α-RETINAL AS A PROSTHETIC GROUP IN BACTERIORHODOPSIN

Paul TOWNER, Wolfgang GAERTNER, Baerbel WALCKHOFF, Dieter OESTERHELT and Henning HOPF*

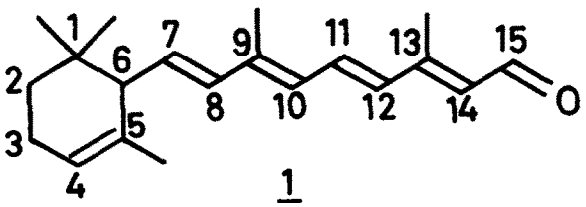
Institut fuer Biochemie, Roentgenring 11, D-8700 Würzburg, and **Institut für Organische Chemie, Technische Universität Braunschweig, D-3300 Braunschweig, FRG*

Received 30 June 1980

1. Introduction

The role of bacteriorhodopsin (BR) as a proton pump is well characterised, but the relationship between the structure of retinal, its interaction with the protein, and consequent proton transfer is not well understood [1]. After illumination BR is found to contain solely all-*trans* retinal, however, the protein undergoes a cyclic sequence of reactions, observed as transient absorption changes. One of the intermediates of this photochemical cycle can be accumulated at low temperature and has an absorption maximum at 412 nm (M intermediate). When this species is denatured 13-*cis* retinal can be extracted indicating that a *trans–cis* isomerization occurs during the cycle [2,3]. Models have been proposed which attempt to explain proton transfer by considering a *trans–cis* isomerization with concomitant protonation changes of the active site Schiff's base [4]. A different proposal to this model is a cycle in which the producton of an intermediate with *retro*-structure leads to proton transfer by exchange at position 4 or the methyl group at position 5 of retinal [5]. Even though *retro*-retinal has not been isolated from bacteriorhodopsin, the possibility of its existence is not yet excluded [6]. To determine whether a *retro*-intermediate occurs during the photochemical cycle we synthesized α-retinal (*1*) (formula 1) which formed a chromoprotein $(BR)_\alpha$ when mixed with bacterio-opsin.

Because α-retinal (*1*) has the double bond of the ring in the 4,5-position, in contrast to retinal (5,6-position) an exchange of protons at position 4 or at the 5-methyl group via a *retro*-structure is impossible.



1

Here we show that the chromoprotein BR_α undergoes a photochemical cycle. Furthermore, when α-retinal was incorporated into the bacteriorhodopsin of mutant cells deficient in retinal, ATP formation took place upon illumination and only α-retinal was re-extracted afterwards. These observations appear to rule out a *retro*-intermediate of bacteriorhodopsin during photocycling.

2. Materials and methods

α-Retinal (*1*) was prepared from α-ionone (Merck) by Wittig-Horner reactions with a C_2-nitrile phosphonate followed by partial reduction with DIBAH and the cycle repeated using a C_5-nitrile phosphonate. The intermediates and the final product were purified by HPLC similarly to procedures in [7]. Illumination of all-*trans 1* in acetonitrile led to the formation of three *cis* isomers, the major component of which was 13-*cis 1*; 13-*cis 1* together with all-*trans 1* were identified by 500 MHz high resolution ^1H NMR spectroscopy (Bruker WM 500).

Bacteriorhodopsin was isolated from *Halobacterium halobium* and bacterio-opsin prepared as in [8,9].

Abbreviations: DIBAH, di-isobutylaluminiumhydride; HPLC, high performance liquid chromatography; TLC, thin-layer chromatography

Present address: Max-Planck-Institut fuer Biochemie, D-8033 Martinsried, München, FRG

α-Retinal was incorporated into the bacteriorhodopsin of whole cells by growing a mutant of *H. halobium* (AO 151), which is deficient in retinal, but contains bacterio-opsin [10]. Growth was over 5 days, during which 600 nmol α-retinal were added in 200 nmol aliquots on days 2,3 and 4 to a 700 ml culture. The cells were centrifuged and resuspended in 10 ml basal salt in a thermostatted cylindric glass cuvette under nitrogen. pH changes of the medium were monitored continuously. ATP levels of the cells were followed by removal of 100 μl samples which were lysed in 5 ml buffer and assayed for ATP by the luciferin–luciferase method [11].

Bacteriorhodopsin containing α-retinal (BR_α) was isolated from cells by lysing them in water with DNAse and centrifuging at 30 000 rev./min for 30 min; the membrane pellet was resuspended in water, layered onto a linear sucrose gradient (25–45%, w/w), and centrifuged at 30 000 rev./min for 16 h (Beckman SW 41). The chromoprotein fraction was then diluted with water and collected by centrifugation. Transient changes in the absorption spectrum of BR_α were measured in a flash photometer at 20°C using a 15 μs Xenon flash as light source.

α-Retinal was extracted from the BR_α membrane by mixing 0.5 ml membrane suspension (10–100 nmol BR_α) with 1.0 ml isopropanol and extracting the aqueous solution twice with 2.0 ml hexane. The hexane phase was dried over Na_2SO_4, evaporated and subjected to HPLC (Knauer, 4.3 × 250 mm column) or TLC using 60:10 hexane–ether (v/v) as developing solvent.

Absorption spectra were recorded on an Aminco DW 2 spectrophotometer. All chemicals were of analytical grade, Carr-Price reagent was prepared by dissolving $SbCl_3$ to saturation in chloroform.

3. Results

When bacterio-opsin at pH 5.5–7.5 was mixed with either all-*trans* or 13-*cis* α-retinal, the absorption at 370 nm decreased and was replaced by a new peak at 490 and 480 nm, respectively. Fig.1 shows that the products were formed with no observable spectral intermediates, and for the all-*trans* isomer, that the reaction had an isosbestic point at 425 nm. At a lower temperature (5°C) the regeneration of the chromoprotein required about twice the time as with retinal. Unlike purple membrane, BR_α reacted with

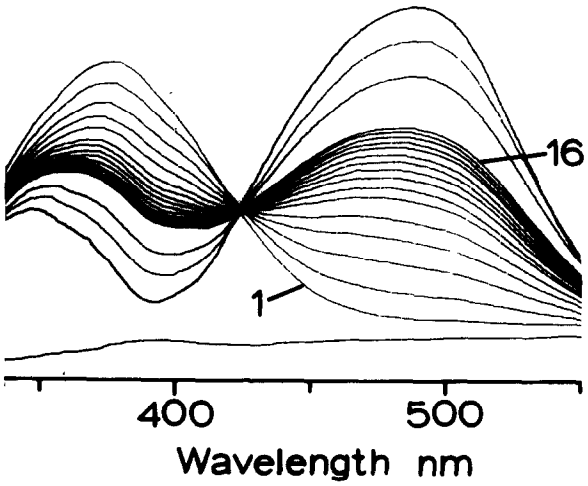


Fig.1. Regeneration of BR. A 2 ml sample of bacterio-opsin (5 μm) divided equally in two cuvettes at 20°C were placed in a spectrophotometer and 5 nmol all-*trans* α-retinal in 10 μl isopropanol stirred into the sample cuvette. Spectra were recorded continuously (1–16, 20 nm/s) and then at 23,56 and 145 min after which time no further increase in absorbance at 490 nm was observed.

hydroxylamine in darkness ($t_{1/2}$ = 40 min, 20°C), the reaction being accelerated when illuminated ($t_{1/2}$ = 7 min, 20°C), yielding en absorption spectrum with λ_{max} at 350 nm due to α-retinal oxime.

We then wished to extract α-retinal from the BR_α samples and measure quantitatively the amount of the different isomers. This was done best with BR_α

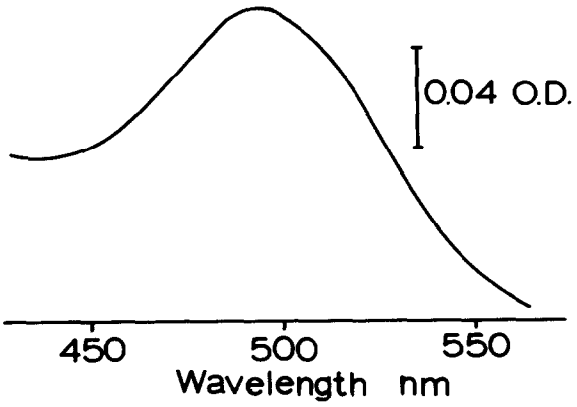


Fig.2. Difference spectrum of the chromoprotein formed by mutant cells grown in the presence of α-retinal. The absorbance spectrum of a suspension of cells was recorded and a further spectrum taken 120 min after addition of hydroxylamine to 50 mM. The difference between these two spectra was then drawn.

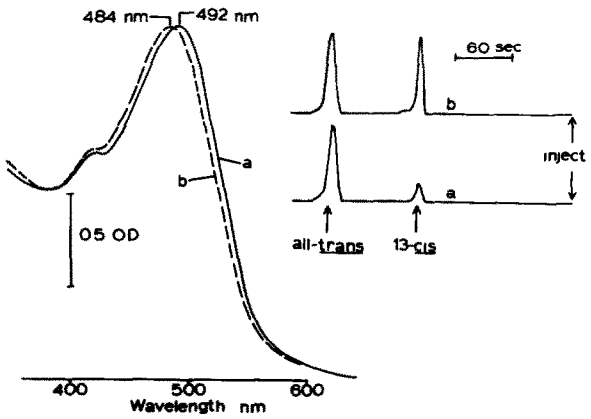


Fig.3. Absorption spectrum of BR_α. Membranes of cells were purified on a sucrose gradient and the membrane rich in BR_α washed and a spectrum recorded after storage in darkness for 24 h (a). The shoulder at 420 nm was present to varying extents in all membranes, whether the cells were grown in the presence or absence of α-retinal. When the sample was illuminated, a hypsochromic shift took place (b). The samples represented by spectra (a,b) were extracted and the α-retinal isomers injected into a HPLC system. The insert shows the recording of the column effluent at 380 nm. The R_F-values of *trans* and 13-*cis* α-retinal are marked on the trace.

prepared from mutant cells, grown in the presence of α-retinal. When an absorption difference spectrum of the cells was recorded, before and after addition of hydroxylamine, a peak at 490 nm corresponding to BR_α was clearly observed, as shown in fig.2. When the cells were lysed and purified on a sucrose gradient, the membrane containing BR was separated into several fractions the lower ones being clearly visible fawn bands (λ_{max} 494 nm). The lowest of the bands had an apparent bouyant density of 1.18 g/cm^3. When the cell membrane before fractionation was extracted and subjected to HPLC and TLC only 13-*cis* and all-*trans* α-retinal were detected. Treatment of the chromatographic plate with Carr-Price reagent revealed two scarlet bands due to the α-retinal isomers. The membranes of cells grown in the absence of α-retinal showed absorbance only at 420 nm due to the presence of cytochromes.

When α-retinal was extracted from dark adapted BR_α-samples, mainly the all-*trans* isomer was found (fig.3,a) whereas with the light adapted membrane the amount of 13-*cis* increased markedly (fig.3,b), which is in contrast to the results found with bacteriorhodopsin. These changes of isomeric configuration in BR_α correlated to a λ_{max} shift of 8 nm. The blue shifted form corresponded to an increase in the amount of the 13-*cis* isomer, which is analogous to the behaviour of bacteriorhodopsin [12]. This change in isomer composition upon light–dark adaption is separate from any transient *cis–trans* isomerisation which takes place in the photochemical cycle.

When BR_α was subjected to flash photometry two species were found as shown in fig.4. One had a difference spectrum with λ_{max} of 370 nm, which was formed within 10 ms, whereas the second product, which had a difference spectrum of 520 nm took 100 ms for maximal formation, and decayed with $t_{1/2}$ = 400 ms. Neither of these species were comparable to the intermediates of the BR photochemical cycle.

To test the role of α-retinal in fulfilling the bioenergetic function of bacteriorhodopsin, cells con-

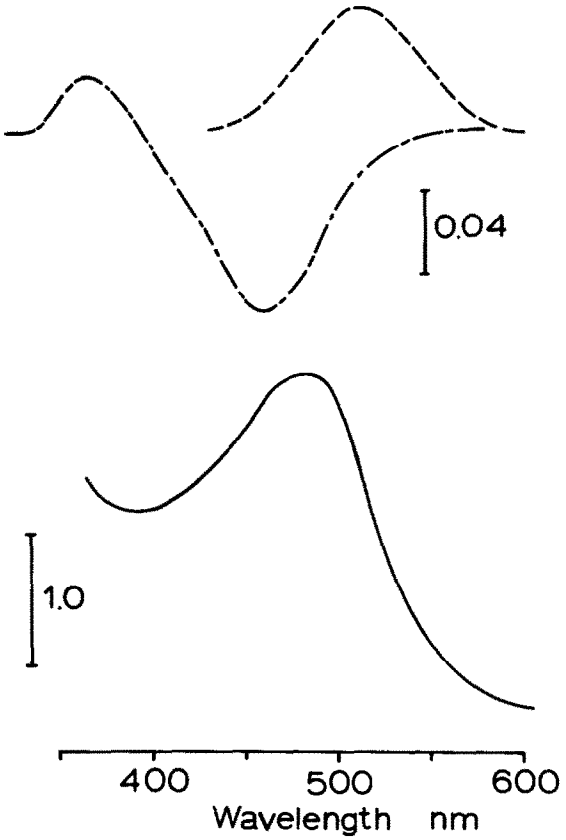


Fig.4. Flash photometry of BR_α. Transient changes in the spectrum of BR_α at pH 6 were recorded and the variations in transmission after 10 ms(–·–) and 100 ms (––) were plotted. The continuous line represents the absorbance spectrum of BR_α. The bar on the left shows the absorbance scale for the transient difference spectra.

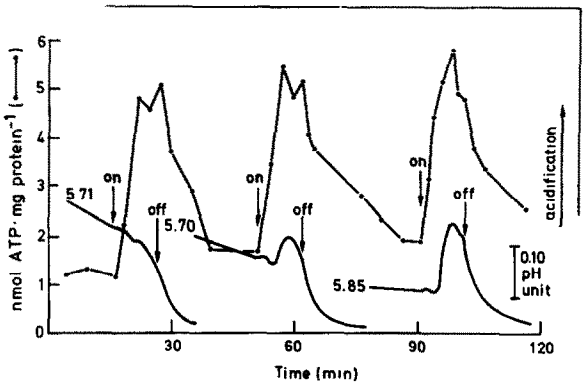


Fig.5. Changes in ATP and pH of cells evoked by light. The pH of cells (10 ml) thermostatted at 20°C in a cuvette was monitored continually. ATP in the cells was measured by removal of samples for lysis and estimation of ATP by luciferin–luciferase (○——○). The points at which illumination was started and stopped are indicated by arrows. The continuous lines represent pH traces, numerical values at the right show the pH prior to illumination. For further details see [12].

taining BR_α were stirred in a thermostatted cuvette under nitrogen in darkness and ATP levels measured. During the first 30 min of the experiment the ATP concentration decreased and reached a basal level. The cell suspension was then illuminated with light from a 150 W slide projector fitted with a 420 nm cut-off filter, 20 cm from the cuvette. Samples of the cells were lysed at strategic time intervals and assayed for ATP. Fig.5 shows that upon illumination the ATP in the cells increased 4-fold and decreased to the basal level when the cells were returned to darkness. These changes were of similar magnitude to those obtained with cells grown in the presence of retinal. The changes in pH were also recorded and show acidification of the medium upon illumination. Cells grown in the absence of α-retinal when subjected to the same experiment gave no observable changes in pH or ATP upon illumination.

4. Discussion

Similarly to retinal the all-*trans* and 13-*cis* isomers of α-retinal combined with bacterio-opsin to form a chromoprotein. Nonetheless, α-retinal must have an altered fit to the binding site, since it readily reacts with hydroxylamine. The λ_{max} shift between light-adapted (482 nm) and dark-adapted (494 nm) BR_α membrane is inverse to the change found with bacteriorhodopsin where light leads to a red shift. However, in both cases all-*trans* is the prevalent isomer of the long wavelength species [13]. From the data in fig.1 it is possible to estimate an extinction coefficient for BR_α. Based on an ϵ_m = 48 800 1 M^{-1} cm^{-1} for α-retinal, BR_α is found to have an extinction coefficient of ~68 000, which is not dissimilar to the value for bacteriorhodopsin itself.

The λ_{max} values of retinal and α-retinal are similar because in solution the double bond of the hexene ring, in the case of retinal, does not contribute significantly to the π-system. However, upon combination with the apo-protein the ring and chain of retinal are forced into a conformation which results in their mutual conjugation [6]. A similar phenomenon with α-retinal cannot extend the π-system because the double bond of the ring is not conjugated to the chain. To some extent this difference must account for the shorter wavelength maximum of BR_α (490 nm) compared to bacteriorhodopsin (560 nm) and also shows that a conjugated double bond in the ring is not necessary for function. Furthermore, proton transfer in BR_α cannot proceed via a *retro*-structured intermediate in which protons at position 4 or 5-methyl are exchanged. If proton transfer occurred instead at position 6, even though in retinal no proton is present at this position, the resulting *retro*-structure would be conjugated with the 4,5-double bond of the ring. This structure is identical to *retro*-retinal, which upon further rearrangement of the π-bond system would form retinal. Mutant cells which were grown with α-retinal were scrupulously analysed for the presence of retinal, which was never discovered.

The frequency of the photochemical cycle in BR_α is considerably slower than that of bacteriorhodopsin. However, the large amounts of opsin present in the cells would saturate the ATP synthase system even at ~1% of maximal velocity in the case of BR and therefore is sufficient in the case of BR_α to explain the observed ATP synthesis [11]. The system we have used to assess the bioenergetic function of BR differs slightly from the method in [16], in that we have used a mutant deficient in retinal, thus avoiding the presence of nicotine during growth.

In conclusion, α-retinal is shown to form a chromoprotein with bacterio-opsin which behaves as a proton pump. If proton transfer in bacteriorhodopsin were mediated via a *retro*-structure, in BR a structure indistinguishable from *retro*-retinal would be formed,

which upon further rearrangement would yield retinal. This could not be detected upon re-extraction of BR_{α} after illumination and therefore the possibility of the intermediate occurrence of a *retro*-structure is reduced to a minimum.

During the preparation of this manuscript a report came to our attention describing an active chromoprotein formed between 5,6-dihydroretinal and bacterio-opsin [17], which further shows the unlikely nature of a *retro*-structure intermediate. Furthermore it could not be shown that tritium was incorporated into retinal or deuterium lost from 4-[^{2}H]retinal during the photochemical cycle of bacteriorhodopsin [18].

Acknowledgements

The authors express their gratitude to Bruker Messtechnik, Karlsruhe, for their NMR facilities and the unfailing help of Dr W. E. Hull. We are indebted to Dr K. Grellmann, Mr E. Kaufmann and Mr H. Weller for their help and instruction in carrying out the flash photometry experiments. This work was supported by the Deutsche Forschungsgemeinschaft.

References

[1] Stoeckenius, W., Lozier, R. H. and Bogomolni, R. A. (1979) Biochim. Biophys. Acta 505, 215.
[2] Pettei, M. J., Yudd, A. P., Nakanishi, K., Henselman, R. and Stoeckenius, W. (1977) Biochemistry 16, 1955.
[3] Tsuda, M. (1980) Fed. Proc. FASEB 39, 1846.
[4] Schulten, K. and Tavan, P. (1978) Nature 272, 85.
[5] Schreckenbach, Th. and Oesterhelt, D. (1975) 10th FEBS Meet., Paris 40, 239.
[6] Schreckenbach, Th., Walckhoff, B. and Oesterhelt, D. (1977) Eur. J. Biochem. 76, 499.
[7] Gaertner, W., Hopf, H., Hull, W. E., Oesterhelt, D., Scheutzow, D. and Towner, P. (1980) Tetrahedron Lett. 21, 347.
[8] Oesterhelt, D. and Stoeckenius, W. (1974) Methods Enzymol. 31/A, 667.
[9] Oesterhelt, D., Schuhmann, L. and Gruber, H. (1974) FEBS Lett. 44, 257.
[10] Bergner, U. (1978) Diplomarbeit, Univ. Würzburg.
[11] Hartmann, R. and Oesterhelt, D. (1977) Eur. J. Biochem. 77, 325.
[12] Sperling, W., Carl, P., Rafferty, Ch. N. and Dencher, N. A. (1977) Biophys. Struct. Mech. 3, 79.
[13] Oesterhelt, D., Meentzen, M. and Schuhmann, L. (1973) Eur. J. Biochem. 40, 453.
[14] Robeson, C. D., Blum, W. P., Dieterle, J. M., Cawley, J. D. and Baxter, J. G. (1955) J. Am. Chem. Soc. 77, 4120.
[15] Hartmann, R., Sickinger, H.-D. and Oesterhelt, D. (1977) FEBS Lett. 82, 1.
[16] Oesterhelt, D. and Christoffel, V. (1976) Biochem. Soc. Trans. 563rd Meet., London, 4, 556.
[17] Mao, B., Ebrey, T. G., Crouch, R., Arnaboldi, M., Balogh-Nair, V. and Nakanishi, K. (1980) Fed. Proc. FASEB 39, 1846.
[18] Goesswein, L. (1976) Diplomarbeit, Univ. Würzburg.