BBA Report

BBA 41206

Biochemical aspects of the visual process. XIX. Formation of isorhodopsin from photolyzed rhodopsin by bacterial action

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SUMMARY

A washed and concentrated suspension of bacteria (*e.g. Proteus mirabilis*) causes a rapid and almost complete conversion of photolyzed rhodopsin to isorhodopsin. Upon ultrasonication of the bacteria the activity is retained in the supernatant. The isorhodopsin formation is very sensitive to oxygen. This suggests that an easily oxidized bacterial product is able to isomerize in darkness all-*trans*-retinaldehyde present in a photolyzed rhodopsin preparation to the 9-*cis*-isomer.

The mechanism of the regeneration of the visual pigment after photolysis is still largely unknown. The crucial step must involve isomerization of the all-*trans*-form of retinaldehyde or retinol to the 11-*cis*-isomer. Hubbard[1] reported that an ammonium sulfate fraction of water-soluble proteins from cattle retina was able to isomerize all-*trans*-retinaldehyde to 11-*cis*-retinaldehyde in the light. Since regeneration of rhodopsin *in vivo* can take place in darkness, we investigated whether retinaldehyde isomerase activity could be demonstrated in the dark. When repeating Hubbard's experiments, we observed that in the presence of this retinal protein fraction a visual pigment was formed in the dark from illuminated rhodopsin[2]. A closer study of the absorption spectrum revealed to our surprise that this pigment was isorhodopsin, an analogue of rhodopsin not occurring *in vivo*. This was indicated by its absorption maximum at 485 nm and was proven by the identification of 9-*cis*-retinaldehyde as its chromophoric group after extraction with 90% ethanol[3]. Even more surprising was the finding that this property of the retinal protein fraction was heat-stable. Furthermore, the formation of isorhodopsin was very slow, requiring from 10 to 20 h. The isorhodopsin formation often showed a lag-time of several h. The isorhodopsin yield varied between 30 and 80% of the amount of rhodopsin originally present, the ratio of the molar absorbancies of isorhodopsin and rhodopsin being taken as 1.06[1].

We began to suspect that the bacteria present in the illuminated rhodopsin

suspension played a role in this tranformation. The experiment was therefore repeated under sterile conditions. After chemical sterilization of rhodopsin with ethylene oxide and incubation of the illuminated pigment under sterile conditions, the formation of photolysable pigment no longer occurred, although the sterilized rhodopsin preparation had retained its ability to form isorhodopsin under non-sterile conditions. Pigment formation was also inhibited by various antibiotics. Replacement of the retinal protein fraction by a typical bacterial growth medium, brain-heart infusion, led to a rapid formation of isorhodopsin in high yields.

To confirm the bacterial influence, bacteria isolated from an isorhodopsin generating system, were cultured overnight at 37°C in 300 ml brain-heart infusion and harvested by centrifugation (8 000 × *g*, 30 min). The pellet was repeatedly washed with 0.067 M phosphate buffer, pH 6.5, and resuspended in 30 ml of the same buffer. Photolyzed rhodopsin was prepared by 10 min illumination of a $2.5 \cdot 10^{-5}$ M rhodopsin[4] suspension in the same buffer through orange (OG 370, Schott-Jena) and infrared (KG 1, Schott-Jena) filters, leaving about 5–10% of the visual pigment originally present as a mixture of photoregenerated rhodopsin and isorhodopsin. Equal volumes of the bacterial suspension and the illuminated rhodopsin suspension were incubated under nitrogen in the dark at 37°C. The amount of isorhodopsin reached a maximum after about 4 h. At that time 65–95% of the rhodopsin originally present had been converted to isorhodopsin. A typical experiment is demonstrated in Fig. 1. Absorption spectra were

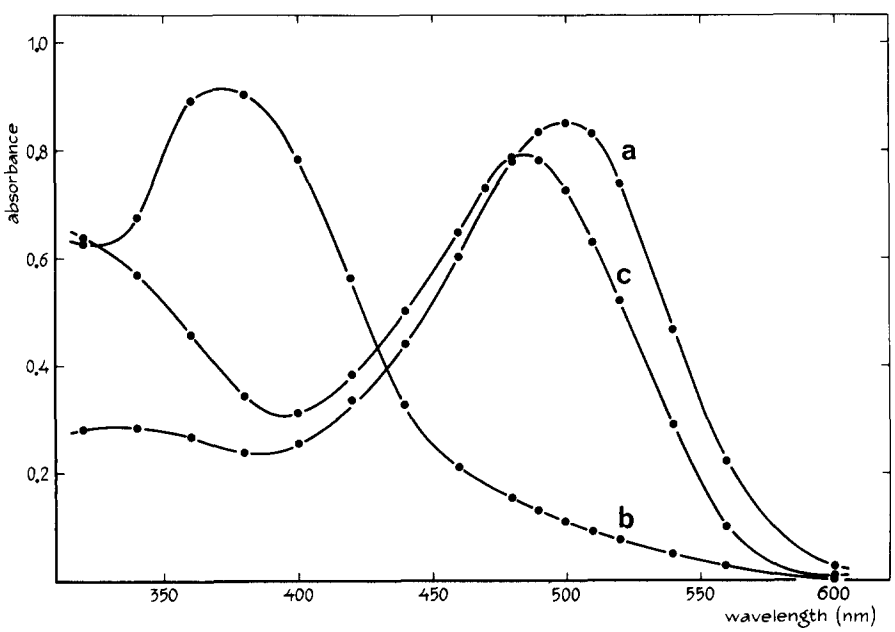

Fig. 1. Spectral evidence for the formation of isorhodopsin. Absorption spectra of rhodopsin (a), photolyzed rhodopsin (b) and isorhodopsin formed by bacterial action (c). The pigment suspensions were solubilized in a 1% Triton X-100 solution in 0.15 M phosphate buffer (pH 6.5) prior to spectral measurement.

obtained after solubilization in detergent solution of an aliquot of the original rhodopsin suspension (Curve a), of the photolyzed suspension (Curve b) and of the isorhodopsin formed after 4 h incubation with bacteria (Curve c). Fig. 2 shows that the isorhodopsin formed by bacterial action has a normal photolytic behaviour. Fig. 3 demonstrates the presence of 9-*cis*-retinaldehyde as the chromophoric group in the newly formed pigment, whereas all-*trans*-retinaldehyde is the isomer resulting from illumination.

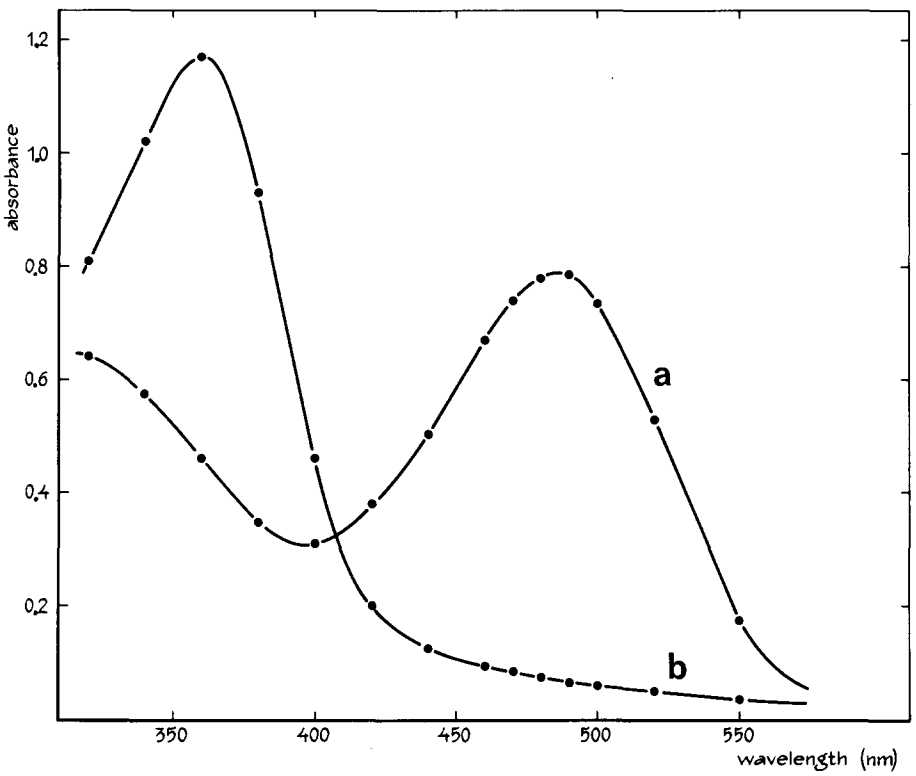

Fig. 2. Photolysis of isorhodopsin formed by bacterial action. Absorption spectra are shown of isorhodopsin formed by bacterial action, before (a) and after (b) exhaustive illumination. The pigment was solubilized in a 1% Triton X-100 solution in 0.15 M phosphate buffer (pH 6.5) containing 0.05 M hydroxylamine.

In further experiments the following properties of the bacterial formation of isorhodopsin were established.

(1) The formation of isorhodopsin is not specific for a distinct microorganism, though differences with respect to velocity and maximal level are observed. In subsequent experiments a facultatively anaerobic organism, *Proteus mirabilis*, has been used.

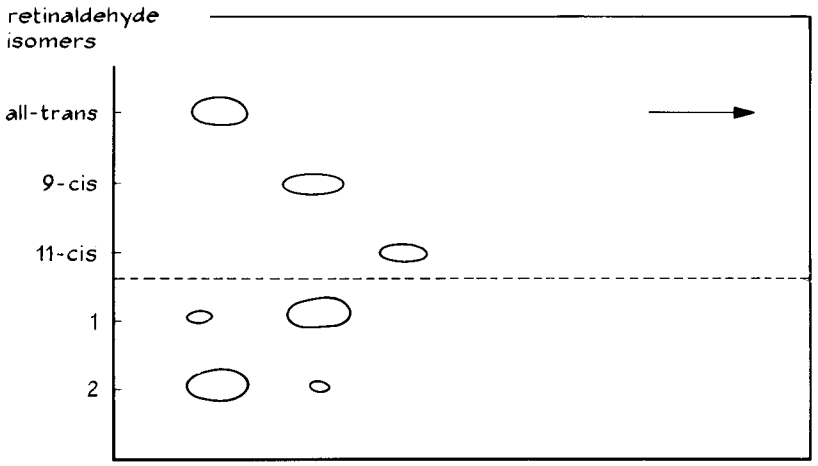

Fig. 3. Thin-layer chromatography on silica gel of chromophore extracted, before (1) and after (2) illumination from isorhodopsin formed by bacterial action. Extraction by 90% ethanol; eluent, hexane–ether (85:15, v/v); detection: spraying with Carr-Price reagent.

(2) Under our conditions the initial velocity of pigment formation after the initial lag phase is first order with respect to photolyzed rhodopsin ($t_{1/2} \approx 35$ min) and proportional to the logarithm of the bacterial concentration.

(3) Addition of antibiotics (penicillin or streptomycin, both 100 mg/l) results in complete inhibition of pigment formation.

(4) At very high bacterial concentrations the maximal yield of isorhodopsin tends to decrease.

(5) Photolyzed rhodopsin is the best substrate, giving up to 95% of isorhodopsin formation. The isorhodopsin yield is independent of the time elapsing between illumination and start of the incubation up to 3 h. Under the same conditions, all-*trans*-retinaldehyde added in a 1:1 ratio to retinaldehyde-free opsin (prepared by NADPH treatment of photolyzed rhodopsin followed by removal of retinol[5]) reacts for up to 40% to isorhodopsin. Rhodopsin itself is not affected by the bacteria. In the absence of opsin, retinaldehyde is converted by the microorganism to colorless products.

(6) Exclusion of oxygen during the incubation is obligatory, since no pigment formation occurs in the presence of even very low oxygen concentrations.

The supernatant of a centrifuged bacterial culture (30 000 × *g*, 20 min) shows some isomerizing activity, but only after concentration of the supernatant. After ultrasonic treatment of a concentrated bacterial suspension, followed by centrifugation (125 000 × *g*, 30 min), the supernatant is still fully active in forming isorhodopsin. In both types of experiments all manipulations have to be carried out in a nitrogen atmosphere. The isomerizing activity of the supernatant is not inhibited by the addition of penicillin and streptomycin.

For a further explanation of our findings three observations are important. First, the synthesis of isorhodopsin occurs in darkness, so a light-activated isomerization of the chromophoric group is excluded. Second, the bacterial system only works in the presence of opsin, but not on free retinaldehyde alone. This indicates a specific role of opsin in the isomerization process. Third, the sensitivity of the pigment formation towards oxygen suggests that an easily oxidized substance produced by bacteria is able to change the opsin conformation in such a way that the adhering all-*trans*-retinaldehyde is isomerized to 9-*cis*-retinaldehyde, followed by formation of isorhodopsin. At present work is in progress to isolate and identify this substance.

Whether or not there is a relationship to the type of regeneration, recently described by Futterman and co-workers[6, 7] is not clear. These authors emphasize the isomerization of all-*trans*-retinaldehyde rather than formation of visual pigment, and they do not state explicitly whether formation of isorhodopsin has been excluded.

Bacterial formation of isorhodopsin obviously cannot occur *in vivo*. Yet, an analogy to the rhodopsin regeneration *in vivo* cannot be excluded, since in both processes isomerization of all-*trans*-retinaldehyde can occur in darkness. Isolation and identification of the oxidizable bacterial product may, therefore, yield a clue to the understanding of the physiological process.

The excellent technical assistance of Mr G.L.M. van de Laar is gratefully acknowledged. Financial support was received from the Netherlands Organization for Basic Research (Z.W.O.) through the Netherlands Foundation for Chemical Research (S.O.N.).

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