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Thermal denaturation of deionized and native purple membranes

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Spectrophotometric and microcalorimetric techniques have been used to study the influence of cations on the thermal denaturation of bacteriorhodopsin. Deionized (blue) membrane at pH 5.0 shows a lower enthalpy of denaturation than native purple membrane, as well as a denaturation temperature about 20C° lower. Divalent cation binding increases both the temperature and enthalpy of denaturation. pH values also affect thermal denaturation of deionized membrane. At pH 6.5, a denaturation temperature about 20C° higher than at pH 4.0 and a much higher enthalpy are obtained. Ultraviolet difference spectra suggest that tryptophan residues are located in more exposed regions in the deionized membrane than in the native membrane. Hg^{2+}, which does not promote the purple form, only slightly affects the temperature and enthalpy of denaturation. The small reversible pretransition observed for native purple membrane at about 80°C, attributed to a disordering of the lattice distribution of bacteriorhodopsin molecules, is absent in the deionized membrane and in that supplemented either with 1 mol of Mn^{2+} or 5–20 Hg^{2+} mol per mol of bacteriorhodopsin. The possible contribution of surface membrane potential and of defined protein conformation to bacteriorhodopsin stability is discussed.

Introduction

Bacteriorhodopsin, the unique protein found in the purple membrane of *Halobacterium halobium* cells, is a light-driven proton pump [1] (for recent reviews see Refs. 2 and 3). One molecule of retinal is bound to a molecule of bacteriorhodopsin (BR) via a protonated Schiff base to a lysine residue [4–6]. Once a photon is captured by the retinal moiety, a photocycle is initiated, which consists of several intermediates and which is coupled to proton transport from inside to the outside of the cell [7–9]. BR molecules are organized in trimers, which in turn are disposed in a hexagonal arrangement of a paracrystalline nature [10]. Native purple membrane is a very stable complex, showing no thermal denaturation of protein until temperatures near 100°C are reached [11–13]. Similarly, the commonly used denaturating agents such as urea or GdnHCl do not induce denaturation of BR, even at high concentrations [14,15], but only

Abbreviations: $AcTrpNH_2$, *N*-acetyl-L-tryptophanamide; BR, bacteriorhodopsin; DSC, differential scanning calorimetry; GndHCl, guanidine hydrochloride; ΔH, calorimetric enthalpy; $\Delta H^{v.H}$, van't Hoff enthalpy; $T_{c,0}$ melting temperature.

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subtle conformational changes [16]. The sole observable effect brought about by GdnHCl is a remarkable slowing down of the photocycle, at the level of the M412 intermediate [15]. It has recently been demonstrated that native purple membrane binds about 1 mol of Ca^{2+} and 4 mol of Mg^{2+} per mol of BR [17,18]. Removal of these cations turns the membrane blue and inhibits the formation of the M412 intermediate [19]. Cations seem essential for the functioning of BR [19], although it has been shown that deionized membrane turns purple with increasing pH (apparent pK of approx. 5.4), and that this purple form discloses the M412 intermediate [20]. Partially delipidated purple membrane cannot be converted to the blue form by deionization, showing an apparent pK of 1.4 for the blue to purple transition [21]. It has also been indicated that deionized membrane has a diminished thermal stability [22]. On the other hand, removal of cations from the membrane leads to a conformational change that exposes some aromatic residues to a more polar environment [20]. This suggests that the BR molecules adopt a more open conformation in the deionized form. This work was undertaken in order to determine the contribution of divalent cations and pH in the structural stability of the purple membrane. Specifically, we have studied the thermal denaturation of blue and purple membranes as well as several degrees of cation-depleted membrane, by using spectrophotometric and microcalorimetric techniques. We take advantage of our previous characterization of the five divalent cation-binding sites, which shows the presence of one site of affinity constant 26 μM^{-1}, three sites of 2 μM^{-1} and one site of 0.6 μM^{-1} at pH 5.0 [23,24].

Materials and Methods

Purple membrane was isolated from *Halobacterium halobium* strain S9 as described [25]. Deionized samples were prepared by passing purple membrane suspensions through a cation-exchange column (Dowex 50W). The pH of the samples (which eluted from the column with a pH value of 3.8–4.4) was adjusted with microliter amounts of concentrated NaOH (analytical quality), prepared with deionized water. For cation binding, membrane suspensions were adjusted to pH 5.0 and cations were added to the desired concentration. The pH was then readjusted to 5.0. All pH measurements were made with 1- or 2-ml aliquots in order to avoid cation contamination by the electrode. All membrane preparations were dark-adapted prior to the experiments.

Spectrophotometric experiments were carried out using a Perkin-Elmer model 320 double-beam instrument. The cuvette temperature was controlled by means of a Coleman digital controller, with a precision of 0.1 C°. At each temperature, 15 min was allowed for thermal stabilization.

Thermal difference spectra were obtained directly with the spectrophotometer, by storing the absorption spectrum at 20°C in the microprocessor memory, and substracting it from each spectrum at higher temperatures.

Calorimetric experiments were performed with the high-sensitivity differential adiabatic scanning microcalorimeter, DASM-4, designed by Privalov [26] with cell volumes of 0.48 ml, at a heating rate of 2 C° · min^{-1}. An overpressure of 2.5 atm was always kept over the liquids in the cells throughout the scans to prevent any degassing during heating. Sample concentrations were always around 1 mg · ml^{-1} of protein with the purple or the blue membranes suspended in water. The reversibility of the thermal transitions was verified by checking the reproducibility of the calorimetric trace in a second heating of the sample. The enthalpy of the transitions was determined from the areas under the heat capacity curves by means of a 25 μW electric calibration mark. The traces were corrected for the calorimetric (instrumental) base line and, to obtain the area, a linear chemical base line was traced between the initial and final temperatures of the transition. The so-called 'van't Hoff enthalpy', $\Delta H^{v.H}$, of the transition was calculated from the equation [27]:

$$\Delta H^{v.H} = 4RT_m^2 \frac{C_p^m}{Q_t}$$

where T_m stands for the temperature at the maximum of the heat capacity curve, C_p^m for the height of the heat capacity at the maximum, R for the gas constant and Q_t for the total heat of the process. The $\Delta H^{v.H}$ value can thus be obtained

without any reference either to the concentration or to the molecular weight of the sample.

Results

Spectrophotometric studies: visible region

Fig. 1A shows the effect of Mn^{2+} binding on the absorbance change at λ_{max} as a function of temperature, in water at pH 5.0. Besides the known decrease in the visible A_{max} of the native purple membrane as temperature increases [11,13], it is clear that the blue deionized membrane loses its colour at a lower temperature than the native purple membrane (approx. 20 C° lower) *. Binding of Mn^{2+} to blue membrane increases the thermal stability of retinal environment, the first bound cation being more effective than the others. Similar results were obtained using Ca^{2+} or Na^+ (the latter at a molar ratio 10–20 times higher) instead of Mn^{2+}. However, Hg^{2+} binding, which does not promote the purple form [28], not only does not increase the temperature of denaturation, but slightly diminishes it by 2 C° for 5 Hg^{2+} added per BR (not shown). Fig. 1B shows the same type of representation for the deionized membrane at pH values of 4.4 and 5.0 (blue), 5.5 (blue-purple) and 6.5 (purple). It is clear that a pH increase, besides promoting the purple form, also makes the retinal environment more resistant to thermal denaturation. A pH value of 6.5 appears to be equivalent, in this respect, to the binding of 2 or 3 Mn^{2+} per BR at pH 5.0.

As previously described, temperature increase also changes the λ_{max} value [11,13]. Deionized membrane supplemented with various Mn^{2+}/BR ratios elicits an initial shift in its λ_{max} to higher wavelengths, followed by a shift to lower values (not shown). This effect is more important for intermediate Mn^{2+}/BR ratios, whereas the native purple membrane or the deionized (blue) membrane only shows a wavelength shift to lower values. A simple interpretation of the shift to higher wavelength values would be that as temperature increases there is a depletion of cations from the membrane and that this occurs before the onset of denaturation. However, a similar effect is observed for deionized (purple) membrane at pH 6.5, where no cations are responsible for the purple colour.

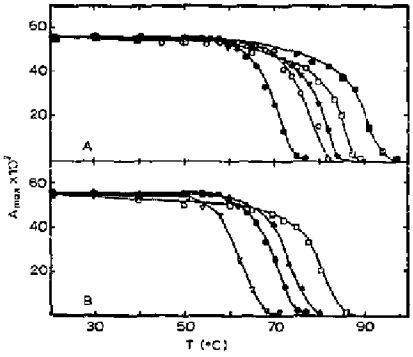

Fig. 1. Plot of A_{max} in the visible region as a function of temperature (BR concentration, 10 μM). (A) ●, deionized membrane; ■, native purple membrane. Deionized membrane with the following amounts of Mn^{2+} ion bound per BR: ○, 1; ▲, 3; □, 5. All samples were at pH 5.0. (B) Deionized membrane at the following pH values: △, 4.4; ●, 5.0; ▲, 5.5; □, 6.5.

Spectrophotometric studies: ultraviolet region

Fig. 2 shows an ultraviolet difference spectrum of the blue membrane upon temperature increase. Similar difference spectra were obtained from the native purple membrane and from the deionized membrane partially regenerated with Mn^{2+}. Fig. 3A shows the effect of cation binding on the absorbance change at 292 nm as a function of temperature in water at pH 5.0. The first part of the plot is clearly linear for all samples (up to 60–65 °C). The difference spectra in this temperature interval are due to the thermal perturbation effect, and represent a direct influence of temperature on several properties of the aromatic aminoacids [29,30]. The shape of these spectra is consistent with thermal perturbation of relatively internal tryptophan residues, as indicated by the negative peaks seen at 292 and 286 nm and the

* The number of water washes (and also the water quality) of the last steps of the purple membrane extraction, influences the denaturation temperature of native purple membrane. This, presumably, is due to the loss of some bound cations as the suspension is washed [18]. In order to obtain comparative results, the purple membrane samples were supplemented with 50 magnesium cations per BR.

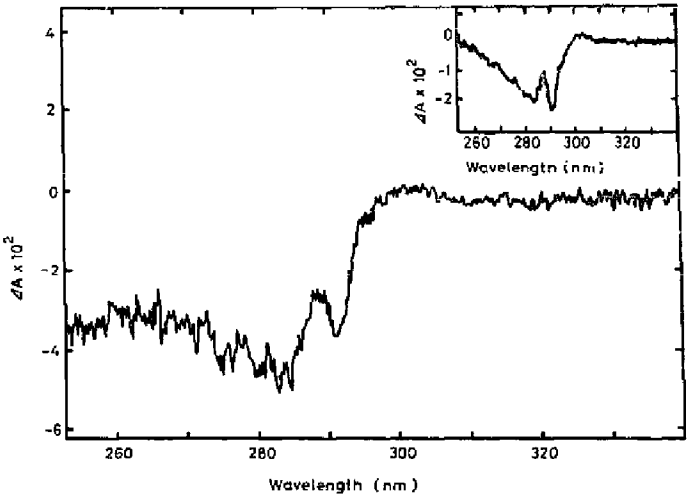

Fig. 2. Ultraviolet difference spectrum of deionized (blue) membrane upon temperature increase (sample at 55 °C minus sample at 20 °C), in water at pH 5.0. BR concentration, 10 μM. Inset, thermal difference spectrum obtained from $AcTrpNH_2$ (50 μM) dissolved in butanol (sample at 50 °C minus sample at 20 °C.

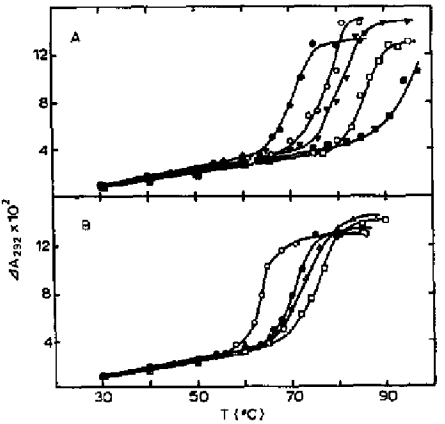

Fig. 3. Influence of Mn^{2+} binding and pH on the ultraviolet difference spectra of the deionized membrane. The plots of ΔA_{292} as a function of the sample temperature are shown. BR concentration, 10 μM. (A) ●, deionized membrane; ■, native purple membrane; deionized membrane with the following amounts of Mn^{2+} ion bound per BR (pH 5.0): ○, 1; ▲, 3; □, 5. (B) deionized membrane at the following pH values: ○, 4.4; ●, 5.0; △, 5.5; □, 6.5.

small positive peak at 300 nm [29,30]. In this case, the spectra are similar to those obtained for $AcTrpNH_2$ dissolved in an apolar solvent such as butanol (Fig. 2, inset). A contribution from tyrosine residues is also likely, as suggested by the increased intensity of the negative broad minimum at 283 nm, as compared with that at 292 nm [30].

Because of the observed linear dependence of ΔA_{292} versus T, no conformational changes are to be expected within this temperature interval [30]. Above 60–65 °C, denaturation is apparent, as indicated by the sigmoidal shape of the curves. Similarly to that observed in the visible region, denaturation occurs at lower temperatures for the deionized membrane or at low levels of Mn^{2+} binding. pH values also influence the T_m, thus rendering the BR molecule more stable as pH increases (Fig. 3B). For 20 Mn^{2+} per BR added to the deionized membrane, a denaturation temperature of 87 °C is observed. In the temperature range where denaturation occurs, the difference spectra are due to a change of aromatic residues from an apolar environment to a more aqueous

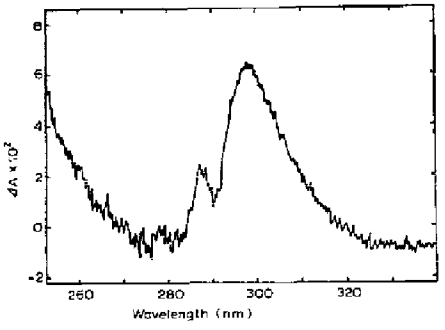

Fig. 4. Ultraviolet difference spectrum of deionized (blue) membrane supplemented with 5 Hg^{2+} per BR (sample at 50 °C minus sample at 20 °C). BR concn., 10 μM (pH, 5.0).

one. This generates a negative difference spectrum, which adds to that produced by thermal perturbation.

Thermal difference spectra of Hg^{2+}-substituted membrane are quite different from those indicated above. Below denaturation, the spectra show a prominent positive peak at about 300 nm, a minor one at about 287 nm and a minimum at about 292 nm (Fig. 4). This shape would be consistent with thermal perturbation of relatively exposed (aqueous) tryptophan residues [29]. At temperatures where denaturation occurs, the presence of a negative component of the difference spectrum is apparent, as indicated by the negative values of ΔA below 290 nm (not shown). However, due to the positive and intense component of the difference spectrum, denaturation of this sample is difficult to follow by ultraviolet difference spectra

Microcalorimetric studies

Fig. 5 shows an original calorimetric curve corresponding to native purple membrane suspended in water, along with an instrumental base line. All differential scanning calorimetry (DSC) base lines were similar to this one, and for the sake of simplicity, all subsequent DSC scans will be depicted with the instrumental base line substracted. As originally reported by Jackson and Sturtevant, native purple membrane exhibits a small transition centered at about 80 °C and a higher one at around 96 °C [11]. In agreement with these authors, the 80 °C transition was found to be highly reversible according to the criterion cited in the Materials and Methods, while the second transition appeared to be irreversible, since it was not seen in a reheat after heating through this 96 °C transition.

As Fig. 6 shows, the deionized membrane in the pH range between 4.0 and 6.5 only discloses the second transition. The T_m of this transition increases with pH from a T_m value of about 71 °C at pH 4.0 to 90 °C at pH 6.5.

Fig. 7 includes the endotherms corresponding to partially regenerated membranes obtained by adding Mn^{2+} to deionized membrane at pH 5.0, from a ratio of 1 to 5 cations per BR molecule. The cation-regenerated membranes show the main transition progressively shifted to higher temperatures as more cations are bound, attaining a T_m of about 87 °C for 5 Mn^{2+} per BR. The first small reversible transition, which is not observed for the deionized membranes, appears between 2 and 3 Mn^{2+} bound per BR.

The thermodynamic parameters of the main transition under the different conditions investigated, are given in Table I. There is a clear difference in the calorimetric enthalpy value between the purple membrane and the deionized blue membrane, both at pH 5.0. The enthalpy of the latter increases with pH, reaching a value at pH 6.5 even higher than that of purple membrane at pH 5.0. On the other hand, partially Mn^{2+}-regen-

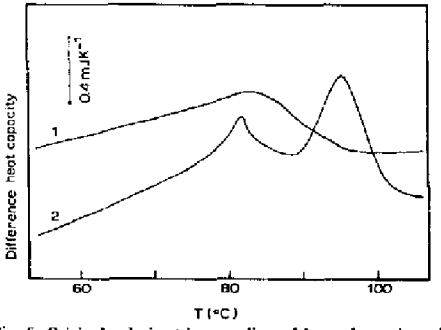

Fig. 5. Original calorimetric recording of heat absorption of native purple membrane in water (pH 5.0). 1, base line; 2, native purple membrane. BR concentration, 1.0 mg·ml^{-1}. Scan rate, 2 C°·min^{-1}.

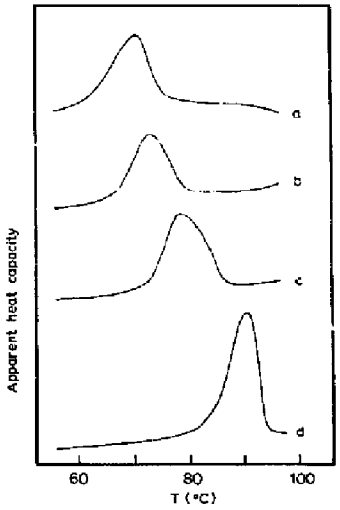

Fig. 6. Effect of pH on the calorimetric profiles of the deionized membrane. The curves correspond to the following pH values: a, 4.0; b, 5.0; c, 5.5; d, 6.5. The instrumental base line has been substracted in all cases. BR concentration 1.0 mg·ml^{-1}. Scan rate, 2 C°·min^{-1}.

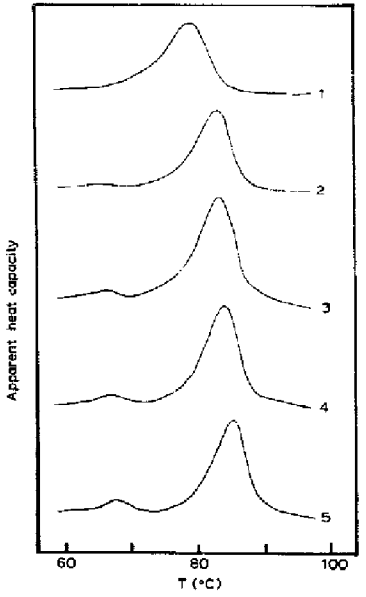

Fig. 7. Effect of Mn^{2+} binding on the calorimetric profiles of the deionized membrane (pH 5.0). The numbers 1–5 for the five traces refer to the ratio of Mn^{2+} per BR. The instrumental base line has been substracted in all cases. BR concentration, 1.0 mg·ml^{-1}. Scan rate, 2 C°·min^{-1}.

TABLE I

DSC THERMODYNAMIC PARAMETERS FOR THE MAIN THERMAL TRANSITION OF PURPLE, DEIONIZED AND CATION-REGENERATED MEMBRANES

Experiments including either Mn^{2+} or Hg^{2+} were carried out at pH 5.0. The data referring to mol of cations were per mol of BR in each experiment. Scan rate, 2 C°·min^{-1}. Molar enthalpies were calculated using a molecular weight of 26400.

Sample	T_m (°C)	ΔH (kJ·mol^{-1})
Purple membrane (pH 5.0)	96	303
Deionized membrane		
pH 4.0	71	235
pH 5.0	73	216
pH 5.5	80	285
pH 6.5	90	360
+1 Mn^{2+}	80	260
+2 Mn^{2+}	83	260
+3 Mn^{2+}	84	260
+4 Mn^{2+}	85	268
+5 Mn^{2+}	87	262
+5 Hg^{2+}	77	226
+20 Hg^{2+}	75	230

erated membranes have enthalpy values between the purple and the blue ones at pH 5.0; there is a net increase when the first Mn^{2+} is bound to the membrane and the enthalpy remains practically constant as more cations are also bound (Table I). Particularly interesting are the results obtained with deionized membrane supplemented with Hg^{2+}. As Table I shows, with either 5 or 20 Hg^{2+} added per BR, both the T_m and ΔH values only display a small increase in relation to those of the blue membrane at the same pH.

The 80 °C transition in the purple membrane has a molar enthalpy of 29 kJ·mol^{-1}. This transition becomes evident and can be quantitatively evaluated in the deionized membrane when 3 or more Mn^{2+} are bound; here, T_m and ΔH values (around 68 °C and 16 kJ·mol^{-1}, respectively) do not seem to depend appreciably on the number of cations bound to the membrane.

Discussion

The results obtained in this work clearly demonstrate that totally or partially deionized membrane (at pH 5.0) possesses a lower thermal stability than native purple membrane, which agrees with a recent report [22]. Both cation addition and pH increase make BR more stable over thermal denaturation. We have studied these processes using two complementary techniques, spectrophotometry and microcalorimetry. It is worth noting that these obviously give information about different domains of the membrane complex. Visible spectroscopy only reports on the retinal environment, a very localized part of the membrane. Ultraviolet difference spectrophotometry monitors a more extensive part of the molecule, owing to the fact that aromatic residues are widely distributed among the polypeptide chain [31,32]. DSC provides relative specific heat of the system during any thermally induced denaturation process, thus giving information about the overall structural domains of the membrane. Therefore, one could expect that the denaturation curves obtained from each technique do not give the same T_m. As a matter of fact, the equality of T_m values obtained using different techniques would only hold for a reversible two-state process. As discussed below, this seems not to be the case for the BR thermal denaturation.

Spectrophotometric results in the visible and ultraviolet regions lead to similar conclusions, as regards the T_m of the denaturation process of retinal and aromatic aminoacid environments. However, ultraviolet difference spectra may provide additional information. The linear part of the plot ΔA_{292} versus T might in principle be used for calculating the number of tryptophan residues exposed to the solvent [29,30]. Using the values given for model compounds by Bello [29] or Nicola and Leach [30], between 50% (native purple membrane) and 66% (blue membrane) exposed tryptophan residues can be calculated. The technique of thermal perturbation has been put forward for water-soluble proteins, and even for these simpler systems, the finding of adequate models for the aromatic residues located in the protein interior becomes problematic [29,30,33,34]. Therefore, the above values for exposed tryptophan residues must be used with caution. For example, proximity of non-surface aromatic residues to phosphate or sulfate lipid groups would contribute in some degree to the difference spectra and, thus, give an overestimation of the number of exposed residues. Nevertheless, we believe that the values above can be used for comparative purposes among the different membrane samples. In this respect, it can be concluded that the blue membrane has a higher proportion of exposed aromatic groups than the native purple membrane.

DSC experiments show the presence of the two thermal transitions previously reported by other authors for native purple membrane [11–13]. The main transition, corresponding to the irreversible BR denaturation, is accompanied by drastic changes in the visible and ultraviolet absorption spectra, due to changes in both the retinal and aromatic residue environments. The specific enthalpy values of this transition under all the conditions investigated (Table I) are clearly lower than the typical ones for non-membrane, water-soluble proteins [35] and compare well with those of other membrane proteins so far investigated by DSC [36]. As stated by these latter authors, the comparatively lower denaturation enthalpy value for membrane proteins should result from the particular hydrophobic environment around most regions of membrane proteins, as well as from the fact that in the denatured state the level of structural organization should be higher than that of the water-soluble proteins. In the case of native purple membrane, this latter assumption has been demonstrated by Brouillette et al. [13], mainly on the basis of far-ultraviolet circular dichroism measurements, which indicated the presence of important residual secondary structure after the denaturation process had been completed.

As determined using spectrophotometric techniques, native purple membrane in water discloses almost no change in T_m over the pH range between 5.0 and 6.5 (not shown). This is in agreement with recent microcalorimetric studies carried out on native purple membrane suspended in buffer [13]. However, pH changes readily influence the denaturation temperature of the deionized membrane. Results show that this membrane denatures at higher temperatures as the pH increases (Table I). Indeed, comparison of both

T_m and ΔH values for cation-substituted samples with those corresponding to the deionized (purple) membrane at pH 6.5 shows that this latter sample is more stable than the 5 Mn^{2+}-regenerated one at pH 5.0. If the same comparison is made with native purple membrane, it can be seen that deionized sample at pH 6.5 shows, in this case, a higher ΔH value and a lower T_m value. Although a certain amount of sodium ions has to be added in the form of NaOH, this does not seem to be the origin of the increased thermal stability, given the minor effect of monovalent cations as compared to the divalent ones. Recent FTIR results show that about 14 carboxylic residues deprotonate on pH increase of deionized membrane films [37]. These could, in turn, interact with positively charged amino acids to form saline bonds, which would increase the thermal stability of BR. In any case, it seems likely that the protein conformation in the deionized purple membrane at pH 6.5 and in native purple membrane at pH 5.0 present some significant differences, as indicated by the lower T_m and higher ΔH of the former.

Comparing the evolution of T_m and ΔH values on pH increase and cation binding (pH 5.0), a different type of behaviour is apparent. Cation binding would probably act by stabilizing specific concrete microenvironments. pH increase, as has been indicated, might stabilize the membrane by promoting saline bonds between carboxyls and other protein groups disseminated in the protein surface. It is interesting to note that, whereas cation binding (at pH 5.0) certainly will diminish the negative surface potential, pH increase would increase the negative surface potential by allowing deprotonation of some surface groups, or at least would leave it unchanged. Although more work is necessary to clarify these points, it seems likely that the average surface potential does not appear to bear the main responsibility for the thermal stability of BR. Defined microenvironments (around cation-binding sites or around deprotonated carboxyls) seem to provide adequate nucleation sites to maintain the BR conformation.

Both spectrophotometric and DSC techniques provide further evidence of the striking effect of Hg^{2+} [28]. As previously indicated, thermal difference spectra of Hg^{2+}-substituted membrane ressemble those spectra obtained from aromatic amino acids fully exposed to the solvent. However, it appears that no conformational change involving aromatic residues exist upon Hg^{2+} binding [20]. Thus, thermal difference spectra of this sample should be similar to that corresponding to the blue membrane. One possible explanation would be that solvent–solute interaction changes elicited by a temperature increase allow a direct effect of some Hg^{2+} ions on tryptophan residues. DSC experiments also show that Hg^{2+} seems to bind to the membrane in a very different manner from Mn^{2+} or native cations. In particular, the enthalpic effect seen on Mn^{2+} binding is absent in the case of Hg^{2+}. The slight increment in the T_m and ΔH values may be due to the decrease in the negative surface charge of the membrane as Hg^{2+} is added.

Special consideration must be paid to the absence of the DSC pretransition in all the deionized membrane samples, including that at pH 6.5 and those supplemented with Hg^{2+}, as well as the blue membrane with 1 Mn^{2+} bound per BR (blue membrane with 2 bound Mn^{2+} shows an incipient unquantifiable minor transition). This reversible transition has been attributed, by Jackson and Sturtevant [11] and Hiraki et al. [38], to a cooperative disordering of the paracrystalline lattice distribution of BR molecules. Apart from requiring a minimum amount of bound Mn^{2+}, this transition shows calorimetric values that are more or less the same in between 3 and 5 bound Mn^{2+}, but they are quite different from those of the native purple membrane. Moreover, since this transition is reversible, the ratio of the van't Hoff to the molar calorimetric enthalpy provides an indication of the size of its cooperative unit. For native purple membrane this ratio is 40 ± 5 while for Mn^{2+}-regenerated membrane it is 50 ± 5, both values calculated per mol of protein. Thus, whereas the size of the unit is of the same order, the T_m and ΔH values are higher for the purple form than for the Mn^{2+}-containing membranes. This indicates that the overall paracrystalline organization of the membrane may be similar in both cases, while there could be differences in the type of intermolecular interactions resulting, for instance, from minor protein conformational changes. In connection with this, binding of the first Mn^{2+} to the membrane increases the denaturation enthalpy,

whereas additional binding of Mn^{2+} does not affect this parameter, but is required for the appearance of the first transition. Therefore, the site for the first Mn^{2+} may well be in the protein itself, thus stabilizing its conformation. An alternate possibility is that this first bound cation is linking a protein group (carboxyl) with the lipid moiety. This would be in accord with recent results from ^{31}P-NMR studies [39], in which a structural relationship between cation binding sites and phospholipid head groups is suggested.

Acknowledgements

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