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Chapter 8

General discussion, conclusions and perspectives.
The work presented in this thesis covers a wide range of classical and paramagnetic NMR techniques applied to diverse protein systems. The C-terminal domain of EiAPR studied in chapter 2 is part of a two domain protein that has evolved to possess both reductase and electron transfer components within the same macromolecule. In many redox pathways, electrons are donated from one biomolecule to another; EiAPR, nitrite reductase and cytochrome P450BM3 are exceptions to this rule. P450cam (Chapters 3 and 5) is the archetypal member of the CYP superfamily and as such has been extensively studied over the past five decades. CYPs are terminal acceptors in a variety of redox pathways involved in a plethora of diverse metabolic processes throughout the three kingdoms of life. The precise molecular mechanisms by which CYPs bind to their redox partners and catalyse the hydrogenation of different substrates still remains to be fully elucidated. The data presented in this thesis provide strong evidence that P450cam remains closed when Pdx binds to it. Recent studies \((195, 196)\) have advocated that P450cam opens or adopts an intermediate conformation when Pdx binds. Further investigation is required to determine which of these theories represents the behaviour of P450cam in solution. Avr2 (chapter 4) is a protein produced by a pathogenic fungus that infects tomato plants and causes leaf mould. However, the tomato plants have evolved to possess a level of innate immunity against such an infection, although the precise mechanism, by which this occurs, remains unclear. T4 lysozyme (Chapter 7) has widely been used as a model protein for the development of spin labels for EPR and during the last decade, the L99A mutant of this protein has been used to develop relaxation dispersion techniques \((150)\). This protein is widely used because homologous expression and purification can produce very high yields and it is extremely stable. The diversity of NMR techniques available make this type of spectroscopy ideally suited to unravel the atomic level details of how these different protein systems behave in solution and this thesis outlines but a few of these, applied to the aforementioned protein systems.

**The use of classical NMR spectroscopic methods in structural biology**

The prerequisite to any detail NMR study is an assignment of the protein nuclei of interest. Nowadays, heteronuclear triple resonance experiments are routinely used to
assign the backbone nuclei of proteins (Chapter 2) and in the case of structure solution, the side chains can be assigned using homo- or heteronuclear experiments (Chapter 4). One of the biggest limitations to the use of classical homo and heteronuclear experiments is the size of the system. As protein size increases, the relaxation rates of the nuclei decrease and in such cases, TROSY techniques can be used overcome this difficulty (Chapter 3). The stability of protein systems can also make assignments challenging to obtain, since classical and TROSY type assignment experiments can last for days, after which time, a protein may have degraded. The introduction of BEST-type pulse sequences (17, 18) has enabled the acquisition times of experiments to be significantly decreased, such that systems such as P450cam can be assigned using BEST-TROSY experiments and a high level of assignment can be obtained (Chapter 3). The assignment of some proteins can still be challenging or even impossible especially if these proteins are especially large and spectra of the uniformly labelled protein would be too crowded for detailed information to be extracted. In such cases, selective labelling of methyl groups (Chapter 3) or the amide nitrogen of a specific residue type (Chapter 5) can yield cleaner spectra. Alternative strategies for the assignment of large proteins have been developed, such as the divide and conquer approach (208) and a method based on systematic mutagenesis (205).

Once an assignment has been obtained, and if the structure of the protein of interest has been solved, detailed atomic level analysis into the behaviour of the protein can commence. Chemical shift perturbation analysis (Chapters 4 & 5) can provide information on how a protein behaves in solution under different conditions at the atomic level. For example when binding to a redox partner or when a specific ligand binds, changes in the chemical shifts of different nuclei are indicative of a change in the chemical environment of those nuclei.

If a structure is not available for the protein of interest, a homology model could be produced, and this could serve as a surrogate until the 3D structure becomes available. Over the past decade software packages, for example TALOS+ (191), have been developed that has the ability to predict secondary structural features on
the basis of Cα and Cβ chemical shifts. If a structure can be found that has a high level of sequence homology, predictions of secondary structure can be compared to the structure of the homologous protein and this can provide information on the nature and behaviour of the protein under study (Chapter 2).

If structural information is not available, NMR spectroscopy can be used to solve the solution structure of a protein using NOESY data and software such as ARIA (225) and CYANA (24). However, structures solved in the manner are often in vacuo structures and these may be over-constrained and thus not representative of the true solution structure of the protein, and therefore, water refinement of the obtained structure should be carried out and the resultant structural bundle should be validated, for example using CING (190) (Chapter 4).

The applications of classical NMR in structural biology are considerable; however, if the assignment of NMR spectra cannot be obtained for a protein system, the utility of NMR for detailed studies diminishes considerably. In such situations, paramagnetic NMR techniques can provide a solution.

**Paramagnetic NMR techniques for the study of proteins and protein-complexes**

Paramagnetic NMR is widely used for the study of proteins and protein complexes; however, its use for protein assignment is less widespread. In 2004, Pintacuda *et al.* published the first piece of software for the assignment of proteins on the basis of pseudocontact shifts (66). This software uses a single paramagnetic centre, whose position is known, and its efficacy was demonstrated on selectively labelled samples. However, the larger the protein, the more complex the search space becomes and if the position of a paramagnetic centre is not known, obtaining an assignment from a single paramagnet can be impossible. These difficulties can be overcome by using more than one paramagnetic centre and using a probe for which the approximate position can be consistently and reliably predicted. Each paramagnetic centre yields an independent set of pseudocontact shifts for the same nuclei and an approximate
position can provide a starting point for an assignment algorithm, from which nuclei can be assigned in uniformly and selectively labelled spectral data (Chapter 6).

As simple as using multiple paramagnetic centres may sound, the biochemical work involved in producing numerous mutants to which probes can be attached is laborious and can be extremely time consuming. The development of a methodology whereby fewer mutants are required for an assignment to be obtained would be highly beneficial. The paramagnetic centres produced by different lanthanide ions can be isotropic and anisotropic and the anisotropic fields can have diversely different magnetic susceptibilities. Isotropic paramagnetic lanthanides cause paramagnetic relaxation enhancements in NMR spectra, which provide distance dependent information, and only the position of the paramagnet needs to be fitted. The fact different magnetic susceptibilities are associated with anisotropic paramagnetic fields means that paramagnetic effects can be observed at different distances. For example, using Tm can yield effects up to 60 Å from the paramagnetic centre, whereas Yb can yield effects up to 30 Å (209). The combination of PRE data with short and long range PCS has the potential to provide sufficient information for an assignment to be obtained using a single probe attachment site.

The power of paramagnetism can also be harnessed for the study of protein dynamics. Traditionally, studies of minor states in proteins have utilised relaxation dispersion techniques, however, interpreting the chemical shifts differences obtained from these experiments, in structural terms, can be very difficult. Paramagnetic relaxation dispersion (133) could provide a manner by which the analysis could be simplified (Chapter 7). If a region of a protein is undergoing a chemical exchange process in a paramagnetic field, such as internal motions relative to the paramagnetic centre, an average PCS value will be observed for the nuclei in the region. Fluctuating PCSs can be analysed using relaxation dispersion techniques; thereby the structure of the minor state of a protein could be obtained. A potential pitfall in this method would be if the paramagnetic centre was also undergoing motion on the same time scale as the exchange process being studied and therefore, the source of the paramagnetic field must be rigid for paramagnetic relaxation dispersion to be of
any utility. The development of rigid paramagnetic probes could overcome this limitation and thereby, enable the dynamics of minor states to be investigated. A recently developed paramagnetic probe, CLaNP-7 (61) was designed for increased rigidity, however the dynamic profile of this probe has not been investigated using paramagnetic relaxation dispersion. Paramagnetism could also be using in conjunction with EXSY spectroscopy. Even if two chemical shifts can be observed for an exchange process, the resolution of the spectrum may not be sufficient to identify, unequivocally, the individual autopeaks and cross peaks in an EXSY spectrum. The use of a paramagnetic centre could yield spectra with superior spectral resolution and aid the analysis of processes occurring on the ms-s timescale, where the chemical shift difference between two populations is rather small. The use of saturation transfer for investigating chemical exchange processes has been developed using methyl groups as probes. The utility of this method for studying protein folding has been demonstrated (226).

The significant advances in classical NMR spectroscopy over the last three decades have enabled detailed studies of increasingly complex protein systems. However, the study of some systems is not possible using purely classical techniques. Paramagnetic NMR in isolation can be of considerable utility in the study of biomolecules and this has become increasingly clear over the past two decades. The combination of classical NMR and paramagnetic NMR techniques has the potential to increase the size limit of systems that can be studied at the atomic level and provide solution state information even on the most complex of protein systems.