Photoinduced Isochromic Rearrangement in Rhodopsin

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Abstract. Two tests have been used to detect and to study conformational rearrangements of cattle rhodopsin, occurring in the process of rhodopsin photolysis and resulting in no change in the visual pigment absorption spectrum. The first test concerns the ability of retinal to react with hydroxylamine. This ability occurs after photoizomerization of retinal with a time constant of 0.3 s at 20 °C reflecting this way a conformational transition demasking the retinal-opsin NC-bond. The other test takes advantage of the ability of rhodopsin to modulate the conductance of artificial lipid membranes. After a bleaching flash such a rhodopsin containing membrane shows a transient change in conductance. One of its characteristic time constants is that of NC-bond demasking. It shows that the "demasking" rearrangement is not an artefact due to presence of hydroxylamine and that it occurs in native rhodopsin. It has been shown that the "demasking" rearrangement is isochromic, not associated with known rhodopsin conformational transitions and, judging by its time characteristics, it may be of a functionally importance. The common scheme of rhodopsin photolysis has been modified to include a new conformational transition.*

Key words: Rhodopsin bleaching — Isochromic rearrangements — Receptor potential

Introduction

From the functional point of view two events are significant in the process of visual pigment photolysis. The first one concerns the "memorizing" of photon absorption by rhodopsin; this seems to be a cis-trans isomerization of retinal, the visual pigment chromophore. The other involves a conformational transition in rhodopsin that gives rise to an electric response of the outer segment. A number of investigators suppose this to be a MI—MII (metarhodopsin I — metarhodopsin II) transition. Two points support this opinion: (1) this is the ultimate transition

* Selected experimental results were presented earlier as short communications (Fesenko and Lyubarsky 1977; Fesenko et al. 1976).

that may result in the rod photoresponse generation according to their kinetic parameters; (2) the conformation of opsin becomes obviously changed upon this transition (see e.g. a review by Ebrey and Honig 1975).

Point (2) is based on following experimental facts: (a) MI—MII transition is accompanied by a capture of a proton and by a substantial increase in entropy (Matthews et al. 1963); (b) MII may react with sodium borohydride as distinguished from MI (Bownds and Wald 1965; Akhtar et al. 1968); (c) conditions of photoregeneration change upon the MI decay (Williams 1970); also absorption at 280 nm (Orlov et al. 1975) and protein fluorescence of rhodopsin (Guzzo 1973; Fesenko et al. 1977) change upon MI decay.

The fact that photolysis of the visual pigment involves a number of spectrally different stages does not rule out the possibility of such rhodopsin conformational transformation of a functional importance that do not change its absorption spectrum.

It may be supposed that, e.g. some of the parameters mentioned may become changed as synchronously with the MI—MII transition; they may however show isochromic conformational rearrangements of the visual pigment.

Following two tests were used in an attempt to detect isochromic transition: (1) detection of the changes in NC retinal-opsin bond availability in the rhodopsin photolysis process for a hydrophilic reagent (hydroxylamine); (2) capacity of the visual pigment to change conduction of model lipid membranes under the influence of light. We succeeded in obtaining evidence for the existence of a previously unknown rhodopsin conformational transition. Its duration agrees with the hypothetic value for triggering the photoreceptor excitation (Penn and Hagins 1972).

Materials and Methods

Preparations of the visual pigment. Rod outer segments (ROS) were obtained by a conventional technique. Cattle eyes enucleated from dead animals were placed into a light-proof container. Further operations were carried out in red light ($\lambda \geqq 670$ nm). The retinae were isolated two hours after the delivery to the laboratory (about 4 h after the death) and placed in a standard buffer solution containing (mmol/l) NaCl 140; KCl 3.5; $CaCl_2$ 0.5; $MgCl_2$ 1.8; Tris-HCl 10; pH 7.4, 1 ml per retina. The reparations were stirred for 20 min. The obtained homogenates were centrifuged at 800 g for 10 min. Supernatants were removed and the procedure was repeated with the sediments. The supernatants were pooled and centrifuged at $16000 \times g$ for 20 min. The sediment was refloated twice in a sucrose solution prepared with the standard buffer (40 %, and 37 % w/v) respectively. ROS were then washed twice with the standard buffer and placed into distilled water at a concentration giving an optical density of about 1 at 500 nm (D_{500}). The resulting ROS preparations were sonicated for 4 min at 0 °C (ultrasonic dispergator UZDN-1, current 0.28 A). The obtained preparations referred further to as rhodopsin, had a purity criterion D_{280}/D_{500} of below 2.2.

Detergent extract of the visual pigment was prepared by solubilizing ROS with 1 % cetyltrimethylammonium bromide (CTAB, BDH) at room temperature.

Optical measurements. Optical density of the samples was measured using a spectrophotometer Specord UV VIS (samples in thermostatted cells were placed in the chamber for scattering probes near the photomultiplier window). The degree of MI—MII transformation was determined according to Matthews et al. (1963).

To study reaction kinetics of rhodopsin with hydroxylamine immediately after photolysis samples containing required concentrations of both rhodopsin and hydroxylamine were placed in a thermostatted chamber with quartz windows. Temperature was maintained on the required level by a jet of evaporating nitrogen and checked by a thermistor. A xenon lamp flash was used for bleaching (duration 1 ms, electrical energy 50 J) through a glass filter transmitting light with $\lambda > 560$ nm. To measure photoinduced changes in sample absorption the test beam was passed through two monochromators flanking the sample, and light intensity was measured using a photomultiplier (FEU-71). The photocurrent was monitored on an electric oscillograph with a photorecorder. The "dead" time of the equipment was 10—20 ms as determined by the amount of scattered light absorbed by the photomultiplier.

Reaction of pre-bleached rhodopsin with hydroxylamine was studied using a stop-flow equipment with a „dead" time of about 2 ms.

Optical density of samples used to study reactions of rhodopsin with hydroxylamine was always below 0.1. It was therefore assumed to be linearly proportional to transmission and changes in absorption were substituted for changes in intensity of transmitted light.

Interaction of hydroxylamine with rhodopsin was tested by transmission decrease measurement at 420 nm reflecting formation of retinaloxime. All the experiments were performed with a large molar excess of hydroxylamine over rhodopsin. The second-order reaction of their interaction was therefore of pseudo-first order and the kinetics of transmission changes was exponential. Rate constants were determined from the slopes of linear anamorphoses of corresponding oscillograms representing transmission changes.

The artificial lipid membrane (ALM) methods. ALM were formed on an aperture (1 mm in diameter) of a teflon cup placed in a glass chamber. The lipid solution used consisted of egg lecithin (18 mg ml^{-1}) and cholesterol (12 mg ml^{-1}) in heptane. Modified membranes were formed in a solution containing rhodopsin ($D_{500} = 0.15$). Current flowing through ALM was measured using an electrometer amplifier (Keithley Model 301) under voltage clamp conditions.

Membranes were illuminated by the light of a super-high pressure mercury lamp through a shutter. The time constant of rhodopsin bleaching of the membrane was below 15 ms.

Chemicals and solutions. "Reanal" (Hungary), analytical grade hydroxylamine, was recrystallized from water. Other chemicals of analytical grade were used without additional purification. Solutions containing hydroxylamine were titrated with NaOH to obtain the required pH.

In experiments on rhodopsin-hydroxylamine interaction a solution of NH_2OH—NaOH was added to rhodopsin preparation in distilled water (NH_2OH—NaOH buffer). For spectrophotometric measurements of the MI—MII transition degree the visual pigment preparation was suspended in a buffer solution containing 10 mmol/l Tris-HCl and 100 mmol/l NaCl. ALM were formed in imidazole-HCl (10 mmol/l) buffer containing 100 mmol/l NaCl.

Results

Kinetics of rhodopsin-hydroxylamine interaction. Fig. 1 shows rhodopsin transmission change at 420 nm after bleaching by flash illumination in the presence of hydroxylamine.

Fig. 1. A spectrophotometric assay for hydroxylamine reaction with rhodopsin. The oscillogram shows a transmission change in a rhodopsin sample after bleaching in the presence of hydroxylamine. pH 7.0, 23 °C, $[NH_2OH] = 0.5$ mol/l. The absorbance observed is partially due to light scattering. The actual optical density of the sample at 500 nm was 0.1.

Since rhodopsin absorption does not change at 420 nm upon MI—MII transition (Sengbusch and Stieve 1971) and since all the earlier stages of the visual pigment photolysis were accomplished at room temperature in several milliseconds, the observed transmission change reflects the interaction of rhodopsin with NH_2OH.

Special control experiments have shown that 1 mol/l hydroxylamine does not bleach rhodopsin upon 24 h incubation at 20 °C. Thus only photolyzed visual pigment was able to react with hydroxylamine.

The apparent rate constant of the pseudo-first order reaction of rhodopsin with hydroxylamine should be proportional to the concentration of the latter. This proportionality was actually observed at $[NH_2OH] \leq 0.4$ mol/l (Fig. 2). The rate constant reached its limit value (3.3 s^{-1} at 23 °C) and remained unchanged at hydroxylamine concentrations of 0.5 mol/l and more. Thus rhodopsin bleached in the presence of hydroxylamine appeared to be capable of reacting with the latter within 300 ms after the light absorption only, though under such conditions MII

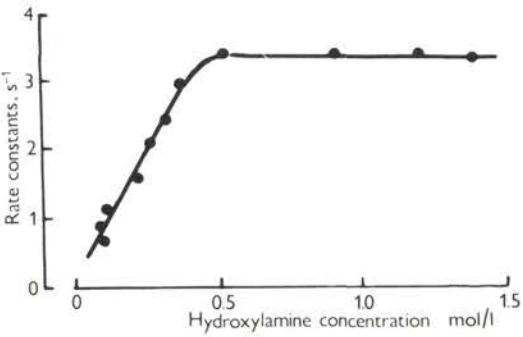

Fig. 2. Dependence of the pseudo-first order reaction rate constant of rhodopsin with hydroxylamine on the concentration of hydroxylamine. Bleaching in the presence of hydroxylamine. 23 °C, pH 7.0.

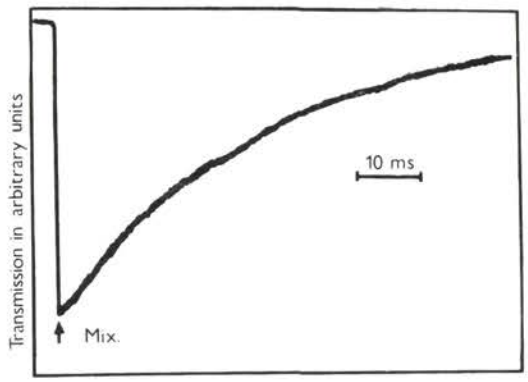

Fig. 3. Change in rhodopsin transmission at 420 nm after the addition of hydroxylamine 2 min after the photolyzing flash (arrow). Conditions: 10 °C, final hydroxylamine concentration 1 mol/l, pH 7.0.

may be formed with a time constant of about 30 ms (Sengbusch and Stieve 1971; Emrich and Reich 1974).

On the other hand, rhodopsin added at 10 °C 2 min after the bleaching (according to our estimations not less than 65 % of it is to be in the MII state) reacts with hydroxylamine more readily with a time constant of about 70 ms (see Fig. 3).

This 2 min after its formation, MII reacts with hydroxylamine much faster than if bleached in the presence of the latter. Hence, newly formed MII cannot react with hydroxylamine. This ability occurs some time later (300 ms at 23 °C) and "old" MII reacts with NH_2OH with no delay. This phenomenon suggests that the retinal-opsin NC-bond to is masked in newly formed MII and it becomes demasked with a time constant of about 0.3 s. Hence, there is a stage in the process of bleaching which limits the rate of rhodopsin-hydroxylamine interaction at high concentrations of the latter.

What kind of a process may it be? In principle, the demasking of NC-bond might be due either to an isochromic conformational rearrangement of opsin after MII formation, or to a change in the position of the protein globule in the lipid membrane. To solve this problem, experiments were carried out with cetyl trimethyl ammonium bromide — solubilized visual pigment, with a broken structure of the photoreceptor membrane, and with almost all the lipids solubilized. The kinetics of interaction of 0.5 mol/l hydroxylamine with solubilized rhodopsin was indistinguishable from that shown in Fig. 1. This means that the ability of solubilized visual pigment to react with NH_2OH occurs as quickly as that of non-solubilized pigment. Hence, the delay in hydroxylamine-rhodopsin reaction after MII formation points to conformational isochromic rearrangement demaskin the binding site of retinal to opsin.

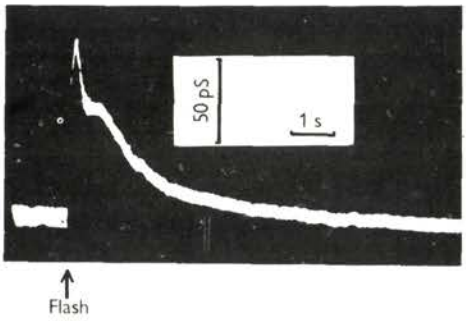

Fig. 4. Photoresponse of an ALM modified by rhodopsin at 11 °C.

Identification of isochromic rhodopsin conformational rearrangements independent of hydroxylamine. The requirement of high concentrations of hydroxylamine is a principal disadvantage of the method used. The study of rhodopsin containing ALM (Fesenko and Lyubarsky 1977) seemed to be an approach to avoid this unphysiological reagent.

Flash illumination of such ALM resulted in a transient increase in conductance with a subsequent decrease to the original or a little higher level (Fig. 4). This photoresponse points to the existence of the two processes occurring with the visual pigment: one of them is responsible for the increase in ALM conductance (i.e. for the front of the response), the other one being responsible for the subsequent conductance drop (i.e. for the trailing edge of the ALM photoresponse). It was shown earlier that the duration of the photoresponse front coincides with the duration of MI—MII transition (Fesenko et al. 1977). The amplitude of the photoresponse was proportional to the amount of MII formed (Fig. 5). No such photoeffect was observed at 2 °C and pH 8; under similar conditions MII did

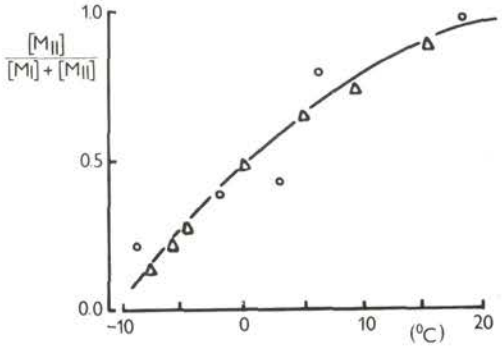

Fig. 5. Temperature dependence of normalized photoresponse amplitude of the model membrane (values of the photoresponse at 25 °C (○) are taken for unity) and the MI—MII transition degree (△) determined from spectrophotometric measurements.

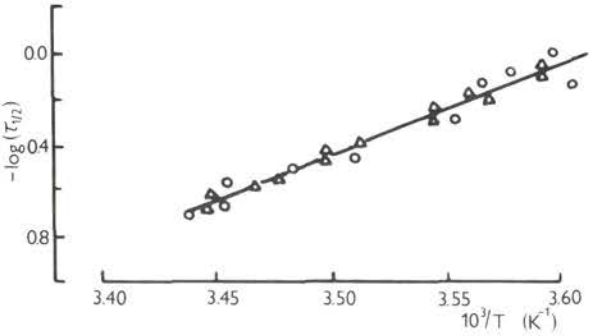

Fig. 6. Temperature dependence of half transformation time for the reaction of rhodopsin with 1 mol/l NH_2OH (△)and a characteristic life-time of photoresponse of the model membrane (○) in the Arrhenius plot, pH 7.0.

practically not form (Emrich and Reich 1974). Consequently, the increase in the membrane conductance is a result of MII formation.

The "half-transformation" time for the process of subsequent conductance recovery to the dark or near to dark level was about 200 ms at room temperature (since the trailing edge of the photoresponse was not strictly exponential, the "half-transformation time" was determined as the period during which the photoinduced component of the membrane conductance diminished from a half to a quarter of the peak value). It coincided with the half-transformation time of the NC-bond demasking process measured using high concentrations of hydroxylamine. Both processes remained synchronous over the entire temperature range studied (see Fig. 6). The apparent activation energy was 19 ± 3 kcal/mol.

Hydroxylamine at concentrations of up to 1 mol/l caused no changes in the duration of the photoinduced increase in ALM permeability although the effective amplitude decreased (see Fig. 7).

Based on this it may be assumed that one and the same conformational rhodopsin rearrangement is responsible for NC-bond demasking and modulation

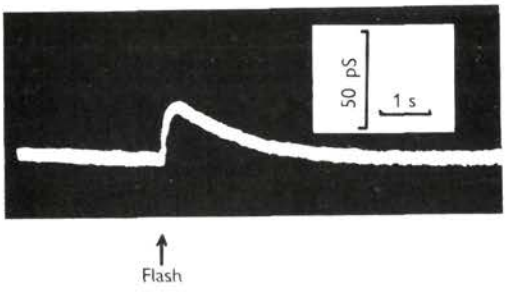

Fig. 7. Photoresponse of ALM modified by rhodopsin after 10 min incubation with 1 mol/l hydroxylamine.

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