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Kinetic study into the irreversible thermal denaturation of bacteriorhodopsin

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Abstract. We report on a differential scanning calorimetry study of native purple membranes under the following solvent conditions: 50 mM carbonate-bicarbonate, 100 mM NaCl, pH 9.5 and 190 mM phosphate, pH 7.5. The calorimetric transitions for bacteriorhodopsin denaturation are highly scanning-rate dependent, which indicates that the thermal denaturation is under kinetic control. This result is confirmed by a spectrophotometric study on the kinetics of the thermal denaturation of this protein. The calorimetric data at pH 9.5 conform to the two-state irreversible model. Comments are made regarding the information obtainable from differential scanning calorimetry studies on bacteriorhodopsin denaturation and the effect of irreversibility on the stability of membrane proteins.

Key words: Bacteriorhodopsin – Irreversible thermal denaturation – Differential scanning calorimetry

Introduction

Bacteriorhodopsin (BR), a light-driven proton pump, is the only protein present in *Halobacterium halobium* purple membrane. The two-dimensional crystalline nature of the purple membrane has allowed researchers to propose secondary structure models for BR on the basis of diffraction data. This fact, together with the accessibility and important function of this protein, explains why bacteriorhodopsin has come to be used as a membrane protein model for studies at the mechanistic and structural level (for a recent review on BR see Khorana 1988).

According to differential scanning calorimetry (DSC) studies of native purple membrane (Jackson and Sturtevant 1978; Brouillette et al. 1987), BR has remarkably high thermal stability. Thus, in the pH range 6–8, the T_m value (temperature corresponding to the maximum heat capacity) of the DSC transitions for the thermal denaturation of BR is close to 100 °C. In addition, a small transition at about 80 °C is often observed; this "pre-transition" has been attributed to a reordering of the crystal lattice (Jackson and Sturtevant 1978).

Thermal denaturation of BR has been reported to be irreversible under a variety of solvent conditions (Jackson and Sturtevant 1978, Brouillette et al. 1987).

Irreversibility in protein thermal denaturation is attributed (see, for instance, Privalov and Medved 1982; Klibanov and Ahern 1987) to alterations to the unfolded state (aggregation, modification of residues...) which lead to a final state that is unable to fold back to the native structure. Until recently it was widely held that these alterations do not significantly distort the observed DSC profiles. Thus, the analysis of DSC transitions corresponding to the irreversible denaturation of many proteins (including bacteriorhodopsin) has often been carried out according to the well-known equilibrium thermodynamics procedure (which involves calculation of the apparent, or van't Hoff enthalpy to obtain information about the cooperativity of the unfolding process and, often, an analysis of the transition shapes in terms of the protein domain organization or the oligomerization state of the native protein). Recent work on the thermal denaturation of soluble proteins (Sanchez-Ruiz et al. 1988; Freire et al. 1990; Galisteo et al. 1991; Conejero-Lara et al. 1991), however, has shown that irreversible alterations are often fast enough to distort significantly the DSC transitions. In these cases, protein thermal stability must be characterized on the basis of kinetic models (for a more detailed discussion about the effect of irreversibility on DSC measurements see Freire et al. 1990 and Sanchez-Ruiz 1992).

Protein structure plays an important role in BR function, and recent studies (Brouillette et al. 1987, and 1989; Cladera et al. 1988; Maglova et al. 1990; Kresheck et al. 1990; Cladera et al. 1992; Kahn et al. 1992) indicate that DSC may be a powerful technique for monitoring the effect of several perturbations on BR structure. This use of DSC requires, however, that the nature of the DSC transition associated with bacteriorhodopsin denaturation be

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elucidated and understood. The aim of this work is to determine whether thermal denaturation of BR (as monitored by DSC) is an equilibrium phenomenon or a process subject to kinetic constraints. The study has been carried out with native purple membranes in 50 mM carbonate-bicarbonate, 100 mM NaCl pH 9.5 and 190 mM phosphate, pH 7.5. These two solvent conditions are similar to those employed by Brouillette et al. (1987) in their study into the pH effect on BR thermal denaturation and will be subsequently referred to as pH 9.5 and pH 7.5.

Experimental

Materials

Purple membrane was isolated from *Halobacterium halobium* strain S9 as described by Oesterhelt and Stoeckenius (1974). Membrane suspensions were prepared by suspending the sample in the desired buffer followed by dialysis against the same buffer. Protein concentrations were determined spectrophotometrically with an extinction coefficient at 568 nm of 63 000 M^{-1} cm^{-1} (Oesterhelt and Hess 1973). Degassed, deionized water was used throughout.

Calorimetric measurements

DSC experiments were performed using a DASM-4 calorimeter, described by Privalov (1980), keeping an additional constant pressure of 2.5 atm over the liquids in the cell. Different scanning rates within the range 0.25–2 K/min were employed. The DSC experiments reported in this work (Fig. 1) were carried out with purple membrane suspensions of BR concentration 2.3 mg/ml (pH 9.5) and 1.9 mg/ml (pH 7.5); additional experiments (not shown) with lower protein concentrations (0.2–1.5 mg/ml) showed that the DSC transitions do not change with protein concentration. The reversibility of the DSC transitions was checked by reheating the solution in the calorimetric cell after cooling from the first run. In all cases, the transition due to BR denaturation ("main transition") was found to be irreversible (no thermal effect in the reheating run); therefore, the thermogram corresponding to the reheating run was used as the instrumental base line. Small "pretransitions" (attributed to a reordering of the BR crystal lattice) were observed at about 20 K below the main ones, but owing to the lower signal to noise ratio at the lower scanning rates, they were clearly evident only in the thermograms obtained at 2 K/min. The main transitions were corrected for the difference in heat capacity between the initial and final states and for the effect of the instrument response time as described by Galisteo et al. (1991).

Kinetics of BR thermal denaturation

Thermal denaturation of BR is accompanied by an irreversible change in retinal absorption [loss of the absorbance maximum at 568 nm (Jackson and Sturtevant 1978); see Fig. 2]. Therefore, the kinetics of thermal denaturation of BR can be studied by following the time dependence of the absorbance at 568 nm at given temperatures within the denaturation range (Fig. 3). The experimental procedure employed is similar to that previously used to study the kinetics of thermal denaturation of soluble proteins (Galisteo et al. 1991; Conejero-Lara et al. 1991). Briefly, 10 µl amounts of a purple membrane suspension at pH 7.5 or 9.5 were introduced into capillary tubes (diameter 0.5 mm) and the tubes were sealed and immersed in a water bath at a chosen temperature within the

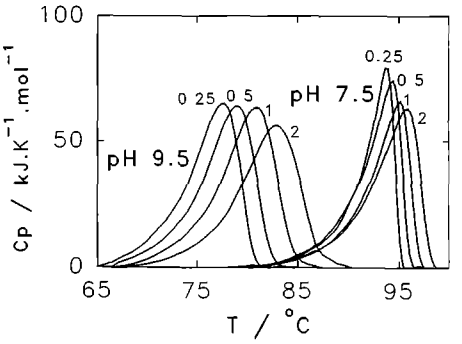

Fig. 1. DSC transitions for bacteriorhodopsin denaturation at pH 7.5 and pH 9.5. The numbers alongside the transitions indicate the scanning rate in K/min. These transitions have been corrected for the instrumental baseline, the chemical baseline and the effect of the instrument response time (see text for details). The denaturation enthalpy values (422 ± 10 kJ/mol at pH 9.5 and 346 ± 22 kJ/mol at pH 7.5) as well as the shape and temperature range of the transitions agree in general with the results of Brouillette et al. (1987) under similar solvent conditions. The results displayed in this figure show, however, that the DSC transitions are highly scanning rate-dependent

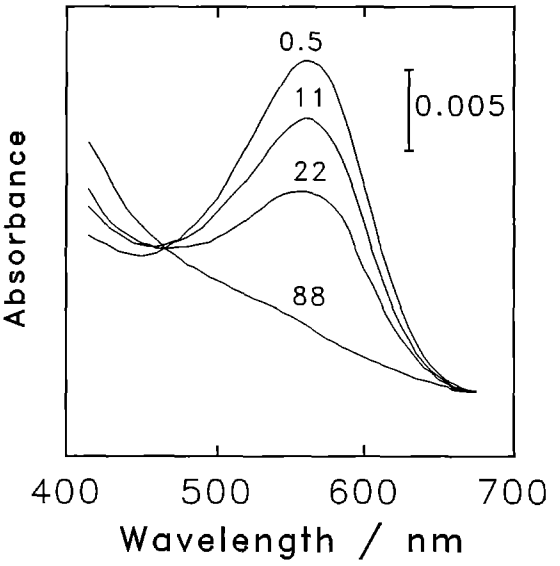

Fig. 2. The kinetics of thermal denaturation of bacteriorhodopsin followed spectrophotometrically (see Experimental for details). The visible spectra shown in the figure correspond to T = 73 °C, pH 9.5 and protein concentration 0.88 mg/ml. The numbers alongside the spectra indicate the time (in minutes) spent by the sample in the high-temperature bath (at T = 73 °C in this case)

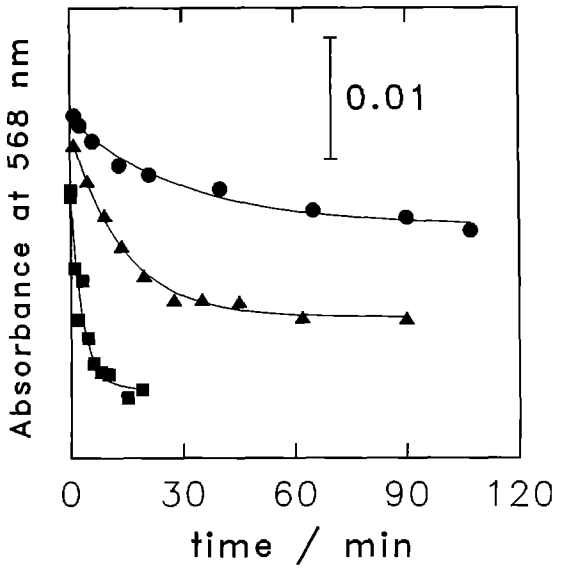

Fig. 3. Absorbance at 568 nm *versus* time profile for the thermal denaturation of BR at the following pH, temperature and protein concentrations: (●), pH 7.5, 90.5 °C, 0.65 mg/mL; (▲), pH 9.5, 75.0 °C, 1.0 mg/mL; (■), pH 9.5, 79.0 °C, 1.0 mg/mL. The lines are the best fits of (1). These profiles were obtained from denaturation experiments such as that shown in Fig. 2

denaturation range. At different times, tubes were extracted from the high-temperature bath and immediately immersed in a low-temperature bath to stop the denaturation process. The samples were then diluted 90-fold in the same buffer and the visible spectra (Fig. 2) were taken using a Beckman DU-70 spectrophotometer. First-order rate constants for BR thermal denaturation (k) were calculated from non-linear, least-squares fitting of the following eq. to the experimental A_{568}/time profiles (Fig. 3):

$$A = A_\infty + (A_0 - A_\infty)\, e^{-kt} \quad (1)$$

where A_0 and A_∞ are, respectively, the absorbances at $t=0$ and $t=\infty$. The rate constants derived from these fittings did not change significantly with protein concentration (within the range 0.7–2.3 mg/ml).

Results and discussion

Under the solvent conditions studied in this work (pH 7.5 and 9.5), the DSC transitions for BR denaturation were found to be irreversible (no thermal effect on the reheating run). The irreversible thermal denaturation of soluble proteins is believed to involve, at least, two steps: a) reversible unfolding of the native protein; b) irreversible alterations to the unfolded protein to yield a state (the final state) that is unable to fold back to the native structure. The simplest scheme that takes into account the two-step character of irreversible denaturation is the well-known Lumry-Eyring model (Lumry and Eyring 1954):

Native State $\rightleftarrows$ Unfolded State $\longrightarrow$ Final State

Note, however, that the reversible unfolding step might involve several significantly populated intermediate states (Freire et al. 1990; Sanchez-Ruiz 1992).

Irreversibility appears to be a general feature in the thermal denaturation of membrane proteins (Sanchez-Ruiz and Mateo 1987; Ruiz-Sanz et al. 1992). Thermal gel analysis studies (Lysko et al. 1981; Rigell and Freire 1987) suggest that, in several cases, the final state is the protein aggregated within the membrane plane. The above two-step scheme might still be applicable to the denaturation of membrane proteins; it must be noted, however, that in this case there is a considerable uncertainty about what is meant by "unfolded state" (Sanchez-Ruiz and Mateo 1987).

The fact that the DSC transitions are irreversible does not *necessarily imply* that the excess heat capacity profile is distorted by the occurrence of the irreversible step (unfolded to final). Thus, the irreversible step might be comparatively slow within the temperature range of the DSC transitions and only occur with significant rate (and little thermal effect) at somewhat higher temperatures. In fact, this point of view has been adopted by several authors in recent literature (Privalov 1982; Privalov and Medved 1982; Manly et al. 1985; Edge et al. 1985; Hu and Sturtevant 1987; Brandts et al. 1989; Bertazzon et al. 1990) and has been experimentally demonstrated for the irreversible thermal denaturation of the B subunit of cholera toxin (Goins and Freire 1988).

In the case of the thermal denaturation of BR, however, the DSC transitions are strongly scanning-rate dependent, at least under the solvent conditions employed in this work (Fig. 1). This indicates (Sanchez-Ruiz et al. 1988; Freire et al. 1990; Galisteo et al. 1991; Conejero-Lara et al. 1991; Sanchez-Ruiz 1992) that the state of the purple membrane suspension at any given temperature (within the denaturation range) depends on the time required to reach that temperature; therefore, the thermal denaturation of BR is under kinetic control and the DSC transitions are distorted by the occurrence of the irreversible step.

This conclusion is further confirmed by our spectrophotometric analysis of the kinetics of thermal denaturation of BR (see Experimental). First-order rate constants for BR denaturation (k) derived from the A_{568}/time profiles were found to be highly temperature-dependent (Fig. 4). An Arrhenius analysis of these temperature-dependencies yields activation energy values of 361 ± 15 kJ/mol for pH 9.5 and 731 ± 36 kJ/mol for pH 7.5. The important point to note here, however, is that the half-lives for irreversible denaturation ($\tau = 1/k$) at the temperature of the maxima of the DSC transitions are within 3–15 min, while the time spent by the protein in the transition region ranges from about 10 min (for scanning rate 2 K/min) to about 1 h (for scanning rate 0.25 K/min). Thus, the irreversible step does taken place during the time the protein spends in the temperature range of the DSC transition. Clearly, the DSC transitions reported in this work must be analyzed on the basis of kinetic models.

It can be shown (Sanchez-Ruiz et al. 1988; Freire et al., 1990; Sanchez-Ruiz 1992) that, when the process responsible for irreversibility is very fast, the concentration of unfolded state becomes very low and only the native and final states are significantly populated during denatura-

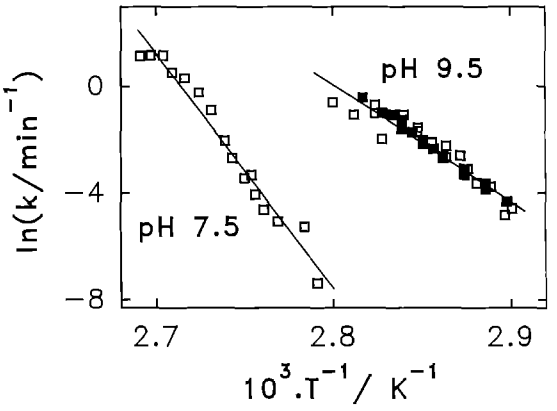

Fig. 4. Temperature dependence of the first-order rate constants for BR thermal denaturation. This figure is an Arrhenius plot of ln k *versus* 1/T. (□), data obtained from the A_{568} *versus* time profiles. (■), data calculated from the DSC transitions at pH 9.5 by using (2) (data calculated from DSC transitions obtained at four different scanning rates are included). The lines are the best fits of the Arrhenius equation

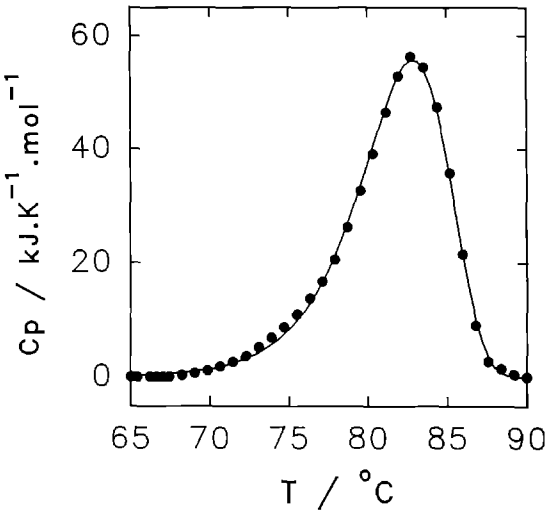

Fig. 5. DSC transition for thermal denaturation of BR at pH 9.5 and scanning rate 2 K/min. (•) experimental excess heat capacity data. (—) best fit of the theoretical curve given by (3)

tion. In this case, the denaturation process can be described by a two-state irreversible model:

Native State ⟶ Final State

The thermal denaturation of several soluble proteins has been found to conform to this model (Sanchez-Ruiz et al. 1988; Freire et al. 1990; Conejero-Lara et al. 1991).

We have found that the thermal denaturation of BR at pH 9.5 follows the two-state irreversible model, as shown by the following analysis:

1) For the two-state irreversible model with first-order kinetics, the rate constant for the native to final conversion is given by (Sanchez-Ruiz et al. 1988):

$$k = \frac{v\, C_p^{ex}}{\Delta H - \langle \Delta H \rangle} \tag{2}$$

where v stands for the scanning rate, ΔH for the total enthalpy of the transition, and C_p^{ex} and $\langle \Delta H \rangle$ for the apparent excess heat capacity and the apparent excess enthalpy at the temperature at which k is calculated. For the model to hold true, there must be agreement between the values of k calculated from DSC transitions obtained at different scanning rates. A good agreement was found for the DSC data at pH 9.5 and, in addition, these calorimetric k values are in acceptable agreement with those determined from the thermal denaturation kinetics (Fig. 4).

2) The shape of the DSC transitions that follow the two-state model with first-order kinetics is given by (Conejero-Lara et al. 1991; Sanchez-Ruiz 1992):

$$C_p^{ex} = \frac{\Delta H\, E}{R\, T_m^2} \exp\left(\frac{E\, \Delta T}{R\, T_m^2}\right) \exp\left[-\exp\left(\frac{E\, \Delta T}{R\, T_m^2}\right)\right] \tag{3}$$

where T_m is the temperature corresponding to the maximum heat capacity and $\Delta T = T - T_m$. The DSC transitions for the thermal denaturation of BR at pH 9.5 are quantitatively described by (3), as shown in Fig. 5.

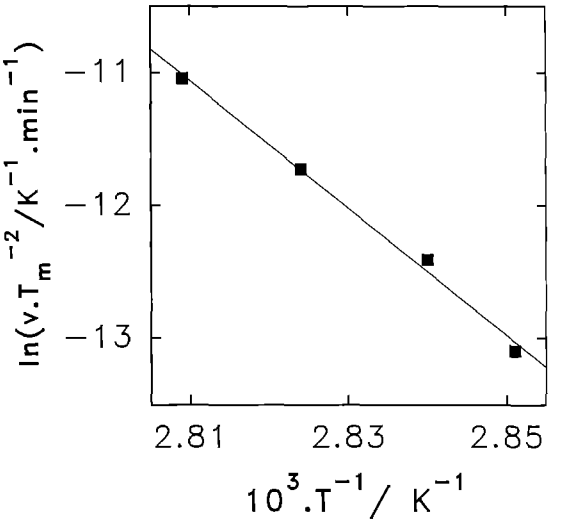

Fig. 6. Plot of $\ln(v/T_m^2)$ *versus* $1/T_m$ (according to (4)) for the thermal denaturation of BR at pH 9.5 (see text for details)

3) Finally, according to the two-state irreversible model, the scanning rate effect on the DSC transitions is given by (Sanchez-Ruiz et al. 1988; Sanchez-Ruiz 1992):

$$\ln(v/T_m^2) = \text{const} - E/R\, T_m \tag{4}$$

The plot of $\ln(v/T_m^2)$ *versus* $1/T_m$ for the DSC data at pH 9.5 is linear (Fig. 6) as predicted by (4), and the activation energy value derived from the slope (398 kJ/mol) is in acceptable agreement with the value obtained from the Arrhenius analysis of the k values (361 ± 15; see Fig. 4).

The fact that the thermal denaturation of BR at pH 9.5 follows the two-state irreversible model has some interesting consequences:

a) The heat absorption is entirely determined by the kinetics of formation of the irreversibly denatured state and no thermodynamic information (other than the total enthalpy for the process) can be derived from the transitions

(Sanchez-Ruiz et al. 1988; Conejero-Lara et al. 1991; Sanchez-Ruiz 1992).

b) The apparent van't Hoff enthalpies calculated from the DSC transitions by using the well-known equation,

$$\Delta H^{vH} = 4\,R\,T_m^2\,C_{p,m}^{ex}/\Delta H \quad (5)$$

$C_{p,m}^{ex} \equiv$ excess heat capacity at the maximum

do not provide any information regarding the cooperativity of the denaturation process and, in fact, they can be shown to be equal to $4E/e$ (Conejero-Lara et al. 1991; Sanchez-Ruiz 1992).

c) The two-state irreversible model, as well as other kinetic models (Sanchez-Ruiz 1992), predicts asymmetrical DSC transitions. Thus, the observed asymmetry is a consequence of the rate-limited character of the transitions and does not imply that a dimer or a trimer of BR molecules denatures as a cooperative unit with concomitant dissociation (see Brouillette et al. 1987). It is interesting to note, nevertheless, that, at pH 9.5, the value of the activation energy (which may be interpreted as an activation enthalpy) is close to that of the denaturation enthalpy per mole of BR ($\Delta H = 422 \pm 10$ kJ/mol at pH 9.5); this would appear to suggest that the transition state is made up of one BR molecule in a situation (structure, environment ...) similar to that of the final state.

On the other hand, the DSC data corresponding to the thermal denaturation of BR at pH 7.5 did not conform to this simple two-state irreversible model. Thus, no acceptable agreement was found between the k values calculated (by using (2)) from DSC transitions obtained at different scanning rates (results not shown) or between the activation energy value derived from the Arrhenius analysis of the denaturation rate constants (Fig. 4) and that obtained from the scanning rate effect on the transitions (4). It appears possible that the thermal denaturation of BR at pH 7.5 may be a complex irreversible process involving more than two significantly populated states. We believe that any specific model proposed to account for the denaturation data at pH 7.5 should be able to explain reasonably the high activation energy found at this pH: 731 ± 36 kJ/mol – about twice the value of the denaturation enthalpy per mole of BR ($\Delta H = 346 \pm 22$ kJ/mol at pH 7.5). This E value would appear to suggest that the transition state is made up of two (or perhaps three) BR molecules and would be consistent with a mechanism in which the irreversible step is preceded by the cooperative (and non-dissociative) unfolding of a dimer (or perhaps a trimer) of BR molecules. We must recognize, nevertheless, that our attempts to fit the DSC data at pH 7.5 on the basis of this kind of kinetic model have been, so far, unsuccessful.

Concluding remarks

Regardless of the specific kinetic models employed to fit the DSC data, the main conclusion of this work is that, under the solvent conditions studied (and possibly under other solvent conditions as well), the thermal denaturation of BR is a rate-limited process. This fact indicates that the DSC transitions must be analyzed on the basis of kinetic models and therefore sets constraints on the kind of information that can be derived from them.

It is also worth pointing out again that irreversibility appears to be a general feature in the thermal denaturation of membrane proteins (Sanchez-Ruiz and Mateo 1987; Ruiz-Sanz et al. 1992) and, in fact, calorimetric transitions in complex membrane systems are usually attributed to protein denaturation (rather than to a lipid phase transition) on the basis of their irreversibility (Sanchez-Ruiz and Mateo 1987). In spite of this, few studies have addressed the effect of irreversibility on the stability of membrane proteins. Recently, Morin et al. (1990) found that the thermal denaturation of membrane-reconstituted cytochrome *c* oxidase is also a kinetically restricted process. These authors suggest the possibility that the stabilization of at least some membrane proteins might not be a thermodynamic phenomenon but a process modulated by kinetic constraints. The results reported in this work support this hypothesis.

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