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Anion selectivity and pumping mechanism of halorhodopsin

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Abstract

Comparison of the amino acid sequences in the A–B and B–C interhelical loop segments in all bacteriorhodopsins and halorhodopsins has shed light on the anion selectivity and pumping mechanism of halorhodopsin. The nucleotide sequences of two haloopsins from two new halobacterial strains, shark and port, have been determined, and shark halorhodopsin was functionally overexpressed in *Halobacterium halobium*. Although a series of six amino acid residues (EMPAGH) in the B–C interhelical loop segment was substituted by QMPPGH, all putative charged residues were conserved. It was also shown that His-95 mutants had lower pumping activity at low chloride concentrations. These results further support the hypothesis that His-95 is important in the halorhodopsin function.

Keywords: Halorhodopsin; Anion pump; Halobacteria; Bacteriorhodopsin; Retinal protein

1. Introduction

Since no mutational approach to elucidating the pumping mechanism of halorhodopsin has been reported, functionally important residues have not been identified. Nonetheless, comparative studies of the amino acid sequences of three halorhodopsins (halobium, pharaonis, and mex) revealed that three positively charged residues (Arg-52, Arg-58, His-95; numbering as in halobium halorhodopsin) in the A–B and B–C interhelical loop segments were conserved only in three halorhodopsins, but not in the bacteriorhodopsin family. Moreover a series of six amino acid residues (EMPAGH) in the B–C interhelical loop segment were conserved in the three halorhodopsins, but the bacteriorhodopsin family lacks these residues altogether. These results suggested that these residues were essential for the anion pumping activity [1]. Further comparison of the length of the B–C interhelical loop segment in all bacteriorhodopsins and halorhodopsins has shed light on the anion selectivity of halorhodopsin. Halorhodopsins containing a longer B–C interhelical loop segment have a lower anion pumping selectivity between chloride and nitrate, suggesting that the length of the B–C interhelical loop segment affected the anion selectivity [1]. Finally a fourth halorhodopsin was recently found in halobacterial strain SG1 [2] and all putative residues were conserved (Table 1). This result strengthened the hypothesis that three positively charged amino acid residues and the series of six amino acid residues (EMPAGH) in the B–C interhelical loop segment are important to the halorhodopsin function.

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2. Results and discussion

2.1. Gene cloning and sequencing of two new halorhodopsins

Light-induced pH changes in the presence of the proton ionophore CCCP were observed to investigate the anion pumping activity of strains port and shark, and no halorhodopsin-like proteins were found. However, a gene homologous to the halobium haloopsin gene observed by the analysis of Southern blot DNA hybridization suggests the presence of a shark haloopsin gene [5]. Using oligonucleotide primers corresponding to the highly conserved regions in the helices C and G of haloopsin genes [1,4,6], the haloopsin genes encoding the C- to G-helix region of halobacterial strains port and shark were successfully amplified. The deduced amino acid sequences were different from those of all other known halorhodopsins, but they were about 65% homologous to the halobium haloopsin gene. Whole structural genes with the flanking regions of strains port and shark were cloned by the cassette gene cloning method, and amplified gene fragments were directly sequenced (in preparation). The deduced amino acid sequences of port halorhodopsin and shark halorhodopsin both showed a 62% homology to halobium halorhodopsin. Almost all the residues near the retinal Schiff base were found to be conserved in the same way as for other halorhodopsins. Fig. 1 shows the amino acid sequence of the A–B and B–C interhelical loop segments for all known bacteriorhodopsins, halorhodopsins and sensory rhodopsins [1,2,4,6]. Although the series of six amino acid residues (EMPAGH) in the B–C interhelical loop segment was substituted by QMPPGH, all putative charged residues in the A–B and B–C interhelical loop segments of halorhodopsin were conserved. These results further supported the hypothesis that the three positively charged amino acid residues (Arg-52, Arg-58, His-95) are important to the halorhodopsin function.

2.2. Expression of shark halorhodopsin in Halobacterium salinarium

Since no halorhodopsin-like protein had been found in the halobacterial strain shark [5], expression of shark halorhodopsin in *Halobacterium salinarium* (*Halobacterium halobium*) was carried out by using the halobacterial transformation system [7]. A fusion gene for shark halorhodopsin overexpression which contained sequences of the first thirteen amino acids of bacteriorhodopsin, plus upstream sequences of putative bacterioopsin promoter was constructed by the recombinant PCR method. Transformation of a

		A-B loop	B-C loop
BR			
	halobium	GMGVSDPDAK	GYGLTMV.....PFGGEQNP..IY
	aR-1 (SG1)	GWGVTDKEAR	GIGLTEV.....QVGSEM..LDIY
	aR-2	GWGVTDKEAR	GIGVTEV.....ELASGTV.LDIY
	mex	GWGVTDKKAR	GIGLTTV.....EVAGMAEPLEIY
	port	GWGETDSRRQ	GFGLTIV.....EFAGEEHP..IY
	shark	GWGETDGRRQ	GFGLTFI.....EFGGEQHP..IY
HR			95
	halobium	GRTIRPGRPR	GLTVGMI**EMPAGH**ALAG.....EMVRSQ
	mex	GRNVEDPRAQ	GLTVGFL**EMPAGH**ALAG....MGAGPEGGVFTP
	pharaonis	TRGLDDPRAK	GLTISVL**EMPAGH**FAEGSSVMLGGEEVDGVVTM
	SG1	GRNVEDPRAQ	GLTVSFL**EMPAGH**ALAG.....QEVLTP
	port	GRDIESPRAK	GLTVGFLQMPPGHALAG.....QEVLSP
	shark	GRDLESPRAK	GLTVGMLQMPPGHALAG.....QEVLSP
SR			
	halobium	LYRSLDGSPHQ	DIGTVIV.....NGNQIV
	SG1	LYAKLGESEDR	GIGTVTV.....NGAELV

Fig. 1. Amino acid sequences of the A–B and B–C interhelical loop segments for bacteriorhodopsins, halorhodopsins and sensory rhodopsins. The underlined amino acids are charged. The series of six residues (EMPAGH) is shown in bold letters.

Table 1
Halorhodopsins found in halobacterial strains

Strain	Amino acid homology (%) [a]	Protein	Nitrate pumping activity	Found by
Halobium	100	purified	one third of the chloride pumping activity	Matuno-Yagi et al. [3]
Pharaonis	52	purified	almost the same as the chloride pumping activity	Lanyi et al. [4]
Mex	(68)	partially purified	one half of the chloride pumping activity	Otomo et al. [1]
SG1	60	not purified	not tested	Soppa et al. [2]
Port	62	not detected	not tested	this work
Shark	62	not detected	not tested	this work

[a] Percentage was obtained by comparing with halobium halorhodopsin.

bacteriorhodopsin- and halorhodopsin-negative strain with this fusion construct yielded a clear blue transformant, showing that the shark halorhodopsin was expressed in *Halobacterium salinarium*. The expression level of shark halorhodopsin was almost the same as that of the halobium halorhodopsin overexpression (data not shown). The anion pumping activity of shark halorhodopsin was monitored by a pH change according to the standard procedure [8]. The transformant cell clearly showed the anion pumping activity in reaction to light, confirming that the overexpressed shark halorhodopsin had physiological

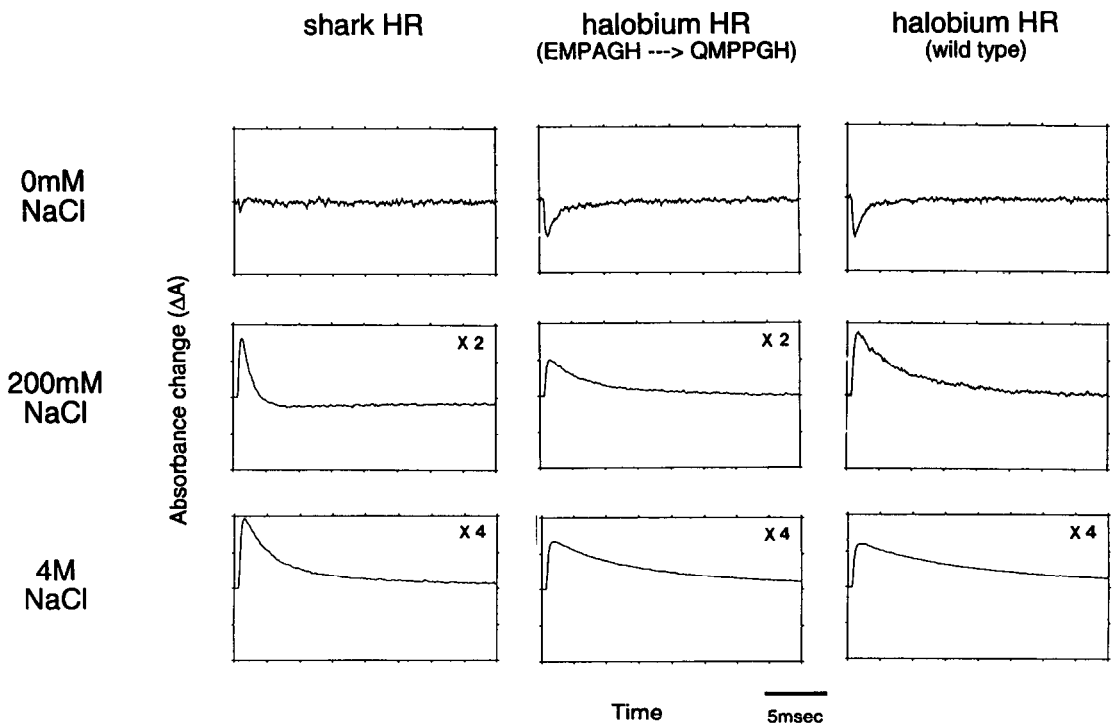

Fig. 2. Flash-induced absorbance changes at 500 nm obtained with shark halorhodopsin and halorhodopsin mutant (EMPAGH → QMPPGH) in different chloride concentrations.

Table 2
Net charges of the interhelical loop segments

Strain	A–B loop	B–C loop
Halobium	+4	0
SG1	0	−1
Pharaonis	+1	−4
Mex	0	−1
Shark	+1	0

activity (data not shown). To clarify whether the difference in the series of six amino acid residues (EMPAGH) in the B–C interhelical loop segment of shark halorhodopsin affected the anion pumping activity and/or selectivity, a halobium halorhodopsin mutant modified (EMPAGH → QMPPGH) in the B–C interhelical loop segment was also expressed. The expression level of this mutant was the same as that of the wild type of halobium halorhodopsin and the light-driven anion pumping activity was also detected, showing that the loop difference (EMPAGH → QMPPGH) was not essential for anion pumping.

Flash-induced absorbance changes of shark halorhodopsin and halobium halorhodopsin mutant (EMPAGH → QMPPGH) were monitored in the presence and absence of chloride (Fig. 2). In 4 M NaCl, the HR_{520} intermediate was generated; in the absence of chloride, no HR_{520} intermediate was monitored for either shark halorhodopsin or the mutant, the same as for the wild type. After addition of 200 mM chloride, the HR_{520} intermediate was again generated. In the presence of 200 mM nitrate, the HR_{520} intermediate was also generated; above dependency of HR_{520} on chloride and nitrate was the same for the wild type (data not shown). On the other hand, the decay rate of the HR_{520} intermediate of shark halorhodopsin was more than twice as fast as that of the wild type. In the mutant (EMPAGH → QMPPGH), the decay rate was the same as that for the wild type. These results showed that the difference in the B–C interhelical loop segment (EM-

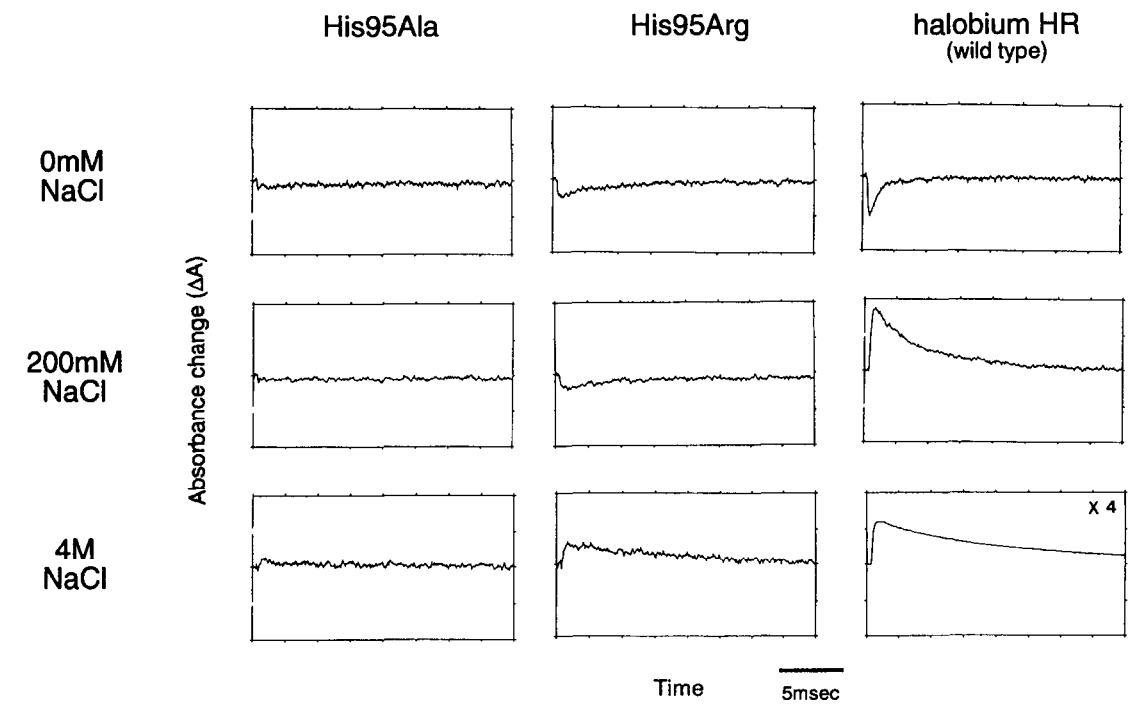

Fig. 3. Flash-induced absorbance changes at 500 nm obtained with halorhodopsin mutants (His95Ala and His95Arg) in different chloride concentrations.

PAGH → QMPPGH) did not affect the photocycle and its anion dependency. The difference in the HR_{520} decay rate must be caused by other amino acid differences between halobium halorhodopsin and shark halorhodopsin. In the A–B interhelical loop segment, shark halorhodopsin is less positively charged residue than halobium halorhodopsin (Table 2). This was probably the cause of the decay rate difference, because no other amino acid difference was found in the helical region.

2.3. Expression and characterization of the His-95 mutants

To clarify whether the series of six amino acid residues (EMPAGH) in the B–C interhelical loop segment are important to the halorhodopsin function, two His-95 mutants (His95Arg and His95Ala) were successfully expressed. Although, the halobium halorhodopsin overexpression vector was used for expression of the mutants, the expression levels of both mutants were lower than that for the wild type. In the case of His95Ala mutant, the transformant cell was not clear blue in coloration, so that it was rather difficult to identify. The expression level of His95Arg was also lower than that for the wild type, but it was higher than that for the His95Ala mutant. These results suggested that a positive charge in this position in the form of His-95 was crucial in the proper expression of the protein.

The anion pumping activities of both mutants in 4 M NaCl were lower than that for the wild type in 200 mM NaCl. Flash-induced absorbance changes in both mutants were monitored in the presence of chloride. Although an HR_{520}-like intermediate was generated in 4 M NaCl, it disappeared in 200 mM NaCl for both mutants (Fig. 3). These results clearly showed that His-95 was important for the formation of HR_{520} in the anion pumping photocycle of halorhodopsin. In 200 mM NaCl, the absorbance at 560 nm decreased for both mutants, showing the presence of a photocycle the same as for the wild type halorhodopsin observed in the absence of chloride. These results indicated that His-95 was important for the anion pumping activity and that it affected the anion binding site of halorhodopsin.

3. Conclusion

Haloopsin genes from the new halobacterial strains port and shark were cloned and sequenced, and shark halorhodopsin was successfully expressed in *Halobacterium salinarium* in a physiological functional form. The decay rate of the HR_{520} intermediate is different for shark halorhodopsin, showing that the net charge of the A–B interhelical loop segment probably affected the decay rate of the intermediate. His-95 mutants had lower pumping activities and chloride binding abilities, indicating that His-95 was important for the anion pumping activity and that it affected the anion binding site of halorhodopsin. The present mutant halorhodopsin study strongly suggests that the A–B and B–C interhelical loop segments are critical for the anion pumping mechanism in halorhodopsin.

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