Alternative translocation of protons and halide ions by bacteriorhodopsin*

(electric signals/isotope effects/photocycle kinetics/halorhodopsin)

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ABSTRACT Bacteriorhodopsin (bR₅₆₈) in purple membrane near pH 2 shifts its absorption maximum from 568 to 605 nm forming the blue protein bR₆₀₅^{acid}, which no longer transports protons and which shows no transient deprotonation of the Schiff base upon illumination. Continued acid titration with HCl or HBr but not H₂SO₄ restores the purple chromophore to yield bR₅₆₄^{HCl} or bR₅₆₈^{HBr}. These acid purple forms also regain transmembrane charge transport, but no transient Schiff base deprotonation is observed. In contrast to bR₅₆₈, no rate decrease of the bR₅₆₈^{acid} transport kinetics is detected in ²H₂O; however, the transport rate decreases by a factor of ≈2 in bR₅₆₈^{HBr} compared with bR₅₆₄^{HCl}. The data indicate that in the acid purple form bR transports the halide anions instead of protons. We present a testable model for the transport mechanism, which should also be applicable to halorhodopsin.

Halobacterium halobium contains two light energy-transducing pigments: bacteriorhodopsin, with an absorption maximum at 568 nm (bR₅₆₈), which transports H⁺ out of the cell; and halorhodopsin (hR), which transports Cl[−] into the cell. In the cell membrane and in the purified state, bR occurs in the form of small membrane patches, the purple membrane, which are two-dimensional crystals of bR and lipid (for review, see refs. 1–4). Both pigments are intrinsic membrane proteins, contain nearly identical all-*trans*-retinylidene chromophores, and show extensive sequence homologies (for review, see ref. 5).

The absorption maximum of bR is red-shifted from 568 to 605 nm at low pH, forming blue bR, or bR₆₀₅^{acid}, and transport activity ceases (6–8). The same effect is obtained by removing residual metal cations with an ion-exchange resin because the high negative surface charge of the purple membrane then drops the surface pH below 1.0 (9, 10). The addition of Cl[−] reverses the red shift and restores charge transport in this “acid purple” form (bR₅₆₄^{HCl}) (11). The absorption changes accompanying charge transport of bR₅₆₈ are changed in bR₅₆₄^{HCl} and resemble those of hR (12). The question arises, which ion is translocated by the bR₅₆₄^{HCl} form?

Direct measurements of the expected changes in Cl[−] or H⁺ concentration under the required low pH conditions are difficult and so far have not succeeded. However, substantial information on the ion transport in bR and hR has been obtained by measuring the kinetics of the absorption changes and charge shifts that accompany ion transport, and these should be sensitive to the properties of the transported ion. We have, therefore, measured current and absorption kinetics of purple membrane after substituting ²H⁺ for H⁺ and Br[−] for Cl[−]. Techniques and results of photoelectric measurements have recently been reviewed comprehensively (13) and will not be described here.

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MATERIALS AND METHODS

The bR-containing purple membrane fragments were prepared from *H. halobium* strain JW-3 (ET 1001) according to the standard procedure (14). After orientation in an electric field, they were immobilized in a polyacrylamide gel (15), and slabs of 10 × 5 × 2 mm were placed in a cuvette. The gel samples were preequilibrated in the desired solution overnight, and the pH was monitored with an OP-0808 electrode (Radelkis, Budapest) and adjusted by adding appropriate amounts of concentrated HCl or HBr solutions (Aldrich). The measured p²H values were corrected by subtracting 0.41 pH unit.

Optical and electrical signals were measured after laser pulse excitation at 532 nm from a frequency-doubled neodymium–yttrium/aluminum garnet (Nd–YAG) laser with 8-ns pulse length and 1.5-mJ pulse energy. To record photosteady-state currents, we used 0.8- to 4-s-long illuminations at 47 mW/cm² from a 250-W tungsten-halogen lamp through a 550-nm glass filter with bandwidth 150 nm. The illuminated area was 0.1 cm². Freshly platinized Pt electrodes were always used, and care was taken not to illuminate the electrodes.

Transient and quasicontinuous photoelectric and absorption signals were amplified by fast homemade amplifiers and were recorded by computer-controlled transient recorders (Thurlby DSA 524 and a homebuilt model with logarithmic time base). For absorption kinetic signals, we used logarithmic time bases, and for electric signals, linear time bases. The vertical resolution of the transient recorders (8 bit) did not allow recording of both the fast and slow components in one trace due to the large difference in their amplitudes. The traces shown in the figures are the average of 200 signals. They were decomposed into first-order kinetic components according to ref. 16. The continuous illumination signal was filtered by a low-pass filter (cutoff frequency, 30 Hz). Absorption spectra were recorded by a Shimadzu UV-160 dual-beam spectrometer.

RESULTS

Near neutral pH, the absorption spectrum of bR shows no significant change when ²H₂O is substituted for H₂O or Br[−] for Cl[−]. Fig. 1 compares the absorption spectra of purple membrane at pH 0.0 in 4 M HCl with that in 4 M HBr when the transition to the acid purple form is essentially complete. The HCl and HBr preparations show maxima at 564 and 568 nm, respectively.

Abbreviations: bR, bacteriorhodopsin (with subscripts indicating the absorption maximum and superscripts for the medium conditions; bR₅₆₄^{acid} refers to all forms in which purple color is restored by halide anions); hR, halorhodopsin.

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FIG. 1. Absorption spectra of bR in 4 M KCl or KBr at pH 0.0 set by the addition of H_2SO_4 . The baselines of the different spectra are offset for clarity. Spectrum a, 4 M KBr in H_2O ; spectrum b, 4 M KBr in $^2\text{H}_2\text{O}$; spectrum c, 4 M KCl in H_2O ; spectrum d, 4 M KCl in $^2\text{H}_2\text{O}$.

Fig. 2 shows the transient electric signals obtained at neutral pH for media containing Cl^- or Br^- , ^1H or ^2H . (For a general description of the signals and their interpretation, the reader is referred to ref. 13.) The derived kinetic constants for these experiments are given in Table 1. No effect of the anion exchange is seen, but in agreement with earlier absorption kinetic and photocurrent measurements (13, 17–20), a significant increase in the time constants is observed with the heavier isotope for the microsecond and millisecond processes, which reflect mainly the transport of ionic charge across the membrane. These are the expected results, if bR at neutral pH translocates protons and the ion mass affects the translocation rate.

At low pH the two halide preparations also give quasicontinuous photoelectric currents (data not shown; see figures 1 and 2 in ref. 50); the blue H_2SO_4 preparation does not (11), confirming that it does not translocate charge across the membrane. Transient electric signals for the HCl, HBr, and ^2HCl preparations are shown in Fig. 3. It is obvious from an inspection of the curves that the HBr preparation shows slower kinetics than the other two. The results of the computer analysis given in Table 2 confirm this impression. No isotope effect is detectable in the ^2HCl data, whereas the time

FIG. 2. Transient photocurrents induced by a 532-nm laser flash. In the soaking solution of the gels, 300 mM NaCl was dissolved in H_2O or in $^2\text{H}_2\text{O}$ (curves a and c), or 300 mM NaBr was dissolved in H_2O (curve b). The pH and $p^2\text{H}$ values were kept at 6.9. The traces depicted were measured with medium time resolution (40 μs per channel). Faster and slower phases of the signal are not shown.

Table 1. Kinetic analysis of photocurrents from Fig. 2

Time constant	NaCl (H_2O)	NaCl ($^2\text{H}_2\text{O}$)	NaBr (H_2O)
τ_1	$5.71 \pm 4.7 \times 10^{-5}$ (38.2)	$2.0 \pm 1.0 \times 10^{-4}$ (29.4)	$6.1 \pm 5.1 \times 10^{-5}$ (56.9)
τ_2	$2.8 \pm 1.8 \times 10^{-4}$ (44.5)	$9.9 \pm 3.0 \times 10^{-4}$ (111)	$3.2 \pm 1.4 \times 10^{-4}$ (34.3)
τ_3	$5.4 \pm 0.2 \times 10^{-3}$ (273)	$7.9 \pm 0.6 \times 10^{-3}$ (620)	$5.4 \pm 0.2 \times 10^{-3}$ (299)

A sum of exponentials was fitted to the time integral of the photocurrent signals. Time constants are given in seconds, and amplitudes in arbitrary units in parentheses. The fast negative components have been omitted.

constants for the two slowest components in HBr are much larger than in HCl. That the isotope effect at neutral pH is largest in the microsecond processes, whereas the anion effect at acidic pH is obvious only in the millisecond processes, may give important clues for modeling the transport mechanism (see *Discussion*).

At neutral pH, there is a close correspondence between the kinetic components for the charge transport and absorption changes of the photoreaction cycle (for review, see ref. 21). We have also recorded the light-induced absorbance changes for the HCl and HBr preparations and, for comparison, the changes at neutral pH (data not shown). An interpretation in terms of a sequence of intermediates is difficult because of the large overlap in intermediate spectra and the uncertainty in the amount of cycling pigment. However, in agreement with earlier observations (ref. 12 and references therein), we find that the sequences of absorbance changes are similar to those seen at neutral pH. The main difference is the lack of a far blue-shifted M-like intermediate with a rise time in the microsecond range and decay time in the millisecond range. The photocycle at low pH in the presence of halide anions thus resembles that of hR.

DISCUSSION

The results show that at low pH the halide ions Cl^- and Br^- can restore light-driven charge transport of bR. The charge could be carried by either H^+ or the halide anion. In the generally accepted model for the bR photocycle at neutral pH, the all-trans chromophore isomerizes to 13-cis in the excited state and relaxes via a series of intermediate states labeled J to O, while the Schiff base proton is released and ejected on the external side of the membrane and is then

FIG. 3. Transient photocurrents of $\text{bR}^{\text{acid}_{\text{purple}}}$ measured with medium time resolution (40 μs per channel). Faster and slower phases of the signals are not shown. pH or $p^2\text{H}$ was set to 0.5 with HCl (curve a), HBr (curve b), or ^2HCl (curve c).

Table 2. Kinetic analysis of photocurrents from Fig. 3

Time constant	HBr	HCl	² HCl
τ_1	$3.0 \pm 1.5 \times 10^{-6}$ (-13.6)	$2.9 \pm 0.5 \times 10^{-6}$ (-28.1)	$3.0 \pm 0.6 \times 10^{-6}$ (-45.3)
τ_2	$5.3 \pm 2.3 \times 10^{-5}$ (1.9)	$5.3 \pm 1.8 \times 10^{-5}$ (8.2)	$8.3 \pm 2.7 \times 10^{-5}$ (20.1)
τ_3	$3.7 \pm 0.9 \times 10^{-3}$ (14.3)	$9.2 \pm 3.3 \times 10^{-4}$ (6.0)	$8.9 \pm 5.9 \times 10^{-4}$ (8.7)
τ_4	$1.7 \pm 0.2 \times 10^{-2}$ (3.1)	$1.1 \pm 0.6 \times 10^{-2}$ (15.1)	$1.1 \pm 0.1 \times 10^{-2}$ (20.4)

Data are presented as in Table 1.

replaced from the medium on the cytoplasmic side. Since the transient 150-nm blue shift of the absorption spectrum due to the Schiff base deprotonation in the M state is not seen in the photocycle of $\text{bR}_{564}^{\text{HCl}}$, this may be taken as evidence for Cl^- transport (11). The argument, however, is not conclusive, because at low pH one would expect deprotonation to be slowed down and reprotonation to be accelerated so that detectable concentrations of the deprotonated state might not accumulate. Conversely, a transient deprotonation of the Schiff base would not exclude Cl^- as the transported ion.

If H^+ is the transported ion, one would expect a slowdown of the kinetics by a factor of nearly 2 or more in $^2\text{H}_2\text{O}$, as is observed for bR at neutral pH. The lack of such an isotope effect in $\text{bR}_{564}^{\text{HCl}}$ is therefore a stronger argument in favor of Cl^- transport. Unfortunately, this argument is not conclusive either, because processes other than the charge transport could be rate-limiting—e.g., the required conformational changes of the protein or chromophore isomerization. However, the reduced rate of transport in HBr shows that the mass of the anion affects the millisecond kinetics in $\text{bR}_{568}^{\text{HBr}}$ but has no effect in bR_{568} . The available evidence thus strongly suggests a switch from proton to anion transport in $\text{bR}_{568}^{\text{acid}}$. This conclusion is obviously important for understanding how bR functions and interesting also because the closely related hR translocates halide anions at neutral pH. It emphasizes the need to develop a realistic model that can account for both

types of transport in very similar proteins, and we outline such a model here. It is based on the concept that a charge pair in a protein can provide a binding site for both anions and cations and that the same change in local electric potential will move cations and anions in opposite directions. Minor changes in the protein affecting the relative binding strength or barrier heights could then determine which ion is transported.

The $\text{bR}_{568} \rightarrow \text{bR}_{605}^{\text{acid}} \rightarrow \text{bR}_{564}^{\text{HCl}}$ transitions can be explained on the basis of the generally accepted model for the bR_{568} chromophore, which assumes that the position of the absorption maximum is mainly determined by the protonation of the Schiff base linkage to Lys-216 and the strength of its interaction with the surrounding charges (reviewed in ref. 3). Nuclear magnetic resonance spectroscopy strongly suggests that the counterion near the Schiff base is a hydrogen-bonded complex comprising several protic amino acid residues and at least one water molecule (22, 23). The recent paper of Henderson *et al.* (24) on the structure of bR and the extensive work on the properties of mutant proteins from Oesterhelt's and Khorana's laboratories (cited in ref. 24) point to Asp-85, Arg-82, and Asp-212 as the main amino acids involved. However, other residues—e.g., Tyr-57 and -185—probably also contribute. In the $\text{bR}_{568} \rightarrow \text{bR}_{605}^{\text{acid}}$ transition the H^+ taken up (7) is presumably inserted into this complex, weakening the interaction with the Schiff base and causing the red shift. This would explain the low pK of 1.7 for the transition (10), which is inconsistent with the protonation of a single, undisturbed amino acid residue. In the $\text{bR}_{605}^{\text{acid}} \rightarrow \text{bR}_{564}^{\text{HCl}}$ transition, the Cl^- is exchanged for an OH^- (or H_2O is exchanged for dissociated HCl), forming a complex with the same number of hydrogen bonds but one fewer hydrogen and a protonated Asp-212 (Fig. 4), so that the original strength of the counterion is approximately restored (23). This model is supported by the observation that the $\text{bR}_{564}^{\text{HCl}}$ concentration is linearly dependent on the product of the Cl^- and H^+ concentrations (Y. Kimura and W.S., unpublished data). Conformational changes in the protein, which also occur in the $\text{bR}_{568} \rightarrow \text{bR}_{605}^{\text{acid}}$ transition (9), can at present not be specified and may not correlate with the absorption changes (23).

FIG. 4. Schematic of the proposed counterion complexes for bR, based on the nuclear magnetic resonance work (22, 23) and the structure proposed by Henderson *et al.* (24). The indicated changes have to be thought of as delocalized to a considerable extent, and additional hydrogen bonding of the aspartic and arginine residues is assumed to exist but is not specified.

The Schiff base is located near the center of the membrane (25), but the rapid exchange of the Schiff base proton in the dark and the effect of azide in a bR mutant indicate that diffusion pathways to and from the membrane surfaces exist (26, 27). We postulate that an increased positive electrical potential near the Schiff base in the L state provides the driving force for H^+ ejection. The change may be caused by changes in the charge distribution around the chromophore and/or by complete or partial isomerization of the retinal around bonds other than the $C^{13}-C^{14}$ double bond, which reverses only after reprotonation to the N state in the $N \rightarrow O$ transition. For details see ref. 3. It has long been recognized that models in which the Schiff base proton is translocated require two consecutive M states (28, 29), and these have recently been derived from an analysis of the photocycle kinetics (30). Such a "reprotonation switch" (31), which disconnects the Schiff base after deprotonation from the external surface and assures reprotonation from the cytoplasmic surface, presumably involves a protein conformational change. Strong evidence for a large protein conformational change in the M state was first provided by time-resolved calorimetry and more recently by Fourier-transform infrared spectroscopy (32–35), but the two states have still not been satisfactorily characterized.

In bR_{564}^{HCl} , the counterion cannot accept an additional proton, and we postulate that, instead, similar to an earlier proposal (36), Cl^- is bound more firmly to the protonated Schiff base and carried with it during the protein conformational change, which connects the Schiff base to the cytoplasmic surface. To complete the cycle, we now require an increase in negative potential at the Schiff base to expel the Cl^- or attract a H^+ from the cytoplasmic surface. Unfortunately, the bR_{568} structure cannot be used with any confidence at this point for identification of groups involved, because of the significant structural change that apparently takes place when the Schiff base connection is switched from the external to the cytoplasmic surface. However, we expect that a counterion is formed that is similar to the complex existing in the L state, because the chromophore structures of the L and N intermediates appear to be nearly the same (31), and some of the same groups may participate. Such a complex would provide an analogous mechanism for alternative H^+ uptake or Cl^- extrusion. Thr-89 may be involved (37). Asp-115 and Arg-175 and -134 of bR could participate (see below), although their replacement in bR only partially inactivates proton transport (38, 39). Asp-96, which is widely assumed to be the donor group for Schiff base reprotonation, is a less likely candidate because it could probably not deprotonate at pH 0.5. We also note that the postulated local potential change at the Schiff base which drives the Cl^- ejection or H^+ uptake need not be caused by shifting charged groups in its immediate vicinity, since any charge shift near the ionone ring will be transmitted to the Schiff base via the conjugated double-bond system of the retinal.

The proposed mechanism explains why in the kinetics of bR_{568}^{HBr} compared with bR_{564}^{HCl} , an ion effect corresponding to the large $^2H^+$ effect in the $L \rightarrow M$ transition of bR_{568} is missing. In bR_{568} the transported ion is ejected from the membrane, whereas in bR_{564}^{acid} the anion is already bound near the Schiff base and does not move over a significant distance when the local potential increases. The subsequent conformational change, which connects the Schiff base to the cytoplasmic surface, is not likely to be strongly affected by the mass of the transported ion. The mechanism requires two consecutive, L-like states for bR_{564}^{acid} . They may, however, be even more difficult to detect and distinguish than the corresponding two M states in the bR_{568} photocycle. Several other observations that are readily explained by the proposed mechanism can be found in the literature, especially in the

work from Khorana's and Heyn's laboratories, (e.g., refs. 40 and 41), but will not be elaborated here.

The proposed model should also be applicable to hR. The sequence of hR can be folded into seven α -helix segments analogous to bR (42, 43). In the resulting structure, Asp-85 of bR is replaced by Thr-111 in hR, whereas the residues corresponding to Arg-82 and Asp-212 are conserved. This should allow formation of a counterion analogous to that in bR_{564}^{HCl} which could support a protonated Schiff base near neutral pH. Thr-89 is replaced by serine in hR, whereas Asp-115, Arg-175, and Arg-134 are conserved. The same double-bond isomerization occurs in the photocycles, and the chromophore structures of the L intermediates are nearly identical (44). As in bR, the millisecond kinetics are significantly affected when Br^- is substituted for Cl^- (45), and the required, second L-like intermediate appears at least in the photocycle proposed for hR from *Natronobacterium pharaonis* (46). However, it remains to be shown, when Br^- is exchanged for Cl^- (47, 48), why the expected effects on the absorption maximum and resonance Raman spectra of hR are missing, and how NO_3^- can fit into the complex counterion, if it is indeed a transported ion (46). One should keep in mind that the 580-nm chromophore cannot be maintained in the complete absence of Cl^- (47) and that residual Cl^- effects may remain a problem.

The model outlined above is of course speculative, incomplete, and oversimplified, but it provides a plausible explanation for the alternative transport of H^+ or anions by nearly identical protein structures. Furthermore, since a high-resolution structure for photocycle intermediates of bR is unlikely to become available soon, testing the transport activity of point mutants for H^+ and Cl^- effects remains the most promising experimental approach, and the model suggests which mutants to select. It may also be interesting to search for evolutionary connections between other anion and cation transporters or for alternative anion and cation transport by the same protein. One example in which the role of intramolecular charge pairs has been postulated has recently come to our attention (49).

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