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Photolysis Intermediates of Human Rhodopsin†

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ABSTRACT: Photochemical studies were conducted on human rhodopsin at 20 °C to characterize the intermediates which precede the formation of metarhodopsin II, the trigger for the enzyme cascade mechanism of visual transduction. Human rhodopsin was prepared from eyes which had previously been used for corneal donations. Time resolved absorption spectra collected from 10^{-8} to 10^{-6} s after photolysis of human rhodopsin in detergent suspensions displayed biexponential decay kinetics. The apparent lifetimes obtained from the data are 65 ± 20 and 292 ± 25 ns, almost a factor of 2 slower than the corresponding rates in bovine rhodopsin. The spectra can be fit well using a model in which human bathorhodopsin decays toward equilibrium with a blue-shifted intermediate (BSI) which then decays to lumirhodopsin. Spectra and kinetic rate constants were determined for all these intermediates using a global analysis which showed that the spectra of the human intermediates are remarkably similar to bovine intermediates. Microscopic rate constants derived from this model are 7.4×10^6 s^{-1} for bathorhodopsin decay and 7.5×10^6 s^{-1} and 4.6×10^6 s^{-1} for the forward and reverse reactions of BSI, respectively. Decay of lumirhodopsin to later intermediates was studied from 10^{-6} to 10^{-1} s after photolysis of rhodopsin in human disk membrane suspensions. The human metarhodopsin I ⇄ metarhodopsin II equilibrium appears to be more forward shifted than in comparable bovine studies.

Rhodopsin, the sensory pigment of scotopic vision, is the most successfully characterized receptor protein, particularly in its bovine form. Extending this level of understanding to human rhodopsin is important because mutations affecting the rhodopsin amino acid sequence have recently been shown to cause human disease. It thus becomes vital to characterize human rhodopsin so that the defects in health-related variants can be understood mechanistically. It is also interesting to compare rhodopsins with different sequences because this photoreceptor protein has emerged as a model for other more elusive membrane receptors, strongly posing the question of how amino acid sequence affects membrane protein function. While bovine rhodopsin has been extensively characterized, previous studies of human rhodopsin have been confined to the slower processes occurring after the signal initiated by light absorption has been transmitted to other proteins in the rod outer segment (ROS).[1] Here we characterize the more rapid stages which lead to the activated form of human rhodopsin which triggers the enzyme cascade in the human eye.

Dramatic spectral changes which follow exposure of rhodopsin to light attracted attention as early as the 19th century (Kuhne, 1878). Modern time-resolved spectral studies have expanded our knowledge of these changes and have shown that photolysis leads to a number of discrete intermediate states of the protein (Ottolenghi & Sheves, 1989; Birge, 1990). The importance of understanding the mechanism of rhodopsin function has gained even wider significance in recent years, first because at least one form of retinitis pigmentosa has been shown to be caused by a variety of point defects in the rhodopsin gene (Dryja et al., 1990, 1991; Heckenlively et al., 1991; Ingelhearn et al., 1991; Sung et al., 1991) and second because

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[1] Abbreviations: BSI, blue-shifted intermediate; EDTA, ethylenediaminetetraacetic acid; ROS, rod outer segments; TBS, Tris-buffered saline.

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it is now clear that rhodopsin belongs to a family which includes at least 10 important receptor proteins which are activated by diffusing chemical transmitters (Henderson & Schertler, 1990). In rhodopsin, different isomers of the retinylidene chromophore function similarly to hormone receptor antagonists (11-cis, in the dark) and agonists (all-trans, formed by light). This analogy is strengthened by the observation that chemical binding of retinal to opsin is not obligate to the functionality of rhodopsin (Zhukovsky et al., 1991). Photolysis intermediates of rhodopsin are relatively easily studied compared to analogous intermediates which may occur in the other receptors of this family because of the absence of the diffusion step involved in chemical signaling systems.

Study of human rhodopsin is difficult because of its extremely limited availability (Wald & Brown, 1958), but sequence differences between bovine and human forms [12 amino acids differ in the transmembrane region alone (Nathans & Hogness, 1984)] suggest that direct studies of the human protein are important. Recent advances in high signal-to-noise nanosecond spectroscopy on small, photolabile samples, used in combination with improved methods which double the amount of rhodopsin which can be extracted from human eyes, make this feasible. We thus report the first measurements of the photolysis intermediates in human visual transduction.

Experimental Procedures

Regenerated human rod outer segments (ROS) were obtained in crude form from eyes which had been used for routine, conventional corneal transplant surgery as described previously (van Kuijk et al., 1991). To purify the ROS, the crude extract from six retinas was diluted 1:3 with Tris-buffered saline referred to hereafter as TBS [10 mM tris(hydroxymethyl)aminomethane, 60 mM KCl, 30 mM NaCl, 2 mM $MgCl_2$, and 0.1 mM sodium EDTA, pH 7.0, with 0.01 trypsin inhibitor unit of aprotinin and ~1 mM fresh dithiothreitol added] and spun at 40000g for 20 min. The pellet was resuspended in 2 mL of TBS containing 33% sucrose. After the density of this solution was measured by using a refractometer, the composition was adjusted to 33% sucrose by adding TBS containing 43% sucrose. The ROS suspension was overlaid with TBS and spun at 16000g for 2 h in a swinging bucket rotor. The carpet of ROS that collected between the layers was harvested from above using a syringe with a coarse gauge, flat-tipped needle and avoiding collection of the underlayer. The resulting material was free from hemoglobin Soret band absorbance. Approximately 13 mg of rhodopsin in purified ROS was obtained from the 60 eyes used in this work. Extrinsic membrane proteins were removed by washing the ROS twice with a 1 mM EDTA solution adjusted to pH 7.0. The resulting disks were either solubilized (0.6 mg rhodopsin/mL) in TBS buffer containing 2% octyl β-D-glucopyranoside to produce detergent suspensions or resuspended (0.2 mg rhodopsin/mL) in low salt buffer [10 mM tris(hydroxymethyl)aminomethane, 2 mM $MgCl_2$, 0.1 mM EDTA, pH 7.0] and sonicated as previously described (Lewis et al., 1981) to produce membrane suspensions.

Optical absorption difference spectra ([postbleach at time t] − [prebleach]) at specific time delays after excitation were collected using an optical multichannel analyzer (Lewis et al., 1987b). For up to ~1 μs after photolysis the bleaching intermediates are the same in both detergent and disk membrane suspensions (Einterz et al., 1987). Detergent suspensions were used to obtain spectra for these times since the short detector exposures needed on this time scale require relatively concentrated, optically clear samples for high signal-to-noise ratio results. At later times, the behavior of bleaching intermediates is dramatically affected by the presence of detergents (Stewart et al., 1977), and these intermediates are best characterized in disk membrane suspensions.

Rhodopsin was photolyzed using vertically polarized 477-nm light (7-ns pulse duration, fluence ~5 mJ/cm^2) produced by a dye laser pumped by the third harmonic of a Nd:YAG laser. Apparatus details were as described previously (Hug et al., 1990) with sample temperature maintained at 20 °C. To eliminate absorbance changes due to rotational diffusion of photoselected molecules (Lewis & Kliger, 1991), absorbance difference spectra were measured for both vertical and horizontal probe beam polarizations. $\Delta\bar{A}$, the average absorbance free from rotational artifacts, was then computed using

$$\Delta\bar{A} = (\Delta A_{\parallel} + 2\Delta A_{\perp})/3$$

and is the quantity reported. For the detergent suspension studies (the early intermediates), an optical multichannel analyzer gate time of 10 ns was used. For the disk membrane suspension studies of the late intermediates, the probe flashlamp pulse was delayed relative to the laser pulse, and a much wider optical multichannel analyzer gate (~500 ns) was used. The wider gate was possible because the wavelength distribution of the probe light was profiled using a photographic filter (Lewis et al., 1987a), which increased the signal-to-noise ratio in the red and violet portions of the spectrum. The optical path length for the probe beam was 1 cm and was 0.2 cm in the actinic pulse direction (Hug et al., 1990). The amount of rhodopsin bleached by the laser was determined using Ammonyx LO detergent as described previously (Albeck et al., 1989) so that the directly measured difference spectra could be converted to absolute spectra. Typically, one-third of the rhodopsin originally present was bleached (or converted to isorhodopsin). The measured bleach spectrum was modeled as the removal of rhodopsin with some production of isorhodopsin (a 485-nm absorber). The amount of isorhodopsin which was required to fit the bleach spectrum is reported in the appropriate figure captions.

The uncertainties in the apparent lifetimes observed after bovine rhodopsin photolysis were determined from the reproducibility of a large number of replicate experiments. For the human pigment, the uncertainties were estimated on the basis of bovine experiments at comparable signal-to-noise ratios.

Results

The early time difference spectra we obtained after photolysis of human rhodopsin in detergent suspensions are shown in Figure 1A. The solid curve in Figure 1A clearly shows the presence of a red-shifted absorber similar to bovine bathorhodopsin 20 ns after photolysis. The difference spectra then evolve toward a more blue-absorbing species over the first microsecond. This is clearer when the bleached material's absorbance is added back as in Figure 1B, producing absolute time evolution spectra.

Interpretation of the difference spectra, and even the time evolution spectra, in terms of actual spectra of photolysis intermediates is complicated because at any given time more than one intermediate, as well as unphotolyzed pigment, is present and contributes to the sample absorption. In studies of bovine rhodopsin it has been shown that this problem can be circumvented by determining the kinetics and spectra of photolysis intermediates using a global analysis technique in which absorptions at all wavelengths and at all times are analyzed simultaneously (Hug et al., 1990). This method uses a singular value decomposition technique to determine how

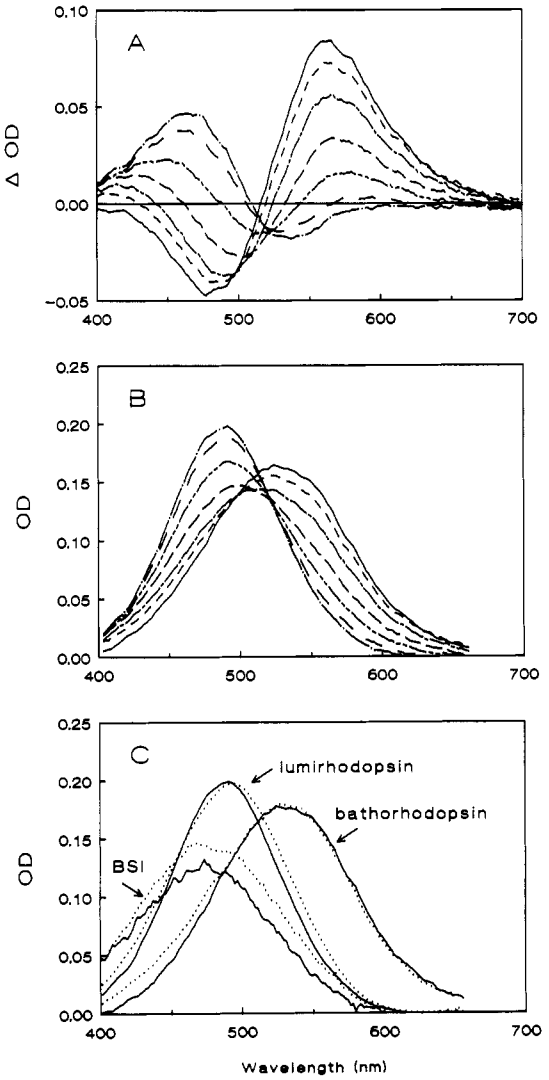

FIGURE 1: Human rhodopsin photolysis in detergent suspensions. (A) Difference spectra collected at 20 ns (—), 40 ns (- - -), 80 ns (– –), 160 ns (– –), 320 ns (– – –), 640 ns (– –), and 1.28 μs (– · –) after photolysis. (B) Time evolution of absolute spectra. These spectra were obtained by adding back the spectrum of the bleached pigment to the difference spectra. Compensation was included for 6% of the original pigment that was converted to isorhodopsin. Line styles are as in panel A. (C) Spectra of the intermediates. Dotted lines show bovine intermediates for comparison. A larger equilibrium constant (1.6) than observed for bovine (1.4) was required to eliminate spurious negative optical density from the BSI spectrum (Randall et al., 1991).

many intermediates contribute to the spectra, what their decay rates are, and, given a proposed mechanism for the interconversion of the intermediates, what the spectra of the intermediates are.

When the difference spectra in Figure 1A are fit with this technique as in previous bovine experiments (Hug et al., 1990), the analysis yields results very similar to those from bovine rhodopsin. As with that system, the absorption spectra of bathorhodopsin, BSI, and lumirhodopsin, the human early photolysis intermediates, are determined (see Figure 1C). Residuals obtained from comparing the fit to the data show that the model adequately describes the human system to within the noise of the measurements. Apparent lifetimes of 65 ± 20 ns and 292 ± 25 ns were obtained for the approach to the bathorhodopsin ⇄ BSI equilibrium and formation of lumirhodopsin, respectively. The microscopic rate constants obtained using an equilibrium constant of 1.6 for the human bathorhodopsin ⇄ BSI equilibrium were 7.4×10^6 s^{-1} and 4.6×10^6 s^{-1}. The rate constant obtained for BSI decay to lumirhodopsin was 7.5×10^6 s^{-1}.

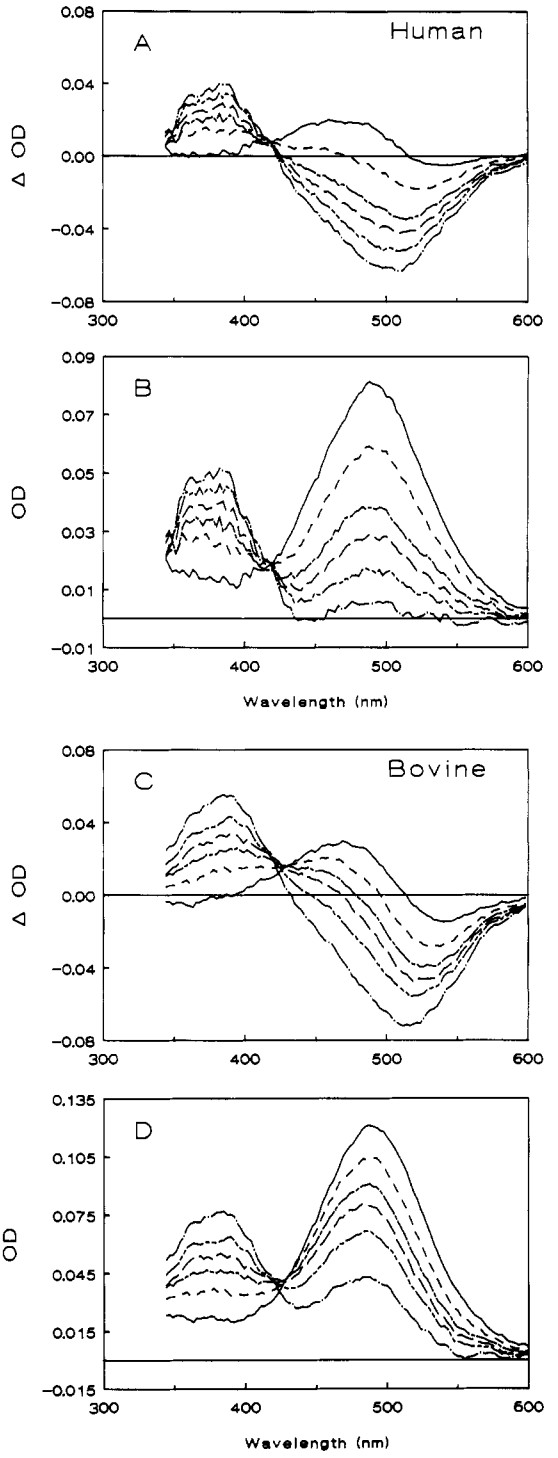

FIGURE 2: Rhodopsin photolysis in disk membrane suspensions. (A) Difference spectra collected 1 μs (—), 2 ms (- - -), 6 ms (– – –), 10 ms (– –), 20 ms (– – –), 100 ms (– · –) after photolysis of human rhodopsin disk suspension. (B) Time evolution of absolute, human disk spectra. These spectra were obtained as for Figure 1B except that 8% of the original pigment was converted to isorhodopsin. Line styles are as in panel A. (C) Difference spectra collected after photolysis of a bovine rhodopsin disk suspension. Line styles are as in panel A. Data was collected on the same day as that in panel A. (D) Time evolution of absolute, bovine disk spectra. These spectra were obtained as for panel B except that 4% of the original pigment was converted to isorhodopsin. Line styles are as in panel A.

Difference spectra obtained at later times after photolysis of human rhodopsin in disk membrane suspension are shown in Figure 2A. Time evolution spectra obtained by adding back the spectrum of the bleached rhodopsin are shown in Figure

2B. Results of comparable experiments on bovine rhodopsin in disk membranes are shown in Figure 2C,D.

Discussion

On the time scale studied here, photolysis of bovine rhodopsin can be discussed in terms of the following scheme of intermediates (Hug et al., 1990; Lewis et al., 1990; Randall et al., 1991; Yoshizawa & Wald, 1963):

$$\underset{498\ nm}{\text{rhodopsin}} \xrightarrow{h\nu} \underset{529\ nm}{\text{bathorhodopsin}} \underset{}{\overset{36\ ns}{\rightleftharpoons}} \underset{477\ nm}{\text{BSI}} \xrightarrow{215\ ns}$$

$$\underset{492\ nm}{\text{lumirhodopsin}} \left[\longrightarrow \underset{477\ nm}{\text{metarhodopsin I}} \rightleftharpoons \underset{380\ nm}{\text{metarhodopsin II}} \right] \ldots$$

Here, bathorhodopsin is the first intermediate which can be shown to contain the isomerized, all-trans form of the chromophore (analogous to the hormone receptor agonist). BSI is a recently discovered blue-shifted intermediate (relative to rhodopsin) which builds up in significant concentrations at room temperature but was not previously detected since it does not accumulate at the cryogenic temperatures originally used to study the early intermediates (Hug et al., 1990). Brackets enclose the intermediates after lumirhodopsin because the scheme shown, based on static, low-temperature measurements (Matthews et al. 1963), has been demonstrated to be inadequate to describe kinetic experiments at physiologically relevant temperatures as used here (Williams, 1975; Applebury, 1984; Lewis et al., 1981; Straume et al., 1990).

One of our first objectives was to determine whether human rhodopsin follows the early part of this scheme up to lumirhodopsin. The data presented in Figure 1 clearly document that the early intermediates of human rhodopsin follow the same general pattern observed in bovine rhodopsin with some quantitative differences. While the lifetimes obtained here are significantly longer than the 36 ± 15 and 215 ± 20 ns observed for bovine rhodopsin, the spectra of the human and bovine intermediates are quite similar (Figure 1C). Given that the kinetics observed for bovine rhodopsin on this time scale do not depend on whether the protein is in detergent micelle or membrane vesicle, the kinetic differences observed between bovine and human forms presumably arise from differences in the more immediate protein environment of the chromophore. Within the transmembrane portion, bovine and human rhodopsins differ at 12 amino acid positions. Of these, 8 of the 12 substitutions in human rhodopsin are to groups with smaller side chains, leading to a net reduction in side-chain bulk which is equivalent to approximately seven carbon atoms. A possible explanation for the slower kinetics of BSI formation and decay observed in human rhodopsin is that the conformational changes in the chromophore are retarded in the more compact human form of the protein. Previous studies of synthetic pigments based on bovine rhodopsin have implied that the BSI decay rate is relatively independent of the chromophore structure and may be controlled by a protein relaxation (Randall et al., 1991). The current results lend support to this idea since the BSI decay rate is reduced 30% by the sequence differences between human and bovine proteins.

The difference spectra at later times in human disk suspensions (Figure 2A,B) are clearly similar to those obtained in bovine disks (Figure 2C,D). Analysis in terms of the "classical" intermediates from the low-temperature model is clearly inappropriate since, as can be seen here for both human and bovine data, the changes from 1 μs to 2 ms do not display the lumirhodopsin → metarhodopsin I difference spectrum that would be reflected in a shifting absorption from 492 to 477. Instead, they show early appearance of a 380-nm-absorbing species. This early 380-nm component has been noted in previous bovine data (Lewis et al., 1981; Straume et al., 1990), and its temperature dependence is incompatible with the existence of a simple metarhodopsin I intermediate as originally proposed. The situation may be analogous to that of the BSI intermediate where low-temperature trapping experiments do not detect an intermediate which is observable at higher temperatures. Given the apparent complexity, it is obvious that more extensive room temperature, full-spectrum studies of bovine disks are required to obtain data of high enough precision to elucidate the exact mechanism on this time scale. What *can* be said regarding the millisecond intermediates of human rhodopsin is that they are quite similar to those of bovine rhodopsin and hence the bovine experiments which implicate metarhodopsin II as the intermediate which activates G-protein (Emeis et al., 1982) are likely to be relevant to the human rhodopsin system as well. It is interesting in this context to note that comparison of the 100-ms traces in Figure 2B,D shows that, within the framework of the historical mechanistic scheme presented above, the metarhodopsin I ⇌ metarhodopsin II equilibrium constant is noticeably greater in the human pigment than in the bovine case. Whether this has significance at the higher temperatures where production of metarhodopsin II should be essentially complete in either species is not clear. Since kinetics on this time scale are affected by more factors than influence earlier time scale data, the observed difference here could be due to either amino acid sequence or membrane composition differences.

Previous studies of human rhodopsin's bleaching intermediates in vitro have been limited to the much slower decays which follow metarhodopsin II (Baumann & Bender, 1973; Crescitelli, 1985). An in vivo technique was able to show that metarhodopsin I decays faster than 2 ms (Pugh, 1975). Although surprising at the time, this result is completely consistent with our conclusions. The early human intermediates are unlikely to be studied in vivo because of the high photon densities required to study shorter lived species. Hence, future studies of these intermediates will require isolation of human rhodopsin in order to provide fundamental information about rhodopsin's role in human visual impairments. Here, we show for the first time that eyes previously used for corneal donation can provide adequate amounts of rhodopsin for these studies.

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