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**RECOLLECTIONS**

# Bacterial rhodopsins: Evolution of a mechanistic model for the ion pumps

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Bacteriorhodopsin (bR) and halorhodopsin (hR) found in the cell membrane of halobacteria are the simplest known light energy-converting systems. They are small proteins and require only light and a lipid bilayer to generate an electrochemical ion gradient. In overall structure, chromophore, and photoreactions, they closely resemble the visual pigments of animals, but those are signal, not energy, transducers and no obvious sequence homologies originally indicated an evolutionary connection. Signal-transducing archaebacterial rhodopsins have also been detected, but they are closely related to the energy transducers. These pigments show the same seven-helix structural motif common to energy-transducing bacterial rhodopsins, visual pigments, and other G-protein-linked sensors. Nevertheless, their sensory function is not mediated by either ion transport or G-proteins, but resembles the chemosensory transduction system of eubacteria. This review, however, will trace only the development of a mechanistic model for the active transport function of bR up to 1995.

Before proceeding, I should clarify the meaning of two terms frequently used in this paper. The word "model" frequently used with modifiers, such as "structural," "photocycle," "kinetic," and "energetic," signifies an interpretation of the experimental data usually involving some simplification and conjecture, to yield a coherent picture of a structure or process. It should allow one to relate it to models derived from sets of data acquired with other techniques. Often different models will fit the experimental data from one technique about equally well. Compatibility with models obtained by different techniques will then determine the choice. The mechanistic model of the title should emerge as the synthesis of all the lower level models. However, a good educated guess of a high level model at the beginning may prove very helpful in choosing techniques and models to try.

Another frequently occurring term is "photocycle"; it was originally—and still is—used as an abbreviated form of "photoreaction cycle" for the sequence of visible- and UV-absorbance changes, which bR undergoes after light absorption. It should, however, be clear that other photocycles exist, e.g., an electric charge shift cycle, a structural cycle, a thermal cycle, etc. . . . with related, but not necessarily identical kinetics. Selecting a model that fits all the different photocycles is a prime example of the process leading to the comprehensive final transport model.

Finally, in this paper I have avoided quoting individual investigators or research groups, because I believe that, ideally, all scientific work should be published anonymously, so that it can be judged unemotionally and without prejudice. Practically, that is clearly impossible, but here it is attempted to help readers focus on the development of a research topic. I am appending a list of selected review articles, which provide access to the primary literature.

## *1970–1978: The basic data*

Bacteriorhodopsin from the archaeon *Halobacterium salinarum* (formerly *salinarium*)[1] was discovered in 1966. After it had been

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**Walther Stoeckenius** received a MD degree at the University of Hamburg, Germany in 1950. After 18 months of clinical work as an intern, he began postdoctoral work on the development of pox viruses at the Institute for Tropical Medicine in Hamburg using mainly electron microscopy techniques. After two years he moved as Assistant Professor to the Department of Pathology at the University of Hamburg and became Docent for Pathology in 1958. In addition to teaching and routine pathology work, he continued to use electron microscopy to explore the fine structure of cells and developed an interpretation of the triple-layered appearance of membranes in electron micrographs in terms of molecular structure and the chemistry of osmium tetroxide fixation.

In 1959 he obtained a position as Research Associate in Keith Porter's laboratory at Rockefeller University. This was changed after a few months to Assistant Professor and he stayed there, later as Associate Professor, for eight years. The work on membrane structure continued, and a model was developed that described the membrane as a lipid bilayer with embedded protein domains. In efforts to isolate such domains, the purple membrane and bacteriorhodopsin were discovered.

In 1966, the lure of California became irresistible and Dr. Stoeckenius accepted a professorship at the University of California at San Francisco. The work on bacteriorhodopsin continued there with the emphasis changing from electron microscopy to spectroscopy and biochemical techniques. He is now Professor Emeritus there in the Department of Biochemistry and Biophysics and the Cardiovascular Research Institute.

[1] The halobacterium strain first used in the isolation and characterization of bacteriorhodopsin and in most of the subsequent work was obtained from a culture collection as *Halobacterium halobium*. It was indistinguishable from other strains obtained, which were named *H. salinarium* or *H. cutirubrum*. The nomenclature was recently reexamined and *H. salinarum* designated as the official name for the type species in the family *Halobacteriacea*. *H. halobium* and *H. cutirubrum* are considered strains incertae sedis in the same family. The originally used salinarium was changed to salinarum, the correct genitive of the Latin word for salt works, salinae.

recognized as a rhodopsin-like protein in 1970, further characterization progressed rapidly. In 1972 its function as an electrogenic, light-driven proton pump, or at least its role as the light energy receptor for intact bacteria had been established and around 1977 most of the basic data on its structure and function had been obtained. It was recognized as an intrinsic membrane protein of approximately 25 kD, which forms crystalline patches in the cell membrane, the "purple membrane" (pm). Its chromophore had been characterized as a protonated Schiff's base (SB) of all-trans-retinal[2] with the amino group of a lysine residue. A low resolution structure of pm obtained in 1975 by electron crystallography showed a cluster of seven alpha-helical segments that cross the 50 Å thick membrane at approximately right angles. The retinal was not resolved but was obviously located in the hydrophobic interior. The apoprotein causes a large redshift of the retinal absorbance, acts as retinal isomerase, and increases the apparent pK of the chromophore-SB from ~7.0 to >13.[3] Kinetic spectroscopy of the photoreaction in the visible and near UV range had identified a sequence of five thermal intermediates in the photocycle, with life times increasing from less than a few pico- to a few milliseconds (the subscripts indicate absorption maxima):

$$bR_{570} \rightarrow K_{590} \rightarrow L_{550} \rightarrow M_{410} \rightarrow N_{(520)} \rightarrow O_{640} \rightarrow bR_{570}.$$[4]

Only the first step of the cycle requires light. The energy for translocating protons against an electrochemical gradient is transiently stored in the protein. Light absorption by intermediates K, L, and M rapidly short-circuits the cycle. Protons are released from the outer membrane surface in the L → M transition and appear in the outside medium after less than 1 ms; they are taken up from the inner medium during N decay a few milliseconds later. A stoichiometry of 1 proton/cycle appeared most likely but higher ratios were also proposed and the problem of "Bohr protons" was debated. The controversy slowly died without being clearly resolved.

Already in 1976, before all of these results were in hand, a heuristic model for the pump mechanism was presented. That bR itself was the light-driven proton pump had been strongly suggested by action spectra and the transient release of protons from pm during the photoreaction cycle. It was validated by reconstituting bR into lipid vesicles and demonstrating photophosphorylation. The mechanistic model assumed that the SB was located in the interior of the protein and would transiently switch its connection from one membrane surface to the other. The model restricted vectorial proton transfer to a relatively small conformational change, because the crystalline protein lattice in pm made any large movements or large conformational changes unlikely. This was confirmed by failure to detect rotational diffusion of bR in pm, large linear dichroism changes, or large changes in the X-ray diffraction pattern in the M intermediate. Permanent, large liquid water-filled channels connecting the SB to the inner and outer membrane surfaces were ruled out, because they were incompatible with the observed inaccessibility of the SB to small water-soluble solutes like $NaBH_4$ or hydroxylamine and because the physiological membrane potentials would have caused dielectric breakdown of the remaining protein barrier. The well-established mechanism for proton conduction in ice and hydrogen-bonded organic crystals suggested chains of hydrogen-bonded groups on adjacent helix surfaces and/or bound water molecules as the alternative. Time-resolved RR spectroscopy showed rapid exchange of the SB proton and demonstrated that at least one such connection exists even in the dark but it proved impossible to determine from which membrane surface the exchange occurred.

The driving force for protons was likely to appear as pK changes of the SB and amino acid residues in the protein, and transport should have been detectable as transient protonation changes of the groups involved. The most likely cause of the pK changes were presumably conformation changes of the chromophore and/or the SB. Since energy input occurred at the retinal chromophore and the 410 nm absorbance maximum of the M-intermediate indicated that the SB transiently deprotonated during the cycle, transport originated there. These considerations prompted the first time-resolved RR experiments, which confirmed SB deprotonation in the M-intermediate. Figure 1 shows the resulting model. This basic model would survive with some modifications, and its crucial postulates would be extensively tested. They were: (1) SB location in the hydrophobic interior of the membrane, (2) transport origin at the SB with a conformational change, (3) later deprotonation and reprotonation of the SB to and from groups in the protein and/or bound water generating two sequential unprotonated SB intermediates, connected to opposite surfaces (later designated MI and MII), (4) requirement for only small conformational changes, and (5) stoichiometry of 1 proton transported per cycle.

The analogy to rhodopsin suggested that light might cause isomerization of the retinal, which was soon shown to be a transient

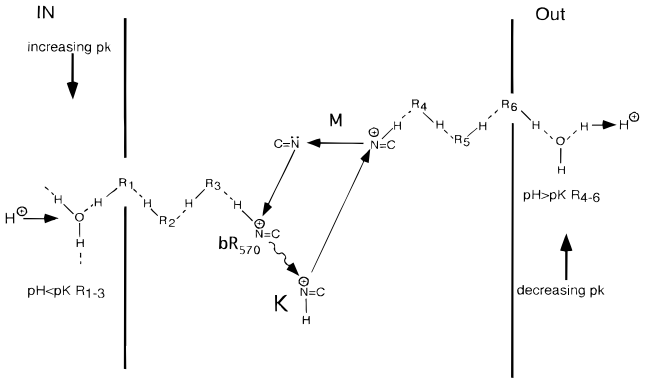


**Fig. 1.** The first proposed model for proton transport in bR (1976). The R-groups represent protein groups and/or bound water. Note that the SB connection in the ground state is to the intracellular side, and consequently the first switch in surface connection is placed between $bR_{570}$ and M. The retinal isomerizations during the cycle been considered at this time but not been established.

[2] We are considering here only the transport-active, "light-adapted" form of the pigment: $bR_{568}$. In the dark the retinal slowly isomerizes to $bR_{558}$, an equilibrium mixture containing about 65% 13-cis retinal with a syn C=N bond. This fraction is not transport-active, but in the light is rapidly converted back to $bR_{568}$. The physiological role of this process, if any, is not known.

[3] This pK value does not indicate denaturation of the protein, because bR with a retinal chromophore modified by replacing the C13-CH3 group by CF3, which lowers its SB pK 5 pH units in model compounds, has a SB pK lowered by the same amount.

[4] The designation K, L, M, . . . etc. was suggested because of the obvious similarity of the second and third intermediate to the photoproducts lumirhodopsin and metarhodopsin of visual pigments. If—as was likely —more intermediates would be discovered, the sequence could then be extended in either direction with letters in alphabetical order. This was suggested by Richard Cone, in whose laboratory the first time-resolved spectroscopic studies were carried out.

change from the all-trans to the 13-cis isomer. This observation and quantum-chemical considerations very soon required one significant modification and produced a refined model. Calculations initially limited to the retinal SB system showed that bond rotations can generate the necessary large pK changes and that the energy barrier for the 13-cis isomerization of an unprotonated SB is very high, but decreases with increasing protonation, whereas the low energy barrier for single bond isomerization correspondingly rises. This suggested that the initial all-trans to 13-cis isomerization occurs in the excited state and is accompanied by a C14–C15 single bond rotation. The single bond isomerization could then reverse in the early unprotonated MI intermediate, producing MII and the first change in connectivity, while the high activation energy for the required C13–14 double bond re-isomerization would be reduced in the reprotonated SB of N or O (Fig. 2). The double isomerization reduced the space and mass movement required to an extent more compatible with the $<5$ ps rise time of the K intermediate and the apparently tight binding site in the protein and shifted connectivity switches and larger conformational changes to the later and slower part of the cycle. Independently, time-resolved transient dichroism measurements and other observations had provided evidence for two different M-states. However, this feature would mostly be disregarded during the following 10 years.

### *1979–1989: Development of techniques and accumulation of data*

A role for proton conduction in biological energy transduction had first been considered in the 1930s and had attracted little attention. But now the kinetics and thermodynamics of proton conduction through hydrogen-bonded chains and networks formed by amino acid residues and water were more thoroughly explored theoretically and in model systems, frequently with explicit reference to bR. They revealed some interesting properties, such as the buffering capacity, pH-dependence, and electric potential distribution and the counterintuitive fact that in these systems high pK groups can donate protons to low pK groups. Several mechanisms for proton pumps based on transmembrane, hydrogen-bonded chains ("proton wires") were proposed, and their calculated parameters proved to be compatible with what was known about the thermodynamics of bR. These studies demonstrated the possibility that such systems could exist in proteins, but early attempts to locate the proton path through bR based on the low resolution structure and amino acid sequence data would fail. Instead this period was characterized by steady progress in exploring the chemistry and kinetics of bR photoreactions and significant improvements of techniques.

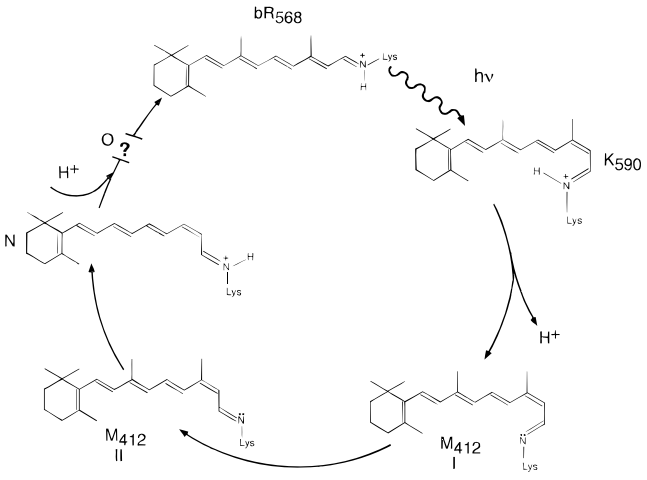


**Fig. 2.** The transport model in 1979. The first significant modifications of the 1976 model postulated connection of the SB in the ground state to the external surface and a simultaneous isomerization of the retinal in the excited state from all-trans to 13- and 14s-cis. The single bond reisomerization to trans was assumed to occur in M state switching connectivity from the exterior to the interior surface. Subsequent SB reprotonation in the N → M transition would then facilitate thermal double bond re-isomerization. Note the change in N-H bond orientation caused by the 14s-isomerizations.

The large redshift of the retinal chromophore absorption spectrum compared to retinal and retinal-SB spectra in solution, the "opsin shift," was explained as mainly due to the SB protonation, modulated by the counterion distance but also with little immediate effect on the bR work. In 1979 two laboratories directly sequenced the very hydrophobic protein. This important advance allowed the tentative connection of the spectroscopic data and the protein structure. Hydrophobicity plots of the amino acid sequence in combination with data on the accessibility to proteolytic enzymes and other reagents roughly identified seven hydrophobic segments in the sequence, which apparently constituted the transmembrane helices seen in the electron density map (Fig. 3). The retinal-binding site at Lys216 was located near the middle of the C-terminal helix G, and Asp212 was identified as the most likely counterion for the SB. While sufficiently high resolution data for an identification of individual helices in the map were still lacking, considerable ingenuity and effort were expended to trace the path of the polypeptide chain through the positions of the helices in the map. The rotational orientations of the helices were deduced by assuming that their hydrophilic side chains would preferentially face the interior of the helix cluster and their positions were chosen to maximize the number of salt bridges. A computer evaluation of all possible positions resulted in a preferred arrangement, which would later turn out to have been correct (Fig. 4). However, because of remaining doubts it did not contribute much to further development of the transport models until it was buttressed by additional data. Most important in this respect were later neutron diffraction results of pm reconstituted with selectively deuterated retinal, which located the SB and beta ionone ring in the map, and the study of mutant proteins, which identified interacting groups in helices C and G directly involved in the transport process (see below).

The photocycle model derived from time-resolved and low temperature, visible light absorption spectroscopy formed the basis for most efforts to understand proton transport, and a large effort went into critical examination of the assumptions used and the limitations of the analysis. New computer programs, which allowed global analyses, were developed and applied to extensive new data sets covering a wide range of temperature and pH values. Photoselection and light-scattering effects were explored and taken into account. Backreactions were explicitly introduced, branching pathways allowed, and the possibility that multiple forms of bR with different photocycles coexisted was investigated. Many cycle modifications were proposed, mostly based on results from a very limited number of experimental conditions, but no generally accepted modification of the cycle evolved, at least not for near-physiological conditions. Except for the inclusion of backreactions and reintroduction of the important N-intermediate, which had

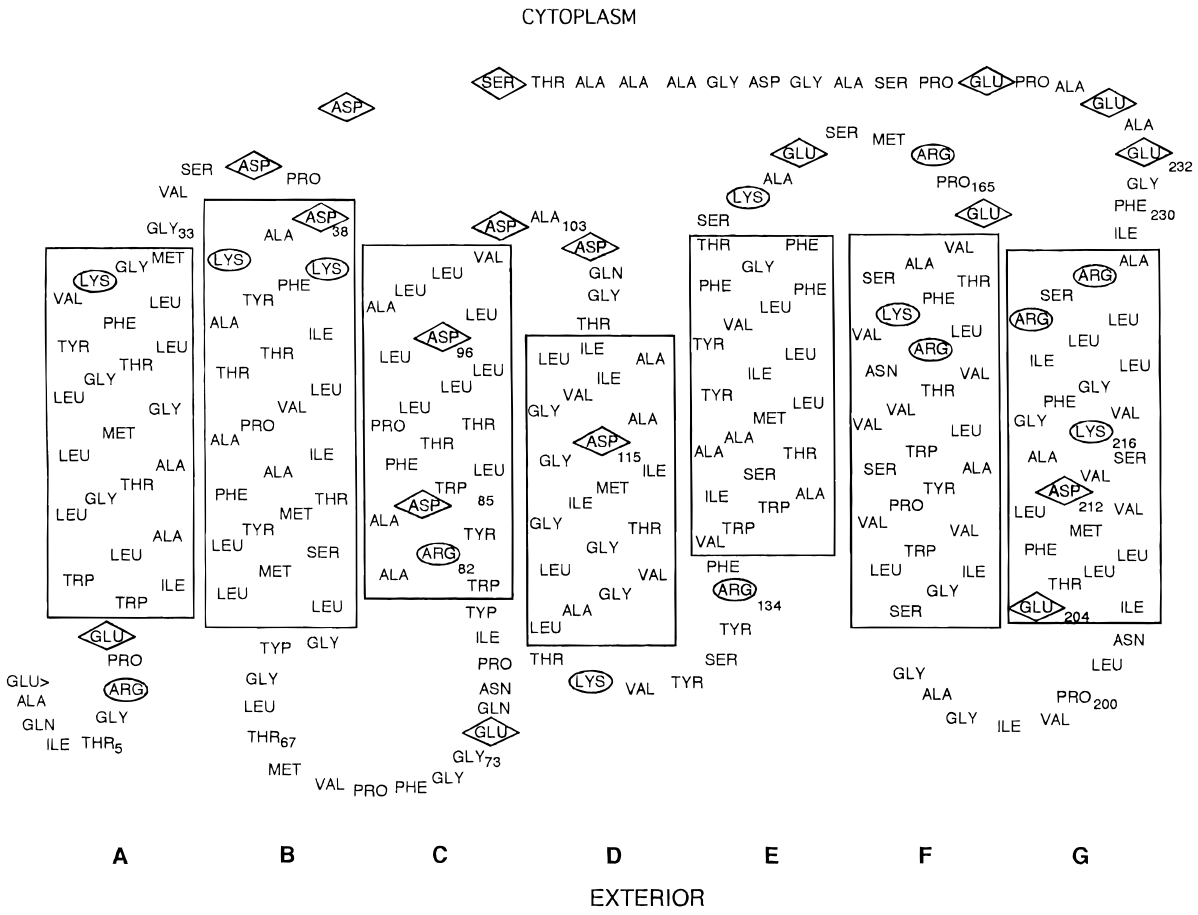


**Fig. 3.** The amino acid sequence of bR and the inferred helical segments proposed in 1979. The exact length of the segments and their vertical positions are taken from a higher resolution electron crystallography map published in 1990. Potentially charged residues are marked.

temporarily been ignored, the originally proposed cycle remained the most useful and generally used version, at least for near physiological conditions.

Complementary studies of the photocycle by time-resolved analysis of photocurrent or photovoltage kinetics, mostly obtained using pm adsorbed to black lipid films or lipid-impregnated support membranes, largely confirmed the optimistic expectation, that distinct steps in the charge translocation would correlate to the spectral transitions. A small, very fast displacement of positive charge toward the cytoplasm was detected in the bR $\rightarrow$ K transition; it is followed by a positive charge motion to the exterior in the L $\rightarrow$ M transition. Together these steps represented about 20% of the total charge transport. The remaining 80% occurred between M and bR. A technique, which orients pm suspensions in an electric field and immobilizes the oriented membranes in an aqueous gel, proved to be very useful because it allows simultaneous optical and electric measurements and easy medium changes avoiding significant disturbance of the sample. This technique helped to establish firmly the general, but not absolute, correlation of absorption changes and intramolecular charge movements and to link the proton transport to the chromophore changes over a wide range of pH and ionic conditions. Since it eliminated rotational diffusion of pm, it also afforded higher precision in photoselection measurements and their

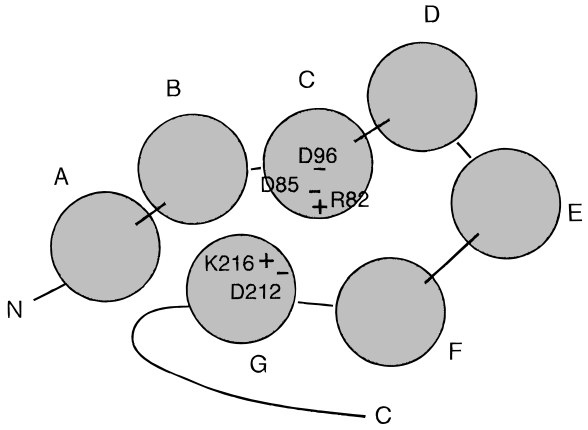


**Fig. 4.** The most likely relative positions of the helices in the membrane deduced from the amino acid sequence in a projection onto the membrane plane and viewed from the cytoplasmic side. Rotational positions of some groups assumed to be important for proton transport are indicated for near-physiological conditions around 1989. The times shown refer to the forward reactions. Neither the various proposed branchings and shortcuts nor the rapid, light-induced backreactions, which have been established for most major intermediates are indicated. The question marks indicate other groups participating in the proton transfer, which could not be specified.

extension to longer times, so that chromophore movements in the late intermediates could also be studied.

Since determination of the quantum efficiency for cycling and the number of transported protons per cycle depended on the assumed cycle model, the remaining uncertainties necessarily impeded progress in understanding the transport mechanism. Only toward the end of this period did reinvestigations begin to result in a consensus in favor of $1H^+$/cycle and a quantum efficiency between 0.6 and 0.7. The question as to whether or not intermolecular interactions in the bR lattice of the pm were involved was not resolved, but it could be shown that the pumping efficiency of bR monomers in lipid vesicles was comparable to that of bR in the pm lattice, justifying transport models based on single bR molecules. Vibrational spectroscopy and NMR data (see below) could be fitted to the same sequence of intermediates as the visible light data, so that retinal conformation and amino acid protonation changes could be assigned to spectral intermediates. Time resolution of spectroscopy advanced into the femtosecond range and allowed direct observation of retinal isomerization in the excited state. It also identified a J-state preceding K with rise and decay times of 0.5 and 3 ps, respectively. Figure 5 shows the cycle as it looked toward the end of this period.

Arguably, during the first years the most important contributions to our knowledge of light-induced, intramolecular changes were provided by RR spectroscopy. A systematic study using substitution of selectively isotope-labeled retinals into the chromophore succeeded in assigning nearly all vibrational frequencies in the highly detailed RR spectra of bR and its main photocycle intermediates. Their retinal configurations were thus characterized and an analysis of the photocycle kinetics by RR spectroscopy became possible. In addition to retinal isomerization in the excited state, it confirmed that SB deprotonation occurred in the L → M, and reprotonation in the M → N transition, and assigned the C13 double bond reisomerization to the N → O transition. The RR spectra also showed that in $bR_{570}$ the C6–C7 single bond had essentially a trans- and the C=N bond an anticonformation. Not confirmed was the postulated additional C14–C15 single bond isomerization in the excited state. A long controversy over this very attractive feature of the transport model ensued, which has not been conclusively settled today, but most researchers do not include the single-bond isomerization in their models. Solid state NMR spectra corroborated and extended many of these findings. RR spectroscopy also yielded the first direct evidence for the presence of water at the SB. It took a long time, however, until the importance of this result was recognized and water was used in the modeling efforts.

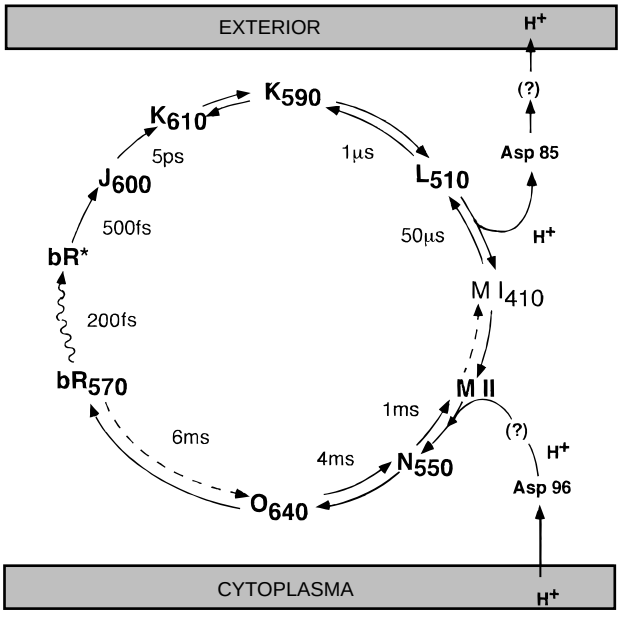


**Fig. 5.** A consensus version of the general photocycle model for near-physiological conditions around 1989. The times shown refer to the forward reactions. Neither the various proposed branchings and shortcuts nor the rapid, light-induced backreactions, which have been established for most major intermediates are indicated. The question marks indicate other groups participating in the proton transfer, which could not be specified.

Whereas RR spectroscopy was limited to the chromophore, IR difference spectra—especially after the introduction of Fourier transform IR spectroscopy (FTIR)—could provide comparable data also for the protein. FTIR with rapidly improving time resolution allowed detection of changes in the protonation state or the environment of single amino acid residues. The IR spectra of the chromophore gratifyingly agreed with RR results, which greatly facilitated the separation of chromophore from protein vibrations in the IR-difference spectra. The much higher resolution of the vibrational spectra now also allowed better characterization of the intermediates in the second half of the photocycle, which had been difficult to resolve with UV-visible spectroscopy.

After 1985, FTIR slowly replaced RR spectroscopy as the most informative spectroscopic technique for the exploration of molecular changes during the transport cycle. The first important observations were still obtained with single wavelength measurements and isotope-labeled bR from bacteria grown on labeled amino acids. They showed that four aspartic acid residues in a hydrophobic environment underwent changes during the photocycle, which could be correlated to transitions of spectral intermediates. The structure model allowed identification of the four residues involved, but could not distinguish between them. Two were unprotonated in the ground state, and one of these was protonated in the L → M transition, whereas one of two protonated carboxyls transiently deprotonated during the M decay. In addition, all four residues experienced changes in their environment. These were obviously key residues in the transport mechanism and their individual identification in the sequence now posed an important challenge.

In the meanwhile the bR gene had been cloned, sequenced, expressed in *Escherichia coli*, and reconstituted with retinal into detergent micelles and lipid vesicles, which allowed generation of mutants with single amino acid substitutions and tests of their functions. It, thus, opened one way to address this problem. However, the same question was also tackled by a different experimental approach. It was based on the expectation that the differences in the sequences of bR mutants containing colored pm, but unable to use light as a source of energy, would identify residues important for proton transport. After random mutagenesis of strains lacking the main carotenoids, purple-colored colonies could be easily recognized on agar plates, and failure to grow under anaerobic conditions would select for functionally impaired pigments. This strategy would always identify functionally important residues, but it had the drawback that it could not be aimed at specific amino acids types, e.g., the four Asp residues in question. The other approach systematically replaced individual amino acids, e.g., Asp residues, by site-directed mutagenesis, and tested the effects on transport.

This method was more labor-intensive, but obviously more powerful, and would also exclude an essential role for specific amino acids. Initially it still had the drawback that the function of the mutant bRs could not be tested in a native environment, because the only available expression system used *E. coli* and the protein had to be renatured in detergent micelles or lipid vesicles, which even for wild-type bR introduced subtle differences in function compared to native wild-type pm from halobacteria. Nevertheless, this soon became the dominant technique and would contribute most to our knowledge of molecular changes during the photoreaction cycle, even before expression in halobacterium was obtained. Both approaches, of course, could be and were used to identify the characteristic vibrational frequencies of residues in the wild-type FTIR-difference spectra and to analyze their changes during the photoreaction cycle.

Both strategies independently and virtually simultaneously identified Asp85 and Asp96 in helix C as the transiently protonated and deprotonated residues. The two Asp residues, which underwent only environmental changes, were identified as Asp115 in helix D and Asp212 in G. However, transient Asp212 protonation changes were tentatively excluded only after considerable controversy; the point is still not definitively settled. The time correlation of protonation and absorption changes, photocurrent kinetics, and the position of the residues in the structure showed that Asp85 must be the acceptor for the SB proton in the extracellular pathway and Asp96 the donor on the cytoplasmic side. Asp85 appeared to be close enough to the SB for a direct proton transfer, but for Asp96 the available structure showed a distance of about 10A, and it was presumably connected to the SB through other groups via hydrogen bonds.

Since the SB and most of the residues, which apparently interact with it, were located on helices C, D, F, and G, and the preferred arrangement of the helices mentioned above places these opposite or next to each other, this structural model began to be generally accepted and prompted the first attempts to model the active site by adjusting the vertical and rotational positions of the helices and arranging the side chains according to the interactions deduced from the FTIR results with refinement by energy minimization. Three tryptophan residues (86, 182, 189) were identified, which define the hydrophobic binding site for the retinal and restrict its movements during isomerization to the vicinity of the SB. Interaction of the SB with Tyr185 and Arg82 were incorporated into the model, but participation of water molecules was mostly neglected. The SB connectivity switch through retinal single bond isomerization was abandoned by most investigators because of the RR results and replaced by a still undefined conformational change of the protein between L and N. Evidence from FTIR spectra corroborated these conclusions and indicated a role for intrahelical prolines in the process. Whereas there was no obvious reason to consider the two switch mechanisms as alternatives, they were nevertheless mostly discussed as such. These first, but still tentative steps beyond the basic model, proposed a plausible, molecularly defined mechanism. However, structural information on the retinal location and orientation was still rather incomplete.

Neutron diffraction, which due to technical limitations had failed in early attempts, became an increasingly important source of structural information after 1984, because in contrast to X-ray and electron crystallographic techniques it could locate the retinal and water in the structure. Difference projection maps between pm substituted with $^{1}$H-retinal and retinals labeled in selected positions with as few as five $^{2}$H clearly identified the ionone ring and SB positions. The results left only two choices for the location of helix G in the map, one of which appeared more likely to be correct, because it again corresponded to the preferred model (Fig. 3B). The problem of the rotational orientation of retinal with respect to its long axis and toward which surface this axis was inclined remained. Polarized light studies in the visible and IR, and RR spectroscopy had already shown that the plane of the retinal ring was normal to the membrane plane, and that its long axis formed an angle of $\sim 20^{\circ}$ with it. Measuring the difference of retinal transition dipole moments in pms reconstituted with retinal and 3,4-dehydroretinal with respect to the membrane normal showed that the ionone ring, and therefore also the N-H bond, must point toward the nearer surface. Early attempts to identify this surface with fluorescence energy transfer had given ambiguous results, but later work using the rapid diffusion limit or second harmonics interference techniques demonstrated, that it must be the extracellular surface. That orientation was also consistent with the distances of 9 and 16 Å from the surface for the ring and SB, respectively, obtained by neutron diffraction and provided the important information missing from the electron diffraction-based map. It forced significant changes in some of the more detailed proposals for the molecular arrangement around the chromophore and proton paths, but the plausibility of the conclusions that the first steps in the transport mechanism are the 13-cis isomerization and proton transfer to Asp85, resulting in release of a proton from the outer surface, were reinforced.

Whereas the protein changes during this first part of the transport cycle were obviously small, and to neglect them at this point could be justified, the same was obviously not true for the second part of the cycle. However, these later changes remained unspecified beyond the statements that a switch in SB connection from the outer to the cytoplasmic surface occurs, and that Asp96 transfers its proton via other groups to the SB in the M $\rightarrow$ N transition. When Asp96 is reprotonated from the cytoplasmic medium, N relaxes to O with reisomerization of the retinal to a still twisted all-trans form. Full relaxation and deprotonation of Asp85(!) occur in the O $\rightarrow$ bR transition. Vibrational spectroscopy also indicated hydrogen bonding changes during these steps. Details of the proposed path necessarily remained controversial and speculative, not only because the participation of water molecules was still largely neglected, but also because information on structure changes of the intermediates was almost entirely missing.

In a major collaborative effort toward the end of this period, three laboratories examined a large number of functionally impaired, but still transport-active point-mutants. The study revealed at first sight surprising differences in photocycle kinetics, the timing of SB and amino acid protonation changes, charge transport steps, and proton release and uptake. The data could, however, be understood on the basis of the developing model and yielded new information on the groups involved in transport and the timing of their interactions. Some of the mutants also accumulated photocycle intermediates, which had been difficult to distinguish in the wild-type cycle and would eventually allow their better structural and functional characterization. Importantly, the data showed that the transport mechanism does not follow an absolutely rigid scheme, but possesses remarkable flexibility. The stage was thus set for rapid advances in understanding the details of the transport mechanism.

Whereas this progress was rather spectacular, it nevertheless followed a course that was not unexpected. However, *H. salinarum* also produced a few surprises in this period. Indications that small

amounts of one or more other pigments with spectroscopic properties similar to bR were present in the cells had already been seen, but ignored. Now an investigator new in the field reported a green-light-driven inflow of protons and ATP-synthesis mediated not by bR, but another retinal pigment. At first this notion, in a typical reaction, was dismissed out of hand by the establishment. The situation was complicated by the fact that pm in intact cells can, and often does, cause secondary influxes of protons and that the bR-independent primary inflow was initially observed only in strains, which contained little if any bR. These had, of course, been selected against by laboratories focusing on bR. However, the phenomenon refused to disappear, and when the proton inflow was shown to increase in the presence of proton ionophores and to disappear upon addition of other cation-specific ionophores, the conclusion that it had to be coupled via the potential to another electrogenic light-driven ion pump similar to bR became inevitable. The pigment, named halorhodopsin (hR), showed an action spectrum similar to bR's with a slightly red-shifted absorption maximum near 580 nm. It was at first assumed to pump sodium outward, which was soon corrected to inward $Cl^-$-pumping. The cause of the initial error, probably attributable to the presence of a $Na^+/H^+$-antiporter in the cells and a general tendency to neglect anion permeation, was never explained, and quietly accepted by the community. The obvious similarity of hR to bR in spectral properties indicated a very similar protein, which was confirmed when the amino acid sequence of hR became available and showed extensive homologies to bR. Its hydrophobicity profile was also compatible with a seven helix structure. The hR photocycle too begins with the C13,14-trans $\rightarrow$ cis isomerization, a rapid red shift of the absorbance, followed by a blue shift corresponding to the K and L intermediates of bR. The striking difference is that hR lacks an M-like intermediate. This is consistent with the postulated role of the SB in bR, but severely hindered analysis of the hR cycle, and consequently attempts to understand the $Cl^-$-transport mechanism.

Phototactic responses of *H. salinarum* had also been observed even before bR was recognized as a retinal protein. Crude action spectra for an attractant response appeared to match the absorption spectrum of pm, and initially bR was, therefore, taken also to be the sensor for the light response. Closer scrutiny, however, revealed discrepancies. The action spectrum maximum for the attractant effect appeared to vary and was often found to be slightly blue- or red-shifted from the position of the bR absorption maximum, and subsidiary maxima were somewhat inconsistently found on both sides of it. Also for large repellent responses in blue and near-UV light, no corresponding absorption peaks exist in the bR spectrum. However, all phototactic responses were retinal-dependent. The problem was further complicated by the discovery of hR, and the fact that at high light intensities bR can, indeed, elicit phototactic responses. A long controversy ensued, during which the existence two additional retinal pigments with absorption maxima at 565 and 370 nm and a fifth "slow-cycling" rhodopsin were postulated. The issue was only resolved, after the unexpectedly difficult selection of a mutant, which contained neither bR nor hR, but was still phototactic. In this mutant a new photosensory retinal pigment, sensory rhodopsin-I (sR-I), with an absorption maximum at 590 nm was discovered. It showed extensive sequence homologies with bR and a spectroscopically similar chromophore. Its photocycle has K- and L-like intermediates and a long-lived M-analog with maximal absorbance at 370 nm, which was identified as the signaling state for a repellent response. Near-UV light rapidly converted this M back to the initial state. Shortly afterward still another, sensory rhodopsin (sR-II), with an absorption maximum at 480 nm was found in *H. salinarum*, and was shown to be the receptor for a photophobic response to blue light. Its chromophore and photoreactions also closely resembled sR-I's except for the shift in wavelength. Sensory rhodopsin-II- and hR-like pigments were also found in alkalophilic extreme halophiles.

Thus, around 1985 three more rhodopsins similar to bR, but with very different functions had been discovered in the same cells. Halorhodopsin, at least, was also an electrogenic ion pump, even though it transported an anion instead of a cation. Its lack of an M-like intermediate fitted the notion that in bR the SB-proton is transported; nevertheless, its discovery briefly revived an earlier discussion, whether bR might not be an $OH^-$ rather than a $H^+$-pump, which, Peter Mitchell had decreed, could not be distinguished experimentally. This possibility never seems to have been pursued very seriously though, and no plausible mechanism for $OH^-$ pumping by bR was ever proposed.

When the amino acid sequences for hR and sR-I became available and could be fitted to the seven-helix structure, sequence comparisons revealed more residues apparently important for bR's function and/or structure. The same turned out to be true even more so for an increasing number of bR-like proton pumps isolated from other strains of halobacteria. In hR the proton donor and acceptor groups Asp96 and Asp85 of bR were replaced by Ala and Thr, respectively, which was not surprising in view of its function. The 590 nm absorption maximum of sR-I was more difficult to understand, because its amino acid sequence showed Asp residues in the positions corresponding to Asp85 and Asp212 in bR.

These newly identified pigments strongly challenged the mechanistic model for the function of bR. With their obvious homologies to bR and their very similar light-induced absorption changes, one would expect basically the same molecular changes during the photoreaction, but it was not clear how these could be linked to their quite different functions. For hR a photocycle and transport model in analogy to the model for bR was proposed. However, its rational suffered from the insufficiently resolved photocycle, the difficulties in determining the $Cl^-$-binding sites and the timing of $Cl^-$ release and uptake. The analogy also relied on the dubious C14,15-single bond isomerization mechanism for the switch in connectivity, and its central assumption, that the approach of a chloride ion to the SB is equivalent to its deprotonation, was difficult to reconcile with the maximal observed blueshift of only 60 nm in its photocycle. Therefore, the model remained unconvincing. For the sRs, which are photosensors, i.e., signal not energy transducers, the obvious guess was that they also were proton pumps and that the change in membrane potential transduced the signals. However, no electrogenic effect of light could be detected and experimental evidence clearly pointed to a diffusion-controlled signal transmission through the cytoplasm. No mechanistic model could be proposed for sR and at this time the transport model for bR was not significantly affected by the newly discovered pigments.

### *1995: Refinement, general acceptance, and generalization of the model*

In 1990, electron crystallography produced an improved three-dimensional map for bR with 3.5 Å resolution in the plane of the membrane and 10 Å normal to it. The crystallographic data fixed the rotational position of the helices, except for helix D, the beta-

ionone ring and the six aromatic amino acids (4Trp, 2Tyr) defining the retinal-binding pocket. The longer, interhelical linking segments contributed sufficient density to determine the sequence in which the helices are connected. The ends of the transmembrane helices, correct to one turn, and their relative positions normal to the membrane plane were given. Including additional information from neutron diffraction and the interactions of functional groups obtained from the mutant work, the map also included the location of the SB (Fig. 6). A vertical shift of helix D by about 4 Å toward the cytoplasmic surface was the only major correction required since. The postulated connections of the SB to both membrane surfaces, at least in the central part of membrane, were deduced from the positions of resolved hydrophilic side chains, which become less certain with increasing distance from the membrane center. The cytoplasmic "channel" mainly determined by the positions of Asp96 and the SB, is narrow, relatively hydrophobic and would probably not conduct protons in this ground state conformation. The wider outer channel shows open spaces near the still unresolved SB, presumably occupied by water, and sufficient hydrophilic groups to connect it to the surface. This structure is consistent with the rapid proton exchange of the SB in the dark and the observation that, in spite of the central SB location, 80% of the total charge movement occurs during the second half of the transport cycle through the cytoplasmic channel. The new map largely confirmed the retinal environment inferred from indirect evidence before; but it also forced some changes in the proposed SB interactions. It therefore held no great surprises, but nevertheless had great impact, because it finally provided a firm and more exact basis for further attempts at refinements of structure and transport mechanisms (Fig. 8). The most serious limitation was not the map's suboptimal resolution, but that it could provide only the ground state structure.

Two other influential papers appeared at that time, which were based mainly on solid state NMR spectroscopy of model SBs and pm with isotope-labeled SB and retinal. The authors concluded that only a complex counterion including water could account for the spectra. As a minimal model they proposed a network of hydrogen bonds connecting two anionic and one cationic group via a central water molecule with the SB (Fig. 7, $bR_{568}$). Such a complex would delocalize the charge, exhibit increased polarizability,

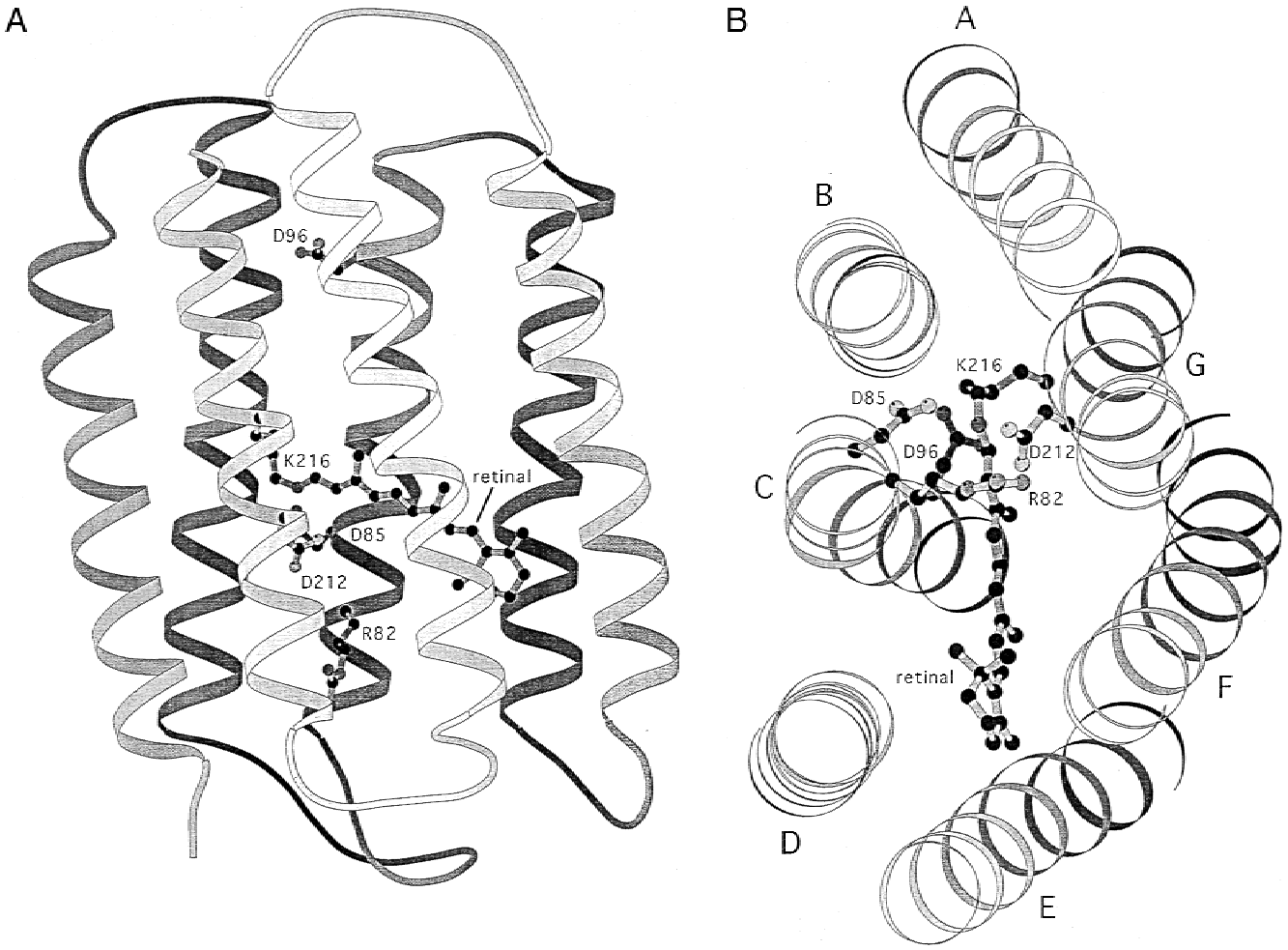


**Fig. 6. A:** The 1990 structure model. The location of retinal and important groups of the transport path are shown in projection onto the threefold axis of the bR trimer normal to the membrane plane. The position of Arg82 was not resolved and is shown here with its end group near the surface; it should, however, be turned toward the interior (see text). **B:** The structure in projection onto the membrane plane, viewed from the external side.

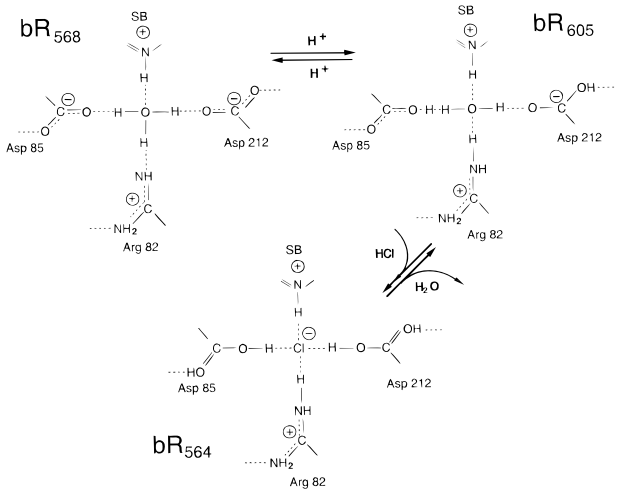


**Fig. 7.** The type of complex counterion structure at neutral and acid pH proposed in 1990. Additional protein groups and water presumably participate and the actual arrangement of groups is probably different. Identification of the amino acids involved is taken from a 1991 publication.

and generate a weak, "soft" counterion. This concept proved to be very useful not only for understanding the NMR data but also for numerous other observations, e.g., the difficulties in assigning "the" counterion, understanding the opsin shift and the high pK of the SB.

Some rather provocative papers also contributed decisively to general acceptance of the transport model. Not only did they rely on unproved, but reasonable assumptions, they also tolerated some differences between calculated and observed data for the sake of plausibility. For instance, in one study shapes of intermediate spectra were assumed and discrepancies between observed and calculated kinetics could not be called small. The existence of an irreversible transition between two consecutive M-intermediates, which could not be distinguished spectroscopically, was deduced from anomalies in the L, M, and N kinetics, even though more rigorous analyses of other studies better fitted the kinetics with only one M intermediate. This new analysis of absorption kinetics and a subsequent study of thermodynamics concluded that a virtually irreversible structural transition occurs in the M state and affects the switch in SB connectivity in the M-state. This had been postulated already in the earliest model but received little attention. The concept was also supported by other early studies, which had found two Ms with different chromophore orientations and a large entropy decrease in M. Surprisingly nobody had followed up on the early experiments, and no one did now. Nevertheless, the two M-states began to be widely recognized and their interpretation accepted.

The results were reinforced by CD and FTIR spectroscopy, which also had very early provided evidence for a relatively large structural change in the M state involving intrahelical Pro residues and extensive changes in hydrogen-bonding. New spectral data at low temperature on partially dehydrated pm and also on mutants lacking the Asp96 donor group, e.g., D96N, in which SB reprotonation is delayed, succeeded in characterizing the spectra for the MI, MII, and N protein conformations. They also described an M intermediate with the same protein conformation as N but a still unprotonated SB, that accumulated when SB-reprotonation was inhibited. Figure 8 shows a sketch of the model incorporating these features (see also the outer reaction cycle in Fig. 9A for another representation).

Attempts to detect the structural changes in intermediates using crystallographic techniques initially encountered the problems that one M could not be accumulated in a reasonably pure state and/or identified. Eventually, FTIR spectra resort to low temperature, partial dehydration, and the use of mutants, coupled with the crystallographic results began to resolve these problems. Whereas none of the observations obtained were conclusive, together they were convincing enough to lead to a general acceptance of these model refinements. Electron crystallography of the M intermediate suggested that the main change in the late M might be an outward tilt of the cytoplasmic end of helix F, probably originating at the Tyr185, Pro184 bond near the SB. This movement could open the cytoplasmic channel and allow water to enter.

The availability of the higher resolution structure and new data from neutron diffraction and solid state NMR emphasizing the role of intramolecular water provided the basis for new efforts to explore by molecular dynamics, electrostatics, and quantum chemical calculations the active center of the molecule, the proton path, and their changes during the photocycle. Compared to the earlier proposals, which had been based on the lower resolution structure and neglected participation of intramolecular water, these efforts now afforded a much more realistic picture of details in the transport mechanism. Whereas the crystallographic analysis had not resolved the Arg82 position in $bR_{568}$, but preferred to place its cationic group at the surface, the calculated structures confirmed the necessity to place the positive charge near the SB and connect it like other groups, e.g., Tyr185, Tyr57, and Thr89, to the counterion, either directly or via water. Helix F, in gratifying agreement with the crystallographic results, developed a bend, which moved its cytoplasmic end outward, opening a channel to the center of the molecule and allowing water to enter, which would connect Asp96 to the cytoplasmic medium and to the SB. Calculated pK changes of the SB during the photocycle were consistent with experimentally estimated values and the requirements for transport.

The structure and transport models now readily provided explanations for many formerly puzzling observations. Their validity was also strikingly demonstrated by the increasing predictability of functional defects in point mutants and their reversal by chromophore modifications, environmental changes, or second mutations. For instance, the disturbed counterion structure in mutant Y57N prevents SB deprotonation during the photocycle, suggesting that either the pK of the SB does not decrease, or the pk of Asp85 does not rise sufficiently for proton transfer. Replacing the retinal in the chromophore by 14-fluororetinal lowers the SB pK by three units and does restore M. Volume changes by uncharged amino acid replacements in the retinal binding site, e.g., A53V and A53G, which were expected to change the relative positions of the SB and Asp85, had the expected effects on the proton transfer reactions. Examples of similar effects in other mutants are rapidly accumulating revealing the contribution of other groups in the transport, strengthening confidence in the model and refining it. If understanding a biological system means being able to predict its reaction to changed conditions, then these results indicate that we are really beginning to understand bR.

Two seminal papers appearing at the beginning of this period initiated extension of the bR transport model to hR and sR. The paper originally proposing the complex counterion for bR also described the counterion at low pH (Fig. 7). It ascribed a well-

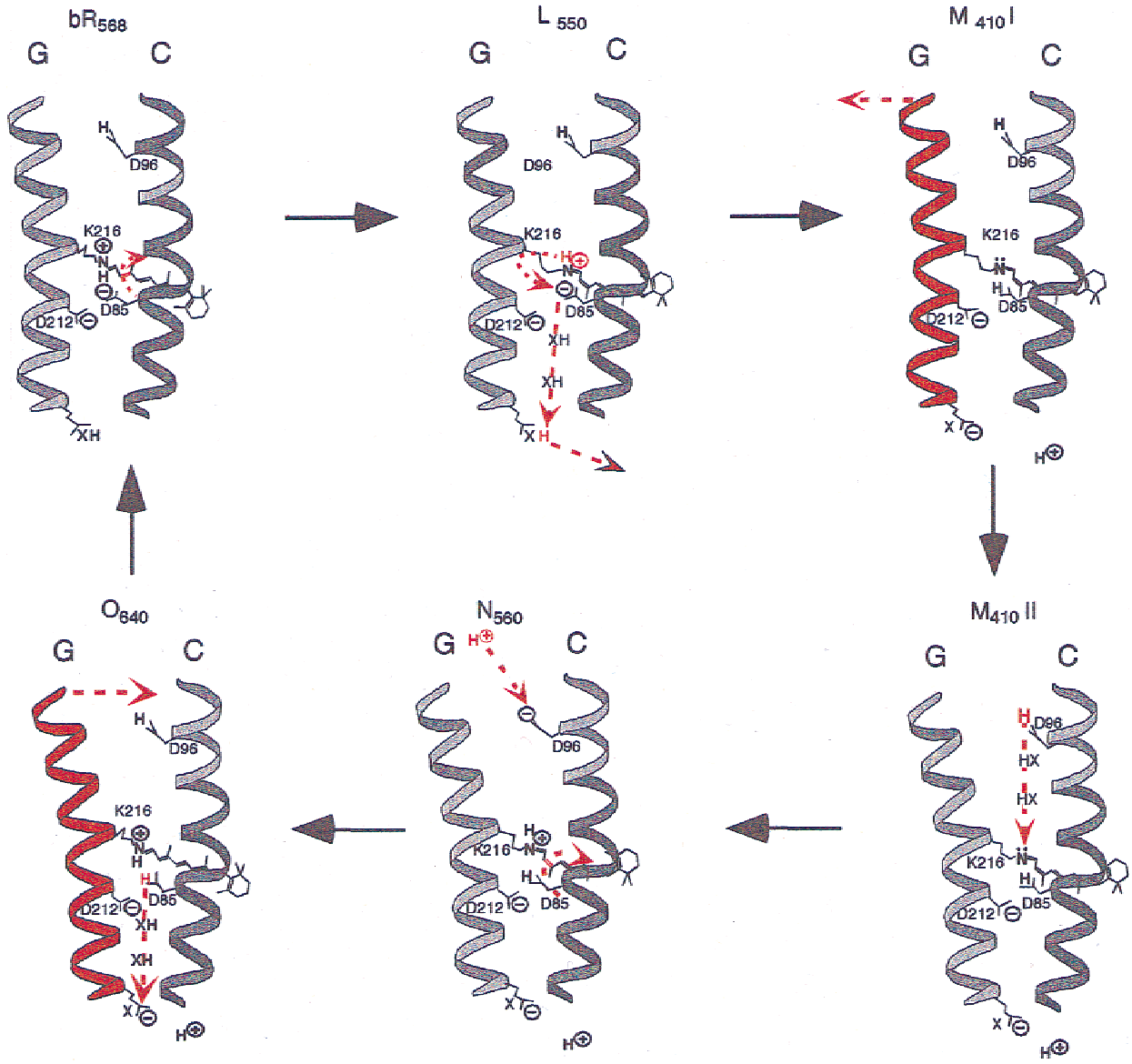


**Fig. 8.** Sketch of the pump model as it appeared around 1990. The view is approximately the same as in Figure 6A. Positions of the retinal, the helices, and side chains in $bR_{568}$ are based on the 3.5 Å resolution structure published in that year. Arrows and color indicate the reactions leading to the next intermediate. X represents unspecified groups or water molecules in the hydrogen bond network, that mediate proton translocation. Arg82, an important group in this network on the external side, is not shown because its position in the published 3.5 Å structure is probably incorrect. The small conformation changes of the early intermediates are ignored, and the ground state structure is maintained up to MII except for the retinal isomerization to 13-cis, which is assumed to affect the SB position only slightly because it is compensated by single bond isomerization in the Lys side chain. The switch in SB connection in the N → M transition is indicated arbitrarily as an increase in the tilt of helix G; a change in helix tilt in M was suggested by early CD spectra. Later electron crystallography of intermediates has shown that the cytoplasmic end of helix F tilts outward in the M → N transition, i.e., away from the trimer axis.

known reversible acid-induced red shift of bR absorbance with a pK near pH 3, which generates the "blue membrane" ($bR_{605}$) to the uptake of a proton into the hydrogen bond network of the counterion, which would introduce a Bjerrum double fault and confer a partial positive charge to the central oxygen and one of the aspartates. At still lower pH and in the presence of $Cl^-$, this transition is followed by a halide anion-dependent return to purple color, generating the "acid purple membrane" ($bR_{565}$). This second transition was attributed to replacing the $OH^-$ of the water molecule in the counterion by $Cl^-$, which removes the surplus proton and restores the ground state hydrogen bond pattern.

An inspection of the bR structure strongly suggested Arg82, Asp85, and Asp212 as candidates for the postulated cationic and anionic groups of the counterion (Fig. 7). Protonation of Asp85 in the $bR_{568} \rightarrow bR_{605}$ transition had been assumed before and was supported by a red shift of the absorption spectrum in the mutant D85N.

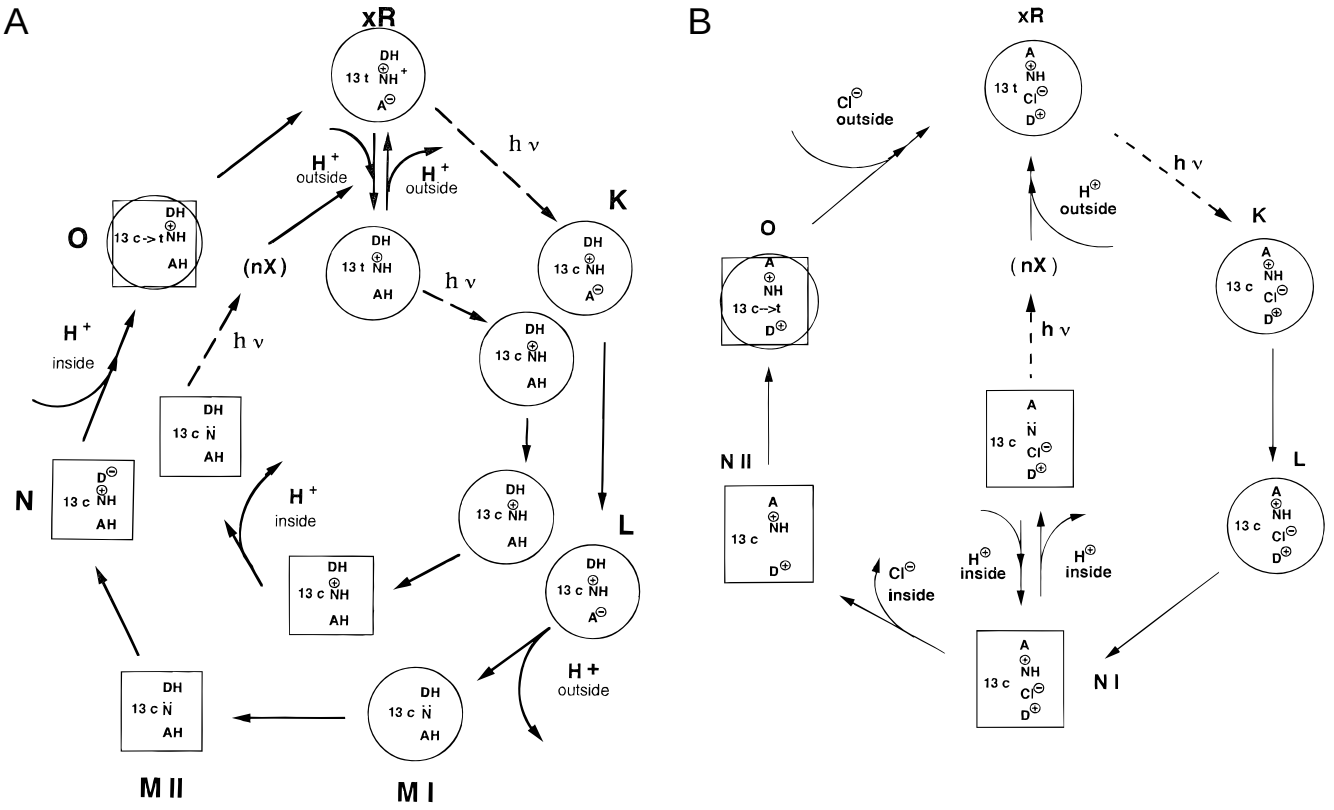


**Fig. 9. A:** Sketch of ion transport reactions by bacterial rhodopsins. The acceptor (A) and donor (D) groups are Asp96 and Asp85 in the case of bR. Small early protein conformation changes have been ignored; the switches in connectivity are indicated by changes of the circle to a square. The outer cycle has been demonstrated in $bR_{568}$ and $sR_{540}$. It is the physiological cycle in bR. An Asp residue is not absolutely required in position 96; in sR it is replaced by Tyr and in hR by Ala, which slows down the SB reprotonation. Modified cycles can be found in some bR mutants. With regard to MII see also the text on p. 455. Note that the inner cycle requires two photons. It has been demonstrated in $sR_{590}$, in hR in the absence of chloride, and in bR mutants, in which deprotonation of the SB toward the external surface is hindered, e.g., D85N. **B:** The corresponding sketch for chloride and proton transport by bacterial rhodopsins hR, $bR_{565}$, and D85T. The details of the transport cycle are still highly speculative and have not been extensively discussed in the literature. Many crucial data are still missing, e.g., the $Cl^-$ release and uptake points in the cycle. The donor molecule in hR is presumably Arg108, corresponding to Arg82 in bR; however, the acceptor molecule is still unknown. The counterion structure in the absence of added chloride is also unclear. D85T and $bR_{565}$ require molar chloride concentrations; but hR may bind chloride firmly enough to retain it even in the absence of added chloride.

It would eventually be confirmed by NMR spectroscopy. The role of $Cl^-$ as the main counterion in $bR_{565}$ was supported by a small red shift of the spectrum when $Cl^-$ was replaced by $Br^-$. However, the concentration of $bR_{565}$ proved to be proportional to the product of the $H^+$ and $Cl^-$ concentrations, which argued that the transition $bR_{605} \rightarrow bR_{565}$ was better understood as the replacement of the central water molecule in the complex counterion by dissociated HCl with protonation of the second anionic group Asp212.

It had been known for some time that both acidic forms of bR show modified photocycles, which lack M-like intermediates, i.e., their SBs do not deprotonate in their photocycles and that $bR_{605}$ and $bR_{565}$ apparently lack $H^+$-transport activity, presumably because the acceptor for the SB proton, Asp85, is protonated. Photocurrent measurements on oriented pm gels now confirmed lack of transport activity for $bR_{605}$; however, $bR_{565}$ supported a light-driven electric current with the same polarity as in $bR_{568}$. Its photocycle like that of hR lacks an M-intermediate, which suggested that $bR_{565}$ transports $Cl^-$ rather than $H^+$. The notion received support when in contrast to $bR_{568}$ no deuterium isotope effect on photocycle kinetics was found in $bR_{565}$, but a slowdown was observed when $Br^-$ was substituted for $Cl^-$. Even though neither these findings nor the proposed counterion structure could be considered to be firmly established, they suggested a plausible, common ion transport mechanism for $bR_{565}$ and $bR_{568}$. It postulates that the SB in $bR_{565}$ undergoes the switch in connection from the outer to the inner surface without deprotonation of the SB, i.e., directly changes from the L- to the N-state. The same local potential changes, which drive the proton release to the outer and the subsequent uptake from the inner surface in $bR_{568}$, would in $bR_{565}$ first bind the chloride anion more firmly to the SB and after the switch drive its release to the inner medium. $Cl^-$ re-uptake would occur with return of the molecule to the ground state (Fig. 9B, outer cycle).

The proposed mechanism extended the transport model from bR to hR. Its photocycle too lacks the M-intermediate. In addition to many other amino acids thought to take part in the proton translocation, Arg82 and Asp212 are conserved in homologous positions 108 and 238, respectively, in hR, whereas Asp85 is replaced by Thr111. A threonine residue should at neutral pH sustain the same hydrogen bonds as the protonated Asp85 in $bR_{565}$, and thus

support essentially the same counterion structure and transport mechanism (Fig. 9B, outer cycle). This proposition implies that in bR as well as in hR the SB will change its connection from outside to inside regardless of its protonation state, and that it can carry a mobile counterion through the switch.

If this interpretation is correct and the low pH causes no other significant changes in the bR molecule, the D85T mutant of bR at sufficiently high NaCl concentrations should transport $Cl^-$ near neutral pH. That expectation has been experimentally confirmed. Thus, a shift in pH or the change of one key amino acid apparently suffices to convert a cation to an anion pump.

The extension of the transport model to hR and the notion that the SB of bacterial rhodopsins will switch its connectivity, even if its deprotonation is prevented, was soon supported by the demonstration that this connectivity switch occurs also in hR and that hR can also transport protons. Whereas its photocycle lacks an M-like intermediate, it had long been known that continuous illumination in the main absorption band causes accumulation of a M-like form ($H_{410}$) in a side reaction and that the SB proton is then released at the cytoplasmic surface. The accumulating $H_{410,}$ which thermally reverts very slowly, progressively decreases the efficiency of the $Cl^-$ transport. Blue light reisomerizes the retinal of $H_{410}$ and returns it quickly to the ground state. Thus, addition of blue light restores the efficiency of $Cl^-$ pumping.

In photocurrent recordings of this pump re-activation by blue light, a small negative current spike of opposite direction occurs, before the $Cl^-$ current increases. This spike suggested that in the rapid return of accumulated $H_{410}$ to the ground state, as in the photoreversal of M in bR, the SB reprotonation occurs from the outer surface. In hR the second photon would thus complete a two photon cycle transporting protons into the cell, and in white light hR could function as a two-photon, reverse proton pump (Fig. 9B, cycle through shunt). Its efficiency would be low, because under physiological conditions little $H_{410}$ is formed and $Cl^-$ transport dominates. However, optimizing conditions for proton transport by omitting $Cl^-$ from the medium and adding azide, which facilitates the SB protonation changes, affords an efficient, reverse, two-photon proton pump. The same reverse cycle is also found in bR mutants where the proton release from the outer surface is blocked, e.g., D85N or D85T.

Obviously, we are still far from understanding the details of these processes. For instance, it has not been explained why efforts to demonstrate proton pumping in the hR mutants in which aspartates have been substituted in the positions corresponding to Asp96 and 85 of bR have failed so far. However, hR from the alkalophilic halophile *N. pharaonis* in the presence of azide does transport $H^+$ outward. An important consequence of these observations for the transport model is that they argue for a constrained conformation of the protein rather than an earlier invoked charge imbalance caused by SB deprotonation as the driving force for the switch in SB connectivity.

The transport model's power to explain light effects on the two ion pumps tended to emphasize our lack of understanding of the function of the homologous sensory pigments. These show bR-like photocycles with all-trans to 13-cis retinal isomerization and M-like intermediates. The best known, sR-I, has preserved most of the amino acids important for transport, including a potential proton acceptor, Asp76, homologous to Asp85 in bR, yet sR-I lacks charge transport activity. FTIR spectra suggested that Asp76 might be protonated in the ground state, which could explain the red-shifted absorption band, but still seemed to conflict with missing proton release in the M-state of its photocycle. Since sRs, as might be expected of signal transducers in contrast to energy transducers, are present in very low concentrations, work was hampered until the pigment could be overexpressed and isolated. Then it was realized that in the cells and membrane preparations used sR-I was firmly complexed with Htr-I, the next protein in the signal transduction chain. After genetic removal of Htr-I the pK of Asp76 decreased from 8.5 to 7.2, and its absorption spectrum and photocycle kinetics became pH-dependent. The high-pH form, $sR_{540,}$ transported protons outward like $bR_{568}$ (Fig. 9A, outer cycle). The transport efficiency was low, because at alkaline pH reprotonation is slow and at lower pH the concentration of the alkaline form rapidly decreases. In the acidic form, $sR_{590}$, the acceptor Asp76 is protonated and sR will pump $H^+$ inward in a two-photon cycle like bR D85N, D85T, or hR in the absence of $Cl^-$. With these observation it was finally shown that the basic mechanism of the light reactions is valid for all bacterial rhodopsins (Fig. 9). How it generates the phototactic signal is, of course, still not solved with these observations. However, the conformational change of the connectivity switch and/or direct transfer of a proton from sR to Htr are obvious candidates.

*Epilogue*

It is gratifying finally to see a generally accepted solution for the mechanism of proton transport in bR, even though it has taken 30 years rather than the 5 many investigators had estimated at the beginning. The unexpected difficulty posed by the discovery of two other closely related proteins with apparently very different functions proved to be a blessing in disguise, because the model's ability to accommodate them proved its value and greatly strengthened confidence in it. This review describes developments only up to 1995. Since then more details of the proton path have been revealed and more of the groups involved have been identified, mostly by studying the functional defects in mutants obtained by site-directed mutagenesis. Crystallography has yielded improved three-dimensional maps for key intermediates and reached atomic resolution for the ground state molecule. It has also begun to reveal the structural changes of the switch in connectivity between L and N. Details of the counterion and reaction path will certainly change when higher resolution structures of the intermediates become available, but the principle of its mechanism seems to be well established. To be sure, the most interesting questions that bR poses, how the light energy is stored in the molecule and how it steers the reaction path, still remain unanswered. Exploration of that problem has barely scratched the surface, but we seem to have attained a good basis from which to proceed.

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