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Low-temperature spectrophotometry of phoborhodopsin

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Photoreactions of the fourth rhodopsin-like pigment, phoborhodopsin (pR480), of *Halobacterium halobium* were studied by low-temperature spectrophotometry. Upon irradiation of pR480 at −80 as well as −170°C with 436 nm light, a new intermediate having a difference absorption maximum at about 520 nm was produced. We designate this intermediate 'P520'. Prolonged irradiation caused the formation of a photosteady-state mixture composed of P520 and pR480, indicating that P520 was photoreversible to pR480 at −80°C. P520 was thermally converted back to pR480 through P350 and P530, both of which were detected previously [(1986) Biochem. Biophys. Res. Commun. 139, 389–395]. Based on these results, a new photochemical reaction cycle of pR is presented.

Phoborhodopsin; Photochemical reaction; Primary intermediate; Low-temperature spectrophotometry; (*Halobacterium halobium*)

1. INTRODUCTION

Recently it was discovered that an archaebacterium, *Halobacterium halobium*, possesses the fourth rhodopsin-like pigment which follows bacteriorhodopsin (bR), halorhodopsin (hR) and sensory rhodopsin (sR). This pigment (absorption maximum: 480 nm) is called phoborhodopsin (pR480) or sR_{II} [1–3]. It acts as a photoreceptor for the phobic response of the bacterium to blue-green light at around 480 nm. The photochemical reaction of pR480 has thus far been investigated by flash photolysis methods on the millisecond time scale at room temperature by which two intermediates were found; one has a difference absorption maximum at 350 nm (P350) [2,3] and that of the other is at 530 nm (P530) [2]. These intermediates may correspond to the M and N or O intermediates of bR, respectively [4].

In order to gain more information on the elementary process of the reaction cycle of pR and discuss its similarity to those of the other rhodopsin-like pigments (bR, hR and sR), we have investigated the photochemical reactions of pR480 by low-temperature spectrophotometry.

2. MATERIALS AND METHODS

The mutant *H. halobium* strain Flx3bl [2] which contains only pR480 was grown in peptone medium [1]. The membrane fraction containing pR480 was prepared from cells as described [4]. For low-temperature spectrophotometry, glycerol was added to the membrane suspension in 20 mM Pipes buffer containing 4 M NaCl to a final concentration of 66%.

Difference spectra of pR480 before and after irradiation at low temperatures were recorded on a Hitachi model 330 spectrophotometer equipped with a special glass cryostat [5]. The temperature of the sample was monitored using a copper-constantan thermocouple connected to a sample holder. The sample was irradiated by light from a 1 kW tungsten-halogen lamp which had passed through an interference filter (436 nm, Nihonshinku) or a glass cut-off filter (VO 58 and VO 56, Toshiba) and through a 5 cm water layer for removing heat radiation. For correction of scattering of the sample, an opal glass was placed on each side of the sample and reference sides. For the sake of high turbidity of the sample, spectra for wavelengths shorter than 440 nm could not be recorded.

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Abbreviation: Pipes, 1,4-piperazinediethanesulfonic acid

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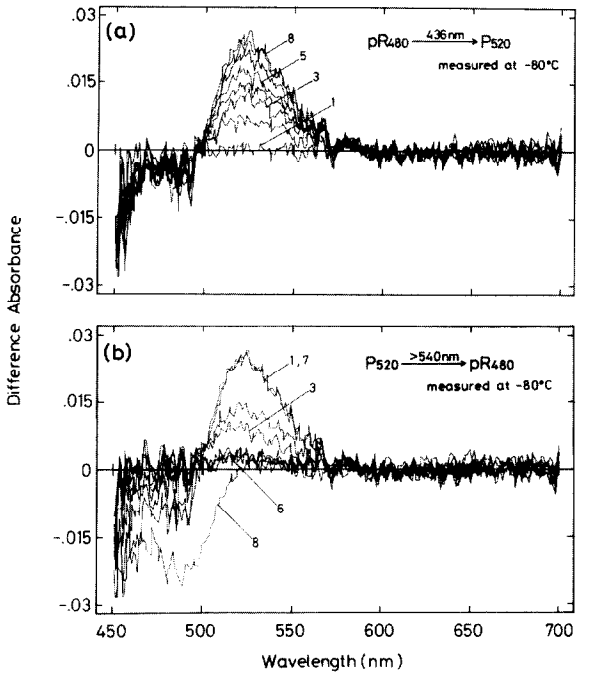

Fig.1. Interconversion between pR480 and P520 by irradiation at −80°C. The absorption spectrum of pR membranes was used as a baseline in measurements. (a) Spectral changes in the course of conversion of pR480 to P520. The pR membrane (curve 1) was irradiated with 436 nm at −80°C for a total of 40, 80, 160, 320, 640, 1280 and 2560 s (curves 2–8, respectively). Curve 8 refers to a photosteady-state mixture composed of pR480 and P520. (b) Spectral change in the course of the conversion of P520 to pR480. The photosteady-state mixture composed of P520 and pR480, produced by irradiation of pR480 with 436 nm light (curve 1, same as curve 8), was irradiated with orange light (>540 nm) at −80°C for a total of 5, 10, 20, 40 and 80 s (curves 2–6). P520 in the photosteady-state mixture reverted almost to pR480. The sample was then re-irradiated with 436 nm light for 2560 s (curve 7). This spectrum coincided with that before irradiation with orange light (curve 1), indicating that photoreversibility between pR480 and P520 occurred at this temperature. The photosteady-state mixture was then warmed to −30°C and the spectrum at −80°C recorded (curve 8).

3. RESULTS

The experiment was started with measurement of the absorption spectrum of pR480 as the baseline (curve 1, fig.1a). On irradiation of pR480 with 436 nm light at −80°C, a remarkable increase in absorbance at about 520 nm with a concurrent decrease at about 480 nm was observed (fig.1). These spectral changes indicate the formation of a bathochromic photoproduct on irradiation of pR480. Prolonged irradiation caused the formation of a photosteady-state mixture composed of the photoproduct and pR480 (curve 8, fig.1a). In fact, the photoproduct was converted back to pR480 on irradiation with orange light (>540 nm) which pR480 absorbed slightly (fig.1b). Re-irradiation of this preparation with light of 436 nm produced the same photosteady-state mixture (curve 7, fig.1b), indicating that photoreversibility between pR480 and the photoproduct took place at this temperature. We designate this photoproduct 'P520', because the difference absorption maximum is located at about 520 nm.

When the photosteady-state mixture was warmed from −80°C, the absorbance at about 520 nm diminished with a concurrent decrease at about 480 nm above −50°C, and at −30°C a difference spectrum exhibiting only a negative absorbance over the wavelength region 440–550 nm was obtained (curve 8, fig.1b). This spectral change appears to indicate the conversion from P520 to P350, although we could not measure an increase in absorbance around 350 nm due to the high turbidity of the sample.

In fig.2, pR480 was irradiated with yellow light (>480 nm) for 1 min at −10°C, where the time courses of the absorbance changes at 480, 510 and 530 nm were measured. Since P520 decayed rapidly to P350 at this temperature, we failed to record any absorbance change due to formation of P520. The immediate decrease in absorbance at 480 nm recovered with half-times of 50 and 160 s (fig.2a), whereas that at 510 nm showed a half-time of only 50 s (fig.2b). On the other hand, the absorbance at 530 nm increased gradually and then declined to the initial level (fig.2c). The 530 nm kinetic curve could be resolved into two components by non-linear least-squares methods; the half-times were estimated to be 53 and 160 s, which are almost equal to those at 480 nm, respectively. These data indicate that P350 which was formed immediately after irradiation under these experimental conditions decayed to P530 with a half-time of 50 s and P530 was then converted back to pR480 with a half-time of 160 s. These absorbance changes at −10°C were quite similar to those at 20°C observed previously [2], although the time scales of the conversion differed from each other.

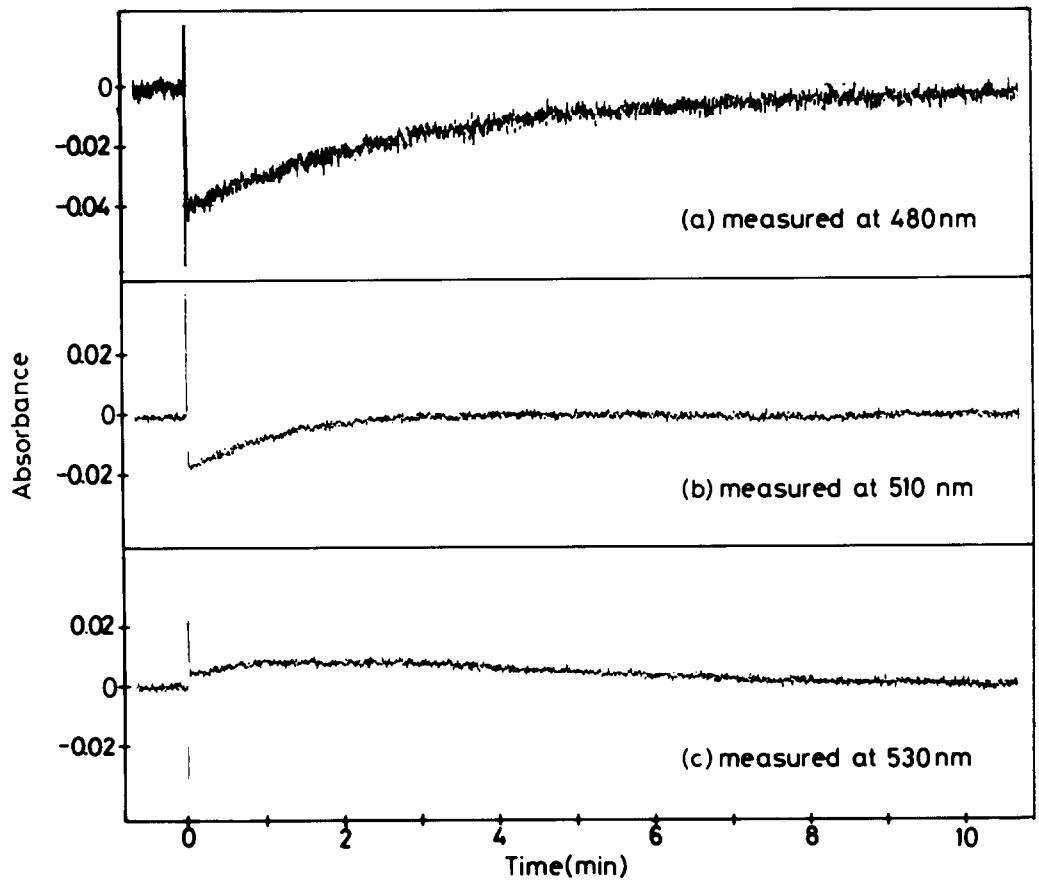

Fig.2. Time courses of absorbance changes at 480, 510 and 530 nm after irradiation of pR480 at −10°C. Immediately after irradiation of pR480 with yellow light (>480 nm) for 1 min at −10°C, the absorbance changes of the sample were continuously monitored at 480 (a), 510 (b) and 530 (c) nm on a time scale of seconds.

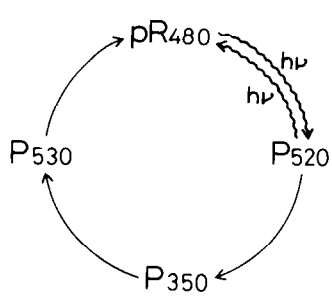

Fig.3. Photocycle of pR480. Wavy lines, photochemical reactions; smooth curves, thermal (dark) reactions. For details see text.

4. DISCUSSION

Based upon the results described above, the photoreaction cycle of pR480 is represented in fig.3. P520 appears to correspond to the K or hR600 intermediate of bR or hR ([6,7]; Ogurusu, T. et al., unpublished), since their absorption maxima are located at longer wavelengths than those of the original pigments. In fact, our preliminary experiments showed that P520 was produced by irradiation of pR480 at −170°C, where K or hR600 is produced photochemically. The thermal stability of P520, however, was different from those of the intermediates; P520 was stable below −50°C, whereas K and hR600 were stable below −120°C ([6,8]; Ogurusu et al., unpublished). Whether an

L-like intermediate exists in the photoreaction cycle of pR480 must await future research.

Our recent low-temperature spectrophotometry of sR demonstrated that no intermediates other than sR373, which may correspond to P350 of pR480, were detected by irradiation at various temperatures ranging from −40 to −190°C [9], although two other intermediates (sR_K and sR_L) have been found at room temperature using flash photolysis [10,11]. On the other hand, irradiation of pR480 readily produced P520 as well as P350 and P530. Therefore, the chromophore-protein interaction is fairly different between two kinds of phototactic pigments, namely sR and pR.

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