Photoisomerization of the Chromophore in Bacteriorhodopsin during the Proton Pumping Photocycle†

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ABSTRACT: The configurations of the 13,14 double bond of the retinylidene chromophore in bacteriorhodopsin (bR) were investigated during the photocycle. The cycle was monitored by measuring the extent of formation of the M intermediate absorbing at 400–420 nm in the light and its rate of decay in the dark. It was inhibited by forming purple membrane films on glass and by using the synergistic ionophores beauvericin and valinomycin, in an equimolar ratio with bR, at 7 °C. These systems afforded enrichment in the M intermediate and a rate of decay that could be conveniently studied. The configuration was analyzed by extracting the chromophore with methylene chloride and measuring the ratio of 13-*cis*- to *all-trans*-retinals by high-performance liquid chromatography. The relationship between the percent bR cycling and percent 13-*cis*-retinal extracted was measured for the films by varying the light intensity; the molar ratio of bR cycling to 13-*cis*-retinal extracted was 1.11. This confirms previous studies which showed photoisomerization of the chromophore during the pumping cycle. The decay kinetics of the blue-shifted intermediate absorbing at 400–420 nm and of the 13-*cis*-retinal extracted were also measured for the purple membrane films and purple membrane suspensions containing the ionophores. The decay of the 13-*cis*-retinal extracted followed first-order kinetics with $t_{1/2}$ = 0.6 times the decay of the fastest measured component of the 400–420-nm decay. We conclude that the proton pumping photocycle of bR contains a photoisomerization about the 13,14 double bond of the chromophore from the all-trans to the 13-cis configuration and that the thermal rearrangement back to the all-trans form may occur more rapidly than the decay of the M photointermediate.

The controversy over whether or not the chromophore during the proton pumping photocycle of bacteriorhodopsin (bR) undergoes a photoisomerization from all-trans to 13-cis has not been settled. There is general acceptance of three intermediates in the early events in the cycle: K_{590}, with a half-life of ~10 ps (Applebury et al., 1978), absorbs maximally at ~590 nm; L_{550} has a half-time of ~50 μs; M_{412} has one of ~1 ms at ambient temperature (Lozier et al., 1975). In addition, Applebury et al. (1978) have reported an intermediate with a decay half-time of less than 6 ps, and Marcus & Lewis (1978) have postulated one between L_{550} and M_{412}. Of these intermediates, only M_{412} is a deprotonated retinylidene Schiff base (Terner et al., 1979). The consensus about the conformation of the chromophore, however, has been vague until quite recently.

Bacteriorhodopsin exists in two states between pH 4 and 8: a dark-adapted form which absorbs maximally around 558 nm (bR_{558}), in which the chromophore is believed to be equimolar in the 13-cis and all-trans configurations, and the metastable light-adapted complex absorbing maximally at 568 nm (bR_{568}), which contains the predominantly all-trans protonated Schiff base (Oesterhelt et al., 1973). Pettei et al. (1977) confirmed these results by extracting the chromophore and quantitating the distribution of isomers by high-performance liquid chromatography. They also extended their studies to photointermediates absorbing maximally at 405–420 nm by suspending purple membrane either in 2 M guanidine, pH 9.8, or in ether-saturated saline solutions and optically pumping bR into the blue-absorbing species prior to extraction. These solutions yielded retinals enriched in the 13-cis isomer whereas samples which were subsequently allowed to stand in the dark prior to extraction were found to revert to a predominantly trans isomeric mixture. Aton et al. (1977) used similar techniques to form a species absorbing at 410 nm and studied it by using resonance Raman spectroscopy. They likewise concluded that their putative M intermediate is 13-cis. Marcus & Lewis (1978) suggested that the complexes studied by the previous workers are not the kinetic M_{412} intermediate in the photocycle and that the latter has the chromophore in the all-trans configuration. However, their contention was recently disputed by Stockburger et al. (1979). El-Sayed & Terner (1979), again basing their arguments on resonance Raman spectroscopy, stressed that the chromophore resonances of the early intermediates differed significantly from either bR_{568} or the 13-cis component of bR_{558}. Consequently, Raman studies of this type are suspect in assigning conformations to the chromophore. Braiman & Mathies (1980) have substantiated this point by performing resonance Raman experiments on bacteriorhodopsin reconstituted with 15-deuterioretinal. Their data unambiguously show that the M_{412} intermediate is 13-cis. Consequently, there is a photoisomerization in the photocycle. However, nothing is known about the lifetime of the 13-cis species. We therefore have initiated the investigation by extracting the chromophore during the photocycle as a function of time.

In order to conduct such experiments, the photocycle must be sufficiently inhibited to allow a buildup of the 410-nm species and to afford the possibility of several extractions over a period of seconds. Korenstein & Hess (1977a,b) showed that purple membrane can be dried on a glass slide and that the bR undergoes a photocycle. At 0–10% humidity, it produces a transient absorbing maximally at 405 nm. The decay can be fitted to a sum of three exponentials, the fastest having a half-life of 2.3 ± 0.7 s (Korenstein & Hess, 1977a). This appeared to be a feasable system for study. However, an alternative was wanted with purple membrane in an aqueous

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suspension. The ionophores beauvericin and valinomycin interact synergistically with bacteriorhodopsin, slowing the rate of 410-nm decay under conditions of low salt and neutral pH (Avi-Dor et al., 1979; Brith-Lindner & Avi-Dor, 1979). The decay curve can be fitted to a sum of two exponentials, the fastest decaying in ~9 ms, the other in ~80 ms (Brith-Lindner & Avi-Dor, 1979). Therefore, this system was studied at low temperature.

Materials and Methods

Extraction of Films. Purple membrane (PM) suspensions from strain S-9 or R_1, isolated as described by Oesterhelt & Stoeckenius (1974), were placed on 8 × 22 mm glass strips and were allowed to dry overnight as described by Korenstein & Hess (1977b). They were then allowed to dry for 24 h in vacuo over P_2O_5 prior to use. The resultant slides had an absorbancy of ~0.6 unit.

The apparatus consisted of a Cary 14 spectrophotometer with side illumination provided by a General Electric Mark 300 lamp. The light beam was collimated to a 1 × 2 cm beam at the sample cuvette and filtered through a Corning 2-73 filter, affording total sample irradiation. The photomultiplier was further protected with a Corning 5-57 filter. Data were collected with a Nicolet 1180 computer. Films were placed at an angle of 45° to both the measuring and the actinic beam, and spectra were taken with and without actinic illumination. The decay of the 400-nm transient was measured at 22 °C before each extraction, and the three exponentials were resolved by hand. The intensity of the actinic illumination was 15.7×10^5 erg cm^{-2} s^{-1}. The percent of M accumulated was estimated by assuming a difference extinction coefficient for the 405-nm transient of 38 000 M^{-1} cm^{-1} (R. A. Bogomolni, unpublished experiments).

The chromophore was extracted by squirting a mixture of 1 mL of water and 1 mL of methylene chloride at the dried film followed by 30-s sonication at 0 °C with an 80-KHz, 80-W bath-type sonicator (Laboratory Supplies Co.). The samples were subsequently handled as described previously (Mowery et al., 1979).

Extraction of Solutions Containing Ionophores. Purple membrane suspensions contained 4 μM bR from strain R_1 [measured by assuming a molar extinction coefficient of 62 000 (Rehorek & Heyn, 1979)], 10 mg/mL valinomycin (Sigma), 4 mg/mL beauvericin (Bachem), 3 mM KCl, and 10 mM *N*-2-(hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid (Hepes), pH 7.0. Ionophores were added as ethanolic solutions (2 μL in 3 mL of purple membrane suspensions), resulting in an equimolar ratio of bR to each ionophore.

The extractions were performed in an apparatus designed for this purpose. The chopped monitoring beam was passed through a Spex double monochromator set at 420 nm and was focused on the sample by using f/4.0 optics. The exit beam was collimated with a lens, passed through a 420-nm interference filter, and measured with a 1P21 photomultiplier. Signals from the photomultiplier and chopper (Ithaco Model 383A) were fed into a lock-in amplifier (Princeton Applied Research Model JB-4), and the output was processed through a Tektronix 3A9 differential amplifier and collected in the Nicolet 1180 computer. The actinic beam was passed through a timed shutter and a 2-73 filter and was collimated with a rectangular lens to a 1 × 2 cm spot to illuminate the entire cuvette at a right angle to the monitoring beam. The intensity was 1.2×10^6 erg cm^{-2} s^{-1}. Both the shutter on the actinic beam and the kinetics of the 420-nm transient were monitored simultaneously with the computer for each point. The sample was quenched during the repetition of a cycle of illumination, thereby providing a timed record of when the extraction solution was added and the duration of the bleaching process. The extractions were performed with 2 mL of 20% ethanolic methylene chloride at 7.0 ± 0.5 °C by syringing the extraction mixture into the stirred PM suspension. The 410-nm transient was found to reach a steady state when the shutter was open 10 s. Times for complete quenching were ≪0.5 s. A typical quench, shown in Figure 3, trace 3 (arrows), was <0.1 s. The samples were analyzed as described previously (Mowery et al., 1979).

Table I: Extraction of Purple Membrane Films and Solutions Containing Ionophores

conditions	% *all-trans*-retinal: light adapted	% *all-trans*-retinal: dark adapted
film, 0% humidity	69	68
film, 0% humidity, light	52	
BV[a] + VL, 23 °C	94	51
BV + VL, 5 °C	83	50
BV + VL, 7 °C, light	61	

[a] BV, beauvericin, VL, valinomycin.

Extraction of Chromophores as the Oximes. Purple membrane suspensions were extracted in the presence of a 1000-fold molar excess of hydroxylamine (relative to bR) as described by Groenendijk et al. (1980). The samples were processed as described before (Mowery et al., 1979), only 9% ether in hexane was used as eluent.

Results

The extraction of films presented several difficulties. Anhydrous methylene chloride did not bleach them, whereas if water was first added time was lost and the isomeric composition of the chromophore could be altered due to wetting. A 1:1 mixture of water–methylene chloride, however, effected rapid bleaching, and a 30-s sonication improved the yield of retinals to ~15% by removing the bleached membrane from the slide without affecting the ratio of isomers extracted. In the dark, the anhydrous films consistently yielded 31–32% 13-*cis*-retinal (Table I). However, the 13-cis isomer will not interfere in the analysis, since only the trans photocycle contains an M intermediate (Sperling et al., 1977). The yield of the 13-cis isomer was increased upon illumination to 48% with the light intensities used. This represents a maximum range of 17% of the 13-cis chromophore undergoing a light-catalyzed trans to cis isomerization under conditions were the 405-nm transient absorbance indicates ~20% cycling. The discrepancy is small and is consistent with 1 mol of 13-*cis*-retinal extracted per mol of bR cycling.

The next problem was to show a proportionality between the fraction of bR cycling and the percent 13-*cis*-retinal extracted over a range of light intensities. To do this, the actinic light was attenuated with neutral density filters, and spectra were recorded in the dark and in the photo steady state, followed by immediate extraction. The percent bR cycling was monitored at 405 nm, and both the fraction cycling and the percent 13-*cis*-retinal extracted were normalized to a range of 0 (in the dark) and 100 (in the absence of neutral density filters). The relationships were nonlinear functions of the light intensity and demonstrated that the system was below light saturation (not shown), but an excellent linear proportionality was found between the fraction of bR cycling and the percent 13-*cis*-retinal extracted (Figure 1).

We next wanted to measure the decay rate of the 412-nm intermediate. To do this, spectra of each film were recorded

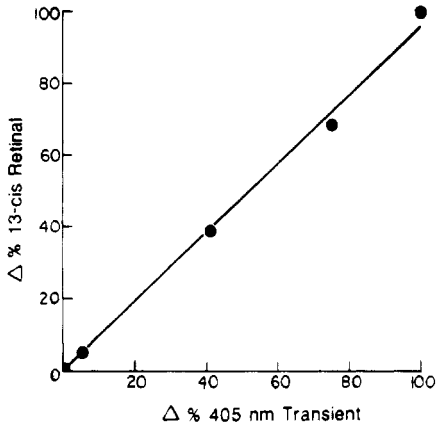

FIGURE 1: Percent 13-*cis*-retinal extracted from purple membrane films in the dark is subtracted from that extracted in the presence of light and is plotted against the percent of 405-nm species cycling. The data were normalized to 100% for light which was unattenuated by interference filters. Correlation coefficient, 0.999.

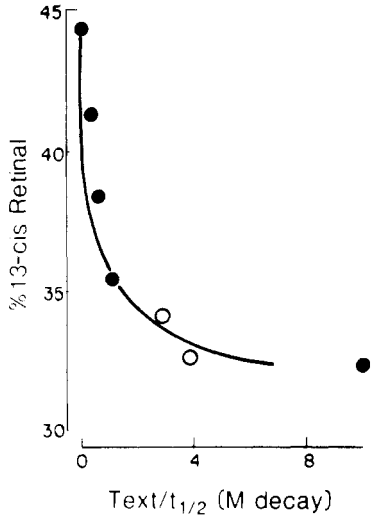

FIGURE 2: Summary of the percent 13-*cis*-retinal extracted from purple membrane films at 22 °C. Samples were allowed to reach a steady-state photocycle, the light was extinguished, and the samples were extracted at various times afterward. The time scale refers to the time the sample was extracted divided by the half-time of decay for the fastest component at 405 nm. (○) Data not used in least-squares fit to a first-order decay; (●) $t_{1/2} = 0.58t_{1/2}$ (405-nm decay), correlation coefficient, 0.999.

in the absence and presence of actinic light, and the rate of decay of the 405-nm transient was measured. The photo steady state was reestablished, illumination was stopped by a shutter, and the time the sample was in the dark prior to extraction was measured. Each sample, therefore, was documented with the percent bR cycling, the extraction time, and a kinetic trace. The traces were fitted to three exponentials, the fastest having a half-time of 2.0 ± 0.2 s, the second, 15 ± 1 s, the third, 150 ± 30 s, in good agreement with the data of Korenstein & Hess (1977a) for films at 0% humidity. A plot of the time after illumination at which the chromophore was extracted divided by the half-time for each sample serves as an internally consistent time scale, since some errors arising from the variability of samples are corrected for. The data, summarized in Figure 2, were treated as first-order kinetics. A good least-squares fit was found for over 2 half-lives of decay of the 13-cis chromophore, the half-time for which is $0.58t_{1/2}$ for the 405-nm transient. With $t_{1/2}$ (405 nm) = 2.0 ± 0.2 s, this translates to a half-time of 1.2 s.

Preliminary experiments suggested that the system employing the ionophores beauvericin and valinomycin could be

Table II: Effect of the Extraction Technique on the 13-Cis/Trans Ratio of Retinals Extracted from Purple Membrane

conditions[a]	extraction system	% *trans*-retinal: light adapted	% *trans*-retinal: dark adapted	% retinal extracted
pH 7.0	CH_2Cl_2	93 ± 1	49 ± 2	10
BV + VL[b]	CH_2Cl_2			0
BV + VL[b]	C_2H_5OH	77 ± 2	60 ± 1	60
BV + VL[b]	1:1 CH_2Cl_2/C_2H_5OH	87 ± 2	57 ± 1	32
BV + VL[b]	1:5 C_2H_5OH/CH_2Cl_2	94 ± 3	48 ± 2	12
pH 7.0	1:5 C_2H_5OH/CH_2Cl_2	92 ± 1	50 ± 1	19
pH 7.0	as oxime[c]	90 ± 3	49 ± 1	91
pH 4.0	CH_2Cl_2	70 ± 1	54 ± 3	3
pH 4.0	as oxime[c]	72 ± 2		87
BV + VL, 5 °C	as oxime[c] (1:5 C_2H_5OH/CH_2Cl_2)	86 ± 1		89

[a] Samples at 22 °C except as noted. [b] Beauvericin and valinomycin solutions described under Materials and Methods. [c] See Groenendijk et al. (1980) for detailed procedure.

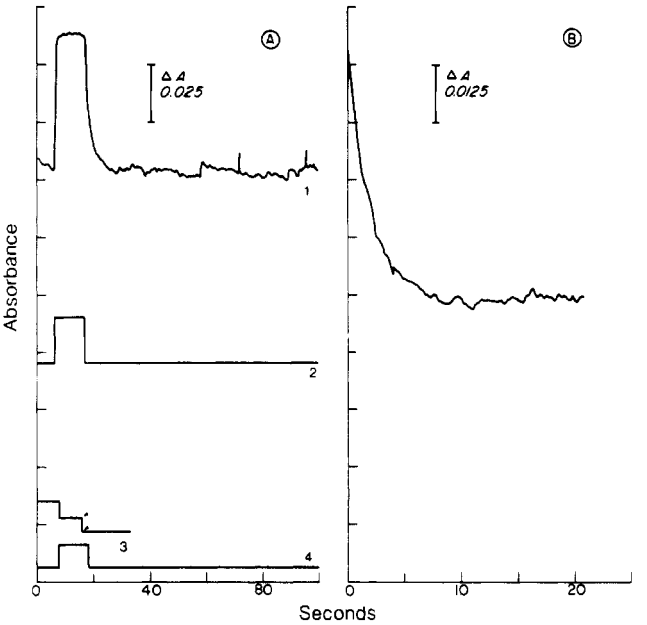

FIGURE 3: (A) Representative data used to monitor the decay of the 420-nm-absorbing transient of purple membrane suspensions containing beauvericin and valinomycin: (1) rise and decay kinetics; (2) history of when shutter was on (10-s plateau) and off in (1); (3) history of chromophore extraction (arrows show quench); (4) history of shutter for (3). (B) Expansion of decay in (A), trace 1.

used. Extractions employing methylene chloride failed to yield retinals. An attempt was made to extract the chromophore with ethanol after the method of Papadopoulos et al. (1978), but some isomer equilibration occurred (Table II). A solution of 20% ethanolic methylene chloride, however, was found to extract 10–15% of the chromophore. In control experiments in which the ionophores were omitted, this mixture was found not to affect the cis to trans isomer ratio.

The decay kinetics of the 412-nm transient are still very fast in the presence of the ionophore at room temperature (Brith-Lindner & Avi-Dor, 1979). We therefore assembled the apparatus described under Materials and Methods for measuring the faster decay rates. We found that the data at 7 °C could be fitted to a single exponential with a half-time of 2 ± 0.3 s (Figure 3B) which is not affected by the 1-ms resolution of the lock-in amplifier. We therefore measured the rates of decay of the 420-nm species for each sample and also obtained records of when the light was turned off, when the sample was quenched, and the duration of the quenching

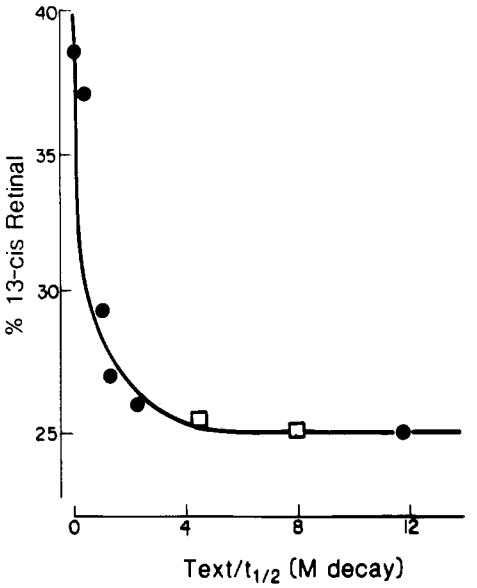

FIGURE 4: Summary of chromophore extraction data for purple membrane suspensions in the presence of ionophores. Data are presented as in Figure 2. (□) Data not used in least-squares fit to a first-order decay; (●) $t_{1/2} = 0.60t_{1/2}$ (420-nm decay, correlation coefficient, 0.979).

procedure (Figure 3A). The percent bR cycling was 15%, which corresponded to a 13% enrichment of the 13-*cis*-retinal extracted. A summary of the extraction data is shown in Figure 4. When the data were replotted as first-order kinetics, the half-life, $0.61t_{1/2}$, for the decay of the 420-nm-absorbing transient, was found to be in excellent agreement with the film data; the half-life for the combined data is $0.6t_{1/2}$ (correlation coefficient = 0.983). The data shown by open symbols were omitted from the least-squares analysis because the errors in the measurement of 13-*cis*-retinal extracted were larger than its enrichment.

A major criticism of the extraction technique has been that the low yields of retinal extracted may allow the preferential extraction of one isomer. Groenendijk et al. (1980) have recently published a technique in which the oxime is isolated in >90% yield by adding hydroxylamine in 1000-fold molar excess to bR. Complications arise because the extinction coefficients of the 13-cis oximes are not known (Groenendijk et al., 1979, 1980), and the major syn peaks overlap for 13-cis and trans oximes (Groenendijk et al., 1980; Pettei et al., 1977). The high yields of oximes extracted allow the well-separated anti peaks to be easily analyzed, and we found the syn to anti ratio to be constant as reported by Groenendijk et al. (1980). We quantitated the data by assuming the ratios of retinal isomers extracted with methylene chloride at pH 7 to be correct. This allowed us to estimate a correction for the ratio of extinction coefficients in the anti isomers, and to use that estimate to calculate the isomer distribution under different conditions of extraction. The correction factor for the 13-cis/trans anti peak ratio at 365 nm, 0.57 ± 0.02, agrees well with that calculated from the data of Groenendijk et al. (1980) for 360 nm. The results, summarized in Table II, strengthen the reliability of the retinal data.

Discussion

Two major criticisms have been made concerning the applicability of using the ratio of 13-*cis*- to *all-trans*-retinal extracted from bR as a criterion for the conformation of the chromophore in situ. The low yield of retinal extracted, generally less than 10%, may hide the preferential extraction of one isomer. It has now been extracted as the oxime in over 90% yield; no effect was seen on the isomer ratios. This suggests that the low-yield data are valid.

The extracted retinals do not reflect the exact conformations of the retinylidene moiety in bR—so far only resonance Raman spectroscopy may be able to do that—but they do appear to reflect the configuration of the 13,14 double bond. The applicability of this criterion to the isomeric composition of light- and dark-adapted membranes has been reviewed recently (Mowery et al., 1979). It appears to be a valid indication of the 13,14 bond configuration in the photocycle, and the ratio between the fraction of bR cycling and the percent 13-*cis*-retinal extracted (Figure 1) is close to one. In addition, Braiman & Mathies (1980) have shown, using resonance Raman spectroscopy, that the intermediate absorbing maximally at 412 nm contains a 13,14-cis double bond. The analysis of the bR photocycle is complex (Lozier & Niederberger, 1977). Furthermore, the cycle is altered in the studies reported here in order to slow it down and to build up the intermediates sufficiently to detect changes in the isomer ratio. The decay of the 400-nm transient appears to be even more complex than that for the normal photocycle. Avi-Dor et al. (1979) showed that the decay of the 412-nm-absorbing transient observed in suspensions containing ionophores can be fitted to two exponentials at ambient temperatures, unlike the data at 7 °C reported here. Furthermore, they reported the absence of the red-shifted O intermediate usually present in the photocycle. In addition, we have confirmed the observation of Korenstein & Hess (1977a) that the 405-nm transient observed in films can be fitted to three exponentials. If the photocycle consists of a unidirectional series of intermediates, each decaying by a first-order process, e.g.

$$K_{590} \xrightarrow{k_1} L_{550} \xrightarrow{k_2} M \xrightarrow{k_3} M \xrightarrow{k_4} O \xrightarrow{k_5} bR_{570}$$

the kinetics may be fitted to a series of exponentials, the relative amplitudes of which are dependent on the experimental conditions and wavelength monitored. In addition, rate constants may be too similar to allow their deconvolution. This is why Lozier & Niederberger (1977) originated their multiple wavelength kinetics studies. Under the conditions reported here, the decays around 410 nm can be fitted to three exponentials for films or one for solutions because the amplitudes or the rate constants for the other exponentials, if present, render them unobservable. The decay of the 13-cis chromophore apparently is faster than the fastest component of the 412-nm transient in both cases. This is at least consistent with mechanisms in which the retinylidene moiety acts as an impeller in the proton pumping cycle. The Schiff base should return to the all-trans configuration before its reprotonation, so that it can be reprotonated from the cytoplasmic side only. We do not consider our data sufficient to establish this firmly; they do, however, confirm that the M intermediate contains 13-*cis*-retinal.

The existence of the thermal rearrangement of the 13-cis to all-trans photointermediates necessitates the search for a mechanism. Four potential mechanisms are known, one of which can already be discarded. Retrointermediates are improbable, because the β-ionone ring can be replaced with a benzene or thiophene ring and the synthetic pigment can still pump protons (Marcus et al., 1977). Sack & Seltzer (1978) have proposed that in solutions a nucleophile may reversibly attack at the 13-carbon of the protonated 13-*cis*-retinylidene Schiff base, thereby catalyzing a 13-cis to all-trans thermal equilibration. We have shown that the more probable route under these conditions is through the general base-catalyzed formation of an enamine intermediate, from the Schiff base

(Mowery & Stoeckenius, 1979). However, both are potential biological mechanisms. Recently, Crouch et al. (1979), in a preliminary communication, have stated that 5,6-dihydro-1,1,5,9,18-pentademethylretinal forms a pigment with bacterioopsin and that it pumps protons. If this is true, the enamine mechanisms may be abandoned. The fourth mechanism is a protein-catalyzed isomerization about the 13,14 double bond. The bond order of this bond may be decreased enough via protein–chromophore interactions to lower the barrier to isomerization. Orlandi & Schulten (1979), using semiempirical molecular orbital calculations, have shown that this is possible for the protonated Schiff bases.

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