Asp76 Is the Schiff Base Counterion and Proton Acceptor in the Proton-Translocating Form of Sensory Rhodopsin I[†]

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ABSTRACT: Both sensory rhodopsin I, a phototaxis receptor, and bacteriorhodopsin, a light-driven proton pump, have homologous residues which have been identified as critical for bacteriorhodopsin functioning. This includes Asp76, which in the case of bacteriorhodopsin (Asp85) functions as both the Schiff base counterion and the proton acceptor. Sensory rhodopsin I exists in a pH dependent equilibrium between two different forms in the absence of its transducer protein HtrI. At pH below 7, it exists primarily in a blue form (λ_{max} = 587 nm) which functions as a phototaxis signal transducer when complexed to HtrI, while at higher pH, it converts to a purple proton-transporting form similar to bacteriorhodopsin (λ_{max} = 550 nm). We report ATR-FTIR difference spectra obtained from both low- and high-pH forms of purified sensory rhodopsin I reconstituted into lipid vesicles. The low-pH species has an ethylenic C═C stretch mode at 1520 cm^{-1} which shifts to 1526 cm^{-1} in the high-pH form. No frequency shift was found for the mutant D76N, in agreement with visible absorption measurements. Weak negative/positive bands at 1763/1751 cm^{-1} previously assigned to a perturbation of the C═O stretch mode of Asp76 during S_{373} formation in the low-pH form are replaced by a single intense positive band near 1749 cm^{-1} in the high-pH form. These results along with the effects of H/D exchange show that Asp76 is protonated in the signal-transducing form of sensory rhodopsin I and is ionized and functions as the counterion and Schiff base proton acceptor in the proton-transporting high-pH form of sensory rhodopsin I similar to bacteriorhodopsin.

Sensory rhodopsin I (SR-I)[1] is one of the two phototaxis receptors found in *Halobacterium salinarium* which control cell swimming behavior through light-activated signal transduction (Bogomolni & Spudich, 1982; Takahashi et al., 1985).[2] Phototaxis is mediated by two spectrally distinct forms of SR-I: SR_{587}, the dark-adapted state pigment, and the photointermediate S_{373} (Spudich & Bogomolni, 1984), with absorbance maxima at 587 and 373 nm, respectively. Signal transduction also involves a closely associated methyl-accepting transducer protein HtrI (Spudich et al., 1988; Yao & Spudich, 1992; Ferrando-May et al., 1993; Spudich & Spudich, 1993) which plays a signal relay role analogous to that of the G-protein, transducin, in the rhodopsin photocascade (Vuong et al., 1984).

Two other retinal proteins from *H. salinarium* have also been extensively studied*:* bacteriorhodopsin (BR) which functions as a light-driven proton pump [see Ebrey (1993), Khorana (1993), Skulachev (1993), and Rothschild & Sonar (1995) for recent reviews] and halorhodopsin (HR) which functions as a light-driven chloride pump (Lanyi, 1986). Despite the different functions of BR, HR, and SR-I, each of the three pigments consists of a protein with seven transmembrane-spanning regions (Blanck & Oesterhelt, 1987) bound to a retinylidene chromophore via a protonated Schiff base linkage (Fodor et al., 1989). In addition, all three pigments have a number of conserved residues (Blanck & Oesterhelt, 1987; Hegemann et al., 1987; Blanck et al., 1989), several of which form a pocket around the retinylidene chromophore which acts to constrain the initial retinal photoisomerization about the 13−14 double bond (Ahl et al., 1989; Rothschild et al., 1989).

In bacteriorhodopsin, the most extensively studied of the three pigments, two residues have been identified as critical for normal proton pumping: Asp85 which functions as the Schiff base counterion and acceptor group for the Schiff base proton during its deprotonation (L → M step of BR photocycle) (Braiman & Rothschild, 1988; Braiman et al., 1988a; Fahmy et al., 1992; Metz et al., 1992) and Asp96 which functions as the proton donor during Schiff base reprotonation (M → N step of BR photocycle) (Otto et al., 1989; Bousché et al., 1991a). In contrast, SR-I contains only an Asp85 analog (Asp76), whereas both the analogs to Asp85 and Asp96 are missing in HR.[3] All three pigments have conserved aspartic acid residues located near the Schiff base (Asp212-BR, Asp201-SR-I, and Asp238-HR) and near the β-ionone ring (Asp115-BR, Asp106-SR-I, and Asp140-HR), although the function of these residues in BR is not clearly established.[4]

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[1] Abbreviations: ATR, attenuated total reflection; BR, bacteriorhodopsin; FTIR, Fourier transform infrared; SR-I, sensory rhodopsin I; SR_{587} and 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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On the basis of FTIR difference spectroscopy and site-directed mutagenesis, it was recently established that a key difference between SR-I and BR is the protonation state of Asp76 (Asp85 in BR) (Rath et al., 1994). In SR-I, this residue is protonated in the SR_{587} state and undergoes an increase in its hydrogen bonding during the $SR_{587} \rightarrow S_{373}$ transition. In contrast, the homologous residue in BR, Asp85, is ionized in the BR_{568} state and accepts a proton from the Schiff base during the analogous $BR_{568} \rightarrow M_{412}$ transition. Importantly, this finding accounts partly for the red shift of the SR-I absorption (587 nm) compared to that of BR (568 nm) and the ability to substitute Asn for Asp76 without greatly altering the SR-I phenotype (Rath et al., 1994).

The existence of a protonated form of Asp76 also provides a possible explanation for the recent discovery that transducer free SR-I converts into a light-driven proton pump at high pH (Bogomolni et al., 1994). This transition occurs at a pK_a of 7.2 and is accompanied by a downshift of the λ_{max} from 587 to near 550 nm (Spudich & Bogomolni, 1983; Bogomolni et al., 1994; Haupts et al., 1995). Assuming that Asp76 deprotonates at high pH, it would be in a position to serve as both the Schiff base counterion and the proton acceptor group which is similar to the role of Asp85 in BR (Braiman et al., 1988a).

In this work, we have tested this hypothesis by measuring structural changes which occur in SR-I upon light activation at both low and high pH and pD. This is possible by using the method of attenuated total reflection-FTIR (ATR-FTIR) difference spectroscopy which allows complete control of the pH, ionic strength, and temperature of the bathing medium (Marrero & Rothschild, 1987). In contrast to earlier FTIR studies of SR-I which were performed on membrane purified from *H. salinarium* (Bousché et al., 1991b; Rath et al., 1994), SR-I and the mutant D76N were affinity purified using a polyhistidine tag on the C-terminal end of the protein (Krebs et al., 1995) and reconstituted at a high protein/lipid ratio into artificial membranes containing phosphatidylglycerol lipids. This new procedure dramatically improved the ability to obtain high-quality difference spectra of SR-I using the ATR-FTIR method. Our measurements confirm that at a pH below 7.0 Asp76 exists predominantly in a neutral form in which the carboxylic acid moiety undergoes an increase in hydrogen bonding upon formation of the S_{373} intermediate. Upon the pH being increased to 8.0, the spectral features in the carboxylic acid region change dramatically. A positive band is identified upon S_{373} formation which is similar to a band that appears during M formation in the BR photocycle. In contrast, this band is completely absent in D76N at high pH. We also find that the ethylenic C═C stretch mode of retinal in wild type SR-I upshifts to a frequency close to that of bacteriorhodopsin when the pH is raised to 8.0. In contrast, no change was observed for the ethylenic C═C stretch mode in D76N. These data establish conclusively that at high pH SR-I is converted to the 550 nm proton-pumping form due to the ionization of Asp76 which in analogy to bacteriorhodopsin now functions as a counterion and Schiff base proton acceptor.

[4] It has been postulated that Asp212 plays a role in Schiff base reprotonation through its participation in a hydrogen-bonded proton wire extending to the Schiff base proton donor, Asp96 (Braiman et al., 1988a).

MATERIALS AND METHODS

Sample Preparation and Purification. His-tagged SR-I was expressed in *H. salinarium* strain TAG1, in which the His-tag-encoding *sopI* gene is integrated at the *bop* locus of strain Pho81Wr$^-$ (Krebs et al., 1995). A mutated *sopI*-encoding His-tagged D76N was constructed by ligating the 8 kbp *Bgl*II-*Sna*BI fragment of plasmid pD76Ntr (Olson et al., 1995) to a 2 kbp *Bgl*II-*Sna*BI fragment of pSO12 (Krebs et al., 1995). The resulting plasmid was integrated at the *bop* locus of Pho81Wr$^-$ to produce strain TAG6, following a procedure similar to that for TAG1. Membranes of TAG1 and TAG6 were extracted with lauryl maltoside, and His-tagged SR-I was purified with Ni^{2+} affinity chromatography and reconstituted into L-α-phosphatidylglycerol proteoliposomes as described (Krebs et al., 1995).

UV-Visible Spectroscopy. Absorption data were recorded on a Hewlett-Packard 8453 diode array spectrophotometer (2 mL in 1 cm path length cuvettes), and pH titration was performed as described (Krebs et al., 1995). Flash-induced absorption changes were measured with a laboratory-constructed cross-beam kinetic spectrophotometer as described (Spudich et al., 1986), except the flash source was a pulsed Nd:YAG laser (6 ns pulse width, 40 mJ) at 532 nm (Surelite I, Continuum, Santa Clara, CA). Flashes were delivered at 0.08 Hz and collected at 2 ms/point for the pH 5.0 transients and at 0.017 Hz and collected at 5 ms/point for the pH 7.8 transients. Eight to fifteen transients were averaged for each measurement.

ATR-FTIR Difference Spectroscopy. ATR-FTIR difference spectra were recorded as previously described (Rath et al., 1994). SR-I samples stored as pellets in pH 6.8 Tris buffer (25 mM Tris and 4 M NaCl) were washed by rinsing the pellet with 2 mL of distilled water. The pellet was then dissolved in 8 μL of phosphate buffer (5 mM sodium phosphate, 5 mM KCl, 2 mM $MgCl_2$, 3 mM $CaCl_2$, and 250 mM NaCl at pH 6.8) and 32 μL of water and sonicated with a microtip sonicator (model W185, Branson Sonic Power Co., New York) at 25 W for a total of 10 s with 2 s bursts. The sample was then quickly transferred onto a 50 × 20 × 2 mm germanium internal reflection element and dried under 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) equipped with a quartz window for sample illumination. A buffer solution (50 mM MES and 4 M NaCl) was injected into the cell and allowed to equilibrate at 10 °C. The pH (or pD) of the buffer was adjusted prior to injection by adding microliter amounts of either 2 N HCl or 4 N NaOH. D_2O buffer was prepared by dissolving MES [2-(*N*-morpholino)ethanesulfonic acid] and NaCl in D_2O. The pD of the buffer was calculated by measuring its pH and using the relation pD = pH (meter reading) + 0.4 (Glasoe & Long, 1960). Each spectrum recorded is the average of 450−1350 scans recorded with a BioRad FTS60A spectrometer (BioRad, Digilab division, Cambridge, MA) at either 2 or 8 cm^{-1} resolution, respectively, and more than 30 light/dark differences were averaged to obtain the final spectrum. A 150 W tungsten/halogen lamp (model 180, Dolan-Jenner Industries, Woburn, MA) with several heat filters and a 500 nm long pass filter (Corion Corp., Holliston, MA) were used for illumination of the sample. Spectral analysis was performed using the software LabCalc (Galactic Industries, Nashua, NH).

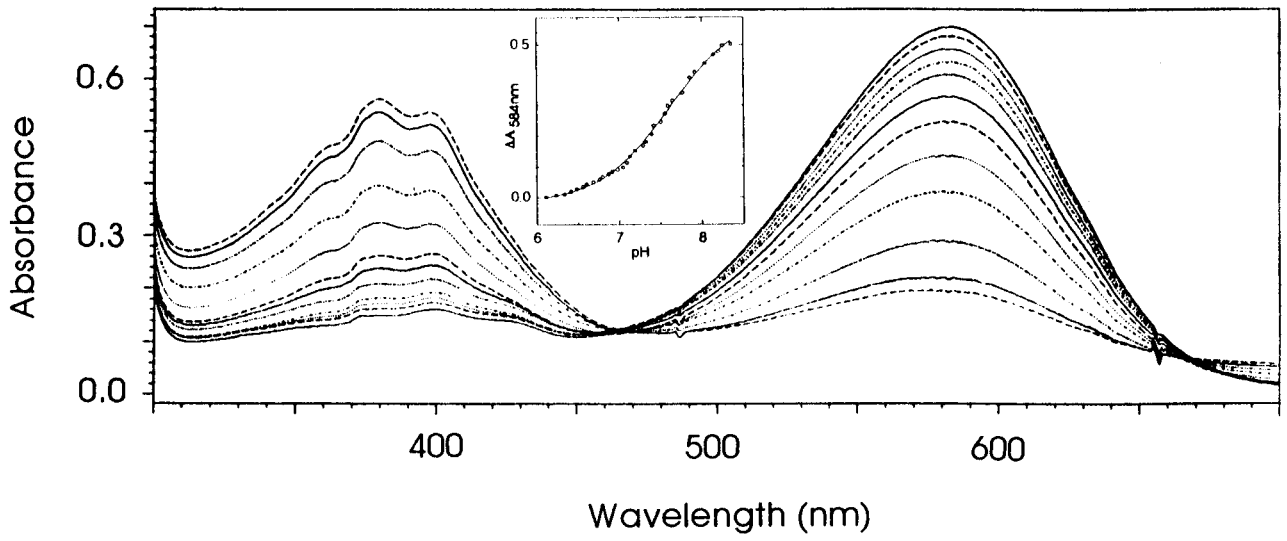

FIGURE 1: pH dependence of the His-tagged D76N absorption spectrum. The pH of purified His-tagged D76N in 50 mM sodium acetate, 25 mM MOPS, 4 M NaCl, and 0.025% lauryl maltoside was adjusted in the dark with dilute NaOH or HCl. The top spectrum is at pH 6.1 and the bottom spectrum at pH 8.4 (top to bottom at 580 nm). Intermediate spectra were collected at 0.2 pH unit increase per spectrum. The inset depicts the decrease in absorbance at 584 nm as a function of pH. The curve corresponds to a fit of the data to titration of a single group.

RESULTS

pH Titration and Flash-Induced Difference Spectra of Purified SR-I and D76N. Like native membranes, His tag-purified SR-I exhibits an alkaline transition characterized by the disappearance of the 587 nm band and simultaneous appearance of two bands near 550 and 400 nm with a pK_a of 6.5 (Krebs et al., 1995). In contrast, titration of D76N (λ_{max} = 582 nm) leads to formation of a structured band similar to the wild type 400 nm species ($\lambda_{max} \sim$ 380 nm in D76N, Figure 1) with a pK_a of 7.6 but not the 550 nm form at any pH between 6.1 and 8.4 (Figure 1). The titration data are consistent with titration of a single group since (i) there is an isosbestic point at 463 nm and (ii) the titration curve fits to a single pK_a (inset, Figure 1). The transition from low to high pH for D76N protein in Figure 1 was reversible and most likely reflects Schiff base deprotonation similar to the alkaline transition of D85N in bacteriorhodopsin (Duñach et al., 1990; Otto et al., 1990; Turner et al., 1993; Nilsson et al., 1995). Flash photolysis of His-tagged D76N confirms the absence of a photoactive 550 nm form (Figure 2) at pH 7.8, unlike His-tagged SR-I, which exhibits a depletion maximum shifted to 550 nm at alkaline pH (Krebs et al., 1995). As in HtrI-free SR-I, the rate of the photocycle in purified D76N is greatly retarded at high pH (Figure 2, insets).

Effects of pH on the FTIR Difference Spectra of Wild Type SR-I. Figure 3 shows the FTIR-ATR difference spectrum obtained from native SR-I at pH 6.0 and 7.9. The pH 6.0 spectrum is similar to previously reported FTIR difference spectra of SR-I (Bousché et al., 1991b; Rath et al., 1994). Under these conditions, light/dark differences should reflect the $SR_{587} \rightarrow S_{373}$ transition. Accordingly, many of the prominent negative bands can be assigned on the basis of resonance Raman spectroscopy (Fodor et al., 1989; Haupts et al., 1994) to vibrations of the SR_{587} *all-trans* retinylidene chromophore. These include the ethylenic C═C stretch mode at 1520 cm^{-1}, the C−C stretch modes at 1197 and 1165 cm^{-1}, and the CH_3 methyl rock at 1005 cm^{-1}. Since the infrared vibrations of a deprotonated retinal Schiff base

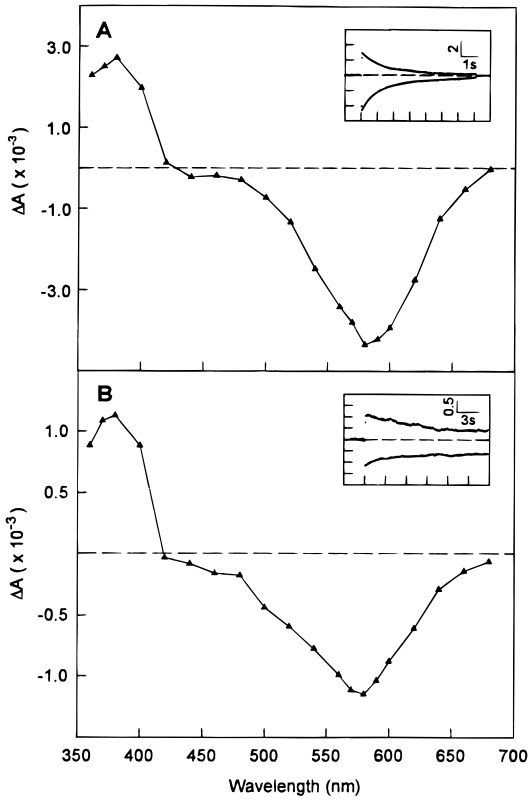

FIGURE 2: Laser flash-induced difference spectra of purified His-tagged D76N at pH 5.0 (A) and at pH 7.8 (B). Amplitudes were measured as the absorbance change 162 ms after the flash. The insets depict absorption transients monitored at 380 nm (upper trace) and 580 nm (lower trace).

are relatively weak compared to those of the corresponding protonated species (Gerwert & Siebert, 1986), positive bands due to the S_{373} chromophore which contains a 13-*cis*-deprotonated Schiff base are not expected to contribute significantly to the difference spectrum.

The C═O stretching region (1720−1780 cm^{-1}) reflects protonation and hydrogen-bonding changes of Asp/Glu carboxylic acid groups (Braiman & Rothschild, 1988). In

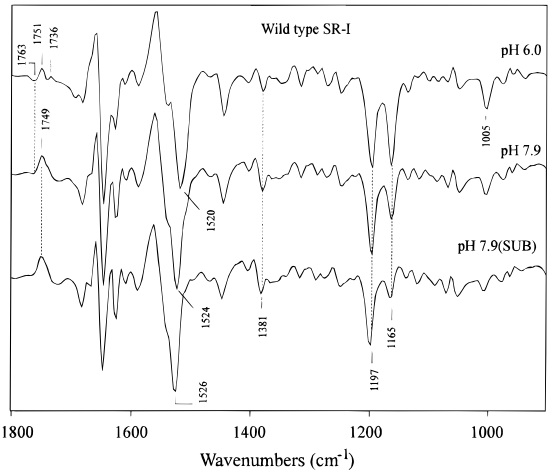

FIGURE 3: Dark → light ATR-FTIR difference spectra of native SR-I (wild type) at low and high pH recorded at 10 °C and 8 cm^{-1} resolution. Each difference spectrum is the subtraction of a dark spectrum (1350 scans, 5 min) and a light spectrum (1350 scans, 5 min); and consists of the average of at least 30 such differences. The pH 7.9 trace (SUB) was obtained by interactively subtracting pH 6.0 data from pH 7.9 data until the shoulder of the ethylenic mode at 1520 cm^{-1} disappeared.

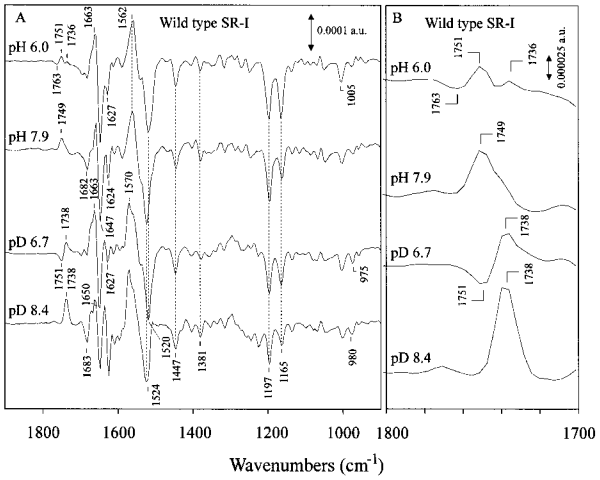

FIGURE 4: (A) Dark → light difference spectra of SR-I at low and high pH and pD. Spectra were recorded at 10 °C and 8 cm^{-1} resolution. All spectra were scaled to the same ethylenic intensities near 1520 cm^{-1} for comparison. (B) Expanded view of the dark → light FTIR difference spectra of SR-I in the region between 1700 and 1800 cm^{-1} from A. The scale bars shown are for the pH 6.0 spectrum.

the case of bacteriorhodopsin, a positive band at 1762 cm^{-1} in the BR → M difference spectrum (Rothschild et al., 1981) was assigned on the basis of site-directed mutagenesis to the protonation of the Asp85 counterion during Schiff base deprotonation (Braiman et al., 1988a; Fahmy et al., 1992). In contrast, only a small negative/positive set of bands at 1763/1751 cm^{-1} (Figure 3, pH 6.0) appear in the SR_{587} → S_{373} transition at pH 6.0. These bands were previously assigned by comparison with the mutant D76N to an increase in hydrogen bonding of Asp76 during this transition (Rath et al., 1994).[5]

In comparison to the pH 6.0 difference spectrum, several distinct changes occur when the pH is raised to near 8.0 (Figure 3). Most noticeable is the appearance of a positive band at 1749 cm^{-1} in the carboxylic acid C═O stretch region. This band is analogous to the positive band at 1762 cm^{-1} in the BR → M difference spectrum of bacteriorhodopsin, although appearing ∼10 cm^{-1} lower in frequency. In analogy to Asp85 in BR, its appearance indicates that Asp76 undergoes a net protonation upon formation of S_{373}. In support of this conclusion, an increase in (negative) intensity is observed near 1381 cm^{-1} in the pH 7.9 spectrum relative to the pH 6.0 spectrum (Figure 3) which falls in the region of the symmetric C═O stretch of carboxylate groups (Bellamy, 1968). Indeed, such a negative band is expected if an Asp/Glu residue is ionized in SR_{587} and protonated in S_{373} and as shown below can be assigned to Asp76.

An increase in pH also causes bands assigned to vibrations of the retinylidene chromophore of SR-I to become more similar to the chromophore vibrations of light-adapted bacteriorhodopsin (BR_{568}). Most apparent is the upshift of the ethylenic C═C stretch mode from 1520 to 1524 cm^{-1} [1527 cm^{-1} in BR_{568} (Roepe et al., 1987)]. Such an increase in frequency is consistent with the empirical inverse correlation between ethylenic stretch and λ_{max} for retinal proteins (Aton et al., 1977) and the blue shift of the visible λ_{max} of transducer free SR-I upon increase of the pH (Spudich & Bogomolni, 1983; Bogomolni et al., 1994; Haupts et al., 1995). A decrease in intensity of the 1165 cm^{-1} C−C stretch mode upon increasing pH is also observed. This effect is similar to the drop in intensity of the 1167 cm^{-1} band in BR_{568} relative to the O intermediate in the BR photocycle (Smith et al., 1983; Bousché et al., 1992).

In an earlier study (Bogomolni et al., 1994), it was found that the alkaline conversion of the 587 nm-absorbing species (low-pH form) to the 550 nm-absorbing species (high-pH form) occurs over a broad pK_a titration centered near 7. Hence, the difference spectrum recorded at pH 7.9 may not reflect the photocycle of a pure high-pH species of SR-I. In fact, this is indicated by the residual negative band at 1763 cm^{-1} at the same frequency as found in the low-pH photocycle. As seen in Figure 3, if this negative band is removed by interactive subtraction of the low-pH form from the high-pH form, a corrected difference spectrum is obtained which displays an ethylenic stretch mode at a similar frequency (1526 cm^{-1}) as in BR_{568}.

Effects of Hydrogen/Deuterium Exchange. As demonstrated previously for BR, information about structural changes of the protein and chromophore can be obtained by measuring band shifts induced by H/D exchange (Braiman & Rothschild, 1988; Rath et al., 1993). For example, the extent of the H/D-induced downshift of the carboxylic C═O stretch mode of Asp and Glu amino acid residues decreases with stronger hydrogen bonding, reflecting the coupling between the OH in-plane bend and the C═O stretch modes (Maeda et al., 1992). In contrast, ester carbonyl groups of phospholipids such as the L-α-phosphatidylglycerol lipids which were used to reconstitute SR-I in these studies are insensitive to H/D exchange. In the case of SR-I, H/D exchange causes the C═O stretch bands at 1763/1751 cm^{-1} (pH 6.0) and 1749 cm^{-1} (pH 7.9) to downshift 11−13 cm^{-1} (Figure 4A,B). This result eliminates the possibility that this band arises from structural changes in reconstituted mem-

[5] Note, however, that, in the earlier studies using transmittance and ATR at pH 6.8, the positive component near 1751 cm^{-1} was more intense relative to the negative component. For this reason, the possibility was raised that this effect may be due to a second high-pH reaction of SR-I which gives rise to a positive band in this region (Rath et al., 1994).

brane lipids. Instead, the shift is similar to the extent of H/D-induced shift (12 cm^{-1}) of the C═O stretch mode of Asp85 in the M intermediate of BR and implies a similar hydrogen-bonding strength. We also note that as expected the increase in intensity of the negative 1381 cm^{-1} band assigned to the symmetric C═O stretch of a carboxylate group is unaffected by D_2O.

In the case of the chromophore, several H/D-induced spectral changes can be attributed to deuteration of the Schiff base. The increased intensity of the negative band at 975 cm^{-1} in the pD 6.7 difference spectrum (Figure 4) is due to the C═N−D bending mode (Haupts et al., 1994). Significantly, this band shifts to 980 cm^{-1} in the pD 8.4 spectrum, closer to the position measured in the resonance Raman spectrum of BR (Haupts et al., 1994). The small negative band at 1627 cm^{-1} has previously been assigned to the C═N stretch mode of the Schiff base on the basis of a 7 cm^{-1} H/D-induced downshift of this band in the resonance Raman spectrum (Fodor et al., 1989). However, a more recent assignment of this mode is to a band which appears in the resonance Raman spectrum at 1634 cm^{-1} and downshifts to 1617 cm^{-1} upon deuteration of the Schiff base (Haupts et al., 1994). This is consistent with the H/D-induced changes we observe in this region in the FTIR difference spectra. While the 1627 cm^{-1} band is still present in the pD 6.7 spectrum, increased intensity is observed near 1635 cm^{-1}, possibly due to the downshift of a negative band near this frequency. H/D exchange also causes a band to appear at 1570 cm^{-1}, close to the frequency of the ethylenic C═C stretch in S_{373} (Haupts et al., 1994). This effect occurs for all pD's measured and also for the case of D76N (see below). This band can also be seen as a shoulder in resolution-enhanced spectra of wild type SR-I at pH 6.0.

Effects of Asp76 → Asn76 Substitution. Site-directed mutagenesis can be used to assign bands in the FTIR difference spectrum of a protein (Braiman et al., 1988a,b). In agreement with an earlier study based on FTIR transmittance difference spectroscopy (Rath et al., 1994), we find that the substitution Asp76 → Asn results in the complete disappearance of the negative/positive bands near 1763/1751 cm^{-1} in the pH 6.0 difference spectrum (Figure 5A,B). This effect confirms the assignment of these bands to the carboxylic acid C═O stretch mode of Asp76 (Rath et al., 1994). The D76N difference spectrum also clearly reveals the existence of bands at 1744 cm^{-1} (negative) and 1737 cm^{-1} (positive) which were partially canceled in the wild type SR-I spectrum (Figure 4A,B). The assignment of these bands is presently unknown, although two possible candidates are Asp201 and/or Asp106 which may undergo a change in their hydrogen bonding or protonation state. We also find in agreement with resonance Raman measurements on D76N (Haupts et al., 1994) that the chromophore is not perturbed by the D76N substitution since the ethylenic and C−C stretch modes of the retinal chromophore at 1520 and 1165 cm^{-1}, respectively, remain the same as those of wild type at pH 6.0. Overall, these effects indicate that at pH 6.0 the Asp side chain exists in a nonionized (*e.g.* neutral) state in both SR_{587} and S_{373} but undergoes an increase in its hydrogen bonding.

In contrast to that of wild type SR-I, the difference spectrum of D76N is almost identical at pH 6.0 and 8.0 (Figure 5A,B). In particular, the positive band at 1749 cm^{-1} which appears in the wild type difference spectrum of SR-I upon the pH being increased to near 8.0 is absent in D76N at pH 8.0. Instead, the small positive/negative bands at 1744/1737 cm^{-1} are present at both pH's. In addition, the alkaline-induced increase in intensity of the negative 1381 cm^{-1} band does not occur in D76N, further indicating that this effect in wild type SR-I occurs due to the existence of an Asp76 carboxylate group in SR_{550} which becomes protonated in S_{373}. In the case of the chromophore, the ethylenic C═C stretch mode of D76N remains near 1520 cm^{-1} in contrast to that of wild type SR-I, where the ethylenic stretch mode increases in frequency from 1520 to 1526 cm^{-1} at pH 8. Together, these effects establish that raising the pH of wild type SR-I converts the carboxylic acid group of Asp76 to a carboxylate counterion and Schiff base proton acceptor.

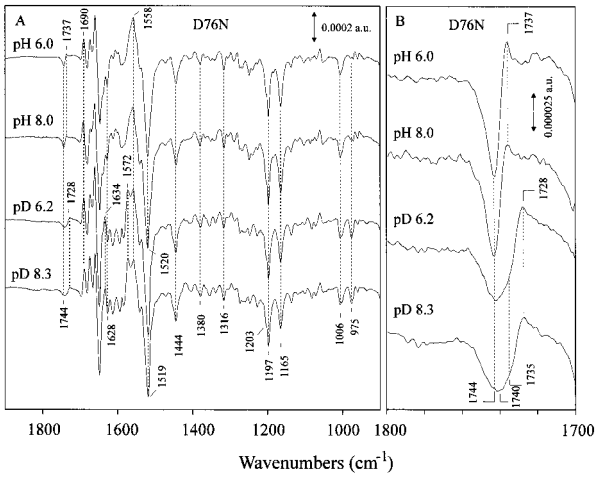

FIGURE 5: A. Dark → light difference spectra of the mutant D76N at low and high pH and pD. Spectra were recorded at 10 °C and 2 cm^{-1} resolution. All spectra were scaled to the same ethylenic intensities near 1520 cm^{-1} for comparison. (B) Expanded view of the dark → light FTIR difference spectra of D76N in the region between 1700 and 1800 cm^{-1} from A. The scale bars shown are for the pH 6.0 spectrum.

The effects of H/D exchange on the FTIR difference spectrum of D76N were also investigated. Almost identical spectra were obtained at pD 6.2 and 8.3. As seen in Figure 5A,B, the 1744/1737 cm^{-1} pair of bands downshift in frequency, with the negative component downshifting approximately 4 cm^{-1} and the positive band 9 cm^{-1}. This may indicate that these bands arise from different Asp/Glu carboxylic acid groups with different hydrogen bond strengths. Alternatively, the increased broadness of the negative component may indicate the existence of two different components at 1744 cm^{-1} which undergo different H/D-induced shifts.

Finally, we note that a new positive band is found near 1690 cm^{-1} (Figure 5A) in the D76N difference spectra at both high and low pH and pD. This frequency is typical for the C═O stretch mode of Asn and may reflect a change in hydrogen bonding of Asn76 during the SR_{587} → S_{373} transition due to Schiff base deprotonation. A similar band has been observed when Asn is substituted for Asp96 and Thr46 in the BR → M difference spectrum (Gerwert et al., 1989; Coleman et al., 1995).

DISCUSSION

In this work, we have continued our FTIR-based studies of the molecular basis for SR-I function (Bousché et al., 1991b; Rath et al., 1994) [for a recent review on FTIR studies

of retinal pigments see Rothschild (1992)]. In the absence of the transducer protein HtrI, SR-I exists in a pH dependent equilibrium between two forms with a pK_a near neutral pH (~7.2) (Bogomolni et al., 1994). At low pH, the dominant form is spectrally identical to the signal-transducing light receptor, SR_{587} with a λ_{max} at 587 nm. At high pH, it converts to a purple form with λ_{max} near 550 nm and exhibits electrogenic light-driven proton transport just as in the case of BR (Bogomolni et al., 1994). Recently, it has been shown (Rath et al., 1994) that a critical difference between BR and SR-I is that the Asp85 is the counterion and Schiff base proton acceptor in BR (Braiman & Rothschild, 1988; Braiman et al., 1988a; Fahmy et al., 1992; Metz et al., 1992), whereas the homologous Asp76 residue in the signal-transducing form of SR-I is not ionized. This raised several related questions. (i) Is Asp76 the titratable group in SR-I which converts the phototaxis receptor protein to a proton pump? (ii) Does Asp76 function as the Schiff base proton acceptor and counterion in the alkaline proton-pumping form of SR-I? (iii) Are other Asp groups such as Asp201 involved in signal transduction and/or proton pumping of SR-I?

The present work establishes two key findings.

(1) Asp76 functions as the Schiff base proton acceptor during the photocycle of the alkaline form of SR-I. On the basis of site-directed mutagenesis, we are able to assign bands in the ATR-FTIR difference spectrum of the alkaline form of SR-I to the protonation of Asp76 during the $SR_{587} \rightarrow S_{373}$ transition. This indicates in complete analogy to the role of Asp85 in the proton pump mechanism of BR that Asp76 functions as the proton acceptor for the Schiff base. In contrast, we find that Asp76 is protonated and undergoes an increase in hydrogen bonding during the photocycle of the low-pH signal-transducing form of SR-I in agreement with an earlier FTIR study (Rath et al., 1994). These findings show that Asp76 is the titratable group which converts SR-I between its phototaxis and proton-pumping forms as suggested in a model of SR-I/HtrI interaction (Spudich, 1994).

(2) Asp76 functions as the Schiff base counterion in the high-pH form of SR-I. The observation of the blue shift of the SR-I absorption from 587 to 550 nm at alkaline pH has led to the prediction that Asp76 functions as the Schiff base counterion (Bogomolni et al., 1994; Haupts et al., 1994). Our present study provides conclusive evidence for this hypothesis since, in contrast to wild type SR-I, we find no shift in the visible λ_{max} to 550 nm or changes in chromophore vibrational modes of D76N at high pH. This is expected if Asp76 serves as the Schiff base counterion, since, unlike the COOH group of Asp, the $CONH_2$ group is not ionizable (at least not in the pH range examined) and should not be therefore convertible to a counterion. In addition, the chromophore bands assigned in the alkaline form of SR-I appear at frequencies similar to those of BR, providing further evidence that Asp76 is the counterion for the Schiff base of SR-I at high pH.

Finally, we note that, since Asp76 is already protonated in the signal-transducing form of SR-I, it cannot function as the Schiff base proton acceptor as we find for the alkaline proton-pumping form. This raises the question about the identity of the Schiff base acceptor as well as the role of Schiff base deprotonation in SR-I signal transduction. One possibility is that a proton acceptor in the intact SR-I/HtrI signaling complex may reside on the transducer. In this case, the proton from the Schiff base would be required to move toward the cytoplasmic side of the SR-I protein, where HtrI is known to interact (Jung & Spudich, 1996). Protonation of a group on HtrI perhaps following initial transfer to a primary acceptor in SR-I during S_{373} formation might serve to trigger the attractant phototactic response. Alternatively, HtrI may sense a conformational change associated with the proton transfer in SR-I.

Recent evidence shows that a proton-conducting path exists between the Schiff base and the extramembraneous environment in the HtrI-free receptor with HtrI binding blocking this path (Olson & Spudich, 1993). An interesting possibility is that some of the bands we observe in the carboxylic C=O stretch region of the SR-I difference spectrum which are not assigned to Asp76 may reflect changes in Asp/Glu groups involved in this proton transfer. One candidate for such a residue is Asp201, which 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). A second possibility is that some of these bands arise from Asp106, which in the case of BR (Asp115) undergoes changes in hydrogen bonding (Braiman et al., 1988a). However, in order to investigate further these possibilities, it will be important to assign other bands in the carboxyl region using site-directed mutagenesis.

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