The secondary structure of bacteriorhodopsin polypeptides comprising two (AB, CD, DE, FG), three (AC, CE, EG), four (AD, DG), or five (AE, CG) of the seven transmembrane segments has been analyzed by circular dichroism spectroscopy. A comparison of the α-helical content with that predicted from the high resolution structure of the native protein revealed that the N-terminal AB, AC, AD, and AE fragments and the C-terminal CG fragment are completely refolded in the presence of mixed phospholipid micelles. In contrast, the DG, EG, CG fragment are completely refolded in the presence of mixed phospholipid micelles. Each of the latter fragments displayed, however, an increased helicity upon lowering the pH to 4. Fluorescence measurements with the CD and FG fragments suggest that this helix formation occurs within transmembrane segments C and G, respectively, and thus is likely to originate from the protonation of carboxyl residues that participate in proton translocation. The partial misfolding at neutral pH observed for the shorter fragments from the central and C-terminal part of bacteriorhodopsin indicates that the conformation of some transmembrane segments is specified by interactions with neighboring helices in the assembled structure. Moreover, the data demonstrate that two stable helices at the N terminus of a multihelical membrane protein are sufficient as a folding template to induce a native conformation to the following transmembrane domains.

Integral membrane proteins generally adopt a regular secondary structure within the lipid bilayer in order to satisfy hydrogen bonding of the peptide backbone in a hydrophobic environment. The physicochemical constraints imposed by the membrane limit the variety of basic protein architectures, and structure prediction is thus expected to be much simpler than for globular proteins. This characteristic has been exploited in the development of different algorithms for the identification of membrane protein secondary structure (1–3). Transmembrane (TM) helices represent the predominant structural element and can be predicted with high reliability by scanning the protein sequence for regions of adequate length and hydrophobicity to span the lipid bilayer in an α-helical conformation. One such algorithm identifies TM α-helices based on an estimate of the free energy of transferring a helical segment from the aqueous environment into a lipid bilayer (3). These thermodynamic calculations predict that the individual TM α-helices should be stable, even in the absence of interactions with other parts of the molecule. The folding and assembly of multihelical membrane proteins could thus proceed according to a sequential two-step mechanism, in which the individual TM helices form during the initial membrane insertion step, and subsequently associate to form the native tertiary structure (4, 5).

Direct structural studies on integral membrane proteins are hindered by the difficulty in obtaining crystals suitable for x-ray diffraction, and by their restricted motion in the lipid environment which prevents the application of solution NMR spectroscopy. As a consequence, alternative techniques, such as circular dichroism (CD), fluorescence, Fourier transform infrared and electron paramagnetic resonance spectroscopy, have been widely used to derive structural features of membrane proteins. The secondary structure components of proteins are often estimated from the CD of the backbone amide absorption band (6, 7). In the case of membrane proteins the CD measurements, however, can be affected by the optical artifacts of differential light scattering and absorption flattening caused by membrane particles (8–10). Thus, for a reliable analysis these proteins have to be solubilized in detergent and/or incorporated into lipid vesicles using conditions that maintain the native conformation. Structural investigations of membrane proteins are further complicated by their generally low abundance and frequently encountered problems with their overexpression in a functional state (11). As an alternative approach, synthetic peptides corresponding to individual TM segments have been used to obtain information on the structure, function, and folding of polytopic membrane proteins (12–14). However, in the absence of a three-dimensional structure the relevance of such studies remains uncertain.

Bacteriorhodopsin (BR), the light-driven proton pump of purple membrane (15), provides an attractive model system for analyses of the conformational properties and folding characteristics of TM domains. First, the structure of BR has recently been solved at high resolution, revealing the detailed arrangement of the seven membrane-spanning α-helices and the connecting surface loops (16–19). The retinal chromophore is linked covalently to Lys-216 in helix G (Fig. 1) and is located in a central cavity where it contacts each of the TM segments. This heptahelical TM topology is shared by the large and functionally diverse superfamily of receptors coupled to guanine nucleotide-binding proteins (20, 21). Second, denatured BO can be spontaneously refolded to the native state with quantitative
recovery of secondary structure, chromophore binding, and proton pumping activity (22, 23). Reconstitution of the native structure has also been accomplished with complementary combinations of recombinant or proteolytic fragments and/or synthetic peptides, comprising one or more of the TM regions (24–30). Furthermore, the folding process of native BR has been analyzed by time-resolved spectroscopic techniques (31, 32). Third, recombinant variants of BR can be produced in sufficient quantities for the necessary biophysical measurements, using heterologous or homologous expression systems (reviewed in Ref. 11).

In recent studies by Hunt et al. (33, 34) the secondary structure of synthetic peptides corresponding to each of the seven TM domains of BR was determined. The peptides containing the sequence of TM regions A, B, D, or E all formed stable TM α-helices in isolation (33), and the peptide corresponding to helix C showed a pH-dependent transition between a membrane-associated, nonhelical state and a TM α-helix (34). In contrast, non-native conformations were displayed by the peptides containing the sequence of the two C-terminal TM segments of BR. Specifically, the peptide reflecting helix D did not form any stable secondary structure, and for the peptide corresponding to helix G a hyperstable β-sheet structure was observed (33). In order to characterize further conformational constraints on the folding and assembly of BR, we have analyzed in the present work the secondary structure of recombinant fragments that are capable to associate in complementary combinations and to regenerate the native chromophore (30). These polypeptide fragments comprise either two (AB, CD, DE, FG), three (AC, CE, EG), four (AD, DG), or five (AE, CG) of the TM segments of BR (Fig. 1). CD measurements in lipid micelles at pH 6 revealed a completely refolded state for the AB, AC, AD, AE, and CG fragments, whereas for the remaining polypeptides the helicity was reduced to different extent relative to that predicted from the high resolution ED structure. A non-native conformation was particularly evident for the FG fragment, which showed a high proportion of β-sheet and β-turn structures. The pronounced misfolding of the short CD and FG fragments could be compensated for by the protonation of internal carboxyl residues or alternatively by their incorporation into an extended polypeptide chain. The data demonstrate that the folding capability of individual TM helices of BR is strongly dependent on the molecular context. The two N-terminal helices are apparently sufficient to promote the proper folding of the remaining TM segments of the protein.

**EXPERIMENTAL PROCEDURES**

**Materials—**DHPC was purchased from Avanti Polar Lipids (Alabaster, AL), and DMPC was obtained from Sigma (Deisenhofen, Germany). L-10-Camphorsulfonic acid was from Aldrich (Steinheim, Germany), and cesium chloride was from ICN (Eschwege, Germany). The suppliers of the other chemicals have been reported (30).

**Expression and Purification of BR Polypeptide Fragments—**The procedures used for the construction, expression, purification, and molecular characterization of the BR fragments AB, AC, AD, AE, CG, DG, and EG, and FG have been described (30). Genes for the three additional fragments CD, CE, and DE were assembled from restriction fragments of previously constructed pSBO2 vectors that encode appropriate deletions in the wild-type protein sequence (30). Specifically, for CD the small pol1-BshIII gene fragment of CG was ligated with the large BshIII-Pou1 gene fragment of AD, for CE the small Pou1-Nor1 gene fragment of CG with the large Nar1-Pou1 gene fragment of AE, and for DE the small Pou1-Nar1 gene fragment of DG with the large Nar1-Pou1 gene fragment of AE (cf. Fig. 1 of Ref. 35). The sequences of the newly constructed genes were confirmed by direct plasmid sequencing using the dideoxy method (36). The cloned BO genes were introduced into the expression vector pPL1 as HindIII-EcoRI fragments (35) and expressed in *Escherichia coli* (30). Subsequently the fragments were extracted from *E. coli* membranes and purified to homogeneity using previously described procedures (30). The purity of the fragments was assessed by SDS-PAGE (12% gel electrophoresis and Western blot analysis), and their N-terminal sequence was verified by Edman degradation (30).

**Preparation of Samples for Spectroscopic Measurements—**Mixed lipid micelles were prepared by vigorously stirring DMPC and DHPC in water for 1 h followed by sonication in a bath sonicator until a clear suspension was obtained (32). The stock solution contained 3% (w/v) total lipid and was used with a DMPC/DHPC ratio of 2:1 (w/w) and was stored at 4 °C for a maximum period of 1 month. For CD and fluorescence measurements the fragments were diluted to a concentration of 0.05 mg/ml in 10 mM sodium phosphate, pH 6.0, or ~10 mM citric acid/sodium phosphate, pH 4.0, containing alternatively 0.2% SDS (for measurements of the partially denatured state) or 1% DMPC and 0.5% DHPC (for measurements of the refolded state). The samples were equilibrated at room temperature for at least 30 min prior to spectroscopic analyses.

Complexes of complementary fragments were formed by mixing equimolar amounts (12–14 μM) of two or three fragments at a total protein concentration of 0.4 mg/ml in the presence of SDS. Following addition of a 3-fold molar excess of all-trans retinal, the solution was equilibrated for 5 min and subsequently diluted to yield a protein concentration of 0.05 mg/ml in the presence of 1% DMPC, 0.5% DHPC, and 10 mM sodium phosphate, pH 6.0. Chromophores were regenerated by incubating the samples in the dark at room temperature for 20 h.

**Absorption Spectroscopy—**UV-visible absorption spectra were recorded at 22 °C in the range from 250 to 750 nm on a Perkin-Elmer Lambda 9 spectrophotometer using 1.0-cm path length quartz cells (Hellma, Mullheim, Germany). Protein concentrations were calculated based on the respective extinction coefficient at 280 nm in 0.2% SDS, determined for the purified fragments by quantitative amino acid analysis (30). The extinction coefficients of the newly prepared CD, CE, and DE fragments amounted to 17,700, 32,800, and 18,100 μM−1 cm−1, respectively. Regeneration yields of fragment complexation were calculated using the previously determined extinction coefficients for the dark-adapted chromophores at pH 6.0 (30).

**Circular Dichroism Spectroscopy—**CD spectra were measured in the range from 185 to 280 nm on a Jasco J-720 spectropolarimeter equipped with a temperature control unit (Lauda, Lauda-Königshofen, Germany). The ellipticity and wavelength were calibrated, respectively, with a standard solution of 1:10-camphorsulfonic acid and a neodymium probe according to the instructions of the manufacturer. During recording the high voltage of the detector always remained below 600 V, thereby minimizing distortions of the spectra. The measurements were carried out at 22 °C using 0.1- and 0.05-cm path length quartz cuvettes (Hellma) for fragments solubilized in SDS or lipid micelles, respectively. At an interval of 1 day, eight consecutive scans were accumulated with a scan speed of 20 nm/min (for samples in SDS) or 10 nm/min (for samples in lipid micelles) and averaged. An appropriate background spectrum (i.e. buffer with SDS or mixed lipids) was subtracted from the raw data. For secondary structure prediction the data were reduced to one point per nm by averaging, and no smoothing algorithm was applied to the spectrum. The secondary structure content was evaluated with the software program CDNN (version 2.0c; Refs. 37 and 38) that uses the artificial neural network technique (39–41). The calculations were based on a data set of 33 reference spectra from water-soluble proteins. By using the entire wavelength range of the spectrum for structure prediction, unreasonable values (i.e. a sum of <90 or >110% for the structure elements) were obtained in several cases. Thus, the analysis was performed with the spectral range from 190 to 260 nm, thereby yielding a total secondary structure content between 95 and 105% in each case.

**Fluorescence Spectroscopy—**Fluorescence measurements were carried out on a Perkin-Elmer LS56B spectrophotometer using 4 × 4-mm quartz cells (Hellma). Protein fluorescence was excited at 285 nm with a bandwidth of 10 nm, and the emission was recorded in the range from 300 to 430 nm with a bandwidth of 4 nm. An appropriate background spectrum (i.e. buffer with SDS or mixed lipids) was subtracted from the raw data. Quenching experiments were performed by adding aliquots of a 4 M CsCl stock solution to the fragments solubilized in SDS or DMPC/DHPC micelles. Fluorescence intensities were calculated as the integrals of the emission band from 300 to 430 nm.
RESULTS

Preparation and Reconstitution of BR Polypeptide Fragments—Eleven different polypeptides that comprise two (AB, CD, DE, FG), three (AC, CE, EG), four (AD, DG), or five (AE, CG) of the seven TM regions of BR (Fig. 1) were prepared by expression in E. coli. Each of these constructs contains the C-terminal tail of the protein (residues 226–248), which is required for purification by anion-exchange chromatography (30). Recently, the four pairs of complementary fragments (AB-CG, AC-DG, AD-EG, and AE-FG) have been shown to assemble and reaggregate the native BR chromophore with high efficiency in DMPC/CHAPS micelles (30). This lipid/detergent system is, however, inappropriate for CD measurements, since the high absorbance of CHAPS obscures key CD bands in the far UV range associated with protein secondary structure. Thus, in the present study a lipid-based refolding system was employed, in which the lipid DHPC is substituted for CHAPS (31). In the case of native BR from purple membrane (32) or eBR a near quantitative regeneration of the chromophore has been observed in DMPC/DHPC micelles for the four shortest polypeptides (AB, CD, DE, and FG), each comprising two of the seven TM regions of BR. A quantitative determination of the secondary structure with the program CDNN (37) revealed for the longer fragments derived from the N terminus (AC, AD, and AE) or C terminus (CG, DG, and EG) of the protein an \( \alpha \)-helix content in the range of 55–67%, compared with the value of 65% for eBO (Table I). For the remaining polypeptides that encode two or three TM regions (AB, FG, CD, CE, and DE) the proportion of \( \alpha \)-helical conformation was found to be reduced to 38–48% (Table I). An analysis of these CD spectra with the variable selection program VARSCLC (43) yielded relatively similar values for the secondary structure components of the different fragments. A generally lower \( \alpha \)-helix content was calculated with procedures that are based solely on the ellipticity at 222 nm (6, 44).

To assess the secondary structure in a membrane mimetic environment, the BR polypeptides were reconstituted into lipid micelles composed of 1% DMPC and 0.5% DHPC at pH 6.0. Except for FG, all of the fragments maintained a predominantly helical conformation in the presence of lipids (Fig. 3). The CD spectra of AB, AC, AD, AE, and CG displayed an increased negative ellipticity at 222 nm, as previously reported for intact BO (32). Based on the quantitative analysis the increase in the \( \alpha \)-helix content of these fragments amounted to 3–14%, compared with the respective values in SDS (Table I). For DG, EG, CD, CE, and DE the qualitative and quantitative evaluation of the CD spectra revealed minimal conformational differences between the detergent and lipid environments (Fig. 3 and Table I). A striking alteration of the secondary structure was observed, however, upon transfer of the FG polypeptide into lipid micelles. The resulting CD spectrum (Fig. 3D) resembled that of proteins containing a substantial amount of \( \beta \)-sheet conformation (6, 45). The quantitative analysis showed...
that in DMPC/DHPC micelles the α-helix content of FG was markedly reduced to 21%, whereas the proportions of β-sheet and β-turn structures were both increased to 22% (Table I).

A comparison of the CD spectra recorded for the individual peptides in the detergent and lipid environments revealed a distinct change in the shape of the curves (cf. Figs. 2 and 3). In the presence of SDS the magnitude of the negative ellipticity at 208 nm was always increased relative to the value at 222 nm, whereas in lipid micelles the 222 nm band was more intense for several of the fragments and eBO. Fig. 4 shows the ratio of the molar ellipticity at 222 versus 209 nm for the peptides in the two different environments. Similar to eBO, refolding of AB, AC, AD, AE, and CG in lipid micelles resulted in a significant increase in the ratio of $\theta_{222}/\theta_{209}$. For the three fragments derived from the central region of BR (CD, CE, and DE), this ratio remained essentially unchanged, whereas in the case of FG a decrease was noticed in DMPC/DHPC micelles (Fig. 4). These observations paralleled the changes in the helicity of the respective fragments (Table I). However, a precise correlation between the absolute α-helix content and the ratio of $\theta_{222}/\theta_{209}$ was found neither in the lipid nor in the SDS environment, as is evident for example from a comparison of these parameters for the AC and EG fragments (Table I and Fig. 4). The present data suggest that a $\theta_{222}/\theta_{209}$ ratio near 1.0 represents an intrinsic property of refolded TM helices of BR.

Based on the quantitative analysis of the CD spectra recorded in lipid micelles at pH 6.0 (Table I), the number of residues present in an α-helical conformation was calculated for each fragment and compared with the value for the respective segment of the native BR structure determined by electron microscopy (Table II). If appropriate, the number derived from the ED map included residues 192–196 that form a loose helical turn at the C terminus of helix F (16). For the N-terminal AB, AC, AD, and AE fragments, and the C-terminal CG fragment the number of helical residues derived from CD spectroscopy was increased by 5–16% compared with the ED data (Table II). An identical result was also obtained for eBO (+10%), indicating that the TM regions of these constructs are completely refolded to a native-like state. On the other hand, the proportion of α-helical residues determined by CD spectroscopy was reduced in the case of DG, EG, FG, CD, CE, and DE relative to the value calculated from the high resolution structure. This effect was particularly pronounced for FG (−64%) and to a lesser extent for CD and DE (−25 and −19%, respectively; cf. Table II). Thus, portions of the TM helices of these fragments obviously are misfolded in DMPC/DHPC micelles at pH 6.0.

**pH-dependent Changes in the Conformation of Polypeptide Fragments**—Several of the TM regions of BR contain acidic residues (Fig. 1) which, by undergoing a change in the protonation state, can induce alterations in the conformation of the respective polypeptide segment (34). Similar to eBO, the secondary structure contents of AB, AC, AD, AE, and CG determined at pH 6.0 and 4.0 in either SDS (data not shown) or DMPC/DHPC micelles (Table I) showed little variation. In contrast, the helicity was notably increased in the lipid environment at pH 4.0 for all of the fragments that are partially misfolded at pH 6.0. For example, the CD fragment displayed an increased α-helix content in SDS (46%) and particularly in DMPC/DHPC micelles (56%) upon lowering the pH to 4.0 (Fig. 5A and Table I). Based on the comparison with the ED structure the TM regions of the CD peptide, which comprise three aspartates (Asp-85 and Asp-96 in helix C, Asp-115 in helix D), are completely folded in the lipid environment at pH 4.0 (Table II). Similarly, acidification to pH 4.0 resulted in complete refolding of the DG and EG fragments (Fig. 5D and Table II). In the case of CE and DE the increase in the helicity was minor (Fig. 5B and Table I), and their conformation remained partially misfolded at low pH in lipid micelles (Table II). The most striking pH-dependent structural difference was noticed for the FG polypeptide. Unlike at pH 6.0, the transfer from SDS into the lipid environment at pH 4.0 did not induce a decrease in the α-helix content and formation of β-sheet structure (Fig. 5C and Table I). The FG fragment is therefore folded to a large extent in DMPC/DHPC micelles at acidic pH (Table II). The pH-induced conformational alterations observed for the different fragments were fully reversible.

**Fluorescence Spectroscopy of Polypeptide Fragments**—All of the 11 polypeptides contain at least two tryptophan residues that are located within the TM regions in the BR structure (Fig. 1). In the presence of SDS the various fragments and eBO displayed fluorescence emission maxima between 334 and 338 nm at both pH 6.0 and 4.0. A significant deviation was observed solely in the case of FG, which showed an emission maximum at 343 nm (Fig. 6C). These wavelength maxima are consistent with a location of the fluorophores within the hydrophobic core of the SDS micelles (46, 47). Following transfer into the lipid environment the fluorescence quantum yield was increased 1.4–2.6-fold for all of the fragments and eBO (Fig. 6). Under these conditions the emission maxima remained unchanged, except in the case of FG. The latter fragment displayed emission maxima that were blue-shifted to 335 and 338 nm at pH 6.0 and 4.0, respectively, in DMPC/DHPC micelles (Fig. 6C). A comparison of the fluorescence spectra recorded at the two different pH values revealed that the emission intensity generally remained unchanged or decreased at low pH in the lipid and detergent environments (Fig. 6). An altered behavior was noticed, however, for the shorter polypeptides that comprise TM segment C of BR. For the AC, CD, and CE fragments the
quantum yield at pH 4.0 was increased in both DMPC/DHPC and SDS micelles relative to that measured at pH 6.0 (Fig. 6, A and B).

In order to investigate whether the pH-dependent changes in the fluorescence emission intensity are caused by an altered accessibility of the fluorophores to the aqueous medium and/or by a change in the microenvironment of the tryptophans, quenching experiments were performed with the CD and FG fragments. These two peptides were chosen as they are representative examples for an increase and a decrease, respectively, in the fluorescence quantum yield upon lowering the pH to 4.0 (Fig. 6, A and C, respectively). Whereas in SDS about 50% of the fluorescence intensity was quenched by CsCl at the two different pH values, the decrease in the emission intensity was negligible for both peptides in DMPC/DHPC micelles (<5% at the final CsCl concentration of 320 mM; data not shown). The very low Stern-Volmer constants of 0.10 and 0.13 M<sup>-1</sup> for CD, as well as 0.08 and 0.01 M<sup>-1</sup> for FG at pH 6 and 4, respectively, in lipid micelles demonstrate the general inaccessibility of the fluorophores to the water-soluble quencher (48). Thus, the enhanced emission intensity at low pH observed for the short peptides containing TM segment C is likely to originate from a structural change in the microenvironment of the fluorophores (49).

CD Spectroscopy of Regenerated Fragment Complexes—Following overnight regeneration the secondary structure of the four complexes formed by a pair of complementary fragments (AB-CG, AC-DG, AD-EG, and AE-FG) was analyzed by CD spectroscopy in DMPC/DHPC micelles at pH 6.0. For the AB-CG and AD-EG complexes the magnitude of the ellipticity in the resulting CD spectrum was qualitatively very similar to the respective composite spectrum calculated from the two individual counterparts (Fig. 7, A and C). On the other hand, a decrease and an increase, respectively, in the helicity was noticed for the regenerated AC-DG and AE-FG complexes relative to the calculated spectrum of the two complementary fragments (Fig. 7, B and D). The results of the quantitative analysis of these CD measurements are summarized in Table III. Compared with eBR, the a-helix contents of the regenerated complexes were found to be reduced in each case. However, for a meaningful comparison it has to be considered that AB, AC, AD, and AE contain the C-terminal tail (residues

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**Table I**

<table>
<thead>
<tr>
<th>Fragment</th>
<th>SDS, pH 6.0</th>
<th>DMPC/DHPC, pH 6.0</th>
<th>DMPC/DHPC, pH 4.0</th>
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<tr>
<td></td>
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<tr>
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</tr>
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**Fig. 3.** Circular dichroism spectra of BR fragments in 1% DMPC, 0.5% DHPC at pH 6.0. A, fragments AB and AC; B, fragments AD and AE; C, fragments CG and DG; D, fragments EG and FG. Each spectrum represents the average of eight scans with the background subtracted. Details of sample preparation and spectroscopic measurements are described under “Experimental Procedures.” The results of the quantitative analysis of the secondary structure content are listed for the individual fragments in Table I.

**Fig. 4.** Ratio of the mean residue ellipticities at 222 versus 209 nm for BR fragments. The ratio was calculated from the spectrum of the respective fragment recorded in 0.2% SDS at pH 6.0 or 1% DMPC, 0.5% DHPC at pH 6.0 (cf. Figs. 2 and 3).
and CG and FG the N-terminal eight amino acids of BR as additional peptide segments (30), which both adopt a nonhelical conformation in the ED structure (16, 18). Thus, for the ABzCG and ADzEG complexes the total number of helical residues was in close correspondence to the wild-type value, indicating that the constituent fragments are completely refolded (Table III). Similar to the native protein (32), a small increase in the proportion of helical residues was observed upon regeneration of the ABzCG complex (cf. Tables II and III). This effect was even more pronounced in the case of ADzEG and particularly AEzFG, if the complementary fragments were incubated at acidic pH prior to the regeneration (Table III). Since FG and to a lesser extent EG are partially misfolded in lipid micelles at pH 6.0 (Table II) it is evident that their native conformation is stabilized by interaction with the complementary fragment and chromophore formation. In contrast, the number of helical residues was reduced for the regenerated ACzDG complex relative to the sum of the two constituent counterparts (cf. Tables II and III). This shows that the previously observed partial denaturation of the AC-DG complex upon extended periods of incubation (30) cannot be completely

![Image](50x271 to 297x498)

**FIG. 5.** pH dependence of the circular dichroism spectra of BR fragments in DMPC/DHPC and SDS micelles. A, fragment CD; B, fragment DE; C, fragment FG; D, fragment EG. Spectra of the individual fragments were recorded in the presence of 1% DMPC, 0.5% DHPC at pH 6.0 (--), 1% DMPC, 0.5% DHPC at pH 4.0 (—), 0.2% SDS at pH 6.0 (- - -), or 0.2% SDS at pH 4.0 (— - -). Each spectrum represents the average of eight scans with the appropriate background subtracted. Details of sample preparation and spectroscopic measurements are described under “Experimental Procedures.”

![Image](297x501 to 555x729)

**FIG. 6.** pH dependence of the fluorescence emission spectra of BR fragments in DMPC/DHPC and SDS micelles. A, fragment CD; B, fragment CE; C, fragment FG; D, fragment EG. Spectra of the individual fragments were recorded in the presence of 1% DMPC, 0.5% DHPC at pH 6.0 (--), 1% DMPC, 0.5% DHPC at pH 4.0 (—), 0.2% SDS at pH 6.0 (- - -), or 0.2% SDS at pH 4.0 (— - -). The spectroscopic measurements were carried out as described under “Experimental Procedures.”

![Image](555x701 to 800x928)

**FIG. 7.** Circular dichroism spectra of regenerated BR complexes formed by pairs of complementary fragments. A, AB-CG; B, AC-DG; C, AD-EG; D, AE-FG. Spectra of the individual fragment complexes were recorded in the presence of 1% DMPC, 0.5% DHPC at pH 6.0 following chromophore regeneration in the dark for 20 h (—). Each spectrum represents the average of eight scans with the background subtracted. In addition, the composite spectrum calculated from the two complementary fragments is shown in each panel (- - -). The results of the quantitative analysis of the secondary structure content are listed for the fragment complexes in Table III.
this 5-residue segment does not form a regular
form a loose helical turn at the end of helix F (16). Although
resolution x-ray and electron diffraction structures (16–19). In
identical to that of purple membrane reconstituted in micelles
77% displayed by eBO in this lipid system (Table I) is virtually
spectrum of the wild-type protein, 4 these variations in the helix
detergent concentration have virtually no effect on the CD
for BO in octyl glucoside (52). As significant alterations in the
content must be attributed to differences in the isolation and/or
denaturation procedure.

Upon dilution of SDS below the critical micellization concen-
tration, denatured BO (31) or eBO can be refolded in mixed
DMPC/DHPC lipids to the native state. The a-helix content of
77% displayed by eBO in this lipid system (Table I) is virtually
identical to that of purple membrane reconstituted in micelles
or small unilamellar vesicles composed of native or synthetic
phospholipids (8, 25, 32, 53). The helicity determined by CD
spectroscopy exceeds the value of 70% derived from the high
resolution x-ray and electron diffraction structures (16–19). In
the latter number we have included residues 192–196 that
form a loose helical turn at the end of helix F (16). Although
this 5-residue segment does not form a regular a-helical con-
formation, it is expected that the backbone exhibits a rotary
strength similar to an a-helix and thus contributes to the
negative ellipticity at 208 and 222 nm. In view of the close
similarity of the CD analyses performed by different in-
vestigators, it has to be considered that in addition to the TM
regions helical structures could be contained in surface regions
of BR. Recent NMR measurements indicate that residues 227–
235 in the C-terminal tail display a helical conformation,
thereby extending helix G on the cytoplasmic surface (54). The
structure of the C-terminal segment of BR (residues 227–248)
has so far not been resolved by high resolution crystallography
(17–19). On the other hand, the discrepancy between CD and
diffraction analyses could arise from a difference in the CD
band intensity between the regular a-helix of water-soluble
proteins and the TM a-helix. The currently available algo-
rithms for the quantitative determination of secondary struc-
ture from CD spectra use data bases of water-soluble proteins
as reference (7), since corresponding data for membrane pro-
teins are highly limited. A recently performed deconvolution of
the CD spectra of membrane proteins suggests that the TM
a-helix displays an increase in the negative ellipticities at 208
and 222 nm, and the positive ellipticity near 195 nm relative to
the a-helix of globular proteins (55). This difference would
result in an overestimation of the a-helix content of membrane
proteins, in case the quantitative analysis is based on reference
spectra from water-soluble proteins.

Like for the intact BR, the DMPC/DHPC lipid system
induced at pH 6.0 a complete refolding of the N-terminal AB, AC,
AD, and AE fragments and the C-terminal CG fragment (Table
II). For AB and CG this observation is consistent with previous
studies that used corresponding fragments produced by chy-
matryptic digestion of purple membrane (24, 25). In contrast,
the shorter fragments derived from the C terminus (DG, EG,
and FG) or the center (CD, CE, and DE) of the molecule did not
form a-helices of the expected lengths (Table II). This effect was
most evident in the case of FG, which displayed a high amount
of non-native b-sheet conformation in the lipid environment
at pH 6.0 (Fig. 3D and Table I). Recently, the secondary structure
of synthetic peptides corresponding to each of the seven TM
helices of BR was analyzed in phospholipid vesicles (33, 34).
Although a slow reconstitution method was used for refolding,
rather than the fast mixing procedure of the present work, a
similar result was obtained regarding the conformation of TM
segments F and G. The peptide corresponding to the F helix did
not form any stable secondary structure, whereas for the pep-
tide corresponding to the G helix a hyperstable b-sheet struc-
ture was observed (33). The combined data demonstrate that
the covalent connection and intermolecular interaction
between TM segments F and G are not sufficient to prevent
misfolding of the two C-terminal helices of BR. Thus, the pre-
ference of helices F and G for a non-native conformation can be
attributed to a unique characteristic of their amino acid
sequence.

A striking observation of this work is that the folding capa-
bility of individual TM helices of BR is strongly dependent on
the molecular context. In the case of the FG fragment, the
helical conformation is stabilized to a large extent if the
polypeptide chain is extended to include the preceding TM
segment E (Table II). Similarly, a comparison of the helicity of
CD with either AD or CG shows that the native structure of TM
regions C and D is maintained in the presence of additional
N-terminal or C-terminal helices. In general, the progressive
extension of the polypeptide chain induces an increase in the
a-helix content of the fragments (Table I). This stabilizing
effect can be explained by the interaction of helix dipoles (56),
the shielding of polar side chains by neighboring TM segments,
and/or the formation of interhelical hydrogen bonds (5). Fur-
thermore, the present CD analyses of the fragments establish
an enhanced structural stability of the N-terminal a-helices of
BR, relative to the C-terminal segments. Comparable data
have also been obtained by in vivo expression studies of lactose
permease from E. coli (57, 58). The efficient folding and assem-

<table>
<thead>
<tr>
<th>Fragment complex</th>
<th>a-Helix</th>
<th>b-Sheet</th>
<th>b-Turn</th>
<th>Random</th>
<th>No. of residues in a-helical conformation</th>
</tr>
</thead>
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<tr>
<td>eBR</td>
<td>80</td>
<td>5</td>
<td>7</td>
<td>8</td>
<td>199</td>
</tr>
<tr>
<td>AB · CG</td>
<td>70</td>
<td>7</td>
<td>8</td>
<td>14</td>
<td>200</td>
</tr>
<tr>
<td>AC · DG</td>
<td>61</td>
<td>8</td>
<td>1</td>
<td>19</td>
<td>166</td>
</tr>
<tr>
<td>AD · EG</td>
<td>73</td>
<td>5</td>
<td>9</td>
<td>13</td>
<td>201</td>
</tr>
<tr>
<td>AE · FG</td>
<td>57</td>
<td>10</td>
<td>12</td>
<td>21</td>
<td>164</td>
</tr>
<tr>
<td>AE · FG, pH 4.0</td>
<td>62</td>
<td>8</td>
<td>12</td>
<td>19</td>
<td>178</td>
</tr>
</tbody>
</table>

a The fragments were incubated in SDS at pH 4.0 prior to the regen-
eration in DMPC/DHPC micelles at pH 6.0.
ently of polytopic membrane proteins in vivo may require the formation of stable TM structures by the segments that are synthesized first, in order to provide a folding template for the following TM regions. In this way, functionally required polar residues, which generally reduce the intrinsic stability of a TM segment (59, 60), can be properly incorporated into a helical bundle, provided that they are not located in the initial folding domain of the molecule. The in vitro studies with the BR fragments provide evidence that TM helices A and B are sufficient to promote the correct folding of the remaining TM domains of the protein.

Helices C, D, and G of BR contain ionizable carboxyl residues (Asp-85 and Asp-96, Asp-115, and Asp-212, respectively) that are located near the center of the respective TM segment (Fig. 1). In the assembled structure these highly polar groups face the interior of the heptahelical bundle and form part of the proton channel and/or retinal-binding pocket (16–19). Within the context of a fragment comprising a small number of TM segments, it is expected that such ionizable residues cannot be completely shielded by neighboring helices from the hydrophobic fatty acid chains of the lipids. For several of the fragments, and most pronounced for the short CD and FG polypeptides, an increase in the α-helix content was observed in lipid micelles upon lowering the pH from 6.0 to 4.0 (Table I and Fig. 5), i.e. in the pKa range of carboxyl side chains. This effect is apparently caused by the protonation of one or more acidic residues, which is expected to result in a higher hydrophobicity of the respective TM domain (61). Studies with model peptides have shown that the relative helicity of a polypeptide segment in lipid environments correlates with the hydrophobicity of the constituent amino acid side chains, rather than the secondary structure propensity in aqueous solutions (51, 59).

The increase in the helicity of the CD fragment upon acidification to pH 4.0 was much more pronounced than that of the DE fragment, which contains solely the protonatable Asp-115 residue within its TM portion (Table I). This suggests that the protonation-induced helix formation occurs within TM segment C of the CD fragment, and not within helix D or the C-terminal tail that are common to the two polypeptides. This interpretation is supported by the associated increase in the fluorescence emission intensity of the CD fragment (Fig. 6A). The very low and invariant Stern-Volmer constants at both pH 6.0 and 4.0 demonstrate the general inaccessibility of the fluorophores to the water-soluble quencher. In addition, a pH-dependent alteration in the polarity of the fluorophore environment can be excluded by the constant tryptophan emission maximum at 337 nm in lipid micelles. Thus, the increased quantum yield observed upon acidification of the CD fragment (Fig. 6A) is likely to originate from the removal of an internal quencher. A possible candidate is a close-by backbone carbonyl group, since its participation in main chain hydrogen bonding upon helix formation is expected to significantly reduce the quenching of the fluorophore (49). Within the relatively polar N-terminal part of TM segment C Trp-86 is directly preceded by Asp-85 (Fig. 1), which therefore represents a primary candidate for the acid-induced protonation.

For the FG fragment the strongly increased helicity upon acidification to pH 4.0 in DMPC/DHPC micelles (Fig. 5C and Table I) was not associated with an enhanced fluorescence emission intensity (Fig. 6C). This altered behavior can be explained by the fact that Trp-182 and Trp-189 are both contained within helix F, whereas the protonatable groups Glu-204 and Asp-212 are located in TM segment G (Fig. 1). Thus, the pH-induced helix formation of the FG fragment is likely to occur within TM region G. In recent studies that used synthetic polypeptides containing the sequence of each of the seven TM domains of BR, a pH-dependent conformational change was reported solely for the peptide reflecting helix C (33, 34). The peptide displayed a transition from a membrane-associated, nonhelical state to a TM α-helix with a pKa of approximately 6.0, and the titration resulted in the protonation of a single amino acid side chain (34).

The CD measurements of integral membrane proteins are known to be influenced by the optical artifacts of differential light scattering and absorption flattening (8–10). Based on the experimental setup and the lipid micelle system used in the present work, both of these effects can be excluded (32, 53). Therefore, the change in the shape of the CD spectra, i.e. the increased θ222/θ209 ratio upon refolding of the BR fragments in DMPC/DHPC micelles (Fig. 4), is likely to reflect an alteration in the helical structure of the TM domains. A θ222/θ209 ratio of >1 is also displayed by isolated TM segments of BR (33), indicating that it represents a property of the individual helix, rather than their assembled state. In previous studies a θ222/θ209 ratio of >1 has been assigned to a 310 helix (62) or alternatively to a coiled-coil structure (63, 64), both exhibiting a steeper helix pitch compared with an α-helix. For TM α-helices a characteristic CD spectrum with a θ222/θ209 ratio of about 1.2 has been derived by deconvolution of the CD spectra of different membrane proteins (55), and from the high resolution ED structure of BR it is known that the ends of several of the TM segments possess a 310 helix conformation (16). The enhanced θ222/θ209 ratio displayed by refolded eBo and N-terminal fragments could thus reflect a special helical conformation that promotes the structural stability of TM helices.

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REFERENCES
Secondary Structure of Bacteriorhodopsin Fragments

Secondary Structure of Bacteriorhodopsin Fragments: EXTERNAL SEQUENCE CONSTRAINTS SPECIFY THE CONFORMATION OF TRANSMEMBRANE HELICES

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