

Only water-exposed carboxyl groups are protonated during the transition to the cation-free blue bacteriorhodopsin

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FTIR (Fourier-transform-infrared) difference spectra between cation-free blue and purple bacteriorhodopsin were recorded. The results indicate that during the blue to purple transition no isomerization of the chromophore takes place. It is further observed that approx. 14 water-exposed carboxyl groups of amino acids are protonated in blue bacteriorhodopsin. The groups were deprotonated during the blue to purple transition. Protonation of carboxyl groups in the interior of the protein, which are not accessible to water, can be excluded.

FTIR: Cation depletion; Blue bacteriorhodopsin; Surface potential; Carboxyl group; Protonation change

1. INTRODUCTION

Recently, it has become evident that the light-driven proton pump bacteriorhodopsin [1] binds several divalent cations with different affinities [2–7]. By removing the cations the absorption maximum is shifted from 560 nm (purple bR) to about 605 nm (blue bR). Blue bR is obtained either at low pH [8–10] or by different deionisation procedures [2,3,11]. Resonance Raman experiments [12–14] indicate that the chromophore structure in both blue species is essentially identical [12].

Based on the results of earlier FTIR experiments, it is suggested that the shifts of the absorption maximum during the photocycle of bR are caused by protonation changes of internal aspartic acids near the protonated Schiff base [15–17]. Here, we report on our investigation of the differences between blue and purple bR by FTIR spectroscopy. This is of interest because the

bathochromic shift of acid blue bR might be caused by protonation of the Schiff base counterion [9,10].

From the presented FTIR experiments it can be excluded that during the purple to blue transition protonation of carboxyl groups in the interior of the protein occurs. Only protonation of about 14 water-exposed carboxyl groups is observed.

2. MATERIALS AND METHODS

Purple membranes (PMs) were isolated from *Halobacterium halobium* according to [18]. Cation-depleted blue PMs were prepared by the cation-exchange method [2]. Papain treatment for removal of the C-terminal amino acids [19] was carried out prior to deionization of the membrane. To control the removal, SDS-polyacrylamide gel electrophoresis was performed.

Blue PMs were dried on AgCl and CaF₂ windows under a nitrogen stream. The films were rehydrated or deuterated to about 100% humidity as described [20]. The blue to purple transition was initiated by injection of NH₃ into the closed sample

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chamber [20,21]. Control experiments in the visible region were recorded on a Perkin Elmer spectrophotometer (model Lambda, 9). IR spectra were recorded with 2 cm^{-1} resolution on a Bruker IFS 48 Fourier transform infrared spectrophotometer equipped with an MCT detector.

For the difference spectra two spectra of the blue PMs were measured, both with 128 scans. The difference between the absorbances of the spectra served as control of the baseline quality. Subsequently NH_3 was added repeatedly in order to induce the blue to purple transition. A time series of spectra were taken, each with 128 scans until no further changes were observed. In section 3, difference spectra between the initial and the final state are shown.

3. RESULTS

In contrast to earlier investigations using FTIR spectroscopy, where light-induced changes of bR were discovered, in this contribution pH-induced changes of the protein were observed. Since water is a strong absorber in the infrared region, we investigated the blue to purple transition in PM films which strongly reduces the effective water layer. Control spectra in the visible regions under these conditions are shown in fig.1a. Injection of NH_3 into the sample chamber causes an immediate shift of the absorption maximum from 605 to 558 nm. Subsequent illumination of the purple species shifts the absorption maximum from 558 to 568 nm and increases its absorbancy. Further illumination of the latter sample releases a full bR photocycle indicating the complete regeneration of the function of bR.

An overview of the infrared difference spectrum between the blue and the purple species is given in fig.1b. Only those groups which change during this transition are reflected. Negative bands arise from the blue species and positive bands from the purple species. To exclude baseline distortions in the difference spectra the same amount of NH_3 was given to a PM film in a further experiment yielding no changes in the spectrum.

Fig.2 shows the difference spectrum between the purple species, which was obtained by the blue to purple transition, and the subsequently illuminated purple species. This difference spectrum is identical with that between dark- and light-adapted bR

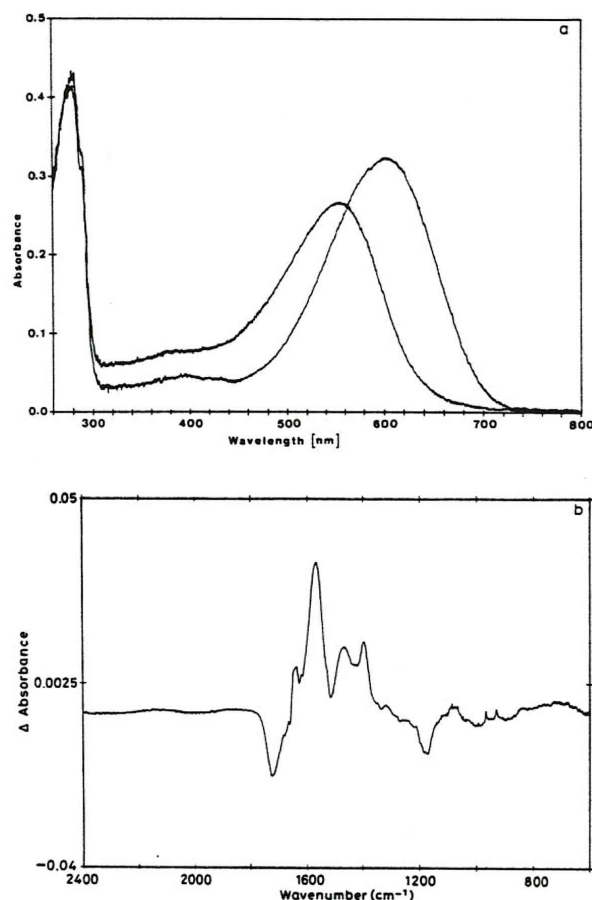


Fig.1. (a) Absorbance spectra of the initial blue bR₆₀₅ and the purple bR₅₅₈, arising after injection of NH_3 into the sample chamber. (b) Difference spectrum between these two species in the infrared region.

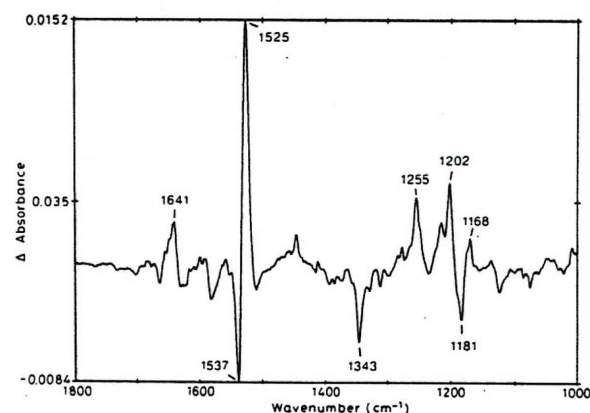


Fig.2. Difference spectrum between the purple bR₅₅₈ and the light-adapted bR₅₆₈.

[20,23]. This indicates that the purple species which forms in the blue to purple transition is identical with the dark-adapted bR.

Comparison of the bR₆₀₅-bR₅₅₈ difference spectrum (fig.3) with resonance Raman spectra of the deionised and dark-adapted bR species [12-14] confirms this observation. From resonance Raman data one expects for the transition from blue bR₆₀₅ to dark-adapted bR₅₅₈ that

- (i) the bands of the C-C stretching vibrations at 1171 and 1184 cm⁻¹ show intensity changes
- (ii) the band of the C=C stretching vibration shifts from 1518 to 1533 cm⁻¹, and
- (iii) the band of the C=N stretching vibration shifts from 1628 to 1640 cm⁻¹.

All these changes are observed in the difference spectra (fig.3). The assignments of the bands arising from the chromophore vibrations are more tentative because additionally much stronger bands are recorded at 1723, 1571, 1470 and 1396 cm⁻¹. Since these bands are not observed in the resonance Raman spectra they must be assigned to vibrations of protein residues. The frequency of the negative bR₆₀₅ band at 1723 cm⁻¹ is typical of the carbonyl stretching vibration of hydrogen-bonded carboxylic acids and the frequencies of the bands at 1571 and 1396 cm⁻¹ are typical of the antisymmetric and symmetric stretching vibrations of deprotonated carboxylic acids, respectively [22].

To confirm these assignments, the difference spectrum for deuterated membrane films was recorded (fig.4). The shift of the negative band

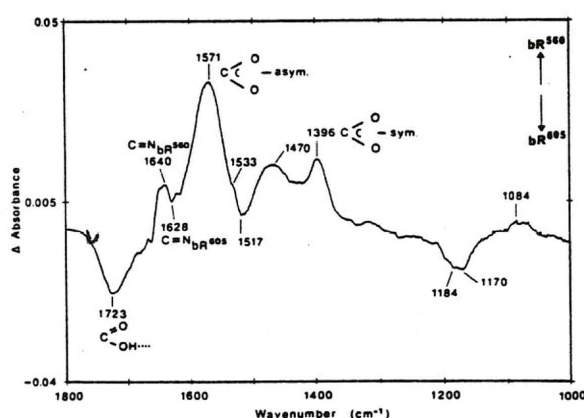


Fig.3. Difference spectrum between blue bR₆₀₅ and dark-adapted bR₅₅₈ in H₂O.

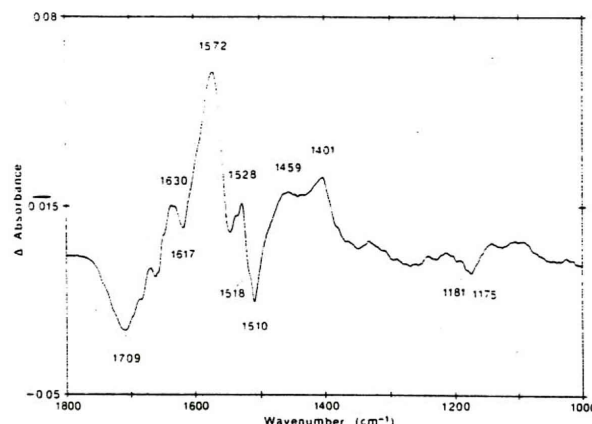


Fig.4. Difference spectrum between blue bR₆₀₅ and dark-adapted bR₅₅₈ in D₂O.

from 1723 to 1709 cm⁻¹ in D₂O corroborates the assignment to a carbonyl stretching vibration. The shifts of the bands from 1640 and 1628 cm⁻¹ to 1630 and 1617 cm⁻¹, respectively, strengthen the assignment of these bands to the Schiff base stretching vibrations of bR₆₀₅ and bR₅₅₈. The positive bands at 1571 and 1396 cm⁻¹ are almost unaffected as expected for the antisymmetric and symmetric stretching vibrations of CO₂⁻ groups. Also, the band at 1170 cm⁻¹ is influenced in D₂O as observed in the resonance Raman experiments [12,13].

The intensity of the broad negative band in the fingerprint region seems somewhat strong for C-C stretching vibrations compared to that of the C=C stretching vibrations. In the fingerprint region vibrations of the phosphoryl groups of the phospholipids are also expected at about 1200 and about 1080 cm⁻¹ [24]. Therefore, part of the broad negative band at about 1200 cm⁻¹ and of the positive band at about 1084 cm⁻¹ (fig.3) could be caused by conformational and/or protonation changes of the polar lipid head groups.

The difference spectra of fig.5 were recorded after successive addition of small amounts of NH₃ to the sample chamber. Fig.5 shows that the carbonyl band increases at 1723 cm⁻¹. There is no indication of an underlying smaller carbonyl band at higher wave numbers.

In order to analyze the contribution of five carboxyl groups of amino acids exposed toward the outside of the PM, the C-terminal segment was removed by papain treatment [25,26]. Fig.6a

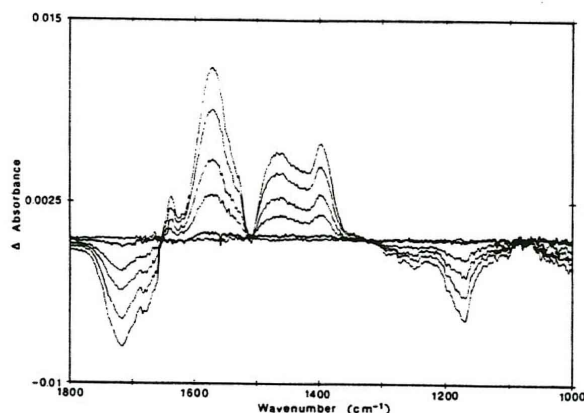


Fig.5. Difference spectra when smaller amounts of NH_3 were given, repeatedly, to the sample chamber.

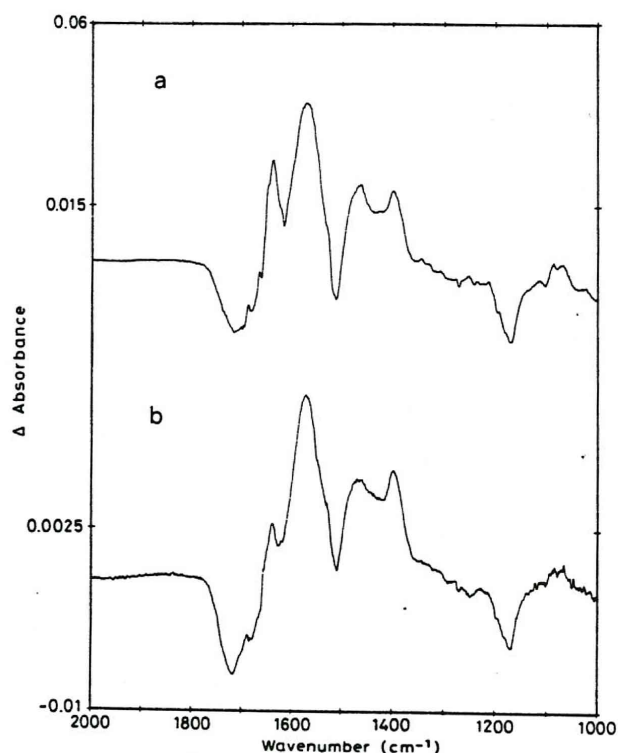


Fig.6. (a) Difference spectrum between bR_{605} and bR_{558} of bacteriorhodopsin without C-terminus. (b) Difference spectrum of the control sample with C-terminus.

shows the $\text{bR}_{605}/\text{bR}_{558}$ spectrum of the papain-treated PMs. The difference spectrum of the control sample from the same preparation, not being papain-treated, is presented in fig.6b. On com-

parison with the bands of the C=C stretching vibration, which indicates the amount of reactive proteins, the carbonyl band at 1723 cm^{-1} is decreased by about 35%. This is the average from five different measurements of papain-treated and native samples, respectively. Also, the band of the CO_2^- symmetric stretching vibration at 1396 cm^{-1} decreases relative to that at 1470 cm^{-1} . The increase of a band at about 1640 cm^{-1} (fig.6a) is caused by instabilities of the amide I band for the papain-treated protein.

4. DISCUSSION

Our experimental results clearly show that no sharp positive bands are observed in the fingerprint region of the IR difference spectrum, excluding isomerization of the retinal during the blue to purple transition in agreement with earlier results [12]. Furthermore, the frequency shifts of the C=N and C=C stretching vibrations indicate that in the blue bR species the positive chromophore charge is less stabilized at the protonated Schiff base by the amino acid groups of the protein environment, compared to the state observed in the purple species. Therefore, changes in the opsin moiety are responsible for the bathochromic shift of the absorption maximum.

The results show further that several hydrogen-bonded carboxyl groups of amino acids are protonated in the bR_{605} species. The frequency of the carbonyl stretching vibration depends on the strength of hydrogen bonding with the environment of the carboxylic acid [22]. Since only one carbonyl band is observed at 1723 cm^{-1} , it can be concluded that all protonated carboxyl groups are exposed to water in the same manner. The carboxyl groups were deprotonated during the blue to purple transition. This is indicated by the decrease of the carbonyl band at 1723 cm^{-1} and the increase of the CO_2^- stretching vibration bands at 1571 and 1401 cm^{-1} , respectively (see fig.5).

If the C-terminal tail of bacteriorhodopsin is removed, a decrease in intensity of the carbonyl band by about 35% is observed (see fig.6). Since this C-terminus contains five carboxyl groups [25,26], the carbonyl band at 1723 cm^{-1} reflects the protonation of about 14 water-exposed carboxyl groups in blue bR. This observation is in agreement with the model [27], which proposed 14

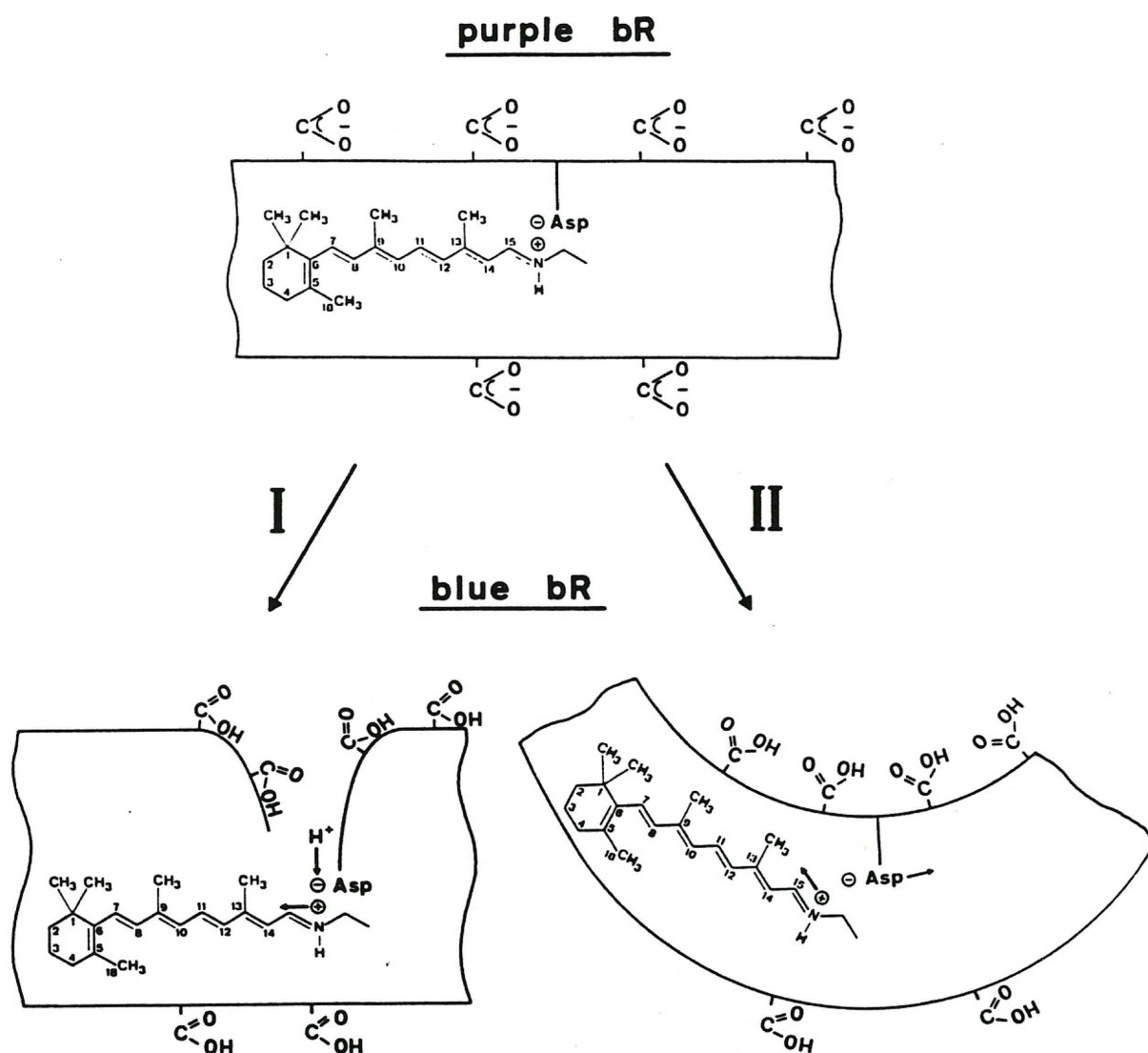


Fig.7. The batho shift of cation-free blue bR is caused by protonation of the water-exposed counterion (scheme I) or by separation of the counterion from the protonated Schiff base by a conformational change of the protein (scheme II). All water-exposed carboxyl groups are protonated in blue bR.

water-exposed carboxyl groups. Thus, a large change in the surface charges of the protein occurs during the blue to purple transition, thereby providing the forces for binding of divalent cations. This explains just as well the mechanism of formation of blue bR upon acidification.

Because only one symmetric and one antisymmetric CO_2^- -stretching vibration band are recorded, the NH_4^+ are all adsorbed in the same manner and no specific binding is observed. In the case of

binding sites with different affinities and coordination, as for divalent cations, one would observe several CO_2^- stretching vibration bands [28].

On the basis of these experimental results we cannot decide whether in the transition to the bR_{605} species the counterion of the protonated Schiff base becomes accessible for water and is deprotonated (fig.7, scheme I) or whether by protonation of water-exposed carboxyl groups a conformational change of the protein is induced which

separates an internal counterion from the protonated Schiff base (fig.7, scheme II). Both effects would cause destabilisation of the positive charge at the protonated Schiff base leading to a more delocalised electron distribution in the retinal.

However, protonation of a carboxyl group in the interior of the protein, as it takes place during the photocycle of bR [15,16], can be excluded. The problem remains as to whether the divalent cations play a functional role during the proton pump mechanism or whether the binding of cations induces the specific structure by which the protein can act as a proton pump. Thus, it would be useful to analyze whether there is a change in the binding constant of the cation during the photocycle of bR.

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