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Control of bacteriorhodopsin color by chloride at low pH. Significance for the proton pump mechanism

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The chromophore of bacteriorhodopsin undergoes a transition from purple (570 nm absorbance maximum) to blue (605 nm absorbance maximum) at low pH or when the membrane is deionized. The blue form was stable down to pH 0 in sulfuric acid, while 1 M NaCl at pH 0 completely converted the pigment to a purple form absorbing maximally at 565 nm. Other acids were not as effective as sulfuric in maintaining the blue form, and chloride was the best anion for converting blue membrane to purple membrane at low pH. The apparent dissociation constant for Cl^- was 35 mM at pH 0, 0.7 M at pH 1 and 1.5 M at pH 2. The pH dependence of apparent Cl^- binding could be modeled by assuming two different types of chromophore-linked Cl^- binding site, one pH-dependent. Chemical modification of bacteriorhodopsin carboxyl groups (probably Asp-96, -102 and/or -104) by 1-ethyl-3-dimethlyaminopropyl carbodiimide, Lys-41 by dansyl chloride, or surface arginines by cyclohexanedione had no effect on the conversion of blue to purple membrane at pH 1. Fourier transform infrared difference spectroscopy of chloride purple membrane minus acid blue membrane showed the protonation of a carboxyl group (trough at 1392 cm^{-1} and peak at 1731 cm^{-1}). The latter peak shifted to 1723 cm^{-1} in D_2O. Ultraviolet difference spectroscopy of chloride purple membrane minus acid blue membrane showed ionization of a phenolic group (peak at 243 nm and evidence for a 295 nm peak superimposed on a tryptophan perturbation trough). This suggests the possibility of chloride-induced proton transfer from a tyrosine phenolic group to a carboxylate side-chain. We propose a mechanism for the purple to acid blue to chloride purple transition based on these results and the proton pump model of Braiman et al. (Biochemistry 27 (1988) 8516–8520).

Introduction

Purple membrane from *Halobacterium halobium* contains the proton pump bacteriorhodopsin [1]. Absorption of light by the retinal chromophore activates this simple ion pump. A cycle of photochemical intermediates has been identified, and many studies have examined the relationship between the photocycle and the proton pump (for a recent example, see Ref. 2). Two photointermediates are red-shifted from the initial 570 nm chromophore, thus drawing one's attention to another red-shifted form of purple membrane called blue membrane (absorbance maximum = 605 nm), which is stable in the dark. Blue membrane may be prepared by acidification [3–6] or deionization [7,8] of purple membrane. Although blue membrane is photochemically active [5], it fails to pump protons [9]. Much interest has centered on the question of the mechanism of formation of the blue membrane, with some experiments pointing to the importance of a surface effect [10] and others suggesting direct involvement of a single titratable group [11].

Remarkably, the acidified blue membrane may be restored to a purple form by treatment with halide [6]. Occasionally, this anion specificity has been attributed to very high acidity, without separation of anion and proton effects [5,12]. The low-pH halide purple chromophore appears to be nearly identical to the neutral-pH purple form [12]. This places severe constraints on models for the chemical environment of retinal in bacteriorhodopsin. We have reexamined the low-pH blue-to-purple transition with the objective of relating this effect to the mechanism of the bacteriorhodopsin proton pump. The results reported in this paper are consistent with a specific halide binding interaction, and

Abbreviations: EDC, 1-ethyl-3-dimethylaminopropyl carbodiimide; FTIR, Fourier-transform infrared.

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tend to support a recently proposed pump mechanism [13].

Experimental methods

Materials. Purple membrane was isolated from *Halobacterium halobium* S9 by the method of Oesterhelt and Stoeckenius [14]. All salts and acids were reagent grade, except trifluoroacetic acid, which was Sequanal grade (Pierce Chemical Co.).

Absorption spectra of purple membrane in polyacrylamide gels. Gels containing purple membrane were cast and spectra measured as previously described [15]. Spectra were measured primarily on a Cary 14 instrument, or on a Cary 14 modified by Aviv Associates. The mole fraction of bacteriorhodopsin in the purple form, X_p, was calculated from spectra of the pure purple form and the pure blue form. Using the molar extinction measured at three wavelengths for the two forms, we obtained the following expression:

$$X_p = 1.344\,(A_{550}/A_{590}) - 0.813\,(A_{640}/A_{590}) - 0.644 \quad (1)$$

where A refers to absorbance at the wavelength indicated in the subscript.

Chemical modification. Purple membrane was modified as previously described with EDC [16], dansyl chloride [17] or cyclohexanedione [18].

Ultraviolet difference spectra. Ultraviolet difference spectra were measured with the Aviv-Cary 14 instrument. Spectra of purple and blue pigments were obtained individually and subtracted by computer to produce difference spectra. Samples were prepared as films air-dried on the quartz window of a cylindrical cell. The films had absorbance at 570 nm of about 0.8. The film was bathed in 1 M H_2SO_4, drained of excess fluid, and sealed with a circular CaF_2 window before the reference spectrum was measured. Subsequently, the same film was bathed in 1 M H_2SO_4 containing 1 M NaCl, drained and sealed, and the sample spectrum was measured.

Fourier transform infrared spectroscopy. Films of purple membrane were dried under a slow stream of nitrogen on a circular silicon window. The films had absorbance of approx. 2 for the amide I vibration. Small drops of H_2SO_4 were gently applied to the surface of the film and carefully spread with the pipet tip over the surface of the film without actually touching the pipet tip to the surface. Additional drops were added if necessary to cover the entire film and convert it to the blue form. Excess sulfuric acid was drained from the film by tilting and removing with a pipet. The window was then mounted in a cylindrical infrared cell and the open end was sealed with a piece of stretched parafilm or a CaF_2 window. The spectrum was measured with a Mattson Polaris instrument. The cell was then removed and drops of 1 M sulfuric acid containing 1 M NaCl were applied to the film as described above. After sealing, another spectrum was measured. The difference spectrum was obtained by computer subtraction. The quality of the resulting difference spectra is obviously less than published spectra in which the difference is achieved by irradiation, where the sample is not disturbed.

For measurement in D_2O, the purple membrane was centrifuged and resuspended in D_2O several times. The final pellet was resuspended in D_2O (pH $\approx$ 6) at a bacteriorhodopsin concentration of 0.1 mM and equilibrated at 5 °C for 18 h in the dark. Prior to preparing a film for FTIR spectra, the sample was equilibrated at pH 10 [18a] (titrated with NaOD) for 20 min. Films were prepared as described above except the H_2SO_4 and NaCl were dissolved in D_2O.

Results

Effect of anions on blue membrane

Purple membrane in polyacrylamide gels was equilibrated with a variety of acids to explore the specificity of the halide effect. The results, summarized in Table I, are similar to those reported by Fischer and Oesterhelt [6]. A significant new finding is that the acid and chloride effects can be separated by using H_2SO_4. Blue membrane is very stable in H_2SO_4, even at pH 0 for 24

TABLE I

Effect of various acids and salts on blue-to-purple transition

Acidified membrane			
Concentration of H_2SO_4 (M)	Other acids or salts	Concentration (M)	Mole fraction purple [a]
1	–	–	0.0
1	NaCl	1	1.01
1	KBr	1	0.69
1	NaI	1	0.45
1	$KClO_3$	1	0.45
–	NH_2SO_3H	1	0.03
–	HNO_3	1	0.44
–	trifluoroacetic	1	0.68
–	trichloroacetic	1	– [b]
–	trichloroacetic	0.01	0.24
–	0.01 M trichloroacetic + 1 M NaCl		0.60

Deionized membrane	Mole fraction purple [a]
Deionized blue membrane	0.0
+ 100 mM Na_2SO_4	1.04
+ 10 mM Na_2SO_4	0.61
+ 20 mM NaCl	0.70
+ 10 mM NH_4F	0.98

[a] Mole fraction purple calculated from Eqn. 1.
[b] Absorbance maximum = 450 nm.

h. By contrast, $HClO_4$ and HNO_3 tend to slowly bleach the chromophore. Blue membrane is also stable in 1 M amidosulfonic acid (pH 1.5). Strong organic acids are unable to maintain the blue membrane at low pH. Trifluoroacetic acid at a concentration of 1 M appears to act in a manner similar to HCl (i.e., regenerates the purple chromophore), whereas 1 M trichloroacetic acid converts the membrane to a 450 nm absorbing yellow pigment.

Various anions were added to purple membrane equilibrated with 1 M H_2SO_4. At a concentration of 1 M, NaCl completely converts blue membrane to the low-pH purple membrane. Bromide, iodide and chlorate all cause incomplete conversion. The effect of fluoride is dificult to study because it is a weak acid. However, at neutral pH, it is essentially fully dissociated. Therefore, we compared the conversion of deionized blue membrane to the purple form by Na_2SO_4, NH_4F and NaCl. The results (Table I) are complicated by the competing effect of the cations discharging the surface potential, which also converts the blue pigment to purple. At a concentration of 100 mM, sodium sulfate completely regenerates purple membrane, while only partial conversion is observed at 10 mM. Sodium chloride at a concentration of 10 mM also partially converts blue membrane to purple. Presumably most of this conversion is due to an increase in the surface pH. By contrast, 10 mM ammonium fluoride completely converts the membrane back to the purple form. These results show that the halide purple pigment is observable in deionized membrane, and that fluoride is more effective than chloride at the conversion of blue to purple.

Apparent dissociation constant of Cl^-

We measured the fraction of purple chromophore, X_p, at varying concentrations of NaCl at pH 0 (1 M H_2SO_4), pH 1 (0.1 M H_2SO_4) and pH 2 (10 mM NH_2SO_3H). The results are shown in Fig. 1. The data appears to follow hyperbolic curves (solid lines) when interpreted as chromophore-linked chloride dissociation from bacteriorhodopsin, suggesting saturation of a single type of non-interacting binding site. The apparent dissociation constants vary strongly with pH, from 35 mM at pH 0, to 0.7 M at pH 1, to 1.5 M at pH 2. The pH dependence is described empirically by:

$$X_p = [Cl^-]/([Cl^-] + 0.7\,pH + 0.035) \quad (2)$$

Eqn. 2, with no adjustable parameters, generates essentially the same solid lines in Fig. 1 obtained from three independently adjusted dissociation constants.

We have attempted to describe the pH dependence in Fig. 1 in more chemically meaningful terms. An indirect type of pH dependence for a chloride-induced chromophore change might arise from surface charge effects. At low pH, the membrane becomes increasingly positively charged, resulting in an apparent increase in chloride binding due to the accumulation of counterions in the diffuse double layer at the membrane surface. In the simplest model of this type, we assume somewhere in the range of 4 to 7 positive charges per bacteriorhodopsin at pH 2. The initial surface charge was used to calculate the surface potential, which, in turn, permitted a calculation of ion binding. The ion binding led to a new value for the surface charge, and the iteration continued until successive rounds agreed to within 1%. Using one or two classes of sites titrating between pH 2 and 0, we obtained fits to the data in Fig. 1 similar to the dashed lines, but only when unreasonably large numbers of sites (50 to 100) were used. We have also considered a series of more complicated models in which surface charge alters the protein conformation by electric field effects on protein dipoles rather than by chloride binding. One of these models gave a satisfactory fit to the data similar to the dashed lines in Fig. 1. However, it requires a large excess of proton binding to the extracellular membrane surface at low pH, and there is no reason to expect this asymmetry.

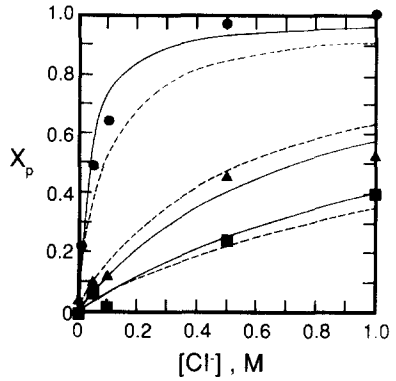

Fig. 1. Effect of chloride and pH on the blue-to-purple transition. Samples of purple membrane cast in polyacrylamide gels were equilibrated with acid and NaCl solutions at 23°C (±1°C) and absorbance spectra were measured from 700 to 400 nm. The mole fraction of purple chromophore (X_p) was calculated according to Eqn. 1. ●, pH 0 (1 M H_2SO_4); ▲, pH 1 (0.1 M H_2SO_4); ■, pH 2 (0.1 M NH_2SO_3H). Solid lines: data fit with apparent dissociation constants for a single chloride binding site: pH 0, 35 mM; pH 1, 0.7 M; pH 2, 1.5 M. Dashed lines: data fit with model for H^+ activation of chloride binding (Eqn. 6).

The pH dependence is not fit by a simple model in which proton binding precedes chloride binding to the same site:

$$PHCl \rightleftharpoons BH^+ + Cl^- \quad (3)$$

$$BH^+ \rightleftharpoons B + H^+ \quad (4)$$

In this model, PHCl is the halide purple pigment at acid pH, BH^+ is blue bacteriorhodopsin with the halide binding site protonated, and B is blue bacteriorhodopsin with the halide binding site unprotonated. This model consistently predicts far wider separation

between the apparent chloride dissociation constants than is observed. For example, when the pH 0 and 1 data in Fig. 1 is fit with lines similar to the solid lines, the predicted pH 2 line lies nearly on the x-axis. A similar model to Eqns. 3 and 4 using two obligatory independent proton binding steps instead of one does not improve the fit. Models combining surface charge effects with obligatory proton binding to the chloride site were also unsuccessful. These models actually have very small pH-dependent surface charge effects because the Boltzmann exponentials cancel in terms containing the product $[H^+][Cl^-]$.

A satisfactory fit is obtained with a model in which there is a chloride binding site directly linked to the chromophore change, but with two different binding constants, one pH-dependent. Eqns. 3 and 4 are combined with the following:

$$PCl^- \rightleftharpoons B + Cl^- \tag{5}$$

There are two purple forms, PCl^- and PHCl, and two blue forms, B and BH^+. The fraction of purple is given by:

$$X_p = \frac{[Cl^-](K_a K_{C2} + K_{C1}[H^+])}{[Cl^-](K_a K_{C2} + K_{C1}[H^+]) + K_{C1}K_{C2}(K_a + [H^+])} \tag{6}$$

where K_a, K_{C1}, and K_{C2} are, respectively, the dissociation constants in Eqns. 4, 5 and 3. The concentration terms in Eqn. 6 are the membrane surface concentrations, which are related to the bulk solution concentrations by the appropriate Boltzmann exponentials. The dashed lines in Fig. 1 were calculated from Eqn. 6 with $pK_a = -0.1$, $K_{C1} = 2.2$ M, and $K_{C2} = 0.061$ M. The surface potential was calculated iteratively from the surface charge density, which in turn was calculated from Cl^- and H^+ binding. For the purpose of calculating the surface charge density, it was assumed that, in addition to the H^+ binding site linked to Cl^- (Eqns. 3 and 4), there are 19 additional similar sites that are not linked to Cl^- binding. The constants are physically reasonable, but of course they cannot be uniquely determined from the data in Fig. 1.

Chemical modification studies

Previous experiments in this laboratory showed that the formation of acid blue membrane is inhibited by treatment of purple membrane with water-soluble carbodiimide [15]. We reacted purple membrane with EDC and found no subsequent effect of this modification on the amount of Cl^--induced blue-to-purple conversion. The results at pH 1 for EDC-modified membrane were superimposable on the data for unmodified blue membrane shown in Fig. 1. We also measured the chloride effect on blue-to-purple conversion after modification of purple membrane with dansyl chloride, which we previously showed specifically reacts with Lys-41 [17]. Like the EDC modification, there was no detectable inhibition of the chloride effect at pH 1. Finally, we modified purple membrane with cyclohexanedione [18]. Again, we found no effect on the chloride-induced blue-to-purple transition at pH 1.

FTIR difference spectroscopy of chloride-induced blue-to-purple transition

The difference spectrum of hydrated films of acid chloride purple membrane minus acid blue membrane is shown in Fig. 2. The spectrum clearly shows the expected change in the ethylenic stretch from blue (trough at 1510 cm^{-1}) to purple (peak at 1527 cm^{-1}). Other prominent features are a peak at 1731 cm^{-1} and a trough at 1392 cm^{-1}. This corresponds to the change of a carboxylate in the blue form (1392 cm^{-1}) to a carboxylic acid in the purple form (1731 cm^{-1}). These features were observed in 16 separate samples. However, between 1600 and 1700 cm^{-1}, interference from water vapor produced considerable variability in different samples. The sharp features between 1460 and 1475 cm^{-1} are due to the parafilm seal on the sample to keep it hydrated. Below 1300 cm^{-1}, sulfuric acid and the silicon windows interfere with the spectrum. As a result of these limitations, it is not possible to obtain information from the difference spectra about the protonation state of tyrosine residues.

Equilibration of purple membrane with D_2O at pH 10 prior to measurement of the FITR spectrum shifts the 1731 cm^{-1} line to 1723 cm^{-1} (not shown). This is consistent with the expected deuterium isotope shift of a carboxylic acid carbonyl vibration.

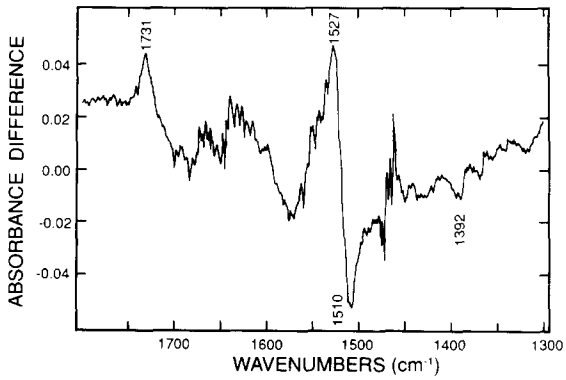

Fig. 2. FTIR difference spectrum, chloride purple membrane minus acid blue membrane. A purple membrane film dried on a silicon window was wetted with 1 M sulfuric acid (pH 0) and the acid blue membrane spectrum was measured. The same film was then wetted with 1 M sulfuric acid containing 1 M NaCl, and the chloride purple spectrum was measured. Spectra were averaged from 128 scans, 2 cm^{-1} resolution. Difference spectrum was obtained by computer subtraction.

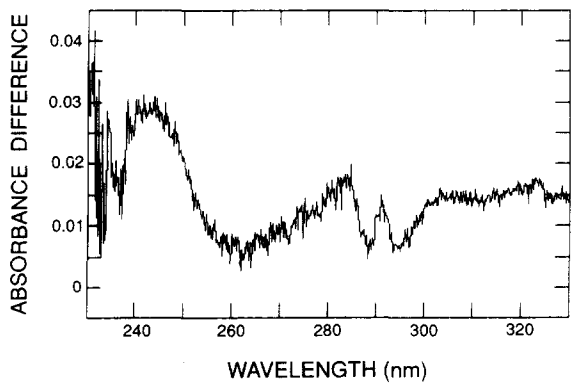

Fig. 3. UV difference spectrum, chloride purple membrane minus acid blue membrane. Similar to Fig. 2, except that the film was dried on a quartz window for measurement of the ultraviolet spectra.

Ultraviolet difference spectroscopy of chloride-induced blue-to-purple transition

The ultraviolet spectrum of acid chloride purple membrane minus acid blue membrane is shown in Fig. 3. The spectrum contains a peak at 243 nm and sharp negative features between 280 nm and 300 nm. The peak at 243 nm is distinctive [19] and suggests the deprotonation of a tyrosine residue along with the conversion of blue to halide purple membrane. A phenolate minus phenol difference spectrum would also be expected to have a smaller positive peak near 295 nm. The sharp troughs at 289 nm and 295 nm appear to be a tryptophan perturbation difference spectrum, which tends to obscure broader features in this region. However, the apparent peaks at 285 nm and 302 nm may be interpreted as the sides of a broad peak centered at 295 nm, from which the negative tryptophan features have been subtracted [20].

Discussion

Effect of anions on blue-to-purple transition

The anion selectivity does not reveal much information about the nature of the interaction with bacteriorhodopsin. Among the halides, the fraction of blue-to-purple conversion at pH 0, 1 M halide (Table I) correlates very well with ionic radius. However, other factors must be involved, since the oxyanions do not show any correlation of molar volume with fraction of conversion (Table I). For example, trifluoroacetate has about twice the molar volume of sulfate, but trifluoroacetate is about as effective as the much smaller bromide in blue-to-purple conversion, whereas sulfate has no effect on the blue-to-purple transition.

The chloride effect on the blue-to-purple transition (Fig. 1) has the appearance of simple dissociation curves, and the points can be fit rather well assuming a single, non-interacting binding site per bacteriorhodopsin. However, the pH dependence requires involvement of hydrogen ion in the chloride binding process. We have explored a variety of models that predict a pH dependence of the chloride effect. All of the models contain too many adjustable parameters to determine the constants from the data in Fig. 1. One model, which combines a Cl^- binding site with different intrinsic dissociation constants in the presence and absence of bound H^+ (Eqns. 3–6) fits the data satisfactorily with physically reasonalble values for the constants. A scheme similar to that in Eqns. 3–6 has been suggested by Varo and Lanyi [20a]. They do not consider the effects of surface charge on the Cl^- and H^+ interactions, and their model requires cooperative H^+ binding.

Nature of side-chains involved in the blue to purple transition

Chemical modification experiments did not reveal any membrane surface side-chains that are essential for the chloride-induced blue-to-purple transition. We previously showed that reaction of purple membrane with EDC inhibits the proton uptake rate of the proton pump and also inhibits the formation of acid blue membrane [16,15]. However, this modification has no effect on formation of the chloride purple membrane at pH 1. We recently have localized the EDC site between residues 72 and 118, the most probable sites being Asp-96, Asp-102 and/or Asp-104 [21]. These groups are on the cytoplasmic side of the membrane and the uptake side of the proton pump. Assuming the pump mechanism involves a single path across the membrane, inhibition by EDC of the color change only in the neutral purple-to-acid blue direction suggests that the chloride effect may be exerted from the extracellular side of the membrane, the opposite side to the EDC site.

Anion binding sites in proteins often utilize Lys or Arg side-chains. However, modification of Lys-41 with dansyl chloride, or modification of surface Arg residues with cyclohexanedione, had no effect on the chloride-induced blue-to-purple transition at pH 1.

Spectroscopic investigations were more informative about groups undergoing changes during the blue-to-purple transition. FTIR difference spectroscopy (Fig. 2) clearly showed disappearance of carboxylate absorbance (trough at 1392 cm^{-1}) and appearance of carboxylic acid absorbance (peak at 1731 cm^{-1}) upon conversion of acid blue pigment to acid chloride purple. Comparison of the amide I absorbance of the films in Fig. 2 with the height of the carboxylic acid difference peak at 1731 cm^{-1} [22,23] suggests that about one carboxylic acid is formed per bacteriorhodopsin during the chloride-induced conversion of blue membrane to purple. If the neutral pH and acid pH purple-to-blue transitions share mechanistic features involving carboxyl groups, then our FTIR results are incompatible with the conclusions reached by Gerwert et al. [24]. In their infrared difference spectra obtained by neutralizing de-

ionized blue membrane with ammonia to form purple membrane, they found more than ten carboxyl groups *dissociated* upon conversion of blue to purple. However, Gerwert et al. observed many carboxyl groups dissociating because their membrane sample was undergoing a drastic increase in surface pH (from less than 3 to neutrality). The dissociation of a large number of surface carboxyl groups could easily mask the protonation of a single interior group that is involved in determining the color of the chromophore. By contrast, our infrared spectra were measured at essentially constant surface pH, making it more likely that the difference features observed are related to the blue-to-purple transition.

A spectrum similar to that in Fig. 2 was previously reported by Marrero and Rothschild [25]. Because HCl was used in their study to adjust the pH, they did not attempt to separate pH and chloride effects. Both sample and reference were at different pH and different chloride concentrations, resulting in mixtures of purple and blue forms.

The ultraviolet difference spectrum of acid chloride purple membrane minus acid blue membrane (Fig. 3) indicates perturbation of tryptophan superimposed on a tyrosine pH difference spectrum. The absorbance difference at 243 nm compared with the expected differential extinction between tyrosinate and tyrosine [19] suggests less than one tyrosine group dissociates upon conversion of acid blue membrane to chloride purple membrane. However, the differential ultraviolet absorbances of the films must be considered less reliable than the FTIR, because the large incident beam size relative to the film diameter makes film inhomogeneities a more serious source of error. Our results are qualitatively similar to the findings of Dunach et al. [11] for the Mn^{2+}-induced conversion of deionized blue membrane to purple membrane. However, in their spectra, the tryptophan difference feature is positive rather than negative, and the spectra appear to display a decrease in light-scattering upon Mn^{2+} binding.

A simple explanation of the ultraviolet and FTIR difference spectra is that the phenolic group of the tyrosine that becomes ionized is the proton donor to the carboxylate group that becomes protonated.

Mechanism of the blue-to-purple transition

Fischer and Oesterhelt [6] proposed that the chloride may bind directly to the retinal Schiff base of blue membrane, thereby replacing the counter ion and regenerating the purple chromophore. This model does not make any predictions about the protonation state of specific protein side-chains. Thus, our results do not prove or disprove their idea. Marrero and Rothschild [25] proposed that the Cl^- effect is entirely due to membrane surface charge. We have tested several surface charge models, and although two give satisfactory fits to the data in Fig. 1, they require large numbers of titratable groups near pH 0. These models could be further tested by a detailed study of the low-pH titration of purple membrane.

Our spectroscopic results suggest an interesting connection to a recently proposed proton pump model of purple membrane [13] (Fig. 4). We suggest that acid blue membrane is formed from the cytoplasmic side of the membrane, through the protonation of a chain of hydrogen-bonded groups. At one end of this chain in the purple form (Fig. 4a) is a protonated carboxyl side-chain (Asp-96, -102 or -104), which is the site of reaction with EDC. At the other end is an ion pair consisting of the protonated retinal Schiff base and an

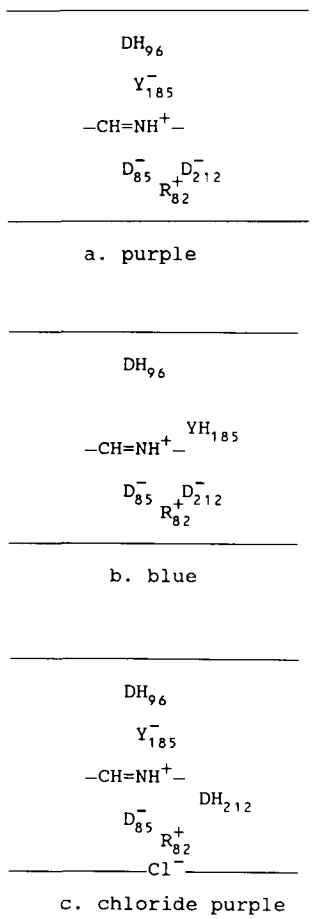

Fig. 4. Proposed mechanism of the purple to acid blue to chloride purple transitions. (a) Purple membrane at neutral pH. Upper line is cytoplasmic surface of membrane, and lower line is extracellular side of membrane. Bacteriorhodopsin side-chains are identified by single letter code and sequence position. Charge state of side-chain is indicated by + or − signs and presence or absence of H for dissociable protons. Protonated retinal Schiff base is shown in the center. (b) Acid blue membrane. Tyr-185 is protonated from the cytoplasmic side of the membrane along a hydrogen bond chain. (c) Chloride purple membrane. Chloride binds to Arg-82, disrupting a hydrogen bond with Asp-212. This carboxyl group removes a proton from Tyr-185, restoring the chromophore to the purple form. This scheme is consistent with the proton pump mechanism proposed by Braiman et al. [13], and it is supported by the spectroscopic evidence in Figs. 2 and 3.

anion (Tyr-185). When the pH is lowered, protons are displaced along the hydrogen-bonded chain, resulting in the protonation of the anion near the Schiff base (Fig. 4b). This hydrogen-bonded chain is the proton uptake pathway of the proton pump. Thus, at acid pH, the proton pump is inactive. When chloride is added (Fig. 4c), it binds to a site on the extracellular side of the membrane (Arg-82). The binding of chloride disrupts hydrogen bonds between the chloride ligand (Arg-82) and another group to which it is ordinarily hydrogen bonded (Asp-212), resulting in the movement of these groups apart. Along with this conformational change is the movement of the protonated counter-ion (Tyr-185) away from its position near the Schiff base. This motion is postulated by Braiman et al. to perform a key proton transfer during pumping. According to the model of Braiman et al. Asp-212 can accept a proton from Tyr-185 during the Schiff base reprotonation step of the proton pump. In our model for the formation of the chloride purple pigment, this same proton transfer occurs from Tyr-185 to Asp-212, regenerating the tyrosinate anion and at the same time returning the chromophore to the purple form. The protonation of Asp-212 between Fig. 4b and 4c explains the protonation of a carboxyl group that we observe by FTIR (Fig. 2). The dissociation of Tyr-185 between Fig. 4b and 4c is consistent with the absorbance rise we observe at 243 nm in the ultraviolet difference spectrum (Fig. 3). The model we propose does not directly explain the observed pH dependence of apparent chloride binding. It is possible that the conformational change that occurs between the blue and purple forms at low pH is linked to a proton binding step involving some other group on the protein which we have not observed spectroscopically.

The model shown in Fig. 4 makes detailed predictions about the blue–purple transitions. Future spectroscopic and mutation experiments could test the proposed roles of Tyr-185, Arg-82 and Asp-212 in the chromophore transitions and chloride binding.

Acknowledgements

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