EPR and NMR spectroscopy of spin-labeled proteins

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Concluding Remarks

The main theme of the present thesis is the application of complementary paramagnetic approaches to obtain restraints for structural and dynamic determination in protein studies.

High-field EPR for detailed investigation of local polarity in proteins

Spin labeled proteins and EPR techniques have long been simultaneously used as tools for the study of structure and dynamics of proteins[1-3]. A spin label specifically attached to the surface of a protein is affected by conformational restrictions arising from sterical constraints or from interactions with the neighboring amino-acid residues. The conformational restrictions are reflected in the spin-label motion that can be detected by EPR techniques. These provide a way to investigate, with high accuracy, the microenvironment around the position to which the spin label is attached[2]. In fact, EPR spectra provide parameters that can be used to explore both the polarity and the propensity of the protein environment to donate hydrogen bonds (i.e. the proticity). When EPR spectroscopy of a spin labeled protein is performed at 95 GHz (W-band EPR), the spectral resolution is significantly improved compared to conventional, 9 GHz EPR (X-band EPR). The polarity profiles of membrane proteins have been determined in this way[2]. Also, the usefulness of multi- and high-frequency EPR to determine the dynamics of biological membranes[4] was demonstrated on a model system using spin-labeled lipids.

To further improve the spectral resolution, J-band EPR has been applied in the present work with the goal to discriminate even small differences in the local polarity. This is the first J-band EPR study made with the aim to investigate local polarity at specific positions on the surface of a protein. In a well known protein, azurin, spin labels were attached at four different surface positions. Indeed, the higher resolving power of J-band compared to W-band EPR made it possible to reveal the subtle differences in proticity. We expect that this work will allow calibrating the result of structure and polarity calculations, and that it will be the basis for investigations of these parameters in other, less well characterized protein systems.

Most remarkable was the finding that all EPR spectra of the surface positions of azurin appeared to be single component in W-band EPR, whereas the higher resolution of J-band EPR revealed two components. It seems likely that many “single component spectra” from lower field EPR could turn out to be multiple component, when investigated at J-band or higher.

Long-range distance measurement through double electron-electron resonance

In the study of biomolecules, distance determination provides crucial information for structural and functional investigations. Dipolar EPR spectroscopy of doubly labeled biological systems offers a

complementary method for distance determination, which is particularly useful when other techniques, like X-ray crystallography, FRET and NMR, are not applicable, or when the results are not completely understood[5,6]. In this situation, the possibility to perform measurements of the distance between spin labels becomes very interesting. Pulsed double electron-electron resonance (DEER, also known as PELDOR[7-10]) has been introduced about 30 years ago but only in the last decade has its application in structural biology increased significantly[5], due to widespread use of site directed mutagenesis. Very recently, the integration of the DEER with PRE NMR and conventional NMR intrachain NOESY-based distance constraints has been used for the structure determination of the homodimer Dsy0195 protein[11].

One of the advantages of DEER is that in addition to being sensitive to long, i.e. nm-distances, it produces distance distributions. The width of the distance distribution reflects different conformations of the proteins, and therefore provides information on the flexibility of the protein. This width, however, contains a contribution deriving from rotational isomers of the spin label linker. To consider this point, different approaches have been used in the past[12-17] in the interpretation of experimental data. The accurate interpretation of the results requires a biological model. In the present thesis we use the azurin protein as model system for which we have produced two doubly spin-labeled proteins. The conformation of the spin labels were modelled using XPLOR-NIH[18], already used in the NMR field. The appropriate conformers were selected from all rotamers by only excluding orientations that clash with other atoms in the protein structure. The distances between the nitrogen atoms of the two spin label linkers of allowed spin label orientations yield the modelled distance distribution. Surprisingly, this simple model gives realistic widths, but these seem to be centered at too long a distances, a clear indication that side chain modelling needs to be improved. The width of the distribution obtained experimentally (3 - 5 Å) is also a concern, since it limits the intrinsic accuracy of structure determination by this approach. Therefore, the use of other spin labels, such as the backbone incorporated TOAC label or shorter tether lablels should be considered. The price to be paid is the more difficult biochemical incorporation of such labels.

Structure of spin labeled proteins by paramagnetic NMR

Protein-protein associations give rise to either stable or transient complexes, which control most of the cellular activities. Electron-transfer protein complexes are an example of macromolecules interacting transiently because of their physiological role. The conditions that must be satisfied for this transient behavior are high reaction efficiency and low affinity. This ensures an adequate reaction rate for the electron transfer. Transient protein complexes are therefore at the boundary between specific and non-specific complexes. The encounter complex, preceding the formation of

the final complex with which is in equilibrium, has been demonstrated to be of dynamic nature. Nevertheless, a comprehensive understanding of the encounter complex is difficult; in fact, its nature is rather elusive, since it is formed by multiple orientations of the partner proteins, which interact in an aspecific way. For this reason, studying the encounter complex requires specific methods, able to detect differently populated states. Using spin labeled proteins and PRE NMR fits well in this framework. In general, the validity of this technique, which is based on paramagnetic tags for intermediate and long range distance measurements was proven[19]; it also demonstrated the complementarity of this approach with other established techniques. This approach exploits the large magnetic dipolar interaction existing between unpaired electrons and nearby nuclear spins.

In the present thesis, the physiological complex between Cyt *f* and Pc from cyanobacterial *Nostoc* sp. has been investigated and the interaction of Pc with five Cyt *f* cysteine mutants has been analyzed. The structure obtained was compared to the results of other NMR based approaches, namely pseudocontact shifts (PCSs) and chemical shift perturbations (CSPs). The conclusion of the present thesis is that a single structure does not adequately represent the complex. To proceed from the results presented here to a real picture of the dynamic encounter complex, the route outlined in several recent studies[20-24] can be taken. The results presented in the present thesis will provide the basis for such an interpretation and should cast a new light on the encounter complex structure.