PEPTIDE-CHAIN SECONDARY STRUCTURE OF BACTERIORHODOPSIN

B. K. JAP, M. F. MAESTRE, S. B. HAYWARD
Donner Laboratory, Lawrence Berkeley Laboratory, University of California, Berkeley, California 94720

R. M. GLAESER
Department of Biophysics and Medical Physics, and Donner Laboratory, University of California, Berkeley, California 94720

ABSTRACT Ultraviolet circular dichroism spectroscopy in the interval from 190 to 240 nm and infrared spectroscopy in the region of the amide I band (1,600 cm^{-1} to 1,700 cm^{-1}) has been used to estimate the α-helix content and the β-sheet content of bacteriorhodopsin. Circular dichroism spectroscopy strongly suggests that the α-helix content is sufficient for only five helices, if each helix is composed of 20 or more residues. It also suggests that there is substantial β-sheet conformation in bacteriorhodopsin. The presence of β-sheet secondary structure is further suggested by the presence of a 1,639 cm^{-1} shoulder on the amide I band in the infrared spectrum. Although a structural model consisting of seven α-helical rods has been generally accepted up to this point, the spectroscopic data are more consistent with a model consisting of five α-helices and four strands of β-sheet. We note that the primary amino acid sequence can be assigned to segments of α-helix and β-sheet in a way that does not require burying more than two charged groups in the hydrophobic membrane interior, contrary to the situation for any seven-helix model.

INTRODUCTION

Bacteriorhodopsin is an integral membrane protein that is found in *Halobacterium halobium* (Stoeckenius et al., 1979) and in some other halophilic bacteria. This protein occurs naturally in two-dimensional crystalline arrays within the cell membrane, and is well suited for crystallographic structure analysis by electron diffraction and high-resolution electron microscope imaging techniques. The purple color of this protein is due to a Schiff-base linkage of retinal to a lysine group of the protein, as in the visual pigment, rhodopsin. The biological function of the protein is to serve as a light-driven proton pump, converting the energy of absorbed photons to the electrochemical energy of a pH gradient across the cell membrane (Stoeckenius et al., 1979).

The three-dimensional structure of bacteriorhodopsin has been determined to a resolution of 7 Å within the membrane plane and ~14 Å perpendicular to the plane using electron crystallographic methods (Henderson and Unwin, 1975). This three-dimensional structure consists of seven roughly parallel rods, each ~35 Å in length, which have been interpreted to represent seven α-helical regions of the peptide chain that span the 45-Å thickness of the membrane. On the basis of this interpretation, more detailed models of the structure have been proposed in which specific segments of the primary sequence are in the α-helical conformation (Ovchinnikov et al., 1979; Engelman et al., 1980; Engelman and Zaccai, 1980; Katre et al., 1981).

More recently, a higher resolution map of the in-plane projection of bacteriorhodopsin has been obtained by Hayward and Stroud (1981). This high-resolution map is clearly consistent with there being four or possibly five α-helical strands in the structure. On the other hand, a significant portion of the structure, designated as helices 1, 2, and 3 by Engelman et al. (1980) and referred to by us in the following as the outer slur, has developed a more narrow, higher contrast appearance, which led us to reconsider the α-helical model for that portion of the molecule.

In the work reported here we have used quantitative image calculations to verify, in a preliminary way, our intuitive impression that the outer slur does not appear to be the projection of three (tilted) α-helices. It is virtually impossible to rule out a three-helical model by such calculations, however, because of the large number of variations of the model that would have to be tested. At the same time we have demonstrated that the projection of four strands of β-sheet is consistent with the experimentally obtained features of the image. On the basis of these image-matching results we carried out a detailed analysis of the ultraviolet circular dichroism spectrum and the infrared absorption spectrum (amide I band) to obtain

S. Hayward's present address is the Air and Industrial Hygiene Laboratory, California State Health Services, 2151 Berkeley Way, Berkeley, CA 94704.

further information about the secondary-structure content of bacteriorhodopsin. The spectroscopic data indicate that as much as 20% or more of the peptide chain of bacteriorhodopsin is in the β-sheet conformation, while the α-helix content appears to be no more than 50%. A structural model consisting of five α-helical strands (containing ~24 residues each) and four β-sheet strands (containing ~11 residues each) would seem to explain these spectroscopic data and the high-resolution electron crystallographic data better than a seven-helix model. Such a model can be built in a way that buries fewer charged and hydrophilic groups within the hydrophobic membrane interior than is the case for any seven-helical model.

MATERIALS AND METHODS

Materials

Papain (activity 21 units/mg; Sigma Chemical Co., St. Louis, MO), Triton X-100 (Fisher Scientific Co., Pittsburgh, PA), 2-mecaptoethenol (Sigma Chemical Co.), cysteine-HCl (Calbiochem-Behring Corp., American Hoeckst Corp., San Diego, CA), and EDTA (J. T. Baker Chemical Co., Phillipsburg, NJ) were used. Tropomyosin was a generous gift from Dr. G. Phillips (Brandeis University). Purple membrane (isolated from the R_1 strain of *Halobacterium halobium*) was kindly provided by Dr. W. Stoeckenius (University of California at San Francisco) as a flash-frozen suspension in 40% aqueous sucrose solution containing 0.2% sodium azide. Frozen purple membrane was thawed and washed five times with distilled water before use. The purity of purple membrane, as determined by polyacrylimide gel electrophoresis, was >99%.

Papain Cleavage of Bacteriorhodopsin

The papain-cleavage method for bacteriorhodoprin is derived from the ones described by Ovchinnikov et al. (1979) and Katre et al. (1981). Purple membrane was suspended at a concentration of 0.5 mg/ml in buffer at pH 6.8. Cleavage buffer was composed of 10 ml of 10 mM EDTA, 10 ml of 50 mM cysteine-HCl, 0.1 ml of 70 mM 2-mecaptoethenol, and 70-ml distilled water. The final pH of this mixture was adjusted by the addition of NaOH. Papain, dissolved in the same buffer at 0.1 mg/ml, was added to the membrane suspension to a final concentration of 0.0065 mg/ml. The final suspension was incubated at room temperature for 2 h, and then washed five times with distilled water. The homogeneity of the purple membrane cleavage products was checked by polyacrylimide gel electrophoresis.

Triton X-100 Solubilization of Bacteriorhodopsin

The basic method used for Triton X-100 solubilization of bacteriorhodopsin follows that described by Hwang et al. (1977). 10-mg purple membrane or papain-cleaved purple membrane in H_2O was centrifuged at 50,000 g for 40 min. The pellet was resuspended in 0.4 ml of 0.1 M acetate buffer, pH 5.0, containing 5% Triton X-100. The suspension was kept in the dark at room temperature for 2 d, and then centrifuged at 100,000 g for 30 min. The pellet, if any, was discarded. Immediately before use, the suspension was diluted with distilled water to a final Triton concentration of 0.2 mg/ml. After the circular dichroism of this suspension was measured, the suspension was centrifuged again at 100,000 g for 30 min to assure, through the absence of any visible pellet, that there had been no aggregation of bacteriorhodopsin at the lower Triton concentration.

Concentration Determination of Bacteriorhodopsin

The concentration of bacteriorhodopsin was measured from the absorbance spectrum of light-adapted purple membrane at 568 nm using a Cary 14 spectrophotometer (Varian Associates, Inc., Palo Alto, CA) with scattering correction attachment in the laboratory of Dr. W. Stoeckenius. The molar extinction coefficient for light-adapted bacteriorhodopsin was assumed to be 63,000 (Rehorek and Heyn, 1979). The bacteriorhodopsin concentrations of papain-cleaved and of Triton X-100 treated purple membrane were calculated from volumetric dilutions. The concentrations of the specimens used in circular dichroism (CD) experiments were also measured by direct amino acid analysis determinations. The agreement of the concentrations determined by these two methods was always within 5%.

Circular Dichroism (CD) and Fluorescence Detected Circular Dichroism (FDCD) Spectroscopy

CD and FDCD spectra were obtained between 190 and 240 nm using a modified Cary 6001 dichrograph (Varian Associates, Inc.) (Dorman et al., 1973; Reich et al., 1980). The absorbance spectra used in the data analysis to obtain the FDCD spectrum were measured using a Cary 14 spectrophotometer (Varian Associates, Inc.) The dichrograph was calibrated using a standard solution of *d*-10-camphorsulfonic acid. CD and FDCD spectra were obtained from the average of several runs and were repeated with at least two different samples. The final spectra, after conversion to molar ellipticities, were fitted by a linear combination of three different basis functions for α-helix, β-sheet, and random coil. Different basis functions for the α-helix spectrum were used, corresponding to different numbers of residues per helix as described by Chen et al. (1974). These basis functions (for 10-residue helices, 20-residue helices, β-sheet, and random coil, respectively) are reproduced in Fig. 1. The percentages of α, β, and random coil were constrained to add up to unity as part of the least-square fitting program. Our goal in using different α-helix basis functions was to determine both how well the experimental data could be accounted for, using the basis function for a 20-residue helix, and to determine the number of residues for which the theoretical spectrum would give the best fit to the data.

Infrared Spectroscopy

Infrared spectra were measured using an infrared spectrometer (283; Perkin-Elmer Corp., Instrument Div., Norwalk, CT). Frequency calibration was checked by using a standard polystyrene film (Perkin-Elmer Corp., Instrument Div.). Purple membrane samples suspended in distilled water were deposited evenly on CaF_2 windows and then air dried. The purple membrane spectrum was measured from 1,100 cm^{-1} to 2,000 cm^{-1} in the normal scan mode. High-resolution scans were also done over the range from 1,600 cm^{-1} to 1,700 cm^{-1}. Tropomyosin, dissolved in 0.6 M KCl at pH 6.5, was also deposited on a CaF_2 window and then air dried. The spectrum was measured in the normal scan mode. High-resolution spectra in the amide I band were decomposed into a sum of Lorentzian functions by a least-square program that determined the peak height, peak position, and peak width, and that also determined the number of Lorentzian functions required to get a good fit to the experimental data.

Computer Simulation of Images of α-Helix and β-Sheet in Projection

The coordinates of the atoms used for both α-helix (Parry and Suzuki, 1969) and β-sheet (Arnott et al., 1967) model structures were those for poly-*l*-alanine. Image simulation was carried out for three different structural models. The first model consisted of three α-helices, 24 residues in length, that were tilted and positioned such that their helix axes passed

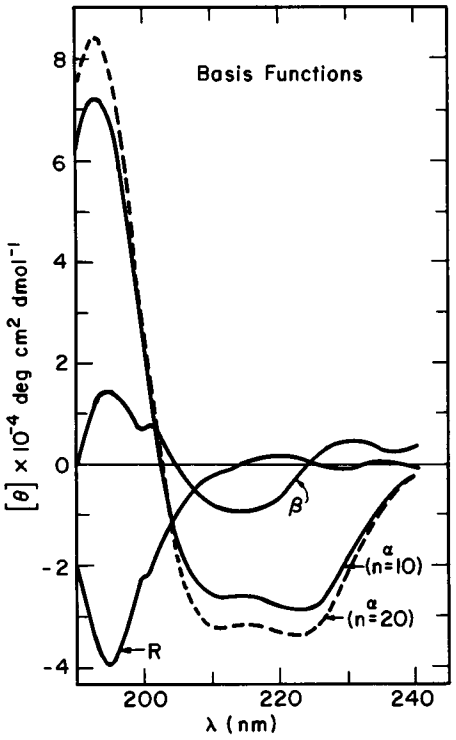

FIGURE 1 Basis functions used for circular dichroism spectra representing beta sheet (β), random coil (R), and alpha helices (α) with chain lengths of 10 and 20 residues obtained from Chen et al. (1974).

through the midpoints of the contour maxima at $Z = 15$ Å and $Z = -10$ Å in the three-dimensional model of Henderson and Unwin (1975) (Unwin, personal communication), in the region we are referring to as the outer slur at the positions referred to as helices 1, 2, and 3 by Engelman et al. (1980). The second model consisted of three identical, parallel coplanar helices containing 24 residues each, separated by a center-to-center distance of 10 Å. The third, β-sheet model consisted of four anti-parallel, coplanar strands consisting of 11 residues each. To get a better idea of how the image might depend upon the tilt angle of the models relative to the membrane plane, model two and model three were rotated in 5° steps about an axis lying in the plane of the membrane, which was perpendicular to the plane of the three helices or the plane of the β-sheet, respectively.

When computing the model images, the Fourier spectrum of the projected potential for model structures was limited to a resolution of 4 Å, the F_0 term was deleted, and the spectrum was weighted by the same resolution-dependent figure of merit that applied to the high-resolution map of Hayward and Stroud (1981). The increment in contour levels was adjusted such that the projected potential for an untilted α-helix in the model image is represented by eight contour levels, which is identical to the inner (untilted) helices of the bacteriorhodopsin trimer in the experimental image of purple membrane.

RESULTS

The projected potential maps for both α-helix and β-sheet models at various tilt angles are shown in Fig. 2 together with (*a*) the experimental image of the outer slur region of bacteriorhodopsin, which has been reproduced from the high-resolution map of Hayward and Stroud (1981), and (*b*) the model image of α-helices fitted to the three-dimensional contour map of Henderson and Unwin (1975). None of the projections for the α-helical models account for the general appearance of the outer slur. In particular, none of these calculated projections reproduce the narrow, sharp peak at the center of the slur. The projected potential for the β-sheet model structure at a tilt angle of between 10° and 15° consists of a fairly narrow, continuous stretch of density that seems qualitatively to be in much better agreement with the right-hand two-thirds of slur region.

CD and FDCD spectra of native purple membrane are shown in Fig. 3. There is very little difference between the two spectra, suggesting that any effects due to light scattering (which would be experimentally compensated in the FDCD spectrum) must already be insignificant in the

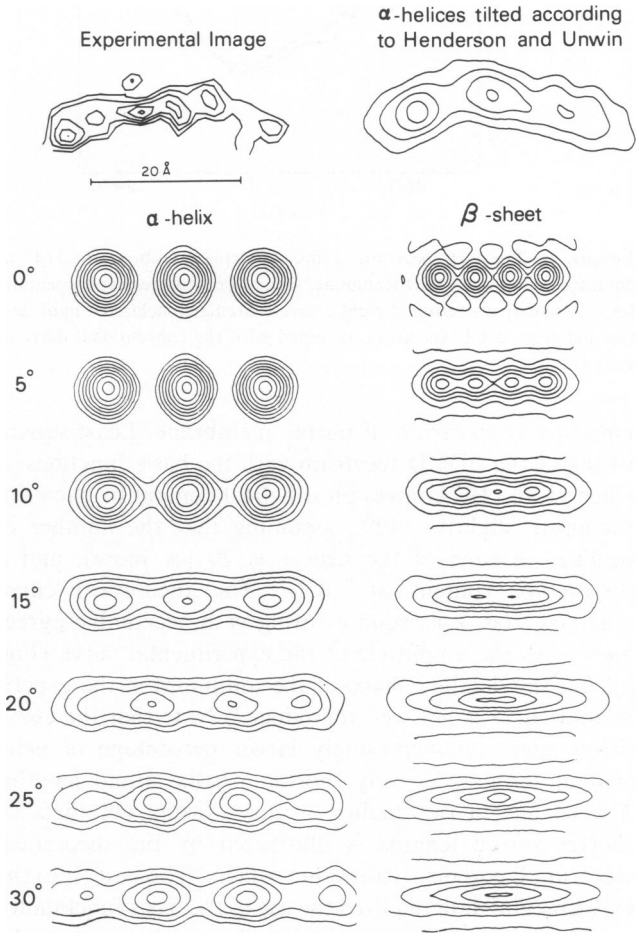

FIGURE 2 Contour display of the outer slur region obtained from the high-resolution map of the in-plane projection of bacteriorhodopsin at a resolution of 3.7 Å, reproduced with minor modifications from the data of Hayward and Stroud (1981), together with contour maps of projections for three types of model structures as described in the text. Tilt angles for the model structures are indicated on the left-hand column. The projections of the model structures were calculated using a 99 by 99 array with an increment corresponding to 0.5 Å. The displayed portions of the contour plots of the model structures have been limited to a rectangle, 40 by 10 Å, for the three-helix model and 30 by 10 Å for the β-sheet model. These dimensions correspond, respectively, to the whole of the outer slur or to the portion that remains if one-third of the outer slur is assigned an α-helical region.

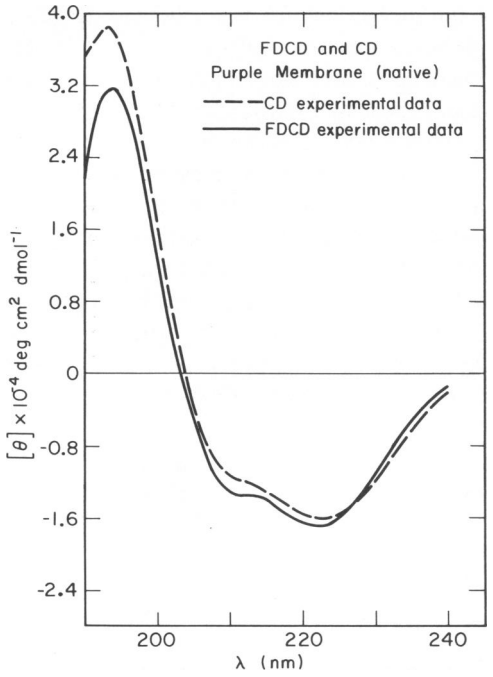

FIGURE 3 The CD spectrum of intact purple membrane in H_2O as obtained using the FDCD technique, which compensates experimentally for differential scattering of right and left circularly polarized light, and the uncorrected CD spectrum, obtained with the conventional detector system.

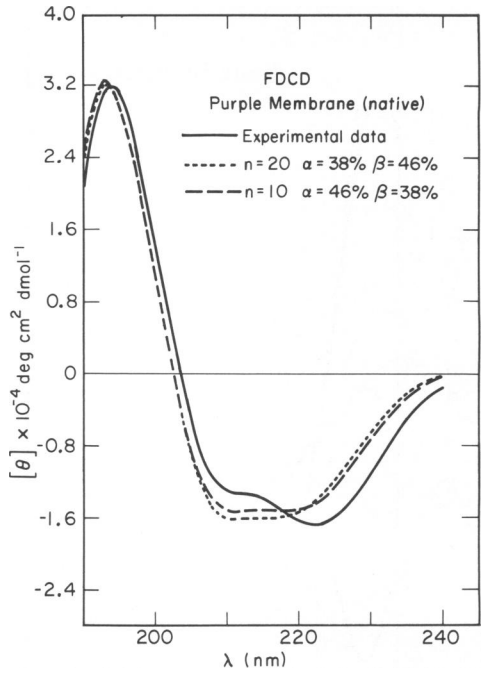

FIGURE 4 FDCD spectrum of purple membrane and the theoretical curves representing the least-square best fit with the Chen et al. (1974) basis functions. The theoretical curves are shown for two different α-helical chain lengths, corresponding to 10 and 20 residues, respectively.

normal CD spectrum of purple membrane. Least-square fitting of the FDCD spectrum with the basis functions of Chen et al. (1974) gives an α-helical content of bacteriorhodopsin slightly <40%, assuming that the number of residues in each of the helices is 20 (or more), and a β-structure content of ~45%. The theoretical curve obtained from least-square fitting is in acceptable agreement with the amplitude of the experimental curve (Fig. 4), but the detailed shape of the minimum in the experimental curve is not well reproduced. Note that the curve fitting gives an increasingly larger percentage of helix content for progressively shorter α-helix strand lengths. The change in the quality of curve fitting obtained for shorter strand lengths is illustrated by the theoretical spectrum based on a 10-residue α-helix. The best fit to the experimental data required the use of the basis function for α-helixes only 5 residues in length, which seems to be physically unreasonable in the case of bacteriorhodopsin. X-ray diffraction, electron microscopy, and amino acid sequence data all suggest that the α-helices in bacteriorhodopsin should be at least 20 residues in length.

CD spectra of purple membrane solubilized in Triton X-100 were obtained down to 190 nm (Fig. 5). CD spectra of detergent-solubilized membrane proteins are expected to have the advantage of not being influenced by light-scattering or absorption-flattening effects, but on the other hand, there is the danger that the conformation of the protein itself can be altered by interaction with the deter-

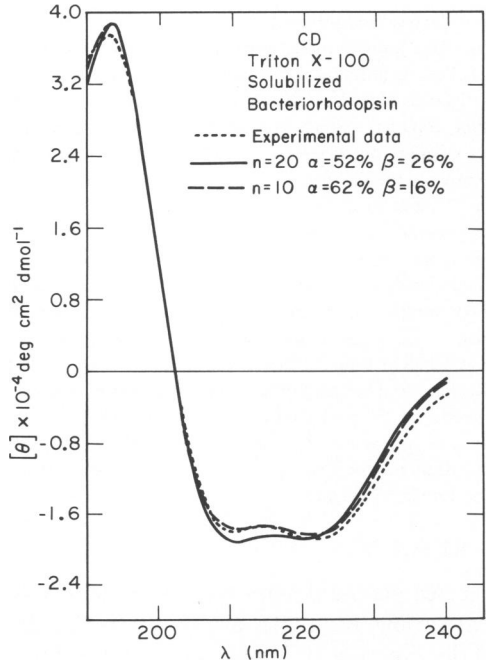

FIGURE 5 CD spectrum of purple membrane solubilized in Triton X-100 and the theoretical, least-square best-fit curves obtained by using the Chen et al. basis functions for α-helical chain lengths of 10 and 20 residues.

gent. The molar ellipticity at 222 nm has a magnitude ~0.8 times that previously reported by Reynolds and Stoeckenius (1977), and ~12% smaller than that reported by Huang et al. (1981). Least-square curve fitting of the spectrum gives an α-helical content of 52% (assuming again that the number of residues in each helix is at least 20) and a β-sheet content of 26%. The least-square best fit to the basis functions is in very good agreement with the observed data in this case (Fig. 5).

The CD spectrum of papain-cleaved purple membrane that was solubilized in Triton X-100 has also been measured down to 190 nm. The molar ellipticity, shown in Fig. 6, was calculated from the observed CD spectrum and the measured amino acid concentration of the papain-cleaved, solubilized specimen. The experimental molar ellipticity was then scaled by the factor 231:248, which is the ratio of amino acid residues in the cleaved and uncleaved proteins, respectively. The scaled, molar ellipticity is shown in Fig. 6, and represents the molar ellipticity expected for a protein with the same number of residues in the α-helix and β-sheet conformation as actually exists in the cleaved, solubilized protein, but that has an additional 17 residues in a completely nonchiral conformation. Polyacrylimide gel electrophoresis of the cleaved proteins confirmed that essentially 100% of the protein had been cleaved, presumably at Glu-231. Least-square curve fitting of the scaled spectrum, assuming there are at least 20 residues in each helix, gives a helix content of ~117 residues for papain-cleaved, Triton solubilized purple membrane. This is ~5% less helix content than obtained for uncleaved purple membrane in Triton X-100 and ~9% more α-helix than was inferred from the FDCD spectrum of intact membranes.

Infrared spectra of the air-dried purple membrane show an amide I peak at 1,662 and an amide II peak at 1,547 cm^{-1} (Fig. 7 *a*). Air-dried tropomyosin has an amide I peak at 1,657 cm^{-1}. As shown in Fig. 7 *b*, the high-resolution spectrum of the amide I band of purple membrane is decomposed into two Lorentzian bands using a least-square curve-fitting technique; the two bands are centered at 1,663 cm^{-1} (full width at half height equal to 33 cm^{-1}) and 1,639 cm^{-1} (width of 38 cm^{-1}).

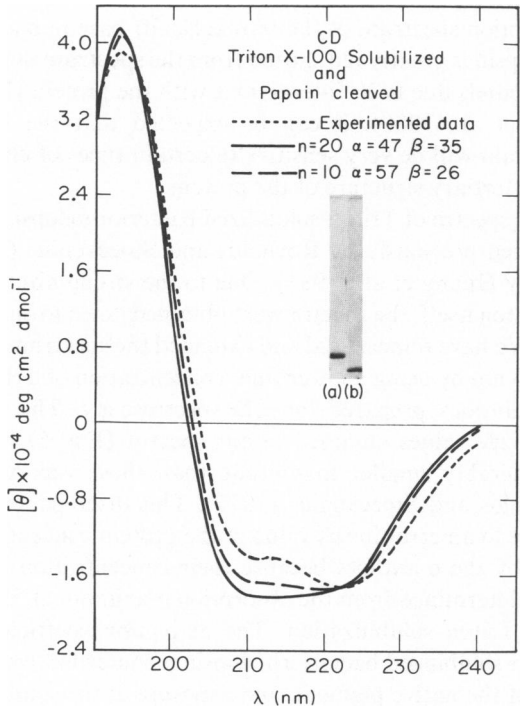

FIGURE 6 CD spectrum of purple membrane after papain cleavage and Triton X-100 solubilization, and the theoretical least-square best-fit curves using the Chen et al. basis functions for α-helical chain lengths of 10 and 20 residues. The experimental spectrum has been normalized by the factor 231:248 residues to provide a direct comparison with the spectrum of the uncleaved, native purple membrane. The insert shows the polyacrylimide gel patterns for (*a*) native purple membrane and (*b*) papain-cleaved purple membrane.

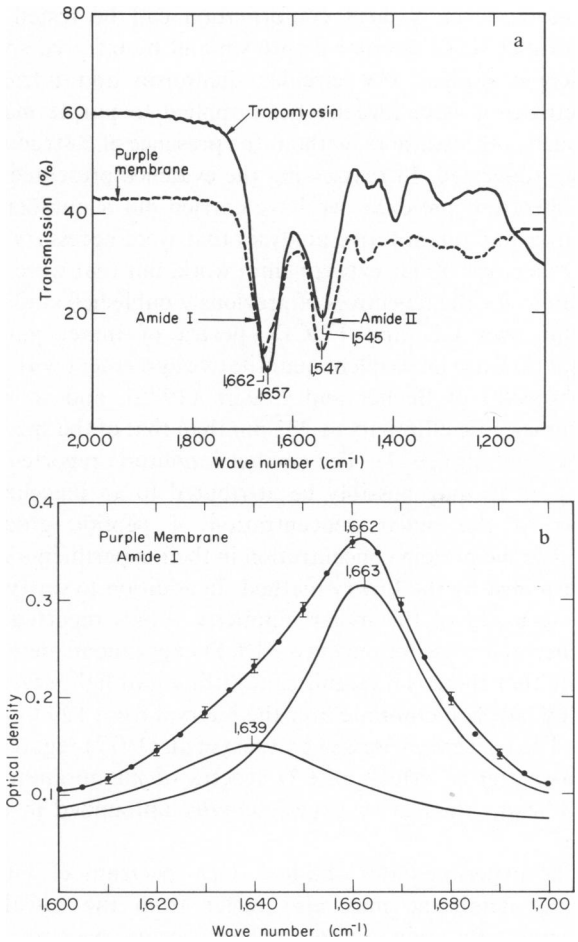

FIGURE 7 (*a*) Infrared spectrum of purple membrane over the range 1,200 cm^{-1} to 2,000 cm^{-1}, together with the spectrum of tropomyosin as a reference. The prominent peaks are the amide I and amide II bands. (*b*) High-resolution spectrum of the amide I band of purple membrane and the theoretical least-square best fit using Lorentzian functions as a basis set. The solid curves are theoretical functions, while the data points and error bars represent the sampled, experimental spectrum.

DISCUSSION

Our simple, three-helix models do not account for the high-resolution experimental image of the outer slur region in the projected structure of bacteriorhodopsin in a very satisfactory way. This fact suggests (but does not in any way prove) that at least two of the rodlike features in the 7 by 14-Å-resolution map of Henderson and Unwin (1975) may not be α-helices, contrary to what has been previously thought. It is not uncommon that β-sheet domains cannot be distinguished from adjacent helices in three-dimensional crystallographic models at 6 or 7-Å resolution. Whether the rodlike features in the 7-Å map are helices or not could presumably be answered unambiguously by extending the high-resolution electron crystallographic structure analysis to three dimensions, but certain technical difficulties must still be overcome before a direct structure analysis of that type will be feasible.

The possibility that a significant fraction of the peptide chain is in the β-sheet conformation can be tested by ultraviolet (UV) circular dichroism and by infrared spectroscopic studies. UV circular dichroism and infrared spectroscopy have already been applied to purple membrane by other authors, without the presence of β-structure being suggested. In reassessing the evidence presented by the spectroscopic data, we have carried out a number of controls and quantitative analyses that were necessary for the objectives of our experimental work, but that were not required for the objectives of previously published studies.

Our own CD and FDCD spectra of intact purple membrane are in excellent quantitative agreement with the CD spectra of Becher and Cassim (1976), and show a larger specific ellipticity at 222 nm than that of the spectra of Long et al. (1977). The smaller amplitude reported by Long et al. may possibly be attributed to an inaccurate value of the molar concentration of peptide groups, because the protein concentration in their experiments was determined by the Lowry method. In addition to verifying the accuracy of the molar ellipticity values reported by Becher and Cassim, our own FDCD experiments demonstrate that there is no significant differential light scattering by purple membrane over the interval from 190 to 240 nm. The concern expressed by Long et al. (1977) regarding light-scattering effects in CD spectra of membrane proteins seems thus to be experimentally unfounded in this case.

The experimentally obtained CD spectrum of intact purple membrane may also suffer from the so-called absorption-flattening effect, which occurs because the chromophore is concentrated within particulate matter rather than being homogeneously distributed in solution (Duysens, 1956). The situation is similar to the case commonly known in infrared spectroscopy of dry films, where it is difficult to obtain specimens with a uniform thickness (Jones, 1952). Long et al. (1977) have applied the "pseudo-reference state" method of Urry (1972) to correct for both light-scattering and absorption-flattening effects. Bacteriorhodopsin that was solubilized in sodium dodecyl sulfate (SDS) followed by dilution with trifluoroethanol was used as the pseudoreference state material. Whether these solvent systems induced a change in the bacteriorhodopsin secondary structure, as they are known to do for some other proteins, was not determined. The results obtained by Long et al. do not, in any event, agree with our own results obtained with the FDCD method, which indicate that light scattering plays a negligible role in the CD spectra of intact purple membrane, or with our results obtained for papain-cleaved, Triton-solubilized purple membrane, which indicate that there is no major distortion of the spectrum due to absorption flattening.

The absorption-flattening effect can be eliminated, of course, by recording CD spectra of detergent solubilized, monomeric membrane proteins. This approach is expected to give valid information about the protein's secondary structure, provided that no change in secondary structure is induced by the detergent. In the case of bacteriorhodopsin, it would seem that the tertiary structure (and thus necessarily the secondary structure) of the protein near the retinal group is only slightly perturbed by solubilization of the protein in Triton X-100, since the visible absorption maximum is only slightly shifted from that of native purple membrane (Casadio et al., 1980). Because the visible absorption spectrum of the retinal Schiff base in bacteriorhodopsin is greatly red shifted from the spectrum of model compounds due to its interactions with the protein (Nakanishi et al., 1980), it can be expected that the visible spectrum will be very sensitive to certain types of changes in the tertiary structure of the protein.

CD spectra of Triton-solubilized bacteriorhodopsin were obtained previously by Reynolds and Stoeckenius (1977) and by Huang et al. (1981). Due to the strong absorption by Triton itself, the spectra were obtained down to only 208 nm. We have remeasured and extended the spectrum down to 190 nm by using a lower final concentration of Triton in the solutions prepared for CD spectroscopy. The molar ellipticity values obtained in our spectra (Fig. 5) have a considerably smaller magnitude than those reported by Reynolds and Stoeckenius (1977). This discrepancy may be due to an error in the value of the protein concentration used by these authors because their concentration values were determined from the absorption maximum at 555 nm after Triton solubilization. The extinction coefficient of Triton-solubilized bacteriorhodopsin is somewhat less than that of the native pigment, and exposure of the solubilized protein to light also leads to bleaching (Casadio et al., 1980). Both factors could lead to an underestimation of the protein concentration with a concomitant overestimation of the molar ellipticity. The molar ellipticity values in our spectra of Triton-solubilized bacteriorhodopsin have also a somewhat smaller magnitude than those reported by Huang et al. (1981). This discrepancy might be due to small inaccuracies in the assumption made by Huang et al.

that the absorbance at 280 nm for bacteriorhodopsin solubilized in SDS is independent of prior exposure of the protein to other detergents (e.g., Triton).

The CD spectrum of Triton-solubilized bacteriorhodopsin has a somewhat larger amplitude than the spectrum of native membranes, and the shape of the CD minimum is significantly different. Curve fitting of the Chen et al. (1974) basis functions to our spectrum of solubilized purple membrane (Fig. 5), using the basis function for a 20-residue helix as before, gives an α-helix content of 52% and a β-sheet content of 26%. The change in the shape and amplitude of the spectrum and in the concomitant estimate of α-helix content may be due to elimination of an absorption-flattening effect. However, it is also necessary to consider that the detergent may induce changes in the secondary structure of the protein, such as a change in conformation of random coil segments of the peptide to α-helix (or β-sheet) conformations or a change in the long-range twist of a preexisting β-sheet domain.

The 17 residue carboxy-terminal segment of bacteriorhodopsin, which can be cleaved by papain, is a possible example of a domain whose secondary structure could be altered without causing a change in tertiary structure that would result in a significant change in the visible spectrum of the protein. As a control experiment we have therefore recorded the CD spectrum of papain-cleaved, Triton-solubilized bacteriorhodopsin. The magnitude of the CD spectrum is indeed decreased by the papain-cleavage step, and the spectrum of cleaved, solubilized bacteriorhodopsin is almost indistinguishable from the spectrum of native purple membrane. The theoretical curves generated by the least-squares fitting routine are noticeably different for the two cases, however. One admissible interpretation of this control experiment would be that the carboxy-terminal chain is indeed in a nonchiral (random coil) configuration in the native protein and is folded into a helical segment in the detergent solubilized form.

A second, admissible hypothesis offered as an explanation for all of the CD spectra is that (*a*) the long-range twist of the β-sheet domain of both the native protein and the papain-cleaved, Triton-solubilized protein are similar to each other, but that (*b*) the long-range twist of this domain in the uncleaved protein is altered by interaction of the protein with detergent. A further requirement of this explanation is that the position of the CD minimum of the β-sheet in the former case must be blue shifted from that of the standard β-sheet spectrum, thereby accounting both for the deepened trough at 222 nm relative to that at 208 nm and for the inability of the standard basis functions to account for the detailed shape of the spectrum. On the other hand, the CD spectrum of the distorted β-sheet in the uncleaved, solubilized protein must be close to that of the standard β-sheet spectrum (see Fig. 1) to account both for the more equal trough depths and for the ability of the standard basis functions to accurately represent the experimental spectrum. Absorption flattening cannot account for the flattened depth of the 208-nm trough (relative to the 222-nm trough) in the cleaved, solubilized protein because the sample is a homogeneous, molecular solution; we are inclined to feel that absorption flattening is also unimportant in the native membranes. CD spectra of three high β proteins (RNase S, elastase, and concanavalin A) clearly demonstrate that the position of the single CD minimum can be considerably blue shifted or red shifted (from the standard position of 217 nm) depending upon the global twist of the β-sheet domain (Chang et al., 1978), and these experimental results are an important reason why we feel the hypothesis discussed above is an admissable one.

Great caution must always be exercised in quantitative estimation of protein secondary structure based upon CD spectra. In addition to the potential occurrence of several random and systematic errors, which have already been discussed above, neither the use of reference values of ellipticity at specific wavelengths nor the use of empirical basis functions gives a perfect fit to either the known secondary structure or the CD spectra of other soluble proteins (Chen et al., 1974). Using either the molar ellipticity at 222 nm or a complete curve-fitting procedure, the α-helix content is usually obtained with an accuracy of a few percent, provided that the average number of residues in the standard is commensurate with that in the unknown (Chen et al., 1974). The estimates of β-sheet content, on the other hand, are often much more unreliable; the percentage of β-sheet can be overestimated by as much as 20% or more of the entire peptide content of individual proteins (Chen et al., 1974). The chirality of β-sheet domains and their concomitant CD spectra can be easily changed, of course, by variations in long-range twist, while the chiral structure of helices has far less flexibility. It is tempting to suggest that these differences may account for the relatively reliable estimates of the helix content and the relatively unreliable estimates of the β-sheet content. We therefore believe that our UV circular dichroism spectra provide reliable evidence that the α-helix content of native bacteriorhodopsin cannot be much greater than ~124 residues (50% of the structure). However, the CD spectrum cannot, by itself, be taken as irrefutable evidence that a significant portion of the structure of bacteriorhodopsin is in the β-sheet conformation.

Qualitative confirmation that bacteriorhodopsin does indeed contain a significant amount of β-structure is provided by the 1,639 cm^{-1} component, occurring as a shoulder on the amide I band in the infrared spectrum. The normal position for the amide I band is at ~1,652 cm^{-1} for α-helix, ~1,656 cm^{-1} for random coil, and ~1,630 cm^{-1} for β-sheet (Miyazawa and Blout, 1961; Susi et al., 1967). The existence of a distinct, long-wavelength shoulder in the amide I band (as well as the anomalous blue shift of the entire amide I band) has been noted previously in spectra of sucrose-embedded (air-dried) purple membrane (Rothschild and Clark, 1979), in spectra of purple membrane suspended in D_2O (Rothschild and Clark, 1979; Cortijo et

al., 1982), and in spectra of purple membrane suspended in H_2O (Cortijo et al., 1982). In the earlier studies other alternative assignments for the origin of the shoulder were proposed (which cannot be invoked in our own case) and the possibility of β-structure was not considered, but Cortijo et al. (1982) have suggested that the shoulder might be at least partially attributed to "a contribution from the anti-parallel β-sheet structure which is small but not negligible." The suggested assignment of the 1,639 cm^{-1} band as being due to strands of β-sheet running across the membrane can be tested by measuring the its linear dichroism (Miyazawa and Blout, 1961). Supercoiling of the α-helices in bacteriorhodopsin apparently cannot account for the observed blue shift because tropomyosin, a coiled-coil α-helical protein, shows an amide I band at the more normal position of 1,657 cm^{-1}. The suggestion has been made recently by Krimm and Dwivedi (1982) that the blue shift of the amide I band may indicate the presence of α_{II}, for which the plane of the peptide group is tilted out from the helix axis.

A model of the secondary structure of bacteriorhodopsin consisting of four α-helices, a possible fifth helix, and four strands of β-sheet would be compatible with our spectroscopic data and with the high-resolution map of Hayward and Stroud (1981). A possible assignment of positions of α-helix and the β-sheet domains, within the high-resolution map of Hayward and Stroud, are identified in Fig. 8. Assuming an average strand length of 24 residues for the α-helices and an average strand length of 11 residues for the β-sheet domains, such a model would consist of ~50% (120 residues) of α-helix and ~18% (44 residues) of β-sheet. Our spectroscopic data are not consistent, however, with structural models containing seven α-helical rods. These models require a helix content of 70% or more, and they do not account for the spectroscopic evidence that suggests that substantial β-sheet is present in this protein. A maximum of six β-turns containing four residues each could exist in a seven-helix model, and the maximum value of 24 such residues is insufficient to account for the observed spectroscopic data. Quantitative interpretation of x-ray diffraction intensities suggests that there are 4 to 7 α-helical rods with an average length of ~30 to 35 Å, which is compatible with both types of model. There apparently is no clear evidence for β-structure in the x-ray patterns, but the meridional reflections from β-structures can be quite weak, while the equatorial reflections fall at about the same place as those from the close packing of the tails of the membrane lipids.

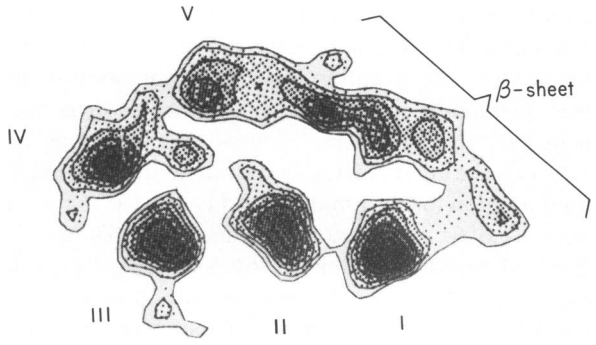

FIGURE 8 Two-dimensional high-resolution projection of a single molecule of bacteriorhodopsin. The map shows a possible domain for the proposed β-sheet structure. I to IV are the regions of α-helices and V could also be a helical region.

Membrane proteins, similar to water-soluble proteins, may be expected to come in different sizes, shapes, and secondary conformations appropriate to their different functional roles. Porin, a bacterial outer-membrane protein, is an example of a protein that has been shown to contain largely β-structure (Nakamura et al., 1974; Rosenbusch, 1974; Garavito et al., 1982). Other membrane proteins may ultimately be found to have a substantial content of both α-helix and β-structure. An hypothesis of peptide-chain folding and insertion into membranes was recently proposed (Engelman and Steitz, 1981), suggesting that only helical conformations are likely to occur in the interior of membranes. It is difficult to assess the general validity of this hypothesis, since the number of examples of membrane proteins whose secondary structure is well characterized is still quite small. While the mechanisms of folding that are discussed by Engelman and Steitz (1981) quite probably play a role in the insertion of some membrane proteins, it is likely that other strategies are employed by β-sheet containing proteins, such as porin, and these alternative strategies would be available to a protein consisting of five helical rods and four strands of β-sheet.

As a final point, we believe that it is premature to build any specific models for the path of the peptide chain through five helical regions and through four strands of β-sheet based on the limited data that can be provided from spectroscopy and a single, high-resolution projection. Inspection of the primary sequence data (Khorana et al., 1979), however, reveals five rather long stretches that are likely to adopt an α-helical conformation (Glu-9 to Lys-30, Lys-41 to Gly-65, a reported papain-cleavage point [Ovchinnikov et al., 1979], Asp-104 to Lys-129, Arg-134 to Lys-159, and Arg-175 to Glu-194). In addition there are four shorter stretches of primary sequence (Asp-85 to Asp-96, Gly-195 to Glu-204, Thr-205 to Ala-215, and Lys-216 to Arg-225) that together with adjacent charged and polar amino acids are less obviously suitable as α-helical domains, but that clearly could be candidates for inclusion in a domain of β-sheet.

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