Removal of the Transducer Protein from Sensory Rhodopsin I Exposes Sites of Proton Release and Uptake During the Receptor Photocycle

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ABSTRACT The phototaxis receptor sensory rhodopsin-I (SR-I) was genetically truncated in the COOH terminus which leads to overexpression in *Halobacterium salinarium* and was expressed in the presence and absence of its transducer, HtrI. Pyranine (8-hydroxyl-1,3,6-pyrene-trisulfonate) was used as a pH probe to show that proton release to the bulk phase results from the SR-I_{587} to S_{373} photoconversion, but only in the absence of transducer. The stoichiometry is 1 proton/S_{373} molecule formed. When SR-I is overexpressed in the presence of HtrI, the kinetics of the thermal return of S_{373} to SR-I_{587} is biphasic. A kinetic dissection indicates that overexpressed SR-I is present in two pools: one pool which generates an SR-I molecule possessing a normal (i.e., transducer-interacting) pH-independent rate of S_{373} decay, and a second pool which shows the pH-dependent kinetics of transducer-free S_{373} decay. The truncated SR-I receptor functions normally based on the following criteria: (i) Truncated SR-I restores phototaxis (attractant and repellent responses) when expressed in a strain lacking native SR-I, but containing HtrI. (ii) The absorption spectrum and the flash-induced absorption difference spectrum are indistinguishable from those of native SR-I. (iii) The rate of decay of S_{373} is pH-dependent in the absence of HtrI but not in the presence of HtrI. The data presented here indicate that a proton-conducting path exists between the protonated Schiff base nitrogen and the extramembranous environment in the transducer-free receptor, and transducer binding blocks this path.

INTRODUCTION

Halobacterium salinarium (formerly known as *H. halobium*) is a motile archaeon capable of photosensory transduction and photoenergy conversion by a family of retinylidene proteins (for review see Bogomolni and Spudich, 1991). Two, sensory rhodopsin I (SR-I) and sensory rhodopsin II (SR-II), are phototaxis receptors which modulate cell swimming behavior (Bogomolni and Spudich, 1982; Takahashi et al., 1985). The other two are light-driven electrogenic pumps: bacteriorhodopsin (BR,[1] a proton pump) and halorhodopsin (HR, a chloride pump) (Oesterhelt and Stoeckenius, 1973; Schobert and Lanyi, 1982).

For BR a near-atomic resolution structure is available (Henderson et al., 1990), and the structure and function of its photoactive site are well characterized. Retinal is covalently bound in a protonated Schiff base linkage to the ϵ-amino group of a lysine in an internal cavity formed by its seven membrane-embedded α-helices (Bayley et al., 1981; Katre et al., 1981). In SR-I, retinal is also bound in a protonated Schiff base linkage (Fodor et al., 1989) and the retinal-binding pocket is structurally similar to that of BR (Blanck et al., 1989; Henderson et al., 1990; Yan et al., 1991).

SR-I exists in two spectrally distinct forms each of which can be photoactivated to generate sensory signals (Spudich and Bogomolni, 1984). The thermally stable species (SR-I_{587}, subscript denotes λ_{max}) is an attractant receptor (its photoexcitation suppresses swimming reversals). The attractant signal is produced by formation of the long-lived, blue-shifted photointermediate S_{373} (Yan and Spudich, 1991). S_{373} is photochemically reactive and functions as a repellent receptor (its photoexcitation induces swimming reversals). Compelling evidence indicates signals from SR-I are relayed to HtrI, an intrinsic membrane methyl-accepting protein (Spudich et al., 1988; Yao and Spudich, 1992; Ferrando-May et al., 1993b; Spudich and Spudich, 1993).

SR-I exhibits similarities to BR in its photocycle; in particular S_{373} is spectroscopically similar and appears at the analogous position in the SR-I photocycle as M_{412}, the deprotonated Schiff base intermediate of BR (Bogomolni and Spudich, 1987). In BR the deprotonation and reprotonation of M_{412} is correlated with vectorial proton translocation. Grzesiek and Dencher (1986) have determined 1.1 ± 0.2 protons are released from the protein into the bulk phase per M_{412} formed (pH 7.0, 20°C) using the pH-sensitive dye pyranine. In the SR-I protein (in which the residues implicated in M_{412} deprotonation (Mogi et al., 1988; Butt et al., 1989; Otto et al.; 1990; Needleman et al., 1991) are conserved) net vectorial proton movement was not detected in the SR-I photocycle (Spudich and Spudich, 1982; Bogomolni and Spudich, 1982; Ehrlich et al., 1984). In addition, in BR^-, phototactically wild-type (i.e., $SR\text{-}I^+HtrI^+$) membrane envelope vesicles, no proton release into the bulk phase was detected with the pyranine method (Olson et al., 1992).

Recently a system has been developed for plasmid-directed expression of the SR-I apoprotein (Krebs et al.,

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[1] *Abbreviations used:* BR, bacteriorhodopsin; SR-I, sensory rhodopsin I; HtrI, halobacterial transducer for sensory rhodopsin I; SR-II, sensory rhodopsin II; SEM, standard error of the mean; SR-I_{587}, S_{373}, SR-I photocycle species with absorption maxima at 587 nm and 373 nm, respectively.

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1993). Comparison of SR-I expressed in the presence and absence of HtrI revealed that the transducer alters the photochemical properties of the receptor (Spudich and Spudich, 1993). Here we extend the analysis of SR-I/HtrI interaction and show that proton release to the bulk phase results from the SR-I_{587}-to-S_{373} photoconversion, but only in the absence of transducer.

MATERIALS AND METHODS

Chemicals

Pyranine (8-hydroxy-1,3,6-pyrenetrisulfonate) was from Eastman Kodak (Rochester, NY), and mevinolin was a gift of Dr. A. W. Alberts (Merck Sharp, Dohme).

Strains and culture conditions

Escherichia coli DH5α cultivation and use in cloning followed standard protocols (Sambrook et al., 1989). *H. salinarium* Pho81r⁻, a strain lacking all known retinylidene proteins is described in Spudich and Spudich (1993) and references therein. Pho81W, a strain which lacks carotenoids, was isolated as a white colony from Pho81. Part of the *sopI* gene and the entire *htrI* gene are deleted in Pho81. Flx15Δ*sopI* contains a targeted deletion of the *sopI* gene which left the *htrI* gene intact (Krebs et al., 1993). *H. salinarium* strains were grown aerobically in complex medium in the dark as described (Spudich et al., 1988). We used an inoculum volume of 5 ml/800 ml medium. After 4 days of growth, we added all-*trans* retinal to a final concentration of 2 μM and incubated for an additional 2 days before harvesting the cells for membrane preparation. We obtained between 30- and 50-fold increase of SR-I over native (Flx15) amounts in both Pho81/pTR2Δ and Flx15Δ*sopI*/pTR2Δ transformants.

Plasmids

High level expression of functional SR-I in *H. salinarium* has been achieved by genetically engineering a truncation of the COOH terminus of SR-I (Ferrando-May et al., 1993a). We prepared a similar COOH-terminal truncation by replacement of the *Nsi*I-*Not*I cassette of the synthetic *sopI* gene described (Krebs et al., 1993). In our construct, the COOH-terminal 15 amino acids were deleted by inserting a stop codon after Ser-224. The truncation differs by one residue from that of Ferrando-May and co-workers which terminates with Glu-223. Oligonucleotides for cassette replacement were synthesized with a 394 Oligonucleotide Synthesizer (Applied Biosystems Incorporated) by the Molecular Core Facility, Department of Microbiology and Molecular Genetics, University of Texas Medical School, Houston, TX. Before reannealing, the oligonucleotides were detritylated and desalted using OPC columns (oligonucleotide purification cartridge, No. 400771, Applied Biosystems Incorporated). The complementary oligonucleotides were reannealed by adding 200 pmol of each to an Eppendorf tube, overlaying with 40 μl mineral oil, and placing in a 80°C water bath. The water bath was turned off and allowed to slowly reach ambient temperature overnight.

The truncated synthetic gene was constructed by modifying the pTZ-derivative *E. coli* vector, pSO5 (Krebs et al., 1993), which contains the full-length synthetic *sopI* gene. A three-piece ligation was performed (to reduce parental background) by ligating the: 1) 665-bp *Aat*II-*Nsi*I fragment from pSO5 (encoding the seven helices of SR-I including seven residues of the COOH terminus extending from the terminal helix); 2) 2832-bp *Not*I-*Aat*II fragment (the bulk of the pSO5-vector containing an *E. coli* origin of replication and ampicillin-resistance marker); and 3) 25-bp *Nsi*I-*Not*I cassette (containing the stop codon at amino acid position 223). DH5α cells were transformed and plated on LB/Amp plates at 37°C overnight.

The plasmid pS07 contains the full-length synthetic *sopI* gene (Krebs et al., 1993) and has elements which allow it to be stably maintained in *E. coli* and, upon removal of a *Sph*I fragment and ligation, in *H. salinarium*. pTR2 is the truncated SR-I-encoding derivative of this plasmid and was constructed by a four-piece ligation. The four fragments were identical with those used to construct pSO7 (Krebs et al., 1993), except the 750-bp *Aat*II-*Not*I fragment from pTR1 was used instead of the *Aat*II-*Not*I fragment from pSO5. In addition, the 6.7-kb *Bam*HI-*Hin*dIII from pMPK54 was treated with alkaline phosphatase and gel-purified to decrease background. DH5α cells were transformed and plated and plasmid DNA from a transformant colony was isolated by Magic Miniprep (Promega, Madison, WI), and digested with *Pst*I and *Not*I. The resulting 150-bp fragment was subcloned into pGEM-5Zf(+) (Promega) and the mutation confirmed. Before transforming *H. salinarium*, a 3.2-kbp *Sph*I fragment containing pAT153 was removed and the remaining 6.9-kbp fragment was circularized in vitro to yield pTR2Δ (Fig. 1). All plasmids used here to transform *H. salinarium* have this fragment removed and hence are designated "Δ." pMPK56 contains the mevinolin resistance gene and no *sopI* gene (Krebs et al., 1993). *H. salinarium* Pho81W and Flx15Δ*sopI* were transformed using the procedure described (Krebs et al., 1993). Strain Flx15Δ*sopI* contains carotenoids. Proton release was detected from Pho81W/pTR2Δ and from carotenoid-containing Pho81r⁻/pTR2Δ transformants, indicating that carotenoids have no effect on this process.

Membrane preparations

The membrane fraction was isolated from sonicated early stationary phase cells as described (Bogomolni and Spudich, 1993). The final suspension (in 4 M NaCl) of membranes was 8–10 mg protein/ml as determined by Lowry assay (Sigma Chemical Co., St. Louis, MO). Membranes were pelleted for 20 min at 75,000 rpm (304,000 *g*) in a Beckman TL-100 tabletop ultracentrifuge (Palo Alto, CA). The pellet was resuspended in 250 mM KCl and washed twice.

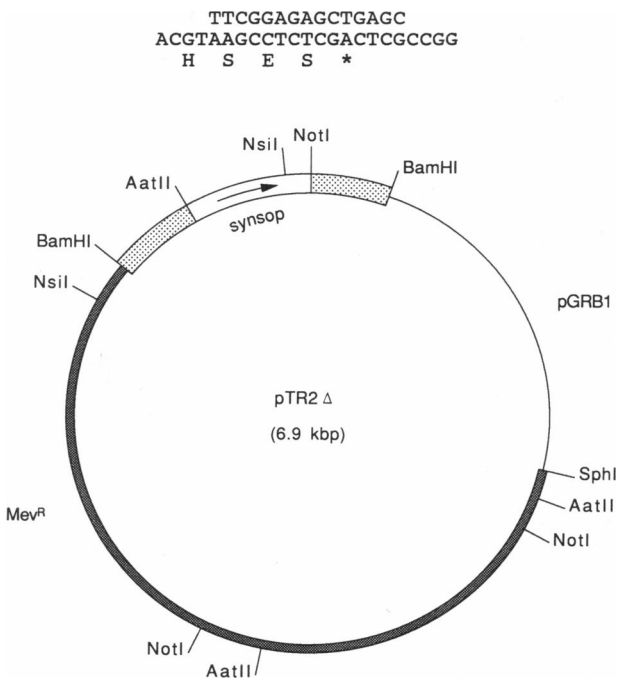

FIGURE 1 *H. salinarium* plasmid (6.9 kbp) showing the location and direction of transcription of the synthetic gene *sopI* (*synsop* in figure) described previously (Krebs et al. 1993). Also shown is the cassette which was inserted at the *Nsi*I-*Not*I site in the gene. The star represents the stop codon. Mev^R is the gene encoding mevinolin resistance.

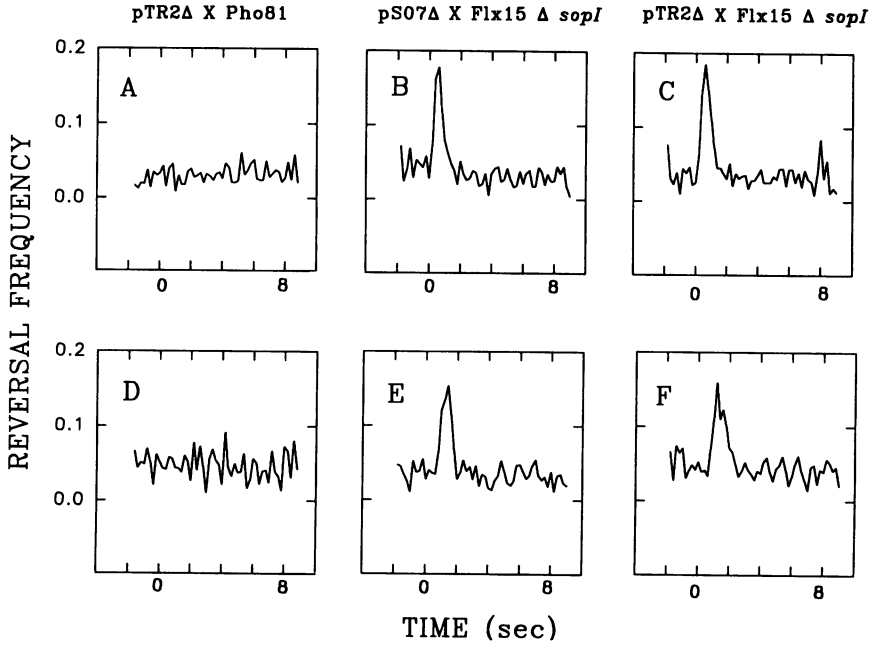

FIGURE 2 Motility responses to photostimuli. The video data were processed at 5 frames/s and population reversal frequencies were measured (i.e., the number of reversals detected within the 200-ms frame interval per number of paths present in that interval). Cell swimming behavior was monitored with nonactinic infrared illumination. At time 0, a continuous 600-nm light was interrupted for 4 s (*a–c*), or a 370-nm light was delivered for 0.5 s in an orange background (*d–f*). (*a* and *d*) Pho81/pTR2Δ; (*b* and *e*) Flx15Δ*sopI*/pSO7Δ; (*c* and *f*) Flx15Δ*sopI*/pTR2Δ.

Spectroscopy

Visible absorption spectra of SR-I were measured in a double-beam UV-vis SLM Aminco DW-2000 spectrophotometer (SLM Instruments, Urbana, IL). Membranes (2 ml in 1-cm-path length cuvettes) were degassed under house vacuum before absorption measurements.

Flash-induced absorbance changes were measured with a lab-constructed cross-beam kinetic spectrophotometer (Spudich et al., 1986). The actinic light generated from an electronic flash (Sunpak Auto544) was passed through two wide-band interference filters (550 ± 20 nm) or a single long pass 620-nm filter, except in Fig. 3. Monitoring light was provided by a 12 V 50 watt tungsten-halogen lamp beam passed through a monochromator (dispersion 4 nm/mm, 2-mm slit), the sample, and two narrow-band interference filters (±5 nm, Ditric Optics, Inc., Hudson, MA) protecting the detector (photomultiplier model R928, Hamamatsu Corp., Bridgewater, NJ) from scattered actinic light. All measurements were made at ambient temperature. Absorbance transients were captured with a Nicolet (Madison, WI) model 4562 analog-to-digital converter. Typically, three flashes were averaged. Each trace was acquired as 3,968 data points, and the acquisition rate was 2 ms/point or 100 μs/point. Flash frequency was 0.05 Hz. Each trace was stored on a Nicolet 4094A digital oscilloscope. In some cases initial points of flash artifact were removed. Rate constants and amplitudes were obtained by fitting the data to single or double exponentials using the Asystant algorithm (Macmillan Software, Inc. New York, NY).

For pyranine measurements, flash-induced absorbance changes were monitored both with and without the indicator dye pyranine (25 μM, pH 7.0, ambient temperature) as previously described (Olson et al., 1992). The pyranine signal is defined as the difference of the flash-induced difference transient with pyranine minus the flash-induced transient without pyranine.

pH measurements

The pH was measured with a Beckman Φ72 pH meter, Altex electrode No. 39522. To confirm that the shift of pH dependence on the rate of decay of S_{373} is not due to different readings of the pH meter in low salt versus high salt, 0.47 g NaCl was added to 2 ml of an aqueous solution of 25 mM Tris buffer (pH 7.88) and the pH was measured to be negligibly influenced (pH 7.96).

RESULTS

Truncated SR-I restores HtrI-dependent phototaxis behavior to a sopI deletion mutant

We subjected the Flx15Δ*sopI*/pTR2Δ transformant to photostimuli which selectively activate the attractant and repellent photoreceptor forms of SR-I, SR-I_{587}, and S_{373}, respec-

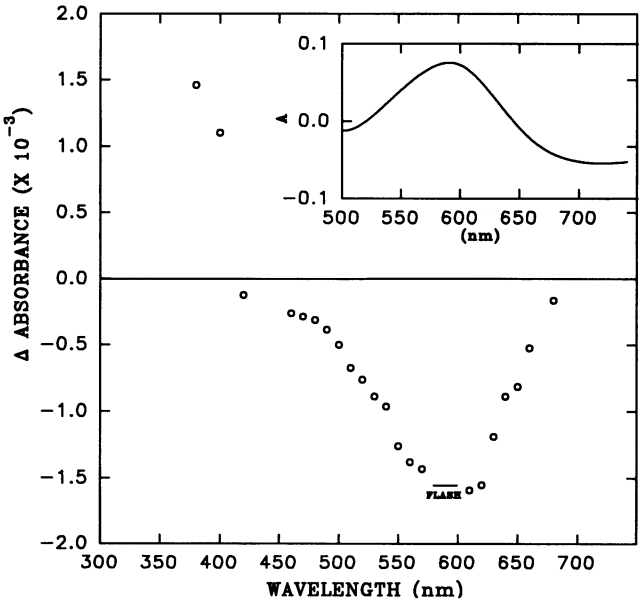

FIGURE 3 Flash-induced absorption difference spectrum of overexpressed SR-I. Membrane envelope vesicles from Pho81W/pTR2Δ were suspended at 1.25 mg protein/ml in 250 mM KCl. Maximum absorption changes after the flash were measured at various wavelengths. The actinic light was a 1-ms flash through a 590 ± 10 nm interference filter. (*Inset*) Absorption spectrum of Pho81W/pTR2Δ minus Pho81W/pMPK56Δ membranes in 250 mM KCl.

tively. As a control, the nontruncated synthetic *sopI* gene expressed in Flx15Δ*sopI* (i.e., Flx15Δ*sopI*/pSO7Δ), previously shown to produce wild-type responses (Krebs et al., 1993), was used. Equivalent responses were seen in native and overexpressed SR-I transformants to a 600 nm step-down (Fig. 2, *B* and *C*) and to a 370 nm step-up stimulus (*E* and *F*). There is no response to step-up or step-down stimuli of transformed Pho81, a strain which lacks the SR-I transducer HtrI (*A* and *D*).

Truncated SR-I exhibits HtrI-modulated photochemical reactions

The flash-induced difference spectrum of truncated SR-I is normal (Fig. 3) and the absorption spectrum has a λ_{max} at ~590 nm (*inset*), as is observed for native SR-I.

The half-life of the thermal return of S_{373} to SR-I_{587} in SR-I^+HtrI$^+$ vesicles is essentially pH-independent over a pH range of ~3 to ~9 (Olson et al., 1992). In contrast, SR-I expressed in the absence of transducer exhibits a pronounced pH dependence in the $S_{373} \rightarrow$ SR-I_{587} thermal transition (Spudich and Spudich, 1993). Truncated SR-I in the absence of transducer also exhibits a pH-dependent S_{373} decay (Fig. 4). As with native SR-I, HtrI confers pH insensitivity to the S_{373} decay of truncated SR-I (Fig. 5).

The pH dependence of S_{373} decay in the absence of HtrI is shifted to higher pH in low salt (250 mM KCl) compared with high salt (4 M NaCl) (Fig. 5). Our interpretation of these data is that the pH at the membrane surface and therefore at the surface of the SR-I protein is altered in low salt. That is, at a given bulk phase pH the effective pH is lower in low salt versus high salt as expected from Gouy-Chapman theory (Szundi and Stoeckenius, 1987). Fig. 5 also shows HtrI controls the S_{373} decay rate in low as well as in high salt.

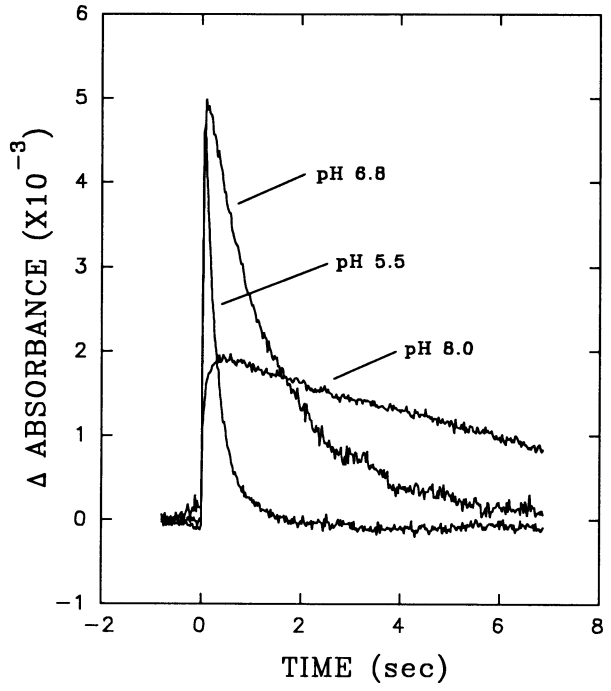

FIGURE 4 Time course of flash-induced absorbance changes of Pho81/pTR2Δ membranes in 250 mM KCl monitored at 400 nm at pH 5.5, 6.8, and 8.0.

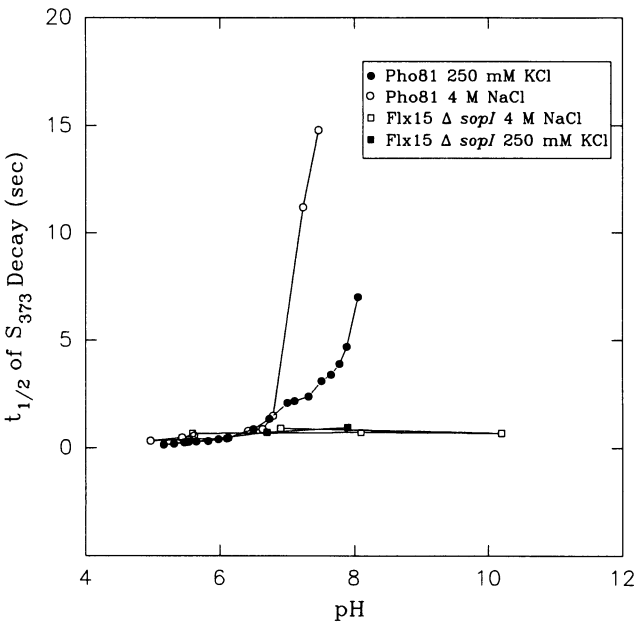

FIGURE 5 The rate of S_{373} decay as a function of pH for Pho81/pTR2Δ (*circles*) and Flx15Δ*sopI*/pTR2Δ (*squares*) in 4 M NaCl (*open symbols*) or 250 mM KCl (*closed symbols*).

Both transducer-complexed and transducer-free receptors are obtained when SR-I is expressed in high levels

Because HtrI is expressed from the chromosome, the SR-I:HtrI ratio is expected to be increased above its native value by overexpression of SR-I. One can envision two possibilities for photochemical behavior of the "excess receptor" system:

1. A fraction of the total SR-I molecules will interact with the limited number of HtrI molecules at the normal fixed ratio. In this case we would expect to observe two distinct populations of receptor: one with the properties of SR-I in Pho81 (i.e., pH-dependent), and one with that of wild-type SR-I in the presence of transducer (i.e., pH-independent). In this case biphasic kinetics would be observed at pH 5.5 and 8.0, the interacting SR-I/HtrI molecules exhibiting normal S_{373} decay kinetics and the remainder of the SR-I molecules showing a pH-dependent rate, faster at pH 5.5 and slower at pH 8.0.
2. There is no defined ratio of SR-I:HtrI molecules, but HtrI samples the photochemical state of the population of SR-I molecules and catalyzes their decay. Because SR-I is in excess, in this case we may detect some intermediate rate of S_{373} decay.

To differentiate between these possibilities, we kinetically dissected the decay of S_{373} at pH 5.5 (low pH) and pH 8.0

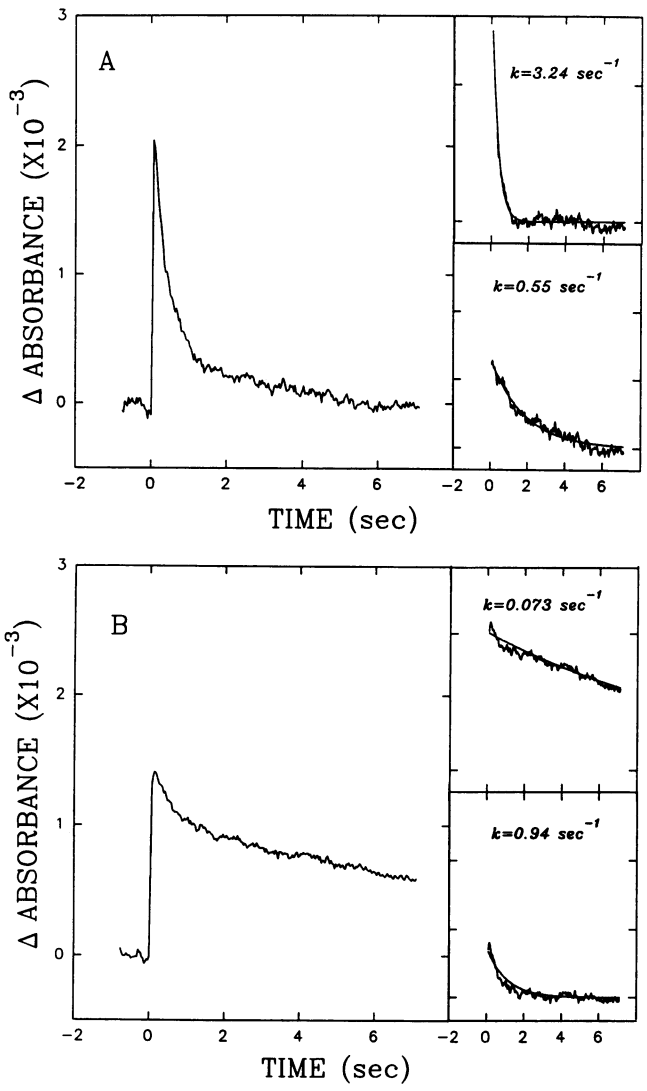

FIGURE 6 (*A*) Membranes from Pho81/pTR2Δ (1.25 mg protein/ml) in 250 mM KCl, 25 mM 4-morpholineethanesulfonic acid, pH 5.5. (*Left*) Time course of flash-induced absorbance change monitored at 400 nm. (*Right*) Kinetic dissection showing the (*1*) fast phase (*top right*) and (*2*) normal phase (*bottom right*). (*B*) Pho81/pTR2 membranes (1.25 mg protein/ml) in 250 mM KCl, 25 mM Tris, pH 8.0. (*Left*) Time course of flash-induced absorbance change monitored at 400 nm. (*Right*) Kinetic dissection showing the (*1*) very slow phase (*top right*) and (*2*) normal phase (*bottom right*).

(high pH). At low pH (Fig. 6 *A*) we observe two phases. One is fast ($t_{1/2}$ = 214 ms) and the other ($t_{1/2}$ = 1.26 s) is approximately normal, i.e., similar to wild-type SR-I in the presence of HtrI. At pH 8.0 (Fig. 6 *B*), we observe a very slow phase ($t_{1/2}$ = 9.5 s) and again a phase with normal kinetics ($t_{1/2}$=737 ms). The amplitudes of the near normal components are similar ($7.2 \times 10^{-4} \pm 0.7 \times 10^{-4}$ and $3.3 \times 10^{-4} \pm 0.6 \times 10^{-4}$ at pH 5.5 and pH 8.0, respectively). These data support possibility (1) and are consistent with overexpressed SR-I being present in two pools: one pool which interacts with HtrI and contains SR-I molecules with a normal pH-independent rate of S_{373} decay, and the second pool which does not interact with HtrI and shows the kinetics of a transducer-free S_{373}-decay expected for that pH (compare rates of Fig. 6 with Fig. 5). Table 1 is a summary of kinetic fits of SR-I ± HtrI at pH 5.5 and pH 8.0. The control Flx15/pMPK56Δ were membrane preparations containing wild-type SR-I and HtrI.

Proton release and uptake occur during the photocycle of transducer-free sensory rhodopsin-I

We have previously observed that in wild-type membranes (i.e., membranes containing native SR-I and HtrI) proton release to the bulk phase was not detected during the SR-I photocycle (Olson et al., 1992). BR-containing vesicles were used as a control to show that a transient proton release could be detected with the pH indicator dye used (pyranine) under similar data-acquisition parameters and equivalent numbers of photocycling molecules. Pyranine shifts its absorption maximum from 450 nm to 400 nm upon protonation, which results in the absorption difference spectrum shown in Fig. 7 *A*. In the absence of HtrI, (i.e., membranes from Pho81/pTR2Δ) acidification of the bulk phase does occur during the SR-I photocycle as evidenced by the pyranine signals at 400 and 450 nm (Fig. 7 *B*). Similar numbers of photocycling SR-I in the presence of HtrI (i.e., membranes from Flx15Δ*sopI*/pTR2Δ) do not cause bulk phase acidification (Fig. 7 *C*). Addition of buffer eliminates the pyranine absorbance changes (Fig. 7 *D*), confirming the absorbance changes in the absence of HtrI are due to protonation of pyranine and not to an alteration of the SR-I photocycle by pyranine.

TABLE 1 Kinetic analysis of SR-I in the presence and absence of HtrI and at various pH values

	pH 5.5		pH 8.0	
Flx15/pMPK54Δ	t_1 1.2 ± 0.2 s	A_1 $6.5 \times 10^{-4} \pm 0.3 \times 10^{-4}$	t_1 1.3 ± 0.01 s	A_1 $4.3 \times 10^{-4} \pm 0.4 \times 10^{-4}$
Pho81/pTR2Δ	t_2 182 ± 16 ms	A_2 $2.9 \times 10^{-3} \pm 0.09 \times 10^{-3}$	t_2 11.3 ± 0.5 s	A_2 $1.5 \times 10^{-3} \pm 0.3 \times 10^{-3}$
Flx15Δ*sopI*/pTR2Δ	t_1 1.7 ± 0.3 s	A_1 $7.2 \times 10^{-4} \pm 0.7 \times 10^{-4}$	t_1 0.9 ± 1 s	A_1 $3.3 \times 10^{-4} \pm 0.6 \times 10^{-4}$
	t_2 185 ± 15 ms	A_2 $1.5 \times 10^{-3} \pm 0.3 \times 10^{-3}$	t_2 9.4 ± 1.0 s	A_2 $9.2 \times 10^{-4} \pm 0.6 \times 10^{-4}$

A_1 and t_1 are the amplitude in absorbance units and the half-time of the normal phase, respectively. A_2 and t_2 are the amplitude and the half-time of the slower or faster component.

We estimated the number of protons (N) released per SR-I photocycle by using BR pumping (known to release one proton per BR photocycle) (Grzesiek and Dencher, 1986) to calibrate the pyranine signals in our system, using the following equation:

$$N = \frac{\Delta A_{SR,400,pyr} \cdot \epsilon_{SR,590}}{\Delta A_{SR,590}} \times \frac{\Delta A_{BR,600}}{\Delta A_{BR,400,pyr} \cdot \epsilon_{BR,600}},$$

where $\epsilon_{SR,590} = 54{,}000\ M^{-1}\ cm^{-1}$ and $\epsilon_{BR,600} = 44{,}100\ M^{-1}\ cm^{-1}$.

Averaging seven independent measurements for SR-I and six for BR, $N = 1.2 \pm 0.3$ (mean ± SEM) protons released per SR-I photocycle (equivalently per S_{373} formed). The measurements of Fig. 7 were made in 250 mM KCl. We have repeated the measurements in 4 M NaCl, the salt concentration at which HtrI effects on SR-I photocycling were initially demonstrated (Spudich and Spudich, 1993). Again we observe proton release from SR-I only in the HtrI-free membranes (data not shown). The flash yield of S_{373} in 4 M NaCl in the absence of transducer is only 20% of that in 250 mM KCl, rendering the pyranine signals more difficult to quantitate. Based on averaging eight measurements in high salt, $N = 1.1 \pm 0.7$ protons per S_{373} formed. Therefore the data indicate there is one proton released per S_{373} produced both in low and in high salt.

It should be noted, however, that in our BR measurements (Olson et al., 1992) we calculate only 0.33 protons released per photocycling BR molecule based on the pyranine absorbance change. We attributed the value of <1.0 to the buffering capacity of vesicle membranes.

No pyranine signal was observed in the case of SR-I in the presence of HtrI (Fig. 7 *C*). An S_{373}-transient from the Flx15Δ*sopI*/pTR2Δ membranes in 4 M NaCl was curve fit to determine the amount of SR-I interacting with HtrI in this particular preparation. We observed a normal S_{373} decay (757 ms) and no slow component (data not shown), indicating negligible transducer-free SR-I was present. We have found that individual pTR2 transformants of Flx15Δ*sopI* exhibit varying amounts of transducer-free SR-I from negligible (Fig. 7 *C*) to >50% of the total SR-I (e.g., as in Fig. 6).

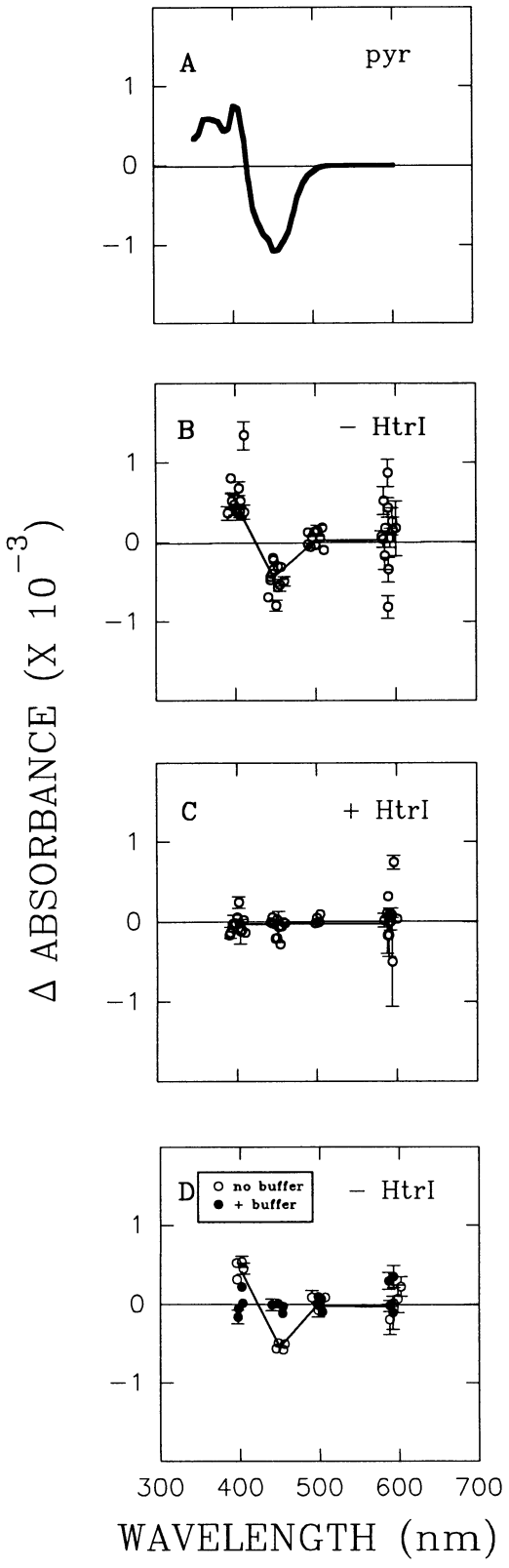

FIGURE 7 The difference spectrum labeled "pyr" (*A*) represents the conversion of unprotonated pyranine (absorbance maximum = 450 nm) to the protonated form (absorbance maximum = 400 nm) upon shifting the pH from 8.6 to 3.8. The bottom three graphs are summaries of the flash-induced absorbance changes due to pyranine at 400, 450, 500, and 600 nm for overexpressed SR-I in Pho81 (*B* and *D*) and Flx15Δ*sopI* (*C*). Data points represent the difference of: [the maximum absorbance change of the sample with pyranine] − [the maximum absorbance change of the sample without pyranine]. Error bars (SEM, n = 3–4) are shown for each point. No bar is shown on points where the error is smaller than the symbol used to represent the point. Data points are slightly offset for clarity. Data points are not normalized and spread over a range. The flash-induced absorbance changes at 590 nm were: *Pho81/pTR2Δ (250 mM KCl) (absorbance × 10³)* mean: −14.1. range: −5.0 to −21.4. N = 14; and *Flx15ΔsopI/pTR2Δ (250 mM KCl) (absorbance × 10³)* mean: −10.5. range: −2.9 to −18.2. N = 12. (*D*) Buffered (25 mM Tris, pH 6.8) Pho81/pTR2Δ membranes in 250 mM KCl. The "no buffer" data points shown here are not included in Fig. 7 *B*. The flash-induced absorbance changes at 590 nm (absorbance × 10³) were: no buffer, mean = −12.1; + buffer, mean = −9.8.

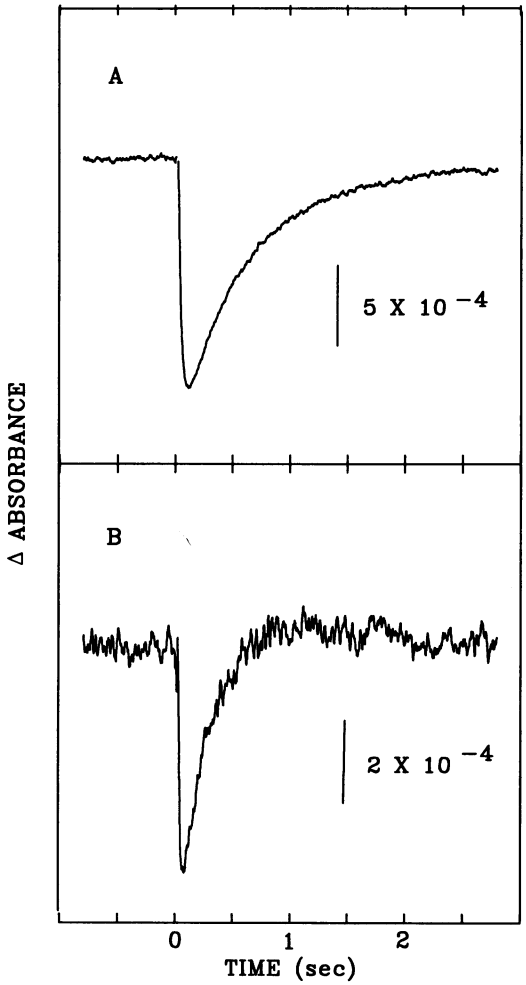

FIGURE 8 (*A*) Flash-induced absorption transient of Pho81/pTR2Δ membranes at 450 nm. (*B*) Pyranine signal at 450 nm determined by subtracting the trace in *A* from the trace obtained after adding pyranine to the membranes. Each trace is the average of three independent flash photolysis measurements as described in the text.

Pyranine signal decay is more rapid than SR-I_{587} reformation

The pyranine signal decay ($t_{1/2}$ = ~350 ms) is faster than the reappearance of SR-I_{587} in the photocycle ($t_{1/2}$ = ~1 s in the measurements reported here). The difference in rates is evident in the absorbance transient at 450 nm due to SR-I_{587} return (Fig. 8*A*) and the pyranine signal decay extracted from the same set of measurements at the same wavelength (Fig. 8 *B*). The decay of S_{373} kinetically matches the return of SR-I_{587} in transducer-free SR-I (data not shown), as it does in transducer-complexed SR-I (Bogomolni and Spudich, 1987). Therefore the faster pyranine signal decay may reflect a spectroscopically silent transition from S_{373} to a previously undetected protonated intermediate before SR-I_{587} reformation.

DISCUSSION

The results above show that removal of transducer exposes sites of release and uptake of protons during the SR-I photocycle. The stoichiometry is 1 proton/S_{373} molecule formed. Under the single-flash conditions used, proton transfers to and from the aqueous bulk phase are not detected in transducer-interacting SR-I. S_{373} formation is believed to require deprotonation of the Schiff base attachment site of the retinal chromophore in the photoactive center of the protein, so S_{373} decay would entail reprotonation of this site. The Schiff base proton is therefore a possible source of the proton we detect.

The truncated SR-I receptor functions normally based on the following observations: (i) Truncated SR-I restores phototaxis (step-up and step-down responses) when expressed in a strain lacking native SR-I, but containing HtrI, as reported for a similar truncation (Ferrando-May et al., 1993a). (ii) The flash-induced difference spectrum and the absolute spectrum are indistinguishable from those of native SR-I. (iii) The rate of decay of S_{373} is pH-dependent in the absence of HtrI but not in the presence of HtrI. This effect of SR-I/HtrI interaction recently reported in 4 M NaCl (Spudich and Spudich, 1993) is evident in 250 mM KCl as well. Because *H. salinarium* is an exteme halophile, the maintenance of SR-I/HtrI interaction in low salt was not necessarily expected. The low-salt condition is especially useful for work with SR-I in native membranes because light scattering is greatly reduced compared with high salt, presumably because low salt disrupts the vesicles formed by sonication in high salt (Blaurock et al., 1976).

Previously we found that S_{373} decay is sensitive to bulk phase pH in the transducer-free but not in the transducer-interacting receptor (Spudich and Spudich, 1993). An hypothesis consistent with all these findings is that *a proton-conducting path exists between the protonated Schiff base nitrogen and the extramembranous environment in the transducer-free receptor.* Formation of S_{373} would release the proton to the bulk phase as detected here, and bulk phase protons would drive the reprotonation during S_{373} decay as observed in the previous report (Spudich and Spudich, 1993). *Transducer binding blocks the proton-conducting path,* preventing proton release to the bulk phase and rendering S_{373} decay pH-independent as observed (Olson et al., 1992).

It is important to note that the data do not completely exclude an alternative scheme in which the proton transfer reactions at the Schiff base nitrogen are conformationally coupled to proton release and uptake from sites on the protein surface. Characterization of the Schiff base proton paths in the presence and absence of transducer would test and possibly refine this hypothesis.

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