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Reason for the lack of light–dark adaptation in *pharaonis* phoborhodopsin: reconstitution with 13-*cis*-retinal

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Abstract The reconstitution of *pharaonis* phoborhodopsin was performed by incubation of its opsin with 13-*cis*-retinal. Spectrum change was very slow, and two phases of the change were observed: the first and second phases are due to the transient formation of *13-cis* pigment and spontaneous isomerization to *all-trans*-retinal, respectively. Slow binding supports an idea that the retinal binding pocket of ppR is highly restricted. Being bent in the configuration, 13-*cis*-retinal cannot be accommodated in the pocket due to the steric hindrance. This is a possible reason for the lack of light–dark adaptation.

Key words: Phoborhodopsin; Isomerization of retinal; Retinal proteins; Light–dark adaptation; *Natrobacterium pharaonis*

1. Introduction

Halobacterium halobium has at least four retinal proteins: bacteriorhodopsin (bR) [1], halorhodopsin (hR) [2,3], sensory rhodopsin (sR or sR-I) [4] and phoborhodopsin (pR) [5,6]. The former two work as light-driven ion pumps and the latter two as sensory receptors. Among them, pR is the least characterized due to its minimal quantity and to its instability against solubilization with detergents.

We succeeded in purifying a pR-like protein from the haloalkaliphilic bacterium [7], *Natronobacterium pharaonis*, and called it *pharaonis* phoborhodopsin (ppR) [8]. A remarkable difference of pR and ppR from other bacterial retinal proteins is their wavelength at maximum absorption and the presence of shoulders in the absorption spectrum: lmax is located near 500 nm (498 nm for ppR and 488 nm for pR) which is close to that of rhodopsin, while those of the other three bacterial rhodopsins are at 560–590 nm. Using a purified sample, we investigated the photochemistry [8–10], chromophore configuration and isomerization on photon absorption [11].

A phenomenon called light–dark adaptation is known for bR [1,12]: Light-adapted bR undergoes slow ($T_{1/2}$=20 min at room temperature) dark adaptation, which results in a 10 nm blue shift of λ_{max} and isomerization of retinal chromophore from *all-trans* to a mixture of *all-trans* and *13-cis*. hR also exhibits light–dark adaptation, and λ_{max} of the dark-adapted hR is blue-shifted from that of the light-adapted form [13]. ppR, on the other hand, exhibits no light–dark adaptation, because incubation in the dark caused no spectral change. In addition, chromophore extraction experiments detected only *all-trans*-retinal from both light- and dark-adapted ppR. To confirm the lack of the light–dark adaptation of ppR, reconstitution of the opsin with 13-*cis*-retinal was attempted, and we pondered why *13-cis* chromophore of ppR does not exist.

2. Materials and methods

2.1. Retinal and 13-cis-retinal

All-trans-Retinal was purchased from Sigma Chemical Co. 13-*cis*-Retinal was isolated from a mixture of isomers produced by irradiation of *all-trans*-retinal and purified using high performance liquid chromatography [14].

2.2. Preparation of ppR-opsin

ppR-opsin was prepared in accordance with a method described previously [15].

2.3. Spectrophotometry

Absorption spectra were recorded by a recording spectrophotometer (MPS-2000, Shimadzu) which was interfaced with a personal computer (PC-9801RA, NEC) for storing and analyzing the spectral data. To keep the sample temperature constant, a thermostatic circulator (Neslab) was installed in the sample compartment of the spectrophotometer.

3. Results and discussion

Fig. 1A shows the spectral changes observed during incubation of ppR-opsin with 13-*cis*-retinal. An increase in absorbance at about 500 nm and an absorbance decrease at 390 nm were concomitantly observed, indicating the regeneration of ppR. Curve 7 of Fig. 1A is the spectrum 4970 min after addition of 13-*cis*-retinal. Further incubation did not change the spectrum. This sample was bleached with hydroxylamine and difference spectrum was shown in curve 1 of Fig. 1B. It should be noted that this spectrum is identical with that of a pigment reconstituted with *all-trans*-retinal (curve 2 in Fig. 1B). Only *all-trans*-retinal was extracted from the pigment reconstituted with *all-trans*-retinal. The spectrum identity shown in Fig. 1B suggests strongly that 13-*cis*-retinal chromophore does not exist stably in the ground state of ppR, because λ_{max} of pigments with *all-trans*- and 13-*cis* retinal differ from each other in bR and hR [1,13].

Fig. 1C shows the time course of 498 nm absorbance change during the incubation of ppR-opsin with 13-*cis*-retinal. Two phases of the absorbance change were observed and they are well fitted by two sequential single-exponentials, the calculated half-times from which were 5.9×10^3 and 10^4 s. Careful inspection of Fig. 1A led to recognition that as incubation time increased, the absorption maximum was shifted to longer wavelength with clearer appearance of an absorption shoulder. We have, therefore, concluded that 13-*cis*-retinal can bind ppR-opsin but that, once bound, it isomerizes spontaneously to

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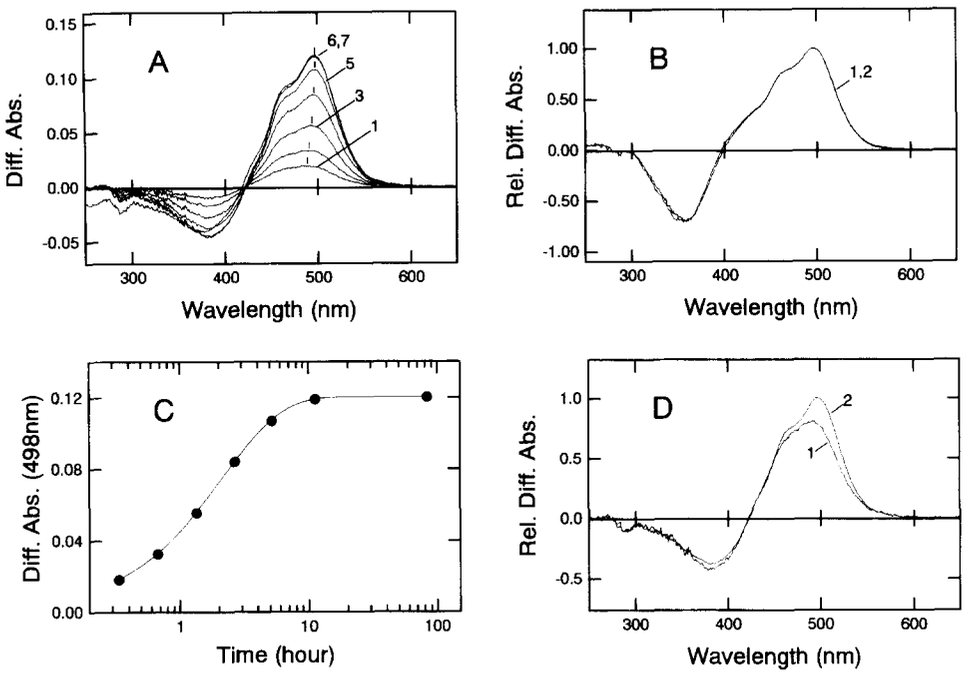

Fig. 1. Pigment regeneration from incubation of ppR-opsin with 13-*cis*-retinal. The opsin was suspended in 4 M NaCl containing 0.5% octyl glucoside buffered with 25 mM Tris-HCl (pH 7.2) at 25°C. (A) Spectral changes observed after addition of 13-*cis*-retinal to ppR-opsin. The baseline is the spectrum immediately after addition of 13-*cis*-retinal. Spectra were taken after various periods of incubation. Periods after the addition of 13-*cis*-retinal were: 20, 40, 80, 160, 310, 670 and 4970 min, respectively, from curves 1–7. (B) Comparison with the spectrum obtained from 13-*cis*-retinal (curve 1) and that from *all-trans*-retinal (curve 2). The sample of curve 7 in (A) was bleached and the difference spectrum between after and before bleaching was shown as curve 1. Spectra were normalized at their maximum. (C) Time course in absorbance at 498 nm during incubation with 13-*cis*-retinal, showing that the reconstitution was composed of two processes. Solid line is a fitted curve which is expressed by the sequential two single-exponentials. (D) Calculated difference spectrum of *13-cis* pigment from ppR-opsin and 13-*cis*-retinal is shown by curve 1. For comparison. difference spectrum of *all-trans* pigment is also shown by curve 2.

all-trans. Chromophore extraction showed that *13-cis* geometry was retained in the early stage of incubation (data not shown).

Using the above time-constants, we calculated that 80 min after incubation the sample contains 44% opsin, 43% *13-cis* pigment and 13% *all-trans* pigment, and the sample at 670 min, 5% *13-cis* pigment and 95% *all-trans* pigment. We can, therefore, calculate the spectrum of *13-cis* ppR which is shown as curve 1 in Fig. 1D. It displays an absorption maximum at 490 nm. Like bR and hR, ppR can have a *13-cis* isomer whose λ_{max} is about 10 nm blue-shifted with the smaller extinction coefficient (81% of *all-trans* pigment). Contrary to bR and hR, however, *13-cis* isomer is not stable, as shown above. The absorption shoulder which is a characteristic of both pR and ppR, also appears in the chromophore of *13-cis* isomer, although it is fainter than that of the *all-trans* chromophore.

It was previously demonstrated [15] that the space of the retinal binding site in ppR is restricted to the plane of the cyclohexenyl ring of the chromophore. $T_{1/2}$ of the reconstitution process from ppR-opsin and *all-trans* retinal was only 180 s [15], while that from 13-*cis*-retinal was as slow as 5900 s. This is also consistent with the tight space of the retinal binding pocket, since 13-*cis*-retinal has a 'bent' structure which gives rise to the steric hindrance.

ppR has the following photocycle [9,10]: ppR(498nm) → ppR_K(~540nm) → ppR_{KL}(512nm) → ppR_L(488nm) → ppR_M (390nm) → ppR_O(560nm) → ppR(498nm). ppR_M has a *13-cis* chromophore [11]. Thermal re-isomerization to *all-trans* chromophore takes place at the conversion from ppR_M to ppR_O which reverts to the original ppR without chromophore isomerization. Due to the tight binding pocket in ppR, the retinal chromophore cannot take conformation other than *all-trans*. This is the reason for the lack of dark-adaptation.

It is shown that the steric interaction between retinal and protein is important for signal transduction of both bovine rhodopsin [16] and sR [17]. The binding pocket of ppR cannot accommodate 13-*cis*-retinal, but ppR_M has a 13-*cis*-retinal. Thus the steric interaction between 13-*cis*-retinal and nearby protein may force the protein to change. This putative conformational change may be the trigger for signal transduction and this awaits experimental confirmation.

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