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Over-expression of a new photo-active halorhodopsin [1] in *Halobacterium salinarium*

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Abstract

The gene of haloopsin (*hop*) from halobacterial strain *shark* was cloned and its nucleotide sequence was determined. The deduced amino acid sequence of shark halorhodopsin (HR) showed that its homology with halobium HR was 62%. The gene product seems to be HR having several positively charged residues that are conserved in all known HRs. The gene encoding shark *hop* as well as that encoding halobium *hop* were successfully expressed in *Halobacterium salinarium* (*halobium*) by using a plasmid shuttle vector containing the bacterioopsin (*bop*) promoter. The expression level of shark HR is almost the same as that for halobium HR with the same shuttle vector containing the *bop* promoter. Under the physiological conditions, the anion pumping activity of the shark HR expressed in *H. salinarium* was almost the same as that for halobium HR; however, the anion selectivity and half-maximal anion transport were different. Furthermore, its absorption maximum in the absence of chloride shifted to approx. 596 nm in contrast to that for halobium HR. The half-lifetimes of HR_{520} formation for shark HR and halobium HR were almost the same; however, the half-lifetime of its decay was approx. 6-times faster for shark HR than it was for halobium HR at a high chloride concentration (1000 mM). Even at a low chloride concentration (50 mM), HR_{520} and HR_{640} intermediates could be detected for shark HR, and the half-lifetime of HR_{640} decay was found to be approx. 25 ms. In the presence of nitrate, the half-lifetime of HR_{565} recovery for shark HR was approx. 10-times slower than that for halobium HR. Some of amino acid substitutions between shark HR and halobium HR may affect the anion selectivity and the photoreaction of HR.

Keywords: Halorhodopsin; Pharaonis halorhodopsin; Bacteriorhodopsin; Halobacterium; Anion pump

1. Introduction

Halorhodopsin (HR) is a member of bacterial rhodopsins which exist in extremely halophilic archaebacteria. The first HR was found in *Halobacterium salinarium* (*halobium*) by Matuno-Yagi and Mukohata [1], and the gene encoding haloopsin (*hop*) has been cloned and sequenced [2]. Second and third HRs have been also isolated in other halophilic archaebacteria, and their amino acid sequences have been determined [3–5]. Comparative studies of the amino acid sequences for the three HRs have suggested that several positively charged residues are important for anion transport. Specifically, Arg-52, Arg-58, and His-95 in the A-B and B-C interhelix loop segments were conserved in the three HRs, but the bacteriorhodopsin (BR) family lacks these residues, suggesting that these residues may be essential only for anion binding [5]. A *hop* gene from halobacterial sp. SG1 has recently been cloned and sequenced, and all of the supposedly positively charged residues were conserved [6].

Changes in the absorption spectrum with the addition of anions have been studied by Ogurusu et al. [7]. In physiological conditions (4 M NaCl) the absorption maximum of halobium HR is known to be around 578 nm. In the complete absence or at low concentrations of chloride, this has been found to shift to around 568 nm. In the presence of nitrate, almost the same shift in the absorption maximum has been observed. Additions of various anions have

Abbreviations: BR, bacteriorhodopsin; HR, halorhodopsin; bp, base pair; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); PCR, polymerase chain reaction.

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[1] The nucleotide sequence data reported in this paper have been submitted to the GSDB, DDBJ, EMBL and NCBI nucleotide sequence databases and have been assigned the accession number D43765.

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been found responsible for shifts in absorption, indicating the presence of a single chloride binding site in halobium HR. Recently, wavelength regulation and photoreaction caused by added anions in purified pharaonis HR were investigated extensively by Scharf and Engelhard [8]. They have found that in the absence of chloride, the pharaonis HR showed a shift of the absorption maximum to around 600 nm, and that this shift can be regulated by changes in the anions added and their concentration. The presence of an anion binding site in pharaonis HR was suggested by these results.

The photochemical properties of halobium HR have been studied by several groups [9–12]. The sequence of intermediates ($HR_{578} \rightarrow HR_{600} \rightarrow HR_{520} \rightarrow HR_{640} \rightarrow HR_{565} \leftrightarrow HR_{578}$) has been proposed as a photocycle in the presence of chloride. From resonance raman study, Ames et al. [11] have suggested that chloride was released during the $HR_{520} \rightarrow HR_{640}$ transition and taken up in the transition between HR_{640} and HR_{578}. A recent FTIR study by Braiman et al. [12] indicated that the arginine–halide interaction is disrupted by photoconversion to HR_{520}. These studies have indicated that HR_{520} and HR_{640} might be the key intermediates for gaining an understanding of the mechanism of HR anion pumping. At low chloride concentrations or the presence of sodium nitrate, HR_{520} has not been detected, suggesting the presence of chloride dissociation from a binding site in the $HR_{520} \rightarrow HR_{640}$ transition [13]. However, a photocycle as it occurs in the absence of chloride or in the presence of nitrate needs to be further characterized to elucidate the nitrate pumping process and the pumping selectivity between chloride and nitrate in HR, because halobium HR and pharaonis HR both have nitrate pumping activity [3].

A transformation system for halophilic bacteria was developed by Cline et al. [14], and BR was subsequently successfully expressed in *H. salinarium* forming a crystalline lattice identical to purple membrane [15,16]. This system has been used to make BR mutants. Both HR and sensory rhodopsin (SR) have been over-expressed in the same strain, *H. salinarium*, using the *bop* promoter system [17,18], and the production of mutants thereof started. This system, therefore, should prove a powerful tool in producing not only mutants of BR, HR and SR from *H. salinarium*, but also for expressing new kinds of bacterial rhodopsins from other halophilic bacteria so as to investigate their structure and function.

In this study, we found a new *hop* gene type in a newly isolated halobacteria and its gene product was expressed in *H. salinarium* to further investigate its function. The deduced amino acid sequence was different from those of all other known HRs. However, almost all the residues near the retinal Schiff base and the three positively charged residues (Arg-52, Arg-58 and His-95) in the A-B and B-C interhelix loop segments were found to be conserved. The features of its gene product were found to be different from those of all other known HRs.

2. Materials and methods

2.1. Microbial strains

H. salinarium strains L33 (BR negative) and HN5 (BR and HR negative), and shuttle-vector plasmid (pEF191) were the generous gifts of Dr. Dieter Oesterhelt. Newly isolated halobacterial strain *shark* was also a generous gift from Dr. H. Tomioka. Competent cells of *Escherichia coli* JM109 were obtained from Takara Shuzo Co., Japan.

2.2. Cloning and sequencing of the haloopsin gene

Chromosomal DNA from strain *shark* was isolated and purified in a process described in the preceding paper [19]. Oligonucleotide primers corresponding to highly-conserved regions in C-helix and G-helix were designed from the nucleotide sequences of the *hop* gene common to those for strains *halobium*, *pharaonis* and *mex* (Sense primer, 5′-TGGGG(C,G)CG(C,G)TA(C,T)CT(C,G)ACGTGG-3′; Antisense primer, 5′-AACGCGAAGA(C,T)GTACT-TCGCGA(A,C)GA(C,T)GTC-3′). With these two primers, a DNA fragment of about 400 bp was amplified directly from the genomic DNA of strain *shark*, and subsequently the amplified product was sequenced directly. From the sequence analysis we concluded that this fragment was the C-helix to G-helix region (C-G region) of the *hop* gene. The flanking regions of the C-G region were cloned by using the 'cassette and cassette primer cDNA, genome walking kit' (Takara Shuzo), which is based on the cassette-ligation-mediated PCR method [20]. The conditions for PCR amplification were as follows: a 100 μl reaction mixture containing 20 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 25 mM KCl, 1 mg gelatin, 50 mM each dNTPs, 1 unit of Taq DNA polymerase (Takara Shuzo), and 20 pmol of each primer. PCR was performed in a programmable heat block (Thermal Cyclic Reactor, Model TC-100, Houei, Japan). The PCR products were fractionated by electrophoresis on a 4.0% NuSieve 3:1 Agarose gel (Takara Shuzo). PCR products were purified from the gel using GENECLEAN II (Funakoshi Co., Japan). Sequence reactions were performed using the dideoxy chain-termination method with Sequenase DNA Polymerase Version 2.0 (US Biochemicals, USA) and the nucleotide sequence of the PCR products was determined with an automated DNA sequencing system (SQ-3000, Hitachi, Japan). The oligonucleotide primers used for PCR amplification and for sequencing were obtained from Sawadei Technology, Japan.

2.3. Construction of plasmid vectors and transformation of Halobacterium salinarium

A fusion gene for shark HR expression, which contained sequences of the first thirteen amino acids of BR, plus upstream sequences of the putative *bop* promoter was

constructed using the recombinant PCR method. The *bop* promoter region was amplified by the sense primer having a *Bam*HI site, 5′-CCGCCTGCAGGATCCGACGTGAAGATG-3′ (~ 400 bp upstream from the ATG codon of *bop*) and by the antisense primer having the sequence of the junction region of the first thirteen amino acids of BR and shark HR, 5′-GTAGTACTGACAGCTGTCATCGATACCCCCTCCACTGC-3′. The structural gene of shark *hop* containing the terminator region was also amplified by the sense primer having the sequence of the same junction region, 5′-GCAGTGGAGGGGGTATCGATGACAGCTGTCAGTACTAC-3′ and the antisense primer, 5′-ACCGAGTGCAACATACCG-3′ (approx. 200 bp downstream from the stop codon of shark *hop*). In the first step PCR, a approx. 400 bp DNA fragment having the *bop* promoter plus the first thirteen amino acids of BR, and a approx. 900 bp DNA fragment having the entire shark *hop* gene plus its terminator region were amplified. In the secondary PCR, the mixture of first PCR DNA products was used as the template to amplify a recombinant DNA fragment with a length of approx. 1300 bp. The recombinant gene was first cloned into pT7Blue T-Vector, which is used for the simple cloning of PCR product, to introduce a new *Hin*dIII site at the end of the termination region. The *Bam*HI-*Hin*dIII-digested fusion gene was then ligated into in the *Bam*HI-*Hin*dIII-digested pEF191 plasmid vector to yield the shuttle vector pEJsHR.

H. salinarium strain HN5 was transformed with the plasmid pEJsHR using the method described previously [16]. Transformants were plated on 1.5% agar plates of a complex support medium containing 25 μM Mevinolin and incubated at 40°C for 2 weeks. Transformants expressing shark HR were detected by color. 10–20% of Mevinolin-resistant transformants were blue, and three of these transformants were analyzed. The plasmid DNA was inserted into the genome at the homologous *bop* promoter *locus* in the three analyzed transformants. The transformant over-expressing the halobium HR (JO17) was also obtained using the same strategy, and was used as a control for wild-type HR (halobium HR).

2.4. Preparation and characterization of cell-envelope vesicles from HR over-expressing strains

The transformant cells were grown in a peptone medium prepared as described by Oesterhelt and Stoeckenius [21].

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TTAACACAGTTATGCCACCTCGTGTCCGGGACTGGTCTTGTCCTTCCGAAACCCGAATCTAACAGCGGATACCGATACCGTTT

GGGTACGCGTCTTTAAGTGTTCTCTTCGTAATTAGTGTAC    ATG ACA GCT GTC AGT ACT ACT GCA ACG ACA
                                             M   T   A   V   S   T   T   A   T   T

GTG CTT CAA GCG ACA CAG TCA GAC GTG TTA CAG GAG ATA CAG TCG AAT TTC CTC CTG AAT TCG
 V   L   Q   A   T   Q   S   D   V   L   Q   E   I   Q   S   N   F   L   L   N   S

TCG ATC TGG GTG AAC ATC GCG CTG GCG GGC GTC GTC ATC CTC CTG TTC GTC GCG ATG GGT CGA
 S   I   W   V   N   I   A   L   A   G   V   V   I   L   L   F   V   A   M   G   R

GAC CTC GAG TCG CCC CGC GCG AAA CTC ATC TGG GTC GCG ACG ATG CTG GTG CCG CTG GTC TCG
 D   L   E   S   P   R   A   K   L   I   W   V   A   T   M   L   V   P   L   V   S

ATT TCC AGC TAT GCC GGC CTC GCC TCC GGG CTG ACA GTC GGG TTT CTG CAG ATG CCG CCG GGC
 I   S   S   Y   A   G   L   A   S   G   L   T   V   G   F   L   Q   M   P   P   G

CAC GCC CTC GCC GGA CAG GAG GTC CTA TCA CCG TGG GGC CGC TAC CTG ACG TGG ACG TTC TCG
 H   A   L   A   G   Q   E   V   L   S   P   W   G   R   Y   L   T   W   T   F   S

ACA CCG ATG ATT CTT CTG GCG CTG GGC CTG CTG GCT GAC ACT GAC ATT GCG TCG CTG TTC ACC
 T   P   M   I   L   L   A   L   G   L   L   A   D   T   D   I   A   S   L   F   T

GCA ATC ACG ATG GAT ATC GGG ATG TGT GTG ACC GGC CTC GCC GCC GCG CTC ATC ACT TCC TCG
 A   I   T   M   D   I   G   M   C   V   T   G   L   A   A   A   L   I   T   S   S

CAC CTC CTC CGC TGG GTG TTT TAC GGC ATC AGC TGT GCG TTC TTC GTC GCA GTG CTG TAC GTC
 H   L   L   R   W   V   F   Y   G   I   S   C   A   F   F   V   A   V   L   Y   V

CTG CTC GTC CAG TGG CCG GCC GAC GCA GAG GCC GCC GGA ACA AGC GAG ATA TTC GGG ACG CTC
 L   L   V   Q   W   P   A   D   A   E   A   A   G   T   S   E   I   F   G   T   L

AAG ATT CTC ACC GTG GTG CTG TGG CTG GGC TAC CCG ATT CTG TGG GCA CTG GGT TCC GAG GGC
 K   I   L   T   V   V   L   W   L   G   Y   P   I   L   W   A   L   G   S   E   G

GTT GCC CTC CTG AGC GTC GGC GTC ACC TCG TGG GGC TAC TCC GGG CTG GAC ATC CTT GCA AAG
 V   A   L   L   S   V   G   V   T   S   W   G   Y   S   G   L   D   I   L   A   K

TAC GTC TTC GCG TTC CTG CTC CTG CGC TGG GTC GCC GCC AAC GAA GGC ACC GTC TCA GGG TCT
 Y   V   F   A   F   L   L   L   R   W   V   A   A   N   E   G   T   V   S   G   S

GGG ATG GGC ATC GGC TCC GGC GGC GCT GCG CCC GCA GAT GAC    TGAGTAGCGCATCGAGACGTGGGC
 G   M   G   I   G   S   G   G   A   A   P   A   D   D

AGTATCCTCCTGCTCGCGTATCCCTGGCCGTATTCTGGCCGTTCTCTATCCCTGCCCGCACAGTAGCAGATGCAGAAAAGATA
AGTTTATGTTGCCCAAGTGACTAGTCTGTATGCCCTCCCCATTCGAAAACCCAGCCATTCGCTACGGTATCCCACTGCTCAGCG
CTACGGTTGTCGCGGCTGTCGCGTTCCTCTTCCTTGAGGGAACAGTACGGTATGTTGCACTCGGTATCGCTGTCCTCGAAGTT
GTG
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Fig. 1. The nucleotide and deduced amino acid sequences of the *hop* gene and its flanking regions from the *shark* strain. This gene was cloned by the cassette-ligation-mediated PCR method and the amplified fragments were directly sequenced. (We have also determined the nucleotide sequence of the *hop* gene from the *port* strain. The deduced amino acid sequence of port HR showed five amino acid substitutions between shark HR and port HR. These nucleotide sequence data have also submitted to the GSDB, DDBJ, EMBL and NCBI nucleotide sequence database, with access number D43766).

35 ml of precultured cells were inoculated into 1.5 L of the same medium in a 3.0 L erlenmeyer. After 6–8 days of cultivation at 40°C, the cells were collected by centrifugation and resuspended in a P-buffer (4 M NaCl, 25 mM pipes/NaOH at pH 7.0) containing DNase. Cell-envelope vesicles were prepared by the freeze-thaw method described by Otomo et al. [19]. The Lowry method, with BSA as a standard, was used for the determination of protein concentration in the cell-envelope vesicles. Light-induced ion pump activity was observed by measuring pH changes using a glass pH electrode (Radiometer GK2321C, USA). A projector lamp (300 W) was used as a light source. The apparatus was almost the same as that described in [19]. Absorption spectra were recorded on a Hitachi U-3210 spectrophotometer. Kinetic flash-spectroscopic measurements were performed with a flash-spectroscopic apparatus (Model USP-500, Unisoku Scientific Instruments Corp., Japan). The monitoring beam provided by a 12 V 100 W tungsten-halogen lamp was passed through a monochromator. The monitoring beam was guided to the sample by glass-fiber cable. After passing though the sample and an additional monochromator, the beam was focused onto a photomultiplier. The photomultiplier output was fed into a current-voltage converter, digitized by an A/D converter and transferred to a microcomputer. The actinic flash was delivered at 90° to the monitoring beam as a Xenon flash passed though a orange filter (O–57) for $\lambda > 570$ nm light. All measurements were performed at 20°C.

3. Results

The genes homologous to the halobium *hop* gene had been detected in the genomic DNA from the newly isolated halobacterial strain *shark* by using Southern blot hybridization analysis [19]. The DNA fragment encoding the C-G region of the *hop* gene was directly amplified from the *shark* strain by using PCR. We cloned the entire shark *hop* structural gene with the flanking regions by using the cassette-ligation-mediated PCR method [20], and its nucleotide sequence was determined (Fig. 1). This sequence showed that shark HR consists of 276 amino acids having 62% homology to halobium HR. The amino acid sequence homologies of shark HR to all other known HRs were less than 70%. Fig. 2 shows an amino acid sequence comparison of HR from the strains *halobium*, *SG1*, *pharaonis*, *mex* and *shark*. Almost all the charged residues in the helix regions, and three supposedly positively charged residues in A-B and B-C interhelix loop segments (Arg-52, Arg-58, and His-95; numbered as in halobium HR [2]) were conserved in the same way as for all known HRs. However, one series of six amino acids (EMPAGH) in the B-C interhelix loop segment, which was conserved in all known HRs, was replaced by QMPPGH for shark HR.

Transformation of strain HN5 with plasmid pEJsHR produced several transformant colonies purple-blue in coloration. This color clearly suggested that these transformants produced shark HR. The PCR analysis of the ge-

strain	N-term	helix A	A-B Interhelix
halobium	MSITSVPGVVDAGVLGAQSAAAVRENALL	SSSLWVNVALAGIAILVFVYM	GRTIRPGRPR
SG1	MIETAAADILAGGMVPLEMTQTQIFEAVQSDTLL	ASSLWINIALAGLSILLFVYM	GRNVEDPRAQ
pharaonis	MTETLPPVTESAVALQAEVTQRELFEFVLNDPLL	ASSLYINIALAGLSILLFVFM	TRGLDDPRAK
mex	???QTEIFQFIQDNTLL	SSSLWVNIALAGLSILLFVYM	GRNVEDPRAQ
shark	MTAVSTTATTVLQATQSDVLQEIQSNFLL	NSSIWVNIALAGVVILLFVAM	GRDLESPRAK

	helix B	B-C Interhelix	helix C	
halobium	LIWGATLMIPLVSISSYLGLLS	GLTVGMIEMPAGHALAG.....EMVRSQ	WGRYLTWALSTPMILLALGLLA	DVDLGS
SG1	LIFVATLMVPLVSISSYTGLVS	GLTVSFLEMPAGHALAG.....QEVLTP	WGRYLTWALSTPMILIAVGLLA	GSNTTK
pharaonis	LIAVSTILVPVVSIASYTGLAS	GLTISVLEMPAGHFAEGSSVMLGGEEVDGVVTM	WGRYLTWALSTPMILLALGLLA	GSNATK
mex	LIFVATLMVPLVSISSYTGLVS	GLTVGFLEMPAGHALAG....MGAGPEGGVFTP	WGRYLTWAFSTPMILIALGLLA	GSNMSK
shark	LIWVATMLVPLVSISSYAGLAS	GLTVGFLQMPPGHALAG.....QEVLSP	WGRYLTWTFSTPMILLALGLLA	DTDIAS

	helix D		helix E	
halobium	LFTVIAADIGMCVTGLAAAM	TTSALLF	RWAFYAISCAFFVVVLSALVTDWA	ASASSAG
SG1	LFTAVVADIGMCVTGLAAAL	TTSSYLL	RWVWYAISCAFFVVVLYILLAEWA	EDAEIAG
pharaonis	LFTAITFDIAMCVTGLAAAL	TTSSHLM	RWFWYAISCACFLVVLYILLVEWA	QDAKAAG
mex	LFTAVVADVGMCITGLAAAL	TTSSYLL	RWVWYGISCAFFVVVLYILLAEWA	KDAEVAG
shark	LFTAITMDIGMCVTGLAAAL	ITSSHLL	RWVFYGISCAFFVAVLYVLLVQWP	ADAEAAG

	helix F		helix G	C-term
halobium	TAEIFDTLRVLTVVLWLGYPIVWAVGV	EGLALVQSVG	VTSWAYSVLDVFAKYVFAFILLRWV	ANNERTVAVAGQTLGTMSSDD
SG1	TADIFNTLKVLTVVLWLGYPIFWALGA	EGLAVL.DVA	ITSWAYSGMDIVAKYLFAFLLLRWV	VNNERTVADVASGLGSGSRGGAAPADD
pharaonis	TADMFNTLKLLTVVMWLGYPIVWALGV	EGIAVL.PVG	VTSWGYSFLDIVAKYIFAFLLLNYL	TSNESVVSGSILDVPSASGTPADD
mex	TADIFNTLKVLTVVLWLGYPIFWALGA	EGLAVL.DIA	ITSWAYSGMD??AKY??A???????	
shark	TSEIFGTLKILTVVLWLGYPILWALGS	EGVALL.SVG	VTSWGYSGLDILAKYVFAFLLLRWV	AANEGTVSGSGMGIGSGGAAPADD

Fig. 2. Alignment of amino acid sequences for HR from strains *halobium* (Blanck and Oesterhelt [2]), *SG1* (Soppa et al. [6]), *pharaonis* (Lanyi et al. [4]), *mex* (Otomo et al. [5]) and *shark* (this work). The single-letter amino acid code is used. Two Arg residues in A-B loop and a His residue in B-C loop, which are suggested to be anion binding sites, are shown in bold letters. The underlined amino acid corresponds to the series of six amino acids in B-C loop.

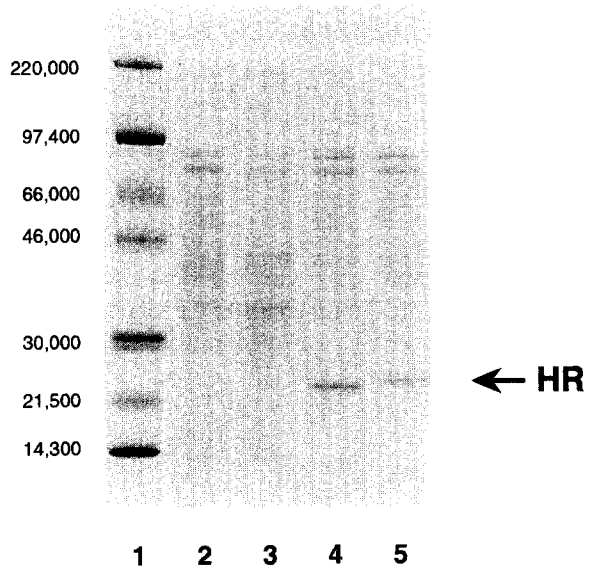

Fig. 3. SDS-PAGE analysis of total membrane fractions of strain HN5 (lane 2), L33 (lane 3), JO17 (lane 4) and JOS5 (lane 5). 10 μg of the total membrane protein was loaded. Lane 1 is marker proteins. The arrow corresponds to Halorhodopsin.

nomic DNA from one of these purple-blue transformants (JOS5) showed that the plasmid pEJsHR was integrated into the *bop* promoter locus (data not shown). A transformant JO17, over-expressing halobium HR, was also obtained by the same way.

Cell-envelope vesicles from strains HN5 (BR^-, HR^-), L33 (BR^-, HR^+), JOS5 (shark HR^{++}) and JO17 (halobium HR^{++}) were prepared and total membrane proteins were monitored via SDS-PAGE analysis (Fig. 3). The vesicles from strains JOS5 and JO17 were deep purple-blue, almost the same as that for BR over-producing strain S9. As shown in Fig. 3, HR was the main product in total membrane proteins both for shark HR and halobium HR unlike the HR production in the strain L33, indicating that shark and halobium HRs were over-expressed in strain HN5 using the expression-vector plasmid carrying the *bop* promoter. The SDS-PAGE showed that the densities of the HR bands of shark and halobium were almost the same. The expression level of shark HR was also estimated from the absorbance at 580 nm as ~ 10 mg per 1 L culture. This level was almost the same as that for halobium HR under the same culture conditions.

Anion pumping activity was monitored using the light-induced passive proton uptake of the cell-envelope vesicles in the presence of 50 μM CCCP. In 3 M NaCl, proton uptake into the vesicles was detected for the strain JOS5, showing that the shark HR expressed in strain HN5 had light-induced chloride pumping activity. Pumping rates of shark HR and halobium HR in 3 M NaCl are approx. 850 nmol/min and approx. 810 nmol/min, respectively (data not shown). That is, both HRs have almost the same chloride pumping activity at high chloride concentrations. The anion pumping activity of the cell-envelope vesicles for both strains JOS5 and JO17 were further investigated in terms of their dependence on chloride and nitrate concentrations. In the 1.5 M sodium sulfate solution, light-induced proton uptake was not detected (data not shown). This showed that shark HR did not have sulfate pumping ability; this is the same as was the case for the halobium HR. Fig. 4 shows light-induced passive proton uptake (anion pumping activity) of the vesicles for shark HR and halobium HR, measured as a function of the concentrations of added chloride and nitrate. Although at high anion concentrations the pumping activities of shark HR and halobium HR were almost the same, at low concentrations the activity for shark HR is higher. The half-maximal anion transport for shark HR was observed at chloride concentrations less than 20 mM; for halobium HR the half-maximal anion transport was reached at chloride concentrations greater than 50 mM. In addition, the JOS5 cell-envelope vesicles showed approx. 2-times higher pumping activity for chloride than that for nitrate. A 3-fold greater specificity in favor of chloride was also detected

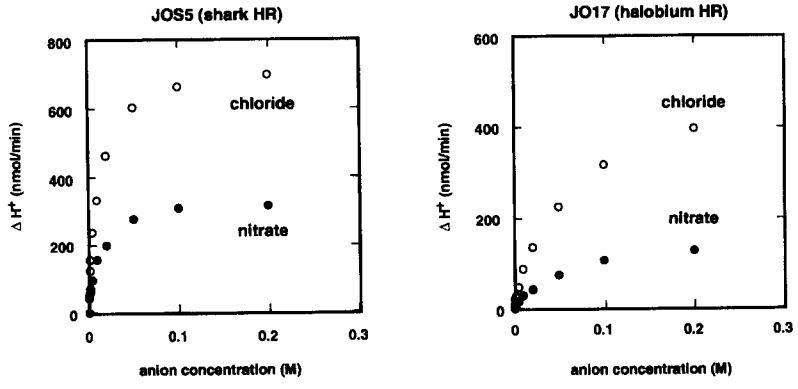

Fig. 4. Anion specificity of light-induced transport by the cell-envelope vesicles from strains JOS5 (shark HR) and JO17 (halobium HR). The rate of the light-driven anions (chloride and nitrate) was plotted as a function of added chloride and nitrate concentrations. The rate was determined by calculating the initial slope of pH change during illumination. The sample (1 mg protein/ml) was suspended in 1.5 M sodium sulfate, 1 mM Pipes (pH 7.0), 50 μM CCCP. A projector lamp (300 W) was used as a light source. The light was passed through a heat-absorbing water bath, an infrared-cut filter and an orange filter (O54).

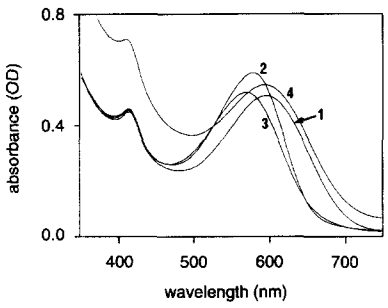

Fig. 5. Absorption spectra of the total membrane fraction from strain JOS5 containing shark HR. The sample containing 2.5 mg/ml total membrane protein was suspended in 20 mM Pipes (pH 7.0). Spectra were measured immediately after illumination with orange light for 1 min (light-adapted). Spectra were measured with various salts at a final concentration of 1000 mM: no salt (1); sodium chloride (2); sodium nitrate (3); sodium sulfate (4). The absorption maxima are as follows: no salt (596 nm); sodium chloride (580 nm); sodium nitrate (570 nm); sodium sulfate (596 nm).

for the JO17 cell-envelope vesicles; this is the same as that for the L33 strain containing wild-type halobium HR. Therefore, the selectivity between chloride and nitrate of the anion transport for shark HR is lower than that for halobium HR.

After dialysis of the solution containing cell-envelope vesicles for JOS5 against the chloride free solution (20 mM Pipes/NaOH, pH 7.0), membrane fractions containing shark HR were obtained. The suspensions of these membrane fractions were clear blue, almost the same as the blue form of purple membrane. Fig. 5 shows absorption spectra of shark HR membrane fractions with various salts at a concentration of 1000 mM. The absorption maximum without salt was approx. 596 nm, in contrast to that for halobium HR whose absorption maximum was known to be approx. 565 nm. As shown in Fig. 5, the addition of chloride or nitrate at a concentration of 1000 mM led to a shift in its absorption maximum. This was not true for sulfate. In addition, changes in their extinction coefficients also accompanied addition of salts. Membrane fractions containing halobium HR were also prepared and their absorption spectra with various salts were measured in the same way. In halobium HR from the strain JO17, the shifts with various salts were almost the same as those for wild-type halobium HR from the strain L33 (data not shown). The shift in absorption maxima for shark HR with the various salts were quite similar to those found by Scharf and Engelhard [8] for pharaonis HR.

Table 1
Time constants of the HR_{520} intermediate for shark HR and halobium HR

HR	Half-lifetime (ms)	
	formation	decay
Shark	0.347	0.513
Halobium	0.358	3.42

Photoreaction cycles of the membrane fraction for shark HR and halobium HR were analyzed using flash-induced absorption changes. Fig. 6 shows the formation and depletion of the intermediates (HR_{640} and HR_{520}) for in the presence of 50 mM and 1000 mM NaCl. Shark HR formed HR_{520} intermediate even in 50 mM NaCl, whereas this intermediate was not detected for halobium HR. In the presence of 1000 mM NaCl, both HRs formed the intermediate; however, their half-lifetimes were different (Fig. 6; HR_{520} at 1000 mM, NaCl). We calculated the half-lifetime constants of their HR_{520} formation and decay in 1000 mM NaCl. The results are summarized in Table 1, and show that for both HRs HR_{520} was formed with almost the same rate constant (approx. 0.35 ms). However, HR_{520} of shark

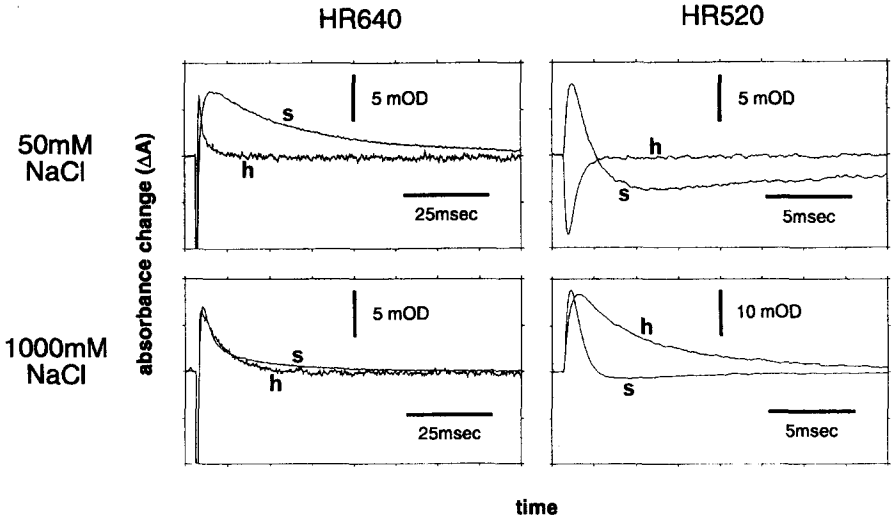

Fig. 6. Flash-induced absorption changes for HR in different sodium chloride concentrations and at different wavelengths. For detection of the HR_{640} intermediate, absorption changes were monitored at 660 nm and 640 nm for halobium HR and shark HR, respectively. For detection of HR_{520} intermediate, absorption changes were monitored at 500 nm for both halobium HR and shark HR. Samples containing a total of 2.5 mg/ml membrane proteins were suspended in 20 mM Pipes (pH 7.0). (h) halobium HR; (s) shark HR.

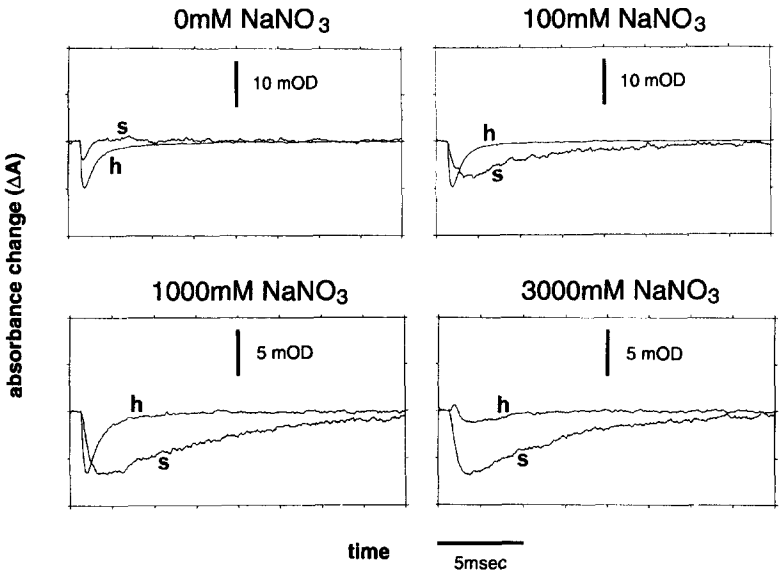

Fig. 7. Flash-induced absorption changes at 500 nm for halobium HR and shark HR at different concentrations of sodium nitrate. Samples containing a total of 2.5 mg/ml membrane proteins were suspended in 20 mM Pipes (pH 7.0). (h) halobium HR; (s) shark HR.

HR decayed with a half-lifetime of approx. 0.569 ms, which was approx. 6-times faster than for halobium HR. Although, the decay-time constant of HR_{640} for shark HR in 1000 mM NaCl was almost the same as that for halobium HR, its constant at 50 mM chloride concentration was more than 25-times slower than that for halobium HR (Fig. 6; HR_{640} at 50 mM, NaCl).

Fig. 7 shows absorbance changes in the presence of sodium nitrate at 0 mM, 100 mM, 1000 mM and 3000 mM concentrations. The half-lifetime of decay to the ground state for shark HR was approx. 5-times slower than for halobium HR in the presence of both 100 mM and 1000 mM nitrate concentrations. A small increase at 500 nm, which is most likely to correspond to the HR_{520} intermediate, was detected in the presence of 3000 mM sodium nitrate for halobium HR; however, no such absorption increase was detected for shark HR. These flash-induced absorption changes clearly indicate that photoreactions in the presence of either chloride or nitrate are different for shark HR and halobium HR.

4. Discussion

Only the function of HR from *H. salinarium* (halobium HR) has so far been extensively studied. Several models of its chloride pumping have been proposed [11,12]. However, several new kinds of HR have recently been discovered in other halophilic bacteria, and their structure and function have also been studied. Pharaonis HR (from strain *Natronobacterium pharaonis*) was first characterized by Duschi et al. [3], and pharaonis HR purified in a detergent of dodecyl maltoside was further characterized photochemically by Scharf and Engelhard [8]. For the third HR (mex HR) only anion pumping activity has been characterized, but not its photoreaction [5]. The gene encoding *hop* of halobacterial strain SG1 has been cloned and sequenced; however, its gene production has not yet been undertaken [6]. In the present study, a new *hop* gene from halobacterial strain *shark* was cloned and sequenced. Furthermore, its gene product was successfully obtained using the halobacterial expression system with a shuttle vector plasmid, pEF191 [16]. This is the first example of the new *hop* gene expression in *H. salinarium*.

The shark HR expressed in *H. salinarium* had not only a photochemical reaction, but also anion pumping activity, showing that its gene was still active. No HR-like product had been detected in the halobacterial strain *shark* [5]. This suggested that HR expression in the strain was repressed for some reason.

Anion transport measurements with envelope vesicles for the transformant JOS5 clearly indicated that shark HR had pumping activities not only for chloride but also nitrate. However, the anion selectivities and the half-maximal transports of anions for shark HR and halobium HR were different. The anion transport activity data for halobium HR over-expressed in *H. salinarium* was almost identical to those for HR in strain L33 [3], indicating that these anion transport activities reflect the specific amino acid sequence of HR, but not to the expressed strain nor to the expression level of HR. The selectivity and the half-maximal transport of the anion for shark HR were very similar to those for mex HR [5]. It had been suggested that the length of the B-C loop segment was correlated to the anion selectivity for HR. However, the data did not support this assumption, because the length of the B-C loop for shark HR was the same as that for halobium HR, but 4 residues shorter than that for mex HR.

Shifts in absorption maxima for shark HR caused by addition of chloride, nitrate and sulfate were found to be almost the same as those for purified pharaonis HR solubilized in detergent [8], but not the same as those for halobium HR. Shifts in absorption maxima induced by changes of anions and pH were investigated extensively for pharaonis HR. The changes in absorption maxima with several anions were found to follow a sigmoidal curve. These data suggested the presence of one anion binding site in pharaonis HR. In this study, such titration curves for anions were not measured for shark HR. Therefore, we could not yet compare the shifts in absorption maximum between pharaonis HR and shark HR. We need further detailed characterization of shark HR.

Flash photolysis measurements with membrane fractions containing shark HR clearly showed that photoreactions in the presence of chloride and nitrate were different from those for halobium HR. Even at low chloride concentration (~ 50 mM), the HR_{520} intermediate was detected for shark HR. Furthermore, the capacity for chloride transport for shark HR was about 3-times higher than that for halobium HR at this chloride concentration, indicating that HR_{520} formation could be a key intermediate for HR chloride transport. On the other hand, this intermediate was not detected in the presence of nitrate, although the pumping ability of nitrate was about a half that for chloride. Therefore, the HR_{520} intermediate might not be related to the nitrate pumping.

At high concentrations of nitrate, the HR_{520} intermediate has been detected for pharaonis HR [8], suggesting that the photoreaction in the presence of nitrate is different for shark HR and pharaonis HR. In pharaonis HR, however, the photochemical analysis was performed only in a detergent of dodecyl maltoside, but not in its natural halobacterial lipid environment. These analytical experiments should be performed under the same conditions for comparison of the photoreaction of shark HR with that of pharaonis HR.

From the comparison of amino acid sequences for several BRs and HRs, the tertiary structure of HR was most likely to be almost the same as that for BR. The three-dimensional structure of HR at 7 Å has recently been obtained by Havelka et al. [22], and was almost identical to that for BR. Based on the structure model of BR, the conserved amino acids in three HRs (halobium, pharaonis and mex) were indicated in black (Fig. 5. from the manuscript by Otomo et al., [5]). In comparing the amino acid sequence of shark HR with three other HRs, only Ala-113 was found not to be conserved; other amino acids conserved in the three HRs were also conserved in shark HR, further supporting the proposition that the amino acids residues near the retinal Schiff base are important for anion pumping in HR.

In A-B and B-C interhelix loop segments, three positively charged residues are also conserved in shark HR; however, a series of six amino acids (EMPAGH) was replaced by QMPPGH. We have assumed that this substitution affects the expression of shark HR in strain *shark*. However, shark HR was over-expressed in *H. salinarium* strain HN5 and its product had anion pumping activity and photoreaction activity, showing that the series of these amino acids was not essential for the expression and function of HR.

Various features of shark HR, such as shifts in absorption maximum caused by added anions, half-maximum anion transport, anion selectivity between chloride and nitrate, and photoreactions in the presence of chloride and nitrate, were different from those for halobium HR. There is no doubt that these differences are caused by differences in electrostatic interactions around the retinal, between shark HR and halobium HR. As already mentioned, all the residues around the retinal Schiff base in helix regions were conserved between two HRs. Only Arg-200 in F helix located near the β-ionone ring of the retinal for halobium HR is replaced by Lys for shark HR. In addition, a few charged amino acid substitutions were found, in the A-B and the B-C interhelix loop segments, between shark HR and halobium HR. Some of these substitutions probably affect the electrostatic interaction around the retinal. However, there is no reason to exclude the possibility of other amino acid substitutions. Photochemical analysis of HR using point mutants would be required to clarify such assumptions.

In summary, we have cloned and sequenced a new HR, and this HR was subsequently expressed. This new HR had a different amino acid sequence and its gene product had different photochemical properties and different anion pumping activities from those for other known HRs. From comparison of amino acid sequences, we proposed that some charged amino acid substitutions between halobium HR and shark HR probably affect the photochemical reactions and anion pumping activities.

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