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Chemistry

Primary photochemical event in vision: Proton translocation

(rhodopsin/prelumirhodopsin/picosecond kinetics/proton tunneling)

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ABSTRACT Picosecond studies of rhodopsin in low-temperature glasses have been carried out in order to observe directly the risetime of prelumirhodopsin, the first intermediate in the visual pathway. Only at 20 K or below can the risetime of this intermediate be resolved and even at 4 K it is astoundingly rapid, about 36 psec. An examination of the Arrhenius dependence on temperature of the rate of formation of prelumirhodopsin shows a strong deviation from linearity at low temperatures, i.e., non-Arrhenius behavior. This marked nonlinear behavior is characteristic of a quantum mechanical tunneling event such as the translocation of hydrogen. An excellent candidate for the tunnelling process is the hydrogen of the protonated Schiff base formed between opsin and its retinal chromophore. Deuterium-exchanged rhodopsin, in which the Schiff base hydrogen is replaced by a deuterium, also shows a marked non-Arrhenius temperature dependence at low temperatures, consistent with tunneling. The rate of formation of prelumirhodopsin in deuterium-exchanged samples is much slower and a deuterium isotope effect $k_H/k_D \simeq 7$ is observed. The data support a model in which the formation of prelumirhodopsin involves translocation of a proton toward the Schiff base nitrogen of the retinal chromophore.

The primary process in visual excitation is initiated by a photochemical event, the absorption of a photon by the photoreceptor, rhodopsin, resulting in the formation of a new species, prelumirhodopsin. The characterization of this new species has been carried out by photostationary studies in low-temperature glasses (1, 2) and by picosecond kinetic studies near room temperatures (3, 4). Prelumirhodopsin is formed within 6×10^{-12} sec (6 psec) following excitation of rhodopsin and has an absorption maximum at 543 nm which is bathochromically shifted compared to that of rhodopsin. This event has been classically described as the isomerization of the 11-*cis*-retinal chromophore of rhodopsin to the *all-trans*-retinal form (5, 6). That full isomerization of a bulky chromophore could occur within this time scale has been questioned (3, 7), and speculation still exists as to the nature of this photochemical event.

The time resolution for most ultrafast kinetic studies is about 6 psec, which is slower than the actual risetime of prelumirhodopsin at room temperature. To overcome this restriction, we have excited rhodopsin in low-temperature glasses and monitored the formation rate of prelumirhodopsin. The kinetics are sufficiently slow at 20 K or below to allow us to measure directly the formation of prelumirhodopsin. Our picosecond data suggest that the initial photochemical step in the visual process is not *cis–trans* isomerization, but rather proton translocation. Plausible models are presented and discussed.

METHODOLOGY

Rod outer segments were isolated from frozen bovine retinas (G. Hormel Co.) and were solubilized in 0.3 M Ammonyx LO (Onyx Chem. Co.), 0.01 M *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes) at pH 7.0 by the method of Applebury *et al.* (8). The final solutions had absorbances at 500 nm of 8.0–11.0. The deuterated rhodopsin (D-rhodopsin) was prepared by methods similar to those of Oseroff and Callender (9). Isolated rod outer segments were pelleted and washed twice with D_2O, buffered with 0.01 M Hepes at pH 7.0. Stock Ammonyx (30%) was freeze dried and resuspended in D_2O. Washed rod outer segments were solubilized in 0.1 M Ammonyx/D_2O/0.01 M Hepes at pH 7.0. The final A_{500} of the deuterated rhodopsin was 7.0.

All kinetic measurements were made with samples in 2-mm optical pathlength cells. Low-temperature studies between 50 K and 4 K were carried out in a variable temperature cryo-tip Dewar flask (Air Products). The temperature, which was measured by a gold/chromel thermocouple, was kept constant within ±0.5 K. The optically clear low-temperature glasses of rhodopsin consisted of one part rhodopsin and two parts distilled ethylene glycol. In the case of D-rhodopsin, deuterium-exchanged ethylene glycol was used.

The spectral changes were observed by a double beam picosecond spectroscopy apparatus similar to that described previously (10). All samples were excited at 530 nm with a single 6-psec pulse with about 5 mJ of total energy. Particular care was taken to eliminate photon saturation of the rhodopsin samples and to avoid multiphoton events. We estimate that less than 25% of the sample in the optical path was bleached per excitation pulse.

RESULTS

Prelumirhodopsin (also called bathorhodopsin) is generated as the first intermediate following the absorption of a photon by rhodopsin. A characteristic difference spectrum at room temperature and the difference spectrum generated by photostationary studies at 77 K (8) and 7 K (2, 11) for prelumirhodopsin-rhodopsin are given in Fig. 1.

At room temperature the kinetic risetime of prelumirhodopsin and the depletion of rhodopsin are faster than can be observed with our picosecond instrumentation (<6 psec). Thus, in order to characterize the origins of prelumirhodopsin and its nature, picosecond studies at low temperatures were undertaken with optically clear glasses of rhodopsin/ethylene glycol. The risetime of prelumirhodopsin as monitored at 570 nm is still within 6 psec at 77 K, and, moreover, no risetime for product appearance can be detected until the glass temperatures are below 30 K (Fig. 2). At 20 K or below the formation of a relatively long-lived species can be observed. Even at 4 K, the long-lived species, prelumirhodopsin, is formed within 36 psec. It arises, however, not directly from ground-state rhodopsin but from an initial transient species (presumably the excited state of rhodopsin) which is formed too fast for us to

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Abbreviations: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; D-rhodopsin, deuterated rhodopsin.

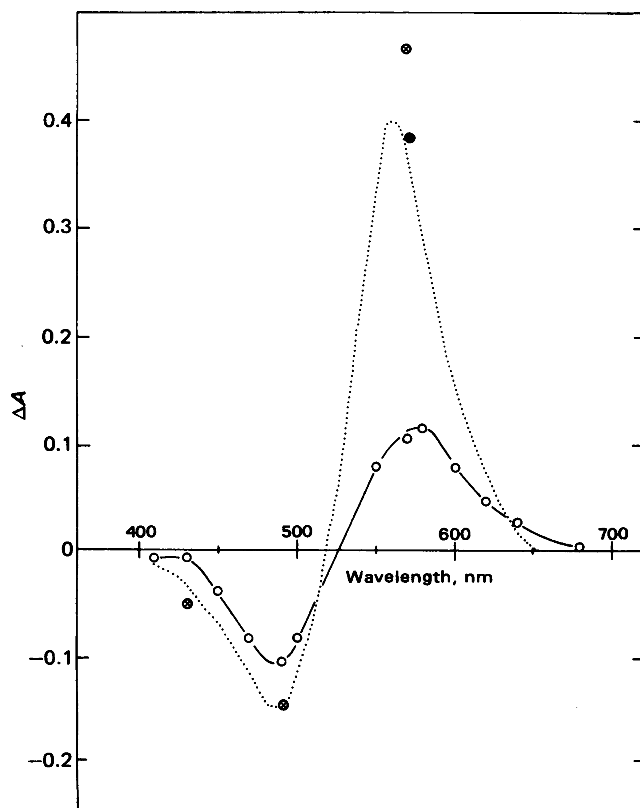

FIG. 1. Prelumirhodopsin difference spectrum. O, Recorded 298 K, 60 psec after excitation with a 5 mJ pulse at 530 nm (4); ●, 77 K and ⊗, 4 K (this work); ·····, difference spectrum generated by photostationary studies of low-temperature glasses for 77 K (8) and 7 K (2, 11). Photostationary studies at 77 K and 7 K give identical difference spectra. The data is normalized to concentrations used for this kinetic study. Rhodopsin was solubilized in 0.3 M Ammonyx/0.01 M Hepes at pH 7.0; A_{500} = 0.73 in 2 mm.

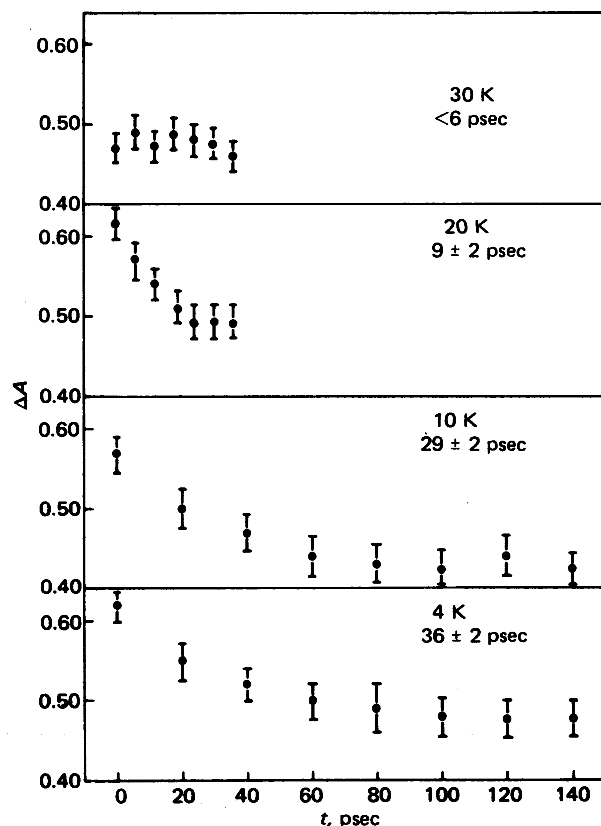

FIG. 2. Kinetics of formation of prelumirhodopsin at various temperatures monitored at 570 nm. Excitation of rhodopsin was with a 5-mJ, 530-nm, 6-psec pulse. The glass for low-temperature study was formed by mixing 1 part rhodopsin, solubilized in 0.3 M Ammonyx/0.01 M Hepes at pH 7.0, A_{500} = 10.0, with 2 parts distilled ethylene glycol. The lifetime for formation, given in the upper right of each panel, is the reciprocal of the rate constant obtained by a least square fit of ln ($A_t - A_\infty$) versus time, t, in psec, in which A is absorbance at 570 nm.

monitor. This transient species decays quite rapidly to prelumirhodopsin. Although there are other possible interpretations for the nature of this transient, we have used this one as a working hypothesis; see *Discussion*.

A notable difference in the amplitude of the absorbance change at 570 nm is observed as the samples are cooled. At room temperature the ratio of prelumirhodopsin produced to rhodopsin depleted, i.e., A_{570}/A_{490}, is 1:1. At 77 K this ratio becomes about 2.5:1, and at 4 K it increases to 3.5:1 (Figs. 1, 2, and 5*B*). The change in this ratio as a function of temperature can be the result of at least two effects: (*i*) alteration in shape and/or maximum of the prelumirhodopsin spectrum, and (*ii*) an alteration in the quantum yield for prelumirhodopsin as discussed below. At 4 K photostationary studies show that the absorption spectrum of rhodopsin is red shifted by 8 nm and the maximum absorption coefficient is enhanced by 15% compared to the room-temperature spectrum (11). A comparable change in the spectrum of prelumirhodopsin as a function of temperature would not be sufficient to account for the changes in ratios noted above. A possible explanation would be that there is a dramatic effect of temperature upon the band shape of prelumirhodopsin.

The remarkably fast formation of prelumirhodopsin at 4 K and the temperature dependence of formation (see below) has led us to consider mechanisms other than *cis–trans* isomerization as the photochemical event in the first step of visual transduction. A plausible alternative for the event would be proton translocation and, if so, replacement of the proton involved with a deuterium should lead to a pronounced deuterium isotope effect. To test this possibility, deuterium-exchanged rhodopsin was prepared by the method of Oseroff and Callender (9). In such samples the proton of the protonated Schiff base has been shown to be exchanged for deuterium, while other protons in the rhodopsin retinal chromophore would not be expected, and are not observed, to be exchanged (9). With these deuterated samples, a strong deuterium isotope effect on the rate of formation of prelumirhodopsin is observed, as shown in Fig. 3. The formation of the long-lived species absorbing at 570 nm is slower in D-rhodopsin samples than in rhodopsin and can be observed at higher temperatures. For example, at 40 K prelumirhodopsin is formed in less than 6 psec, while prelumirhodopsin from D-rhodopsin is formed within 17 psec. This fast formation rate of the long-lived species at 570 nm is observed even at 4 K, where it is found to be 257 psec for deuterated samples and 36 psec for the protonated rhodopsin. Over the temperature range in which the production of metastable prelumirhodopsin was measured, the isotope effect on the rate k_H/k_D was found to be approximately 7. The observed absorbance changes shown in Fig. 3 are smaller than those displayed for rhodopsin in Fig. 2 due to sample dilution.

The dependence of the rate of formation of prelumirhodopsin upon temperature is plotted in Fig. 4. The data for both rhodopsin and D-rhodopsin show non-Arrhenius behavior. At very low temperatures the rate of formation of prelumirhodopsin is nearly independent of temperature and has a finite

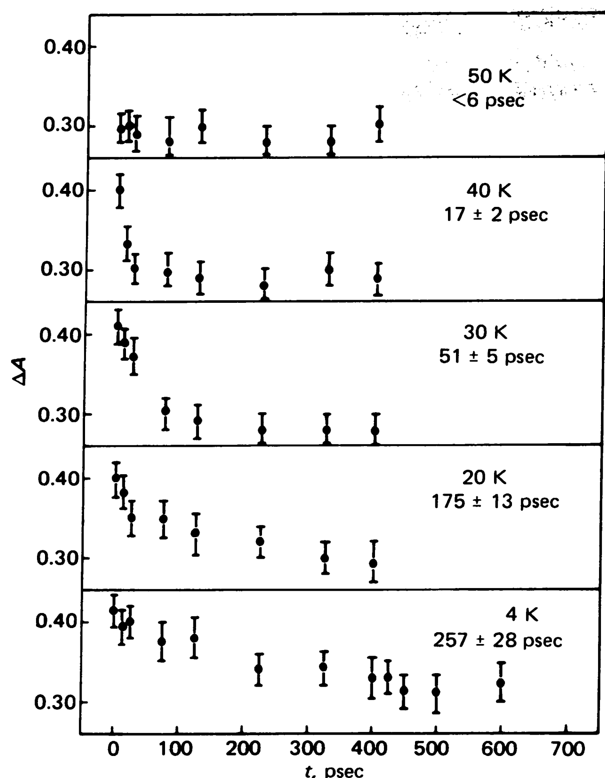

FIG. 3. The kinetics of formation of deuterium-exchanged prelumirhodopsin at various wavelengths. The excitation of deuterium-exchanged rhodopsin was with a 5-mJ, 530-nm, 6-psec pulse. The glass for low-temperature study was formed by mixing 1 part D-rhodopsin in deuterium-exchanged 0.1 M Ammonyx/0.01 M Hepes at pH 7.0, A_{500} = 7.0, with 2 parts deuterium-exchanged ethylene glycol. The lifetime of formation was calculated as for Fig. 2.

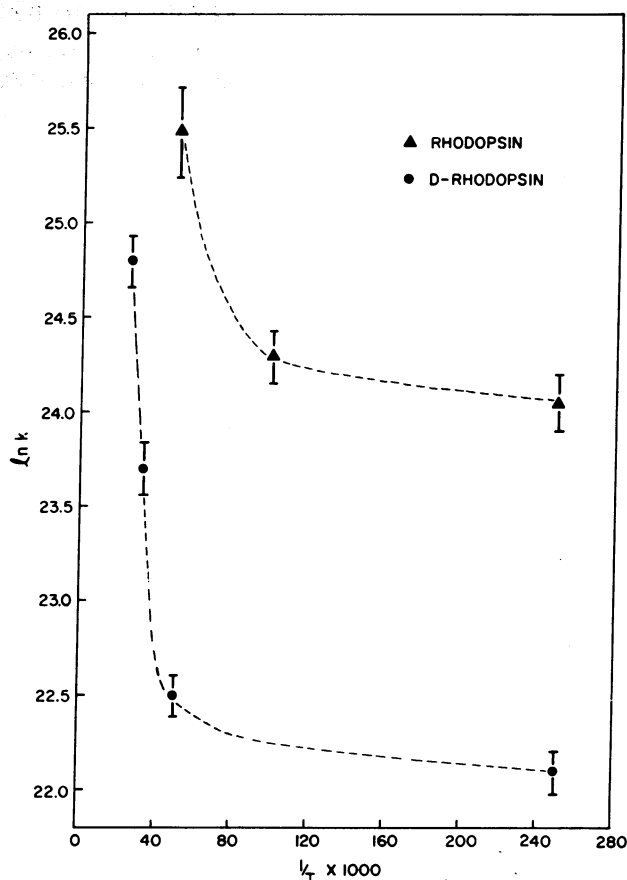

FIG. 4. An Arrhenius plot, ln k for formation of prelumirhodopsin versus $1/T$ (K) × 10^3, of the kinetic data in Figs. 2 and 3. The value ln k = 25.84 corresponds to a lifetime of 6 psec, our minimal time resolution

value as the temperature approaches 0 K rather than vanishing as predicted for a normal Arrhenius temperature dependence. A rough extrapolation of the temperature-dependent rate to 298 K would predict that the risetime of prelumirhodopsin at room temperature is about 0.3 psec or less.

To investigate the possible presence of other early intermediates at 4 K, we monitored kinetic changes at 440 nm and 490 nm as shown in Fig. 5. The absorbances observed at 440 nm (Fig. 5) reflect a minimal change and are consistent with the small decrease in absorbance of the tail of the rhodopsin band. We observe no new intermediates in this spectral region at 4 K. The decrease in absorbance at 490 nm upon excitation (Fig. 5) reflects an immediate decrease in ground-state population of the rhodopsin. The observation that at 4 K there is no apparent increase in the rhodopsin ground-state population during the first 100 psec after excitation, indicates that all excitation energy is channelled to production of other species. As indicated by the error bars in Fig. 5, it must be noted that we could not reliably detect a repopulation of ground state of less than 20%.

DISCUSSION

Formation of Prelumirhodopsin at Low Temperatures. Only at 20 K or below are we able to observe directly an apparent risetime of prelumirhodopsin—the first metastable intermediate produced upon excitation of rhodopsin. We assume that prelumirhodopsin arises from a transient precursor on the following basis: At 30 K, as at room temperature, a species absorbing at 570 nm is formed within 6 psec and is stable during the remaining time of observation (Fig. 2). At 20 K, however, we observe a new transient species also absorbing at 570 nm which decays relatively rapidly, revealing the long-lived species, prelumirhodopsin, absorbing at 570 nm. From studies at 570 nm we cannot determine whether the decay of this transient gives rise to prelumirhodopsin or if prelumirhodopsin is formed within 6 psec and the transient decays into another state not observed at 570 nm. A third possibility is that the transient decays into both prelumirhodopsin and some other unknown intermediates. If the transient does decay to some other species,

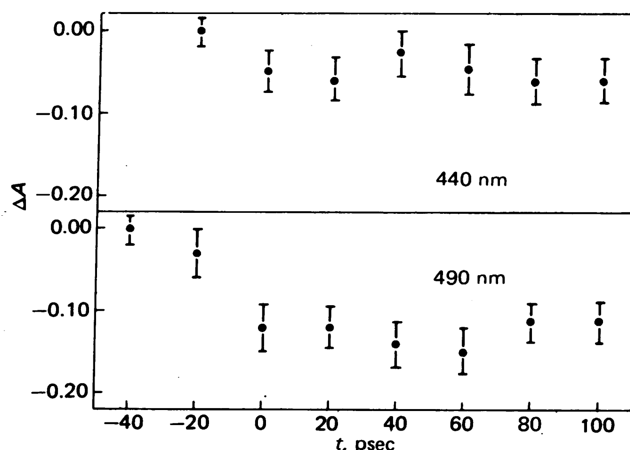

FIG. 5. The bleaching kinetics of rhodopsin monitored at 540 nm and 490 nm at 4 K. The excitation pulse and low-temperature glass were as described in Fig. 2.

it does not decay into one absorbing at 440 nm or 490 nm because there is no evidence for either formation or decay of states at these wavelengths (Fig. 5). This excludes the possibility that the transient is hypsorhodopsin or decays to hypsorhodopsin, an intermediate that is reported to have an absorption maximum at 440 nm and has been suggested as a precursor of prelumirhodopsin on the basis of photostationary studies of low-temperature glasses at 4 K (2, 11). A reasonable alternative is to assign the initial transient that decays to form at least prelumirhodopsin to the first excited singlet state of rhodopsin. As a working hypothesis we have thus assumed the transient to be the precursor of prelumirhodopsin.

Temperature-Dependent Formation of Prelumirhodopsin (Proton Tunneling). In a classical Arrhenius form, the temperature dependence of the formation of prelumirhodopsin (PL) may be plotted as ln k_{PL} versus $1/T$. When the data is plotted in this fashion (Fig. 4), two outstanding features become apparent: (*i*) this plot is nonlinear, and (*ii*) as the temperature approaches 0 K, k_{PL} becomes temperature independent and asymptotically approaches a finite constant value. In contrast, if the reaction followed a classical Arrhenius behavior, the rate k_{PL} would have a linear temperature dependence and vanish as $T \rightarrow 0$ K.

This nonclassical temperature dependence of the formation of prelumirhodopsin is characteristic of a tunneling phenomenon. The temperature dependence of tunneling itself is complicated and depends upon the nature of the barrier through which the particle must pass (12–14). As shown in Fig. 4, at higher temperatures, the temperature dependence obeys an approximate power law and would suggest that the predominant process throughout the temperature region of our measurements is tunneling (13).

Several lines of evidence have suggested that proton translocation is an attractive model to account for the appearance of prelumirhodopsin (15–17). The red-shifted absorption spectrum of prelumirhodopsin, compared to that of rhodopsin, suggests that prelumirhodopsin might be an even more highly protonated Schiff base form. Model studies of Waddell and Becker indicate that translocating the H^+ towards the Schiff base nitrogen could account for such a red shift (18). Resonance Raman data suggest that the prelumirhodopsin species is a protonated species like rhodopsin (9). The phenomenon of proton transfer in excited states has been well established in azole compounds (19) and azaindole dimers (20). Considering this body of evidence, an excellent candidate for proton translocation in rhodopsin would be the hydrogen of the protonated Schiff base that is formed between the lysine residue of opsin and the retinal chromophore.

Deuterium-Isotope Effect. If the proton translocated is the Schiff base hydrogen, our model would predict that deuteration of the Schiff base should result in a marked isotope effect. Our kinetic observations demonstrate that the rate of formation of deuterated prelumirhodopsin is markedly slower than protonated prelumirhodopsm which is reflected in the ratio of k_H/k_D being approximately 7, as seen in Figs. 2 and 3. Any secondary effects of the deuterium solvent on the protein structure seem unlikely because the rate of metarhodopsin I → metahodopsin II transition is totally unaffected by deuterium exchange (M. Resh, A. Eisenberg, and M. L. Applebury, unpublished results). Oseroff and Callender have shown that in deuterium-exchanged rhodopsin, the Schiff base proton is replaced by a deuterium (9). A deuterated Schiff base would be expected to have no effect on a *cis–trans* isomerization process and thus the isotope effect is additional strong evidence for proton translocation.

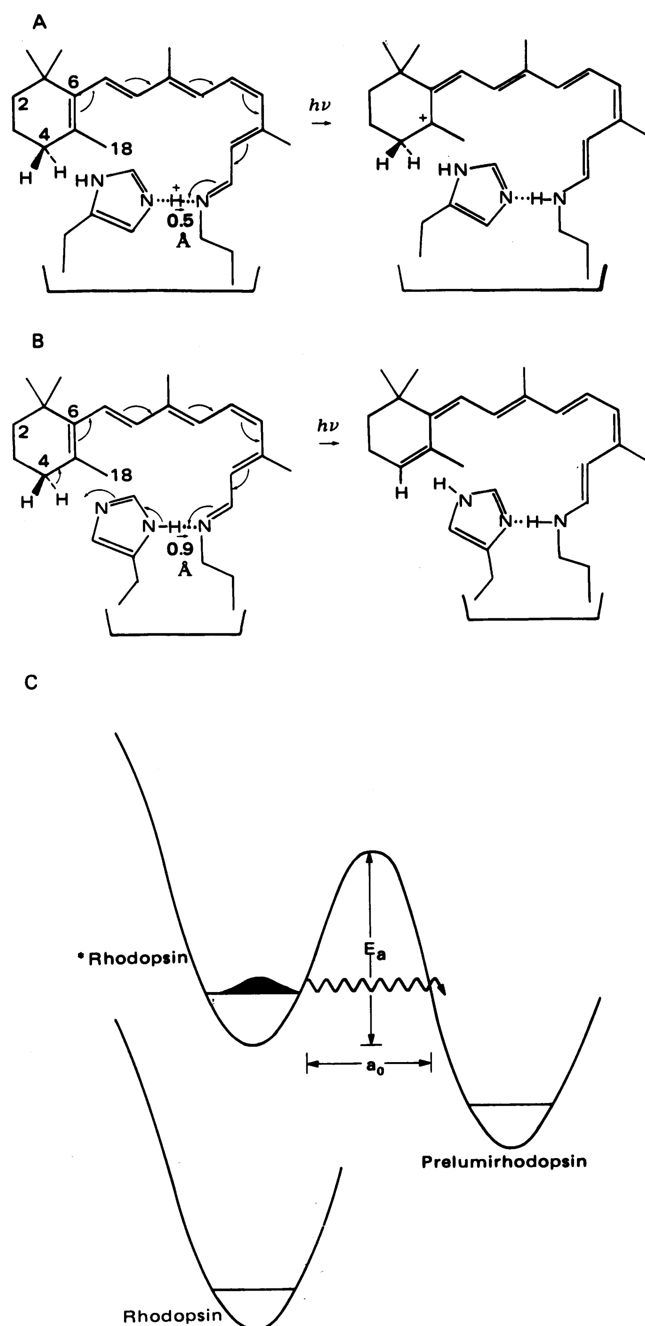

FIG. 6. Models for proton translocation to form prelumirhodopsin. (Model *A*) Single proton translocation with carbonium ion formation. (Model *B*) Concerted double proton translocation with retro-retinal formation. (*C*) Tunneling potential energy barriers for formation of prelumirhodopsin. The energy barrier E_a for proton tunneling calculated for model *A* with a_0 = 0.5 Å is 4.5 kcal/mol, while for model *B* with a_0 = 0.9 Å is 1.4 kcal/mol.

Models for Proton Translocation. Our kinetic data is consistent with at least two models for proton translocation leading to the formation of prelumirhodopsin. These models, as depicted in Fig. 6, show that such translocation may proceed by either (*A*) a single proton translocation to the Schiff base nitrogen producing a carbonium ion as proposed by Salem (21, 22) or Mathies and Stryer (23), or by (*B*) a concerted double hydrogen transfer leading to a retro-retinal structure as proposed by van der Meer *et al.* (16) although here modified. In both cases some amino acid side chain is postulated to mediate

the proton donation and an excellent amino acid candidate, although by no means exclusive, would be histidine. Translocation should occur along the hydrogen bond stretch coordinate. For a normal N-H···N bond distance of 3.0 Å, the proton would be predicted to move a maximum of 0.9 Å toward the Schiff base nitrogen. If, however, the hydrogen is equally shared by the two nitrogens, the translocation distance would be less.

In the case of single proton translocation the carbonium ion produced would be relatively long lived; in the case of double translocation the carbonium ion would be stabilized by the removal of a hydrogen from an adjacent carbon. As originally proposed by van der Meer *et al.* (16), a C-18 hydrogen would be removed, but studies of Kropf (24) with the 5-desmethyl retinal as a rhodopsin chromophore indicate that prelumirhodopsin is still observed and hence must not involve the removal of this moiety. (There is no C-18 in this chromophore.) A proton could, however, be removed from the C-4 position. Model building with space filling structures suggests that histidine would fit very nicely between the nitrogen of the Schiff base and the C-4 of the ionone ring; it does not fit well, however, between the nitrogen and C-18. For opsins containing the 3,4-dehydroretinal as a chromophore (25) the second translocation would be predicted from a hydrogen of the C-2 carbon. In the dehydroretinal chromophore the ionone ring is flattened and, with a torsion about the 6–7 bond, histidine spans the space between the Schiff base nitrogen and the C-2 carbon.

Using these models as a guide for proton tunneling, we can calculate the energy barrier for the formation of prelumirhodopsin. An expression for the rate of tunneling (k_t) (12) is:

$$k_t = \nu_0 \exp \frac{-\pi^2 a_0 \kappa}{h} (2mE)^{1/2}.$$

Using the observed rate constant (2.8×10^{10} sec^{-1}) at 4 K, an N—H bond stretching frequency ν_0 of 1500 cm^{-1}, a maximum translocation distance a_0 of 0.9 Å predicted by the models, κ, the fraction of energy measured from the top of the barrier equal to 1 at 4 K (12), h, Planck's constant, and m, the mass of a proton, we calculate the barrier E_a to be 1.4 kcal/mol (1 cal = 4.184 J). E_a is not strongly dependent on the frequency ν_0 used, i.e., with ν_0 of 3000 cm^{-1} E_a becomes 1.6 kcal/mol. It is interesting to note that this calculated value of 1.4 kcal/mol for E_a is the same as the barrier calculated for photoinduced proton transfer in azaindole dimers (20).

For a translocation distance as short as 0.5 Å and with the rate constant used above, the barrier would be 4.5 kcal/mol. Therefore, a shorter translocation distance a_0, which might be suggested for a shared proton (model A, Fig. 6), would enhance the barrier.

Our proton translocation model (tunneling) is supported further by the rapid kinetic appearance of prelumirhodopsin at 4 K, which suggests that it is extremely unlikely that the formation of prelumirhodopsin results from *cis-trans* isomerization of the retinal chromophore. No experimental evidence exists, to our knowledge, that supports the isomerization of an olefin at 4 K. Experimental studies with stilbene (26, 27) show that isomerization ceases to take place at 77 K. A more relevant study of the isomerization of retinal analogs and retinal Schiff bases also indicates that as temperature is decreased the quantum yield for isomerization diminishes until at 77 K this process no longer occurs (28). The formation of prelumirhodopsin above 50 K cannot be unambiguously ascertained from our present data and it is possible that a change in the mechanism could take place at higher temperatures.

We can, however, describe the kinetic nature of prelumirhodopsin formation in low-temperature glasses with certainty. Indeed, this first intermediate was classically identified by photostationary studies at these low temperatures (2, 11). The non-Arrhenius dependence of the rate of formation on temperature and the strong deuterium isotope effect observed demonstrate that the mechanism of the initial photochemical event at low temperatures leading to prelumirhodopsin involves proton translocation via tunneling.

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