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NEUTRON DIFFRACTION STUDIES OF BACTERIORHODOPSIN

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Abstract

Neutron diffraction studies of bacteriorhodopsin have utilized the entire range of deuterium labeling techniques that are commonly used in biological neutron scattering experiments. We will review the work published in this area and report on current projects.

Bacteriorhodopsin is an integral membrane protein of relatively small size (m.w. approx. 26000) that is found in the plasma membranes of halophilic bacteria. It functions as an energy transducer, converting light energy into an electrochemical potential for use by the micro-organism in metabolism (1). Bacteriorhodopsin exists in discrete patches of the plasma membrane (the "purple membranes") in a two-dimensional array, and electron diffraction studies have given a low resolution three-dimensional structure of the protein showing a bundle of seven rods traversing the membrane bilayer (2). Considerable complementary structural information has been obtained from neutron diffraction studies.

Contrast variation studies of purple membranes (3,4) have given information concerning the areas of hydration in the membrane. Neutron diffraction data were collected from the same sample of stacked, oriented purple membranes in different relative humidities of H_2O and D_2O . This data was interpreted to show the locations of the exchangeable 2 protons as a function of total hydration. Hydration was found predominantly around the lipid headgroups and the protein had no bulk water associated with it. When the membrane is totally dry, the protein structure is maintained, but the lipids are dehydrated, resulting in a smaller unit cell (by a few percent). Mechanisms of proton pumping involving bulk water in the protein were thus eliminated.

It has been shown that the photocycle is greatly slowed, and can even be trapped in intermediate states either by drying the membranes, or cooling them (4). Recently, data have been collected from purple membranes at liquid nitrogen temperatures (unpublished data, Zaccai) and the structure and hydration in these conditions were compared to those of the dry state. In both cases a similar reduction in the unit cell is observed. Contrary to the drying case, however, the H_2O/D_2O exchange experiments clearly show that the water is not removed from the membrane upon cooling and that the contraction is entirely accounted for by the lowering of the temperature.

Specific deuteration of bacteriorhodopsin has been used to derive information concerning the folding of the polypeptide chain when it is in the membrane bilayer. The amino acid sequence was determined in 1979 (5), but it has not been possible, to date, to make a definitive assignment of the sequence to the features observed in the structural map.

The earliest studies involving specific deuteration depended on biosynthetic incorporation of deuterated amino acids that were obtained either from acid hydrolysis of deuterated protein extract from *E. Coli*, or by chemical synthesis (6,7,8). By growing halobacteria on a fully defined medium and supplementing with a given deuterated amino acid it was possible to obtain high levels of incorporation of the deuterated amino acid with little or no spread of the label to other amino acids. Two-dimensional neutron diffraction powder patterns were collected from stacked, oriented multilayers of partially dried purple membranes with different deuterated amino acids. Difference Fourier's were used in early attempts to interpret such data in terms of the projected structure in the plane of the membrane bilayer. It was concluded from the difference Fourier analyses that bacteriorhodopsin was 'inside-out' compared to water soluble protein structures. That is, the polar amino acid side

chains appeared to project toward the interior of the molecule, while the non-polar sidechains faced the hydrophobic lipid environment (6). This is consistent with the observation in the contrast variation studies that there was more H/D exchange in the center of the protein than on its periphery (4).

In an effort to interpret the deuterated amino acid data in a more quantitative way a model building analysis was developed (7). The modeling studies were based on the assumption that the membrane spanning segments of the molecule were approximately alpha-helical. The locations of the membrane spanning segments in the sequence were deduced from theoretical calculations combined with the results of studies involving enzymatic digestion and/or chemical modification of the linking regions between the helices that are accessible to soluble agents. Different models were constructed by assigning specific helices to locations in the structural map and varying their rotational orientations about their axes. Models were evaluated by comparing the predicted neutron intensity data against the observed data with the aid of a weighted residual factor. This work led to the assignment of two specific amino acid sequence segments to locations in the structural map (7), but further sequence to structure assignments were not possible due to the combined effects of the deuterium label being spread over the entire molecule and insufficient contrast between the different labelled parts.

In order to make further sequence to structure assignments a reconstitution experiment was undertaken (these authors, unpublished data). Bacteriorhodopsin was chymotryptically cleaved into two fragments, one small and one large fragment containing two and five helices, respectively. This cleavage was performed on hydrogenated preparations as well as bacteriorhodopsin that had been grown in D_2O with nutrient obtained from hydrolyzed extract from deuterated E. Coli. Bacteriorhodopsin was then reconstituted with the two helix fragment deuterated and the five helix fragment hydrogenated. The two-dimensional lattice was reformed with the reconstituted molecule (9) and neutron diffraction data were collected. We are in the process of interpreting this data to give the location of the two deuterated sequence segments in the structure. If we were able to locate these helices unambiguously then it is conceivable that by combining this information with the previous assignments and what is known concerning the length of the connecting regions between the membrane spanning segments, a pattern of folding for all seven segments could be deduced.

Finally, it has also been possible to obtain purple membranes for which the retinal chromophore has been incorporated in vivo in deuterated form (10). Nicotine inhibits the biosynthesis of retinal. By growing halobacteria in a medium containing nicotine as well as fully deuterated oxidation products of beta-carotene, Jubb and co-workers were able to obtain purple membranes with biosynthetically incorporated deuterated retinal. Neutron diffraction data were then interpreted (using both difference Fourier and modeling analyses) to give the location of the retinal chromophore in the projected plane of the membrane bilayer.

Figure 1 summarizes the results obtained from neutron diffraction studies of bacteriorhodopsin. In each case the power of the technique

lies in the fact that bacteriorhodopsin has been particularly amenable to being labeled in a variety of ways by deuterium. Relatively little is currently known about the structure of membrane proteins at this time, mainly due to the fact that these proteins have proven difficult to crystallize in three dimensions for study by traditional crystallographic techniques. Membrane systems are naturally planar and there are a number of examples of membrane proteins that occur naturally in (or can be induced to form) two dimensional crystals. In bacterial systems the deuterium labeling studies can be undertaken with relative ease and it is therefore possible that these types of studies will prove useful in studying other membrane protein structures.

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Figure 1. A unit cell of the purple membrane lattice, showing a protein trimer with the seven helices numbered conventionally. The clear areas around the protein molecules are lipid.

Structure and Hydration
Maintained Upon Freezing