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Unfolding of monomeric bacteriorhodopsin in water-urea solution

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The theoretical equations for unfolding of globular proteins are applied to the unfolding of monomeric bacteriorhodopsin by the action of urea. The optical properties of the monomeric bacteriorhodopsin detergent (MBD) complex demonstrate an equilibrium between two conformations – unique native and unfolded. The stability of the MBD complex is significantly changed in urea buffer solution and the destabilizing energy of the protein is estimated to be about 20 kcal/mol. The model of unfolding used proves the existence of two steps – 'molecular swelling' – a process of low cooperativity and a lower stability of about 36 kcal/mol and the 'true unfolding' – a highly cooperative characterized with stability of about 55 kcal/mol. The mechanism of unfolding demonstrates a value of 6 for the ratio of the theoretical to the observed sharpness of the transition which is too large to be assumed as an existence of intermediate forms.

Introduction

Bacteriorhodopsin (BR) the light-dependent proton pump of the plasma membrane of *Halobacterium halobium* contains one polypeptide chain of 248 amino-acid residues with known sequence [1–5] and a retinal in a protonated Schiff base linkage with the e-amino group of Lys-216 as a chromophore [6,7]. The secondary structure of BR based on diffraction data [8–10] leads to the currently accepted model of the protein in which the peptide sequence traverses the membrane seven times in the form of alpha helices [9,13]. Together with some lipids (25% w/w), the protein is arranged naturally in two-dimensional hexagonal lattices which form sheets of the membrane, well known as the purple membrane.

All thermodynamic investigations of BR show the presence of protein in purple membrane or in liposomes [11,12,14–16]. The investigation of these systems by differential-scanning calorimetry allows the evaluation in detail of the quantitative characteristics of the disintegration of the crystalline lattice structure in purple membrane [14–16], or the interaction of the protein with artificial lipids in liposomes [11,12].

It will be of interest to study the conformational stability and the mechanism of unfolding of the single BR molecule. In order to solve this problem we use:
(1) A monomeric bacteriorhodopsin detergent complex (MBD complex) well-characterized by Dencher et al. [17–26] in order to avoid the protein–protein interactions.
(2) The Tanford concept for isothermal unfolding of proteins in urea solution applied to the MBD complex [27,28].

We analyse the mechanism and the kinetic of the MBD complex unfolding and evaluate quantitatively the difference in the free energy $\Delta F^{O}_{U,H_2O}$ between the unique native and unfolded conformations of the MBD complex in water buffer solution.

Materials and Methods

Triton X-100 and urea were purchased from Merck. All other reagents were of high purity grade.

Purple membranes were isolated from *Halobacterium halobium*, strain ET 1001, following the procedure of Oesterhelt et al. [30,31]. The preparation of the MBD complex was carried out according to the procedure of Dencher et al. [17,22,29] with some modifications. Purple membrane (0.70 mg) was solubilized in 0.9 ml 0.1 M phosphate buffer (pH 7.0) containing 0.4% Triton X-100 at a detergent-to-protein ratio of about 4 (w/w). After sonification for 2 min, the samples were kept in the dark at 25 °C for 20 h. The protein concentration was determined assuming an absorption coefficient of 62 700

Abbreviations: MBD, monomeric bacteriorhodopsin detergent; BR, bacteriorhodopsin.

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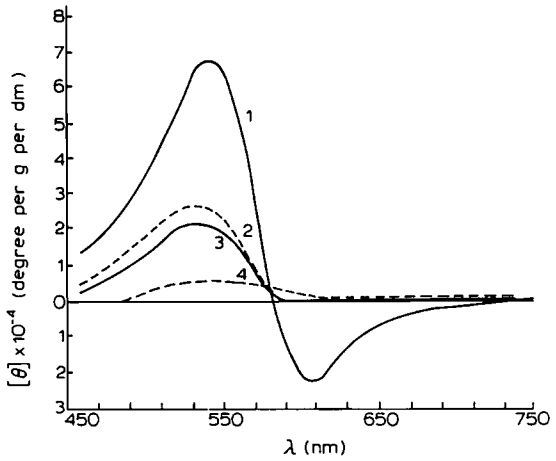

Fig. 1. Visible CD spectra of purple membrane as a function of the solubilisation procedure. 1, purple membrane (0.07 mg protein in 0.1 M phosphate buffer (pH 7.0) at 25° C; 2, MBD complex (0.07 mn protein in 0.1 M phosphate buffer (pH 7.0)/0.4% (w/w) Triton X-100 at 25° C; 3 and 4, MBD complex in 3 and 9 M urea, respectively, after 1 h incubation with urea.

$M^{-1} \cdot cm^{-1}$ at $\lambda_{max} = 555$ nm after light adaptation [17,22].

The absorption spectra were recorded on a double-beam Specord UV-VIS Spectrophotometer (C. Zeiss, Jena, G.D.R.) at λ_{max} of 0.05 absorbance units in order to avoid aggregation and light scattering.

The circular dichroism (CD) spectra were taken on a Mark III dichrograph (Jobin Yvon, France) in a 1 cm path quarz cell at 25° C.

The pH of the solution was measured with a precise digital pH-meter Model OP 101 (Radelkis, Hungary). The set of data for the change of the free energy of transfer ($\Delta f_{t,i}$) for individual amino acids was taken from Tanford [26,27].

Results

The solubilisation of purple membrane was controlled by visible CD spectra. The disappearance of the exciton effect at 600 nm indicates the homogenity of the preparation [17,19,22,25]. This is demonstrated by the comparison of two preparations: purple membrane fraction in 0.1 M phosphate buffer without detergent (Fig. 1, curve 1) and MBD complex prepared as described in Materials and Methods (Fig. 1, curve 2). The transfer of MBD complex from water buffer into urea buffer solution at concentrations up to 3 M shows only a weak decrease in the ellipticity at 535 nm (Fig. 1, curve 3), which is strongly influenced by the increasing urea concentration up to 9 M (Fig. 1, curve 4).

The urea-induced unfolding reaction was monitored by absorption spectroscopy. The decrease of the absorption coefficient at 555 nm (from 61 950 to 23 140 $M^{-1} \cdot cm^{-1}$) reflects the changes in retinal moiety as the protein unfolds. The results at pH 7.0 and 25° C and 1 h incubation time are shown in Fig. 2, curves 1–9. The unfolding transition is insignificant at low, 1–3 M urea concentrations and the native conformation is stable in this region. The main spectral changes are observed in the concentration interval 3–9 M, after 1 h incubation. The presence of an isobestic point at 424 nm can be shown as convincing evidence of the transition from unique native (N) to unfolded (U) state.

The observed sigmoidal decrease of the absorption at λ_{max} between 3 and 9 M urea (Fig. 3A, curve 1) indicates that the cooperative unfolding transition is a complicated process. It is known [34] that the kinetic study of the unfolding transition can define more precisely the process of unfolding. The time-dependence (1–120 h) of ΔA_{max} is observed in Fig. 3A, curves 1–7. These transition curves can best be compared by normalizing the data in terms of the apparent fraction unfolded, $\bar{Y}_{app}$:

$$\bar{Y}_{app} = \frac{A_{obs}^{555} - A_N^{555}}{A_U^{555} - A_N^{555}}$$

where A_{obs} refers to the observed absorption at $\lambda_{555\,nm}$ and A_N and A_U refer to the absorption of native and

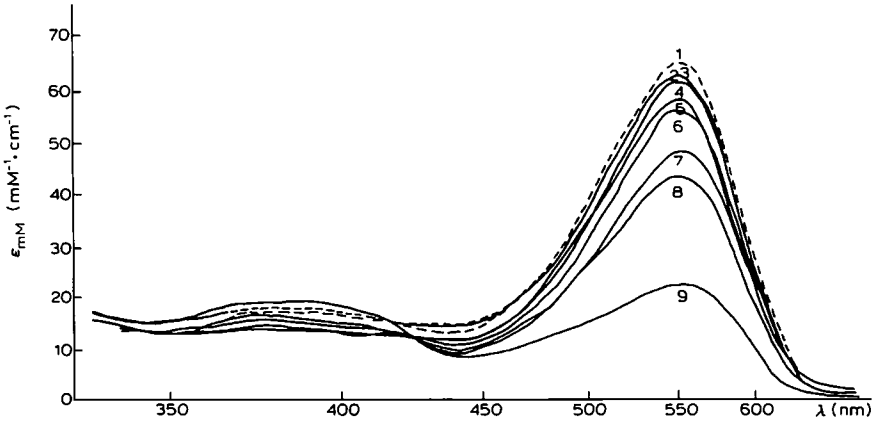

Fig. 2. Absorption spectra of the MBD complex (0.07 mg protein in 0.1 M phosphate buffer (pH 7.0)/0.4% Triton X-100 at 25° C) in different urea concentrations (1–9 M) after 1 h incubation.

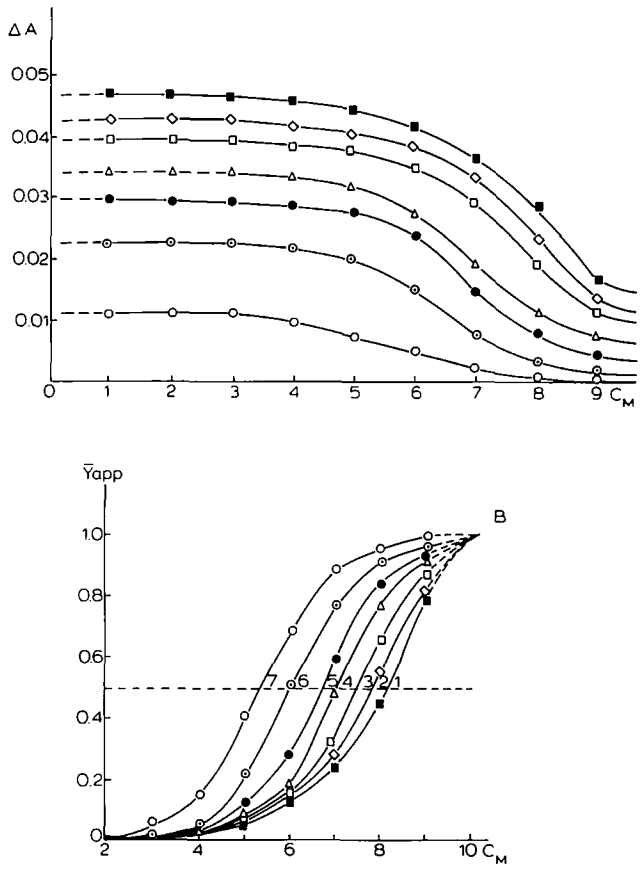

Fig. 3. (A) Absorption change at λ_{max} of the MBD complex as a function of urea concentration by varying the time of the incubation – 1, 3, 5, 9, 18, 60 and 120 h. (B) Dependence of the fractional change $\bar{Y}_{app}$ at λ_{max} on urea concentration as determined by data in (A) and after normalization.

unfolded forms, respectively at the appropriate urea concentration. These latter values were obtained by linear extrapolation of A_N and A_U from the native and unfolded base-line regions.

The plots of $\bar{Y}_{app}$ as a function of urea concentration for 1, 3, 5, 9, 18, 60 and 120 h incubation time are shown in Fig. 3B, curves 1–7. The value of the urea concentration at $\bar{Y}_{app} = 0.5$ varies between 8.3 M during the first hour of incubation and 5.3 M after 120 h. The effect can be a result of a slow rate of approach to the equilibrium, which probably proceeds together with a decrease in the stability of the MBD complex in urea, as was shown for another proteins [32,33].

The evaluation of the rate constants at every concentration of urea can be calculated from the slopes of the plots showing log $\tilde{Y}_{app}$ as a function of the incubation time (Fig. 4A, curves 4–9) where

$$\tilde{Y}_{app} = \frac{\Delta A_t}{\Delta A_{max}}$$

and ΔA_{max} is normalized for every curve at given C_M. The linearity of every curve demonstrates that the mechanism of unfolding at each urea concentration is monomolecular with respect to the protein concentration. If the unfolding mechanism is constant and non-urea concentration-dependent, then log K as a function of log C_M has to be a linear single curve but the testing of the experimental data (Fig. 4B) shows some nonlinearity. The correlation coefficients of lines passing through experimental points are separated into two statistical domains which allows us to assume the presence of different stochiometrical coefficients of urea binding below and above 6 M urea.

According to the experiments and their analysis described above, the unfolding transition of the MBD complex can be viewed as a change in the structure of the complex under increased urea concentration. On this basis, the changes of $\bar{Y}_{app}$ at every C_M for curve 1 in Fig. 3B are corrected and the result is plotted in Fig. 5, curve B. It is a typical two-step curve (B_I and B_{II}) characterized with $C^*_M = 4.8$ and 7.8 M urea concentration for the first and the second step of the transition, respectively. The linearisation of each of these steps of transition in log coordinates is shown in the insertion of Fig. 5 and allows the calculation of the slopes ν_{obs} equal to 9 and 15 for B_I and B_{II}, respectively.

All the data presented make it possible to apply the well-developed approach by Tanford [27,28] for each step. On the basis of the already known amino-acid sequence of BR [3–5], and the currently accepted tentative two-dimensional model of BR [8–10] and assuming the structure of BR in the MBD complex is the same as in the membrane, the contribution of each amino acid to the $-\delta\Delta F_U$ can be calculated using Tanford's parametrization [27,28]. The maximum possible contribution to $-\delta\Delta F_U$ is shown in Fig. 6, where 61% of the protein stability comes from nonpolar residues, 3% from the buried peptide groups and the contribution of all other groups is not more than 7%, as follows from the ratio of $-\delta\Delta F_U$ for each class of group between themselves. The sum of all these contributions is plotted against C_M in Fig. 7. The slope of this curve represents the theoretical sharpness ν_{th} of the transition. For the observed two steps, ν_{th} is equal to 56 and 84, respectively. Accordingly to the experimental evaluated C^*_M for the first and second step and from the theoretical calculated sum of contributions of all groups to the BR stability (Figs. 5 and 7), the value of $\Delta F^{O}_{U,H_2O}$ can be estimated. For the first step, $-\Delta F^{O}_{U,H_2O}$ is equal to 36 kcal/mol and for the second step to 55 kcal/mol.

Discussion

The optical properties of the MBD complex at low concentrations of urea (1–3 M) are the same as in water buffer solution. The added (1–3 M) urea does not change the CD and optical spectra significantly (Fig. 1, curve 3 and Fig. 2, curves 1–3), an effect which is very similar to that observed by the addition of more polar

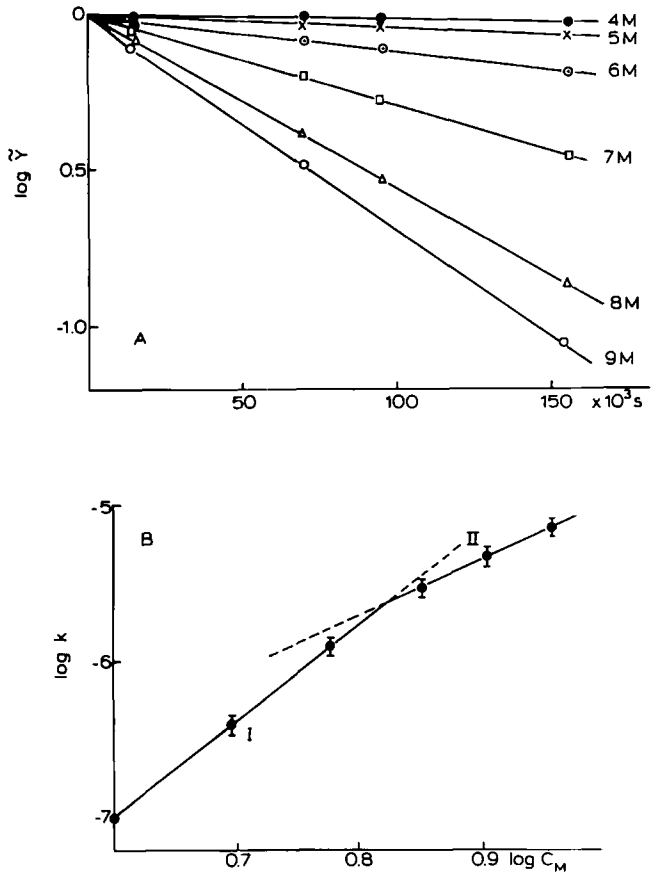

Fig. 4. (A) Time-dependence of the fractional changes, $\bar{Y}_{app}$, on the indicated final urea concentrations. (B) Concentration dependence of the rate constant calculated by the data in (A).

solvents. Further increase of the urea concentration up to 9 M changes the ellipticity of the visible CD spectra drastically and can be explained as a disappearance of

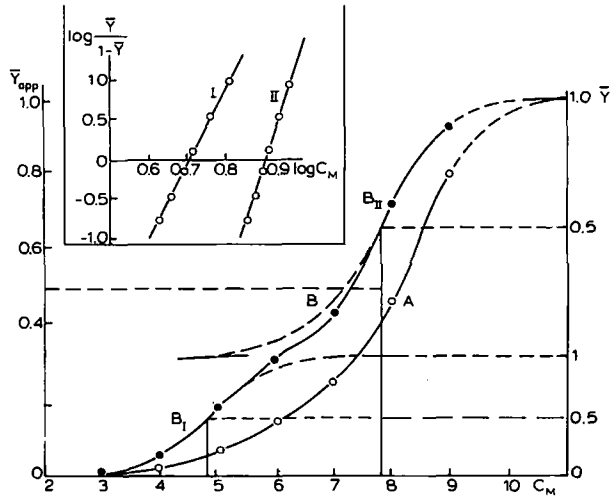

Fig. 5. (A) Normalized experimentally observed curve of the unfolding process. (B) The same curve after correction with kinetic parameters. I represents the first step of the transition with sharpness $\nu_{I\,obs} = 9$; II is the second step of the transition with $\nu_{II\,obs} = 15$. The insert shows the dependence of the equilibrium constant on urea concentrations in log coordinates.

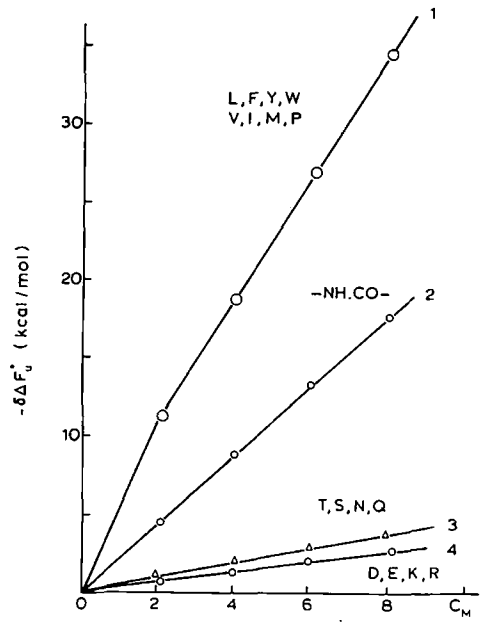

Fig. 6. Maximal possible contribution of different amino acids (1, 3, 4) and peptide groups (2) for the molecule of BR to $-\delta\Delta F_U$.

the asymmetrical arrangement of the electronically interacting amino-acid residues around the retinal chromophore. This is in agreement with the changes in the absorption spectra for the 4–9 M urea concentration interval (Fig. 2, curves 4–9). The observed isobestic point at 424 nm represents the equilibrium between two conformations – native and unfolded.

The stability of the MBD complex in urea buffer solution is significantly changed in comparison to that in water buffer solution. The data shown in Fig. 3, curves 1–7, and the analysis in Fig. 4 demonstrate indirectly the lower stability of the MBD complex in urea after 1 h of incubation, the effects of which increase the penetration of the solvent into the hydro-

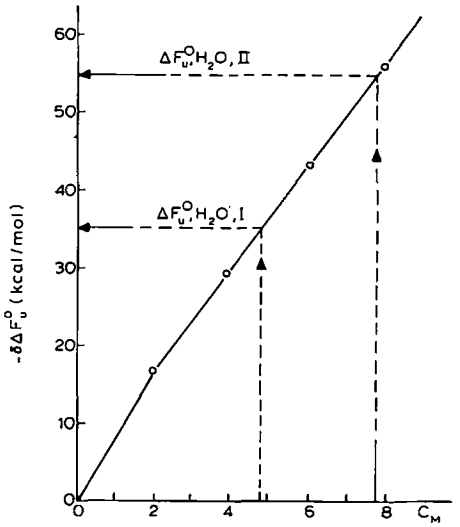

Fig. 7. Dependence of the sum of all possible contributions to the free energy ($-\delta\Delta F_U$) of the BR molecule on the urea concentration (C_M).

phobic parts of the MBD complex and the destabilisation of the protein can be calculated to be about 20 kcal/mol estimated from the C_M^* of curves 1–7 in Fig. 3B and the theoretically evaluated value of $\Delta F^O_{U,H_2O}$ in Fig. 7.

The kinetics of the transition demonstrate a dependence of the unfolding model on the urea concentration which is evaluated by the nonlinearity of the curve in Fig. 4B. The calculated value of the sharpness $n_I = 6$ and $n_{II} = 9$ can be observed as a change in the molecules approaching the active state of unfolding. So the change of the model of unfolding appears at a urea concentration equal to 6 M, which can be explained by different solvation forces. The kinetic study allows a correction of the unfolding process. The new calculated equilibrium process (Fig. 5, curve B) divides into two steps – B_I and B_{II}. The first step, by which the optical properties change by not more than 10–15% (Fig. 2, curves 3–6), can be explained by the increased accessibility of the retinal moiety to the polar solvent. Thus, the initial process of penetration of the solvent to the chromophore can be regarded as a molecular swelling. This process exhibits a relatively low cooperativity which is in agreement with the experimentally obtained sharpness $\nu_I = 9$ (Fig. 5, curve 1) and less stability $C_M^* = 4.8$ M (Fig. 5, curve B_I), in comparison with the behaviour of other proteins in urea [22,27,28,33,34].

The second step in which the main optical changes are detected (Fig. 2, curves 6–9) shows a high level of cooperativity (Fig. 5, curve B_{II}), whose characteristics of $\nu_{II} = 15$ and $C_M^* = 7.8$ M describe the process of true unfolding.

The mechanism of unfolding and the nature of the MBD-complex stability in urea solution can be shortly discussed by the comparison of the theoretically calculated and experimentally observed sharpnesses of the two steps of unfolding. The value of $r = \nu_{th}/\nu_{obs}$ can be calculated and its value is about 6 for both steps (Fig. 5, curve B_I and B_{II}). As a first tentative interpretation, it is possible to relate the number of the calculated intermediates to each of the seven alpha helices. However, this value is too large to be assumed for the existence of intermediate forms through the swelling and true unfolding of the protein molecule. Analysing the data in Fig. 7, it is clear that the main contribution to the protein stability is due to the hydrophobic interactions. These stabilities can be quantitatively evaluated as 36 and 55 kcal/mol, respectively, for the molecular swelling and true unfolding. In spite of the partial destabilisation of the MBD complex, these stabilities are about three times higher in comparison with such characteristics of the globular proteins [28,32,33].

In conclusion, the quantitative evaluation of the stability of the MBD complex allows the understanding of the structural stability of the BR molecule, which increases significantly after its incorporation in a selforganized structure such as purple membrane.

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