Proton Circulation During the Photocycle of Sensory Rhodopsin II

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ABSTRACT Sensory rhodopsin II (SRII) in *Halobacterium salinarum* membranes is a phototaxis receptor that signals through its bound transducer HtrII for avoidance of blue-green light. In the present study we investigated the proton movements during the photocycle of SRII in the HtrII-free and HtrII-complexed form. We monitored sustained light-induced pH changes with a pH electrode, and laser flash-induced pH changes with the pH indicator pyranine using sealed membrane vesicles and open sheets containing the free or the complexed receptor. The results demonstrated that SRII takes up a proton in M-to-O conversion and releases it during O-decay. The uptake and release are from and to the extracellular side, and therefore SRII does not transport the proton across the membrane. The pH dependence of the SRII photocycle indicated the presence of a protonatable group ($pK_a \sim 7.5$) in the extracellular proton-conducting path, which plays a role in proton uptake by the Schiff base in the M-to-O conversion. The extracellular proton circulation produced by SRII was not blocked by HtrII complexation, unlike the cytoplasmic proton conduction in SRI that was found in the same series of measurements to be blocked by its transducer, HtrI. The implications of this finding for current models of SRI and SRII signaling are discussed.

INTRODUCTION

Sensory rhodopsin II (SRII; λ_{max} 487 nm), is one of the photoreceptors present in the plasma membrane of the archaeon *Halobacterium salinarum* and functions as a sensor for phototactic avoidance of blue-green light (Takahashi et al., 1985, 1990). The other phototaxis receptor SRI (λ_{max} 587 nm) mediates attractant responses to orange light as well as repellent responses to violet light (Spudich and Bogomolni, 1984). Both receptors transmit their signals through integral membrane transducer proteins HtrI (Yao and Spudich, 1992) and HtrII (Zhang et al., 1996), which are present in multi-subunit signaling complexes with SRI and SRII, respectively (reviewed by Hoff et al., 1997).

SRI and SRII are structurally similar to bacteriorhodopsin (BR) and halorhodopsin (HR), which are light-driven pumps for protons and chloride, respectively (Lanyi, 1997; Oesterhelt, 1998). These proteins share a common architecture that consists of seven transmembrane helices surrounding a retinal chromophore covalently bound in a protonated Schiff base linkage to a lysine residue in the seventh helix (helix G). They also exhibit similar light-induced reaction cycles that are initiated by photoisomerization of the chromophore from all-*trans* to 13-*cis* and produce a series of spectrally distinct intermediates that recover thermally to the unphotolyzed state (photocycles).

SRI, when free of HtrI, has been found to carry out electrogenic proton transport like BR (Bogomolni et al., 1994; Haupts et al., 1996). To explain this observation, phototaxis signaling by the SRs was proposed to be brought about by the same processes as occur in the electrogenic pumps (Spudich, 1994). Analysis of mutants (Spudich et al., 1997; Jung and Spudich, 1998) has led to a more detailed model in which the second transmembrane helix of the Htr proteins physically interact with SR helices to detect a conformational change involving movement of the SR cytoplasmic regions of helices F and G (Spudich, 1998), which in BR opens a cytoplasmic channel for proton uptake during the pumping process (Lanyi, 1995).

Consistent with the model, functional interaction of SRI with HtrI, and SRII with HtrII, occurs through their transmembrane helices (Zhang et al., 1999), and light-activation of SRI causes movement of the second transmembrane helix (TM2) of HtrI (Zhang and Spudich, 1998). The work presented here establishes that HtrI complexation blocks light-driven proton pumping by SRI, consistent with HtrI coupling to the receptor's cytoplasmic channel, and extends the analysis of proton movements to SRII, which we found to take up and release protons from and to the extracellular side of the molecule, without transmembrane transport. Therefore, SRII differs from SRI in that the presumed conformational change on the cytoplasmic side does not enable proton uptake on that side. The HtrII transducer does not block the *extracellular* proton circulation in SRII.

MATERIALS AND METHODS

Membrane preparation

H. salinarum membranes containing SRI in the absence and presence of HtrI, and SRII in the absence and presence of HtrII, were produced by transformation of the mutant Pho81Wr$^-$ (Perazzona et al., 1996), which lacks the four archaeal rhodopsins (BR, HR, SRI, and SRII) and the two transducers (HtrI and HtrII) by plasmid expression of *sopI*, *htrI* · *sopI*, *sopII*, and *htrII* · *sopII* genes, respectively. Expression of transducer-free SRI and SRII used the *bop* promoter as described (Krebs et al., 1995), and expression of both SR-Htr complexes used the *htrI* promoter as described with the native *htr-sop* gene pairs (Spudich et al., 1997; Zhang et al., 1999).

Membranes containing both SRI and SRII were produced by transformation with expression plasmid pKJ70, which contains the *sopII* gene

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under the control of the *bop* promoter in the *H. salinarum* strain ENS112, which has a chromosomal copy of the *sopI* gene under the control of the *bop* promoter at the *bop* locus (Krebs et al., 1995).

Membrane vesicles were prepared by sonication and membrane sheets were prepared by dialysis of the cells against 250 mM KCl at 4°C for >12 h, as described (Olson and Spudich, 1993). The membranes were finally pelleted by centrifugation at 48,000 rpm (Beckman, rotor type 70 Ti), for 60 min at 4°C, resuspended in 25 mM Tris buffer (pH 6.8) containing 4 M NaCl, and stored at 4°C.

Proton pumping measurements

Membranes were washed with 4 M NaCl three times by centrifugation as described above and finally suspended in 4 M NaCl, 10 mM MgCl, and 0.1 mM MES buffer at pH values indicated in the figures. The light source for the illumination of the samples was a 24 V 150 W tungsten-halogen projection lamp. The light was passed through a 3-cm-thick $CuSO_4$ solution and three heat filters to remove infrared wavelengths, and one interference filter or cutoff filter to select the desired visible region of the spectrum, and then focused onto the sample by a lens. pH was probed by a pH electrode (Beckman Electrode 39532) and monitored by a pH meter (Beckman F72 pH meter), which was connected to a personal computer through an RS232C cable.

Flash photolysis

Flash-induced absorption transients in the millisecond to seconds time domain were acquired on a digital oscilloscope (Nicolet, Integra20) following an Nd-YAG laser flash (Continuum, Surelight I; 532 nm, 6 ns, 40 mJ) in a lab-constructed flash photolysis system as described (Sasaki and Spudich, 1998); 32–256 transients were collected for each measurement.

For the measurement of pH changes during the photocycle, the membranes were pelleted by centrifugation at 75,000 rpm (Beckman TL100) and suspended in 4 M NaCl. This process was repeated three times. Flash-induced absorbance changes were measured at 500, 450, and 400 nm, 1:20 vol 500 mM pyranine (8-hydroxy-1,3,6-pyrenetrisulfate acid trisodium salt, Kodak) in 4 M NaCl was added to the membrane suspension, and the absorption changes at 500, 450, and 400 nm remeasured. Finally, 1:20 500 mM MES in 4 M NaCl (pH 7) was added and the measurements were repeated.

RESULTS

Proton release and uptake by SRI and SRII during illumination

Proton pumping activity of SRI driven by light was measured by monitoring the pH of a membrane vesicle suspension with a pH electrode (Fig. 1). Membrane vesicles at neutral pH containing HtrI-free SRI expressed in strain Pho81 exhibited a pH decrease when illuminated with yellow (>520 nm) light (Fig. 1 *a, trace 2*), indicating light-induced proton ejection from the vesicles during the illumination. The number of protons pumped per SRI molecule during the illumination was ~5, in agreement with the value reported previously by Bogomolni et al. (1994). The pH change from SRI in nonvesicular membrane sheets, produced by exposing the membrane vesicles to a low salt solution (250 mM KCl) and returning them to high salt, corresponded to ~1 proton per SRI (Fig. 1 *a, trace 3*), as expected from stoichiometric release of the proton from the Schiff base, demonstrating that the value of 5 derives from

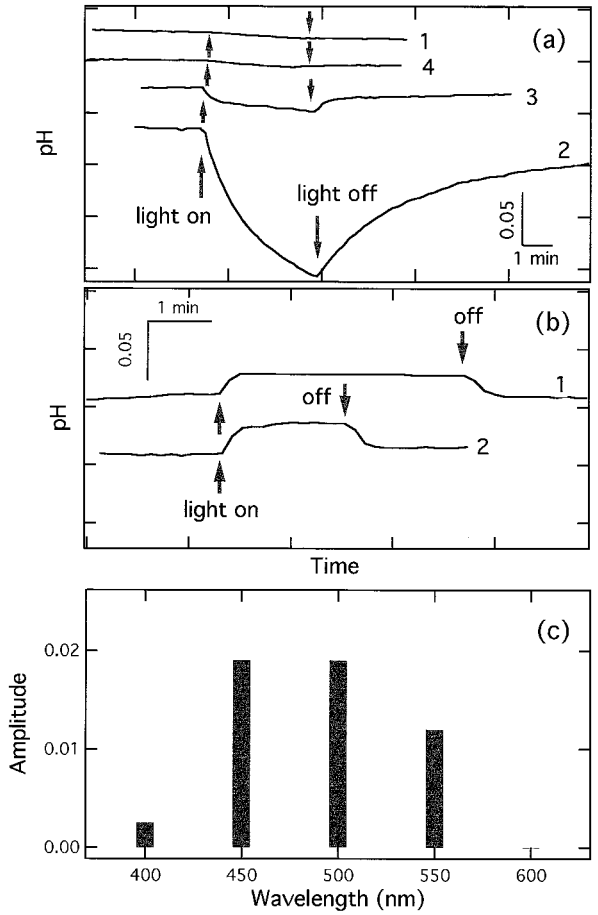

FIGURE 1 (*a*) pH changes of membrane suspensions upon illumination with yellow light (>520 nm). *Trace 1:* membrane vesicles of Pho-81; *traces 2* and *3:* membrane vesicles and sheets, respectively, containing free SRI without HtrI; *trace 4:* membrane vesicles containing SRI · HtrI complex. The onset and the end of the illumination are shown with up and down arrows, respectively. (*b*) pH changes of membrane suspensions upon illumination with 500-nm light. *Trace 1:* membrane vesicles containing free SRII; *trace 2:* membrane sheets containing free SRII. (*c*) Wavelength-dependence of the amplitude of the pH changes of membrane sheets containing free SRII.

transport. As a control, membrane vesicles of *H. salinarum* Pho81 (that lack all of the archaeal rhodopsins) exhibited no light-induced pH change (Fig. 1 *a, trace 1*). Also, SRI coexpressed with HtrI showed negligible pH change during the illumination (*trace 4*), confirming the previous report that HtrI binding suppresses SRI pumping activity (Bogomolni et al., 1994).

Similar measurements were carried out for membranes containing SRII prepared with the same method. In contrast to free SRI, illumination of membrane vesicles containing SRII at neutral pH with blue-green light (500 ± 20 nm) caused a small pH increase without further change during the prolonged illumination (Fig. 1 *b, trace 1*). The pH returned to the initial value when the light was turned off. The same measurement using nonvesicular membrane sheets showed the identical result (Fig. 1 *b, trace 2*), demonstrating that the pH change derives from passive uptake

and release rather than transmembrane proton transport. Our interpretation is that SRII takes up protons from outside the vesicles when photocycle intermediates are accumulated by continuous illumination and releases them back to the same side with the decay of the intermediates. The wavelength dependence of the pH increase from illumination of the SRII-containing vesicles roughly matches that expected for SRII, which absorbs maximally in the 450–500 nm range (Fig. 1 *c*).

In order to prove that SRII was present in sealed vesicles oriented in the same manner as the vesicles containing SRI, we prepared *H. salinarum* cells that produce both SRI and SRII by transforming a *sopI* integrant of Pho81 with a plasmid encoding SopII, based on the assumption that SRI and SRII insert into the membrane in the same orientation, as is expected from their high homology. Laser flash-induced absorption transients of membranes prepared from a transformant colony shows both pigments present with SRI at ~20% the amount of SRII (Fig. 2 *a*). Yellow light (>520 nm) induced acidification of membrane vesicles from this transformant (Fig. 2 *b, trace 1*) in a similar manner as from yellow light photoexcitation of SRI shown in Fig. 1 *a*. Accordingly, we attribute this signal to SRI. When illuminated with blue light (450 ± 20 nm), which is predominantly absorbed by SRII, a pH increase at the onset and decrease at the end of the illumination are superimposed on a small acidification signal (Fig. 2 *b, trace 2*). Our interpretation is that light induces a pH increase through SRII and a decrease through SRI in the same vesicles.

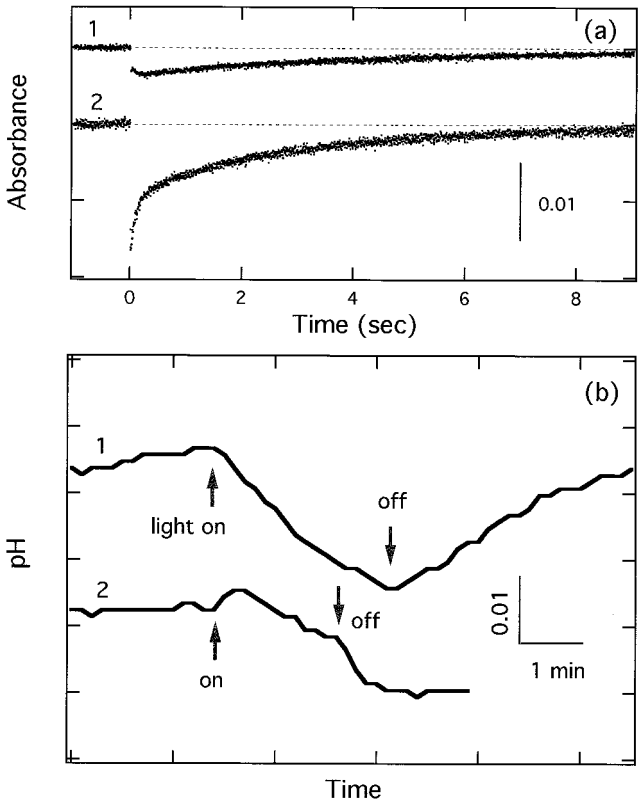

FIGURE 2 (*a*) Flash-induced absorption changes of the membrane suspension containing both free SRI and free SRII. The transients measured at 590 (*trace 1*) and 490 nm (*trace 2*) represent the depletion and recovery of SRI and SRII, respectively, upon the excitation. (*b*) pH changes of the suspension of membranes containing SRI and SRII upon illumination with >520-nm light (*trace 1*) or with 450-nm light (*trace 2*). The onset and the end of the illumination are shown with up and down arrows, respectively.

The kinetics of proton uptake and release in the photocycle of SRII

The pH increase during the illumination of SRII membranes suggests photostationary state accumulation of a photocycle intermediate whose formation correlates with proton uptake. Accordingly, we monitored formation and decay of the two known intermediates in the SRII photocycle, M at 360 nm and O at 540 nm, and simultaneously monitored pH changes with pyranine as previously described for membranes containing SRI (Olson et al., 1992). In our previous measurement, no other kinetic components due to the evolution of an N intermediate between M and O were observed, although the spectral shape of O, which was unusually broad in the free-SRII photocycle, indicated the coexistence of N and O in equilibrium (Sasaki and Spudich, 1998). Here we denote the kinetic component that arose concomitantly with the decay of M as O, as in earlier publications. Flash-induced absorption transients of HtrII-free SRII in membrane vesicles at neutral pH show the first intermediate after the flash at the data acquisition rate used is the unprotonated Schiff base intermediate M. M decays with ~80-ms half-life, as is evident in the 360-nm trace (Fig. 3 *a, trace 1*) with concomitant formation of the O intermediate seen as the rise at 540 nm (Fig. 3 *a, trace 2*). O decays to the initial state with 1.2-s half-life, as is seen in the decay of 540-nm absorbance and the recovery at 490 nm (Fig. 3 *a, trace 3*). These observations are in agreement with previous measurements of free SRII in 4 M NaCl and 25 mM Tris buffer at pH 6.8 (Sasaki and Spudich, 1998).

Absorption changes due to pyranine protonation changes were obtained by subtraction of absorption transients in the presence and absence of 25 mM pyranine at 400 and 450 nm. Pyranine signals at 450 nm are shown in Fig. 3 (*trace 4* in each panel); negative transients with opposite features were observed at 400 nm (not shown). The difference transients at 500 nm, where pyranine has negligible absorbance, exhibited no signal, confirming that the signals at 450 and 400 nm are due to pyranine but not to the perturbation of the SRII photocycle by pyranine. The rate of pyranine signal increase and decrease matched O formation and decay (Fig. 3). After these measurements, the membrane suspensions were buffered by adding 1:20 vol of 500 mM MES buffer, and the light-induced transients at 500, 450, and 400 nm were remeasured to confirm that the pyranine signal had vanished, confirming that the pyranine signals were caused by pH changes. The results demonstrate that the pH changes are ascribable to ~1 proton taken up and released per SRII molecule with the M-to-O conversion and the O decay, respectively.

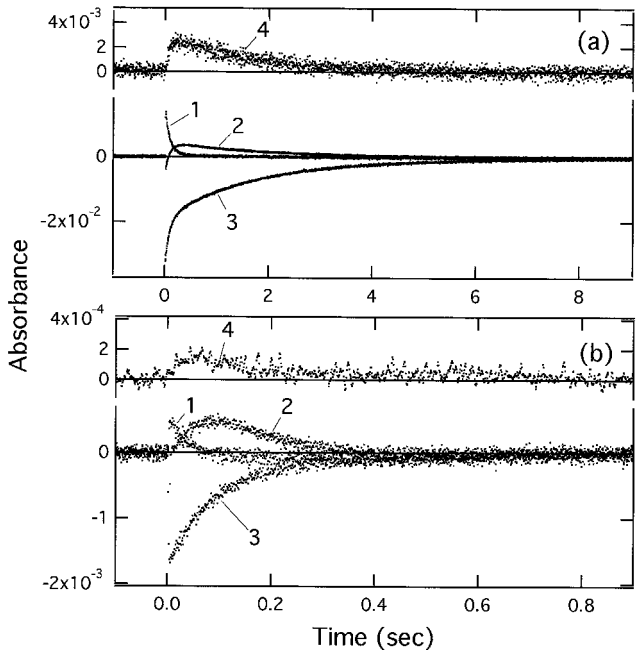

FIGURE 3 (*a*) Flash-induced absorption changes of free SRII measured at 360 nm (*trace 1*), 540 nm (*trace 2*), and 490 nm (*trace 3*) in 4 M NaCl at pH 6.8. Measurements were made also at 450 nm before and after 25 mM pyranine was added. The pyranine signal was obtained by subtraction of the former from the latter (*trace 4*). (*b*) Flash-induced absorption changes of the HtrII-complexed SRII measured at 360 nm (*trace 1*), 540 nm (*trace 2*), and 490 nm (*trace 3*) in 4 M NaCl at pH 6.6, and the pyranine signal at 450 nm (*trace 4*).

The kinetic profile of the pyranine signal was similar to those of membrane sheets containing BR mutants R82Q, E194Q, and E204Q, in which residues involved in fast proton release are substituted, causing a delayed proton release until the decay of the O intermediate (Govindjee et al., 1996; Brown et al., 1995; Balashov et al., 1997; Dioumaev et al., 1998). However, in the case of SRII, the pyranine signal was observed in membrane vesicle suspensions in which only extracellular side proton exchange was detected. We conclude that in SRII the proton uptake in M-to-O conversion as well as the release in O-decay occurred at the extracellular side of the molecule.

The pyranine measurements were also performed using HtrII-complexed SRII in membrane vesicles. The photochemistry in 4 M NaCl at pH 6.6 (Fig. 3 *b*) agrees well with our previous data in 25 mM Tris buffer and 4 M NaCl at pH 6.8 (Sasaki and Spudich, 1998). The M intermediate produced by photoexcitation converts to the O with a 37-ms half-time (Fig. 3 *b, traces 1* and *2*) followed by the decay to the initial state with 70-ms half-time (Fig. 3 *b, traces 2* and *3*). The rate of conversion is considerably accelerated in the complex compared to the free form, as described previously (Sasaki and Spudich, 1998). The pyranine signal was observed in this case also showing a pH transient that coincides with O formation and decay, respectively (Fig. 3 *b, trace 4*). The results demonstrate that proton uptake and release also occur in the complexed SRII in the same direction as in free SRII.

pH dependence of the photocycle of SRII

Inasmuch as SRII undergoes proton exchange with the outer milieu during its photocycle, it is expected that the proton uptake process would be affected by the external pH. As

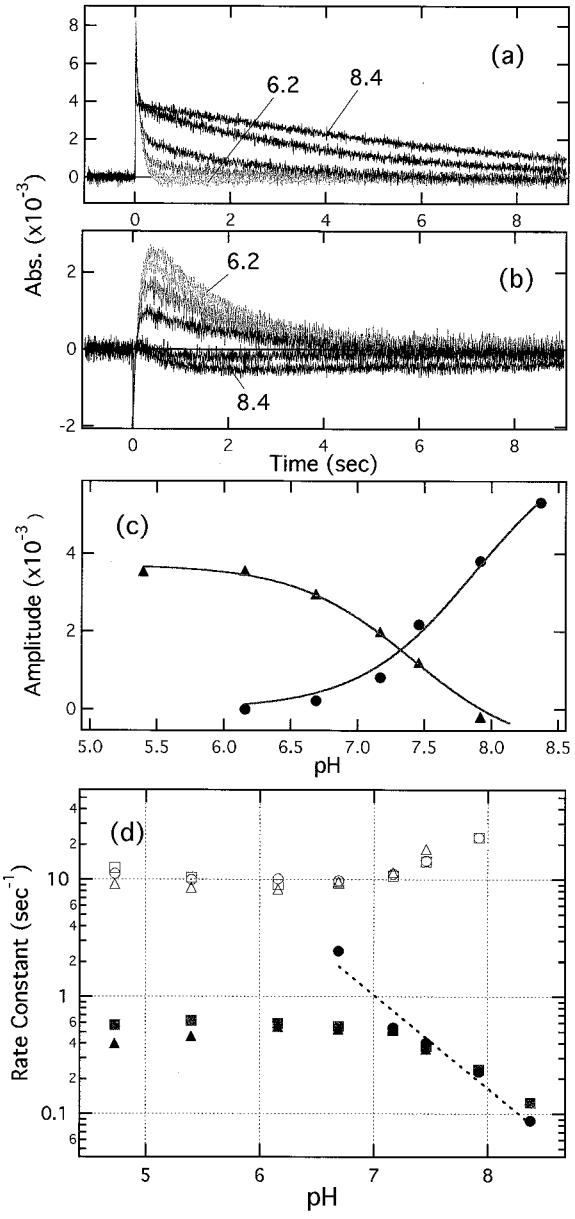

FIGURE 4 (*a*) Flash-induced absorption changes of free SRII at 360 nm measured at pH 6.2, 6.7, 7.2, 7.9, and 8.4 in the order of increasing amplitude of the slower phase. (*b*) Flash-induced absorption changes of free SRII at 540 nm measured at pH 6.2, 6.7, 7.2, 7.9, and 8.4 in the order of decreasing amplitude. (*c*) Traces in (*a*) and (*b*) were fit with two exponentials, and the amplitude of the slower components at 360 nm (*filled circles*) and 540 nm (*filled triangles*) plotted as a function of pH. (*d*) Rate constants obtained as the result of two exponential fits of the 360-, 490-, and 540-nm traces, plotted as a function of pH. The rates of faster components are shown with open circles (360 nm), open squares (490 nm), and open triangles (540 nm), while those of slower components are shown with filled circles (360 nm), filled squares (490 nm), and filled triangles (540 nm).

shown in Fig. 4 *a*, above pH 7 a portion of M began to show a slower decay rate and the amplitude of the slow M increased as the pH increased. Concomitantly, the amplitude of O decreased as the pH was raised (Fig. 4 *b*). However, even at pH values at which the slow M appears, O formation coincided with the fast M-decay, which appears to be pH-independent, suggesting that O accumulates after the fast M but not after the slow M. This may indicate the presence of parallel pathways for the reactions in which the fast M decays to O independently of the pH and the pH-dependent slow M decays to SRII without measurable accumulation of O, probably because the O decay is faster than that of slow M. Apparently, the two different pathways arise from two different substates of SRII, the conversion between them being pH-dependent.

The transients at 540, 490, and 360 nm at various pH values were fit with two exponentials, and the amplitude of the slower components of the 540-nm and 360-nm traces, which represent the amount of O and the slow M, respectively, were plotted as a function of pH (Fig. 4 *c*). The conversion from one substate of SRII to the other, which produces the fast M and the slow M, respectively, occurs with a single pK_a of near 7.5, indicating deprotonation of a residue (X-H in Fig. 5) with pK_a near 7.5 is responsible for the latter pathway.

The rate constants of the fast M and the slow M decay reactions from the 360-nm traces and the rise and decay of O from the 540-nm traces, together with the two rate constants of the 490-nm traces, which represent the recovery of the initial state (SRII), are plotted as a function of pH in Fig. 4 *d*. Between pH 5 and 7, all the traces consist of two kinetic components (M-to-O and O-to-SRII conversion), which are pH-independent. Above pH 7, where the slow M appears, the rate shows a linear pH dependence that fits with the equation ($M + n \cdot H^+ \rightarrow SRII$) in which $n = 0.8$ proton taken up per molecule.

The pH dependence of O-decay is less obvious because of the decreasing amplitudes above pH 7 that make the fitting ambiguous. The rate of the fast M decay to O is increased ~2-fold as the pH is raised.

These results suggest the presence of a residue X-H at acid or neutral pH that functions as a relay for reprotonation of the Schiff base from the extracellular side in the conversion of M to O, making this process pH-independent. The parallel reaction pathways between pH 7 and 8 would then be attributable to the coexistence of the protonated and the unprotonated form of this residue. In this interpretation, the unprotonated X^-, which becomes significant above pH 7, renders the reprotonation of the Schiff base dependent on the bulk pH and gives rise to the slow M.

DISCUSSION

BR and HtrI-free SRI, when illuminated under similar conditions as used here, translocate protons from the cytoplasm to the extracellular side of the membrane. In contrast, the profile of pH changes we observed upon the illumination of SRII in membrane vesicles or sheets revealed that SRII does not pump protons across the membrane, but rather that it takes up a proton from the extracellular side when an intermediate is accumulated by the light and releases it to the same side after the light is turned off. Time-resolved measurement of pH changes near neutral pH by use of pyranine during the SRII photocycle revealed that the formation and decay of the O intermediate coincide with proton uptake and release, respectively. Similar pH changes were also observed for SRII in complex with HtrII in membrane vesicles, demonstrating that the binding of transducer does not block the proton uptake and release on the extracellular side.

In HtrI-free SRI, but not in HtrI-complexed SRI, the Schiff base proton is transferred to Asp-76 according to FTIR spectroscopy and mutant analysis (Rath et al., 1996). Flash spectroscopy and mutant analysis indicate that the Schiff base proton is transferred to the homologous Asp-73 in M formation both in the free and complexed forms of SRII (Spudich et al., 1997; Zhu et al., 1997). Based on the similar behavior of BR, the formation of O, observed as the rise of absorption bands with a red-shifted λ_{max} (near 520

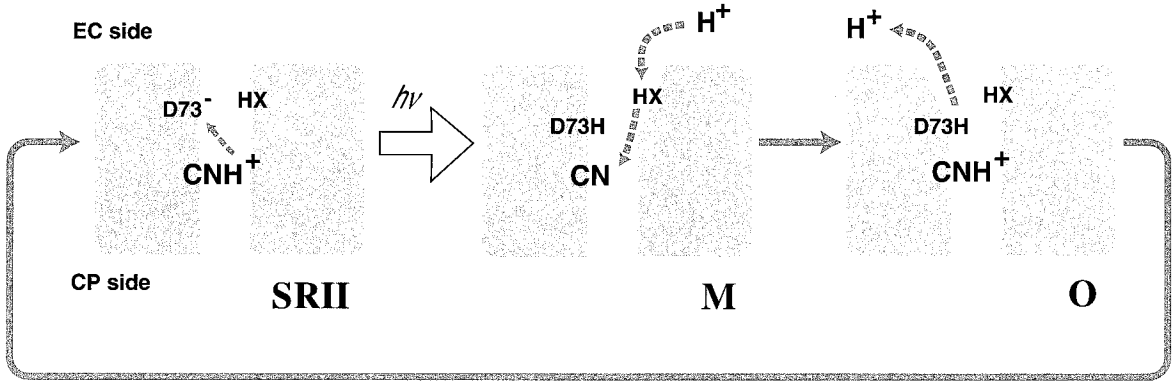

FIGURE 5 A model showing proton transfers coupled with the conversion of the intermediates during the photocycle of SRII. The arrows with broken lines show the proton translocations that occur in the next steps. EC, extracellular; CP, cytoplasmic; CN, the retinylidene Schiff base; X, Schiff base proton donor with pK_a near 7.5 (see text).

nm), is expected to entail the reprotonation of the Schiff base with Asp-73 remaining protonated. This process is postulated to be responsible for the proton uptake we observe (Fig. 5). The following shift of the absorption maximum from 520 nm back to 487 nm in O-decay is in our model largely attributable to the deprotonation of Asp-73 by releasing the proton to the extracellular side. Hence, during SRII photocycling internal protons are circulated in the extracellular half of the protein by alternating the pathway for the uptake and release, corresponding to conformational changes between M and O.

At alkaline pH, instead of slowing the M-to-O conversion, an additional kinetic pathway becomes evident in which an M-like species (the slow M) decays in a pH-dependent manner so that proton uptake occurs concomitantly with the decay process, while the other M-decay (the fast M) is slightly accelerated. This phenomenon may be explained by the existence of a residue (X-H) below pH 7 that relays a proton from the extracellular bulk water to the Schiff base in M-to-O conversion, rendering this process pH-independent as long as X is protonated. When X is deprotonated at alkaline pH, protons must be taken up directly from the bulk with M decay, which becomes progressively slower as the pH is raised.

Positioning the amino acid residues of SRII based on the 3-D structure of BR suggests candidates for X. Based on their pK_a values, Asp-63 and Glu-189 (corresponding to positions 75 and 202 in BR) are candidates, although they are predicted to be distant from the expected proton conduction channel. Other candidates are Arg-67, Arg-73, and Tyr-193 (corresponding to positions 76, 82, and 204 in BR), which are situated well within the postulated channel, although their pK_a values in an aqueous environment are >10. It is also possible that complex hydrogen-bonding networks in the channel play a role as a functional group with pK_a near 7.5. The identification of X as a specific site awaits future investigation by mutagenesis and a crystal structure of SRII.

The occurrence of both proton uptake and release at the extracellular side indicates either inaccessibility of the Schiff base to the cytoplasmic side or reduced conductivity for protons in the cytoplasmic half-channel compared to BR and SRI. A similar extracellular proton circulation was reported in BR expressed in *Xenopus laevis* oocyte membranes voltage-clamped with an electric field of opposite polarity to the one BR would generate (Nagel et al., 1998). The interpretation was that the electric field inhibits cytoplasmic-side proton uptake, and therefore the M intermediate decays very slowly by taking up protons from the extracellular side. This was explained as voltage-dependent inhibition of a "molecular switch" defined as the conversion between two substates of M (M1 and M2, whose Schiff bases are accessible to the extracellular side and cytoplasmic side, respectively). Therefore, one explanation of the extracellular proton circulation of SRII is that its M intermediate corresponds to M1 of BR in the model of Nagel et al. Alternatively, in BR the proton conductivity in the cytoplasmic half-channel may be voltage-dependent and reprotonation from this side would not occur even if the Schiff base were oriented toward the cytoplasmic side.

In contrast, the M of SRI (S_{373}) in the absence of HtrI corresponds to M_N in D96N of BR (Sasaki et al., 1992; Kamikubo et al., 1996), or equivalently M2 of Nagel et al., in which the Schiff base is connected to the cytoplasmic side, since M decay is coupled to proton uptake from that side. It should be noted that in SRI the residue corresponding to Asp-96 is a Tyr and the reprotonation of the Schiff base is from the cytoplasm milieu, as is the case in D96N of BR.

In bacteriorhodopsin, cryoelectron microscopy and x-ray and neutron diffraction have detected a conformational change that involves tilting of helices F and G on the cytoplasmic side. This conformational change has been found in N of wild-type BR (Vonck 1996; Kamikubo et al., 1996) and M_N of D96G or D96N (Subramaniam et al., 1993; Kamikubo et al., 1996), and is thought to be important in opening the cytoplasmic channel for proton uptake during the second half of the photocycle (Spudich and Lanyi, 1996).

The attractant receptor SRI is presumed to assume this conformation, because it is capable of taking up protons from the cytoplasmic side when free of its transducer. Motion on the cytoplasmic side of SRI in the SRI-HtrI complex is also consistent with the observation that the two HtrI subunits of the complex approach one another at residue 64 in the cytoplasmic region of transmembrane helix 2 (TM2) when SRI is activated by orange light to produce its M intermediate (Zhang and Spudich, 1998). Since HtrI and SRI appear to interact through their transmembrane helical domains (Zhang et al., 1999), the outward tilting motion of helices F and G may be communicated by direct interaction with TM2 of HtrI. Inverted mutants of SRI, which produce the opposite phototaxis signals from wild-type, and form protonated Schiff base intermediates rather than M, cause movement of TM2 in HtrI in the opposite direction (Zhang and Spudich, 1998).

Our finding that the active repellent signaling states M and O (Yan et al., 1991) of SRII exhibit Schiff base connectivity to the extracellular side, whereas the attractant signaling state SRI-M shows connectivity to the cytoplasmic side, raises the question of whether this difference is responsible for the opposite signals from SRI and SRII (possibility 1). Alternatively, the cytoplasmic helix movements may occur in SRII, but unlike in SRI, be insufficient to increase the proton conductivity to out-compete proton uptake from the cytoplasmic side (possibility 2). This latter possibility would be predicted by the model for SR signaling presented recently, which proposes that SRI and SRII exhibit the same conformational change, entailing the cytoplasmic side helix movements, and that the Htr transducers specify the sign of the signal as attractant or repellent (Spudich, 1998).

In the context of the relationship between SRI and SRII signaling conformations, it is interesting to conjecture on the structure of M and O of SRII based on the corresponding

intermediates of BR. X-ray and cryoelectron crystallography revealed the projection structure of BR-M stabilized in low water content or with arginine treatment (Kamikubo et al., 1996) and of BR-O in L93A (Subramaniam et al., 1997) in which the movement of the outward tilt of the helix F was less pronounced compared to that in N. Therefore, if possibility 1 were the case, SRII may be designed to skip the formation of an N-like structure so as to mediate only repellent signaling to the flagellar motor. We cannot, however, discard the alternative possibility that the SRII-O has the structure with the cytoplasmic side open like D85N/D96N of BR (Kataoka et al., 1994), whose structure shows the noticeable F-helix tilt as in BR-N even in the unphotolyzed state probably as the result of the neutralization of the residues at 85 and 96, which is the case in SRII-O. Distinguishing these alternatives will likely require crystallography of SRI and SRII.

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