slow deprotonation of HR_{520} or HR_{640} can occur producing HR^{L}_{410} and that the strong inorganic base azide catalyzes this deprotonation.

In summary, HR undergoes a photocycle which, as in BR, is characterized by a red-shifted *trans* to *cis* isomerized photoproduct and a *trans* to *cis* reisomerization during dark reactions not including, however, deprotonation of the Schiff base.

Approach of an anion in halorhodopsin is equivalent to deprotonation in bacteriorhodopsin

The similarity of HR and BR with respect to chromophore absorption, resonance Raman spectra and stereodynamics suggests that explanations regarding the mechanism of BR may apply also to HR. The explanations of the chromophoric properties of BR are briefly summarized as follows:

BR as well as other retinal proteins which carry red-shifted chromophores experience strong electro-

static interactions with their protein environment. These interactions originate from the specific location of one or more negatively charged amino acid side groups which regulate two important properties of the protonated Schiff base: *(i)* the absorption maximum, *(ii)* the activation barriers for C–C bond rotation. The influence of local electric fields on rotational barriers of the retinal chromophore was demonstrated by quantum chemical MNDOC (modified neglect of diatomic overlap correlated version) calculations (Thiel 1981). A most important result was that upon protonation of the retinal Schiff base the rotation around the $C_{13}-C_{14}$ bond becomes thermally allowed (Orlandi and Schulten 1979; Tavan et al. 1985a). Furthermore, it was shown that substituents, especially at the C_{13} position will influence the rotational energy barrier (Tavan et al. 1985b). Experimental work with retinal analogue Schiff bases and with these analogues incorporated in BR have confirmed this prediction (Sheves and Baasov 1984; Gärtner et al. 1983).

The lowering of the $C_{13}-C_{14}$ double bond rotational energy barrier in the protonated retinal Schiff base is accompanied by an increased rotational energy barrier of the adjacent single bonds. The $C_{14}-C_{15}$ bond, for example, under these conditions becomes stronger than the $C_{13}-C_{14}$ bond and can only be rotated at the expense of up to 20 kcal/Mol (Tavan et al. 1985a).

These properties, which should apply to both BR and HR, led to a model of the BR photocycle in which the primary reaction is an all-*trans* to 13-*cis*,14*s*-*cis* photoisomerization (Schulten and Tavan 1978; Schulten et al. 1984). The thermal reisomerization around the $C_{13}-C_{14}$ and the $C_{14}-C_{15}$ bonds cannot take place simultaneously but rather must proceed in two steps: *(i)* deprotonation of the Schiff base enables the rotation around the $C_{14}-C_{15}$ bond by lowering the respective rotational barrier from 20 kcal/mol to about 5 kcal/mol; *(ii)* reprotonation enables then the 13–14 bond rotation by lowering the activation barrier from the 48 kcal/mol value of the unprotonated chromophore to a value of about 13 kcal/mol. This three isomer model, i.e. all-*trans* → light → 13-*cis*,14*s*-*cis* → deprotonation → 13-*cis* → reprotonation → all-*trans* accounts for both, the thermal reversion of the photochemical reaction and the irreversible de- and reprotonation of the chromophore in BR. The protons released and taken up can but do not have to be the translocated protons.

This mechanism of BR invokes a rotation around the $C_{14}-C_{15}$ bond. A proof for the occurrence of such a rotation must be based on observations which are sensitive to such structural details. Suitable in this respect are vibrational spectra observed by Smith et al. (1985) by means of resonance Raman spectroscopy and by Gerwert and Siebert (1986) by means of infrared absorption experiments. The interpretation of such spectra, however, requires the assignment of geometries to vibrational frequencies. Two theoretical approaches assigned the frequencies differently and consequently resulted in support for (Tavan and Schulten 1986) and reject of the suggested mechanism (Smith et al. 1985).

To determine if the function in HR could involve isomerization around the $C_{14}-C_{15}$ bond even in the absence of deprotonation we asked whether the approach of an anion to the positively charged nitrogen of the Schiff base in HR might substitute mechanistically for deprotonation. Such approach would neutralize partially the positive charge of the proton at the Schiff base nitrogen and, thereby, have the same effect on the rotational barriers as a deprotonation step. Figure 2 shows the result of a quantum chemical calculation. The approach of an anion towards the nitrogen from infinity to 3 Å lowers the $C_{14}-C_{15}$ rotational energy barrier from about 13 kcal/mol to 6 kcal/mol. As the distance decreases further the barrier disappears completely. Due to intramolecular steric interaction the 14*s*-*cis* state lies 4 kcal/mol above the 14*s*-*trans* state. This energy difference can provide, in fact, a driving force of about 4 kcal/mol for a 14*s*-*cis* to 14*s*-*trans* isomerization during the HR photocycle. The approach of an anion could reconcile three facets of the HR photocycle *(i)* the stereodynamics of the chromophore of HR involves the same three isomers as in BR, i.e. all-*trans* → light → 13-*cis*,14*s*-*cis* → anion approach → 13-*cis* → anion removal → all-*trans* and is also steered by charge movements *(ii)* the 13-*cis*, 14*s*-*cis* → 13-*cis* reaction is accompanied only by a partial

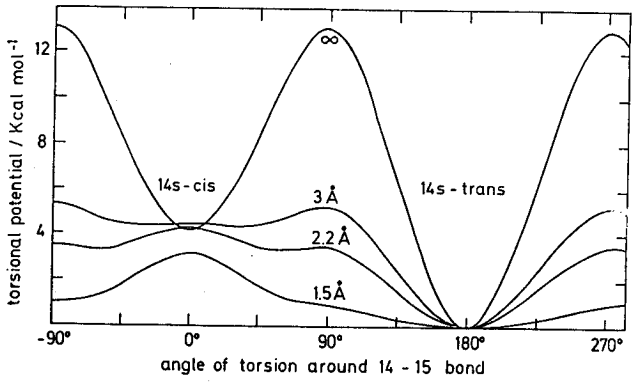

Fig. 2. Torsional potential of the rotation around the 14–15 bond in retinal. The energy barriers shown are for a protonated Schiff base with an anion at infinite distance (∞), and at distances of 3 Å, 2.2 Å and 1.5 Å. The potentials were calculated using the same methods and geometries as described by Tavan et al. (1985a)

blue shift, i.e. to 520 nm, as the chromophore remains protonated: *(iii)* the 13-*cis*, 14*s*-*cis* → 13-*cis*, 14*s*-*trans* reaction transports a Cl^- ion between two binding sites.

Model for the photocycle

The suggested photocycle of HR is illustrated in more detail in Fig. 3. The sequence of intermediates is justified by results described by Oesterhelt et al. (1985). The primary reaction leading to HR_{600} rotates all-*trans* retinal into the 13-*cis*, 14*s*-*cis* conformation. From this conformation all-*trans* cannot be formed directly by a thermal process as the respective activation energy should be above 40 kcal/mol (Tavan et al. 1985a), but rather by an indirect pathway which reaches first the intermediate HR_{520}. This intermediate is assumed to comprise two spectroscopically indistinguishable forms, HR'_{520} and HR_{520}. HR'_{520} and HR_{520} differ in the isomeric state relative to the 14–15 bond of retinal, the latter being energetically more stable (see Fig. 2) (Schulten et al. 1984). HR_{520} or HR_{640} has a pK of 4.9 which is shifted from the value of 9.6 as found under the same conditions for HR_{578} (Oesterhelt et al. 1985). HR_{520} or HR_{640} deprotonates slowly, the reaction can be accelerated more than hundred fold by azide, the respective half maximal concentration measuring 50 m*M*. This behaviour indicates that the Schiff base becomes more easily accessible to anions in the HR_{520} stage, in comparison to the HR_{578} or HR_{600} stages. Apparently only strong bases, such as azide (pK 4.72) or cyanate (pK 3.66), but not chloride, are able to react with the proton at the nitrogen.

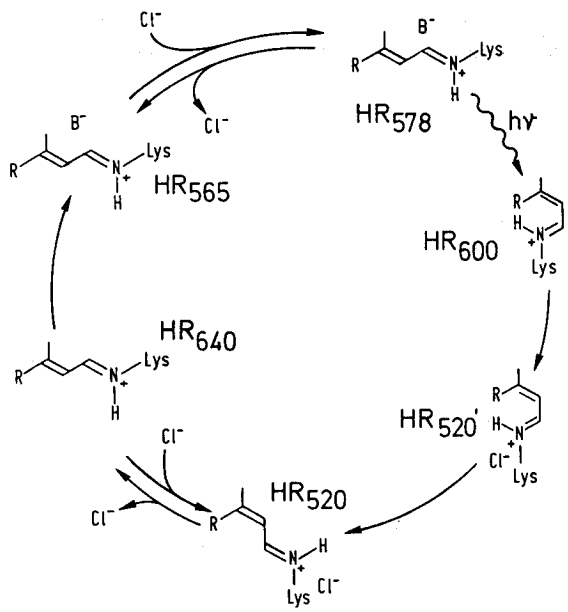

Fig. 3. Suggested photochemical cycle of halorhodopsin. The sequence all-*trans* (HR_{578}) → 13-*cis*, 14*s*-*cis* (HR_{600}, HR'_{520}) → 13-*cis*, 14*s*-*trans* (HR_{520}) → all-*trans* (HR_{640}, HR_{565}, HR_{578}) of isomeric structures of retinal are assigned to the kinetic scheme of the spectroscopically distinct intermediates as established by Oesterhelt et al. (1985). The stereodynamics of retinal accompanying the photocycle follow the model for bacteriorhodopsin (Schulten and Tavan 1978). The blue shifted HR_{410} species occurring as deprotonated side products in darkness and light are omitted. The chloride ions in the scheme indicate the approach and release from the protonated nitrogen but not necessarily transport steps

The movement of the protonated nitrogen connected with the isomerization during the photocycle and motions of negative charges in the vicinity of the chromophore, as depicted in Figs. 3 and 4, can also explain the spectral shifts 578 nm → 600 nm → 520 nm. Upon formation of HR_{600} the nitrogen moves away from its intrinsic anion B^- resulting in a red shift. The approach of a Cl^- ion towards the protonated nitrogen upon formation of HR_{520} produces a blue shift. The fact that the formation of this salt does not lead to an absorption around 440 nm as expected for a retinylidene ammonium salt in solution can be explained by interactions with other charges near retinal's binding site, e.g. those near the cyclohexane moiety of retinal as in BR (Spudich et al. 1986; Harbison et al. 1985).

In the photocycle of HR the intermediate HR_{520} equilibrates with HR_{640} by a chloride-dependent process and the strongly red-shifted absorption of the latter intermediate should be due to an arrangement of anionic charges being even more distant from the protonated nitrogen than in HR_{600}. The analogy with BR is striking considering the similar absorption properties of HR_{640} and of the O-intermediate of BR. The O-intermediate of BR was shown by resonance Raman spectroscopy to contain all-*trans* retinal and, if the analogy holds true, HR_{640} should also be all-*trans*, as depicted in Fig. 3. HR_{640} and HR_{520} are the most likely candidates for reaction with hydroxylamine since under the conditions of light-induced bleaching HR_{600} does not accumulate to any appreciable extent. Observation of a mixture of 13-*cis* and all-*trans* retinal oximes in the experiment of Fig. 1 corroborates the assumption of HR_{640} being all-*trans*. According to this picture for the HR_{520} → HR_{640} conversion the movement of the Cl^- ion through an ion channel of the protein away from the protonated Schiff base catalyzes the thermal isomerization of retinal around the 13–14 double bond by reducing the activation energy for this reaction and by destabilizing the strained 13-*cis* configuration upon the loss of Coulomb attraction. The spectral shift connected with HR_{640} to HR_{565} should reflect the formation of a salt bridge between the protonated all-*trans* retinal Schiff base and a negatively charged amino acid side group.

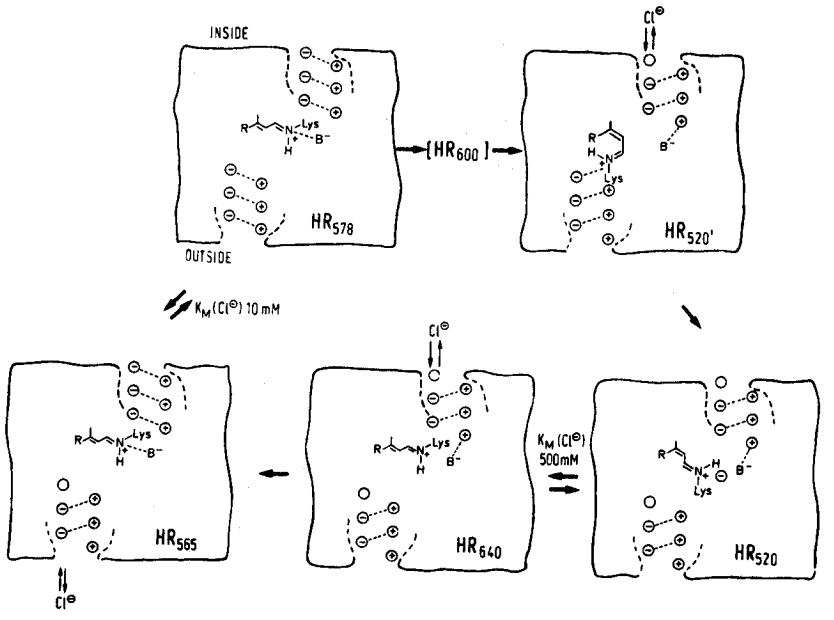

Fig. 4. Model for ion translocation in halorhodopsin. A retinal switch connects two halves of a salt bridged ion-conducting system. The charge movement induced by the primary reaction which is thought to be an all-*trans* → 13-*cis*,14*s*-*cis* isomerization leads to a release of a chloride ion on the inside upon formation of HR'_{520}. Rotation around the 14–15 bond to HR_{520} is allowed by the concerted movement of the positive nitrogen with a chloride ion from the nearby binding site. HR_{640} is formed in a step which removes the chloride from its positive counter-charge and transfers it to the surface binding site under concomitant 13-*cis* to *trans* isomerization. A large red shift of the absorption maximum and a chloride-dependent equilibrium with a low affinity for chloride of 500 m*M* (Lanyi and Vodyanoy 1986) for HR_{640} is the result. After rearrangement of the salt bridges in HR_{565} the final step to HR_{578} is a chloride uptake on the outer side

The chloride-dependent equilibrium between HR_{578} and HR_{565} was analyzed in some detail, and two photocycles were postulated (Bogomolni et al. 1981) to explain the chloride-dependent absorbance changes and decay times in flash experiments. However, a single unbranched photocycle as shown in Fig. 3 including a chloride-dependent equilibrium between HR_{640} and HR_{520}, the former main intermediates of two photocycles, can also explain all the observations. Calculations of a one-photocycle model with spectroscopic data of flash photolytic and stationary illumination experiments justify the cycle in Fig.3 which includes HR_{640}. For symmetry reasons, a second chloride-dependent step and thus HR_{565} must be included into the cycle (Oesterhelt et al. 1985; Lanyi and Vodyanoy 1986).

At low chloride concentrations the HR_{520} to HR_{640} transition becomes monophasic and the equilibrium lies far to the side of HR_{640}. Under these conditions, the photocycle runs about 5–10 times faster as analyzed by flash photolysis (Weber and Bogomolni 1981). At the same time, chloride transport ceases. At present the reason for this lack of chloride transport activity is not known. Although HR_{565} is photochemically active its photochemical activity might not lead to ion translocation. The HR_{578} dependent activity, on the other hand, might become negligible because the HR_{565} to HR_{578} transition is slowed down and the equilibrium concentration of HR_{578} becomes too small.

Model for chloride transport

The main features of the photocycle described in Fig. 3 are three states of the retinal chromophore which can be used to devise a model for chloride transport in halorhodopsin as shown in Fig. 4.

In HR_{578}, which is the most stable state in the presence of chloride, retinal has an all-*trans* configuration and the intrinsic anion B^- serves as counterion. Although chloride binding sites are known in HR (see below) we cannot attribute any geometry to their arrangement. It is assumed that a salt-bridged system, serving a similar function as a hydrogen bridge system for proton conduction in bacteriorhodopsin connects the Schiff base to the inner and outer surfaces of the molecule. No indications of the relative position of the Schiff base with respect to the surfaces have been obtained so far and, therefore, the number of salt bridges on either side is arbitrary. From the band III protein of erythrocytes (anion transporter) it is known that anion binding sites are preferably made by arginine (Brock et al. 1983). Indeed, one of the segments of HR has the sequence Gly-*Arg*-Thr-Ile-*Arg*-Pro-Gly-*Arg*-Pro-*Arg*-Leu-Ile- (Hegemann 1984) which, if folded to a helical structure, might well serve the purpose of Cl^- conduction schematically shown in Fig. 4. Recent experiments showed that 9 out of 12 arginine residues can be modified by phenyl glyoxal in HR without affecting the chromophore spectrum (Ariki et al. 1986). Whether this modification also inactivates ion translocation is not known.

Light is assumed to induce isomerization around the 13–14 and 14–15 bonds leading to a state where the protonated nitrogen can form a salt bridge with a chloride ion of the transport chain. The group B^- which is left behind might pair with a positively charged group of the other half chain. As a result of such a pairing, a chloride ion leaves HR on the cyto-

plasmic side (inside) and a chloride binding site becomes available at the extracellular side (outside) in HR'_{520}. The transition from HR'_{520} to HR_{520} rests on the binding of a chloride ion to the protonated nitrogen enabling the 14–15 bond rotation. After this rotation the chloride ion is transferred to the half chain of salt bridges connected to the cytoplasmic side. This transfer catalyzes the transition from HR_{520} to HR_{640} which involves the reisomerization of retinal around the 13–14 bond. The subsequent reformation of the salt bridge between B^- and the protonated nitrogen is connected to the formation of HR_{565} and the rearrangement of the chloride ions with respect to the positive counterparts regenerates HR_{578}.

Conclusions

The model presented here for the photocycle of HR does not intend to describe in detail the absorption maxima of the intermediates or to localize the anion binding sites. This must wait until more knowledge on the molecular structure of HR is available. However, the model unifies and extends the view that retinal bound to the lysine residue of a protein as a protonated Schiff base serves as a molecular switch for protons and anions. This behaviour originates from its specific photochemistry and its predictable behaviour with respect to thermal bond rotations. The ion specifity of the switch, i.e. for protons or anions, is conferred by the protein sequences. It should be stressed that the retinal switch does not have to participate directly in transfer as suggested in Fig. 4, but could by its movement, trigger a translocation event somewhere else in the protein. In any case, our model predicts that the thermoreversibility of the photochemical reaction in BR is mediated by a reversible deprotonation of the Schiff base and in HR by a reversible chloride binding to the Schiff base.

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