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Isolation of photochemically active archaebacterial photoreceptor, pharaonis phoborhodopsin from *Natronobacterium pharaonis*

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Abstract

A photoreceptor, pharaonis phoborhodopsin for the negative phototaxis of extremely halophilic and alkalophilic archaebacterium, *Natronobacterium pharaonis* was isolated in a photochemically active state. A detailed examination of the chromatographic separation made it possible to separate contaminating proteins, such as cytochromes. The procedure resulted in a 2938-fold enrichment with a yield of 15.5%. The isolated pharaonis phoborhodopsin had an absorption maximum at 498 nm, an A_{280}/A_{498} ratio of 1.27 and a single band near 24 kDa on the SDS-polyacrylamide gels. The isolated pharaonis phoborhodopsin underwent a photochemical reaction after flash excitation. The photocyclic reaction closely resembled that of the membrane-bound pharaonis phoborhodopsin.

Keywords: Phototaxis; Haloalkalophile; Halobacterium; Archaebacterium; Phoborhodopsin; (*N. pharaonis*)

1. Introduction

Extremely halophilic archaebacteria [1] have evolved photoenergy and photosensory reception systems. Vitamin A aldehyde, retinal, is commonly used in the chromophore of the photoreceptors for the systems. Four retinal-containing protein pigments have been identified in the cell membrane of *Halobacterium salinarium* (formerly called *Halobacterium halobium*): bacteriorhodopsin (bR) [2], halorhodopsin (hR) [3], sensory rhodopsin (sR) [4,5] and phoborhodopsin (pR) [6,7]. When these pigments are photoexcited, they return thermally to their original state via a series of photointermediates with distinct absorption maxima [4,7–9]. This photochemical reaction cycle, spectroscopically detected, is called photocycle. These chromoproteins are categorized into two groups according to the photocycling rate: one with several ms which includes bR and hR [4,8,9], and the other with several hundred ms which includes sR and pR [4,7]. The fast-cycling proteins, bR (λ_{max} = 568 nm) and hR (λ_{max} = 578 nm), function as light-driven ion pumps (for proton and chloride, respectively) [8,10]. The slow-cycling proteins, sR (λ_{max} = 587 nm) and pR (λ_{max} = 490 nm), are photoreceptors for the phototactic responses [4–7,11,12]. sR_{587} mediates a positive phototactic response to light in the yellow-red range. A long-lived photointermediate of sR, S_{373} (373 indicates λ_{max}), is a photoreceptor for the negative phototactic response to light in the near-UV. Another negative phototactic response to blue-green light is mediated by pR.

A haloalkalophilic archaebacterium, *Natronobacterium pharaonis* has been isolated and characterized [13,14]. Spectroscopic analysis revealed the presence of two retinal-containing pigments in *Natronobacterium pharaonis* cell membrane: one is a fast-cycling retinal pigment with an absorption peak around 580 nm and the other is a slow-cycling retinal-pigment with a maximum absorption around 500 nm [15]. The photochemical properties and ion transport properties of the 580 nm pigment are affected with chloride anions. The 580 nm pigment was proposed to be a halorhodopsin-like chromoprotein [15]. The halorhodopsin-like protein from *Natronobacterium pharaonis* was isolated, sequenced and named pharaonis halorhodopsin [16,17]. The 500 nm pigment has been investigated spectroscopically and purification procedures have been developed [15,18–21]. The 500 nm pigment was named pharaonis phoborhodopsin (ppR) from the similarity to phoborhodopsin in *Halobacterium salinarium*. The ppR preparations by the purification procedures previously

Abbreviations: bR, bacteriorhodopsin; hR, halorhodopsin; sR, sensory rhodopsin; pR, phoborhodopsin; ppR, pharaonis phoborhodopsin; Pipes, 1,4-piperazine-di-2-ethanesulfonic acid.

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reported have contaminating proteins detected spectroscopically and electrophoretically. We examined intensively the condition of chromatographic fractionation. The contaminating proteins were removed from ppR and the purified sample gave a single band on SDS-polyacrylamide gels. We report here the procedure of ppR isolation and the spectroscopic properties of the isolated ppR.

2. Materials and methods

2.1. Materials

Sodium cholate was obtained from Sigma. Octylglucoside and Pipes were from Dojindo. Octyl-Sepharose CL-4B was purchased from Pharmacia. Hydroxyapatite was from Mitsui-Toatsu-Chemicals.

2.2. Strain and culture conditions

The type strain of *Natronobacterium pharaonis*, DSM2160 was obtained from the culture collection DSM (Deutsche Sammlung von Mikroorganismen). The culture medium (1 l) contains: 10 g yeast extract, 7.5 g casamino acids, 3 g trisodium citrate, 2 g KCl, 1 g $MgSO_4 \cdot 7H_2O$, 360 mg $MnCl_2 \cdot 4H_2O$, 50 mg $FeS0_4 \cdot 7H_20$, 200 g NaCl and 50 g $Na_2CO_3 \cdot 10H_2O$. The cells were grown in a 20 l container at 40° C. The culture was aerated vigorously by an air pump (14 l/min). The aeration was reduced (5 l/min) when the culture entered the stationary phase. After 5–6 days culture, the cells were harvested and suspended with about 800 ml of 4 M NaCl. The cell suspension was frozen with liquid nitrogen and stored at −70° C.

2.3. Membrane preparation

The frozen cells were thawed in a flow of tap water. The viscosity of the suspension was reduced with a Waring blender (SM-25, Sanyo, Japan) for 60 s (six 10-s blendings at intervals of 1 min). The suspension was centrifuged at $10\,000 \times g$ for 15 min at 4° C to remove undisrupted cells. Supernatant was centrifuged at $140\,000 \times g$ for 60 min at 4° C. Red precipitate was suspended in 4 M NaCl with stirring at 4° C. The membrane fraction was washed with 4 M NaCl until red color of the supernatant disappeared. The washed membrane fraction was suspended in about 40 ml of 4 M NaCl and stored at 4° C.

2.4. Flash-spectrophotometry

The monitoring beam was provided by a 12 V 100 W tungsten-halogen lamp (Philips, 7027, Netherlands) passed through a monochromator (Otsuka Electronics, UM-001, Japan). After passing through the sample and an additional UM-001 monochromator, the beam was focused onto a photomultiplier (Hamamatsu Photonics, R374, Japan). The photomultiplier output was fed into a current-voltage converter and then digitized with an A/D converter (Datel, ADC-HZ12B, USA) and transferred to a microcomputer (Sharp, MZ-2200, Japan). The data were stored on a floppy diskette. The actinic flash was delivered at 90° to the monitoring beam with a xenon flash (Sunpak, auto622, Japan) passed through a 487 ± 6 nm interference filter (Irie, KL-48, Japan) and a short-cut filter (Irie, Y-46, Japan). A 556 nm flash was obtained through an interference filter (Kenko, BC-600, Japan) and glass filters (Kenko, O-53 and CM-500, Japan). Data were collected and averaged over 5 to 50 sweeps at 25 s intervals. The sample was thermostated at 20° C with a water jacketed cuvette holder.

2.5. Quantitation of pharaonis phoborhodopsin

Pharaonis phoborhodopsin was assayed by flash-spectrophotometry. A flash-light (486 ± 6) was used for the excitation of pharaonis phoborhodopsin. The flash-induced absorbance change was monitored at 450 nm. Five flashes were averaged for each assay. After the second column separation, absorption spectra of the eluted fractions were also measured for assay of pharaonis phoborhodopsin.

2.6. Quantitation of protein

Protein assays were by the colorimetric method of Lowry et al. [22]. Sample was dialyzed against pure water to remove detergents since the detergents affect the colorimetry.

2.7. Buffers for isolation of pharaonis phoborhodopsin

Buffer A: 2 M NaCl, 25 mM Pipes (pH 7.2); cholate buffer: 0.4% sodium cholate, 2 M NaCl, 25 mM Pipes (pH 7.2); octylglucoside buffer: 0.280% octylglucoside, 2 M NaCl, 25 mM Pipes (pH 7.2).

2.8. Solubilization of pharaonis phoborhodopsin from the membrane fraction

The washed membrane was resuspended in a final volume of 200 ml with buffer A. The membrane suspension was placed in a 500-ml beaker on a magnetic stirrer and 67 ml of 20% (w/v) sodium cholate was added throughout 1 min. After 30-min gentle stirring, the mixture was centrifuged at $140\,000 \times g$ for 60 min at 15° C. The supernatant containing the solubilized pharaonis phoborhodopsin was decanted and saved, while the pellet was discarded.

2.9. Octyl-Sepharose chromatography I

5.0 cm diameter × 3.6 cm height (71 ml) of octyl-Sepharose CL-4B column was prepared and equilibrated with buffer A. The membrane extract was applied onto the

column and washed with cholate buffer until red color of the eluate disappeared (about 4.5 l). Pharaonis phoborhodopsin is adsorbed on the gel in the cholate buffer. After the washing with cholate buffer, the adsorbed fractions which included pharaonis phoborhodopsin were eluted with 1.8 l of octylglucoside buffer. The eluted ppR-rich fractions were pooled for the next chromatographic separation.

2.10. Hydroxyapatite chromatography I

One part dried hydroxyapatite was added to six parts buffer A. The mixture was swirled gently and settled for 30 min. The cloudy upper level and the fines at the top of the settled bed were removed by decantation. The slurry of hydroxyapatite was poured into a glass column of 5.0 cm diameter and packed to a height of 3.5 cm (69 ml) under air pressure. The gel was equilibrated with buffer A. The pooled ppR fractions were diluted with buffer A and the concentration of octylglucoside was adjusted to 0.20%. The diluted sample was loaded on the gel. Pharaonis phoborhodopsin was desorbed with octylglucoside buffer.

2.11. Hydroxyapatite chromatography II

2.5 cm diameter × 2.5 cm height (12 ml) of hydroxyapatite column was prepared and equilibrated with buffer A. The concentration of octylglucoside of the pooled fractions were adjusted to 0.20% with buffer A. The diluted sample was applied onto the gel. ppR was eluted with octylglucoside buffer.

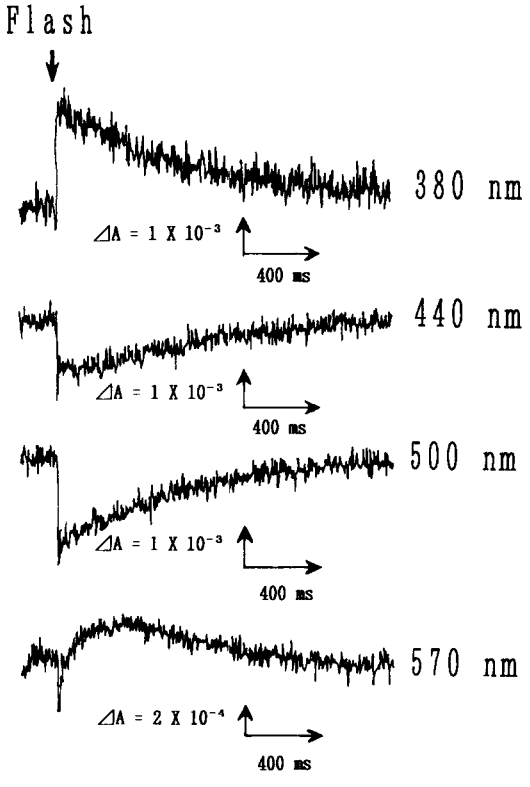

Fig. 1. Blue-green flash-induced absorbance changes in the membrane of *Natronobacterium pharaonis* at 380, 440, 500 and 570 nm. The flash (487 ± 6 nm) was delivered at the time indicated by arrows. The membrane was suspended (2.0 mg protein/ml) with 3.42 M NaCl-20 mM Pipes/NaOH (pH 7.0). The absorbance changes were monitored at 20° C.

2.12. Octyl-Sepharose chromatography II

A 6 ml of octyl-Sepharose gel was packed in a glass column of 2.5 cm diameter. The gel was equilibrated with buffer A. The pooled fractions from hydroxyapatite column II were diluted with buffer A and the concentration of the octylglucoside was adjusted to 0.14%. The diluted sample was loaded onto the gel. ppR was eluted with octylglucoside buffer.

2.13. SDS-polyacrylamide gel electrophoresis

The buffer of Laemmli [23] was used to run 8–16% polyacrylamide gel (8 cm). Protein samples were desalted and dried up. The dried samples were mixed with an sample buffer (20 μl) which contained 2% SDS and 5% of 2-mercaptoethanol. Solubilization was carried out at room temperature for 20 min. Samples were electrophoresed at 18 mA (constant current). Proteins in the gel was visualized by staining with Coomassie blue R-250.

3. Results

3.1. Photocycle of membrane-bound pharaonis phoborhodopsin

We examined pharaonis phoborhodopsin in the membrane of the type strain of *Natronobacterium pharaonis* by flash-spectrophotometry. The membrane suspension was excited by a blue-green flash and absorbance changes were monitored in a 2000 ms time window. The flash brought about absorbance changes in the range from 350 nm to 600 nm. Fig. 1 shows the time-courses of the absorbance changes at selected four wavelengths. Wavelength dependence of the flash-induced absorbance changes at 100 ms and 400 ms after the flash was shown in Fig. 2. The flash-induced absorbance difference spectra have a negative band around 500 nm and two positive bands around 390 nm and 560 nm. The absorbance change around 500 nm indicates a flash-induced fast decrease and the following recovery of the original state of pharaonis phoborhodopsin. The absorbance change around 390 nm shows that a blue-shifted intermediate is produced within 1 ms and decayed gradually. The flash-induced absorbance change around 560 nm indicates that another red-shifted intermediate generated before 200 ms and then decayed. In the later time range than 1 ms, three states in the photocycle of pharaonis phoborhodopsin were detected, which has been proposed by several groups [15,18–21].

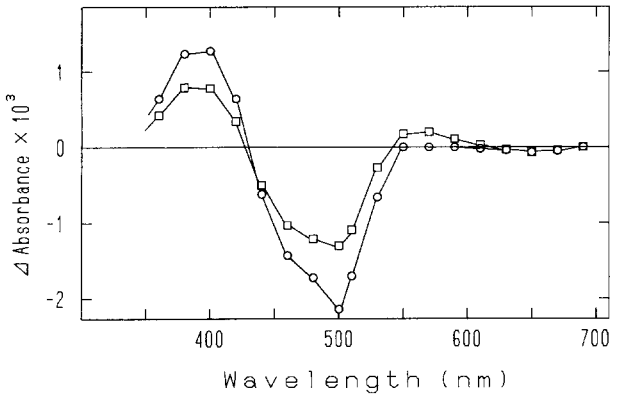

Fig. 2. Blue-green flash induced absorbance difference spectra in the membrane of *Natronobacterium pharaonis* at 100 ms (○) and at 400 ms (□) after flash. 460 nm, 480 nm, and 500 nm points were obtained by 556 nm flash and normalized to the 487 nm flash-induced absorbance changes at 510 nm. The absorbance changes were monitored as shown in Fig. 1.

3.2. Biosynthesis of pharaonis phoborhodopsin

Synthesis of bR, hR and sR apoproteins increase as *H. salinarium* cells enter the stationary phase [8,24,25], whereas that of pR apoprotein decreases [7,26]. We compared the amount of pharaonis phoborhodposin in *N. pharaonis* cells in the exponential phase and that in the stationary phase. The cells in the stationary phase contained pharaonis phoborhodopsin at approx. 2-fold the amount found in the exponential phase (Table 1). Pharaonis phoborhodopsin was synthesized more in the stationary phase than in the exponential phase.

As *Halobacterium salinarium* cells enter stationary phase, biosynthesis of retinal increases [25,27]. *H. salinarium* cells in the exponential phase do not synthesize enough retinal to bind to all the apoproteins (opsins) in the cell membrane. Addition of retinal to the membrane generates the holoprotein, as indicated by increase of the amplitude of the flash-induced absorbance changes. The membrane from *Natronobacterium pharaonis* cell did not have the apoprotein of pharaonis phoborhodopsin both in the stationary phase and in the exponential phase (Table 1). It indicates that *Natronobacterium pharaonis* cells synthesize enough retinal even in the exponential phase.

Table 1
Pharaonis phoborhodopsin amounts in the membrane from the exponential phase cell and the stationary phase cell

Growth phase	$\Delta A_{500} \times 10^3$	
	+ ethanol	+ retinal
Exponential phase	1.09	1.07
Stationary phase	1.93	1.90

Amounts of pharaonis phoborhodopsin were determined by blue flash-spectrophotometry and expressed as 500 nm absorbance changes at 100 ms after 556 nm flash. The membrane suspensions at 2 mg protein/ml were flashed at 2 h after addition of 5 μl of 17.6 mM all-*trans*-retinal in ethanol (right column) or 5 μl of ethanol (left column). The absorbance changes were monitored as in Fig. 1.

3.3. Isolation of pharaonis phoborhodopsin

Pharaonis phoborhodopsin was stably solubilized with several detergents in a photochemically active state. We chose two detergents, sodium cholate and octylglucoside in our isolation procedure. ppR was solubilized first with sodium cholate from the cell membrane. High concentration of the sodium cholate (final 5.0%) brought about 97% extraction of ppR from the membrane. The solubilized membrane was applied onto the first hydrophobic chromatography. Octyl-Sepharose column was used for the first chromatographic separation. Octyl-Sepharose was chosen among six hydrophobic gels examined. ppR with sodium cholate buffer was adsorbed on the octyl-Sepharose gel. In the condition for the adsorption, the octyl-Sepharose gel was washed with 4.5 l sodium cholate buffer. After red pigments was washed off with the sodium cholate buffer, adsorbed proteins were eluted with octylglucoside buffer. ppR was eluted after cytochromes which had the Soret absorption band near 417 nm (Fig. 3A). The pooled ppR-rich fractions contain 95.0% of ppR loaded on the gel, resulting in a 16.1-fold purification. In this stage, the absorption spectrum of ppR was not detected (Fig. 4A)

The pooled ppR-rich fractions from the first octyl-Sepharose column were further purified on a hydroxyapatite column. The octylglucoside concentration of the pooled fractions was adjusted to 0.20%. The diluted sample was applied onto the hydroxyapatite column. In this condition, pigments were adsorbed on top of the gel about 5 mm in height. The adsorbed proteins were eluted with octylglucoside buffer. Most of cytochromes were still adsorbed on the gel. Some cytochromes were eluted before ppR (Fig. 3B). Absorption of ppR was detected near 500 nm in the eluted fractions (Fig. 4B). This hydroxyapatite column purified ppR 58.2-fold but 51.9% of ppR loaded on the gel was lost. An A_{280}/A_{498} value of the pooled ppR-rich fractions is 2.40.

The pooled fractions from the first hydroxyapatite column had a small amount of cytochromes as judged by the Soret absorption band (Fig. 4B). The cytochromes were separated on the third purification step, another hydroxyapatite column. The pooled ppR-rich fractions were diluted with 2 M NaCl-25 mM Pipes buffer and the octylglucoside concentration of the pooled fractions was adjusted to 0.20%. The diluted fractions were applied onto the column. At this concentration of octylglucoside, ppR was adsorbed on the top part of the gel within 1 mm. Elution with octylglucoside buffer separated ppR from the cytochromes (Fig. 3C). ppR-rich fractions were collected and their absorption spectrum was measured (Fig. 4C). The pooled fractions did not have the Soret absorption band. The A_{280}/A_{498} value of the ppR fractions is 1.49.

The final purification step is hydrophobic chromatog-

raphy on another octyl-Sepharose. The pooled fractions from the hydroxyapatite column II were diluted and the octylglucoside concentration was adjusted to 0.14%. The diluted ppR-rich sample was applied on the octyl-Sepharose column II. At 0.14% octylglucoside, the ppR was adsorbed on the top half of the octyl-Sepharose gel. ppR

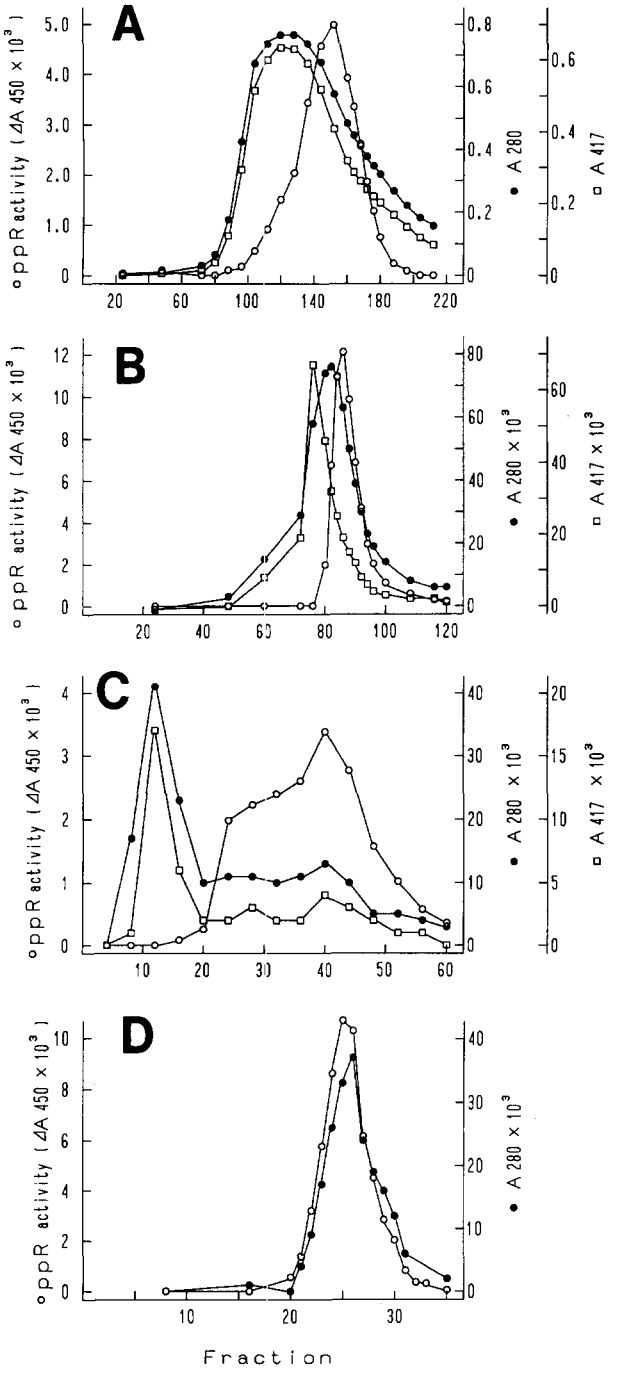

Fig. 3. Elution profiles from four column chromatography steps. (A) Octyl-Sepharose CL-4B column I. (B) Hydroxyapatite column I. (C) Hydroxyapatite column II. (D) Octyl-Sepharose CL-4B column II. ○, pharaonis phoborhodopsin measured by flash-spectrophotometry. ●, protein determined by absorption at 280 nm. □, cytochrome monitored by absorption at 417 nm.

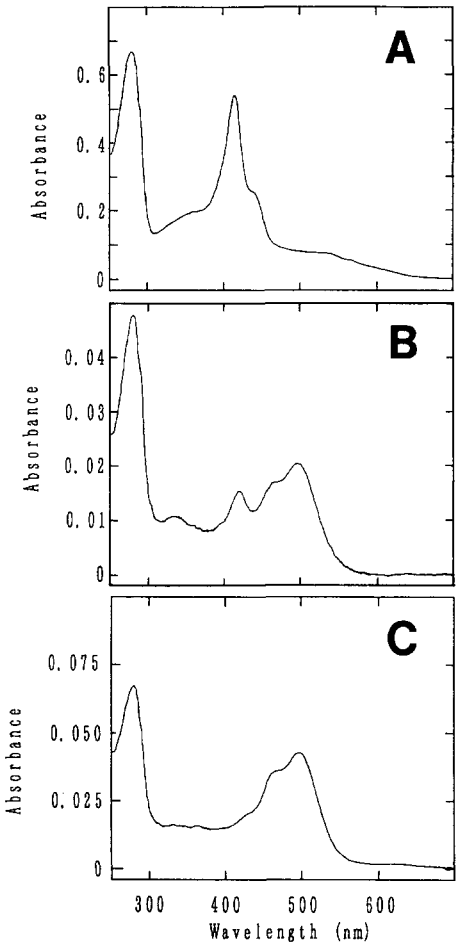

Fig. 4. Absorption spectra of the pooled fractions eluted from each column. (A) Octyl-Sepharose CL-4B column I. (B) Hydroxyapatite column I. (C) Hydroxyapatite column II.

was then eluted with octylglucoside buffer. The best six fractions contain 48.7% of ppR applied to this column, resulting in a 3.05-fold purification. The collected six fractions was analysed by SDS-polyacrylamide gel electrophoresis shows a single band near 24 kDa (Fig. 5). Table 2 shows an overview of the purification of the pharaonis phoborhodopsin from the membrane fractions of *Natronobacterium pharaonis* cells. The purification procedure produced 0.184 mg of the purified ppR from the membrane obtained by a 20 l culture. The ppR is purified 2938-fold from the membrane. The overall yield of the ppR is 15.5%. An absorption spectrum of the purified ppR shows a maximum at 498 nm and a shoulder near 470 nm (Fig. 6). The A_{280}/A_{498} value of the purified ppR is 1.27, indicating a high degree of purity by the criterion often used for retinal-containing chromoproteins. The isolated pharaonis phoborhodopsin was excited with blue-green flash and the flash-induced absorbance changes were measured (Figs. 7 and 8). These absorbance changes indicate that photocycle of the isolated pharaonis phoborhodopsin closely resembled that of the membrane-bound pharaonis phoborhodopsin (Figs. 1 and 2), except that the red-shifted

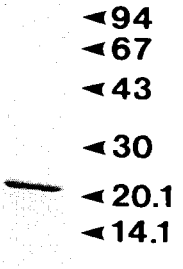

Fig. 5. SDS-polyacrylamide gel electrophoresis of the purified pharonis phoborhodpsin.

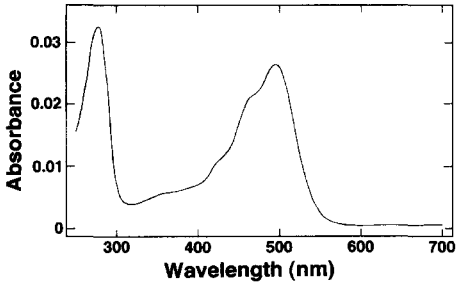

Fig. 6. Absorption spectrum of the purified pharaonis phoborhodopsin in 0.280% octyl glucoside, 2 M NaCl, 25 mM Pipes (pH 7.2).

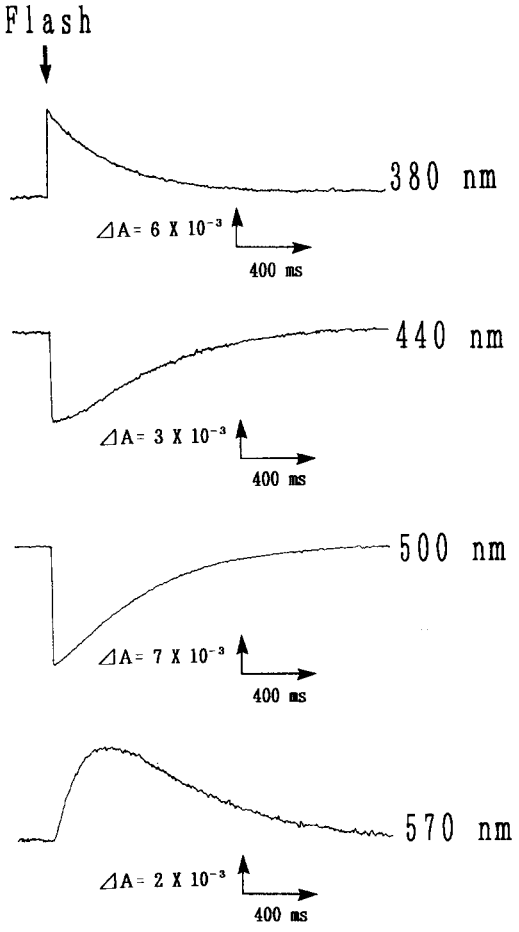

Fig. 7. Blue-green flash-induced absorbance changes of the purified pharaonis phoborhodopsin at 380, 440, 500 and 570 nm. The flash (487 ± 6 nm) was delivered at the time indicated by arrows. Purified ppR is in 0.280% octyl glucoside, 2 M NaCl, 25 mM Pipes (pH 7.2). The absorbance changes were monitored at 20° C.

intermediate (around 560 nm) is particularly enhanced in the isolated state.

4. Discussion

Study on the retinal proteins in the haloalkalophilic archaebacteria have been opened by Bivin and Stoeckenius [15]. They reported that pharaonis phoborhodopsin is photochemically active in the presence of a detergent, octylglucoside. Purification procedures of pharaonis phoborhodopsin have been developed. Purified samples which have been reported so far have cytochromes detected spectroscopically and impurities observed by SDS-polyacrylamide gel electrophoresis [18,28]. The procedure reported here removed the remaining cytochromes and impurities. Strictly controlled detergent concentration in every chromatographic separation made possible to remove the contaminating proteins. The removal of cytochromes and impurities was confirmed by the absence of

Table 2
Isolation of pharaonis phoborhodopsin

Step	Protein (mg)	Amount of ppR [a]	Specific ppR activity	Purification factor	Yield (%)
Membrane	3488	1.458	0.418	1.00	100
Cholate extract	1153	1.420	1.23	2.94	97.4
Octyl-Sepharose I	200	1.349	6.75	16.1	92.5
Hydroxyapatite I	1.65	0.649	393	940	44.5
Hydroxyapatite II	1.15	0.464	403	964	31.8
Octyl-Sepharose II	0.184	0.226	1228	2938	15.5

[a] ΔA_{450} cm^{-1} × total volume of sample (ml). ΔA_{450} cm^{-1} was the amplitude of 450 nm absorbance change at 100 ms after flash (487 ± 6 nm).

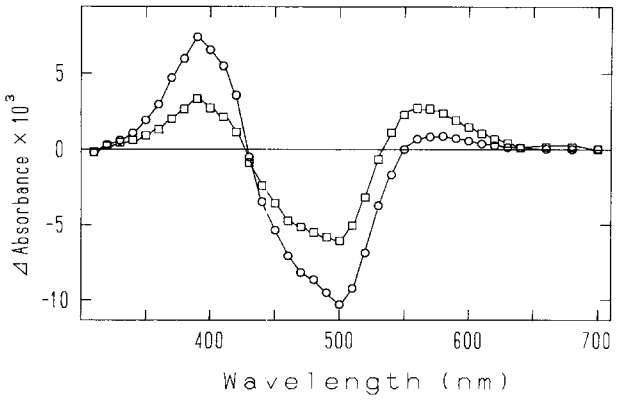

Fig. 8. Blue-green flash induced absorbance difference spectra of the purified pharaonis phoborhodopsin at 50 ms (○) and at 400 ms (□) after the flash (487 ± 6 nm). The absorbance changes were monitored as shown in Fig. 7.

the Soret absorption band and by the absence of other bands than 24 kDa band on SDS-polyacrylamide gel. The A_{280}/A_{498} ratio, 1.27, also supports the high purity of the isolated ppR. The photochemical properties of the isolated pharanois phoborhodopsin closely resemble those of membrane-bound pharaonis phoborhodopsin, suggesting that pharaonis phoborhodopsin is largely unaffected by our isolation processes.

Pharaonis phoborhodopsin is more synthesized by *N. pharaonis* cells in the stationary phase than those in exponential phase. Phoborhodopsin is more synthesized by *H. salinarium* cells in the exponential phase [7,26]. Although pharaonis phoborhodopsin and phoborhodopsin have the same physiological function, the biosynthesis of pharaonis phoborhodopsin is different from that of phoborhodopsin in the culture growth.

Our separation of the contaminating proteins from pharanois phoborhodpsin may make possible to study more precisely pharaonis phoborhodopsin spectroscopically, structurally and functionally.

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