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# A SPECTROFLUOROMETRIC STUDY OF THE ENVIRONMENT OF TRYPTOPHANS IN BACTERIORHODOPSIN

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The emission spectrum of intact purple membranes of *Halobacterium halobium* has a very short wavelength position (the main maximum at 314 nm) and can be fitted by two spectral components, one of which (component A) corresponds to the fluorescence of buried tryptophan residues located in a highly hydrophobic rigid environment (like the single tryptophan residue in azurin), the other (component I) being due to the emission of buried tryptophan residues located in a rather polar environment. Treatment of bacteriorhodopsin by $NaBH_4$, fragmentation of the membranes and thermal formation of vesicles result in a decrease in the contribution of component A, an increase in that of component I and the appearance of spectral components corresponding to the emission of surface tryptophan residues. Temperature induces at least two distinct changes of the fluorescence parameters of the protein: one change occurs from 45 to 65°C, the other from 65 to 90°C. The spectral changes correlate with the peaks of heat sorption caused by thermal transitions in the purple membrane structure and conformational changes in the protein structure. Alkaline denaturation of bacteriorhodopsin registered by tryptophan fluorescence begins at pH > 11.0.

## 1. Introduction

Bacteriorhodopsin is a retinal-protein complex of the purple membrane of *Halobacterium halobium* which is responsible for a light-driven proton pump of the membrane (for a review, see ref. 1). The apoprotein is a single polypeptide chain ($M_r$ 26 000) organized into seven $\alpha$-helical segments [2]. The retinal moiety is attached to one of these $\alpha$-helical segments via a protonated Schiff-base linkage with a lysine $\epsilon$-amino group [3,4]. Most of the studies on bacteriorhodopsin have been devoted to light-induced events in the protein. The illumination of bacteriorhodopsin results in a rapid cyclic photochemical reaction of the pigment and in a series of conformational changes in the protein structure [1,5].

Bacteriorhodopsin contains seven tryptophan residues per molecule [6]. This allows the study of its properties by the intrinsic fluorescence method. However, the data obtained by various investigators on tryptophan emission of bacteriorhodopsin are in some contradiction with each other [7–9]. Even the shapes and positions of the bacteriorhodopsin fluorescence spectra determined by various authors are different.

In the study reported here we have attempted to measure and analyse the tryptophan fluorescence of bacteriorhodopsin in more detail and to reveal changes in the tryptophan residue environments induced by temperature and pH. The results show that the emission spectrum of intact purple membranes has a very short-wavelength position and can by fitted by two spectral components, one of which (component A) corresponds to the emission of buried tryptophan residues located in a highly hydrophobic rigid environment (like the single tryptophan residue in azurin), the other one (com-

ponent I) to the emission of buried tryptophan residues located in a rather polar environment. Fragmentation of the membranes and formation of vesicles result in a decrease in the contribution of component A to the total fluorescence spectrum. Treatment of bacteriorhodopsin with $NaBH_4$ which leaves retinal covalently bound also causes a decrease in the contribution of component A and the appearance of a component corresponding to the emission of surface tryptophan chromophores. Temperature and pH affect the environment of tryptophan residues in bacteriorhodopsin. Temperature-induced changes of bacteriorhodopsin fluorescence have a two-stepped character and take place within the regions from 45 to 65°C and from 65 to 90°C.

## 2. Materials and methods

The Pushchino 353 strain of *H. halobium* was used in our work. Purple membranes were isolated and purified according to Oesterhelt and Stoeckenius [4]. The spectroscopic measurements were carried out in 0.1 M borate, pH 9.55. Concentrations of bacteriorhodopsin were evaluated spectrophotometrically using $\epsilon_{570} = 63\,000\ M^{-1}\ cm^{-1}$ [10] and were $2-6 \times 10^{-6}$ M in all experiments. All chemicals used were of analytical grade.

Fluorescence spectra were recorded from the front cell surface of a laboratory-made spectrofluorimeter described earlier [11]. All fluorescence spectra were corrected for the instrumental spectral sensitivity. In order to correct the protein fluorescence spectra for screening and reabsorbing inner filter effects of retinal, the derived correction factor was used for each fluorescence wavelength, $\lambda$ [12]:

$$w(\lambda) = \frac{1 - T_p}{1 - T_p T_e T_r} \cdot \frac{D_p + D_e + D_r}{D_p}$$

where $T$ and $D$ are transparency and absorbance ($T = 10^{-D}$), respectively. Subscripts p and e refer to the protein and screening agent at the excitation wavelength (280.4 nm), respectively, and r to the reabsorbing agent (retinal) at the fluorescence wavelength $\lambda$. To minimize effects of light scattering, rather low concentrations of the protein were used and the sample and reference cells in spectrophotometric measurements were placed near the photomultipliers. In all experiments the light scattering was low and no special corrections were needed. Intensities in the corrected spectra are proportional to the number of photons emitted per unit wavelength interval. Spectral resolution was 0.7–1.1 nm.

Protein fluorescence quantum yield was evaluated by comparing areas under fluorescence spectra of a protein sample with that of an aqueous tryptophan solution (quantum yield 0.23 at 20°C [13]) with the same absorbance at the excitation wavelength. The position of the middle of a chord drawn at the 80% level of the maximal intensity ($\bar{\lambda}$) was taken as the position of the spectrum.

The temperature dependence of the fluorescence spectrum characteristics was investigated using thermostatically controlled water circulating in a hollow brass cell holder. The temperature in the sample cell was monitored by means of a copper-constantan thermocouple.

Ultraviolet absorption spectra were measured with a Specord UV-VIS spectrophotometer (Carl Zeiss, Jena, G.D.R.).

Calorimetric measurements were carried out by means of a DASM-1M scanning adiabatic microcalorimeter (U.S.S.R.) with a 1 ml cell volume (heating rate 1 K $min^{-1}$).

Circular dichroism measurements were performed with a J-20 spectropolarimeter (Jasco, Japan).

pH values of solutions were measured to an accuracy of $\pm 0.05$ pH units.

The fitting of the experimental spectra with theoretical spectral components was carried out with an M-4030 computer using a standard optimization program [14]. The quality of the fit was judged from the weighted root mean square residual and by visual inspection of the randomness of the weighted residual plots. Adequacy of the curve fitting to the experimental points was checked by the fitting of model spectra composed from known contributions of the spectral components. When the contributions of the components are within the range from 0.1 to 0.9 the optimization program determines the contributions quite well.

## 3. Results

Fig. 1 shows the spectrum of intact bacteriorhodopsin fluorescence excited at 280.4 nm. The spectrum has a very short wavelength position (the main maximum at approx. 314 nm and a shoulder at approx. 303 nm). Excitation at 289.7 nm gives practically the same spectrum. It must be borne in mind that the fluorescence spectra of most tryptophan-containing proteins have maxima within the region from approx. 330 to approx. 353 nm [15]. Only a few proteins demonstrate positions at shorter wavelengths of their tryptophan fluorescence spectra. The maxima of the emission spectra for some proteins are located within the region from approx. 316 to 320 nm (L-asparaginase of *Escherichia coli*, ribonuclease $T_1$ and $C_2$, parvalbumin of whiting [15–17]). The fluorescence spectrum of azurin has the shortest wavelength position (the main maximum at approx. 307 nm) and a well resolved vibrational fine structure [18].

According to the model of discrete states of tryptophan residues in proteins [15,16,19], there are several most probable physical states of tryptophan residues. Each of the states is characterized by its fluorescence spectrum. According to Burstein's classification [16], there are, at least, five most probable states of tryptophan residues in proteins. The fluorescence spectrum of class A (fig. 2, curve A) corresponds to the buried location of tryptophan residues unperturbed by any interactions with an extremely rigid and hydrophobic environment (like the single tryptophan residue in azurin [18]). The fluorescence spectra of class S and I (fig. 2, curves S and I) also belong to the buried tryptophan residues in a rather rigid environment which contains some polar groups with different mobilities. The spectra of classes II and III (fig. 2, curves II and III) correspond to the location of tryptophan residues at the protein molecule surface in contact with bound (class II) or free (class III) water molecules.

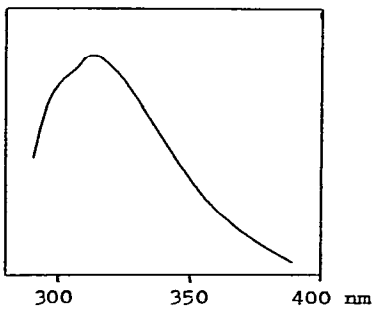


Fig. 1. Fluorescence spectrum of intact purple membranes. 0.05 M borate, pH 9.55; 20°C; excitation wavelength 280.4 nm.

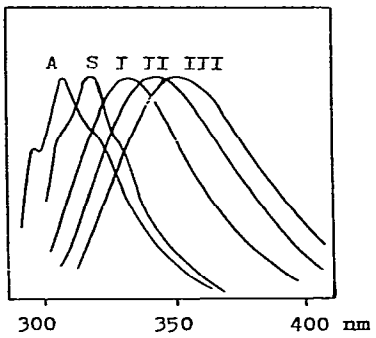


Fig. 2. Normalized spectra of classes A, S, I, II and III [16] corresponding to the model of discrete states of tryptophan residues in proteins.

We have attempted to analyse the emission spectrum of the intact purple membranes on the basis of the model of discrete states of tryptophan residues in proteins. The spectrum was fitted by the spectral components A, I, II and III and the tyrosine fluorescence spectrum T [20,21]. Component S was not used in the fit because the presence of both components A and S in the protein spectrum seems to be unlikely, since there are few proteins containing even one component A (or S) in their fluorescence spectra. The best fit is achieved by components T, A and I (fig. 3) which suggests that in intact bacteriorhodopsin all tryptophan residues are located in the interior of the molecule and that some of them have a rather unusual extremely rigid and hydrophobic environment which is similar to that of the tryptophan residue in azurin. The relative contributions of the components which give the best fit of the experimental spectrum and parameters of the spectrum are presented in table 1.

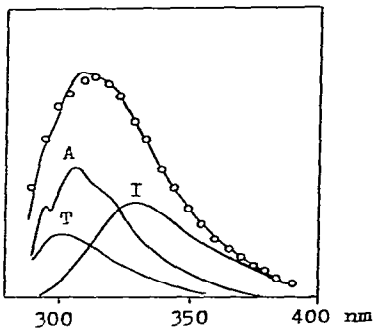


Fig. 3. Fitting of the experimental fluorescence spectrum of intact bacteriorhodopsin (points) by theoretical one (curve) which is the sum of the spectral components T, A, I, II and III.

Treatment of the intact purple membranes with $NaBH_4$ results in a decrease in the contribution of component A and in an increase in those of components I and III (table 1). This suggests the appearance of some tryptophan residues at the protein surface in contact with water molecules.

Formation of vesicles caused by heat treatment of intact or $NaBH_4$-treated purple membranes [22] also leads to a decrease in the contribution of component A and an increase in those components I and II. The complete heat denaturation of the purple membranes results in a very pronounced decrease in the contribution of component A. In this case component I gives the main contribution to the total emission spectrum. The fluorescence quantum yield value of the heat-denatured purple membranes is higher than that of the native preparation, though it is much lower than that of free aqueous tryptophan.

### *3.1. Thermal denaturation*

Fig. 4 shows the temperature dependence of the fluorescence parameters of the intact purple membranes. Heating of the preparation from 7 up to approx. 45°C does not significantly change the fluorescence spectrum position (fig. 4A, curve 1). A shift of the spectrum towards longer wavelengths begins above 45°C. At least two distinct steps can be seen on the curve of $\bar{\lambda}$ vs. temperature. One step occurs from approx. 45 to approx. 65°C, the other from approx. 65 to approx. 90°C. The main shift (by about 15 nm) occurs in the region from 65 to 90°C and is accompanied by a rise of the fluorescence quantum yield value (fig.

Table I

Parameters of the fluorescence spectra of bacteriorhodopsin in different states

$\bar{\lambda}$, position of the spectrum; $\Delta\lambda$, spectrum width; $q$, fluorescence quantum yield; T, A, I, II and III, relative contributions of the emission of tyrosine and tryptophan residues of spectral classes A, I, II and III, respectively, to the experimental spectrum. 0.05 M borate, pH 9.55; 20°C; excitation wavelength 280.4 nm.

| State of bacteriorhodopsin | $\bar{\lambda}$ (nm) | $\Delta\lambda$ (nm) | $q$ | T | A | I | II | III |
|---|---|---|---|---|---|---|---|---|
| Intact purple membranes | 314.2 | 55 | 0.014 | 0.19 | 0.39 | 0.02 | 0.00 | |
| Vesicles from intact membranes | 319.0 | 56 | 0.014 | 0.19 | 0.26 | 0.51 | 0.00 | 0.05 |
| Heat-denatured membranes | 330.2 | 56 | 0.028 | 0.09 | 0.08 | 0.73 | 0.00 | 0.11 |
| Purple membranes treated with $NaBH_4$ | 316.7 | 60 | 0.014 | 0.17 | 0.30 | 0.37 | 0.00 | 0.16 |
| Vesicles from membranes treated with $NaBH_4$ | 322.8 | 64 | 0.015 | 0.16 | 0.22 | 0.39 | 0.08 | 0.15 |
| Heat-denatured membranes treated with $NaBH_4$ | 327.3 | 63 | 0.016 | 0.16 | 0.11 | 0.55 | 0.00 | 0.19 |

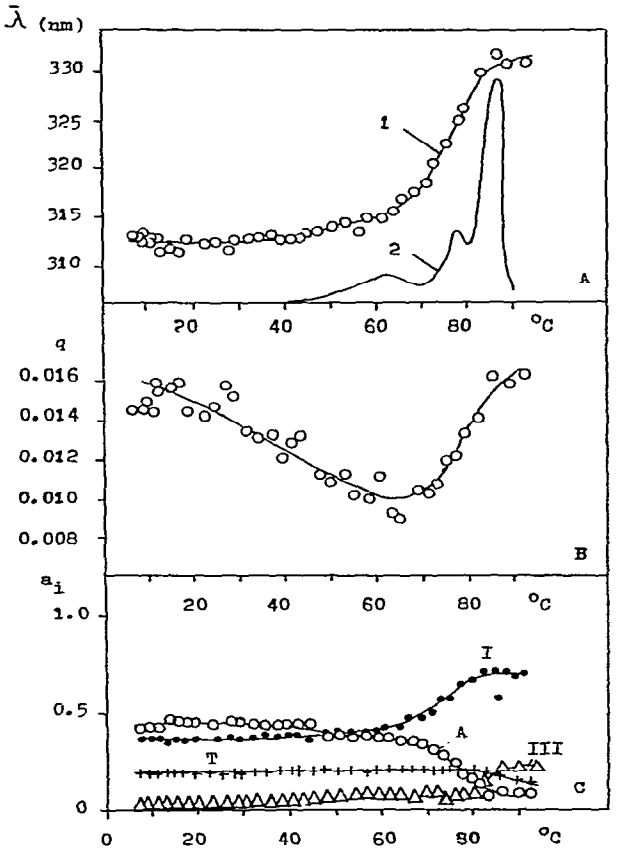


Fig. 4. Temperature dependence of the fluorescence parameters of intact bacteriorhodopsin. (A) Spectrum position $\bar{\lambda}$ (curve 1); (B) fluorescence quantum yield, $q$; (C) contributions of spectral components T, A, I, II and III to the total protein spectrum. Curve 2 in A is a calorimetric record. 0.05 M borate, pH 9.55; excitation wavelength 280.4 nm.

4B). The analysis of the data on the basis of the model of discrete states of tryptophan residues in proteins shows that the spectral shift corresponds to a decrease in the contribution of component A, an increase in that of component I and the appearance and increase in that of component III (fig. 4C). This suggests that temperature induces a transition of some buried tryptophan residues from a rigid polar environment into a more polar or even an aqueous environment. The two-stepped character of the thermal denaturation curve is seen well from the phase diagram in fig. 5A [15,17,23]. The first part of the phase plot of $I(\lambda_{360})$ vs. $I(\lambda_{320})$ (10–45°C), which can be extrapolated to the origin, corresponds to ordinary thermal quenching, without any changes in shape or position of the spectrum, due to the thermal activation of intramolecular collisions between excited indole groups and neighbouring quenching groups [15]. The second (45–65°C) and third (65–93°C) linear parts seem to correspond to at last two successive conformation changes affecting the structure around some tryptophan residues. The existence of two segments in the phase plot indicates that the denaturation process proceeds via an intermediate, the maximal population of which is observed at about 65°C under our conditions. The fluorescence spectra of the protein at 10, 65 and 93°C are presented in fig. 5B.

Curve 2 in fig. 4A is a calorimetric record for the intact purple membranes. One can see three peaks of the heat sorption at 62, 78 and 87°C. The first peak corresponds to the lower temperature spectral change, and the other two correlate with the higher temperature spectral shift.

### 3.2. Acidic and alkaline denaturation

Fig. 6 depicts the pH dependence of the fluorescence spectrum characteristics of the intact purple membranes measured at 20°C. In the pH range 6–11 all the parameters remain essentially constant. The long-wavelength shift of the fluorescence spectrum at pH > 11 could be due to the alkaline denaturation of bacteriorhodopsin caused by deprotonation of arginine residues and at least partially by titration of the phenolic –OH groups of some tyrosine residues with abnormal $pK_a$ values. The appearance of tyrosine at pH > 11 is seen well in the absorption spectrum of the protein (fig. 6B, curve 2). The drop in quantum yield value at pH > 11 seems to be due to long-distance tryptophan-tyrosine energy transfer [15]. Analysis of the data on the basis of the model of discrete states of tryptophan residues in proteins shows that the alkaline pH-induced spectral changes correspond to the transition of some buried tryptophan chromophores to the surface of the

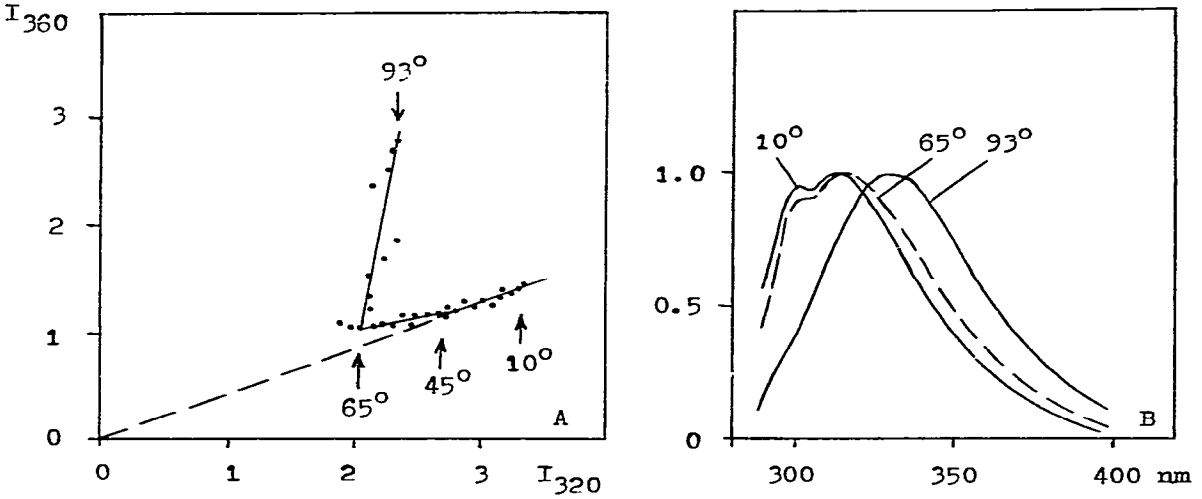


Fig. 5. (A) Fluorescence phase plot corresponding to the temperature dependence of the intact bacteriorhodopsin fluorescence in fig. 4. (B) Fluorescence spectra of bacteriorhodopsin at different temperatures. Conditions as in fig. 4.

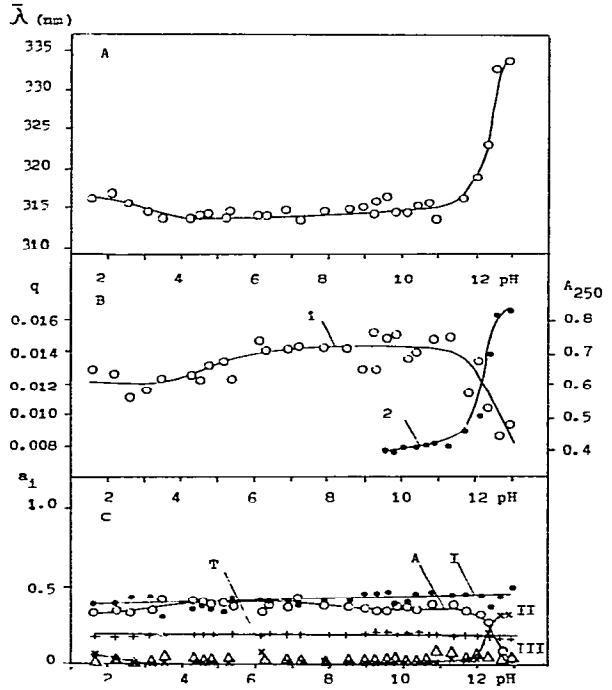


Fig. 6. pH dependence of the fluorescence parameters of intact bacteriorhodopsin. (A) Spectrum position, $\bar{\lambda}$; (B) 1, fluorescence quantum yield, $q$; 2, absorbance at 250 nm; (C) contributions of the spectral components T, A, I, II and III to the total protein spectrum. 0.05 M borate; 20°C; excitation wavelength 280.4 nm.

protein, resulting in contact with water molecules (fig. 6C).

In the acidic parts of the plots a small long-wavelength shift of the fluorescence spectrum takes place at pH $< 3.5$ and a decrease in the fluorescence yield occurs between pH 6.0 and 3.5. The changes seem to be caused by titration of some carboxyl groups of the protein.

## 4. Discussion

The fluorescence spectrum of intact bacteriorhodopsin measured in this study differs from those registered in the works of other investigators. The spectrum registered with a spectral resolution of approx. 1 nm and corrected for the instrumental spectral sensitivity and the screening and reabsorbing inner filter effects has the main maximum at approx. 314 nm and a shoulder at approx. 303 nm. According to Bogomolni et al. [7], the fluores-

cence spectrum of bacteriorhodopsin is relatively featureless and has the maximum at 330 nm and a slight shoulder at 350 nm. The limitations of their measurements are poor spectral resolution (13.2 nm) and the right-angle geometry of their spectrofluorimeter which did not allow correction of the spectrum for screening and reabsorbing inner filter effects. The fluorescence spectrum of the native bacteriorhodopsin registered by Fukumoto et al. [9] with a higher spectral resolution (2.5 nm) has the main maximum at approx. 319 nm and a shoulder at approx. 340 nm. They also did not correct the spectrum for screening and reabsorbing inner filter effects. The longest wavelength position of the emission maximum for intact bacteriorhodopsin was measured by Sherman [24] ($333 \pm 2$ nm). One of the reasons for the differences between the data of various authors could be a difference in nativeness of their membrane preparations. The long-wavelength position of the emission maximum, large width of the spectrum ($64 \pm 2$ nm in the work of Sherman [24]) and the existence of the long-wavelength shoulders in the protein spectrum seem to suggest the presence of some portion of denatured protein in the bacteriorhodopsin preparation.

There is some uncertainty about a tyrosine contribution to the bacteriorhodopsin spectrum. Fukumoto et al. [9] suggested the existence of the tyrosine emission at the excitation at 288 nm but Kaliski et al. [8] presented some reasons for assuming that only light absorbed by tryptophan residues is responsible for the observed emission. Due to the extremely short wavelength position of the bacteriorhodopsin spectrum and the rather high turbidity of the purple membrane suspension we failed to measure the protein fluorescence spectrum at a long-wavelength excitation where only tryptophan residues absorb. It does not allow us to evaluate the tyrosine emission contribution directly. The analysis of the spectrum measured at 280.4 nm excitation on the basis of the model of discrete states of tryptophan residues in proteins shows the existence of some tyrosine contribution to the protein emission spectrum.

The fluorescence quantum yield of bacteriorhodopsin is rather low (0.014 for the intact preparation). According to the data of Kaliski et al. [8], five to six tryptophan residues are completely quenched by energy transfer to the retinyl chromophore, while one tryptophan residue is unquenched and one is partially quenched. Thermal denaturation of the purple membranes leads to a decrease in the energy-transfer efficiency and a subsequent rise of the fluorescence yield.

The results of this work show that the tryptophan fluorescence of bacteriorhodopsin could be used to test for protein nativeness. Treatment of the protein with $NaBH_4$ which leads to disruption of intramolecular protein-pigment interactions results in the appearance of long-wavelength spectral components corresponding to the emission of the surface tryptophan residues. Thermal treatment of the protein which causes only formation of the purple membrane vesicles also changes the tryptophan residue environment making it more polar and more mobile. Both modifying procedures applied practically do not change the circular dichroism spectrum of the protein.

Some words are in order concerning the analysis of the protein spectra on the basis of the model of discrete states of tryptophan residues in proteins. It must be borne in mind that the model is statistical and that the real location of the tryptophan residues in a protein molecule may be somewhat different from that obtained by this analysis. Therefore, the analysis of the protein spectra in terms of the discrete states of tryptophan residues is arbitrary to some extent. However, the model is very demonstrative and allows elucidation of the principal character of changes in chromophore environment.

The temperature dependence of fluorescence parameters for intact bacteriorhodopsin has a biphasic character. One shift of the fluorescence spectra takes place from approx. 45 to approx. 65°C and the other from approx. 65 to approx. 90°C. The first spectral shift corresponds to the lower temperature heat sorption peak and the second correlates with two other higher temperature calorimetric peaks. The heating of the purple membranes induces a transition of the $D_{570}$ form of retinal to the $D_{500}$ one [22]. The transition begins at approx. 40°C and is completely reversible up to 80°C. The origin of the lower temperature heat sorption peak remains unclear. Electron

microscopy data show that heating up to 60–65°C converts the plane purple membranes to semispheres. The heat sorption peak at 78–85°C seems to be due to the formation of purple membrane vesicles [22] and that at 80–90°C can be explained by a conformational transition in the protein molecule which leads to destruction of a part of its α-helical segments and by subsequent destruction of the purple membranes [22,25]. Thus, the first two heat sorption peaks are due to a thermal transition in the purple membrane structure and the third is due partially to conformational changes in the protein structure. One can see that the tryptophan fluorescence of bacteriorhodopsin is sensitive to all these processes. Even transitions in the membrane structure result in changes of the environment of some tryptophan residues in the protein. Our data are in some contradiction with the results of Sherman [24] who registered a nonmonotonic temperature dependence of the bacteriorhodopsin emission yield with a transition between 20 and 40°C which was explained by a melting process in the interstitial membrane lipids. Perhaps these differences are due to differences in nativeness of the purple membrane preparations used in our work and in that of Sherman.

Alkaline denaturation of bacteriorhodopsin registered by tryptophan fluorescence beings at pH > 11. Electron microscopy data show that a pH > 12 the purple membranes are subjected to fragmentation and form spherical vesicles [22]. According to the fluorescence and circular dichroism data, the states of bacteriorhodopsin in alkaline pH- and temperature-denatured forms are somewhat different. Our results on pH denaturation of bacteriorhodopsin are in a good agreement with the data of Sherman [24].

Thus, the results of the present work demonstrate that the environment of the tryptophan residues in bacteriorhodopsin is sensitive to any changes of the protein structure and even to any changes of the membrane structure. Tryptophan fluorescence could be used successfully for a study of bacteriorhodopsin states.

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