RECONSTITUTION OF BACTERIORHODOPSIN

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Received 17 May 1974

1. Introduction

In the preceding paper [1] we described a method for removal of retinal from bacteriorhodopsin. Under intense light retinal is trapped as retinaloxime by reaction of hydroxylamine with the 412-nm complex, which is an intermediate of the photochemical cycle in bacteriorhodopsin. Purple membrane suspensions become white upon this treatment now containing bacterio-opsin instead of bacteriorhodopsin. Addition of retinal to such a membrane suspension reconstitutes the chromophore (purple complex) typical for bacteriorhodopsin.

2. Materials and methods

Growth of *Halobacterium halobium* NRL R_1M_1 and isolation of the purple membrane was carried out as described in [2]. Membrane suspensions containing 2×10^{-5} M bacteriorhodopsin and 0.2 M NH_2OH pH 7 were illuminated with light from a Xenon high pressure lamp (900 W) filtered through 25 cm water, 2 cm 5% $CuSO_4$ solution and an orange glass filter (OG 4, Schott, Mainz). When the sample was completely discolored (1–2 hr) it was centrifuged for 30 min at 50 000 *g* and the precipitate washed three times with water. In some experiments hydroxylamine was replaced by 1% sodium borohydride (final concentration). Aliquots containing approximately 10^{-5} M bacterio-opsin were placed in a cuvette and an ethanolic solution of retinal (1–5 μl) was added in the dark. Spectra were recorded with a Zeiss DMR 21 or an Aminco DW2 spectrophotometer. All operations were carried out at room temperature.

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3. Results and discussion

Fig. 1 compares the absorption spectra of the purple membrane (spectrum 1) and its apo-membrane (spectrum 2) produced by treatment with hydroxylamine and light. Retinaloxime remains in the membrane during the washing procedure and causes absorption around 360 nm. It can be destroyed by near ultraviolet light irradiation (spectrum 3) but was not removed in most of the experiments described because reconstitution is more efficient without prior UV-light treatment. Spectrum 4 in fig. 1 indicates that almost complete reconstitution of bacteriorhodopsin occurs when a slight excess 13-cis retinal over bacterio-opsin is used. The buoyant densities of the native purple membrane (1), its apo-membrane (2) and the reconstituted purple membrane (3) are apparently the same (fig. 1, insert). Within a minute at room temperature most of the purple complex is formed, followed by a slower increase in absorption and a shift in the absorption maximum towards longer wavelengths (fig. 2A). This was shown earlier to be due to the formation of an equilibrium mixture of the 13-cis purple complex and the all-trans purple complex in the dark [2]. Light converts the purple complex completely into its all-trans form [2]. Regeneration with all-trans retinal occurs at a slower rate than with 13-cis retinal and, as expected, a small shift of the absorption maximum towards shorter wavelengths is observed (fig. 2B). In time an identi-

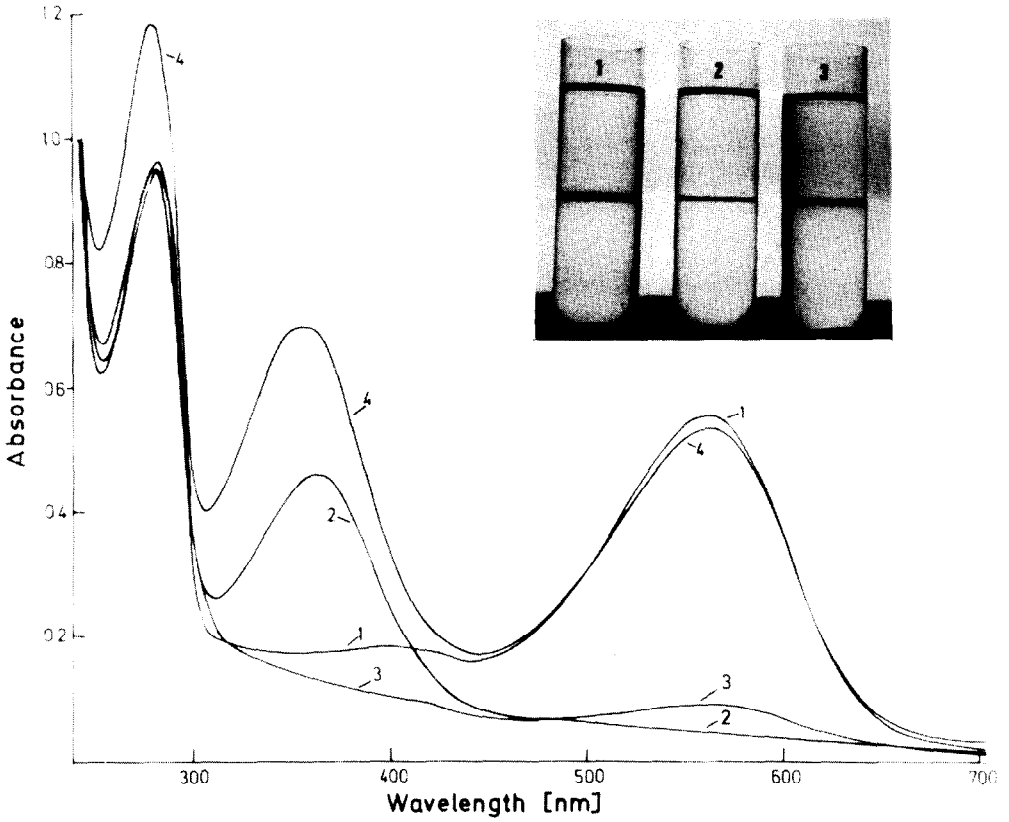

Fig. 1. Spectra of the purple membrane (1), its apo-membrane (2 and 3) and the reconstituted membrane (4). Bacteriorhodopsin concentration appr. 10^{-5} M. The apo-membrane was prepared as described under Materials and methods and its concentration adjusted to that of the purple membrane sample with respect to the optical density at 280 nm. Spectrum 3 is that of an apo-membrane suspension (2) irradiated for 30 min with white light from the Xenon lamp (900 W). To obtain spectrum 4 a slight excess of 13-cis-retinal was added to the sample of spectrum 2. The increased absorption at 280 nm is due to the overlap of this band with the absorption band of retinaloxime and excess 13-cis-retinal. The spectra were recorded with an Aminco DW 2 spectrophotometer. Insert: sucrose density gradients (30–50% see [1]) of the purple membrane (1), its apo-membrane (2) and the reconstituted membrane (3). A light yellow color appears in (2) due to retinaloxime.

cal purple complex absorption band is obtained independently whether 13-cis or all-trans retinal are used in the regeneration experiment indicating that the dark equilibrium of the isomeric forms is reached. Regeneration of the purple complex with all-trans retinal apparently involves an intermediate showing the same spectral characteristics and the same isosbestic point with the purple complex as does the 460-nm complex described in [2] which equilibrates with the purple complex in dimethyl-sulfoxide–water mixtures. Whether this intermediate is identical to the 460-nm complex has to be proven.

Neither 11-cis nor 9-cis retinal are able to reconstitute bacteriorhodopsin in the dark. Light, however, allows regeneration of the purple complex presumably via isomerization of the sterically hindered 11-cis isomer to the all-trans form (fig. 3). An intermediate, absorbing maximally around 440 nm is observed which absorbs in the same region as protonated Schiff bases of retinal. Also elevated temperatures (60°C) allow reconstitution of bacteriorhodopsin with 11-cis to some extent in the dark. Using 9-cis retinal neither light nor temperature produce amounts of the purple complex comparable to those obtained with the other retinal isomers.

Retinal and its binding site in bacterio-opsin show

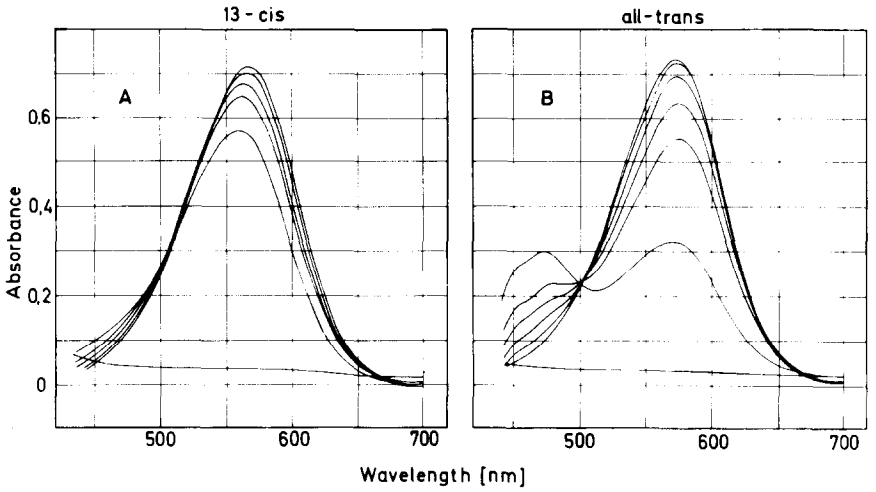

Fig. 2. Time dependence of the reconstitution of bacteriorhodopsin with 13-cis and all-trans-retinal. A stoichiometric amount of retinal was added to the apomembrane suspension (1.2×10^{-5} M) and the spectrum recorded with a Zeiss DMR 21 spectrophotometer using a sample without added retinal as reference. A: 13-cis-retinal; the spectra were recorded before addition of retinal and after 0.5, 4, 10, 25 and 35 min. B: all-trans-retinal; the spectra were recorded before addition of retinal and after 0.3, 2, 4, 8, 15 and 30 min.

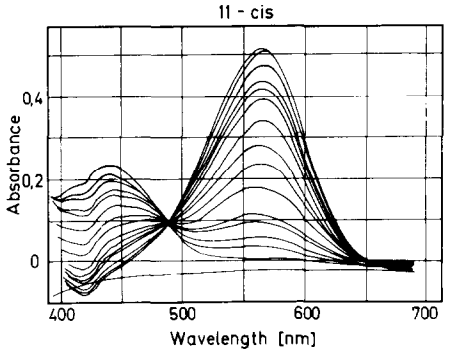

Fig. 3. Reconstitution of bacteriorhodopsin with 11-cis-retinal and light. Conditions as in fig. 2. The cuvette was irradiated with light (380 nm) of the spectrophotometer (Zeiss DMR 21) with fully opened slit for 2, 12, 25, 40 and 55 min and the spectra recorded afterwards (the five spectra with higher absorbance at 440 nm than at 560 nm). Then white light from a 60 W tungsten filament lamp was shined on the cuvette for 1, 2, 3, 4, 5, 6, 7, 10, 15 and 25 min and the spectra recorded again.

the key-lock relationship postulated long ago by Emil Fischer for substrates and enzymes catalyzing their chemical reactions. We expect that modified retinal as well as modified bacterio-opsin put into the reconstitutive system described above will give us detailed information about the chemical nature of the purple complex and will allow us to prove or disprove the interpretation of the purple complex absorption band as a charge transfer band [2,3].

Some time ago the covalent attachment of retinal to the amino group of a lysin residue within bacteriorhodopsin was analyzed [4]. Since similar experiments with visual pigments have led to results indicating binding of retinal to the amino group of a phosphatidyl ethanolamine these results have been critically checked by Bonting and coworkers. They re-established the amino group of lysine as the binding site of retinal [5,6].

If purple membrane suspensions are illuminated in the presence of borohydride they become discolored as in the presence of hydroxylamine. The pigment is now linked to the protein by a single C–N bond and the membrane shows the typical retinylprotein fluorescence. No reconstitution of bacteriorhodopsin with retinal can be detected under these conditions indicating that the retinyliden moiety was reduced at its original binding site.

Acknowledgement

This work was supported by the Deutsche Forschungsgemeinschaft.

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