Bacteriorhodopsin Thermal Stability: Influence of Bound Cations and Lipids on the Intrinsic Protein Fluorescence

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Z. Naturforsch. **55c,** 355–360 (2000); received December 27, 1999/February 23, 2000

Bacteriorhodopsin, Deionization, Delipidation

Temperature – induced changes in protein intrinsic fluorescence of native, delipidated and deionized purple membranes are investigated. It is found that the removal of cations most strongly affects the protein and its thermal stability. The denaturation of dei-BR completes at 70 °C, while delipidated and native BR still maintain their native structure at this temperature. Both, the quantum yield and the fluorescence maximum suggest correlation between the Trp-retinal coupling and protein structural stability. The low red shift of the fluorescence maximum caused by increasing of temperature indicates limited unfolding of bacteriorhodopsin upon denaturation.

Introduction

The retinal protein, bacteriorhodopsin (BR) is found in the cell membranes of *Halobacterium salinarium* and functions as a light-driven proton pump. It consists of a single polypeptide chain of 248 residues, folded in seven roughly parallel transmembrane α-helical segments separated by small loop regions on the membrane surfaces (Henderson and Unwin, 1975; Lanyi, 1993; Henderson *et al.*, 1990). Bacteriorhodopsin molecules form trimers, which are arranged in highly ordered two-dimensional hexagonal lattice, so called purple membranes. The purple colour of the membranes, with an strong absorption band at 570 nm, is due to a retinal chromophore covalently attached to the e-amino group of Lys 216 via a protonated Shiff base (Grigoriev *et al.*, 1996).

The removal of divalent cations by deionization or by acidification of purple membranes results in formation of a blue form of BR (λ_{max} = 603 nm) with an altered photocycle and inhibited proton pump (Kobayashi *et al.*, 1983; Kimura *et al.*, 1984; Chronister *et al.*, 1986; Chang *et al.*, 1986). Partial delipidation of purple membranes changes slightly the absorption maximum (λ_{max} = 561 nm), reduces the rate and the efficiency of the Shiff base deprotonation and affects the purple to blue spectral transition (Szundi and Stoeckenius, 1987; Jang *et al.*, 1988; Jang and El-Sayed, 1988).

The intrinsic protein fluorescence of BR originates from Trp residues and the contribution from Tyr residues is negligible (Sherman, 1981; Sherman, 1982). The low quantum yield of protein fluorescence is accounted for a strong quenching by energy transfer to the retinal chromophore (Permyakov and Shnyrov, 1983; Acuña *et al.*, 1984; Palmer and Sherman, 1985). Recent structural data of BR (Grigoriev *et al.*, 1996) show that four of all eight Trp residues are in close contact with the retinal, thus they are not expected to contribute to the protein fluorescence. It is well established that the fluorescence parameters are very sensitive to the fluorophore environment and they can report on small conformational changes in the proteins (Burstein *et al.*, 1973).

In the present work the protein stability of BR after membrane perturbations induced by removal of bound cations and endogenous lipids, and by low pH is studied by using intrinsic protein fluorescence. It is found that deionization and low pH destabilize the protein and its thermal stability. A strong correlation between Trp-retinal coupling and the protein native state is suggested.

Materials and Methods

Purple membranes (PM) were isolated from *Halobacterium salinarium* (strain S9) as described

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previously (Oesterhelt and Stoeckenius, 1974). Delipidated (dL-BR) membranes were prepared by treatment with the zwiterionic surfactant 3-[(3-cholamidopropyl) dimethylammonio]-1-propane sulfonate (CHAPS) as in (Szundi and Stoeckenius, 1987). Deionized (dei-BR) membranes were prepared by passage of purple membranes through cation exchange column (Dowex AG 50) according to Kimura *et al.*, 1984. Protein concentrations were evaluated spectrophotometrically using extinction coefficients, $\lambda_{568\,nm} = 63000\ M^{-1}\ cm^{-1}$ for PM and $\lambda_{603\,nm} = 60000\ M^{-1}\ cm^{-1}$ for dei-BR (Kimura *et al.*, 1984; Oesterhelt and Stoeckenius, 1971). Steady-state fluorescence spectra were recorded by a Jobin-Ivon JY3 spectrofluorimeter using 4 and 2 nm bandpass slits and 1 cm quartz cuvette. The spectra were not corrected for the spectral response of the detection system. The sample absorption at 286 nm was adjusted to be less than 0.1 in order to minimize light scattering. Fluorescence quantum yield (QY) was estimated by comparing the areas under the fluorescence spectra of PM with that of aqueous Trp solution with the same absorption at the excitation wavelength (Aldashev and Efremov, 1983). The position of the emission fluorescence spectrum (λ_{max}) was evaluated as described in (Permyakov and Shnyrov, 1983). The temperature of the sample was maintained using thermostatically controlled circulating water in the sample holder and was monitored by flexible thermistor. Analysis of both the fluorescence emission and the difference spectra between the samples being at different temperatures (in the range from 25 ° to 90 ° C) and samples at 25 °C was performed for native, delipidated and deionized purple membranes. The difference spectra enable us to estimate the changes in the location of Trp emitters upon temperature increase.

Results

The fluorescence parameters, emission maximum and quantum yield of native (nBR), delipidated, deionized and acid – BR samples measured at room temperature are listed in Table I. The relatively short wavelength of intrinsic protein fluorescence of nBR samples indicates that the Trp emitters are located mainly in a hydrophobic environment (Burstein, 1977). Delipidation of purple membranes leads to a reduction of the quantum yield and to a subtle red shift of the emission maximum as compared to native BR. The acidification of the medium to pH 3.2 at low ionic strength gives values similar to that at neutral pH, suggesting no significant alteration in the protein structure. This finding agrees with the absence of secondary and minor tertiary structural changes found in native BR at this pH (Muccio and Cassim, 1979; Brouilette *et al.*, 1987). The deionization, however, results in an apparent enhancement of quantum yield values and in a red shift of the λ_{max} in comparison with nBR sample (Table I).

Fig. 1. represents the temperature dependence of the fluorescence parameters of native and delipidated BR samples at neutral pH. The fluorescence maximum does not change significantly upon heating up to 70 °C and up to 90 °C for nBR and dL-BR, respectively (Fig. 1A). Further increase of temperature results in a red shifted maximum, due to exposure of Trp emitters to more hydrophilic environments (Burstein *et al.*, 1973; Burstein, 1977). The temperature induced changes in the Trp environment can be unambiguously followed in the calculated difference spectra. The difference spectrum obtained by subtracting the spectrum at 75 °C from that at 25 °C, along with the fluorescence spectra of nbR sample at both temperatures are shown on Fig. 2A. The dif-

Table I. Protein fluorescence parameters of perturbed BR samples at room temperature.

Sample	Native bR pH 7 (nBR)	Native bR pH 3.2 (acid-BR)	Delipidated bR pH 7 (dL-BR)	Deionized bR pH 4.4 (dei-BR)	Trp
λ_{max} (nm)	321	323	323	327	356
QY	0.014	0.014	0.012	0.019	0.23*

QY – fluorescence quantum yield.
λ_{max} – position of the emission maximum.
* – data for Trp QY are according to Aldashev and Efremov (1983).

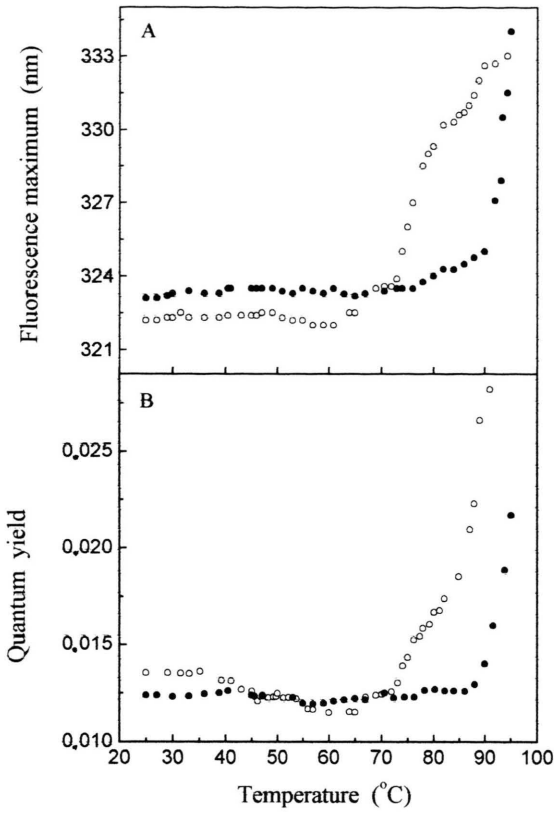

Fig. 1. The temperature dependence of the fluorescence maximum λ_{max} (A) and the quantum yield QY values (B) of fluorescence of native (nBR) (○) and delipidated BR (dL-BR) (●), suspended in 3 mM HEPES (*N* -2-hydroxylethyl piperazine-*N'*-2-ethanesulfonic acid), pH 7. Excitation wavelength 286 nm. The concentration of BR was 0.8 mM.

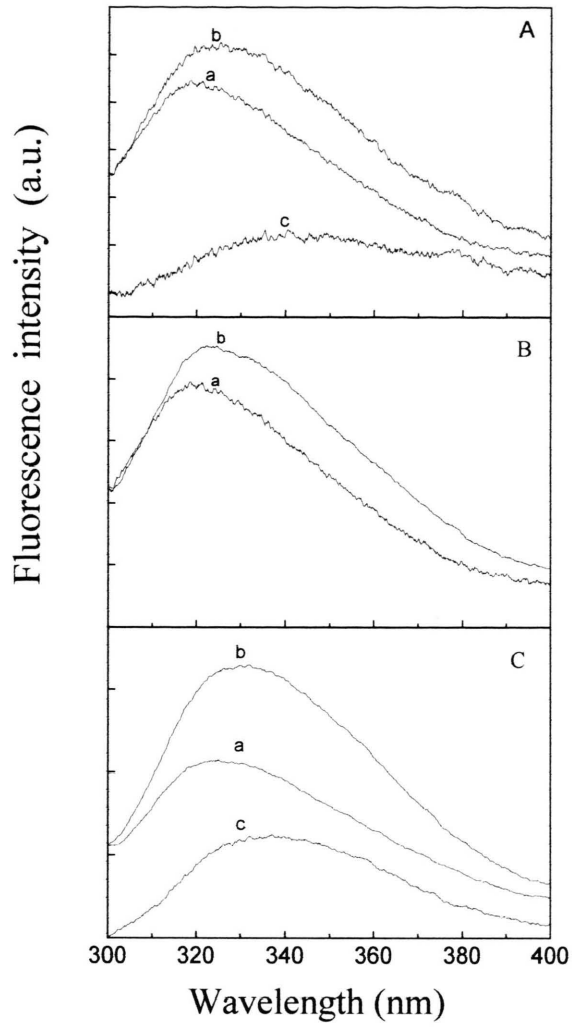

Fig. 2. (A) The fluorescence emission spectra of nBR suspended in 3 mM HEPES pH 7 at 25 °C (a) and 75 °C (b), and the difference spectra (c) between curves b and a. The concentration of BR was the same as in Fig. 1.; (B) Comparison of the fluorescence spectra of nBR (pH 7) (a) and dei-BR (b) at room temperature; (C) The fluorescence spectra of dei-BR at 25 °C (a) and 75 °C (b); curve c is the difference spectrum between curves b and a.

ference spectrum displays a maximum at 340 nm, suggesting a more polar environment of BR emitters at higher temperature. The contribution of the long wavelength component at 340 nm in the difference spectra increases with further increase of temperature and becomes dominant above 85 °C and 90 °C for nBR and dL-BR samples, respectively (spectra not shown). The quantum yield values and the fluorescence maximum follow similar temperature dependence for both samples (Fig. 1A and B). The slight decrease of the fluorescence intensity observed for nBR sample in the temperature range from 20 ° to 70 °C might account for thermal activation of intermolecular collisions between excited indols and the neighbouring quenching groups (Burstein, 1977). Heating nBR sample above 75 °C leads to significant enhancement of the quantum yield, which starts at higher temperature for dL-BR sample (Fig. 1B). Moreover, temperature dependence of the quantum yield reveals some differences between both samples (Fig. 1B). For nBR sample it consists of two regions, characterized by a gradual increase in the range 70 °C to 85 °C and a steep increase

above 85 °C. In contrast, the quantum yield values of dL-BR sample exhibits only an abrupt one-step increase above 90 °C.

At room temperature dei-BR sample exhibits altered fluorescence parameters compared to the nBR sample (Fig. 2B, Table I). Increase of temperature results in further red shift of the maximum and appearance of component at 340 nm in the difference spectra of dei-BR, similar to that seen in the nbR difference spectrum (Fig. 2C). Comparison between dei-BR – and nBR difference spectra (Fig. 2C and Fig. 2A), calculated for the same temperature gradient, shows that the 340 nm component has a higher contribution in dei-BR difference spectra suggesting a more loosely protein structure after removal of cations. Further evidence for perturbed protein structure induced by removal of bound cations comes from thermal denaturation experiments. Fig. 3 displays the temperature dependencies of the fluorescence parameters of dei- and acid-BR samples. Despite that acidification merely induces some changes in fluorescence parameters of BR at room temperature (Table I), the thermal stability of the protein is reduced at low pH (Fig. 3). The increase of the quantum yield above 70 °C is accompanied by a red shift of fluorescence maximum and it reaches a plateau above 85 °C. Dei-BR sample is less resistant to temperature raise. The fluorescence intensity changes began at about 55 °C and they are completed at about 75 °C (Fig. 3B). It is worth to mention that both parameters, the absorption at 292 nm (as reported previously by Cladera *et al.*, 1988) and fluorescence quantum yield values of dei-BR samples (Fig. 3B) obey the same temperature dependence. Similarly to the quantum yield values, the emission maxima shift towards longer wavelengths at temperatures above 60 °C for acid-BR and 50 °C for dei-BR, respectively (Fig. 3A).

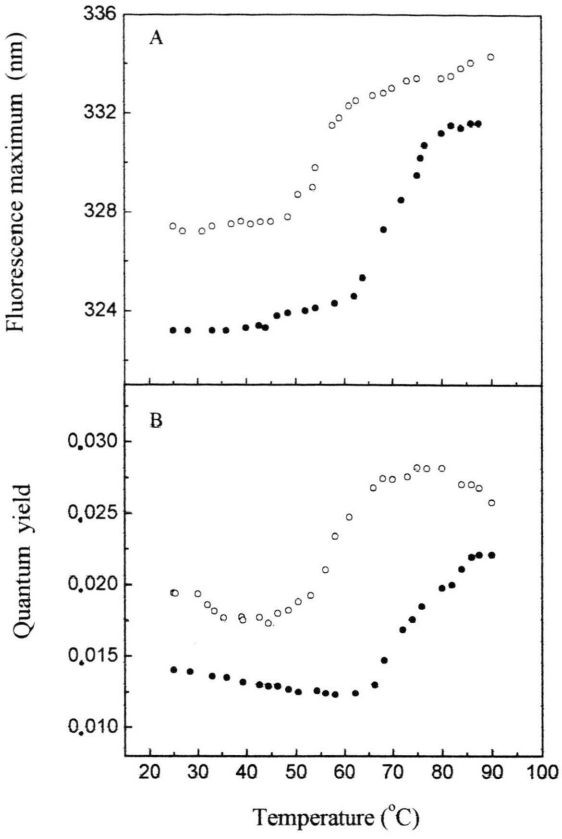

Fig. 3. Temperature dependence of the fluorescence maximum λ_{max} (A) and the quantum yield QY (B) of dei-BR (○) and acid- BR (●) in 3 mM acetate buffer, pH 3.2.

Discussion

We found that among the perturbants used herein the deionization has the strongest effect on protein structure. The removal of divalent cations induces conformational changes, which result in exposure some of Trp emitters to a more hydrophilic environment and in decoupling between the chromophore and fluorescence emitters (Jang *et al.*, 1988, 1990; Mercier *et al.*, 1988). Thermal stability of dei-BR is also strongly reduced and the maximum of fluorescence intensity is reached at 70 °C. Partial delipidation of purple membranes causes some changes in fluorescence parameters, but mainly affects the quantum yield. Lower quantum yield of dL-BR samples, measured at room temperature suggests an enhanced coupling between the aromatic residues and the retinal, which in turn leads to stronger fluorescence quenching. This is most likely due to an altered size of the paracrystalline lattice of BR trimers after detergent treatment, which may influence Trp-retinal coupling (Glaeser *et al.*, 1985).

Using FTIR and DSC (differential scanning calorimetry) methods it has been established that secondary and tertiary structures of native BR remain stable in wide temperature range at neutral pH (Brouillete *et al.*, 1987; Cladera *et al.*, 1992). In

accordance with these data, the obtained fluorescence parameters (λ_{max} and QY) do not reveal any changes upon heating of nBR samples up to 70 °C (Fig. 1). The alterations in fluorescence parameters measured above 80 °C indicate conformation changes in protein structure, at least near to Trp residues and/or to the retinal, both being responsible for the protein fluorescence intensity (Sherman, 1981; Acuña *et al.*, 1984). It is worth to mention that above 80 °C a sharp increase in the UV-absorbances at 280 nm was detected (Brouillete *et al.*, 1987) and an endothermic peak was recorded by DSC (Kresheck *et al.*, 1990; Taneva *et al.*, 1994). Delipidation of purple membranes does not reduce the thermal stability of the protein. In contrary, the sharp increases of fluorescence intensity starts at higher temperature than in the native BR sample (Fig. 1B). Moreover, as already mentioned in the Results, the thermal dependence of fluorescence parameters is different for nBR and for dL-BR samples. The quantum yield increases for the nBR sample in the temperature range 75 °–85 °C, which closely correlates with the "premelting transition" temperature range of native BR (at about 80 °C) recorded by DSC (Kresheck *et al.*, 1990; Taneva *et al.*, 1994). Lack of any changes in quantum yield values of dL-BR sample in this temperature range might be accounted for the absence of pretransition in DSC scans of dL-BR. On the other hand the abrupt rise of the protein fluorescence at about 90 °C for dL-BR and above 85 °C for nBR correlates with the rise of the heat sorption of both samples upon denaturation. The different thermal behaviour of both samples suggests that lipid – protein interactions most likely control the cooperativity of denaturation. Previous DSC data show that the main transition (T_m) for both samples take place at rather high temperatures, 96 °C for nBR and 99 °C for dL-BR (Cladera *et al.*, 1988; Kresheck *et al.*, 1990; Taneva *et al.*, 1994). Because of restrictions in equipment, we were not able to follow the accomplishment of the thermal denaturation in these samples. However, the obtained fluorescence parameters reflect closely all conformational changes prior to this process.

The process of BR denaturation in all samples is accompanied by enhance of the fluorescence intensity and red-shift of the emission maximum. However, red-shifted values of fluorescence maxima recorded (Fig. 1A, Fig. 3A) are much shorter than the values (350 nm) for Trp exposed to aqueous environment, characteristic for unfolded proteins (Burstein *et al.*, 1973). Thus, the low red shift of λ_{max} obtained for all samples indicates that hydrophobic groups are not completely exposed to the aqueous environment during denaturation or that there is limited unfolding of BR when it denatures. This interpretation is in accordance with the presence of α helical structure (Cladera *et al.*, 1988) and with calculated lower values of heat capacity in denaturated BR (Brouillete *et al.*, 1987).

In conclusion: both, the quantum yield and the fluorescence maximum suggest a close relation between the Trp-retinal coupling and protein structural stability. The low red shift of the fluorescence maxima caused by temperature increase indicates limited unfolding of bacteriorhodopsin upon denaturation.

Acknowledgements

This work was supported by a contract K-621 from Bulgarian National Fund Science Research. We are thankful to Dr. M. Velitchkova for critical reading of the manuscript.

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