AN EFFECTIVE COMPETITIVE INHIBITOR OF BACTERIOOPSIN–RETINAL RECOMBINATION

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1. Introduction

Bacteriorhodopsin (BR), a chromoprotein from the purple membrane of *Halobacterium halobium* contains as a prosthetic grouping retinal in the form of a protonated aldimine with the Lys_{41} ϵ-amino group [1–3] Of interest as an approach to elucidation of the polyene environment is the search for non-aldehydic retinal analogs capable of occupying the place of this prosthetic group in BR and being held there only by non-bonded interactions. By investigating the properties of such complexes, not only can the contribution of the interactions to the retinal-protein binding energy be evaluated, but light could be shed on their effect on the conformation and electronic structure of the polyene molecule. Analogs forming stronger complexes with bacterioopsin (BO), the apo-protein of BR, should at the same time be higher competitive inhibitors of BO–retinal recombination, in other words of the regeneration of BR. Such inhibitors could be effective tools in the elucidation of the light-induced or dark reactions of BR that are accompanied by reversible cleavage of the aldimine bond. It is shown here that all-*trans* 6-methyl-8-(1′,5′,5′-trimethylcyclohexen-5′-yl-6′)-octatrien-3,5,7-one-2 (for short, C_{18}-ketone) is a highly effective competitive inhibitor of BR regeneration.
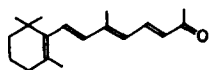
 C_{18}-ketone

2. Materials and methods

Purple membranes were isolated from *Hal. halobium* strain R_1 as in [1]. The membranes were solubilized in 2% Triton X-100 according to [4]. Apo-membranes were prepared by the procedure in [5] followed by gel filtration on a Sephadex G-25 (coarse) column, at pH 8.2. The BR regeneration in alkali-denatured purple membranes was triggered by small amounts of Triton X-100 as in [6].

The purity of all compounds listed in table 1 and of all-*trans* retinal was checked by gas–liquid or thin-layer chromatography immediately before use. They were used as ethanolic solutions, the resulting concentration of ethanol in the protein-containing media being < 1.5%.

A high pressure mercury arc (500 W; Hg546 + Hg577 was used for the light-induced transformations of BR.

The BR concentration was estimated spectrophotometrically, the molar absorption coefficient for the 570 nm peak being taken $6 \times 10^4 \, mol^{-1} \cdot cm^{-1}$ (c.f. [7]). To determine the amount of BO in the apo-membrane suspensions their absorption at 570 nm was measured 1 h after treatment with excess retinal.

3. Results and discussion

C_{18}-Ketone strongly inhibits BR regeneration independent of the method of preparing the BO-containing specimen. by hydroxylaminolysis of BR in purple membranes to yield the so-called apo-mem-
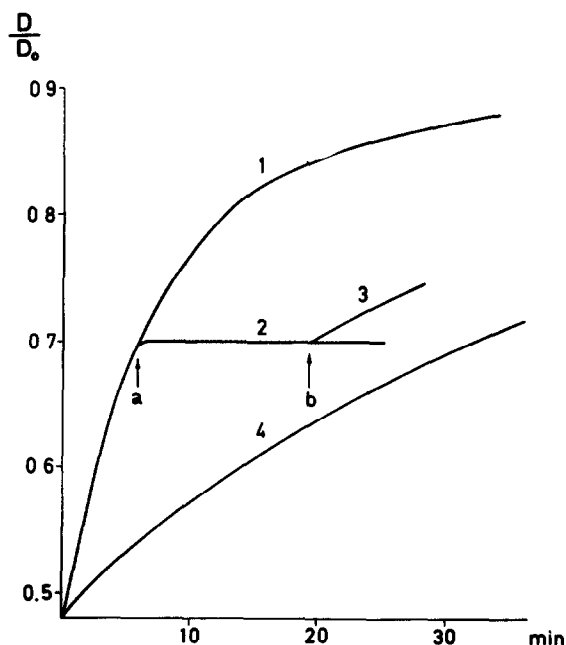
Fig.1. Effect of C_{18}-ketone on solubilized bacteriorhodopsin regeneration after irradiation for 5 min (Triton X-100 2%, retinal 10^{-4} M; MES 10^{-2} M, BR 8.5×10^{-6} M; pH 6.5; 20°C). 1, control, 2, + C_{18}-ketone, 5×10^{-4} M (a), 3, + C_{18}-ketone 5×10^{-4} M (a), + retinal 5×10^{-4} M (b), 4, + C_{18}-ketone 10^{-4} M before irradiation.

Fig.2 Effect of C_{18}-ketone concentration (c) on the V_0/V_1 ratio, where V_0 and V_i are the initial rates of regeneration in the absence and presence of the ketone, respectively (BO in apo-membranes 4.2×10^{-6} M; MES 10^{-2} M; pH 6.5; 20°C). Retinal concentration $\times 10^6$ M: 1, 20; 2, 9.8; 3, 6.75.

branes [5,8], alkali denaturation of purple membranes [6] or by light-induced hydrolysis of the retinal aldimine in BR solubilized by 2% Triton X-100 [9]. It can be seen from fig.1, that in the last case regeneration of BR is practically completely blocked by C_{18}-ketone, and is deblocked only on adding excess retinal. C_{18}-Ketone not only inhibits the regeneration but protects BO from denaturation which it suffers in the absence of exogenic retinal [9]. If retinal is only added to the medium 1.5 h after irradiation of the specimen, the degree of regeneration of BR does not exceed 20%, whereas it attains a value of 85–90% when the light-induced hydrolysis is carried out in the presence of $\sim 10^{-4}$ M C_{18}-ketone. Both the overcoming of the inhibiting action of C_{18}-ketone by excess retinal and the anti-denaturation effect of the ketone lead to the conclusion that the regeneration inhibition by C_{18}-ketone is of a competitive nature.
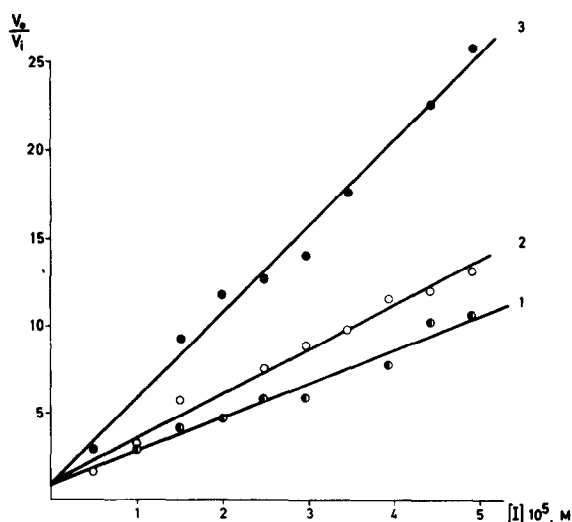
This conclusion is confirmed by experiments on apo-membranes. The regeneration kinetics in apo-membranes measured spectrophotometrically at 570 nm is satisfactorily described by the Michaelis-Menten equation ($K_m = (2.5 \pm 0.5) \times 10^{-6}$ M; 20°C, pH 6.5). This gives grounds to regard the regeneration as a process involving reversible binding of retinal followed by transformation of the BO–retinal complex into BR (c.f. [10]).

The family of straight lines represented in fig.2 reflects the dependence of the degree of inhibition of BR regeneration upon the concentration of C_{18}-ketone for differing retinal concentrations. In the coordinates used, such dependences are typical of competitive inhibition [11]. Such plots were used to calculate the values of K_i and of the K_i/K_m ratio for C_{18}-ketone and a number of other compounds listed in table 1. The K_i and K_i/K_m values, strictly speaking, differ somewhat from the true values, because apo-membranes contain retinal oxime-complexed BO rather than BO alone and dissociation of the complex must precede interaction of BO with either retinal or its competitive analogs. This systematic error should be small, however, for the following reasons. First, the value of K_i for retinal oxime is 1–2 orders of

Table 1
Retinal analogs as competitive inhibitors of BR regeneration

Compounds	$K_1 \times 10^6$ (M)	K_1/K_m
C_{18}-ketone	0.59 ± 0.03	0.24 ± 0.05
5-*cis* isomer of C_{18}-ketone	3.5 ± 0 3	1.4 ± 0.3
4-keto-C_{18}-ketone[a]	11 ± 1	4.4 ± 1
all-*trans* retinol	13 ± 1	5.2 ± 1
all-*trans* retinal anti-oxime[b]	130 ± 10	52 ± 11
β-ionone	minor effect	
ψ-ionone	inactive	
all-*trans* retinol acetate	inactive	

[a] m.p. 104–105°C; λ_{max}^{MeOH} (log ϵ): 275 (3.94) nm, 343 (4.59) nm

[b] m.p. 144–146°C

[a,b] Syntheses of the compounds will be published elsewhere

magnitude higher than for any other of the investigated compounds for which this constant could be determined. From this it follows that the binding equilibrium is usually shifted in the direction of retinal oxime displacement by retinal, retinol or C_{18}-ketone On the other hand, direct experiments described below show that the formation rate of BO—retinal or BO—inhibitor complexes is much higher than that of BR regeneration (c.f. [10]) so that dissociation of the BO—oxime complex is not kinetically significant.

The binding of C_{18}-ketone to BO is accompanied by a bathochromic shift of the polyene chromophore absorption which is nicely brought out by means of difference spectra (fig.3). The difference spectra were recorded not only on interaction of C_{18}-ketone with apo-membranes or with alkali-denatured purple membranes, but also in the light-induced hydrolysis of solubilized BR in the presence of C_{18}-ketone. The cause of the effect is, therefore, not incorporation of the C_{18}-ketone in the lipid matrix of the membrane, but its direct interaction with the protein molecule.

This assumption is confirmed by the following experimental facts. First, induced circular dichroism appears in the absorption region of the bound C_{18}-ketone (fig.4). This effect which is due to the chirality of the C_{18}-ketone environment attains a limiting value at ketone/protein ratios very close to
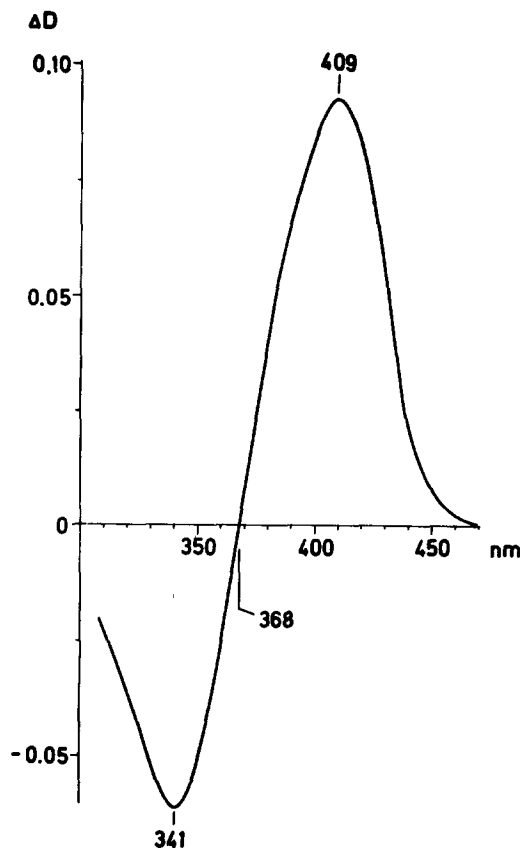

Fig.3. Difference spectrum reflecting the binding of C_{18}-ketone to bacterioopsin in the light-induced bleaching of solubilized bacteriorhodopsin (Triton X-100 2%; MOPS 10^{-2} M, BR 1.2 × 10^{-5} M ($A_{0.5cm}^{555}$ 0.35), pH 7 2; 20°C; irradiation for 5 min). Main beam cell 1, 2% Triton X-100, cell 2, sample bleached in the presence of 8.6 × 10^{-5} M C_{18}-ketone. Reference beam cell 1, 8.6 × 10^{-5} M C_{18}-ketone in 2% Triton X-100; cell 2, control bleached sample.
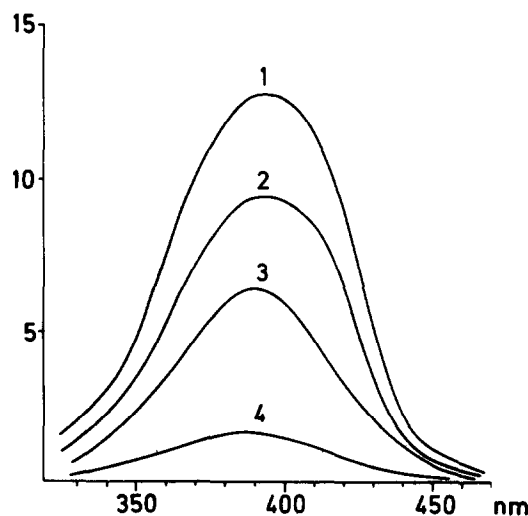
$[\theta] \cdot 5000$

Fig.4. CD spectra of the C_{18}-ketone bound by solubilized bacterioopsin which was produced as in fig.3 1, initial curve; 2, 3 and 4, curves recorded 10, 25 and 40 min after adding 1 mol retinal/mol ketone

ΔD

Fig 5 Difference spectra reflecting the displacement of retinol by C_{18}-ketone from its complex with bacterioopsin in apo-membranes (BO in apo-membranes 5.7×10^{-6} M, MES 10^{-2} M, pH 6.5, 20°C). Additives in mol/mol protein 1, retinol 0.75 in the suspension in main beam; 2, 3, C_{18}-ketone 0.45 and 0.75, respectively, into suspensions in both beams

unity and disappears either on denaturation of the BO (heating, strong alkalization, ionic detergents) or on retinal-added regeneration of BR. This is just the result that would be expected if C_{18}-ketone occupies the same site in the protein molecule as retinal.

Since retinol is a much less effective regeneration inhibitor than C_{18}-ketone, one could expect that the former would be displaced by the latter from the protein Judging from disappearance of the 'tripronged' band characteristic of bound retinol [10] in both absorption and CD spectra, such displacement in fact already takes place at C_{18}-ketone/retinol ratios close to unity (fig.5)

The data obtained show that both lengthening of the chain relative to that of retinal and removal of the cyclohexene moiety hinder the BO binding of the retinal analogs. Judging from the K_1/K_m value, C_{18}-ketone forms a stronger complex with BO than even retinal. This makes one look for particular reasons for such an effective binding. The 'tripronged' nature of the absorption band of bound retinol was ascribed [10] to distortion of its natural conformation such that the cyclohexene ring becomes coplanar
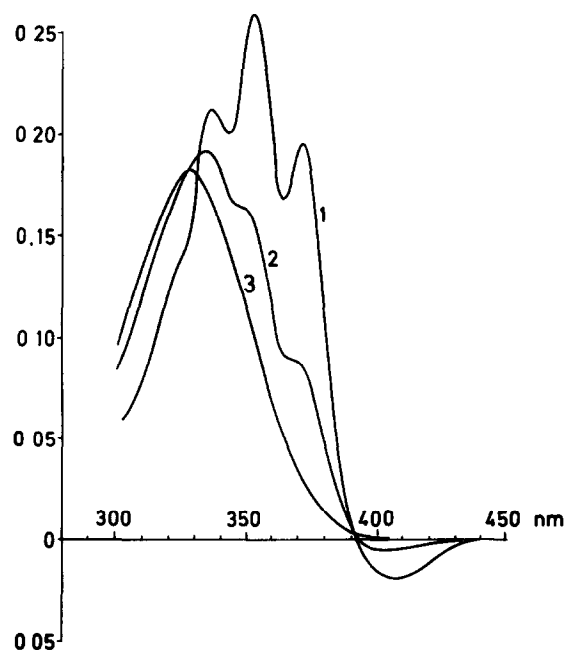
with Δ^7-bond. The energy source for such a distortion must be the overall non-bonded interaction energy, inevitably leading to a relative decrease in complex stability. The spectrum of the bound C_{18}-ketone displays no fine structure in the absorption band. This might mean that binding of 'shortened' analogs is not accompanied by distortion of their molecules.

An alternative explanation is that the C_{18}-ketone complex is additionally stabilized by a hydrogen bond between the carbonyl O-atom and some functional group of the protein. This explanation is attractive as in BR such a group should be near the retinal Δ^{13}-bond and could thereby take part in its conversion in the process of the photochemical cycle. Further investigation in this direction is thus of considerable interest from the viewpoint of the mode of action of BR.

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