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Dual Roles of DMPC and CHAPS in the Refolding of Bacterial Opsins *In Vitro*[1]

Yasuo Sugiyama[2] and Yasuo Mukohata

Department of Biology, School of Science, Nagoya University, Furo-cho, Chikusa-ku, Nagoya, Aichi 464-01

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The bacterial opsins can be refolded to regenerate the chromophore by transfer from SDS to DMPC/CHAPS/SDS mixed micelles in the presence of retinal. A sequential refolding model has been proposed for bacterioopsin [Booth *et al.* (1995) *Nature Struct. Biol.* 2, 139-143]. However, the roles of DMPC and CHAPS in the refolding process are not clear. In this study we measured the effects of DMPC and CHAPS on the refolding of bacterial opsins *in vitro* by CD and fluorescence spectroscopy. In contrast to in experiments in the presence of large amounts of DMPC, the process of retinal binding pocket formation was a rate-determining step in overall chromophore regeneration with relatively low concentrations of DMPC. CHAPS triggered α-helix formation and long-range interactions between the helices within 1 s by providing a suitable hydrophobic environment for bacterial opsins. This CHAPS-induced transient molten globule-like structure would be identical to I_1 postulated by Booth *et al.*, to which DMPC bound and induced the proper packing of the side chains to form a retinal binding pocket. If DMPC was not present, CHAPS induced another conformation change in bacterial opsins, which led to denaturation. DMPC dependence of chromophore regeneration and the maintenance of the retinal binding pocket suggested that retinal binding pocket formation was part of the large structure changes during stable apoprotein formation.

Key words: archaeopsin, bacterioopsin, long-range interaction, refolding process *in vitro*, retinal binding pocket.

Bacteriorhodopsin (bR) comprises a single polypeptide chain of 248 amino acid residues, which traverses the membrane 7 times as α-helical rods in a simple up-and-down fashion. These 7 helices are arranged so as to pack together and form an internal retinal binding pocket (*1*). BR can be refolded from a fully denatured state to regenerate a native chromophore in either mixed DMPC and bile salt or Triton X-100 micelles (*2, 3*). Since the pioneering studies of Khorana, the effects of pH, the CHAPS (cholate) concentration, and the DMPC/CHAPS ratio on chromophore regeneration have been extensively studied (*2-4*). The structure and function of this reconstituted bR are essentially the same as those of the purple membrane, although the intermolecular and intramolecular interactions are somewhat weakened in mixed micelles (*5*). Furthermore, two proteolytic fragments of bacterioopsin (bO) can be separately refolded in mixed micelles, where they reassociate to regenerate the chromophore (*2, 4, 6-8*). Thus bR has provided a suitable experimental system with which to study the refolding dynamics, the details and significance of the helix-helix interactions, and the structural stability of the integral membrane proteins (*9, 10*). Booth *et al.* detected transient intermediates during folding, on stopped-flow fluorescence spectroscopy, and proposed a sequential folding model for bR in mixed detergent and lipid micelles (*11*). In their scheme, the folded apoprotein, I_0, is formed with a rate constant of 0.067 s^{-1} in the absence of retinal in 1% DMPC, 1% CHAPS, and 0.1% SDS. I_0 is a conformation state of bO with a "vacant" retinal binding pocket to which retinal binds to regenerate the chromophore. They suggested that this kinetic phase reflects the *cis-trans* isomerization of proline residues or rearrangement of the secondary structure necessary to form a retinal binding pocket. At present this remains moot and, furthermore, the roles of phospholipids and detergents in the refolding process are not yet clear. Here, we measured the folding of bacterial opsins [bO and archaeopsin-1 (aO-1)] with various concentrations of DMPC and CHAPS, and found that the initial intermediate was formed upon transfer of the bacterial opsins from SDS to CHAPS/SDS, and that the rate of I_0 formation changed, depending on the concentration of DMPC. On the basis of these observations we discussed the roles of DMPC and CHAPS in the refolding process, in particular, the first half of the chromophore regeneration of bacterial opsins.

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[2] To whom correspondence should be addressed. Tel: +81-52-789-2971, Fax: +81-52-789-2968, E-mail: ysugiya@bio.nagoya-u.ac.jp

Abbreviations: ANS, anilinonaphthalene sulfonate; aO-1, archaeopsin-1; aR-1, archaerhodopsin-1; bO, bacterioopsin; bR, bacteriorhodopsin; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; CMC, critical micellar concentration; DMPC, dimyristoyl phosphatidylcholine.

EXPERIMENTAL PROCEDURES

Materials—BR and aR-1 were prepared as purple and claret membranes from *Halobacterium salinarium* R_1M_1 and *Halobacterium* sp. aus-1, respectively (*12, 13*). BO and aO-1 were prepared as described (*14*), and stored in 0.2% (w/v) SDS and 0.01% (w/v) NaN_3 at 4°C. DMPC was purchased from Sigma Chemicals. CHAPS was from Wako Pure Chemicals.

Chromophore Regeneration—BO or aO-1 was incubated with all-*trans* retinal in medium comprising DMPC, CHAPS, SDS, and 20 mM Na-acetate (pH 6.0) at 20°C in the dark. Absorption increases were measured with a Shimadzu UV300 spectrophotometer at 20°C.

Spectroscopy—CD was measured with a Jasco J-720 spectropolarimeter at 20°C. Far-UV and near-UV CD spectra were recorded at a scanning speed of 10 nm/min. Base line correction for the respective solvents and signal averaging of CD spectra were performed with an NEC 98 computer. Samples were analyzed in either 0.1 or 1 cm optical path cells. Molar ellipticity was expressed in units of degree $\times$ cm^2 $\times$ dmol^{-1} of amino acid residues. Stopped-flow CD measurements were performed with an Applied Photophysics SX.18MV at 20°C. Trp fluorescence was measured at 331 and 333 nm for bO and aO-1, respectively, with a Hitachi F4010 spectrofluorimeter, with excitation at 290 nm and 20°C, with a time constant of 0.5 s (5 nm slit for both excitation and emission).

RESULTS

Effects of the DMPC and CHAPS Concentrations on the Chromophore Regeneration of Bacterial Opsins—The chromophore regeneration of bacterial opsins has been historically performed in a medium containing 0.5% (w/v) DMPC, 0.5% (w/v) CHAPS, and 0.1% SDS at 20°C. Under these conditions, the bacterial opsins exist as mixed detergent and phospholipid micelles. We re-examined the effects of DMPC and CHAPS on the chromophore regeneration of bO and aO-1. With 0.2% CHAPS, the extent of chromophore regeneration of bO increased with an increase in the DMPC concentration and was saturated at 0.05% DMPC (Fig. 1A). The concentration of DMPC for half maximal chromophore regeneration was 0.008% in 0.2% CHAPS. With increasing concentrations of CHAPS, a plot of the extent of chromophore regeneration as a function of the DMPC concentration gave a sigmoidal curve. The maximal yields of chromophore regeneration of bO obtained in the presence of saturating amounts of DMPC were estimated to be 80, 90, and 95% with 0.2, 0.4, and 0.6% CHAPS, using a molar absorption coefficient of 52,000 $M^{-1} \cdot cm^{-1}$ at 555 nm (*15*). The chromophore regeneration of aO-1 showed similar profiles for DMPC and CHAPS dependence (Fig. 1B), although aO-1 had a lower affinity for DMPC than bO. The concentration of DMPC for the half maximal chromophore regeneration of aO-1 was 0.025% in 0.2% CHAPS.

It has been reported that the incubation of bO with 0.5% DMPC and 0.5% CHAPS before the addition of retinal does not affect the rate of chromophore regeneration (*3*). In contrast to this finding, bO chromophore regeneration occurred after a time lag when 0.05% DMPC, 0.2% CHAPS, and retinal were added to bO simultaneously (Fig. 2). On the other hand, it proceeded without a time lag when bO

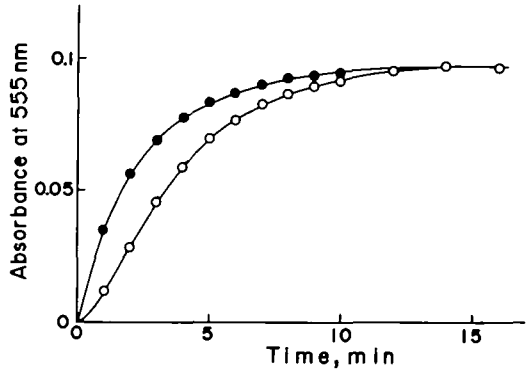


Fig. 2. **The effect of prior DMPC/CHAPS incubation on the kinetics of chromophore regeneration.** Bacterioopsin (70 μg) was incubated in 1 ml of 0.05% DMPC, 0.2% CHAPS, 0.08% SDS, and 20 mM Na-acetate (pH 6.0) for 10 min, and then 5 μl of 1 mM all-*trans* retinal was added to start chromophore regeneration at 20°C (●). As a control, the chromophore regeneration of bO in 0.08% SDS and 40 mM Na-acetate (pH 6.0) was started by adding an equal volume of 0.1% DMPC, 0.4% CHAPS, 0.08% SDS, and retinal (○). The time course of the chromophore regeneration was recorded as the increase in absorbance at 555 nm.

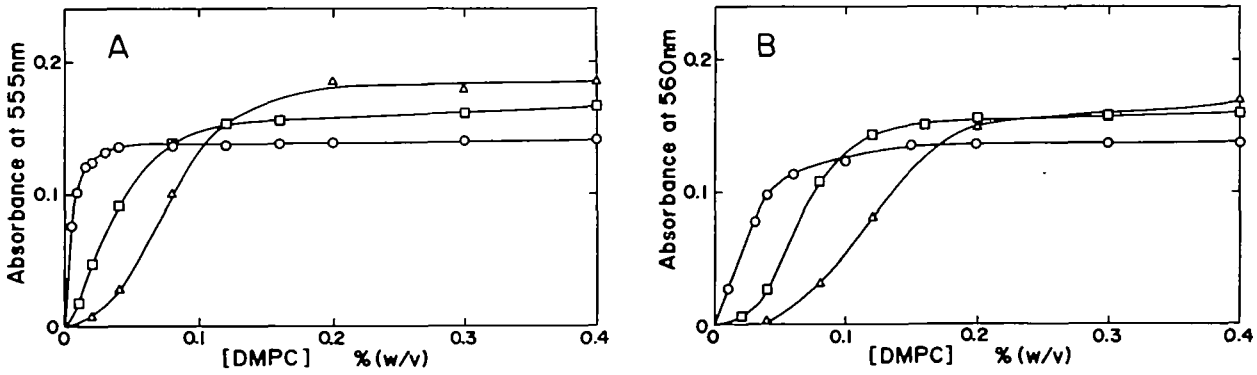


Fig. 1. **The effects of DMPC and CHAPS on the extents of bO (A) and aO-1 (B) chromophore regeneration.** One hundred micrograms of bO and aO-1 was incubated with all-*trans* retinal in 1 ml of 0.08% SDS, 20 mM Na-acetate (pH 6.0), various concentrations of DMPC, and 0.2% (○), 0.4% (□), or 0.6% (△) CHAPS, at 20°C in the dark. After 20 h, the increases in the absorbance at 555 and 560 nm were measured for bO and aO-1, respectively.

was incubated with DMPC and CHAPS for 10 min before the addition of retinal. Upon increasing the concentration of DMPC to 0.2%, the chromophore was regenerated without a time lag, irrespective of prior incubation. These results indicated that the rate-determining step was the formation of the retinal binding pocket, which was affected by the DMPC concentration.

Effect of Incubation with CHAPS on Chromophore Regeneration—The apoprotein, which was formed upon transfer from SDS to 0.5% DMPC and 0.5% CHAPS, was so stable that it could bind retinal to regenerate the chromophore even after 24 h (*4*). This apoprotein is believed to be the intermediate, I_0, during chromophore regeneration (*11*). However, it remains unknown whether or not bacterial opsins are stable in CHAPS/SDS. When bO and aO-1 were incubated with 0.2% CHAPS/0.1% SDS, the extent of chromophore regeneration decreased with time (Fig. 3). Complete abolition of the chromophore regeneration of bO required 40 h. In contrast, aO-1 in CHAPS/SDS rapidly lost the ability to regenerate the chromophore (Fig. 3B). When DMPC was added to the preincubation mixture, the loss of chromophore regeneration was depressed, depending on the concentration of DMPC. For complete depression, bO and aO-1 required 0.1 and 0.2% DMPC respectively.

Detection of Structural Changes during Formation of the Apoprotein by Far-UV and Near-UV CD Spectroscopy—Far-UV CD spectra indicated that the secondary structure of bO in SDS differed from that in mixed DMPC/CHAPS/SDS micelles (*2*). We measured the time course of the changes in the $-[\theta]_{222}$ values of bacterial opsins upon transfer from SDS to CHAPS/SDS or DMPC/SDS or DMPC/CHAPS/SDS.

Figure 4 shows representative traces of CD changes for bO and aO-1 at 20°C. $-[\theta]_{222}$ of bO was 15,200 in 0.08% SDS. Upon transfer to 0.2% CHAPS/0.08% SDS, it increased by 15% within 30 s, and then gradually decreased to a value of 13,200 after 24 h (Fig. 4A, trace a). Stopped-flow CD measurements indicated that the increase in $-[\theta]_{222}$ occurred within 1 s (not shown). The slow decrease in $-[\theta]_{222}$ occurred with a rate constant of 0.016 min^{-1}. The same experiments were repeated at various wavelengths (205-228 nm), and the $[\theta]_{222}$ values at 30 s were plotted (Fig. 5). Difference far-UV CD spectra before and after transfer from SDS to CHAPS/SDS indicated that a region(s) of the bO polypeptide was immediately refolded as an α-helix. After 24 h, the far-UV CD spectrum of bO showed one negative peak at 216 nm in 0.2% CHAPS/0.08% SDS (Fig. 5). $-[\theta]_{222}$ of aO-1 increased by 8% within 30 s upon transfer from SDS to CHAPS/SDS. However, the following CD decrease occurred much more rapidly than with bO (Fig. 4B, trace a). The rate constant of this process was 0.085 min^{-1}.

Upon transfer from SDS to 0.05% DMPC/0.08% SDS, $-[\theta]_{222}$ of bO increased with a rate constant of 0.11 min^{-1} and reached a value of 16,000 at 30 min (Fig. 4A, trace c). The increased CD signal did not change on 24 h incubation. Furthermore, the far-UV CD spectrum of bO in DMPC/SDS had the same shape as that in SDS. Similar observations were made for aO-1.

Upon transfer to 0.05% DMPC/0.2% CHAPS/0.08% SDS, $-[\theta]_{222}$ of bO increased within 30 s to the same level as that upon transfer to 0.2% CHAPS/0.08% SDS, and it

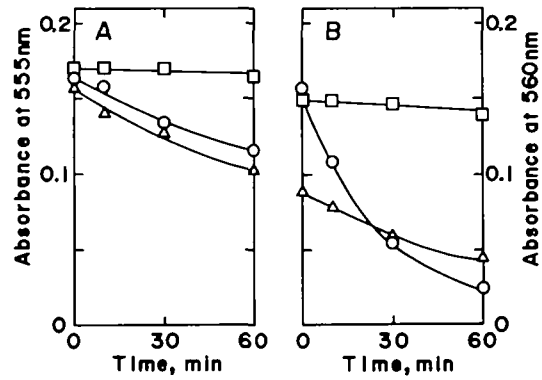


Fig. 3. **The effect of preincubation with CHAPS on the chromophore regeneration.** Bacterioopsin (A) and aO-1 (B) in 0.08% SDS and 20 mM Na-acetate (pH 6.0) were incubated with 0.2% CHAPS (○) for the indicated periods at 20°C. DMPC (final, 0.1%) and all-*trans* retinal were added to regenerate the chromophore. As a control, bO and aO-1 were incubated with 0.025% (△) or 0.1% DMPC (□) containing 0.2% CHAPS, 0.08% SDS, and 40 mM Na-acetate (pH 6.0) for the indicated periods, and then retinal was added to regenerate the chromophore. Protein concentration, 100 μg/ml.

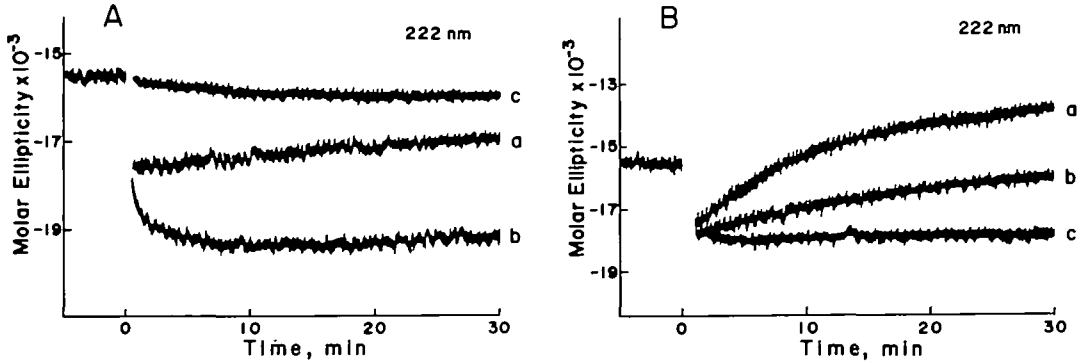


Fig. 4. **Far-UV CD changes of bO (A) and aO-1 (B) upon transfer from SDS to CHAPS/SDS or DMPC/CHAPS/SDS.** Bacterioopsin or aO-1 0.8 ml; 100 μg/ml) in 0.1% SDS and 25 mM Na-acetate (pH 6.0) was mixed with 0.2 ml of 1% CHAPS (trace a), 0.25% DMPC, and 1% CHAPS (trace b), or 0.25% DMPC and 0.05% SDS (trace c for bO), or 0.5% DMPC and 1% CHAPS (trace c for aO-1) at 20°C. After 30 s, the ellipticity at 222 nm was recorded for 30 min. The ellipticity in SDS was measured after mixing 0.8 ml of bO or aO-1 (100 μg/ml) in 0.1% SDS and 25 mM Na-acetate with 0.2 ml of distilled water in a cell of a 0.1 cm path length.

further increased and reached a constant value of 19,500 after 7 min (Fig. 4A, trace b). The addition of retinal did not affect the time course of the increase in $-[\theta]_{222}$ within the duration of the experiments (30 min). The fast increase in $-[\theta]_{222}$ of aO-1 occurred upon transfer to 0.05% DMPC/0.2% CHAPS/0.08% SDS, like that of bO. However, it was not followed by a further increase but rather by a slow decrease (Fig. 4B, trace b). Upon transfer to 0.1% DMPC/0.2% CHAPS/0.08% SDS, only the fast CD change was evident and the increased CD signal did not change further (Fig. 4B, trace c). In contrast to bO, a slow increase in the CD signal was not evident for aO-1, even upon transfer to 0.4% DMPC/0.2% CHAPS/0.08% SDS.

The tertiary structure changes during the formation of the apoprotein have remained obscure. $[\theta]_{290}$ of bO was 22.8 in 0.08% SDS, 0.05% DMPC/0.08% SDS, and 0.2% CHAPS/0.08% SDS, which changed to a value of -24.3 upon transfer from SDS to 0.05% DMPC/0.2% CHAPS/0.08% SDS (Fig. 6A). This process occurred with first order kinetics and a rate constant of 0.53 min^{-1}. This is the first observation of the changes in the tertiary structure during the formation of the apoprotein (the intermediate, I_0). Higher concentrations of DMPC accelerated this process and increased the amplitude of the CD changes. Similar experiments were carried out for aO-1 by monitoring the changes of $[\theta]_{280}$ (Fig. 6B). Upon transfer from SDS to 0.05% DMPC/0.2% CHAPS/0.08% SDS, $[\theta]_{280}$ of aO-1 immediately changed to the value in CHAPS/SDS and then gradually changed to -56.6. This process occurred with first order kinetics and a rate constant of 0.22 min^{-1}.

The near-UV CD spectra of bO and aO-1 in 0.08% SDS did not change upon transfer to 0.05% DMPC/0.08% SDS. Upon transfer from SDS to 0.2% CHAPS/0.08% SDS, the near-UV CD spectrum changed within the dead time for measurements (a few seconds), and did not change further after 24 h in both bO and aO-1. The near-UV CD spectra measured after 24 h incubation indicated that bO and aO-1 maintained the tertiary structure even in SDS, but almost

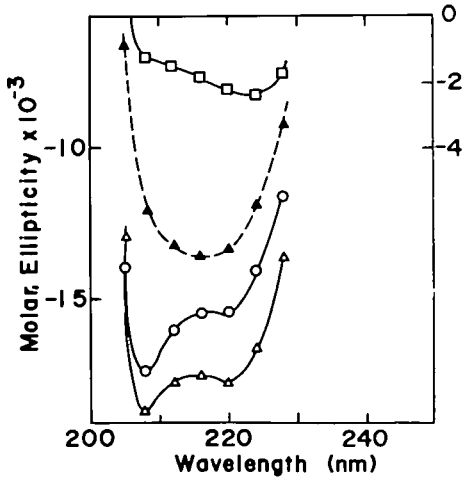


Fig. 5. **Far-UV CD spectral changes of bO upon transfer from SDS to CHAPS/SDS.** Bacterioopsin (0.8 ml; 100 μg/ml) in 0.1% SDS and 25 mM Na-acetate (pH 6.0) was mixed with 0.2 ml of 1% CHAPS, and after 30 s the ellipticities at the indicated wavelengths were monitored at 20°C. (○) Ellipticity in SDS. (△) Ellipticity at 30 s after transfer to CHAPS/SDS. (▲) Ellipticity after 24 h in CHAPS/SDS. (□) Difference in the ellipticity before and after transfer to CHAPS/SDS (scale indicated on the right ordinate).

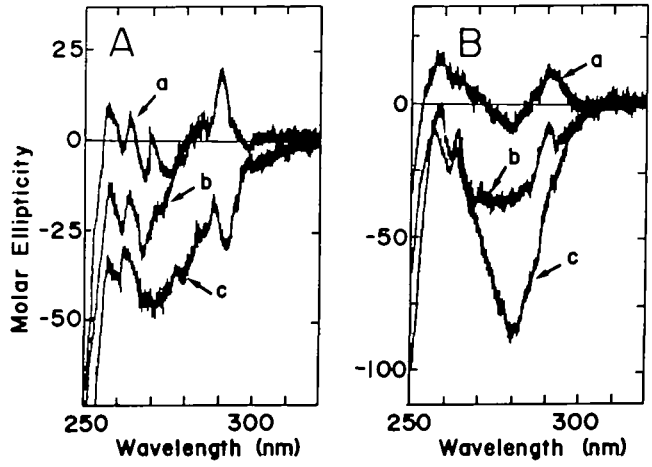
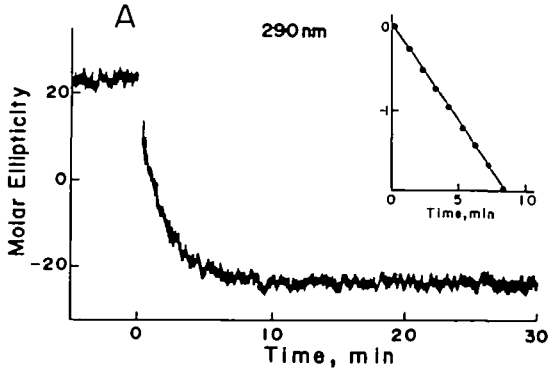


Fig. 7. **Near-UV CD spectra of bO (A) and aO-1 (B) in SDS, CHAPS/SDS, and DMPC/CHAPS/SDS.** CD spectra of bO or aO-1 were measured one day after incubation with 0.08% SDS (trace b), 0.2% CHAPS/0.08% SDS (trace a), or 0.2% DMPC/0.2% CHAPS/0.08% SDS (trace c). Each spectrum is the average of 6 scans.



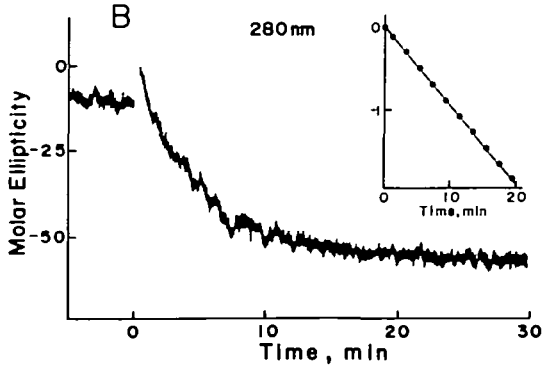


Fig. 6. **Near-UV CD changes of bO (A) and aO-1 (B) upon transfer from SDS to DMPC/CHAPS/SDS.** Bacterioopsin or aO-1 (1.6 ml; 350 μg/ml) in 0.1% SDS and 25 mM Na-acetate (pH 6.0) was mixed with 0.4 ml of distilled water (trace before time zero) or 0.25% DMPC and 1% CHAPS in a 1 cm path length cell, and then the ellipticity was monitored at 290 nm for bO or 280 nm for aO-1 for 30 min at 20°C. (Inset) Kinetic analysis of the CD changes. Log($1-\Delta CD/\Delta CD_{max}$) was plotted against time.

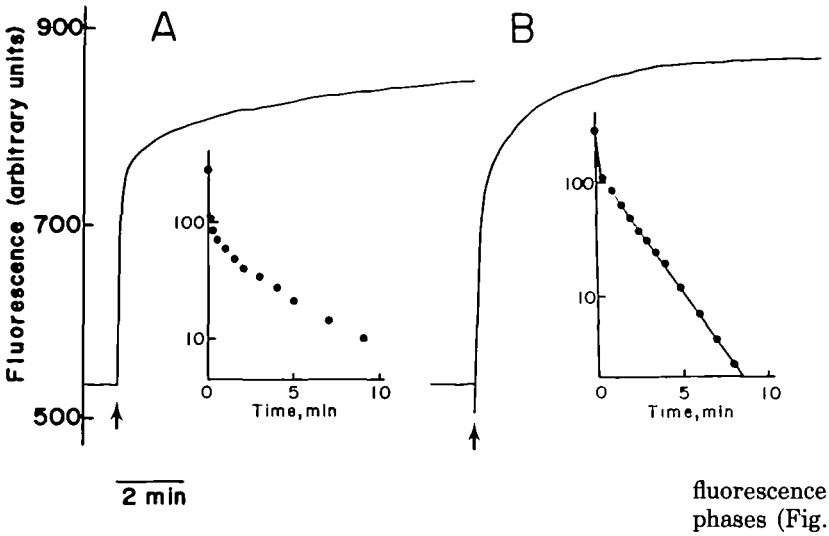


Fig. 8. **Time courses of Trp fluorescence changes of bO after transfer from SDS to CHAPS/SDS (A), and DMPC/CHAPS/SDS (B).** To 2 ml of bO (200 μg) in 0.1% SDS and 25 mM Na-acetate (pH 6.0), 0.5 ml of 1% CHAPS (A), or 0.25% DMPC and 1% CHAPS (B) was added under continuous stirring at 20°C at the time indicated by the arrow. The excitation and emission wavelengths were 290 and 331 nm, respectively. (Inset) Kinetic analysis of the fluorescence changes. Log_{10} ($\Delta F_{max} - \Delta F$) was plotted against time.

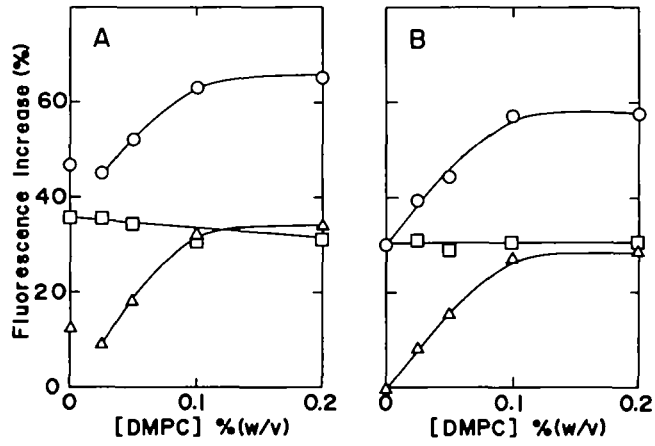


Fig. 9. **The effects of the DMPC concentration on the extents of the fast and slow phases of Trp fluorescence changes upon transfer from SDS to CHAPS and DMPC.** Trp fluorescence of bO (A) and aO-1 (B) was monitored after adding 0.2% CHAPS containing various amounts of DMPC (final %) as in Fig. 8. The extents of the fast (□) and slow (△) phases were estimated graphically as in Fig. 8 inset, and plotted against the concentration of DMPC. The total intensity changes (○) are the sums of the fast and slow phases.

totally lost it in CHAPS/SDS (Fig. 7). It is notable that the near-UV CD spectrum of bO in DMPC/CHAPS/SDS differs from that of aO-1, although all aromatic amino acid residues of the retinal binding pocket (*1*) are conserved in bO and aO-1 (*16*).

Trp Fluorescence Changes during Formation of the Apoprotein—CD measurements indicated that the secondary and tertiary structures of bO changed upon transfer from SDS to CHAPS/SDS and DMPC/CHAPS/SDS. We then measured the kinetics of intrinsic Trp fluorescence changes during the refolding of bO and aO-1. Upon transfer to 0.2% CHAPS/0.08% SDS, the Trp fluorescence of bO increased by 35% within a few seconds, followed by a slow increase (Fig. 8A). The fast phase may occur within the dead time of measurements (a few seconds), and kinetic analysis indicated that the slow phase proceeded through at least two kinetic processes (Fig. 8A, inset). Upon transfer from SDS to 0.05% DMPC/0.2% CHAPS/0.08% SDS, fluorescence intensity changes occurred with fast and slow phases (Fig. 8B). In contrast to in the case of transfer to CHAPS/SDS, the slow phase proceeded with first order kinetics and a rate constant of 0.52 min^{-1} (Fig. 8B, inset). Similar experiments were performed for bO and aO-1 by varying the concentration of DMPC in 0.2% CHAPS, and the extents of the fast and slow phases of fluorescence increase were plotted as a function of the concentration of DMPC (Fig. 9). For aO-1, only the fast phase was evident upon transfer from SDS to CHAPS/SDS. DMPC did not affect the extent of the fast phase in bO or aO-1. The extent of the slow phase increased with increasing DMPC concentration in 0.2% CHAPS, and was saturated at 0.1% DMPC for both bO and aO-1. These profiles did not coincide with the DMPC dependence of the extent of the chromophore regeneration for bO and aO-1 (Fig. 1), but with that of maintenance of the apoprotein (Fig. 3). It should be noted that the fluorescence of the bacterial opsins increased by 4% upon transfer from SDS to 0.2% DMPC/0.1% SDS.

DISCUSSION

DMPC and CHAPS Exerted Different Effects on the Chromophore Regeneration of Bacterial Opsins—In contrast to in the case of soluble globular proteins, there are many experimental restrictions to the study of the folding of membrane proteins *in vitro*. Firstly, these proteins are not soluble in aqueous media, therefore, it is necessary to use detergent(s) to avoid aggregation in all procedures. Secondly, denaturants such as urea, guanidine-HCl, and some anionic detergents and organic solvents cannot be used as solubilizers because they usually cause irreversible denaturation. Thirdly, it is difficult to prepare large amounts of membrane proteins. BR has been extensively studied. Its denaturation is reversible and large scale preparation is feasible, although many components such as detergents, lipid, and retinal are necessary to regenerate the chromophore from the SDS-denatured apoprotein (the D-state), which complicates the system. However, the stable native-like apoprotein (the N-state) accumulates in DMPC/CHAPS/SDS, and it is equivalent to the intermediate, L_0, of the sequential folding scheme for bR proposed by Booth *et al.* (*11*). This allows retinal to be omitted from the refolding medium, and the kinetics of the transition from the D- to the N-state can be investigated. However, since their experiments were carried out with high concentra-

tions of DMPC and CHAPS, namely in mixed micelles, the interactions of DMPC and CHAPS with the bO polypeptide and their roles in the refolding of bO could not be revealed. This was partly due to a lack of a procedure for analyzing the CHAPS or DMPC concentration dependence of the refolding process in micelles. In this study, we found that the rate and the extent of chromophore regeneration increased with increasing DMPC concentration with low concentrations of CHAPS (below the CMC) (Fig. 1). Therefore, the folding process can be described as the sequential binding of CHAPS and DMPC to a bacterial opsin polypeptide. Specific binding sites for DMPC might be present on bacterial opsins in CHAPS/SDS, although the properties and the number of binding sites for DMPC have not been determined. The difference in the affinity for DMPC between bO and aO-1 may be related to the properties of the hydrophobic faces that interact with DMPC and/or the free energy change in this process. That the affinity for DMPC decreased with a high concentration of CHAPS (above the CMC) would be due to the dilution of the effective DMPC concentration in CHAPS micelles.

We found that CHAPS affected the refolding of bacterial opsins. As shown in Fig. 3, CHAPS inactivated or denatured bacterial opsins if DMPC was not present, although CHAPS is an essential component for refolding and chromophore regeneration. CHAPS thus has two effects on bacterial opsins. One leads to folding and the other leads to denaturation. Our results also show that the chromophore regeneration and maintenance of the apoprotein are dependent on two distinct concentrations of DMPC (Figs. 1, 3, and 9). Relatively low and high concentrations of DMPC were required for the former and the latter, respectively.

Structural Changes during the Transition from the D- to the N-State—Far-UV and near-UV CD spectra show that the polypeptide of a bacterial opsin is denatured but not "fully" unfolded in SDS. We thus designated this state of the bacterial opsins in SDS as the D-state. That the rate of the transition from the D- to the N-state decreased with low concentrations of DMPC and CHAPS allowed the structural changes during the refolding of bacterial opsins to be followed using standard spectroscopic instruments. In this study we analyzed, in particular, the first half of chromophore regeneration; namely, the process of N-state formation. Kinetic CD measurements indicated that a part of the random coil in the D-state immediately refolded upon transfer to CHAPS/SDS or DMPC/CHAPS/SDS, but not to DMPC/SDS (Fig. 4). Thus, CHAPS triggers the refolding process and this CHAPS-induced conformation state (the D_2-state) is transient prior to the formation of the N-state. The D_2-state would be the same as the partially folded intermediate, I_1, postulated by Booth *et al.* (*11*). The CHAPS-induced increase in fluorescence intensity (quantum yield) suggested that Trp residues moved to a more apolar region during the transition from the D- to the D_2-state, where the motions of the side chain were somewhat restricted (Fig. 8). To address the role of CHAPS in this process, we compared the hydrophobic property of SDS with that of CHAPS by measuring the fluorescence of ANS, a hydrophobic probe (*17*). The fluorescence of ANS in an SDS solution was similar to that in water, and it dramatically increased in CHAPS/SDS together with a blue shift of the fluorescence maximum (not shown). The presence of a bacterial opsin did not affect the intensity or spectrum of ANS fluorescence. These observations suggested that CHAPS provides bacterial opsins with a hydrophobic environment mimicking a biomembrane due to its unique amphiphilic properties (*18*). Our preliminary studies indicated that the distances between Trp residue(s) and the fluorophore-labeled carboxyl residue located in the helical region decrease during the transition from the D-state to the D_2-state (Y. Sugiyama *et al.*, manuscript in preparation). Therefore, the hydrophobic interaction with CHAPS would induce a long-range helix-helix interaction. It has not yet been determined whether the CHAPS-induced α-helix formation, the helix-helix interactions and the movement of Trp residues to an apolar region proceed simultaneously or sequentially. Millisecond time-resolved CD and fluorescence measurements will clarify these issues.

We found that the secondary structure of the D_2-state slowly changed from an α-helix to a β-like structure (the D_3-state) in the absence of DMPC (Figs. 4 and 5). This slow process is another pathway from the D_2 state and would be related to the decrease in the chromophore regeneration of bacterial opsins incubated in CHAPS/SDS (Fig. 3). The rate constant of the process from the D_2-state to the D_3-state for aO-1 (0.085 min^{-1}) was larger than that for bO (0.016 min^{-1}). This is consistent with the finding that aO-1 in CHAPS/SDS lost the ability to regenerate the chromophore faster than bO (Fig. 3). However, the rate of this structural change was slightly faster than that of the decrease in chromophore regeneration. One explanation for this discrepancy is that the process from the D_2-state to the D_3-state is reversible. The D_2- and D_3-states of bO and aO-1 almost totally lack the tertiary structure (Fig. 7). The far-UV and near-UV CD spectra of bO and aO-1 in 8 M urea were the same as those of their D_3-states. Further experiments will be necessary to characterize the structures of the D_2- and D_3-states by other techniques, such as NMR and vibrational spectroscopy.

Why the secondary structure changed on the transition from the D_2- to the N-state for bO but not for aO-1 remains unclear (Fig. 4). However, Fig. 6 shows that the tertiary structure changed from the D_2- to the N-state through a two-state transition for both bO and aO-1. The same rate constants were obtained on near-UV CD and fluorescence measurements. Therefore, there is no intermediate between the D_2- and N-states. Since the rate of this transition changed depending on the DMPC concentration, this comprises the process of binding of DMPC to bacterial opsins and/or the replacement of DMPC with CHAPS bound to bacterial opsins. In contrast to the transition from the D- to the D_2-state, the polarity around Trp residues did not significantly change on the transition from the D_2- to the N-state, particularly when small but sufficient amounts of DMPC were added to form the retinal binding pocket. Rather, Trp residues moved to another asymmetrical region created on the packing of the side chains in this transition (Fig. 6).

The near-UV CD spectra suggested that the microenvironments and the packing of the side chains of the aromatic amino acid residues in the N-state differed between bO and aO-1 (Fig. 7). This would be supported by the observation that the chromophore regeneration of aO-1 occurred *via* a metastable intermediate (435/460 nm), but that of bO did not at 20°C *in vitro* (*14*). In contrast to the

chromophore regeneration (retinal binding pocket formation), relatively large amounts of DMPC were required to stabilize the preformed retinal binding pocket in which large Trp fluorescence intensity changes were observed (Figs. 3 and 9). These findings suggested that retinal binding pocket formation is part of the large structural changes that occur during N-state formation.

Finally, we illustrate the first half of the refolding process for bacterial opsins based on the present results. A tentative scheme for the folding process is as follows:

$$\begin{array}{l} D \rightarrow D_2 \rightarrow N \\ \quad\downarrow \\ \quad D_3 \end{array}$$

Where D, D_2, and N are the conformational states of bacterial opsins in SDS, CHAPS/SDS, and DMPC/CHAPS/SDS, respectively. D_3 is another state in CHAPS/SDS. The transition from D to D_2 comprises α-helical structure formation that occurs within 1 s. CHAPS provides the hydrophobic environment that triggers the long-range interactions between the seven helices. However, D_2 did not exhibit the same structural ellipticity in the aromatic region as the native form, and thus it may be a so-called transient molten globule of globular proteins (*19*). In the absence of DMPC, D_2 gradually transformed into D_3 (probably a β-structure). The transition from D_2 to N comprises a process of apoprotein formation and the rate-determining step of the overall reaction under our experimental conditions. The rate of this transition increased with increasing DMPC concentration. When small amounts of DMPC bind to D_2, the side chains pack to form the retinal binding pocket. Relatively large amounts of DMPC are required to maintain the retinal binding pocket in the absence of retinal.

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