Infrared Spectroscopic Detection of Light-Induced Change in Chloride–Arginine Interaction in Halorhodopsin†

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ABSTRACT: A light-induced transient change in the ionic interaction between chloride and arginine in the transmembrane anion pump halorhodopsin (hR) is detected with infrared absorption spectroscopy. In the IR difference spectrum of hR and one of its photoproducts (hL), only a few bands have frequencies that depend on the particular halide ion (Cl^-, Br^-, or I^-) present. Three of the halide-sensitive negative difference bands (at 1695, 1610, and 1170 cm^{-1}) correspond in frequency to arginine C–N vibrations and undergo anion-dependent shifts that match those seen in ethylguanidinium halide model compounds. These shifts reflect the different strengths of the ionic interactions formed with the various halides. We conclude that a halide–arginine ion pair is present in the hR state; this interaction appears to be disrupted by photoconversion to hL.

Vibrational spectroscopy has previously permitted the direct detection of transient light-induced proton binding and release at side chains of specific residues in the proton transport protein bacteriorhodopsin (bR)[1] (Braiman et al., 1988; Gerwert et al., 1989). Analyzing proton binding sites with IR spectroscopy is relatively simple, because protonation/deprotonation reactions involve covalent bond changes and therefore result in large shifts of molecular vibrational frequencies. In contrast, noncovalent binding interactions with larger ions are expected to lead to relatively small shifts in the vibrational frequencies of protein residues. Here we demonstrate that FTIR vibrational spectroscopy is nevertheless capable of detecting these relatively weak interactions in a membrane transport protein.

The protein examined is halorhodopsin (hR, MW = 27 000), which is structurally homologous to bR and is also found in *Halobacteria.* However, hR utilizes light energy to pump anions—specifically, halide ions—instead of protons (Lanyi, 1990). In the process of being ferried across the low-dielectric membrane, halides are expected to interact with positively charged groups of the protein. One such group is the protonated Schiff base formed between the *all-trans*-retinal chromophore and lysine-242. This is the only lysine in the primary sequence of hR obtained from *Halobacteria halobium* (Blanck & Oesterhelt, 1987). Another cationic residue in hR from this species is histidine-95, which is thought to be situated at the extracellular membrane surface and therefore unlikely to play a central role in the transport mechanism (Lanyi, 1990). The only other positively charged groups in this hR are 11 arginines, five of which are conserved or mutated to lysine in hR obtained from a related species, *Natronobacterium pharaonis*, and three of which (Arg-60, -108, and -200) are thought to be located in membrane-spanning α-helical portions of the protein (Lanyi et al., 1990). Presumably some of these arginines play a role in binding anions transiently as they are transported across the membrane.

A specific model for the halide-transporting photocycle of hR involving one of these arginine residues is shown in Figure 1. In this model, photoisomerization of retinal around its C_{13}=C_{14} bond translocates an ion pair, consisting of the protonated Schiff base group of the chromophore and a halide counteranion (X^-), away from an initial location accessible to the extracellular side of the membrane to another location accessible to the cell interior. This model predicts that, in the starting (hR) state, the halide ion participates in weakly hydrogen-bonded ionic interactions with both the protonated Schiff base and arginine-108. In the principal photoproduct state (hL), the arginine–halide interaction is disrupted. This specific prediction can be tested with IR vibrational spectroscopy, in combination with a novel "halide perturbation" approach. IR spectra of hR in suspensions containing Cl^-, Br^-, or I^- are examined for halide-dependent vibrational frequencies. The different radii of the halides are expected to result in changes in interaction strength with any protein residues they contact, with concomitant frequency shifts in their localized molecular vibrations. Indeed, halide perturbation effects are observed for hR vibrations that are attributable to arginine based on spectra of model compounds. These halide perturbation effects support the mechanism shown in Figure 1.

EXPERIMENTAL PROCEDURES

HR Samples. Achieving 0.5-cm^{-1} reproducibility for peak frequencies in hR→hL difference spectra required samples with identical lipid to protein ratios. To this end, protein/lipid vesicle samples used for comparing spectra in the presence of the three different halides came from a single preparation of hR and lipids in aqueous detergent solution (3% octylglucoside, 4 M NaCl, 25 mM Tris, pH 7.2). The hR had been purified as described previously (Duschl et al., 1988; Walter & Braiman, 1994) and then combined in the detergent solution with halobacterial polar lipids (Kates et al., 1982) in a ~1:5 ratio by weight, to give an hR concentration of ~0.5 mg/mL. Similar-sized portions of the hR/lipid/detergent solution were then dialyzed extensively against buffered 3 M potassium halide buffers (pH 7.2), in order to remove detergent and form hR/lipid vesicles in the presence of the desired halide.

Model Compounds. Ethylguanidinium (EG) salts, used to

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[1] Abbreviations: hR, halorhodopsin; bR, bacteriorhodopsin; EG, ethylguanidinium; TR, time-resolved; FTIR, Fourier transform infrared; PTFE, poly(tetrafluoroethylene).

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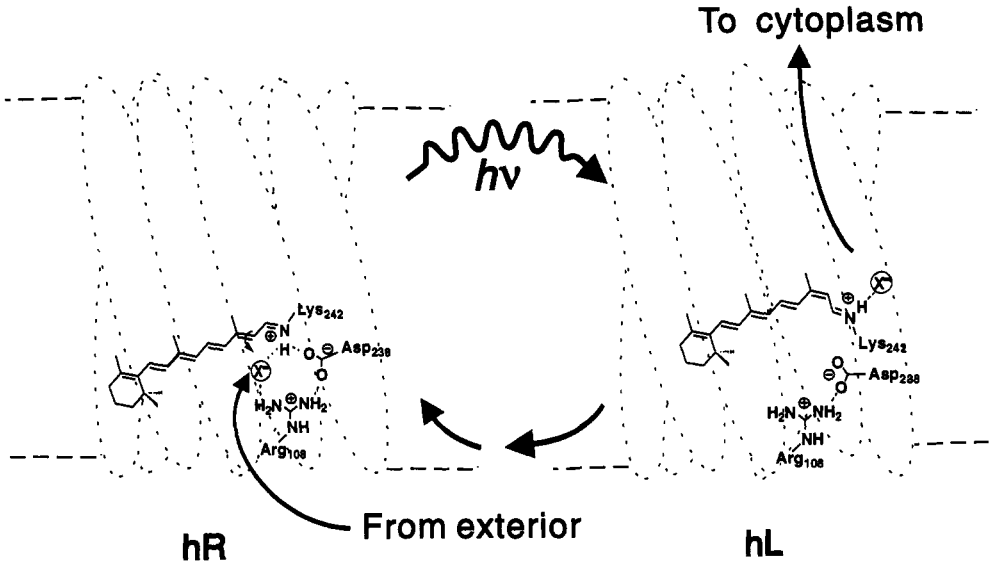

FIGURE 1: Model for coupling of hR chromophore photoisomerization to translocation of a halide ion (X^-) during the hR→hL photoreaction, modified from a proposal by Ames et al. (1992). The specific hypothesis tested in the current work is whether the indicated hydrogen-bonded ionic interaction between halide and arginine exists in the hR state, and whether this interaction is disrupted when hL is formed.

model arginine interactions with various anions, were prepared as follows. EG·Cl (Aldrich) was converted to the carbonate salt by dissolving 0.1 g of it in 2 mL of H_2O, adding a 4× excess of Ag_2CO_3, and bubbling CO_2 through the stirred mixture for 20 min. After raising the pH to 7 with concentrated ammonia, AgCl precipitate and unreacted Ag_2CO_3 were removed by filtration. Residual silver ions were removed by passing the EG-carbonate solution through an ion-exchange column (Bio-Rad Chelex, 100, ammonium form, 0.5 g of resin/g of ethylguanidine) and then lyophilizing the eluant to remove H_2O and $NH_4{\cdot}HCO_3$.

Addition of an excess of HCl, HBr, HI, or HOAc to the EG-carbonate in H_2O led to replacement of the carbonate by another anion, as evidenced by production of CO_2 bubbles. The resulting EG salts were alternately dried under vacuum and dissolved in absolute ethanol to remove excess acid and residual water, and finally recrystallized from dry ethanol. Crystallization was promoted by addition of diethyl ether to the cold ethanol. The colorless crystals were washed in cold ether. The hydrochloride salt prepared in this fashion had an IR spectrum identical with that of the commercially obtained starting material and clearly distinct from the carbonate salt intermediate.

Deuterated EG salts were prepared by alternately dissolving the undeuterated salts in methanol-O-D (Cambridge Isotope Labs) and evaporating this solvent under vacuum (3× total). The measuring solvent for deuterated samples was $CHCl_3$:MeOD, 97:3 (v/v).

FTIR Spectroscopy and Data Analysis. Spectra of EG salts dissolved in organic solvents were measured in a Nicolet 740 spectrometer, using a demountable liquid cell (Harrick) with 19-mm diameter CaF_2 windows separated by a 0.25-mm PTFE spacer. Reagent-grade $CHCl_3$ and MeOH were kept dry by storage over 4-Å molecular sieves.

Low-temperature difference spectra of the hR→hL and bR→L photoreactions were measured in a Heli-Tran cryostat, mounted in a Nicolet 60SXR spectrometer equipped with a broad-band HgCdTe detector, using previously published procedures (Rothschild et al., 1988; Chen & Braiman, 1991; Walter & Braiman, 1994). Spectral data were plotted and fitted using Lab-Calc software from Galactic Industries. See figure and table captions for additional details.

RESULTS AND DISCUSSION

Bands in the hR→hL FTIR difference spectrum (Figure 2A) arise from groups whose structure or environment changes when hR is photolyzed by visible light to form the hL intermediate (Rothschild et al., 1988; Walter & Braiman, 1992, 1994). The clear similarity of the hR→hL difference spectra measured in the presence of Cl^-, Br^-, and I^- (Figure 2A) indicates that the same hR→hL structural transformation is taking place. This is consistent with earlier reports that these three anions are all tightly bound and efficiently pumped by hR (Steiner et al., 1984).

Against this nearly invariant background, a few hR→hL difference bands show small but reproducible anion-dependent changes in frequency. These bands most likely represent groups directly involved in halide binding. In particular, all the bands in Figure 2A that show Cl^-/Br^- frequency shifts of 1 cm^{-1} or more are labeled with bold arrows, along with the peak frequency measured in the presence of Br^-. In all cases, the frequency in Br^- was lower than in Cl^-; these peaks also all showed a further downshift of at least 1 cm^{-1} in the presence of I^-.

As an internal control, none of the 32 peaks labeled with light tick marks in Figure 2A shifts by more than 0.5 cm^{-1} when Cl^- and Br^- spectra are compared. When the I^- spectrum is added into the comparison, the maximum spread among each of the 32 sets of three values is 1.5 cm^{-1}; the average spread was 0.67 cm^{-1}, with a standard deviation of 0.5 cm^{-1}. The halide-dependent shifts of the peaks marked with bold arrows are thus all at least 4 times the expected standard error of FTIR frequency measurements; the Cl^-/I^- shift of the 1695-cm^{-1} peak (see Figure 3 and Table 1) is about 6 times this standard error.

Additional control experiments on bR show that, unlike the hR→hL spectrum, the bR→L difference spectrum (Figure 2C) has no halide-dependent peak frequencies. More specifically, no bands as large as those labeled with light tick-marks in Figure 2A show Cl^-/Br^- frequency shifts greater than 0.5 cm^{-1}. This is as expected, since bR is a proton pump and, unlike hR, does not bind halide ions under physiological conditions (Falke et al., 1984).

Essentially all of the halide-dependent frequencies in the hR→hL difference spectrum (Figure 2A) can be accounted

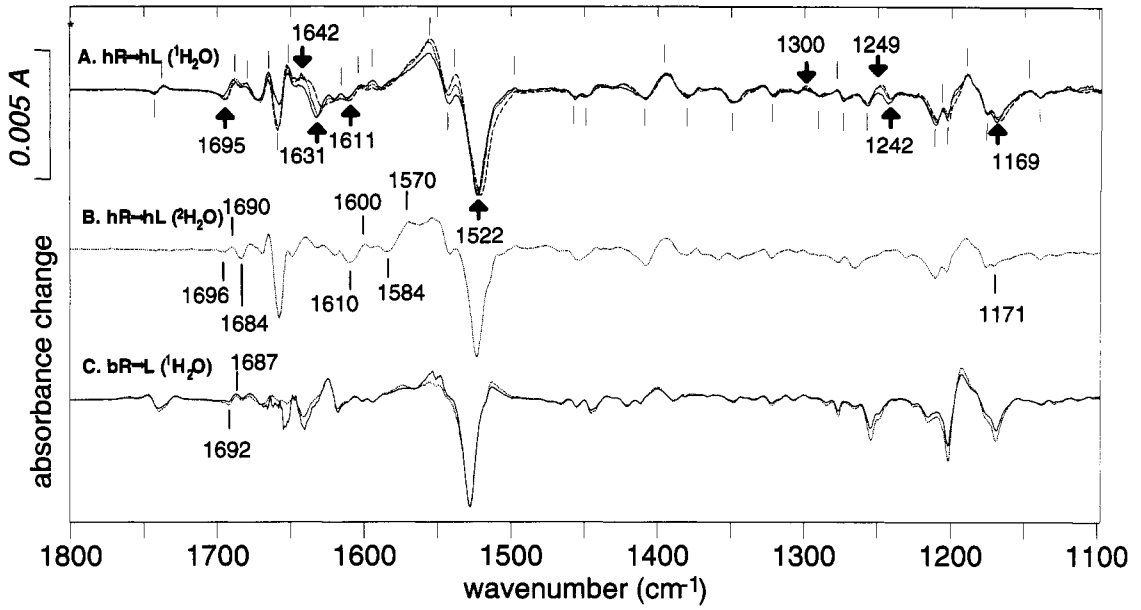

FIGURE 2: Halide dependence of light-dark difference spectra of undeuterated hR (A), deuterated hR (B), and undeuterated bR (C), measured in the presence of saturated KCl (solid curves), KBr (dotted), or KI (dashed). The hR→hL difference spectra in spectra A and B were measured at 250 K and 1-cm^{-1} resolution as in Rothschild et al. (1988) and Walter and Braiman (1994). Positive signals are due to new structures formed in the hL state, while negative signals represent photolysis-depleted hR structures. The bR→L difference spectra in C were measured at −100 °C as in Braiman et al. (1988) and Chen and Braiman (1991). Peaks are labeled with numbers only if referred to in the text. HR samples were purified and reconstituted as reported elsewhere (Walter & Braiman, 1994). The lipid to protein ratio in each sample was approximately 5:1 (w/w) and was identical among the three samples in spectrum A. Water concentration was ~50% by weight, and the pH was ~7.2. Spectra in each group were shifted vertically by different constants in order to bring the absorbance in the range 1750–1800 cm^{-1} to the same baseline and then scaled relative to each other (by a factor of 4 at most) to give identical peak heights for the strongest negative band near 1525 cm^{-1}. The y-axis scale bar representing this peak height is thus an average value of the true scales of six different plots.

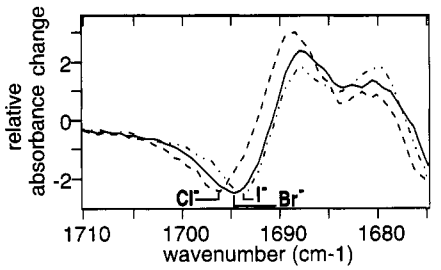

FIGURE 3: Expansion of a portion of the difference spectra shown in Figure 2A, showing more clearly the anion dependence of the ~1695-cm^{-1} negative band attributed to an arginine C=N vibration of hL. The three spectra were scaled to give identical y-minima for this peak. The ordinate gives the absorbance relative to the maximum of the positive band at 1740 cm^{-1}, which is due to the carbonyl stretch absorbance of a single group, Asp-141 (Rothschild et al., 1988).

for by interactions of halide ions with the two cationic groups shown in the model in Figure 1 as follows:[2]

(1) The three negative halide-sensitive difference bands at ~1695, ~1611, and ~1169 cm^{-1} are likely due to arginine side chain vibrations, specifically to C–N stretching modes. Characteristic group vibrations near these frequencies have been observed and assigned in Raman and IR spectra of arginine and other substituted guanidinium compounds (Chirgadze et al., 1975; Venyaminov & Kalnin, 1990; Sension et al., 1990).

(2) The anion-dependent negative hR peaks near 1631 and 1522 cm^{-1} are due to chromophore vibrations. Likewise, the anion-dependent positive band near 1642 cm^{-1} is due to the C=N stretch of the hL photoproduct. The anion dependence of these chromophore Schiff base vibrations is discussed in greater detail elsewhere (Walter & Braiman, 1994). An additional anion-dependent difference band (with both positive and negative components) near 1245 cm^{-1} can be attributed to a vibration of the lysine participating in the Schiff base linkage to the chromophore. The C–N vibration of this single bond has been calculated to have a frequency near 1250 cm^{-1} (Smith et al., 1987). Furthermore, a negative shoulder at 1250 cm^{-1} in FTIR difference spectra of bR photointermediates (Figure 2C) has been definitively assigned to such a vibration on the basis of specific isotope labeling of lysines (McMaster & Lewis, 1988).

This report focuses on the three halide-dependent vibrations attributable to arginine. It is important first to understand

Table 1: Anion Dependence of hR → hL Difference Bands near 1695 cm^{-1} [a]

sample	frequency (cm^{-1})	bandwidth (cm^{-1})	intensity[b]
hR·Cl⁻	1695.7	10.3	−2.5
	1688.8	6.8	+3.9
	1680.9	4.1	+1.2
hR·Br⁻	1694.3	10.4	−2.6
	1688.0	5.7	+3.5
	1681.0	6.9	+1.7
hR·I⁻	1693.0	11.0	−2.3
	1688.2	5.6	+2.9
	1680.7	6.1	+2.0

[a] Based on the hR → hL difference spectra shown in Figure 3. There were a total of 44 x,y pairs in each spectrum. For each sample, the nine parameters given in the table were adjusted during the fit; they converged to identical values in repeated runs with different initial guesses. Peak shapes were constrained to be Lorentzian for the highest frequency (negative) band and Gaussian for the two lower frequency (positive) bands. Assuming other combinations of band shapes always gave worse fits but also always increased the separation between the fitted frequencies of the negative band near 1695 cm^{-1}. Fitted frequencies are estimated to have a standard error of ±0.3 cm^{-1}. [b] Fitted absorbance intensities are given as a fraction of the peak value at the maximum near 1738 cm^{-1}. Using the same intensity scale, the root-mean-square deviation of the fitted and measured values was in the range 0.08–0.12 for each of the three fits.

[2] The sole exception is the small positive band at ~1300 cm^{-1} for which we currently have no specific assignment.

the logic of this spectral assignment, since amide groups, which are represented in hR by glutamine and asparagine side chains, and by peptide linkages, have also been known to give rise to vibrations near 1695 (or 1610) cm^{-1} as well as 1170 cm^{-1}. Assignment of most of the intensity in these three bands to arginine makes better sense for several reasons. First, the ~1695- and ~1170-cm^{-1} bands in hR are at the extremes of the usual ranges for the carbonyl stretch (or amide I) and C–N (or amide II) stretch modes, respectively, of primary or secondary amides (Venyaminov & Kalnin, 1990). On the other hand, while the observed hR frequencies of ~1695, ~1611, and ~1169 cm^{-1} are somewhat different than those observed for arginine model compounds reported previously (Sension et al., 1990; Venyaminov & Kalnin, 1990), new model compound spectra are shown below which essentially duplicate the hR frequencies.

Furthermore, assignment of hR bands near 1695, 1611, and 1170 cm^{-1} to arginine is consistent with shifts observed in the spectrum upon deuterium exchange (Figure 2B), indicating that these vibrations must be due to a group or groups with exchangeable proton(s).[3] Other workers' model compound data (Sension et al., 1990; Venyaminov & Kalnin, 1990), as well as our own (see below), indicate that $^1H/^2H$ exchange with deuterated solvent should shift arginine C–N vibrations from around 1675, 1625, and 1180 cm^{-1} to around 1620, 1590, and 1135 cm^{-1}. In fact, in the deuterium-exchanged hR→hL difference spectrum (Figure 2B), a negative/positive pair of bands near 1690 cm^{-1} and a negative band near 1169 cm^{-1} show significant decreases in relative intensity. At the same time, a difference band with negative/positive peaks at 1584/1570 cm^{-1} appears to grow in. No single amide group, nor any other individual protein or chromophore group except arginine, can account for these shifts. Only simultaneous shifts of multiple amide bands with unusual frequencies could conceivably produce the observed deuteration sensitivities in the 1690- and 1580-cm^{-1} spectral regions. Nevertheless, assignment of 1695-, 1611-, and 1169-cm^{-1} hR→hL difference bands to arginine can not be considered proved until they are observed to shift as a function of more specific labeling.

A final reason for assigning all three of these hR frequencies to arginine rather than an amide group is that there is no simple explanation for why amide carbonyl vibrations would show the observed dependence on external anions, whereas a simple model system for arginine–halide ion pairs closely mimics the observed anion dependence of hR vibrations, as described below.

Modeling the Anion Dependence of the Guanidino C–N Vibrations. It has been known for some time that the C–N vibrations of guanidinium salts in the solid state show strongly anion-dependent frequencies (Bonner & Jordan, 1976). However, the observed shifts are somewhat larger than the 1–3-cm^{-1} halide perturbations that we observe in the hR→hL difference spectrum. For example, the strongest (and highest frequency) C–N stretch band in a phenylguanidinium iodide solid film was 11 cm^{-1} lower than the corresponding band of the chloride salt (Walter & Braiman, 1992). However, interpretation of such solid-state spectra is complicated because, in solids, each guanidinium ion is expected to be involved in hydrogen-bonding contacts with multiple anions in a shared fashion, as demonstrated in the crystal structure of methylguanidinium chloride (Curtis & Pasternak, 1955). The halide-perturbation effect on arginine frequencies in hR is expected to be smaller than for solid-state guanidinium salts, because in the former only a single hydrogen-bonding ionic interaction with halide is proposed. To model this weaker interaction, it is desirable to find a solvent system in which substituted guanidinium halides exist, at least in part, as isolated ion pairs.

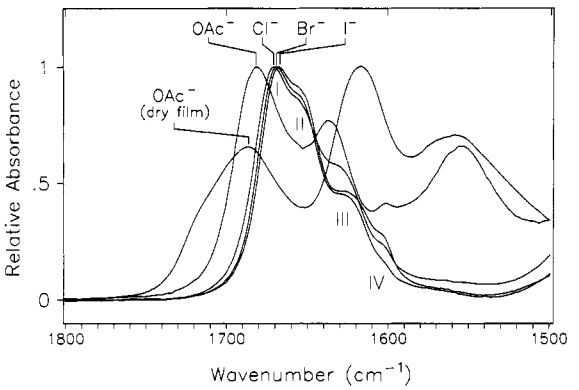

FIGURE 4: FT-IR spectra of the indicated salts of EG, measured as near-saturated (12 mM) solutions in 97:3 chloroform:methanol (v/v) and, for the OAc^- salt only, as a film dried from 15 μL of saturated aqueous solution. The EG-OAc film was noncrystalline, indicating that some water may have remained in it. Note that both solution and film spectra of EG-OAc salt show a broad band near 1555 cm^{-1}, corresponding to the antisymmetric C–O stretch of the anion. Spectral resolution was 2 cm^{-1}. Spectral curves were ratioed against the solvent-filled cell (or a single clean window, in the case of the EG-acetate film) and were left unsmoothed. Baselines were adjusted by subtraction of a linear function, and then the spectra were rescaled to give identical maxima for the strongest peak near 1675 cm^{-1}. To remove imperfectly nulled H_2O-vapor absorption bands, an appropriate fraction of a reference water vapor absorbance spectrum was added to or subtracted from each sample spectrum.

Figure 4 establishes that an ethylguanidinium (EG) salt dissolved in a dry organic solvent consisting of chloroform:methanol (97:3, v/v) produces the desired model system of weakly solvated ion pairs. The EG salts were not sufficiently soluble in pure chloroform to obtain satisfactory IR spectra and indeed are only slightly soluble (~15 mM) in the stated binary system. In this solvent, however, the frequencies of all three of the C–N stretching vibrations of EG are measurably different in the presence of different counteranions (see also Table 2). For example, in Figure 4 the bands labeled I and III (near 1670 and 1630 cm^{-1}) are very close in frequency to previously observed IR and Raman bands of arginine in aqueous solution (Venyaminov & Kalnin, 1990; Sension et al., 1990). The IR frequencies of these two bands are clearly counteranion-dependent; the size and direction of halide-dependent shifts are very similar to those seen for the ~1695- and ~1611-cm^{-1} bands of hR (Figures 2 and 3). We attribute bands I and III in the EG solution spectra in Figure 4 to C–N frequencies of hydrogen-bonded ion pairs exhibiting an interaction strength that depends on the counteranion radius.

The observation of four instead of two bands in the 1700–1600-cm^{-1} region suggests the presence of two distinct populations of EG molecules. The minor population has frequencies near 1651 and 1602 cm^{-1} labeled as bands II and IV in Figure 4. The fitted frequencies of these peaks exhibited no significant shifts with different halide ions. These two

[3] The halide-sensitive hR→hL band near 1170 cm^{-1} is not due to, but rather overlaps with, a C–C stretch band of the chromophore. The latter vibration is expected to be deuteration-insensitive, based on analogies with the 1170-cm^{-1} negative band in the bR→L difference spectrum (Figure 2C). This bR→L difference band, due to the C_{10}–C_{11} vibration of the chromophore (Smith et al., 1987), was previously shown not to exhibit any shifts in frequency or intensity upon deuterium exchange (Gerwert & Siebert, 1986).

Table 2: Anion Dependence of C–N Vibrational Frequencies of Ethylguanidinium Model Compounds in $CHCl_3$/MeOH[a]

	^1H			^2H[b]		
sample	$\bar{\nu}$[c] (cm^{-1})	$\Delta\bar{\nu}$[d] (cm^{-1})	A_{max}[e]	$\bar{\nu}$[c] (cm^{-1})	$\Delta\bar{\nu}$[d] (cm^{-1})	A_{max}[e]
EG·Cl^-	1672.7	26	0.83	1619	32	0.73
	1627.2	31	0.41	1583	24	0.40
	1181[f]	30	0.07	1137[f]	24	0.07
EG·Br^-	1671.3	24	0.83	1618	39	0.91
	1623.9	29	0.34	1584	24	0.37
	1179[f]	34	0.08	1136[f]	29	0.09
EG·I^-	1670.0	24	0.84	1619	37	0.72
	1623.0	35	0.37	1589	35	0.33
	1179[f]	40	0.11	1136[f]	26	0.06
EG·OAc^-	1684.3	28	0.66	1627	54	0.50
	1634.7	24	0.40	1598	62	0.59
	1187[f]	27	0.07	1140[f]	42	0.08

[a] Obtained from fitted parameters of peaks I and III in the scaled absorbance spectra of Figure 4 and of the most intense peak in the 1130–1180 cm^{-1} range of the same spectra. Deuterated spectra were obtainedly similarly. Fits were to four Voigtian peaks in the 1725–1570-cm^{-1} range for the EG-halide spectra or five Voigtian peaks in the 1725–1540-cm^{-1} range for the EG-OAc spectra, and to two Voigtian peaks in the 1170–1180 range (or one Voigtian peak in the 1130–1140-cm^{-1} range for the deuterated samples). Lorentzian and Gaussian components of broadening were constrained to be equal; only the summed bandwidth was optimized by the fit. For undeuterated EG, fitted peak centers are reproducible to ~0.2 cm^{-1} on different preparations of each salt. For deuterated EG, fitted peak centers are reproducible only to ~1 cm^{-1} due to greater band overlap. [b] Deuterons present at exchangeable positions only. [c] Fitted center frequency. [d] Fitted bandwidth at half-height. [e] Fitted peak heights are given as a fraction of the highest absorbance in the same spectrum. Using this intensity scale, the root-mean-square residual difference between the fitted and measured spectra was <0.004 for each of the fits. [f] These bands are weak and on the edge of a strong IR absorption of $CHCl_3$, making them difficult to observe reliably in the binary solvent used here. The band centers and optimum shapes shift somewhat depending on the choice of subtraction parameter for the solvent reference spectrum, and the rms residual of the fits was a significant fraction of the peak height. Therefore, we claim only 5-cm^{-1} accuracy for these frequencies.

bands may therefore arise from a form of EG in which the anion and cation are fully solvated by the weakly dipolar chloroform.

In addition to the two C–N stretch frequencies above 1600 cm^{-1}, EG is expected to have a third vibration with significant C–N stretch character. This vibration is expected near 1180 cm^{-1} on the basis of the observation of a UV-excitation-enhanced band at this frequency in the Raman spectrum of aqueous methylguanidinium (Sension et al., 1990). In the EG salts dissolved in the binary organic solvent, an IR band near this frequency shows a discernable anion dependence (Table 2). Furthermore, this vibration downshifts in deuterated samples to ~1140 cm^{-1}; this 40-cm^{-1} deuterium shift is nearly identical to that seen in the methylguanidinium Raman spectra (Sension et al., 1990).

Additional observations with model compounds establish that halide-dependent frequency shifts in the hR→hL difference spectrum are expected only for non-solvent-exposed arginine side chains. Solvation by protic or strongly dipolar solvents interferes with the interaction between EG and its counteranion. With pure methanol as a solvent, we observe little or no counterion dependence for any of the three C–N bands of EG; at most a slight band-shape change is detectable. For example, the highest C–N stretch band of EG in MeOH peaks at 1676 cm^{-1} regardless of counterion (spectra not shown). A lack of anion dependence was also observed previously for IR spectra of a variety of unsubstituted guanidinium salts in water, dimethyl sulfoxide, and sulfolane (Bonner & Jordan, 1976). This indicates that, in these solvents, the cation and anion are completely and independently solvated, disrupting their interaction.

Structural Basis for the Halide Dependence of Guanidino Group Vibrations. The observed anion dependence of the C–N vibrations of the EG model compounds is very similar to that observed previously for retinylidene protonated Schiff bases (Walter & Braiman, 1994), which have a C=N stretching vibration near 1650 cm^{-1}, as well as for unconjugated protonated Schiff bases, for which the C=NH vibration is near 1700 cm^{-1} (Lussier et al., 1986). That is, the smaller the anion and the greater its tendency to form strong hydrogen bonds, the higher the frequency of the C=N vibration in the protonated Schiff base. Likewise, in protonated Schiff base salts as in EG salts, cation solvation by hydrogen-bond-accepting solvents such as methanol raises the frequency and removes the counteranion dependence of the C=N frequency. By contrast, chloroform solvates cations poorly, and it is therefore one of the best "nonleveling" solvents for observing counteranion-dependent behavior of protonated Schiff base salts (Baasov et al., 1987).

Extensive experimental and theoretical analysis has indicated that the counteranion and solvent sensitivity of the C=N stretch frequency in protonated Schiff bases is due principally to two factors. The first is that this stretch vibration is coupled with the C=N–H bending motion near 1350 cm^{-1} and hydrogen-bonding affects the bending force constants (Smith et al., 1984; Maeda et al., 1985; Diller et al., 1987; Rodman-Gilson et al., 1988). The second is that counteranion separation distance affects the degree of polarization of the π electrons, which in turn affects the C=N bond order. For monatomic counteranions the two effects are expected to be in the same direction, leading to decreases in C=N frequency with increasing halide size.

The substituted guanidino group has a local bonding structure closely related to that of a protonated Schiff base; all three C–N bonds in the guanidino group have partial double-bond character. It is therefore likely that the observed anion dependence of the guanidino group C–N frequencies arises from effects on the vibrational force field that are analogous to those seen in protonated Schiff bases. In the limited vibrational calculations performed to date on substituted guanidinium, such counteranion effects have not been investigated in any detail. However, it was predicted that at least the C–N stretch vibration near 1180 cm^{-1} would show an environment-dependent frequency (Sension et al., 1990).

Interpretation of the hR→hL Spectrum Based on EG Model Compounds. Assignment of halide-dependent hR vibrational bands near 1695, 1610, and 1170 cm^{-1} (Figures 2 and 3) to arginine side chain vibrations is supported by our EG model compound data, because the size and direction of halide-perturbation shifts in hR spectra are well modeled by the anion dependence of bands near 1675, 1625, and 1180 cm^{-1} in spectra of the EG binary salts in organic solvent (Figure 4, Table 2). In contrast, with the alternative assignment of these bands to an amide carbonyl, it is not clear that interactions with halides could lead to the observed frequency shifts.

Our data also help to explain the frequency discrepancy between model compound C–N vibrations and the hR→hL difference bands attributed to arginine. For example, the ~1695-cm^{-1} band of the latter (Figure 3) is about 20 cm^{-1} above the highest C–N frequency observed for aqueous arginine (Venyaminov & Kalnin, 1990) or for EG-Cl in $CHCl_3$ (Figure 4, Table 2). However, it must be remembered that the protein environment around the Arg–Cl^- pair in hR probably differs from the solvent environment in either of the two foregoing models, as a result of the involvement of at least

one carboxylate group (e.g., Asp-238, as indicated in Figure 1) in additional ionic interactions with the arginine. The effect of multiple carboxylate interactions on arginine C–N frequencies was modeled simply by evaporating the solvent from the EG-acetate sample (Figure 4). Condensing multiple carboxylate groups around EG in this manner shifts the intensity peak of the highest C–N vibration up from 1683 to 1690 cm^{-1}; this band also broadens, suggesting that, in some environments present in the amorphous solid film, EG molecules have a C–N frequency above 1700 cm^{-1}. In contrast, the next highest C–N band shifts down from 1635 to ~1615 cm^{-1}. Finally, the 1187-cm^{-1} C–N frequency observed for the dissolved EG-acetate salt shifts down to 1156 cm^{-1} for the dry film (spectral region not shown). Thus, the observed anion-dependent hR→hL band frequencies of 1695, 1610, and 1170 cm^{-1} are within a range that might be expected for a buried arginine participating in ionic interactions with carboxylate(s) as well as a halide.

The observed halide-dependent shifts in the hR→hL difference spectrum are admittedly close to the limits of detection. It would be desirable to induce larger shifts, perhaps with more strongly hydrogen-bonded anions. For example, the model compound data in Figure 4 indicate that putting acetate into the halide-binding site of hR would lead to larger perturbation of the ~1695-cm^{-1} band frequency than is seen in the presence of the halides; alternatively, F^- would be expected to form an even stronger hydrogen bond. Unfortunately, hR cannot pump F^-, and the only small oxyanion that supports even a moderate level of functionality is NO_3^- (Duschl et al., 1988). FTIR difference spectra of the hR→hL reaction measured in the presence of NO_3^- are difficult to interpret because multiple forms of hR are observed, with only about half of the sample undergoing a normal photoreaction (Walter & Braiman, 1994). Nevertheless, the ~1695-cm^{-1} negative band appears to shift up to 1705 cm^{-1} in such spectra. This upshift supports our conclusions with the halides, since it indicates a substantial strengthening of the ionic interaction with arginine, as would be expected for such an oxyanion based on the OAc^- model compound data (Figure 4).

The Arginine Environment in the hL State. As discussed above, anion-dependent negative bands in the hR→hL difference spectrum signal the presence of ion pairing between arginine and halide in the hR state. What happens to these pairs in the hL state can be gleaned from positive peaks in the same spectral regions. For example, the simplest interpretation of the ~1700-cm^{-1} spectral region is that this C=N stretch vibration shifts down from ~1695 cm^{-1} in the hR state to 1688.5 cm^{-1} in the hL state, giving a positive band at the latter frequency in the difference spectrum (Figures 2A and 3A; Table 1). In support of this interpretation, the positive band at 1688.5 cm^{-1} shows a sensitivity to deuterium exchange that is very similar to that of the ~1695-cm^{-1} negative band, as evidenced by an apparent shift of positive intensity to 1570 cm^{-1} in D_2O (Figure 2B).

Thus, the ~1690-cm^{-1} C–N stretch vibration appears to downshift by ~6.5 cm^{-1} during the hR→hL reaction, suggesting that an arginine residue experiences an overall weakening of its hydrogen-bonded interactions. Furthermore, the arginine C–N frequency of 1688.5 cm^{-1} in the hL state shows essentially no halide dependence (Table 1). The 0.6-cm^{-1} range observed is close to the error limits of the fits and is 4–5 times smaller than the halide-dependent shifts observed for the ~1695-cm^{-1} peak in the hR state (Table 1). Thus, in hL the halide–arginine interaction appears to be disrupted.

The light-induced change in halide sensitivity of the ~1690-cm^{-1} arginine peak contrasts with what is observed for the ~1635-cm^{-1} Schiff base vibration, which changes in frequency during the hR→hL reaction but shows similar halide-dependent shifts in both hR and hL states (Walter & Braiman, 1994). Taken together, the FTIR spectral results provide experimental support for the hypothetical model of the hR→hL reaction shown in Figure 1. That is, they indicate that an ion pairing interaction with the chromophore protonated Schiff base carries the mobile halide ion away from its starting location in contact with an arginine, to a new location in the hL state where only the interaction with the protonated Schiff base is maintained.

Conclusion. We have demonstrated that it is possible to detect spectroscopically a transiently altered interaction of a transported anion with an arginine side chain. An arginine critical for normal halide transport function has been identified in a putative membrane-spanning domain of at least one mammalian protein, the cystic fibrosis gene product (CFTR) (Dean et al., 1990). Techniques for performing FTIR difference spectroscopy on such nonphotoreactive transport proteins are currently being developed by us and others. For example, ligand gating of acetylcholine receptor has recently been studied with FTIR difference spectroscopy, by changing the ligand concentration surrounding receptor-containing membranes that were adsorbed to the surface of an attenuated total reflection optical element (Baenziger et al., 1993). By altering concentrations of ligands and/or halide ions in contact with CFTR in situ in a spectrometer, the halide-perturbation/FTIR approach developed for hR may make it possible to detect halide interactions with specific residues in these channels.

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