Characterization of the Recombination Reaction of Rhodopsin†

Robert A. Henselman‡ and Michael A. Cusanovich*,§

ABSTRACT: The kinetics of recombination of 11-*cis*-retinal with bleached rod outer segments and sodium cholate solubilized rhodopsin have been investigated. At neutral pH, it was found that bleached rod outer segments in the presence of an excess of 11-*cis*-retinal follow pseudo-first-order kinetics. The results suggest the second-order formation of an intermediate addition compound followed by a first-order dehydration step to form a protonated aldimine linkage. In addition, at pH values above 7.5 or below 6.5 the kinetics of recombination are complex, indicating the formation of a molecular species inactive in recombination which is in equilibrium with the active form of opsin. Based upon the observed rate constants as a function of pH, a scheme is presented to describe the recombination reaction in bleached rod outer segments. The kinetics of recombination of sodium cholate solubilized opsin were also analyzed. In terms of formation of an intermediate addition compound and subsequent dehydration, the values for the individual rate constants for both bleached rod outer segments and cholate-solubilized opsin were found to compare very favorably. These results demonstrate that the sodium cholate (2 mg/ml) maintains opsin in a conformation very similar to that in the rod outer segment membrane and suggest that the cholate–opsin complex is an excellent model system for studies on opsin–membrane interactions.

Rhodopsin, the visual pigment protein, has been the subject of a large number of studies designed to determine its chemical and physical properties. Photolyzed rhodopsin is capable of undergoing recombination with 11-*cis*-retinal only if the apoprotein opsin is present in isolated rod outer segment (ROS[1]) membranes (DeGrip et al., 1972; Futterman and Rollins, 1973) or if the protein is solubilized with digitonin (Wald and Brown, 1956), Tween-80 (Zorn and Futterman, 1973), or sodium cholate (Henselman and Cusanovich, 1974). Preparations of rhodopsin which have been solubilized with Triton X-100 (Johnson and Williams, 1970), cetyltrimethylammonium bromide (Heller, 1968), laurylamine *N*-oxide (Ebrey, 1971), Emulphogene BC 720 (Shichi et al., 1969), dodecyltrimethylammonium bromide (Hong and Hubbell, 1973) are all incapable of recombination with 11-*cis*-retinal, even though the characteristic absorption spectrum of rhodopsin is retained.

In order to eclucidate the mechanism of action of rhodopsin in vision, it should be useful to understand the reaction of opsin with 11-*cis*-retinal. This derives from the fact that any bonds or interactions broken during visual excitation (photolysis) must be formed during recombination. It is well established that in rhodopsin the chromophore is buried in the protein with little direct access to the solvent (Bownds, 1967). However, upon photolysis, the chromophore (*all-trans*-retinal) is released, implying substantial conformational changes. In light of these observations, it appears that the rod outer segment membrane must stabilize both rhodopsin and opsin in a manner that facilitates recombination. Further, it has been suggested that the lack of recombination in some detergents results from an alteration of the conformation of opsin, due to the fluidity of the micelle (Hong and Hubbell, 1973).

To date, studies on the recombination reaction have focused on the extent of recombination. Little information is available on the kinetics of the reaction. We are reporting here kinetic studies designed to obtain insight into the mechanism of recombination and to compare quantitatively recombination by bleached rod outer segments and sodium cholate solubilized rhodopsin.

Materials and Methods

Preparation of ROS Membranes and Sodium Cholate Solubilized Rhodopsin. Procedures involving rhodopsin or ROS membranes were done either in total darkness or under dim red light (Kodak Wratten Series 1A Filter) at 4 °C, unless otherwise stated. ROS membranes were prepared from frozen and dark adapted bovine retinas (George Hormel Co., Austin, Minn.) by repeated flotation in 1.02 M sucrose, 67 mM potassium phosphate buffer at pH 6.5, as described earlier (Henselman and Cusanovich, 1974). The isolated ROS membranes were washed three times with distilled water, lyophilized to dryness, and stored at −10 °C in light-proof containers. For kinetic experiments, the lyophilized membranes were homogenized in a glass–Teflon homogenizer in the presence of 0.10 M potassium phosphate buffer (pH 7.0) containing 1 mM DTE. The ROS membranes were suspended at a concentration of 1 mg of dry material/ml to yield solutions which were 5–6 μM in rhodopsin.

Sodium cholate solubilized rhodopsin was prepared by homogenizing the lyophilized ROS membranes in 0.10 M potassium phosphate buffer (pH 7.0) containing 20 mg/ml of sodium cholate and 1 mM DTE, as previously described (Henselman and Cusanovich, 1974).

Recombination of Rhodopsin. Experiments on the recombination of exogenous 11-*cis*-retinal and bleached ROS membranes were carried out as follows. Equivalent 1-ml samples of the homogenized ROS membranes in 0.10 M potassium phosphate buffer containing 1 mM DTE and at the pH values indicated under Results were used to record a zero

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‡ Supported, in part, by a training grant from the U.S. Public Health Service (GM 01982). Present address: Cardiovascular Research Institute, University of California, San Francisco, Calif. 94122.

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[1] Abbreviations used are: ROS, rod outer segment; DTE, dithioerythritol.

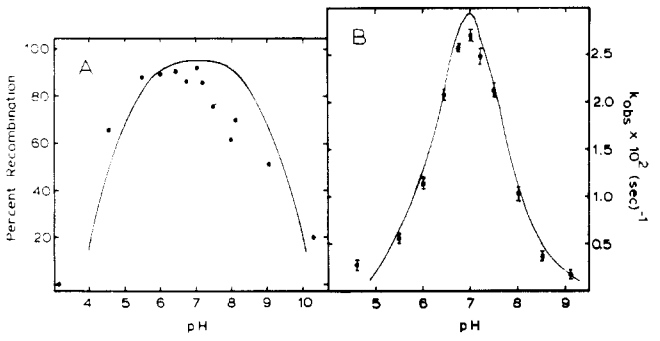

FIGURE 1: (A) pH dependence of the extent of rhodopsin recombination in isolated ROS membranes. Recombination reactions were carried out as described under Materials and Methods. (B) pH dependence of the rate of rhodopsin recombination in isolated ROS membranes. The concentration of exogenous 11-*cis*-retinal was 53 μM and the temperature was 28 °C. The solid lines in A and B were calculated, as described under Discussion, using the following constants: $k_3 = 0.075\ s^{-1}$, $K_3 = 37.5$, $K_1 = K_2 = 10^{-7}$ M, $K_4K_5 = 1.4 \times 10^5\ M^{-1}$.

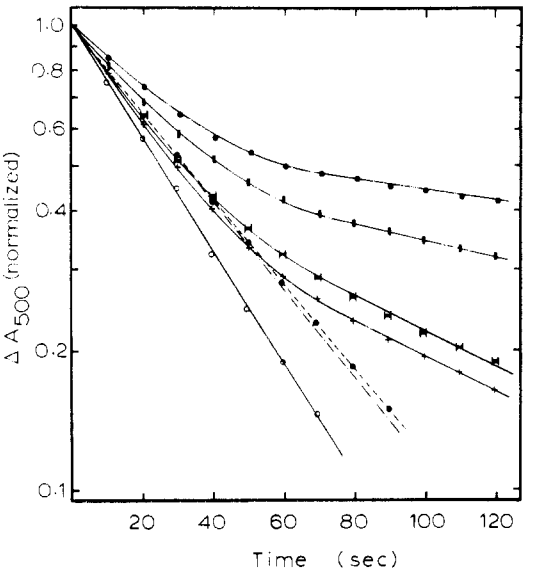

FIGURE 2. Effect of pH on the pseudo-first-order kinetic plots of rhodopsin recombination in isolated ROS membranes. The concentration of exogenous 11-*cis*-retinal in these experiments was 53 μM, the temperature was maintained at 28 °C, and the pH of each reaction mixture was as follows: pH 4.6 (-●-); pH 6.0 (-+-); pH 6.45 (- -●- -); pH 7.0 (—○—); pH 7.4 (— • —); pH 8.0 (-H-); pH 8.5 (■-).

baseline (625–350 nm) with a Cary 118 recording spectrophotometer. Hydroxylamine was added to the reference cuvette to a final concentration of 20 mM and the reference solution was bleached with white light from a microscope illuminator for 15–20 min. The difference spectrum was recorded and the observed ΔA_{500} was used to calculate the initial rhodopsin concentration, assuming an extinction coefficient of 40 000 M^{-1} cm^{-1} at 500 nm. The sample solution was then bleached for at least 20 min, resulting in the loss of greater than 90% absorbance at 500 nm. The recombination of each sample was initiated by the addition of 1–10 μl of exogenous 11-*cis*-retinal carried in 1,4-dioxane. Addition of exogenous 11-*cis*-retinal carried in ethanol gave identical results. The sample was immediately placed in the Cary 118 and the increase of absorbance at 500 nm was recorded as a function of time. In the figures presented under Results, ΔA_{500} at time zero was normalized to 1. In those experiments where only the overall extent of recombination was determined, the samples were incubated in the dark at room temperature for 1 h. After incubation, the reactions were terminated by the addition of hydroxylamine to a final concentration of 20 mM, the difference spectrum of each sample was again recorded before and after a second bleaching, and the observed percent recombination was calculated in the same manner as previously described (Henselman and Cusanovich, 1974).

Experiments on the recombination reaction utilizing sodium cholate solubilized rhodopsin were carried out as above. The sodium cholate concentration in the samples was 2 mg/ml, since this concentration of sodium cholate permits opsin to undergo its largest extent of recombination with exogenous 11-*cis*-retinal (Henselman and Cusanovich, 1974).

Other Methods. Concentrations of sodium cholate solubilized rhodopsin were calculated using a molar extinction coefficient at 498 nm of 41 400 M^{-1} cm^{-1} (Henselman and Cusanovich, 1974). The concentrations of 11-*cis*-retinal used were determined using the extinction coefficient in ethanol (24 900 M^{-1} cm^{-1}) at 376 nm, as given by Morton (1972).

Chemicals. Cholic acid was obtained from Sigma Chemical Co. and was purified as described previously (Henselman and Cusanovich, 1974). Crystalline 11-*cis*-retinal was a generous gift of Hoffman-La Roche Inc., Nutley, N.J., and was used as received. The 11-*cis*-retinal was at least 95% pure as determined by thin-layer chromatography. All other chemicals used in this study were of reagent grade quality.

Results

Recombination of Rhodopsin in Isolated Rod Outer Segment Membranes. The observed extent of rhodopsin recombination in isolated ROS membranes at pH 7 was typically in the range of 90–95% of that theoretically expected. The dependence of the overall extent of rhodopsin recombination in the isolated membranes as a function of pH is shown in Figure 1A and indicates the pH optimum for the extent of reaction is very broad and lies between pH 5.5 and 7.2.

The effect of pH on the rate of the recombination reaction with isolated ROS membranes was assessed by measuring the pseudo-first-order rate constant for the reaction as a function of pH. Representative kinetic plots of these experiments are presented in Figure 2 and illustrate that between pH 6.45 and 7.4 the reaction follows pseudo-first-order kinetics as the plots were linear. At pH values higher than 7.4 or lower than 6.45, the kinetics were biphasic and could be resolved into a fast and a slow phase (sum of two exponentials). The fast phase of the reaction below pH 6.45 was essentially independent of pH, with values of 0.04–0.05 s^{-1}, while the fast phase of the reaction above pH 7.4 was also independent of pH and with values of 0.04–0.06 s^{-1}. The dependence of the observed rate constant (k_{obsd}) upon pH is presented in Figure 1B and indicates the reaction had a sharp pH optimum at pH 7.0. In Figure 1B, the values of k_{obsd} used were obtained from the linear (ln ΔA_{500} vs. time) plots for the pH range 6.45–7.4 and from the slow phases of these plots at pH values lower than 6.45 or higher than 7.4.

The determination of the second-order rate constant for the recombination of opsin in isolated ROS membranes was carried out at pH 7.0. In these experiments, each reaction mixture was maintained at 25 °C and contained 0.10 M potassium phosphate buffer (pH 7.0), 1 mM DTE, ROS membranes equivalent to a rhodopsin concentration of 5 μM. At this pH, plots of ln ΔA_{500} vs. time were linear for at least three half-lives at all 11-*cis*-retinal concentrations used. The second-order plot (k_{obsd} vs. [11-*cis*-retinal]) for the reaction at pH 7.0 is given

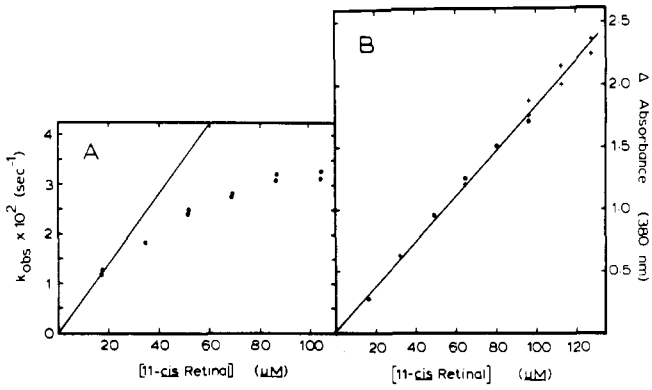

FIGURE 3: (A) Second-order kinetic plot for the recombination of opsin in isolated ROS membranes. For the determination of the individual values of k_{obsd} at the indicated ligand concentrations, each reaction mixture was maintained at 25 °C and contained 0.1 M potassium phosphate (pH 7.0), 1 mM DTE, and ROS membranes at a concentration equivalent to 5 μM rhodopsin prior to bleaching. (B) Solubility of exogenous 11-*cis*-retinal in the presence of ROS membranes. ROS membranes (unbleached) were suspended at a concentration equivalent to 5 μM rhodopsin in 0.10 M potassium phosphate, pH 7.0, containing 1 mM DTE. The increase in absorbance at 380 nm was determined after the addition of increasing amounts of 11-*cis*-retinal.

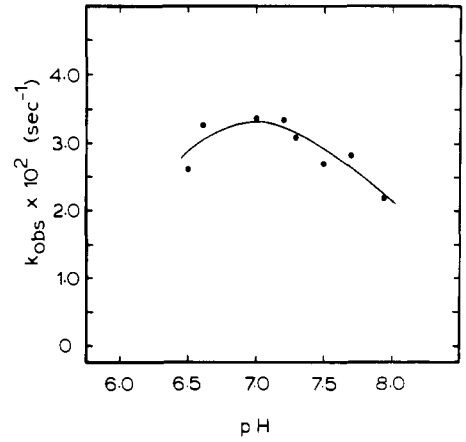

FIGURE 4: pH dependence of the rate of rhodopsin recombination in sodium cholate solutions. Recombination reactions were carried out as described under Materials and Methods. The concentration of exogenous 11-*cis*-retinal in these experiments was 53 μM and each reaction mixture contained 5 μM rhodopsin (before bleaching), 0.10 M potassium phosphate buffer at the indicated pH, 1 mM DTE, and 2 mg/ml of sodium cholate.

in Figure 3A and indicates that at high concentrations of 11-*cis*-retinal (>50 μM) the value of k_{obsd} becomes independent of ligand concentration. Control experiments demonstrated that exogenous 11-*cis*-retinal was apparently solubilized by ROS membranes, as reflected by the linear relationship between the increasing absorbance at 380 nm and increasing concentrations of 11-*cis*-retinal in the presence of unbleached ROS membranes (Figure 3B). Thus, the kinetic data in Figure 3 suggested the recombination reaction was proceeding via a mechanism involving an intermediate species, as illustrated by eq 1.

$$11\text{-}cis\text{-retinal} + \text{opsin} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{INT} \xrightarrow{k_2} \text{rhodopsin} \quad (1)$$

Solution of eq 1 for the condition that 11-*cis*-retinal is in excess yields eq 2

$$1/k_{obsd} = 1/k_2 + 1/(k_2 K_{eq}[11\text{-}cis\text{-retinal}]) \quad (2)$$

where $K_{eq} = k_1/(k_1 + k_2)$. Consistent with a reaction mechanism involving a single intermediate species, we find a plot of $1/k_{obsd}$ vs. $1/[11\text{-}cis\text{-retinal}]$ is linear.

From Figure 3A (solid line), and assuming k_{-1} is small, we can estimate from the initial slope a value of 690 M^{-1} s^{-1} for k_1. From plots according to eq 2, values of 5.5×10^{-2} s^{-1} for k_2 and 1.7×10^4 M^{-1} for K_{eq} at pH 7.0 and 25 °C are found.

Recombination of Rhodopsin in Sodium Cholate Solutions. The pH optimum for the maximum extent of rhodopsin recombination in solutions of sodium cholate was previously shown to be between pH 7.0 and 7.4 (Henselman and Cusanovich, 1974). The effect of pH on the rate of the recombination reaction was determined by measuring the pseudo-first-order rate constant for the reaction as a function of pH. The kinetic plots (not shown) were linear over the pH range 6.5–8.0, indicating the reaction followed pseudo-first-order kinetics for the experimental conditions employed. The observed rate constants (k_{obsd}) are plotted against the reaction mixture pH in Figure 4, yielding an optimum rate between pH 6.8 and 7.2. The reaction could not be studied below pH 6.5, due to precipitation of cholic acid.

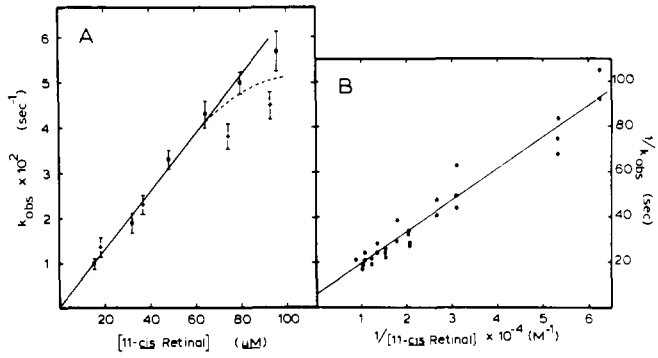

FIGURE 5: (A) Second-order kinetic plot for the recombination of rhodopsin in sodium cholate solutions. In these experiments, solutions of rhodopsin in (5 μM) 0.10 M potassium phosphate buffer, pH 7.0, containing 1 mM DTE and 2 mg/ml of sodium cholate were bleached with white light for at least 20 min. The recombination of each sample was initiated by the addition of exogenous 11-*cis*-retinal to the indicated final concentration. The values of k_{obsd} were determined from linear plots of ln ΔA_{500} vs. time, as indicated under Materials and Methods. Each point and error bar represents the average and range of at least two kinetic determinations and the different symbols represent rhodopsin samples from different preparations of ROS membranes. (B) Reciprocal plot for the recombination of rhodopsin in sodium cholate solutions. The experimental conditions and data were the same as indicated in A.

The determination of the second-order rate constant for the recombination of sodium cholate solubilized rhodopsin was carried out at pH 7.0 in 0.10 M potassium phosphate buffer containing 1 mM DTE and 2 mg/ml of sodium cholate. The initial concentration of rhodopsin (before bleaching) in these experiments was 5 μM and the reaction mixtures were maintained at 25 °C. Figure 5A illustrates the second-order plot (k_{obsd} vs. [11-*cis*-retinal]) for the recombination reaction of sodium cholate solubilized rhodopsin. The data in Figure 5A are plotted according to the reaction mechanism given by eq 3 and yield a value of 660 M^{-1} s^{-1} for k_1.

$$11\text{-}cis\text{-retinal} + \text{opsin} \xrightarrow{k_1} \text{rhodopsin} \quad (3)$$

At high concentrations of 11-*cis*-retinal (>60 μM) the data in Figure 5A appears to layover but not as dramatically as was observed in the case with isolated ROS membranes (Figure

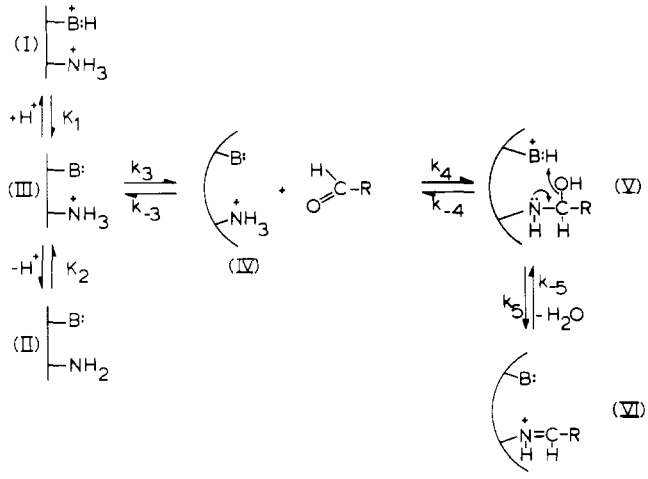

FIGURE 6: Proposed mechanism for the recombination of rhodopsin from 11-*cis*-retinal and opsin. R-CHO represents 11-*cis*-retinal and structure VI represents the product rhodopsin. The group −B: is postulated to be an amino acid residue of opsin which acts as a general acid/general base during the recombination reaction.

3A). The kinetic data can be replotted in a reciprocal form (eq 2, Figure 5B) yielding values of 0.18 s^{-1} for k_2 and 4.45×10^3 M^{-1} for K_{eq}. However, it must be noted that kinetic data obtained on the recombination of rhodopsin in solutions of sodium cholate do not allow us to state with certainty whether the reaction is best described by the mechanism given by eq 1 or 3.

Discussion

Based on the kinetic data presented here, the known chemical and physical properties of rhodopsin, the aldimine linkage between the apoprotein opsin and 11-*cis*-retinal (Bownds, 1967; Fager et al., 1972), and mechanisms for the reaction of simple amines and aldehydes (Jencks, 1969) a tentative scheme describing recombination can be proposed (Figure 6). In the following discussion, the scheme will be analyzed in terms of the reaction of 11-*cis*-retinal and bleached ROS and, subsequently, the data with cholate solubilized opsin will be dealt with.

It is generally agreed that 11-*cis*-retinal is bound to opsin via a protonated aldimine bond (Morton, 1972). Formation of this type of a bond would be expected to proceed, as shown in Figure 6, with an unprotonated amino group attacking the carbonyl oxygen of 11-*cis*-retinal. At neutral pH, ϵ-amino groups of lysine are generally protonated; hence, we propose a proton acceptor (−B:) in close proximity which facilitates formation of the intermediate addition compound (species V). The protonated acceptor (−B:H) subsequently serves as a proton donor to drive dehydration and formation of the protonated aldimine product (VI), which is rhodopsin. At neutral pH, we find the rate of formation of rhodopsin is not directly proportional to ligand concentration (Figure 3A), indicating a change in the rate-determining step has taken place. Such an observation is consistent with the formation of an intermediate species and, if k_4 [11-*cis*] $\sim k_5$ (Figure 6), eq 4 can be applied.

$$k_{obsd} = \frac{k_4 k_5 [11\text{-}cis]}{k_{-4} + k_5 + k_4[11\text{-}cis]} \quad (4)$$

Thus, linear plots of $1/k_{obsd}$ vs. $1/[11\text{-}cis]$ were obtained. As the ligand concentration approaches zero, $k_5 \gg k_4[11\text{-}cis]$ and assuming k_{-4} is not significant compared to k_5, $k_{obsd} = k_4[11\text{-}cis]$. Hence, the limiting slope of a plot of k_{obsd} vs. [11-*cis*], as shown in Figure 3A, yields k_4. The kinetic data obtained is consistent with the scheme given in Figure 6 with $k_5 = 0.055$ s^{-1} and $k_4 = 690$ M^{-1} s^{-1}.

The change in the rate-limiting step reported here could result from a limiting rate of transport of the 11-*cis*-retinal in the membrane. This point is difficult to address experimentally. We have looked for spectral changes resulting from aggregation of 11-*cis*-retinal not soluble in the membrane (Figure 3B) and observed no deviation from Beers law for the concentration range used in the kinetic experiments. Moreover, support for a rate-limiting dehydration step comes from studies on the reaction between carbonyl compounds and simple amines (Jencks, 1959). For example, in the reaction of hydroxylamine and acetone, the dehydration step was the rate-limiting step of the reaction at neutral pH with a rate constant of approximately 0.13 s^{-1} (Jencks, 1959), a value very similar to what we find. Finally, the mobility of rhodopsin in the ROS membrane suggests that transport should not be a limitation (Poo and Cone, 1974).

For the effect of pH on the observed rate of recombination by ROS, the basic observations that must be explained are: (1) the reaction at any ligand concentration is described by a single exponential in the pH range 7.5–6.5 but by two exponentials above and below this pH region, and (2) the slow kinetic species is pH dependent and the fast kinetic species is pH independent. In addition, the kinetic scheme must be consistent with the observed extent of regeneration (Figure 1A).

In terms of the chemistry of the ligand binding reaction, the formation of a fully protonated species (I) at low pH and a deprotonated species (II) at high pH should result in nonreactive forms of opsin. However, as protonic equilibria (K_1, K_2 in Figure 6) are generally rapid, these molecular species (I, II) would not be expected to limit the availability of IV. Hence, we propose species III, which is envisioned to be chemically identical to IV but not in a conformation capable of binding 11-*cis*-retinal. Thus, the rate of formation of IV can be written as given in eq 5. Species IV can be expected to be in equilibrium with protonated and deprotonated forms; however, these equilibria would not limit the availability of IV and hence, do not affect the analysis given below.

$$\frac{d[IV]}{dt} = k_3[III] - k_{-3}[IV] \quad (5)$$

The total opsin present can be represented by the equation $(O_T) = [I] + [II] + [III] + [IV]$ and K_1 and K_2 are rapid equilibria. Hence, eq 6 and 7 can be written.

$$\frac{d[IV]}{dt} = \frac{k_3 O_T}{\left(1 + \frac{[H^+]}{K_1} + \frac{K_2}{[H^+]}\right)} - [IV]\left(k_{-3} + \frac{k_3}{1 + \frac{[H^+]}{K_1} + \frac{K_2}{[H^+]}}\right) \quad (6)$$

$$k_{obsd} = k_{-3} + \frac{k_3}{1 + \frac{[H^+]}{K_1} + \frac{K_2}{[H^+]}} \quad (7)$$

At the pH extremes ($[H^+] > K_1$ or $K_2 > [H^+]$) $k_{obsd} \Rightarrow k_{-3}$, which must be the limiting rate (Figure 3) and can be estimated from the data (Figure 1A) to be 0.001–0.002 s^{-1}. Using eq 7, k_{-3} derived above, and the observed rate constants

for the slow kinetic species, estimates can be obtained for k_3, K_1, and K_2. These estimates do not represent unique solutions, as we do not have sufficient information; however, they do serve to demonstrate the feasibility of application of eq 7. Using the values of $k_3 = 0.075\ s^{-1}$, $k_{-3} = 0.002\ s^{-1}$ ($K_3 = 37.5$), $K_1 = K_2 = 10^{-7}$ M and $K_4K_5 = 1.4 \times 10^5\ M^{-1}$, the solid lines shown in Figure 1A and 1B were calculated with the extent of regeneration determined from eq 8.

$$\text{extent} = \frac{\text{rhodopsin}}{\text{opsin} + \text{rhodopsin}} = \frac{K_3K_4K_5[11\text{-}cis]}{K_3K_4K_5[11\text{-}cis] + K_3 + 1 + \frac{K_2}{[H^+]} + \frac{[H^+]}{K_1}} \quad (8)$$

The observed rate constant vs. pH curve (Figure 1B) is described by the scheme given in Figure 6 quite well. However, the extent of regeneration (Figure 1A) is not described quantitatively at alkaline pH. Nevertheless, considering the inaccuracies in the measurements and the possibility that some irreversible denaturation takes place at high pH, the mechanism proposed adequately describes available data. To summarize, we propose that at neutral pH two processes are taking place ((dIV/dt), (dV/dt)); however, these have similar rates over the concentration range of ligand used and, hence, are not resolvable in the kinetic experiments. However, as the pH is raised or lowered relative to pH 7, the rate of conversion of III to IV becomes limiting and a slow kinetic phase is observed. Nevertheless, that fraction of opsin which exists as species IV at a particular pH reacts via k_{obsd}, as defined by eq 4, and a fast kinetic phase is also observed.

The kinetics of the reaction of cholate-solubilized opsin with 11-*cis*-retinal at pH 7 also suggest a change in the rate-limiting step at high ligand concentrations. Moreover, plots of $1/k_{obsd}$ vs. $1/[11\text{-}cis]$ are linear, supporting the application of eq 2 and 4. The rate constants derived by this analysis are $k_5 = 0.18\ s^{-1}$, $k_4 = 660\ M^{-1}\ s^{-1}$ in good agreement with those for bleached ROS, strongly suggesting that the cholate complex provides a chemical and physical environment similar to that provided by the rod disk membrane. The effect of pH on the reaction of cholate-solubilized opsin and ligand does not show the biphasic kinetics observed with ROS. However, only a restricted pH range has been investigated, due to the insolubility of cholic acid at pH values less than 6.5.

Hong and Hubbell (1973) have proposed that the lack of regeneration by opsin solubilized by most detergents results from a highly fluid and disordered micelle interior which does not stabilize a regenerable conformation. Thus, in terms of the suggestion of Hong and Hubbell (1973), the cholate complex should be ordered and provide the appropriate stabilization for the regenerable conformations of opsin. Combining the kinetic properties reported here with the physical and chemical characteristics of the cholate–opsin complex (Henselman and Cusanovich, 1974), cholate-solubilized opsin provides a most acceptable model system for studies on physiological protein–membrane interactions.

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