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A calorimetric study of white and purple membranes

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The thermal properties of bacteriorhodopsin, arranged in purple and white membranes, have been investigated by differential scanning microcalorimetry. Only a single endotherm was observed for unfolding of bacterioopsin before and after reconstitution with all-*trans*-retinal. The transition midpoint temperature is 79 °C for retinal free protein and 101 °C for retinal bonded bacterioopsin. The comparison of all the thermodynamic parameters, quantitatively evaluated, indicates that the retinal reconstitution stabilizes the native conformation of the protein but does not influence the intramolecular organization. The detailed analysis of the obtained thermodynamic characterization of bacteriorhodopsin, relative to that respective values of the thermodynamic parameters of the globular water-soluble proteins, reveals the complicated unfolding behaviour of this membrane protein as the values of the specific enthalpy change ($\Delta h^{110\,°C} = 4.6$ cal $\cdot$ g^{-1}) and the denaturation change of the heat capacity ($\Delta c_p^d = 0.088$ cal $\cdot$ g^{-1} $\cdot$ deg^{-1}) are significantly reduced and inapplicability the two-state transition model ($\Delta H^{cal}/\Delta H_{vH} = 0.69$) is demonstrated. The results are discussed in terms of presence of partial transition of the protein in this temperature interval.

Introduction

Bacteriorhodopsin, the light-driven proton pump of the plasma membrane of *Halobacterium halobium,* is a retinal-containing protein with known sequence and a unique organization in the membrane [1–3].

Although low-resolution electron diffraction image shows the topology of the protein as seven roughly parallel rods [4], presumably α-helices [5,6], the three-dimensional structure [7] as well as the position of the retinal chromophore [8] and the functional role of the intramolecular organization [9,10] have not been fully described. Unfortunately, there are only a limited number of reports on calorimetric, spectroscopic and circular dichroic investigations of thermal [11–15] and isothermal [16] denaturation giving direct insight into the stability of bacteriorhodopsin as they run into the complex character of the unfolding process of this membrane protein [12,13].

The application of the concepts for organization of globular water-soluble proteins [17,18] in this case is only an approach to the unfolding mechanism and the nature of forces stabilizing the bacteriorhodopsin structure and their study requires the measurement and comparison of all thermodynamic parameters to the same values of the soluble protein class.

The aim of the present paper is to measure the bacteriorhodopsin thermodynamic parameters in purple membrane and bacterioopsin in white membrane before and after reconstitution with all-*trans*-retinal and to analyse the evaluated results, thus providing further insight into the thermal unfolding of bacteriorhodopsin.

Material and Methods

The purple and white membranes were isolated from *Halobacterium halobium,* strain P-359 and JW-5, respectively, according to the procedure of Oesterhelt et al. [19] with some modifications.

The BR concentration was determined spectrophotometrically with the extinction coefficient at 568 nm of 63 mM^{-1} $\cdot$ cm^{-1} [20]. BO concentration was measured after 3 h incubation at room temperature with all-*trans*-retinal under the same conditions as for BR. The process of reconstitution was followed by the A_{280}/A_{568} ratio, equal to 2.4 [21].

The calorimetric studies were carried out with DASM-4 differential scanning microcalorimeter

Abbreviations: DSC, differential scanning calorimetry; BR, bacteriorhodopsin; BO, bacterioopsin.

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(U.S.S.R.) equipped with matched 0.47 ml platinum cells. DSC scans were run with a protein concentration of 115–125 μM at scan rate $1C° \cdot min^{-1}$. The membrane fractions were suspended in 0.1 M phosphate buffer (pH 7.0 and dialysed 12 h against the same buffer.

The molar enthalpy change (ΔH^{cal}) was found from the area under the peak corresponding to the excess heat capacity by using of a sigmoidal base line traced between the initial and final temperature of the transition [17,18] and after correction of DSC traces with the calorimetric base line. The observed area was converted into units of calories by using an electrical pulse as shown elswhere [17].

The ratio of the calorimetric to the van't Hoff enthalpy changes was calculated according to the following equation [17]:

$$\frac{\Delta H^{cal}}{\Delta H_{vH}} = \frac{Q_d}{2T_m}\sqrt{\frac{M}{R\Delta c_d}} \quad (1)$$

where T_m is the temperature of the heat absorption peak maximum and Δc_d is the peak height determined at T_m. All the parameters were obtained from the same experimental curve. The DSC traces presented in Fig. 1 were typical curves from a group of five or six experiments.

The van't Hoff enthalpy change (ΔH_{vH}) was evaluated indirectly from the ratio observed above, as the thermal absorption curve is asymmetrical. In addition, the substitution of T_m for T_d may lead to an inaccuracy if one uses for determination of ΔH_{vH} the ratio of T_m (degrees kelvin) to the half-width, $\Delta_{1/2}$ [17,18]:

$$\Delta H_{vH} = 4RT_m/\Delta_{1/2} \quad (2)$$

The partial heat capacity ($c_{p,pr}$ (T)) at 25 °C was evaluated by the equation [17]:

$$c_{p,pr}(T) = c_{p,sol}(T)\frac{V_{pr}(T)}{V_{sol}(T)} - \frac{\Delta c_p^{app}(T)}{m_{pr}} \quad (3)$$

Results

Fig. 1 shows the temperature dependence of the partial heat capacities of three samples, containing bacterioopsin in white membrane (BO) (curve I), bacteriorhodopsin in purple membrane (BR) (curve II) and reconstituted with all-*trans*-retinal white membrane (curve III). The DSC profiles demonstrate that the BO and reconstituted BO in white membrane undergo only one thermal transition. BR in purple membrane shows the presence of two endothermic transitions centered at about 80 °C and 100 °C, as was published earlier [11].

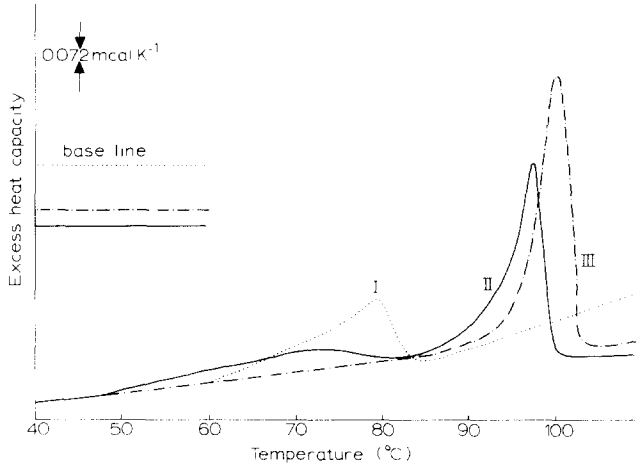

Fig. 1. DSC curves for the thermal unfolding of: (I) bacterioopsin in white membrane; (II) bacteriorhodopsin in purple membrane; (III) reconstituted with all-*trans*-retinal bacterioopsin. All the samples are in 0.1 M phosphate buffer (pH 7.0), protein concentration of 3–3.25 $mg \cdot ml^{-1}$.

The peaks of thermoabsorption of reconstituted BO and BO are shown at quite different temperature under the same experimental conditions, which is the first direct evidence for a different thermal stability of the protein containing or not containing retinal.

The experimental obtained DSC curves allow more detailed analysis of bacteriorhodopsin stability and reveal the contribution of the retinal chromophore on the base of the main thermodynamic parameters evaluated.

The value of Δc_p^{app} (T)$/m_{pr}$ from Eqn. 3 measured experimentally (see Material and Methods) at room temperature is equal to 0.42 ± 0.01 cal $\cdot g^{-1} \cdot deg^{-1}$ for BO and increases slowly – 0.47 ± 0.01 cal $\cdot g^{-1} \cdot deg^{-1}$ for BR to a value of 0.58 ± 0.01 cal $\cdot g^{-1}$, deg^{-1} for BO reconstituted with retinal in white membrane.

On the base of Eqn. 3 and using the experimentally measured Δc_p^{app} (T)$/m_{pr}$ mentioned above it is possible to calculate the value of $c_{p,pr}$ for BR, which is equal to 0.33 cal $\cdot g^{-1} \cdot deg^{-1}$. Assuming the value of $c_{p,pr}$ for all samples to be the same and determining the variability of the value of $\Delta c_p^{app}(T)/m_{pr}$ in the same experimental conditions the change of the protein partial volume can be evaluated roughly and indirectly as following:

$$V_{wh.m}^{BO} > V_{pm}^{BR} \geqslant V^{BO_{reconst.}}$$

The temperature increase results in linear increase of the partial heat capacity ($d(c_{p,pr})/dT$) to the temperature region, where the thermal transition is initiated. For the most stable reconstituted BO, where this parameter is conveniently measurable, it has a value of $(1.9 \pm 0.5) \cdot 10^{-3}$ cal $\cdot g^{-1} \cdot deg^{-2}$. For the other samples (BR and BO) this slope is the same only in the temperature region of 10–50 °C (not shown in Fig. 1) as at higher temperatures it is altered by the presence of pretransi-

tion (Fig. 1, curve II) or by shifting of the transition to a lower temperature (Fig. 1, curve I).

The enthalpy change of the pretransition ($\Delta H'^{cal}$), measured only for BR (Fig. 1, curve II), is equal to 17 ± 1 kcal · mol^{-1}. For other samples no pretransition is detected (Table I).

The enthalpy change of main transition (ΔH^{cal}) or the value of the specific enthalpy (Δh) increases from 50 ± 3 kcal · mol^{-1} (1.9 ± 0.1 cal · g^{-1}) (Fig. 1, curve I; Table I) for BO to a value of 104 ± 7 kcal mol^{-1} (4.0 ± 0.3 cal · g^{-1}) for reconstituted BO (Fig. 1, curve III; Table I). The values of the same characteristics for BR are 86 ± 6 kcal · mol^{-1} (3.3 ± 0.2 cal · g^{-1}) (Fig. 1, curve II; Table I).

T_m of the main transition is significantly shifted from 79° C for BO to 101° C for reconstituted BO.

The T_m dependence of ΔH^{cal} or Δh is shown in Fig. 2, curve A). It is a linear curve with a slope of 0,088 cal · g^{-1} · deg^{-1} and a value of $\Delta h^{110°C}$ equal to 4.6 cal · g^{-1}.

The data of the measured enthalpy change for the main transition of every sample allow the calculation of the entropy changes (Fig. 2, curve B, Table I).

The protein unfolding is characterized by the change in the partial heat capacity (Δc_p^d). The data in Fig. 1, curves I–III, show a value of 0.13 cal · g^{-1} · deg^{-1} for BO which decreases to 0.084 cal · g^{-1} · deg^{-1} for reconstituted BO and BR.

The calculated value of $\Delta H^{cal}/\Delta H_{vH}$ ratio is equal in average to 0.69 ± 0.05 and it is the same in all samples.

Discussion

The DSC study of bacterioopsin in white membrane demonstrates absence of endothermic transition at about 80° C which coincides with the suggestion of a low state of intramolecular organization in the protein molecules not containing retinal [22]. The increase of the partial volume evaluated indirectly is consistent with this. The enthalpy of unfolding for the main phase transition (Table I) is about 1.6–2-times lower for BO (50 kcal · mol^{-1}) compared to BR and reconstituted BO and the temperature of the maximum (T_m) for this transition is shifted to lower temperatures of about 20° C. These data demonstrate the low stability of the protein molecule in absence of retinal as a chromophore.

The BO calculated $\Delta H^{cal}/\Delta H_{vH}$ ratio is equal to 0.7, which is somewhat higher than the value demonstrated by Jackson et al. [11] and Brouillette et al. [13] ($\Delta H^{cal}/\Delta H_{vH} = 0.5$) for BR in purple membrane but too far from the predicted value of 1 for an all-or-nothing mechanism of unfolding [17]. It is possible that these data can be connected with the unfolding of more complicated cooperative unit, or with a presence of a temperature-induced aggregation which requires a more detailed study of the asymmetry of the main phase-transition peak.

The reconstituted with all-*trans*-retinal bacterioopsin in white membrane shows a significant increase of T_m and absence of pretransition (Fig. 1, curve III). The unfolding enthalpy increases about 2-times (Table I) and the peak of the main transition remains asymmetrical. The value of $\Delta H^{cal}/\Delta H_{vH}$ is not changed and is equal to 0.68. The calculated value of the entropy change decreases about 2-times and the evaluated value of $\Delta c_p^{app}(T)/m_{pr}$ demonstrates a decrease in the sample partial volume.

A comparison of the thermodynamic characteristics of BO and reconstituted BO with those of BR in purple membrane shows that the molecules arranged in the crystalline lattice demonstrate a partial volume between BO reconstituted and BO. The thermal destruction of this macroscopic organization is evaluated with the observed pretransition enthalpy of 17 kcal · mol^{-1} (Table I), which includes additionally the changes in the retinal environment as shown by the absorption spectroscopy [11,13]. The enthalpy and entropy changes of the main phase transition of BR in native membrane are equal to 86 kcal · mol^{-1} and 232 cal · mol^{-1} · deg^{-1}, respectively, and these values are between the values observed for the same thermodynamic characteristics of BO and reconstituted BO. The T_m for this sample is 97° C. The value of $\Delta H^{cal}/\Delta H_{vH}$ is very close to that of BO and reconstituted BO.

Analysing all the observed results from the comparison of the thermodynamic characteristics of each sample, between themselves and related to the same characteristics of the soluble proteins, it is evident that: (1) BO in white membrane appears to have a noncompact

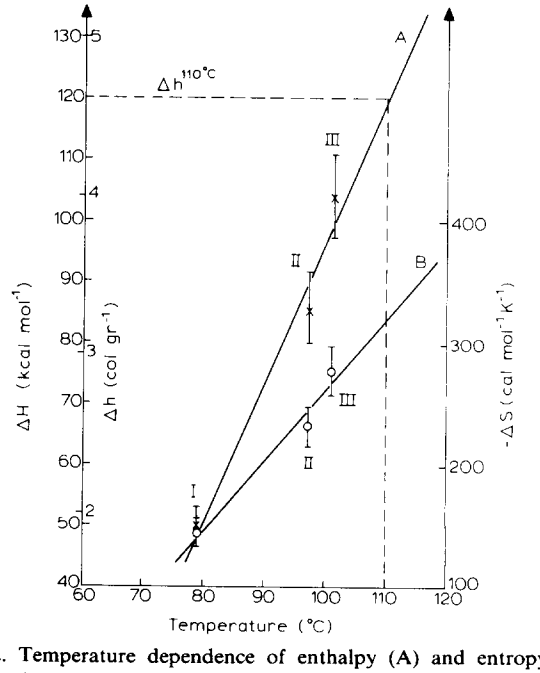

Fig. 2. Temperature dependence of enthalpy (A) and entropy (B) changes in BO. (I); BR in purple membrane (II); reconstituted BO in white membrane reconstituted with all-*trans*-retinal (III).

TABLE I

Calorimetric data for bacteriorhodopsin in purple and white membranes

Compound	T_m' (°C)	$\Delta H'^{cal}$ (kcal·mol^{-1})	$\Delta h'$ (cal·gr^{-1})	$\frac{\Delta H^{cal}}{\Delta H_{vH}}$	$\Delta H_{vH}'$ (kcal·mol^{-1})	$\Delta S'$ (cal·K^{-1} mol^{-1})	T_m (°C)	ΔH^{cal} (kcal·mol^{-1})	Δh (cal·g^{-1})	$\frac{\Delta H^{cal}}{\Delta H_{vH}}$	ΔH_{vH} (kcal·mol^{-1})	ΔS (cal·K^{-1}·mol^{-1})
BO in white membrane	–	–	–	–	–	–	79±0.5	50±3.0	1.92±0.1	0.7 ±0.05	71± 8.0	142± 9.0
BR in purple membrane	74±0.5	17±1.0	0.65±0.04	0.56	30±4.0	49±3.0	97±0.5	86±6.0	3.3 ±0.2	0.69±0.05	125±16	232±15
BO reconstituted	–	–	–	–	–	–	101±0.5	104±7.0	4.0 ±0.3	0.68±0.05	155±20	278±20

organization of low stabilized molecules. (2) The retinal as a chromophore significantly influences the stability of the protein molecule but the macroscopic organization of the protein molecules was not observed. (3) The unfolding mechanism of all samples is complex and the main phase transition asymmetry can be a result of a temperature-induced aggregation or dissociation of di- or trimers as a cooperative unit but it requires a further investigation. (4) All the data shown above, together with the low value of the plot slope of the enthalpy change versus T_m (Fig. 2, curve A) as well as the low value of $\Delta h^{110°C}$ in comparison to the same values of the globular water-soluble proteins [17], suggest the presence of only partial protein unfolding in this temperature interval or a reduction of the absolute value of all the thermodynamic characteristics as a result of the complicated unfolding character connected with the factors inhibiting the protein refolding after the partial transition [23,24].

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