CHROMOPHORE EQUILIBRIA IN BACTERIORHODOPSIN

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ABSTRACT An investigation of the dark equilibria between different chromophores of bacteriorhodopsin (BR) and studies of the kinetics of their interconversion and photochemical activity have led to the following conclusions. (*a*) A component of the 605-nm chromophore of BR decays in the millisecond range and is likely to be identical to the intermediate O of the photochemical cycle of BR and is assumed to be formed from the purple complex (PC) by the binding of one proton to BR. (*b*) An acidic form of the PC, $PC_a^{L^-}$, arises from the 605-nm chromophore by selective binding of anions L^- ($F^- > Cl^- > Br^- > I^- > ClO_4^-$) to BR. (*c*) The isomeric equilibrium between 13-*cis* and all-*trans* retinal is ~0.15/0.85 in $PC_a^{Cl^-}$, 0.3/0.7 in the 605-nm chromophore as compared to 0.5/0.5 in the PC. (*d*) The 500-nm chromophore is formed from the PC by release of nearly one proton from BR. (*e*) The pH range in which the PC exists is reduced in a high-temperature structure of the purple membrane as compared to its low temperature structure. A model for the chromophore structure is proposed as a hypothesis, which allows a comprehensive interpretation of the results. In this model the absorption spectrum of the retinylidene lysine Schiff base is modulated by its protonation state and the interaction with an anionic group.

INTRODUCTION

Bacteriorhodopsin in the purple membrane (PM) of Halobacteria acts as a light-driven proton pump (1, 2). This function is mediated by the thermoreversible photochemical reaction of its native chromophore, which is called the purple complex (PC). At neutral pH in the dark the PC is a mixture of two species from which 13-*cis* and all-*trans* retinal are extractable (3). We assigned these species as all-*trans* PC and 13-*cis* PC (4). A detailed knowledge of the chromophore structure is necessary for an understanding of its photochemical reaction and those after dark reactions which lead to proton transport.

In this context it is of interest that the PC equilibrates in the dark with other chromophores under various conditions; at acidic pH, PC gives rise to a 605-nm chromophore (5) and at alkaline pH to a 500-nm chromophore (4). The absorption spectra of BR under conditions where each of the different chromophores is dominant are shown in Fig. 1. This paper describes some properties of these chromophores and their interconversion.

DEFINITIONS

A chromophore is defined as the retinylidene structure and those parts of the bacterio-opsin moiety that contribute to the characteristic absorption spectrum of bacteriorhodopsin (4). A

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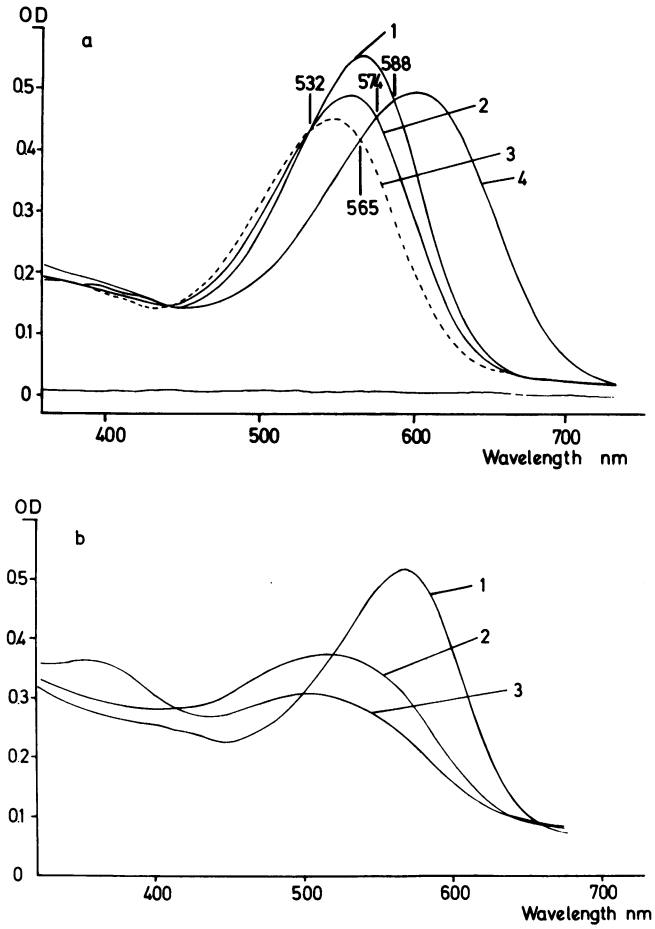


FIGURE 1 Absorption bands of bacteriorhodopsin. (*a*) Comparison of the 605-nm chromophore (4) with the PC (1–3). A PM containing gel (see Materials and Methods) was equilibrated for 30 min in 10 mM HCl at 25°C. Under these conditions mainly the 605 nm chromophore contributes to the absorption spectrum (4). The dark-adapted PC (2) was obtained 70 min after exchange of 10 mM HCl against 10 mM phosphate buffer of pH 7 in the reservoir (Fig. 2 B). After illumination of the dark-adapted PC for 1 min with green light from a 200-W, high-pressure Hg-xenon lamp, the absorption spectrum of the all-*trans* PC (1) was recorded. The dotted curve (3) was calculated as the absorption spectrum of the 13-*cis* PC with the assumption that (2) represents an equimolar mixture of 3 and 1. (*b*) Comparison of the 500-nm chromophore (2) with the PC (1). If a light-adapted PM suspension (1) (10 mM glycine-NaOH buffer, pH 10.5, 25°C) is heated to 65°C, the 500-nm chromophore (2) is obtained transiently. Within ~ 1 h after reaching 65°C this chromophore starts being destroyed irreversibly (3). The denatured chromophore has an absorption maximum at 370 nm which upon titration to acidic pH shifts to ~ 430 nm (data not shown), indicating that it is a retinylidene compound.

chromophore is characterized by its approximate absorption maximum, e.g., 500 nm chromophore, and may be specified by further properties such as the isomeric configuration of the chromophore or the state of protonation. The term BR-chromophore, e.g., BR-605 or BR-PC, is used if we want to refer to the whole bacteriorhodopsin molecule comprising both chromophore and protein.

MATERIALS AND METHODS

PM was isolated according to Oesterhelt and Stoeckenius (5). All reagents used were of analytical grade.

Gels Containing PM for Spectroscopic Investigation

PM were polymerized into polyacrylamide gels to prevent aggregation arising at low pH or high ionic strength. To a homogenized PM sample (70–100 μM BR) suspended in 10% acrylamide and 0.5% N,N′-methylene-bis-acrylamide solution, N,N,N′,N′-tetra-methyl-ethylene-diamine (TEMED), and $(NH_4)_2S_2O_8$ were added to a final concentration of 0.5%. The mixture was quickly poured into the holes of a polyvinylchloride (PVC) template (Fig. 2 A) lying on a piece of glass and the template was then covered with another piece of glass until polymerization was complete, usually in 2 min. Removal of the glass plates leaves the gels in the holes due to their wedge-shaped rim. The gels can be removed and reinserted into the holes without damage. The flow arrangement for spectroscopic measurements is shown in Fig. 2 B. A change in solvent composition within the gels was achieved by solvent exchange in the reservoir (Fig. 2 B) and was complete within 30 min as could be judged from the color changes of the PM within the gels. Absorption spectra were recorded in an Aminco DW 2 spectrophotometer (American Instrument Co., Silver Spring, Md.) at a bandwidth of 3 nm.

Stopped Flow Experiments

Experiments with low time resolution were performed as described in Fig. 7 (see below) with the stopped flow accessory of an Aminco DW 2 spectrophotometer used in dual wavelength mode. For measurements of fast kinetics, a single beam stopped flow spectrophotometer, kindly made available to us by K. Feldmann from the Institute of Physiological Chemistry, University of Würzburg, was used. With a cuvette of 2 mm optical path, a deadtime of 0.7 ms for the instrument was determined. For rapid pH jumps from lower to higher pH values, PM suspensions at pH 3.1 were mixed with a phosphate-citrate buffer. The buffer was made from 10 mM citric acid and 20 mM di-sodium phosphate. To avoid aggregation at low pH, PM was washed twice and resuspended by homogenization in distilled water before adjustment of pH to 3.1 with HCl.

Determination of Isomeric Equilibria

A PM suspension (0.25–0.5 mM BR) was equilibrated in the dark for more than 1 h at 40°C. Still in the dark, an aliquot of 0.15 ml was then rapidly (~1 s) neutralized with 2.3 ml ice-cold phosphate buffer (10 mM, pH 7) and mixed with 2.3 ml cetyl trimethyl ammonium bromide (CTAB) solution (40°C). Subsequent preparation of retinal oximes and extraction for TLC analysis was performed according to Oesterhelt et al. (3) under dim red light (using Kodak safelight No. 1 filter; Eastman Kodak Co., Rochester, N.Y.). A quantitative analysis of the fluorescent retinal oximes was done by evaluation of photographs taken of the plates illuminated with exciting light of 360 nm (Camp type Min U VIS, C.

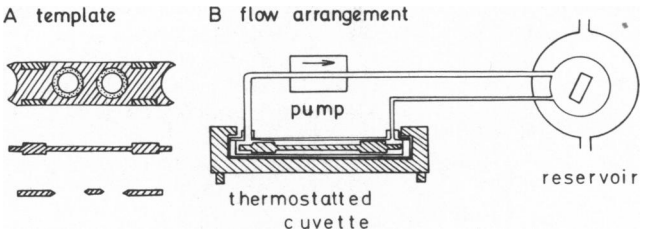


FIGURE 2 Set up for spectroscopic measurements of polyacrylamide gels containing PM. The gels are prepared in the template (A), which fits into a demountable flow cuvette (136 OS, 2-mm optical path, Hellma GmbH & Co., Müllheim, West Germany). The bathing solution was sucked through the cuvette by a peristaltic pump (B).

Desaga, Heidelberg, West Germany) and photographed through a 530 OG (Schott & Gen., Mainz, West Germany) cutoff filter. From a densitogram (Microdensitometer MK III CS; Joyce Loebl & Co., Ltd., Gateshead, England) of the photographs, the relative amounts of the different isomers were determined.

Photochemical Experiments in Salt Ether

1.7 ml of a PM suspension containing 4 M NaCl in a cuvette (d = 1 cm) was treated with peroxide-free ether by gentle mixing at room temperature. The suspension was illuminated with focused actinic light (520–600 nM) from a 200 W Hg-Xe lamp (901 B0011; Engelhard Hanovia Inc., Newark, N.J.) directed onto the cuvette via a light guide. A layer of ether was maintained over the stirred suspension and the pH monitored continuously using a micro pH electrode (Ingold L OT-402-M3-6235; Ingold Electrodes, Lexington, Mass.) connected to a Philips PW 9413 ion activity meter (Philips Electronic Instruments, Inc., Mahwah, N.J.) during recording of spectra.

RESULTS

Equilibrium between the PC, the 605-nm Chromophore, and an Acidic Form of the PC (PC_a)

Absorption spectra of the different forms of the PC, compared to the spectrum of BR under conditions where the 605-nm chromophore is dominant, are shown in Fig. 1 *a*. The maximum difference in absorption between the PC (spectrum 2) and the 605-nm chromophore (spectrum 4) is found at ~640 nm, and therefore absorption changes at this wavelength best reflect shifts in the equilibrium between these two species. Fig. 3 shows the pH dependence of the 605-nm chromophore concentration at different NaCl concentrations. Increasing salt concentrations lowers 605-nm chromophore formation, but independent of salt, the maximal concentration of the 605-nm chromophore is reached at pH 1.8. At pH 0.2 the chromophore exhibits spectral characteristics (Fig. 4, spectrum 3) almost identical to those of the all-*trans* PC (spectrum 2). The 605-nm chromophore is formed from PC by protonations of the membrane. It is difficult to conceive that further protonations would shift the equilibrium

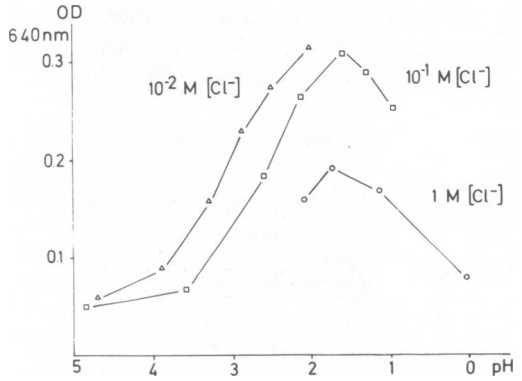


FIGURE 3 Spectroscopic titration of bacteriorhodopsin at constant ionic strength with transient formation of the 605-nm chromophore. Measurements were performed in gels at 25°C with dark-adapted samples 30 min after adjustment of pH in the reservoir (Fig. 2 B) by mixing equimolar NaCl and HCl solutions.

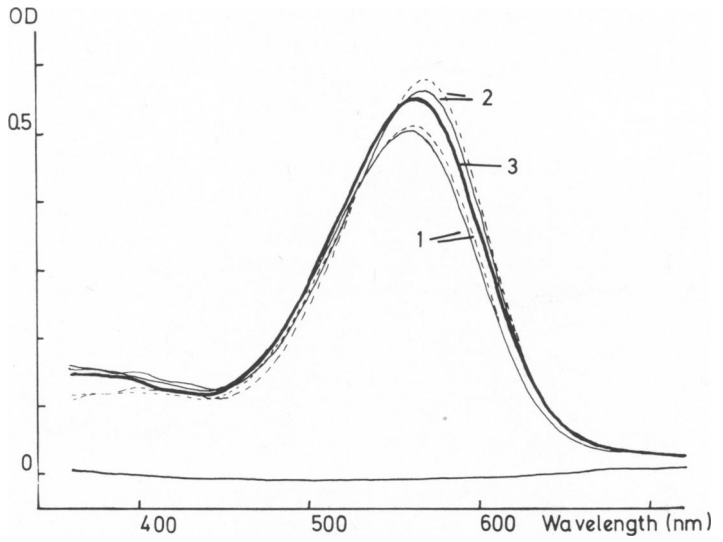


FIGURE 4 Comparison of the PC and its acidic form PC_a. Spectra were recorded from a gel containing PM suspended in 4 M NaCl at 36°C as described in Materials and Methods: (- - -, 1) Dark-adapted sample at pH 6.5 (1:1 13-*cis* and all-*trans* PC); (----, 2) after light adaptation at pH 6.5 (all-*trans* PC); (—, 3) 30 min after adjustment of pH 0.2 by addition of 4 M HCl in the reservoir (see Fig. 2). (—, 1) Dark adapted for 1 h after attainment of pH 6.5 by extensive washing with 4 M NaCl (pH 6.5); (—, 2) as before, after light adaptation. Small differences in the spectra at pH 6.5 taken before and after acidification resulted most probably from light-scattering changes due to aggregation of membranes within the gel. No light-dark adaptation is observed below pH 1 (see p. 90 of reference 7).

back to PC again. Therefore we tentatively call the species that arises at pH 0.2, PC_a (acidic form of the PC). The equilibria between the species are represented by the following scheme:

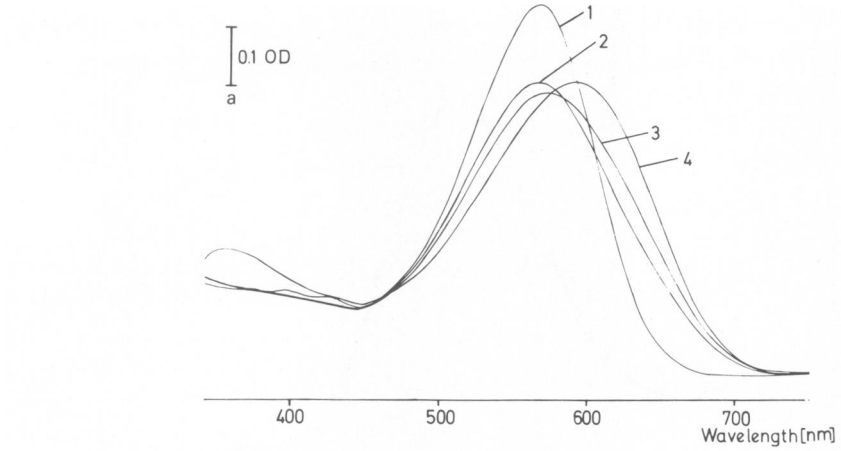
$$\overset{\text{I}}{\text{PC} \rightleftharpoons} \text{ 605-nm chromophore } \overset{\text{II}}{\rightleftharpoons} \text{PC}_a.$$

The dependence of these two equilibria on pH and salt concentration reflects proton and/or ion binding to the membrane; this binding will also be influenced by the charge density of the membrane and charge shielding by ions. In this paper we assume that the binding of one

TABLE I
INFLUENCE OF pH AND ANION CONCENTRATION ON THE CHROMOPHORE EQUILIBRIA

pH	Anion concentration [mM]							
	Cl^-				ClO_4^-			
	10^3	10^2	10^1	10^0	10^3	10^2	10^1	10^0
	λ_{max}							
3	560	570	—	605	560	570	—	605
2	582	602	603	—	603	—	604	—

The absorption maximum of gels containing dark-adapted PM is shown as a function of pH and anion concentration. pH was adjusted by exchange of Na^+ against H^+ ions. *T*, 21°C.


0.1 OD
a
1
2
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600
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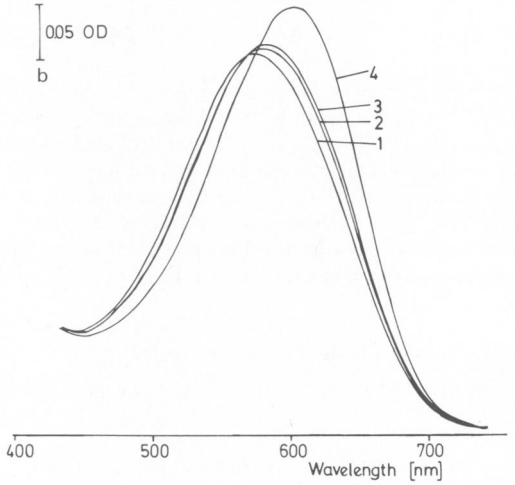
Wavelength [nm]


0.05 OD
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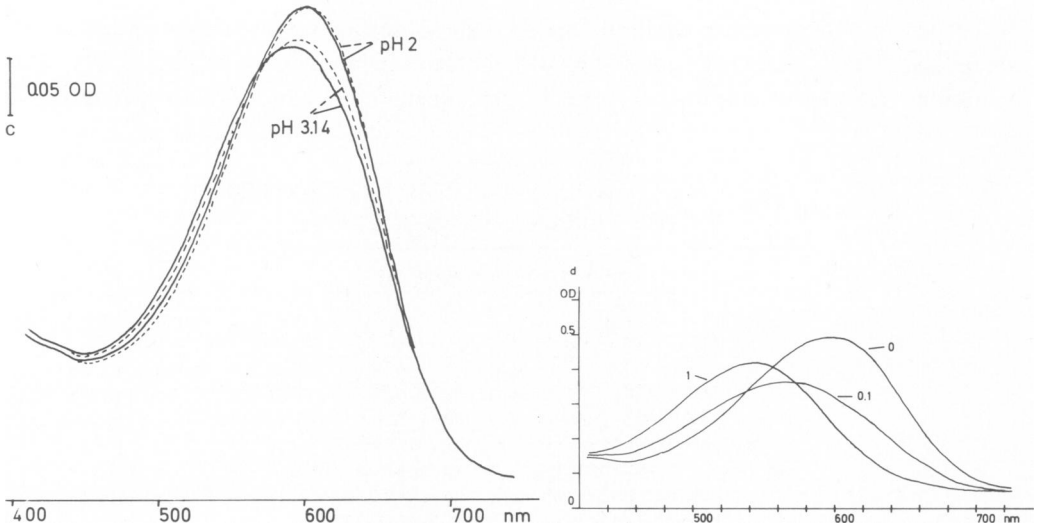
Wavelength [nm]


0.05 OD
c
pH 2
pH 3.14
400
500
600
700 nm
d
OD
0.5
0
1
0
0.1
500
600
700 nm

proton to BR-PC leads to the formation of BR-605 (see below). A change in the electrostatic charge of the membrane seems to have an influence on equilibrium I, since low concentrations of the positively charged detergent CTAB inhibit the formation of the 605-nm chromophore (5), whereas its formation is favored to a significant degree by the negatively charged detergent sodium dodecyl sulfate (SDS) in less than stoichiometric amounts (unpublished observation). Thus a decrease in negative charge seems to impede the formation of the 605-nm chromophore; an increase in ionic strength has a similar effect shown in Table I (observe line "pH 3"). This may be due to a shielding of negative charges above the isoelectric point. Only a weak influence of anions on equilibrium I is observed above pH 2 as is shown in Fig. 5 *c*.

Our interpretation of the results pertaining to pH- and ion effects on equilibrium I are summarized in the following scheme:

$$PC + 1\ H^+ \overset{I}{\rightleftharpoons} 605\text{-nm chromophore.}$$

Formation of PC is favored by an increase of ionic strength (above pH 2) and decrease of the negative membrane charge.

Compared to equilibrium I, equilibrium II is affected by pH and ions in a rather different way. The salt-induced formation of PC_a depends on the anion, as shown in Table I. At pH 2 sodium chloride induces the formation of PC_a, as indicated by a blue shift of λ_{max}, whereas sodiumperchlorate does not. On the other hand, at pH 3 chloride and perchlorate induce PC formation to approximately the same extent.

The anion-induced blue shift at pH 2 becomes larger in the sequence, ClO_4^-, I^-, Br^-, Cl^-, of decreasing anion radius, as shown in Fig. 5 *a* and *b*. No comparable influence of cation radius is observed, as shown in Fig. 5 *b*. The anion dependence of the blueshift indicates that the binding of anions to BR-605 leads to PC_a formation. Since PC_a formation is half maximal at ~1 M chloride concentration (Fig. 3, pH 2), a dissociation constant in the order of

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FIGURE 5 The influence of anions on the formation of PC_a from the 605-nm chromophore. Measurements were performed on gels containing PM as described in Materials and Methods. (*a*) Influence of halogen anions; T, 25°C. (1) Absorption spectrum of all-*trans* PC in water at pH 6. The subsequent spectra were obtained by exchange of the bathing solution which consisted of 10 mM HCl and (2) 0.9 M KCl, (3) 0.9 M KBr, (4) 0.9 M KI. 1 mM NaH_2PO_2 was present throughout, and nitrogen was flushed constantly through the solution to prevent extensive oxidation of iodide. In spectrum 4, traces of iodine led to an absorption at 360 nm. This disappeared upon exchange of the bathing solution with 10 mM phosphate buffer (pH 7), and upon light adaptation an absorption spectrum identical to spectrum 1 was obtained. (*b*) The influence of univalent cations. Spectra taken at pH 2, 21°C in the presence of (1) 1 M LiCl, (2) 1 M NaCl, (3) 1 M CsCl, and (4) 1 M $NaClO_4$ are compared. (*c*) Formation of the 605-nm chromophore in the presence of (—) 10 mM Cl^- and (---) 10 mM ClO_4^- at 21°C. (*d*) Influence of fluoride on the absorption spectrum of BR at 25°C. A slice of a gel (1 × 10 × 8 mm) containing PM was equilibrated for 30 min in a solvent mixture of 1 M $NaClO_4$, 1 M $HClO_4$, 1 M NaF, and 1 M HF of pH 2, ascertained by means of an indicator paper (Acilit, Merck No. 9560; Merck Sharp & Dohme pH range 0.5–5). The fluoride concentration was adjusted by exchange of $NaClO_4$ and $HClO_4$ against NaF and HF. For recording of the spectra, the gel was layered on a small perspex plate, which was fitted at an angle of 45° to the bottom of a perspex cuvette filled with the solvent. Spectra are shown of PM at pH 2 in various concentrations (0.0, 0.1, and 1 M) of [NaF] + [HF] in the presence of decreasing concentrations of [$NaClO_4$] + [$HClO_4$] (1, 0.1, and 0.0 M, respectively). They were recorded in a Shimadzu spectrophotometer MPS 5000 (Shimadzu Scientific Instruments, Inc., Columbia, Md.)

1 M has to be assumed for $PC_a^{Cl^-}$. Concentrations of up to 1 M ClO_4^- do not affect the absorption spectrum of BR at pH 2. When PM is incubated in 1 M $HClO_4$, $PC_a^{ClO_4^-}$ arises as indicated by a λ_{max} = 572 nm, which is close to the $\lambda_{max} \simeq$ 565 nm of $PC_a^{Cl^-}$. $PC_a^{ClO_4^-}$ is, however, not stable and decays irreversibly within approximately 1 h to a species with $\lambda_{max} \simeq$ 440 nm (data not shown), which is characteristic for the protonated Schiff's base of retinylidene compounds (21). We conclude that $PC_a^{ClO_4^-}$ has a λ_{max} value that is close to that of $PC_a^{Cl^-}$ and that it has a considerably higher dissociation constant than $PC_a^{Cl^-}$ at pH 2.

Because the fluoride ion is smaller than chloride, one would expect $PC_a^{F^-}$ to have a smaller dissociation constant than $PC_a^{Cl^-}$. An experimental difficulty arises in performing fluoride titrations at low pH since fluoride has a pK_a value of 3.45 (24). Therefore, HF (and other species such as HF_2^-, H_2F^-, and H_2F_2 [25]) are present in the solution together with the fluoride ion; the free fluoride concentration is not accurately known.

A gradual exchange of a mixture of 1 M $NaClO_4$ and 1 M $HClO_4$ of pH 2 as a bathing solution against a mixture of 1 M NaF and 1 M HF at pH 2 results in a blueshift of λ_{max} from 600 to 540 nm of a gel containing PM (see Fig. 5 *d*). The λ_{max} of BR has a minimum between pH 2 and 3 with a mixture of 1 M HF and NaF as solvent (Table II).

The following conclusions are drawn from these results of the blueshift induced by fluoride. (*a*) The blueshift is due to anions rather than to acid conditions, because lowering the pH eventually leads to a decrease of the blueshift (Table II). (*b*) Addition of fluoride ions to a gel containing PM below pH 4 leads to the formation of $PC_a^{F^-}$ that has a λ_{max} value of ~540 nm. Since this value is smaller than that of the PC, $PC_a^{F^-}$ and PC must be distinct species. (*c*) No quantitative determination of a dissociation constant for $PC_a^{F^-}$ is possible from the results shown in Fig. 5 *d*, since the absence of an isosbestic point in the spectra implies the participation of more than two species with different absorption spectra, nor is the fluoride concentration accurately known. Nonetheless, the optical density (OD) decrease above 600 nm, which is half saturated at a concentration of $[HF] + [F^-] = 0.1$ M, does allow us to estimate the dissociation constant of $PC_a^{F^-}$ to be considerably smaller than 0.1 M. (*d*) An increase in $PC_a^{L^-}$ formation upon lowering pH below pH 2 occurs for $L^- = Cl^-, ClO_4^-$, but not for $L^- = F^-$. Therefore, we consider the pH influence on PC_a to be due to an indirect induction of anion binding by positive charging of the membrane upon progressive protonation, rather than to a direct acid-induced formation of PC_a. In the case of fluoride at low pH values this induction may well be overcome by a reduction of the free fluoride concentration.

Our interpretation of the results of ion and pH influences on equilibrium II is summarized in the following scheme:

$$\text{605-nm chromophore} + L^- \overset{\text{II}}{\rightleftharpoons} PC_a^{L^-}.$$

λ_{max} and the dissociation constant of $PC_a^{L^-}$ decreases with decreasing radius of the anion L^-. In this equilibrium the 605-nm chromophore is favored by decreasing positive charge via deprotonation of the membrane.

In summary we conclude that PC_a is formed from BR-605 by anion binding and that PC is formed from BR-605 by deprotonation. The similarity in the absorption spectra of PC and $PC_a^{Cl^-}$ further suggests that an extrinsic anion, which is bound in $PC_a^{L^-}$, is replaced upon formation of PC by an intrinsic anion and that upon protonation of the intrinsic anion PC

TABLE II
pH DEPENDENCE OF λ_{max} OF GELS CONTAINING PM IN A BATHING SOLUTION CONSISTING OF HF AND NaF AT A COMBINED CONCENTRATION $[F^-] + [HF] = 1$ M

pH...	1	2	3	4
λ_{max}	555	542	545	553

Absorption spectra were recorded with a set up for gels as described in the legend of Fig. 5 *D. T*, 25°C.

leads to formation of the 605-nm chromophore. The intrinsic anion may well be a negatively charged amino-acid residue.

Isomeric Configuration of Retinal in the Various Chromophores

The absorption spectra of all-*trans* PC and $PC_a^{Cl^-}$ are much alike (Fig. 4), suggesting that $PC_a^{Cl^-}$ may contain the retinylidene moiety in its all-*trans* configuration. For analysis we made use of the fact that the isomer equilibrium is attained within seconds at low pH (6, 7), but slowly at neutral pH ($t_{1/2}$ = 14 min at 35°C, pH 7, 10 mM NaCl). Thus after incubation of PM suspensions at low pH and rapid mixing (<1 s) with ice-cold buffer of pH 7, the isomer equilibrium is frozen in its original state. Retinal isomers were then extracted and analyzed as described in Materials and Methods. In Table III the results of this experiment are summarized. The all-*trans* isomer is present to the extent of 83% at pH 0, 40% at pH 3 (4M NaCl), and 50% at pH 7 (3, 8, 9).[1] The same values are found by analysis of the pH dependence of the OD at 565 nm, the wavelength where the spectra of 13-*cis* PC and the 605-nm chromophore intersect (Fig. 1 *a*). In addition, this titration was performed in 4 M NaCl to suppress BR-605 formation. Thus, OD changes at 565 nm will be mainly due to a shift in the *cis-trans* equilibrium of the PC. Only negligible changes are expected for shifts in the BR-PC/BR-605 equilibrium and shifts in the equilibrium between the different possible forms of BR-605. When TLC analysis is performed after incubation at low ionic strength (10 mM NaCl) it appears that at approximately pH 2.5, where the 605-nm chromophore is now formed, a higher percentage of all-*trans* retinal is found. A dominance of all-*trans* retinal in BR-605 is also seen from analysis of its decay kinetics, as shown below.

The Protonation State of Bacteriorhodopsin in the 500-nm Chromophore, the 605-nm Chromophore, and the PC

The 500-nm chromophore was characterized by Schreckenbach et al. (4). It is formed at alkaline pH in suspensions saturated with ether containing 4 M NaCl (salt-ether system, Fig. 6 A). Similar chromophoric species are also observed if the PM structure is perturbed by

[1]Similar results about the pH-induced formation of $PC_a^{Cl^-}$ and its isomeric configuration were obtained independently by R. H. Lozier et al., and influences of anions were mentioned by the same authors. Lozier, R. H., Q. Chae, P. C. Mowery, and W. Stoeckenius. 1978. Flash photolysis studies of bacteriorhodopsin species formed at extreme low pH. *In* Developments in Halophilic Microorganisms. S. R. Caplan and M. Ginzburg, editors. Elsevier North-Holland, Inc. Vol. 1 297. Mowery, P. C., R. H. Lozier, Q. Chae, Y.-W. Tseng, M. Taylor, and W. Stoeckenius. 1979. The effect of acid pH on absorption spectra and photo reactions of bacterorhodopsin. *Biochemistry*. In press.

TABLE III
ISOMERIC EQUILIBRIUM OF THE CHROMOPHORE AS A FUNCTION OF pH

pH	Q* (TLC) 4 M NaCl	Q (TLC) 10 mM NaCl	Q (spectr) 4 M NaCl	OD (pH)[d] 4 M NaCl
7	0.51	0.53	0.5	0.47
4	—	0.51	—	—
3.5	—	—	0.46	0.46
3	0.4	—	—	—
2.5	—	0.68	—	—
2	—	—	0.38	0.45
1.5	—	—	0.62	0.48
1	—	—	0.72	0.5
0	0.83	—	0.85	0.52

TLC after retinal oxime extraction was carried out as described by Oesterhelt et al. (3) and isomer distribution analyzed as described under Materials and Methods. Spectroscopic determination at 565 nm is based on the known isomer distribution of 1:1 at pH 7 in the dark (3, 6, 14). Isomer distribution (Q [spectr]) at different pH values was calculated as $Q = 0.5 \times [OD\ (pH)^d - OD\ (pH\ 7)^d/OD\ )pH\ 7)^1 - OD\ (pH\ 7)^d] + 0.5$, where OD $(pH)^d$ is the OD at 565 nm of the dark-adapted sample at a given pH; subscript 1 indicates light-adapted samples and OD $(pH\ 7)^1$ was 0.542. Temperature, 35°C.

*Q = ([*trans*]/[*trans*] + [13 − *cis*])

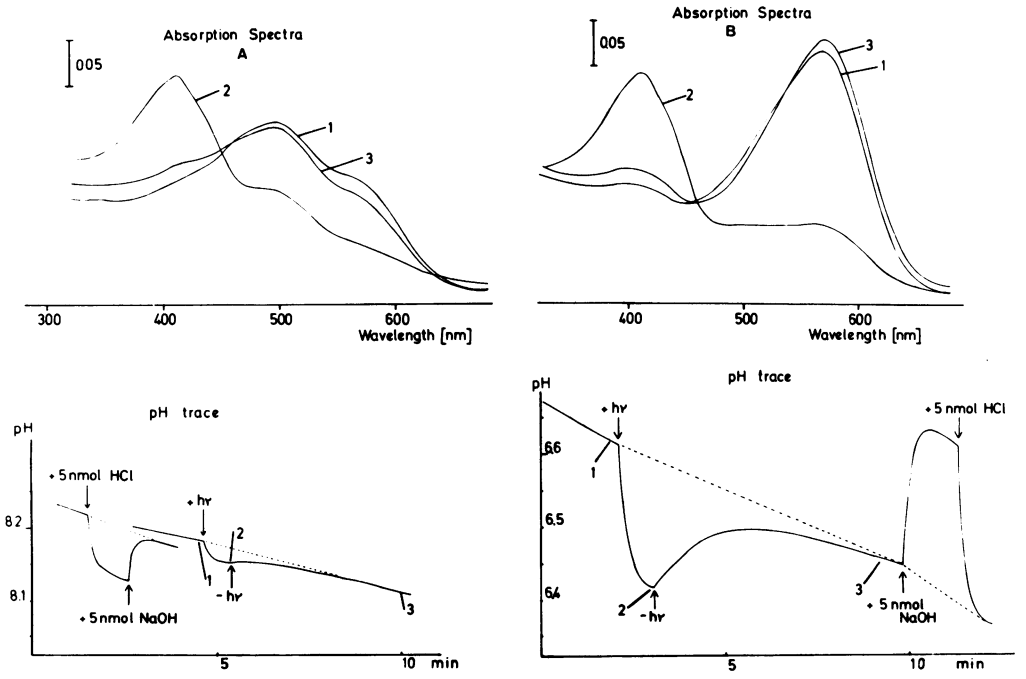

FIGURE 6 Light-induced protonation changes of the 500-nm chromophore (A) and the PC (B). pH traces and absorption spectra were recorded at 0°C of a PM suspension saturated with ether as described in Materials and Methods. (A) pH 8.2 was attained by addition of NaOH. Within ~30 min the 500-nm chromophore slowly developed. Spectra were recorded within 20 s before turning on the actinic light (1), shortly after turning off the actinic light (2), and ~10 min later in the dark (3). (B) The same sequence of measurements was performed after backtitration to pH 6.6 by addition of HCl. The dotted lines indicate pH drifts that could not be avoided in the unbuffered solutions under the conditions of the measurements.

other solvents such as DMSO or by changes in lipid environment (3, 10). Although differences in the absorption maxima of these chromophores are found, they all react in the dark with hydroxylamine to form retinal oxime, a reactivity not shown by the PC. At high pH and elevated temperatures a blue-shift occurs in the absence of solvent perturbation, followed by chromophore denaturation as shown in Fig. 2 *b*. For analysis of the protonation state of BR-500 as compared to BR-PC, the equal photochemical activity (4) of both forms was exploited. Upon irradiation of BR-PC or BR-500 in the salt/ether system, the 412-nm intermediate of the photochemical cycle of BR is accumulated in the photostationary state (4, 11). Fig. 6 shows the light-induced transient changes of absorption spectra and pH of the suspension. At pH 8.2 (mainly 500-nm chromophore) and at pH 6.6 (PC) approximately equal amounts of the 412-nm intermediate accumulate. From the results shown in Fig. 6 we calculate that the pH change of 0.175 pH U at pH 6.6 (0.022 at pH 8.2), accompanied by an OD increase of 0.11 (0.102) at 412 nm, corresponds to a number of 0.83 (0.34 at pH 8.2) protons being released upon the formation of 1 molecule of 412-nm intermediate. For this calculation an extinction difference coefficient, $\epsilon_{412} = 3.7 \times 10^4\ M^{-1}\ cm^{-1}$ (11), for the 412-nm intermediate was assumed in both cases, and the light-induced pH change was compared to a pH change of 0.21 at pH 6.6 (0.069 at pH 8.2), which was induced by addition of 5 nmol of acid to the volume of 1.7 ml. Since the spectra at pH 8.2 represent a mixture of BR-PC and BR-500, part of the light-induced pH change must be due to the bleaching of BR-PC. Thus we conclude that considerably less than half as many protons are released with the formation of BR-412 from BR-500 than from BR-PC. The results are qualitatively consistent with the assumption that the protonation change at pH 8.2 is entirely due to formation of BR-412 from BR-560. Care had to be taken that no extensive photodestruction of the chromophore occurred at pH 8.2, which leads to an irreversible proton release. Due to the unfavorable conditions the reversible part of the protonation change could not be determined more accurately. It is suggested that one proton is released in the transition BR-PC/BR-412 at pH 6.6 and that no proton is released in the transition BR-500/BR-412 at pH 8.2. Our interpretation of the results is summarized in the following scheme:

$$\begin{array}{ccc} \text{BR-PC (pH 6.6)} & \xrightarrow{h\nu} & \text{BR-412} + \text{H}^+\text{(pH 6.6)} \\ -(n+1)\,\text{H}^+ \Big\downarrow\Big\uparrow +(n+1)\text{H}^+ & & -n\text{H}^+ \Big\downarrow\Big\uparrow +n\text{H}^+ \\ \text{BR-500 (pH 8.2)} & \xrightarrow{h\nu} & \text{BR-412 (pH 8.2).} \end{array}$$

In the transition from BR-PC (pH 6.6) to BR-500 (pH 8.2) nearly one proton more ($n + 1$) per chromophore is released from the PM than in the transition from BR-412 at pH 6.6 to BR-412 at pH 8.2. The group that is additionally dissociated in the transition BR-PC to BR-500 may or may not be identical with the group that dissociates during the light-induced transition from BR-PC (pH 6.6) to BR-412 (pH 6.6).

A similar procedure, outlined in Fischer's Ph.D. thesis (7), was applied to assess the protonation state of BR-605. That publication showed that in the photostationary state of BR an intermediate of the photochemical cycle is accumulated at low pH whose difference spectrum is similar to the difference spectrum between all-*trans* PC and BR-605. The accumulation of this intermediate is accompanied by the uptake of protons by BR; the results are consistent with the assumption that one proton is taken up per molecule of intermediate. A

further publication of these results is in preparation. In the present paper we assume that one proton is bound with the formation of BR-605 from BR-PC. Here we give evidence only that the kinetics of the decay of BR-605 to BR-PC are consistent with BR-605 being an intermediate of the photochemical cycle (presumably the 0 intermediate) [13]; see below).

We conclude that BR-500 is likely to be a species that contains one proton less than BR-PC, and we assume that BR-605 contains one proton more than BR-PC.

Decay of the 605-nm Chromophore to the PC

In the case that the 605-nm chromophore and the 0 intermediate are identical, both species should decay with the same kinetics to the all-*trans* PC. An investigation of the conversion of BR-605 to BR-PC upon a rapid pH jump from acidic to neutral pH reveals the presence of a fast decaying component of BR-605 which may be identical with the 0 intermediate.

As shown in Fig. 7, BR-605 decays to BR-PC with very complex kinetics extending from 10^{-3} to 10^{4} s. The slowest component in this decay occurs with a half time of ~150 s (Fig. 7 *b*), which is identical with the half time for attainment of the 13-*cis*/all-*trans* equilibrium under comparable conditions (7). Furthermore, this component does not appear at the isosbestic point of the spectra of 13-*cis* and all-*trans* PC, which is close to 528 nm (see Fig. 4), but is most prominent at 577 nm where this equilibration is expected to yield the highest contribution in comparison with the BR-605 to BR-PC transition (Fig. 2 *a*). Therefore the slowest component in the decay represents an all-*trans*, 13-*cis* equilibration of the PC. This result is in accordance with the observation that the 605-nm chromophore contains more all-*trans* retinal than the dark-adapted PC (see above).

A second slow phase occurs in the decay of BR-605 which can clearly be seen from

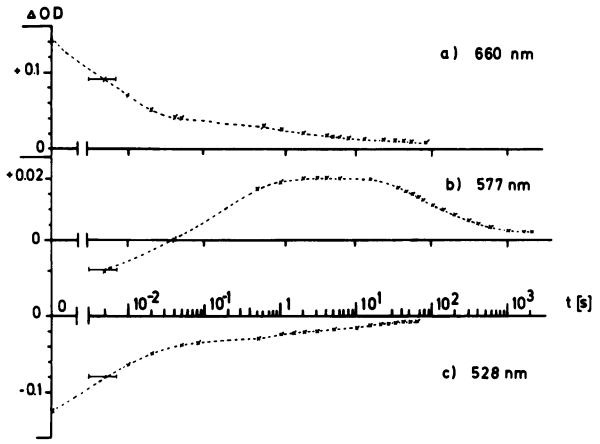

FIGURE 7 Time-course of the decay of BR-605 to BR-PC at pH 5. The absorption changes after a pH-jump from pH 3.1 to pH 5 at 36°C were measured in an Aminco DW 2 spectrophotometer equipped with a stopped flow accessory (time resolution, 4 ms indicated by a bar). The dual wavelength mode was applied, using 750 nm as reference wavelength. Kinetics extend from 10^{-3} to 10^{4}s. Absorption changes within 500 mn were recorded on an oscilloscope and from 0.5 to 10^{4}s on the chart recorder of the instrument. The time-zero reference signal was obtained by mixing the sample (pH 3.1) with buffer of the same pH. The absorption changes at 660 (*a*) and 528 nm (*c*) indicate the formation of PC (independent of *cis-trans* state; see Fig. 1) and concomittant decay of the 605-nm chromophore. Changes at 577 nm (*b*) preferentially reflect a *cis-trans* transition of the PC (see Fig. 1).

TABLE IV

pH DEPENDENCE OF THE RELAXATION TIME FOR THE FAST COMPONENT IN THE DECAY OF THE 605-nm CHROMOPHORE TO THE PC

pH ...	3.66	4.16	4.65	5.15	5.65	6.1	6.6	7.7
				ms				
τ	18.6	15	13.5	10	5.5	4.4	2.3	2.3

Measurements were performed with a single beam, stopped flow spectrophotometer as described in Materials and Methods. Transmission changes at 640 nm were recorded on a storage oscilloscope. Transmission (Tr) at t = 0 ms was 80% and at t = 100 ms 88% between pH 7.7 and 5.2. At pH 7.7 and 6.6 the transmission change during the deadtime of 0.7 ms was 2%. Below pH 5 the amplitude Tr (t = 0 ms) − Tr (t = 100 ms) decreased linearily from 8 to 4%. The relaxation times were evaluated from the slopes of the plots of $\log_{10}\{\log_{10}[\mathrm{Tr}\ (t = 0\ \mathrm{ms})/\mathrm{Tr}\ (t = 100\ \mathrm{ms})]\}$ vs. time (Fig. 16 in reference 7). T, 18°C.

absorption changes at 528 nm and 660 nm (Fig. 7 *a* and *c*). From the results shown below it will become plausible that this second slow phase involves a cooperative change in membrane structure. Moore et al. suggested that a slow component in the formation of BR-605 from BR-PC may be related to a cooperative change in membrane structure (12).

The fastest component with the largest amplitude in the decay curve of Fig. 7 (*a* and *c*) occurs in the millisecond range and is pH dependent (Table IV). At pH 7.7 a relaxation time of 2.3 ms is found and therefore the component causing this phase of decay is a candidate for an intermediate of the photochemical cycle which has a frequency of ~200 s^{-1} at room temperature. Evaluation of the relaxation time as a function of pH reveals a decrease by a factor of 8 going from pH 3.7 and pH 6.6. On the other hand the decay of the 412-nm intermediate occurs in the same time range but is nearly independent of pH between pH 4 and 8 (16). One can assume a nearly constant time of formation for the 412-nm intermediate and an increasing or constant rise time for the redshifted 0 intermediate of the photochemical cycle (17).

These results obtained with the 605-nm chromophore, the 412-nm (intermediate M in reference 13) and, the 0 intermediate, taken together, explain that lowering the pH of a suspension with BR in the photostationary state leads to the preferential accumulation of the 0 intermediate (7) if an identity of 0 with the 605-nm chromophore is assumed. In summary we suggest the identity of 0 with the fast decaying component of the BR-605. An identity of intermediate 0 with BR 605 was also suggested by Moore et al. (12).

Temperature Dependence of Absorbance at Various pH Values.

The slow phase in the decay of the 605-nm chromophore to the PC (Fig. 7) suggested to us a possible involvement of membrane structural changes. Detailed calorimetric and spectroscopic measurements at pH 7 have already shown that such changes exist (18). We have analyzed chromophore equilibria as a function of temperature and pH. Dark-adapted membrane suspensions were subjected to temperature scans at different pH values. In Fig. 8 *a*, high- and low-temperature spectra at pH 4.55 and pH 7 are shown. At pH 7 a blue shift of the spectrum is observed with increasing temperatures. The spectrum 2 in Fig. 2 *b* taken at pH 10.5 and 65°C further indicates that this blue shift is due to the formation of BR-500. At pH 4.55 increasing temperature induces a red shift indicating the formation of BR-605. As a result the existence of the PC is narrowed to the region around pH 6 upon increase in

temperature. Fig. 8 *b* shows the dependence of OD on temperature at various pH's with a constant heating rate of 0.5°C/min. Care had to be taken that no gross destruction of the chromophore occurred at high temperatures, which resulted in a sharp decline of OD between 500 and 700 nm. A small irreversibility of the temperature-induced changes could not be avoided as is shown in Fig. 8 *a*, where two series of spectra are shown taken at the beginning and at the end of a temperature scan and after cooling to the initial temperature. At pH 7 a rather abrupt break in the temperature profile is observed at 79°C (also reported in reference 18). At pH 9.1 a less-pronounced break occurs at a lower temperature. Below pH 7 no such breaks are observed, but inflection points in the temperature scans still occur.

The reason for the temperature-induced shift of the chromophore equilibrium in favor of

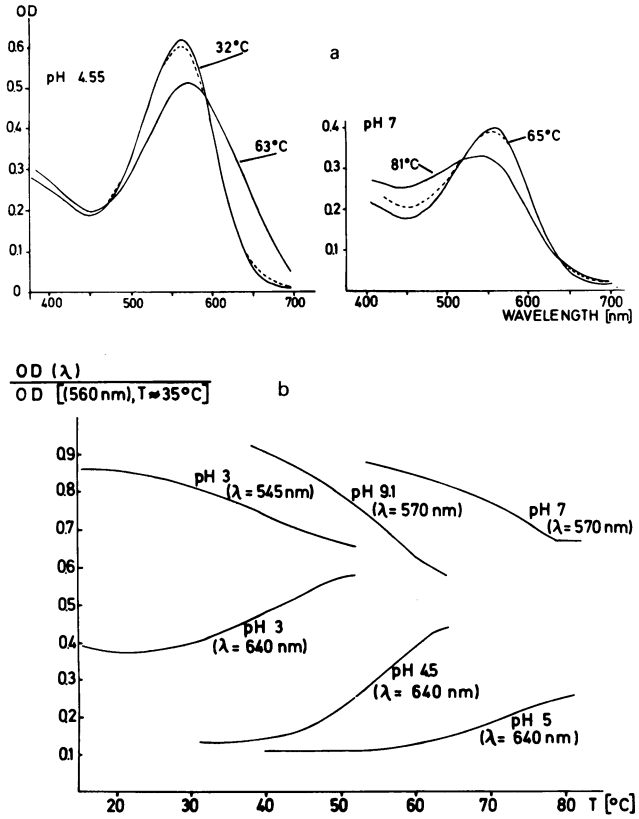

FIGURE 8 Evidence for temperature and pH-induced changes of chromophore equilibria and membrane structure. Measurements were performed in a grating spectrophotometer (DB-G, Beckman Instruments, Inc., Fullerton, Calif.) with a "TM-analyzing" accessory. The suspensions were buffered either with 10 mM K-phosphate (pH 7), 10 mM K-phthalate (pH 4.55), 10 mM borax (pH 9.1), 10 mM phosphate-citrate buffer (pH 5), or 1 mM HCl (pH 3). Only the measurements at pH 3 were performed using gels (see Materials and Methods). (*a*) Shift of chromophore equilibrium in favor of the 605-nm chromophore (pH 4.55), and the 500-nm chromophore (pH 7) at high temperature. Spectra were recorded at low and high temperature (—) and again at low temperature (---). (*b*) Temperature- and pH-induced changes of PM structure. With different samples, temperature scans of absorption at a given wavelength and pH were performed at a heating rate of 0.5°C/min starting from dark equilibrated samples. This heating rate allows equilibration (18) and at the same time prevents extensive denaturation.

the 605-nm chromophore at low pH and the 500-nm chromophore at high pH is unclear. The sharp transition point at neutral pH indicates a cooperative structural change, which according to a suggestion by Jackson and Sturtevant (18) may be due to a protein conformational change preceding denaturation.

The points of inflection in the temperature scans of Fig. 8 *b* shift to lower values with decreasing and increasing pH, starting from pH 7. Therefore, the presumed transition seems to occur at temperatures lower than 80°C for pH values other than 7. With this interpretation of the results we can account for the slow phase in the transition from BR-605 to BR-PC (see above). Thus at 36°C and pH 3.1 the membrane may exist partly in the structure arising at high temperatures, whereas at pH 5 one would expect it to be completely in the structure occurring at low temperatures. Therefore a structural change would have to occur upon a pH-jump from pH 3.1 to pH 5. This structural change could account for the slow phase observed in the kinetics of the BR-605 to BR-PC decay. We conclude that the state of the membrane that arises at high temperatures favors the 605- and 500-nm chromophores at the expense of the PC.

DISCUSSION

For a comprehensive interpretation of our results we propose as a hypothesis a model for the structure of the BR chromophore. The model consists of the following elements. (*a*) In the PC, a protonated group A interacts with an ionized group B^- and forms a complex designated as ($AH^+ \ldots B^-$). (*b*) In the 605-nm chromophore, group A is protonated and no complex is formed with either B^- or other anions. The 605-nm chromophore arises from the PC either by protonation of B^- or by removal of B^- from the site of interaction with AH^+. Two possible forms of the 605-nm chromophore exist that are designated (AH^+, B^-) and (AH^+, BH). (*c*) In the 500-nm chromophore, group A is deprotonated. Therefore it can be formed from the 605-nm chromophore or from the PC by deprotonation of AH^+. The two forms of the 500-nm chromophore are designated as (A, B^-) and (A, BH). We further assume an interaction with a protonated group A'H (see below), which, however, is not considered in the formal treatment of the equilibria. (*d*) PC_a is formed from the 605-nm chromophore by binding of a water soluble anion L^-. The possible forms of PC_a are ($L^- \ldots AH^+$, B^-) and ($L^- \ldots AH^+$, BH). Thus, upon formation of PC_a, the intrinsic anion B^- is replaced by the extrinsic anion L^- in the complex. (*e*) All chromophores can exist with the retinylidene moiety in its all-*trans* or 13-*cis* configuration.

The above model in plausible molecular terms is outlined in Fig. 9. From current suggestions of retinal protein chromophore structure (19, 20, 21) we follow the one of Blatz et al. (21), which is based on studies of model compounds. λ_{max} of the protonated Schiff's base between retinal and an amino group (AH^+) is modulated by the counterion. A decrease in the distance of the anion (L^- or B^-) to the positive charge of the retinylidene structure leads to a blue shift of λ_{max} from 600 nm for the cation to ~ 440 nm for the salt. B^- could be a carboxyl group of an amino acid side chain. Blatz et al. also suggest that the λ_{max} shift is due to an interaction of the anion with the ground state of the chromophore leading to a higher stability of those complexes in which the distance between the charges is smaller. This stabilization may contribute to the decrease of the dissociation constant K_L- (see below) with decreasing anion radius which we found. The additional group AH', e.g., a tyrosine or protonated lysine

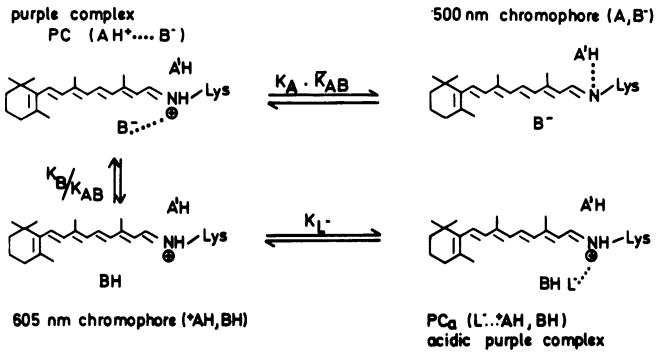

FIGURE 9 Suggested realization of the model for chromophore structure. The acid AH^+ of our model is the protonated Schiff base. B^- is a negatively charged base in the vicinity of the retinylidene moiety, allowing for an interaction (...) with the protonated Schiff base in the PC. B^- is protonated or it is shifted to a different position in the other chromophore forms such that the interaction of B^- with the Schiff's base is broken. In the 500-nm chromophore an interaction with a further protonated group A'H is assumed. In the PC_a, B^- of the PC is replaced by an anion L^-. The equilibrium constants, $K_{(B^-)}$, $K_{(L^-)}$, $K_{(A)}$, and $K_{(\overline{AB})}$, which describe the chromophore equilibria, were introduced in the text.

residue was introduced to account for the red-shifted absorption maximum of the 500-nm chromophore as compared to a deprotonated Schiff base (λ_{max} 360–370 nm [21]). A'H may induce a partial positive charge on the Schiff base and thus induce a red shift. The interaction of A'H with the Schiff base is neglected in the formal treatment. A consideration of this interaction would lead to a modified intrinsic dissociation constant $K_{\overline{AB}}$ in the transition from PC to the 500-nm chromophore as compared to the constant K_{AB} introduced below.

For a mathematical description of the model we adhere to the treatment of Laskowski and Sheraga (22, 23). The following equilibrium constants are introduced. (*a*) $K_{(A)} = \{[(A,BH)] \cdot [H^+]/[(AH^+, BH)]\} = \{[(A,B^-)] \cdot [H^+]/[(AH^+, B^-)]\}$ is the dissociation constant for AH^+ when it is not perturbed by the interaction with B^-. (*b*) $K_{(B^-)} = \{[(A, B^-)]\ [H^+]/[(A, BH)]\} = \{[(AH^+, B^-)]\ [H^+]/[(AH^+, BH)]\}$ is the dissociation constant of the unperturbed group BH. (*c*) $K_{(AB)} = \{[(AH^+, B^-)]/[(AH^+ \ldots B^-)]\}$ is the dissociation constant for the intrinsic anion. (*d*) $K_{(L^-)} = \{[(AH^+, BH)]\ [L^-]/[(L^- \ldots AH^+, BH)]\} = \{[(AH^+, B^-)]\ [L^-]/[(L^- \ldots AH^+, B^-)]\}$ is the dissociation constant of the complex of AH^+ with L^-. (*e*) The isomeric equilibrium is close to 1 in PC. $K_{(AB)}$, $K_{(L^-)}$, and $K_{(A)}$ may be different for the all-*trans* and 13-*cis* forms. (*f*) Due to the lack of a detailed knowledge of the membrane's charge distribution and the position of the binding site, we introduce phenomenological parameters to take into account effects of membrane charge and ionic strength on the equilibria. For the protonation equilibria we write $K = K^0 \exp(\beta Z)$ (22). Z is the net charge of the binding site and β is an empirical electrostatic interaction factor. Z will be a function of pH. For simplicity a linear relationship is assumed: $Z = \epsilon(pH - pH_0)$. Z becomes zero at $pH = pH_0$, which for the binding site is equivalent to an isoelectric point. Because the charge is expected to become more negative with increasing pH, ϵ must be a negative factor. Thus we obtain $K = K^0 \exp(\beta\epsilon\,[pH - pH_0])$ or $K = K^0 \cdot ([H^+]/[H^+_0])^\alpha$, where $\alpha = \beta\epsilon\ \ln 10$ is a positive parameter which increases with decreasing ionic strength (22). Correspondingly, we put $K_{(L^-)} = K^0_{(L^-)}\ ([H^+_0]/[H^+])^\alpha$. For simplicity the parameter α is assumed to be equal for both anion and proton binding.

For clarity, the interpretation of the experimental results within the model above is discussed separately with respect to: (*a*) the protonation state of the various chromophores, (*b*) the temperature dependence of the equilibria, (*c*) the equilibria PC $\overset{I}{\rightleftharpoons}$ 605-nm chromophore $\overset{II}{\rightleftharpoons}$ PC_a and their relation to anion binding, and (*d*) the isomer equilibrium in the various chromophore states.

(*a*) The results on the protonation state of BR-605 and BR-500 are in accord with the model. At room temperature BR-605 and BR-500 appear at pH values which are sufficiently separated from each other so that only one form of each chromophore, i.e., (AH^+, BH) of the 605-nm chromophore and (A, B^-) of the 500 nm chromophore, will be present at a significant concentration. Therefore BR-605 binds one proton more and BR-500 binds one proton less than BR-PC.

(*b*) With increasing temperature, the pH range in which PC can exist is attenuated. This may simply be due to an increase of the intrinsic dissociation constant K_{AB} with rising temperature, as is illustrated in Fig. 10, for a selected choice of the parameters $K_{(A)}$ and $K_{(B^-)}$. A value of $\sim 10^{-8}$ M for $K_{(A)}$ is derived from a spectroscopic titration of the Schiff base (unpublished observation) which is obtained after destruction of the chromophore at high pH (see Fig. 1 *b*). If a value of $10^{-5.5}$ M for $K_{(B^-)}$ is chosen, the maximal stability of PC at pH 7 is accounted for. A value of 10^{-3} at room temperature for $K_{(AB)}$ has to be chosen because the 605-nm chromophore arises at pH 3. For the qualitative interpretation we neglect effects of the charge *Z*. The temperature dependence of $K_{(AB)}$ may be related to the temperature-dependent conformational change of protein and membrane structure proposed by Jackson and Sturtevant (18). This structural change may well increase the distance between AH^+ and B^- and thus lessen the interaction of these groups, i.e., cause an increase in $K_{(AB)}$. On the other

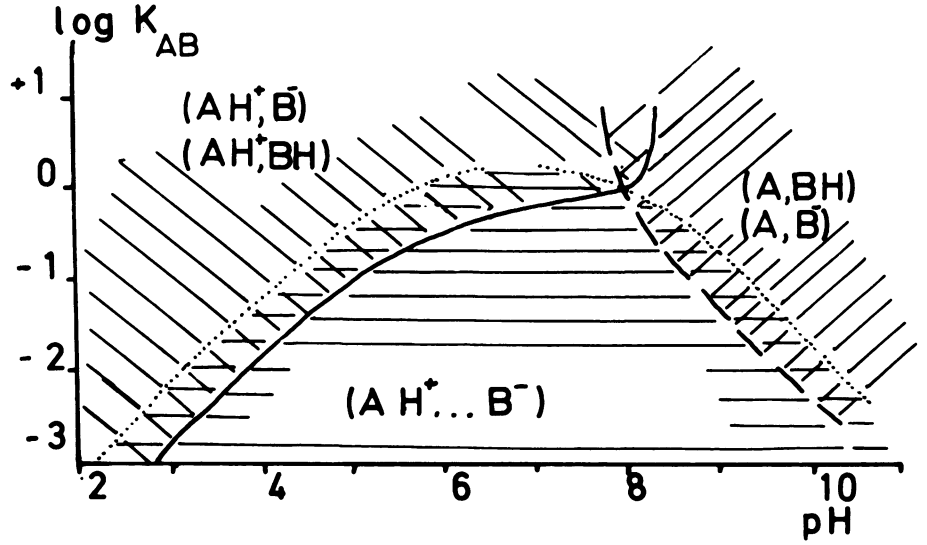

FIGURE 10 pH range for the existence of the various chromophores. At the indicated lines the concentration (—) [(AH^+, BH)] + [(AH^+, B^-)] of the 605-nm chromophore, (----) [(A, B^-)] + [(A, B H)] of the 500-nm chromophore, (····) [($A^+H...B^-$)] of PC is calculated to be one third of the total chromophore concentration; $K_{(B^-)} = 10^{-5.5}$ M and $K_{(A)} = 10^{-8}$M. Within the areas limited by the lines, the concentration of the inscribed species is more than one third of the total concentration.

hand, a rupture of this interaction by protonation of B^- or deprotonation of AH^+ could in turn favor the high-temperature structure and lead to the observed decrease in the transition temperature range for the cooperative change of membrane structure (see Fig. 8 *b*).

(*c*) An apparent dissociation constant, $K'_{(B^-)} = K_{(B^-)}/K_{(AB)}$, describes the transition $(AH^+...B^-) \underset{\rightleftharpoons}{I} (AH^+, BH)$ with sufficient accuracy because $K_{(AB)}$ is small and (A, B^-) does not contribute in the pH-range of this transition. The transition between the 605-nm chromophore and PC_a is determined by $K_{(L^-)}$. With the assumption of an isoelectric point of the binding site at pH 1.5, theoretical curves for the dependence of the equilibria on pH, ionic strength, and anion (L^-) concentration can be calculated and are shown in Fig. 11. The experimental results shown in Fig. 3 compare well with the characteristic features of our model.

The pH range for the transition from $(AH^+...B^-)$ to (AH^+, BH) is shifted to more acidic pH values with increasing ionic strength at constant concentration of the anion L^- (Fig. 11 *a*). This influence of ionic strength is due to the effect of the charge Z on the dissociation constant $K_{(B^-)}$, and may well account for the influence of ionic strength on the 605-nm chromophore $\underset{\rightleftharpoons}{I}$ PC equilibrium. With increasing concentration of L^- PC_a is formed (Fig. 11 *c*). Due to protonations the binding site becomes more positively charged with lower pH and therefore anion binding increases (Fig. 11 *b*).

(*d*) Most interestingly, the isomer equilibrium shifts with the chromophore equilibria. Within the model, a difference in the dissociation constants $K_{(AB)}$ and $K_{(L^-)}$ for the different isomers is likely to be the reason for these shifts in the isomer equilibria. Thus, in the 605-nm

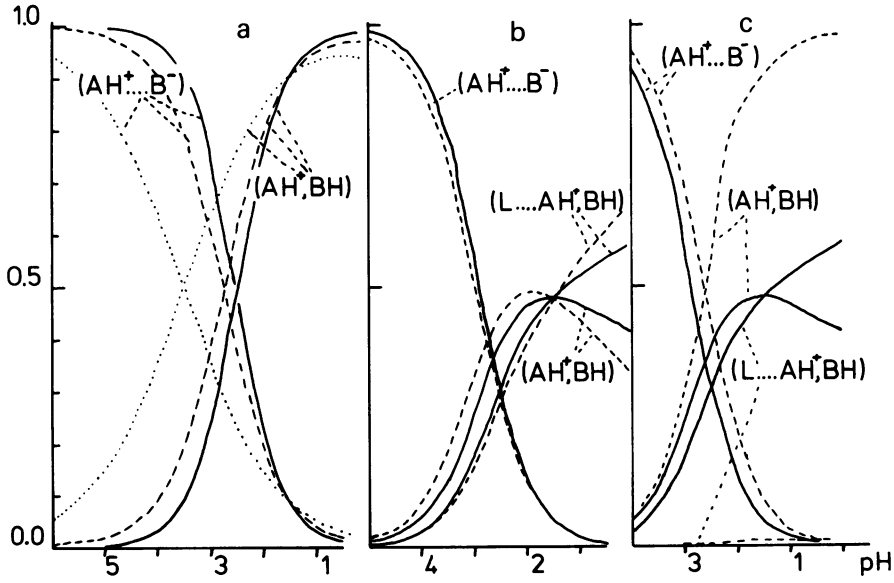

FIGURE 11 pH dependence of the $(AH^+...B^-) \underset{\rightleftharpoons}{I} (AH^+, BH) \underset{\rightleftharpoons}{II} (L^-...AH^+, BH)$ equilibria. $K'_{(A)} = 10^{-2.5}$ M; $K_{(L^-)} = 1$ M; $pH_0 = 1.5$. (*a*) $[L^-] = 10^{-2}$ M; (—)$\alpha = 0$; (----) $\alpha = 0.2$; (····) $\alpha = 0.5$. (*b*) $[L^-] = 1$ M; (—) $\alpha = 0.1$; (----) $\alpha = 0.2$. (*c*) (—) $[L^-] = 1$ M, (----) $[L^-] = 10^{-2}$ M; $\alpha = 0.1$. $K'_{(A)}$ is the dissociation constant for the protonation equilibrium I; $K_{(L^-)}$ is the dissociation constant of the anion binding equilibrium II for the anion L^-; α is a positive empirical parameter that increases with decreasing ionic strength; pH_0 is the isoelectric point. For further explanations see text.

chromophore where no interaction is assumed, Q = [all-*trans*]/{[(all-*trans*)] + [(13-*cis*)]} ~ 0.7 is found, a value which incidentally also was shown to hold for free retinal (8). Thus $K_{(AB)}$ (all-*trans*) > $K_{(AB)}$ (13-*cis*) could be the reason for the shift of isomer equilibrium in the PC and $K_{(Cl^-)}$ (all-*trans*) < $K_{(Cl^-)}$ (13-*cis*) for the shift in the isomer equilibrium of $PC_a^{Cl^-}$. A dominance of all-*trans* retinal would also be expected for the 500-nm chromophore; this has yet to be checked.

As a concluding remark we point out that the observed anion binding may also be of importance for the light-induced transport process associated with the photochemical cycle of BR. The anions may serve as mobile carriers for a proton transport from the Schiff base to the external water phase. If the sequence of strength in anion binding also holds for OH^-, one has to assume that OH^- has a high affinity to the binding site.

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