The Schiff Base Counterion of Bacteriorhodopsin Is Protonated in Sensory Rhodopsin I: Spectroscopic and Functional Characterization of the Mutated Proteins D76N and D76A†

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ABSTRACT: Both sensory rhodopsin I (SR-I), a phototaxis receptor, and bacteriorhodopsin (BR), a light-driven proton pump, share residues which have been identified as critical for BR functioning. This includes Asp76, which in the case of bacteriorhodopsin (Asp85) functions both as the Schiff base counterion and proton acceptor. We found that substituting an Asn for Asp76 (D76N) in SR-I has no effect on its visible absorption unlike the analogous mutation (D85N) in BR which shifts the absorption to longer wavelengths. The mutated proteins D76N and D76A are also fully functional as phototaxis receptors in contrast to BR, where the analogous substitutions block proton transport. D76N was also found to exhibit a spectrally normal $SR_{587} \rightarrow S_{373}$ transition. However, FTIR difference spectroscopy reveals that two bands in the $SR_{587} \rightarrow S_{373}$ difference spectrum at 1766/1749 cm^{-1} (negative/positive), assigned to the C=O stretch mode of a carboxylic acid, disappear in D76N, although no changes are observed in the carboxylate region. In addition, the kinetics and yield of this photoreaction are altered. On this basis, it is concluded that, unlike Asp85 in bacteriorhodopsin, Asp76 is protonated in SR-I and undergoes an increase in its hydrogen bonding during the $SR_{587} \rightarrow S_{373}$ transition. This model accounts for the difference in color of SR-I and BR and the finding that Asn can substitute for Asp76 without greatly altering the SR-I phenotype. Interestingly, parallels exist between this residue and Asp83 in the visual receptor rhodopsin which has recently been found to exist in a protonated form and to undergo an almost identical change in hydrogen bonding during rhodopsin activation.

Sensory rhodopsin I (SR-I)[1] is one of the two phototaxis receptors found in the archaeon *Halobacterium salinarium* which modulate cell swimming behavior through light-activated signal transduction (Bogomolni & Spudich, 1982; Takahashi et al., 1985).[2] It consists of a protein with seven membrane spanning regions (Blanck et al., 1989) bound to a retinylidene chromophore via a protonated Schiff base linkage (Fodor et al., 1989). The phototaxis response by SR-I is mediated by two spectrally distinct forms of the molecule: SR_{587}, the ground-state pigment of SR-I, and the photointermediate S_{373} (Spudich & Bogomolni, 1984), with absorbance maxima at 587 and 373 nm, respectively. Compelling evidence indicates that signals from SR-I are directly relayed to the methyl-accepting transducer protein HtrI (Spudich et al., 1988; Yao & Spudich, 1992; Ferrando-May et al., 1993b; Spudich & Spudich, 1993) which is the second component in the signaling path and in this sense is analogous to the G-protein transducin in the rhodopsin photocascade (Vuong et al., 1984).

In addition to the sensory rhodopsins, two other retinal-containing membrane proteins are found in the cytoplasmic membrane of *H. salinarium*: bacteriorhodopsin (BR), the light-driven proton pump (see Ebrey, 1993; Khorana, 1993; Skulachev, 1993; Rothschild & Sonar, 1994 for recent reviews), and halorhodopsin (HR), the chloride pump (Lanyi, 1986). Bacteriorhodopsin is the most extensively studied of the four pigments and several of its residues have been identified as critical for proton pumping including four buried Asp residues: Asp85, Asp96, Asp115, and Asp212. A three-dimensional structure has also been derived for BR through cryoelectron diffraction (Henderson et al., 1990) which reveals the location of these residues as well as others lining the retinal binding pocket. Importantly, most of the residues in the retinal binding pocket are also present in the SR-I and HR amino acid sequences (Blanck & Oesterhelt, 1987; Hegemann et al., 1987; Blanck et al., 1989).[3] Thus, a key question is what function, if any, do these conserved residues play relative to their functions in BR.

In this study we have focused on the role of Asp76 in the signal-transduction mechanism of SR-I. The homologous residue in BR, Asp85, has been found to be critical for proton transport and color regulation in BR. The structural model shows that it is located close to the protonated Schiff base and in a position to serve as a counterion (Henderson et al., 1990). In agreement, several spectroscopic studies show that it is ionized and serves as the proton acceptor for the Schiff base during M formation (Braiman & Rothschild, 1988; Braiman et al., 1988a; Fahmy et al., 1992; Metz et al., 1992). In addition, the Asn and Ala substitutions of Asp85 abolish proton pumping and cause a red-shift in the visible absorption (Otto et al., 1990; Marti et al., 1991; Fahmy et al., 1992; Greenhalgh

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[1] Abbreviations: ATR, attenuated total reflection; BR, bacteriorhodopsin; FTIR, Fourier transform infrared; SR-I, sensory rhodopsin I; SR_{587}, S_{373}, SR-I species with absorption maxima at 587 and 373 nm, respectively; WT, wild type.

[2] A second phototaxis receptor pigment is known as sensory rhodopsin II.

[3] Asp96 of BR, which is believed to be a part of the reprotonation pathway (Braiman et al., 1988a; Rothschild, 1992), is replaced by a Tyr in the SR-I sequence.

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et al., 1992) consistent with the hypothesized direct role of this residue in an extracellular proton-release mechanism (Braiman et al., 1988a).

Because of recent progress in the synthesis and cloning of a cassette *sopI* gene (Krebs et al., 1993), and methods developed for mutagenesis and expression of SR-I in the native *H. salinarium* (Krebs et al., 1993; Olson & Spudich, 1993; Spudich & Spudich, 1993), we have been able for the first time to combine Fourier-transform infrared (FTIR) difference spectroscopy with site-directed mutagenesis to study SR-I. This approach has been used previously to study BR (Braiman & Rothschild, 1988; Braiman et al., 1988b; Gerwert et al., 1989; Fahmy et al., 1992; Maeda et al., 1992) and more recently rhodopsin (Fahmy et al., 1993; Rath et al., 1993a). In the case of BR these studies have revealed detailed information about the structural changes which occur in the retinal chromophore, protein backbone, and individual amino acid residues during the different steps in the BR photocycle (Rothschild et al., 1981, 1992; Gerwert, 1992; Rothschild, 1992; Rothschild & Sonar, 1994). We have also applied FTIR attenuated total reflection (ATR) spectroscopy for the first time to study SR-I. In contrast to more conventional transmittance studies, ATR allows complete control of the pH and ionic strength of the bathing medium (Marrero & Rothschild, 1987), an important requirement due to the recent finding that the SR-I photoreaction cycle is extremely sensitive to pH when the receptor is not bound to its transducer, HtrI (Spudich & Spudich, 1993).

Our measurements show that unlike BR, where Asp85 serves as the Schiff base counterion and obligate proton acceptor for its function, the homologous residue in SR-I (Asp76) is not essential for its function. Moreover, evidence from FTIR studies indicates that Asp76 is protonated in both the SR_{587} and S_{373} states, undergoing an increase in hydrogen bonding strength upon S_{373} formation. This would account for the difference between the visible absorption of SR-I near 587 nm relative to that of BR near 570 nm as well as the weaker hydrogen-bonding interaction detected for the Schiff base of SR-I (Fodor et al., 1989). Since the counterion by serving as an obligate proton acceptor is an essential component of the proton pump in BR, the absence of a proton transfer to Asp76 would also explain why in contrast SR-I function is intact in D76N.[4] Indeed, the substitution of this residue by Asn or Ala does not greatly affect the spectral properties or prevent function of SR-I, indicating that Asp76 plays no direct role in the SR-I signal transduction mechanism. Instead, the proton-accepting group for the Schiff base may reside on the transducer protein, HtrI, the transfer serving to activate it in the process of signal transduction [one possible mechanism described by Spudich (1993)].

MATERIALS AND METHODS

Strains and Culture Conditions. *Escherichia coli* DH5α cultivation and use in cloning followed standard protocols (Sambrook et al., 1989). *Halobacterium salinarium* Pho81W is a strain which lacks carotenoids and was isolated as a white colony from Pho81, a mutant with part of the *sopI* gene and the entire *htrI* gene deleted (Spudich & Spudich, 1993). 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).

[4] Designations of the mutations make use of the standard one-letter abbreviations for amino acids. Thus, D76N signifies the mutated protein in which the aspartic acid at position 76 has been replaced by asparagine.

Plasmids

High level expression of functional SR-I in *H. salinarium* has been achieved by genetically engineering truncations of the COOH terminus of SR-I (Ferrando-May et al., 1993a; Olson & Spudich, 1993). Each of the Asp76 mutated proteins used in this work lacks the C-terminal 15 amino acids (termination at Ser224). Oligonucleotides for cassette replacement were synthesized with a Model 394 oligonucleotide synthesizer (Applied Biosystems Incorporated; Foster City, CA) by the Molecular Core Facility, Department of Microbiology and Molecular Genetics, University of Texas Medical School, Houston, TX. Prior to reannealing, the oligonucleotides were detritylated and desalted using OPC columns (oligonucleotide purification cartridge, 400771, Applied Biosystems Incorporated).

pD76Atr. Asp76 was substituted with Ala by modifying the pTZ-derivative *E. coli* cloning vector, pTR1 (Olson & Spudich, 1993), which contains the truncated synthetic *sopI* gene. A four-piece ligation was performed by ligating (i) the 2832 bp *Not*I–*Aat*II fragment (the bulk of the pTR1-vector containing an *E. coli* origin of replication and ampicillin-resistance marker), (ii) the 447 bp *Spe*I–*Not*I fragment (encoding the final four helices of SR-I including seven residues of the C-terminus extending from the terminal helix), (iii) the 217 bp *Aat*II–*Dde*I (encoding the first two helices of SR-I) and (iv) the *Dde*I–*Spe*I cassette (encoding the mutation Asp76→Ala, i.e., GAC→GCC).

The plasmid pTR2 contains the truncated SR-I synthetic *sopI* gene 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* (Olson & Spudich, 1993). The four fragments used to construct the D76A expression vector were identical to those used to construct pTR2 (Olson & Spudich, 1993) and pSO7 (Krebs et al., 1993), except the 685 bp *Aat*II–*Not*I fragment from the *E. coli* cloning vector containing the Asp76→Ala mutation was used. DH5α cells were transformed and plated and plasmid DNA from a transformant colony was isolated by using the Magic Mini Plasmid Prep kit (Promega, Madison, WI) and digested with *Bam*HI and *Hin*dIII. The resulting 323 bp fragment was subcloned in pBluescriptII KS (+) (Stratagene, La Jolla, CA) and the mutation confirmed by sequencing. Prior to transforming *H. salinarium*, pD76Atr2 was digested in *Sph*I and the resulting 6.9 kbp fragment was circularized *in vitro* to yield pD76AtrΔ.

A single *H. salinarium* transformant was picked and grown in the presence of mevinolin (1 μg/mL). The mutation was reconfirmed by isolating the plasmid from 1 mL of *H. salinarium* and amplifying by PCR the 323 bp fragment between the *Bam*HI and *Hin*dIII sites of the synthetic gene. This fragment was subcloned into pBluescript. The primers used for PCR were as follows: *Bam*HI site, 5′TACCGGTCCCTCGATGG3′; *Hin*dIII site, 5′AGGTACGCGAACAAGC3′.

pD76Ntr. The mutation was initially introduced in the full-length (i.e. nontruncated) form. The cloning vector was constructed by a four-piece ligation as described above, except the *Dde*I–*Spe*I cassette contained the mutation Asp76→Asn (GAC→AAC) and the 447 bp *Spe*I–*Not*I fragment was from the synthetic gene in pSO5 (Krebs et al., 1993). The resulting plasmid was cut with *Aat*II–*Not*I and the 730 bp fragment was isolated. A four-piece ligation was completed to construct the expression vector as described previously (Olson & Spudich, 1993), using a 730 bp *Aat*II–*Not*I fragment containing the *sopI* gene with the D76N mutation. A 162 bp

*Bam*HI–*Spe*I fragment was isolated from a transformant and subcloned into pBluescript and sequenced prior to *H. salinarium* transformation.

The truncated pTR2-like derivative of Asp76→Asn (pD76Ntr) was prepared by a two-piece ligation of (i) the 8 kbp *Sna*BI–*Bgl*II from D76N containing the Mev^R gene and the first three helices of SR-I and (ii) the 2 kbp *Bgl*II–*Sna*BI from pTR2 containing the final four helices of SR-I, including the truncation. *H. salinarium* was transformed as previously described (Krebs et al., 1993). One-half of a colony was picked and used as an inoculum in 2 mL of complex medium (CM) and Mev (1 μg/mL). The other one-half of the colony was resuspended into 200 μL of resuspension buffer from the Magic Mini Prep kit and the plasmid isolated. The mutation was confirmed by amplifying and sequencing a fragment of the synthetic gene with the *Bam*HI and *Hin*dIII primers described above. Five microliters of the plasmid suspension was used in the PCR reaction.

For the purposes of discussion in this paper, we will refer to the protein products of pD76AtrΔ and pD76NtrΔ expressed from the *bop* promoter containing the extended N-terminus as described by Krebs *et al.* (1993) as D76A and D76N, respectively. The similarly expressed form from pTR2Δ, which has been shown to have wild type SR-I properties, will be refered to as "native" SR-I in this paper.

Motion Analysis. Motility responses to SR-I photoactivation were assayed by computer-assisted cell tracking and motion analysis as described (Krebs et al., 1993).

Flash Photolysis. Flash-induced absorption changes were recorded with a laboratory constructed cross-beam flash spectrometer as described (Spudich & Spudich, 1993, and references therein), except that the actinic flash was delivered at 0.1 Hz with a pulsed Nd:Yag laser (6 ns pulse width) at 532 nm (Continuum, Santa Clara, CA).

FTIR Difference Spectroscopy

Transmission Measurements. Stock suspension of membranes (10 mg/mL) containing SR-I or the mutated protein D76N in 4 M NaCl at pH 6.8 (25 mM Tris buffer) were diluted (16×) with distilled water and centrifuged at 80000*g* for 1 h. The pellet formed was resuspended with the supernatant solution to the original volume, thus resulting in a final salt concentration of 250 mM. SR-I films were prepared by depositing 10–20 μL of this suspension onto an AgCl window and drying with a slow stream of argon gas. The film was then rehydrated and mounted in a temperature-controlled IR cell (Model TFC, Harrick Scientific Corp., Ossining, NY). Sample temperature was maintained at 10 °C. Spectra were recorded as previously reported (Rath et al., 1993a) with a Bio-Rad FTS-60A FTIR spectrometer (Bio-Rad, Digilab Division, Cambridge, MA) using an MCT detector. A Dolan-Jenner (Woburn, MA) Model 180 illuminator (150 W, tungsten–halogen) and a fiber-optic light-guide was used for sample illumination in combination with a 505-nm long-pass filter (Corion Corp., Holliston, MA). Each light and dark spectrum consisted of 3000 scans at 8-cm^{-1} resolution, and several of the individual light–dark differences were averaged to obtain the final spectrum. Large-scale random intensity fluctuations were observed between 1600 and 1700 cm^{-1} which averaged out upon increasing the number of data sets.

Attenuated Total Reflection (ATR) Measurements. Approximately 80 μL of SR-I stock solution was dried onto a 50 × 20 × 2 mm internal reflection germanium crystal by a slow stream of argon gas. The crystal was then mounted in a modified temperature-controlled ATR cell (MEC-1TC, Harrick Scientific Corp., Ossining, NY) with quartz window for sample illumination. The SR-I film was equilibrated with a pH 6.8 buffer (5 mM sodium phosphate, 5 mM KCl, 2 mM $MgCl_2$, 3 mM $CaCl_2$, and 250 mM NaCl) at 15 °C flowing at a rate of 1.5 mL/min for several hours prior to FTIR measurements. Each spectrum is the average of 3000 scans at 8-cm^{-1} resolution, and several light–dark differences were averaged to obtain the final difference spectrum. A baseline spectrum was interactively fit (sixth-order polynomial) and subtracted from the averaged difference spectrum using the software provided with Lab Calc (Galactic Industries, NH).

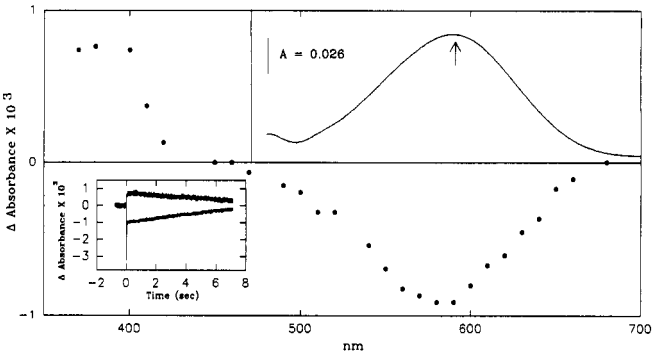

FIGURE 1: Flash-induced absorption difference spectrum of the D76N receptor. Membrane envelope vesicles from Pho81W/D76NtrΔ were suspended at 1.25 mg protein/mL in 4 M NaCl, 25 mM TRIS-HCl, pH 6.8. Maximum absorption changes following the flash were measured at various wavelengths. (Upper right) Absorption spectrum of Pho81W/D76NtrΔ membranes minus Pho81W membranes; arrow indicates maximum absorption at 590 nm. (Inset, lower left) Time course of absorbance changes of D76N monitored at 590 nm (lower transient) and 400 nm (upper transient) following a flash at time 0 (35 mJ, 532 nm, 6-ns pulse).

RESULTS

Spectroscopic Characterization of D76N and D76A. The absorption spectrum of the D76N receptor in Pho81 membranes (λ_{max} of 590 nm, Figure 1) is indistinguishable from that of native SR-I (with the same extended N-terminal and C-terminal truncation, see Materials and Methods) which exhibits a λ_{max} of 590 ± 2 nm in Pho81W membranes.

Flash photolysis of D76N produces an S_{373}-like intermediate with a flash-induced difference spectrum (Figure 1) which is the same as that of native SR-I. However, two differences of D76N from native SR-I were found from flash-photolysis data. First the yield of S_{373} in Pho81W (transducer-free membranes) is about 20% of that of native SR-I in Pho81W. Second the half-time of decay of S_{373} is >6–10 s, which is slower than the return ($t_{1/2}$ = 4–6 s) shown by native SR-I in the transducer-free membranes at the pH of the measurement (pH 6.8) (Spudich & Spudich, 1993). The flash-induced difference spectrum of D76N in Flx15Δ*sopI* (transducer-containing membranes) is also essentially the same as that of native SR-I in Pho81 or Flx15Δ*sopI*. However, in the presence of transducer the $t_{1/2}$ of S_{373} decay in D76N is 4–6 s, considerably slower than that of transducer-complexed native SR-I (0.5–1 s). A significantly lower yield of S_{373} from D76N compared to native SR-I is also evident in Flx15Δ*sopI* membranes.

The absorption spectrum of the D76A protein is slightly red-shifted from that of D76N with λ_{max} near 600 nm (data not shown) measured under the same conditions as in Figure 1.

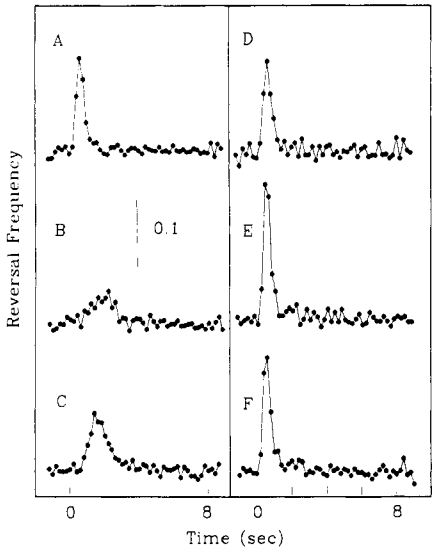

FIGURE 2: Motility responses to photostimuli. Dark field microscopy video data were processed at 5 frames/s and population reversal frequencies were measured (i.e. the number of reversals detected within the 200 ms frame interval per number of paths present in that interval). Cell swimming behavior was monitored with nonactinic infrared illumination. At time 0, a continuous 600-nm light was interrupted for 2 s (A–C) or a 370-nm light was delivered for 0.5 s in a ≥600-nm background (D–F). A and D, Flx15 Δ*sopI*/pTR2Δ; B and E, Flx15Δ*sopI*/pD76NtrΔ; C and F, Flx15Δ*sopI*/pD76AtrΔ.

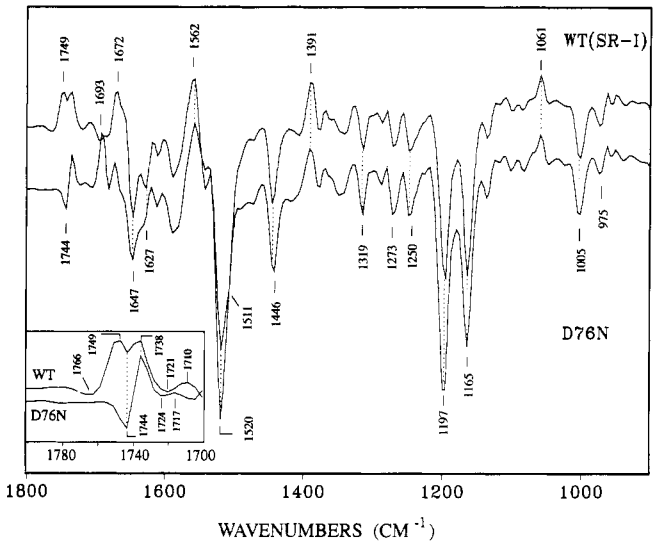

FIGURE 3: Dark→light FTIR difference spectra of native SR-I (WT) and D76N films recorded at 10 ° C and 8-cm^{-1} resolution. Each difference spectrum is the subtraction of a spectrum in the dark (3000 scans, 10 min) and a spectrum in the light (3000 scans, 10 min) and consists of the average of several such differences. Both the native SR-I and D76N spectra were scaled using the intensity of chromophore bands (e.g. at 975, 1005, 1165, and 1520 cm^{-1}). Inset: Expanded view of the dark→light FTIR difference spectra of SR-I and D76N in the region between 1700 and 1800 cm^{-1}.

Note that in transducer-free D76N a fast component ($t_{1/2}$ = 16 ms) is evident at 590 nm (Figure 1, inset) in addition to a slower component that corresponds more closely to S_{373} return (bottom left, Figure 1). A similar fast component of comparable amplitude is also observed in native SR-I in transducer-free membranes. This fast phase is not observed at 400 nm and evidently corresponds to a reaction cycle which does not include an S_{373}-like intermediate. Our measurements indicate this aberrant fast reaction cycle is characteristic of transducer-free receptor. Its absorption difference spectrum (not shown) indicates formation and decay of a blue-shifted intermediate(s) (λ_{max} between 520 and 560 nm) without S_{373} formation.

Phototaxis Signaling by D76N and D76A. When D76N and D76A are expressed in Flx15Δ*sopI* which contains the SR-I transducer HtrI, the cells exhibit phototaxis responses (Figure 2). Responses mediated by the native SR-I attractant form (SR_{587}) and repellent form (S_{373}) are shown in panels A and D. Delayed responses to a step-down in attractant light were observed in cells containing the D76N and D76A receptors (panels B and C), while the repellet responses (panels E and F) are at least as great as those in cells containing native SR-I. These data prove that the carboxyl group of D76 is not required for either repellent or attractant signaling by SR-I. The disappearance of S_{373} has been proposed to produce the reversal-inducing signal in response to a step-down in attractant (600 nm) light (panels A–C), on the basis of retinal-analogue receptors with altered S_{373} decay kinetics (Yan & Spudich, 1991). The delayed attractant responses in B and C are therefore expected since the S_{373} decay rates are reduced to 4–6 and 1.8–3 s in D76N and D76A, respectively, compared to 0.5–1 s in native SR-I. The equivalent (or enhanced) repellent responses in E and F are also consistent with the longer lifetimes of the S_{373}-like intermediates in the mutants, since photoexcitation of S_{373} is known to mediate the repellet response (Spudich & Bogomolni, 1984).

The SR_{587}→S_{373} FTIR Difference Spectra. Figure 3 compares the SR_{587}→S_{373} difference spectra obtained from native SR-I and D76N. Both spectra are very similar to previously reported FTIR difference spectra from SR-I (Bousché et al., 1991b) with a few exceptions (see Discussion).

As previously discussed (Bousché et al., 1991b), many of the negative bands in the SR_{587}→S_{373} difference spectrum can be assigned to vibrations of the SR_{587} chromophore on the basis of resonance Raman spectroscopy and are characteristic of the *all-trans* structure of the retinal (Fodor et al., 1989). These include the ethylenic C=C stretch at 1520 cm^{-1} (with a shoulder near 1511 cm^{-1}),[5] C–C stretch modes at 1197 and 1165 cm^{-1}, CH_3 methyl rock at 1005 cm^{-1}, and hydrogen-out-of-plane wag at 975 cm^{-1}. The C=N stretching mode of the retinal Schiff base appears at 1627 cm^{-1}, much lower than for BR (1642 cm^{-1}), and reflects a relatively weak hydrogen-bonding strength of the Schiff base (Fodor et al., 1989).

Despite the close similarity of the native SR-I and D76N difference spectra, a dramatic change occurs in the 1700–1800-cm^{-1} region (Figure 3, inset), with the disappearance of the negative/positive pair of bands at 1766/1749 cm^{-1}. This disappearance uncovers the negative 1744-cm^{-1} and positive 1738-cm^{-1} bands which are partially canceled in the native SR-I spectrum (Figure 3, inset). Bands in this region are characteristic of the C=O stretch mode of carboxylic acid groups and in the case of BR, have been assigned on the basis of site-directed mutagenesis to individual Asp residues (Braiman et al., 1988a). In this case, the simplest explanation for the disappearance of the bands would be their assignment to Asp76, which is replaced by Asn in D76N. The 1766/1749-cm^{-1} pair could arise from Asp76 if its hydrogen-bonding strength increased between SR_{587} and S_{373}. Indeed, a pair of bands at an almost identical frequency (1767/1748 cm^{-1}) has recently been assigned to a hydrogen-bonding change of Asp83 in rhodopsin during the Meta I→Meta II transition (Rath et

[5] A peak at 1513 cm^{-1} was observed in studies done at 2-cm^{-1} resolution (Bousché et al., 1991b) and may be due to the ethylenic stretch of a second red-shifted species based on the inverse correlation between ethylenic frequency ($\nu_{C=C}$) and absorption maximum (λ_{max}).

al., 1993a). In contrast, a single positive band at 1762 cm^{-1} has been assigned to the protonation of the Asp83 counterion during the L→M transition in BR (Braiman et al., 1988a; Fahmy et al., 1992).

Although the intensities of the two bands at 1766 and 1749 cm^{-1} are not equal, with the 1766 cm^{-1} being less intense, this asymmetry in intensity is not unexpected. In fact, a very similar pattern is also observed due to a hydrogen-bonding change of Asp83 in rhodopsin during the Meta I→Meta II transition (Rath et al., 1994). Such asymmetry in intensity could arise from several factors. Changes in hydrogen bonding of a carboxylic acid group are not only expected to induce frequency shifts but also changes in the intensity and half-width of the C=O stretch band. For example, a broadening of the 1766-cm^{-1} band (where the integrated intensity remains unaltered) relative to the 1749-cm^{-1} band would account for the observed intensity difference. Since membranes which are dried onto AgCl windows are normally oriented (Rothschild & Clark, 1979; Clark et al., 1980; Rothschild et al., 1980), intensity changes can also occur if the carboxyl group undergoes a change in orientation relative to the membrane plane due to dichroism effects. Finally, the possibility also exists that a positive band overlies the negative 1766-cm^{-1} band, thereby reducing its apparent intensity. In particular, recent studies (Bogomolni et al., 1994) show that a BR-like form of SR-I exists which is capable of pumping protons. We expect, in analogy with BR, that Asp76 in this BR-like form of SR-I will exist in a deprotonated state and undergo protonation during the SR-I photocycle. This protonation may occur in a minor fraction of the SR-I molecules and could therefore give rise to a positive band near 1766 cm^{-1}.

Several other features of the D76N difference spectrum support the assignment of the 1766/1749 cm^{-1} pair to Asp76. A new band is found at 1693 cm^{-1} (Figure 3) which is characteristic of Asn vibrations (Venyaminov & Kalnin, 1990) and may reflect a change in hydrogen bonding to the Asn76 residue in the SR_{587}→S_{373} transition. In fact, a similar band has been observed when Asn is substituted for Asp96 in the BR→L and BR→M difference spectra (Gerwert et al., 1989). The intensity of the band at 1391 cm^{-1}, in the region of the symmetric C=O stretch of carboxylate groups (Bellamy, 1968), is unaltered in the D76N mutant, indicating that Asp76 does not undergo changes in protonation during the SR_{587}→S_{373} transition. In contrast, a band has been assigned in this region to deprotonation of Asp-96 during the M→N transition of bacteriorhodopsin (Bousché et al., 1991a). A relatively small change in intensity is observed near 1425 cm^{-1}; however, this is outside the normal range for carboxylate groups.

An alternative explanation is that these bands arise from two different Asp and/or Glu residues which undergo a simultaneous deprotonation/protonation reaction, both of which are blocked in D76N. However, we consider this possibility unlikely since only small changes were found in the carboxylate stretch region near 1400 cm^{-1}. Since the SR-I samples measured were expressed in an HtrI-free strain, we also made FTIR measurements on SR-I expressed in the presence of HtrI. Similar results were obtained (data not shown) with both bands at 1766/1749 cm^{-1} still present. Finally, we note that a small change occurs between native SR-I and D76N difference spectra in the region of the C=N stretch mode (1627 cm^{-1}) which might reflect a change in the Schiff base environment of D76N (see Discussion).

ATR Difference Spectra. Since the photocycle kinetics of SR-I in the absence of the transducer *Htr*I has been found to be sensitive to pH (Spudich & Spudich, 1993), we obtained FTIR difference spectra of native SR-I using attenuated total reflection (ATR) spectroscopy. In contrast to more conventional transmittance measurements made on thin hydrated films, this approach allows a more accurate control of the sample pH and ionic concentration. A thin film deposited on the surface of a germanium internal reflection element (IRE) crystal is exposed to a flowing aqueous medium whose pH and ionic content are externally controlled. As in the case of earlier studies on BR (Marrero & Rothschild, 1987) and acetylcholine receptor (Baenziger et al., 1992), we found that drying a suspension of the SR-I sample resulted in strong binding of the membranes to the germanium IRE surface. Almost no detectable loss of sample was detected after flowing of a buffer past the film for several hours, as determined by monitoring the FTIR absorption spectrum.

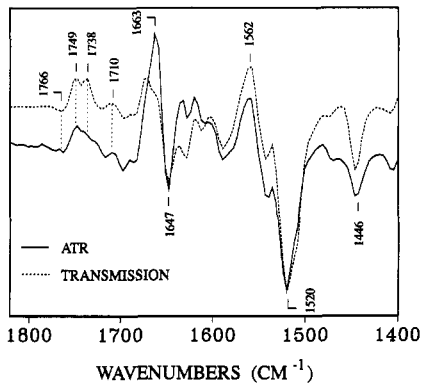

FIGURE 4: Comparison of normal transmission and ATR dark→light FTIR difference spectra of SR-I using a flowing buffer. The ATR difference spectrum was obtained at 15 °C and 8-cm^{-1} resolution. Each difference spectrum is the subtraction of a spectrum in the dark (3000 scans, 10 min) and a spectrum in the light (3000 scans, 10 min) and consists of the average of several such differences. For additional details see the Materials and Methods section.

Figure 4 compares the SR_{587}→S_{373} difference spectra obtained from native SR-I using the transmittance and ATR techniques. In the case of ATR, the flowing buffer was adjusted to have a pH of 6.8. Under these conditions, the two spectra are very similar with the 1766/1749-cm^{-1} pair of bands assigned to Asp76 clearly present. Thus, we conclude that the two bands at 1766/1749 cm^{-1} assigned here to Asp76 are not an artifact due to nonphysiological pH or absence of an aqueous medium.

DISCUSSION

On the basis of sequence conservation, Asp76 in SR-I is in a position which corresponds to Asp85 in BR (Blanck et al., 1989). It is therefore likely that Asp76 is located close to the protonated Schiff base of retinal, consistent with the electron diffraction derived structure of BR (Henderson et al., 1990). It has previously been established that Asp85 serves as the proton acceptor from the Schiff base (Braiman et al., 1988a) and is required for the proton-pumping function of BR (Mogi et al., 1988; Butt et al., 1989; Subramaniam et al., 1990). Proton transfers to and from the Schiff base also appear to be important in the generation of SR-I signaling states (Spudich, 1993). However, the data reported here demonstrates that protonation of Asp76 during the SR-I photocycle is not required for SR-I phototaxis signaling function. The fundamental differences in the essential proton transfers in SR-I and BR revealed by these measurements may help us understand the molecular basis of the differing functions of these two proteins.

The FTIR Difference Spectra Indicate That Asp76 Is Protonated in Both the SR_{587} and S_{373} States. This interpretation is based on the disappearance of two bands at 1766/1749 cm^{-1} in the carboxylic acid stretch region of the SR_{587}→S_{373} difference spectrum due to the substitution Asp76→Asn along with the absence of any changes in the region characteristic of carboxylate groups. The appearance of these negative/positive bands in the wild type spectrum is consistent with Asp76 undergoing an increase in hydrogen bonding between SR_{587} and S_{373}. *Thus, unlike BR, where the homologous residue, Asp85, serves as the Schiff base counterion and its proton acceptor, Asp76 in SR-I appears to remain neutral and therefore unable to serve either function.* This conclusion was previously suggested in an earlier FTIR study of SR-I (Bousché et al., 1991b) on the basis of the absence of a strong band at 1762 cm^{-1}, characteristic of Asp85 protonation in BR.[6]

We also found that SR-I with the D76N substitution is fully functional in mediating the SR-I color-discriminating phototaxis behavior of an $HtrI^+$ strain. We conclude that the substitution does not impair the ability of SR-I to function as a signal transducer. Similarly, no changes were found in the λ_{max} of the flash-induced absorbance difference spectrum. The decay of S_{373} is slower in D76N than in the wild-type, but the major functional steps in the SR-I photocycle are unaltered by the mutation. In contrast to SR-I, the corresponding mutation, D85N, in BR abolishes proton pumping (Mogi et al., 1988; Butt et al., 1989; Subramaniam et al., 1990), produces a major red-shift in the λ_{max} from 570 to ca. 590 nm (Subramaniam et al., 1990), and drastically alters the photocycle, almost completely blocking flash-induced M formation (Stern et al., 1989; Otto et al., 1990). The changes observed in the phenotype of SR-I due to the Asp76→Asn substitution most likely reflect the proximity of this residue to the Schiff base as expected on the basis of the similarity of SR-I to BR. In particular, while our work demonstrates that this residue is not required for transduction, its replacement is expected to alter the microenvironment of the Schiff base which probably causes the changes in the kinetics and S_{373} yield in the photocycle.

Importantly, a neutralization of the BR counterion in SR-I is entirely consistent with the subtle phenotypic effects of D76N in contrast to the drastic effects in BR. In the latter case a red-shift occurs and the ethylenic stretching mode of the retinal downshifts, consistent with the substitution of a negatively charged residue (Asp) with a neutral residue (Asn) (Rath et al., 1993b). The BR function and photocycle are expected to be completely disrupted since Asp85 can no longer function as the Schiff base proton acceptor. In contrast, the substitution of an Asn residue for an electrostatically neutral (protonated) Asp76 in SR-I is not expected to greatly perturb the color, photocycle, or function of the protein since Asn is also neutral. In fact, due to its isomorphic structure, the carboxyamide group of Asn is expected to be able to substitute for the carboxylic acid group of Asp and establish a hydrogen-bonding interaction with the Schiff base (Rath et al., 1993b).

The C=N stretching frequency is very sensitive to the electrostatic and hydrogen-bonding interactions of the Schiff base (Kakitani et al., 1983; Baasov et al., 1987; Ottolenghi & Sheves, 1989) and is expected to reflect a difference in charge near the Schiff base lysyl nitrogen between SR-I and BR. Indeed, as previously discussed (Fodor et al., 1989), the lower C=N stretching frequency and hydrogen/deuterium exchange shift (isotope shift) observed in SR-I indicates a hydrogen bonding even weaker than in BR (De Groot et al., 1989). This change in hydrogen bonding may also reflect a neutralization of Asp76.[7]

Since Asp76 in SR-I can be excluded as the Schiff base proton acceptor, the question arises as to the identity of the Schiff base proton acceptor as well as the role of Schiff base deprotonation in the SR-I signal transduction mechanism. In BR, Asp85 accepts the Schiff base proton during M formation, thereby triggering simultaneous ejection of a proton into the outer medium. Three other Asp residues are also active during the BR photocycle: Asp96, which deprotonates during the M→N transition acting as the Schiff base proton donor; Asp115, which is predominantly protonated and responds to retinal isomerization; and Asp212, which is also located close to the Schiff base and may undergo a partial protonation during M formation (see Rothschild, 1992; Rothschild & Sonar, 1994 and references therein for a recent discussion).

In SR-I, Asp96 of BR is changed to a tyrosine; thus, it is excluded from functioning as the Schiff base proton acceptor under physiological conditions. Asp201 is in a good position to function as the proton acceptor, since its homologous residue in BR (Asp212) is located close to the Schiff base (Braiman et al., 1988a; Henderson et al., 1990). Asp106 should also be considered since its protonation state has not yet been determined although the analogous residue in BR (Asp115) is protonated (Braiman et al., 1988a). Alternatively, it is possible that a group residing on the transducer, HtrI, acts in the native system as the Schiff base proton acceptor. This possibility is particularly attractive, since it would provide a mechanism to explain how the signal is transmitted from the receptor (SR-I) to the transducer (HtrI). It would also account for the transient proton release and uptake observed when transducer is removed from SR-I (Olson & Spudich, 1993). In order to further investigate these possibilities it will be important to assign other bands in the carboxyl region using site-directed mutagenesis.

A recent study of the visual pigment rhodopsin (Rath et al., 1993a) reveals an interesting parallel between Asp83, which is highly conserved and present in helix-B of rhodopsin, and Asp76 of SR-I. As in the SR_{587}→S_{373} difference spectrum, two bands in the Rho→Meta II difference spectrum appear at 1767/1748 cm^{-1} (positive/negative). These bands were assigned to Asp83 on the basis of their disappearance in the mutant D83N. It was concluded that Asp83 undergoes an increase in its hydrogen bonding during the Meta I→Meta II transition. In addition, the D83N mutation does not appear to block Meta II formation. Thus, both Asp76 in SR-I and Asp83 in rhodopsin appear to respond in very similar ways to receptor activation. Although a detailed structural model is

[6] The bands at 1766/1749 cm^{-1} were not detected in the previous study, despite the similarities of the overall spectrum in other regions, suggesting that SR-I existed in a different form than studied here. This may be due to the much lower concentrations of SR-I in its membrane relative to the overexpressed SR-I samples used in this study or due to partial dehydration of the sample which can cause an increased pH (and possible deprotonation of Asp76).

[7] Note however that a small change in the relative Schiff base/Asp76 distance and/or orientation in SR-I relative to Asp85 in BR could also affect this C=N stretch frequency and its isotope shift. In particular, acid blue membrane also has a neutral Asp85 (Subramaniam et al., 1990), yet its C=N stretch frequency is higher (1630 cm^{-1}) and hydrogen/deuterium exchange shift larger than in SR-I (Fodor et al., 1989). In addition, a recent resonance Raman study of the BR mutated protein D85N shows that the substitution of a negative Asp for a neutral Asn causes a relatively small downshift in frequency, possibly because of an increased hydrogen-bonding interaction caused by the Asn residue (Rath et al., 1993b).

not yet available for rhodopsin, the most likely explanation is that both Asp76 in SR-I and Asp83 in rhodopsin are protonated and undergo a similar response to Schiff base deprotonation.

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