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INDUCTION OF THE BLUE FORM OF BACTERIORHODOPSIN BY LOW CONCENTRATIONS OF SODIUM DODECYL SULFATE

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The effects produced on bacteriorhodopsin by low concentrations of several detergents have been studied by absorption and fourth-derivative spectrophotometry. Sodium dodecyl sulfate induces the appearance of the blue form of bacteriorhodopsin (λ_{max} = 600 nm) at pH values up to 7.0 in a reversible manner. The apparent p*K* of the purple-to-blue transition raised with increasing concentration of SDS. Of the other detergents tested, only sodium dodecyl-*N*-sarcosinate showed a slight red-shift of the absorption band to 580 nm, whereas sodium taurocholate, Triton X-100 and cetyltrimethylammonium bromide did not favour the appearance of the blue form. The effect of SDS was found to be consistent with a localized conformational change that moves away the counter-ion of the protonated Schiff base.

Introduction

Bacteriorhodopsin from the purple membrane of *Halobacterium halobium* acts as a light-driven proton pump through a photocycle that is initiated when retinal absorbs a photon [1]. The precise interactions between retinal and its environment give rise to the notable red-shift of the visible absorption maximum with respect to the free protonated Schiff base (about 120 nm). These interactions are now a subject of great interest, since the elucidation of their nature may aid in the resolution of the proton-pump mechanism [2–6].

The blue form of bacteriorhodopsin ($\lambda_{max} \approx 600$ nm) appears as a consequence of the modification of these interactions by acidic pH [7]. The purple-to-blue transition is a reversible process with an apparent p*K* in the absence of salt of 3.2 [7]. Several systems have been described in which the p*K* of the transition is altered. For example, the addition of salts, the interaction with polyelectrolytes or cross-linking with carbodiimides diminishes the apparent p*K* [8–10]. On the other hand, it has been shown that low concentrations of SDS, interaction with polar hydrophobic molecules or the acetylation of lysine residues increases the apparent p*K* [6,11,12]. Moreover, the reconstitution of purple membranes with endogenous polar lipids or with the white membrane causes the apparent p*K* of the transition to be 4.2 and 5.3, respectively [13,14].

In this communication, we report studies on the induction of the blue form of bacteriorhodopsin by low concentrations of SDS. We have studied this effect, as well as the effect of other detergents on the purple membrane properties, by using absorption and fourth-derivative spectrophotometry in the visible and the near-ultraviolet respectively. This latter technique has been shown to be useful for the study of the environment of aromatic residues in proteins [15,16].

Abbreviations: CTAB, cetyltrimethylammonium bromide; sarcosyl, sodium dodecyl-*N*-sarcosinate.

Materials and Methods

Purple membranes were obtained from the R_1M_1 strain of *Halobacterium halobium* as described [17]. In some preparations, pellet fractionation on a 25–50% sucrose gradient was replaced by repeated washings, each followed by centrifugation. The detergents employed (Fig. 1) were from the following sources: CTAB from Serva; SDS and Triton X-100 Sigma; sarcosyl and taurocholate from Fluka. All the reagents were of analytical grade. Spectrophotometric measurements were obtained with a Perkin-Elmer 320 spectrophotometer, interfaced to a Sharp MZ 80B microcomputer. For the fourth-derivative spectra, a scan rate of 30 nm/min, a slit width of 1 nm, a time constant of 1 s and a derivative interval of 4 nm were selected [15,16]. All measurements were done at 20°C.

Irradiation of samples was performed with red light ($\lambda > 600$ nm) or blue light ($\lambda_{max} = 505$ nm) using Wratten filters and a slide projector (light intensity at the sample, 480 $W \cdot m^{-2}$).

pH measurements were performed with a combined electrode GK2321C connected to a Radiometer pH-meter PHM 84. The pH of the samples was measured before and after the addition of detergents.

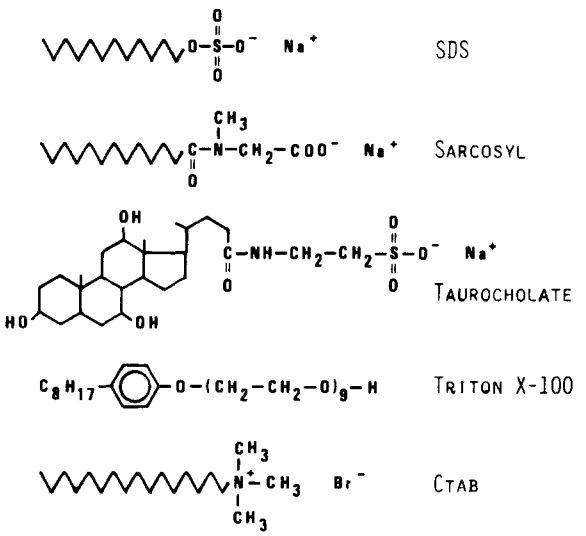

Fig. 1. Structural formulae of the detergents employed in this work.

Results

Visible spectral region

Addition of low concentrations of SDS to a buffered purple membrane suspension at a pH between 3.5 and 7.0 shifts the visible absorption spectrum to longer wavelength and decreases its intensity (Fig. 2). The suspension thus appears blue, like the form induced at acidic pH. This effect is instantaneous, showing a maximal shift to 600 nm. The 600-nm band first converts slowly to a new band centered at 440 nm, and finally to a 390-nm band. The concentration of SDS to obtain the blue form must reach a minimum value; if not, a slight red-shift and a decrease in the intensity of the retinal band is observed, followed by the appearance of the 440-nm species, and later by the 390-nm ones. The 440-nm species can also be generated directly by the addition of 4-fold the concentration of SDS necessary to obtain the blue form. The same results were obtained for both light-adapted and dark-adapted purple membranes.

The SDS-induced red-shift of the retinal band is a reversible effect. A blue sample (1.7 mM SDS, pH 6.0) was applied to a Sephadex G-25 column (1×20 cm) equilibrated with 10 mM citrate/phosphate buffer at pH 6.0, in order to remove the SDS. As the membrane fragments progressed

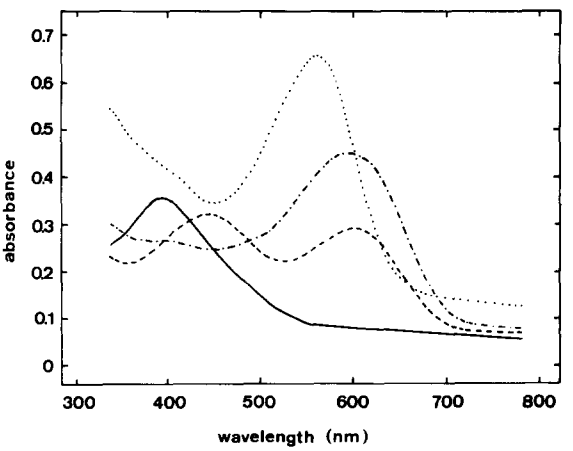

Fig. 2. Absorption spectra of purple membrane in 10 mM citrate buffer, pH 6.0. ·····, purple membrane; ·–·–·, SDS-blue membrane (1.38 mM SDS); - - - - -, after 15 min; ———, after 24 h.

through the column, the blue colour gradually changed to purple. The absorption spectrum of the eluted sample was indistinguishable from that of the native purple membrane, except for a slight shoulder at 390 nm, as a consequence of the initial effect of SDS.

Similar results were obtained at all pH values studied between 3.5 and 7.0, except for the increased SDS concentration needed to obtain the blue form as the pH was raised. At alkaline pH, it was no longer possible to produce the blue form, irrespective of the SDS concentration essayed; instead, the addition of SDS produced a band at 440 nm, with a shoulder at 580 nm. These forms slowly transformed into the 390-nm species. Fig. 3 shows the dependence of the ratio A_{600}/A_{max} on the pH, at various SDS concentrations. In these plots, the apparent pK of the SDS-induced purple-to-blue transition can be estimated. For example, 1.02 mM SDS gives rise to an apparent pK of 6.0, whereas in 0.17 mM SDS the apparent pK moves to 4.2.

Accessibility of the retinal Schiff base was tested by adding hydroxylamine in the dark (final concentration 0.2 M) to a buffered purple membrane or SDS-treated suspensions at various pH values. Whereas the purple membrane did not react with hydroxylamine, as expected [7], the SDS-treated membranes reacted at higher SDS concentrations than that necessary to obtain the blue form. In this case the 440-nm band did not appear, giving rise directly to the 360-nm band, characteristic of retinal oxime. This suggests that hydroxylamine reacts with the 440-nm form. On the other hand, the recovered purple membrane (obtained from the SDS-blue form after passage through a Sephadex G-25 column) did not react with hydroxylamine.

The influence of salts on the SDS-blue membrane was found to be similar to that observed on the acidic blue membrane [8]. Thus, at pH 6.2, 0.1 M NaCl inhibited the formation of the blue form by SDS, giving rise to a shift of the retinal band to 460 nm, and later to 390 nm.

Fig. 4 shows the effect of irradiation of the SDS-blue form with red light ($\lambda > 600$ nm). It can be seen that short time periods of irradiation of 1 min greatly accelerated the generation of the 440-nm species. The action of SDS with time and the effect of illumination with red light (in the presence of SDS) thus appear to be additive factors that increase the yield of the 440-nm species at the expense of the 600-nm form.

In order to see whether the described effects were exclusive for SDS, other detergents at low concentrations were also studied. Fig. 1 shows their structural formulae. Addition of sarcosyl, a weak ionic detergent similar to SDS, to buffered purple membrane suspensions at pH 6.0 produced no detectable shift in the 560-nm band, and the slow appearance of the 390-nm band. At pH 4.0 a

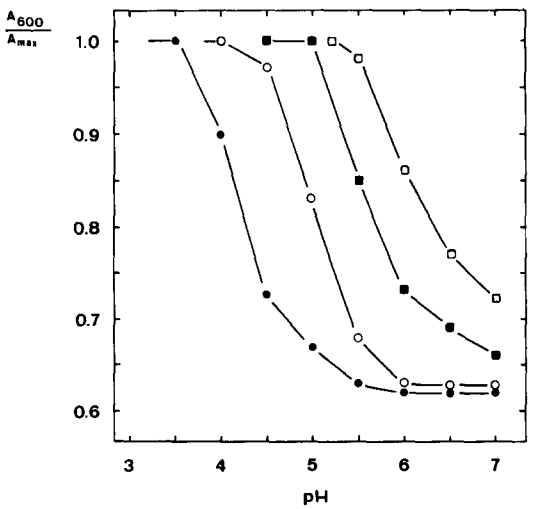

Fig. 3. Dependence of A_{600}/A_{max} on the pH, at different SDS concentrations, for 8.7 μM purple membrane. ●, 0.17 mM SDS; ○, 0.34 mM SDS; ■, 0.69 mM SDS; □, 1.02 mM SDS.

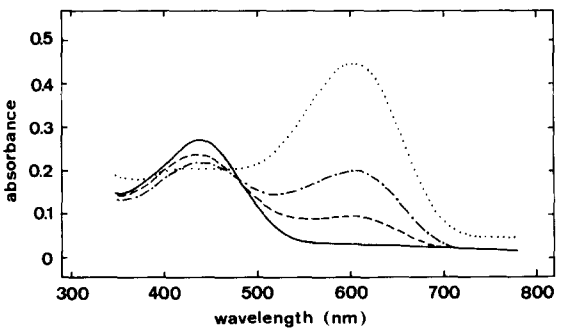

Fig. 4. Light-induced spectral change of the SDS-blue form. ·····, the spectrum of the SDS-blue form (purple membrane in 10 mM citrate/phosphate buffer, pH 6.0, 1.7 mM SDS); after irradiation of the SDS-blue form with $\lambda > 600$ nm light for 1 min (·–·–·), 2 min (- - - - - -) and 5 min (———).

red-shift of the 560-nm band to 580 nm, and the slow appearance of the 440 and the 390-nm bands were observed (Fig. 5). The retinal Schiff base did not react in the dark with hydroxylamine, except at pH 4.0 (in 0.36 mM sarcosyl). This reaction was, however, slower than in the presence of SDS. Sodium taurocholate carries a negative charge similar to that of SDS, but it has a rigid hydrophobic region. Its addition to buffered membrane suspensions at different pH values did not affect the 560-nm band, even after 24 h at a taurocholate concentration of 18.6 mM. The 440- or 390-nm bands did not appear in any case, nor did hydroxylamine react in the dark with the Schiff base.

The non-ionic detergent Triton X-100 produced a shift of the absorption maximum to 555 nm, in accordance with the observations made by other authors [18]. After a long time period, the 390-nm band appeared. The positively charged detergent CTAB did not influence the 560-nm band, but slowly generated the 390-nm one. Neither of these detergents facilitated the reaction of hydroxylamine with the Schiff base in the dark, even at a concentration of 1% (w/v).

The effect of detergents on the acid-induced blue form was also investigated by adding small amounts of detergents to a sample of the blue form of bacteriorhodopsin (in 10 mM citrate/phosphate buffer, pH 3.0). The anionic detergents did not restore the purple form, whereas Triton X-100 and CTAB induced a shift of the retinal maximum back to 555–570 nm.

Near-ultraviolet spectral region

As we have previously shown, fourth-derivative spectrophotometry is a convenient technique for monitoring the state of aromatic residues in proteins [15,16]. We have thus applied this technique as a means of following the conformational changes of bacteriorhodopsin upon detergent treatment. Fig. 6 shows the fourth-derivative spectra of bacteriorhodopsin in its native and SDS-treated forms. The effect of 1.7 mM SDS (pH 6.0) is a small but definite blue-shift of some parts of the fourth-derivative spectrum. In particular, the value of λ_1 shifts from 294.0 to 293.3 nm, whereas the R parameter changes from 1.03 to 1.10. The 440-nm form of bacteriorhodopsin does not show any further shift of the fourth-derivative spectrum (see also Ref. 16). On the basis of previous work, we interpret these changes as being due to an exposure of some tryptophan residues to the solvent [16].

A closer examination of the alterations in the fourth-derivative spectra showed that the observed changes are dependent on the SDS-concentration: increasing the concentration of SDS also leads to an increased blue-shift of the fourth-derivative

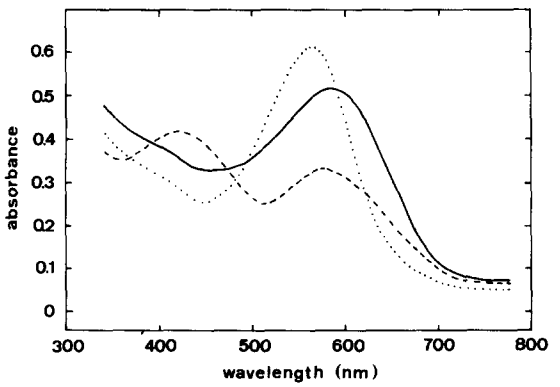

Fig. 5. Effect of sarcosyl on the purple membrane spectra. ·····, the spectrum of purple membrane in 10 mM citrate/phosphate buffer, pH 4.0; ———, after the addition of 0.36 mM sarcosyl; - - - - - -, the same, after 30 min.

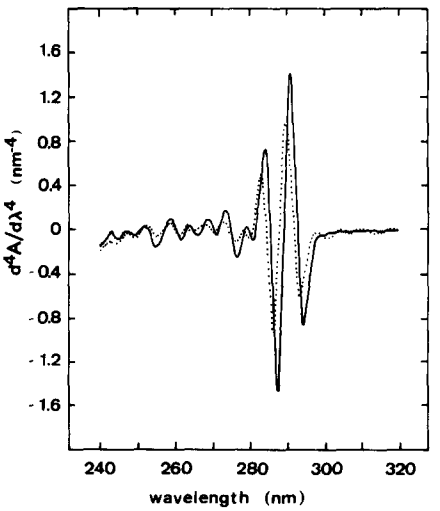

Fig. 6. Fourth-derivative spectra of 6 μM bacteriorhodopsin in 10 mM citrate/phosphate buffer, pH 6.0. ———, native form; ·····, plus 1.7 mM SDS, blue form.

spectra. Anyway, no correlation was observed between the changes in the fourth-derivative spectra and the generation of the blue form by SDS.

The cationic detergent CTAB and the anionic detergents sarcosyl and taurocholate showed effects similar to those of SDS. Low detergent concentrations did not alter the fourth-derivative curves. However, as the detergent concentration increased, the λ_1 values progressively displaced to shorter wavelengths.

In order to obtain more information about possible conformational changes involving the aromatic residues upon appearance of the blue form, we also examined the acid-induced blue form (pH 3.0). Its fourth-derivative spectrum did not show any differences from that corresponding to the purple species (pH 6.0).

Discussion

In order to put on a molecular basis the effect of SDS on the purple-to-blue transition, we have searched for a model of the chromophore environment that could best explain the results obtained. Several models, based on the appearance of the blue form of bacteriorhodopsin at acidic pH and on the studies of reconstitution of bacteriorhodopsin with retinal analogues, suggest that retinal interacts with one or more negative charges [2–4,6]. In all these models, a counter-ion of the protonated Schiff base is postulated. In the purple-to-blue transition, it has been suggested that the neutralization of this counter-ion or its removal from the site of interaction with the Schiff base leads to the appearance of the blue form [6,19]. On the other hand, another negative charge interacting with the β-ionone ring or with the C_{11}-C_{12} bond seems to be necessary to explain the observed spectral characteristics of bacteriorhodopsin [2,3].

In our hands, the model postulated by Fischer and Oesterhelt [6] is the one that explains best the SDS results. Following this model, an equilibrium constant that accounts for the interaction between the protonated Schiff base (N^+H) and its counter-ion (B^-) can be defined, $K_{NB} = [(N^+H, B^-)]/[(N^+H \ldots B^-)]$. Assuming a p$K$ for the free counter-ion of 5.5, these authors obtained a pH range for the existence of the blue and purple forms of bacteriorhodopsin. This is shown in Fig. 7, which is a modified version of Fig. 10 from Ref. 6. At each pH value, the solid line gives the log K_{NB} at which there exists 50% of the blue form. At room temperature the percentage of each form of bacteriorhodopsin as the pH is changed is indicated by a horizontal line at the level of log $K_{NB} = -3$ [6]. Taking into account the observed pK values of the purple-to-blue transition in the presence of different SDS concentrations (Fig. 3), the corresponding values of log K_{NB} can be obtained, and are indicated in Fig. 7. Thus, increasing the SDS concentration leads to increments in K_{NB} values, and correspondingly the pH range in which the blue form exists is expanded. In this respect, the effect of SDS appears to be similar to that produced by increased temperatures [6]; that is, a local conformational change that lessens the interaction between the Schiff base and the counter-ion B^-.

In connection with this conformational change, the results obtained from the fourth-derivative experiments show that the exposure of some tryptophan residues upon detergent treatment apparently does not bear any relationship to the appearance of the blue form, but to the detergent concentration. Furthermore, although the anionic detergent taurocholate really exposes some tryptophan residues, it does not induce the blue form. It thus appears clear that the generation of the SDS-blue form does not require the exposure of

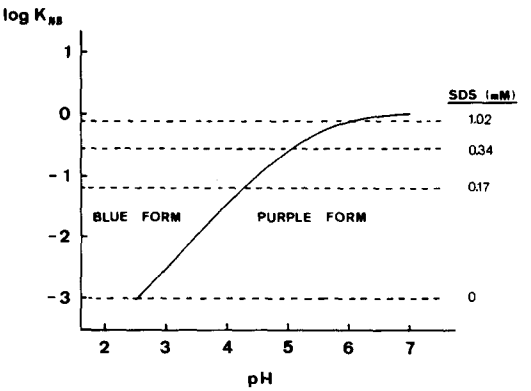

Fig. 7. pH range for the existence of the purple and blue forms of bacteriorhodopsin. At each pH, the solid line gives the log K_{NB} at which there exists 50% of each form. The horizontal discontinuous lines correspond to different concentrations of SDS, and give the pH range for the existence of the two forms.

some aromatic residues, as seen by the fourth-derivative technique.

Other interpretations are, of course, possible for the effect of SDS on the purple membrane. For example, a direct interaction of the negative charge of SDS with the β-ionone ring would shift the absorption spectrum to the red, as observed. Alternatively, the promoting effect of SDS might be merely due to an increase of the negative charge surface. In the former case, however, it is difficult to account for the shift to higher values of the apparent pK of the purple-to-blue transition as the concentration of SDS is increased (Fig. 3). On the other hand, the second possibility seems to be unlikely, because the negatively charged detergent taurocholate would exhibit the same effect as SDS. Moreover, the inhibitory effect of CTAB on the acidic blue form [7] is also shown by the neutral detergent Triton X-100. This argues against the role of the negative charge of the membrane surface in the induction of the blue form.

The 440-nm species appearing in the time after the 600-nm species have been already described by London and Khorana [20]. They adhered to the interpretation given by other authors, in that these species consist of a form of protonated Schiff base that has largely lost its interaction with the protein [21]. It is interesting to note that the 440-nm species can also be generated by irradiating the SDS-blue form with red light (Fig. 4). Moreover, we have observed that the 440-nm species cannot be regenerated to the native 570 form either by eliminating the SDS, or by irradiating with blue light. Thus, it can be concluded that in the 440-nm species the conformation of the retinal environment has been altered irreversibly in a way that exposes the Schiff base to the solvent.

Other systems in which the blue species are favoured have also been described. Reconstitution with negative polar lipids [13] or interaction with hydrophobic polar molecules [11] constitutes a similar situation to that reported in the present work, that is, the interaction of anionic hydrophobic molecules with the retinal environment. In our case, the results obtained with the anionic detergent taurocholate suggest that its bulky rigid region would prevent its access to the retinal environment. Acetylation of lysine residues of bacteriorhodopsin also increases the pK of the purple-to-blue transitions [12]. Whereas this case is very different from the others, the possibility of the induction of some structural distortion within the membrane must be borne in mind, as indicated by the authors [12]. We think that the increase in the pK of the purple-to-blue transition observed in all these cases can be explained by a similar effect to that assumed for SDS, that is, a localized conformational change that moves away the counterion of the protonated Schiff base. The effect of SDS in producing the 440-nm species would also be in accordance with such a hypothesis.

The above discussion can also be related to the presence of sulfated lipid components in the purple membrane [22]. They might provide an adequate negative environment for the retinal molecule, thus contributing in part to the shift of the visible absorption maximum from 440 nm (free protonated Schiff base) to 570 nm.

Acknowledgements

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