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Research Article

Specific isomerization of rhodopsin-bound 11-cis-retinal to all-trans-retinal under thermal denaturation

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Abstract. The natural ligand of the retinal photoreceptor rhodopsin, 11-cis-retinal, is isomerized to its all-trans configuration as a consequence of light absorption in the first step of the visual phototransduction process. Here we show, by means of difference spectroscopy and high-performance liquid chromatography analysis, that thermal denaturation of rhodopsin induces the same type of isomerization. This effect is likely due to thermally induced conformational rearrangements of amino acid residues in the retinal-binding pocket – possibly implying helical movements – and highlights the tight coupling between 11-cis-retinal and opsin. This effect could have implications in the instability and functional changes seen for certain mutations in rhodopsin associated with retinal disease, and in the stability of the different conformers induced by mutations in other G protein-coupled receptors.

Key words. G protein-coupled receptor; membrane protein; thermal stability; rhodopsin; 11-cis-retinal; all-trans-retinal; retinal-binding pocket.

11-cis-retinal is the universal chromophore responsible for light absorption in the visual process [1]. This chromophore is covalently bound to a lysine in the seventh transmembrane helix of opsin through a protonated Schiff base linkage to form rhodopsin, the photoreceptor protein of the vertebrate retina [2, 3] that belongs to the G protein-coupled receptor (GPCR) superfamily [4–6]. 11-cis-retinal is the photoreactive part of the molecule that captures a photon of light, resulting in isomerization of the chromophore to its all-trans configuration, and yielding the active rhodopsin photointermediate metarhodopsin II capable of binding and activating the G protein transducin [7, 8]. This stereochemical change is of key importance and can be considered the initial physicochemical event that ultimately leads to visual perception in the brain. 11-cis-retinal bound to opsin forms a very stable molecule, in the dark state, keeping a very low level of spontaneous thermal isomerization of the chromophore. Recent crystallographic studies have shown that the retinal-binding pocket of rhodopsin is remarkably compact [9–11]. In some cases, reduced thermal stability of the chromophore has been proposed to be the basis of the molecular mechanism of certain eye diseases, such as congenital night blindness (CNB) [12, 13]. We have investigated the status of the retinal chromophore upon thermal denaturation of rhodopsin. By means of difference ultraviolet spectroscopy and high-performance liquid chromatography (HPLC) analysis, we show that the isomer recovered after the thermal bleaching process of rhodopsin is all-trans-retinal. In contrast, free 11-cis-retinal is stable at 55 °C and does not isomerize to the all-

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trans form. Thus, 11-cis-retinal is more stable in its free form than inside the rhodopsin-binding pocket, at 55 °C. This finding indicates that retinal isomerization can be induced by a thermal process, when 11-cis-retinal is in the retinal-binding pocket bound to the opsin apoprotein, and that this forces the retinal to leave the binding pocket. The results reported may have implications in our understanding of the mechanism of photoreceptor noise.

Materials and methods

Materials

Bovine retinas were from J. A. Lawson Co. (Lincoln, Ne.). n-Dodecyl β-D-maltoside (DM) detergent was from Anatrace (Maumee, Ohio). All-trans-retinal was purchased from Sigma (St. Louis, Mo.). 11-cis-retinal was a gift of Prof. P. P. Philippov (Moscow State University). Stock solutions were prepared by dissolving retinals in absolute ethanol (HPLC grade), and the concentrations were determined by ultraviolet spectroscopy (11-cis-retinal, $\varepsilon_{376.5\,nm}$ of 24,935 $M^{-1}cm^{-1}$ [14] all-trans-retinal, $\varepsilon_{383\,nm}$ of 42,900 $M^{-1}cm^{-1}$ [14]). Rod outer segment (ROS) membranes were prepared under dim red light from frozen bovine retinas using a sucrose gradient method. The membranes were suspended in isolation buffer (buffer A): 70 mM potassium phosphate, 1 mM $MgCl_2$, and 0.1 mM EDTA (pH 6.9) and pelleted by centrifugation. The membrane pellets were resuspended in 5 mM Tris-HCl (pH 7.5) and 0.5 mM $MgCl_2$ (hypotonic buffer). Two alternating washes with these buffers were carried out to reduce any further contaminating proteins. Typically, 1 mg of ROS membranes was solubilized in 2 ml of buffer A containing 1% DM. Solubilization was done at 4 °C in the dark for 1 h and the solution was then centrifuged for 30 min in a T865 Sorvall rotor at 30,000 rpm. The supernatant was used to purify the protein by means of immunoaffinity chromatography using the Rho1D4 monoclonal antibody as previously described [15, 16]. Purified rhodopsin was eluted in 2 mM Na_2HPO_4 buffer, pH 6.0, 0.05% DM (buffer B) and was usually stored in the dark at 4 °C. The rhodopsin concentration was determined using a molar extinction coefficient value ε_{500} of 40,600 $M^{-1}cm^{-1}$, and a molecular weight of 40,000 Da. The preparations showed ultraviolet-visible (UV-vis) absorbance ratios (A_{280}/A_{500}) in the 1.6–1.8 range.

UV-vis spectra of rhodopsin

All measurements were made on a Cary 1E spectrophotometer (Varian, Australia), equipped with water-jacketed cuvette holders connected to a circulating water bath. Temperature was controled by a Peltier accessory connected to the spectrophotometer. All spectra were recorded in the 250–650 nm range, with a bandwidth of 2 mm, a response time of 0.1 s and scan speed of 250 nm/min. The experiments were performed in triplicate. Difference spectra were calculated by subtracting the corresponding absorption spectra using Grams/32 software (Galactic Industries).

Thermal stability assay

Rhodopsin thermal stability in the dark was followed by monitoring the rate of A_{500} loss, and the appearance of A_{380} as a function of time, at 55 °C. The process was carried out as previously described [17]. Complete absorption spectra, in the 250–650 nm range, were recorded at 5-min intervals.

Extraction of retinals from solubilized rhodopsin

All procedures were carried out under dim red light, or in aluminum foil-covered vials. After 1 h of thermal denaturation at 55 °C, retinal was extracted with hexane. Two milliliters of hexane followed by 0.1 ml of butylated hydroxytoluene as antioxidant agent were added to 400–500 µl (per tube, 18.8 µM by A_{500}) of rhodopsin in buffer B, and homogenized with vortex. Then, 2 ml of methanol and 1 ml of milli-Q water were added. The mixture was homogenized with vortex and centrifuged at 3000 rpm for 5 min at 4 °C to break the phases. The upper phase (hexane phase, 2 ml) was collected and dried with a gentle flow of nitrogen directed to the surface of the liquid with a Pasteur pipette, and dissolved with vortex in 200 µl of methanol-equilibration buffer (v/v) (see below for equilibration buffer composition) for reversed-phase HPLC analysis [14]. Retinals (11-cis and all-trans, at 36.8 and 33.3 mM, respectively) were prepared by dilution in equilibration buffer. A 2.5-µl aliquot of retinal was diluted in 122.5 µl of equilibration buffer (dilution factor was 50) and homogenized with vortex. All samples were injected with a Hamilton syringe. In all cases, a sample injector that delivers 20 µl in the loop was used.

HPLC analysis

HPLC analysis was performed essentially following a described procedure [14], with a Perkin-Elmer instrument equipped with a solvent conditioning module, quaternary pump and diode array detector. The latter feature allows the acquisition of spectra 'on the fly' and simultaneous measurement of two wavelengths during elution. For reversed-phase chromatography a C-18 (25 × 0.46 cm, 5-µm particle size) analytical column (Tracer) was employed. Briefly, the narrow-bore reversed-phase column was equilibrated at a flow rate of 3 ml/min with 72% aqueous acetonitrile (v/v), 0.015 M in ammonium acetate, pH 5.5 (obtained with glacial acetic acid). After injection of the sample in aqueous acetonitrile, retinals were eluted with the equilibration buffer. The effluent was monitored simultaneously at 350 and 325 nm. The chromatograms were analyzed with Turbochrom Navigator software (Perkin-Elmer).

Results and discussion

Thermal bleaching of rhodopsin

Figure 1 shows the UV-vis spectra of the thermal bleaching process of rhodopsin at 55 °C. The thermal denaturation of the protein was followed by measuring the decrease in the 500-nm absorption band with time. After 1 h at this temperature, most of the 500-nm visible band had been converted to a 380-nm band. The thermodynamic parameters of this process have been studied in detail in a recent study where the effect of zinc upon the process was analyzed [18]. The spectrum obtained upon acidification of the sample at the end of the thermal experiment (fig. 1, spectrum 6) clearly indicates that the retinal was already detached from opsin and there was no presence of Schiff base-linked material. To determine if we recovered 11-cis or all-trans-retinal, we performed several spectroscopic and chromatographic experiments as described below.

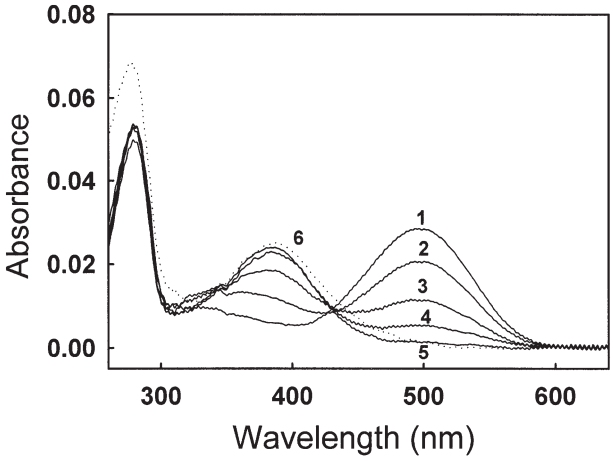

Figure 1. Thermal bleaching of rhodopsin at 55 °C. UV-vis absorbance spectra at different times of a rhodopsin sample at 55 °C. Rhodopsin from ROS membranes was in 2 mM Na_2HPO_4 buffer pH 6.0, 0.05% DM. Sample was measured at the indicated times and finally acidified with H_2SO_4 to a final pH of 1.9. 1, 0 min; 2, 10 min; 3, 30 min; 4, 60 min; 5, 110 min, and 6 (- - -), acidified.

Difference spectroscopy upon photobleaching

We first investigated the spectroscopic properties of free 11-cis-retinal and all-trans-retinal upon photobleaching and upon thermal exposure to 55 °C, as with the protein samples and in the same buffer conditions. The results obtained for the experiments with the free retinals are shown in figure 2. When 11-cis-retinal was illuminated with white light, the corresponding spectrum changes because it was being photoconverted mainly to all-trans-retinal, and the corresponding difference spectrum is shown in figure 2 (trace A). Thus, in this case, the difference spectrum (light minus dark) gave a main positive band with a maximum at about 382 nm. In contrast, illumination of all-trans-retinal, in the same way as the 11-cis isomer, gave a main negative band centered at about 392 nm in the photobleaching difference spectrum. These experiments indicate that 11-cis-retinal and all-trans-retinal can be differentiated taking advantage of the completely different behavior of the two isomers upon illumination. Also of interest was to see the effect of maintaining a free 11-cis-retinal sample at 55 °C during the same period of time as rhodopsin in the experiment shown in figure 1. Surprisingly, there was no difference spectrum (fig. 2, trace D) when the spectrum at the end of the thermal experiment was subtracted from the spectrum at the beginning of the thermal experiment. No aggregation was detected in the time course of the experiment in the detergent conditions used. This clearly shows that keeping free 11-cis-retinal at 55°C for more than 1 h does not promote its isomerization to the all-trans configuration, despite the strong dependence of the conformational stability of 11-cis-retinal on temperature and solvent environment [19]. This result is consistent with early investigations on the stereoisomerization of free 11-cis-retinal in several solvents including the detergent digi-

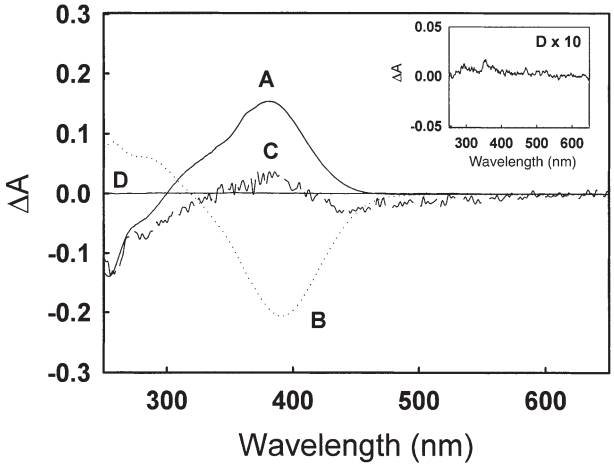

Figure 2. Difference spectra of free 11-cis-retinal and all-trans-retinal. Retinals in ethanol were measured at 20 °C. Curve A, difference spectrum between 11-cis-retinal after illumination for 20 s with maximum intensity of light of a Fiber-lite MI-150 Dolan Jenner light source equipped with an optic fiber guide, and the same sample before illumination. Curve B, difference spectrum between all-trans-retinal after illumination for 20 s with maximum intensity of light of a Fiber-lite MI-150 Dolan Jenner light source equipped with an optic fiber guide, and the same sample before illumination. Curve D, difference spectrum between 11-cis-retinal at 55 °C for 1 h and the sample at 55 °C at the beginning of the experiment (0 min). Curve C, the 11-cis-retinal sample used in D and kept at 55 °C was subsequently illuminated using the same conditions as in A and B (× 25 to better visualize the difference). The difference spectrum shows the features corresponding to 11-cis-retinal changing to an all-trans configuration. Inset, trace in D has been multiplied by 10 for better visualization.

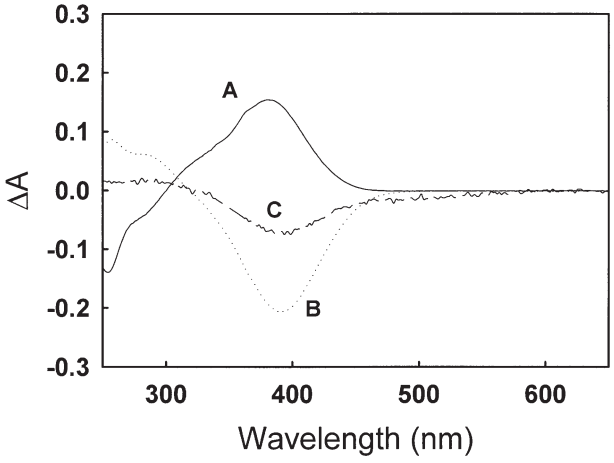

Figure 3. Difference spectra of free retinals and rhodopsin sample after thermal bleaching. A and B are difference spectra corresponding to the spectra seen in fig. 2, i.e. photobleahing difference spectra of 11-cis-retinal and all-trans-retinal, respectively. Curve C, rhodopsin in 2 mM Na_2HPO_4 buffer, pH 6.0, 0.05% DM after thermal bleaching at 55 °C (corresponding to the sample in fig. 1) was illuminated in the cuvette with white light and the difference spectrum (light minus dark) recorded.

tonin [20]. In addition, further proof that no isomerization to all-trans had taken place was derived from the experiment shown in trace C of figure 2. In this case, a sample of comparable absorbance to that of the protein sample studied in figure 1 was used. When this sample was illuminated with white light, the corresponding difference spectrum indicated that there was photoconversion to all-trans-retinal (compare traces C and A in fig. 2), although the difference spectrum was much noisier due to the lower concentration of 11-cis-retinal used in this case. This result provides further evidence that 11-cis-retinal remains in this configuration after being kept at 55 °C for more than 1 h. Thus, the 380-nm species obtained in figure 1 after maintaining the rhodopsin sample at 55 °C was possibly still 11-cis-retinal. However, the results presented in figure 3 rule out this possibility. In this figure, the difference spectra (light after illumination minus dark) for free 11-cis-retinal and all-trans-retinal (traces A and B, respectively) are the same as those in figure 2. Trace C in this figure corresponds to the difference spectrum between the rhodopsin sample at 55 °C at the end of the thermal experiment (sample corresponding to spectrum 5 in fig. 1) and the spectrum obtained after illumination of this sample. The difference spectrum obtained clearly shows that the retinal recovered from the thermal bleaching experiment was in its all-trans configuration.

HPLC analysis of thermally bleached samples

Figure 4 shows the HPLC profile for 11-cis-retinal, all-trans-retinal and the protein sample recovered from the thermal bleaching experiment. 11-cis-retinal and all-trans-retinal can be clearly separated with retention times

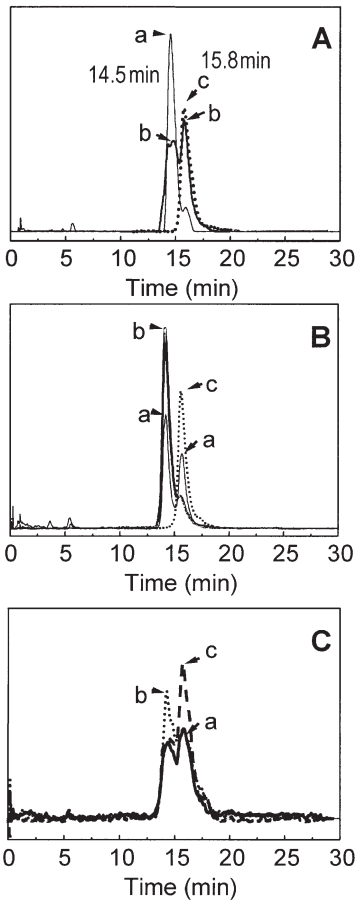

Figure 4. Retention times of retinals by HPLC. (*A*). 11-cis-retinal was eluted at 14.5 min (a, –) and all-trans-retinal at 15.8 min (c, ---). A sample of 11-*cis*-retinal was partially bleached with white light (60 W) and analyzed by HPLC; under these conditions, two peaks were eluted: the first peak corresponded to 11-cis-retinal and the second peak to all-trans-retinal (b, –). (*B*). A mixture of 11-cis-retinal and all-trans-retinal was resolved by HPLC (a, –). Free 11-cis-retinal (b, –) and all-trans-retinal (c, ---) were incubated for 1 h at 55 °C in the dark; each retinal isomer maintained its retention time with regard to the mixed sample without thermal treatment. (*C*). A mixture of free 11-cis-retinal and all-trans-retinal was resolved by HPLC (a, –). Retinal recovered from dark rhodopsin (not heated), was loaded with the mixture of 11-cis-retinal and all-trans-retinal (b, ····). Retinal was also recovered from rhodopsin after thermal bleaching at 55 °C (corresponding to the sample in fig. 1) and loaded with the mixture of 11-cis-retinal and all-trans-retinal (c, ---).

of 14.5 and 15.8 min, respectively (traces a and c, fig. 4A). When 11-cis-retinal is partially bleached, a mixture of the two isomers is observed (trace b, fig. 4A). Figure 4B shows that neither 11-cis (trace b) nor all-trans (trace c) are affected by keeping the sample at 55 °C for 1 h before running the chromatographic column in the dark. A mixture of 11-cis and all-trans (at 20 °C) is shown as reference for the retention of the free isomers and proper comparison with the heated sample (comparison between trace a and traces b and c). Finally, figure 4C clearly shows that the isomer recovered from the rhodopsin sam-

ple in figure 1 after thermal bleaching is all-trans-retinal. A mixture of free 11-cis-retinal and all-trans-retinal is shown in figure 4C (trace a). The same sample plus retinal extracted from dark rhodopsin (not heated) gives an increase at the retention time corresponding to 11-cis-retinal (fig. 4C, trace b). An analogous sample but with retinal extracted from thermally treated rhodopsin gives an increase in the peak eluting as all-trans-retinal (fig. 4C, trace c).

Physiological implications of the observed results in the case of receptor mutants

Thermal isomerization of an unprotonated Schiff base-linked pigment has been proposed as the cause of photoreceptor noise [21, 22]. However, a recent study has proposed that thermal pigment activation does not necessarily require prior Schiff base deprotonation [23]. Our findings reinforce the current notion that there is a tight coupling between the retinal chromophore and the binding pocket in the case of rhodopsin and indicate that thermal denaturation of rhodopsin forces the retinal to isomerize to the all-trans configuration and to leave the binding pocket. Recent studies have provided further evidence of tight coupling of the retinal chromophore and the opsin moiety [24, 25], and strengthen the role of the specific interaction of the amino acids forming part of the retinal-binding pocket in the retinal configuration [26]. These results may be relevant to the molecular mechanism of some retinal diseases like CNB [12, 13, 27] caused by mutations that would mimic the destabilizing effect of temperature observed in the present work. In the case of the T94I mutant associated with CNB [28], a clear thermal instability could be observed [17]. This mutant was also shown to be constitutively active [29]. Structural instability has been shown to be associated with constitutive activity of GPCRs [30]. In the case of GPCR mutants, this constitutive activity is likely due to the fact that these mutations may be inducing a relaxed tertiary structure that would also explain the reduced thermal stability. This effect has been observed in the case of mutants at Phe303 in transmembrane VI of the α_{1b}-adrenergic receptor [31]. These mutations induce helix movements that result in conformational rearrangements of the structure around the binding pocket of the ligand [31], and may also affect the formation of different receptor conformers. Our results may have implications for the stability of the different conformations of other GPCRs and may be relevant to the molecular mechanism of disease caused by mutations in this type of receptor, particularly those of the rhodopsin subfamily.

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