brief communication

What spectroscopy can still tell us about the secondary structure of bacteriorhodopsin

R. M. Glaeser, K. H. Downing, and B. K. Jap
Division of Cell and Molecular Biology, Lawrence Berkeley Laboratory, University of California, Berkeley, California 94720 USA

ABSTRACT The recently published model of the structure of bacteriorhodopsin (bR), developed by fitting the peptide chain to a high-resolution, three-dimensional density map, rules out the existence of transmembrane β-sheet and provides an accurate estimate of the helix content. The precise geometry of the dihedral angles in the helical regions of the polypeptide cannot yet be specified from the diffraction data, however. Published data on the circular dichroism (CD) spectrum between 190 and 240 nm, and the infrared (IR) spectrum in the amide I band suggest that the helical conformation in bR may be, for the most part, a rather unusual one. The precise structural model, which specifies the number of residues in transmembrane helices, can now be used as an additional constraint in seeking models of the helical conformation that are in quantitative agreement with the CD and IR spectroscopic data. Further spectroscopic measurements can also be used to determine whether there are changes in the unusual dihedral-angle conformation within the helices during the photocycle.

INTRODUCTION

The recent construction of a three-dimensional model of the polypeptide chain of bacteriorhodopsin (Henderson et al., 1990) leaves no doubt that the seven transmembrane segments previously seen in lower resolution maps (Henderson and Unwin, 1975; Leifer and Henderson, 1983; Tsygannik and Baldwin, 1987) are exclusively helical in nature, and that there is no transmembrane β-sheet present in this structure. The accurate estimation of secondary structure content made possible by the molecular model of bacteriorhodopsin (bR) raises a number of questions, however, of how to account for some unique features of the spectroscopic information that is determined by secondary structure.

The high-resolution projection image of bR exhibits very narrow, high-density feature in the positions of rods one and two (Hayward and Stroud, 1981) and these features are not replicated in calculated images of a polyalanine model of bR. This observation was the original basis for performing further circular dichroism and infrared spectroscopic measurements, which together led to the proposal that "rods one and two" might be transmembrane β-sheet rather than helices (Jap et al., 1983). The narrow, high-density features are even more clearly shown in the projection image derived from the recent three-dimensional data set of Henderson et al. (1990), shown here in Fig. 1 *a*. As one can see in Fig. 1 *b*, similar features are again not retained when the molecular model of Henderson et al. (1990) is simplified by reducing all residues except glycine and proline to alanine residues. This simplified molecular model, like the earlier polyalanine model, shows significantly broader, flatter density features than does the experimental image in the region of rods one and two. From this we now conclude that the comparison of an experimental projection image to that of a polyalanine model can give a misleading indication of the secondary structure content, when the direction of projection in the image is not parallel to a helix axis, the reason being that side-chain densities can significantly alter and even narrow, rather than broaden, the positive contours. On the other hand, projection images very clearly reveal long, transmembrane helices when the direction of the projection is close to being parallel to the helix axis. This point is brought home by using the three-dimensional data set of structure factors of bR (Henderson et al., 1990) to calculate the projection image at a tilt angle of 21°, looking roughly down the axis of rods one and two. As shown in Fig. 1 *c*, the tilted helices now become well resolved from one another in this orientation, and they take on an appearance equivalent to that seen for helices 5, 6, and 7 in the projection image of untilted specimens.

Circular dichoism (CD) spectra in the far ultraviolet (190–240 nm) and infrared (IR) spectra in the amide I band both contain unusual features which indicate that the helical secondary structure conformation in bR is in some ways unlike that of standard α-helices or, for that matter, of any protein which has been extensively studied up to this point. Whereas it was proposed that one possible explanation for some of these features

0006-3495/91/04/934/05 $2.00

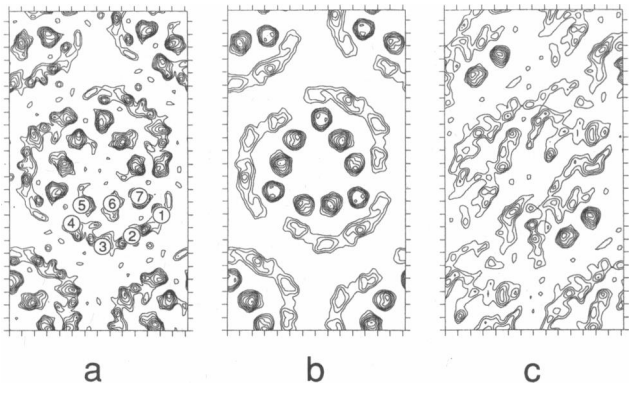

FIGURE 1 Projection images of bR (positive contours only). Projected density of an untilted specimen at 3.5 Å resolution, calculated from the experimental structure factors in the three-dimensional data set of Henderson et al. (1990). The approximate positions of helices 1–7, referred to in the text, are labeled on the figure. (*b*) Projected density of a molecular model of bR in which all amino acids except for glycine and proline have been converted to alanine. (*c*) Projected density of a specimen tilted by 21 degrees, looking roughly parallel to the axis of helices 1 and 2, calculated from the experimental structure factors in the three-dimensional data set of Henderson et al. (1990).

would be the presence of transmembrane β-sheet (Jap et al., 1983), that hypothesis is now ruled out by the chain-tracing model of the protein. The transmembrane β-sheet model is also ruled out by the recent analysis of the 1,640 cm^{-1} component of the amide I band, which fails to show the expected negative linear dichroism, and which is further shown to contain a contribution from the C = N stretch of the Schiff base (Earnest et al, 1990). It seems likely, therefore, that the anomalous features in this spectroscopic data might instead be used to tell us a great deal about the precise helical conformation, i.e., the dihedral angles of the residues making up the helices in bR, and thus aid in the ultimate refinement of our understanding of the structure and function of this protein.

Unique characteristics of the CD and IR spectra

We would point out, in particular, the following unique features of the CD and IR spectra: (*a*) the experimentally measured molar ellipticity of bR at 222 nm in purple membrane sheets (Becher and Cassim, 1976; Jap et al., 1983), or in detergent-solubilized form (Jap et al., 1983; Jap and Kong, 1986), appears to be in the range −16,000 to −19,000 deg-cm^2-$dmol^{-1}$, provided that an accurate measurement of the concentration of the specimen, rather than a computational renormalization procedure, is used to scale the experimental data. This is ~ 15–30% lower than one would expect if the helices had a molar ellipticity of 33,000, which is characteristic of an α-helical run of 20 residues or more (Chen et al., 1974). This comparison assumes that there are 165 residues in transmembrane helices and as many as six more residues in a short surface helix, as proposed by Henderson et al. (1990). It should be noted, in this regard, that a higher molar ellipticity, ~ −21,000 deg-cm-$dmol^{-1}$, is reported by Wallace and Teeters (1987) for bR reconstituted into small unilamellar vesicles (SUV). This higher value is attributed by Wallace and Teeters to the absence of absorption flattening and light scattering artifacts, but there is a rather good case that neither effect causes a significant distortion of the spectrum in the case of intact purple membrane as well (Glaeser and Jap, 1985).

(*b*) The amplitude of the CD band at 208 nm appears to be sensitive to small changes in the environment, and therefore presumably in the tertiary structure of bR. The 208 nm band is similar in magnitude to the CD band at 222 nm for detergent solubilized bR (Jap et al., 1983; Jap and Kong, 1986) and for some specimens of previously oriented films after the membrane orientation has been randomized by the addition of an organic solvent (Gibson and Cassim, 1989). The 208 nm band is significantly weaker than the 222 nm band in native purple membrane, even when the fluorescence-detected CD technique is used to rule out the possibility of light scattering as the cause (Jap et al., 1983). The 208 nm band is similarly depressed in papain-cleaved bR even after solubilization in detergent (again, eliminating light scattering or absorption flattening as an explanation) (Jap et al., 1983), in bR reconstituted into SUV (Wallace and Teeters, 1987), and in certain other films where the membrane orientation was again randomized by organic solvent (Gibson and Cassim, 1989).

(*c*) The CD band at 222 nm (more correctly, at 225 nm) should be extinguished for conventional α-helices in samples that are oriented with the helix axis parallel to the the optical beam. This band is actually very strong, however, in oriented films of purple membrane, and Gibson and Cassim (1989) report that an "average" helix tilt angle of 49° would be required to account for the measured amplitude of this band in oriented films. Such a large helix-tilt angle is incompatible with the known protein structure. Furthermore, one can rule out that the helices are so highly tilted due to a large angular/mosaic spread, because such a large range of tilt angles would introduce a large amplitude into the 207 nm band, which itself *is* completely extinguished in the oriented films. Thus, there is no question but that the orientation-dependence of the CD spectra of the helices

in bR indicates something unusual in the backbone conformation of these helices.

(*d*) The amide I band of the IR spectrum, assigned to a carbonyl stretching mode, is also anomalous in the sense that (*a*) the peak frequency is markedly blue shifted in comparison to other proteins (Rothschild and Clark, 1979*a,b*), (*b*) the amide I band is markedly asymmetric in shape and readily lends itself to decomposition into a major peak centered at 1663 cm^{-1} and a minor peak centered at 1,639 cm^{-1} (Jap et al., 1983), and (*c*) the peak frequencies of the parallel (A) and perpendicular (E_1) modes of the amide I band show a much larger splitting than is found in typical helices (see the discussion in Krimm and Dwivedi, 1982). To our knowledge, the peak position > 1,660 cm^{-1} is unique among natural proteins, the normal position for the α-helix being at 1,652 cm^{-1} and for the random coil being 1,656 cm^{-1}. Whereas the amide I band is blue-shifted beyond 1,660 cm^{-1} in some synthetic polypeptides, and relatively weak contributions to the amide I band are made at frequencies > 1,660 by β-sheet and β-turn amide groups (Holloway and Mantsch, 1989), we are unaware of such a high-frequency amide I peak for a complete, natural polypeptide other than alamethicin (Haris and Chapman, 1988). In 60% dimethylsulfoxide (DMSO) the amide I band of purple membrane shifts even further, to 1,665 cm^{-1}; the shoulder (i.e., asymmetric peak shape) is lost, indicating that the contribution made by the Schiff base (Earnest et al., 1990) must be a relatively small one; and the band width is greatly narrowed (Pande et al., 1989). In this case there is also clear evidence of a change in tertiary structure, in that the absorption peak in the visible shifts from 568 to 480 nm. This shift is reversible, however; the all-*trans* retinal itself remains bound in the form of a protonated Schiff base; and the protein remains in a two-dimensional crystalline lattice. As a result, *large* changes in tertiary structure, let alone changes in secondary structure content, would seem to be ruled out.

Krimm and Dwivedi (1982) proposed that the anomalous amide I peak position and the anomalous A, E_1 splitting might be explained by the majority of the bR helices being in the α_{II} rather than the α_I conformation. Gibson and Cassim (1989) have further noted that an α_{II}-like conformation would account for the simultaneous extinction of the 207 nm CD band and the retention of a high molar ellipticity in the 225 nm CD band of oriented helices because the plane of the peptide group is tilted away from the helix axis rather than being parallel to the axis as in the conventional, α_I conformation.

Polyalanine in hexafluoroisopropanol (Parrish and Blout, 1972), cited by Gibson and Cassim (1989) as an example of an α_{II}-like helix, is in fact a poor model of the secondary structure of the helices in bR, however. The amide I band of this polymer has a peak at 1,643 cm^{-1}, and thus this particular helical conformation could only account for, at most, a small portion of the polypeptide, corresponding perhaps to the minor band at 1,639 cm^{-1} in the bR spectrum. The CD spectrum of this model polypeptide has far too weak an amplitude at 222 nm to account for the molar ellipticity of bR at this wavelength, and the molar ellipticity of the 208 nm band is actually much stronger than that of the 222 nm band, which is the opposite of what is found for the helices in native purple membrane. Alamethicin is similarly a poor model of the secondary structure of bR, in spite of the good agreement in the amide I peak position of 1,662 cm^{-1} *and* in the occurrence of a shoulder at 1,640 cm^{-1} (Haris and Chapman, 1988), because both the 208 nm band and the 225 nm band in the CD spectrum are very nearly extinguished in oriented samples (Wu et al., 1990), unlike the case of oriented purple membrane films. Perhaps in the case of alamethicin the high frequency of the amide I band has something to do with the high content of α-aminoisobutyric acid, an amino acid which is not found in proteins.

Some future directions

The molecular model of the secondary structure content (percent helix and exact helix orientation) that has been derived from high resolution electron microscopy can be used to substantially increase the power of IR and CD spectroscopy to define the precise secondary structure conformation of the helices in bR. To begin with, the interpretation of spectroscopic data can now take the helix content to be a known parameter, ~165–170 residues out of 248, rather than an adjustable parameter to be determined from spectroscopy itself. Furthermore, in experiments with oriented membranes, the precise tilt angles of various helices and the number of residues in each such helix can be taken as known parameters, and quantitative attempts to explain the spectroscopic data must be compatible with these known, structural parameters. The challenge for biophysical spectroscopy thus shifts from an effort to estimate the percent helix, β-sheet, and random coil, and even the average orientation of components, to the more detailed question of the dihedral angles or the helix type within the identified helical regions. Given the marked anomalies in both the IR and the CD spectra of bR described above, there is some hope that a quantitative accounting of the anomalies could be a powerful tool in establishing helix conformation more precisely than perhaps crystallography alone may be able to do. For this reason it will be

important in future work to measure both the CD and the IR spectra, so that the explanation of anomalies that may be quantitatively accounted for in one of the measurements can also be shown to be quantitatively compatible with the second type of measurement.

It will be especially important in future work to determine whether there are changes in helix conformation, i.e., changes in dihedral angles, that occur during the photocycle and which may therefore play a role in protein function. Existing time-resolved Fourier transform infrared (FTIR) data (Braiman et al., 1991) as well as FTIR on the M-state intermediate trapped at low temperature (Ormos, 1990) show only very small changes in the amide I band, suggesting that there is little change in the helix conformation during the photocycle. Even so, it will be worthwhile to investigate further whether changes in the dihedral angles occur during formation of the M-state intermediate, which may account for the weak emergence of a 207 nm band in the CD spectra of oriented films. Data on this point may help to resolve the question of whether there are relatively large changes in helix tilt angle upon formation of the M-state intermediate (Draheim and Cassim, 1985), which could be due either to a change in the average helix tilt angle relative to the plane of the membrane or to an increased mosaic spread of the oriented sample, or whether changes in the amount of helix tilt are very small (Glaeser et al., 1986; Earnest et al., 1986; Dencher et al., 1989) but the helical secondary structure itself changes in an important way.

We thank Dr. Richard Henderson for providing the atomic coordinates of the model structure of bacteriorhodopsin and the three-dimensional structure factors before publication. We also thank Dr. Thomas Earnest for valuable contributions to our discussion of unusual features of the spectroscopic data.

This work was supported by the Office of Health and Environmental Research, United States Department of Energy, under contract no. DE-AC03-76SF00098 and by National Institutes of Health Program Project grant GM36884.

Received for publication 1 October 1990 and in final form 14 January 1991.

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