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Predicted bacteriorhodopsin from *Exiguobacterium sibiricum* is a functional proton pump

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ABSTRACT

The predicted *Exigobacterium sibiricum* bacterirhodopsin gene was amplified from an ancient Siberian permafrost sample. The protein bacteriorhodopsin from *Exiguobacterium sibiricum* (ESR) encoded by this gene was expressed in *Escherichia coli* membrane. ESR bound all-trans-retinal and displayed an absorbance maximum at 534 nm without dark adaptation. The ESR photocycle is characterized by fast formation of an M intermediate and the presence of a significant amount of an O intermediate. Proteoliposomes with ESR incorporated transport protons in an outward direction leading to medium acidification. Proton uptake at the cytoplasmic surface of these organelles precedes proton release and coincides with M decay/O rise of the ESR.

1. Introduction

Bacteriorhodopsins are bacterial retinal-containing membrane proteins with various functions including ion transport and photosensory activity [1]. The prototype member of the family, bacteriorhodopsin from *Halobacterium salinarum* (BR), is a photoactivated proton pump extensively studied by genetic and biophysical methods [2,3]. Since the first discovery of a proteorhodopsin gene in metagenomic DNA library from the Red Sea [4] it was found that diverse groups of microorganisms possess bacteriorhodopsin homologs [5]. Majority of these new bacterio- and proteorhodopsin genes were isolated from marine-derived microbial communities. The recent studies revealed the presence of proteorhodopsin-expressing bacteria also in non-marine environments [6,7]. These findings pose a question about the functional significance of such "non-typical" BR representatives.

Siberian permafrost contains the unique microbial community adapted to extreme conditions including long-term freezing, cumulative radiation and high water osmolarity [8,9]. *Exiguobacterium sibiricum* is one of Gram-positive microorganisms widely present in permafrost samples and withstanding wide range of growth conditions [10]. Recently the presence of potential bacteriorhodopsin gene had been revealed in the genome of *E. sibiricum* [11]. However, the functional state of the predicted protein and its possible role in adaptation to extreme environmental conditions remained unclear. Here we describe the heterologous expression of *E. sibiricum* bacteriorhodopsin gene in *Escherichia coli* membrane, purification of the protein and the primary investigation of its functional characteristics.

2. Materials and methods

2.1. Sample collection

The permafrost samples for this study were collected in north-eastern Arctic tundra (69°29′N, 156°59′E, Kolyma lowland). The

Abbreviations: BR, bacteriorhodopsin from *Halobacterium salinarum*; ESR, bacteriorhodopsin from *Exiguobacterium sibiricum*; DDM, *n*-dodecyl-β-D-maltopyranoside; OG, *n*-octyl-β-D-glucopyranoside

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cores were extracted from the mid Pleistocene lake-alluvium sandy loam strata buried on 25-m depth. This is a syngenetically frozen horizon formed 200–400 thousand years ago and never thawed till now. Contamination with organisms non-indigenous to the frozen sediment sample can be excluded on the basis of previous method validation [8].

2.2. DNA isolation

Total genomic DNA was extracted from approximately 0.25–0.5 g of frozen sediment from the interior of subcore using the UltraClean soil DNA isolation kit (MoBio Laboratories, USA) following to the manufacture's protocol.

2.3. Gene cloning

E. coli strain XL-1 Blue (Stratagene) was used for all DNA manipulations. To amplify bacteriorhodopsin from *Exiguobacterium sibiricum* (ESR) gene from environmental sample two gene-specific primers ESR1 5′ATCATACATATGGAAGAAGTCAATTTACTCG and ESR2 5′ACATCTCGAGGGACGTCAGCGTTTTTCCTT were designed according to the published genomic DNA sequence of *E. sibiricum* [11] and synthesized by Evrogen (Moscow, Russia). *Nde*I and *Xho*I restriction sites (underlined) were introduced at 5′ and 3′ ends of the gene respectively. The termination codon was omitted from the sequence. PCR was carried out with *Pfu* DNA-polymerase (Fermentas, Lithuania) as recommended by supplier. The amplified fragment was cloned into *Nde*I and *Xho*I sites of pET32a plasmid (Novagen). DNA sequencing was carried out by Genome Centre (Moscow, Russia). The resulting plasmid pET-ESR contains entire ESR gene with LEHHHHHH-coding extension at 3′ terminus.

2.4. Protein purification

E. coli BL21(DE3)pLysS cells freshly transformed with pET-ESR were grown for 2 days at 30 °C in 2XZY5052 autoinduction media [12] supplied with 100 μg/ml ampicillin and 34 μg/ml chloramphenicol. Upon harvesting the cells were resuspended in 100 mM Tris–HCl, pH 8.0, 20% sucrose and 5 mM EDTA, incubated with 0.2 mg/ml lysozyme and diluted with 5 volumes of cold deionized water. After ultrasonication the insoluble material was removed by centrifugation at 6000×*g* for 30 min. Membrane fraction was sedimented at 100 000 × g for 1 h and after addition of 5 μM all-*trans*-retinal ESR was extracted by incubation with 50 mM Tris–HCl, 200 mM NaCl, 1% *n*-dodecyl-β-D-maltopyranoside (DDM), 10 mM imidazole, pH 8.0, for 18 h at 4 °C. Solubilized fraction was applied on Ni Sepharose FastFlow resin (GE Healthcare) and extensively washed with buffer 50 mM NaH2PO_4, 200 mM NaCl, 0,1% DDM, 30 mM imidazole, pH 6.0. ESR was eluted with buffer 50 mM NaH_2PO_4, 200 mM NaCl, 0,2% DDM, 300 mM imidazole, pH 7.4 and concentrated using Amicon centrifugal filter device (10 kDa MWCO). To obtain protein for reconstitution into liposomes 5% and 1% *n*-octyl-β-D-glucopyranoside (OG) was used for the extraction and purification steps instead of DDM.

2.5. Spectroscopic characterization

Absorption spectra were obtained on Biowave II spectrophotometer (WPA). Difference absorption spectra (light minus dark) were recorded on phosphoroscopic device as described in [13]. Flash-induced absorption changes were detected with home-made flash-photolysis system as described in [14]. Flash was from Quantel Y6-481 Nd-YAG Q-switched laser (532 nm, 10 ns). Photoinduced changes in the protein absorbance at 400 nm were determined in 1 mM MOPS, 200 mM NaCl, 0,2% DDM, pH 7.0 in the absence and presence of 60 μM *p*-nitrophenol. The spectral response of the pH indicator itself was obtained by subtraction. Averaging of 10–100 single kinetic responses was used to improve signal-to-noise ratio.

2.6. Proton transport measurements

ESR in OG micelles was reconstituted into proteoliposomes containing Type II-S phospholipid from soybean (Sigma) as described in [15]. 150 μl of proteoliposome suspension (0.25 mg/ml of ESR) was added to 2.1 ml of 2 M NaCl and pH was adjusted to 7.0. The measurements were conducted in the thermostated cell at 25 °C under rapid stirring. pH was monitored with Radiometer PHM82 pH-meter.

3. Results and discussion

3.1. Gene cloning, protein expression and purification

ESR gene encodes a protein of 252 aa displaying less than 40% identity with published sequences of bacterio- and proteorhodopsins (excluding bacteriorhodopsin from closely related *Exiguobacterium sp*. AT1b). Alignment of ESR sequence with BR from *H. salinarum* revealed conservation of several residues in the retinal-binding pocket including K225 which corresponds to K216 participating in the retinal binding in BR (Fig. 1). It should be noted that predicted retinal-binding pocket of ESR exhibits relatively high level of homology with xanthorodopsin and can potentially accommodate carotenoid [16]. The question whether ESR contains additional chromophore(s) in its native host remains for future investigation.

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BR     20   MGLGTLYFLVKGMGVSDPDAKKFYAITTLVPAIAFTMYLSMLLGYGLT--MVPFGGEQNP  77
            M  GTLYFLV+   ++ P+ +    +  LV  +A   Y  M    G +  +    G
ESR    22   MAAGTLYFLVERNSLA-PEYRSTATVAALVTFVAAIHYYFMKDAVGTSGLLSEIDGFPTE  80

BR     78   IYWARYADWLFTTPLLLLDLALLVDADQGT----ILALVGADGIMIGTGLVGALTKVYSY  133
            I   RY DWL TTPLLL+   LL+          +  LV AD IMI  G +G  +   +
ESR    81   I---RYIDWLVTTPLLLVKFPLLLGLKGRLGRPLLTKLVIADVIMIVGGYIGESSINIAG  137

BR     134  RF----VW-WAISTAAMLYILYVLFFGFTSKAESMRPEVASTFKVLRNVTVVLWSAYPVV  188
             F    +W + I   A +YI+Y+LF   T  AE+    +      +R   ++ W+ YP+
ESR    138  GFTQLGLWSYLIGCFAWIYIIYLLFTNVTKAAENKPAPIRDALLKMRLFILIGWAIYPIG  197

BR     189  WLIGSEGAGIVPLNIETLLFMVLDVSAKVGFGLILLRSRAIFGEAEAPEPSAGDGAAATS  248
            + +     G+    +  L++   D++ KVGFGLI     A F        S+  G   TS
ESR    198  YAVTLFAPGVEIQLVRELIYNFADLTNKVGFGLI-----AFFAVKTMSSLSSSKGKTLTS  252
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Fig. 1. Alignment of BR and ESR amino acid sequences using BLAST. Positions corresponding to BR primary proton acceptor (D85), proton donor (D96) and the Schiff base (K216) are marked with grey.

No signal sequence was detected at the ESR N-terminus with the help of SignalP tool [17]. Therefore we have designed primers for amplification of the entire coding region of ESR according to the published genomic DNA sequence. *E. sibiricum* strain used in the sequencing project [11] was isolated from the sample which was preserved in frozen condition for 3 million years. We have failed to directly amplify ESR gene from this sample, probably because of the nucleic acids degradation and crosslinking [18]. That's why PCR was conducted on the DNA isolated from the younger geological layer (200 000–400 000 years). Nevertheless sequencing of the cloned gene revealed 100% conservation of its primary structure in comparison with the published data probably reflecting the functional significance of this protein in *E. sibiricum* metabolism.

To construct an efficient expression system we have cloned ESR gene into pET32a vector providing for hexahistidine tag at the protein C-terminus. ESR expression in *E. coli* cells was independent of externally added *all-trans*-retinal and led to the accumulation of protein with apparent MW of ~23 kDa in the membrane fraction (Fig. 2). The MALDI mass fingerprinting of the fragments obtained after trypsinolysis of the corresponding gel band revealed the sequence coverage ~48% with ESR amino acid sequence derived from genome sequencing data (data not shown). Purity of the ESR extracted from *E. coli* membranes in the presence of DDM after Ni2+-affinity chromatography was about 90% (Fig. 2) with the yield of 10–15 mg/L culture. The absorption maximum of purified ESR at pH 8.0 was 534 nm (Fig. 3a) without noticeable shifts of absorption maximum (dark adaptation) after 12 h incubation (data not shown).

3.2. Photocycle kinetics

Difference absorption spectra of DDM-solubilized ESR and of isolated membranes from ESR-expressing *E. coli* cells were recorded in the range of 350–700 nm. In both cases spectra characteristic for bacteriorhodopsin with depletion and regeneration of the ground state, rise and decay of O intermediate at >500 nm and subtle changes at <400 nm (absorption of M intermediate) were obtained (Fig. 3b). In order to compare ESR and BR photocycles flash-photolysis experiments were conducted at characteristic wavelengths of 410, 550 and 610 nm. Formation of M intermediate was clearly observed at 410 nm (Fig. 4). Its accumulation was ~100 times faster than in BR (τ = 0.5 μs). M decay kinetics (τ = 3.5 ms) was the same order as in BR [3]. Absorption changes of O intermediate observed at 610 nm were ~4 times greater than of M. The decay of O (τ = 26 ms) corresponded to the return of the ground state observed at 550 nm.

In contrast to BR proton uptake at the cytoplasmic surface of ESR precedes proton release and coincides with M decay/O rise (Fig. 4). This "late release" phenomenon is an indicator of blocked proton-releasing complex and consistent with the absence of extracellular analogues of E204 and E194 in BR [19–21]. More rapid M-formation and increased amount of O intermediate are also

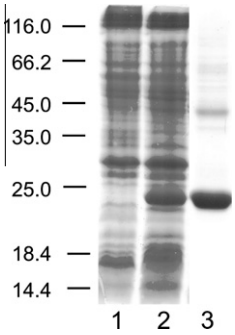

Fig. 2. SDS–PAGE of ESR in *E. coli* membranes (2) and purified ESR (3). 1 – *E. coli* membranes without ESR.

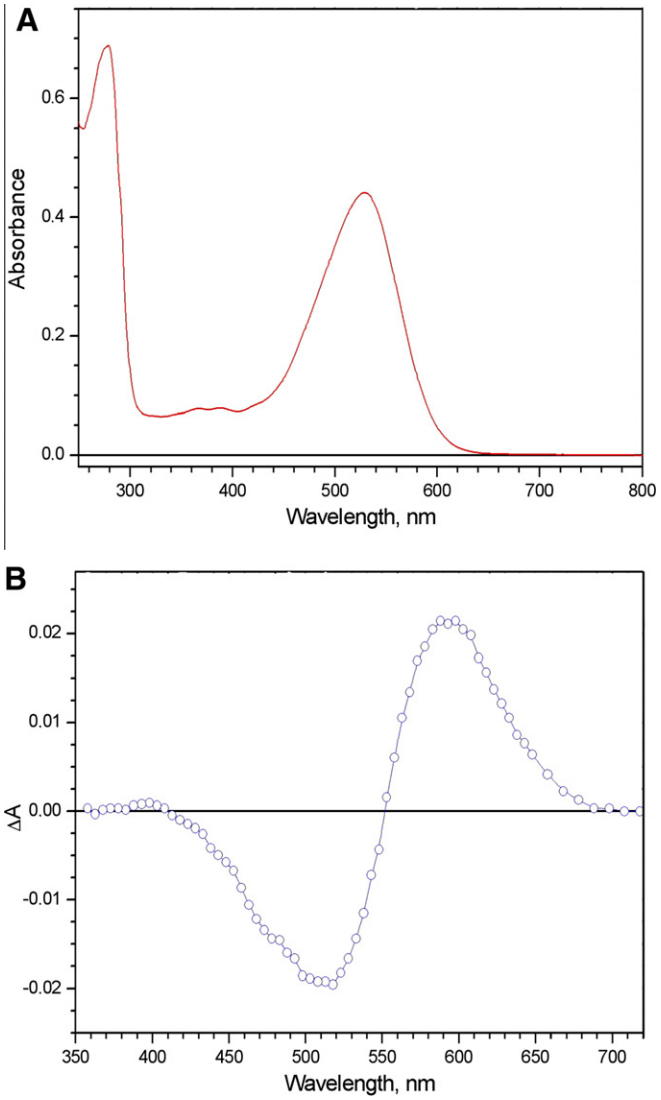

Fig. 3. (A) Absorption and (B) absorption difference (light minus dark) spectra of purified ESR in DDM micelles. Protein concentration was 0.4 mg/ml.

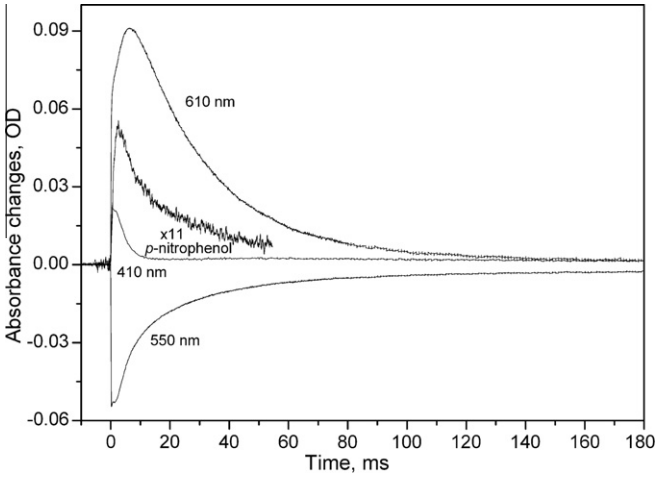

Fig. 4. Flash-induced transient absorption changes of ESR in DDM micelles recorded at 410, 550 and 610 nm.

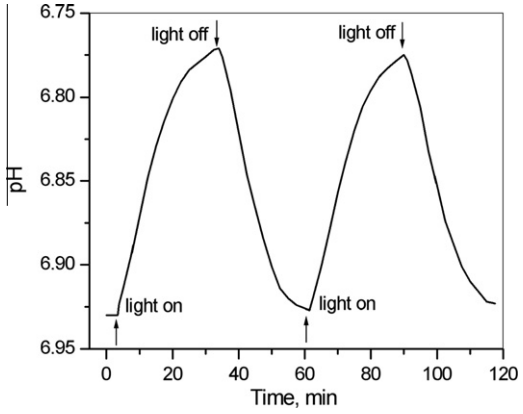

Fig. 5. Light-induced proton fluxes in ESR-containing liposomes. Samples were illuminated with 500 W halogen lamp (OSRAM) from 35 cm distance.

characteristic for E204Q mutant of BR [19]. Indeed position corresponding to E194 in BR is occupied with F203 in ESR. Location of R213 adjacent to E214 (analog of E204 in BR) can potentially impair its ability to deliver protons (Fig. 1).

The presence of R82, D85 and D221 of ESR at the positions corresponding to R82, D85 and D212 of BR points to the structural similarity of proton translocation channels of ESR and BR (Fig. 1). Intriguing feature of ESR is the presence of K96 residue at the position corresponding to D96 which serves as a proton donor in BR and is occupied by carboxylic acid almost in all known proton pumping bacteriorhodopsins [22]. BR mutant D96N and homologous proteorhodopsin mutant E108Q both have a very slow decay of the M intermediate due to reprotonation of the Schiff base from the medium [21,23]. This is not the case in ESR, suggesting the existence of an alternative intramolecular proton donor whose position should be the matter for further investigation.

3.3. Proton transport measurements

After illumination we have observed net acidification of the suspension of ESR-containing liposomes corresponding to the proton pumping from the inside to the outside of liposomes (Fig. 5). The rate of proton translocation by ESR is ~10 times slower than in BR-containing liposomes measured in the same conditions (data not shown) but correlates with the time-course of light-induced acidification in proteorhodopsin liposome suspension [24]. The same effects were observed when pH changes were measured for washed *E. coli* cells expressing ESR confirming that ESR is incorporated into liposomes in the same direction as in the *E. coli* membrane. Therefore we can conclude that ESR unlike bacteriorhodopsin [25] is oriented in the proteoliposomes with its C-terminus facing the inner surface of the bilayer.

This report is the first functional characterization of a bacteriorhodopsin gene from *Firmicutes*. Experimental data obtained confirm that ESR heterologously expressed in *E. coli* membranes demonstrates light-induced photocycle and proton transport. ESR photocycle at neutral pH was relatively fast (<100 ms) enabling attribution of this protein to the transport bacteriorhodopsins. Future studies should clarify the details of the proton translocation mechanism conducted by ESR as well as its role in *E. sibiricum* physiology and adaptive response to environmental stimuli.

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