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Artificial Pigments of Halorhodopsin and Their Chloride Pumping Activities

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ABSTRACT: Halorhodopsin (HR), the light-driven chloride pump of *Halobacterium halobium*, was bleached with hydroxylamine and regenerated with *all-trans*-retinal under several different conditions. The largest recovery of the pigment was found with apoprotein obtained from detergent-free HR [HR(BB)]. To compare the chloride-pumping mechanism of HR with that of bacteriorhodopsin (BR; the light-driven proton pump of the same bacteria), HR pigment analogues were reconstituted with the bleached HR(BB) and retinal analogues. The corresponding BR pigment analogues have previously been shown to have little or no proton-pumping activity, except for retinal$_2$ (3,4-dehydroretinal). Pigment analogues with 13-demethylretinal or retinal$_2$ showed an "opsin shift" similar to that of the *all-trans*-retinal pigment of both HR and BR. Opsin shifts of the pigments of 9-12-phenylretinal and 3,7-dimethyl-2,4,6,8-decatetraenal and haloopsin are slightly different from those of the corresponding BR pigment analogues, presumably reflecting differences of the chromophoric structures in HR and BR. In addition to the spectral properties, the effect of chloride ion on deprotonation of the Schiff base was measured. These pigment analogues showed the "chloride effect" (a shift of the pK value for deprotonation of the Schiff base), but a smaller one than that seen in HR. For a measurement of the chloride-pumping activity, each retinal analogue was added to a culture of L07 cells (BOP^-, HOP^+, Ret^-), and the activity was measured with the cell suspension. Only cultures with retinal or retinal$_2$ showed chloride-pumping activity, as is true for proton pumping by BR. This suggests that a similar retinal–protein interaction is necessary for both ion pumps.

Halorhodopsin (HR)[1] is a light-driven chloride pump found in the cytoplasmic membrane of *Halobacterium halobium* (Lanyi, 1986; Oesterhelt & Tittor, 1989). Like bacteriorhodopsin (BR), the light-driven proton pump found in the purple membrane of the same bacteria, HR has a retinal bound to a Lys residue via a protonated Schiff base as a chromophore. HR has been purified by several groups (Steiner & Oesterhelt, 1983; Taylor et al., 1983; Ogurusu et al., 1984; Sugiyama & Mukohata, 1984). The amino acid sequence has been deduced from the nucleotide analysis of the haloopsin gene (Blanck & Oesterhelt, 1987). From comparison of the primary sequences of both light-driven pumps, about 35% of the total amino acids seem to be similar in the putative membrane domain. This should imply a similar mechanism for converting light energy into chemical energy.

Recently, several single amino acid mutants of BR were made in an *Escherichia coli* expression system and were investigated by Khorana's group (Khorana, 1988). By the mutagenesis of *Halobacterium* GRB strain, several mutants defective in the proton-pumping activity were obtained (Soppa & Oesterhelt, 1989). Both studies indicate the importance of Asp85 and Asp96 in the proton-pumping mechanism (Mogi et al., 1988; Butt et al., 1989). Henderson et al. (1990) reported the structural model for BR based on high-resolution cryomicroscopy, which shows seven membrane-spanning α-helices, bulky aromatic side chains, and the β-ionone ring of retinal. Thus, it is possible to assign a role to certain amino acids in the proton-pumping mechanism and in chromophoric structure on the basis of the molecular structure. In the case of HR, however, such mutants have not been reported yet. The naturally occurring alternative of HR was found in *Natoronobacterium pharaonis*, an alkaline-halophilic bacterium (Bivin & Stoeckenius, 1986) whose gene has been cloned and

[1] Abbreviations: HR, halorhodopsin; Bis-Tris propane, 1,3-bis[tris(hydroxymethyl)methylamino]propane; BR, bacteriorhodopsin; BOP, bacterioopsin; CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; HOP, haloopsin; HR(OG), halorhodopsin solubilized in a 1% octyl glucoside solution; HR(BB), halorhodopsin incubated with Biobeads SM2; MES, 2-(*N*-morpholino)ethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; Ret, retinal; SOP, sensoryopsin; SR, sensoryrhodopsin; λ_{max}, the wavelength of the maximum absorbance.

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sequenced (Lanyi et al., 1990). Following comparison of the primary structures of HR, pharaonis-HR, and BR, the possible role of the amino acid residues in HR was discussed (Oesterhelt & Tittor, 1989; Lanyi et al., 1990). Another possible approach for understanding the mechanism is to use synthetic pigments having artificial chromophores. In the case of BR, artificial pigments were synthesized with various retinal analogues and have been investigated [Iwasa et al., 1984, 1988; Towner et al., 1981; Gaertner et al., 1983; Koelling et al., 1984; for review, Crouch (1986)], and recently several analogue pigments of sensoryrhodopsin have been synthesized (Spudich et al., 1986; Baselt et al., 1989). However, not many artificial pigments have been synthesized from the apoprotein of HR and retinal analogues, probably due to the difficulty in pigment regeneration (Lanyi et al., 1988; Baselt et al., 1989).

In the present paper, pigment was regenerated from the HR apoprotein (HOP) and *all-trans*-retinal under different conditions. Under the most effective conditions, artificial pigments were formed and tested for the chloride-pumping activity. The largest recovery of the pigment was found with the apoprotein of the detergent-free HR [HR(BB)]. Using retinal analogues, which could not form a BR pigment analogue active in proton pumping (Gaertner et al., 1983; Koelling et al., 1984), the bleached HR(BB) formed corresponding HR pigment analogues. These pigment analogues, except that formed with retinal$_2$, did not show detectable activity of chloride pumping in L07 cells, suggesting that a similar retinal–protein interaction is necessary for both light-driven pumps.

Materials and Methods

Preparation of HR(OG) and HR(BB). *H. halobium* OD2 or L33 was cultured and HR was purified according to Steiner and Oesterhelt (1983). The purified HR solubilized in 1% octyl glucoside solution (100 mM NaCl, 10 mM MOPS, pH 7.0) will be referred as HR(OG) in this paper. HR(OG) was dialyzed at 4 °C against a 500-fold buffer solution (10 mM MOPS, pH 7.0, 100 mM NaCl). The outer solution was exchanged twice a day. After the dialysis for more than 3 days, HR(OG$^-$) was obtained. When HR(OG) was mixed with the total lipids of *H. halobium* JW5 and then dialyzed as described above, HR(L) can be obtained. The total lipids were extracted from *H. halobium* JW5 cells according to the method of Bligh and Dyer (1959) and stored as chloroform solution (20 mg/mL) at −80 °C. HR(OG) was incubated with the total lipids of *H. halobium* JW5 [400 μg of lipids/(A_{580} unit·mL of HR)] dispersed in a 10% octyl glucoside solution [8 μL/(A_{580} unit·mL of HR)] and Biobeads SM2 [0.25 g/(A_{580} unit·mL of HR)] for at least 3 days in the dark at room temperature. After enough incubation, HR became like a membrane sheet and was easily precipitated by centrifugation (2000 rpm, 2 min). Such a preparation is called HR(BB).

Synthesis of Pigment Analogues. Samples at different stages of HR preparation were irradiated by yellow light (OG 515 filter) from 1-kW W-lamp after mixing with hydroxylamine (neutralized with 4 M sodium hydroxide; final concentration 500–750 mM), sodium chloride (0.1–4 M), and DTT (1 mM). The pH values of the samples were adjusted to a desired value between 6 and 8. After irradiation, the sample was dialyzed against a 500-fold buffer solution with three changes of the outer solution to remove residual hydroxylamine. The purple membrane was bleached and dialyzed in the same way as that for HR.

All-trans isomers of retinal and retinal analogues were a gift from Dr. E. Koelling [Max-Planck-Institut für Biochemie, FRG; retinal, 13-demethylretinal, 9-12-phenylretinal, and 3,7-methyl-2,4,6,8-decatetraenal (analogues **1**, **3**, **4**, and **5** in Figure 1, respectively)] and Dr. M. Ito [Kobe Women's College for Pharmacy, Japan; retinal$_2$ (analogue **2** in Figure 1)]. The retinal analogues were identified by absorption spectral measurements and purified by high-performance liquid chromatography. The Schiff bases of retinal and retinal analogues were formed with *n*-butylamine in methanol solution and protonated by HCl.

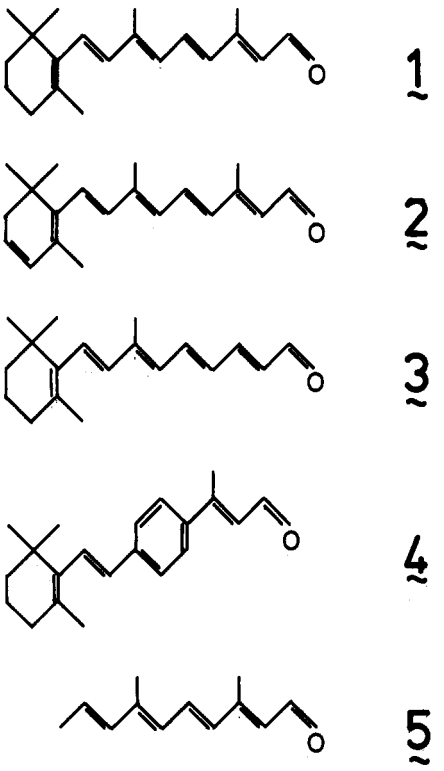

FIGURE 1: Retinal and retinal analogues used in this study. We used all-trans isomers: **1**, retinal; **2**, 3,4-dehydroretinal (retinal$_2$); **3**, 13-demethylretinal; **4**, 3-[4-[2-(2,6,6-trimethylcyclohexenyl)vinyl]-phenyl]-2-butenal (9-12-phenylretinal); **5**, 3,7-dimethyl-2,4,6,8-decatetraenal. All the retinal analogues are referred to as numbers in the text except retinal$_2$.

A 2-propanol solution of each retinal or retinal analogue was added to the bleached preparations. After an overnight incubation at 4 °C, the mixture was washed several times with buffer containing 2% BSA to remove residual retinal or retinal analogue. Absorption spectra were measured with an Aminco DW-2 or a Shimadzu UV-300 spectrophotometer.

pH Titration of Regenerated Pigments. The pH titration of regenerated HR(BB) and pigment analogues was carried out according to Schobert et al. (1986). A regenerated sample (1 mL) was dialyzed against 2 × 500 mL of 50 mM Bis-Tris propane and 50 mM Mes or 50 mM Bis-Tris propane and 50 mM CAPS for 4 h at 4 °C. Both buffers contained 0.2 M NaCl and 0.3 M Na_2SO_4 or 0.5 M Na_2SO_4 as salt. The sample was then put into an optical cuvette, and 2 N NaOH was added stepwise. The absorbance spectrum was measured, and the relative absorbance change at 570 nm was plotted as a function of pH. In the case of pigment analogues, the absorbance change at about 460 nm, the isosbestic point of the spectral change due to the pH change in HR(BB), was plotted to avoid the absorbance change due to unbleached residual HR (less than 7%). The pH after the addition of NaOH was estimated from the calibration curve of the buffer used for dialysis. The buffering capacity of HR or the pigment analogues was negligible compared to that of the buffer.

Measurement of Chloride-Pumping Activity of Pigment Analogues. *H. halobium* L07 (BOP$^-$, HOP$^+$, Ret$^-$) was cultured in a 2-L Erlenmyer flask. At a given time, 30 nmol

Table I: Bleaching and Regeneration of HR at Different Stages of Preparation

sample	bleach[a]	regeneration[b]
cell membrane	L33, 4 °C, 62 h, 67%	ND
Tween-washed membrane	OD2, RT, 69 h, 100%	ca. 30% (SR + HR)
	L33, RT, 5 h, 65%	ca. 30% (SR + HR)
	L33, 4 °C, 53 h, 100%	ca. 20% (SR + HR)
HR(OG)	RT, 2 h, 100%	(−)
HR(OG⁻)	RT, 2 h, 100%	(+)
HR(L)	RT, 2 h, 100%	(+)
HR(BB)	RT, 26 h, 93%	25%

[a] The bacterial strain, temperature, irradiation period for bleaching, and the extent of bleaching are given. The extent of bleaching was calculated as (A_{580} after bleaching/A_{580} before) × 100. [b] The percent of regeneration was estimated as (A_{580} after addition of retinal/decrease of A_{580} by bleaching). Abbreviations and symbols: ND, not determined; (SR + HR), regeneration products were the mixture of HR and SR; (−), absorbance increase was not observed; (+), absorbance increase was observed but was less than 10%.

of each retinal analogue was added to each culture medium (700 mL). The same volume of 2-propanol was added to the control culture at the same time. The increase in cell numbers was monitored by measuring the absorbance at 650 nm. The chloride-pumping activity of the cells was monitored according to Oesterhelt (1982). The cells were incubated with CCCP (50 μM) and irradiated with light from a 100-W tungsten lamp through an OG515 (>490 nm) or a L41 (>390 nm) filter. The initial slope of the transient proton inflow (alkalization of the medium) was measured (in nanograms of H^+ per minute at A_{650}) and used as an indicator of the chloride-pumping activity of pigment analogues.

Preparation of the Membrane Fragment from L07 Cells and Measurement of the Absorption Spectrum. L07 cells in the 700-mL culture were harvested, and the membrane fraction was prepared with ultracentrifugation (100000*g*, 30 min). The absorption spectra of membrane fractions from the cultures containing retinal or a retinal analogue were measured with an Aminco DW2 spectrophotometer using the membrane fraction from the control culture as the reference.

Results and Discussion

Regeneration Conditions of HR. The regeneration of HR was tested at different stages of purification. The results are summarized in Table I. In the early stage of purification, two different strains of *H. halobium* (L33 and OD2) were tested, as we used both strains to prepare HR.

The pigments, HR and SR, are so stable in the membrane structure that with 500 mM hydroxylamine an irradiation with yellow light for 62 h decreased only 67% of the initial absorbance at 580 nm at 4 °C (Table I). In the case of the Tween-washed membranes from OD2 or L33 cells, the absorbance at 580 nm was bleached completely by irradiation for 60 h. After several washes to remove residual hydroxylamine, the absorbance at 580 nm was recovered to 20–30% of the original level by the addition of *all-trans*-retinal. The recovery of the absorbance resulted from regeneration of HR and SR.

After further purification with detergent solubilization HR(OG), HR solubilized in a 1% octyl glucoside solution was easily bleached completely within 2 h by irradiation at room temperature. The sample, however, did not show any increase in absorbance due to pigment regeneration upon the addition of *all-trans*-retinal. In the case of the solubilized purple membrane, the regeneration of BR pigment was less than that from the unsolubilized one. When BR was bleached in a 1% octyl glucoside solution, dialyzed extensively to remove residual hydroxylamine, and mixed with *all-trans*-retinal, the amount of regeneration was only 6% of that from BR without octyl

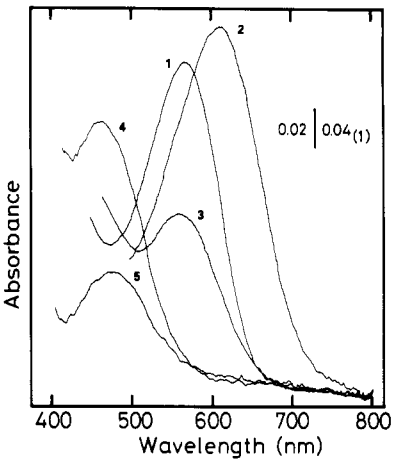

FIGURE 2: The absorption spectra of regenerated HR (curve 1) and pigment analogues (curve 2, retinal$_2$; curve 3, analogue **3**; curve 4, analogue **4**; curve 5, analogue **5**). The bleached HR(BB) was mixed with the excess amount of retinal or retinal analogue. After overnight incubation in the dark at 4 °C, the mixture was washed several times with 2% BSA and the absorption spectra were measured using the bleached HR(BB) as the reference. The bar represents 0.04 absorbance for curve 1 and 0.02 absorbance for curves 2–5.

glucoside. This suggests that detergent prevented the regeneration of pigment. Thus, I tried to remove the detergent from the sample by extensive dialysis with [HR(L)] or without [HR(OG⁻)] the total lipids of *H. halobium*. After extensive dialysis, however, regeneration of the pigment was scarcely observed (less than 10%). It seemed that the detergent was not completely removed from the sample. This was suggested also by the similar bleaching rate after the dialysis.

After incubation of HR(OG) with Biobeads SM2 and the total lipids of *H. halobium*, the detergent seemed to be removed. HR(BB) thus obtained again becomes stable to hydroxylamine. It took about 26 h at room temperature to bleach 93% of HR(BB). After an extensive dialysis to remove residual hydroxylamine, about 25% of the initial absorbance was recovered upon mixing with retinal. It is obvious that the regenerated pigment is only HR. The largest recovery of the initial absorbance was less than 30%. The reason of this small recovery is not yet clear.

Formation of Pigment Analogues of HR. The retinal analogues shown in Figure 1 all formed pigment analogues with bleached HR(BB). The absorption spectra of the pigment analogues are shown in Figure 2. Although the same amount of bleached HR(BB) was used, the absorbances of the pigment analogues were smaller than that of the regenerated HR (curve 1 in Figure 2) (less than half of the regenerated HR). I think that the difference in absorbance does not result from a difference in the molar extinction coefficients of the pigments but rather is due to the difference in the recovery and stability of the pigments because the absorbance recoveries are not very different in the cases of the corresponding BR pigment analogues; the absorbance recovery was about 65% (analogue **3**) to 47% (analogue **4**) of that of BR.

Table II summarizes the optical properties of analogue retinals and pigments formed with bacterioopsin or bleached HR(BB). The opsin shift (OPS in Table II), proposed by Nakanishi et al. (1980), is the difference in wave number (cm^{-1}) between the λ_{max} of the pigment and that of the protonated Schiff base of retinal or the retinal analogue. The value of OPS should reflect the retinal–protein interaction. The pigment formed from analogues **2** and **3** showed an opsin shift of a similar value as that from retinal (see the values listed in column OPS). The ratios of the opsin shift in BR and HR

Table II: Absorption Maxima and Opsin Shifts (OPS) for Retinal Analogoues and Pigment Analogues

	Ret[a] (nm)	PSB (nm)	+BOP[b] maximum (nm)	+BOP[b] OPS[c] (cm^{-1})	+HOP[b] maximum (nm)	+HOP[b] OPS[c] (cm^{-1})	HO/BO[d]
1	378	440	568	5121 (1.00)	578	5426 (1.00)	1.06
2	398	464	603	4968 (0.97)	620	5423 (1.00)	1.09
3	370	432	555	5130 (1.00)	565	5449 (1.00)	1.06
4	325	376	475	5542 (1.08)	465	5090 (0.94)	0.92
5	353	414	458	2321 (0.45)	475	3102 (0.57)	1.34

[a] In methanol. [b] Data from reconstitution in this study. BOP: bacterioopsin in 10 mM MOPS, pH 7.0. HOP: haloopsin in 10 mM MOPS, 0.5 M NaCl, 1 mM DTT, pH 7.0. [c] The relative opsin shift values compared to that with retinal are given in parentheses. [d] Ratio of opsin shift with retinal or the retinal analogues between the pigment with HOP (HO) and that with BOP (BO).

Table III: Amino Acids Forming the Retinal Pocket[a]

helix	BR		HR	
A	20	M	I	42[b]
B	49	V	I	69
	53	A	S	73
	57	Y	Y	77
C	85	D	T	111
	86	W	W	112
	89	T	S	115
	90	T	T	116
	93	L	I	119
D	115	D	D	141
	118	M	M	144
	122	G	G	148
E	138	W	Y	165
	141	S	S	168
	145	M	F	172
F	182	W	W	207
	185	Y	Y	210
	186	P	P	211
	189	W	W	214
G	212	D	D	238
	216	K	K	242

[a] Amino acids forming retinal pocket are according to Henderson et al. (1990). [b] The numbering for amino acids of HR was according to Oesterhelt and Tittor (1989).

system (HO/BO) were quite similar (1.06 in retinal and analogue **3** and 1.09 in analogue **2**), in agreement with the correlation of the opsin shifts of HR and BR found by Lanyi et al. (1988). This indicates that analogues **2** and **3** interact with the proteins (HOP and BOP) in the same way as retinal, and it also indicates that the chromophoric structure of HR causes a larger red shift of the λ_{max} than that of BR, suggesting a difference in chromophoric structure between HR and BR.

It can be expected that an analogue can distinguish a structural difference between HR and BR; it will interact differently with both opsins if the modified site of the retinal analogue is near the chromophoric site, which is different between the pigments. Pigments from retinal analogues **4** and **5** showed different HO/BO values; in the case of analogue **4** the ratio is 0.92, and it is 1.34 for analogue **5**. I attribute the difference to the larger steric changes in analogues **4** and **5** compared to those of analogues **2** and **3**. The larger difference in the molecular structure such as the loss of the β-ionone ring or the addition of a 6-membered ring in the polyene chain affects the BR and HR chromophores differently.

Recently the chromophoric structure, the retinal pocket, of BR was elucidated by means of electron cryomicroscopy (Henderson et al., 1990). The amino acid residues of the retinal binding pocket of BR were listed and compared with the corresponding amino acid residue of HR (Table III). Many of them either are the same or are a conservative change. The important changes were D(85) → T111 in helix C and W(138) → Y165 and M(145) → F172 in helix E. According to a helical wheel model, helix E is near the β-ionone ring and helix C is near the center of polyene chain. Analogues **4** and **5** should detect the difference in the chromophoric structure between BR and HR at helices C and E, respectively. Whether this idea is true or not can be clarified by a combined study of the retinal analogue and site-directed mutagenesis of HR.

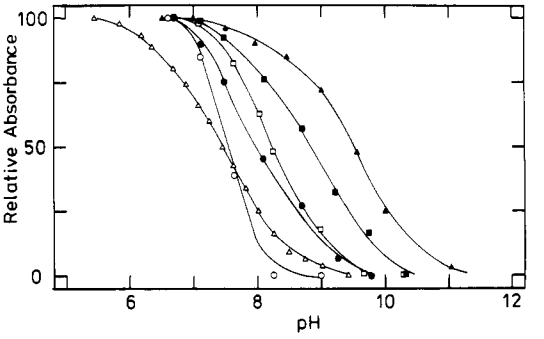

FIGURE 3: Titration curves of the regenerated HR(BB) and pigment analogues with or without chloride ion. Absorption spectra were measured at different pH values, and the absorbance change at 570 nm [HR(BB), triangles] or at 460 nm (analogue **2**, squares; analogue **4**, circles) was estimated. Open symbols are for data obtained without chloride ion (0.5 M Na_2SO_4) and closed symbols are for data obtained with chloride ion (0.2 M NaCl, 0.3 M Na_2SO_4).

pH Titration of Pigments with or without Chloride Ion. At high pH values, the Schiff base of HR deprotonates in the dark, the absorbance at 570 nm decreases, and that at 410 nm increases (Schobert et al., 1986). The pK_a value of this spectral change is affected by anions in the medium. This effect is attributed to the interaction between the Schiff base nitrogen and the anion binding to HOP. The binding site of the anion is not known but was designated site 1 by Schobert et al. (1986). To elucidate the effect of replacement of chromophore and to survey the position of site 1, we measured the absorption spectra of the pigments at different pH in the presence or absence of chloride ion (Figure 3). The absorbance change of regenerated HR(BB) was estimated at 570 nm, the maximum of the absorbance difference. In the case of the pigment analogues, the absorbance change around 460 nm was plotted to avoid problems arising from the absorbance changes due to the residual unbleached HR(BB) because the spectral change of HR(BB), depending on the pH, has an isosbestic point at 460 nm. The titration curve for pigment analogue **3** could not be obtained because the λ_{max} of the pigment analogue was very similar to that of HR(BB) and the absorbance change at 460 nm was very small.

The pK_a shift due to chloride ion was more than 2 pH units in the regenerated HR(BB), which is larger than the value observed in HR(OG). With chloride ion the pK_a of HR(OG) was nearly pH 9 but that of the regenerated HR(BB) was 9.8. In the case of the pigment analogues, however, such a large effect of chloride ions on the pK_a was not observed. These were only 0.5–1 pH unit. In the case of analogue **5**, addition of

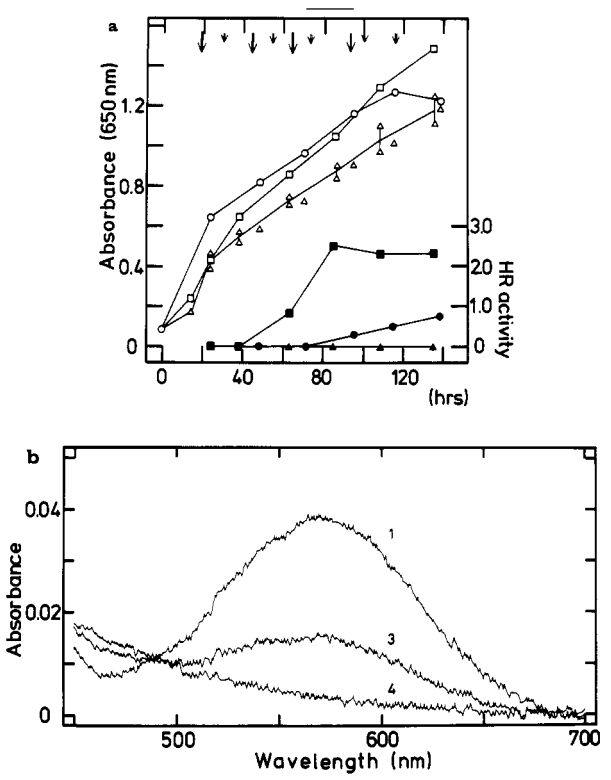

FIGURE 4: (a) Growth and HR activity of L07 cells with retinal or retinal analogues. To the cultures of L07 cells (BOP^-, HOP^+, SOP^+, Ret^-) was added retinal or retinal analogue (30 nmol) at the time indicated by arrows (long arrows for $retinal_2$ and short arrows for retinal and the other analogues). The growth of cells was monitored by the increase in absorbance at 650 nm. The chloride-pumping activity of pigments was measured according to Oesterhelt (1982) and was presented as (nanograms of protons)/(minute·A_{650} unit). Squares: cultures with retinal. Circles: cultures with $retinal_2$. Triangles: cultures with analogues **3**–**5** and the control cultures. The cultures with retinal analogues except $retinal_2$ showed similar growth and almost no chloride-pumping activity. (b) The absorption spectra of membrane suspension of L07 cells. The membrane fraction was prepared from L07 cells cultured with retinal (curve 1), analogue **3** (curve 3), and analogue **4** (curve 4). The membrane suspensions containing the same amount of proteins were sonicated (15 s × 4), and the absorption spectra were measured. The membrane suspension from the culture with only 2-propanol was used as reference.

chloride ions seemed to have no effect on the pK_a (data not shown). The chloride binding to site 1 caused a small blue shift of the absorption maximum (Schobert et al., 1986). The regenerated HR(BB) showed a shift of about 5 nm in its λ_{max}, and the other pigment analogues also showed a small blue shift (data not shown).

The results show an interaction between site 1 and the Schiff base even in the pigment analogues. But the chloride effect on the shift in pK_a is less in the pigment analogues. I could not encompass the position of site 1 with the use of retinal analogues. The chloride effect was observed independently of the site of modification of retinal: near the ring portion (analogues **2** and **5**) or the center of the polyene chain (analogues **3** and **4**).

Chloride-Pumping Activities of Pigment Analogues. The chloride-pumping activities of pigment analogues were measured in the intact cells (L07; retinal-deficient mutant). The cell growth was monitored with absorbance at 650 nm. L07 cells with retinal (open squares in Figure 4a) or $retinal_2$ (open circles in Figure 4a) showed slightly larger growth than those with the other retinal analogues (retinal analogues **3**–**5** in Figure 1) and the control cultures (with only 2-propanol) (open triangles in Figure 4a). The chloride-pumping activity (see HR activity in Figure 4a) was detected only in two cultures: those with retinal and $retinal_2$ (closed squares and circles in Figure 4a, respectively). The other cultures did not show any detectable activity (closed triangles in Figure 4a).

To confirm the pigment formation in the cultures, the membrane fraction was prepared from each culture and the absorption spectra were measured. As shown in Figure 4b, cultures with retinal (curve 1) and 13-demethylretinal (curve 3) showed the absorbance maximum of the respective pigments (compare with Table II). The culture with $retinal_2$ also showed an absorption maximum similar to that of the regenerated pigment (data not shown). In the case of analogues **4** (curve 4) and **5** (data not shown), the membrane fractions did not show a clear peak due to the intense scattering. The larger absorbance of curve 4 in the shorter wavelength region suggests the formation of pigment of analogue **4** because the absorbance difference between curves 4 and 1 in Figure 4b becomes largest around 450 nm (compare curve 4 with curve 1 in Figure 2). All analogue retinals formed pigment analogues in vitro (Figure 2 and Table II). It seemed, however, that not enough of the chromophore was formed in vivo in the case of analogues **4** and **5** to be able to detect it by absorption measurement. Therefore, we could not know whether the pigment analogues of analogues **4** and **5** really pump chloride ion or not. However, enough of the analogue pigment of 13-demethylretinal was formed *in vivo*, and it did not show the detectable pumping activity. Absorption spectra, trans–cis isomerization during the photochemical cycle (Oesterhelt et al., 1986), and the conformational change due to photoreaction observed by the Fourier transform infrared spectroscopy (Bousche et al., 1991) are similar in the HR and BR systems. Therefore, the retinal–protein interactions essential for the pumping mechanism should be similar in both ion pumps.

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Simultaneous Internalization and Binding of Calcium during the Initial Phase of Calcium Uptake by the Sarcoplasmic Reticulum Ca Pump†

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ABSTRACT: The kinetics of Ca^{2+} transport mediated by the sarcoplasmic reticulum (SR) Ca-ATPase were investigated by rapid kinetic techniques that either measure the disappearance of Ca^{2+} from the medium [stopped-flow photometry of Ca^{2+} indicators or rapid filtration (method 1)] or directly detect the changes in the accessibility of Ca^{2+} to the exterior of the membrane, i.e., occlusion of Ca^{2+} within the Ca pump and Ca^{2+} transport into the lumen of SR vesicles [EGTA quench (method 2)]. SR vesicles were preincubated in micromolar Ca^{2+} to form the $E{\cdot}2Ca_{cyt}$ intermediate of the Ca-ATPase, and then Ca^{2+} transport was initiated by addition of ATP. It was found that Ca^{2+} uptake measured by method 1 began with no lag phase, in spite of the prediction of kinetic models of the Ca-ATPase. Instead, the time course of Ca^{2+} uptake was found to have two components: a fast and a slow phase, similar to that obtained using method 2, although the rate constant of the fast phase determined by method 1 was considerably lower than that measured by method 2. The fast phase of Ca^{2+} uptake measured by method 1 was not influenced by either Ca^{2+} ionophore or detergent treatment, whereas the slow phase was diminished. These findings indicate that, upon formation of the phosphorylated intermediate (EP) of the Ca-ATPase, two events occurring simultaneously contribute to Ca^{2+} transport: (1) the internalization of Ca^{2+} that is detectable by method 2, but not by method 1, i.e., Ca^{2+} occlusion within the interior of the Ca-ATPase polypeptide and the following translocation of Ca^{2+} from occluded to internally oriented sites and (2) the binding of Ca^{2+} that is detected by method 1, but not by method 2, to the newly available high-affinity sites that are vacated by Ca^{2+}, as event 1 proceeds. This suggests that, during the Ca-ATPase reaction cycle, Ca^{2+} sites do not convert from high to low affinity, i.e., from outward- to inward-oriented sites; Ca^{2+} instead moves through the membrane from site to site.

The mechanism of Ca^{2+} transport by the sarcoplasmic reticulum (SR)[1] Ca-ATPase (Ca pump) is usually described by a cycle of sequential reaction steps, as first formalized by deMeis and Vianna (1979), with two major states of the enzyme, E_1 and E_2 [for reviews, see Inesi (1985) and Tanford (1984)]. According to this model (E_1–E_2 model), the movement of Ca^{2+} against its concentration gradient is achieved by the simultaneous alteration of both the affinity and the location of a set of Ca-binding sites on the pump. The affinity of the binding sites for Ca^{2+} is high when they are exposed to the cytoplasmic surface of the enzyme (E_1) but low when they face the lumenal side (E_2). Both the translocation and the affinity change of the Ca^{2+} binding sites are driven by a

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[1] Abbreviations: SR, sarcoplasmic reticulum; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; TCA, trichloroacetic acid. E·Ca and EP, Ca-loaded and phosphorylated, respectively, intermediates of the Ca-ATPase.

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