Low-Temperature Photoreactions of Halorhodopsin. 1. Detection of Conformational Substates of the Chromoprotein†

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ABSTRACT: Absorption spectra of halorhodopsin (HR), a retinal protein in the halobacterial membrane, and its photostationary states were determined at 80 K. The absorption lines appear to narrow upon cooling, thereby revealing complex spectral fine structure of the main absorption band in the visible region, characteristic of conformational substates of HR. Illumination causes (1) the redistribution of these substates and consequent changing of the fine structure ("hole-burning") and (2) the appearance of a hypsoproduct of undefined nature, in addition to the previously described bathoproduct HR_{600}. Bacteriorhodopsin, a related retinal pigment, gives rise only to the bathointermediate (i.e., K_{590}) under these conditions. After warming of illuminated HR to 110 K, and recooling to 80 K, relaxation of the illumination-induced change in spectral fine structure, and decay of the hypsoproduct but not the bathoproduct, was observed. The results are explained with a model in which one ensemble of HR conformational substates at 80 K is converted to another in a photoequilibrium via the excited state, which also produces the batho- and hypsoproducts. The original ensemble can be regained through thermal pathways at a somewhat higher temperature, and only the bathoproduct will decay thermally into the next intermediate of the HR photocycle.

Halorhodopsin (HR),[1] the light-driven chloride pump in the cytoplasmic membrane of *Halobacterium halobium*, contains retinal in either the all-trans configuration (enriched by adaptation with blue light) or the 13-cis configuration (enriched by adaptation with red light). The photocycle of *all-trans*-HR has been described before (Weber & Bogomolni, 1981; Tsuda et al., 1982; Schobert et al., 1983; Hazemoto et al., 1983; Bogomolni, 1984; Hegemann et al., 1985; Oesterhelt et al., 1985; Polland et al., 1985; Lanyi & Vodyanoy, 1986; Oesterhelt et al., 1986; Tittor et al., 1987). It includes the formation of a bathoproduct, HR_{600}, and subsequent thermal reactions which generate HR_{520}, HR_{640}, and finally HR again. The bathoproduct is observed either at ambient temperature on a picosecond time scale (Polland et al., 1985) or at liquid nitrogen temperature in the photostationary state (Weber & Bogomolni, 1981; Ogurusu et al., 1984), similarly to K_{590}, the bathoproduct of a related protein, BR [for example, see Becher et al. (1978) and Iwasa et al. (1979, 1980)]. As part of our further investigations of the HR photocycle, we have undertaken a systematic study of the low-temperature photoreactions of this pigment [cf. also the following paper (Zimányi & Lanyi, 1989)]. We made use of the fact that the HR bathoproduct is identifiable by a characteristic difference spectrum upon its formation; at low temperature, this consists of a large maximum near 620 nm and a much smaller, shallow, minimum centered near 540 nm (Ogurusu et al., 1984). A difference spectrum can be reconstructed also from published absorption spectra of HR_{578} and HR_{600} at ambient temperature (Tittor et al., 1987), which yields a maximum at 620 nm and a minimum of about the same magnitude and shape at 540 nm.

In this study, we have obtained information on the formation of the HR bathoproduct at 80 K and its thermal stability but found that the pigment has two additional photoreactions so far not described. The first produces a blue-shifted (hypso) form of an uncertain nature, while the second appears to be the redistribution of conformational substates of HR as revealed by alteration of spectral fine structure. The latter is consistent with the postulated multiplicity of interconvertible conformational substates in proteins (Frauenfelder et al., 1988).

Materials and Methods

HR was purified from *Halobacterium halobium* strain OD-2W (provided by J. L. Spudich), using a newly described method (Duschl et al., 1988). The preparations were stored at 4 °C in 4 M NaCl, 25 mM Tris·HCl, pH 7.2, and 0.5% octyl glucoside, in the dark. Before use, 20% sodium cholate (in H_2O) was added to a final concentration of 0.5%, and the samples were concentrated in an AMICON concentrator (filter YM 10). Lubrol-solubilized HR was prepared by adding Lubrol PX (Pierce Chemicals, peroxide-free) to a final concentration of 0.5% and dialyzing 2–3 times against 100–200 volumes of the desired buffer solution, each time for approximately 12 h at 4 °C in the dark, in order to remove the octyl glucoside. Purple membrane was purified according to a published procedure (Oesterhelt & Stoeckenius, 1974). Blue membrane was prepared by deionization of purple membrane, as described before (Ariki & Lanyi, 1986). Prior to the measurements, 1 part of HR sample was mixed with 2 parts (w/w) of glycerol.

Absorption spectra were measured in a Shimadzu UV-250 spectrophotometer, whose sample chamber was modified to accommodate a liquid nitrogen cryostat (Oxford Instruments DN1714), controlled by a digital temperature control unit (Oxford Instruments ITC 4). The output of the spectrophotometer was recorded in a desktop computer (XPC), and the

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[1] Abbreviations: HR, halorhodopsin; BR, bacteriorhodopsin; Tris, tris(hydroxymethyl)aminomethane; MES, 2-(*N*-morpholino)ethanesulfonic acid; octyl glucoside, *n*-octyl β-D-glucopyranoside.

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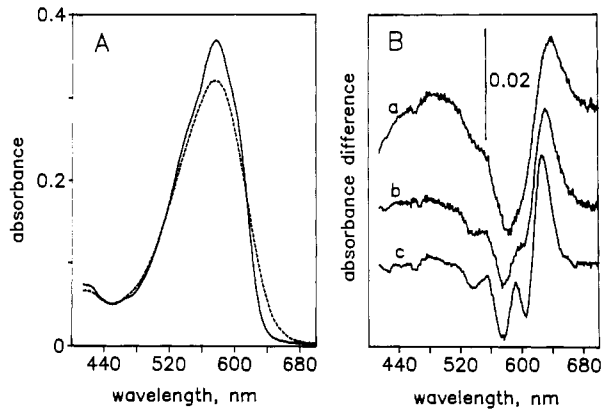

FIGURE 1: (A) Absorption spectra of HR at 273 K (---) and 80 K (—) in chloride buffer. An appropriate base line corresponding to light scattering was subtracted. (B) Difference of spectra taken at different temperatures in chloride buffer. Curve a, 273 K – 200 K; curve b, 200 K – 140 K; curve c, 140 K – 80 K. For both (A) and (B), the HR was solubilized in 0.5% octyl glucoside, 0.5% cholate, 4 M NaCl, and 25 mM Tris·HCl, pH 7.2, and mixed with 2 parts (w/w) of glycerol.

data were analyzed with Lotus 123 software. Properly normalized light-scattering spectra taken under similar conditions, but using buffer–glycerol mixtures without HR, were subtracted from the low-temperature absorption spectra. Both light adaptation and actinic illuminations at low temperature were performed with a 200-W lamp, through a 50-cm-long fiber optic which served also as an effective heat filter, and an appropriate color filter. The filters used were as follows: "blue", 420-nm broad-band glass filter (half bandwidth 110 nm); "red", 610-nm high-pass filter; and "deep red", 670-nm high-pass filter.

The sample in a 1-mm path-length cell, constructed from Plexiglass, was placed into the cryostat and after light adaptation at 273 K was cooled to 200, 140, and finally to 80 K in the dark, while absorption spectra were recorded at these reference temperatures. The rate of cooling was approximately 5–10 K/min. The preillumination spectrum at 80 K was then acquired, and subsequent illuminations were performed followed by measurements of spectra. Each illumination was continued for up to 5 min, unless otherwise mentioned, which was sufficient to reach photostationary states; i.e., no further spectral changes were observed. Retinal extraction to determine the isomeric composition of retinal in blue- and red-adapted HR was performed after adaptation at 273 K, as described earlier (Zimányi & Lanyi, 1987).

Results

Effects of Cooling on the Spectrum of HR. When HR was cooled from room temperature to 80 K, the maximum extinction of the main visible band gradually increased, the bandwidth decreased, and the appearance of vibrational fine structure was observed (Figure 1A). However, only negligible red shift was detected, unlike the 10-nm red shift seen under these conditions for visual rhodopsins and BR (Hurley et al., 1977; Becher et al., 1978; Tsuda et al., 1980). The differences between the absorption spectra of blue light adapted HR, taken at different temperatures (Figure 1B), reveal that the resolution of fine structure is most pronounced below 140 K. The 140 K – 80 K difference spectrum (curve c) is characterized by a positive peak at 628 nm and three negative peaks, at 605, 578, and 536 nm. The appearance of spectral fine structure can be attributed to the narrowing of spectral lines in an ensemble of increasingly confined retinal bond torsional angles and/or protein conformations at lower temperatures, as discussed below. The shape of the difference spectra upon cooling was the same for red light adapted HR, except for a slightly smaller amplitude. Inasmuch as retinal extraction data showed that the isomeric composition in our red light adapted sample was not very different from the blue light adapted sample (76% and 84% all-trans, repsectively), the smallness of the difference between the two samples is not surprising. Similar resolution of fine structure can be seen in published spectra of cooled purple membrane samples (Iwasa et al., 1979, 1980), an observation we have confirmed in this study (not shown).

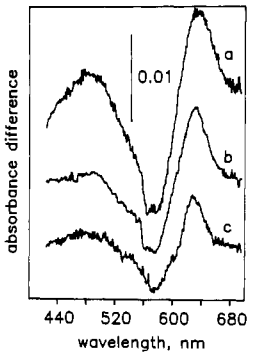

FIGURE 2: Difference of spectra of HR taken at different temperatures in nitrate buffer. Curve a, 273 K – 200 K; curve b, 200 K – 140 K; curve c, 140 K – 80 K. The HR was solubilized in 0.5% Lubrol, 370 mM Na_2SO_4, 200 mM $NaNO_3$, and 25 mM MES, pH 6.0, and then mixed with 2 parts (w/w) of glycerol. Room temperature absorption of the sample at maximum was 0.22.

The effect of cooling on the absorption spectrum was similar in octyl glucoside solubilized and Lubrol-solubilized HR (data for the latter not shown). Unlike octyl glucoside, Lubrol allows the use of anions other than chloride without precipitation of the pigment. When chloride was thus replaced with nitrate in Lubrol-solubilized HR, only the narrowing of the absorption band was observed on cooling; i.e., the prominent fine structure was absent (Figure 2). In the presence of 67 mM nitrate, as used here, only one of the two important anion binding sites, site I, which is proposed to be in the vicinity of the Schiff base, is occupied (Schobert et al., 1986; Lanyi, 1986; Lanyi et al., 1988). Therefore, in the absence of chloride, no anion is bound to site II, which is thought to be located close to the β-ionone ring of the retinal. It would seem to follow from the results in Figures 1 and 2 that the factors leading to the narrowing of spectral lines depend on either electrostatic interaction between the negative charge of the bound anion and the ionone ring of the retinal or structural alteration of the protein upon anion binding to site II.

Effects of Illuminating HR at 80 K. The photoreactions of HR at 80 K were investigated by using different colors of actinic light. Figure 3 demonstrates spectral changes at photoequilibria, produced by different illumination sequences at 80 K, using octyl glucoside solubilized HR in chloride buffer. Similar results were obtained with Lubrol-solubilized HR in chloride (not shown). Figure 3 curve a shows that the difference spectrum after blue illumination is complex. It contains a contribution from a red-shifted photoproduct (the HR bathointermediate) and, in addition (and unexpectedly), changes in the fine structure similar to the 140 K – 80 K difference spectrum (cf. Figure 1B, curve c). Illuminations at greatly reduced light intensities by means of neutral density filters (1% of the intensities used in Figure 3) produced a rough time course for the appearance of these spectral changes (not shown): the rise of the bathoproduct occurred well before the change of the spectral fine structure. With red illumination (Figure 3, curve c) an apparent blue shift of the spectrum was

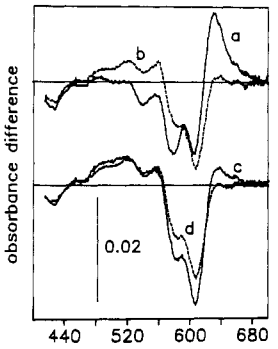

FIGURE 3: Difference spectra of HR at 80 K after various illuminations vs the preillumination sample. Blue illumination (curve a) was followed by deep red illumination (curve b). Red illumination (curve c) was followed by deep red illumination (curve d). The same sample as in Figure 1.

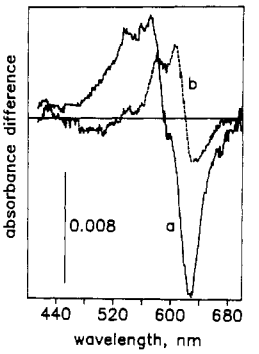

FIGURE 4: Effect of deep red light after blue (curve a) and red (curve b) illumination. Difference spectra shown as after vs before the deep red illumination. The same sample as in Figure 1.

observed, as well as a change of the fine structure. In addition, this sample contained much less bathoproduct than the blue-illuminated sample. This is as expected, since the HR bathoproduct is itself photosensitive (Ogurusu et al., 1984). In the low light intensity experiments, the appearance of the blue shift was slower than the change in the fine structure (not shown), suggesting that a so far not described hypsochromic form of HR, distinct from the other photoproducts, accumulates. The origin of the spectral blue shift will be discussed in detail below.

As for the observation of illumination-dependent changes in vibrational fine structure, a trivial explanation which occurred to us was that it is caused by heating of the sample by the actinic light and subsequent rapid cooling. This is unlikely considering the efficiency of cooling by the cryostat, and the fact that the light was well filtered for heat. Moreover, difference spectra obtained after lengthy illuminations (up to 2 h) at light intensities (and therefore rates of heat input) reduced by a factor of 100 were essentially the same as those in Figure 3 (not shown). In another series of experiments, we used the same cooling and illumination regime for BR samples (purple membranes in 67% glycerol). While formation of the BR bathoproduct (the K_{590} intermediate) was evident, no change in the vibrational fine structure was seen, in accordance with earlier published spectra [for example, see Becher et al. (1978) and Iwasa et al. (1979, 1980)]. Blue membrane preparations (deionized purple membranes) also did not show a change in spectral fine structure after illuminations at 80 K. The earlier published difference spectrum of the illumination of HR with blue light at liquid nitrogen temperature (Ogurusu et al., 1984) does not contain the change in fine structure we observe, probably because the sample was cooled too rapidly to reach the low-temperature thermal equilibrium of conformations (cf. Discussion).

As the next step, we used a second illumination with deep red light after either red or blue illuminations of HR. This was expected to reconvert the bathoproduct of HR (Ogurusu et al., 1984). Additionally, if the changes in vibrational structure reflect repopulation of different states in an ensemble of protein/chromophore conformations, it might be expected that deep red light would cause further redistribution, i.e., a further change in the spectral fine structure.

The effect of deep red light after blue illumination (Figure 3, curve b) was that the peak characteristic of the bathoproduct (near 630 nm) disappeared, and the absorption below 600 nm increased, with the appearance of a positive region around 500 nm. When the deep red light followed the red illumination (Figure 3, curve d), the small positive peak around 630 nm diminished, and the amplitude of the depletion signal between 550 and 620 nm decreased. Figure 4 demonstrates more clearly the effect of deep red light after blue (curve a) and red (curve b) illuminations, by showing difference spectra, after vs before the deep red illumination. From Figure 4 curve a, it is evident that deep red illumination after blue light caused mainly the reconversion of the bathoproduct to HR, as well as some, poorly resolved, changes in the vibrational structure of the spectrum. Further thermal treatment of this sample produced an estimate for the pure bathoproduct *minus* HR_{578} difference spectrum (cf. below), and comparison of this spectrum [as well as the published difference spectrum from Ogurusu et al. (1984)] with Figure 4 curve a strengthened this interpretation. The disappearance of the bathoproduct of the blue illumination had a zero-crossover point somewhat below 600 nm (curve a).

Consistently with the much smaller amount of bathoproduct after red illumination (cf. Figure 3, curve c), the second illumination of this sample with deep red light caused little change which was related to the bathoproduct. Instead, as shown in Figure 4 curve b, the intensity of two bands, at 578 and 605 nm, increased at the expense of a third band at 628 nm. The shape of this difference spectrum and its red-shifted zero-crossover point around 620 nm appears very unlike the difference spectrum of the reconversion of the bathoproduct to HR. On the other hand, the bands in this difference spectrum coincide with some of those seen for the low-temperature vibrational fine structure (cf. Figure 1B, curve c). Thus, it appears that the dominant contribution to the spectral alteration by deep red illumination after red illumination is the change in the vibrational fine structure of the absorption band.

The changes in vibrational fine structure during these illuminations greatly complicate the interpretation of the spectra. Moreover, since the bands of the bathoproduct and the blue-shifted photoproduct postulated as a result of red illumination should overlap strongly with both the parent species and probably each other, it is not obvious whether the blue-shifted product is produced only by red illumination or also by blue light illumination. One way to distinguish between these possibilities is the examination of the thermal conversions of the two photoproducts.

By appropriate illumination regimes, we produced (1) a combination of vibrational fine structure alteration and the hypsoproduct (red illumination followed by deep red light) and (2) a combination of vibrational fine structure alteration and potentially both the hypsoproduct and the bathoproduct (blue illumination). In Figure 5, these illuminations at 80 K were followed by warming the samples to 110 K and then recooling to 80 K. Comparison of curves a and b in this figure shows that warming completely reversed the effect of the red and

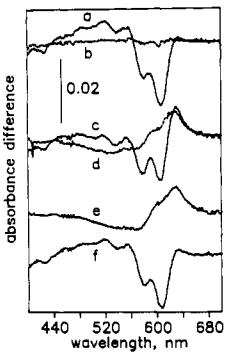

FIGURE 5: Difference spectra of HR at 80 K after various illuminations, and warming and recooling to 80 K. Curve a, the spectrum of a red and then deep red illuminated sample vs the spectrum of the preillumination sample; curve b, the spectrum taken after the red and deep red illumination sequence, warming to 110 K, and recooling after 1 h at the higher temperature vs the spectrum of the preillumination sample; curve c, the spectrum of a blue illuminated sample vs the spectrum of the preillumination sample; curve d, the spectrum taken after blue illumination, 1-h dark period at 110 K, and recooling to 80 K vs the spectrum of the preillumination sample; curve e, the spectrum taken after blue illumination, 1-h dark period at 110 K, and recooling to 80 K vs the spectrum after a subsequent deep red illumination; curve f, the spectrum taken after blue illumination vs the spectrum taken when blue illumination was followed by a 1-h dark period at 110 K and recooling to 80 K. The HR sample was similar to that in Figure 1. Room temperature absorption at maximum was 0.3.

deep red illumination sequence; i.e., both fine structure changes and the blue-shifted photoproduct disappeared. Comparison of curves c and d shows, however, that the warming of the blue-illuminated sample left a difference spectrum characteristic of the depletion of HR_{578} and the creation of bathoproduct. This is because (1) curve d resembles the published difference spectrum for the HR bathoproduct (Ogurusu et al., 1984), (2) after the warming to 110 K, deep red illumination at 80 K, which converts all of this intermediate to HR_{578}, produces a very similar difference spectrum (compare curves d and e), and (3) that portion of the spectrum which had disappeared on warming (curve f) is virtually identical with what is seen after red and deep red illumination (curve a), which contains little of the bathoproduct (i.e., the bathoproduct did not decay on warming). Point 3 also shows that blue illumination had produced changes observed after both red and deep red illumination, i.e., the vibrational changes and the hypsoproduct, in addition to the bathoproduct. It is noteworthy that the sample had to be kept at 110 K for about an hour before recooling to 80 K, in order to completely reverse the changes in the fine structure induced by illumination.

The effect of illuminating HR with blue light is thus 2-fold. On the one hand, it forms a red-shifted intermediate (bathoproduct) that is stable at 110 K, therefore, it cancels out from the difference of the two spectra taken after and before heating (Figure 5 curve f) but can be photoconverted back by deep red light (Figure 5 curve e). On the other hand, it produces similar alterations in the fine structure and gives rise to the hypsoproduct as does the red–deep red illumination sequence. Since at 110 K only the bathoproduct is stable, it must be the precursor of all further intermediates of the photocycle, as assumed by other authors who reported on low-temperature spectra (Weber & Bogomolni, 1981; Ogurusu et al., 1984). It should be mentioned that the deep red illumination had no effect on the spectrum, unless red or blue light was applied previously.

Lubrol-solubilized HR in nitrate buffer provided results similar to the above, except that the changes in the vibrational fine structure were smaller (not shown).

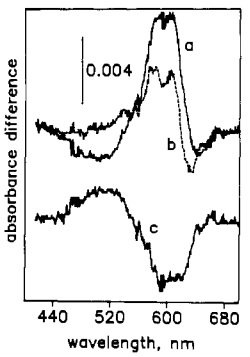

FIGURE 6: Difference spectra of HR at 80 K after various illuminations, which reveal the difference spectrum of the formation of hypso-HR. Curve a, the spectrum taken after blue and a subsequent deep red illumination vs the spectrum taken after a red illumination; curve b, the spectrum taken after red and a subsequent deep red illumination vs the spectrum taken after a red illumination; curve c, difference of curves b and a. Similar sample as in Figure 1.

Determination of the Difference Spectrum Due to Hypso-HR. In the following, we tried to find experimental conditions where a pure difference spectrum between HR and its postulated hypsoproduct could be obtained. It appears from Figure 3 that the amount of the hypsoproduct formed is different after red and blue illuminations. Moreover, we have seen that the bathoproduct could be eliminated from the photostationary mixtures by deep red light, while no significant changes were caused in the spectral region characteristic of the hypsoproduct (Figure 3 curves b and d). Therefore, we produced four photostationary states sequentially: (1) after blue, (2) after blue and then deep red illumination, (3) after red, and (4) after red and then deep red illumination. In Figure 6, the results of such an illumination sequence are shown. Curve a is the difference between states 2 and 3; curve b corresponds to the difference between states 4 and 3. These two spectra are then subtracted (curve c), providing a difference spectrum that is virtually free of the fine structure changes and the bathoproduct, and thus can be considered as a pure hypsoproduct *minus* HR_{578} difference.

Temperature Dependence of the Yield of the HR Bathoproduct. When the photostationary mixture produced by blue light at 80 K was warmed to 140 K, the difference spectrum taken at 140 K indicated the presence of the bathoproduct (not shown). This difference spectrum resembled that produced by blue light illumination at 140 K except that the amount of bathoproduct was about twice at this temperature as at 80 K. Since the HR bathoproduct decays only over several hours at 140 K [cf. following paper (Zimányi & Lanyi, 1989)], this implies that the quantum efficiencies of the forward and reverse photoreactions, which produce the equilibrium $HR_{578} \leftrightarrow$ bathoproduct, are temperature dependent, contrary to what was observed for rhodopsin and BR (Hurley et al., 1977; Iwasa et al., 1979, 1980).

Discussion

At room temperature, retinal proteins are characterized by single broad, diffuse visible absorption bands. There are at least three possible reasons for the absence of fine structure in the spectra. First, torsional freedom around the C6–C_7 single bond was experimentally shown to be responsible for the spectral broadening in retinyl polyenes (Christensen & Kohler, 1973). Theoretical calculations demonstrated that this intrinsic spectral broadening originates from the very shallow potential well for the C6–C7 bond torsion in the ground state, and a much deeper potential surface in the excited state, with a minimum significantly displaced relative to the ground-state

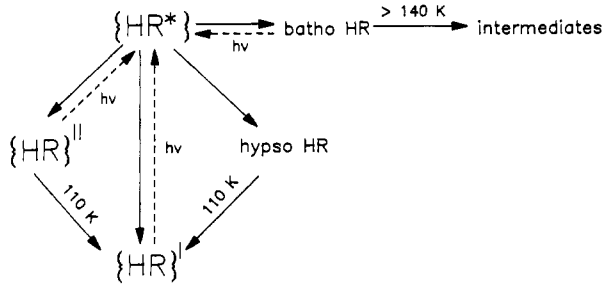

FIGURE 7: Tentative scheme of reaction pathways of HR at low temperature. Brackets symbolize the ensemble of conformational substates for a distinct chromophore state. $\{HR\}^{I}$ is the equilibrium distribution of conformational substates at 80 K. {HR*} stands for the excited state. The first intermediate of the HR photocycle, HR_{600}, is designated as batho-HR, and the blue-shifted form detected in this work is indicated as hypso-HR. $\{HR\}^{II}$ stands for the light-induced distribution of conformational substates of the parent species at 80 K. Solid arrows are thermal steps; interrupted arrows refer to photoreactions.

minimum. This allows a whole range of average angles between the planes of the polyene chain and the ionone ring, and also high-intensity transitions to the higher energy, closely spaced, anharmonic vibrational levels of the excited state (Warshel & Karplus, 1974; Birge et al., 1982). Second, Birge et al. (1982) suggested that the intrinsic broadening of the spectrum in visual pigments and BR originates from the barrierless excited-state potential surface for double bond isomerization of the retinal, rather than from the C6–C7 single bond torsion. Third, there are a large number of possible conformational substates in a protein, which are rapidly interconvertible at room temperature (Austin et al., 1975). It is reasonable to suppose, therefore, that there may be a variation of environmental constraints on the retinal, manifested in slightly different steric hindrance and electrostatic interaction experienced by the chromophores of different molecules in the ensemble. This kind of an effect accounts for an estimated 12% of the broadening of the absorption band in BR, as demonstrated recently by hole burning in the distribution of conformational substates and subsequent monitoring of the retinal ethylenic stretch frequency of the ground state in the infrared region (Ormos et al., 1987). In each of these alternatives, the broadening of the absorption band and the concomitant loss of fine structure are expected to be temperature dependent.

The predicted narrowing of the absorption band and appearance of shoulders upon cooling were indeed demonstrated for HR (Figure 1A). Unexpectedly, the results also show that the vibrational structure in the spectra diminishes and changes on illumination at 80 K. Figure 7 provides a tentative scheme to describe the complex photoreactions of HR at 80 K. No discrimination is made between the 13-cis and all-trans chromophores, since we could not resolve differences between red- and blue-adapted samples.

Let us suppose that as the temperature is decreased to 80 K, a characteristic ensemble of conformational substates of HR becomes populated (designated as $\{HR\}^{I}$ in Figure 7). At this temperature, no thermal interconversion between substates is possible. The broadening of the absorption spectrum is less pronounced at low temperature, thereby allowing the resolution of some of the vibrational structure of the retinal. When the sample is illuminated, a photostationary state which includes the bathointermediate is reached via the excited state, {HR*}. The concentration of batho-HR depends on the color of the light, but remains low compared to the concentration of the parent species. During the illumination, however, a dynamic equilibrium between these two species allows multiple forward and backward reactions. Let us suppose that the photoreactions facilitate transitions between conformational substates of the protein, thereby altering their distribution. [In fact, fast relaxation of excess vibrational energy from the excited state of retinal to the protein moiety in rhodopsin has been proposed by Birge and Hubbard (1980).] The new distribution, $\{HR\}^{II}$, will be frozen again after the light is turned off. Deep red illumination appears to produce hole burning in this new distribution at the red edge of the spectrum, an effect that is most conspicuous after exposure to red light when little of the bathointermediate is formed. When the sample is warmed and kept at 110 K, the conformational substates eventually relax into an ensemble characteristic of this temperature, from which $\{HR\}^{I}$ can be regained by cooling back to 80 K.

We suggest that it is the altered ensemble of available protein conformations that is monitored by the changes of the spectral fine structure of the chromophore. This model is corroborated by the results of Ormos et al. (1987), who have shown that in BR the transition range from "dynamic" to "frozen" distributions of conformational substates is between 100 and 150 K, the temperature range in which our changes occur also (Figure 1). Theoretical calculations show that subtle variations of the interaction of retinal and its environment can cause dramatic changes in the fine structure of the chromophore spectrum (Warshel & Karplus, 1974). Specifically, the constraint on the equilibrium torsional angle of the C6–C7 single bond of retinal, and the allowed range of this torsional angle by environmental effects, can significantly modify the fine structure of the spectrum. In this context, it is especially interesting that in buffer containing nitrate but not chloride, where the negative charge bound to site II in the vicinity of the β-ionone ring is presumed missing, we found much less variation of the fine structure of the absorption band upon both cooling and illumination. However, the broadening upon illumination was observed in this case also, as well as the hole burning effect of deep red light after red or blue illumination. Therefore, we conclude that, although similar structural changes may take place during illumination at 80 K in the chloride- and nitrate-containing samples, electrostatic interaction with the negative charge of the anion occupying site II makes the retinal chromophore a more sensitive indicator of these changes.

It is interesting to speculate on the nature of the HR hypsoproduct. It is not likely to be a precursor of the HR bathoproduct, since (1) it arises at longer times of illumination than the bathoproduct, and (2) if the hypsoproduct could be thermally converted to the bathoproduct at 110 K, the new equilibrium at this temperature would have been shifted toward the bathointermediate. One possibility is that the illumination at 80 K populates yet another set of conformational substates of the protein where special constraints or charge distribution induces an overall blue shift of the absorption band. Although such an explanation was proposed for hypsorhodopsin (Cooper, 1983), it is not likely for hypso-HR because a structureless difference spectrum characterizes this form (Figure 6, curve c). Another possibility is that hypso-HR is a distinct photointermediate, as argued by others for hypsorhodopsin [for example, see Tsuda et al., (1980)]. The magnitude of the blue shift cannot be, of course, estimated from the difference spectrum for the hypsoproduct in Figure 6, since the fraction of protein converted is not known.

The amount of the bathoproduct in the photostationary states appears to be temperature dependent: at 80 K, substantially less bathointermediate was produced than at 140 K under similar illumination conditions. In contrast, in ver-

tebrate rhodopsin and BR, essentially the same amount of bathoproduct is formed at 10 K and 77 K, and the difference spectra are also similar (Iwasa et al., 1979, 1980). No light-induced vibrational changes like the ones we report here for HR are seen in BR at 77 K (Iwasa et al., 1979; this study). Thus, we believe that the photoisomerization leading to the formation of the bathointermediate in HR takes place in a more confined environment than in other retinal proteins. At low temperatures, where relaxation between conformational substates of opsin is not possible, the quantum efficiency of the HR → batho-HR transition may be decreased by the relative rigidity of the protein.

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