Volume 257, number 1, 63–67 FEB 07722 October 1989

11-*cis* retinal formation in the light catalyzed by a retinal-binding protein from the honeybee retina

C. Cugnoli, R. Mantovani, R. Fioravanti and I. Pepe

Institute of Cybernetics and Biophysics of CNR, via Dodecaneso 33, 16146 Genoa, Italy

Received 26 July 1989; revised version received 1 September 1989

A retinal-binding protein purified from honeybee retina was able to combine with various isomers of retinal and to catalyze their photoisomerization mostly to 11-*cis* retinal, the isomer necessary for rhodopsin regeneration. A model of the enzymatic reactions was developed which best fits the experimental curves. The resulting values for the kinetic constants confirm that the isomerization from all-*trans* retinal to 11-*cis* retinal is the favoured reaction. The results are discussed in terms of a possible physiological role of this protein which, similarly to retinochrome of cephalopods, may be involved in visual pigment regeneration.

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

1. INTRODUCTION

Photoreceptor cells contain the visual pigment rhodopsin whose chromophore, upon absorption of a photon, is isomerized from 11-*cis* retinal to all-*trans* retinal. This is the first step in the visual process, triggering a dark-reaction sequence leading to the production of the photoreceptor potential. As visual pigments of both vertebrate and invertebrate are eventually destroyed by light absorption, vision is assured by a continuous renewal of rhodopsin molecules which takes days to be completed [1,2]. A necessary step of this renewal is the isomerization of all-*trans* retinal to 11-*cis* retinal. In the fly's visual system, this isomerization has been clearly demonstrated to occur only in the light, with maximal efficiency in the blue/violet region [3]. In the compound eye of the honeybee, a retinal-binding protein (RBP) was isolated which absorbs light with a maximum at 440 nm and is able to direct the photoisomerization of all-*trans* retinal almost exclusively to 11-*cis* retinal [4–7]. We show here that this protein, similarly to retinochrome of cephalopods [8,9], can combine with various isomers of retinal and catalyze their photoisomerization mostly to 11-*cis* retinal, the isomer required for visual pigment regeneration.

Correspondence address: I. Pepe, Institute of Cybernetics and Biophysics of CNR, Via Dodecaneso 33, 16146 Genoa, Italy

Abbreviations: RBP, retinal-binding protein; HPLC, high-performance liquid chromatography

2. MATERIALS AND METHODS

2.1. *Protein extraction and purification*

Honeybees (*Apis mellifera*) were obtained from the Apiculture Society at Abazia Benedettina in Finale Ligure (Italy). Bees were stored in the dark at −20°C, until used. RBP, first isolated from the retina of a honeybee drone [4], was extracted and purified from honeybee heads by preparative electrophoresis and ion-exchange chromatography as previously described in two articles where it was named RBP-B [6,7].

2.2. *Binding of retinal isomers with RBP*

As only a small percentage (about 5%) of the RBP purified from honeybee retina was found to contain bound retinal and the Schiff-base between retinal and the protein is easily hydrolyzed in aqueous buffer, a three-fold molar excess of each retinal isomer (Sigma) was added to solutions of 2×10^{-6} M RBP in 0.1 M phosphate buffer, at pH 7. The mixtures were incubated in the dark and the Schiff-base linkage formation between each retinal isomer and RBP was monitored by measuring the absorbance of the sample at 440 nm with a double beam spectrophotometer (Shimadzu, model UV-160) with automatic baseline-correction.

2.3. *Photoisomerization of retinal: extraction and identification of retinal isomers*

After 1 h incubation in the dark, each sample containing one retinal isomer and RBP was irradiated with an actinic lamp (Xenophot HLX 64634, 15 V, 150 W, Osram) and an interference filter with maximal transmission at 531 nm (Balzer Ch 526/5979). Extraction of retinal isomers from the irradiated samples was performed in dim red light with an equal volume of a cold mixture of methylene chloride/methanol (1:1, v/v). After stirring, the resulting emulsion was centrifuged at 4000 × *g* for 10 min, the methylene chloride layer containing the retinals was drawn off and evaporated under nitrogen. In order to increase the extraction yield, this procedure was repeated twice. Hexane (30 μl) was then added to dissolve the residue and the solution was analyzed by HPLC (isocratic Beckman model 330), on an Ultrasphere-Si column (5 μm particle size; 4 × 250 mm; Beckman) used in normal phase with 5% ether in

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/89/$3.50

n-hexane as eluent at a constant flow rate of 1 ml/min. The elution sequence of standard retinal isomers was: 13-*cis*, 11-*cis*, 9-*cis* and all-*trans* retinal.

This procedure yielded about 70% of the total retinal present and caused no unspecific isomerization [10].

2.4. *Calculation of kinetic constants*

A model describing the enzymatic reactions was developed which consists of a system of five differential equations of the second order (see text). This system was solved by using a predictor-corrector method on a computer Victor-286 equipped with a mathematical co-processor. By using the Monte-Carlo method, the kinetic parameters were changed from different initial conditions until a local minimum of the least square difference was reached between the solutions of the theoretical model and the experimental data of fig.2a,b,c.

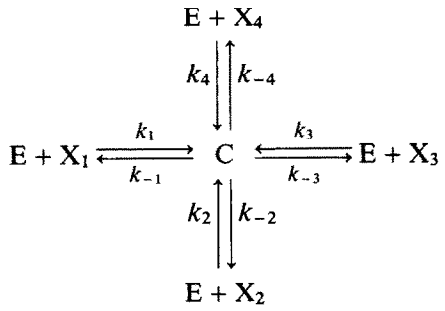
3. RESULTS

In fig.1 the formation in the dark of the binding between the various isomers of retinal and RBP was monitored by measuring the increase in the optical absorbance at 440 nm typical of the Schiff-base linkage of retinal to the protein [6]. The time constants of the binding were approx. 3 min and 10 min for all-*trans* and for 13-*cis*, respectively.

After 1 h incubation in the dark, a green light of 531 nm was used to irradiate the samples, as this wavelength is still absorbed by the retinal bound to the protein, even though with low efficiency, but not by free retinal.

The geometrical form of the isomers produced during irradiation was analyzed by HPLC. Fig.2a shows that continuous irradiation with green light of all-*trans* retinal in the presence of the protein, led mainly to the formation of 11-*cis* retinal. The most abundant product of the irradiation of 9-*cis* and 13-*cis* retinal (fig.2b and c) was still 11-*cis* retinal, nevertheless its amount was reduced with respect to that produced during all-*trans* retinal irradiation. Control experiments were carried out by irradiating with the same green light each retinal isomer either in the absence or in the presence of the denatured protein. No appreciable change of the initial isomeric composition of retinals was observed in the former conditions, whereas a slight decrease was observed in the latter, as fig.2d shows for the case of the all-*trans* retinal.

The following simplified reaction scheme can explain the experimental data:



where E is the enzyme; X_1, X_2, X_3, X_4 are all-*trans*, 11-*cis*, 9-*cis*, 13-*cis* retinal, respectively, and C is the enzyme-substrate complex made up of a mixture of all isomeric states of retinal respectively bound to the protein. k_1, k_2, k_3, k_4 are the usual thermal rate constants whereas k_{-1}, k_{-2}, k_{-3}, k_{-4} include also the respective absorption cross sections of the various C species as well as the quantum yields for conversion of the respective isomeric retinals.

The above enzymatic reactions are governed by the following system of second order differential equations:

$$\frac{dX_i}{dt} = k_{-i}C - k_iEX_i$$

$$\frac{dC}{dt} = \sum_{i=1}^{4} k_iX_iE - \sum_{i=1}^{4} k_{-i}C$$

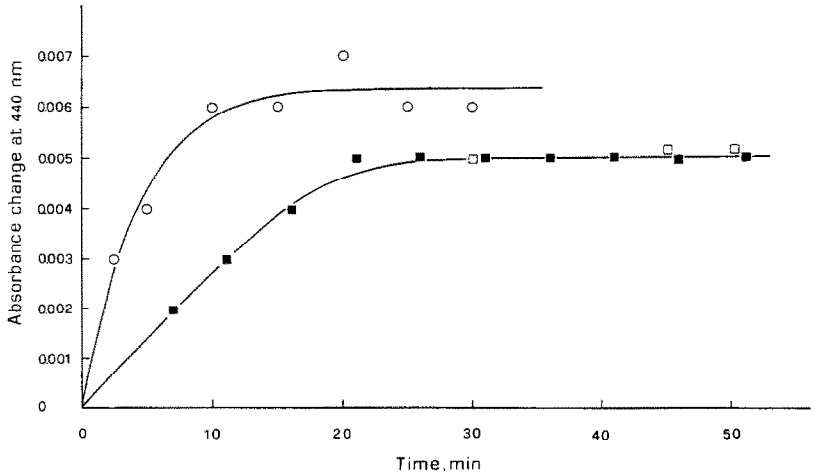
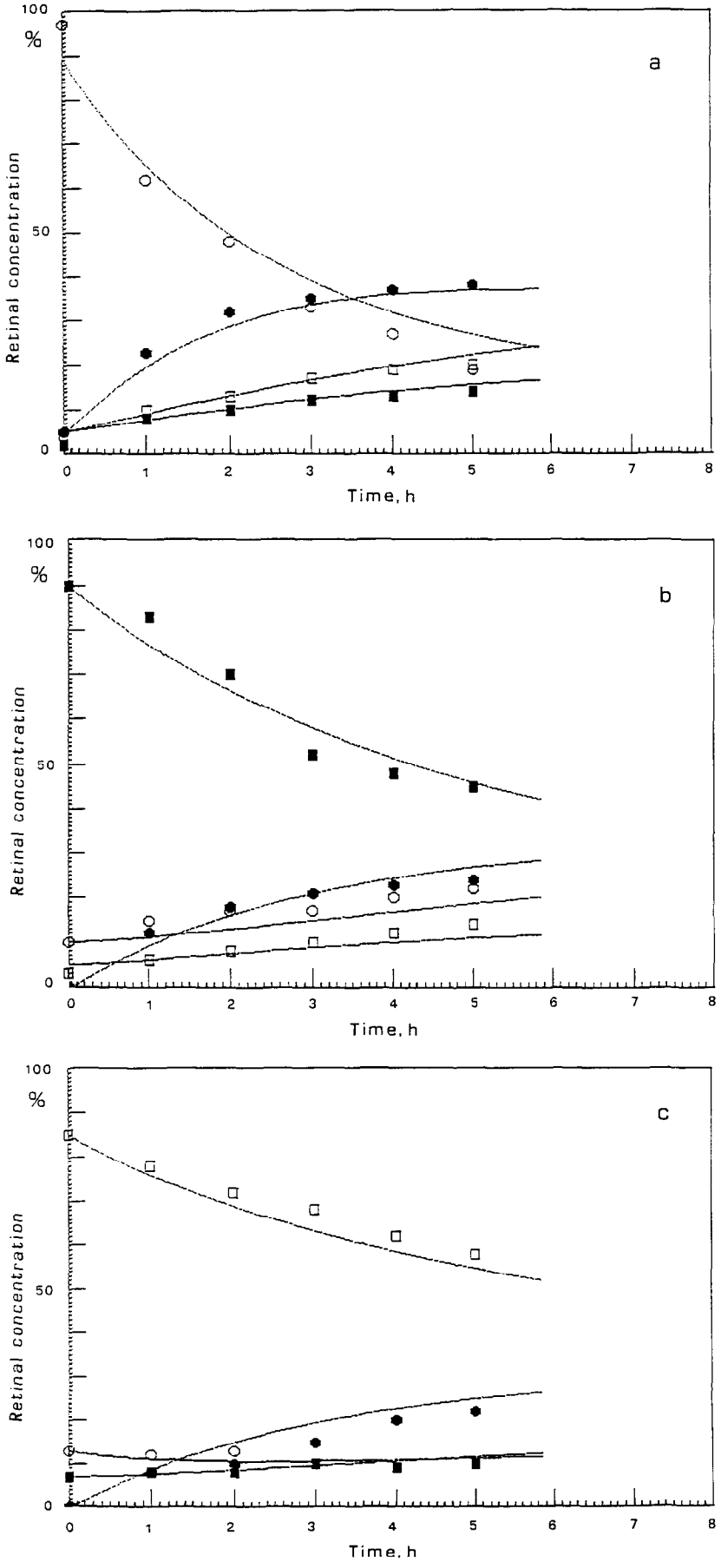


Fig.1. Kinetics of the Schiff-base linkage formation between RBP and all-*trans* (○), 13-*cis* (■) and 9-*cis* retinal (□) in the dark, at room temperature. A three-fold molar excess of all-*trans*, 13-*cis*, and 9-*cis* retinal was added to solutions of 2×10^{-6} M RBP in 0.1 M phosphate buffer at pH 7.


100
%
a
Retinal concentration
50
0
0 1 2 3 4 5 6 7 8
Time, h
100
%
b
Retinal concentration
50
0
0 1 2 3 4 5 6 7 8
Time, h
100
%
c
Retinal concentration
50
0
0 1 2 3 4 5 6 7 8
Time, h

The computed solutions of the system that best fit the experimental data as a whole are shown by the continuous curves of fig.2a, b and c. The values for the relative kinetic constants resulted as follows: k_1 = 1.04 $\mu mol^{-1} \cdot h^{-1}$; k_2 = 1.7 $\mu mol^{-1} \cdot h^{-1}$; k_3 = 0.36 $\mu mol^{-1} \cdot h^{-1}$; k_4 = 0.46 $\mu mol^{-1} \cdot h^{-1}$; k_{-1} = 1.62 h^{-1}; k_{-2} = 6.02 h^{-1}; k_{-3} = 1.35 h^{-1}; k_{-4} = 1.0 h^{-1}. These values clearly confirm that the photoisomerization from all-*trans* retinal to 11-*cis* retinal is the most favoured reaction.

4. DISCUSSION

The isomerization of retinals (absorbance maximum at about 380 nm) is well known to take place after irradiation in the near UV region. A photoequilibrium is rapidly reached and the resulting isomer mixture – containing the all-*trans* isomer as the major component – has a constant composition, irrespective of which isomer is used [11]. In the presence of the RBP from bees, instead, irradiation with a green light of 531 nm, which is absorbed only by retinal molecules bound to RBP, mainly produced the 11-*cis* isomer with a rate that increased in the order: 9-*cis*, 13-*cis* and all-*trans* retinal.

One should consider the fact that in vivo the formed 11-*cis* retinal is continuously used to regenerate rhodopsin from opsin and for this reason the equilibrium is shifted toward 11-*cis* formation until all other isomers are exhausted. Besides, in retinula cells, either 13-*cis* or 9-*cis* retinal are probably much less abundant than all-*trans* retinal, which comes either from an external contribution or from rhodopsin degradation. Therefore the reaction all-*trans* ⟶ 11-*cis* retinal is presumably the only reaction that actually occurs in photoreceptor cells of the honeybee compound eye.

Therefore, there seems to be a close similarity between the physiological role of the RBP from bees and of retinochrome extracted from the cephalopod retina, as the latter can convert the retinal isomers into the 11-*cis* form with different reaction rates [8]. Namely not only does it probably serve for storing retinal, but also for supplying the photoreceptor cell with 11-*cis* retinal [12]. This hypothesis can also be consistently supported by the observation that rhodopsin regeneration in flies, whose eyes were injected with all-*trans* retinal, occurred only in the light, with maximal efficiency in the violet/blue region [3,13]. This could be due to the fact that the injected all-*trans* retinal binds to a compound, possibly a protein, via a Schiff-base linkage – thus shifting its absorbance maximum from 380 nm to 440 nm – and is then selectively photoisomerized to 11-*cis* retinal [3]. The protein from bee (named after the Hara's honeybee retinochrome [14]) seems to fit this hypothesis well. Recent evidence appears to indicate that also in the eyes of crayfish rhodopsin regeneration is photosensitive, in the blue/violet region [15]. Further research in other invertebrate visual systems is clearly needed before extending this model of light-dependent renewal of rhodopsin based on a retinal-photoisomerase to all invertebrates.

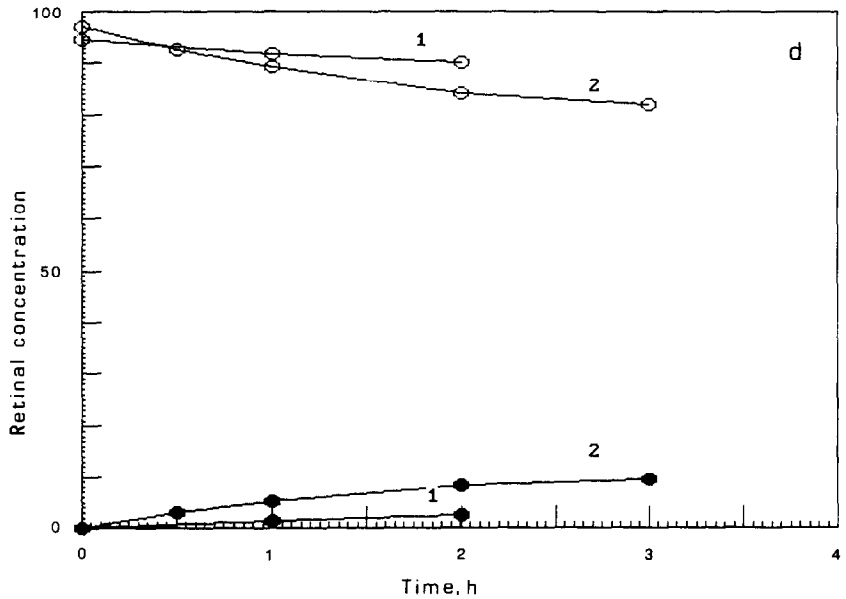


Fig.2. Photoisomerization of retinal isomers by continuous irradiation with 531 nm light at room temperature: all-*trans* retinal (a), 13-*cis* retinal (b) and 9-*cis* retinal (c) in the presence of RBP; all-*trans* retinal (d) without RBP (1) and with RBP denatured by boiling for 5 min (2). For clarity, only 11-*cis* retinal is shown as a product in d. The samples a, b and c were the same of fig.1. The samples in d were in 0.1 M phosphate buffer, pH 7. Aliquots were collected at different times during irradiation, and the extracted retinal isomers were analyzed by HPLC. The following symbols represent the various retinal isomers: (○) all-*trans*; (●) 11-*cis*; (■) 13-*cis*; (□) 9-*cis*.

REFERENCES

[1] Roof, D.J. (1986) in: The Molecular Mechanism of Photoreception. Dahlem Konferenzen (Stieve, H. ed.) pp.287–302, Springer, Berlin.
[2] Schwemer, J. (1986) in: The Molecular Mechanism of Photoreception. Dahlem Konferenzen (Stieve, H. ed.) pp.303–326, Springer, Berlin.
[3] Schwemer, J. (1984) J. Comp. Physiol. 154, 535–547.
[4] Pepe, I.M., Perrelet, A. and Baumann, F. (1976) Vision Res. 16, 905–908.
[5] Pepe, I.M. and 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] Hara, T. and Hara, R. (1973) Nature 242, 39–43.
[9] Hara, T. and Hara, R. (1982) Methods Enzymol. 81, 827–833.
[10] Pepe, I.M. and Schwemer, J. (1987) Photochem. Photobiol. 45, 679–681.
[11] Kropf, A. and Hubbard, R. (1970) Photochem. Photobiol. 12, 249–260.
[12] Seki, T., Hara, R. and Hara, T. (1980) Photochem. Photobiol. 32, 469–479.
[13] Schwemer, J. (1983) Biophys. Struct. Mech. 9, 287–298.
[14] Ozaki, K., Terakita, A., Hara, R. and Hara, T. (1987) Vision Res. 27, 1057–1070.
[15] Cronin, T.W. and Goldsmith, T.H. (1984) J. Gen. Physiol. 84, 63–81.