The Regeneration of Rhodopsin from all-trans-Retinal: Solubilization of an Enzyme System Involved in the Completion of the Visual Cycle

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Studies on visual receptors both *in vivo* and *in vitro* have highlighted the existence of at least two broad biochemical processes. The first involves the combination through a Schiff-base linkage (Akhtar *et al.*, 1967, 1968; Hirstenstein & Akhtar, 1970; Bownds, 1967) of 11-*cis*-retinal with the protein opsin to give rhodopsin ($\lambda_{\max}$, 498 nm). In the second process on absorption of light rhodopsin participates in the transmission of impulses responsible for the visual sensation, and itself is converted into opsin and all-*trans*-retinal (for reviews see Bridges, 1970; Morton & Pitt, 1969). In order to complete the visual cycle there must be present *in vivo* an enzyme system that catalyses the conversion of all-*trans*-retinal into 11-*cis*-retinal. Hubbard (1956) suggested the involvement of a light-dependent isomerase. However, attempts to repeat this particular work have been unsuccessful in this laboratory and elsewhere (Plante & Rabinovitch, 1972). We have since shown (Amer & Akhtar, 1972) that carefully prepared rod outer segments possess the ability to regenerate rhodopsin from all-*trans*-retinal in a completely dark reaction and at a physiologically significant rate. This observation has provided the first convincing evidence for the existence of the retinal isomerase system that catalyses the conversion of all-*trans*-retinal into 11-*cis*-retinal. Several properties of this system within the rod outer segments have been studied. We now report on the solubilization of the enzyme system participating in the formation of rhodopsin from all-*trans*-retinal.

Experimental

Purification of all-trans-retinal. all-*trans*-Retinal was obtained from Eastman (Kodak) Ltd. (Liverpool, U.K.) and was purified by t.l.c. on silica gel G in light petroleum (b.p. 40–60°C)–acetone (20:1, v/v). The upper band (R_f 0.53) pertaining to 11-*cis*-retinal was discarded, and the lower band of all-*trans*-retinal was carefully scraped off and extracted with light petroleum. The all-*trans*-retinal gave a peak at $\lambda_{\max}$, 381 nm with no subsidiary peak at 255 nm.

The concentrations of compounds were determined by assuming ϵ 43 400 at $\lambda_{\max}$, 381 nm for all-*trans*-retinal, ϵ 24 900 at $\lambda_{\max}$, 376 nm for 11-*cis*-retinal and ϵ 40 400 at $\lambda_{\max}$, 498 nm for rhodopsin (Morton & Pitt, 1957).

Preparation of rod outer segments. All operations were carried out in dim red light at 4°C. In all experiments 67 mM-sodium phosphate buffer, pH 7.0, was used.

One hundred fresh retinæ were homogenized in 45% (w/v) sucrose in the buffer and floated by centrifugation at 1600g for 1 h. The floating crust of rod outer segments was removed, diluted with the buffer and recentrifuged at 38 000g for 1 h. This gave the rod-outer-segment pellet.

Solubilization and fractionation of the isomerase system. The rod-outer-segment pellet was suspended in 25 ml of the buffer and frozen at –15°C for 24 h. After being thawed the suspension was centrifuged

Fig. 1. Regeneration in intact and extracted rod outer segments

Intact or extracted rod outer segments from seven bovine retinæ were suspended in a final volume of 0.8 ml of the buffer. Measurements were made with 2 mm-light-path cells in a Unicam SP.8000 spectrophotometer. Curve 1a, intact rod outer segments and 0.088 μ mol of 11-*cis*-retinal at zero time; curve 1b, after 4 h. Curve 2a, intact rod outer segments and 0.08 μ mol of all-*trans*-retinal at zero time; curve 2b, after 4 h. Curve 3a, extracted rod outer segments, 0.08 μ mol of all-*trans*-retinal and 0.3 ml of the rod-outer-segment supernatant at zero time; curve 3b, after 4 h. When the rod-outer-segment supernatant was omitted in this last experiment no regeneration was observed.

at 38 000g for 1 h. This yielded a supernatant (referred to below as the rod-outer-segment supernatant) and a rod-outer-segment pellet, which was washed twice. These washed and extracted rod outer segments are referred to below as extracted rod outer segments.

Ultrafiltration was carried out in a Chemlab C50 cell, with a UM 20E membrane, which excludes compounds of molecular weights below 20 000. By using this apparatus 25 ml of the rod-outer-segment supernatant together with 5 ml of distilled water was concentrated to 10 ml of concentrate and 20 ml of filtrate.

Incubation. Intact or extracted rod outer segments from 100 retinæ were stored in the buffer, and an appropriate sample was bleached with a 500 nm light immediately before use. All incubations were carried out at 37°C in the buffer containing the substrate, intact or extracted rod outer segments (bleached) and the retinal isomerase system. The course of the reaction was monitored by recording spectra (350–650 nm) with a Unicam SP.800 or SP.8000 spectrophotometer. The reference cells always contained all the components of the reaction mixture except the substrate.

Results and discussion

Rod outer segments prepared from fresh bovine retinæ were separately incubated with either 11-*cis*-retinal or all-*trans*-retinal. Fig. 1 shows the almost quantitative conversion of both isomers into rhodopsin. The stoichiometric conversion of all the all-*trans*-retinal into rhodopsin demonstrated that the regeneration of rhodopsin in this experiment was predominantly due to all-*trans*-retinal and not due to any contaminants.

The extracted rod outer segments prepared as described above under 'Experimental' retained the ability to synthesize rhodopsin from 11-*cis*-retinal but not from all-*trans*-retinal. However, the bio-synthesis of rhodopsin from all-*trans*-retinal in extracted rod outer segments could be re-established on supplementation with rod-outer-segment supernatant (Fig. 1). The rod-outer-segment supernatant lost its activity when heated at 100°C for 5 min. Attention is drawn to the fact that, under the conditions of the experiments of Fig. 1, the turbid suspension of rod outer segments gave variable $\lambda_{\max}$ for regeneration from both 11-*cis*-retinal and all-*trans*-retinal. In order to make a more reliable spectral comparison,

Fig. 2. (a) Rates of and (b) pH optima for regeneration of rhodopsin from extracted rod outer segments

(a) All experiments contained extracted rod outer segments from five to ten retinæ, 0.08 μmol of all-*trans*-retinal in a final volume of 0.8 ml of the buffer and other additions (or modifications) as indicated. Curve 1, no addition. Curve 2, 0.3 ml of the original rod-outer-segment supernatant. Curves 3a and 3b, 0.1 ml and 0.3 ml of the concentrate respectively (see under 'Experimental' in the text). Curve 4, 0.3 ml of the concentrate in the presence of 5 mM-iodoacetamide. Curve 5, excess of 11-*cis*-retinal added to the experiment of curve 4 at the time indicated (arrow). Curve 6, boiled rod-outer-segment supernatant. Curve 7, 0.3 ml of filtrate obtained by ultrafiltration (see under 'Experimental' in the text). Curve 8, regeneration when extracted rod outer segments were incubated with 0.11 μmol of 11-*cis*-retinal instead of the all-*trans* isomer. The theoretical conversion of all the added substrate into rhodopsin was taken as 100%. (b) Experimental conditions were similar to those described in (a). Sodium phosphate buffers (67 mM) of different pH values were used. —, Regeneration from all-*trans*-retinal; ---, regeneration from 11-*cis*-retinal.

therefore, the rhodopsin regenerated in the rod outer segments from either 11-*cis*-retinal or all-*trans*-retinal was extracted into digitonin, and examination of their spectra revealed that in both cases the light-sensitive material had λ_{max} 498 nm.

Further studies involved ultrafiltration of the rod-outer-segment supernatant, which gave two fractions, (a) concentrate and (b) filtrate. When these fractions were separately used for the synthesis of rhodopsin from all-*trans*-retinal and extracted rod outer segments all the activity was found to be in the concentrate; the filtrate supported no detectable regeneration of rhodopsin (Fig. 2a). In addition, Fig. 2(a) shows the effects of various amounts of the concentrate on the rate of rhodopsin synthesis and also compares the effectiveness of the concentrate with the original rod-outer-segment supernatant (curves 2 and 3a in Fig. 2a). The formation of rhodopsin from all-*trans*-retinal was inhibited by 5 mM-iodoacetamide. Since this conversion involves the participation of two biochemical reactions (all-*trans*-retinal $\rightarrow$ 11-*cis*-retinal and 11-*cis*-retinal + opsin $\rightarrow$ rhodopsin), the precise site for this inhibition was studied. Curve 5 in Fig. 2(a) shows that the incubation of extracted rod outer segments with all-*trans*-retinal in the presence of iodoacetamide for 1.5 h gave no detectable regeneration; however, the addition of 11-*cis*-retinal at this stage resulted in the formation of rhodopsin.

These experiments conclusively prove that iodoacetamide inhibits the reaction catalysed by the retinal isomerase system. We have previously shown (Amer & Akhtar, 1972) that in intact rod outer segments the rhodopsin formation from all-*trans*-retinal

but not from 11-*cis*-retinal is also inhibited by formaldehyde. Further investigation involved the demonstration that the pH optimum for rhodopsin formation from all-*trans*-retinal was at 7.0, whereas the pH optimum for rhodopsin synthesis from 11-*cis*-retinal was at 5.0 (Fig. 2b).

The cumulative evidence provides the first authentic demonstration of the solubilization of an enzymic component that is involved in the regeneration of rhodopsin from all-*trans*-retinal. That the present observations may be relevant to dark adaptation *in vivo* has crossed our minds.

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