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Denaturation of bacteriorhodopsin by organic solvents

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The denaturation of bacteriorhodopsin by various organic solvents was studied using absorption, circular dichroism (CD) and fluorescence measurements. Organic solvents with a hydrogen-bonding group caused the release of retinal. The CD measurements showed that the helical structure was maintained even in the denatured state, whereas its tertiary structure was destroyed. The change in fluorescence intensity of tryptophan and fluorescent retinal also confirmed that the tertiary structure was destroyed. Comparison of the denaturation efficiency of various organic solvents showed that the concentration at denaturation was inversely proportional to the partition coefficient of the denaturant. This inverse proportionality clearly indicated that denaturation was determined by the concentration of denaturants which partitioned into the hydrophobic region of the membrane. It was discussed from the experimental results that the tertiary structure of bacteriorhodopsin was stabilized by the hydrogen-bonding networks between side chains of the helices. The results obtained from analysis of the amino acid sequence were also consistent with the hydrogen-bonding mechanism for the formation of the tertiary structure.

1. Introduction

Bacteriorhodopsin is a small intrinsic membrane protein which exists in crystalline patches in the plasma membranes of *Halobacterium halobium*. Its function is to translocate protons across the membrane according to the light absorption around 570 nm [1]. This protein has several advantages for the study of structure formation of membrane proteins, as follows: (1) It is very easy to purify bacteriorhodopsin in the form of purple membranes whose protein content is as high as 75% [2]. (2) A single polypeptide of 26 kDa shows sufficient activity of light-driven proton pumping without any complex subunit structure [1]. (3) various kinds of structural analysis have revealed that the major part of the polypeptide is composed of seven α-helices and embedded in the lipid bilayer region of the membrane [3,4]. The position of retinal has also been ascertained [5,6]. (4) The amino acid sequence has been determined in two laboratories and indicates that this protein is quite hydrophobic in nature [7,8], i.e., bacteriorhodopsin is one of the typical membrane proteins.

It is considered that there are three stages in the structure formation of membrane proteins: (1) penetration into the membrane, (2) formation of α-helices spanning the membrane and (3) binding between helices [9]. In order to resolve the question as to how the three-dimensional structure of a membrane protein is formed from its primary structure, one requires an understanding of the

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interaction which stabilizes each stage in structure formation. The mechanism involved in the first stage, i.e., penetration into the membrane, has been discussed by various authors, and it has been established that almost all polypeptide segments which penetrate into the lipid bilayer membrane are highly hydrophobic [9–11]. Therefore, hydrophobic interactions should make the greatest contribution to penetration of hydrophobic helices into membranes. The most important problem hence concerns the mechanism of formation of the tertiary structure of a membrane protein. Although Engelman and Steitz [9] have discussed the possibility that binding between helices is due to some polar forces, no direct experimental evidence in favor of this proposal has been obtained thus far.

Denaturation phenomena should provide insights into the mechanism of structure formation of proteins [12]. If one is able to carry out the denaturation of bacteriorhodopsin in which only the tertiary structure is destroyed, then the nature of the interaction stabilizing this structure should be clarified. In the present work we have studied the denaturation of bacteriorhodopsin by organic solvents, using optical absorption, CD and fluorescence measurements. Various organic solvents in fact caused incomplete denaturation of bacteriorhodopsin in which the tertiary structure was destroyed but the helical structure was preserved. Comparison of the denaturation efficiency of the organic solvents strongly suggested that the binding between helices is due to hydrogen bonds of the side chains. The mechanism of formation of the tertiary structure of membrane proteins is discussed from the viewpoint of the experimental results as well as information on the amino acid sequence.

2. Materials and methods

2.1. Chemicals

Organic solvents for denaturation experiments were purchased from Wako Chem. Reagents of the best grade commercially available were used without purification.

Sodium borohydrate for reduction of the Schiff base of retinal was obtained from Wako Chem. Limited proteolysis of the carboxyl terminus was performed using papain obtained from Sigma.

N-Methylpicolinium (MePic) for fluorescence quenching was synthesized from 4-picoline and methyl iodide according to the method of Shinitzky and Rivnay [13]. The purity of MePic was checked by thin-layer chromatography, showing a single spot.

2.2. Preparation of purple membranes

Purple membranes of *Halobacterium halobium*, strain R1M1, were prepared according to the established method [14]. After sucrose-gradient centrifugation, membranes were washed three times with water and stored in liquid nitrogen. Before use, purple membranes were suspended in 55 mM Tris-glycine (pH 7) or distilled water and sonicated for several minutes. Appropriate amounts of aqueous and organic solvents were added to purple membrane suspensions in denaturation experiments.

2.3. Limited proteolysis of carboxyl terminus

Limited proteolysis of the carboxyl terminus was performed by treatment with papain (papain/bacteriorhodopsin ratio, 1 : 50) for 4 h at 37 °C. The product was examined on SDS electrophoresis, yielding a slightly smaller polypeptide than native bacteriorhodopsin. It is known that treatment for longer periods with papain at greater concentrations results in much smaller polypeptide segments [15]. However, there was no trace of smaller polypeptides or native bacteriorhodopsin in our preparations. Therefore, complete proteolysis of the carboxyl terminus had been achieved, as reported by Abdulaev et al. [15].

2.4. Reduced membranes

Purple membranes in which the retinal chromophores were converted to fluorescent derivatives were prepared according to the method of Schreckenbach et al. [16]. Purple membrane fragments were reduced with 1% $NaBH_4$ at 0 °C

under illumination by visible light (520–600 nm), until complete reduction was attained. The reduced product was washed and resuspended in distilled water and irradiated with ultraviolet light (including an L39 Toshiba filter) under nitrogen. By means of this procedure, completely ultraviolet-converted membranes, which were fluorescent, were obtained.

2.5. Absorption spectra

Absorption spectra were recorded on a double-beam spectrophotometer (Hitachi 557). In denaturation experiments, the stock solution of purple membranes was diluted with buffer solution and an organic solvent, adjusting the protein concentration to 6–7 μM. Absorption measurements were carried out after incubation in the solvent for 24 h at room temperature.

2.6. CD measurements

A Jasco J-40as recording spectropolarimeter was used for the CD measurements. Because the magnitude of the ellipticity was quite different below and above 250 nm, we recorded two sets of measurements on the same sample, changing the wavelength range and sensitivity of the apparatus. The protein concentration was about 10 μM for CD measurements.

2.7. Fluorescence measurements

The fluorescence spectra and fluorescence quenching were measured with a Hitachi F-3000 fluorescence spectrophotometer. Tryptophan fluorescence was excited at 280 nm, whereas measurements of fluorescent retinal were made at an excitation wavelength of 382 nm. In order to minimize inner filter effects, the bacteriorhodopsin concentration was reduced to about 1 μM in fluorescence measurements.

The efficiency of quenching by the soluble quencher, MePic, was monitored by adding concentrated MePic solution to a purple membrane suspension. The results were analyzed through Stern-Volmer plots.

3. Results

3.1. Release of retinal by organic solvents

The denaturation of bacteriorhodopsin has been studied by mixing various organic solvents with purple membrane suspensions. Purple membrane suspensions containing organic solvents were incubated at room temperature for 24 h before measurements. Fig. 1a shows the absorption spectra in the presence of various concentrations of methanol. Native bacteriorhodopsin gives rise to an absorption band at 570 nm due to retinal. When methanol was added, the absorption band of retinal shifted from 570 to 380 nm through an intermediate band of 550 nm. It is clear from the sigmoidal change in fig. 1b that the denaturation occurs cooperatively at a methanol concentration of about 12.5 M. The release of retinal from the inside of the protein was demonstrated by centrifugation of the suspension. A white precipitate

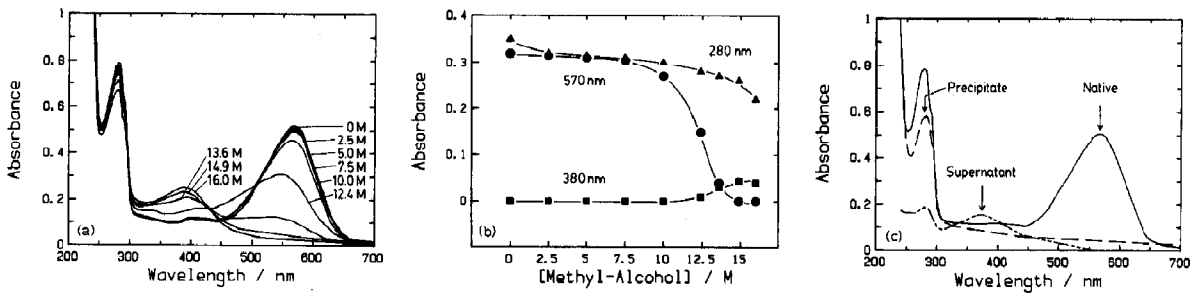

Fig. 1. (a) Absorption spectra of bacteriorhodopsin in the presence of various concentrations of methanol. (b) Absorbance at 280, 380 and 570 nm plotted as a function of methanol concentration. (c) Centrifuged suspension of bacteriorhodopsin with 15 M methanol showing the release of retinal from the protein fraction.

and slightly yellow supernatant were obtained when the denatured sample with 15 M methanol was centrifuged at 40000 rpm for 60 min, these conditions being the same as those for native purple membranes. As the absorption bands at 280 and 380 nm were separated by centrifugation, it was evident that the white pellet contained most of the protein fraction absorbing at 280 nm, while retinal, with an absorption band at 380 nm, was recovered in the supernatant (fig. 1c).

Aldehydes and esters also showed significant denaturation with a shift of the absorption wavelength just before denaturation (data not shown). A blue shift from 570 to 500 nm was observed for aldehydes, while a red shift to 600 nm occurred in the case of esters. Although the structure in these intermediate states has not yet been elucidated, the appearance of the band at 380 nm clearly indicated that retinal was finally released from the protein.

Here, we are concerned with the following two questions as regards the mechanism of structure formation. (1) What kind of structure does bacteriorhodopsin assume in the denatured state? (2) Which form of interaction is weakened by the addition of organic solvents? The answers to these questions should provide information on the mechanism of structure formation.

3.2. *Structure in the denatured state*

In order to elucidate the structure in the denatured state, we have measured the CD and fluorescence spectra of purple membrane suspensions with and without organic solvents. Fig. 2 shows the change in CD spectra elicited by methanol. The denatured sample was washed three times with distilled water in this measurement in order to remove methanol and free retinal. The molar ellipticity per residue was plotted below 250 nm, whereas the CD was determined via the molar ellipticity per molecule at longer wavelengths. The CD spectrum of native bacteriorhodopsin showed several characteristic peaks as reported previously [17]: negative peaks at 222 and 315 nm, a positive peak at 257 nm with a shoulder at 280 nm and an anomalous dispersion around 570 nm. The large peak at 222 nm is assigned to the helical structure,

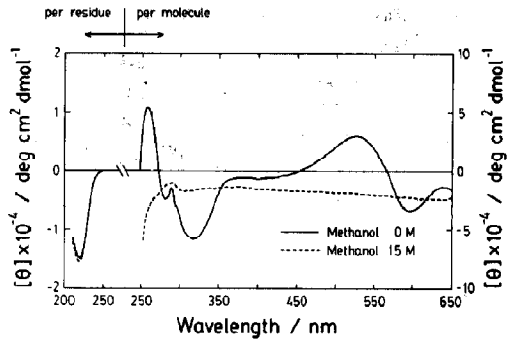

Fig. 2. CD spectra of native bacteriorhodopsin and a sample denatured with 15 M methanol. Methanol was removed by washing three times with water before measurement of the denatured sample. Ellipticity scale is changed above and below 250 nm.

which is the predominant secondary structure in this protein, whereas the anomalous dispersion around 570 nm and the negative peak at 315 nm are due to retinal in bacteriorhodopsin. The signals between 250 and 300 nm are usually considered to be due to the tertiary structure [17], although the possibility of some contribution by retinal cannot be ignored [18]. Therefore, the fact that most of the CD peaks disappeared except for that at 222 nm strongly suggests that all ordered structure other than the helical type no longer exists in the denatured state. The same behavior was also observed for formaldehyde (data not shown).

The dependence on the methanol concentration of the molar ellipticity at various peaks is plotted in fig. 3a–c. In measurements of the methanol concentration dependence, we did not wash the samples, since the difference resulting from the removal of methanol was only minor. The negative peak at 222 nm showed no change at all, indicating that the helical structure was maintained despite denaturation (fig. 3a). However, the molar ellipticity values at 315, 520 and 585 nm, which are assigned to retinal in bacteriorhodopsin, decreased abruptly at about 12.5 M methanol (fig. 3b). This is quite reasonable in view of the fact that retinal was released from Lys 216 under denaturation conditions as revealed by absorption measurements. The CD behavior over the wavelength range 250–300 nm is usually considered to

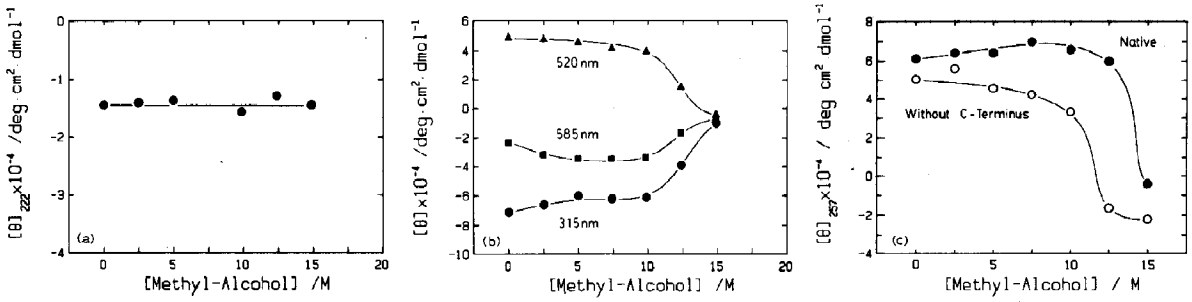

Fig. 3. Dependence on methanol concentration of (a) ellipticity per residue for the band at 222 nm, and (b) ellipticity per molecule at 315, 520 and 585 nm. (c) Ellipticity per molecule at 257 nm is also shown for bacteriorhodopsin (●———●) and the polypeptide fragment lacking the carboxyl terminus (○———○) as a function of methanol concentration.

be due to the tertiary structure of proteins and to be proportional to the content of aromatic side chains [17]. Bacteriorhodopsin contains about 12% aromatic side chains which is sufficient to give rise to the positive peak at 257 nm and the shoulder at 280 nm. Therefore, the disappearance of the positive peak at 257 nm in fig. 3c, which is correlated well to the release of retinal, strongly suggested that the tertiary structure of bacteriorhodopsin was destroyed by the organic solvents.

We have also measured the CD of bacteriorhodopsin without the carboxyl terminus of about 20 residues. Fig. 3c exhibits the dependence on alcohol concentration of the molar ellipticity at 257 nm for native bacteriorhodopsin together with the polypeptide fragment lacking the carboxyl terminus. When the carboxyl terminus is cleaved off, denaturation occurs at a slightly lower concentration of methanol than in the case of native bacteriorhodopsin. It is known that the carboxyl terminus does not have a substantial effect on the proton-pumping activity [19]. The change in denaturation behavior shown in fig. 3c indicates that the interaction between the carboxyl terminus and the other part contributes only slightly to the stability of the three-dimensional structure of the protein.

If the tertiary structure is destroyed, the intrinsic fluorescence spectrum of tryptophan residues is expected to change more or less at the denaturation point [20]. Fig. 4a shows the fluorescence spectra of bacteriorhodopsin at various concentrations of methanol. The fluorescence intensity of tryptophan increased drastically by a factor of greater than 10 (fig. 4b). This change in intensity is anomalously large, suggesting that all tryptophan residues are influenced by denaturation. Bacteriorhodopsin contains eight tryptophan residues which are scattered in the hydrophobic region of the primary structure. Kalisky et al. [21]

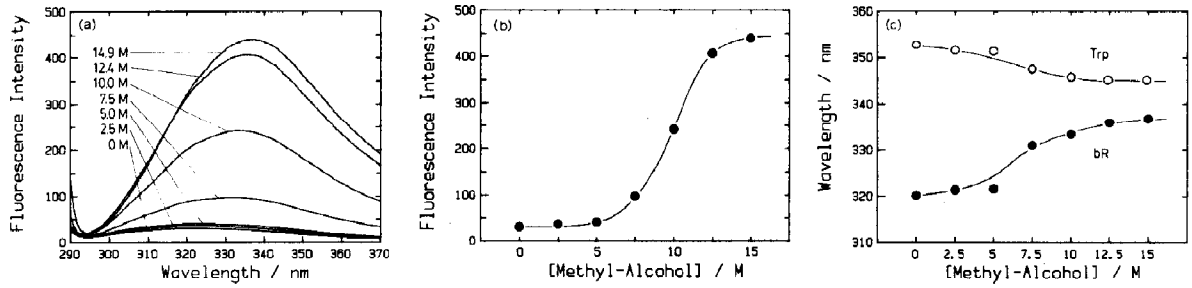

Fig. 4. (a) Tryptophan fluorescence spectra of bacteriorhodopsin at various concentrations of methanol. Excitation wavelength, 280 nm. Methanol concentration dependence of (b) tryptophan fluorescence intensity and (c) maximum wavelength, as a consequence of denaturation. (○———○) Reference measurement for an aqueous solution of tryptophan.

have previously suggested that the quenched tryptophan fluorescence of native bacteriorhodopsin is due to the energy transfer from tryptophan to retinal. However, energy transfer to retinal must be part of the process of quenching of tryptophan fluorescence, since bacterioopsin lacking retinal prepared with hydroxylamine also indicated similar behavior with respect to methanol concentration [22]. Therefore, the most plausible explanation for the large increase in tryptophan fluorescence is that all tryptophan residues experience changes in local environment due to destruction of the tertiary structure.

The tryptophan fluorescence of native bacteriorhodopsin has a maximum at about 320 nm, which characterizes the highly hydrophobic environment of tryptophan residues [20]. This means that tryptophan residues are located in the hydrophobic region of membranes, as expected from the primary structure. The maximum in the tryptophan fluorescence spectra shifted toward longer wavelengths due to denaturation. As shown in fig. 4c in which the maximum wavelength is plotted as a function of methanol concentration, the maximum increased to about 337 nm in the denatured structure. In spite of this red shift, the fluorescence wavelength of about 337 nm is much shorter than the value of 355 nm for tryptophan in water and even shorter than that for tryptophan exposed to methanol solutions. Therefore, it is believed that tryptophan residues in denatured bacteriorhodopsin are buried in the lipid phase. This is consistent with the presence of the helical structure in the denatured state, which is considered to span the lipid bilayer membrane. The midpoint of the sigmoidal change in intrinsic fluorescence (fig. 4b and c) was about 10 M which is a little lower than that observed in the absorption and CD measurements. The local structure of tryptophan may change prior to denaturation of the tertiary structure of the entire bacteriorhodopsin molecule. It should be noted that similar forms of behavior of tryptophan fluorescence were observed for various organic solvents, although for some solvents, such as acetone, which have a strong absorption band in the ultraviolet region, measurements could not be made.

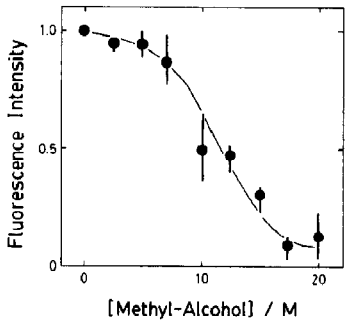

Fig. 5. Fluorescence intensity of ultraviolet-converted bacteriorhodopsin as a function of methanol concentration. Excitation wavelength, 382 nm; emission was monitored at 488 nm.

The fluorescence of converted bacteriorhodopsin also showed significant change on addition of methanol. The configuration of retinal in ultraviolet-converted bacteriorhodopsin is considered to be the same as that of the native structure [5]. In measurements on fluorescent retinal, the excitation and emission wavelengths were 382 and 488 nm, respectively. The fluorescence intensity decreased greatly on addition of methanol, as shown in fig. 5. The decrease in intensity occurred at around 12.5 M in accord with other measurements for methanol denaturation, although the denaturation curve appears broader. Fluorescent retinal is covalently bound to the protein and a possible reason for the fluorescence decrease is the greater freedom of the protein structure around retinal, leading to greater accessibility of retinal to water or quenching molecules such as oxygen.

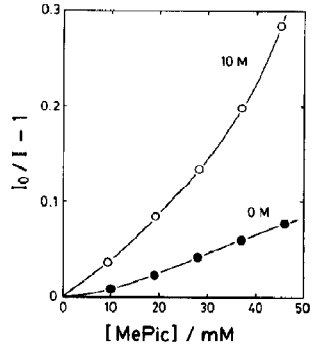

Fig. 6. Stern-Volmer plot of the fluorescence quenching of converted bacteriorhodopsin by a water-soluble quencher, MePic, in the presence and absence of 10 M methanol.

In order to confirm this conclusion, we followed the quenching of fluorescent retinal by using a water-soluble quencher, MePic [23]. Fig. 6 shows a Stern-Volmer plot of fluorescent bacteriorhodopsin and without 10 M methanol. It is clear that the quenching rate is 3-times greater in the presence of 10 M methanol than in its absence. Therefore, fluorescent retinal which is covalently bound to Lys 216 must have become exposed to the soluble quencher, which again confirms destruction of the tertiary structure as well as the enhanced freedom of intramolecular motion.

3.3. Interaction which stabilizes the tertiary structure

Our spectroscopic measurements have shown that the denaturation of bacteriorhodopsin by organic solvents is associated with the disappearance of tertiary structure. Namely, the denatured state of bacteriorhodopsin maintains a helical structure in the hydrophobic region of membranes and only the binding between helices is destroyed. Therefore, if the interaction which is responsible for denaturation is ascertained, the final stage of folding of bacteriorhodopsin should become clear. In this respect, membrane proteins like bacteriorhodopsin possess advantages over soluble globular proteins, because a series of organic solvents may be used for measurements of incomplete denaturation.

Organic solvents may be classified according to three categories with respect to the denaturation of bacteriorhodopsin: (1) hydrophilic solvents (glycerol and diols), (2) quite hydrophobic solvents (chloroform, benzene and other hydrocarbons) and (3) solvents of intermediate hydrophobicity (alcohols, aldehydes, esters and ketones). Solvents of the first category showed no denaturation ability. Therefore, a considerable degree of hydrophobicity must be necessary for the denaturation of bacteriorhodopsin. On the other hand, according to the work by Eisenbach et al. [24], quite hydrophobic solvents do not destroy the structure of bacteriorhodopsin in the absence of water. Therefore, it is considered that organic solvents possessing no hydrogen-bonding group do not give rise to denaturation of bacteriorhodopsin. Solvents in the last category contain a hydrogen-bonding group as well as hydrocarbon chains and exhibit a significant degree of denaturation. This indicates the importance not only of hydrophobic interactions but also of hydrogen bonds.

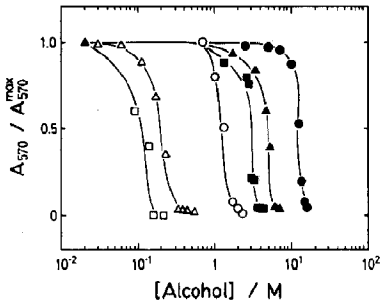

Fig. 7. Absorbance of bacteriorhodopsin plotted as a function of alcohol concentration: (●) methanol, (▲) ethanol, (■) 2-propanol, (○) 1-propanol, (△) butanol, (□) 1-pentanol.

The denaturation curves obtained with a series of alcohols are shown in fig. 7, in which the absorbance at 570 nm is plotted as a function of the concentration of alcohols. The alcohol concentration at which the denaturation occurred could be readily deduced from the sigmoidal change in absorbance. The sharp sigmoidal change indicates the cooperative nature of the denaturation. It is clear that the critical concentration decreases for alcohols with longer hydrocarbon chains. Similar effects were also observed for al-

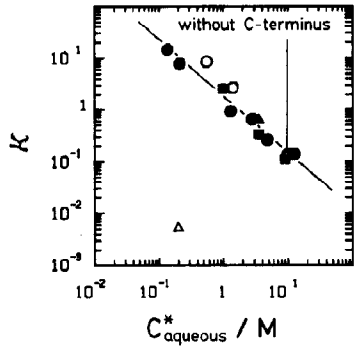

Fig. 8. Partition coefficient of denaturants plotted as a function of critical concentration of organic solvents in aqueous solution at the point of denaturation of bacteriorhodopsin: (●) alcohols, (■) aldehydes, (○) esters, (▲) acetone, (△) hydroxylamine. (◎) Denaturation of bacteriorhodopsin without the carboxyl terminus.

dehydes and esters (data not shown). This led to the conclusion that the more hydrophobic denaturants are more efficient in destroying the tertiary structure of bacteriorhodopsin. The question that arises from this fact concerns the intramolecular interaction which stabilizes the tertiary structure.

In order to clarify the role of the hydrophobic interaction as well as hydrogen bonds in tertiary structure formation, we have examined in fig. 8 the relationship between the hydrophobicity of organic solvents of the third category and the corresponding efficiency in denaturation. The ordinate represents the partition coefficient [25] and the abscissa denotes the concentration of denaturant in aqueous solutions at the point of denaturation. Both the partition coefficient and the critical concentration ranged over two decades and showed very good inverse proportionality. The partition coefficient, κ, is defined as the ratio of the concentration in a hydrophobic environment, C_h, to that in the aqueous phase, C_w, at equilibrium,

$$\kappa = \frac{C_h}{C_w}. \quad (1)$$

Therefore, the inverse proportionality between the partition coefficient of denaturants and the critical concentration indicated that denaturation occurred at the same concentration of denaturants in the membrane. Furthermore, the critical concentration in the membrane could be estimated from fig. 8 to be 1–3 M. This concentration corresponds to several dozens of organic solvent molecules per bacteriorhodopsin molecule. This value is almost the same as the number of hydrogen bonding residues in the helical region of bacteriorhodopsin which may be estimated from the amino acid sequence. Therefore, it is reasonable to assume that denaturation occurs cooperatively, when the hydrogen bonds in bacteriorhodopsin are attacked by the same number of hydrogen-bonding groups of organic solvents, as depicted in fig. 9.

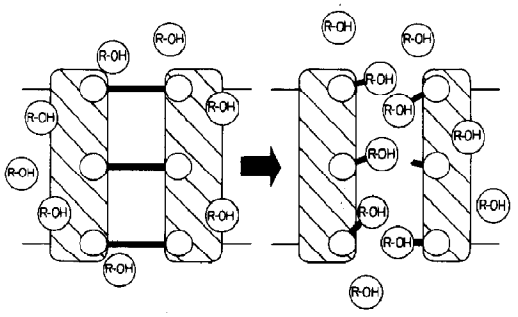

Fig. 9. Schematic representation of the mechanism of denaturation of bacteriorhodopsin by organic solvents. Alcohol molecules, for example, attack the hydrogen bonds between helices and cause denaturation cooperatively, when the concentration of alcohol in the membrane reaches that concentration of the hydrogen-bonding side chains in bacteriorhodopsin.

Thus, all forms of denaturation behavior with organic solvents are quite reasonably explained by the existence of hydrogen-bonding networks which stabilize the tertiary structure of bacteriorhodopsin. First, organic solvents without any hydrogen-bonding group do not destroy the structure of bacteriorhodopsin, as reported by Eisenbach et al. [24]. The fact that only solvents possessing a hydrogen-bonding group can cause denaturation provides clear evidence in support of a significant role for hydrogen bonds in the tertiary structure. Second, the critical concentration of denaturants in the membrane was fairly constant despite the wide variety in denaturants. For example, the effect of methanol was the same as that of 1-pentanol, therefore, the number of hydrogen-bonding groups in a membrane must be of far greater importance than hydrocarbon chains. Third, the critical concentration in the membrane was 1–3 M, corresponding to several dozens of denaturant molecules per bacteriorhodopsin molecule. This number is of the same order as that of hydrogen-bonding side chains which are embedded in the hydrophobic region of the membrane. Therefore, the breaking of hydrogen bonds between helices should occur when the denaturant concentration exceeds the number of intrinsic hydrogen bonds of this protein. Fourth, glycerol and diol did not cause denaturation of bacteriorhodopsin. The reason for this is simple. These organic solvents are so hydrophilic that they do not penetrate into the membrane more than 1 M. Fifth, there was one exception to the inverse proportionality relationship illustrated in fig. 8: retinal was released from bacteriorhodopsin at 0.2 M hydroxylamine under

illumination [26]. In this case the critical concentration in the membrane calculated from the partition coefficient was three orders of magnitude lower than that of other solvents. It is known that hydroxylamine reacts specifically with the aldehyde group of retinal, the tertiary structure of the protein being considered to be maintained. Namely, hydroxylamine modifies only the Schiff base of retinal, while organic solvents like alcohols possibly attack all hydrogen bonds in bacteriorhodopsin. Therefore, the low critical concentration in the case of hydroxylamine demonstrates that binding of retinal and tertiary structure formation are independent phenomena. (The structure of bacterioopsin prepared with hydroxylamine is reported elsewhere [22].)

4. Discussion

It is known that various interactions, i.e., ion-pair, hydrogen-bond and hydrophobic interactions, are responsible for the three-dimensional structure of proteins. The denaturation measurements on bacteriorhodopsin in this work have shown that its tertiary structure is stabilized by hydrogen bonds as well as ion pairs. In contrast, the penetration of a polypeptide into a membrane is considered to be the consequence of hydrophobic interactions. Therefore, what is the physical basis underlying the specialization of each type of interaction in the structure formation of membrane proteins?

The answer to this question appears simple, at least qualitatively. Both ion pairs and hydrogen bonds may be regarded as being due to the forces of electrical attraction between charges of opposite signs. Therefore, the polarity of the environment exerts a negative effect on the strength of the interaction. In other words, the electrical potential of hydrogen bonds as well as ion pairs is inversely proportional to the dielectric constant. Since the value of the dielectric constant of water is 80 and that of the hydrocarbon region of a membrane lower than 10 [23], the corresponding forces in the case of charges separated by the same distance should be almost an order of magnitude greater in a membrane than in water. Hence, hydrogen bonds and ion pairs exert very significant effects in the hydrophobic region of membranes [9].

On the other hand, the hydrophobic interaction acts only in aqueous medium as reviewed by Tanford [27]. The free energy for the transfer of a hydrophobic group, e.g., a hydrocarbon chain or an aromatic ring, from the aqueous phase to an organic solvent is proportional to the surface area of the group. This effect is not due to the actual attractive force which works directly between the hydrophobic groups. It is believed that the structure of water molecules around hydrophobic groups increases the free energy and causes their aggregation only in the presence of water, leading to the conclusion that no hydrophobic interaction occurs within the nonpolar interior of a membrane. The best evidence in support of this feature of hydrophobic interactions is provided by the membrane fluidity, deduced from the free diffusion of various hydrophobic molecules within membranes.

Consequently, polar forces and the hydrophobic interaction show striking contrast in their characteristics. Polar forces are dominant in the nonpolar region of membranes, whereas the hydrophobic interaction is strong only in the aqueous phase and responsible for the formation of the membrane structure itself. Therefore, the conclusion drawn from the present experiments that hydrogen bonds (and ion pairs) stabilize the tertiary structure of bacteriorhodopsin in membranes is quite reasonable from the physical point of view.

It is believed that all information on the higher order structure of proteins is contained in the amino acid sequence. Therefore, the amino acid sequence of bacteriorhodopsin should be consistent with the concept of hydrogen-bonding networks being the basis for tertiary structure formation. Namely, the angular distribution of hydrogen-bonding residues around helices should be in accord with the hydrogen-bonding mechanism. The spatial distribution of hydrogen-bonding residues within a helix has been previously revealed in an approximate manner by neutron diffraction of specifically deuterated bacteriorhodopsin [28]. Engelman and Zaccai [28] have studied the position of valine and phenylalanine residues

within the bacteriorhodopsin molecule and compared the result with helical segments of the amino acid sequence. Their conclusion was that hydrophobic residues are positioned at the periphery of the protein, whereas hydrogen-bonding residues are located in the interior. This is quite reasonable as the external part faces the lipid phase which is uniformly hydrophobic and the functional site must be located within the molecule. The question is how many hydrogen-bonding residues there are in the region between neighboring helices. If hydrogen-bonding networks stabilize the tertiary structure, the interface between helices should comprise many hydrogen-bonding residues.

A helical wheel projection is a useful diagrammatic method of elucidating the spatial distribution of residues, particularly when a helix is amphiphilic [29]. Fig. 10a shows the wheel diagrams of the seven helices of bacteriorhodopsin, which is similar to the diagram of Engelman and Zaccai [28]. All helices are viewed from the cytoplasmic side and the adjacent side chains are separated by 100 degrees of arc on the wheel. Residues are indicated by the single-letter notation. Hydrogen-bonding residues are represented by filled circles except for two residues at both ends of the helices. Because residues at the edges may be exposed to the aqueous medium and also because there is some ambiguity in the secondary structure, hydrogen-bonding residues at the edges are represented by partially filled circles. The exact position of the secondary structure for bacteriorhodopsin is not yet known. However, various secondary structure predictions are in agreement with each other, if we allow only a minor difference of several residues [10,30–32]. Therefore, we have used the prediction of Huang et al. [32] for the sake of convenience in fig. 10a. Although the distribution of hydrogen-bonding residues is rather diffuse, the amphiphilic nature is clearly observed in fig. 10a. The significant feature of the helical wheels is the wide arc of hydrogen-bonding residues. Helices A–C and E–G show arcs with an angle of more than 180°. Only in helix D are the hydrogen-bonding residues distributed within an angle of 140°. Helix E shows a very wide arc of hydrogen-bonding residues which are almost all arranged around the helical wheel. Therefore, the observed hydrogen-bonding arcs of more than 180° strongly suggest that many hydrogen-bonding residues are located at the interface between helices.

When several helices form a polygon, the interior angle of the polygon, namely, the angle θ

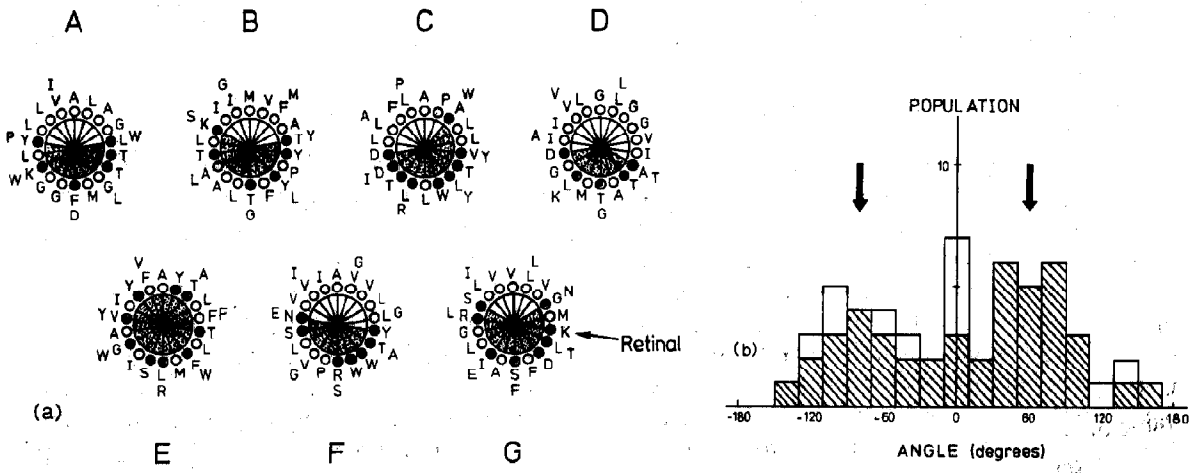

Fig. 10. (a) Helical wheel projections of seven helices in bacteriorhodopsin. Amino acids are represented using the single-letter notation. Closed circles denote hydrogen-bonding residues within the helical regions and half-filled circles represent those at the edge of the helices. (b) Histogram of hydrogen-bonding residues obtained by superposing seven helices with the amphiphilic dipoles maintained the same. Hatched area corresponds to the population of hydrogen-bonding residues in the helical region except for two residues at the ends of helices. The origin of the axis giving the angles is at the bottom of the helical wheels.

between two directions to neighboring helices, must be slightly smaller than 180%. The average angle $\langle\theta\rangle$ is written as:

$$\langle\theta\rangle = 180 - 360/n, \quad (2)$$

where n is the number of angles of a polygon. For a heptagon of bacteriorhodopsin ($n = 7$), the average angle is about 130°. Therefore, if hydrogen bonds are of primary importance in the attractive interactions between helices, the population of hydrogen-bonding residues should statistically exhibit two peaks which are separated by 130°, corresponding to the connections with two neighboring helices.

When the seven helical wheel projections were superposed with the direction of the amphiphilic dipole of helices being maintained constant, a population of hydrogen-bonding residues was obtained as depicted in fig. 10b which is plotted as a function of the angle from the bottom of the wheels in fig. 10a. This figure actually exhibits two peaks for the population. Furthermore, the separation of peaks is about 140°, in accordance with the value of 130° calculated from eq. 2. Therefore, the primary structure of bacteriorhodopsin also confirms the validity of the hydrogen-bonding mechanism for tertiary structure formation, which was revealed by the denaturation experiments.

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