FEBS Letters 420 (1997) 54–56 FEBS 19635

Functional expression of *pharaonis* phoborhodopsin in *Eschericha coli*

Kazumi Shimono, Masayuki Iwamoto, Masato Sumi, Naoki Kamo*

Laboratory of Biophysical Chemistry, Faculty of Pharmaceutical Sciences, Hokkaido University, Sapporo 060, Japan

Received 30 October 1997

Abstract ***Pharaonis*** **phoborhodopsin, the photoreceptor of the negative phototaxis of archaebacterial** ***Natronobacterium pharaonis*****, was functionally expressed in the heterologous system of** ***Escherichia coli*****. Flash-photolysis on a millisecond time scale indicated that the photochemical properties of** ***p*****R expressed in** ***E. coli*** **were the same as those of the native** ***p*****R in** ***N. pharaonis*****. We concluded that the integral membrane protein** ***p*****R is correctly folded in vivo in the eubacterial** ***E. coli*** **membrane.**

Key words: *pharaonis* phoborhodopsin (*p*pR); *pharaonis* sensory rhodopsin II (*p*sRII); Eubacterial expression; Photocycle; Flash-photolysis

1. Introduction

Halobacterium salinarium has at least four retinal proteins; bacteriorhodopsin (bR) [1,2], halorhodopsin (hR) [3,4], sensory rhodopsin (sR or sRI) [5,6] and phoborhodopsin (pR) [7–11] (also called sRII). The former two work as light-driven ion pumps and the latter two as photoreceptors of this bacterium. pR is a photoreceptor of negative phototaxis whose absorption maximum is located at around 500 nm [7–11]. The maximum wavelength is significantly different from the other three proteins (around 570 nm). In addition, pR shows a pronounced shoulder at 460 nm [12]. This pigment, therefore, requires a detailed investigation. The photochemistry was investigated although the S/N ratio was not high [8,13,14]. Further progress has not advanced because of its small content and its inactivation by solubilization with detergents.

We succeeded in the purification of a phoborhodopsin-like protein from the haloalkaliphilic bacterium, *Natronobacterium pharaonis* and named it *pharaonis* phoborhodopsin (*p*pR; also called *pharaonis* sensory rhodopsin II, psRII) [15–19]. The existence of this pigment was first reported by Bivin and Stoeckenius [20]. This pigment also works as a photoreceptor of negative phototaxis [21]. Recently, the sequence of pR and *p*pR was determined [22,23]. Although several studies were done, for further advancement, the expression (possibly large-scale) system is indispensable; the content of *p*pR in *N. pharaonis* is not high, the purification procedure is not easy as well as time-consuming, and the growth rate of the bacterium is slow.

The present communication reports the success in the functional expression of *p*pR in *Escherichia coli*, as is revealed from photochemical measurements. As far as we know, this is the first functional expression of recombinant *p*pR. This system has advantages for studying the photochemistry of *p*pR at a molecular level.

2. Materials and methods

2.1. Bacterial strains

E. coli JM109 was used as host for DNA manipulation, and BL21 was used for expressing the genes. Cells were grown in 2×YT medium supplemented by ampicillin (final concentration of 50 μg/ml). Cells of *N. pharaonis* (NCIMB 2191) were grown as previously described [15].

2.2. PCR and construction of expression plasmid, pFEppR

Genomic DNA from *N. pharaonis* was prepared as described in [24]. For PCR, a sense (5′-CATATGGTGGGACTTACGACC) and an antisense (5′-AGAATAACGACGGGACGTTCG) oligonucleotide were designed based on the nucleotide sequence in the GenBank data base (accession no. Z35086). Underlined are the added restriction site *Nde*I. The reaction mixture (100 μl) for PCR contained 350 ng of template (*N. pharaonis* genomic DNA), 0.5 μM of each primer, 200 μM of the dNTP mixture (TaKaRa, Tokyo), and 2.5 units of *TaKaRa Ex Taq* in a buffer provided by a TaKaRa kit. PCR reactions were performed at 94°C for 1 min, at 57°C for 1 min, at 72°C for 1.5 min and were repeated for 30 cycles (the first step at 94°C and the final step at 72°C were extended for 5 min). A PCR product containing *psopII* gene of 760 bp was obtained, purified and subcloned into the plasmid vector pGEM-T Easy (Promega). DNA sequencing was carried out using a DNA Sequencing Kit (Applied Biosystems). The PCR product was analyzed using an automated sequencer (377 DNA sequencer, Applied Biosystems).

The approximately 800-bp *Nde*I and *Not*I fragment containing the *psopII* gene from this plasmid was gel-purified and ligated to *Nde*I and *Not*I sites of pET21c (Novagen). The plasmid was named pFEppR.

2.3. Expression and preparation of membrane

E. coli BL21 harboring pFEppR was grown to an OD_{660} of 0.4–0.6 in a 37°C shaking incubator, followed by the addition of 1 mM IPTG and 10 μM *all-trans* retinal. Cells were harvested at 120 min post-induction by centrifugation at 4°C, suspended in buffer A (50 mM Tris-Cl (pH 8.0) and 5 mM $MgCl_2$) and stored frozen at −20°C. Thawed cells were disrupted by sonication for 5 min at 4°C with a power of 160 W (duty cycle 50%, UP200H, Kubota). Crude membranes were collected by centrifugation (100 000×*g* for 120 min at 4°C) and washed with buffer A.

2.4. Flash spectroscopy

Crude membranes were resuspended in an appropriate medium. After centrifugation (15 000×*g*, 120 min at 4°C), the supernatant was used for measurements. The medium was composed of 4.0 M NaCl and 0.5% octylglucoside with a buffer: 20 mM citrate for pH 5.0 and 50 mM CHES for pH 9.0. The apparatus and procedure were essentially the same as in [17]. Short flashes were provided by an Xe-flash lamp (duration 200 μs) in combination with a 540-nm interference filter (±10 nm, KL54, Toshiba) and a 523-nm cut-off filter (Toshiba). For a single time scan, 500 data points were stored in a microcomputer (PC-386M, Seiko EPSON) and 30 flash data were averaged for each wavelength.

3. Results

By a PCR, we obtained a fragment containing a full length of the *psopII* gene coding region. A *Nde*I site was created at

*Corresponding author. Fax: (81) (11) 706-4984.
E-mail: nkamo@pharm.hokudai.ac.jp

Abbreviations: *p*pR, *pharaonis* phoborhodopsin; IPTG, isopropyl-1-thio-β-galactoside; CHES, 2-cyclohexylamino-ethanesulfonic acid

PII S0014-5793(97)01487-7

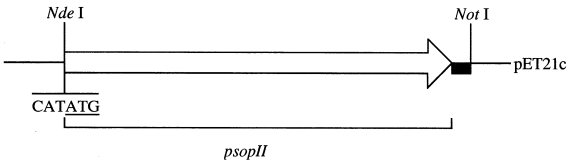

Fig. 1. Construction of pFEppR. The large arrow is the *psopII* coding region. The 5′ terminal of *psopII* is modified for generating the *Nde*I site. The closed box contains the noncoding region, the *psopII* stop codon TAA downstream and approximately 20 bp multiple cloning sites of pGEM-T Easy.

the translation initiation site in the PCR product using the oligonucleotide primer containing the *Nde*I site. The PCR product was subcloned into pGEM-T Easy. By sequencing, it was confirmed that mutagenesis had not occurred during the PCR amplification. To construct pFEppR (Fig. 1), the *Nde*I and *Not*I fragment of pGEM-T Easy containing the PCR product was cloned into the *Nde*I and *Not*I sites of pET21c.

Induction of *E. coli* BL21 harboring pFEppR with 1 mM IPTG caused a growth inhibition, suggesting the expression of the desired protein. Actually, by addition of retinal, the color change of the transformed cells was recognized and its color was definitely different from that of cells transformed only by the vector pET21c.

To confirm the expression, flash-photolysis measurements were done, and we observed the photocycle of *E. coli* *p*pR (*p*pR expressed in *E. coli*) (data not shown). *E. coli* *p*pR was solubilized with 0.5% octylglucoside to increase the S/N ratio without inactivation. The solubilized samples were, therefore, measured and results are shown in Fig. 2, where the time courses of the absorbance change at selected wavelengths are drawn for pH of 5.0 (left column) and 9.0 (right column). Depletion by the flash and recovery of the original pigment were observed (C and F). The formation and decay of an intermediate ppR_M are shown in A and D, where the negative deflection caused by overlapping the change of the original *p*pR appears (see A). This negative deflection was also recorded in native *p*pR derived from *N. pharaonis* [17]. Fig. 2B clearly shows the existence of an intermediate ppR_O in an acidic medium and this intermediate was scarcely detected in an alkaline medium (see E). The photocycling rate of native *p*pR (from *N. pharaonis*) is slow in an alkaline medium [17] and this feature was also recognized in *E. coli* *p*pR (compare the time scale of the left and right columns).

Fig. 3 shows a typical flash-induced difference spectrum of *E. coli* *p*pR. One negative absorption band was caused by the depletion of the original *E. coli* *p*pR. It is noteworthy that the absorption maximum approximately locates at 500 nm with a pronounced shoulder at 470 nm [15,17]. The two positive bands in Fig. 3 have difference absorption maxima at 390 and 560 nm; the former results from ppR_M and the latter, ppR_O. These wavelengths were the same as those of the native *p*pR [15,17].

The photocycle of *p*pR in ms and a longer time range is as follows: $ppR \rightarrow ppR_M \rightarrow ppR_O \rightarrow ppR$ [15,17]. Fig. 2A and B indicate that the decay of ppR_M almost matches the rise in ppR_O. The inset of Fig. 3 shows the flash-induced difference spectrum in the time range where only ppR_O and *p*pR appear. The isosbestic point at 530 nm was formed and is the same as that of the native *p*pR [17]. This indicates the conversion of ppR_O to the original *p*pR, and that the photocycle described above is also valid for *E. coli* *p*pR. Also, the addition of azide accelerated the photocycling rate (data not shown).

4. Discussion

Native *p*pR has the following photochemical properties [17,18]: (i) The photocycling rate is significantly slower than that of bR or hR. (ii) The rate is slower in alkaline solution, and ppR_O is appreciable in acidic solution. (iii) The absorption spectrum of *p*pR has its maximum at 498 nm with a pronounced shoulder at 470 nm. (iv) For the time range of ms or longer, there exist two intermediates whose absorption maxima are 390 and 560 nm. (v) The cycle is $ppR \rightarrow ppR_M \rightarrow ppR_O \rightarrow ppR$. (vi) The photocycling rate is ac-

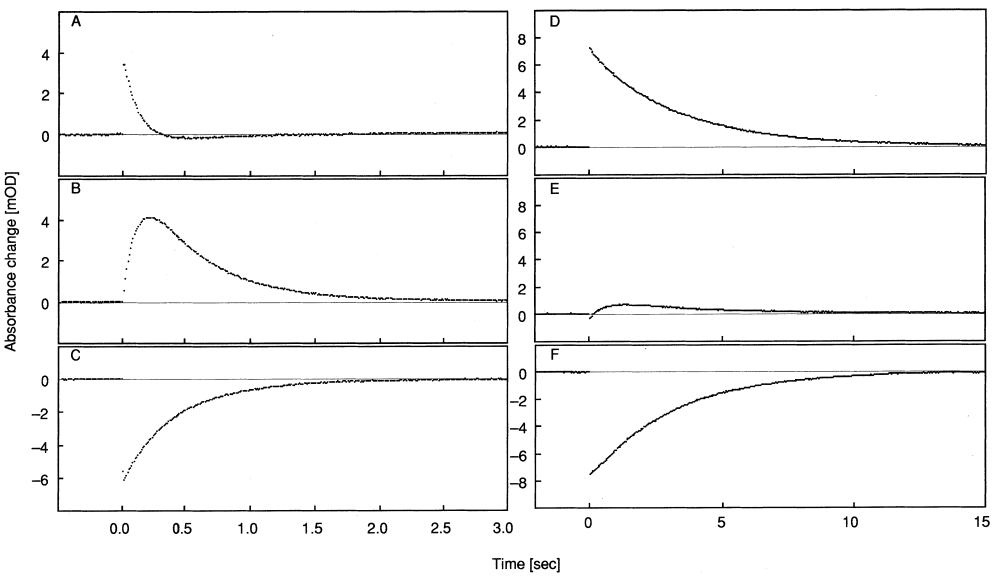

Fig. 2. Typical flash-photolysis kinetic data at three selective wavelengths. The upper two are data at 390 nm (that mainly monitor ppR_M), the middle, 560 nm (ppR_O) and the lower, 500 nm (the original *p*pR). The left three show data obtained at pH 5.0 adjusted with 20 mM citrate. The right three, at pH 9.0 with 50 mM CHES. The solution contained 4.0 M NaCl and 0.5% octylglucoside, and the temperature was 20°C.

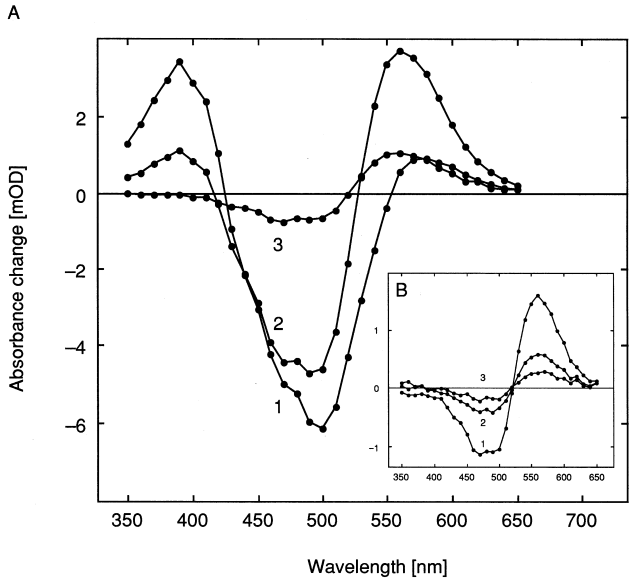

Fig. 3. Flash-induced difference spectrum of recombinant *E. coli* *p*pR. A: Curve 1, spectrum at 20 ms after the flash; curve 2, 130 ms; curve 3, 1000 ms. B: Inset: Conversion of ppR_O to the original *p*pR. Curve 1, spectrum at 800 ms after flash; curve 2, 1300 ms; curve 3, 1700 ms. Flash light was provided through a combination of an interference filter (540 ± 10 nm) and a cut-off filter (> 523 nm). The solution contained 4.0 M NaCl whose pH was adjusted to 5.0 with 20 mM citrate. Temperature was 20°C.

celerated by the addition of azide. These properties are observed in *E. coli* *p*pR, indicating that the conformation of expressed *p*pR in the *E. coli* membrane may be the same as that of native *p*pR in the *N. pharaonis* membrane.

In alkaline solution, the ppR_M decay does not seem to match the ppR_O rise. One possible reason may be the existence of an N-like intermediate which has not been discovered yet in spite of much effort using the native *p*pR.

Since the cell conditions (e.g. salt concentration, membrane structure or pH) are significantly different between *E. coli* and *N. pharaonis*, it is generally doubtful whether the membrane protein expressed in *E. coli* is incorporated into its membrane as it is in *N. pharaonis*. As a matter of fact, bR is expressed as an inclusion body in *E. coli* and a certain treatment was necessary to obtain a proper conformation [25]. Thus, the functional expression of *p*pR in *E. coli* cells is interesting.

The pET system is the most powerful system yet developed for the expression of recombinant proteins in *E. coli*. SDS-PAGE suggests, however, that the *p*pR expression seemed to be not on a high level (data not shown). We are now trying to use other expression vectors so as to increase the level of expression. Although the expression is not on a high level, the present system opens the investigation relating the structure to the photochemical properties of *p*pR using the site-directed mutagenesis. Because of its high growth rate, *E. coli* *p*pR has an advantage. Other interesting work is whether *E. coli* *p*pR can couple with *p*HtrII (*pharaonis* halobacterial transducer for sR-II) if those are co-expressed.

Acknowledgements: This work was performed as part of the Research and Development Project of the Industrial Science and Technology Frontier Program supported by NEDO (New Energy and Industrial Technology Development Organization), and was supported in part by a Grant-in-Aid for Scientific Research from the Japanese Ministry of Education, Science, Sports and Culture.

References

[1] Oesterhelt, D. and Stoeckenius, W. (1971) Nature New Biol. 233, 149–152.
[2] Stoeckenius, W. and Bogomolni, R.A. (1982) Annu. Rev. Biochem. 51, 587–615.
[3] Matsuno-Yagi, A. and Mukohata, Y. (1977) Biochem. Biophys. Res. Commun. 78, 237–243.
[4] Lanyi, J.K. (1986) Annu. Rev. Biophys. Biophys. Chem. 15, 11–28.
[5] Spudich, J.L. and Bogomolni, R.A. (1988) Annu. Rev. Biophys. Biophys. Chem. 17, 193–215.
[6] Spudich, J.L., Zacks, D.N. and Bogomolni, R.A. (1995) Isr. J. Chem. 35, 495–513.
[7] Takahashi, T., Tomioka, H., Kamo, N. and Kobatake, Y. (1985) FEMS Microbiol. Lett. 28, 161–164.
[8] Tomioka, H., Takahashi, T., Kamo, N. and Kobatake, Y. (1986) Biochem. Biophys. Res. Commun. 139, 389–395.
[9] Wolff, E.K., Bogomolni, R.A., Scherrer, P., Hess, B. and Stoeckenius, W. (1986) Proc. Natl. Acad. Sci. USA 83, 7272–7276.
[10] Scherrer, P., McGinnis, K. and Bogomolni, R.A. (1987) Proc. Natl. Acad. Sci. USA 84, 402–406.
[11] Marwan, W. and Oesterhelt, D. (1987) J. Mol. Biol. 195, 333–342.
[12] Takahashi, T., Yan, B., Mazur, P., Derguini, F., Nakanishi, K. and Spudich, J.L. (1990) Biochemistry 29, 8467–8474.
[13] Imamoto, Y., Shichida, Y., Yoshizawa, T., Tomioka, H., Takahashi, T., Fujikawa, K., Kamo, N. and Kobatake, Y. (1991) Biochemisty 30, 7416–7424.
[14] Scharf, B., Pevec, B., Hess, B. and Engelhard, M. (1992) Eur. J. Biochem. 206, 359–366.
[15] Hirayama, J., Imamoto, Y., Shichida, Y., Kamo, N., Tomioka, H. and Yoshizawa, T. (1992) Biochemistry 31, 2093–2098.
[16] Imamoto, Y., Shichida, Y., Hirayama, J., Tomioka, H., Kamo, N. and Yoshizawa, T. (1992) Biochemistry 31, 2523–2528.
[17] Miyazaki, M., Hirayama, J., Hayakawa, M. and Kamo, N. (1992) Biochim. Biophys. Acta 1140, 22–29.
[18] Hirayama, J., Imamoto, Y., Shichida, Y., Yoshizawa, T., Asato, A.E., Liu, R.S.H. and Kamo, N. (1994) Photochem. Photobiol. 60, 388–393.
[19] Hirayama, J., Kamo, N., Imamoto, Y., Shichida, Y. and Yoshizawa, T. (1995) FEBS Lett. 364, 168–170.
[20] Bivin, D.B. and Stoeckenius, W. (1986) J. Gen. Microbiol. 132, 2167–2177.
[21] Scharf, B. and Wolff, E.K. (1994) FEBS Lett. 340, 114–116.
[22] Zhang, W., Brooun, A., Mueller, M.M. and Alam, M. (1996) Proc. Natl. Acad. Sci. USA 93, 8230–8235.
[23] Seidel, R., Scharf, B., Gautel, M., Klein, K., Oesterhelt, B. and Engelhard, M. (1995) Proc. Natl. Acad. Sci. USA 92, 3036–3040.
[24] Charlebois, R.L., Hofman, J.D., Schalkwyk, L.C., Lam, W.L. and Doolittle, W.F. (1989) Can. J. Microbiol. 35, 21–29.
[25] Karnik, S., Doi, T., Molday, R. and Khorana, H.G. (1990) Proc. Natl. Acad. Sci. USA 87, 8955–8959.