Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 2583–2587, March 1991
Biophysics

The reaction of hydroxylamine with bacteriorhodopsin studied with mutants that have altered photocycles: Selective reactivity of different photointermediates

(membrane protein/proton pump/M intermediate/water accessibility/time-resolved spectroscopy)

SRIRAM SUBRAMANIAM*, THOMAS MARTI*, SUSANNE J. RÖSSELET*, KENNETH J. ROTHSCHILD†, AND H. GOBIND KHORANA*

*Departments of Biology and Chemistry, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge, MA 02139; and †Department of Physics and Program in Cellular Biophysics, Boston University, Boston, MA 02215

Contributed by H. Gobind Khorana, December 21, 1990

ABSTRACT **The reaction of the retinylidene Schiff base in bacteriorhodopsin (bR) to the water-soluble reagent hydroxylamine is enhanced by >2 orders of magnitude under illumination. We have used this reaction as a probe for changes in Schiff base reactivity during the photocycle of wild-type bR and mutants defective in proton transport. We report here that under illumination at pH 6, the D85N mutant has a 20-fold lower rate and the D212N mutant has a >4-fold higher rate for the light-dependent reaction with hydroxylamine compared with wild-type bR. In contrast, the reactivities of wild-type bR and the D96N and T46V mutants are similar. It has been previously shown that the D96N and T46V replacements have no significant effect on the kinetics of "M" formation but have dramatic effects on rate of the decay of M. We therefore conclude that the hydroxylamine reaction occurs before formation of the M intermediate. Most likely it occurs at the "L" stage of the cycle and reflects increased water accessibility to the Schiff base due to a light-driven change in protein conformation.**

Bacteriorhodopsin (bR) is a retinal-based integral membrane protein that functions as a light-driven proton pump in the extreme halophile *Halobacterium halobium*. A model for the secondary structure of the protein is shown in Fig. 1. A central component in the mechanism of proton transport is the protonated Schiff base formed between retinal and the ε-amino group of Lys-216. Absorption of a photon initiates a photocycle that involves the formation and decay of at least five distinct intermediates: K, L, M, N, and O (2). This photocycle is coupled to the translocation of protons, which occurs in two stages: (*i*) a fast proton release from the extracellular side ($t \approx 50$ μsec) that coincides with deprotonation of the Schiff base and the formation of the "M" photointermediate and (*ii*) a slow proton uptake from the cytoplasmic side ($t \approx 10$ msec), which occurs after decay of the M intermediate (3).

Recent site-specific mutagenesis studies with bR have identified Asp-85, Asp-96, and Asp-212 as key components in the mechanism of proton translocation (4). Fourier transform IR experiments have indicated that, in addition to the Schiff base, the above residues undergo transient protonation changes during the photocycle (5–7). Time-resolved studies of the photocycle have further clarified the role of these residues in proton transport (8–10). Asp-96 is protonated in bR (5, 6) and functions in the latter half of the photocycle as a proton *donor* to the deprotonated Schiff base (11, 12). A number of experiments with Asp-85 mutants have shown that Asp-85 is deprotonated in bR (5, 13, 14) and indicate that the

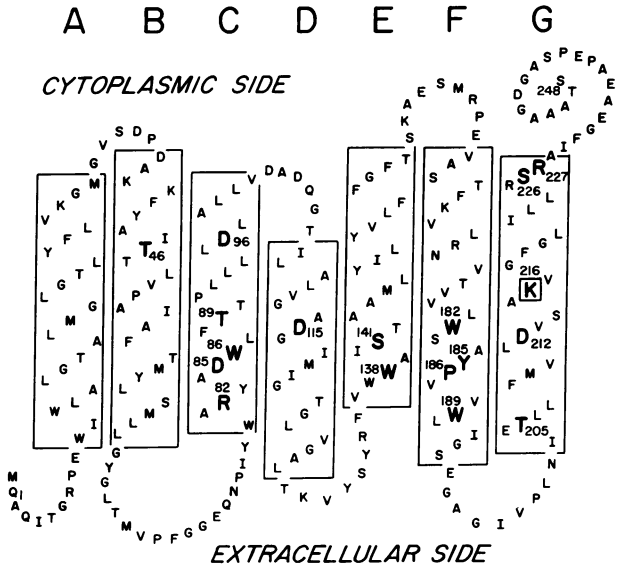

FIG. 1. Model for secondary structure of bR from Henderson *et al.* (1). Locations of the amino acids replaced are indicated in boldface letters; single-letter code is used. The site of retinal attachment, Lys-216, is boxed.

Asp-85 carboxylate functions as a proton acceptor during the first part of the photocycle leading to proton release into the extracellular medium.

The Schiff base in bR is susceptible to reaction with reagents such as hydroxylamine and sodium borohydride. In purple membrane, where bR is present in the form of a two-dimensional hexagonal lattice, the reaction with hydroxylamine requires illumination (15). bR solubilized in lipid/detergent micelles also shows a light-dependent reactivity with hydroxylamine; however, there is detectable reactivity to hydroxylamine even in the absence of illumination (T. Mogi and H.G.K., unpublished work). Oesterhelt and colleagues (16, 17) have proposed that the acceleration of the reaction under illumination in the case of purple membrane is due to specific reaction of hydroxylamine with the M intermediate.

Here, we have studied the rates of the hydroxylamine reaction in lipid/detergent micelles both in the dark and in the light for a variety of bR mutants. For the dark reaction, which

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: bR, bacteriorhodopsin; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate: [Myr_2]PtdCho, dimyristoylphosphatidylcholine; bR mutants are designated by the wild-type amino acid residue (single-letter code) and its position number followed by the substituted amino acid residue (for example, in the mutant D85N, Asp-85 is replaced by asparagine).

is a probe of structural perturbations in the vicinity of the Schiff base caused by different mutations, we find that mutants that display blue-shifted chromophores generally show higher reactivity to hydroxylamine than the wild type, whereas mutants with red-shifted chromophores show lower reactivity. For the light-dependent reaction, we conclude that (*i*) the M intermediate does not react significantly with hydroxylamine, (*ii*) the reaction is probably specific to the "L" stage of the photocycle, and (*iii*) there is increased accessibility of water to the Schiff base during the photocycle due to a conformational change after all-*trans* → 13-*cis* retinal isomerization.

METHODS

Preparation of bR Mutants. The construction, cloning, expression, and purification of wild-type and mutant bacterioopsins have been described (4, 18). Chromophores were formed in 1% dimyristoylphosphatidylcholine ([Myr_2]PtdCho)/1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS)/0.2% SDS/1 mM phosphate buffer, pH 6.0.

Reaction of All-*trans* and 13-*cis* Retinal with Hydroxylamine. The reaction of hydroxylamine (100 mM) with 13-*cis* and all-*trans* retinal in 1% [Myr_2]PtdCho/1% CHAPS/60 mM phosphate buffer, pH 6, results in formation of the corresponding retinal oximes. The reaction is complete in <10 sec. As reported (15), both oximes show structured peaks that are very similar: a main peak at ≈360 nm and a lower-wavelength shoulder.

Reaction of bR with Hydroxylamine in the Dark and Under Steady Illumination. Hydroxylamine reactions were carried out at pH 6 in 0.8% [Myr_2]PtdCho/0.8% CHAPS/0.16% SDS/100 mM hydroxylamine. All experiments were done at 22 ± 2°C. For the light reaction, samples were illuminated by a 100 W fiber-optic illuminator equipped with a long pass 495-nm cut-off filter (GG 495 from Melles Griot, Irvine, CA). The kinetics of retinal oxime formation was monitored by recording absorption spectra of the micellar protein solutions.

Reaction of bR with Hydroxylamine Under Pulsed Illumination. Protein samples were illuminated at 1-sec intervals with a 7-nsec long, 532-nm pulse from a DCR-11 Nd-YAG laser (Spectra-Physics). The laser was operated at ≈100 mJ per pulse. Absorption spectra were recorded between flashes with a 1420 UV-enhanced optical multichannel analyzer and model 1460 controller (Princeton Applied Research) interfaced with a 340S monochromator (Spex Industries, Edison, NJ) as described (19). Final concentrations in the reaction mixture were 0.75% [Myr_2]PtdCho/0.75% CHAPS/0.15% SDS/250 mM hydroxylamine/37.5 mM phosphate buffer, pH 7.0 ± 0.1. Higher hydroxylamine concentrations were used in these experiments compared with the steady-state experiments only to accelerate the formation rate of retinal oxime. The samples were excited with a short laser pulse at intervals of 1 sec, which is longer than the lifetimes of the photocycles of wild-type bR and the mutants studied. This technique ensures that there is no significant excitation of photointermediates that were produced by the previous pulse. Control experiments were done with illumination in the absence of hydroxylamine and with hydroxylamine in the absence of illumination.

RESULTS

Light-Induced Reaction of Hydroxylamine with Wild-Type bR. The time course of the reaction of wild-type bR in mixed micelles at pH 6 with hydroxylamine under steady illumination is shown in Fig. 2. The formation of retinal oxime is characterized by an isosbestic point at 395 nm. The kinetics of the reaction was determined by following changes in absorbance at 360 and 550 nm, although only changes in the latter are displayed in Fig. 2 *Inset*. The half-time for the light-induced reaction is 1.8 min, which is ≈200-fold faster than the corresponding reaction in the dark (Table 1).

Light-Induced Reaction of Hydroxylamine with Aspartic Acid Mutants. Fig. 3 shows the effects of site-specific replacement of Asp-85, Asp-96, or Asp-212 by asparagine on the light-induced reactivity to hydroxylamine under steady illumination. All three replacements have profound effects on the proton transport activity (4). At pH 6, formation of retinal oxime is slowed down by >1 order of magnitude in the D85N mutant ($t_{1/2}$ ≈ 40 min), compared with wild-type bR ($t_{1/2}$ ≈ 2 min). In contrast, the D212N mutant shows enhanced reactivity to hydroxylamine; complete conversion of the chromophore to retinal oxime occurred in <30 sec. The light-dependent reactivity of the D96N mutant is, however, comparable to that of wild-type bR. This result is significant because the lifetime of the photocycle of the D96N mutant at pH 6 is ≈1 order of magnitude longer than that of wild-type bR. This increase is known to be due to the presence of a long-lived M intermediate (9). When the reaction was done in

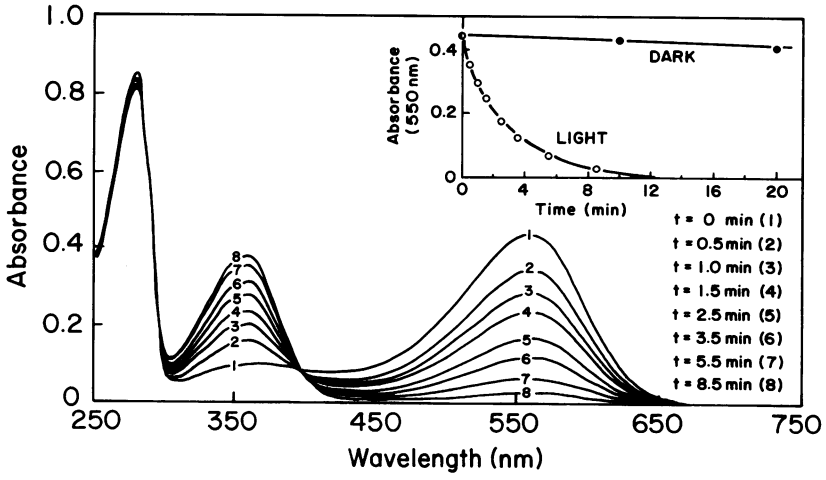

FIG. 2. Time course of the light-enhanced reaction of the Schiff base in wild-type bR with hydroxylamine in [Myr_2]PtdCho/CHAPS micelles at pH 6.2. The sample was illuminated with yellow light for the indicated time periods. Loss of the bR chromophore is matched by formation of the retinal oxime chromophore. (*Inset*) Comparison of rates of retinal oxime formation with (○) and without (●) illumination as measured by changes in *A* at 550 nm.

Table 1. Accessibility of bR mutants to hydroxylamine in the dark at pH 6.0

Mutant	Absorption maximum, nm	$t_{1/e}$, hr
S141C	466	0.49
W182F	465	0.53
D212A	537	0.87
D85E	584	0.87
W189F	526	0.94
W138C	531	1.13
P186G	472	1.36
D212N	545	1.41
T89A	545	1.51
W86F	530	2.00
Y185A	554	2.48
D115N	544	2.66
S141A	540	3.13
T46V	550	4.10
T89D	556	4.89
D96A	554	5.59
D96N	554	5.97
D96A + 100 mM azide	554	6.17
S226A	551	8.25
T205V	550	8.54
D115E	541	8.91
Wild-type bR	551	9.42
R227Q	552	9.86
Y185F	556	10.8
R82Q	567	26.5
R82A	572	26.9
D212E	580	34.0
D115A	542	57.5
D85A	599	273.0
D85N	589	355.4

The $t_{1/e}$ values are derived from best-fit single-exponential curves.

the presence of 100 mM azide, the half-times of the hydroxylamine reaction for D96N and wild-type bR were found to be 1.9 min and 2 min, respectively. Under these conditions the lifetime of the M intermediate is reduced by ≈1 order of magnitude in the D96N mutant relative to that of wild-type bR (11, 20). We therefore conclude that the M intermediate does not react significantly with hydroxylamine.

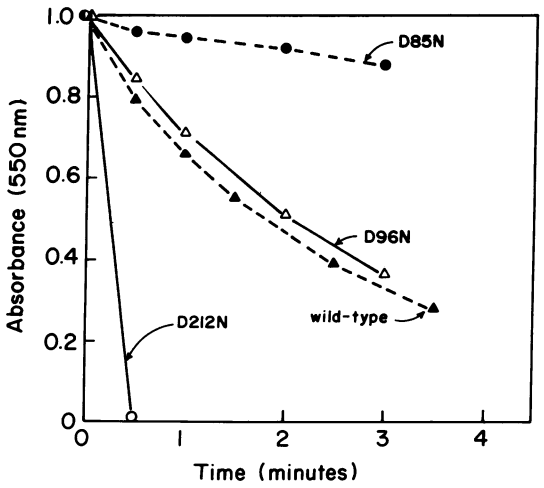

FIG. 3. Comparison of reaction rates of the chromophores in wild-type bR (▲) and D85N (●), D96N (△), and D212N (○) mutants with hydroxylamine as measured by changes in *A* at 550 nm. Reaction conditions are as for Fig. 2. The abscissa represents the total length of illumination time. Absorbances at 550 nm have been scaled to equal 1.0 at t_0.

We have used our knowledge of the time-resolved photocycles of the D96N and T46V mutants to further test the above conclusion. Both mutants display absorption maxima and light/dark adaptation features comparable to those of wild-type bR (4, 18). However, at pH 7, the decay of the M intermediate is 30-fold slower in the D96N mutant and 6-fold faster in the T46V mutant, compared with wild-type bR (13, 18). We have verified that under the conditions of the present experiments, hydroxylamine does not alter these differences in the rate of M decay. If the hydroxylamine reaction occurred primarily at the M stage of the photocycle, it is expected that the number of photocycles required to bleach the D96N mutant would be considerably lower and the number for the T46V mutant would be considerably higher than for the wild-type protein. Fig. 4 shows the kinetics of the decrease in chromophore absorbance as a function of the number of pulses delivered to the sample for wild-type bR and the D96N and T46V mutants. It is clear that the number of pulses required for 50% chromophore loss in the D96N and the T46V mutants is essentially identical. This result further demonstrates that the M intermediate has no significant rate of reaction with hydroxylamine.

Reaction of bR Mutants with Hydroxylamine in the Dark. The reaction of hydroxylamine in the dark at pH 6 with the wild-type protein is characterized by a single exponential rate constant ($t_{1/e} \approx 9.42$ hr). In Table 1, the rates of reaction of a representative set of mutants that affect the properties of the chromophore are presented. The set includes many of the amino acid replacements that have been shown to have significant effects on proton transport activity. The locations of these residues in the protein are marked in the model for the secondary structure shown in Fig. 1. The D85N and the D85A mutants are most resistant to hydroxylamine with $t_{1/e}$ values of 355 and 273 hr, respectively. Both these mutants are completely inactive in steady-state proton translocation (4, 13). The reactivities of mutants T46V, D96N, and D96A (with and without azide) are, however, similar to that of wild-type bR.

At pH 6, the λ_{max} values of these mutants range from 466 nm for the S141C mutant to 599 nm for the D85A mutant (Table 1). Most of the replacements that affect the absorption maximum also affect the rate of hydroxylamine reactivity. All of the residues whose replacements result in proteins with altered absorption maxima are part of the retinal-binding pocket in the model suggested by Henderson *et al.* (1) on the basis of electron microscopic data and by Rothschild *et al.* (21) on the basis of spectroscopic data. There is a significant

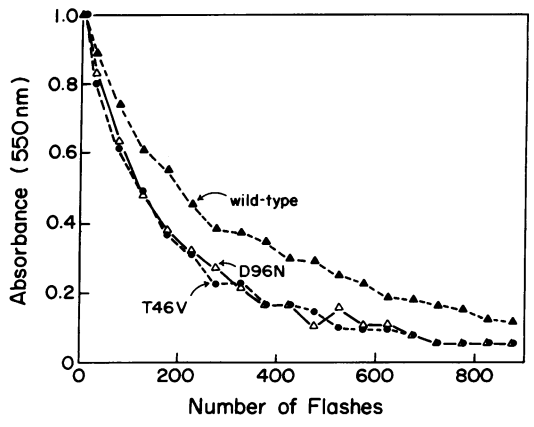

FIG. 4. Rates of hydroxylamine reaction of wild-type bR (▲), D96N mutant (△), and T46V mutant (●) under pulsed illumination. Samples were pulsed at a frequency of 1 Hz as described, and absorption spectra from 400 nm to 700 nm were collected at 700 msec after the laser pulse using a 10-μsec window. To obtain adequate signal/noise ratios, spectra were signal-averaged in blocks of 50.

correlation between a red-shift in the absorption maximum and a decrease in the rate of reactivity to hydroxylamine, although there are a few exceptions (e.g., D85E). Thus, the most reactive mutant, S141C, has a λ_{max} at 466 nm, whereas the least reactive mutant D85N has a λ_{max} at 589 nm.

There is a 30-fold enhancement in the rate of reaction of wild-type bR with hydroxylamine between pH 5 and 8 (Fig. 5). The λ_{max} is essentially unchanged over this pH range, and the enhancement is most likely due to the increased concentration of the unprotonated form of hydroxylamine ($pK_b \approx 6.2$). A similar enhancement of reactivity is seen with the D96N mutant between pH 5 and 8. However, a much steeper pH dependence is seen for the R82A and D85E mutants, which show rate enhancements of 130 fold and 160 fold, respectively, between pH 5 and 8. In contrast, the D85N mutant shows only a 5-fold increase in reactivity to hydroxylamine between pH 5 and 8.

DISCUSSION

The retinylidene Schiff base plays a key role in the mechanism of proton translocation by bR. Studies of changes in the local environment of the Schiff base during the photocycle are therefore of fundamental interest in understanding the molecular mechanism of proton transport. The reaction of the Schiff base with hydroxylamine reflects both the intrinsic reactivity of the Schiff base as well as its accessibility to hydroxylamine. As discussed below, the present study of the reactivities of wild-type bR and different mutants in the dark and under illumination has provided some insights into the above reactions and the structural changes that may occur in bR during the photocycle.

Reaction of bR Mutants with Hydroxylamine in the Dark. Time-resolved $H_2O/^2H_2O$ exchange experiments with purple membrane have shown that the protonated Schiff base in bR can undergo rapid proton exchange with bulk water on a time scale of <3 msec in the dark (22). NMR experiments have suggested that this exchange can be as rapid as 0.1 msec (23). Although purple membrane is largely resistant to the water-soluble reagent hydroxylamine in the dark (15), the micellar suspensions of monomeric bR used here show a detectable rate of reaction with hydroxylamine in the absence of illumination. This is most likely due to the presence of a more flexible conformation in the monomeric state as compared with the crystalline purple membrane lattice.

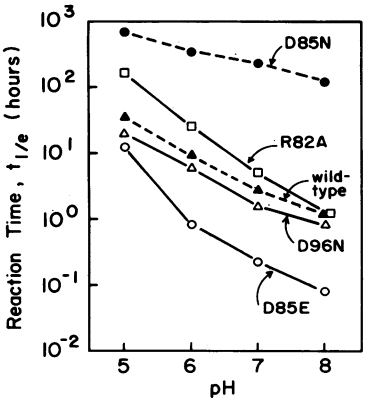

FIG. 5. Dark reactivity of wild-type bR (▲) and mutants R82A (□), D85N (●), D85E (○), and D96N (△) to hydroxylamine at different pH values. The respective λ_{max} values at pH 5 are 552 nm, 586 nm, 588 nm, 606 nm, and 554 nm and at pH 8 are 548 nm, 546 nm, 588 nm, 534 nm, and 548 nm. Reaction times are the inverse of rate constants derived from single exponential fits of the kinetics of retinal oxime formation.

The results shown in Table 1 indicate that bR mutants with red-shifted chromophores appear to have lower rates of reactivity than those with blue-shifted chromophores. One interpretation of this finding is that a smaller "opsin shift" compared with wild-type bR corresponds to weaker retinal–protein interactions and greater water accessibility to the Schiff base. For some of the blue-shifted mutants (e.g., W182F and W189F) there is evidence that the blue-shifted chromophore exhibits greater structural heterogeneity (24), consistent with the higher hydroxylamine reactivity.

It is clear from Table 1 and Fig. 1 that the amino acids whose replacements affect hydroxylamine reactivity do not appear to be localized to one particular side of the membrane. However, the results show that the mutations that have large effects on the kinetics of proton release to the extracellular medium (D85N, D212N, and R82A) also have large effects on the reactivity to hydroxylamine. Furthermore, in the structural model of Henderson *et al.* (1), the region between the Schiff base and the extracellular medium is hydrophilic and loosely packed, whereas the region between the Schiff base and the cytoplasm is hydrophobic and tightly packed. Therefore, the hydroxylamine reaction probably occurs preferentially from the extracellular side of the protein.

We have shown (14) that the R82A and D85E mutants have pK_a values of 6.4 and 6.2, respectively, for the purple-to-blue transition. Both mutants show much higher rate increases for the hydroxylamine reaction between pH 5 and 8 than wild-type bR. This result suggests that the transition from a blue-to-purple form is accompanied by an enhancement in the reactivity of hydroxylamine to the Schiff base. For the D85N mutant, only a 4-fold increase in reactivity is seen over the same pH range. No purple form is seen in the D85N mutant between pH 5 and 8, but instead the Schiff base deprotonates with a pK of 7.0 (13). This result implies that the deprotonated Schiff base has lower reactivity to hydroxylamine than does the protonated Schiff base, which is consistent with the mechanism reported (25) for this class of reactions.

Reaction of bR Mutants with Hydroxylamine Under Illumination. Time-resolved spectroscopic measurements have established that replacement of either Asp-85 or Asp-212 by asparagine has profound effects on the kinetics and quantum yield of proton release and the associated formation of the M photointermediate. The recent structural model for bR (1) and an earlier model (5) place both Asp-85 and Asp-212 at approximately the same distance from the protonated Schiff base. It is therefore interesting that replacement of Asp-85 as asparagine reduces reactivity to hydroxylamine, whereas replacement of Asp-212 by asparagine enhances reactivity to hydroxylamine. The D212N mutant shows significant bleaching under illumination, even in the absence of hydroxylamine (4, 14). The enhanced light sensitivity of this mutant with and without hydroxylamine suggests greater susceptibility of the Schiff base to attack by water and water-soluble reagents.

Decay of the M intermediate is slowed down by >1 order of magnitude in the D96N mutant (9, 11, 20, 26), whereas this decay is accelerated ≈10-fold in the photocycle of the T46V mutant (18). However, the rates of hydroxylamine reaction in the D96N and T46V mutants are essentially identical. This result shows that no significant reaction of the Schiff base with hydroxylamine occurs at the M stage of the photocycle. Because M is the only intermediate in the photocycle in which the Schiff base is deprotonated, we conclude that the intermediate most reactive to hydroxylamine contains a protonated Schiff base. Time-resolved experiments have also indicated that there are differences in the later steps ($\tau > 1$ msec) of the photocycles of wild-type bR and the above mutants. For D96N, decay of the M intermediate appears to correlate with formation of the initial bR state with no detectable accumulation of other intermediates. In contrast, decay of the M intermediate in the photocycle of the T46V

mutant results in the accumulation of a red-shifted intermediate (18). The lifetime of this intermediate increases steeply with an increase in pH. Because the rates of hydroxylamine reaction with the T46V and D96N mutants are identical, it appears unlikely that the reaction occurs at a stage after formation of the M intermediate. In contrast to the differences in decay of M, the kinetics of M formation in D96N and T46V mutants are similar and are slowed down by a factor of ≈3 compared with the kinetics of M formation in wild-type bR. If the hydroxylamine reaction occurred preferentially before formation of the M intermediate, the reactivities of both mutants would be expected to be slightly enhanced compared with wild-type bR. The slight enhancement in the reactivities of D96N and T46V mutants relative to wild-type bR suggests strongly that the conformation most reactive with hydroxylamine occurs *before* rather than *after* the M intermediate and indicates that the reaction is selective to the K and/or L stage of the photocycle.

CONCLUDING REMARKS

The light-induced reactivity of hydroxylamine with bR during the photocycle suggests that there is an increase in the accessibility of water and, therefore, hydroxylamine to the Schiff base. We conclude that the reaction occurs as a consequence of a conformational change caused by the light-catalyzed all-*trans* → 13-*cis* isomerization of retinal but before deprotonation of the Schiff base. Because the K intermediate is formed on a time scale too short for a significant change in protein conformation, the L intermediate is the most likely candidate for the reaction with hydroxylamine. The Schiff base is also protonated in the N and O intermediates, but our experiments suggest that these intermediates do not react significantly with hydroxylamine.

Different protein conformations are very likely involved in different stages of proton translocation—i.e., proton release and proton uptake (27). The present experiments bear on the changes in accessibility of water to the Schiff base early in the photocycle. Further changes that occur in water concentration around the Schiff base in the later steps of the cycle remain to be investigated. The role of structural water molecules in the reprotonation of the Schiff base by Asp-96 is an especially important question because Asp-96 is located ≈10–12 Å from the Schiff base (1). Additional probes of permeability to water and of conformational changes are desirable for a detailed understanding of the molecular mechanism of proton transport.

We thank Dr. M. Krebs for helpful discussions and Ms. J. Carlin for patiently typing many versions of the manuscript. This work was supported by Grants GM28289 and AI 11479 from the National Institutes of Health and Grant N00014-82-K-0668 from the Office of Naval Research, Department of the Navy, to H.G.K. and by Grant DMB-8806007 from the National Science Foundation to K.J.R. S.S. and T.M. are recipients of fellowships from the Damon Runyon–Walter Winchell Cancer Research Fund (DRG-937) and the Swiss National Science Foundation, respectively.

1. Henderson, R., Baldwin, J. M., Ceska, T. A., Zemlin, F., Beckmann, E. & Downing, K. H. (1990) *J. Mol. Biol.* **213**, 899–929.
2. Stoeckenius, W., Lozier, R. H. & Bogomolni, R. A. (1979) *Biochim. Biophys. Acta* **505**, 215–278.
3. Stoeckenius, W. & Bogomolni, R. A. (1982) *Annu. Rev. Biochem.* **51**, 587–616.
4. Mogi, T., Stern, L. J., Marti, T., Chao, B. H. & Khorana, H. G. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 4148–4152.
5. Braiman, M. S., Mogi, T., Marti, T., Stern, L. J., Khorana, H. G. & Rothschild, K. J. (1988) *Biochemistry* **27**, 8516–8520.
6. Gerwert, K., Hess, B., Soppa, J. & Oesterhelt, D. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 4943–4947.
7. Rothschild, K. J., Braiman, M. S., He, Y. W., Marti, T. & Khorana, H. G. (1990) *J. Biol. Chem.* **265**, 16985–16991.
8. Marinetti, T., Subramaniam, S., Mogi, T., Marti, T. & Khorana, H. G. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 529–533.
9. Holz, M., Drachev, L. A., Mogi, T., Otto, H., Kaulen, A. D., Heyn, M. P., Skulachev, V. P. & Khorana, H. G. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 2167–2171.
10. Butt, H. J., Fendler, K., Bamberg, E., Tittor, J. & Oesterhelt, D. (1989) *EMBO J.* **8**, 1657–1663.
11. Otto, H., Marti, T., Holz, M., Mogi, T., Lindau, M., Khorana, H. G. & Heyn, M. P. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 9228–9232.
12. Braiman, M. S., Bousché, O. & Rothschild, K. J. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 2388–2392.
13. Otto, H., Marti, T., Holz, M., Mogi, T., Stern, L. J., Engel, F., Khorana, H. G. & Heyn, M. P. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 1018–1022.
14. Subramaniam, S., Marti, T. & Khorana, H. G. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 1013–1017.
15. Oesterhelt, D., Meentzen, M. & Schumann, L. (1973) *Eur. J. Biochem.* **40**, 453–463.
16. Oesterhelt, D. & Schumann, L. (1974) *FEBS Lett.* **44**, 262–265.
17. Oesterhelt, D., Schumann, L. & Gruber, H. (1974) *FEBS Lett.* **44**, 257–261.
18. Marti, T., Otto, H., Mogi, T., Rösselet, S. J., Heyn, M. P. & Khorana, H. G. (1991) *J. Biol. Chem.* **266**, in press.
19. Duñach, M., Berkowitz, S., Marti, T., He, Y. W., Subramaniam, S., Khorana, H. G. & Rothschild, K. J. (1990) *J. Biol. Chem.* **265**, 16978–16984.
20. Tittor, J., Soell, C., Oesterhelt, D., Butt, H. J. & Bamberg, E. (1989) *EMBO J.* **8**, 3477–3482.
21. Rothschild, K. J., Braiman, M. S., Mogi, T., Stern, L. J. & Khorana, H. G. (1989) *FEBS Lett.* **250**, 448–452.
22. Doukas, A. G., Pande, A., Suzuki, T., Callender, R. H., Honig, B. & Ottolenghi, M. (1981) *Biophys. J.* **33**, 275–280.
23. Harbison, G. S., Roberts, J. E., Herzfeld, J. & Griffin, R. G. (1988) *J. Am. Chem. Soc.* **110**, 7221–7223.
24. Ahl, P. L., Stern, L. J., Mogi, T., Khorana, H. G. & Rothschild, K. J. (1989) *Biochemistry* **28**, 10028–10034.
25. Morton, R. A. & Pitt, G. A. J. (1955) *Biochem. J.* **59**, 128–134.
26. Stern, L. J., Ahl, P. L., Marti, T., Mogi, T., Duñach, M., Berkowitz, S., Rothschild, K. J. & Khorana, H. G. (1989) *Biochemistry* **28**, 10035–10042.
27. Mathies, R. A., Lin, S. W., Ames, J. B. & Pollard, W. T. (1991) *Annu. Rev. Biophys. Biophys. Chem.*, in press.