Evidence For a Bound Water Molecule Next to the Retinal Schiff Base in Bacteriorhodopsin and Rhodopsin: A Resonance Raman Study of the Schiff Base Hydrogen/Deuterium Exchange

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ABSTRACT The retinal chromophores of both rhodopsin and bacteriorhodopsin are bound to their apoproteins via a protonated Schiff base. We have employed continuous-flow resonance Raman experiments on both pigments to determine that the exchange of a deuteron on the Schiff base with a proton is very fast, with half-times of 6.9 ± 0.9 and 1.3 ± 0.3 ms for rhodopsin and bacteriorhodopsin, respectively. When these results are analyzed using standard hydrogen-deuteron exchange mechanisms, i.e., acid-, base-, or water-catalyzed schemes, it is found that none of these can explain the experimental results. Because the exchange rates are found to be independent of pH, the deuterium-hydrogen exchange can not be hydroxyl (or acid-)-catalyzed. Moreover, the deuterium-hydrogen exchange of the retinal Schiff base cannot be catalyzed by water acting as a base because in that case the estimated exchange rate is predicted to be orders of magnitude slower than that observed. The relatively slow calculated exchange rates are essentially due to the high pKa values of the Schiff base in both rhodopsin (pKa > 17) and bacteriorhodopsin (pKa ~ 13.5). We have also measured the deuterium-hydrogen exchange of a protonated Schiff base model compound in aqueous solution. Its exchange characteristics, in contrast to the Schiff bases of the pigments, is pH-dependent and consistent with the standard base-catalyzed schemes. Remarkably, the water-catalyzed exchange, which has a half-time of 16 ± 2 ms and which dominates at pH 3.0 and below, is *slower* than the exchange rate of the Schiff base in rhodopsin and bacteriorhodopsin. Thus, there are two anomalous results, the inconsistency of the observed hydrogen exchange rates of retinal Schiff base in the two pigments with those predicted from the standard exchange schemes and the enhancement of the rate of hydrogen exchange in the two proteins over the model Schiff base in aqueous solution. We suggest that these results are explained by the presence of a structural water molecule (or molecules) at the retinal binding sites of the two pigments, quite close, probably-hydrogen bonded, to the Schiff base proton. In this case, the rate of exchange can be faster than that found for the model compound due to an "effective water concentration" near the Schiff base that is increased from that found in aqueous solution.

INTRODUCTION

Rhodopsin, the protein responsible for sensing light in vision, consists of a chromophore, the 11-*cis* isomer of the aldehyde of vitamin A, retinal, and the colorless apoprotein, opsin. The chromophore is covalently attached by a protonated Schiff base, —C=NH^+-, linkage to an ϵ-amino group of a lysine in opsin. The absorption of light results in the very rapid photoisomerization of the 11-*cis* chromophore to a *trans* form (Schoenlein et al., 1991; Yan et al., 1991). In this process, about half of the energy of the absorbed photon is converted to chemical energy by forming a high energy chemical species called bathorhodopsin (Honig et al., 1979). Bacteriorhodopsin (bR), a protein located in the cell membrane of the bacterium, *Halobacterium salinarium* (formerly *Halobacterium halobium*), has as its chromophore the *trans* isomer of retinal, which is also attached to its apoprotein by a protonated Schiff base. The photophysics of the bound chromophore is quite similar to rhodopsin, although there are differences in detail. For example, the photoconversion of bR to K, the primary photoproduct analogous to bathorhodopsin, is a *trans* to *cis* isomerization, takes somewhat longer, and converts less of the photon's energy to chemical energy. This photophysical behavior of rhodopsin and bacteriorhodopsin is very unusual. In contrast to the pigments, model compounds of protonated Schiff bases of retinal in solution, for example, differ in energy by less than a kcal/mol amongst their *cis* and *trans* forms compared to the about 35 kcal/mol difference in energy between rhodopsin and bathorhodopsin (cf., Birge 1990; Mathies et al., 1991).

For some time it has been supposed that the protein structure around the bound retinal, particularly near the protonated Schiff base linkage, is the key to this remarkable photochemical behavior. Certainly, calculations show that the specific arrangement of the group or groups that "solvate" the positively charged protonated Schiff base linkage have direct and strong effects on the ground and excited states of the chromophore, on the reaction coordinates, and on the dynamics of the excitation process (cf., Birge et al., 1988; Birge 1990; Mathies et al., 1991). Also, it has been supposed that electrostatic considerations, the separation of the positively charged protonated Schiff base from its putative negative counter-ion or solvating groups, is a major factor in the energy storage shown by these pigments (Honig et al., 1979;

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Deng and Callender 1987; Birge et al., 1988). High resolution structures for rhodopsin and bacteriorhodopsin would help immensely in understanding these questions, but unfortunately none exist.

One method for studying the structure of a group with a labile proton, like that of the protonated Schiff base linkage of rhodopsin and bacteriorhodopsin, is hydrogen-deuterium exchange. Such studies have been used extensively to study various properties of proteins, especially protein folding (for recent reviews, see Kim 1986; Englander and Mayne 1992). The standard reaction mechanism for hydrogen-deuterium exchange in aqueous solution involves acid/base catalysis (cf. Eigen 1964). For a protonated Schiff base, only the base (hydroxyl or water acting as base)-catalyzed hydrogen exchange reaction is significant (see Results). The hydroxyl-catalyzed exchange reaction, for example, proceeds via two steps given by the following scheme:

$$\text{—C=N}^{+}\text{D—} + \text{OH}^{-} \xrightarrow{kd} \text{—C=N—} + \text{HDO} \qquad (a)$$

$$\text{—C=N—} + \text{H}_2\text{O} \xrightarrow{kr} \text{—C=N}^{+}\text{H—} + \text{OH}^{-}$$

In some cases the factors that influence the exchange rate have been well characterized. For example, in studies of amide hydrogen exchange in proteins, a decrease in exchange rate by 2 to 10 orders of magnitude compared to model compounds has been observed and attributed to stronger hydrogen bonding of the amide hydrogen (e.g., in an α-helix structure) and/or the change in the accessibility of the amide to the solvent (Kim 1986; Jeng and Englander 1991; Englander and Mayne 1992).

The stretching frequency of the protonated Schiff base, at 1657 cm^{-1} in rhodopsin and at 1640 cm^{-1} in bR, is easily observed in the resonance Raman spectroscopy of these pigments and undergoes a downward frequency shift of 32 and 16 cm^{-1}, respectively, upon deuteration. It is thus quite easy to determine the amount of either the protonated or deuterated form. The hydrogen-deuterium exchange rate of the retinal Schiff base in bR has been measured previously by continuous flow resonance Raman measurements (Ehrenberg et al., 1980; Doukas et al., 1981). A theoretical analysis of the results suggested that the experimentally determined exchange time, on the order of a few milliseconds or less and independent of pH, is 3 orders of magnitude faster than the hydroxyl-catalyzed reaction and 5 orders of magnitude faster than the water-catalyzed reaction (Doukas et al., 1981). On the basis of this analysis, a new reaction mechanism was proposed for the hydrogen exchange reaction. This mechanism did not involve proton dissociation from the protonated Schiff base, but rather consisted of the direct exchange of a proton with a water molecule in a concerted reaction.

We have revisited this problem here. The time resolution of the exchange apparatus has been improved substantially so that we are now able to resolve the exchange time for bacteriorhodopsin. The exchange time of bovine rhodopsin as a function of pH has also been determined in order to explore its similarity or difference with bacteriorhodopsin. In addition, we have performed the hydrogen-deuterium exchange experiments on a Schiff base model compound which was sufficiently stable in aqueous solution so that we could quantitatively compare its exchange reaction with that of the pigments.

MATERIALS AND METHODS

An aqueous suspension of bR was prepared as previously described (Becher and Cassim 1975) and deuterated by centrifugation and resuspension in D_2O. The concentration of the sample was adjusted to about 4 OD at 570 nm. Before the exchange experiment, the bR sample was light-adapted with 568.2 nm laser light from a krypton ion laser for about half an hour. The initial pD of bR was about 6.5 (uncorrected pH meter reading); no buffer was used.

Bovine rhodopsin containing rod outer segment membranes were prepared (Papermaster and Dryer 1973). and kept frozen at −60°C. Just before the Raman experiments, the membranes were thawed and pelleted by centrifugation. The pellet was washed with D_2O once, and then the rhodopsin was solubilized with 10 mM CHAPSO (Boerhinger Mannhaim Co., Indianapolis, IN) in D_2O. The concentration of the rhodopsin was about 4 OD at 500 nm. The pD of the rhodopsin sample was 6.5 (uncorrected pH meter reading) in the unbuffered solution.

The deuterated Schiff base model compound, 3-methyl-2-butene butylamine, ($CH_3C(CH_3)$=CH—CH=NH^+—$CH_2CH_2CH_2CH_3$), was prepared as follows. 2.5 ml of 3-methyl-2-butenal (Sigma Chemical Co., St. Louis, MO) and 2.8 ml of butylamine (Sigma) were mixed on ice. The reaction mixture, mostly unprotonated Schiff base, was then dissolved in 25 ml of n-hexane, followed by centrifugation to remove the water formed in the reaction. After 2.5 ml of a 37% aqueous DCl solution was added to the unprotonated Schiff base in hexane on ice, the mixture was shaken vigorously for a few min. The deuterated Schiff base, separated from hexane by centrifugation, was diluted by D_2O to a final volume of about 40 ml, and its pD was adjusted by NaOD to about 2.5 (uncorrected pH meter reading). The Schiff base hydrolyzed slowly to aldehyde at a rate of about 5–10% per h under these conditions. The time required for the D-H exchange experiment was about 2–3 h, so up to 25% of the Schiff base could have hydrolyzed by the end of the experiment.

A mixing chamber was constructed with two jets meeting at a small angle (about 15°), and the mixed sample exited through a 0.5-mm diameter glass capillary at a flow rate of 60 ml/min. The dead time of the flow apparatus, 0.9 ± 0.3 ms, was calculated from the measured flow rate and the volume of the flow cell. This dead time was verified by following the reaction of potassium ferricyanide with ascorbic acid at pH 8 ($t_{1/2}$ = 6.5 ms; Tonomura et al., 1987) and by the reduction of 2,6-dichlorophenolindophenol with ascorbic acid at pH 3 ($t_{1/2}$ = 0.65 ms; Tonomura et al., 1987). The error in the delay time was mainly from the fluctuation of the pump flow rate because the experiments allow only a short stabilization time after initiating the mixing, due to sample limitations. Therefore, estimated error at longer delay times was about 10%, rather than a fixed time.

Continuous flow experiments were carried out by mixing the deuterated pigments or model compound with 20-fold aqueous solution. For bR, the pD value was always kept at 6.5 (uncorrected pH meter reading), but the water pH was adjusted to 2.5, 6.5, or 10.5 in the three sets of D-H exchange experiments for bR. In the pH 6.5 experiment, distilled water was used. In the pH jump experiments from pD 6.5 to pH 2.5 and pH 10.5, 1 M NaCl was added to the water to stabilize the pH after mixing. In the D-H exchange experiments with rhodopsin, the pD value of rhodopsin was kept at pD 6.5 (uncorrected pH meter reading), and a dilute phosphate buffer (1 mM) was used to stabilize the pH at 3 or 7, in two separate exchange experiments. The following were used as the aqueous mixing solutions in the D-H exchange experiments of the Schiff base model compound: 1 M HCl; 0.1 M HCl; 10 mM NaCl, pH 2; 10 mM phosphate buffer, pH 2; 10 mM NaCl, pH 3; 10 mM phosphate buffer, pH 3; 10 mM formate, pH 4; 10 mM acetate, pH 5; 10 mM phosphate, pH 6 and 6.5.

The resonance Raman spectra of bacteriorhodopsin were measured with a 530.1-nm line from a krypton ion laser at a power level of 40 mW, and those of rhodopsin were measured with 488.0-nm line from an argon ion laser at the same power level. A cylindrical lens was used to focus the laser beam onto the sample so that more laser power could be used to enhance the Raman signal without introducing too much sample photolysis. Under these conditions, less than 15% of the bacteriorhodopsin or rhodopsin undergoes photolysis (Callender et al., 1976). The Raman spectrum of the protonated Schiff base model compound was measured with 514.5-nm line from an argon laser at a power level of 200–300 mW. All experiments were conducted at room temperature.

The Raman spectra were taken with an optical multichannel analyzer (OMA) system consisting of a triplemate spectrometer (Spex Industries, Metuchen, NJ), a model DIDA-1000 reticon detector connected to a ST-100 detector controller (Princeton Instruments, Trenton, NJ), which was interfaced to a MAC II computer (Apple Computer, Cupertino, CA). With the above laser excitation wavelengths, a spectral window of about 1000 cm^{-1} with resolution of 8 cm^{-1} could be detected. The Raman band positions were calibrated against the known Raman peaks of toluene and are accurate to ± 2 cm^{-1}.

RESULTS

Bacteriorhodopsin

Resonance Raman spectra of deuterated bacteriorhodopsin (Fig. 1) were obtained at various delay times after mixing with a 20-fold excess H_2O at pH 6.5. Also shown in Fig. 1 are bacteriorhodopsin spectra in D_2O and H_2O, respectively. The intensity of the protonated Schiff base, C=NH^+, stretch mode at 1640 cm^{-1} increases with delay time whereas the deuterated, 1624 cm^{-1}, C=ND^+, stretch band decreases with delay time. Because there was a 20-fold excess of H_2O over D_2O after mixing, the D—H exchange reaction should depend little on the concentration of water. We assume below that the reactions approximately follow first order reaction kinetics. At a delay time, t, after mixing, the intensity, $I(t)$, of the protonated Schiff base C=N stretch band at 1640 cm^{-1} and the deuterated Schiff base C=N stretch band at 1624 cm^{-1} can then be expressed by the following two equations:

$$I_{1640}(t) = I_{1640}(1 - e^{-kt}) \quad (1)$$

and

$$I_{1624}(t) = I_{1624} \cdot e^{-kt}. \quad (2)$$

The maximum intensities, I_{1640} and I_{1624}, are obtained from the spectra of bacteriorhodopsin in H_2O and D_2O, respectively. Taking the ratio of $I_{1640}(t)$ and $I_{1624}(t)$, it can be shown that

$$\ln\left[\frac{I_{1624} \cdot I_{1640}(t)}{I_{1640} \cdot I_{1624}(t)} + 1\right] = kt. \quad (3)$$

Fig. 2 shows a plot of the left-hand side of Eq. 3 versus t. The biggest problem with determining the accuracy of the peak intensities has to do with the determination of the background levels. All of the spectra contain a sloping background. This has been removed by approximating the background as a straight line and assuming that the Raman intensity is zero at 1470 and 1720 cm^{-1}. The latter value is perfectly justified because it is known that no Raman intensity (apart from very small overtone bands) lies in this region (see, e.g., Callender et al., 1976; Aton et al., 1977). There is no Raman structure at or near 1470 cm^{-1} either, so this value also seems well justified. In addition, the accuracy of each data point has been improved by comparing each spectrum in Fig. 1 with a series of resonance Raman spectra of bacteriorhodopsin in solution with different D_2O/H_2O ratios, which were measured in separate experiments. These controls employed the same samples so that their backgrounds are the same as those found in the kinetic measurements. Thus, only a matching of the

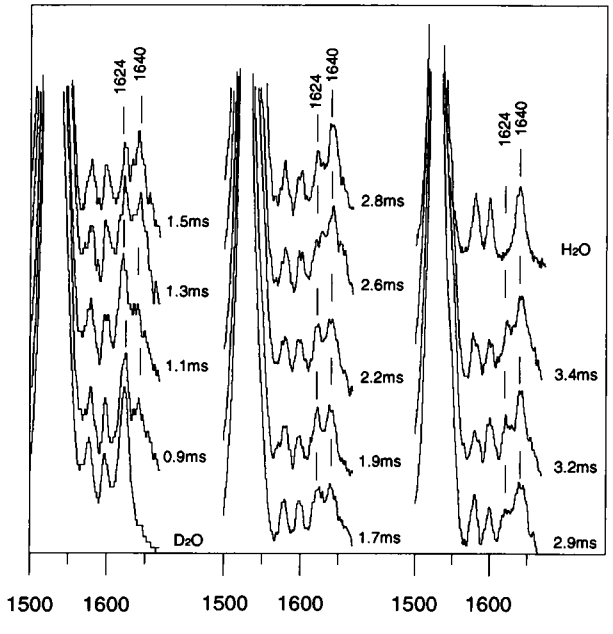

FIGURE 1 Resonance Raman spectra of bR at various delay times after mixing the bR sample in D_2O with 20-fold excess of H_2O at pH 6.5. The spectra of bR in H_2O and D_2O are also shown for comparison. The spectra were obtained with 530.1 nm laser line from a krypton laser at a power level of 40 mW; a cylindrical lens was used to reduce sample photolysis while maintaining efficient Raman scattering. The spectra had a resolution of 8 cm^{-1}.

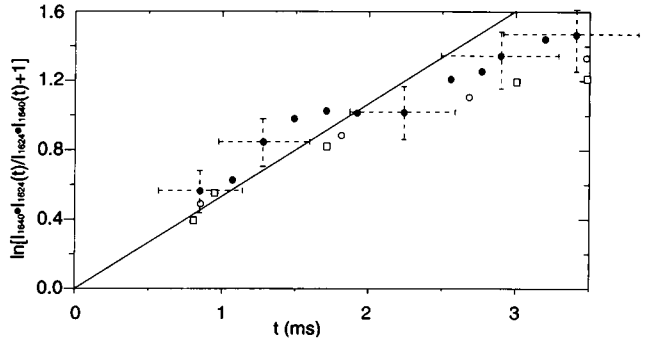

FIGURE 2 Kinetic plot of the deuterium-hydrogen exchange of the Schiff base nitrogen of bacteriorhodopsin with a jump from pD 6.5 to pH 6.5 (●), to pH 2. 5 (□), and to pH 10.5 (○). The straight line is a linear fit of the data obtained at pH 6.5. I_{1640} and I_{1624} are the band intensities (relative to the C=C stretching band intensities) of protonated and deuterated Schiff base, respectively, in the resonance Raman spectra of bR in H_2O and D_2O. $I_{1640}(t)$ and $I_{1624}(t)$ are the band intensities of protonated and deuterated Schiff base of bR, respectively, as a function of time after mixing.

relative peak heights of a particular kinetic experiment with the control series is needed, and this minimizes the problem of ambiguity of the poistion of the background. Using a non-linear least-squares fitting procedure, these data are adequately fit to a linear function, which has been plotted in Fig. 2. However, the data points in Fig. 2 appear to deviate from first-order kinetics at the later time points. A fit to a second-order function also yielded satisfactory results, but the errors in the data points are such that it is not possible to determine whether the kinetics are first or second order (note estimated error bars in the figure). The ambiguity in the value of the background is the essential determinant in the error in the data points along the y-axis. The accuracy of the data points along the x-axis in the plot is limited by the fluctuations in pump velocity (see Methods) yielding $a \pm (0.3 + (t - 1) \cdot 10\%)$ ms of the time value. The rates quoted below arise from calculating the slope of the best straight line fitted to the data of Fig. 2. Values of $t_{1/2}$ can be calculated from this rate. Also, $t_{1/2}$ is the time at which half-exchange occurs, and this can be also determined from a comparison of the series of kinetic runs (Fig. 1) with the spectrum of bacteriorhodopsin in a 50:50 mixture of H_2O and D_2O in a steady-state measurement. Because the baseline is the same for the kinetic runs and the steady-state run, a comparison only of relative peak heights in the raw specta is required. We estimate that the accuracy of the half-times obtained in this way is probably higher than that obtained from the calculated rate, and is limited by the uncertainity in the pump flow rate. In any case, the $t_{1/2}$ values obtained from either method agree within our estimated errors.

The first-order exchange reaction rate thus obtained was $k = 530\ s^{-1}$, and a half-time of $t_{1/2} = 1.3\ (\pm 0.3)$ ms. A similar analysis, performed on data obtained in D-H exchange experiments with a pH jump from either pD 6.5 to pH 2.5 or to pH 10.5, yielded exchange rate constants of 460 s^{-1} ($t_{1/2}$ = 1.5 ± 0.4 ms) and 440 s^{-1} ($t_{1/2}$ = 1.6 ± 0.4 ms), respectively. Therefore, there is no obvious pH dependence of D-H exchange rate of the retinal Schiff base hydrogen in bR.

The pH-independent nature of the D-H exchange reaction in bR has been observed previously (Ehrenberg et al., 1980; Doukas et al., 1981). The reaction time constant obtained here is consistent with the latter (who reported kinetics had $t_{1/2}$ less than 3.0 ms, the instrument deadtime in that report) but is about 3 times faster than the former (who reported a $t_{1/2}$ = 4.7 ms exchange time). We found that under conditions favoring protein aggregation (very high salt concentration and/or repeated usage of the sample) the D-H exchange time was slowed by about 2 to 4 times (data not shown).

Rhodopsin

Fig. 3 shows the resonance Raman spectra of rhodopsin in D_2O at various delay times after mixing with 1 mM phosphate buffer at pH 3. The bands at 1657 cm^{-1} and at 1625 cm^{-1} have been assigned to the protonated and deuterated Schiff base C=N stretch mode, respectively. As in the D-H exchange experiments of bR, the intensity of the 1657 cm^{-1} band increases, and that of the 1625 cm^{-1} band decreases with the delay time. A similar analysis to that used for bacteriorhodopsin is shown in Fig. 4 and yields pseudo-first-order reaction rates of k = 120 s^{-1} ($t_{1/2}$ = 5.8 ± 0.8 ms) at pH 3 and k = 100 s^{-1} ($t_{1/2}$ = 6.9 ± 0.9 ms) at pH 6.5. Thus, the kinetics, like those of bacteriorhodopsin, show no pH dependence over this range.

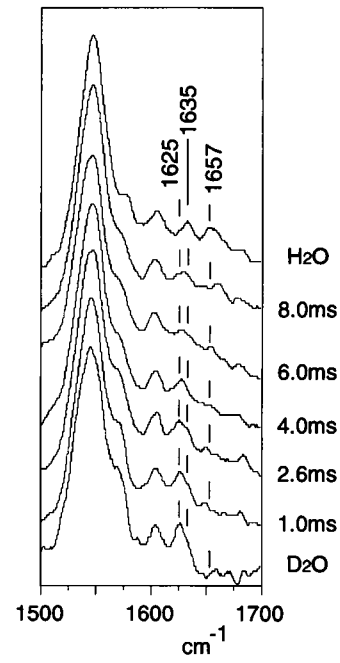

FIGURE 3 Resonance Raman spectra of rhodopsin at various delay times after mixing the rhodopsin sample in D_2O (dissolved with 10 mM CHAPSO) at pD 6.5 (pH meter reading) with 20-fold excess of H_2O at pH 3. The spectra of rhodopsin in H_2O and D_2O are also shown for comparison. The spectra were obtained with 488.0 nm laser line from an argon laser at a power level of 40 mW; a cylindrical lens was used to reduce sample photolysis. The spectra had a resolution of 8 cm^{-1}.

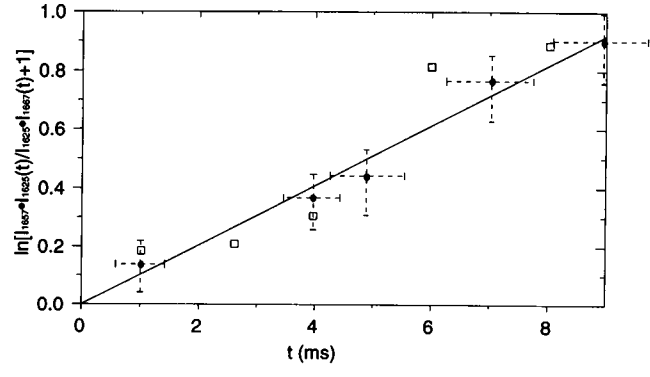

FIGURE 4 Kinetic plot of the deuterium-hydrogen exchange of the Schiff base nitrogen of rhodopsin with the jump from pD = 6.5 to pH 6.5 (●), or to pH 3 (□). The straight line is a linear fit of the data obtained at pH 6.5. I_{1657} and I_{1625} are the band intensities (relative to the C=C stretching band intensities) of protonated and deuterated Schiff base, respectively, in the resonance Raman spectra of rhodopsin in H_2O and D_2O. $I_{1657}(t)$ and $I_{1625}(t)$ are the band intensities of protonated and deuterated Schiff base of rhodopsin, respectively, as a function of time after mixing.

Model Schiff base

Retinal Schiff bases would be the ideal model; however, these are quite unstable in aqueous solution (hydrolyzing very fast to the aldehyde). Therefore, we have turned to measurement of 3-methyl-2-butene butylamine, which is just stable enough for measurement. The chemistry of this compound is very similar to that of a retinal Schiff base, being a polyene joined to an alkane group via the —C=NH$^+$- linkage. Most important, the pK_a of 3-methyl-2-butene butylamine and a retinal Schiff base are virtually the same (Favrot et al., 1978; see below). In Fig. 5, Raman spectra taken of 3-methyl-2-butene butylamine in D_2O are shown at various delay times after mixing with H_2O. In series A, the deuterated Schiff base in D_2O was mixed with a 20-fold excess of 10 mM phosphate buffer at pH 3. In series B, the same sample was mixed with 20-fold excess 10 mM NaCl at pH 3. Up to three bands are observed in these spectra. The spectrum in H_2O contains the protonated Schiff base band, C=NH$^+$ stretch, at 1672 cm^{-1} (top spectrum of panels *A* and *B*) and also contains a band at 1629 cm^{-1} that can be assigned to the butene C=C stretch. The spectrum in D_2O is dominated by the in-phase combination of the C=C and C=ND$^+$ stretches at 1647 cm^{-1} (bottom spectrum of panels *A* and *B*). The delay time spectra, however, are also complicated by the presence of hydrolyzed aldehyde product, 3-methyl-2-butenal, whose spectrum contains strong bands at 1630 and 1650 cm^{-1} (data not shown). We have estimated that up to 25% of the model protonated Schiff base may form hydrolyzed product within the 2 to 3 h time period required to obtain the delay time data (see Materials and Methods). Therefore, in the Raman spectra taken after mixing, the band intensity at 1647 cm^{-1} is significantly higher than the expected C=N stretch band intensity. After correction for this artifact, the data was analyzed as above, and the exchange time as a function of pH is plotted in Fig. 6.

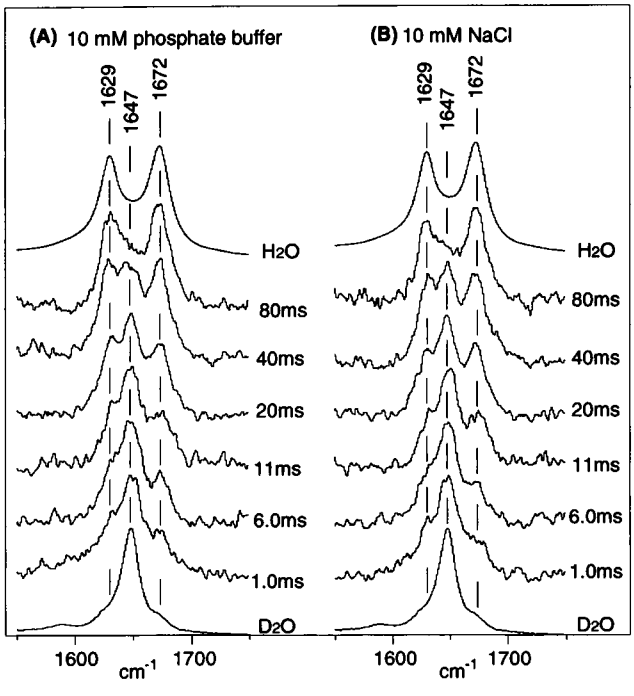

FIGURE 5 Raman spectra of Schiff base model compound (3-methyl-2-butene butylamine) at various delay times after mixing the Schiff base in D_2O at pD 2.5 (pH meter reading) with 20-fold excess of H_2O at pH 3 in the presence of 10 mM phosphate (series *A*) and 10 mM NaCl (series *B*), respectively. The spectra of the compound in H_2O and D_2O are also shown for comparison. The spectra were obtained with 514.5 nm laser line from an argon laser at a power level of 200 mW, with a resolution of 8 cm^{-1}.

It is evident from the data in Fig. 5, by comparing the data in series A (with buffer) with the no-buffer conditions of series B, that the D-H exchange reaction of the Schiff base model compound was not affected significantly by the presence of buffer at pH 3.0. Similar results were found for measurements performed for pH values less than 3.5. However, the pH of the mixed sample could not be reproducibly measured when the buffer concentration was lower than 10 mM at pH higher than 3.5. We believe that the observed exchange rate for pH > 3.5 likely contains a component of buffer catalysis (see below).

The three standard mechanisms for hydrogen exchange involve acid-, base-, or water-catalyzed reactions. Thus, the pH-dependent total exchange reaction rate k, can be expressed by the following equation:

$$k = k_H[H^+] + k_{OH}[OH^-] + k_w[H_2O] \quad (4)$$

where k_H and k_{OH} are the second-order acid- and base-catalyzed exchange rate constants, respectively. $k'_w = k_w[H_2O]$ is the water-catalyzed exchange rate and is independent of pH. Buffer in general may also contribute to the exchange reaction (cf. Eigen 1964; Englander et al., 1972). Fitting the data in Fig. 6 to Eq. 4 yields: $k_{OH} = 1.1 \cdot 10^{12}$ M^{-1} s^{-1} and $k'_w = 43$ s^{-1}; k_H is negligible in the pH range of our study. A general expression to estimate the second-order rate constants, $k_{(H, OH, w)}$ on the right side of Eq. 4 is given by the following equation (Englander et al., 1972):

$$k_{(H, OH, w)} = k_r[10^{\Delta pK_s}/(10^{\Delta pK_s} + 1)] \quad (5)$$

where ΔpK_s is equal to the pK_a of proton acceptor (e.g., OH^-)

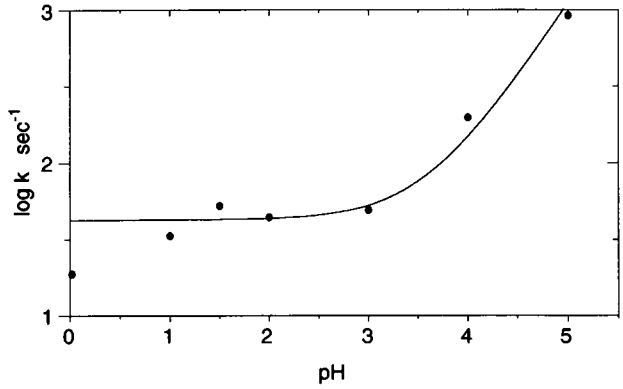

FIGURE 6 The pH dependence of D-H exchange rate of the Schiff base model compound. The exchange rates (k) are obtained by the linear curve fitting procedure outlined in the legend of Fig. 2 at various pHs (shown as *solid circle*). The line is a curve fit of Eq. 4 to the data points (less the pH = 0 point).

minus that of proton donor (e.g., the Schiff base). The recombination rate constant, k_r (see Scheme a in Materials and Methods), is limited to the maximum diffusion rate, which is of the order of 10^{10}–10^{11} M^{-1} s^{-1} (Eigen 1964). The pK_a of our Schiff base model compound is about 7 (Favrot et al., 1978), which is very close to that of retinal Schiff bases (Sheves et al., 1986). Using this and $pK_a(H_2O) = -1.7$ and $pK_a(OH^-) = 15.7$ and the limiting value of $k_r < 10^{10}$–10^{11} M^{-1} s^{-1}, we find that k'_w and k_{OH} are constrained so $k'_w < 10^3$–10^4 s^{-1} and $k_{OH} < 10^{10}$–10^{11} M^{-1} s^{-1}. The observed k'_w of 43 s^{-1} is well below this upper limit and, therefore, is consistent with the conventional reaction mechanism. However, the experimentally derived value of $k_{OH} = 1.1 \cdot 10^{12}$, is one to two orders of magnitude higher than the estimated limit. For this reason, we believe that the observed exchange rate in Fig. 6 above pH 3.5 may have a contribution from buffer catalysis and that this effect is masking the true value of k_{OH}.

DISCUSSION

The measured H-D exchange time constants for bacteriorhodopsin ($t_{1/2} = 1.3 \pm 0.3$ ms) and for rhodopsin ($t_{1/2} = 6.9 \pm 0.9$ ms) are very fast, and the rates are essentially independent of pH over the range of our measurements. As we have shown previously for bacteriorhodopsin (Doukas et al., 1981), these fast exchange times cannot be explained using standard-base catalyzed mechanisms involving either OH^- or the general base H_2O (see Scheme a in Materials and Methods). An upper bound on the OH^--catalyzed reaction rate is given by $k_{OH^-} = k_r[OH^-]$, where the recombination rate is limited by diffusional encounters with the protonated Schiff base, i.e., $k_r < 10^{10}$–10^{11} M^{-1} s^{-1} (Eigen 1964). At pH 3, the lowest pH studied for rhodopsin, and at pH 2.5, the lowest value for the bacteriorhodopsin experiments, the use of the diffusion-controlled upper bound for k_r yields an exchange time no faster than 1–10 s, some three orders of magnitude slower than the observed rates in the two proteins. Moreover, an OH^--catalyzed reaction is pH-dependent, whereas that observed for the two pigments is not. Thus, neither rhodopsin's nor bacteriorhodopsin's Schiff base exchange reaction is base (or acid-)-catalyzed.

Although the standard mechanism for a water-catalyzed reaction is pH-independent, it can also be excluded. In this case, Eq. 5 collapses to the familiar form of a pseudo-first-order deprotonation rate, $k'_w = k_w[H_2O]$, as given by $k'_w = k_r 10^{-pK_a}$. Again, an upper limit of k'_w can be determined once the pK_a of the Schiff base is known by taking a diffusion rate limiting value for k_r. The pK_a of the Schiff base, as complexed in the retinal binding site of the two proteins, is 13.5 for bacteriorhodopsin (Druckmann et al., 1982; Sheves et al., 1986) and may be as high as 17 in rhodopsin (Steinberg et al., 1993). Using a pK_a value of 13.5 and $k_r < 10^{10}$–10^{11} M^{-1} s^{-1}, then $k'_w < 3 \times 10^{-3}$ s^{-1} or $t_{1/2} > 230$ s. The observed exchange time is five orders of magnitude faster than this estimated upper limit. And the use of the pK_a appropriate for rhodopsin obviously yields an even slower upper limit of the exchange time, on the order of 10^{-5} or less, or $t_{1/2} > 7 \times 10^4$ s. On the other hand, similar analysis of the model Schiff base yields an upper limit of the water-catalyzed exchange rate of 10^3 s^{-1}, which is entirely consistent with the observed value, 43 s^{-1}. We conclude, therefore, that neither hydroxyl- nor water-based-catalyzed reactions can explain the fast exchange times observed for the Schiff base proton in bacteriorhodopsin and rhodopsin.

We emphasize that at pH 2–3, the hydrogen exchange rate in rhodopsin (120 s^{-1}) and bR (460 s^{-1}) is *higher* than that of the model compound at the same pH (43 s^{-1}), where the water-based catalysis dominates the exchange in solution. This observation is in clear contrast with all previous studies on the exchange of protein amide hydrogens. Compared to model amides, it was found that the amide hydrogen exchange rate in proteins can be delayed substantially by hydrogen bonding (Kim, 1986; Englander and Mayne, 1992; Jeng and Englander, 1991) or virtually eliminated by a hydrophobic pocket (Maeda et al., 1992). The real enhancement of the Schiff base deuteron exchange with water in these two pigments must be enormous because it has not only overcome the delays caused by possible protein dependent mechanisms, but also the unfavorable pK_a increase of the Schiff base.

A plausible explanation for the rate enhancement of the Schiff base H-D exchange in rhodopsin and bR is that there is a water molecule next to the Schiff base hydrogen that is well oriented and positioned for the reaction. The rate acceleration induced by the proper positioning of the reactants should be similar to that observed in a monomolecular enzymic or intramolecular reaction compared to a bimolecular reaction. In bimolecular reactions, the transition state of the reaction is necessarily monomolecular; therefore, three translational and up to three rotational degrees of freedom are lost, which corresponds to a large entropy loss and contributes to the transition barrier of the reaction in solution. However, when the reactants are bound together by an enzyme or joined by a chemical bond *before* the reaction occurs, the entropy loss will not contribute to the transition barrier of the reaction (but to a barrier of a non-rate-limiting step, such as the diffusion of the water molecule to the Schiff base in rhodopsin or bR), and substantial rate enhancements can then be achieved (Page and Jencks 1971). It has been shown that such entropic effects can contribute to a rate enhancement of up to $10^{6.5}$ (cf. Page and Jencks, 1971).

The proposed water molecule can be constrained to the proper position by hydrogen bonding to protein residues and/or to the retinal Schiff base. A number of studies point towards to the conclusion, with more or less certainty, that a water molecule or molecules are at the binding site of rhodopsin and bacteriorhodopsin, some suggesting that the putative water molecule is hydrogen bonded directly to the protonated Schiff base linkage. For example, rhodopsin (Rafferty and Shichi 1981) and bacteriorhodopsin (Hildebrandt and Stockburger 1984) have been shown to undergo substantial changes in their respective absorption maxima upon dehydration; for both pigments the changes in

absorption maxima are fully reversible upon rehydration. Because the position of the absorption maxima of these proteins is quite sensitive to the binding arrangement at the protonated Schiff base linkage, varying strongly with the nature of the hydrogen bond of the apoprotein to the Schiff base proton and with nearby charges, such behavior has been taken to suggest that water forms part of the structure of the binding site. Birge and co-workers have performed extensive semiempirical and quantum mechanical calculations modeling the results of two-photon spectroscopy (Birge and Zhang 1990) and kinetics of the primary photophysical event of these pigments (Birge et al., 1988; Birge, 1990). To satisfactorily model these results and a number of the spectroscopic properties of the binding site, they have found it necessary to place a water molecule at the binding site, hydrogen-bonded to the Schiff base proton. Finally, the position of the protonated Schiff base, $C{=}NH^+$ stretch, frequency and its change upon deuteration of the pigment and the Schiff base suggest that a water molecule interacts strongly with the protonated Schiff base. In bacteriorhodopsin, the width of the observed $C{=}NH^+$ stretch is relatively broad for this band and narrows considerably upon deuteration of the protein (see, e.g., Fig. 1). This was interpreted by Hildebrandt and Stockburger (1984) as arising from a vibrational energy exchange transfer between the $C{=}NH^+$ stretch, which lies at 1641 cm^{-1}, and the bending mode of H_2O, which lies at 1635 cm^{-1}. This coupling is largely abolished when H_2O is replaced by D_2O because the D_2O bend lies at 1205 cm^{-1}. This type of coupling is a short-range effect so that this analysis places a water molecule very close to the protonated Schiff base. In rhodopsin, the shift in frequency between the protonated $C{=}NH^+$ stretch and the deuterated $C{=}NH^+$ stretch, some 32 cm^{-1}, is generally believed to imply a strong hydrogen bond to the imine proton of the protonated Schiff base (Bagley et al., 1985; Deng and Callender, 1987; Palings et al., 1987). What is unusual is that this large isotope shift decreases either very slightly or not at all upon the photochemical formation of the primary photoproduct, bathorhodopsin. This suggests that the strength of the hydrogen bond is largely unaffected despite the photoisomerization of the bound retinal chromophore that takes place in the rhodopsin (an 11-*cis* chromophore) to bathorhodopsin (a *trans* chromophore) transformation: this despite the fact that the photoisomerization would cleave a putative salt bridge between the protonated Schiff base and a negative counterion and despite the rather large red shift in absorption maximum that distinguishes bathorhodopsin from rhodopsin. One way of explaining such results is that a water molecule is hydrogen bonded between the Schiff base and its counter-ion in rhodopsin and that this molecule and its hydrogen bond follows the retinal chromophore during photoisomerization (Deng and Callender, 1987; Birge et al., 1988).

Assuming that a water molecule is at the binding site of the two pigments and hydrogen bonded to the Schiff base imine proton, the rate of H-D exchange can be cast as the sum of two physically distinct steps. The first is the rate of diffusion of water to the Schiff base site and binding of this water molecule to the proper position. The second step is the rate at which hydrogens of the structural water molecule exchange with the Schiff base hydrogen. Our current results cannot distinguish which of the two steps is rate limiting, although there is some hint that the diffusion of the water molecule to the Schiff base is rate-limiting because the exchange time in bacteriorhodopsin slows down somewhat under experimental conditions where the protein aggregates. However, more experiments, such as kinetic isotope effect of the hydrogen exchange experiments, are needed for such a conclusion.

Finally, the exchange results of this work bear on recent titration studies of rhodopsin that suggest that the pK_a of the protonated Schiff base linkage in rhodopsin is greater than 17 (Steinberg et al., 1993). Given that the pK_a of model retinal Schiff bases are around 7 in solution, an increase of 10 pH units is a remarkable, if not a spectacular, result. However, the analysis of the titration studies assumed explicitly that the binding site is accessible to water. Clearly, the results presented here show that the Schiff base binding site is quite accessible to aqueous solvent. In fact, if the molecular model presented here is correct, the diffusion of water to the Schiff base binding site in rhodopsin and bacteriorhodopsin occurs on the millisecond time scale (or faster).

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