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Bacteriorhodopsin: a paradigm for proton pumps?

Janos K. Lanyi *

Department of Physiology and Biophysics, University of California, Irvine, CA 92717, USA

Abstract

Recent studies of the photochemistry of wild type and mutant bacteriorhodopsins, their proton release and uptake kinetics, and their X-ray diffraction structure have suggested a hypothesis for the way energy is coupled in this light-driven proton pump. The first and critical step in converting light energy to a vectorial proton potential is the transfer of the Schiff base proton to D85 which causes dissociation of the Schiff base–counterion complex. Removal of this primarily coulombic interaction destabilizes the protein structure, and results in transition to an alternative conformation in which the two proton conduction pathways between the active site and the membrane surfaces are reorganized. Recovery of the initial charge state of the Schiff base and D85 must therefore occur through a series of unidirectional proton transfers that create a transmembrane electrochemical proton gradient. Passage of the transported proton through the two peripheral protein domains appears to utilize hydrogen bonded networks containing aspartate, arginine and bound water. This kind of mutual interaction between the active site and the protein conformation that determines the conductive pathways to the two membrane surfaces may have relevance to ion pumps in general.

Keywords: Bacteriorhodopsin; Transport mechanism; X-ray diffraction; Schiff base proton; Protein conformation

1. Introduction

Bacteriorhodopsin is an energy-transducing retinal–protein. Absorption of a photon causes isomerization of the retinal from all-*trans* to 13-*cis* (energy input to the active site), that causes pK_a's to be changed and proton conductive pathways to be rearranged (transfer of free energy from the active site to the rest of the protein), and the resulting proton displacements cause release and uptake of a proton at the two membrane surfaces (conservation of free energy in a transmembrane electrochemical gradient). Recent work motivated by this view of bacteriorhodopsin has produced a fairly detailed conceptual and mechanistic hypothesis for the proton transport. This short account will convey this hypothesis, and attempt to point out where the areas of ignorance lie. There was no reason to discuss the large literature of the spectroscopic measurements of the sequence of K, L, M, N and O intermediates of the photocycle, and their kinetics, except where needed. Instead, the emphasis is on the proton transfers between Schiff base and D85, and through the extracellular and cytoplasmic proton channels to and from the two membrane surfaces, and on what appears to be a correlation of the protein conformation with the charge state of the Schiff base, and with the connectivity of the Schiff base to the two channels.

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* Corresponding author.

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2. Proton transfer from Schiff base to D85

The positively charged retinal Schiff base and a hydrogen-bonded counter-ion domain that contains a variety of groups: the anionic D85 and D212, the positively charged R82, and bound water [1–4], constitute a stable complex with zero net charge. There is a mobile proton distributed in this unphotolyzed chromophore that is associated mostly with the Schiff base. Although it would seem that a buried neutral Schiff base–D85 pair would have lower energy than the ion pair, there is a considerable free energy gap that prevents transfer of the proton to D85. It was calculated to be about 30 kJ/mol [5]. The kinetics of the L ⇔ M equilibrium indicate that after photoexcitation the free energy gap is narrowed to 3–4 kJ/mol [6–8]. Since the K photointermediate has an enthalpy gain of about 50 kJ/mol over the unphotolyzed state [9], a large part of its acquired excess energy must be utilized to change the relative proton affinities in the complex. How is this accomplished by the photoisomerization of the retinal?

Two explanations have been given, that are not mutually exclusive. First, rotation around the C_{13}–C_{14} double bond was suggested to introduce a twist into the retinal skeleton [10–12], causing disruption of the π-system and withdrawal of electron density from the Schiff base nitrogen that results in decreased proton affinity. Indeed, the FT-IR spectrum of the L (but not the N) photointermediate indicated steric conflict between C_{12} and C_{15}, as expected from the twist [13,14]. Second, the isomerization changes the shape of the retinal chain from straight to bent, and this can be expected to displace the Schiff base relative to the other groups in the complex. The change of geometry would, in a way not yet understood, destabilize the Schiff base proton.

The second possibility was examined by introducing small perturbations into the vicinity of the Schiff base and following their consequences on the proton transfer process in the photocycle [15]. The changes were in the volume and/or shape of V49 and A53, residues uniquely suited for this purpose because their side-chains buttress the hydrocarbon chain of K216, the residue that contributes the nitrogen to the Schiff base. By site-specific mutagenesis, V49A, V49L, V49M, A53G, and A53V were produced in a homologous (*Halobacterium salinarium*) expression system [16,17]. None of these mutants had greatly shifted absorption maxima that would have suggested strong perturbation of the Schiff base region. Visual examination of the structural model of the protein based on electron diffraction [1] yielded a set of expected changes for the protonation (i.e. [L]/[M]) equilibrium in the photocycle. Simply put, the equilibrium was thought to shift either toward greater or lesser proton transfer depending on whether the Schiff base was moved either into closer alignment with D85 or farther away.

The photocycle of V49A, where lesser deprotonation was expected was examined in the greatest detail. Indeed, the amplitude of the M state, as measured by the transient absorbance change at 410 nm, was about 1/5 of the wild type. While this did suggest that the [L]/[M] equilibrium had shifted in favor of the L state, the conclusion assumed the validity of the kinetic scheme involving L and M that we had proposed before [7]. The scheme linked L and two M substates by the sequence, $L \Leftrightarrow M_1 \rightarrow M_2$, where at $pH < 6$ an $M_2 \rightarrow M_1$ back-reaction would begin to appear. The pH dependence of the M kinetics of V49A was consistent with the conclusion that the amplitude of M was lowered because of a changed L to M equilibrium. This would lower the concentration of M_1, and make the $M_1 \rightarrow M_2$ reaction so slow that it approached the decay rate of M_2. The phenotypes of other mutants confirmed this. Thus, introducing D96N as a second mutation, that would slowly decay M_2 [18–21], strongly increased the amount of M that accumulated. By the same token, introducing D115N as a second mutation that would increase the rate of the $M_2 \rightarrow M_1$ back-reaction [7] had the opposite effect. In fact, at pH 4 little or no M accumulated in V49A/D115N, yet proton transport was undiminished, suggesting that the photocycle passes through M even though it, for kinetic reasons, could not be observed.

These observations gave some support for the idea that what was measured in the photocycle of V49A was indeed a shifted protonation equilibrium. The extent of this shift was to widen the gap in the pK_a's of the Schiff base and D85 by 0.7 units. Another way to confirm this conclusion was with a retinal analogue. Substitution of the retinal with 13-trifluoromethyl retinal has been shown to lower the Schiff base pK_a independent of the counter-ion [22], and as expected it restored the amplitude of M nearly to what is observed in the wild type [15].

In a similar way, the equilibration of proton in the photocycles of V49M and A53V were found to be shifted toward less deprotonation of the Schiff base, while no difference was seen in V49L, and a shift to greater deprotonation of the Schiff base occurred in A53G [15]. Remarkably, all of these shifts were consistent with the predictions from the structure. The results strongly suggested therefore that a cause of the proton transfer in the photoexcited protein is a small but important change in the geometry of the groups around the Schiff base. FT-IR spectra of O–H stretching in the L state [23,24] and studies of model compounds [25] have indicated that bound water, and probably its changed hydrogen bonding, must play an important role in this rearrangement.

3. Reprotonation switch: the connectivity of the Schiff base

After protonation of D85, that is located to the extracellular side of the Schiff base, the unprotonated Schiff base regains the proton from D96, that is located near the cytoplasmic surface. This change in connectivity [26], an essential component of the transport cycle of any ion pump, is referred to as the 'reprotonation switch.' It is the crucial reaction in the photocycle, the step where the conditions are set for energy conversion. The proton affinity change at the active site results in the unidirectional movement of the proton only because the proton conduction pathways leading to the two membrane surfaces are suitably reorganized. How does the active site interact with the proton conduction channels in the protein to accomplish this?

Circumstantial evidence has suggested that the reprotonation switch is what is detected kinetically and spectroscopically as the $M_1 \rightarrow M_2$ reaction [6,27–29], and that it is accompanied by the protein conformation change detected by diffraction in the M state [30–33]. It is not yet clear if this conformation change is the same as the changes in the amide bands detected by FT-IR [34–38]. The structural change is complex, and might not occur as a single step, but the diffraction suggests that it extends over the whole protein. Its most obvious features consist of tilt of the cytoplasmic end of one of the transmembrane helices away from the proton channel, and reorganization of the residues in the helix to which the retinal is linked [33]. A large decrease in entropy, that occurs in the photocycle uniquely at the $M_1 \rightarrow M_2$ reaction [6], might originate from these structural changes.

The relationship between the two alternative proton conduction pathways and the two alternative global protein conformations, as well as the causes of the conformational change, were explored in mutants containing the D85N residue replacement [39]. These mutants were well suited for such studies because in the absence of the negative charge of D85 the pK_a of the Schiff base is lowered from about 13 [40] to 8–9 [5,41–43], and thus the details of the protonation exchange reactions of the Schiff base could be determined in both photolyzed and unphotolyzed samples.

Whether proton exchange was with the extracellular or the cytoplasmic surface was decided on the basis of the consequences of replacing D96, a residue located at about 6 Å from the cytoplasmic surface [1], on the rate of protonation or deprotonation of the Schiff base. It is well known from studies of single mutants that the passage of a proton from the cytoplasmic surface to the Schiff base depends very strongly on D96: in the photocycle of D96N the vectoriality of the proton transfer is preserved but its rate is greatly slowed [18–21]. Even more useful in this regard was the related observation that azide accelerates the shuttling of protons to the Schiff base through the cytoplasmic channel very effectively in D96N, but poorly in D96A (or D96G, cf. [44]), and hardly if at all in the wild type. On the other hand, the rate of proton transfer through the cytoplasmic channel, identified in this way, is not influenced by the D85N mutation [39]. Utilization of the cytoplasmic proton channel predicted therefore distinct phenotypes for D85N mutants containing D96N and D96A replacements as second mutations. On the other hand, passage of a proton through the extracellular channel is not influenced by replacement of D96 or by azide, but will be affected by replacement of D85. Comparing the expected effects of D85 replacement with the wild type was possible after replacing the retinal of the wild type protein with 13-trifluoromethyl retinal, which lowers the pK_a of the Schiff base to about the same value as in D85N containing authentic retinal [22]. In these comparisons the rate of proton exchange of the Schiff base was found to be nearly two orders of magnitude faster when residue 85 was an aspartate as compared with asparagine. Utilization of the extracellular proton channel was identified by this phenotype.

The rate of the protonation or deprotonation of the Schiff base in unphotolyzed D85N mutants was followed after pH jump (e.g. from 7 to 8, or from 9 to 8) with stopped flow spectroscopy [39]. These rates were unexpectedly slow and in the tens of seconds range. Access of the Schiff base proton in these proteins was uniformly to the cytoplasmic side only. The same conclusion could be drawn for the protonation events in the photocycles of the D85N chromophore with either initially protonated Schiff base (measured at low pH) or unprotonated Schiff base (measured at high pH). In the first case, the Schiff base transiently lost its proton to the cytoplasmic side, either to the (at this time) deprotonated D96 as acceptor or directly to the cytoplasmic surface. Upon recovery of the initial state this proton was regained from the cytoplasmic side. In the second, case, the Schiff base transiently gained a proton, either from D96 or directly from the cytoplasmic surface. Upon recovery of the initial state the proton was lost again to the cytoplasmic side. Unlike in the wild type, therefore, when residue 85 was an asparagine the extracellular proton channel was not utilized, in either unphotolyzed or photolyzed protein.

The observed blockade of the extracellular conduction pathway in these mutants became particularly meaningful when the structures of the protein at high pH and low pH were compared. X-ray diffraction, using a synchrotron source, was performed on bacteriorhodopsin films before and after pH jump (from pH 7 to about 11, using the same films for both measurements) produced by exposure to NH_3 vapor [39]. Numerous reflections in the Bragg diffraction of D85N showed intensity changes at the higher pH, and these were highly correlated with the changes in the reflections observed when D96N (or wild type) samples were converted to the M state by illumination. In D85N/D96N the diffraction indicated that the structural transition had occurred at the lower pH already. Since in the arrangement of proton conduction pathways and the global protein conformation (but perhaps not in the isomeric configuration of the retinal), D85N with deprotonated Schiff base is thus equivalent to the M photointermediate of the wild type photocycle, it was concluded that (1) the alternative protein conformation of the M state is achieved simply upon deprotonation of the Schiff base, and (2) the global protein conformation determines the access of the Schiff base proton.

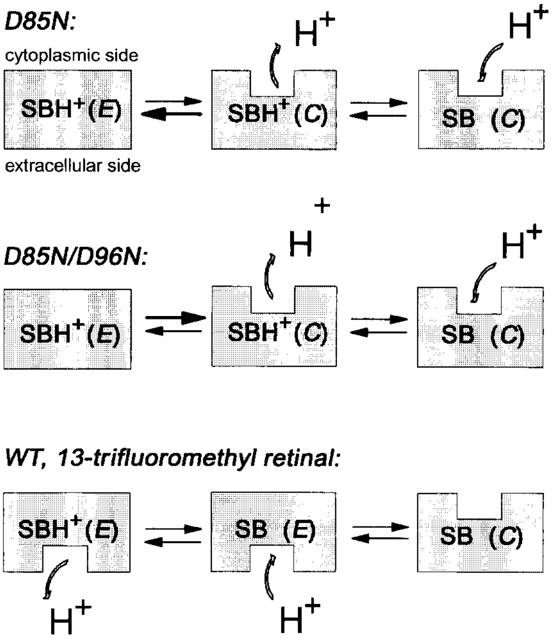

Fig. 1. Schematic representation of how the global protein conformation and the proton transfer pathways of the Schiff base appear to interact in bacteriorhodopsins containing either asparagine (D85N and D85N/D96N) or aspartate (WT) as residue 85. As explained in the text, the global conformation (from measurements of X-ray diffraction) and access of the Schiff base proton (from measurements of absorbance changes in the photocycle) in D85N mutants depend on pH [39]. Conformations that allow cytoplasmic or extracellular access are indicated by upward and downward openings, respectively. SB^+ and SB refer to the protonated and unprotonated forms of the Schiff base, respectively. *E* and *C* refer to the two alternative protein conformations (cf. text). The conformations drawn for the wild type protein containing 13-trifluoromethyl retinal are speculative since they are not based on diffraction.

Fig. 1 shows a schematic model for interpreting the results in this way. Raising the pH to deprotonate the Schiff base causes a structural equilibrium in D85N to shift from largely conformation *E* to conformation *C*, while in D85N/D96N the protein is already in conformation *C*. This must be because conformation *E* of the single mutant is somewhat stabilized by interaction of the protonated Schiff base with its weakened counterion that lacks the negative charge of D85 but still contains D212. In the double mutant some additional stabilization by D96 is removed. That the equilibria in D85N and D85N/D96N do not lie very far apart is shown by the fact that the apparent pK_a of the Schiff base is only 0.6 units lower in the double mutant. Although in conformation *E* access of the Schiff base is to the extracellular side, in these mutants proton transfer through this pathway is blocked by the D85N replacement. The wild type protein (shown in Fig. 1 with 13-trifluoromethyl retinal to allow direct comparison with the D85N mutants) is also in conformation *E* until deprotonation of the Schiff base allows it to pass into conformation *C*, but D85 assures that proton release to the extracellular surface is possible. Thus, it was concluded that while proton release from the Schiff base in the wild type is rapid and utilizes the extracellular channel, in the D85N mutants it is slow and utilizes the cytoplasmic channel. As discussed below, this scheme has strong implications for the nature of the reprotonation switch.

4. Release of a proton at the extracellular surface

At the time of the proton transfer from the Schiff base to D85 in the photocycle (the $L \rightarrow M_1$ reaction) the connectivity of the Schiff base is to the extracellular side. Protonation of D85 results in disruption of the arrangement of the active site [45,46], and release of a proton to the extracellular surface. This proton is not the one acquired by D85 because this residue remains protonated until well after the release [13,34,36,47,48]. Analysis of the pH dependency of the first half of the photocycle in terms of the scheme $L \Leftrightarrow M_1 \Leftrightarrow M_2$ indicated that the proton uptake reaction (that becomes more rapid with decreasing pH) is the $M_2 \rightarrow M_1$ back-reaction, rather than the $M_1 \rightarrow L$ back-reaction [7]. This suggested that the proton release is associated not directly with the deprotonation of the Schiff base but with the subsequent event that is the proposed reprotonation switch. Independence of the proton release at the surface from the internal proton transfer was further demonstrated by the findings that in R82Q or R82A [49–52] and D85E [53] the proton release was considerably delayed relative to the deprotonation of the Schiff base. Even in the wild type, at $pH < 6$ the proton release is delayed (much like in R82 mutants) to beyond the proton uptake on the cytoplasmic surface so that the net proton loss during the photocycle at higher pH is replaced by net proton gain [7]. Although these observations demonstrate that protonation of D85 is by itself not sufficient to trigger the proton release, they leave the nature of the trigger unexplained.

The kinetics of the chromophore reactions and the rate and extent of proton release at various pH both indicated that the pK_a of the release group, termed XH, is about 6 [7]. Presumably, the pK_a of this group in the unphotolyzed state is much higher so it can serve as the source of protons, and its proton affinity decreases so as to release the proton in a timely manner during the wild type photocycle. On the other hand, it is not required for the transport that the proton release occur at its usual time in the first half of the cycle, since transport is undiminished in all examples listed above where the release was delayed.

The identity of the release group XH has been the subject of much speculation. R82 is an attractive candidate because it strongly interacts with D85 [5,51,54,55] and its pK_a must be lowered when this aspartate is protonated [51]. On the other hand, arginine residues do not generally have a pK_a as low as 6, and calculations based on the titration of mutant proteins suggested that from coulombic interactions alone the pK_a of R82 will not decrease below 11 [5]. Y57 is another candidate, with a more suitable pK_a [56], but there is good evidence against deprotonation of a tyrosine in the photocycle [57–59]. Water liganded to R82 [45] would be a reasonable alternative. The presence of water in this protein domain has been demonstrated, and FT-IR spectra indicate perturbation of the hydrogen bonding of water near D85 in the L state [23,24,60–62].

5. Uptake of a proton at the cytoplasmic surface

At the time of the reprotonation of the Schiff base by D96 in the photocycle (the $M_2 \rightarrow N$ reaction) the proton connectivity is to the cytoplasmic side. Since the distance of D96 from the Schiff base is about 12 Å, it seems likely that this proton transfer and the subsequent reprotonation of D96 are made possible by the global protein conformational changes that have occurred earlier in the photocycle. Indeed, it has been demonstrated that deprotonation of D96 in the photocycle is not dependent on whether the Schiff base is protonated or not [38]. In D212N, where conditions can be created to prevent deprotonation of the Schiff base, D96 loses a proton about the same time as in the wild type photocycle but the proton is released to the cytoplasmic surface instead. In the photocycle of D85N this has the consequence that D96 becomes the proton acceptor to the Schiff base, i.e. here the proton movement is retrograde and cannot result in net transport because the subsequent reprotonation of the Schiff base is also from the cytoplasmic direction.

The passage of proton through the cytoplasmic domain appears to be as complex as its passage through the extracellular domain. The dipole environment of water bound in this region is a major factor in the deprotonation of D96, as shown by the fact that the reprotonation of the Schiff base in the wild type, but not in D96N, is strongly diminished by osmotic agents that withdraw internal bound water from the protein [21]. Transient increase of the hydration of this region might be the consequence of the protein conformation change detected by diffraction. However, the deprotonation and the subsequent reprotonation of D96 are influenced also by the neighboring T46 and R227 residues. Replacing T46 with valine causes more rapid deprotonation of D96 but slower reprotonation from the cytoplasmic surface, and replacing R227 with glutamine has the opposite effects [52,63]. In the double T46V/R227Q mutant the two effects compensate one another.

It is usually assumed that what is detected as the N → O chromophore reaction represents not only the reisomerization of the retinal from 13-*cis* to all-*trans*, but also proton uptake and thus the reprotonation of D96. It had become clear, however, that under some conditions the uptake of proton from the cytoplasmic surface does not coincide with decay of the N state [50,52,64]. Moreover, time-course of the proton uptake in R82Q indicated that the proton uptake did not coincide with reprotonation of D96 either. Thus, as proton release in the extracellular region, proton uptake in the cytoplasmic region is not directly coupled to a chromophore reaction. It seems furthermore that the direct proton acceptor for the uptake is not D96, but an unknown group termed Y [52]. Like the proton release group XH on the extracellular side, all protonable residues could be eliminated as possibilities leaving liganded water as the most likely candidate for Y. The experimental findings with mutants [52], as well as structure prediction by molecular dynamics [65] both suggested that there is a water molecule coordinated by both T46 and D96 that could play this role.

6. Discussion

In an active ion pump the central question to be asked concerns the interaction of the energy-yielding events at the active site with the conductive pathways that lead to the two membrane surfaces. 'Energy coupling' consists of transferring excess free energy from the active site to the rest of the protein so as to reorganize the conductive pathways. Thus, recovery of the initial state is by a different pathway from the first part of the reaction cycle. If this will occur in such a way that the system can return to the initial state only by net loss and gain of the transported species from the two membrane sides, the scalar energy input will be converted into the vectorial transmembrane potential energy of the transported ion.

Bacteriorhodopsin is a small protein in which the active transport of a proton is based entirely on transient changes in pK_a's and in proton transfer pathways. The input of energy at the active site is well defined since its immediate result is the all-*trans* to 13-*cis* isomerization of the retinal. For these reasons, and because the structure of the protein is known to a good approximation [1], it is hardly surprising that the above described principles for ion pumps are easier to discern in this system than in other, more complex systems. As shown by the foregoing, recent progress with this protein, particularly through the use of site-specific mutations, has produced the outlines of a hypothesis for how the absorption of light drives the active transport of protons. There is little in this hypothesis that has not been discussed before in speculations about how pumps must function. What is new is the fact that the hypothesis is based on a set of experimental observations in which driving force, vectoriality, protein structure, and proton conduction mechanism are related to one another in a self-consistent way. On the other hand, many of the mechanistic details that would account for each proton transfer event at a molecular level are still missing, and as in other proton pumps some of this information is lacking entirely at this time.

Central to the hypothesis is that the critical step for energy coupling is the dissociation of the Schiff base–counterion complex that occurs in the transport cycle once the Schiff base is deprotonated and thus loses its positive charge. Although the all-*trans* to 13-*cis* isomerization of the retinal might have other consequences (originating e.g. from the changed shape of the retinal skeleton relative to the shape of the binding pocket), its primary effect is to shift the relative proton affinities of the donor Schiff base and the acceptor D85 so as to accomplish the proton transfer and thus the dissociation. Because of this dissociation the protein structure of the unphotolyzed state becomes unstable. Transition to an alternative conformation results, and this reorganizes the proton conduction pathways so as to ensure that return the system to its initial state is through proton release on the extracellular side and reprotonation of the Schiff base from the cytoplasmic side, i.e. through translocation of a proton.

One of the suggested consequences of the reprotonation switch is that once the alternative protein conformation is assumed, the proton conduction pathways leading to the two membrane surfaces will become independent from one another. It is a particularly interesting finding therefore that in various single and double mutants the wild type sequence of proton uptake and release in the photocycle can be reversed and reestablished entirely as predicted from their individual rate constants [52], i.e. the release and the uptake do in fact appear to be independent of one another.

Although neither the details of the two protein conformations nor the way they would influence proton conductivity are as yet known, the hypothesis defines the flow of energy through the system. The protein conformation (conformation E) in the unphotolyzed state is stabilized by the Schiff base–counterion interaction. Absorption of a photon causes proton transfer in this complex and abolishes this stabilization. In this sense the high-energy state that results after photoexcitation originates from the protein. By spontaneous relaxation to the alternative proton conformation (conformation C) the system reaches a state of intermediate energy, which returns to the initial low-energy state through proton release and uptake from the two membrane surfaces. As illustrated in Fig. 2, the coupling of energy in this pump is accomplished by the fact that the proton transfer pathways are different in the two proton conformations. Thus, the relationship of the active site to the protein conformation is mutual: interaction at the active site controls the global conformation while the conformation controls the two pathways of access to the active site. It remains to be seen whether this hypothesis applies to other pumps.

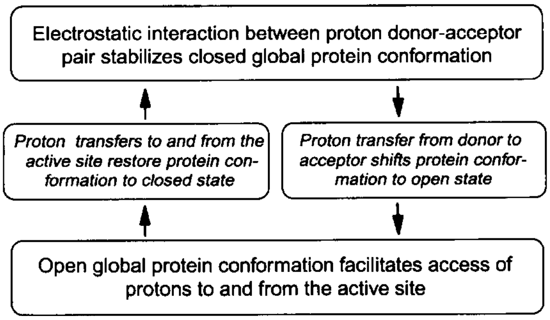

Fig. 2. Conceptual representation of the hypothesis suggested for the proton transport mechanism in bacteriorhodopsin. Open and closed protein conformations denote those determined for the M photointermediate and for the unphotolyzed system, respectively.

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