A pathway for the thermal destabilization of bacteriorhodopsin

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Abstract A variety of structural techniques, including IR spectroscopy, reveals that thermal denaturation of bacteriorhodopsin follows a given pathway (successively rearrangement of helical structures, extensive deuterium exchange, and finally protein aggregation) irrespective of heating rate, pH or ionic strength conditions. In all cases, thermal denaturation leads to a 'compact denatured state' which retains a large proportion of ordered structure.

Key words: Protein denaturation; Protein unfolding; Infrared spectroscopy; Compact denatured state; Bacteriorhodopsin

1. Introduction

The thermodynamic stability of integral membrane proteins is an important issue in membrane biophysics. The intramembranous regions of those proteins are believed to be stable mainly because of hydrogen bonds formed within the hydrophobic membrane matrix [1–3]. Calorimetric studies of membrane protein stability have shown that, at least in some cases, thermal denaturation is a kinetically controlled irreversible process, in which the observed transition temperature is a function of the experimental heating rate [4,5]. Recent data on the thermal denaturation of bovine and *Paracoccus* cytocrome *c* oxidases [2,3] suggest that most of the intramembranous secondary structure is maintained even above denaturation temperatures.

Purple membranes from *Halobacterium halobium* contain bacteriorhodopsin, an integral protein 70–80% of whose mass is intramembranous [6]. Aqueous dispersions of purple membranes exhibit high stability to thermal denaturation, with a 'pre-transition' at 74–78°C and a main thermal transition, i.e. denaturation, at 97–100°C [7–11]. Cladera et al. [12] performed infrared spectral measurements of purple membrane heated to 99°C, that are compatible with the idea that the intramembranous portions of membrane proteins do not become unfolded upon thermal denaturation. Shnyrov and Mateo [11] have used an annealing procedure to study thermal transitions in purple membranes, showing as many as five successive transitions, of which only one (at ≈72°C) is reversible. Moreover, Galisteo and Sánchez-Ruiz [13] have demonstrated that the calorimetric transitions for bacteriorhodopsin denaturation are scanning-rate dependent, indicating that also for this protein thermal denaturation is under kinetic control.

In this paper, we examine the thermal behaviour of bacteriorhodopsin using infrared spectroscopy, quasi-elastic light scattering and electrokinetic techniques, in order to test and complement the previous calorimetric results. We have been able to characterize the structural changes brought about by thermal denaturation in bacteriorhodopsin, we have confirmed the kinetically controlled character of the process, and we have found that the structural alteration follows a given pathway that is independent of the heating conditions.

2. Materials and methods

Purple membrane fragments were isolated from *Halobacterium halobium* S9 strain according to a standard procedure [14] and resuspended in 190 mM phosphate buffer, pH 7.5. Occasionally, the purple membranes were studied in a 10 mM phosphate, pH 7.5 buffer, or in a 50 mM bicarbonate, 100 mM NaCl, pH 9.5 buffer, as indicated in each case.

The hydrodynamic diameter (B_{ave}) and electrophoretic mobility (u_e) of the membrane fragments were measured on a suspension 2 μM in bacteriorhodopsin, using a Zeta Sizer 4 apparatus (Malvern Instruments Ltd.).

Samples for infrared spectroscopy were made up in the buffers described above, with either H_2O or D_2O as solvent. Spectra acquisition and resolution enhancement were performed as described previously [2]. Protein concentration was 12–14 mg/ml (≈0.5 mM). For thermal treatment, the samples were placed in closed capillary tubes and heated as required in each case; the samples were afterwards equilibrated at 20°C, and all measurements were carried out at this temperature. H_2O/D_2O exchange was carried out by submitting the samples to three centrifugation-resuspension cycles in D_2O buffer with identical salt composition as the original H_2O buffer. The samples were then left to equilibrate in D_2O buffer for 15 h at room temperature prior to spectral acquisition.

3. Results

Samples in 190 mM phosphate buffer (D_2O) were heated for 5 min, each at a different temperature between 25 and 97°C, then equilibrated at 20°C and the corresponding IR spectra recorded. Representative deconvolved spectra of the amide I and II bands of bacteriorhodopsin are shown in Fig. 1. Heating at temperatures up to 66°C produces hardly any change in the IR spectra, whose main features are two complex signals, with maxima at 1,665 cm^{-1} (amide I) and 1,547 cm^{-1} (residual amide II), respectively. The maximum at 1,665 cm^{-1} together with the partially unresolved peak at 1657 cm^{-1} are rather characteristic of bacteriorhodopsin, and correspond to two forms of α-helices [15]. At 82°C, above the 'pretransition' temperature [7] a difference in shape of the amide I band begins to be seen; in turn, the intensity of the 1,547 cm^{-1} band also starts to decrease. The observed changes are more clearly detected after incubation at 92°C; at this temperature the 1,665 cm^{-1} signal is no longer resolved. Finally, after heating the sample at 97°C, under irreversible denaturation conditions [7], three phenomena are clearly distinguishable: the virtual disappearance of the residual

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amide II signal at 1,547 cm^{-1}, due to deuterium exchange, the shift of the helical components of amide I into a single signal at 1,654 cm^{-1}, and the appearance of two side peaks in the amide I band, at 1,623 and 1,685 cm^{-1}, that have been associated to protein denaturation [2,16].

Since H/D exchange is facilitated by the heating treatment, the spectral changes observed in Fig. 1 may not be entirely due to the thermal events detected by other authors by DSC, being instead the mixed result of both heating and deuteration. In order to check this point, a similar study was performed in H_2O buffer, of which only the spectra at 25 and 97°C are shown (Fig. 2). The same effects as seen in D_2O are now visible in the amide I band: shift of α-helical signals, now at 1,659 cm^{-1}, and presence of protein denaturation components at 1,628 cm^{-1} and 1,694 cm^{-1}. A comparison between the H_2O and D_2O results reveals that deuteration induces a downward shift of about 5 cm^{-1} of the amide I components, thermal effects being however very similar, if not identical, in both solvents. Thus the results in H_2O buffer indicate that the modifications observed in the amide I band in Fig. 1 are due to the thermal treatment, and not to deuteration. Consequently, all further studies were carried out in D_2O for reasons of technical simplicity.

The next step was to test from the structural point of view the calorimetric prediction of a kinetically controlled denaturation process. According to that hypothesis, denaturation could be achieved below the 97–100°C temperature range, only heating for a longer time than the 5 min used in the experiments in Figs. 1 and 2. In our case, samples were heated at 92°C for various lengths of time, then equilibrated at 20°C and examined by FT-IR. The corresponding spectra are shown in Fig. 3. The spectrum taken after 10 min (0.16 h) is very similar, as expected, to the one shown in Fig. 1 after 5 min (0.08 h) heating; at longer times, the denaturation signals at 1,632 and 1,685 cm^{-1} become detectable, and are fully visible after 5 h. After 23 h heating at

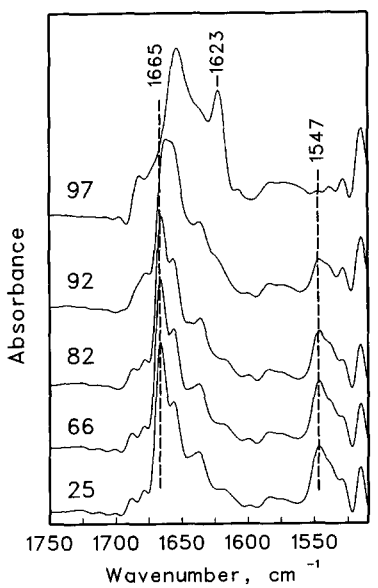

Fig. 1. Thermal denaturation of bacteriorhodopsin in D_2O buffer. Samples were incubated for 5 min at the temperature indicated by each curve (°C), then equilibrated at room temperature for spectral recording. The curves correspond to deconvolved infrared spectra of purple membrane, in the 1,500–1,750 cm^{-1} region, and have been autoscaled. Deconvolution parameters were hw = 18, k = 2.

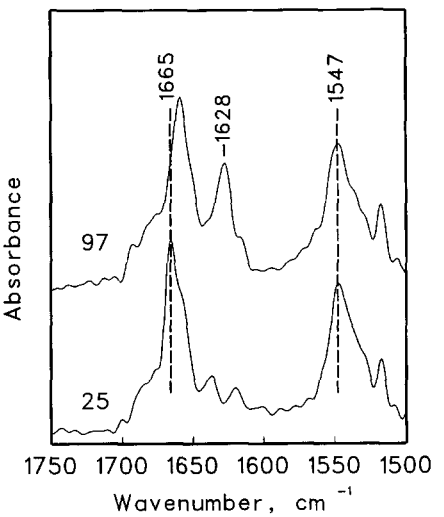

Fig. 2. Thermal denaturation of bacteriorhodopsin in H_2O buffer. Other details as in Fig. 1.

92°C, the IR spectrum is virtually identical to the one taken after 5 min heating at 97°C (the relative intensities of the 1,623 and 1,654 cm^{-1} components at 97°C will vary with small changes in heating time). Thus the same structural end-point is achieved by a long period of heating at 92°C than by a short period at 97°C, confirming the calorimetric prediction.

Thermal treatment modifies as well other parameters of purple membrane structure, as shown in Fig. 4. The average diameter of the fragments (B_{ave}) decreases markedly after heating at 92°C (Fig. 4A) perhaps because of an increased curvature of the membrane sheets and partial membrane fragmentation [17]. Changes in fragment size and curvature are probably reflecting, at a macroscopic scale, the molecular conformation changes detected by IR. The tight quasi-crystalline architecture of the purple membrane patches facilitates a cooperative conversion of mechanical forces from the molecular to the cell level. The behaviour of the electrophoretic mobility (u_e) as a function of temperature is interesting because it reaches a maximum at 92°C, then decreases at 97°C (Fig. 4B) suggesting that the conformational changes detected in the IR spectra of Fig. 1 include variations in the net negative charges of the purple membrane fragments (changes in u_e are more clearly visible in a low ionic strength buffer, see below). The correlation of B_{ave} and u_e with IR spectral changes is facilitated by plotting the amide I bandwidth at half-height vs. temperature in Fig. 4C. Denaturation is indicated by an increase in bandwidth, that is already apparent at 92°C. Changes in B_{ave} do not always occur in the same temperature range as those in u_e or bandwidth, either because small changes in protein or lipid conformation, not detected by IR, may cooperatively induce changes in purple sheet curvature, or because factors not explored in this paper may also concur in modelling the apparent size of the membrane fragments.

In order to facilitate comparison with previous studies [9,13], all the above experiments were repeated in a 50 mM bicarbonate, 100 mM NaCl, pH 9.5 buffer. IR spectra reveal that the end-point of the thermal denaturation process at pH 9.5 is undistinguishable from the equivalent phenomenon at pH 7.5, but the denaturation process appears to be completed after

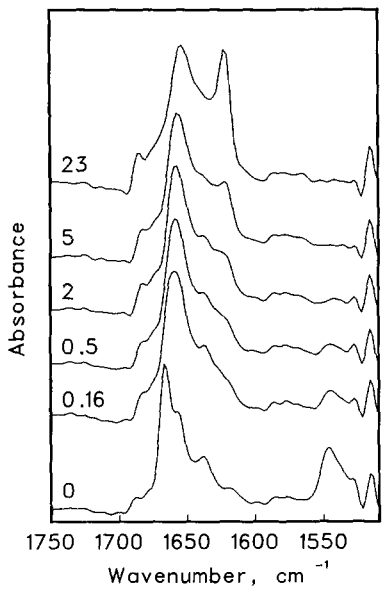

Fig. 3. Time course of bacteriorhodopsin thermal denaturation at 92°C. Samples were incubated in D_2O buffer at 92°C for the lengths of time indicated by each curve (hours), then equilibrated at room temperature for spectral recording. Other details as in Fig. 1.

5 min at 92°C when the pH is 9.5 (data not shown). The pH dependence of denaturation temperature is in agreement with previous data [9]. The average diameter of the purple membrane fragments, and their electrophoretic mobility, follow at pH 9.5 very similar patterns to those at pH 7.5, only the corresponding thermal events occur at a lower temperature, in accordance with the IR data.

The effect of a low ionic strength buffer (10 mM phosphate, pH 7.5) on the various thermal events described above was also tested. Only quantitative differences are seen between this and the 190 mM phosphate buffer: for obvious reasons, the changes in electrophoretic mobility are more clearly seen at low ionic strength (Fig. 4B); moreover, signs of denaturation that can be detected through changes in bandwidth are only visible at 97°C, but not at 92°C (Fig. 4C).

4. Discussion

The results in this paper strongly indicate that thermal denaturation of bacteriorhodopsin follows a given pathway irrespective of heating rate or buffer conditions. This pathway consists of (i) changes in the helical segments of the protein, leading to shifts of the two IR signals attributed to helices in the native protein, that are no longer resolved, (ii) extensive H-D exchange, as shown by the disappearance of the 1,547 cm^{-1} residual amide II signal, and (iii) protein aggregation, as indicated by the signals at 1,623 and 1,685 cm^{-1}. The three stages appear to occur in this order, either when temperature is stepwise increased (Fig. 1) or when the protein is heated over a long time at a given temperature (Fig. 2). Completion of one step is not a prerequisite for the next one to start, e.g. the early part of stage (ii) may occur simultaneously with stage (i). The same three stages are seen when pH or ionic strength are changed (data not shown). These structural changes in the bacteriorhodopsin molecule are tightly linked to macroscopic changes in shape and net electric charge of the purple membrane sheets (Fig. 4). Although previous thermodynamic studies were suggestive of a fixed denaturation pathway, our structural data provide the first experimental support to this idea. It should be noted that a given pathway for bacteriorhodopsin thermal denaturation, as described here, is compatible with small quantitative differences in structure between proteins denatured under varying conditions (heating rate, buffer, etc.).

Other aspects of the present work are also related to contemporary problems in protein studies. The kinetically controlled character of irreversible protein denaturation had been deduced from calorimetric studies for this and other membrane proteins [4,5,13]; however, the present paper provides direct structural evidence that this is the case (see mainly Fig. 3). Another point of debate is the persistence of the secondary structure elements in denatured proteins: this was hinted at by Brouillette et al. [9], more clearly shown by Cladera et al. [12], and extended here to a variety of conditions (Figs. 1–3). Thus

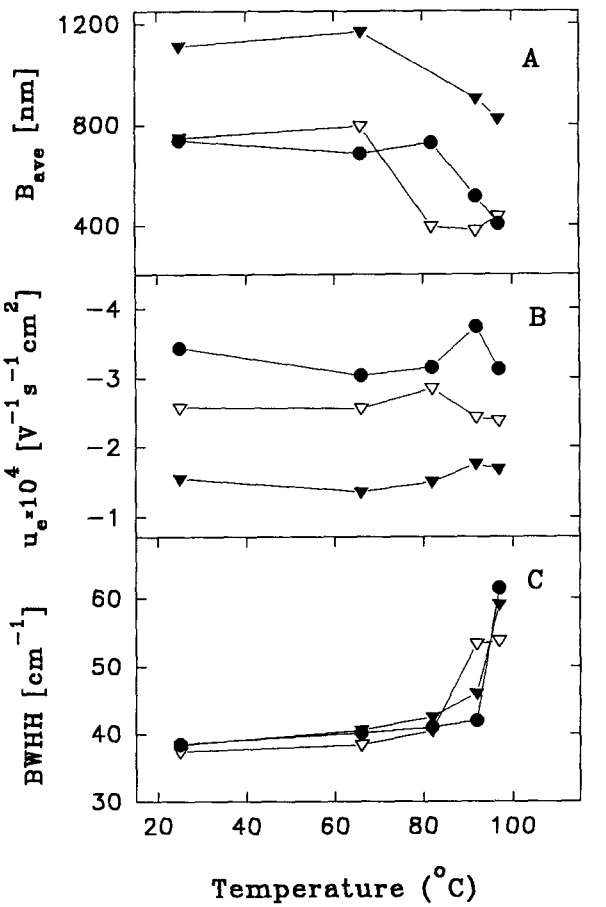

Fig. 4. Structural effects of temperature on purple membrane. (A) Average size of purple membrane sheets, as measured by quasi-elastic light scattering, as a function of incubation temperature. (B) Electrophoretic mobility of purple membrane sheets as a function of incubation temperature. (C) Width at half-height of the amide I infrared band, as a function of incubation temperature. Samples were incubated for 5 min at each temperature, then equilibrated at room temperature before the measurements took place. (▼) 190 mM phosphate buffer, pH 7.5 (●) 10 mM phosphate buffer, pH 7.5; (▽) 50 mM bicarbonate, 100 mM NaCl buffer pH 9.5. The data points correspond to average values of 4 measurements (panels A and B) or 2 measurements (panel C). Errors are of about the size of the symbols.

bacteriorhodopsin joins the steadily growing list of membrane proteins for which a 'compact denatured state' has been observed [2,3,18,19].

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