EMISSION FROM SECONDARY INTERMEDIATES IN THE PHOTOCYCLE OF BACTERIORHODOPSIN AT 77°K

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1. Introduction

It is known that bacteriorhodopsin in the purple membrane of *Halobacterium halobium* gives rise to at least four intermediates in a light induced reaction sequence [1–5].

Emission studies on purple membranes have so far been focused on bacteriorhodopsin (bR) and its primary photoproducts [6–8]. To our knowledge no emission spectra have been published dealing with the intermediates formed in the course of the thermal reaction cycle of bR following the first photochemical step.

In this letter we present emission from two of these intermediates, which have been trapped in purple membrane samples cooled to 77°K under strong illumination.

2. Experimental

The luminescence spectrometer used for recording the emission from purple membrane (from R_1M_1 strain of *Halobacterium halobium*) suspensions in H_2O (protein concentration $3.3 \cdot 10^{-5}$ M) consists of a 2.5 kW Xe-lamp (Osram XBO), two double grating monochromators (Spex 1402) and a photon-counting detection system including an ITT photomultiplier tube (F 4013) with extended S-20 response.

Samples of trapped intermediates of the reaction cycle of the retinal chromophore were prepared in quartz tubes. These tubes containing purple membrane suspensions in H_2O were steadily lowered into liquid nitrogen and simultaneously illuminated with focused light ($\lambda > 515$ nm) from a 1000 W tungsten lamp. Yellow-looking samples were obtained when the lowering rate was 2 mm/min. Because of the change of colour we will call these samples 'bleached samples'. These samples were used for the emission measurements and could be kept in the dark at 77°K for weeks without any detectable change in the luminescence properties.

The purple membrane fragments used in these experiments were prepared according to the procedure given by Oesterhelt and Stoeckenius [9].

Absorption spectra at ~ 80°K were recorded with a Perkin-Elmer (Coleman 575) spectrometer. For these measurements special absorption cells of 1 mm pathlength inserted in a quartz Dewar were used. Bleaching of these samples ($6.6 \cdot 10^{-5}$ M in glycerol–H_2O, 1:1, v/v) was not as complete as for those used in the emission measurements. The absorption spectra given in figs 1 and 2 correspond to a greenish-looking bleached sample.

3. Results

In the long wavelength range of fig.1, an emission spectrum from a bleached sample of purple membrane at 77°K is shown. The excitation wavelength was 640 nm. The emission is seen to have a maximum around 705 nm and a shoulder around 740 nm. The emission at about 740 nm originates from a species which is present also in unbleached purple membrane

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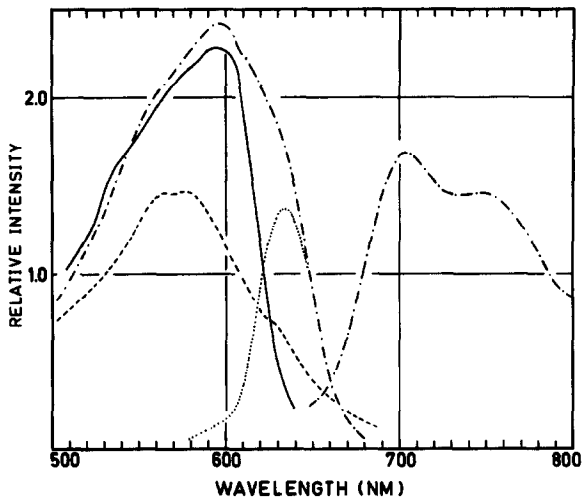

Fig.1. This figure represents our spectral data in the wavelength range 500–800 nm as obtained from purple membrane suspensions in H_2O which were cooled down to 77°K under strong illumination with light of $\lambda > 500$ nm (bleached samples). To the right an emission spectrum with an excitation wavelength of 640 nm is shown. To the left an absorption spectrum in H_2O–glycerol (1:1, v/v, dashed line) and an excitation spectrum in H_2O (emission wavelength 695 nm, dashed–dotted line) are represented. For comparison the excitation spectrum for an unbleached but light-adapted sample (solid line) has been added. An estimate of the excitation spectrum of the species responsible for the emission at 705 nm is obtained (dotted line) by fitting the two excitation spectra (solid and dashed–dotted line) in the wavelength range 500–550 nm and taking the difference between these spectra.

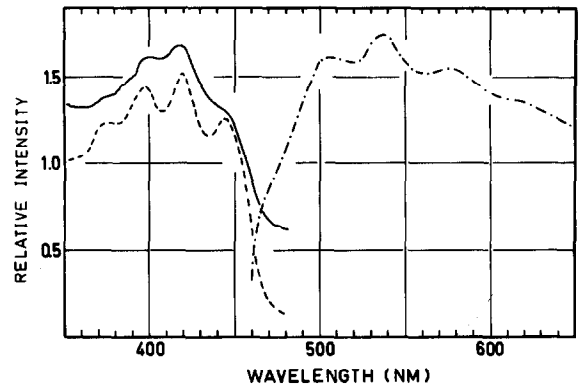

Fig.2. This figure represents the spectral data of the same samples as in fig.1 but in a different wavelength range. The solid, dashed, and dashed–dotted lines represent corresponding absorption, excitation and emission spectra, respectively (see text).

[6,7] and is discussed in detail in another paper [8]. The excitation spectrum from the bleached sample with emission wavelength 695 nm is also shown in fig.1 (dashed–dotted line) together with the excitation spectrum from an unbleached sample (solid line) [8]. The difference spectrum between these two curves (dotted line) has a maximum close to 635 nm. In the absorption spectrum from a bleached sample of purple membrane a shoulder at about 630 nm is observed.

Excitation of the bleached sample with 454.5 nm light yields a structured emission (fig.2). The corresponding excitation spectrum (emission wavelength 530 nm) is also presented in fig.2 (broken line) together with an absorption spectrum (solid line) from the same sample that was used for recording the absorption spectrum in fig.1. The close resemblance between the excitation and the absorption spectra as well as the almost perfect mirror symmetry between the excitation and the emission spectra suggest that they have their origin in the same species.

The emission and excitation spectra showed some wavelength dependence. This can be explained by a broad emission with a flat maximum at $\lambda \simeq 460$ nm which is present even in unbleached samples. The corresponding excitation spectrum shows a broad maximum at about 380 nm.

4. Discussion

Characteristic for the procedure of preparing the samples is that considerable amounts of bacteriorhodopsin are pumped into the reaction cycle (high light intensities). Cooling the sample simultaneously slows the reaction cycle down and finally stops it so that varying amounts of thermal intermediates become trapped. We found emission from two intermediates which were trapped during this procedure.

First, we discuss the origin of the emission peaking at 705 nm. This emission comes from at least two species, since the excitation spectrum is dependent on the emission wavelength. If we compare the excitation spectrum obtained from a bleached sample for the emission wavelength of 695 nm (fig.1) with the corresponding excitation spectrum from an unbleached

sample (fig.1) (which is wavelength independent) we find that the spectrum from a bleached sample extends to the red. The difference between the spectra in fig.1 should closely represent the excitation spectrum of the intermediate emitting at 705 nm. The difference spectrum peaks at 635 nm, which is close to the shoulder at 630 nm in the absorption spectrum from a bleached sample. We therefore assign the emission at 705 nm to an intermediate absorbing at 630–635 nm. Two species occurring in the reaction cycle of bacteriorhodopsin are known to absorb in this wavelength region. These are the batho-form ($\lambda_{max} \simeq 630$ nm [3,4]) and the *O*-form ($\lambda_{max} \simeq 640$ nm [2,4]). Since emission from the batho-form was not detected in the 700–800 nm region even in samples which are known to contain the batho-form, we conclude that the emission at 705 nm originates from the *O*-form.

The second intermediate to be discussed is the one most easily identified, since this is the *M*-form [2] with an absorption maximum at 412 nm in water at room temperature[+] [10]. In the excitation and the absorption spectrum (fig.2) we clearly recognize four peaks at 444 nm, 419 nm, 397 nm and ~ 375 nm, which most likely form a vibrational progression of $\Delta\nu = 1380$ cm^{-1}. We conclude this because the same structure can be recognized in the emission spectrum in fig.2. Presumably due to the underlying broad emission[++], however, the vibrational structure is not so well-resolved. Three peaks at 500 nm, 537 nm and 575 nm are separated by $\Delta\nu \approx 1300$ cm^{-1}.

Warshel and Karplus [11] have calculated the vibrational structure of the transition from the ground state to the $\pi\pi^*$ excited state in several retinal isomers. They found that the vibrational structure of all-*trans*-retinal is very sensitive to rotational conformational changes around the C_6–C_7 bond. According to their results a planar conformation gives more resolved vibrational structure than a twisted conformation. If one accepts these results to be applicable to the *M*-form one is led to conclude that this intermediate is characterized by an essentially planar structure. The vibrational frequency that we observe would then result from C–C or C=C bond stretching modes. Of course it is worth investigating whether this specific structure correlates with the deprotonated state of the *M*-form. For instance, Heyde et al. [12] have found that deprotonated Schiff bases of all-*trans*-retinal have a characteristic Raman band at approx. 1330 cm^{-1}, which might contribute to the rather well-resolved vibrational structure of the *M*-form.

The structure observed around 400 nm in some absorption spectra of light-adapted purple membrane [13,14] agrees with that of the spectrum of the *M*-form presented in this work. It can thus not be excluded that some *M*-form is present in light-adapted samples of purple membrane even if these are cooled in the dark.

Finally we want to stress that the Stokes shift observed in the spectra of the *O*- and *M*-form respectively (1.650 cm^{-1} and 1.700 cm^{-1}) are small as compared to that of isolated all-*trans*-retinal (~ 7.000 cm^{-1}) and related molecules. It is therefore not necessary to assume that the emission in these intermediates originates from a forbidden low-lying state or from an excited state with a geometry which is strongly distorted from that of the ground state [15].

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[+]Our data clearly indicate that the *M*-form does not give rise to a second absorption maximum at longer wavelengths ($\lambda \approx 520$ nm) [2,3,5].

[++]In ref. [14] it has been suggested that the absorption observed for dark- and light-adapted purple membrane (see fig.1 in ref. [14]) in the range of 400 nm is due to a higher $\pi\pi^*$-transition of bR. Our results show that absorption in this region can be explained at least in part by the presence of two molecular species not identical with bR. One is the *M*-form giving rise to a structured absorption. Its presence would depend on the experimental conditions. The second molecule gives rise to an unstructured emission ($\lambda_{max} \simeq$ 460 nm) and excitation spectrum ($\lambda_{max} \simeq 380$ nm) and therefore presumably to an unstructured absorption. The nature of this species is unknown, but its spectral properties resemble strongly those of isolated Schiff bases of retinal. Since emission from higher $\pi\pi^*$ states in condensed media is very exceptional, the unstructured excitation spectrum cannot belong to bR. Our results do not rule out that in addition a higher $\pi\pi^*$ transition of bR contributes in part to this absorption at 400 nm.

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