UNRAVELLING CONFORMATIONAL CHANGES OF BACTERIORHODOPSIN USING PAIRS OF SPIN LABELS

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Membrane proteins present a difficult problem for structure determination. With a few exceptions, X-ray diffraction data are not available due to unsolved problems of crystallization. Two-dimensional NMR techniques are not applicable due to the lack of high resolution spectra. Electron paramagnetic resonance (EPR) in combination with site-directed spin labeling (SDSL) is an alternative approach which can be applied on soluble proteins, on the crystal and on membrane proteins. Using genetic techniques, spin labels are introduced at nearly any desired site of the protein by cysteine substitution mutagenesis followed by selective modification of the sulfhydryl group. The basic SDSL is developed so far that it is possible to routinely define structural elements as well as their orientations, to characterize movements of segments with a time resolution in the ms-time range and to determine membrane protein topography by measuring the accessibilities to paramagnetic quenchers [1,2]. The technique of double site-directed spin labeling (D-SDSL) is a tool to study interspin distances in proteins with two or more nitroxide spin labels attached to specific sites to obtain structural information as well as dynamic features under conditions relvant to function [3,4].

The attachment of two or more spin labels to a macromolecule with an interspin distance of less than ~2.5 nm leads to considerable EPR spectral changes due to dipolar interaction. This line broadening is in turn a measure of the interspin distances, which can be determined by fitting simulated EPR powder spectra to the experimental spectra, measured at temperatures below 200K to freeze the protein motion [5]. Special attention is paid to the fact that the interspin distances in the frozen states will be distributed around an average value due to the conformational fluctuations of the proteins and the flexibility of the spin labeled side chains.

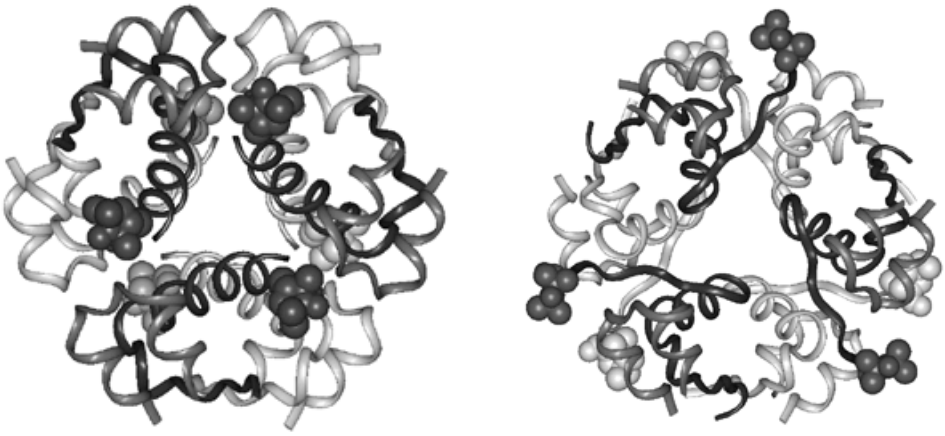

Figure 1: The B2SL insulin hexamer in the R6 state (left) and in theT6 state (right) as seen along the three fold axis. The strands show the peptide backbone of the chains, the spin labels attached to the B-chains are plotted space filled. For the T6 state, the respective spin probe positions were modelled, X-ray data of the spin labeled hexamers reveal the structure of the R6 state [5].

Evaluation of the method:
D-SDSL has been applied to spin labeled insulin in the T6 and R6 conformation, in solution and in the crystal [5]. The nitroxides were attached at the amino termini of the B-chains of the insulin hexamers at position B1 respectively B2 (Fig. 1). Resulting interspin distances determined for the crystals are in excellent agreement with the structural data obtained by X-ray cristallography, see table 1.

	EPR spectroscopy		X-ray
Structure	Solution	Crystal	Crystal
R6 (B1SL)	1.0 ± 0.6		
R6 (B2SL)	1.4 ± 0.6	1.7 ± 0.3	1.8
T6 (B1SL)	1.3 ± 0.6		
T6 (B2SL)	1.4 ± 0.6	1.1 ± 0.4	1.0

Table 1: Average distance and distance distribution, (r ± σ)/nm, determined by EPR dipolar interaction analysis and X-ray crystallography.

The distances between the attached spin labels are determined by a fitting procedure. Due to the location of the amino termini of the B-chains in the R6 hexamer (see Fig. 1), an interaction model consisting of three equally spaced spin agrees better with the molecular structure than an interaction model of pairs of spins. This structural property was considered by calculating the resulting distances for the R6 structure using this model. The values of the measured distances and distance distributions (see table 1) reveal different conformations in crystal and in solution. Those differences are well known from comparisons between data revealed by NMR spectroscopy and X-ray crystallography. The location of the termini are additionally determined by inter-hexamer contacts in the crystal. In solution, the amino termini may occupy a number of different orientations due to its different environment. In addition, the chain termini are expected to be more flexible in the dissolved protein than in the crystallized sample. The different flexibility is reflected in the distance distribution parameter σ, which is significantly smaller for the crystallized than in the dissolved samples.

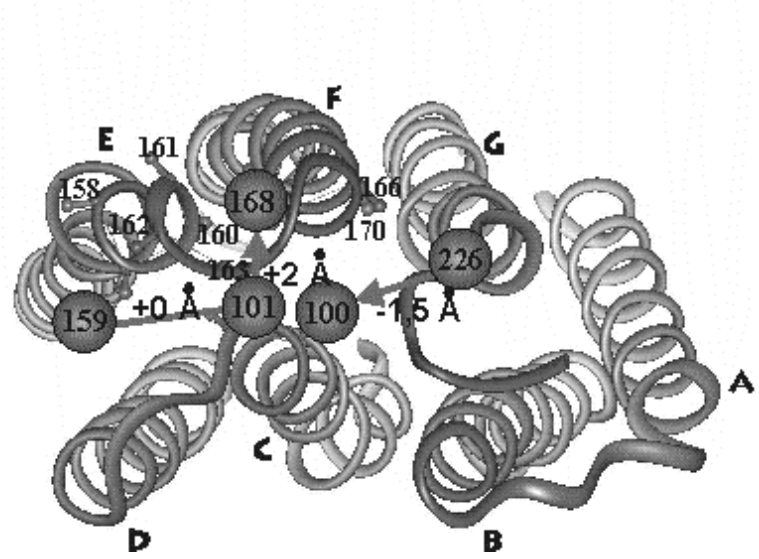

Figure 2:
A view on the cytoplasmic surface of bacteriorhodopsin shows the locations of the modifications in the C-D loop, the E-F loop and in helix G. Distances were determined between position 101 and each marked amino acid in the E-F loop or helix G, respectively. Additionally, two pairs of spin labels were introduced in combination with position 100. Distances changes between the marked amino acids are discussed in the text.

Application:
Bacteriorhodpsin (BR) is a seven α–helical transmembrane protein, which undergoes a proton pumping photo-cycle after light excitation [6]. This proton transport requires structural changes during the catalytic cycle, the extend of this conformational change is still unclear. Changes of the spin label mobility during the BR photocycle due to changes of the interaction of the spin label side chain with the tertiary structure are found in the loop regions A-B, C-D and E-F on the cytoplasmic surface of the protein [7]. The results of the single mutants indicate a transient outward movement of the cytoplasmic part of helix F during the M-intermediate. For quantitative determination of the structural change pairs of spin labels were introduced at the indicated positions (Fig. 2).

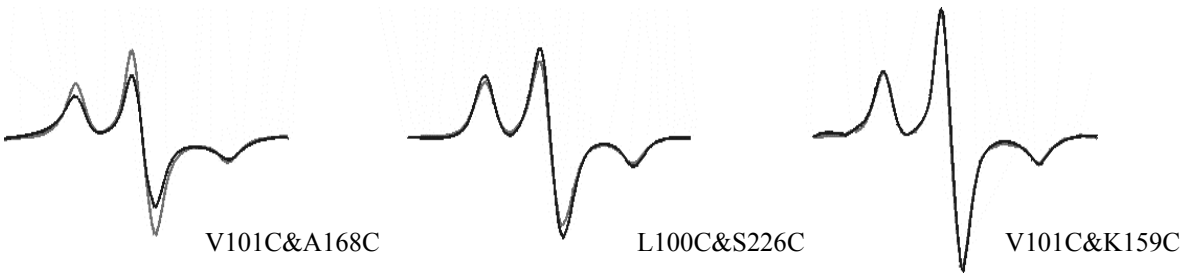

Figure 3:
Spectra of the BR ground state (black) compared to the spectra of the stabilized M intermediate (grey), measured at 170 K. The spectra are normalized to equal spin number.

Distance changes between the spin labels attached to 101 and 168 in the cytoplasmic loops C-D and E-F were detected after freezing of the BR in different intermediates (Fig. 3). The intermediate showing the largest variation was stabilized by illuminating the probe at 230 K with $\lambda > 580$ nm and fast cooling to 170 K. Characterization of the frozen intermediate by FT-Raman spectroscopy revealed that the conformational change of the labeled loops occurs during the M to N transition. The distance between the spin label pair 101 and 168 increases from 1.45 nm in the ground state to 1.65 nm in the M intermediate. Concurrently the distance between the spin labels at position 100 and 226 decreases from 1.6 nm to 1.4 nm. This is evidence that the cytoplasmic end of helix G moves into the direction of the proton channel. There is no distance change detectable between the positions 101 and 159, hence helix E is most probably not affected in a large conformational change.

In combination with the freeze quenching technique conformational changes of BR can be detected. The method allows for measurement of intramolecular distances in the range of 1.0 to approximately 2.0 nm with an accuracy of 0.1 nm. The use of deuterated spin labels with their smaller intrinsic linewidth will increase this upper limit.
The sensitivity of the spectral shape for dipolar interaction and the nitroxide mobility allows to follow structural changes at room temperature with a resolution in the ms time range. The EPR dipolar interaction analysis method thus opens a wide field in the study of the structure and dynamics of proteins.

References:

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