Chymotrypsin cleaves bacteriorhopdopsin to two fragments: C-1, amino acids 72–248, and C-2, amino acids 1–71. Denaturation and renaturation of these fragments have been studied. Following denaturation in sodium dodecyl sulfate, both C-1 and C-2 regain secondary structure in phospholipid/cholate/sodium dodecyl sulfate mixed micelles. When combined, they form a complex with a secondary structure resembling that of bacterio-opsin. However, on further incubation in phospholipid/cholate/sodium dodecyl sulfate, separate fragments as well as the C-1 and C-2 complex denature again. Retinal binds tightly to the C-1 and C-2 complex ($K_d > 10^7 \text{ M}^{-1}$) and stabilizes the folded conformation. The formation of the complex of C-1, C-2, and retinal is maximal at pH 6.0. The ternary complex contains two species: one which absorbs similarly to the light-adapted purple membrane and a second with a $\lambda_{max}$ between 450 and 500 nm. The formation of the latter species is favored at higher temperatures and is reversible. Vesicles formed from the ternary complex of C-1, C-2, and retinal translocate protons at a level close to that of intact bacteriorhodopsin.

BR, the single protein present in the purple membrane of Halobacterium halobium, catalyzes light-driven vectorial proton translocation (1–3). BR consists of one polypeptide chain of 248 amino acids whose sequence is known both by amino acid and gene sequencing (4–6). A single retinal molecule linked as a Schiff’s base to the ε-amino group of Lys 216 serves as the chromophore (7–9). The polypeptide chain is believed to traverse the membrane seven times as α-helical segments which are largely embedded in the membrane (10).

We have previously shown that denatured BR refolds to a completely native structure in lipid/detergent mixtures and in some detergents alone (11–12). Upon reconstitution into phospholipid vesicles, the renatured BR gives full proton-translocating activity (12–15). Furthermore, the two fragments, C-1 (amino acid residues 72–248) and C-2 (amino acid residues 1–71), formed by chymotryptic cleavage of BR, also recombine to generate the native BR chromophore and to form proton-translocating vesicles (12, 16, 17). The ability to form native BR-like complexes from the fragments provides opportunities for studies of many aspects of the structure and function of BR (16, 17). Therefore, we have carried out a further study of the properties of the chymotryptic fragments and their combination. We find that C-1 and C-2 denatured in SDS renature upon the addition of a DMPC/cholate mixture. The renatured fragments form a complex whose secondary structure resembles that of BR. Retinal binds tightly to this complex, thereby stabilizing the folded conformation. The formation of the ternary complex occurs maximally at pH 6.0. The spectral properties of the complex are very similar to those of the intact BR. These studies show for the first time that denatured fragments of a membrane protein can fold independently into a conformation very close to that of the native state and then reassociate to form a functional protein.

**EXPERIMENTAL PROCEDURES**

**Materials**

Purple membrane was isolated as previously described (18) from H. halobium (strain S9, originally obtained from W. Stoeckenius, University of California, San Francisco). Cholic acid was obtained from Sigma and was recrystallized from aqueous acetone. SDS (electrophoresis grade) was obtained from Bio-Rad and CHAPS (3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate) was obtained from Calbiochem–Behring. Soybean phospholipids were obtained from Associated Concentrates, Woodside, NY, and treated as described by Kagawa and Racker (19).

**Buffers**

For Buffer A (DMPC/cholate), DMPC (80 mg) was added to 1.8 ml of 0.1 M NaPi (pH 6.8) containing 0.025% (w/v) NaN3, and the mixture was vortexed at 25°C until the phospholipid formed a homogeneous dispersion. To the milky suspension was added 0.2 ml of 10% (w/v) sodium cholate (pH 8.0), and the mixture was sonicated briefly at 20°C. The resulting clear solution which contained 4% (w/v) DMPC and 1% (w/v) cholate in 0.1 M NaPi (final pH 7.0) was stored at 4°C. This solution becomes cloudy after prolonged storage at 4°C but clarifies rapidly at room temperature. DMPC/CHAPS and soybean phospholipid/CHAPS mixtures in phosphate buffer were prepared by the same procedure. Buffer B, consisted of 0.2% (w/v) SDS, 10 mM NaPi (pH 7.0), and 0.025% (w/v) NaN3. Buffer C consisted of 50 mM NaPi (pH 6.0) and 0.025% (w/v) NaN3.

**Methods**

Preparation of Delipidated Bacterio-opsin and Chymotryptic Fragments C-1 and C-2—Delipidated bacterio-opsin and fragments C-1...
and C-2 were prepared in SDS as described (12, 16) except that the final protein solution contained 0.2% (w/v) SDS, SDS-gel electrophoresis of each purified fragment, performed by the method of Swank and Munkres (20), showed no contamination by either the other fragment or intact protein, and that the COOH terminus of C-1 was intact to >90%.

Regeneration of BR Chromophore—The following procedure is typical. To solutions of delipidated bacterio-opsin (0.2 ml, 5 nmol) or a mixture of fragments C-1 (0.1 ml, 3 nmol) and C-2 (0.1 ml, 3 nmol) in Buffer B was added 1 µl of a 4.5 mm solution of all-trans-retinal in ethanol (retinal concentration was determined spectroscopically using η°C = 43,000 in ethanol (21)). The resulting solution was then mixed with 0.2 ml of Buffer A and incubated in the dark overnight at 23 °C.

Reconstitution of C-1 and C-2 into Phospholipid Vesicles and Proton Pumping Assays—C-1 fragment (75 µl, 6 nmol) was mixed with C-2 (75 µl, 6 nmol) in Buffer B and all-trans-retinal (2 µl, 4.5 mm in ethanol) was added. To this mixture was added 50 µl of 8% (w/v) soybean phospholipid, 2% CHAPS in Buffer C containing 0.15 M KCl and 0.025% NaN₃ (2 x 1 liter) for 2 days and then dialyzed against 0.15 M NaCl and 0.025% NaN₃ (2 x 1 liter) for 2 days. Proton pumping activity was assayed as described previously (13, 22).

Protein Determination—Protein concentration was determined from absorbance at 280 nm in SDS (11). Values of η°C were calculated from the literature values for extinction coefficients of tryptophan and tyrosine (23) and the tryptophan and tyrosine content of each fragment. Since we find that chymotryptic digestion of bacterio-opsin into a mixture of C-1 and C-2 does not affect absorbance at 280 nm in SDS, the calculated values were then normalized to a total η°C of 66,000 cm⁻¹m⁻¹, the extinction coefficient of bacterio-opsin in SDS (11). The resulting values are within about 5% of that found for the renatured intact BR (11).

Absorbance Measurements—Absorbance measurements were made in 1-cm light path quartz cells using a Cary 15 spectrophotometer. The reference cell, protein, and retinal were omitted. For studies at variable temperature, constant temperature semimicrocuvettes were used for both the sample and reference cells. The temperature was controlled to ±1 °C. Each spectrum was obtained after the sample solution was equilibrated at the desired temperature for at least 10 min.

Circular Dichroism Measurements—CD spectra of samples containing 0.2-0.32 mg of protein ml⁻¹ and buffer blanks in 1-mm light path quartz cells were measured at 29 ± 2 °C in a Cary 60 instrument with a 6002 CD attachment. Instrument calibration has been described previously (12). The ellipticity (θ) due to lipids, detergent, and buffer has been subtracted in the spectra shown. The samples prepared for CD measurement contained no NaNO₃. θ is reported in units of degrees cm²d mole⁻¹ of amino acid residues.

Fluorescence Measurements—Fluorescence intensities were measured in 4-mm light path quartz cells using a Perkin-Elmer MPF-44 spectrophotometer. The excitation wavelength was 285 nm (alit band width 5 nm) and the emission wavelength was 335 nm (alit band width 5 nm).

RESULTS

Secondary Structure of the Chymotryptic Fragments in DMPC/Cholate/SDS

It has been shown previously that bacterio-opsin denatured in SDS regains its native secondary structure in DMPC/cholate/SDS (11). Solutions of the fragments C-1 and C-2 in SDS were similarly treated and the CD spectra were measured as a function of time. As shown in Fig. 1A, the ellipticity of C-1 increases dramatically upon addition of DMPC/cholate to the SDS solution of the fragment. The initial ellipticity of C-1 upon addition of DMPC/cholate as determined by extrapolation to time 0 (θC,1 = 18,000) is close to that of the intact protein (θC,1 = 20,000). The decrease in ellipticity observed on further incubation is discussed below. Fig. 1B shows that C-2 in DMPC/cholate/SDS also possesses a molar ellipticity (θC,2 = 20,000) similar to those of C-1 and intact protein, although some differences in spectral shape are evident.

Significantly, when the initial ellipticity of a 1:1 (mol/mol) mixture of C-1 and C-2 is calculated, the spectrum obtained is within about 5% of that found for the renatured intact BR (11). This suggests that the initial secondary structure of the reenatured fragments is nearly the same as their secondary structure in BR. Also, Fig. 1A and B shows that the secondary structure of C-1 and C-2 in SDS is very close to that of intact delipidated bacterio-opsin in SDS (11).

Fragments C-1 and C-2 Individually Do Not Bind Retinal

Solutions of C-1 and C-2 were prepared in DMPC/cholate/SDS as described above and retinal was added immediately to each solution. No change in absorbance maximum from that of free retinal (λmax = 380 nm) was observed in either

![Fig. 1](http://www.jbc.org/)
case within 30 min. This suggests that although the fragments undergo renaturation of secondary structure under these conditions, they do not bind retinal. However, it is possible that retinal binding occurs to one of the fragments, but that no shift in the absorbance spectrum occurs unless the other fragment is also present. Therefore, retinal binding was examined further by fluorescence quenching. Our previous studies have shown that efficient quenching (>80%) of tryptophan fluorescence of renatured BR results from energy transfer to bound retinal (11). Schiff's base formation is not necessary for this quenching because retinal also binds to renatured bacterio-opsin and efficiently quenches bacterio-opsin fluorescence. 3 Fig. 2 shows that no significant quenching of protein fluorescence is observed upon addition of retinal to either renatured C-1 or C-2. Therefore, neither fragment binds retinal. In contrast, addition of retinal to a mixture of renatured C-1 and C-2 results in strong quenching due to retinal binding which develops at about the same rate as the increase of absorbance at 550 nm due to chromophore regeneration (see below).

**Binding of Retinal to Fragments C-1 and C-2 and Regeneration of BR-like Chromophore**

Fig. 3 shows the results of experiments in which retinal binding by a mixture of C-1 and C-2 and by bacterio-opsin in DMPC/cholate/SDS was compared. Two significant aspects were noted. First, the formation of the C-1-C-2-retinal ternary complex led to the regeneration of the BR-like chromophore, (17) and the extent of chromophore regeneration was close to 90% of that with bacterio-opsin. This shows that the fragments can fold to BR-like native conformation. Second, the titration of the C-1-C-2 complex and of bacterio-opsin with retinal showed very tight binding of retinal (Kb > 107 M⁻¹). The equivalence point of the titration indicated that more than 85% of both C-1 and C-2 molecules can bind retinal. Therefore, the 1:1 C-1-C-2 complex (17) binds 1 eq of retinal. Furthermore, the extinction coefficient of the regenerated chromophore can be calculated by dividing the maximal absorbance at 550 nm by the concentration of retinal at the equivalence point. The ε550 thus determined is 47,000 for the complex formed from C-1 and C-2 and 48,000 for intact BR. The latter value is 10% lower than that previously reported for intact BR (11). This is probably due to the higher pH and dark adaptation used in the present work.

**Time-dependent Denaturation of Chymotryptic Fragments in DMPC/Cholate/SDS**

The time-dependent denaturation of individual chymotryptic fragments and their mixture was studied.

**CD Measurements**—The measurements described above (Fig. 1) show a time-dependent loss of secondary structure in C-1 and to a much smaller extent in C-2. In the case of C-1, a loss of 25% of molar ellipticity at 224 nm is observed during the first half-hour and in 3 days, ellipticity drops to the value of 11,500. Assuming that [θ]224 = 33,000 for 100% helical structure (24), the above decrease in [θ] corresponds to a decrease of α-helical content from 55 to 35% in 3 days. 4 Thus, most of the gain in α-helical content observed upon addition of DMPC/cholate to C-1 in SDS is lost on prolonged incubation. However, the secondary structure of C-2 is less sensitive to incubation in DMPC/cholate/SDS. The initial α-helical content of about 61% remains essentially the same for at least 2 days.

**Denaturation As Studied by Chromophore Regeneration**—Denaturation of the fragments was further studied as shown in Fig. 4. In these experiments, C-1 (Fig. 4A) and C-2 (Fig. 4B) were preincubated in DMPC/cholate/SDS for different times, after which the complementary fragment and retinal were added and chromophore regeneration was monitored. The extent of chromophore regeneration decreased as the preincubation time was increased. It follows that C-1 and C-2 undergo changes in conformations such that they no longer combine with each other and retinal. Without preincubation, the kinetics of chromophore formation can be described by a predominant (78% of protein) fast phase (t½ = 2.5 min) and a much slower phase(s). After prolonged preincubation of either fragment, the proportion of the slow component of

---


3 The actual α-helical content of renatured C-1 could be higher when the small amount of unrenatured fragments is taken into account. In addition, the assumed value for 100% α-helix may not be correct for BR, so the calculated α-helical content should be used for comparison only.
Regeneration of Bacteriorhodopsin from Fragments

The regeneration of bacteriorhodopsin from fragments is studied. Time-dependent denaturation of C-1 and C-2 in DMPC/cholate as measured by chromophore regeneration upon addition of complementary fragment and retinal is observed. Maximum regeneration requires up to 1 week.

The data on the extent of chromophore regeneration at 30 min after addition of retinal and the complementary fragment are plotted as a function of preincubation time in Fig. 5. It is seen that the denaturation of C-1 is relatively rapid. In contrast, the denaturation of C-2 is slower, composed of a fast first order phase (30% of protein) with $t_{1/2} = 30$ min and a slower first order phase with $t_{1/2}$ of roughly 15 h. These experiments were repeated for the mixture of C-1 and C-2 preincubated in the absence of retinal. Again, denaturation of the fragments occurred during preincubation, but the denaturation was slower than would be expected if C-1 and C-2 were to denature independently. If denaturation of each fragment had been independent, then at most, chromophore regeneration would have been that observed for C-1 in Fig. 5. This result confirms that C-1 and C-2 interact to form a complex in the absence of retinal. The complex is more stable to denaturation than the individual fragments. For comparison, the effect of preincubation of bacterio-opsin, in the absence of retinal, was determined. As shown in Fig. 5, 90% of the renatured bacterio-opsin molecules were still active in chromophore regeneration after 1 day of preincubation, showing that the intact protein is much more stable than the complex of the fragments.

The absorbance spectrum of the chromophore regenerated from C-1, C-2, and all-trans-retinal in the DMPC/cholate/SDS mixture was found to vary as a function of temperature. Fig. 6A shows the temperature dependence of visible absorbance spectra for renatured C-1 and C-2. An increase in temperature from 3 to 35 °C caused an absorbance decrease of 30% at 550 nm. This was accompanied by a blue shift of $\lambda_{max}$ from 555 to 530 nm. The isosbestic point observed at 505 nm was quite different from the one at 445 nm which is...
observed when the chromophore is regenerated from bacterio-
opsin and free retinal (11). Since the amount of free retinal
remains constant as monitored by absorbance at 380 nm, the
isosbestic point at 505 nm suggests that a species with \( \lambda_{\text{max}} \)
in the region of 450-500 nm increasingly contributes to absorb-
ance at higher temperatures. These absorbance changes were
reversible up to 35 °C. Above 35 °C, slow irreversible bleaching
occurred. Interestingly, chromophore regenerated from bac-
terio-opsin and retinal also exhibits this phenomenon with the
same isosbestic point at 505 nm (Fig. 6B), but the change
in absorbance and \( \lambda_{\text{max}} \) are smaller within the temperature
range studied.

Optimal Conditions for Chromophore Regeneration from C-1
and C-2 and from Bacterio-opsin

Effect of Detergent Concentration—We have examined the
conditions for optimal regeneration of chromophore from
bacterio-opsin and from C-1 and C-2 mixtures in the DMPC/
cholate/SDS mixture. The effect of cholate concentration is
shown in Fig. 7A. At low cholate concentration (0.4% (w/v)
cholate), the extent of regeneration of chromophore from the
C-1 and C-2 mixture is almost the same as that from intact
BR. As cholate concentration is increased, the extent of
chromophore regeneration decreases in both cases, but the
regeneration of chromophore from the C-1 and C-2 mixture
is much more sensitive to cholate than the intact protein.
Below 0.4% (w/v) cholate, the chromophore could not be
measured due to an abrupt increase in turbidity of the DMPC/
cholate/SDS mixtures, presumably due to a change in micellar
structure (see “Discussion”).

We also examined the effect of cholate upon the stability of
the chromophore regenerated from C-1, C-2, and retinal. The
chromophore was regenerated at 0.5% (w/v) cholate
concentration and additional amounts of cholate were then
added. As seen in Fig. 7A, the amount of chromophore that
remained in 1 day was well above that obtained when the
reconstitution of the fragments was carried out at the corre-
sponding cholate concentrations. Nevertheless, after 1 week,
the preformed chromophore was unstable and reached the
same level as that when high cholate concentrations were
present during renaturation.

The other detergent present in our samples was SDS. Fig
7B shows that maximal extent of chromophore regeneration
was obtained at 0.1% (w/v) SDS. With an increase in SDS
ccentration, the extent of chromophore regeneration de-
creased both for intact BR and for the C-1 and C-2 mixture.
Again, renaturation of the fragments was more sensitive to
the SDS concentration than for intact protein.

Effect of pH—The effect of pH upon the regeneration of
the purple chromophore from bacterio-opsin and from the
C-1-C-2 complex was investigated in DMPC/CHAPS/SDS,
DMPC/cholate/SDS, and in Triton X-100/SDS mixtures (Fig. 8).
We used CHAPS, a recently introduced zwitterionic
derivative of cholate (25), to extend the results for cholate
over the entire pH range. In the comparable pH range,
identical renaturation was observed for DMPC/cholate/SDS
and DMPC/CHAPS/SDS. Fig. 8 shows that the maximal
chromophore regeneration in DMPC/CHAPS/SDS and Tri-
ton X-100/SDS was observed at about pH 6.0 both for C-1. C-2
and for bacterio-opsin. At pH 6.0, virtually 100% renat-
uration was observed in DMPC/CHAPS/SDS, although it
should be noted that 10% excess C-1 was used in the C-1-C-2
samples. In contrast, in Triton X-100/SDS, the maximal
extent of regeneration was much less than in DMPC/
CHAPS/SDS both for the intact protein (70%) and, espe-
cially, for the fragments (12%). In addition, Fig. 8 shows
chromophore regeneration occurs over a wider pH range in
DMPC/CHAPS/SDS than in Triton X-100/SDS.

Reconstitution of Proton-translocating Vesicles

Reconstitution of the C-1-C-2 fragment complex in soybean
phospholipid vesicles as described previously (17) gave low

\[^{4}\text{It is not possible to eliminate SDS entirely because SDS is needed initially to solubilize the denatured protein (12).}\]

\[^{5}\text{The DMPC/cholate mixture becomes turbid below pH 6.7 at 23 °C presumably due to the precipitation of cholic acid.}\]
pumping activity (initial rate, 0.32 H⁺/(C-1 + C-2)/s and total protons pumped, 17 H⁺/(C-1 + C-2)) relative to the reconstituted BR (initial rate, 0.8–1.0 H⁺/BR/s and total protons pumped, 40–50 H⁺/BR). It also gave lower yield (12%) of chromophore regeneration than reconstituted BR (55–60%). The limited renaturation of fragments in the soybean lipid/cholate system was probably the major factor in this difference. DMPC is a better lipid system for regeneration of the chromophore but it forms poor vesicles in terms of proton pumping (13). Therefore, we have devised a modified protocol in which the chromophore is reconstituted in DMPC/CHAPS solution at pH 6.0 and this is followed by addition of soybean lipid/CHAPS and dialysis (see “Methods”). Using this protocol, at least 30% of the chromophore regenerated initially in DMPC/CHAPS mixture was retained during a 4-day dialysis. The pumping activity of this preparation showed an increase of at least 2-fold, 0.55–0.6 H⁺/(C-1 + C-2)/s for initial rate and 30–40 H⁺/(C-1 + C-2) for the extent of H⁺ pumped, which is close to the values given above for reconstituted BR under the same conditions.

**DISCUSSION**

**Secondary Structures of Fragments C-1 and C-2—**CD was used to probe the secondary structure of the fragments in DMPC/cholate/SDS. Individually and in combination, the two fragments were found to possess secondary structures very close to that of native BR (11). In addition, the two fragments recognize and bind tightly to each other. A minimum binding constant for association of C-1 and C-2 (Kₛ = 3 × 10⁷ M⁻¹) can be estimated from the fact that in the presence of C-2, 50% of C-1 is protected from denaturation within 1 h (Fig. 5). This reassociation of the fragments suggests (see also below) that C-1 and C-2 fragments acquire conformations in the DMPC/cholate/SDS micelles similar to those they possess in native, intact BR molecules.

Furthermore, since C-1 and C-2 exhibit a nearly equal percentage of α-helix. This suggests that the α-helical rods of BR are proportionately distributed along the polypeptide chain in the native state, with two of the putative α-helices observed in electron diffraction maps (1) in C-2 and five in C-1 (cf. the proposed model of BR structure in Ref. 10).

Following their initial gain of secondary structure, both C-1 and C-2 gradually denature in the DMPC/cholate/SDS mixture. Denaturation of the fragments is indicated by the loss of the ability to bind the complementary fragment and retinal, and in the case of C-1, a considerable decrease in α-helical content. Presumably, in the denatured state, the interhelical interactions are replaced by lipid-protein, detergent-protein, or new intra- or intermolecular interactions between polypeptide chains. The latter may result in aggregation of the fragments.

**Formation of the BR-like Complex from C-1, C-2, and Retinal—**Retinal is attached to C-1 by formation of a Schiff’s base (7–9). Cross-linking results show that at its distal end it approaches helix 6 (26). However, free retinal does not bind to C-1. This suggests that either the retinal binding pocket is shared between C-1 and C-2 or that the binding pocket of C-1 is unable to form a stable complex with retinal.

Therefore, since C-1 and C-2 do form a complex in the absence of retinal (see “Results”), a likely mechanism for the formation of the stable, BR-like complex from C-1, C-2, and retinal is as follows:

\[
C-1 + C-2 + \text{retinal} 
\rightarrow (C-1 \cdot C-2 - \text{retinal}) 
\rightarrow (C-1 \cdot C-2 - \text{retinal}) 
\]

Indeed, retinal binds to the C-1·C-2 complex as tightly (Kₛ > 10⁷ M⁻¹) and as rapidly (17) as it binds to intact bacteriorhodopsin. The resulting complex of C-1, C-2, and retinal efficiently pumps protons, and the λ_max, ε and light adaptation properties (17) of its chromophore are very similar to that of intact BR. In addition, resonance Raman studies show that the vibrational configurations of the light-adapted chromophore and M-intermediate of the photocycle are close to those found in the intact protein (16). These properties indicate that the formation of the C-1·C-2·retinal complex must be very close to that of intact BR.

However, comparison of the absorbance properties of reconstituted BR and the BR-like complex regenerated from C-1, C-2, and retinal shows a subtle difference in their absorbance properties. Studies of the temperature dependence (Fig. 6) reveal that, in both cases, the spectra of the regenerated chromophore are probably a composite of two species, one identical with purple membrane with λ_max close to 570 nm and the second with λ_max somewhere between 450–500 nm. Furthermore, the fragment complex forms more of the blue-shifted species. Similar blue-shifted species have been observed at high pH in the spectrum of purple membrane (28) and reconstituted BR (29). The existence of this blue-shifted species means that absorbance spectra of some forms of BR cannot be interpreted solely in terms of light and dark adaptation (30). For example, the presence of the blue-shifted species probably explains the lower λ_max (540 nm) and ε_max (40,000) of delipidated BR in deoxycholate (11). Furthermore, the presence of merely 10–20% of the blue-shifted component

---

7 Indeed, in the previously studied case of denaturation of delipidated BR by photobleaching in deoxycholate (11, 13), we have detected aggregation: the latter may occur subsequent to the primary denaturation event.

8 Our preliminary results, which show that retinal covalently attached to denatured C-1 can be trapped in a chromophore (λ_max = 515 nm) upon renaturation, suggest that the binding pocket for retinal is composed of the C-1 portion of the polypeptide, but is somehow altered or deficient without C-2 (27).
could account for the fact that the resonance Raman spectrum of the fragment complex is very close to that of BR in light-adapted purple membrane (16).

Effect of Detergents and pH upon Renaturation—Maximal renaturation gauged by chromophore regeneration occurred at low detergent concentration. Therefore, the media used in the present studies consisted of DMPC/cholate/SDS mixtures with a low cholate content and a very low SDS content (2-4 mol % of mixture). This medium most likely forms structures like the “mixed disc” micelle proposed for phosphatidylcholine/bile salt mixtures (31, 32). At the low detergent concentration used, the mixed disc micelles should be more bilayer-like. Although the causes of the detrimental effect of detergents upon renaturation are not certain, it is noteworthy that high cholate content which does reduce renaturation (Fig. 7A) also reduces micelle size (32).

A study of the pH dependence of chromophore regeneration showed a bell-shaped profile (Fig. 8). It is very likely that regeneration of chromophore involves both equilibrium factors, such as the binding constant of retinal, equilibrium for Schiff’s base formation, and equilibrium between folded and unfolded states and also kinetic factors, such as irreversible denaturation and the secondary denaturation which occurs after folding (Fig. 5). Our preliminary results indicate that the pH dependence of the extent of folding and chromophore regeneration is dominated by kinetic factors in Triton X-100/SDS but not in DMPC/CHAPS/SDS. Interestingly, we find that the bell-shaped pH profile observed is consistent theoretically with kinetic control as well as equilibrium control of chromophore regeneration.

If, indeed, refolding of the protein is pH-dependent, then the ionization state of the charged amino acids must be critical in the folding process and thus for the association of C-1 and C-2. Clearly, it is not possible at this stage to identify the amino acids, embedded in or outside the bilayer, that are important in the association of the fragments. It is also not possible to obtain their pK, values in our system because they cannot be determined simply by inspection of the pH profile as in the case of simple equilibrium control. Therefore, in further work, we hope to separate the above processes to study the actual pH dependence of protein folding. It should also then be possible to interpret in a meaningful way the change in pK, when specific alterations in ionizable groups are made in the protein.

Finally, it may be noted that the observations made in this study, namely the spontaneous folding of C-1 and C-2 and their subsequent interaction may have relevance to the events involved in the biosynthetic integration of BR into purple membrane. Folding in micelles and membranes should be similar since both contain polar and nonpolar regions and are surrounded by an aqueous environment.

a Supporting this interpretation we find a phase change from a clear solution to a turbid, viscous suspension above a 2:1 molar ratio of DMPC/cholate even in the presence of SDS. Previously, the same ratio at the phase boundary has been found for egg phosphatidylcholine/cholate micelles (30).

REFERENCES

Regeneration of the native bacteriorhodopsin structure from two chymotryptic fragments.
M J Liao, E London and H G Khorana


Access the most updated version of this article at http://www.jbc.org/content/258/16/9949

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/258/16/9949.full.html#ref-list-1