Rhodopsin reconstitution in bleached rod outer segment membranes in the presence of a retinal-binding protein from the honeybee

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The physiological role of a retinal-binding protein from honeybee is investigated. This protein, upon previous loading with all-*trans* retinal and subsequent irradiation with monochromatic light of wavelength 490 nm, is able to promote the reconstitution of rhodopsin when added to a suspension of opsin membranes from bleached bovine rod outer segments. In this respect this retinal-binding protein could have a role very similar to that postulated for the well-known cephalopod retinochrome, that serves to catalyze the formation in the presence of light of 11-*cis* retinal in photoreceptor cells and to provide it for the reconstitution of rhodopsin during the visual cycle.

Rhodopsin regeneration; Visual pigment renewal; Retinal photo-isomerase; Retinal-binding protein

1. INTRODUCTION

In order to maintain continuous visual function, the visual pigment rhodopsin, once it has been converted into a physiologically inactive photoproduct by light, has to be regenerated. In photoreceptors of invertebrates, such regeneration occurs through the absorption of light by the photoproduct metarhodopsin [1]. However, accumulating evidence suggests that in addition to this fast pathway, visual pigment is continuously renewed through a much slower process: the breakdown and biosynthesis of the pigment [2,3]. In fly photoreceptor it has been demonstrated that metarhodopsin, which contains all-*trans* retinal as chromophore, is selectively degraded and, furthermore, that the biosynthesis of visual pigment requires the presence of the 11-*cis* chromophore [4]. Thus, the all-*trans* retinal released during the breakdown of the photoproduct has to be isomerized to 11-*cis* retinal. In fly photoreceptors, this isomerization has been shown to be mediated by light with maximal efficiency in the blue/violet spectral range, and it was argued that a protein may be involved that binds the all-*trans* chromophore and ensures the stereospecificity of the photoreaction [4].

A water-soluble retinal-binding protein (RALBP) isolated from honeybee heads seems to have the required properties: it binds all-*trans* retinal via a protonated Schiff-base linkage, absorbs maximally at about 440 nm and is able to direct the photoisomerization of all-*trans* retinal almost exclusively to the 11-*cis* form [5-7].

Further strong support for the involvement of the RALBP in the visual pigment cycle of the honeybee is gained from the experiments described in this paper, showing that the regeneration of rhodopsin in bleached bovine rod outer segments occurs in the presence of the previously irradiated RALBP.

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Abbreviations: ROS, rod outer segment; RALBP, retinal-binding protein; HPLC, high-performance liquid chromatography

2. MATERIALS AND METHODS

RALBP was extracted and purified from honeybee heads by preparative electrophoresis and ion-exchange chromatography as previously described [5,6]. In order to saturate the RALBP, 1×10^{-4} M all-*trans* retinal was added to the homogenate from about 300 bee heads. Retinal which was not bound to the protein was retained by the stacking polyacrylamide gel during electrophoresis.

Measurements of the absorbance spectra were carried out with a computer-controlled double-beam recording spectrophotometer (Shimadzu, model UV-160); baseline correction was automatic. Illumination of the samples was carried out with an actinic light source (Xenophot HLX 64634, 150 W, Osram), optical fibers, and an interference filter with maximal transmission at 490 nm (Balzer Ch 526/5816).

Retinal isomers were extracted from RALBP-samples with dichloromethane/methanol [8] and then analyzed by straight-phase isocratic HPLC (Beckman, model 330; column: Ultrasphere-Si, 5 μm particle size, dimensions: 4×250 mm). Retinals were eluted with 5% ether in *n*-hexane at a flow rate of 1 ml/min, and the peaks were recorded at 365 nm. The elution sequence of retinal standard was: 13-*cis*, 11-*cis*, 9-*cis* and all-*trans* retinal.

Opsin membranes were prepared from freshly excised bovine eyes. The eyeballs were hemisected, the retinae removed and the ROS isolated following a standard method based on density gradient cen-

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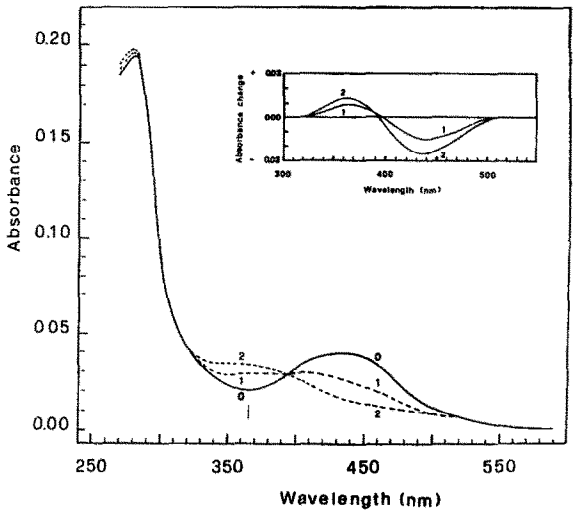

Fig. 1. Absorbance spectra of a solution containing about 0.6×10^{-6}M RALBP in phosphate buffer (pH 7.0) at room temperature before (solid line 0) and after 20 min (dashed line 1) or 40 min (dashed line 2) of monochromatic illumination (490 nm). The inset shows the absorbance difference between curve 0 and curves 1 (1) and 2 (2).

trifugation [9]. The resulting ROS suspension was homogenized with 1 ml of 5 mM Tris-glycine, pH 8.5, containing 5 mM dithiolthreitol and 40% sucrose. The resulting disk membrane suspension was mixed with 50 mM hydroxylamine and exposed for 15 min to monochromatic light (530 nm; 150 W Xenophot lamp with optical fibers, interference filter Balzer Ch 526/5979) at room temperature. After the total bleaching of bovine rhodopsin, free hydroxylamine was removed by washing with a large amount of Tris-glycine buffer and centrifuging for three times.)

3. RESULTS AND DISCUSSION

Since less than 10% of the native purified RALBP was found bound to retinal [5] (all-*trans*:11-*cis* = 3:1), the homogenated bee heads were incubated with an excess of all-*trans* retinal before the protein purification, so as to saturate the binding site. The spectral absorbance of the purified pigment showed a maximum at 440 nm, which decreased upon irradiation with light of wavelength 490 nm (Fig. 1). Concomitantly, the absor-

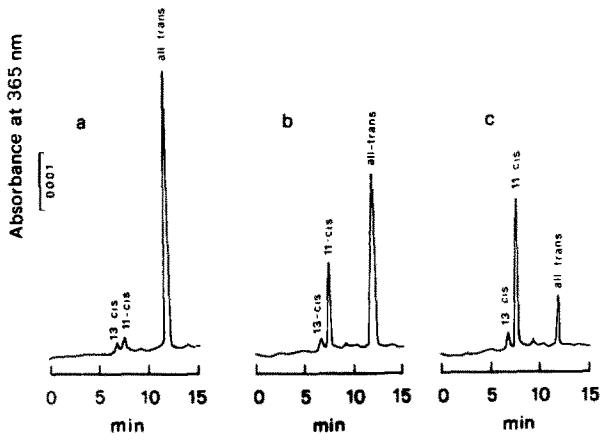

Fig. 2. HPLC analysis of the geometric isomers of retinal before (a) and after 20 min (b) or 40 min (c) of irradiation of the RALBP-sample shown in Fig. 1.

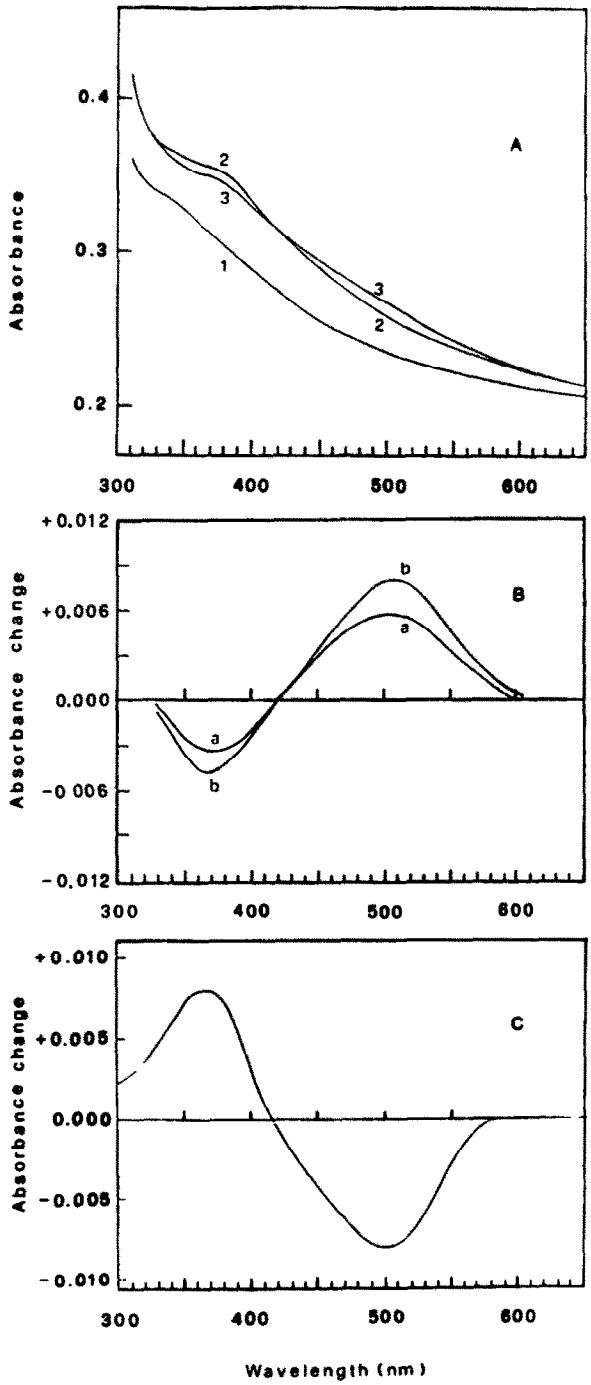

Fig. 3. Reconstitution of bovine rhodopsin upon incubation of opsin membranes with illuminated RALBP. (A) 100 μl of RALBP solution which had been illuminated for 40 min with monochromatic light (490 nm) were added to opsin membranes from bovine ROS suspended in 400 μl of 5 mM Tris-glycine pH 8.5 (curve 1) which contained 40% sucrose. Spectra of the mixture were taken at the start (curve 2) and 3 h after incubation (curve 3) in the dark at room temperature. (B) Absorbance changes after 1 h (curve a) and 3 h (curve b: difference between curves 2 and 3 of A) of incubation. (C) Absorbance change after 15 min irradiation of the sample in A, curve 3, in the presence of 50 mM hydroxylamine.

bance in the near ultraviolet (maximally at about 370 nm) increased due to the formation of a photoproduct with a lower absorbance coefficient than the parent pigment (Fig. 1; inset). Fig. 2 shows the highly stereospecific isomerization of all-*trans* retinal to the 11-*cis* form which accounts for the observed absorbance changes.

After 40 min of irradiation, when about 80% of the all-*trans* retinal was converted to its 11-*cis* isomer, the sample containing the irradiated RALBP was added to a suspension of disrupted bovine rod outer segments (ROS) which had been bleached prior to incubation (opsin membranes). The mixture containing approximately 0.6×10^{-6} M RALBP, 0.5×10^{-6} M 11-*cis* retinal and 1.0×10^{-6} M opsin was then incubated in darkness and the expected reconstitution of bovine rhodopsin was monitored by measuring the absorbance changes (Fig. 3A,B). After 3 h incubation, when saturation was attained, 50 mM hydroxylamine was added in darkness to the sample of Fig 3A (curve 3) in order to bind the 11-*cis* retinal which had not reacted with bovine rhodopsin in the sample. Afterwards, a baseline was recorded, the sample was exposed to monochromatic light (530 nm) for 15 min and the absorbance change recorded (see Fig. 3C). The absorbance decrease at about 500 nm clearly indicates that rhodopsin has been formed during incubation of honeybee RALBP with bovine opsin membranes.

On the basis of the absorbance decrease at 500 nm (Fig. 3C : $A = 0.008$ O.D.) and the molar absorbance coefficient of rhodopsin (40 600 $M^{-1}.cm^{-1}$ the regenerated rhodopsin amounts to 0.2×10^{-6} M, i.e. about 40% of the 11-*cis* retinal bound to RALBP led to rhodopsin formation. Since the concentration of opsin used was about twice that of 11-*cis* retinal, opsin was not the limiting factor. Indeed, the environment seems to somehow influence or restrict the transition of 11-*cis* retinal from RALBP to opsin, although the incomplete reconstitution could be ascribed also to the presence in opsin membranes of traces of hydroxylamine (see section 2), that produces the 11-*cis* retinal oxime, unable to recombine with opsin.

Despite the incomplete nature of reconstitution, the result clearly shows that 11-*cis* retinal, presumably still bound to RBP after photoisomerization, reacts with opsin-containing ROS membranes so as to yield bovine rhodopsin. In this respect, this retinal binding protein resembles the well-known, membrane-bound retinochrome which catalyzes the formation in the light of 11-*cis* retinal in cephalopod photoreceptors [10] and is able to provide it for the reconstitution of squid rhodopsin [11]. As a similar system is postulated also in the crayfish [12], it seems to us that the presence of a retinal-photoisomerase activity in the process of visual pigment renewal might be extended to other invertebrate visual systems.

Although RBP was isolated from honeybee heads, it is quite likely that it is located in the eyes, especially because its properties are very similar to those of the RALBP found in the honeybee drone compound eyes [5,13].

Besides acting as a photoisomerase, thereby providing the retinal isomer required for the biosynthesis of visual pigment, the RALBP may also act as a carrier for shuttling retinal within the eye and/or photoreceptor cells as suggested by the water solubility of the protein. Furthermore, a transfer of chromophore may occur between RALBP and the *meta*-state of the visual pigment in bee photoreceptor, namely the 11-*cis* chromophore of RALBP may be exchanged for all-*trans* retinal bound to *meta*-state. A comparable pathway has been proposed for squid photoreceptors [14].

REFERENCES

[1] Hamdorf, K. and Schwemer, J. (1975) in: Photoreceptors Optics (Snyder A.W. and Menzel R. ed) pp. 263–289, Springer, Berlin.
[2] Schwemer, J. (1986) in: The Molecular Mechanism of Photoreception, Dahlem Konferenzen (Stieve, H. ed.) pp. 303–326, Springer-Verlag, Berling.
[3] Stein, P.J., Brammer, J.O. and Ostroy, S.E. (1979) J. Gen. Physiol. 74, 565–582.
[4] Schwemer, J. (1984) J. Comp. Physiol. 154, 535–547.
[5] Pepe, I.M., Cugnoli, C. (1980) Vision Res. 20, 97–102.
[6] Pepe, I.M., Schwemer, J. and Paulsen, R. (1982) Vision Res. 22, 775–781.
[7] Schwemer, J., Pepe, I.M., Paulsen, R. and Cugnoli, C. (1984) J. Comp. Physiol. 154, 549–554.
[8] Pepe, I.M. and Schwemer, J. (1987) Photochem. Photobiol. 45, 679–681.
[9] Schnetkamp, P.P. and Daemen, F.J. (1982) Methods Enzymol. 81, 110–116.
[10] Hara, T. and Hara, R. (1982) Methods Enzymol. 81, 827–833.
[11] Seki, T., Hara, R. and Hara, T. (1980) Photochem. Photobiol. 32, 69–479.
[12] Cronin,T.W. and Goldsmith, T.H. (1984) J. Gen. Physiol. 84, 63–81.
[13] Pepe, I.M., Perrelet, A. and Baumann, F. (1976) Vision Res. 16, 905–908.
[14] Terakita, A., Hara, R. and Hara, T. (1989) Vision Res. 29, 639–652.