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THE C-TERMINUS OF BACTERIORHODOPSIN IS A RANDOM COIL

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The 21 amino acids which can be selectively removed from the carboxyl terminus of bacteriorhodopsin by proteolytic treatment are disordered in 2-dimensional arrays of the protein present in purple membranes. This C-terminal portion of the molecule may be involved in the efficiency and rate of light-driven proton uptake, although its presence is not required for pumping activity. In this study, the secondary structure of the C-terminus of bacteriorhodopsin has been determined by examining circular dichroism (CD) difference spectra derived from native and digested samples. In low ionic strength media, this part of the molecule appears to form a random coil-like structure. To examine if this structure is related to the structure found under the high ionic strength condition present in halobacteria, the CD spectra of native purple membranes in water and in 4 M salt solutions were compared. They were found to be identical, suggesting the conformation of the C-terminus in vivo may also be a random coil.

Introduction

Twenty-one C-terminal amino acids of the integral membrane protein bacteriorhodopsin are exposed at the cytoplasmic surface of the purple membrane of *Halobacterium halobium*, and are susceptible to proteolytic cleavage [1–3]. They can be selectively removed by tryptic digestion under controlled conditions which do not result in further cleavage of the protein [1]. This part of the molecule is not required for proton pumping, but may influence the rate of proton uptake and pumping efficiency [4], so its structure is of interest. The three-dimensional structure of the intact molecule has been determined at 7 Å resolution by electron microscopy and diffraction studies [5]. Difference Fourier analyses of electron and X-ray diffraction patterns of native and digested specimens have determined the location of the C-terminal 21 amino acids to be at one end of the molecule [1], described as near helices 1 and 2 [6]. Other studies by neutron diffraction [7] have suggested the C-terminal helix may be helix 3, but this does not bear on the location of the bulk of the 21 C-terminal amino acids. A major conclusion of the electron diffraction work [1] was that under low ionic strength conditions, the C-terminal portion of the molecule was almost completely disordered, being free to take up many positions relative to the rest of the molecule. NMR studies have also indicated the flexibility of this region of the molecule (Wallace, B.A., unpublished data). However, simply because the C-terminus is disordered, does not necessarily mean that it folds as a random coil. Rather, the diffraction and NMR data would also be consistent with a regular secondary structure, such as an alpha-helix or beta-sheet, which is connected to the membrane by a flexible hinge region. However, because this portion of the mole-

Abbreviation: CD, circular dichroism.

cule is disordered, the determination of its secondary structure has not been possible by electron or X-ray diffraction methods. Therefore, circular dichroism (CD) spectroscopy has been used in this study to elucidate the secondary structure of the C-terminus of the molecule in purple mebranes.

In addition, since the organism from which this protein is isolated, *H. halobium*, grows in 4 M NaCl [8] while maintaining a high ionic strength (mostly K^+) cytoplasm, it was also of interest to determine if the structure which is found under the low ionic strength conditions used in the diffraction [1,5,9] and spectroscopic experiments, pertains to the high salt condition in vivo. To examine this, CD spectra of native purple membranes in water and in 4 M salt solutions were compared; they were found to be identical, suggesting the conformation of the C-terminus in vivo may also be random coil.

Materials and Methods

Purple membranes were prepared from *H. halobium* as described earlier [8]. The native bacteriorhodopsin concentration was established by E_{568} (1 mg/ml, 1 cm) = 2.35 [10]; the digested bacteriorhodopsin concentration was estimated by Lowry assay using native bacteriorhodopsin as a standard. The concentrations determined for the digested samples were confirmed by absorption measurements at 568 nm (using 2.53 as the extinction coefficient for the digested protein).

Digested membranes were prepared and characterized as previously described [1], according to method II. They had 21 amino acids removed, without cleavage at any other points in the molecule.

For the parallel samples in deionized water and in 4 M NaCl or 4 M KCl (Fisher, certified grade), 0.1 ml of native purple membrane suspension (1.0 mg/ml) was diluted into 0.4 ml of either deionized water (pH 7.0) or 5 M NaCl (pH 6.7) or 5 M KCl solution (pH 7.0).

CD spectra were recorded on a Cary 60 spectropolarimeter fitted with a Model 6001 CD attachment and a variable position detector. The wavelength range scanned was usually 300–190 nm. However, solvent absorption did not permit accurate detection of spectra below 202 nm for the 4 M salt solutions, so the spectra of the parallel samples in deionized water were also collected only from 300–202 nm. The instrument was calibrated with (+)d-camphorsulfonic acid at 290 nm. The calibration at low wavelengths was checked with whale myoglobin. Spectra were obtained with the photomultiplier tube directly adjacent to the sample cell, resulting in an acceptance angle of approx. 90°, which limits differential scattering effects [11]. Some membranes were sonicated for 2 min in a circular bath sonicator (Laboratory Supplies), after flushing with N_2, to permit examination of the effects of differential scattering and flattening on the results [11]. Blank runs of deionized water or 4 M NaCl or KCl were subtracted from the appropriate sample spectra. In separate experiments, three different preparations of native and digested purple membranes were examined. The spectra reported are the average of three or four scans, calculated every 1 nm. Measurements were routinely made at 21°C using either 1 or 0.5 mm pathlength cells. Although lower wavelength measurements could have been made using shorter pathlength cells, this was not done because it would have required higher purple membrane concentrations; due to the tendency of the membranes to stick together at high concentrations [1], it was deemed unwise to subject them to these conditions. Under the conditions employed in this study, the membranes (as revealed by electron microscopy in the absence of stain) are isolated single membrane sheets. The light scattering characteristics of the native and digested samples were compared using absorption spectra recorded on a Cary 15 spectrophotometer in the wavelength range from 310 to 400 nm, where chromophore absorption is negligible [12].

The mean residue ellipticity of the fragment, $[\theta_f]$, is reported as:

$$[\theta_f] = (248[\theta_N] - 227[\theta_D])/20$$

where $[\theta_N]$ and $[\theta_D]$ are the mean residue ellipticities of the native and digested samples, respectively; 248 and 227 are the numbers of residues in the native and digested proteins; and 20 is the number of peptide bonds in the fragment.

Secondary structure was estimated by a non-linear (constrained) least-squares curve fitting proce-

dure [13], using three different reference sets based on data from either soluble proteins [14,15] or poly(L-lysine) [16]. The constrained fit required the fraction of each conformation to be non-negative, but did not require the sum of the fractions to be unity. The results were normalized to 100% by dividing each fraction by the sum of the fractions [13]. A normalized root-mean-square deviation (NRMSD) for each curve fitting was calculated as:

$$\mathrm{NRMSD} = \left\{ \frac{\sum_{N} (\theta_{exp} - \theta_{cal})^2}{\sum_{N} \theta_{exp}^2} \right\}^{1/2}$$

where θ_{exp} and θ_{cal} were the experimental and calculated mean residue ellipticities, respectively, and N was the number of data points used.

Results and Discussion

The C-terminal amino acids of bacterorhodopsin form a random coil

At least 19 of the 21 C-terminal amino acids of bacteriorhodopsin are disordered in purple membranes, such that they cannot be visualized by electron diffraction methods [1], and thus do not appear in the 3-dimensional model of the protein [5]. They could either adopt a floppy random coil conformation, or a more ordered alpha- or beta-type structure, but be joined to the remainder of the protein by a flexible hinge-type region. To distinguish between these two possibilities, circular dichroism spectroscopy has been employed to determine the secondary structure of the C-terminal tail of bacteriorhodopsin.

Twenty-one amino acids were quantitatively removed from the C-terminus of the molecule by tryptic digestion, without otherwise affecting the structure [1]. CD spectra of native and digested purple membranes were obtained (Fig. 1). The difference between these spectra, when properly scaled to reflect the different number of amino acids present in the two samples, is the spectrum of the C-terminal 21 amino acids (Fig. 2). It is a somewhat unusually shaped curve, clearly unlike that of either an alpha-helix or beta-sheet. Attempts have been made to fit the different spectrum to a standard reference data set of secondary

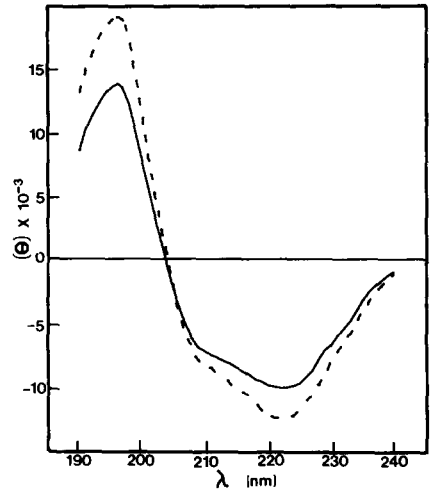

Fig. 1. Circular dichroism spectra of native purple membranes (———) and digested (minus 21 C-terminal amino acids) purple membranes (- - - - - -) at 21 °C. These spectra have not been corrected for absorption flattening effects, but a sample collection geometry has been utilized which minimizes light-scattering effects.

structural types. The results indicated the fragment is random coil in nature (Table I). However, the large values calculated for the normalized root-mean-square deviation fit parameter [10], indicated a relatively poor correspondence with the reference data. This result was not totally unexpected, since the random coil is not a structure

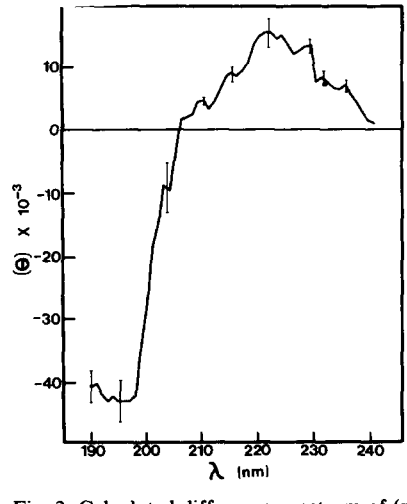

Fig. 2. Calculated difference spectrum of (native-digested) purple membranes (———). Error bars indicate standard deviations in measurements.

with unique angles ϕ and ψ, as is either the alpha-helix or beta-sheet, and thus its spectrum will be highly dependent on the exact geometry of the polypeptide backbone. This is evident from comparisons of the 'standard' curves for random coils obtained from different sets of soluble proteins [14,15] and poly(L-lysine) [16]. While the standard helix and sheet curves from these disparate sources are very similar, the random coil curves differ substantially. However, the best fit of the difference spectrum for the C-terminus to all three of these reference sets produces nearly identical results: it is almost entirely random coil in nature. Furthermore, the general shape of these curves (particularly that of random coil poly(L-lysine) [16]), are similar to the difference spectrum obtained. Thus, it is apparent that the C-terminus is not either beta sheet or alpha helix-like in structure, but is consistent with a random coil conformation. Furthermore, Chou- and Fasman-type calculations [17,18] were done to examine the tendency of this amino-acid sequence to form the different types of secondary structure. These calculations predict that this part of the molecule will be 28% helical (six residues), with the remainder (72%) being disordered, reasonably consistent with the experimental results obtained. Although such empirical prediction methods, derived using soluble protein data bases, should not be expected to give accurate results for hydrophobic membrane proteins, they may produce appropriate results because this is an aqueous-exposed portion of the molecule.

Since purple membranes are particulate structures, the extent of light-scattering and absorption-flattening effects on the spectra should be considered. The instrument detection geometry was chosen such that differential light scattering would be minimized and can be considered to be negligible [11]. That the scattering properties of both samples were identical was confirmed by measurements of the unpolarized absorption spectra in the 310–400 nm range [11,12]. Brief sonication under (presumably) non-denaturing conditions results in very small particles which do not exhibit substantial scattering [11]. While the magnitude of the resulting difference spectrum for the sonicated specimen is changed slightly from that in Fig. 2, the shape is not, so the secondary structure producing it must also be random coil. Since the same results are obtained for the intact membranes and the sonicated samples, this suggests the resulting random coil is not produced by the sonication procedure, nor is it an artifact of the scattering properties. The extent of the absorption flattening effect will depend on the particle size and the concentration of chromophores in each particle. Since the membrane size is unchanged by proteolysis (based on electron microscopy data, not shown) and the concentration of proteins in the membrane is unaltered (no disruption of the lattice [1]), the flattening will be the same for both native and digested samples, and no scaling factor is needed for the two spectra. The absolute magnitudes of the individual spectra, however, may be somewhat depressed due to the flattening, but the difference between these spectra will be largely unaffected.

In order to determine if the difference spectrum could be due merely to an inappropriate scaling of the data (despite careful protein assay vs. a bacteriorhodopsin standard), the digested data was scaled to the native by a factor such that:

$$\frac{[\theta_D]^{222}}{[\theta_N]^{222}} = 1$$

Under these conditions, if the difference were merely the result of an improper scaling factor or concentration, the difference curve should be featureless with $[\theta_f] = 0$ at all wavelengths. This was not the case, so the difference must be real.

Tryptic digestion under the conditions employed in this study does not result in cleavage of any other part of the molecule [1]. Furthermore, electron diffraction studies have shown that removal of the C-terminus changes the structure factor of the whole molecule by approx. 1% (total change = difference minus error level); all of this difference can be accounted for by the removal of one ordered amino acid with the remainder of the molecule being essentially unchanged by the proteolysis [1]. Therefore, the difference spectrum obtained in this study must be due only to the conformation of the portion of the molecule which was removed, the C-terminal 21 amino acids, and not to any other rearrangement within the molecule. Unlike studies on small polypeptide frag-

ments, which tend to be floppy and adopt different conformations as isolated fragments in solution than as part of intact molecules, this difference study examined the conformation of the 21 amino acid polypeptide fragment as it is found as part of the native protein.

The conformation of the C-terminal tail is unaffected by ionic strength

To date, the conformation of bacteriorhodopsin has been examined under low salt conditions. X-ray diffraction [1,9], circular dichroism (see above) and NMR spectroscopic studies were done on samples in deionized water; electron diffraction studies [1,5] employed samples which had been dehydrated from a 0.5% glucose solution in water. However, halobacteria grow in high-salt media (up to saturated NaCl) [8] and the ionic strength of their cytoplasm is similarly high (mostly as the potassium ion). Hence, the C-terminus is exposed to high ionic strength conditions in the cell interior [2] and thus, its conformation in deionized water might be altered from that in vivo. The negatively charged amino acids in this region of the molecule (4 out of 21) could be repelled by the negatively charged head groups of the lipid molecules [19] and the net negative charge of the remainder of the protein molecule on this surface [6]. This repulsion would be expected to be diminished in the high ionic strength medium, thus possibly fostering a more ordered structure. Conversely, hydrophobic interactions would be expected to increase with ionic strength so this type of attraction of the C-terminal amino acids with the cell membrane would be expected to increase under high salt conditions, also possibly resulting in increased order.

To examine if any conformational change occurs with increased ionic strength, CD spectra of membranes in deionized water and 4 M NaCl and 4 M KCl were examined in parallel experiments (Fig. 3). While the samples were unbuffered, the difference in pH of the three specimens was less than 0.3 pH units, so pH is unlikely to be a factor in these experiments. No differences above the error level (approx. 2%) were detected between the high and low ionic strength samples, thus suggesting no significant net change in the secondary structure of the molecule occurs, and indicating the conformation determined under low ionic strength conditions is similar to that in vivo (although, since CD detects the linear sum of all secondary structures present, the unlikely possibility of exactly compensating opposing changes must also be acknowledged).

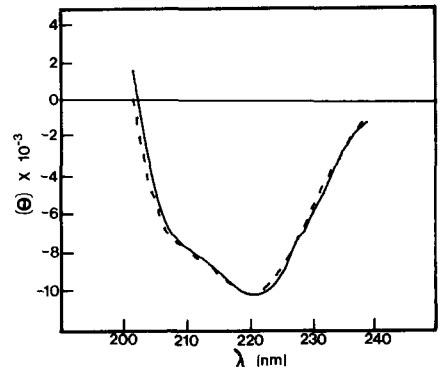

Fig. 3. Circular dichroism spectra of native purple membranes in deionized water (———) and in 4 M KCl (- - - - -).

In conclusion, the C-terminal 21 amino acids of bacteriorhodopsin were found to adopt a random coil conformation. This disordered portion of the molecule appears to be unaffected by ionic strength and so suggests strong electrostatic or hydrophobic interactions with the membrane do not impose constraints on its structure.

TABLE I

CALCULATED SECONDARY STRUCTURE OF BACTERIORHODOPSIN C-TERMINUS

NRMSD, normalized root-mean-square deviation.

Reference data set used	Normalized fractional secondary structures			NRMSD
	α-helix	β-sheet	Random coil	
Chen et al. [14]	0.00	0.00	1.00	0.48
Saxena and Wetlaufer [15]	0.00	0.04	0.96	0.47
Greenfield and Fasman [16]	0.00	0.00	1.00	0.43

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