The Incorporation of Tritiated Retinyl Moiety into the Active-Site Lysine Residue of Bacteriorhodopsin

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Purple membranes were isolated from *Halobacterium halobium* bleached and regenerated with all-*trans*-[15-³H]retinal. The incorporation of label was 1.2 mol of retinal/mol of bacterio-opsin. The [³H]retinyl-bacterio-opsin obtained from regeneration was hydrolysed to give tritiated retinyl-lysine, which, on hydrogenation to *N*- ϵ -perhydro[³H]retinyl-lysine and reaction with 1-fluoro-2,4-dinitrobenzene, gave bis-(2,4-dinitrophenyl)-*N*- ϵ -perhydro[³H]retinyl-lysine. This result confirmed that the retinyl moiety of the chromophore is attached to an ϵ -amino group of lysine.

The approach originally designed in this laboratory to characterize the retinyl-binding site in bovine rhodopsin (Akhtar *et al.*, 1965, 1967, 1968) has now been extended to show that in bacteriorhodopsin, as in rhodopsin, the retinyl moiety is attached to an ϵ -amino group of lysine. Previously this logical conclusion had been deduced either by analogy with the bovine rhodopsin system or through an indirect approach utilized by Bridgen & Walker (1976), who showed that all but one of the lysine residues of bacteriorhodopsin were modified by succinic anhydride.

Experimental

Preparation of purple membranes

Purple membranes from the bacterium *Halobacterium halobium* were prepared by the method of Oesterhelt & Stoekenius (1974). The purified membranes showed a single band of approx. mol.wt. 26000 on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis.

Bleaching of purple membranes

A suspension of purple membranes (5.7 mg; 0.219 μ mol, calculated from ϵ_{568} 63000 litre $\cdot$ mol⁻¹ $\cdot$ cm⁻¹; Oesterhelt & Hess, 1973) in 0.5 M-hydroxylamine (10 ml), adjusted to pH 7.0, was sonicated for 2 s. The suspension was irradiated for 10 h with a 1000 W tungsten/halogen lamp, the light being filtered through aqueous solutions of K₂Cr₂O₇ (0.2%, w/v) and CuSO₄ (2.2%, w/v). The vessel containing the material to be bleached was placed approx. 15 cm from the lamp. The suspension was stirred continuously, and the temperature maintained at approx. 10°C.

Abbreviation used: Dnp, 2,4-dinitrophenyl.

Regeneration of bacteriorhodopsin

Bleached purple membranes were centrifuged at 80000g for 10 min. The pellet was resuspended in water and re-centrifuged. It was then washed twice more by resuspension in water and re-centrifugation. The final pellet was resuspended in 20 mM-potassium phosphate buffer, pH 7.0 (12 ml). An ethanolic solution of 5 mM-all-*trans*-[15-³H]retinal (0.2 μ mol; 3 $\times$ 10⁷ d.p.m./ μ mol) (Akhtar *et al.*, 1968) was added to the bleached material. The mixture was sonicated for 2 s and left in the dark overnight. Regeneration was measured by the appearance of the 568 nm absorption band of bacteriorhodopsin.

Fixation of the chromophore and calculation of the incorporation of all-*trans*-[15-³H]retinal into bacterio-opsin

The [³H]bacteriorhodopsin obtained as described above was centrifuged at 80000g for 10 min, resuspended in aqueous hydroxylamine solution (2%, w/v) and re-centrifuged. The pellet was then washed once in the hydroxylamine solution and twice more in water by resuspension and re-centrifugation. The final pellet was resuspended in water (2 ml). NaBH₄ (15 mg) was added at intervals while the sample was irradiated for 1 h with a 1000 W light-source. During bleaching, the temperature of the stirred suspension was maintained at 10°C. The fixed material was centrifuged at 80000g for 10 min, and the pellet was washed twice in water by suspension and centrifugation. The pellet was dissolved in 5% (w/v) sodium dodecyl sulphate solution (0.5 ml) and the protein was precipitated with methanol (2 ml). The precipitate was centrifuged at 80000g for 10 min and washed twice in water by suspension and centrifugation. The final pellet was suspended in water (3 ml).

Samples of this material (4nmol of protein; 57000d.p.m.) were electrophoresed on polyacrylamide gels (see below, and Fig. 1).

Analysis of tritiated retinyl-bacterio-opsin on polyacrylamide-gel electrophoresis

Samples of the tritiated retinyl-bacterio-opsin were solubilized at 20°C in 5% (w/v) sodium dodecyl sulphate containing 50mM- Na_2CO_3 to give a final protein concentration of approx. 2mg/ml. Protein was applied to gels (6.3mm×90mm) containing 15% (w/v) acrylamide, 0.416% (w/v) *NN'*-diallyltartardiamide, 0.1% (w/v) sodium dodecyl sulphate and 6M-urea. Electrophoresis was performed at 5mA/gel for 6h. The gels were stained with Coomassie Blue, destained by the method of Weber & Osborn (1969) and scanned at 590nm in a Joyce-Loebl Chromascan mark II densitometer. Gels were sliced into 2mm discs, which were dissolved in 2% (w/v) NaIO_4 (1.6ml) and counted for radioactivity.

Hydrolysis and analysis of [15- ^3H]retinyl-bacterio-opsin

This was carried out by the method of Akhtar *et al.* (1968). The tritiated retinyl-bacterio-opsin obtained above (3.1×10^6 d.p.m.) was admixed with carrier retinyl-lysine (25 μmol) and hydrolysed overnight at 110°C, in the absence of O_2 , in 7.5M-NaOH (2ml). The hydrolysate was subjected to the usual work-up and t.l.c. in butan-1-ol/acetic acid/water, 3:1:1, by vol.). This material was converted into *N*- ϵ -perhydroretinyl-lysine by hydrogenation in the presence of pre-reduced PtO_2 and run on t.l.c. in butan-2-one/2-methylpropan-2-ol/aq. NH_3 (sp.gr. 0.880)/water (18:18:7:9, by vol.). The material was further purified by thick-layer chromatography in the same solvent system.

The sample of this material was treated with 1-fluoro-2,4-dinitrobenzene, and the Dnp derivative was subjected to t.l.c. analysis in chloroform/methanol/acetic acid (95:5:1, by vol.).

Results and Discussion

Purple membranes were isolated from *Halo-bacterium halobium* by the method already described in the literature and bleached in the presence of hydroxylamine to dissociate the retinyl moiety from bacteriorhodopsin. Compared with its animal counterparts, bacteriorhodopsin is extremely slowly photolysed, and irradiation from a 1000W tungsten/halogen lamp for 10h was required to bleach a 0.5mg/ml suspension of bacteriorhodopsin to the extent of 70–75%. Incubation of the bleached preparation with an excess of all-*trans*-[15- ^3H]-

retinal resulted in the regeneration of 56% of the bacterio-opsin, as measured by the recovery of the 568nm-absorption band of bacteriorhodopsin. Regeneration was complete within 2h. The un-regenerated protein was probably bacterio-opsin denatured during bleaching. After the reduction of the Schiff-base linkage of the labelled bacteriorhodopsin with NaBH_4 , the membranes were dissolved in an aqueous sodium dodecyl sulphate solution and the resulting [^3H]retinyl-bacterio-opsin was precipitated by the addition of methanol. This procedure removed any unbound tritiated retinyl derivatives from the precipitated protein. From the knowledge of three parameters, i.e. (a) the specific molar radioactivity of the all-*trans*-[15- ^3H]retinal originally used, (b) the extent of regeneration as measured spectroscopically (0.88 μmol was re-generated from 0.219 μmol of purple membranes) and (c) the fixed radioactivity in the retinyl-bacterio-opsin (3.1×10^6 d.p.m.), 1.2mol of retinal was found to be incorporated/mol of bacterio-opsin. When a sample of [^3H]retinyl-bacterio-opsin was analysed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, more than 66% of the radioactivity originally applied to the gel was found in a single peak corresponding to the position of bacterio-opsin (Fig. 1).

Alkaline hydrolysis of [^3H]retinyl-bacterio-opsin and the subsequent work-up of the hydrolysate gave

Fig. 1. Polyacrylamide-gel electrophoresis of tritiated *N*-retinyl-bacterio-opsin

Tritiated *N*-retinyl-bacterio-opsin was prepared as described in the Experimental section. Samples (4nmol; 57000d.p.m.) were electrophoresed in the presence of sodium dodecyl sulphate. The gels were stained, scanned at 590nm (a) and sliced into 2mm discs for the determination of radioactivity (b).

n 30% overall yield a purified fraction (9×10^5 d.p.m.) that ran as a single band on t.l.c. (R_F 0.55) corresponding to the position of α - or ϵ -*N*-retinyl-lysine (these derivatives are known to co-chromatograph; Akhtar *et al.*, 1968). For further characterization, the retinyl-lysine was subjected to hydrogenation. The resulting material ran as a single radioactive peak (5.7×10^5 d.p.m.), which corresponded to the position of authentic perhydroretinyl-lysine on t.l.c. (R_F 0.41). The radioactive band was further purified by thick-layer chromatography to yield 3.6×10^5 d.p.m. This material was converted into the corresponding Dnp derivative. It was now possible to differentiate between α - and ϵ -*N*-perhydroretinyl-

lysine, since these two species yield two distinct derivatives that are readily separated by t.l.c. (Akhtar *et al.*, 1968). The derivative obtained from retinyl-bacterio-opsin gave all the radioactivity (1.8×10^5 d.p.m.) in a single band (R_F 0.71) corresponding to the position of authentic bis-Dnp- ϵ -*N*-perhydroretinyl-lysine (see Scheme 1), showing that the retinyl moiety in bacteriorhodopsin is attached to an ϵ -amino group of lysine.

Major advances in the field have been made through the elucidation of the primary sequence of bacteriorhodopsin (Ovchinnikov *et al.*, 1977, 1978) and the delineation of its organization within the membrane (Henderson & Unwin, 1975). The present paper

Scheme 1. Diagram showing the steps involved in the characterization of *N*- ϵ -^{[3]H}retinyl-lysine isolated from tritiated bacteriorhodopsin

The recovery of radioactivity in the purified material is given for each step. Step 1, bleaching; step 2, regeneration with all-*trans*-[15-³H]retinal followed by reduction with NaBH₄; step 3, hydrolysis with 7.5M-NaOH; step 4, hydrogenation and purification on thick-layer chromatography; step 5, reaction with 1-fluoro-2,4-dinitrobenzene and isolation of Dnp derivative of perhydro^{[3]H}retinyl-lysine.

complements these studies, confirming the previous suggestion that in bacteriorhodopsin the retinyl moiety is attached to an ϵ -amino group of lysine (Oesterhelt & Stoeckenius, 1971; Bridgen & Walker, 1976), and also provides a convenient protocol for the introduction of a specific label at the active site of the pigment. A related approach for the introduction of label at the active site has been reported in an abstract form by Russian workers (Mitsner *et al.*, 1978).

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