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Removal of transducer HtrI allows electrogenic proton translocation by sensory rhodopsin I

(seven-helix receptors/bacteriorhodopsin/phototaxis/signal transduction/ion translocation)

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ABSTRACT **Sensory rhodopsin I (sR-I) is a phototaxis receptor in halobacteria, which is closely related to the light-driven proton pump bacteriorhodopsin and the chloride pump halorhodopsin found in the same organisms. The three pigments undergo similar cyclic photoreactions, in spite of their different functions. In intact cells or isolated membranes sR-I is complexed with protein HtrI, the next link in the signal transduction chain, and does not function as an electrogenic ion pump. However, illumination of sR-I in membranes lacking HtrI causes pH changes in the medium, and its photoreaction kinetics become pH-dependent. We show here that in closed vesicles, near neutral pH it functions as an electrogenic proton pump capable of generating at least −80 mV transmembrane potential. The action spectrum shows a maximum 37 nm below the 587-nm absorption maximum of the native pigment. This apparent discrepancy occurs because the 587-nm form of HtrI-free sR-I exists in a pH-dependent equilibrium with a 550-nm absorbing species generated through deprotonation of one group with a pK_a of 7.2, which we have tentatively identified as Asp-76. We interpret the results in terms of a general model for ion translocation by the bacterial rhodopsins.**

Halobacteria contain a family of retinal proteins, the bacterial rhodopsins, that function as light energy converters and as light energy sensors. The light-driven proton pump, bacteriorhodopsin (bR) (1, 2), and the light-driven chloride pump, halorhodopsin (hR) (3), convert light energy into electrochemical potential energy. The sensory rhodopsin I (sR-I) (4) transiently stores light energy in an activated molecular conformation, that triggers a signal transduction chain, which affects the reversal frequency of the flagellar motor (for reviews see refs. 5–8).

The amino acid sequence of sR-I is homologous to that of bR and hR (9), as are its chromophore (10) and predicted secondary and tertiary structure (11). The polypeptide chain in all three pigments folds into seven transmembrane helices, labeled A through G, which are arranged in two parallel rows. All-*trans*-retinal is located between the two rows and bound as a protonated Schiffs base (SB) to a lysine residue near the center of the C-terminal helix G (12). The retinal environments in sR-I, bR, and hR also are similar. All three rhodopsins have absorption maxima that are 130–150 nm red-shifted compared to protonated retinal SBs in solution. After photoexcitation the pigments return spontaneously to the original state via a similar series of thermally activated transitions. These photocycles in bR and sR-I involve transient states with 13-*cis* unprotonated SBs, the M intermediate in bR, and S_{373} in sR-I. In the hR photocycle deprotonation of the SB occurs only as a side reaction. In bR, the intramolecular proton transfer reactions result in release and uptake of protons at opposite sides of the membrane, producing a net proton transport from the cytoplasm to the medium and an inside-negative membrane potential. During the sR-I photocycle in the wild-type membrane neither proton concentration nor membrane potential changes have been detected (4, 13), and the sR-I photocycle was found to be independent of external pH in the range 3.5–8.5 (14). The SB proton transfer reactions appear to occur entirely within the membrane.

sR-I in its native environment is associated with a membrane-bound signal transducer, HtrI a 57-kDa methyl-accepting protein, which is homologous to eubacterial chemotaxis receptors (15). Transfer of radioactive retinal to HtrI during treatment with a reducing agent suggested physical proximity of sR-I and HtrI (16). sR-I expressed in cells devoid of HtrI has altered photochemical kinetics; decay of S_{373} becomes highly pH-dependent, and protons are transiently released from the membrane (17, 18). These results indicate that the two proteins exist in the native membrane as a molecular complex, which prevents proton exchange between sR-I and the medium.

It has recently been shown that under special conditions bR may also translocate chloride (19) and hR protons (20), and this switching between anion and cation transport has been explained on the basis of a common model for the light-driven protein dynamics and ionization changes in these retinal proteins. We show here that the modified proton transfer reactions, when HtrI is removed, can convert sR-I into a proton pump and that the same dynamics and mechanisms proposed for bR and hR can explain this function of HtrI-free sR-I. The proton transfer reactions in the membrane may also be an important element for receptor/transducer coupling during signal transduction. This work has been presented in abbreviated form (42, §).

MATERIALS AND METHODS

Native and mutant forms of sR-I were expressed by transformation with plasmids pTR2Δ and pD76NtrΔ, respectively, in *Halobacterium salinarium* strain Pho81W, a strain that was isolated as a white (carotenoid-deficient) colony from Pho81, a mutant lacking the ion pumps hR and bR, both phototaxis receptors sR-I and sR-II, and HtrI (17). These expression plasmids, which contain a synthetic *sopI* gene (21) mutated by cassette mutagenesis, have been described in detail (18, 22). Transformants were grown aerobically in the dark at 37°C. In this paper we refer to the protein product of

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Abbreviations: bR, bacteriorhodopsin; hR, halorhodopsin; sR-I, sensory rhodopsin I; HtrI, halobacterial transducer for sR-I; sR_{587}, sR_{550}, S_{373}, species of sR-I with subscript indicating absorption maximum; H410, species of hR with 410-nm absorption maximum; K, L, M, N, photocycle intermediates of bR; FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone; TPP^+, tetraphenylphosphonium; SB, Schiff's base.

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pD76NtrΔ expressed from the *bop* promoter containing the extended N terminus and truncated C terminus as D76N. The similarly expressed form from pTR2Δ has been shown to have wild-type sR-I properties (18).

Preparation of Envelope Vesicles. Right-side out membrane envelope vesicles were prepared from cells producing sR-I or the mutated form D76N¶ by sonication, following standard procedures (23).

Spectroscopy and Electrochemistry. Light-induced pH and absorbance changes were measured simultaneously in a 1-cm pathlength, stirred and thermostated square quartz cuvette, in the four-port optical sample compartment of a homemade, single-beam spectrometer. One hundred-watt quartz-halogen and 75W Xe arc lamps with mechanical shutters provided continuous actinic illumination in the visible and UV range through appropriate heat-absorbing and wide-band color filters. Beams were combined by a beam splitter and focused on the sample cuvette through one of the optical ports. The measuring beam from a 55-W quartz-halogen lamp at 90° to the actinic beam was collimated with quartz optics, passed through 10-nm bandpass filters (Ditric Optics, Hudson, MA), and focused on the center of the cuvette. The photocurrent was monitored with a Hamamatsu (Middlesex, NJ) 928R photomultiplier and Keithley 610C electrometer amplifier and fed to one channel of a strip chart recorder. A semi-micro pH electrode (Beckman 39525) positioned close to the measuring beam recorded the pH via a Corning 110 pH meter in the second channel. Response times of the pH and absorbance measuring systems were about 1 s and 200 ms, respectively. Actinic light fluxes were measured with a Kettering Instruments (Riviera Beach, FL; model 68) internally calibrated thermopile detector.

Difference spectra from 350 to 750 nm in the 0.5- to 300-s time window were recorded with a Hewlett–Packard 8452A diode array spectrophotometer provided with a fiber optic illuminator (model 375; Dyonics, Andover, MA) for side actinic illumination and appropriate bandpass excitation and detector blocking filters (23).

Membrane potential changes in envelope vesicles were monitored by following redistribution of tetraphenylphosphonium (TPP^+) with a TPP^+ electrode, filled with a 10^{-3} M solution of TPP^+ in 3 M NaCl, and an Ag-$AgCl_2$ reference electrode in the extravesicular medium (24). The electrode response was near Nernstian in the range 10^{-3}–10^{-6} M TPP^+. The signal was amplified with a Keithley 610 electrometer and digitized with a Nicolet 4094 digital oscilloscope. To determine total intravesicular volume we measured the ESR signal amplitude of the permeant nitroxide spin label tempol before and after addition of impermeant chromate ions (24) and calculated the change in membrane potential as:

$$\Delta\Psi = \frac{RT}{F}\ln(v/V) - \frac{RT}{F}\ln\{\exp[F(E - E_o)/RT] - 1\}, \quad [1]$$

Where F is the Faraday constant, R is the gas constant, v and V are the internal vesicle volume and the extravesicular volume, respectively, E is the electrode potential difference, and E_o is the electrode potential at standard TPP^+ concentration. We used vesicle concentrations of 2–10 mg of protein per ml and modified the vesicle interior milieu by diluting vesicle suspensions from 4 M to 3 M NaCl, in the presence of the desired combinations of salts and/or buffers. Carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) was added in ethanol solution keeping the final ethanol concentration in the sample below 1%.

¶To characterize mutants we use the one-letter code for amino acids; thus D76N designates a mutant in which Asp-76 has been replaced by asparagine.

RESULTS

Cell envelope vesicles containing HtrI-free sR-I under constant illumination with orange light show an acidification of the suspending medium (Fig. 1) and an absorbance increase at 380 nm. The pH signal rises in two phases, a fast, small acidification followed by a slower further decrease in pH to a photo-steady state. Its maximum amplitude amounts to 5 or 6 H^+/S_{373} estimated from the 380-nm absorbance. Upon addition of FCCP only a small acidification of about 1 H^+/S_{373} remains. The extent of the pH change and the inhibitory effect of the proton ionophore show that in the light protons are translocated across the membrane. The extent of the acidification in the presence of uncoupler is comparable to that observed earlier (18) for suspensions of membrane sheets using pyranine as a pH indicator dye and reflects the stoichiometric proton release upon formation of S_{373}. The absorbance change at 380 nm (Fig. 1 *Inset*) was measured simultaneously with the pH change after addition of FCCP. Neither the decay kinetics of the absorbance signal nor its amplitude was significantly affected by addition of the ionophore (data not shown). Apparently these vesicles, in the absence of FCCP, can maintain a proton gradient of 4 or 5 protons per sR-I molecule cycling under the given experimental conditions.

A substantial light-induced transmembrane electrical potential (negative inside) confirms that the acidification is caused by vectorial proton transport into the medium. The potential rises to a steady-state level appreciably faster than the pH signal and reaches a maximal amplitude of 80 mV at the moderate actinic light intensity used (Fig. 2). The proton ionophore FCCP completely inhibits the light-induced electrical potential (data not shown), indicating that the residual acidification observed in the presence of FCCP reflects transient changes in proton binding (and/or electroneutral proton exchange across the membrane).

The light-induced change of external pH is maximal near pH 7 and is undetectable above pH 8.5 or below pH 6.0. Near pH 6 we observed virtually no light-induced pH changes and a very small accumulation of S_{373} in the steady state, as expected from its accelerated thermal decay (17). A stoichiometric proton release from this small fraction of photocon-

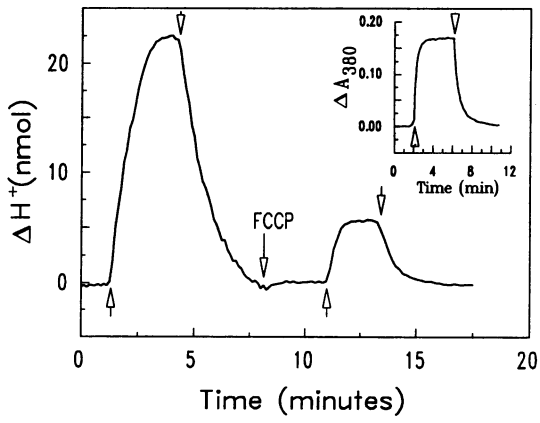

FIG. 1. Light-induced acidification in an unbuffered envelope vesicle suspension of *H. salinarium* Pho81 containing sR-I but not HtrI. (*Inset*) Corresponding absorbance changes at 380 nm for the same sample in the presence of FCCP. The absorbance change in its absence was virtually identical (data not shown). Medium, 4 M NaCl (pH 7.1); pathlength, 1 cm; actinic light, 560–700 nm, 5×10^5 erg/cm²·s (1 erg = 0.1 μJ). Vesicle concentration, 7 mg of membrane protein per ml. From the data we calculate that ≈5 H^+/sR-I are translocated. The residual acidification after addition of ionophore is attributable to the net proton release from S_{373} in the photo-steady state. Calculated stoichiometry: $S_{373}/H^+ = 0.95 \pm 0.15$, using: e_{380} (S_{373}) = 47,000 $M^{-1}\cdot cm^{-1}$ (25).

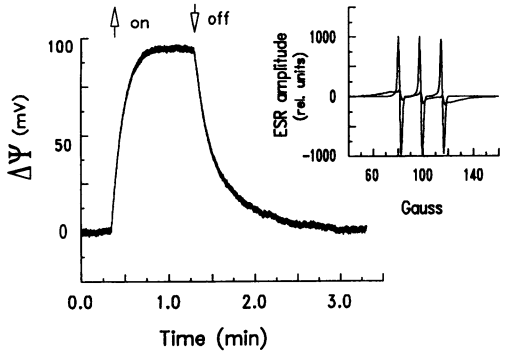

FIG. 2. Light-induced uptake of the permeant tetraphenylphosphonium (TPP^+) cation by an envelope vesicle suspension measured with a TPP^+-sensitive electrode (conditions as in Fig. 1, but with 2.5 mg of protein per ml). (*Inset*) Quenching of the ESR signal from the permeant tempol probe after adding nonpermeant chromate ions to the suspension. The ratio of amplitudes equals the ratio of total to internal volume, because the broadening effect of the paramagnetic chromate ions abolishes the extravesicular signal. The data were used to calculate the internal volume and to calibrate the electrical potential difference scale with the assumption that the vesicle volume is much smaller than the total volume.

verted pigment would be undetectable in the noise of the pH trace. Near pH 7 we converted 15–20% of sR-I to S_{373} in the photo-steady state at the highest available light intensity, which still did not saturate the pump, and observed an external increase of 7–9 H^+/S_{373}, clearly more than one proton per total sR-I molecule present. The fraction of sR-I converted into S_{373} at pH 8.0 increased significantly, and the magnitude of the proton concentration change was roughly stoichiometric with this amount. A decrease in pumping activity at high pH is expected, because of the much slower S_{373} decay. It is compounded by the much higher buffering capacity, which probably prevents buildup of a measurable proton gradient. However, a small electric photopotential could still be detected at this pH as well as at pH 6.0. (see *Discussion*).

The action spectrum for the light-induced membrane potential at neutral pH peaks near 550 nm (Fig. 3), well below the 587-nm absorbance maximum of the vesicle preparation, indicating that proton translocation is mediated not by sR_{587}, but by another photoactive species. In the membranes containing HtrI alkalinization generates a 552-nm absorbing species from sR-I with a pK of ≈8.5 (26). In HtrI-free sR-I this spectral transition is shifted to pH 7.2 (Fig. 4), and the titration curve indicates deprotonation with a single pK. Therefore, in our envelope vesicle suspension at pH 6.0 the 587-nm form predominates while at pH 7.9 the pigment is

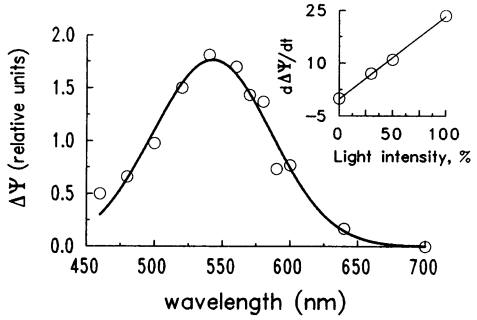

FIG. 3. Action spectrum for the initial rates of TPP^+ uptake by the sample of Fig. 2. The actinic wavelengths were defined by narrow-band (10 nm) interference filters. (*Inset*) Linearity of responses in the range of actinic light intensity used, shown for the 580-nm point.

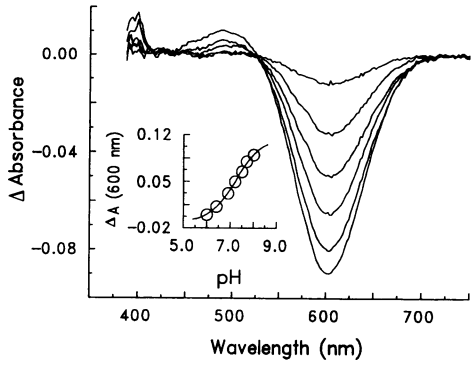

FIG. 4. pH-induced, dark difference spectra between pH 6.1 and 7.9 for the sample of Fig. 1, using pH 6.1 as the reference. From top to bottom (in the depletion) the traces correspond to pH 6.4, 6.9, 7.2, 7.5, 7.7, and 7.9. (*Inset*) The 600 nm amplitude as a function of pH. The solid line between points fits the titration of a single group.

nearly completely converted to the short-wavelength form. The isosbestic point at 525 nm is blue-shifted with respect to that for the alkaline transition in the native pigment, which is at 560 nm (26). A 540- to 550-nm absorbance depletion of another HtrI-deficient preparation has recently been reported, but interpreted differently (27).

Both forms of HtrI-free sR-I are photochemically active and generate spectroscopically identical S_{373} intermediates (Fig. 5). The depletion maximum shifts from 590 nm at pH 6.0 to 550 nm at pH 7.9. Because of the large spectral separation between the parent forms and the photoproduct these wavelengths must be very close to their absorption maxima. We followed kinetically the decay of S_{373} in the range 350–750 nm at the two pH values, and, as previously reported (17), $t_{1/2}$ increases by almost two orders of magnitude over that pH range. The S_{373} decay is monophasic even at intermediate pH values where the parent acid and alkaline forms coexist. Therefore, the external proton concentration must affect the S_{373} product of both species equally, and the good isosbestic points observed at low and at high pH (data not shown) indicate photoconversion between only two species.

At neutral pH the two spectral forms are present in roughly equal concentrations. Since the action spectrum for potential generation has a maximum near the absorption maximum of sR_{550}, it, and not sR_{587}, apparently is the proton-pumping species. To exclude that deprotonation of an sR-I group, without removal of HtrI, is sufficient to confer proton-pumping activity to sR-I, we adjusted the total pigment concentration in retinal-regenerated Flx5R vesicles, which contain the sR-I/HtrI complex (28), to be comparable to that of the HtrI-free sR-I vesicles and checked for light-induced

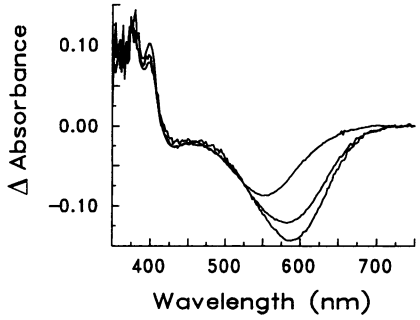

FIG. 5. Light–dark difference spectra at pH 6.1, 7.4, and 7.9 with maximal depletion at 590, 580, and 550 nm, respectively. The traces are the measured spectra, normalized at the 525-nm isosbestic point for the acid–base transition (Fig. 4), so that the correct ratios for the absorbance changes are shown.

membrane potential generation. At pH 8.7 these vesicles had a maximum absorbance depletion near 550 nm under constant light, indicating that the alkaline form is the dominant species. Neither at this nor any other pH in the range 5–8.7 did we observe light-induced membrane potentials or proton release.

An efficiency estimate of the translocation process indicates a yield of 0.23 H^+/light quantum absorbed by sR_{550}. It is based on the incident light fluxes used, the sR_{550} absorbance at the actinic wavelength, the initial rate of proton release, and the assumption that these are pumped protons (see *Discussion*).

DISCUSSION

The results reported here demonstrate that an alkaline form of sR-I can function as a moderately efficient, light-driven, electrogenic proton pump and that in the native membrane complex formation with HtrI prevents release of protons. They support the concept that the different physiological functions of the bacterial rhodopsins can be understood as variations of the same fundamental mechanism (19).

The main groups known to mediate proton pumping in bR are the proton donor Asp-96 near the cytoplasmic surface of the membrane, and the proton acceptor Asp-85, which together with Asp-212, Arg-82, and other groups forms a complex counterion for the protonated SB, that connects it to the external surface of the membrane during the first half of the photoreaction cycle (29–32). At the homologous positions in sR-I, Asp-76 (D85 in bR), Asp-201 (D212 in bR), and Arg-73 (R82 in bR) are conserved, whereas Asp-96 in bR is replaced by Tyr-87 in sR-I. However, it is known that Asp-96 is not essential and that bR still pumps protons at a reduced rate if Asp-96 is replaced by Asn or Ala, and full pumping activity can be restored by the addition of azide (33). Azide does not significantly affect reactions of sR-I complexed with HtrI, but it does accelerate S_{373} decay in its absence (E. N. Spudich and J.L.S., unpublished data). The apparently crucial difference between bR and sR-I is that in the native membrane under physiological conditions Asp-76 is protonated (22, 34) and thus cannot function as a proton acceptor. Protonation of D85 in the blue membrane or its replacement by a nonionizable residue—e.g., in the D85N[1] mutant—also inhibits proton pumping in bR (19, 35). This is true also when D85N is expressed in *Halobacterium* (J. K. Lanyi, personal communication). It follows that D76 presumably is the group that deprotonates in the alkaline 587 → 550 nm transition of HtrI-free sR-I and this conclusion is confirmed by the observation that the sR-I mutant D76N even in the absence of HtrI does not undergo this transition (tested up to pH 8.7; K.D.O. and J.L.S., unpublished).

The action spectrum shows that the alkaline form of HtrI-free sR-I pumps protons and that the proton-pumping activity of the protonated form, if any, is not significant. Nevertheless, the latter forms a S_{373} intermediate and in the absence of HtrI transiently releases protons with a stoichiometry of 1 H^+/S_{373}; so does the mutant D76N. This release and reuptake apparently occur on the cytoplasmic side, because release to the medium after a light flash is detected only if the vesicles are disrupted by low salt or by specific detergents (unpublished data). Furthermore, azide, which at least in bR and hR mediates proton conduction only from the cytoplasmic side, increases the rate of S_{373} decay. Proton release on the cytoplasmic side is observed also after SB deprotonation in H410 formation of hR, at alkaline pH and/or in the presence of azide (36). Light absorption by H410, the species of hR having a 410-nm absorption maximum, as by the M intermediate of bR (37), causes a rapid SB reprotonation from the external side (20). The analogous photoreaction apparently takes place in HtrI-free sR-I, because addition of near-UV light to orange illumination causes a decrease in

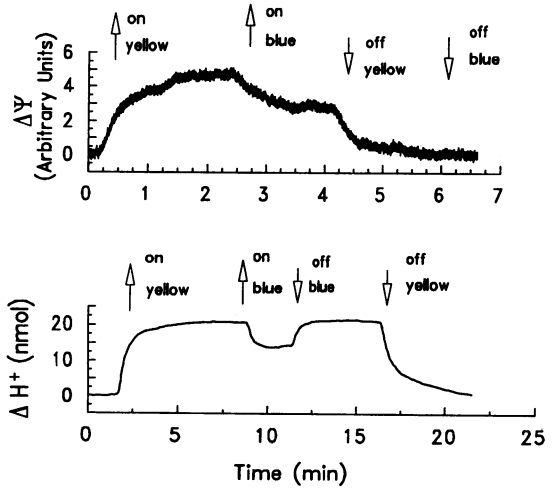

FIG. 6. Blue light reduction of the proton gradient and membrane potential generated by orange illumination. Blue irradiation (350–420 nm, 2×10^5 ergs/cm^2-sec) was superimposed on yellow actinic light (550–650 nm, 5×10^5g/cm^2-sec) for electrical potential (upper trace) and pH (lower trace) measurements. Blue illumination alone at this intensity has no effect (now shown).

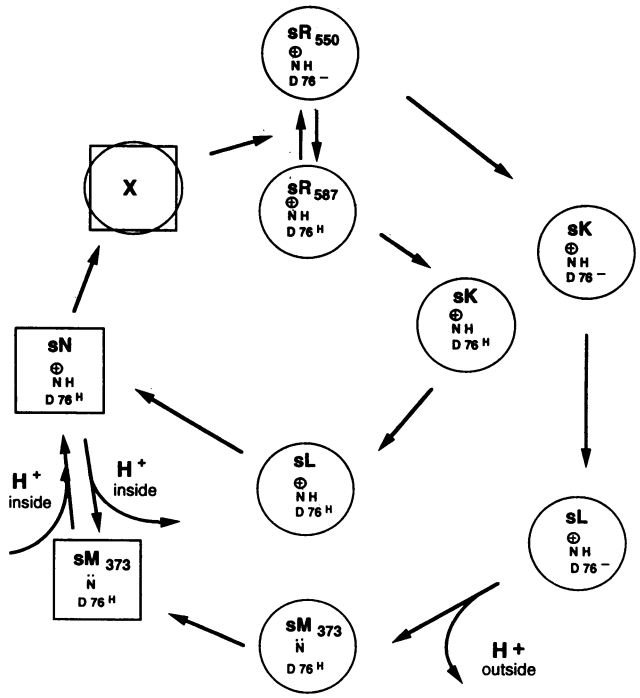

FIG. 7. Proposed photocycles for the acid and alkaline forms of sR-I that fit a general scheme for the photoreactions of halobacterial retinal pigments. The protonation changes for Asp-76 and the SB are indicated. To emphasize the analogy to bR (and hR), the corresponding intermediates are labeled as in the bR cycle: K, L, N, and M, but preceded by s, so that S_{373} becomes sM_{373}, etc. We introduce sN here to indicate that it, like N or bR, has undergone the conformational change that switches the SB connection from the extracellular surface ("out") of sL to the cytoplasmic surface ("in"). The switch is symbolized here by the change from circles to squares. Only the sM intermediates have a deprotonated SB. In the sR_{550} cycle the switch in surface connection occurs between the first and second sM intermediate. The sM_{373} intermediate of the sR_{587} cycle arises only in a side reaction, analogous to the H410 intermediate of the hR cycle (19, 20).

proton pumping, whereas near-UV light alone is without effect (Fig. 6).

The observation of proton pumping by sR-I thus emphasizes again a key feature in the function of bacterial rhodopsins, which has been invoked before to explain chloride pumping by bR and hR (19) and proton pumping by hR (20); the molecules can undergo the conformational change that switches the SB connection from the external to the cytoplasmic surface with an unprotonated or protonated SB and can release the proton on either side. The protonated SB can also carry a mobile counterion with it, and release it on the cytoplasmic side. A scheme for the photocycles of the HtrI-free acid and alkaline sR-I forms, that emphasizes the similarity to bR and hR, is given in Fig. 7. It is still speculative but has already proved to be of heuristic value.

The wild-type sR-I/HtrI complex does not pump and the mutant D76N is active in phototaxis (22). Therefore, neither proton pumping nor protonation changes of D76 are necessary for sensory signaling; only generation of the signaling state S_{373} in the presence of HtrI appears to be required. The proton released from the SB and prevented from leaving the membrane may transiently bind to HtrI and trigger its signal transmission via cytoplasmic components to the flagellar motor. Alternatively, the conformational change of sR-I in the S_{373} state may trigger it.‖ Further studies are needed to distinguish these possibilities. Interestingly, bovine rhodopsin also exhibits protonation changes in its photoactive site upon light activation (40) and other seven-helix membrane receptors—e.g., cholinergic and adrenergic receptors—may undergo charge rearrangements by binding their agonists (41).

‖We note that blue shifts and additional peaks at wavelengths shorter than 590 nm have been reported in phototaxis action spectra for various *H. salinarium* strains, which could not be conclusively attributed to the pigments known to be present (discussed in ref. 5), and that high light intensity shifts the action spectrum maximum to shorter wavelength (38). If sR_{550} is formed under the conditions of these experiments and can activate HtrI, it could explain these results, and may argue against signal transmission by the proton. However, signaling by a generated proton electrochemical gradient would also have to be considered in these cases, because it has recently been shown to initiate a phototactic response by a different pathway (39). These observations may also provide interesting clues for understanding the evolution of phototactic responses.

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