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Formation and Decay of Prelumirhodopsin at Room Temperatures

(bovine rhodopsin/laser/picosecond spectroscopy)

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ABSTRACT **We have excited detergent-solubilized bovine rhodopsin at room temperature with 530-nm light pulses from a mode locked laser, and have observed the appearance and decay of a transient species that absorbs more strongly at 560 nm than does ground-state rhodopsin. Our data show that the absorbing intermediate appears in a time that is at least as short as the experimental resolution (about 6 psec) and decays with a life time of about 30 nsec. The extremely fast risetime supports the hypothesis that prelumirhodopsin is the product of the primary photoprocess.**

Several intermediates after excitation of bovine rhodopsin have been detected by following changes in the visible and near ultraviolet absorption spectrum, which is due to the retinyl prosthetic group (1, 2). Spectral changes that occur very rapidly near physiological temperatures have been observed by cooling the preparations to low temperatures where the rates are compatible with conventional flash photolysis techniques, or by freezing preparations in glass-forming solvents at temperatures where interconversion rates are so slow that ordinary absorption spectroscopy suffices.

The first spectral change during photobleaching of rhodopsin (3) has been interpreted in terms of the formation of an intermediate, prelumirhodopsin (4), having a maximum absorption at 543 nm. This process is considered to be the primary photochemical step for the geometrical isomerization of the polyene chromophore (1, 2, 5).

Experimental data concerning the rate of formation of prelumirhodopsin have not been reported. The decay of prelumirhodopsin in preparations of bovine rhodopsin has been measured at temperatures between −50 and −67° (6, 7). Extrapolation of these values gives an expected lifetime at 37° of <1 μsec (1). Only recently has there been a report of a measurement of the decay near room temperatures (8). In fact, even the existence of prelumirhodopsin as a discrete intermediate at physiological temperatures has been questioned (1, 9).

We have used psec spectroscopy (10, 11) at physiological temperatures to detect transients absorbing at longer wavelengths than rhodopsin on time scales from a few psec to 0.1 μsec. The results indicate that prelumirhodopsin is an intermediate in the bleaching of bovine rhodopsin excited with 530-nm light at physiological temperatures and are consistent with the notion that prelumirhodopsin is the primary photochemical product. In this paper, we describe the methods and results obtained concerning the kinetics of prelumirhodopsin at physiological temperatures.

Abbreviations: ROS, rod outer segments; LDAO, lauryldimethylamine oxide; SHG, second harmonic.

PREPARATION OF BOVINE RHODOPSIN

Frozen bovine retinas were obtained from G. Hormel Co., Austin, Minn. and stored at −60°. Samples were prepared in total darkness or in dim red light near 4°. Bovine retinas were ground to a smooth paste, diluted with cold buffer [0.1 M NaCl–10 mM imidazole-Cl (pH 7)], and homogenized with a Potter–Elvehjem homogenizer. The homogenized retinas were centrifuged for 15 min at 1000 rpm (100 × *g*), and the supernate was decanted very carefully. Crude rod outer segments (ROS) were collected as a pellet by centrifugation for 20 min at 15,000 rpm (27,000 × *g*). An initial sucrose flotation was done by homogenizing the ROS pellet in 40% sucrose (w/w), 0.1 M imidazole-Cl (pH 7). After centrifugation at 15,000 rpm for 20 min the floating material was loosened from the tube walls, diluted with buffer, and collected. Crude ROS were purified by density gradient centrifugation (12). ROS were layered on a continuous sucrose density gradient [20–40% sucrose, w/w in 10 mM imidazole-Cl (pH 7)] and centrifuged in a Spinco model L (SW25.1 rotor) at 22,500 rpm (51,505 × *g*) for 2 hr at 5°C. ROS, the upper band, were collected and used in the subsequent extractions. ROS were washed once with buffer and twice with distilled water. The washed pellets were solubilized with lauryldimethylamine oxide (LDAO)*. The pellet was extracted in 2% LDAO, 0.1 M imidazole-Cl (pH 7), and 1 mM dithiothreitol (Cleland's Reagent, CalBiochem.) (7 ml of solution per 100 retinas) on a slowly rotating mixer for about 12 hr. The extract was centrifuged at 45,000 rpm in Spinco model L-50. The clear supernate had the absorbance ratios:

$A_{400\,nm}/A_{500\,nm} = 0.20 \pm 0.02$; $A_{280\,nm}/A_{500\,nm} = 1.9 \pm 0.2$.

ROS samples solubilized by LDAO with $A_{500\,nm}$ of 3.0–5.0 per cm were used. When necessary, the material was concentrated to an absorbance of 5.0 by vacuum dialysis with Schlichter and Schull collodion bags. The rhodopsin may be further purified by column chromatography. These methods will be reported elsewhere (M. L. Applebury, in preparation). Studies with both purified LDAO and solubilized ROS material showed the same results; therefore, the LDAO-solubilized ROS were used in subsequent experiments.

RESULTS

Measurements in psec range

The expected ultrashort, and until now, unknown rate and mechanism of energy dissipation in the primary step of rhodopsin photobleaching was measured by psec spectros-

* We thank the Onyx Chemical Co. for providing us with LDAO (tradename Ammonyx LO).

copy (13–15). A mode locked Nd^{+3} glass laser (7 × 9/16 inch rod) generated the psec pulses, which were measured by the two (16, 17) and three-photon methods (18), and had an average width (FWHM) of 6 psec. Preliminary experiments showed that the risetime of an intermediate of rhodopsin absorbing at 561 nm as a consequence of excitation with a 530-nm laser pulse is faster than 20 psec, while the decay lifetime of this intermediate is three orders of magnitude longer. This wide range of rates necessitated the use of two different experimental arrangements, one that measures the rate of formation of the primary species and a second that measures its comparatively long decay.

The complete train of pulses from the mode locked laser was not appropriate for excitation in these experiments. First, conversion of rhodopsin to prelumirhodopsin and subsequent intermediates is irreversible, so that pulses late in the train will not create as large change in absorbance as the early ones. In other experiments where we used the entire train (19), the film records the average absorbance change over all the pulses. This averaging would result in weaker signals in the present experiments. Second, the interpulse separation within the train should be large compared to the relaxation time of the process being studied. Then each set of interrogating pulses, which follows each of the exciting pulses, will find a time-dependent change in absorbance that is due only to that pulse and not to previous ones. The approximate 30-nsec decay time found in these experiments for prelumirhodopsin does not satisfy this requirement. To overcome these obstacles, and also to eliminate the variation in width and intensity within a psec pulse train, a single pulse was extracted from the train (G. E. Busch and P. M. Rentzepis, to be published) by switching a Pockels cell that rotates the polarization of a previously selected single pulse in the train. The single pulse, thus polarized 90° to the train of pulses, passes through a polarizer that rejects the rest of the train. The pulse then passes through

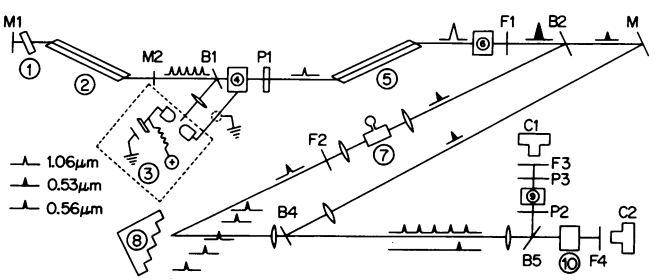

FIG. 1. Diagram of apparatus used to observe the risetime for formation of prelumirhodopsin. A single pulse (about 6 psec) is extracted from the mode locked train of pulses of the Nd^{3+} laser (1.06 μm). The pulse is amplified and used to generate the second harmonic (0.53 μm), which in turn is split into two components by a 50% beamsplitter. Half the pulse is used to excite rhodopsin in the sample cell. The remaining half is Raman shifted to 0.56 μm and split into a set of pulses by reflection at the echelon before entering the sample cell. The path lengths are adjusted so that the set of 0.56-μm pulses interrogates the absorbance in the cell both slightly before and for some time after the passage of the 0.53-μm exciting pulse.

Components: (*1*) Cell containing saturable absorbing dye, (*2*) Nd^{+3} glass-laser rod, (*3*) spark gap, (*4*) Pockels cell, (*5*) Nd^{+3} glass-amplifier rod, (*6*) KDP SHG crystal, (*7*) 15-cm cell containing benzene, (*8*) echelon reflector, (*9*) CS_2 cell, (*10*) sample cell, (*M*) mirror, (*B*) beamsplitter, (*F*) filter, (*C*) camera, (*P*) polarizer.

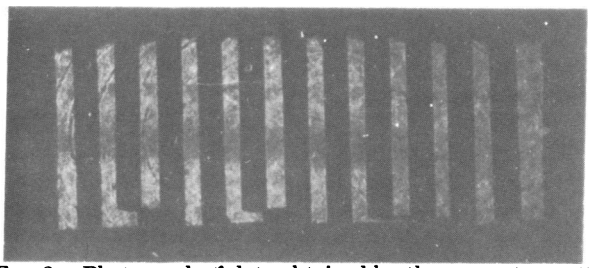

FIG. 2. Photograph of data obtained by the apparatus of Fig. 1. The image is that of the echelon reflector, the 0.56-μm light that has passed through the sample cell and interrogated the absorbance within the cell. Since the light reflected from each segment of the echelon has a unique, incremented optical path length, it samples the absorbance at a particular time with respect to the exciting pulse. The approximate 6-mm intrasegment path difference yields a time separation of about 20 psec. The light from the *fourth segment from the left* enters the cell simultaneously with the exciting pulse, and an absorbing species is immediately generated, as shown by a dark region near the middle of the segment.

a 12-inch amplifier rod, which enhances the intensity about 10-fold. The optical path of the pulse is shown in Fig. 1.

A KDP crystal generates the second harmonic (SHG) 530 nm from the fundamental (1060 nm). A fraction of the 530-nm light then generates the stimulated Stokes Raman wavelength at 561 nm in a 15-cm path of benzene. The stimulated Raman pulse passes through the appropriate filters, where the other Stokes and 530-nm SHG laser light is eliminated. The interrogating 561-nm pulse is subsequently collimated on the echelon (20), then recombined with the intense 530-nm exciting pulse before being focussed into the sample cell (21). Finally, the image of the echelon is displayed by the camera onto photographic film (3000 Polaroid). In Fig. 1, the 530-nm single pulse excites the rhodopsin solution (the temperature was about 25° except when noted) simultaneously with the second echelon segment of the 561-nm interrogating light. In the first set of experiments, the echelon segment time separation was 20 psec; that is, the time corresponding to the difference in length that the light pulse had to travel by reflection from adjacent echelon segments. This is equivalent to a 6-mm increment from one echelon segment to the very next one. The width of the pulse was measured simultaneously by determination of the birefringence time of CS_2 (22).

Typical data of these experiments are reproduced in Fig. 2. The light segments correspond to the reflection of the echelon segments, while the dark ones display the time differential between the successive echelon generated pulses. The dark portion that crosses the light segments is due to the absorption of the interrogating light after excitation by the 530-nm pulse. It is obvious that (Fig. 2) the absorption starts within the first segment after excitation and no decrease in the absorption intensity occurs during the time of the echelon (about 200 psec). By advancing the arrival time of the 530-nm pulse, the variation in intensity of the 561-nm pulse could also be examined in the 200- to 400-psec range. We found no decay during this period. Numerical data extracted from negatives of the photographs by a densitometer are shown in Fig. 3. A strongly absorbing transient is observed that rises in less than 20 psec and that does not decay appreciably over 0.2 nsec.

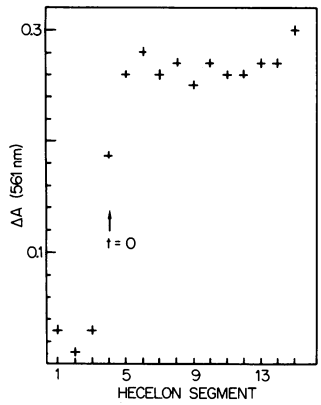

FIG. 3. A plot of absorbance against time (one segment equals 20 psec) obtained from the data of Fig. 2 by a photodensitometer. The results of three laser shots have been averaged to improve the signal-to-noise ratio. Note the sharp rise and then nearly constant absorbance for the remainder of the interrogating pulses (about 200 psec).

We next substituted the 20-psec echelon with one composed of 2-psec segments. The other conditions remained the same. Again the absorption of interrogating light was detected within the first segments after the arrival of the exciting pulse (Fig.4). Thus, the risetime of prelumirhodopsin at room temperature is equal to or shorter than the pulse width, about 6 psec.

Appropriate control experiments were performed in which water, detergent solutions, or bleached rhodopsin samples were present in the sample cell. No photoinduced absorption was ever observed at 561 nm in these samples. Also, we did not observe transient absorption in unbleached rhodopsin samples in the absence of the 530-nm exciting pulse.

Generally the rhodopsin samples (about 0.3 ml, 1-cm path) were bleached significantly only after about 20 laser shots; beam sizes were kept at about 1 mm^2, and each shot could, at most, bleach about 3% of the sample. The sample was stirred between shots. The laser experiments were one in dim red light and with careful shielding of the sample from the pumping flash. The interrogating light was much less intense than the 530-nm exciting pulse and should not have caused complications.

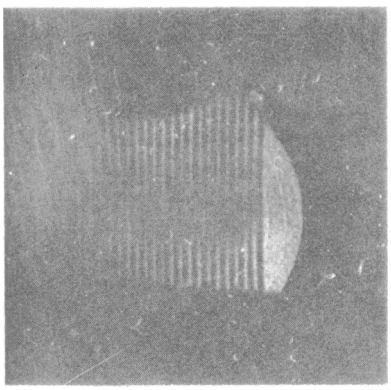

FIG. 4. A photograph similar to that in Fig. 2, but with about 2.5-psec intrasegment time separation. The absorption rises within about 2 segments, which is approximately the risetime of the exciting pulse itself.

The strongest absorption signals were obtained when the more concentrated samples (A about 5/cm at 500 nm) were used and when the 530-nm exciting pulse energy was about 10^{-3} J.

Photoselection

Since the laser light is polarized due to the Brewster optics, photoselection effects may become important in this kind of measurement, and one must consider the relative polarizations of the exciting and interrogating pulses. Indeed we observed that the absorption obtained when the polarization of the 530- and 561-nm pulses were parallel to each other was much stronger than when they were orthogonal. This is expected if both 530-nm absorption by rhodopsin and 561-nm absorption by the transient have nearly the same polarization, and no rotation of the molecules occurs on the time scale of the experiment. Because the 530-nm intensity differs from shot to shot we did not calculate a dichroic ratio at this time. However, it is clear to us that it is large.

nsec decay of the 561-nm transient

Because of the short time span of the echelon this technique was not suitable for measurements of the decay of the transient; therefore we used a method similar to that for the radical in a cage recombination (P. M. Rentzepis, to be published).

The Pockels cell shutter was also used in this experiment. The pulse train exiting from the Pockels cell had all pulses with the same polarization (parallel) except for one near the begin-

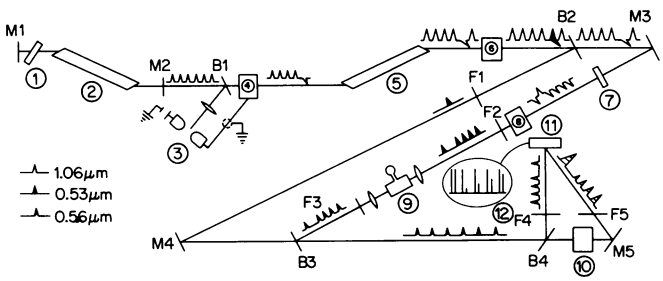

FIG. 5. Diagram of the apparatus used to observe the decay of prelumirhodopsin. A pulse early in the mode locked train of pulses from the Nd^{+3} laser (1.06 μm) has its polarization rotated by the Pockels cell so that it can be doubled (to 0.53 μm) by the KDP crystal. This pulse is then split off, by means of a dielectric reflector, from the remaining pulses in the train and used to excite the rhodopsin in the sample cell. The remaining pulses pass through the 0.53-μm reflector and are used to generate the second harmonic, which is Raman shifted in benzene to 0.56 μm and focussed into the sample cell. Appropriate filters are used so that only 0.53-μm light excites the sample and only 0.56-μm interrogates. Beamsplitters placed before and after the sample cell reflect a fraction of the 0.56-μm light to a fast photodiode, providing a measure of I_0 and I, from which absorbance is computed. The optical path difference for the I_0 and I beams and the finite velocity of light allows the same photodiode to resolve the signal into pulse pairs (see Fig. 6) for each pulse in the modelocked train.

Components: (*1*) Cell containing saturable absorbing dye, (*2*) Nd^{+3} glass-laser rod, (*3*) spark gap, (*4*) Pockel's cell, (*5*) Nd^{+3} glass-amplifier rod, (*6*) KDP SHG crystal, (*7*) 1.06-μm 90° polarization rotator, (*8*) KDP SHG crystal, (*9*) 15-cm cell containing benzene, (*10*) sample cell, (*11*) fast photodiode, (*12*) fast oscilloscope.

ning, which had its polarization rotated by 90° during the shutter operation. The subsequent polarizer, which in the previous experiment rejected all but the single pulse, was removed A KDP crystal was oriented with its axis such that only the rotated pulse generated second harmonic (530 nm). The remaining pulses in the train passed through the crystal without significant SHG. A 530-nm dielectric mirror reflected the exciting 530-nm pulse but transmitted the remaining pulses, which travel along the optical paths shown in Fig. 5. The long train then passed through a 90° polarization rotator and then into a second KDP crystal oriented in a manner that generated the second harmonic. Subsequently, the stimulated Stokes Raman pulse at 561 nm was produced in the benzene cell. Appropriate filters removed all except the 561-nm pulse train. The exciting 530-nm single pulse and the first of the 561-nm pulses of the train were recombined at a beamsplitter and simultaneously entered the rhodopsin cell. Another set of beam-splitters situated before and after the cell reflected a fraction of the interrogating light onto a fast photodiode, the output of which was fed into a Tektronix 519 oscilloscope. Since the optical path from the first beamsplitter to the photodiode is about 30 cm shorter than from the one situated behind the cell, pairs of spikes representing the "I_0" and "I" intensities are displayed on the oscilloscope. The ratio of the intensities per pair gives a direct measure of the absorption, while the time scale of this process may be obtained from either the interpulse separation or the oscilloscope sweep time. The time separation of the pulses was calculated from twice the laser cavity length, $t_{exp} = 2L/c$, where L = length of cavity and c = speed of light. A typical oscilloscope trace is shown in Fig. 6, and a plot of the logarithm of the absorption against time is reproduced in Fig. 7.

The data were analyzed in terms of an exponential decay and the lifetime of prelumirhodopsin decay near room temperature was found to be about 30 nsec. The decay rates at 17.5, 22.5, and 29.3° are $2.7 \pm 0.2 \times 10^7$, $3.7 \pm 0.2 \times 10^2$, and $4.1 \pm 0.5 \times 10^7$ sec^{-1}, respectively. These values yield $\Delta H = 6$ cal/mol and $\Delta S = -5$ cal/mol-deg.

DISCUSSION

Irradiation of detergent-solubilized bovine rhodopsin in aqueous glasses near liquid nitrogen temperatures with light of wavelengths 400–600 nm leads to a mixture of rhodopsin (λ_{max} 500 nm) and prelumirhodopsin (λ_{max} 543 nm) (3–5).

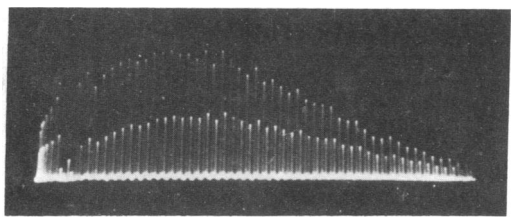

FIG. 6. Photograph of oscilloscope trace showing photodiode response to 0.56-μm interrogating light. Each pulse in the train (5.5-nsec separation) is split into two parts for the absorption measurements. The tall pulse in each pair is the I_0 reference, while the lower intensity pulse following it has sampled the absorbance within the sample cell. Full scale is about 300 nsec. The pulse(s) missing near the beginning of the train were used to generate the 0.53-μm exciting pulse.

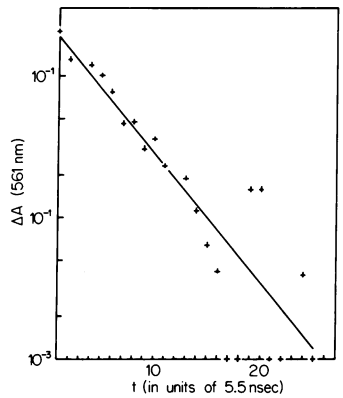

FIG. 7. A logarithmic plot of the data from Fig. 6 converted to relative concentration of transient absorbing species (prelumirhodopsin) against time. The lifetimes obtained from the slopes of such plots are about 30 nsec at room temperature.

Excitation with light <500 nm favors prelumirhodopsin, whereas light >550 nm favors rhodopsin. In addition to photoconversion back to rhodopsin, prelumirhodopsin undergoes thermal transformation to lumirhodopsin. The latter is completely suppressed in frozen glasses at 77°K, but proceeds rapidly at temperatures above −140°C (3–5). Grellman *et al.* (6, 7) measured the rate of thermal decay of prelumirhodopsin between −25 and −75°C using conventional flash photolysis. They reported that the decay consists of three simultaneous first-order processes with rates ranging from 10^4 sec^{-1} to 10^2 sec^{-1} over the temperature range. Extrapolation of an Arrhenius plot of their data to 38°C predicts decay rates between 10^6 and 10^7 sec^{-1}.

We observed no appreciable decay of the transient absorption within 400 psec, the total time range of the echelon (psec) experiment. Within experimental accuracy, the lifetime of the transient species must be greater than several nsec. However, we also observed no short-lived ($\leq$ 10nsec) component in the decay time (nsec) measurements. Thus, the psec measurement and the nsec measurement appear to indicate that a single transient intermediate is being observed over the range of time scales. Within the resolution of the experiment, the onset of absorption at 561 nm followed the laser pulse (about 6 psec). Thus, we conclude that the risetime of the transient is less than 6 psec.

Although we have essentially confined our measurements to one interrogating wavelength (561 nm), we interpret these observations in terms of the formation and decay of prelumirhodopsin. We base this on the following arguments: First, prelumirhodopsin is the only intermediate during the bleaching of bovine rhodopsin known to absorb strongly at 561 nm. Our attempts to observe transients at 626 nm, another wavelength easily available through Raman scattering generated by the doubled Nd^{3+} glass laser frequency, were without success. This is consistent with the low-temperature absorption spectrum of prelumirhodopsin for which $\epsilon(561)/\epsilon(626)$ is about 10 (3, 4). Secondly, the decay rate is consistent with the extrapolated values of low temperature decay data, and the activation parameters are in agreement (1, 6, 7).

The extremely fast risetime of prelumirhodopsin is strong support that this species is the product of the primary photochemical event in the bleaching process. That is, relaxation of

electronically excited rhodopsin leads directly to prelumirhodopsin†.

The psec experiments in which the relative polarization of the exciting and interrogating pulses were varied indicate that the transition moment directions for the visible absorption bands of rhodopsin and prelumirhodopsin do not differ greatly. Similar transition moment directions are to be expected in the absence of any geometry change severe enough to destroy the linear polyene nature of the chromophoric group.

An interpretation of the absorption spectra of rhodopsin, and the several intermediates in the bleaching of rhodopsin, has not been established because virtually nothing is known about the structure of the lipoprotein, which is part of the environment of retinyl chromophore and probably contributes significant perturbations (1). Although our kinetic data do not lead to structural information, it seems to us that the extreme rapidity of the formation of prelumirhodopsin could scarcely allow a major structural change between rhodopsin and prelumirhodopsin, e.g., complete geometric isomerization of the retinyl group from a truly 11-*cis* isomer to an *all-trans* isomer, with concomitant accomodating change in the opsin structure. Rather, a transformation involving only restricted changes in the geometry of the retinyl group and its local environment is more probable‡. The thermal decay of prelumirhodopsin may, however, involve relaxation to the *all-trans* isomer.

The rate of formation of prelumirhodopsin (about 2×10^{11} sec^{-1}) is comparable to the rate of the ultrafast radiationless electronic relaxation of the lowest allowed excited electronic level of azulene to the ground state (1.3×10^{11} sec^{-1}) (25), and to the rate of intersystem crossing in benzophenone (2×10^{11} sec^{-1}) from the origin of the first excited singlet state (26, 27). The processes in azulene and benzophenone are comparable in rate to electronic and vibrational relaxation in dense media (10^{11}–10^{12} sec^{-1}), and have been considered (25–27) to be due to strong coupling between vibronic upper state levels and resonant lower state levels. Similarly, the transformation from electronically excited rhodopsin to prelumirhodopsin may well involve such direct electronic relaxation.

Finally, our values for the decay rate of bovine prelumirhodopsin measured in detergent extracts are larger than the value reported for intact frog retinas recently measured by Cone (8). We intend to investigate by the same methods the decay rate in bovine rod outer segments, to determine any effect of intact membrane structure.

We thank R. P. Jones for valuable technical assistance, and Dr. D. C. Douglass for stimulating discussions.

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† Taking 5 psec for the (nonradiative) lifetime of the rhodopsin singlet state, one would expect a fluorescence yield of 10^{-5} if the radiative lifetime were comparable to that measured for retinals (0.5 μsec) (23). Guzzo and Pool (24) have reported a fluorescent emission from rhodopsin preparations having a quantum yield of about 5×10^{-3} and have assigned it to rhodopsin fluorescence. We (M. L. Applebury, J. Eisinger, and A. A. Lamola, unpublished results) have not been able to record the spectrum reported by Guzzo and Pool (24).

‡ We thank the referee for pointing out to us that recent studies on crystalline 11-*cis* retinal show it to be 11-*cis*, 12-s-*cis;* and have raised the possibility that the first change, forming prelumirhodopsin is to 11-*trans*, 12-s-*cis* [Honig and Karplus (1971) *Nature* **229**, 558; Gilardi *et al.* (1971) *Nature* **232**, 187].