Biochimica et Biophysica Acta, 682 (1982) 446–451
Elsevier Biomedical Press

BBA 41210

EFFECTS OF CHLORIDE ON THE ABSORPTION SPECTRUM AND PHOTOREACTIONS OF HALORHODOPSIN *

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(Received July 5th, 1982)

Key words: Chloride effect; Halorhodopsin; Absorption spectrum; Photoreaction; (H. halobium)

The absorption maximum of halorhodopsin in a membrane fraction prepared from the cells of *Halobacterium halobium* under low-salt conditions shifted to longer wavelenghts upon addition of NaCl (Ogurusu, T., Maeda, A., Sasaki, N. and Yoshizawa, T. (1981) J. Biochem. (Tokyo) 90, 1267–1273). This bathochromic shift was due to chloride, not sodium. Bromide and iodide were also effective. The bathochromic shift of the absorption maximum was not accompanied by any change in the isomer composition of retinal in halorhodopsin. The same ionic species were essential for the formation of the hypsochromic photoproduct at −75°C. These effects of NaCl on halorhodopsin are discussed in terms of the presence of the two forms of halorhodopsin, a form binding chloride and a chloride-free form.

Introduction

Halorhodopsin [1] is one of the photoreceptive proteins in the cell membrane of a highly halophilic microorganism, *Halobacterium halobium*. It has been reported that halorhodopsin in the envelope vesicles prepared by sonication from cells extrudes sodium from the inside to the external medium with the aid of light energy absorbed by the chromophore [2–4]: Consequently, halorhodopsin may be a light-driven sodium pump. Addition of all-*trans*-retinal to the apoprotein of halorhodopsin in cell envelope vesicles regenerated the light-driven sodium pump, as the absorbance around 588 nm increased [5]. Several photointermediates of halorhodopsin have been found by flash photolysis and low-temperature spectrophotometry [6,7].

We have extracted halorhodopsin from the bacteriorhodopsin-deficient mutant cells under low-salt conditions and studied its photoreaction at 0°C [7]. The absorption maximum (λ_{max}) estimated from the difference of spectra after irradiation was 566 nm and this value shifted to 576 nm upon addition of NaCl. In the present paper, this previous work has been extended to study the effects of various kinds of salts on halorhodopsin. It now becomes evident that the λ_{max} shift to longer wavelengths is due to Cl^-, and is independent of the species of countercation. A chloride dependence has also been shown in the photoreactions at low temperatures. Bromide and iodide were also effective for both the bathochromic shift of the λ_{max} and the photoreactions at low temperatures.

Abbreviations: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-ethanesulfonic acid; λ_{max}, the wavelength of maximum absorbance.

* This work has been published in abstract form (Ogurusu, T., Maeda, A., Sasaki, N. and Yoshizawa, T. (1981) in Abstracts of the 7th Annual Meeting of Japan Bioenergetic Group, pp. 8–9, Okayama, Japan (in Japanese).

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Materials and Methods

Strain Y_1, a bacteriorhodopsin-deficient mutant strain [7] of *H. halobium* R_1M_1, was used as a source of halorhodopsin. The membrane fraction containing halorhodopsin was prepared by linear sucrose density gradient centrifugation under low-salt conditions [7]. Then, the membranes were collected by centrifugation for 5 h at $92000 \times g$ and finally suspended in a small volume of 10 mM Hepes buffer (pH 6.8). For experiments at 0°C, various kinds of solutions of salts, dissolved in 10 mM Hepes buffer (pH 6.8), were then added. For low-temperature spectrophotometry that requires concentrated membrane suspensions, crystals of each salt were directly added to the membrane suspension containing 67% (v/v) glycerol. The pH was then adjusted to 6.8 by adding 1 M NaOH or 1 M HCl. The amount of halorhodopsin was estimated from the decrease in absorbance at 570 nm after denaturation by mixing with SDS, using a molar extinction coefficient at 588 nm of $4.8 \cdot 10^4$ $M^{-1} \cdot cm^{-1}$ [5]. Protein concentrations were determined by a modified biuret method [8] using bovine serum albumin as a standard. The content of halorhodopsin in our preparation was approx. 0.4 nmol/mg membrane protein *, being almost the same as those used by other workers [9].

Absorption spectra were measured in a Hitachi 330 recording spectrophotometer equipped with a double monochromator. The difference absorption spectrum was directly recorded by subtracting the stored spectrum before irradiation from that after irradiation. For conventional spectral measurements, the sample was kept at 0°C by circulating ice-cold water through a water jacket in a cuvette holder. Absorption spectra at temperatures below 0°C were measured as follows: The sample was put into an optical cell (light path 3 mm) consisting of a silicone rubber ring sandwiched between two quartz glass plates. Then the optical cell was fixed in a copper sample holder in a cryostat [10] and mounted in the photometer. The temperature was monitored with a copper-constantan thermocouple connected to the sample holder.

The light source for irradiation of the sample was a 1 kW halogen-tungsten lamp in a slide projector. The wavelength of light was selected by inserting a cut-off filter (Toshiba) or a combination of a cut-off and an interference filter (Toshiba, KL series), together with 5 cm of water for the removal of heat.

Results

Effects of salts on the λ_{max} of halorhodopsin

In order to examine whether Na^+ or Cl^- is responsible for the bathochromic shift of the λ_{max} of halorhodopsin in the membrane fraction due to addition of NaCl, the λ_{max} was studied by varying the composition of either cation or anion in a systematic way. After irradiation of halorhodopsin in the membrane fraction at 0°C with red light (greater than 590 nm), its absorbance in the range 500–600 nm decreased with a concomitant appearence of a peak at about 400 nm owing to the formation of a blue-shifted photoproduct [7]. From these spectral changes, it may be reasonable to assume that the photoproduct does not absorb light of wavelengths longer than 550 nm. Thus, the minimum wavelength of the difference spectrum recorded after irradiation is probably the λ_{max} of halorhodopsin [7].

As shown in Table IA, the λ_{max} was located at or above 575 nm in the presence of chloride, irrespective of the species of cation. Bromide also showed an effect similar to that of chloride on the λ_{max} of halorhodopsin (Table IB and C) and iodide was somewhat less effective than chloride and bromide (Table IB, C and E). Fluoride, nitrate, perchlorate, acetate and sulfate were essentially ineffective in this respect (Table IB and D). Thus, the effect of NaCl is primarily due to chloride, not sodium. Furthermore, the effects of salts on the λ_{max} are not ascribable to just ionic strength or lyotropic effects.

Retinal isomer analysis of halorhodopsin in the presence and absence of NaCl

It is known that the λ_{max} of bacteriorhodopsin, another retinal-based pigment in this bacterium, is dependent on its retinal isomer composition

* The values of the amount of halorhodopsin and bacteriorhodopsin in Y_1 cells, which were erroneously described in Ref. 7, are $4.4 \cdot 10^{-11}$ mol and less than $1.1 \cdot 10^{-12}$ mol/mg total cellular protein, respectively.

TABLE I

THE λ_{max} OF HALORHODOPSIN IN VARIOUS SALT SOLUTIONS

All the samples contained 2.8 mg protein/ml in 10 mM Hepes buffer (pH 6.8).

Salt			λ_{max} (nm)[a]
Species		Concentration (M)	
None			566 ± 2
(A)	LiCl	1	578 ± 2
	NaCl	1	576 ± 1
	KCl	1	578 ± 1
	RbCl	1	578 ± 2
	CsCl	1	575 ± 3
	Tris-HCl	1	575 ± 2
(B)	NaF	1	566 ± 1
	NaCl[b]	1	576 ± 1
	NaBr	1	582 ± 1
	NaI	1	572 ± 0
	$NaNO_3$	1	570 ± 0
	$NaClO_4$	1	568 ± 1
	Sodium acetate	1	564 ± 1
(C)	KCl[b]	1	578 ± 1
	KBr	1	583 ± 1
	KI	1	576 ± 1
(D)	$MgSO_4$	1	568 ± 2
	$MgSO_4$	0.4	568 ± 2
	Na_2SO_4	0.4	570 ± 1
(E)	NaCl	0.2	574 ± 1
	NaBr	0.2	572 ± 1
	NaI	0.2	570 ± 3

[a] An average of three to five determinations.
[b] The same datum is described in A.

[11,12]. The retinal isomer composition in halorhodopsin was analyzed before and after addition of 1 M NaCl, by the method of CH_2Cl_2 extraction followed by HPLC analysis [13] on the sample, treated as previously described [7]. The chromophore of dark-adapted halorhodopsin in the presence of 1 M NaCl was composed exclusively of all-*trans*- and 13-*cis*-retinal at a molar ratio of 82 : 18. The same value was obtained in the absence of NaCl. Thus, the shift of the λ_{max} of halorhodopsin on addition of NaCl is not ascribable to a change in isomer composition of its chromophore.

Photoreaction of halorhodopsin at 0°C in the presence of NaCl

Irradiation of halorhodopsin at 0°C with red light in the absence of NaCl produced a blue-shifted photoproduct and in the presence of NaCl a similar spectral change was observed [7]. These results suggest that this photoproduct is formed independent of NaCl concentration. The absorbance decrease at 570 nm was studied at an early time of the photoreaction. The extent of the absorbance decrease became progressively smaller with an increase in the concentration of NaCl (Fig. 1). Throughout the photoreaction, at a given NaCl concentration, the shape of the difference spectrum was invariant.

Photoreaction of halorhodopsin at 0°C in the presence of sulfate

Subsequent irradiation of the blue-shifted photoproduct with blue light (410 nm) can restore the original absorption spectrum of the unirradiated halorhodopsin. The regeneration was not completely reversible, however (cf. Fig. 2A and B in Ref. 7), probably owing to an irreversible loss of photoactivity of the product during the process of irradiation.

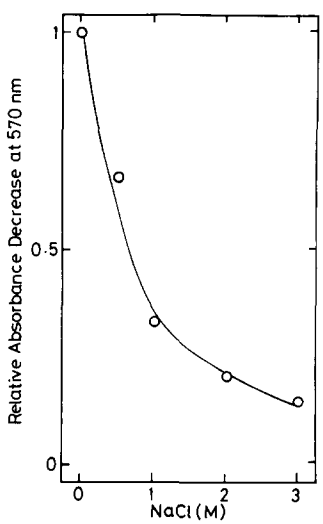

Fig. 1. The dependence of the absorbance decrease at an early time of the photoreaction of halorhodopsin at 0°C on the concentration of NaCl. All samples contained 2.7 mg protein/ml. The sample was irradiated with red light ($\lambda > 590$ nm) for 5 min at 0°C and then the absorbance decrease at 570 nm was measured.

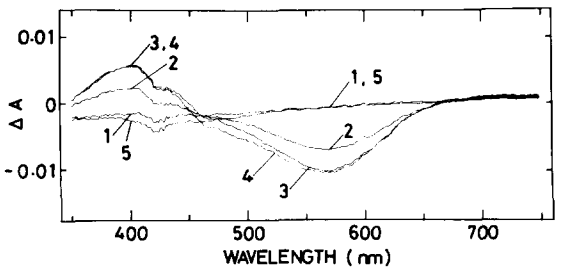

Fig. 2. The photoreaction of halorhodopson in 0.4 M $MgSO_4$ at 0°C. The concentration of the protein was 1.4 mg/ml. Curve 1, halorhodopsin in 0.4 M $MgSO_4$ in the dark. Curves 2, 3 and 4, products after irradiation with red light ($\lambda > 590$ nm) for a total of 10, 40 and 70 min, respectively. Curve 5, a product by irradiating the sample of curve 4 with 410 nm light for 2 min.

H. halobium requires high concentration of $MgSO_4$ along with NaCl for growth. As shown in Fig. 2, a complete restoration of the original spectrum by blue light was attained by irradiation of the blue-shifted photoproduct in a medium containing 0.4 M $MgSO_4$ (curves 1 and 5 in Fig. 2). This reversible photoreaction could be repeated at least four times by alternative irradiation with red and blue light. In spite of this effect, no changes were observed in the shape of the difference spectrum in the presence of $MgSO_4$. A similar reversible photoreaction occurred in the presence of 0.4 M Na_2SO_4, but not in 0.1 M $MgSO_4$ or 1 M NaCl. These results indicate that high concentration of sulfate can make the photoreactions at 0°C reversible, probably by keeping the photoproduct stable with respect to thermal agitation.

Effects of glycerol on the λ_{max} of halorhodopsin in the presence and absence of NaCl

Glycerol is useful to reduce the light scattering of the sample and prevent the formation of cracks when samples are frozen. Addition of glycerol to the membrane fraction containing halorhodopsin (50%, v/v) in the absence of NaCl shifted the λ_{max} from 566 to 578 nm. Upon addition of NaCl to a final concentration of 1 M, the λ_{max} shifted to shorter wavelengths, from 578 to 558 nm. The midpoint of NaCl concentration for the blue shift of the λ_{max} was around 0.1 M, being close to that for the bathochromic shift in the absence of glycerol [7].

Photoreaction of halorhodopsin in the presence of NaCl at low temperatures

In our previous paper [7], we described that, in the absence of NaCl, irradiation of halorhodopsin with 500 nm light at −196°C yielded a bathochromic photoproduct, which reverted to the original pigment after irradiation with 650 nm light; and also that irradiation with red light at 0°C yielded the blue-shifted photoproduct. Other photoproducts have not been observed at any temperature we have studied.

In the presence of 1 M NaCl, irradiation of

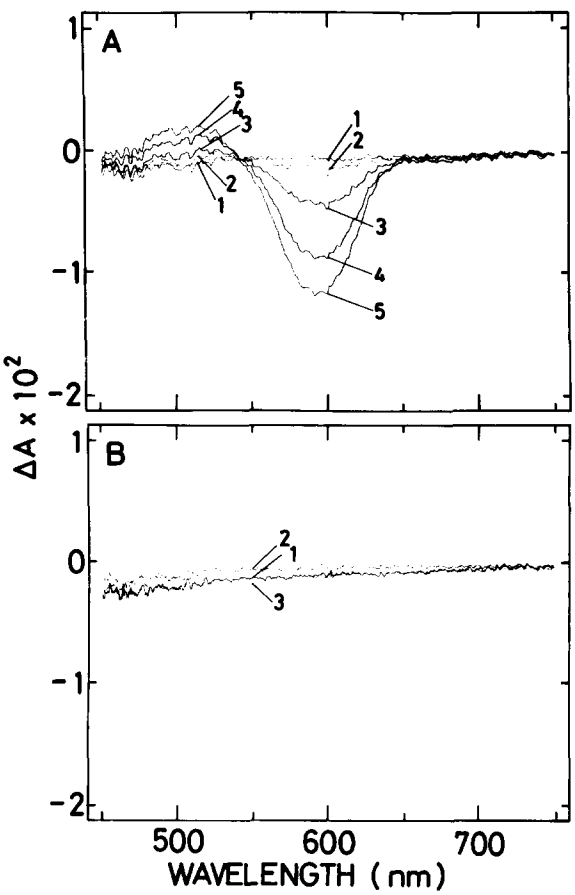

Fig. 3. Photoreaction of halorhodopsin in 67% (v/v) glycerol in the presence or absence of NaCl at −75°C. All the spectra were the difference of the spectra before and after irradiation of halorhodopsin with 650 nm light. All samples contained 6.9 mg protein/ml. (A) In the presence of 1 M NaCl. Curve 1, a baseline. Curves 2, 3, 4 and 5, after irradiation for a total of 15, 65, 164 and 465 s, respectively. (B) In the absence of NaCl. Curve 1, a baseline. Curves 2 and 3, after irradiation for a total of 15 and 315 s, respectively.

halorhodopsin with 650 nm light at −75°C caused a hypsochromic shift due to the formation of a hypsochromic photoproduct (Fig. 3A). The maximum and minimum of the difference spectrum were located at 505 and 593 nm, respectively, with an isosbestic point at 540 nm. It is obvious that this photoproduct is distinct from the blue-shifted photoproduct at 0°C. We have never observed such a photoproduct in the absence of NaCl at any temperature including −75°C (Fig. 3B). The amount of the hypsochromic photoproduct at −75°C depended on the concentration of NaCl (Fig. 4); saturation was attained above 0.5 M and the midpoint was at about 0.15 M NaCl. This value is close to 0.1 M, the value for the midpoint of the NaCl concentration for the bathochromic shift of the λ_{max} at 0°C [7]. The difference spectrum of the formation of this photoproduct was in agreement with that of P_{500} described by Weber and Bogomolni [6] as a short-lived intermediate of halorhodopsin present in the envelope vesicles in 4 M NaCl at room temperature.

Table II shows that this photoreaction occurs by irradiation with 650 nm light at −75°C in the presence of chloride, bromide or iodide, independently of the species of cation. The difference spectrum of each photoreaction was identical. Fluoride, acetate, nitrate and sulfate were ineffective.

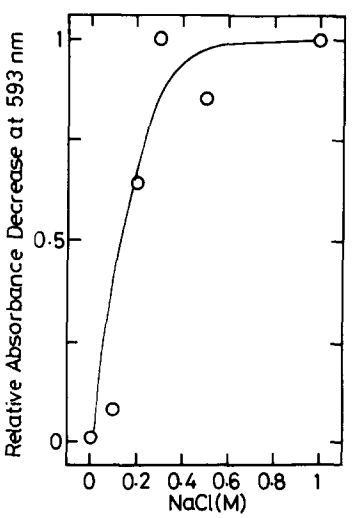

Fig. 4. The relative amount of the photoproduct at −75°C depending on the concentration of NaCl. All samples contained 6.9 mg protein/ml and 67% glycerol. Absorbance at 593 nm was measured after establishment of photosteady state with 650 nm light.

TABLE II

RELATIVE AMOUNT OF PHOTOPRODUCTS FROM HALORHODOPSIN IN VARIOUS SALT SOLUTIONS AT −75°C

All samples contained 6.9 mg protein/ml in 3.3 mM Hepes buffer (pH 6.8) and 66% (v/v) glycerol. The final concentration of each salt was 1 M unless otherwise specified. The amount of absorbance decrease at 593 nm in 1 M NaCl was taken to be 100.

Salt	Relative absorbance decrease at 593 nm (%)
NaF (0.3 M)	9
NaCl	100
NaBr	130
NaI	91
Sodium acetate	25
$NaNO_3$	0
NH_4Cl	100
KCl	110
$MgSO_4$ (0.7 M)	10
None	6

The addition of 0.4 M NaCl to the sample in 0.6 M $NaNO_3$ conferred an ability to form the hypsochromic photoproduct under the same irradiation conditions at −75°C. Thus, the inefficacy of nitrate was not due to an irreversible inactivation of halorhodopsin by nitrate.

Discussion

The effect of chloride on halorhodopsin was most clearly demonstrated in the photoreaction observed at −75°C. Halorhodopsin gave a hypsochromic photoproduct in the presence of NaCl, but not in its absence. These results indicated the presence of two forms of halorhodopsin, one which binds chloride and one which does not. At −75°C, thermal interconversion between the two forms of halorhodopsin cannot be possible, because on forming the photosteady state with 650 nm light the decrease in absorbance at 593 nm (Fig. 4) depended on the concentration of NaCl used. If the two forms were interconvertible, at any NaCl concentration 650 nm light could drive the reaction completely to the hypsochromic photoprod-

uct. This is not the case. The midpoint for the interconversion between the two forms of halorhodopsin was at about 0.1–0.15 M of the NaCl from both the NaCl concentration dependences of the λ_{max} of halorhodopsin [7] and of the amount of photoproduct at −75°C (Fig. 4).

At 0°C, the blue-shifted photoproduct was formed more rapidly at lower concentrations of NaCl (Fig. 1) but the wavelength at the maximal absorbance decrease remained unchanged during the course of the photoreaction of halorhodopsin at any concentration of NaCl tested. These results could be reconciled qualitatively with the concept that the blue-shifted photoproduct is mainly formed from the chloride-free form and interconversion between the two forms of halorhodopsin is faster than the rate of the photoreaction at 0°C.

These two kinds of effects of salts on halorhodopsin were specifically induced by either chloride, bromide or iodide. All these anions are halogens. The inefficacy of another halogen, fluoride, could be due to its extremely small ionic radius, 1.36 Å, as compared with that of 1.81 Å for Cl^-, 1.95 Å for Br^- and 2.16 Å for I^- [14]. A halogen anion-induced spectral shift was reported on the acidified form of bacteriorhodopsin in purple membranes by Fischer and Oesterhelt [15].

Chloride and bromide cause bathochromic shifts in the λ_{max} of gecko visual pigment [16] from 490 to 521 nm and of a chicken cone pigment, iodopsin, from 520 to 562 nm [17]. The association constant of iodopsin with Cl^- ions was $5.7 \cdot 10^2$ M^{-1} [17], whereas that of halorhodopsin was about $1 \cdot 10$ M^{-1} from the dependence of the λ_{max} of halorhodopsin on the NaCl concentration [7]. The addition of iodide somewhat changed the λ_{max} of halorhodopsin but not that of the gecko visual pigment and iodopsin.

Recently, another group reported on the effects of chloride and bromide on halorhodopsin in a preliminary form [18,19]. However, their scheme for the photocycle did not include the blue-shifted photoproduct in the absence of NaCl [19]. Photoreaction of halorhodopsin at low salt concentrations should be examined further.

The peak of action spectrum of halorhodopsin has been reported to be located around 580–600 nm [2,20]. The present results show that the λ_{max} of halorhodopsin becomes close to 580 nm only when chloride, bromide or iodide is present. It may be that the form of halorhodopsin which binds chloride has a crucial role in the light-driven Na^+ transport by halorhodopsin.

Acknowledgements

We thank Professor T.G. Ebrey of the University of Illinois for his kind of reading of the manuscript and the correction of English during his stay at our laboratory. This work was supported by Grants-in-Aid for scientific research from the Japanese Ministry of Education, Science and Culture to A.M. (57470118) and T.Y. (57440096)

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