(5-demethyl)-Bacteriorhodopsin analogue: its formation and light-driven proton pump action

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The binding of the isomers (all-*trans*, 13-*cis*, 11-*cis* and 9-*cis*) of 5-demethylretinal to bacterioopsin and the light–dark adaptation as well as the light-driven proton pump action of the resulting bacteriorhodopsin analogue were studied. The (5-demethyl)-bacteriorhodopsin is formed ~3-times faster than unmodified bacteriorhodopsin and shows an efficient light-driven proton pump action. These findings show that upon binding of retinal to bacterioopsin the protein forces the chromophore to adopt a more planar ring-chain conformation than in free retinal.

Halobacterium halobium *Purple membrane* *Proton pump* *Light–dark adaptation* *Phospholipid vesicle* *Light energy conversion*

1. INTRODUCTION

Chromoproteins with a retinylidene chromophore such as bacteriorhodopsin, visual pigments and retinochrome, play an important role in photobiology [1]. Bacteriorhodopsin (hereafter bR), the light energy converting protein of the purple membrane of the halophilic microorganism *Halobacterium halobium*, occurs in a light- (λ_{max} = 570 nm) and in a dark-adapted form (λ_{max} = 560 nm) [2]. The chromophore of the light-adapted form is an all-*trans* retinylidene moiety. In the dark-adapted form a 1:1 equilibrium between 13-*cis* and all-*trans* isomers exists [2].

The colourless apoprotein bacterioopsin (hereafter bO) is obtained by irradiation of bR with visible light in the presence of hydroxylamine [3]. bO combines with both all-*trans* and 13-*cis* retinal with formation of the so called 430–460 nm intermediates (λ_{max} = 430 nm) leading respectively to all-*trans* (λ_{max} = 570 nm) or 13-*cis* bR (λ_{max} = 550 nm) [4–6]. Also 11-*cis* retinal forms a 430–460 nm complex with bO which however is stable in the dark and does not lead to bR [4]. 9-*cis* retinal does not bind to bO [4]. In continuation of our study on the effect of the different methyl groups in retinal upon the binding properties and the light-driven proton pump action of bR [7], we now report on the binding properties of 5-demethylretinal (fig.1) (in its all-*trans*, 13-*cis*, 11-*cis* and 9-*cis* form) to bO. The light-dark adaptation together with the light-driven proton pump action of (5-demethyl)-bR are also discussed.

2. MATERIALS AND METHODS

2.1. *Preparation of 5-demethylretinal*

All-*trans* 5-demethylretinal (fig.1) was synthesized by known methods [8] from 5-demethyl-β-ionone (unpublished). Irradiation of all-*trans* 5-demethylretinal in acetonitrile with tungsten light and HPLC separation of the resulting mixture of isomers gave the *cis* isomers in pure form. The absorption spectra were measured in ethanol: all-*trans* λ_{max} = 388 nm, 13-*cis* λ_{max} = 381 nm, 11-*cis* λ_{max} = 384 nm and 9-*cis* 5-demethylretinal λ_{max} = 380 nm.

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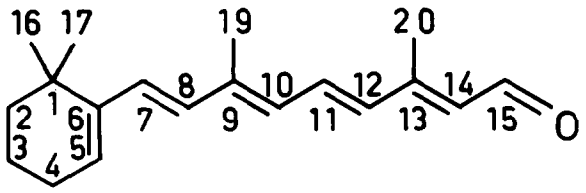

Fig.1. All-*trans* 5-demethylretinal.

2.2. *Preparation of purple membrane and bO*

Halobacterium halobium (strain R1S9) was grown and the purple membrane was isolated as in [9]. bO was prepared following known procedures [3,4].

2.3. *Binding- and kinetic experiments*

Binding experiments were performed as in [3,7] at room temperature and at 1.5°C. The regeneration was followed in 1 cm pathlength cuvettes on a Cary 219 spectrophotometer.

For the kinetic experiments an ~2-fold molar excess of all-*trans* retinal (or all-*trans* or 13-*cis* 5-demethylretinal) was incubated with bO; after complete regeneration no free bO was present. The experiments were carried out at 1.5°C. The increase in absorption was followed spectroscopically at the λ_{max} of the respective (modified) bR. The regeneration was followed until no further increase of absorption was observed.

2.4. *Light–dark adaptation and denaturation experiments*

Light–dark adaptation was performed according to [7]. The denaturation experiments and HPLC analysis of the isolated isomers of 5-demethylretinal were performed as in [2,7].

2.5. *Incorporation of bR (analogue) into phospholipid vesicles*

The incorporation of bR (analogue) into phospholipid vesicles was performed and the light-driven proton pump action was determined as in [7,10]. Nigericin was added to 2.5 μg/mg bR and valinomycin to 1 μg/mg bR.

3. RESULTS

Addition of all-*trans* or 13-*cis* 5-demethylretinal to bO at room temperature results in a rapid formation of the corresponding (5-demethyl)-bR analogues (λ_{max} = 548 and 535 nm, respectively). Upon addition of these retinals to bO at 1.5°C the corresponding 420–460 nm intermediate complexes (not observed at room temperature) are immediately formed which are converted into bR analogue (λ_{max} = 542 nm, the shift in λ_{max} is very probably due to the formation of the dark-adapted form, section 3.2) (fig.2). 11-*cis* 5-Demethylretinal forms also a complex with bO (λ_{max} = 425 nm) but this complex is stable in the dark and is not further converted into bR analogue; 9-*cis* 5-demethylretinal does not react with bO (either at room temperature, or at 1.5°C).

The formation of all-*trans* and (5-demethyl)-bR (the latter formed by bO and all-*trans* or 13-*cis* 5-demethylretinal) shows a biphasic behaviour,

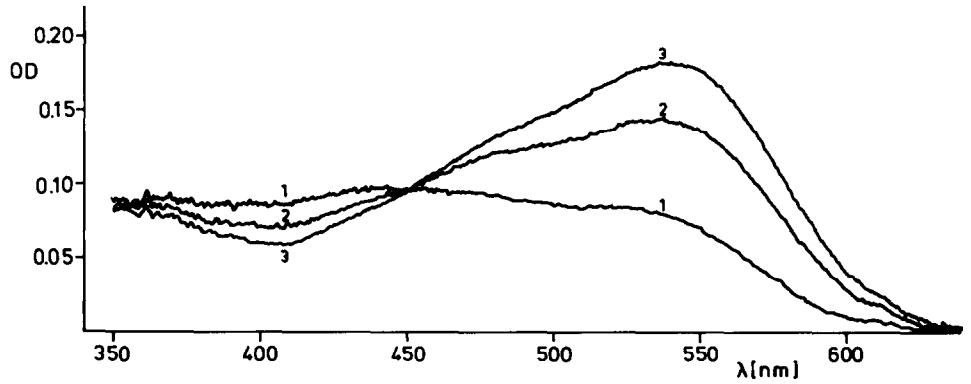

Fig.2. Reaction of all-*trans* 5-demethylretinal with bO at 1.5°C. Conversion of immediately formed 420–460 nm intermediate complex into (5-demethyl)-bR. Immediately (1), 5 min (2) and 15 min (3) after addition of all-*trans* 5-demethylretinal to bO.

with a rapid initial reaction converging into a final slower phase (fig.3). The initial reaction rate of the (5-demethyl)-pigments is at 1.5°C ~3-times larger than that of all-*trans* bR itself. On a much longer time scale both reactions go to completion ((5-demethyl)-bR formation after 15 min, that of all-*trans* bR after 30 min).

After addition of all-*trans* retinal to all-*trans* or 13-*cis* (5-demethyl)-bR (no free bO present) no absorption increase at 570 nm (λ_{max} of all-*trans* bR) is observed indicating that 5-demethylretinal (all-*trans* or 13-*cis*) binds to the same bO binding site as all-*trans* retinal.

3.2. *Light-dark adaptation*

Exposure (30 min) of the dark-adapted (5-demethyl)-bR (λ_{max} = 540 nm, this dark-adapted form is the same for 13-*cis* and all-*trans* (5-demethyl)-bR to visible light results in a red shift of the absorption maximum to 548 nm with a concomitant increase of the absorption in this region. From such light-adapted (5-demethyl)-bR all-*trans* 5-demethylretinal is isolated upon denaturation whereas the dark-adapted form yields upon denaturation 13-*cis* and all-*trans* 5-demethylretinal in an about 1:1 ratio.

After storing the light-adapted (5-demethyl)-bR for 2 h in the dark (at room temperature) it becomes again dark-adapted (λ_{max} = 540 nm) and the absorption reaches its original dark-adapted value.

3.3. *Light-driven proton pump action*

bR when incorporated into phospholipid vesicles still functions as a light-driven proton pump, pumping protons from the outside to the inside of the vesicles [11].

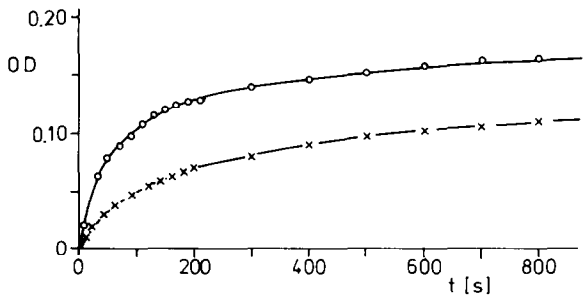

Fig.3. Formation of all-*trans* bR and (5-demethyl)-bR (the latter formed by bO and all-*trans* 5-demethylretinal) at 1.5°C. The absorption increase was recorded at λ_{max} of (modified) bR: (××) all-*trans* bR; (○○) (5-demethyl)-bR.

With illumination of (5-demethyl)-bR vesicles with visible light the pH of the external medium increases rapidly leading to a final maximal value of light-driven proton uptake of 35 nmol H^+/mg protein; i.e., ~70% of the value (53 nmol H^+/mg protein) found with unmodified bR vesicles (fig.4). The initial proton uptake was found to be stimulated by valinomycin (for bR, as well as (5-demethyl)-bR, vesicles). This is in agreement with electrogenic character of the proton movement [12]. The light-driven proton uptake could be destroyed by switching off the light or by adding the nigericin which has an uncoupling effect at the used concentration.

4. DISCUSSION

The results of the binding experiments of bO with the isomers of 5-demethylretinal (9-, 11-, 13-*cis* and all-*trans*) parallel those of bO and unmodified retinal isomers, i.e., the 9-*cis* isomers do not bind to bO, the 11-*cis* isomers form with bO stable (in the dark) complexes (λ_{max} = 425 and 430 nm, respectively), which do not further convert into (modified) bR, 13-*cis* and all-*trans* isomers bind to bO, each with formation of a 420–460 nm (or 430–460 nm for bR) intermediate, which convert into (modified) bR.

The omission of 5-CH_3 in retinal has a distinct electronic effect; e.g., λ_{max} of both all-*trans* and 13-*cis* (5-demethyl)-bR are, respectively, 22 and 15 nm hypsochromically shifted compared to λ_{max} of all-*trans* and 13-*cis* bR. Electrostatic interactions between the apoprotein and the chromophore located near the β-ionone ring have an influence on the colour of the pigment [13].

The absence of 5-CH_3 has a pronounced effect on the bR formation rate. It is to be expected that the conformation around the C_6–C_7 single bond in 5-demethylretinal is much more planar than in retinal itself (~60°) [14,15] due to the absence of the steric hindrance between the 5-CH_3 and the 8-H. This is in agreement with the suggestion [5,16] that upon binding of retinal to bO the protein forces the chromophore to adopt a more planar ring-chain conformation than in free retinal. Thus starting with an initial more planar

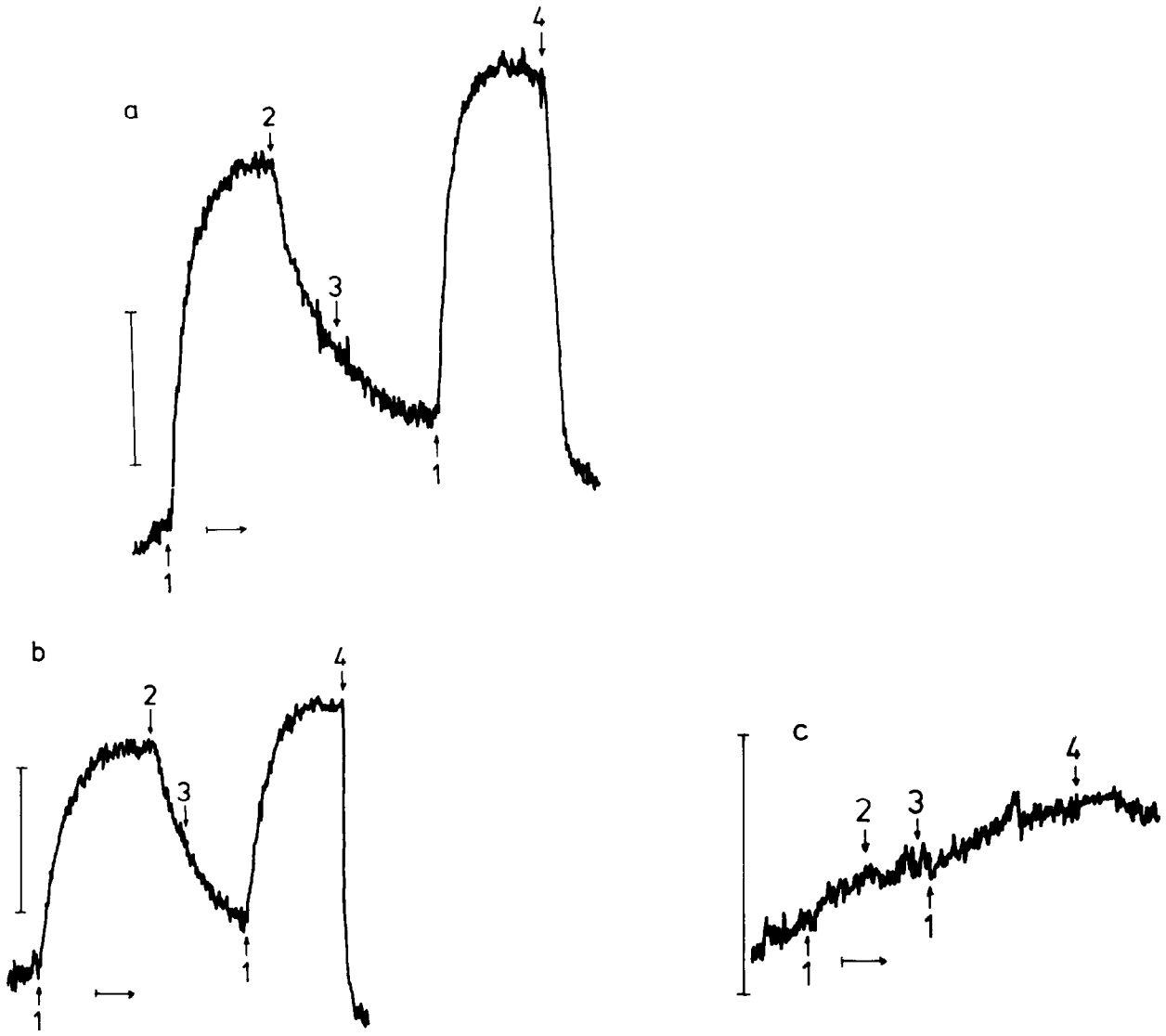

Fig.4. Light-driven proton transport in phospholipid vesicles containing 0.2 mg of: bR (a), (5-demethyl)-bR (b) and bO (c); (1) light on; (2) light off; (3) valinomycin addition; (4) nigericin addition; horizontal scale, time 1 min 20 s); vertical scale, ΔpH by addition of 5 nmol H^+.

retinal would lead to a faster binding process. The light–dark adaptation of (5-demethyl)-bR is very similar to that of unmodified bR as well as the light-driven proton pump action: 70% of the efficiency of the light-driven proton transport in unmodified bR.

It is clear that the 5-methyl group in the chromophore of bR is an important factor in the chromophore–protein interaction.

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