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Secondary Structure of Halorhodopsin†

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ABSTRACT: Ultraviolet circular dichroism (CD) spectroscopy in the interval from 190 to 240 nm has been used to estimate the secondary structural content of halorhodopsin (hR), a light-driven chloride pump isolated from the membranes of *Halobacterium halobium*. Least-squares curve fitting of the CD spectrum for hR solubilized with octyl glucoside yields an α-helical content of $\sim$50% and a β-structure content of $\sim$30%. The CD spectrum of hR is unaffected by the absence or presence of chloride ions or by the ionic strength of the medium. The CD spectrum of halorhodopsin is very similar to that of bacteriorhodopsin, indicating that these light-driven pumps possess nearly identical fractions of α- and β-secondary structures.

Halorhodopsin (hR) is one of three retinal-containing proteins found in *Halobacterium halobium*. The biological function of hR is to serve as a light-driven, electrogenic chloride pump, transporting chloride ions inward across the membrane (Schobert & Lanyi, 1982). Very little is known about the precise physiological role of hR, about its structure, or about the molecular mechanism by which chloride is translocated across the membrane.

Halorhodopsin has many similarities and dissimilarities when compared to bacteriorhodopsin (bR). Both proteins contain a retinal moiety that is apparently in a similar environment as suggested by resonance Raman spectroscopy (Smith et al., 1984; Alshuth et al., 1985), indicating that structures surrounding the retinal in both of these proteins are very similar. On sodium dodecyl sulfate (SDS) gel electrophoresis, hR shows a slightly greater mobility than that of bR, suggesting that hR has a similar molecular mass (i.e., 26 000 daltons). The amino acid compositions of hR and bR are quite similar when residues are grouped as being hydrophobic, neutral, and polar (Ariki & Lanyi, 1984; Sugiyama & Mukohata, 1984). Proteolytic fragments of hR and bR are also similar when the proteins are partially digested with *Staphylococcus aureus* V8 protease (Hegemann et al., 1982). However, hR also shows distinct spectroscopic and photochemical properties. For example, in contrast to the case of bR, the photocycle intermediates and kinetics, as well as the position of hR absorption maximum, are affected by the presence of chloride ions (Ogurusu et al., 1982; Stoeckenius & Bogomolni, 1982; Lanyi & Schobert, 1983; Steiner et al., 1984). There is evidence that hR has a distinct chloride binding site (Schobert et al., 1983; Falke et al., 1984) involving a sulfhydryl group (Ariki & Lanyi, 1984). Furthermore, hR and bR lack immunological cross-reactivity (Hegemann et al., 1982), suggesting a significant difference in their primary sequences.

It was not known whether there is any similarity between the secondary structure of bR and hR. In this paper, we show from ultraviolet CD spectroscopy that these two proteins possess a similar secondary structural content.

Materials and Methods

The procedures for growth of *Halobacterium halobium* (JW-12 and R_1) and for isolation of purple membrane are those described by Oesterhelt & Stoeckenius (1974). The purification of hR follows the method described by Taylor et al. (1983). To ensure that a highly purified protein was obtained, isolated hR was rerun on a small hydroxylapatite column as described by Taylor et al. (1983). The purity of the isolated hR was determined by SDS gel electrophoresis. Fractions with no visible impurity as observed on SDS gels were collected and dialyzed in the dark against several changes of 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes)/3 M NaCl buffer (pH 7) at 4 °C. After removal of octyl D-β-glucopyranoside (Calbiochem) by dialysis, hR was dialyzed at 4 °C in the dark against two changes of 1 L of 20 mM sodium phosphate buffer (pH 7). The dialyzed hR was resolubilized for 6 h in 20 mM sodium phosphate, pH 7, containing 15 mM octyl D-β-glucopyranoside (octyl glucoside) and then immediately used for spectral studies.

The basic method used for solubilization of purple membrane follows that described by Dencher & Heyn (1978). Five milligrams of purple membrane in 20 mM sodium phosphate buffer (pH 6.9) was centrifuged at 50000*g* for 40 min. The pellet was resuspended in 20 mL of 20 mM phosphate buffer, pH 6.9, containing 1.3% octyl glucoside. The suspension was kept in the dark at room temperature ($\approx$22 °C) for 30 h and then centrifuged at 150000*g* (averaged value) for 30 min. The pellet, if any, was discarded.

Circular dichroism was measured with a Jasco J-500A spectropolarimeter. The dichrograph was calibrated with a

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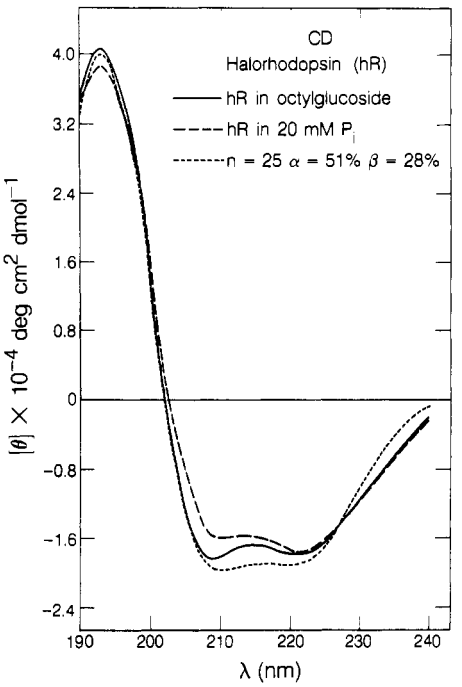

FIGURE 1: The CD spectra of halorhodopsin (hR) in 20 mM phosphate buffer (pH 7), with and without 15 mM octyl glucoside. The theoretical curve represents the least-squares best fit to the spectrum of hR in octyl glucoside, with the basis functions derived empirically by Chen et al. (1974) for α-helical chain lengths of 25 residues. $[\theta]$ and λ are respectively the mean residue ellipticity and the wavelength.

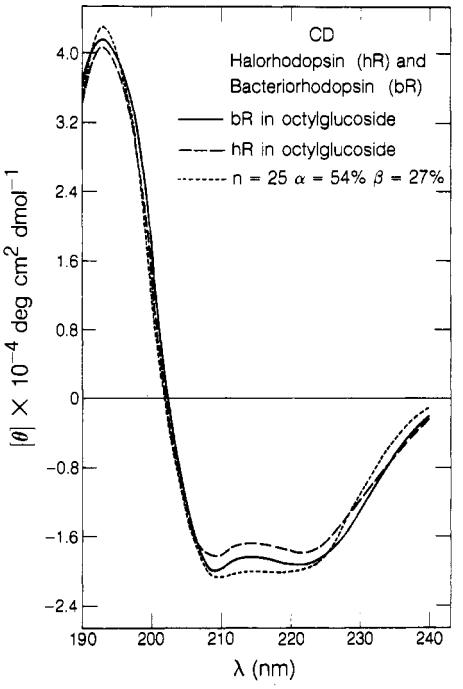

FIGURE 2: The CD spectra of hR and bR solubilized in octyl glucoside and the theoretical least-squares best fit curve to the bR spectrum obtained by using Chen et al. (1974) basis functions and assuming a helical chain length of 25 residues. The spectra of hR and bR show a substantial similarity, indicating that their secondary structural contents are similar.

standard solution of *d*-10-camphorsulfonic acid. The spectrum of each given hR (or bR) sample was obtained from an average of at least two scans at 5 °C. The spectra were scanned from 250 to 190 nm whenever possible. Spectra of hR in solution containing 3 M NaCl were scanned to only 203 nm because the solution has a very high absorbance below 203 nm. Samples after having undergone at least two scans gave no significant change in their CD spectra, indicating that hR is quite stable under our recording conditions.

The concentrations of hR and bR used in CD experiments were measured by direct amino acid analysis. The agreement of the final spectra obtained from different hR (or bR) samples as compared to the averaged spectra was within 5%. The concentration of bR in octyl glucoside was also determined volumetrically from the native bR concentration, which was obtained from the absorbance spectrum of light-adapted bR in 20 mM phosphate buffer, pH 6.9, at room temperature. The extinction coefficient of native bR under these conditions was assumed to be 63 000 (Rehorek & Heyn, 1979). The agreement of the concentrations of bR in octyl glucoside determined by the above two different methods was always within 5%.

The spectra, after conversion to molar ellipticity, were fitted by a linear combination of three different basis functions for α-helix, β-sheet, and unordered structure. Different α-helical basis functions corresponding to different helical chain lengths, as described by Chen et al. (1974), were used in our calculation. As a part of the least-squares fitting program, the sum of the fractions of α, β, and unordered structures were constrained to unity.

RESULTS

Circular dichroism spectra of hR, with and without octyl glucoside, are shown in Figure 1. The spectra are very similar in magnitude, but the detailed shapes of the curves are somewhat different. The spectrum in octyl glucoside is expected to have the advantage of being free from light scattering and absorption-flattening artifacts. The slightly larger CD magnitude at the region of 208 nm of hR in detergent could be the result of a small change in the structure of hR during solubiliztion and/or an artifact in the detergent-free sample due to light scattering and absorption flattening. Since hR without detergent forms aggregates that can be sedimented by centrifugation, the minor difference in the spectrum is probably due mainly to light scattering and absorption-flattening artifacts. Larger ellipticity values aroung the 208-nm peak have also been observed in detergent-solubilized bacteriorhodopsin as compared to that of the native protein (Jap et al., 1983).

Least-squares curve fitting of the spectra, with the basis functions of Chen et al. (1974), gives an α-helix content of 51% and β-structure of 28% for hR solubilized in octyl glucoside. The theoretical curves obtained from the least-squares fitting are in an acceptable agreement with the experimental data (Figure 1). The curve fitting was performed by using an α-helix basis function with an average chain length of 25 residues. When a 10-residue α-helix basis function is used, least-squares curve fitting of the spectrum yields an α-helix content of 60% and β-structure content of 17%. Note that the use of a progressively shorter helical basis function would give an increasingly larger percentage of helix.

The CD spectrum of hR was measured in buffer containing 3 M NaCl and 20 mM phosphate (pH 7) to examine if any conformational change was induced by an increase in ionic strength. The spectra of hR with and without 3 M NaCl are virtually identical (data not shown), indicating that low ionic strength and the absence of chloride ions have no significant effect on the secondary structure of hR.

The CD spectra of bR and hR solubilized in octyl glucoside are shown in Figure 2. The spectra are very similar in magnitude and in their detailed shapes, suggesting that hR and bR have very similar secondary structure contents.

Least-squares curve fitting of the CD spectrum of bR in octyl glucoside yields an α-helical content of 54% and β-structure content of 27%, assuming that the helices in bR have an average length of 25 residues. These percentage values are in a good agreement with those reported for bR in Triton X-100 (Jap et al., 1983). The α-helical content of bR in octyl glucoside reported here is smaller than the 69% reported by Dencher & Heyn (1978). The value reported by Dencher & Heyn (1978) was derived from the ellipticity at 222 nm, but neither the value of ellipticity at this wavelength nor the basis functions used were given in their paper. The ellipticity at 222 nm of bR in octyl glucoside measured here has a magnitude about 1.2 times larger than that previously reported by Mao & Wallace (1984), and the ratio of the magnitudes at 193 to 222 nm is about 2.2, which is significantly larger than that derived from the spectra of Mao & Wallace (1984).

Discussion

CD spectra obtained from detergent-solubilized membrane proteins can be expected to be free of light scattering and absorption-flattening artifacts. The spectra can, therefore, be expected to give a valid secondary structure content for the native proteins, provided that solubilization with detergent does not result in major structural changes. The spectroscopic and photochemical properties (such as absorption spectrum and photocycle) of octyl glucoside solubilized hR (or bR) resemble to those of hR (or bR) in the native state (Taylor et al., 1983; Dencher & Heyn, 1978), suggesting that the minor differences in the spectroscopic and photochemical properties may be the results of only a small shift in their tertiary structures and that their secondary structures may not be significantly affected. There remains a possibility, however unlikely, that the portions of the proteins exposed to the aqueous environment may be significantly altered during solubilization without grossly affecting their spectroscopic and photochemical properties.

The CD spectrum of bR solubilized in octyl glucoside has been reported previously by Mao & Wallace (1984). Because their spectrum is significantly different from that of bR solubilized in Triton X-100 (Jap et al., 1983), we have remeasured the spectrum of bR solubilized in octyl glucoside to verify this difference. The molar ellipticity values of our spectrum for bR in octyl glucoside (Figure 2) are considerable larger in magnitude than those reported by Mao & Wallace (1984). This discrepancy may be due in part to an error in the value of the protein concentration used by the authors. In addition, it seems likely that the CD spectrum of the octyl glucoside solubilized bR that was published by Mao & Wallace (1984) is distorted somewhat due to aggregation of the sample material. The smaller ratio of the ellipticity at 193 and 222 nm of their spectra could be explained by some degree of aggregation of their sample. A properly dispersed sample of bR should have ellipticity values close to 40 000 deg cm^2 dmol^{-1} at around 193 nm.

The CD spectra of hR and bR in octyl glucoside are very similar in both magnitude and shape, suggesting that the secondary structure content of these two retinal binding proteins is very similar. Least-squares curve fitting of the spectra for hR and bR solubilized in octyl glucoside gives about 50% α-helix and about 30% β-structure, assuming that the average number of residues in the helical strands is 25. Such long helical strands are expected for membrane proteins; a substantial number of integral membrane proteins are believed to have helical strands that span across the membrane bilayer (Foster et al., 1983; Hoppe & Sebald, 1984; Nathans & Hogness, 1983). The assumption that bR, an integral membrane protein, has helices that are 25 residues long is reasonable since X-ray diffraction and electron microscopy indicate that the helical strands in bR span across a membrane of $\approx$45 Å thickness. However, no such data are available for hR. Assuming that the molecular masses of hR and bR are indeed similar, the number of helical strands in hR that can transverse a membrane of $\approx$45 Å thickness is no more than five, similar to that of bR (Jap et al., 1983).

Caution should always be used in quantitative estimation of protein secondary structure on the basis of the CD spectra. Aside from the potential errors from absorption flattening and light scattering artifacts mentioned before, long-range flexibility such as bending and twisting of β-structures may sometimes result in a rather unreliable estimate of the β-structure content. This point has been discussed previously in somewhat more detail by Jap et al. (1983). Normally, the helical content of proteins, particularly those having high helical contents, can be obtained from CD spectra to within a few percent accuracy (Chen et al., 1974).

It will be intriguing to know whether there is a similarity in the pumping mechanism between hR and bR, since hR pumps chloride ions and bR pumps "protons". It is not yet known whether the chemical species being transported by bR is protons (H^+), hydroxyls (OH^-), or H_3O^+ etc. If bR indeed transports hydroxyl or H_3O^+ through a channel, it is then quite possible that the molecular mechanisms of operation are quite similar for bR and for hR. The current evidence that the ultraviolet CD spectra of these two protein are very similar is consistent with—but does not prove—that the folding of these two proteins is similar. If a similarity in the three-dimensional molecular structures of these proteins does indeed exist, these proteins may share a common mechanism of ion transport. It is, therefore, important to determine their three-dimensional molecular structures directly by diffraction techniques.

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Proteolytic Processing of Human Factor VIII. Correlation of Specific Cleavages by Thrombin, Factor Xa, and Activated Protein C with Activation and Inactivation of Factor VIII Coagulant Activity

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ABSTRACT: Human factor VIII was isolated from commercial factor VIII concentrates and found to consist of multiple polypeptides with molecular weights ranging from 80 000 to 210 000. Immunological and amino acid sequence data identified these polypeptides as subunits of factor VIII. N-Terminal amino acid sequence analysis determined that the M_r 210 000 and 80 000 proteins are derived from the N- and C-terminal portions of factor VIII, respectively; M_r 90 000–180 000 polypeptides are derived from the M_r 210 000 polypeptide by C-terminal cleavages. Treatment of purified factor VIII with thrombin resulted in proteolysis of M_r 80 000–210 000 proteins and the generation of polypeptides of M_r 73 000, 50 000, and 43 000. Maximum coagulant activity of thrombin-activated factor VIII was correlated with the generation of these polypeptides. The proteolysis as well as activation of factor VIII by thrombin was found to be markedly dependent on $CaCl_2$ concentration. Proteolysis of factor VIII with activated protein C (APC) resulted in degradation of the M_r 90 000–210 000 proteins with the generation of an M_r 45 000 fragment. This cleavage correlated with inactivation of factor VIII by APC. The M_r 80 000 protein was not degraded by APC. Factor Xa cleaved the M_r 80 000–210 000 factor VIII proteins, resulting in the generation of fragments of M_r 73 000, 67 000, 50 000, 45 000, and 43 000. Factor Xa was found to initially activate and subsequently inactivate factor VIII. Activation by factor Xa correlated with the generation of M_r 73 000, 50 000, and 43 000 polypeptides while inactivation correlated with the cleavage of M_r 73 000 and 50 000 polypeptides to fragments of M_r 67 000 and 45 000, respectively. The cleavage sites in factor VIII of thrombin, factor Xa, and APC were identified by amino acid sequencing of the fragments generated after cleavage of factor VIII by these proteases. Interestingly, factor Xa was found to cleave factor VIII at the same sites as APC and thrombin. This may explain why factor Xa activates as well as inactivates factor VIII.

Purification of factor VIII (antihemophilic factor) from plasma indicates that its coagulant activity is associated with multiple polypeptide chains having molecular weights ranging from 80 000 to 210 000 (Vehar & Davie, 1980; Fass et al., 1982; Fulcher & Zimmerman, 1982; Rotblat et al., 1985). Recently, cDNA clones encoding the entire factor VIII protein sequence have been obtained (Toole et al., 1984; Wood et al., 1984). The amino acid sequence deduced from such clones predicts a mature single-chain protein (2332 amino acids) having a molecular weight of ~300 000 (Wood et al., 1984; Toole et al., 1984). Sequence data obtained from the protein chains of purified factor VIII preparations have been shown to overlap with the sequence predicted from the cDNA clones (Toole et al., 1984; Vehar et al., 1984), and the purification of a single-chain precursor having a M_r >300 000 has been reported (Rotblat et al., 1985). Thus, if factor VIII circulates in plasma as a single-chain form, it is partially degraded during its purification, yielding a form with multiple polypeptide chains.

Amino acid sequence analyses also revealed the orientation of the protein chains associated with factor VIII to the single-chain precursor deduced from the cDNA sequence (Vehar et al., 1984; Toole et al., 1984). Such data show that the M_r 210 000 and 80 000 proteins represent the N-terminal and C-terminal portions of factor VIII, respectively (Vehar et al., 1984; Toole et al., 1984). It is proposed that several proteolytic cleavages on the C-terminal side of the M_r 210 000 protein generate a series of proteins with molecular weights between 90 000 and 180 000 (Vehar et al., 1984; Toole et al., 1984).

Recently, thrombin activation of factor VIII coagulant activity has been shown to be associated with specific proteolysis of factor VIII protein chains (Vehar & Davie, 1980; Fass et al., 1982; Fulcher et al., 1983, 1984; Loller et al., 1984; Rotblat et al., 1985). During thrombin activation of purified

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