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Minireview

Photointermediates of Visual Pigments

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Much progress has been made in recent years toward understanding the interactions between various proteins responsible for visual transduction which are initiated by an activated state of visual pigments. However, the changes which take place in the visual pigments themselves to convert them to the activated state are more poorly understood. Many spectroscopic techniques have been applied to this problem in recent years and considerable progress has been made. A major goal of these efforts is to understand at which stages protein change occurs and to characterize its structural features. In the visual system evidence is accumulating, for example, that chromophore independent protein change begins immediately prior to lumirhodopsin formation. Considerable insight has been gained recently into the early intermediates of visual transduction and the stage is set to achieve similar understanding of the later intermediates leading to rhodopsin's activated state.

KEY WORDS: Rhodopsin; bathorhodopsin; lumirhodopsin; BSI; BL; heptahelical receptors; vision; retinal; bleaching intermediates.

INTRODUCTION

The photoreceptor protein rhodopsin consists of one molecule of the 11-*cis* isomer of retinal covalently bound through a protonated Schiff's base linkage to a lysine of the apoprotein, opsin. Rhodopsin occurs as a transmembrane protein in disk vesicles stacked in the rod outer segments of photoreceptor cells which cover the retina. When a molecule of rhodopsin is photolyzed, changes occur in the protein beginning at the retinal chromophore but propagating eventually to rhodopsin's cytoplasmic surface where molecules of G-protein are activated. These in turn activate phosphodiesterase which depresses cyclic GMP concentrations in the cell. This results, finally, in the closure of sodium channels in the cell plasma membrane, an act which constitutes the neural response to light. The entire process involved in converting a light signal to a neural signal is referred to as visual transduction. While the last decade has seen remarkable progress in understanding these intermolecular processes of transduction, the primary processes of visual transduction occur within the photoreceptor protein itself, and it is these features that we address here.

The specific action of light on rhodopsin is confined to isomerization of the bound retinal to the *all-trans* form. When this occurs, the spectroscopic properties of the protein change, and these changes can be used to probe structural features resulting from isomerization. Succeeding relaxations lead to structures whose evolution can also be followed spectroscopically. These characteristic structures which occur after photolysis constitute the photolysis intermediates, or what has traditionally been called the bleaching intermediates, of rhodopsin. The process is referred to as bleaching because the metarhodopsin II (meta II)[2] intermediate, the form which ultimately activates the G-protein, has essentially lost the visible color originally present in rhodopsin. While other

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[2] Abbreviations used: BSI, blue-shifted intermediate (of rhodopsin photolysis); batho, bathorhodopsin; HOOP, hydrogen out of plane; lumi, lumirhodopsin; meta I, metarhodopsin I; meta II, metarhodopsin II; photo, photorhodopsin (or other red-shifted precursor to batho).

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changes occur after meta II, on the way to the final products, opsin and *all-trans* retinal, here we focus on the intermediates up to and including meta II since they have a direct role in the visual transduction process.

Meta II has assumed disproportionate significance relative to other intermediates because we recognize that it has undergone conformational change which alters its interactions with other proteins such as G-protein and rhodopsin kinase. However, interest should not be restricted to meta II because knowledge of the intermediates which precede it will ultimately provide a mechanistic understanding of the visual transduction process. The earlier intermediates have received less attention only because they remain in the initial phases of characterization. While the current state of characterization of these intermediates may seem primitive in terms of structural detail, it should be recognized that in the vast majority of related receptor systems even the detection of intermediates has proven elusive.

Rhodopsin is one member of a family of heptahelical transmembrane receptor proteins (Henderson and Schertler, 1990). Many of these proteins are stimulated not by a physical process which alters the conformation of a bound prosthetic group but rather by the reception of an agonist molecule from the surrounding medium. The nature of their diffusional activation is such that preparation of high concentrations of homogeneous intermediates is difficult, making their spectroscopic characterization impossible. Nevertheless, the similarities within this heptahelical family make it plausible that intermediate stages may exist in all its members, and for the present we must infer their character from analogy with the visual system.

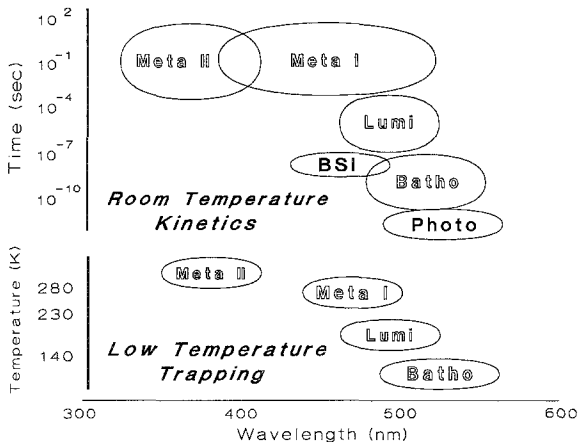

Fig. 1. Comparison of bovine rhodopsin photolysis intermediates observed at low temperature and at room temperature. The bottom of the figure shows the progression of intermediates observed when rhodopsin is photolyzed at cryogenic temperatures and then allowed to warm up slowly, noting the appearance of new intermediates spectrophotometrically. Plotted there are approximate areas corresponding to the temperature stability ranges of the intermediates versus the approximate absorbance range of the intermediates. The top of the figure shows the progression of intermediates observed when rhodopsin is photolyzed at room temperature and the intermediates are detected by time-resolved absorbance measurements. Plotted are approximate areas corresponding to the time ranges during which the intermediates occur versus their absorbance ranges. Two new intermediates are present in room temperature measurements which have never been trapped at low temperature, photorhodopsin and BSI. There is a certain amount of uncertainty in the top left portions of both plots since the meta I and meta II intermediates are dramatically affected by the presence of detergents while the batho and BSI decays are not. Definitive data on these intermediates can only come from studies of the more physiologically relevant but experimentally more challenging membrane system.

SIGNIFICANCE OF INTERMEDIATES

Any complex process can be broken down into more elementary subprocesses. Chemical reactions are analyzed in terms of subprocesses connecting discrete states having relatively well-defined structures. In simple chemical systems such intermediate structures can often be postulated before they are actually observed. When the nature of a chemical intermediate is proposed, it can often be observed directly using chemical approaches specifically designed to stabilize the intermediate itself. For more complex biophysical systems the intermediates have usually been detected before structures could be proposed. In either case, however, the goal is to understand the system in mechanistic terms. In biophysical systems, experimental observation of intermediates benefits from the fact that free-energy barriers separate the intermediate structures, and thus they are stable over limited ranges of temperature and/or time. It is important to keep in mind that these free-energy barriers are themselves temperature dependent and so at some temperatures particular intermediates may not build up sufficient concentrations to be detected. Figure 1 compares the rhodopsin intermediates detected in low-temperature trapping experiments to those observed at room temperature.

A variety of spectroscopic techniques have been used to characterize intermediates, but all of the rhodopsin photolysis intermediates were first detected using UV/visible spectroscopy. This technique has the

advantages of high sensitivity and outstanding time resolution. The disadvantage of UV/visible spectrophotometry, low information content, can be overcome by subsequent application of other methods such as resonance Raman, FTIR, circular dichroism, or NMR spectroscopies once the stability ranges of intermediates have been characterized using UV/visible absorbance. Given the potential structural complexity of the photolysis intermediates, it is important that a detailed understanding of them be based on a wide variety of monitoring techniques. Unfortunately some techniques are applicable only over a more limited time/temperature range than are UV/visible measurements, and for these a promising approach is to modify rhodopsin so that a particular intermediate is stabilized, making it accessible to study by more techniques. In such cases, though, it is important to keep in mind that UV/visible characterization of samples, to determine which intermediates are present, must precede the use of other methods, and hence in this article we will concentrate on UV/visible results.

A further limitation we impose on the scope here is to restrict ourselves mainly to the necessary and sufficient intermediates for visual transduction. To be a necessary intermediate, the structure must occur under conditions approximating physiological ones. Thus we exclude products such as hypsorhodopsin which occur *only* at high photon fluxes and/or low temperatures. Further, to be a necessary intermediate the structure must occur at or before the meta II stage where the transduction process leaves the rhodopsin molecule. Finally, to be sufficient an intermediate must be shown to bleach, i.e., it must go on to produce meta II rather than to fall back to rhodopsin.

In the following sections we will discuss intermediates which have been discovered to exist in different time regimes following absorption of light by rhodopsin. It may be useful to keep in mind the following "classical" mechanism of phototransformations based on low-temperature trapping studies.

$$\text{rhodopsin} \xrightarrow{h\nu} \text{batho} \longrightarrow \text{lumi} \longrightarrow \text{meta I}$$

$$\rightleftharpoons \text{meta II} \ldots$$

It will be clear that in recent years new intermediates have been found through the use of time-resolved spectroscopy which show that the true mechanism is more complex than this classical picture.

PICOSECOND INTERMEDIATES

There have been persistent reports of a red absorbing intermediate which decays in picoseconds to bathorhodpsin (batho). Since batho is also a red absorber (see Fig. 1) the absorbance changes accompanying this very early process are small. Unfortunately there seems to be little consensus among different observers that the same very early species is being monitored. If multiple, red absorbing precursors to batho exist, differences between them certainly seem beyond the resolution of currently available data. Consequently it seems reasonable here to call them all by the most widely accepted name, photorhodopsin (photo). At this point there seems to be no certainty that there either are or are not multiple, red-shifted precursors to batho.

The earliest report of photo observed it only at temperatures below 20 K (Peters *et al.*, 1977). The absence of photo at higher temperatures in that work (disagreeing with more recent observations) may have been due to limitations in signal-to-noise ratio of the experiments. Similarly, different results obtained subsequently for the photo lifetime may be within experimental uncertainties (Monger *et al.*, 1979; Kandori *et al.*, 1989; Yan *et al.*, 1991). Support for this idea comes from the most recent measurement made with a laser pulse duration which was short compared to the observed lifetime. The lifetime value so obtained, 3 ps (Yan *et al.*, 1991), is consistent with the idea that previous experiments which used longer excitation pulses could have agreed with this value if deconvolution using the laser pulse shape had been performed.

At this point it remains to be proved whether photo is actually a sufficient bleaching intermediate in the sense that it does not return to rhodopsin. The original photo observations concluded that an excited state of rhodopsin might account for photo. Such a species might not quantitatively produce the *all-trans* conformation. Results from the 11-*cis* locked synthetic pigment provide evidence in this regard, but the interpretation is still not clear. Yoshizawa *et al.* (1984) did not observe photo in the locked pigment, consistent with the suggestion that formation of photo requires chromophore isomerization. Thus, photo would be considered a pigment intermediate rather than an excited state. Buchert *et al.* (1983), however, observed a red-shifted absorber with photo-like properties in the locked pigment system but interpreted it as an

excited state species rather than a bleaching intermediate. If the identification of this intermediate with photo is correct, this implies that photo is not a sufficient intermediate. Hopefully the recent improvements in time resolution on the picosecond time scale will lead to clarification of the properties of photo. Theoretical ground work has been laid for interpretation of changes on this time scale by the calculations of Birge *et al.* (1988).

BATHORHODOPSIN, BSI, AND LUMIRHODOPSIN

Batho is the first intermediate which definitely contains the *all-trans* form of the chromophore. Evidence for this conclusion comes from the experiments with the 11-*cis* locked synthetic pigment, resonance Raman (Mathies *et al.*, 1976), and from comparison of the optical spectra and decay rates of the batho products formed from 11-*cis* and 9-*cis* (isorhodopsin) pigments (Hug *et al.*, 1988). Both rhodopsin and isorhodopsin form batho products with virtually identical properties. Since only the *trans* isomer can be reached by a single *cis–trans* isomerization from both 11-*cis* and 9-*cis* pigments, the identity of their batho products is strong evidence for batho's *trans* character.

There is also little doubt that batho contains a conformationally strained chromophore since resonance Raman (Palings *et al.*, 1987) and FTIR (Bagley *et al.*, 1985) show hydrogen out-of-plane (HOOP) bending frequencies which are characteristic of a strained *trans* polyene (Eyring *et al.*, 1982). The large negative circular dichroism observed for batho (Yoshizawa and Shichida, 1982) could also be explained by the chromophore being twisted so that the double bonds follow a left-handed helix. Such a structure formed by 20° twists in the same direction about consecutive retinal single bonds has been proposed to account for the resonance Raman spectrum of batho (Palings *et al.*, 1987). A twisted structure for batho is attractive for a number of reasons, one of which is that batho is known to store a large fraction of the original photon energy. The potential energy involved in the bond distortions of a twisted molecule is an excellent vehicle for such storage. Recent NMR results (Smith *et al.*, 1991) as well as theoretical grounds (Birge *et al.*, 1988) support the idea that the energy storage in batho is preponderantly in the form of distortion of the chromophore.

A helically twisted structure at the batho stage is also supported by an important boundary condition of isomerization, i.e., that the retinal must somehow accommodate 180° of torsion until the ends of the molecule can move sufficiently to release it. Movement of the β-ionone ring is presumably slow because of the large number of contacts in that region. Further, resonance Raman (Palings *et al.*, 1987) and NMR (Smith *et al.*, 1991) also show little movement of the Schiff's base charge at the batho stage, possibly since it is hindered by forces associated with the large electrostatic interaction. A uniformly twisted chromophore at the batho stage, for example, would allow the 180° torsion of isomerization to be disposed of in 20–30° twists of the single bonds of the chromophore, allowing the ends of the chromophore to relax at a later stage. While a single *s-cis* configuration could also allow the 180° isomerization torsion to be accommodated, as has been proposed (Liu and Asato, 1985), this one-bond solution seems less likely to be able to store the energy required at this stage without a major contribution from electrostatic effects which NMR (Smith *et al.*, 1991) shows are small. The presence of HOOP modes in batho (Palings *et al.*, 1987; Bagley *et al.*, 1985) also seems inconsistent with such a solution. However, the decay product of batho may involve an *s-cis* conformer and, as discussed below, the features of this decay provide further support for a helically twisted model of batho.

It is now clear that at room temperature batho does not decay directly to lumirhodopsin (lumi), the next intermediate observed in low-temperature trapping experiments. Instead, it first passes through a blue-shifted intermediate (BSI) before reaching lumi (Albeck *et al.*, 1989; Hug *et al.*, 1990; Einterz *et al.*, 1990; Randall *et al.*, 1991). The mechanism which describes events in the first microsecond following excitation is therefore

$$\text{rhodopsin} \xrightarrow{h\nu} \text{photo} \longrightarrow \text{batho} \rightleftharpoons$$

$$\text{BSI} \longrightarrow \text{lumi} \ldots$$

Spectra of the batho, BSI, and lumi intermediates are shown in Fig. 2. It is interesting that BSI is not seen at low temperatures. This is due to the fact that there is a substantial back-reaction rate from BSI to batho and at low temperatures the equilibrium constant shifts so that BSI never accumulates. Thermodynamically this arises because BSI is higher in both enthalpy and entropy relative to batho (Hug *et al.*, 1990). At room temperature the free energy due to the entropy increase overcomes the unfavorable

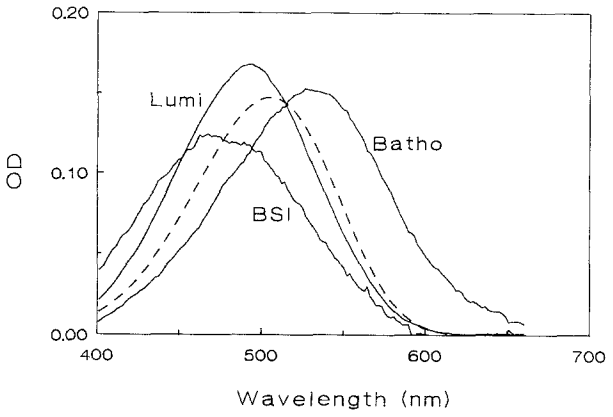

Fig. 2. Absorption spectra of bathorhodopsin, BSI, and lumirhodopsin. The curves show the spectrum that would result if the quantity of rhodopsin whose spectrum is shown by the dashed line were converted completely into the particular intermediate which labels each curve. Of course at most times a mixture of intermediates coexists and hence the spectra observed in practice are composites of these.

enthalpy term and K_{eq} is 1.4. Even with this K_{eq}, at room temperature lumi decay is fast enough that the presence of BSI is only indicated by spectral perturbations rather than the appearance of entirely new peaks in the spectrum. While these perturbations were reported some time ago (Horwitz *et al.*, 1983), they were only interpreted in terms of a new intermediate (Hug *et al.*, 1990) after synthetic pigments containing 13-demethyl (Einterz *et al.*, 1990) and 5,6-dihydroretinals (Albeck *et al.*, 1989) were studied and found to stabilize the BSI intermediate at room temperature. Previously, low-temperature measurements of the 13-demethyl pigment had also managed to trap the equilibrated mixture of batho and BSI, but at that time it was designated as the BL intermediate (Shichida *et al.*, 1981). Since then BSI has been shown to occur in a wide range of synthetic pigments (Randall *et al.*, 1991), as well as after photolysis of human rhodopsin (Lewis *et al.*, 1991). Direct observation of the back-reaction from BSI to batho has been accomplished in the rhodopsin system by using a second light pulse to photolyze away the batho from a pre-equilibrated batho/BSI mixture and then observing return of the red, batho absorbance backflowing from BSI (Lewis *et al.*, 1990).

A notable feature of the BSI intermediates observed in a variety of artificial pigments (in which the retinal moiety is replaced by a retinal analog) is that while their formation rates seem to depend strongly on structural details of the chromophore, the decay rate seems relatively chromophore independent (Randall *et al.*, 1991). These observations have led to the idea that the rate-limiting step in BSI decay is a protein relaxation. This interpretation is supported by observations on human rhodopsin, the only species other than bovine which has been studied in sufficient detail to test the effects of protein structure on BSI kinetics. Human rhodopsin differs in 12 amino acid positions within the transmembrane helical sections of the protein. Thus, it is interesting that the BSI decay rate was reduced 30%, consistent with the idea of a protein relaxation governing the rate at this stage. Mechanistic understanding of how the specific amino acids which differ in these two proteins affect this rate depends on the specific nature of the protein change, a point as yet unclear. Improved description of the state of the chromophore at the BSI stage (which presumably has removed a barrier to the protein change) should help to characterize this protein change in more detail.

Linear dichroism studies of rhodopsin have led to an understanding of how the transition dipole moment of the chromophore changes as the early intermediates progress up to lumi (Lewis *et al.*, 1989). Since the transition dipole of the retinal chromophore should, to a large degree, follow the long axis of the molecule, information about how the chromophore moves can be inferred from these data. A projection of these results onto the conventional heptahelical model of the protein is shown in Fig. 3. From the lower part of that representation, it can be seen that formation of batho involves a rotation of the chromophore out of the plane of the membrane. Formation of BSI then rotates the chromophore in the opposite direction, bringing the transition dipole nearly into the plane of the membrane. This is followed by a return of the transition dipole at the lumi stage to roughly the original position it had in rhodopsin. The fact that the rotation of the chromophore out of the membrane plane is correlated with a red shift of its spectrum is suggested in the lower part of the figure by a hypothetical counterion position which blue-shifts the spectrum as the Schiff's base moves toward it. However, other mechanisms might account for this as well. The motions of the chromophore as viewed from above the membrane plane are also shown in Fig. 3, but these seem uncorrelated with the λ_{max} of the intermediates. This viewpoint is more interesting when considered in terms of the progression of intermediates, as it seems that more or less systematic movement of the chromophore axis is occurring as the

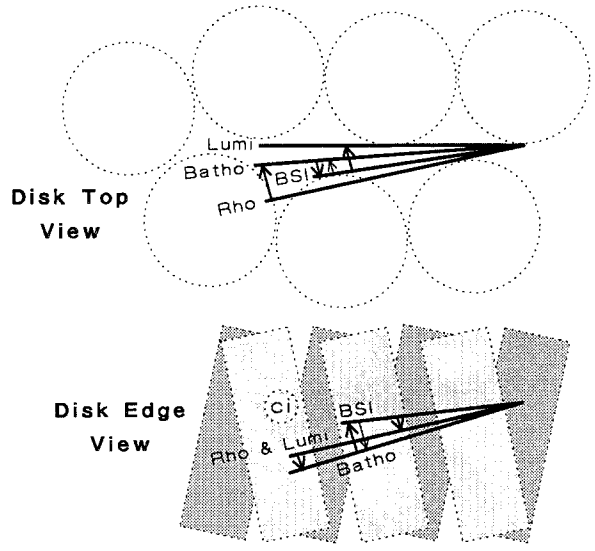

Fig. 3. Transition dipole directions in the early photointermediates observed after rhodopsin photolysis. Linear dichroism measurements after photolysis of rhodopsin in membrane suspensions allow the directions of the transition dipole moments of the intermediates to be located relative either to the main band transition dipole direction (photolysis at 477 nm) or to the *cis* band transition dipole moment direction (photolysis at 355 nm) (Lewis *et al.*, 1989). Since the original transition dipole moment directions of rhodopsin have already been determined relative to the membrane plane (Liebman, 1962; Michel-Villaz *et al.*, 1982; Chabre and Breton, 1979), the intermediate transition dipole moment directions can be projected onto the conventional heptahelical model of the molecule. Given that there is an apparent correlation of the spectral shift in absorbance of the intermediates with the rotation of the transition dipole moment out of the membrane plane, a Schiff's base counterion position is shown which could account for this effect.

bleaching sequence proceeds. One model which could account for these movements is that the chromophore needs to rotate in the disk membrane plane to reach lumi. In the process it encounters a barrier which it must overcome (rotating into the plane of the disk) at the BSI stage, at which point protein change can occur allowing final progress to lumi. Alternatively, the chromophore can fall back to the original position, returning the system to the batho intermediate (i.e., to establish the batho–BSI equilibrium).

The key to understanding any such protein change is a better picture of the BSI intermediate. Progress in this direction has already been made by FTIR studies of the stabilized BSI formed from the synthetic 5,6-dihydro (Ganter *et al.*, 1991) and 13-demethyl (Ganter *et al.*, 1990) pigments. Prominent in both results is the reduction of HOOP modes, revealing a relaxation of the chain torsion present at the batho stage. Relaxation of torsion at the BSI stage also can explain the peculiar thermodynamics of the BSI intermediate relative to batho. One mechanism by which BSI can be higher in entropy than batho is if the chromphore has more vibrational modes which are thermally populated at the BSI stage. The helical distortion which we propose to exist at the batho stage may stiffen vibration of the chromophore's bending modes, moving them above the energy of room-temperature excitation. At the BSI stage, when the torsion is relaxed, these modes become thermally accessible, providing the entropic driving force of BSI formation. This notion can be combined with the motions inferred from the linear dichroism data discussed above. If the barrier traversed at the BSI stage is conceived of as a steric one, BSI's capability to absorb more thermal energy due to its lower vibrational energy spacings may play a direct role in the mechanism. This thermal energy may exert an actual displacing force on the barrier, much as water vaporized in a cylinder can displace a piston.

Any test of these ideas requires a clearer structural picture of BSI. If the 180° twist of isomerization has not been relieved, an obvious candidate for the BSI structure is an *s-cis* conformation. It is interesting to speculate even further that the 10-*s-cis* conformer might be involved. Grounds for these idea come from the reported photostability of the 13-demethyl BSI intermediate (Shichida *et al.*, 1981; Lewis, J. W., Hug, S. J., and Kliger, D. S., unpublished results). Photostability would be expected for the 10-*s-cis* conformer since isomerization about either the 9–10 or 11–12 double bonds results in a very hindered conformation if the 10-*s* bond has a *cis* configuration. Obviously more experiments are needed to test this hypothesis, but it is encouraging to see how FTIR has supplemented the original UV/visible results on BSI.

LATER INTERMEDIATES

Unfortunately, at this point the route between lumi and meta II is not as clear as the events up to lumi. The simple picture proposed by Matthews *et al.* (1963) that lumi (λ_{max} = 497 nm) decays to meta-rhodopsin I (meta I, λ_{max} = 478 nm) which then decays into equilibrium with meta II (λ_{max} = 380 nm) was principally based on low-temperature measurements. Room-temperature measurements conducted with sufficient signal-to-noise ratio on membrane suspensions of rhodopsin have consistently found that

the low-temperature mechanism is inadequate (Stewart *et al.*, 1977; Lewis *et al.*, 1981; Straume *et al.*, 1990), but there has been little agreement about how to improve the reaction scheme. It is worthwhile discussing the difficulties that have been encountered because, in the process of trying to solve these problems, interesting properties of the system have been discovered.

While the submicrosecond kinetics and spectra observed after rhodopsin photolysis are unaffected by whether rhodopsin is in the native membrane or in detergent suspension, the decays following lumi are dramatically speeded up in most detergents. This means that physiologically relevant measurements must be made in the presence of membranes which scatter light. While the light scattering can be reduced by sonication of membrane samples, this also affects the observed kinetics somewhat (Lewis *et al.*, 1981). Both the detergent and sonication effects are consistent with the idea that these later decays involve protein changes which are sensitive to rhodopsin's lipid environment. Further evidence for this idea comes from the pressure dependence observed for the meta I–meta II equilibrium constant (Lamola *et al.*, 1974) which indicates a large volume increase on meta II production.

The fact that lumi is formed within a microsecond at room temperature dictates that measurements of lumi decay must be made with microsecond or better time resolution. To obtain reasonable signal-to-noise ratios in measurements, shot noise considerations associated with this time constant require very bright probe sources, particularly since most photons are lost due to light scattering and go undetected. Samples must be probed by this bright source for approximately 100 ms since time constants of 20 ms are usually obtained for the slowest process associated with meta II production. It is easy to show that if a continuous probe light source is used, at the intensities required for signal-to-noise ratios of ~ 200 (the level required to observe deviations from the simple model), significant bleaching by the probe source can occur. If a continuous probe source is used, care must be taken in experimental design that this does not perturb the kinetic results. Alternatively, a pulsed probe source can be used.

Rotational diffusion is a second source of potential errors in measurements of lumi decay. Rhodopsin rotates in the membrane with a time constant of $\sim 5\,\mu s$ (Cone, 1972) and the membranes themselves rotate with time constants on the order of milliseconds or longer. The resulting decay of linear dichroism could be misinterpreted as decay of an intermediate unless extreme care is taken (Lewis and Kliger, 1991). However, at this point it appears that neither the above-mentioned artifacts nor a range of others involving heterogeneity of rhodopsin seems to explain the observed deviations from the simple model described above. While some of the deviations are confined to restricted portions of the spectrum (further discussed below), others are broadly apparent. In particular, rather than two exponentials decays being observed over the range from $1\,\mu s$ to ~ 1 s, corresponding to lumi and meta I decays, careful studies find at least three exponentials are required to describe the data. The fact that similar results have repeatedly been obtained in different laboratories implies that the observed behaviour is an intrinsic property of rhodopsin in disk membrane suspension.

Numerous explanations have been proposed for deviations from the simple lumi $\rightarrow$ meta I $\rightleftarrows$ meta II model, usually invoking additional intermediates. In most cases these proposals have been made to reconcile mechanistic aspects of the system, such as the fact that the presumed deprotonation of retinal in meta II formation is associated with net proton *uptake* by the system. Only in a very few cases have the models been based on high-quality kinetic measurements, so it is not surprising that none of the proposed models fits such kinetic data in detail.

Recently a model was reported (Straume *et al.*, 1990) which fit high signal-to-noise ratio kinetic absorbance measurements at 380 nm with the following scheme:

$$\ldots \text{ lumi} \longrightarrow \text{meta I} \begin{array}{l} \rightleftharpoons \text{meta II}_f \\ \rightleftharpoons \text{meta II}_s \end{array}$$

In this model, meta II_f and meta II_s are assumed to be isochromic. Data from the fit were then found to be consistent with separate independent measurements on the final meta I–(meta II_f + meta II_s) equilibrium. The ability to fit both equilibrium and kinetic results shows clear progress since the last major review (Hofmann, 1986). While it was a major advance to find a mechanism which fits the data of Straume *et al.*, there are indications that alternate mechanisms must be considered. One aspect of the above scheme that needs to be addressed is that the extinction coefficient of the meta I intermediate at 380 nm is quite temperature dependent (going from 4%

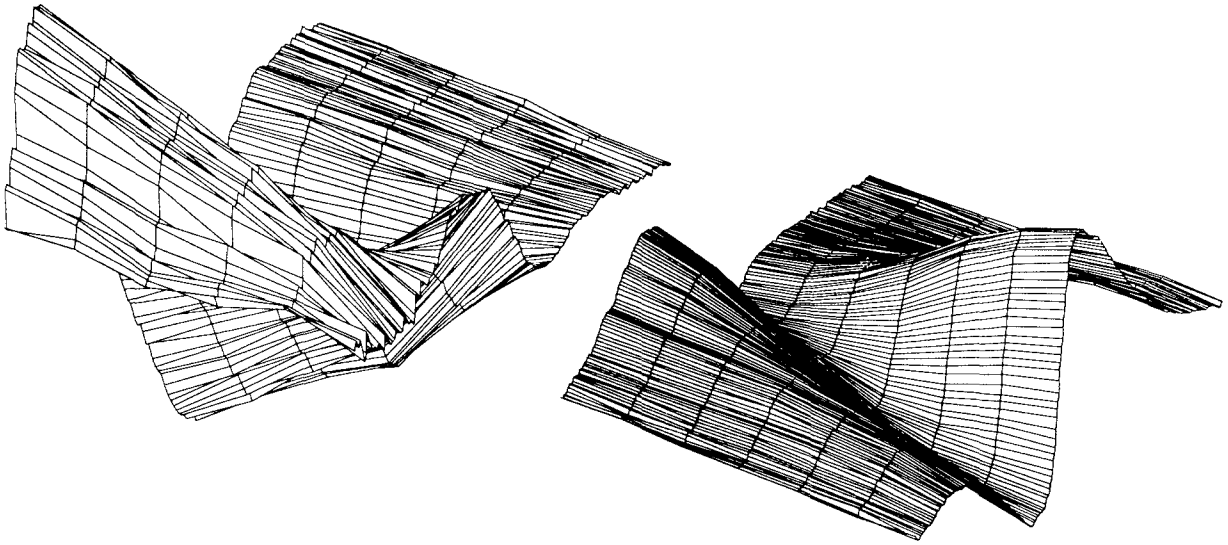

Fig. 4. Optical absorbance changes which follow photolysis of human rhodopsin. The right surface shows (from right to left) difference spectra collected at 20 ns, 40 ns, 80 ns, 160 ns, 320 ns, 640 ns, and 1.28 μs following photolysis of detergent suspensions of human rhodopsin. The spectra run from 400 nm (in the foreground) to 700 nm (in the background). The large positive band in the 20 ns spectrum is due to batho. The small positive band in the foreground is due to BSI. The left surface shows difference spectra collected 1 μs, 2 ms, 6 ms, 10 ms, 20 ms, and 100 ms following photolysis of rhodopsin in sonicated human disk membrane suspension. These spectra run from 360 nm (in the foreground) to 600 nm (in the background). The peak in the 1 μs difference spectrum occurs at $\sim$480 nm and is due to lumi. The fact that a steady decay of absorbance from 1 μs to 2 ms is observed is difficult to reconcile with the lumi decaying to meta I, which should have its absorbance maximum near 480 nm. Results for photolysis of bovine rhodopsin are very similar to the human rhodopsin results presented in this figure.

of the original rhodopsin 498 nm absorbance at 10°C to 32% at 37°C). Similar behavior has been observed previously (Lewis *et al.*, 1981), but this property seems more typical of an equilibrium mixture of meta I and some additional 380 nm absorbing species rather than a well-behaved meta I intermediate.

Measurements of absorbance kinetics at other wavelengths (Lewis *et al.*, 1981, 1991), particularly in the 460–480 nm region, lead to similar conclusions about meta I. Rather than showing the expected increase in absorbance in this region as lumi converts to meta I, a steady decrease in absorbance is observed. This can be seen in the data presented in Fig. 4. Paradoxically, the spectra would seem not to suggest the presence of a new intermediate, but that one of the traditional intermediates, meta I, seems to be absent. Of course meta I must eventually appear since it is detected in equilibrium mixtures at later times, but it seems that the mechanism which best fits all the data will involve new intermediates earlier than have been proposed previously. Clearly more extensive UV/visible measurements on membrane samples will be required before the sequence of intermediates which appears at these later times are fully characterized.

PARALLELS WITHIN THE RECEPTOR PROTEIN FAMILY

Given the intermediates of rhodopsin photolysis described above, what insight can be gained into the as yet undetected intermediates of the other heptahelical receptor proteins? First, it is important to note that rhodopsin itself should be considered to be analogous to a receptor intermediate and not assigned a parallel place with the unbound receptor. Although both these states are the quiescent form of the protein, the binding site has already been sequestered in rhodopsin from chemical attack by hydrophilic molecules like hydroxylamine. This is in distinct contrast to the receptor for a freely diffusing transmitter, since in this case the binding site is presumably exposed. Proceeding, then, to the assumption that it is opsin and the quiescent receptor which are analogous, rhodopsin is already an intermediate which has been stabilized.

While batho has been an extremely important intermediate for the visual pigments, it may not prove significant for chemical receptors. So far as we understand the structural features of batho, it seems to be extremely strained and thus unlikely to occur thermally. If we consider rhodopsin analogous to a

stabilized intermediate of the hormone receptor system, we should probably view batho as an artifact of the sudden resumption of the normal course of reception. Of course, this conclusion is open to reinterpretation later if evidence to the contrary becomes available, but at this point it is plausible that this intermediate is associated with the unique aspects of the photoreceptor. BSI, on the other hand, may prove to be present in other systems. To a large degree this depends on its mechanistic role. If, as we propose here, it has structural features which play a direct role in overcoming a barrier to protein motion, it seems likely to be a universal intermediate. In such a case it becomes crucial to determine the characteristics of the barrier which is involved.

CONCLUSIONS

Recent years have seen significant progress in characterizing early intermediates of visual pigments. Time-resolved absorption spectroscopy has made it possible to detect new intermediates not evident from low-temperature trapping studies. This, added to the information provided by other spectroscopic methods and the use of synthetically modified pigments, has enabled us to gain significant insight into structural features of these early intermediates.

Late intermediates of visual pigments are much more poorly characterized than early intermediates, but recent improvements in measurement techniques show promise for resolving the questions about the transformations of the late intermediates leading to meta II formation. It is quite clear already that the classical picture of the lumi to meta I to meta II transitions is not an accurate description of the physiologically relevant mechanism. Further work, analogous to recent studies of the early intermediates, is needed to elucidate the true mechanism for meta II production.

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