BRIEF COMMUNICATION

ON THE MECHANISM OF HYDROGEN-DEUTERIUM EXCHANGE IN BACTERIORHODOPSIN

A. G. DOUKAS, AJAY PANDE, T. SUZUKI, R. H. CALLENDER, *Department of Physics, City College of the City University of New York, New York 10031*
BARRY HONIG, *Department of Physiology and Biophysics, The University of Illinois, Urbana, Illinois 61801 U.S.A.*
MICHAEL OTTOLENGHI, *Department of Physical Chemistry, The Hebrew University, Jerusalem, Israel*

ABSTRACT Continuous-flow resonance Raman experiments carried out in bacteriorhodopsin show that the exchange of a deuteron on the Schiff base with a proton takes place in times shorter than 3 ms. Exchange mechanisms based on a base-catalyzed deprotonation followed by reprotonation of the Schiff base are excluded. A mechanism is suggested in which a water molecule interacts directly with the Schiff base deuteron in a concerted exchange mechanism. It appears that in the dark, the binding site is more accessible to neutral water molecules than to charged protons.

INTRODUCTION

Bacteriorhodopsin (bR), the purple membrane protein of *Halobacterium halobium*, uses light to actively transport protons from the inside to the outside of the cell membrane. The chromophore of bacteriorhodopsin is all-*trans* retinal bound in the form of a protonated Schiff base to the ϵ amino group of one of the lysines in the protein (1). The Schiff base deprotonates and then reprotonates during the course of the light-driven photocycle of bR (2), and it is likely that these events are directly associated with the proton pumping mechanism. It is known from Raman studies that the Schiff base proton exchanges for a deuteron, resulting in a downward frequency shift of ~25 cm^{-1} in the well characterized 1,645 cm^{-1} C=N stretching vibration (2).

In this paper we use the resonance Raman technique to study the hydrogen-deuterium exchange kinetics over a wide pH range. The rate of exchange can in principle provide important information about the proton pumping mechanism, since it depends on the accessibility of the Schiff base to water molecules and to protons in the aqueous phase. There has been a preliminary report of a 4.7-ms exchange time at pH 7 (3). The results presented below indicate that the exchange (for light-adapted bR) may be even faster over the pH range

Dr. Suzuki's present address is Department of Pharmacology, Hyogo College of Medicine, 1-1 Mukogawa-cho Nishinomiya, Hyogo, 663, Japan.

of 4–10. Given the high pK of the Schiff base within the protein matrix, it is clear that these fast exchange times cannot be accounted for by the standard exchange mechanism involving deprotonation of acidic groups. A mechanism involving a concerted exchange with one or more water molecules in the binding site is presented.

EXPERIMENTAL

Aqueous suspensions of bR were prepared as previously described (4). Deuterated samples were prepared by subsequent centrifugation and resuspension in D_2O. Deuteration of the Schiff base was confirmed by the appearance of the C=ND vibration at 1,621 cm^{-1}, as shown in the stationary Raman experiment of Fig. 1 *A*. Sample pD was controlled by the addition of DCl and NaOD; its value was determined by pD = pH (meter reading) + 0.40 (5).

Continuous-flow experiments were carried out by mixing the deuterated bR suspensions with a distilled water solution of the same pH [1 part bR(D_2O) to 50 parts H_2O]. The mixing chamber was constructed with two jets meeting head-on (e.g., reference 6). The mixed sample exited through a 0.5-mm Diam glass capillary at a flow rate of 100 ml/min, and the Raman spectrum was measured using a 568.2-nm line from a krypton ion laser at a power level of 5.0 mW, as previously described (7). Under these conditions, <15% of the bR undergoes photolysis (5).

The dead time of the flow apparatus, 3.0 ± 0.5 ms, was calculated from the measured flow rates (100 ml H_2O/min, 2 ml bR(D_2O)/min), and from the volume of the flow cell (5 μl). The dead time and the completion of mixing during that time were verified by following the reduction of potassium ferricyanide with L-ascorbic acid (8).

RESULTS AND DISCUSSION

Fig. 1 *A* shows the resonance Raman spectrum of deuterated bR at pD 4. Identical spectra were also obtained at pD 7 and pD 10. Figs. 1 *B*, *C*I, and *D* show spectra recorded for light-adapted bR 3 ms after mixing at pH 4.0, 7.0, and 10.0, respectively. Fig. 1 *C*II is for dark-adapted bR at pH 7, 15 ms after mixing. The intensity of the 1,646-cm^{-1} line relative to that of the 1,530-cm^{-1} ethylenic stretching line was always consistent with observations on

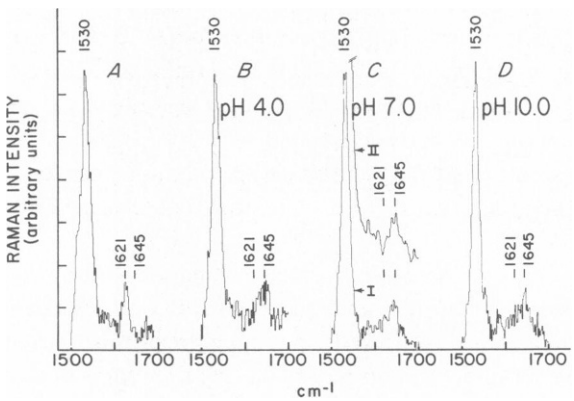

FIGURE 1 Resonance Raman spectra showing hydrogen-deuterium exchange in bacteriorhodopsin at various pH values (resolution 6 cm^{-1}). (*A*) Deuterated sample (light-adapted). (*B*,*C*I,*D*) Deuterated sample (light-adapted) 3 ms after (1:50) mixing with H_2O. (*C*II) Deuterated sample (dark-adapted) 15 ms after mixing (1:50) with H_2O.

stationary samples. From the complete disappearance of the C=ND line at 1,621 cm^{-1} and the appearance of the 1,646-cm^{-1} C=NH line, it is evident that after 3 ms the exchange process is essentially complete. (The broadening of the 1,646-cm^{-1} line in Fig. 1 *B–D* is due to the 1,650-cm^{-1} water line and possibly to traces of unexchanged deuterons.)

The standard mechanism for hydrogen-deuterium exchange involves a base catalyzed dissociation followed by a reprotonation of the acidic group. The general reaction scheme for the exchange of a deuteron on an acid AD with a proton is (9):

$$AD + B \xrightarrow{k_d^B} A^- + BD^+ \qquad (k_d^B = k_d^{OH^-} \text{ or } k_d^{H_2O})$$

$$A^- + BH^+ \xrightarrow{k_r} AH + B$$

where in the present system, the base B may be either OH^- or H_2O, and $k_d^{OH^-}$ and k_d^W are the respective bimolecular rate constants.

The OH^- mechanism can be excluded, since the observed exchange rate cannot exceed the rate of deprotonation which is given by $k_d = k_d^{OH^-}\,[OH^-]$ (where $k_d^{OH^-}$ is limited by the diffusion controlled rate constant which is of the order of $10^{10}\ M^{-1}\ s^{-1}$). At pH 4 this yields an exchange time of ~1 s, which is at least three orders of magnitude slower than the experimental result.

The water catalyzed reaction can also be excluded. In this case, the observed pseudo first order rate of dissociation, $k_d = k_d^W\,[H_2O]$, which is independent of pH, is given by $k_d = k_r\ 10^{-pK}$, where k_r is close to the diffusion controlled rate constant for recombination of protons, i.e., of the order of magnitude of $10^{10}\ M^{-1}\ s^{-1}$ (9). An estimate of the pK of the Schiff base proton in bR is required to calculate k_d. Since the absorption spectrum of bacteriorhodopsin is essentially unchanged up to pH ~ 12 and since deprotonation is associated with large spectral blue shifts (10), it is clear that the Schiff base is fully protonated at pH 12. Protonation at pH 12 has in fact been measured directly in a Raman experiment (11). The pK of the Schiff base must thus be >12, implying that for H_2O catalysis, $k_d \sim 10^{-2}\ s^{-1}$. Since this sets a limit for the exchange time at least five orders of magnitude slower than the observed rate, H_2O catalyzed deprotonation cannot be the mechanism responsible for exchange. This conclusion is also supported by the observation (12) that the formation of the acid bR_{605} species (attributed to the neutralization of an acidic group adjacent to the chromophore) takes place in times longer than 20 ms. Since the observed exchange rate is at least one order of magnitude faster, base

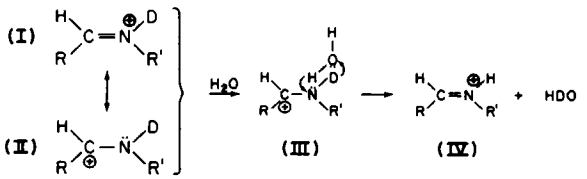

FIGURE 2 Suggested reaction mechanism for the hydrogen-deuterium exchange reaction of the protonated Schiff base of bacteriorhodopsin.

catalyzed (by either H_2O or OH^-) deprotonation and subsequent reprotonation can be excluded.[1]

Since the observed exchange time is too fast to be accounted for by a mechanism involving deprotonation, it is worthwhile considering examples reviewed by Grunwald and Eustace (15) in which hydrogen-deuterium exchange rates are much faster than acid dissociation rates (see also reference 16). It was always necessary to invoke specific exchange mechanisms which do not involve complete proton dissociation, but rather require direct participation of solvent molecules in a concerted exchange reaction.

Fig. 2 describes such a mechanism for bR in which a water molecule donates a proton to the Schiff base nitrogen, which in turn transfers its deuteron to the same water molecule. As shown in the figure, the intermediate state is one in which the nitrogen carries an electron lone pair which allows it to interact with the proton on the water. In this structure, the adjacent carbon atom assumes a positive charge. This mechanism is strongly supported by theoretical calculations (10, 17) which find a positive charge of 0.244 on 15C (carbon 15 in the retinal chromophore is adjacent to the nitrogen) and thus indicate that the resonance structure (II) makes an important contribution to the ground state of the protonated retinal Schiff base. The intermediate state could be stabilized by the presence of a nucleophilic group on the protein (possibly the Schiff base counter-ion) interacting with 15C. It is worth pointing out that the hydrolysis of Schiff bases is believed to proceed via the nucleophilic attack of a water molecule on the Schiff base carbon atom (18), thus suggesting that the above exchange mechanism may be generalized to free Schiff bases in solution. In bR, the resistance of the Schiff base to hydrolysis may be due to the protection of the carbon atom by the adjacent nucleophilic group.

The observed hydrogen-deuterium exchange rate in bR is determined by diffusion of water to the retinal binding site followed by a reaction with the Schiff base according to Fig. 2. Our data do not allow us to establish which of the two processes is rate limiting, but indicate that both steps are complete in milliseconds or less. Because down to pH $\sim$ 3, the diffusion of protons to an acidic group in the vicinity of the chromophore takes place in times $>$20 ms (12), it appears that the diffusion of protons to the binding site is much slower than that of neutral water molecules.

The authors are indebted to Doctors W. Englander, N. Leonard, K. Nakanishi, and B. Perlmutter-Hayman for helpful discussions, and to Ms. I. Efremov for help in the experiments. The large quantity of bR used in this study was a generous gift of Prof. T. Ebrey.

This work was supported in part by the National Science Foundation (PCM79-02683), by the National Institutes of Health (EY03142), by a City University PSC-BHE Faculty Award at the City College, and by the National Science Foundation (PCM80-10931) at the University of Illinois.

Received for publication 6 September 1980 and in revised form 20 October 1980.

[1]The shift in the pK of the Schiff base from the solution value of ~6.5 (13) to above 12 in bR is larger than the shifts encountered for acidic and basic amino acids in proteins. Such an anomolous shift is probably due to the stabilizing effect of a negative counter-ion in the vicinity of the protonated nitrogen (14).

Note added in proof: In a recent paper (1980, *Proc. Natl. Acad. Sci. U.S.A.*, 77:6571–6573), B. Ehrenberg, A. Lewis, J. K. Porta, J. F. Nagle, and W. Stoeckenius have also studied exchange kinetics in bacteriorhodopsin. The two papers differ in the experimental results and, in addition, present a substantially different analysis. In particular, Ehrenberg et al. conclude that the observed exchange times can be understood in terms of the extraction of a proton from a group with high pK, whereas we take the high pK of the Schiff base in bacteriorhodopsin as a given and suggest that (for either experimental result) the observed rate is too fast to be accounted for with this standard exchange mechanism.

REFERENCES

1. STOECKENIUS, W., R. H. LOZIER, and R. A. BOGOMOLNI. 1979. Bacteriorhodopsin and the purple membrane of halobacteria. *Biochim. Biophys. Acta.* **505**:215–278.
2. LEWIS, A., J. SPOONHOWER, R. A. BOGOMOLNI, R. H. LOZIER, and W. STOECKENIUS. 1974. Tunable laser resonance Raman spectroscopy of bacteriorhodopsin. *Proc. Natl. Acad. Sci. U.S.A.* **71**:4462–4466.
3. EHRENBERG, B., and A. LEWIS. 1979. The exchange rate of deuterons by protons at the Schiff base in bacteriorhodopsin. *Biophys. J.* **25**:78*a*. (Abstr.)
4. BECHER, B., and J. CASSIM. 1975. Improved isolation procedures for the purple membrane protein of *Halobacterium Halobium*. *Prep. Biochem.* **5**:161–168.
5. GLASOE, P. K., and F. LONG. 1960. Use of glass electrode to measure acidities in deuterium oxide. *J. Chem. Phys.* **64**:188–190.
6. CHANCE, B., R. H. EISENHARDT, Q. H. GIBSON, and K. K. LONGBERG-HOLM, editors. Rapid Mixing and Sampling Techniques in Biochemistry. 1969. Academic Press, Inc., New York. 400.
7. CALLENDER, R. H., A. G. DOUKAS, R. CROUCH, and K. NAKANISHI. 1976. Molecular flow resonance Raman effect from retinal and rhodopsin. *Biochemistry.* **15**:1621–1629.
8. TONOMURA, B., H. NAKATANI, M. OHNISHI, J. YAMAGUCHI-ITO, and K. HIROMI. 1978. Test reactions for a stopped flow apparatus. *Anal. Biochem.* **84**:370–383.
9. EIGEN, M. 1964. Proton transfer, acid-base catalysis and enzymatic hydrolysis. *Angew. Chem. Int. Ed. Engl.* **3**:1–19.
10. HONIG, B., A. GREENBERG, U. DINUR, and T. G. EBREY. 1976. Visual pigment spectra: implications of the protonation of the retinal Schiff base. *Biochemistry.* **15**:4593–4599.
11. EHRENBERG, B., and A. LEWIS. 1978. The pK of Schiff base deprotonation in bacteriorhodopsin. *Biochem. Biophys. Res. Commun.* **82**:1154–1159.
12. DRUCKMAN, S., A. SAMUNI, and M. OTTOLENGHI. 1979. Dynamics of pH induced spectral changes in bacteriorhodopsin. *Biophys. J.* **26**:143–146.
13. FAVROT, J., C. SANDORFY, and D. VOCELLE. 1978. An infrared study of the basicity on non-aromatic imines: relation to visual pigments. *Photochem. Photobiol.* **28**:271–272.
14. HONIG, B., T. G. EBREY, R. CALLENDER, U. DINUR, and M. OTTOLENGHI. 1979. Photoisomerization, energy storage and charge separation: a model for light energy transduction in visual pigments and bacteriorhodopsin. *Proc. Natl. Acad. Sci. U.S.A.* **76**:2503–2507.
15. GRUNWALD, E., and D. EUSTACE. 1975. Participation of hydroxylic solvent molecules. *In* Proton Transfer Reactions. V. Gold and E. Caldin, editors. Chapman Hall (London), John Wiley & Sons (New York). 103–120.
16. ENGLANDER, S. W., N. W. DOWNER, and H. TEITELBAUM. 1972. Hydrogen exchange. *Ann. Rev. Phys. Chem.* **41**:903–924.
17. BIRGE, R. R., and L. M. HUBBARD. 1980. Molecular dynamics of *cis-trans* isomerization in rhodopsin. *J. Am. Chem. Soc.* **102**:2195–2205.
18. CORDES, E. H., and W. P. JENCKS. 1964. The mechanism of hydrolysis of Schiff bases derived from aliphatic amines. *J. Am. Chem. Soc.* **85**:2843–2848.