CHANGES IN THE PROTONATION STATE OF BACTERIO-OPSIN DURING RECONSTITUTION OF BACTERIORHODOPSIN

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ABSTRACT Protonation changes of the protein occur during the reconstitution of bacteriorhodopsin from bacterio-opsin and all-*trans* retinal in the purple membrane of *Halobacterium halobium*. The protonation changes are conveniently determined from measurement of the pH changes after photoisomerisation of 9-*cis* retinal in apomembrane preparations, which induces the reconstitution. In addition to the ϵ-amino group of the lysine which is involved in the condensation of retinal and bacterio-opsin, the dissociation equilibria of at least two other amino acid residues are changed during the reconstitution. The results are consistent with a proposed model of chromophore structure, in which an interaction of the Schiff's base occurs with two protonable amino acid residues.

INTRODUCTION

By condensation of all-*trans* or 13-*cis* retinal with bacterio-opsin, the functional chromophore is formed in the purple membrane (PM)[1] of *Halobacterium halobium*. This chromophore, which is called the purple complex (PC), consists of a protonated (1) Schiff's base between retinal and the ϵ-amino group of a lysine residue (2). Theoretical models for the redshifted absorption maximum of retinal-protein complexes have been suggested (e.g., in reference 9) and a proposal for the structure of the PC, based on spectroscopic and titration experiments, was recently given (3). There the protonated Schiff's base is suggested to interact with two protonable groups of the protein, and these interactions satisfactorily explain the pH and temperature dependent color shifts of the bacteriorhodopsin chromophore. To test this model of chromophoric structure, we have investigated the difference in the protonation state of the purple membrane and its chromophore free counterpart, called the apomembrane (4). This can be done best by using apomembrane prepared as in reference 5 with the binding site occupied by 9-*cis* retinal which is photoisomerized to all-*trans* retinal to induce reconstitution of the PC (5). Changes in the protonation state of the membrane during reconstitution are determined from light induced pH changes of apomembrane suspensions containing 9-*cis* retinal.

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[1] *Abbreviations used in this paper:* AM(*hν*), apomembrane prepared with light and without NH_2OH; AM(NH_2OH), apomembrane prepared with light and NH_2OH; Δh, number of protons bound per chromophore formed; $=(N^+H)-$, protonated Schiff's base between retinal and bacterio-opsin; PC, purple complex; PM, purple membrane.

MATERIALS AND METHODS

PM was prepared as described (6). 9-*cis* retinal was obtained from Sigma Chemie GmbH (Muenchen, West Germany) and purified by thin layer chromatography before use. Irradiating light was filtered by means of broad band dielectric interference filters (Hugo Anders KG, Nabburg, F.R.G.); the 655- to 685-nm and the 355- to 385-nm bands were selected as red and blue light, respectively. Apomembrane (AM) was prepared from PM either by irradiation in the presence of NH_2OH (AM[NH_2OH]) as described in reference 4 or by light induced release of 9-*cis* retinal (AM[$h\nu$]) as described in reference 5. AM($h\nu$) was prepared by first irradiating a stirred PM suspension (2 ml, 30 μM bacteriorhodopsin, 25°C, pH 2.8) for 80 min with focused red light of a 900-W, high pressure Xenon lamp (XBO 900, Osram AG, Muenchen), with a 500 mW/cm light intensity hitting the front of the cylindrical vessel. By this procedure a 9-*cis* chromophore is produced (5). The following steps were performed in the dark. After several washes by centrifugation for 30 min at 50,000 *g* with distilled water, to remove the acid, the suspension was kept for 4 h at 35°C to release 9-*cis* retinal from the binding site (5). Measurements of pH were performed with aliquots of 0.8 ml of AM (20 μM, 0.1 M KCl, 18°C) which were flushed with nitrogen in a small thermostated cylindrical glass vessel to which a pH microelectrode (LOT-402-M3-6235, Dr. W. Ingold KG, Frankfurt, West Germany) was fitted by a ground glass stopper. The pH value of the stirred suspension was plotted continuously on a chart recorder (type Servogor, Metrawatt AG, Nuernberg, West Germany) connected to the pH meter (type PW 9413, Philips Electronic Instruments Inc., Mahwah, N.J.). To AM (NH_2OH) suspensions 15 μl of a 0.9 mM solution of 9-*cis* retinal in *i*-propanol was added under otherwise identical conditions. ~20 Min after adjustment of the desired pH by addition of 0.1 M HCl or NaOH the pH drift had settled to a small and constant value. Reconstitution of the PC was started by irradiation of the sample with the focused blue light of a 200-W, high pressure Hg-Xe lamp (type 901B-11, Engelhard Hanovia Inc., Newark, N.J.) with a 50 mW/cm intensity at the front of the vessel. Care was taken that no direct light hit the Ag/AgCl reference electrodes. When the light induced pH change (ΔpH) had reached a constant value irradiation was stopped and the proton concentration change $\Delta(H^+)$ determined from the calibration of ΔpH on addition of 10 mM HCl or NaOH (1–3 μl). Absorption spectra were taken before and after reconstitution and the concentration change Δc(PC) of the chromophore was calculated assuming an extinction coefficient $\epsilon = 6.3 \times 10^4\ cm^{-1}\ M^{-1}$ at the absorption maximum of 568 nm (7). The number of protons bound per chromophore was obtained as $\Delta h = \Delta(H^+)/\Delta c$ (PC). It should be mentioned that for unknown reasons, photoisomerisation of 9-*cis* retinal in AM($h\nu$) proceeds only with 50% yield in suspensions whereas almost 100% yield are found for acrylamide gel-embedded preparations (5).

RESULTS AND DISCUSSION

Typical pH traces obtained with AM($h\nu$) suspensions are shown in Fig. 1 for two selected pH values. Protons are taken up by the membrane above pH 5.8 and are released below this pH value. No accurate determination of pH changes was possible above pH 9.5 or below pH 3.5. There, due to the buffering of the suspension, pH changes become small and indistinguishable from pH drift. pH traces similar to those as shown in Fig. 1 were also obtained with AM(NH_2OH) suspensions above pH 6. The pH dependence of Δh is shown in Fig. 2. At pH 8, Δh approaches the value of $+0.8$ and at pH 4 it is close to -0.65 for AM($h\nu$) suspensions. Above pH 8 Δh declines towards zero. The pH dependence of Δh for AM(NH_2OH) is similar as for AM($h\nu$) above pH 6. Proton release below pH 5.8 could not be ascertained for AM(NH_2OH) suspensions because, in contrast to AM($h\nu$), AM(NH_2OH) is liable to photodestruction during reconstitution by blue light below pH 5, leading to further irreversible protonation changes. Therefore, low levels of irradiation have to be applied for an appreciable reconstitution of PC. In this case, however, light induced pH changes become very slow and hardly distinguishable from pH drift. We believe these differences in the properties

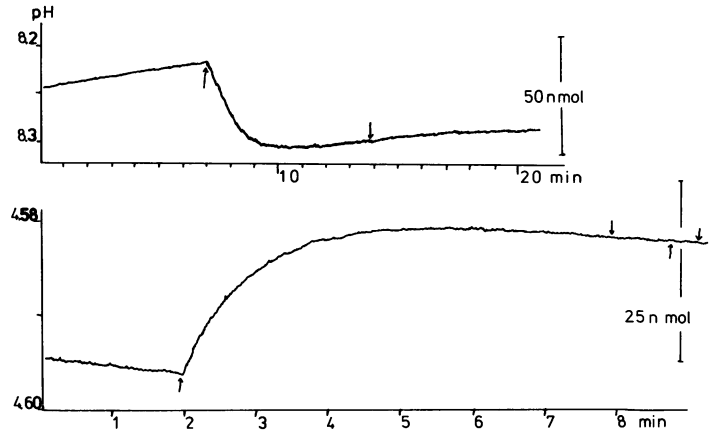

FIGURE 1 Continuous traces of the pH changes occurring on the light induced reconstitution of PM from AM(*hν*) suspensions at two selected pH values. Measurements were performed as described in Materials and Methods. Bars indicate pH changes due to addition of the given amount of acid or base after reconstitution. Arrows indicate onset (↑) and stop (↓) of irradiation.

of the AM(NH_2OH) and AM(*hν*) systems to be due to the retinal oximes which are still present in the PM reconstituted from AM(NH_2OH) as a membrane structure perturbing agent. In PM reconstituted from AM(*hν*) no such agents are present.

The results presented here clearly demonstrate that the dissociation equilibria of at least two groups are changed during the reconstitution reaction, which explains a change in sign of Δh (Fig. 2). One is tempted to assume that one of these groups is the ϵ-amino group of the lysine which condenses with retinal to form the Schiff's base of the PC. One could explain

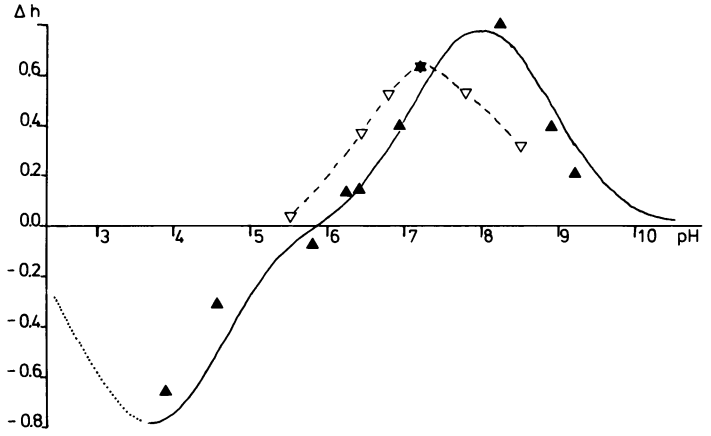

FIGURE 2 The pH dependence of Δh. Δh was determined for AM(*hν*) (▲) and AM(NH_2OH) (▽) suspensions from measurements as shown in Fig. 1. by the procedure which is described in Materials and Methods. The solid line represents Δh values which were calculated on the basis of the proposed protonation states of AM and PM (see Fig. 3) from values for dissociation constants as given in the text. The dotted line represents calculated values extended beyond the experimentally accessible pH-range. No significance is assigned to the broken line.

proton uptake above pH 6 if the lysine in AM would have an unusually low pK value (pK 7) as compared to a lysine in solution (pK 10.5). Condensation with retinal to form the protonated Schiff's base of the PC, which is stable up to pH 12, would then lead to proton uptake between pH 6 and 12. Several reasons lead us to reject this possibility. Both the protonated Schiff's base of the PC (pK > 12) and of heat denatured bacteriorhodopsin (pK ≈ 8, unpublished result) have a higher pK than a model Schiff's base (pK = 5.7 [11]). There is strong evidence that stabilization of the protonated Schiff's base is partly brought about by an interaction with anionic side groups of the protein (3). It is likely that the protonated ϵ-amino group of the lysine in AM has a similar local environment as the Schiff's base and therefore is stabilized by the same kind of interactions. This is also expected from the high negative charge density of the purple membrane (12). For these reasons it is unlikely that the lysine has an unusually low pK in AM. The observed protonation changes are then most likely due to shifts in the dissociation equilibria of at least two amino acid residues of the bacterio-opsin molecule other than the ϵ-amino group of that lysine. Furthermore, if an interaction of two protonable groups with the protonated Schiff's base in the ground state of the chromophore is essential for its structure, as was suggested by Fischer and Oesterhelt (3), a change in the pK of the same groups must contribute to overall changes in the protonation state of the protein during reconstitution. Thus we are lead to test whether the protonation changes during reconstitution are consistent with their being only due to changes in the dissociation equilibria of the two amino acid residues, which are involved in the proposed chromophore structure, irrespective of their as yet unestablished chemical identity.

The scheme of Fig. 3 compares our proposal for protonation states of AM and PM with respect to the lysine moiety, the protonable groups A'^-, B^-, and the protonated Schiff's base represented by the symbol $=(N^+H)-$ (A^+H in reference 3). This proposal explains the data presented here and is consistent with the protonation states of the chromophore according to our model of chromophore structure presented recently (3). Qualitatively, the interaction of

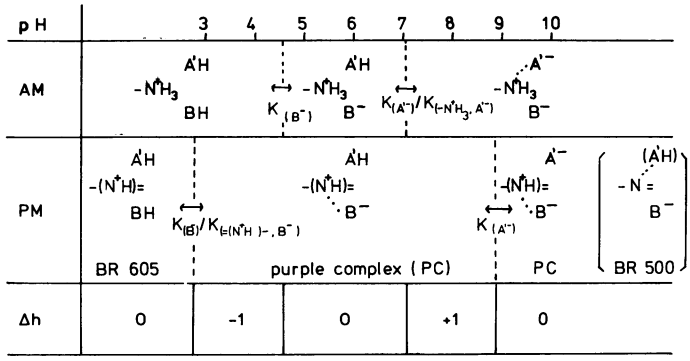

FIGURE 3 The proposed structures and their protonation states of the retinal binding site in AM and of the chromophore in PM. The structure of the chromophore and of the retinal binding site are made up of the ϵ-amino group of the lysine or the Schiff's base together with the surrounding groups A'^- and B^-, whose spatial arrangement is arbitrarily chosen. The structures are identical to the ones of reference 3 and are correspondingly identified. Two tautomeric alkaline chromophores, PC and the 500-nm chromophore, are supposed to coexist in an equilibrium which is not further specified. The dissociation constants are inserted at pH values where protonations occur. The differences in the protonation states of the binding site and of the chromophore is indicated in the last line.

B^- with $=(N^+H)-$ lowers the pK of BH in PM and the dissociation of A'H is facilitated in AM due to the interaction with $-N^+H_3$. The chromophore exists in two tautomeric forms at alkaline pH which are spectroscopically distinguishable. An alkaline form of the PC, (A'^-, $=(N^+H)- \ldots B^-$) is assumed to have the same absorption spectrum as its neutral form (A'H, $=(N^+H)- \ldots B^-$), whereas the form (A'H $\ldots$ $=N-$, B^-) represents the 500-nm chromophore ($\lambda max = 500$ nm). The scheme of Fig. 3 explains qualitatively the pH dependence of Δh as found experimentally and is also consistent with our previous assumption that one proton is bound in the transition PC $\rightarrow$ BR-605 at pH 2.8 (3).

An attempt was made to reproduce this dependence quantitatively as shown in Fig. 2 by the solid line connecting the experimental points on AM($h\nu$), which is extended to acidic pH as a dotted line, to cover the range for the transition from PC to BR-605. For this calculation we assigned to the relevant dissociation constants (for definitions see references 3, 10) the following numerical values:

$$K(A'^-) = \frac{[A'^-][H^+]}{[A'H]} = 10^{-8.9}\ M;\ K(B^-) = \frac{[B^-][H^+]}{[BH]} = 10^{-4.6}\ M.$$

The intrinsic dissociation constants for the complex between the ϵ-amino group of the lysine and A'^- and for the complex between $=(N^+H)-$ and B^- in PM are for simplicity chosen to be equal:

$$K(-N^+H_3, A'^-) = \frac{[(-N^+H_3, A'^-)]}{[(-N^+H_3 \ldots A'^-)]} = K = (N^+H)-, B^-)$$

$$= \frac{[= (N^+H)-, B^-]}{[=(N^+H)- \ldots B^-]} = 10^{-1.8}.$$

On the basis of these figures the net change in protonation during reconstitution is reproduced over the full range of the experimentally obtained data. The same set of dissociation constants with the same range of numerical values may be applied for a description of chromophore and protonation equilibria of bacteriorhodopsin in a similar way as previously attempted (3). Thus our results support the chemical structure of PC suggested in (3).

In the scheme of Fig. 3, the protonated Schiff's base is stabilized by its interaction with B^-. A value for the dissociation constant $K[=(N^+H)-] = 10^{-10.2}$ M of the hypothetical unstabilized Schiff's base is predicted if a dissociation constant $K[=(N^+H)- \ldots B^-] = 10^{-12}$ M is tentatively assumed for the stabilized Schiff's base. This is not an unreasonable value if compared to the pK = 8 of the Schiff's base in heat denatured bacteriorhodopsin which was mentioned above.

An important point in the model of chromophore structure presented here is that an alkaline form of the PC, (A'^-, $=(N^+H)- \ldots B^-$), exists in a tautomeric equilibrium with a 500-nm chromophore, (A'H $\ldots$ $=N-$, B^-), explaining that a proton release from bacteriorhodopsin may occur with or without a concomittant change in the absorption spectrum in the chemical reactions: (A'H, $=(N^+H)- \ldots B^-$) $\rightarrow$ (A'^-, $=(N^+H)- \ldots B^-$) + H^+ and (A'H, $=(N^+H)- \ldots B^-$) $\rightarrow$ (A'H $\ldots$ $=N-$, B^-) + H^+, respectively. At present, no detailed description can be given of the equilibrium between the PC and the 500-nm chromophore

which was shown to be associated with a complex transition in protein or membrane structure (3), a description of which is not within the scope of our simple model.

Finally, we point out that the protonation equilibria which are affected in the reconstitution reaction seem to play an important role in the light induced protonation change of bacteriorhodopsin during its photochemical cycle. Evidence for this suggestion is obtained from a determination of the number of protons released or taken up by bacteriorhodopsin in the photostationary state, at light saturation of the photochemical cycle. This will be the subject of a further publication.

Note added upon revision: A referee pointed out that Honig et al. (13) proposed a similar model for chromophore structure which differs from the one of Blatz et al. (9) by having the counterion at a distance of 3 Å instead of 10 Å from the Schiff's base. We have no means of discriminating between the two on the basis of our results. Honig et al. (13) suggest that a charge separation at the Schiff's base is responsible for color changes during the primary events of the photochemical cycle. In a previous paper (14) they stated that the position of the same anionic group is invariant in the ground state and that color modulations between different photoreceptors are brought about by variations in the distance of a second anion "pulling the positive charge into the chain." The results presented in our previous paper (3) and in this paper suggest, however, that a variation of the distance of the counterion to the Schiff's base and of its protonation state determine color modulations of the ground state of the chromophore, whereas no implications were made concerning the primary events of the photochemical cycle.

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REFERENCES

1. LEWIS, A., J. SPOONHOWER, R. BOGOMOLNI, R. LOZIER, and W. STOECKENIUS. 1974. Tunable laser resonance Raman spectroscopy of bacteriorhodopsin. *Proc. Natl. Acad. Sci. U.S.A.* **71**:4462–4466.
2. OESTERHELT, D., and W. STOECKENIUS. 1971. A rhodopsinlike protein from the purple membrane of *Halobacterium halobium*. *Nat. New Biol.* **233**:149–152.
3. FISCHER, U. CH., and D. OESTERHELT. 1979. Chromophore equilibria in bacteriorhodopsin. *Biophys. J.* **28**:211–230.
4. OESTERHELT, D., L. SCHUHMANN, and H. GRUBER. 1974. Light dependent reaction of bacteriorhodopsin with hydroxyleamine in cell suspensions of *Halobacterium halobium:* demonstration of an apo-membrane. *FEBS Lett.* **44**:257.
5. FISCHER, U. CH., P. TOWNER, and D. OESTERHELT. 1979. Photoreversible release of 9-*cis* retinal from bacteriorhodopsin. Manuscript submitted for publication.
6. OESTERHELT, D., and W. STOECKENIUS. 1974. Isolation of the cell membrane of *Halobacterium halobium* and its fractionation into red and purple membrane. *Methods Enzymol.***31A**:667–678.
7. OESTERHELT, D., and B. HESS. 1973. Reversible photolysis of the purple complex of *Halobacterium halobium*. *Eur. J. Biochem.* **37**:316–326.
8. SCHRECKENBACH, T., B. WALCKHOFF, and D. OESTERHELT. 1977. Studies on the retinal-protein interaction in bacteriorhodopsin. *Eur. J. Biochem.* **76**:499–511.
9. BLATZ, P. E., I. H. MOHLER, and H. V. NAVANGUL. 1972. Anion induced wavelength regulation of absorption maxima of Schiff bases of retinal. *Biochemistry.* **11**:848.
10. LASKOWSKI, M., JR., and H. A. SCHERAGA. 1954. Thermodynamic considerations of protein reactions. I. Modified reactivity of polar groups. *J. Am. Chem. Soc.* **76**:6305.

11. MORTON, R. A., and G. A. J. PITT. 1955. Studies on rhodopsin. IX. pH and the hydrolysis of indicator yellow. *Biochem. J.* **59**:128.
12. NEUGEBAUER, D., Ch., D. OESTERHELT, and H. P. ZINGSHEIM. 1978. The two faces of the purple membrane. II. Differences in surface charge properties revealed by ferritin binding. *J. Mol. Biol.* **125**:123.
13. HONIG, B., T. EBREY, R. H. CALLENDER, U. DINUR, and M. OTTOLENGHI. 1979. Photoisomerisation, energy storage, and charge separation: a model for light energy transduction in visual pigments and bacteriorhodopsin. *Proc. Natl. Acad. Sci. U.S.A.* **76**:2503–2507.
14. HONIG, B., A. D. GREENBERG, U. DINUR, and T. EBREY. 1976. Visual-pigment spectra: implications of the protonation of the retinal Schiff base. *Biochemistry.* **15**:4593.