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Exchange kinetics of the Schiff base proton in bacteriorhodopsin

(purple membrane/halobacteria/proton pump/resonance Raman spectroscopy/Schiff base pK_a)

BENJAMIN EHRENBERG*, AARON LEWIS*†, THOMAS K. PORTA*, JOHN F. NAGLE†‡, AND WALTHER STOECKENIUS†§

*School of Applied and Engineering Physics, Cornell University, Ithaca, New York 14853; ‡Physics Department, Carnegie-Mellon University, Pittsburgh, Pennsylvania 15213; and §Cardiovascular Research Institute, University of California, San Francisco, California 94143

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ABSTRACT Using rapid mixing techniques and resonance Raman spectroscopy, we have found that the $^1H/^2H$ exchange time for the Schiff base proton of bacteriorhodopsin in purple membrane is 4.7 msec, when experiments are carried out at pH 2 or pH 7 at room temperature in the dark. We argue that diffusion of neutral water into the membrane is fast on this time scale. Also, model Schiff bases in solution have a pK_a between 6 and 7, and we show that such model Schiff bases have much faster exchange rates. Therefore, we conclude that the Schiff base proton in bacteriorhodopsin is protected from interaction with the medium, probably by interaction with a protein group, and this would account for a pK_a considerably higher than 6–7.

Bacteriorhodopsin is the only protein found in the purple patches that develop in the plasma membrane of *Halobacterium halobium* when this bacterium is grown under illumination and at low oxygen pressure (1). Bacteriorhodopsin is a chromoprotein composed of retinal complexed to the ϵ-amino group of a lysine residue (1) by a protonated Schiff base (2), and it is well established that the biological role of this membrane protein is to serve as a light-driven proton pump (3, 4). Thus, the purple membrane has generated considerable interest in bioenergetics.

It has been suggested (5) that the Schiff base may play an important role in the proton pumping mechanism. This suggestion arises principally from resonance Raman experiments that demonstrated that the Schiff base is transiently deprotonated (2) 10–20 μsec after excitation of the proton pumping cycle (6). In addition, it has been noted in agreement with this suggestion that alterations in the chromophore around the Schiff base seriously alter the proton pumping activity (7). Therefore, in this paper we focus on the Schiff base proton by studying its rate of exchange in the dark as a function of the pH of the external medium.

For these measurements the purple membrane fragments, which were prepared and purified by the method of Kanner and Racker (8), were suspended in ^2H_2O, and thus the Schiff base was deuterated. This was demonstrated by resonance Raman spectroscopy, in which the 1642 cm^{-1} $C{=}N^+{-}^1H$ stretching mode of bacteriorhodopsin (bR_{570}) shifts to 1621 cm^{-1} upon suspension in ^2H_2O (2). The ^2H_2O suspension was mixed with ^1H_2O by using a continuous-flow rapid mixing technique. The Raman scattering of the mixture was measured at various points downstream, and the disappearance of the 1621 cm^{-1} $C{=}N^+{-}^2H$ band and the accompanying formation of the 1642 cm^{-1} $C{=}N^+{-}^1H$ band were monitored. The mixing was performed by using the eight-jet mixer from a Durrum stopped-flow instrument. The calibration of the mixer's dead time was performed in the following way: Solutions of ascorbic acid and $K_3Fe(CN)_6$ were mixed in the same apparatus and the kinetics of reduction of $Fe(CN)_6^{-3}$ were measured by following the decrease of its Raman band at 2130 cm^{-1}. These measurements were repeated with different concentrations of ascorbic acid and the reaction curves obtained were extrapolated back to intersection. From this we obtained a mixing time of 0.9 ± 0.3 msec at a total flow rate of ≈300 ml/min.

In Fig. 1 resonance Raman spectra are shown for a few mixing experiments after different delay times. These experiments were performed with the pH of the bacteriorhodopsin suspensions in both ^2H_2O and ^1H_2O adjusted to 7 (Fig. 1*A*) or 2 (Fig. 1*B*). As can be seen, the intensity of the 1640 cm^{-1} band increases with time relative to the 1621 cm^{-1} band, indicating the exchange of a deuteron with a proton. The equilibrium position of the exchange reaction cannot be observed here correctly, because the rate of ^1H_2O flow into the mixer was 12 times larger than the flow rate of the membrane ^2H_2O suspension in order to drive the exchange. Therefore, at equilibrium the amount of deuterated Schiff base linkages is less than 10% of the total.

Because of the 12-fold excess of ^1H_2O over ^2H_2O after mixing, we analyzed the exchange reaction as a pseudo-first-order reaction. Under such conditions, one expects the intensities A of the 1640 and 1621 bands (after the backgrounds have been subtracted) to behave as

$$A_{1640} = a_{1H}(1 - e^{-kt}) \quad \text{and} \quad A_{1621} = a_{2H}e^{-kt},$$

in which a_{1H} and a_{2H} are the intensities, respectively, of bacteriorhodopsin species with pure protonated and pure deuterated Schiff bases, t is time, and k is the rate constant of the exchange of the deuteron by a proton. Further, one expects a_{1H} and a_{2H} to be nearly equal for such small changes in vibrational frequency (1640 cm^{-1} to 1621 cm^{-1}). Therefore,

$$\log_{10}\left[\frac{A_{1640}}{A_{1621}} + 1\right] = 0.4343kt. \qquad [1]$$

We analyzed the exchange experiments in this way, using a fitting program to calculate the areas of the relevant Raman bands, and the results are shown in Fig. 2. As can be seen in this figure, the data points at pH 7 can be fitted adequately with a linear function. The data points for pH 2 are in substantial agreement with those for pH 7. The value obtained for the pseudo-first-order reaction rate is k = 146 sec^{-1}, yielding a half-time, $t_{1/2}$, for this exchange reaction of 4.7 msec. The second-order rate constant can be extracted by putting in the ^1H_2O concentration, and the value that is obtained is 2.9 M^{-1} sec^{-1}.

These values for the reaction rate are surprisingly long if the pK_a of the Schiff base nitrogen is assumed to be between 6 and

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† To whom reprint requests should be addressed.

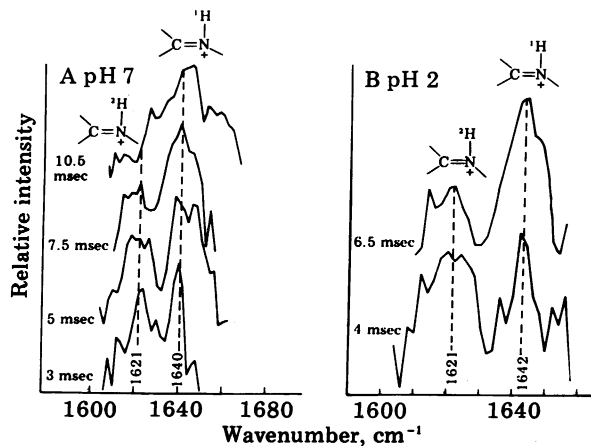

FIG. 1. Resonance Raman spectra of bacteriorhodopsin suspended in ^2H_2O and mixed with ^1H_2O, at the indicated delay times after mixing. The pH of the solutions was 7 (*A*) and 2 (*B*). Spectra were obtained with 514.5-nm excitation; laser power was 50 mW; spectral resolution was 2 cm^{-1}.

7, as it is in other Schiff bases formed from simple amines and aldehydes (9). For such an acid, one would expect a dissociation rate between 10^4 and 10^5 sec^{-1} (10). This would then be the rate-determining step in a reaction involving dissociation of the acid and recombination with another proton. The rates measured in these experiments are 1/100th to 1/1000th of this expected value.

To check this, we compared the above results with similar experiments that we performed with model compounds. We formed the Schiff base of *all-trans*-retinal with butylamine with a deuteron on the nitrogen atom and dissolved it in $C_2H_5O^2H/^2H_2O$ mixtures. We performed the exchange experiment by mixing those solutions with $C_2H_5O^1H/^1H_2O$ mixtures of the same alcohol/water composition. The Raman spectra were measured at a delay time of 1 msec and the results are shown in Fig. 3. As can be seen, even at this short time, the

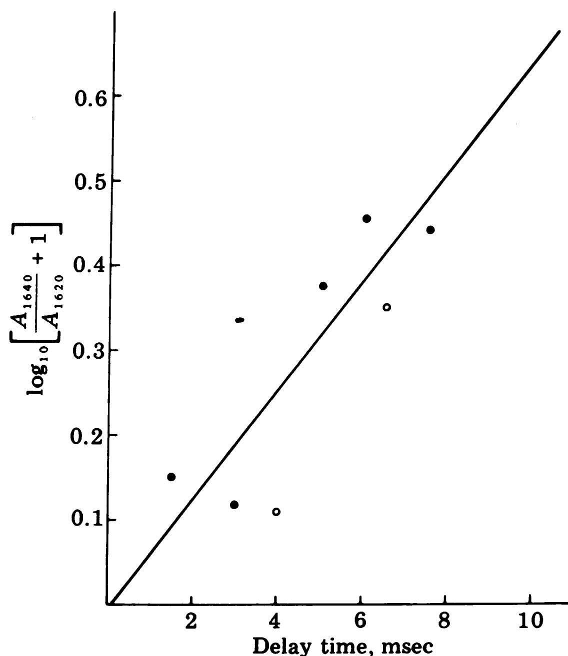

FIG. 2. Kinetic plot of the exchange of the deuteron of the Schiff base nitrogen of bacteriorhodopsin by a proton, performed at pH 7 (●) and 2 (○). A_{1640} and A_{1620} are the areas of the Raman bands of a protonated and deuterated Schiff base, respectively.

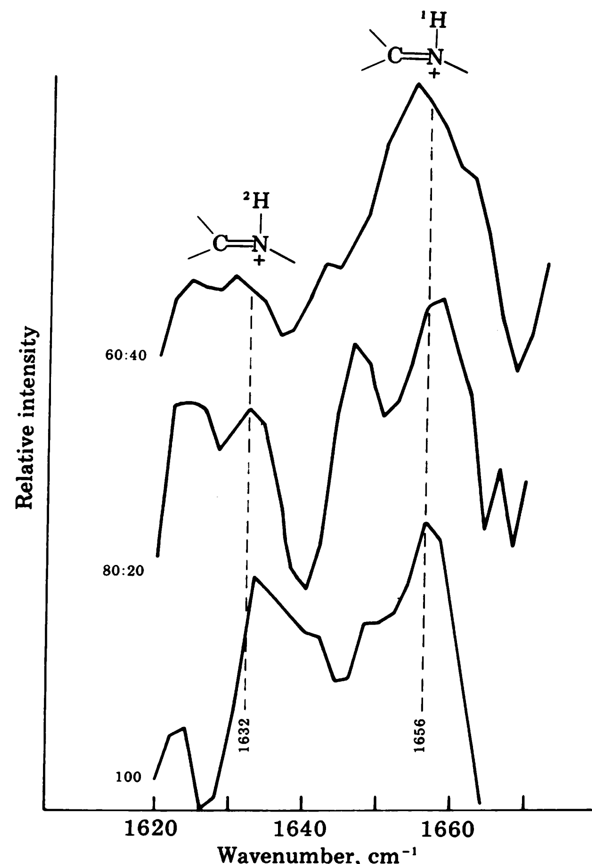

FIG. 3. Resonance Raman spectra of *all-trans*-retinylidene-*n*-butylamine initially dissolved in 100% $C_2H_5O^2H$ or in $C_2H_5O^2H/^2H_2O$ mixtures (80:20 or 60:40, vol/vol), measured 1 msec after mixing with a $C_2H_5O^1H/^1H_2O$ mixture of the same composition. The spectra were obtained with 476.5-nm excitation; laser power was 50 mW; spectral resolution was 2 cm^{-1}.

exchange reaction approaches completion up to the equilibrium situation as the water content of the solution grows. We could not use larger water percentages, which would better mimic the situation in the membrane suspensions, because hydrolysis of the Schiff base linkage occurred. Therefore the exchange of a deuteron on a Schiff base of retinal with an amine moiety that is similar to that in bacteriorhodopsin occurs faster than 1 msec, which is our shortest observation time. Thus the exchange kinetics in bacteriorhodopsin are indeed slow.

The rate of $^1H/^2H$ exchange is comparable to cycling times for proton pumping, and this raises the possibility that the same pathway is used for both processes. But a proton pumping pathway consisting of a single hydrogen-bonded chain of the sort discussed by Nagle and Morowitz (11) would not be conducive to $^1H/^2H$ exchange because it would require a rather rare fluctuation involving a sequence of proton hops in the same direction with no driving force (in the dark). On the other hand, if the single chain were generalized to a more extended network of hydrogen bonds, then the exchange rate along this structure would be considerably enhanced and could account for the measured exchange. The measured rate would be useful in distinguishing such fine points of the proton pathway if one could be certain that the measured $^1H/^2H$ exchange is due to it. However, there is the possibility, realized for instance in ice (12) that isotope exchange proceeds by a mechanism independent of the mechanism for charge transport. Although this is unfortunate as regards learning about the proton pathway, it may yield information about the environment of the Schiff base, as we discuss below.

We will now argue that the experimental exchange rate supports the hypothesis that the Schiff base proton is protected in the bacterio-opsin matrix and that this gives the Schiff base a pK far higher than the 6–7 that it would have in solution. The idea is to explore one possible mechanism that would yield faster $^1H/^2H$ exchange than the measured one if the conclusion above were false. We emphasize that the argument is independent of other mechanisms of exchange, which would operate in parallel and could only further increase the theoretical exchange rate. The mechanism we explore is diffusion of neutral water into the membrane with subsequent $^1H/^2H$ exchange at the site of the Schiff base, similar to the mechanism that controls the isotope exchange in ice. The fact that the kinetics at pH 2 are the same as at pH 7 is consistent with such a neutral transport mechanism, although it does not rule out exchange along a proton wire unless it can also be shown that the states of protonation of side chains composing the wire are different at pH 2 than at pH 7.

First, the time for water molecules to reach the retinal region should be short compared to $t_{1/2}$ of 4.7 msec. This is based upon a diffusion formula:

$$Z_{1/2}^2 = 2t_{1/2}D_{H_2O} \text{ in bR} \quad [2]$$

in which $Z_{1/2}$ is the distance perpendicular to the membrane and D_{H_2O} in bR is a coefficient of diffusion in bacteriorhodopsin roughly related to the membrane permeability by $P = D/d$, in which the thickness d is about 5 nm. The effective Ds or Ps for diffusion in the bacteriorhodopsin molecule are not known, but it seems improbable that they should be much smaller than water permeability in lipid bilayers, $P = 10^{-3}$ cm/sec or $D = 5 \times 10^{-10}$ cm^2/sec, which is already 4 or 5 orders of magnitude smaller than in most liquid systems. Using this value of D in Eq. 2 and assuming the most unfavorable case, that the Schiff base is located at a distance $Z_{1/2}$ of 2.5 nm from the aqueous medium gives $t_{1/2} = 6 \times 10^{-5}$ sec. To obtain a $t_{1/2}$ as large as the measured one requires $D = 5 \times 10^{-12}$ cm^2/sec, which is an order of magnitude smaller even than the coefficient of diffusion of water in ice (13) and several orders of magnitude smaller than water diffusion in solid polymers (14, 15). Thus, it appears that whatever water is in the membrane should be exchanged in less than 0.1 msec. Therefore, the rate-limiting step is the $^1H/^2H$ exchange between the Schiff base and the water in the membrane.

The second part of the argument considers a specific model for the environment of the Schiff base and shows that this model leads to a contradiction. The model is that the Schiff base proton is exposed to a small pool of intramembranous water. Such a model is reasonable *a priori* because it could satisfy hydrogen bonding tendencies of the Schiff base and would lower the electrostatic energy of the charged Schiff base. But, according to this model, the rate of $^1H/^2H$ exchange with the water in the pool should be comparable to the rate of $^1H/^2H$ exchange of Schiff bases in solution. To estimate this rate we use Eigen's formula (10)

$$k_{\text{dissociation}} = k_{\text{recombination}} \times 10^{-pK},$$

in which $k_{\text{recombination}}$ usually has the diffusion-limited value 10^{10}–10^{11} M^{-1} sec^{-1} (ref. 10). Because $k_{\text{dissociation}}$ is slower than $k_{\text{recombination}}$ it would have to be the rate-limiting step for this model and therefore should be set equal to the measured second-order rate constant $k_2 = (146 \text{ sec}^{-1})/55$ M water $= 2.7$ M^{-1} sec^{-1}. Thus, the formula requires that the pK of the Schiff base be between 10 and 11. However, as has already been mentioned, the pKs of Schiff bases in aqueous environments are between 6 and 7. Thus, the model is inconsistent in that it would be expected to undergo $^1H/^2H$ exchange much more quickly than is observed.

A model that is consistent with the $^1H/^2H$ exchange rate result and that appears chemically plausible is that the Schiff base is strongly hydrogen bonded to the bacterio-opsin. This has previously been suggested on the basis of other resonance Raman data (5), which indicated that the Schiff base proton is strongly interacting with a protein group in the bacterio-opsin matrix. The proton in such a model would be less dissociable than in water, and this would account for the slower exchange and would give rise to a higher pK than in solution. This is consistent with the result of kinetic resonance Raman spectroscopy (5, 16) that even at pH 12 the majority of Schiff base linkages are protonated, indicating a pK value ≥ 12.

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