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Is the purple color of bacteriorhodopsin maintained by lipid–protein interactions?

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When bacteriorhodopsin is delipidated and purified in detergents, its purple chromophore can be reversibly titrated to a red one. The pK_a of this equilibrium depends on the nature of the detergent in which bacteriorhodopsin is dispersed. In the absence of solvating amphiphiles, lipid-free detergent-free bacteriorhodopsin is red (λ_{max} = 480 nm) at pH higher than 3.5.

The bacteriorhodopsin molecule contained in the purple membrane of *Halobacterium halobium* bacterial cells has a visible absorption maximum at 560 nm in the dark-adapted state. Upon solubilisation of purple membranes by detergents, photochemical activity of the pigment is only slightly affected [1], but its maximum absorbance is shifted by approx. 20 nm towards the blue end of the spectrum [2]. It has been shown that this blue shift is due to interaction of detergent with the apoprotein rather than to destruction of the native trimeric structure of bacteriorhodopsin [1]. In order to know to which extent the spectral parameters of bacteriorhodopsin could be modulated by interaction with amphiphilic molecules, we have dispersed the purified pigment with some detergents in which it is stable, namely, Triton X-100 and a series of alkyl sucrose esters. We find that, in the purified state, bacteriorhodopsin absorbs at 480 nm. It can be reversibly titrated back to the 540 nm pigment with an apparent pK_a ranging from 2.5 to 12, depending on the detergent used for dispersion. We propose that the pK_a value reflects the strength of the interactions of bacteriorhodopsin with the amphiphilic molecules. Accordingly, lipid–protein interactions would prevent the formation of the 480 nm species in the purple membrane.

The acid-base equilibrium of the two spectral forms of bacteriorhodopsin is shown in Fig. 1. In that experiment, bacteriorhodopsin was first delipidated according to the method of Huang et al. [3] and chromatographied in a column of Phenyl-Sepharose CL-4B (Pharmacia Fine Chemicals, Uppsala, Sweden) in the presence of the detergent lauryl sucrose, according to the procedure previously described [4]. This method proved to yield very stable purified samples. At pH 7, the purified bacteriorhodopsin dispersed in the lauryl sucrose ester shows a broad absorption spectrum centered at 510 nm which is rapidly displaced towards 480 or 540 nm upon alcalinisation or acidification of the medium. The apparent pK_a of this equilibrium can be obtained by measuring the relative absorbances at 480 and 540 nm of the sample at different pH or simply by plotting maximum absorbance wavelength against pH. Both methods give the same result.

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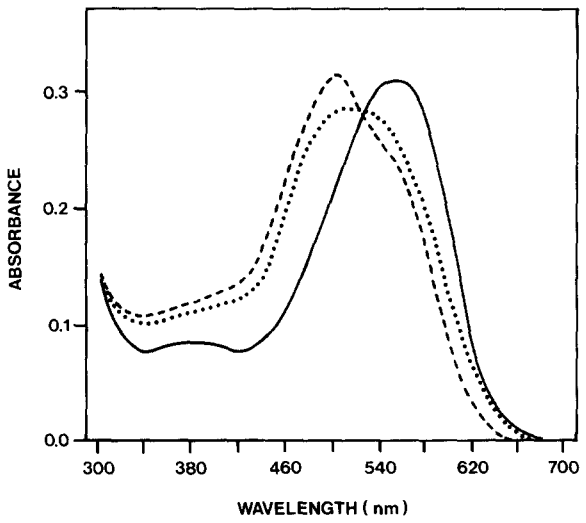

Fig. 1. Absorption spectra of bacteriorhodopsin after delipidation and chromatography on phenyl-Sepharose CL-4B. Spectra were recorded at room temperature, while bacteriorhodopsin was in 50 mM phosphate buffer (pH 7.0) containing 0.2% lauryl sucrose (· · · · · ·), after alcalinisation to (pH 8.0) by addition of small amount of 0.1 M NaOH (- - - - -) and after acidification to pH 6.0 by addition of 0.1 M HCl (———). Spectra were normalised for sample dilution by NaOH and HCl.

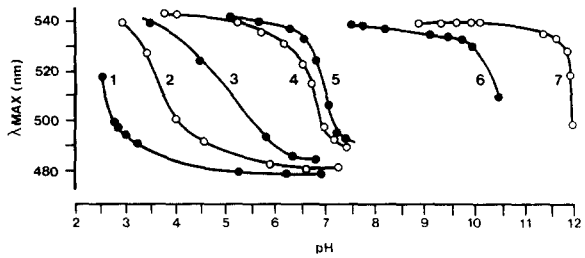

Fig. 2. Titration curves of the acid-base equilibrium observed between the 540 and the 480 nm chromophores of bacteriorhodopsin in different dispersing media. Curve 1 stands for purified bacteriorhodopsin aggregates from which detergent was dialysed out. Curve 2–6 were obtained by solubilisation of the above sample in 0.1% (w/v) octyl sucrose (2), oleyl sucrose (3), lauryl sucrose (4), stearyl sucrose (5) and Triton X-100 (6). Curve 7 was obtained when purple membranes were solubilised in 2% (w/v) Triton X-100 or lauryl sucrose. The sucrose esters were synthesized at the Mitsubishi Chemical Co. (Yokohama, Japan). They all contained less than 0.1% free fatty acids. Lauryl sucrose contained 80% monoesters, while others contained more than 95% monoesters.

While we established the environmental dependence of the acid-base equilibrium of the two spectral forms of the pigment by changing the detergent used during the hydrophobic interaction chromatography (phenyl-Sepharose column), we most often used a faster method which consisted in doing this chromatography in the presence of octyl glucoside followed by extensive dialysis against water or buffer. In the presence of octyl glucoside, bacteriorhodopsin is less stable than in sucrose esters, but the procedure was fast enough to avoid significant pigment denaturation. In addition, the dialysis step (30 ml sample per 2 l of water during 36 h with 2 water changes) had the advantage to yield concentrated (precipitated) lipid-free detergent-free samples which could be added with the desired detergent easily and rapidly.

The titration curves of the purified bacteriorhodopsin dispersed in different media are given in Fig. 2. Curve 1 corresponds to aggregated bacteriorhodopsin in water. This pigment has maximum absorbance at 480 nm within the slightly acidic to highly alcaline pH range. Under pH 3.5, its λ_{max} gradually shifts towards higher wavelengths up to 520 nm at pH 2.5. Lowering the pH more than that results in denaturation of the pigment and appearance of the 367 nm retinal absorbance band. Dispersion of such aggregates in detergents displaces the titration curve towards higher pH values. In the octyl sucrose ester (curve 2), the titration midpoint is at pH 3.5. It still increases to pH 6.8 and 7 in lauryl and stearyl sucrose esters, respectively (curves 4 and 5). Myristyl and palmitoyl sucrose esters yield curves which locate between curves 4 and 5. Interestingly, introduction of a double bond in the hydrophobic chain of the detergent brings back the titration curve to lower pH values, as shown by curve 3 which was obtained in the oleyl (C 18 : 1) sucrose ester. When dispersed in Triton X-100, the purified bacteriorhodopsin aggregates show a titration curve in the alcaline pH range with a midpoint at about pH 10.5 (curve 6). When purple membranes are only solubilised in Triton X-100 or in lauryl sucrose, the 480 and 540 nm chromophore equilibrium has midpoint titration at pH 12 (curve 7).

Except for those which were dispersed in the octyl sucrose ester, all purified bacteriorhodopsin samples were found to be stable for several weeks

at 4°C, and their titration could be repeated back and forth several times, provided that extreme pH conditions were avoided. In addition, they could be reversibly displaced from one titration curve to another by simply changing the dispersing medium. For example, at pH 7, solubilisation of a detergent-free pigment in Triton X-100 immediately shifted its maximum absorbance from 480 to 540 nm (see Fig. 3), while solubilisation with octyl sucrose or octyl glucoside had no effect on pigment λ_{max}.

Blue-shifted spectral forms of bacteriorhodopsin can be obtained under various conditions. Namely, under moderately alcaline conditions, the maximum absorbance of bacteriorhodopsin locates between 460 and 500 nm in purple membranes added with dimethyl sulfoxide [5] or diethyl ether [6], in purple membranes immobilised in polyacrylamide [7], and in bacteriorhodopsin-lecithin recombinants [8]. At neutral pH, chromophores having comparable λ_{max} are formed by incorporation of volatile anesthetics into purple membranes [9] or by solubilising and cooling them to −30°C.

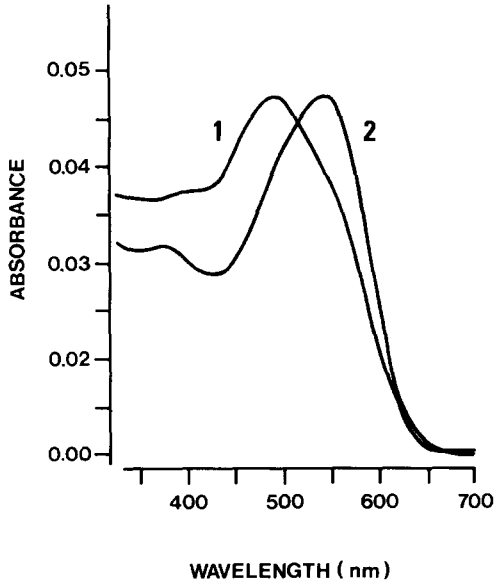

Fig. 3. Effect of dispersing medium on the purified bacteriorhodopsin absorption spectrum. Curve 1 is the absorption spectrum of detergent-free purified bacteriorhodopsin aggregates. Upon addition of 0.2% Triton X-100 (curve 2), aggregates are solubilised, they scatter less light, as seen from the decreased absorbance in the 350–450 nm region and the maximum absorbance is simultaneously shifted to 540 nm.

in Triton X-100 [10]. All these experimental conditions correspond to drastic modification of the membrane organisation and destabilise the native bacteriorhodopsin structure, giving rise to its purple to red transition. The results presented here show that, in the purified state, bacteriorhodopsin spontaneously adopts the 480 nm spectral form. In the absence of lipids and detergents, the pigment has maximum absorbance at 480 nm at pH higher than 3.5. Association of this pigment with detergents regenerates the original 540 nm pigment and its stability towards alcalinisation increases as the detergent hydrophobic chain becomes larger, the most stable lipid-free purple pigment being obtained in Triton X-100.

From the data presented above, it is not possible to precise the real state of the 480 nm pigment although we have shown that it has a complete photocycle [4]. The titrable group which is responsible for the acid-base equilibrium between the 480 and 540 nm pigments can hardly be identified with the nitrogen of the Schiff base link between retinal and bacterioopsin since its pK_a was reported to be 13.3 [11]. Moreover, evidence has been obtained, from resonance Raman spectroscopy, that both red and purple chromophores have a protonated Schiff base (to be published elsewhere). In the lipid-free detergent-free bacteriorhodopsin, the apparent pK_a of the group which controls the equilibrium is approx. 2.5. This is likely to be the pK_a value of the group when freely accessible from the aqueous phase. When the pigment is associated with detergents having increasingly longer hydrophobic tails, the titrable group would progressively becomes less and less accessible to titration and, accordingly, its apparent pK_a would rise considerably. In the purple membrane, the titration site is probably highly isolated from the aqueous phase such that acid-base equilibrium is not easily observed. However, it is likely that not only the membraneous structure, but also lipid–protein interactions do contribute in the stabilisation of the native chromophore. As a matter of fact, in solubilised purple membranes (see curve 7 of Fig. 2), monomeric [2,12] bacteriorhodopsin is not separated from lipids, and under these conditions, the 480 nm pigment only appears at pH higher than 11.5. We thus propose that interaction of bacteriorhodopsin

with membrane lipidic components might be, at least partially, responsible for the maintenance of the purple color of the pigment.

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