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NMR at 900 MHz*

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The very first high-resolution NMR spectra recorded at 900 MHz in July 2000 have demonstrated the benefits of increased magnetic field strength for studies of large biomolecules such as proteins and nucleic acids. Increased sensitivity and resolution for such molecules can only be observed in experiments that are optimized for transverse relaxation (TROSY). Substantial effects of magnetic alignment can easily be observed not only in paramagnetic proteins, but even in small molecules, such as chloroform. Such effects can be very useful for structural studies of biopolymers.

The extreme resolution allows studies of very weak interactions in proteins. For instance, long-range H/D isotope effects are easily observed in H–N correlation experiments. The first systematic studies of relaxation properties of N-15 nuclei have been carried out for proteins at 500, 600, 800, and 900 MHz.

Keywords: cytochrome C, fibronectin, lysozyme, phenylalanine, proteins, 900 MHz, NMR, isotope shifts, molecular alignment, relaxation, resolution, sensitivity, transverse relaxation optimized spectroscopy (TROSY).

The increase in sensitivity and dispersion in NMR spectra with increasing magnetic field strength has always been regarded as one of the major advantages of increase in the magnetic field strength in the 50 year history of NMR spectroscopy. Over that time the working frequency of NMR spectrometers has increased from 30 MHz in the early days to the 600, 750, or even 800 MHz that are fairly common nowadays. It is largely due to this increase in magnet technology that NMR scientists are able to solve the structure of larger and larger molecules. With the new challenges revealed by the advances in genomics and proteomics, the recent demonstration of the first 900 MHz NMR spectra [1] was anticipated particularly eagerly by the NMR community. As shown in this work, not only the fundamental NMR parameters improve at these very high magnetic field strengths, but molecules start to behave differently, revealing information that is particularly useful in determining the structures of large biomolecules.

1. Sensitivity and Resolution

Increasing the magnetic field not only provides a better spectral resolution but also improves the overall *sensitivity*. The photon energy (ΔE) required to excite a transition between two Zeeman states, and hence the intrinsic sensitivity of the NMR method, is directly proportional to the magnitude of the static magnetic field, B_0:

* Dedicated to Professor E. Lukevics on his 65th birthday.

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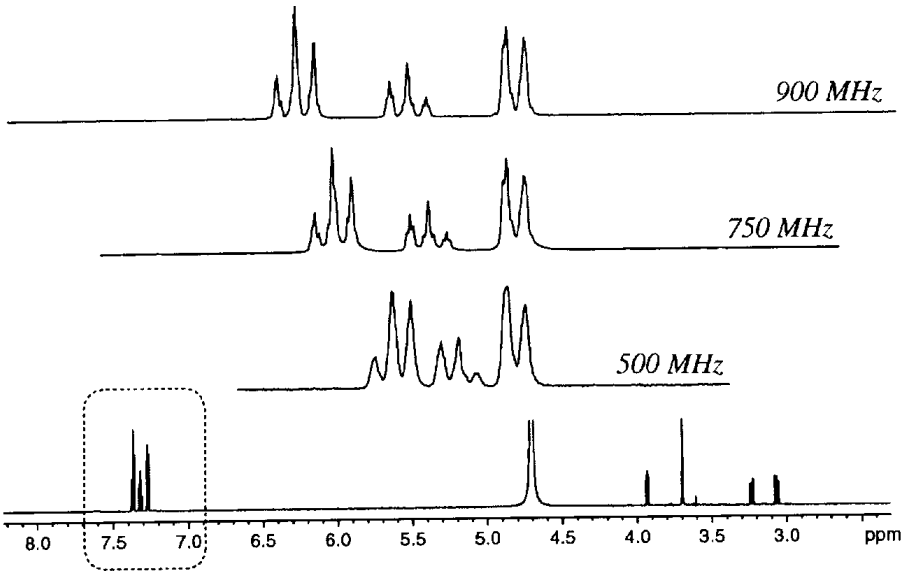

Fig. 1. The proton NMR spectrum of phenylalanine at 900 MHz. The expansions show the additional dispersion and spectral simplification that occurs in the aromatics region as the magnetic field strength is increased from 500 to 900 MHz.

$$\Delta E = \hbar \gamma B_0 \,, \tag{1}$$

where γ is the gyromagnetic ratio of a given nucleus. The effect of increasing magnetic field strength is revealed in the proton spectra of a relatively small molecule, phenylalanine, shown in Fig. 1.

Due to peculiarities of the noise properties [2], the total signal to noise ratio (*S/N*) in an NMR experiment increases much faster as compared to an increase in resolution and is proportional to $B_0^{3/2}$:

$$S/N \propto B_0^{3/2} . \tag{2}$$

For instance, an increase in the field strength on a 900 MHz instrument as compared to that on a 600 MHz spectrometer improves the S/N figure by aproximately 84 percent. Even a modest increase in the field strength when comparing 800 MHz and 900 MHz instruments gives an advantage of almost 20 percent in the *S/N* ratio.

The signal to noise ratio can further be improved by reducing the *noise* level. The introduction of superconductive probe technology [3, 4] potentially can improve the overall *S/N* ratio of the 900 MHz instrument by another factor of four. This new technology is by no means an alternative to increasing the magnet field strength, but rather a complementary technique. While the first increases the separation between the Zeeman energy levels and hence addresses the *sensitivity* issue of the NMR experiment, the superconductive probe technology addresses a principally different problem – reduction of *noise*.

The introduction of 2D NMR at the beginning of the eighties dramatically increased the resolving power of NMR, making it possible to study complex biomolecules such as proteins and DNAs. However, the spectral dispersion and sensitivity still very much depends on the resolving power of the magnetic field (see Fig. 2).

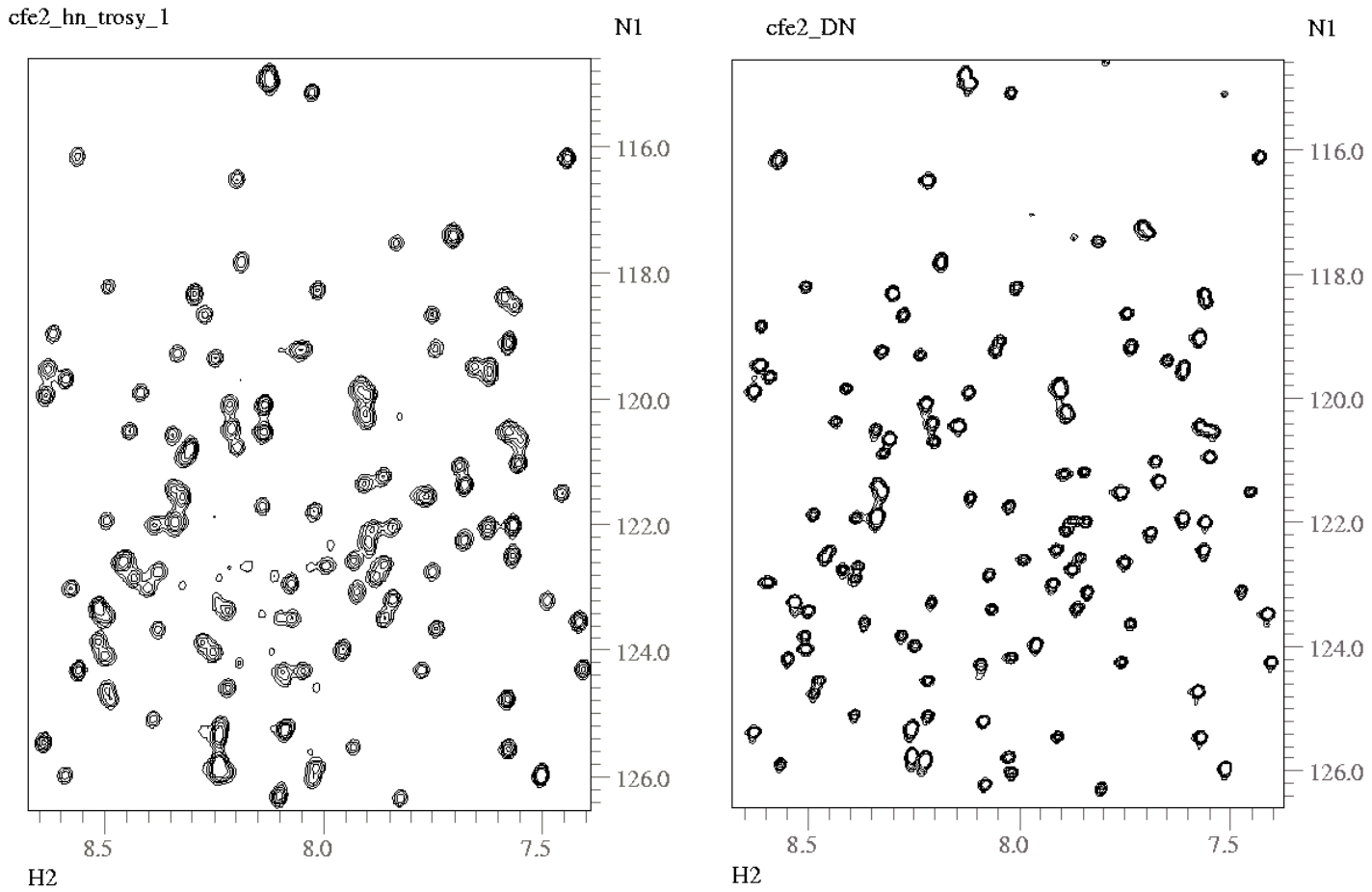

Fig. 2. Comparison of 2D N-15 TROSY spectra of a H-2 and N-15 labelled protein acquired at 600 MHz (on the left) and 900 MHz (on the right) Varian INOVA spectrometers. Note the considerably improved resolution and sensitivity of the spectrum recorded at 900 MHz.

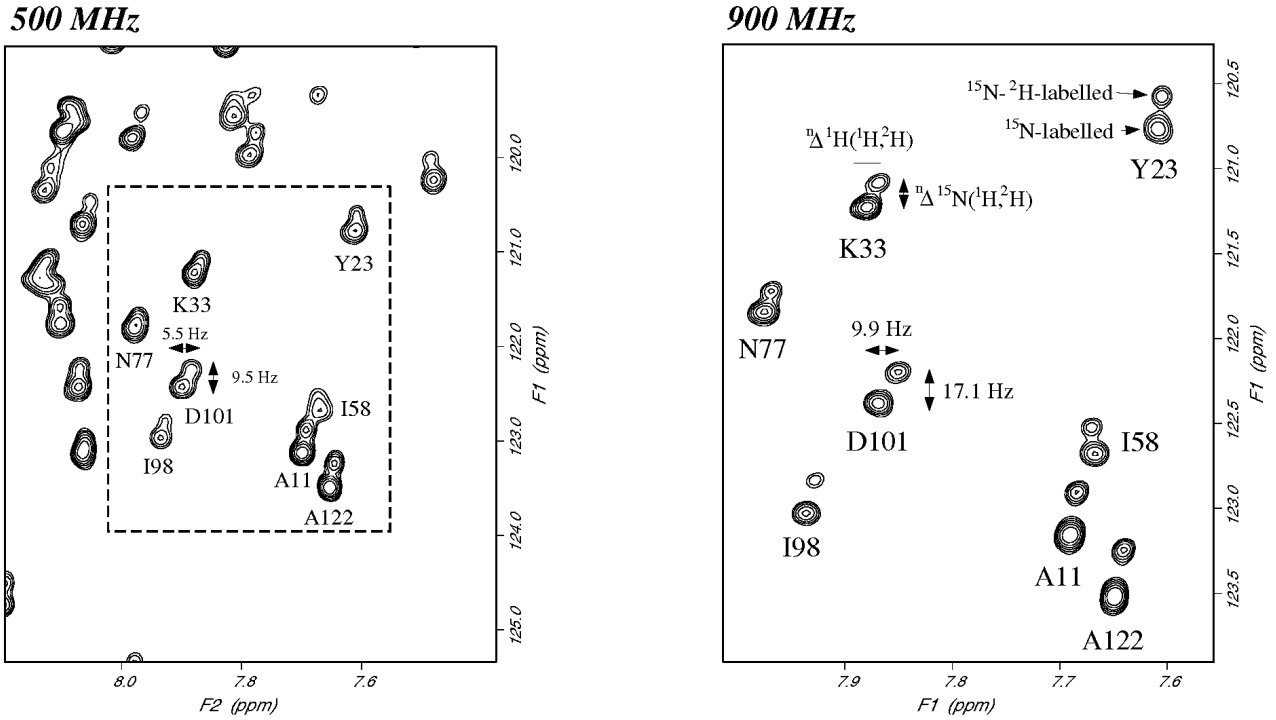

Fig. 3. Comparison of N-15 TROSY spectra of a mixture of H- and D-Lysozyme recorded at 500 and 900 MHz. The splittings in the H-1 and N-15 dimensions are due to the long-range H/D isotope effects.

The high magnetic field is also very important in studies of weak perturbations, such as isotope-induced chemical shift variation (isotope shifts) or perturbations caused by weak intermolecular interactions. The ultimate resolving power of the 900 MHz instrument is compared in Fig. 3 with that of a 500 MHz spectrometer routinely used nowadays.

The long-range isotope shifts $^{n}\Delta^{1}H(^{2/1}H)$ and $^{n}\Delta^{15}N(^{2/1}H)$ are barely resolved in the mixture of deuterated and protonated lysozyme at 500 MHz while the 900 MHz spectrum for the first time allows accurate measurement of these tiny effects in large proteins. The ability to detect such weak perturbations is very important in drug screening, where interactions of proteins with potential drugs and their binding sites and mechanism are studied.

2. Transverse Relaxation Optimized Spectroscopy (TROSY)

NMR relaxation studies provide very detailed information about protein backbone dynamics in solution [5]. Nuclear relaxation rates largely depend on the magnetic field strength, molecular size, and mobility. A qualitative comparison of the longitudinal relaxation rates ($1/T_1$) of N-15 nuclei in a well-resolved region of hen lysozyme reveals that the T_1 relaxation times are indeed very different at 500 and 900 MHz (see Fig. 4).

The experimental relaxation data are typically interpreted in terms of the spectral density function, $J(\omega)$ [5]. In order to improve the accuracy of the calculated $J(\omega)$, such studies often involve recording the spectra at several magnetic field strengths. Since many proteins cannot be studied below 500 MHz, the available range of the magnetic field strengths is often quite limited. The availability of 900 MHz spectrometer substantially extends the available range of field strength for such studies, allowing more accurate studies of molecular properties.

The relaxation also has a strong impact on spectral resolution. Short transverse relaxation times (T_2) result in broader resonances, thus reducing the effective spectral resolution. Unfortunately, the transverse relaxation rates ($1/T_2$) tend to increase with the magnetic field strength and also with molecular size. Protein deuteration is nowadays a routine technique for increasing the proton relaxation times (by ~40%) in large proteins [6].

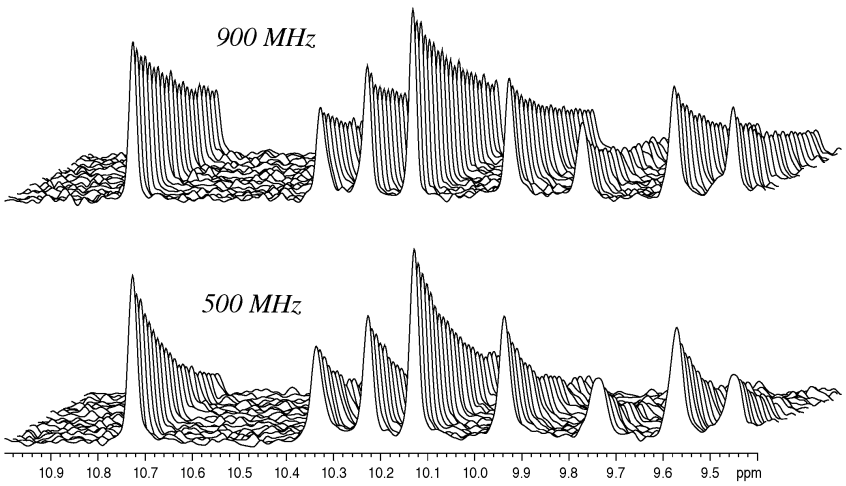

Fig. 4. Qualitative comparison of longitudinal relaxation rates of N-15 nuclei in hen lysozyme at 500 and 900 MHz.

The two most important relaxation mechanisms in proteins are the dipole-dipole interaction (*DD*) and the chemical shift anisotropy (*CSA*) [7]. The magnitude of the CSA contribution for spin *I* depends directly on the magnetic field strength:

$$CSA \propto T_2(\mathbf{B_0}, \mathbf{I}) \tag{3}$$

On the other hand, the dipole-dipole relaxation mechanism is independent of B_0, but rather depends on the mutual orientation of the interacting spins *I* and *S*:

$$DD \propto T_2(\mathbf{I}, \mathbf{S}) \tag{4}$$

Interference between these two relaxation mechanisms gives rise to different transverse relaxation rates (R_2) of the two components of a N–H doublet [7]:

$$R_2^{(1)} = \lambda + \eta$$
$$R_2^{(2)} = \lambda - \eta \tag{5, 6}$$

where λ and η are spectral density functions. This leads to a phenomenon known as differential line broadening (see Fig. 5). Quantitative measurements of relaxation interference yields detailed information related to the ^{1}H, ^{13}C, and ^{15}N *CSA* tensors. Since the spectral density functions λ and η depend also on the molecular correlation times (tumbling rates), the effect is amplified in larger molecules. This opens a whole new avenue for studying extremely large, from NMR point of view, proteins.

The two significant terms that contribute to ^{15}N relaxation are of opposite sign and are field dependent, raising the possibility of optimizing the NMR experiments for transverse relaxation. The experiment that selects the narrow component of the relaxation matrix is called TROSY (Transverse Relaxation Optimized SpectroscopY) [8]. At some "magic field", properly constructed ^{15}N correlation experiments could be performed with relatively narrow lines, even for proteins of 100 kDa! It turns out that the "magic field" is about 1 GHz.

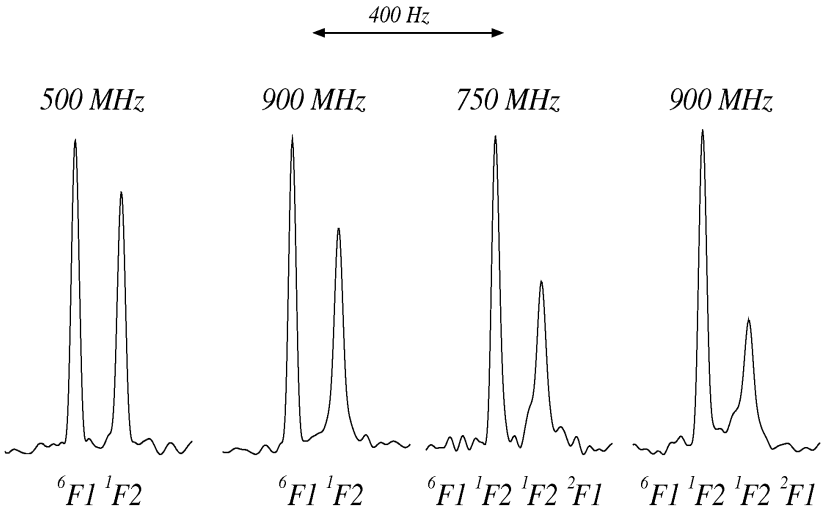

Fig. 5. Comparison of the differential line broadening effect in ^{6}F1^{1}F2 and ^{6}F1^{1}F2^{1}F2^{2}F1 modules of the gelatin binding domain of fibronectin at 500, 750, and 900 MHz. The samples are courtesy of Prof. I. D. Campbell, J. Boyd, J. Werner, and A. Pickford, Oxford University, UK.

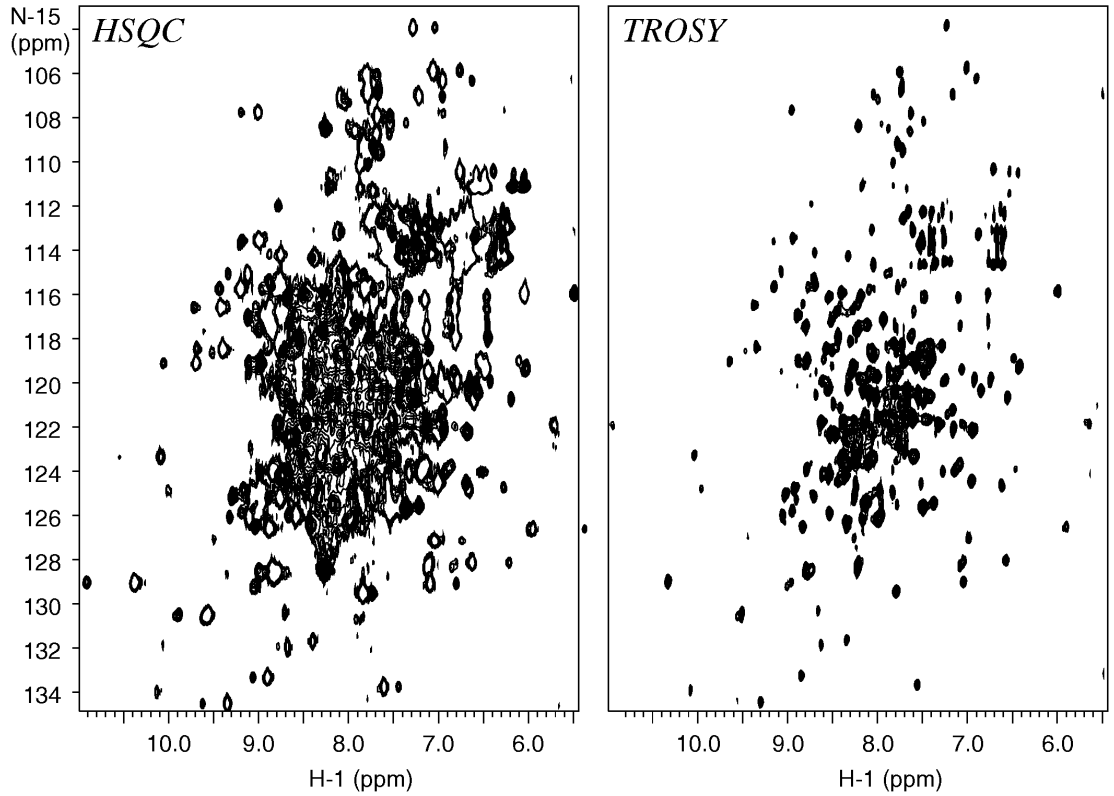

Fig. 6. Comparison of $^{1}H–^{15}N$ 2D HSQC and TROSY correlation spectra of a 78 kDa protein aldolase recorded on Varian INOVA 900 spectrometer. The sample is courtesy of Prof. S. Homans and C. Hilcenko, Leeds University. Note the dramatic improvement of resolution in the TROSY spectrum.

In Fig. 6 two heteronuclear correlation spectra of a 78 kDa protein acquired at 900 MHz are compared. The HSQC spectrum on the left uses conventional techniques to obtain the NH correlations and "decouples" any proton-nitrogen interactions. The spectrum on the right is a relaxation-optimized TROSY spectrum with no N-15 decoupling applied.

3. Molecular Alignment

At high magnetic fields the molecular mobility may be affected by the magnetic field due to anisotropy of magnetic properties of molecules [9]. For instance, the magnetic susceptibility of paramagnetic proteins, DNA, and RNA is largely anisotropic, which can induce partial alignment of these molecules with the magnetic field. Such effects are very interesting, since they reveal important structural information. The dipolar interactions between spin-1/2 nuclei (D) contribute to the line splittings that are observed due to the scalar spin-spin couplings (J). Normally these contributions are negligible because in solution the geometric factor $r^{-3}(3\cos^2\theta - 1)/2$ that scales these dipolar couplings averages out due to fast molecular tumbling. However, if the magnetic field is very strong, the molecular alignment with the field can no longer be neglected. In weakly aligned systems the increase in the observable residual dipolar couplings is proportional to B_0^2. For instance, the contributions from dipolar couplings are by a factor of 3.24 larger on a 900 MHz instrument as compared to that on a 500 MHz system. The residual dipolar couplings due to partial alignment with the magnetic field are clearly visible in the expansions of N–H correlated spectrum of a paramagnetic metalloprotein Cytochrome C (see Fig. 7) [10].

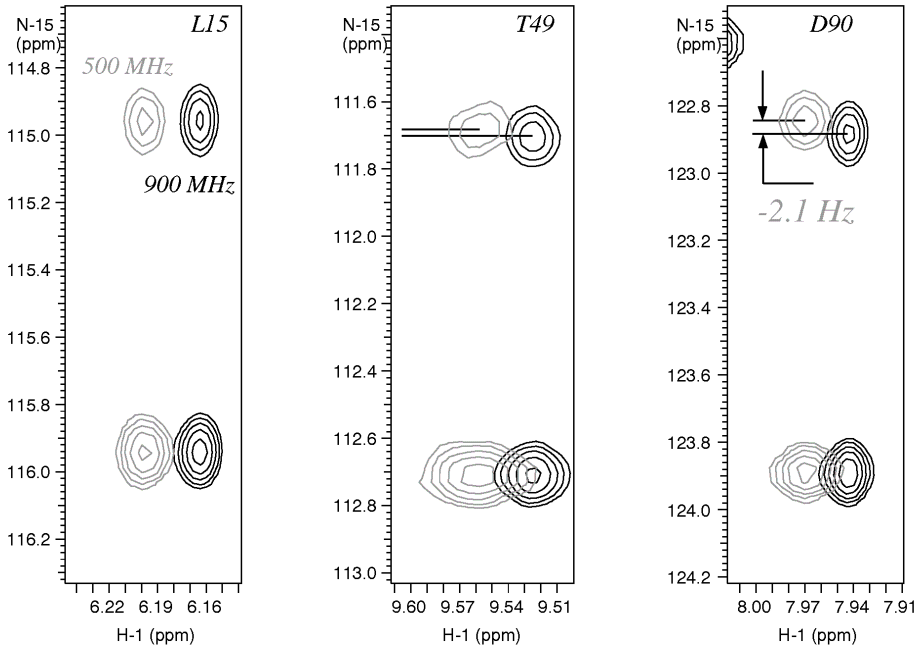

Fig. 7. Residulal dipolar couplings observed due to magnetic molecular alignment in paramagnetic metalloprotein cytochrome C (103 residues) containing Fe^{3+} [Ref.: A. S. Morar, D. Kakouras, G. B. Young, and G. J. Pielak, J. Biol. Inorg. Chem., vol. 4, pp. 220-222, (1999)]. The spectra were recorded on Varian Unity INOVA spectrometers at 500 MHz (in gray) and 900 MHz. The sample is courtesy of Dr. G. J. Pielak, University of North Carolina, USA.

The different magnitudes of the residual dipolar couplings reflect the angles between the magnetic susceptibility vector and the corresponding N–H bond vectors, providing a valuable structural information. This information is new and principally different from the traditional parameters used for structure determination, such as NOEs, *J*-couplings, and chemical shifts, which provide local information regarding torsion angles and distances relative to other atoms in close proximity.

The nuclear Overhauser effect (NOE) is the main source of geometrical information used in protein structure determination. The NOEs have a very strong distance dependence ($\sim r^{-6}$) and provide very accurate through space distance information between protons separated up to 6 Å.

For medium size molecules with $\gamma B_0 \tau_c \sim 1.12$ no NOE can be observed. Availability of high-resolution instruments with different field strength is crucial in such studies.

It is not only the spin-spin couplings that are affected by the strong magnetic field. The chemical shifts are also known to vary due to molecular alignment. Such orientation-dependent changes allow accurate measurements of the chemical shift anisotropy in biomolecules [11].

The molecular alignment can further be amplified by using dilute liquid crystal media that are very strongly oriented, such as bicelles and phages [12]. Working with a higher degree of allignment allows more accurate measurement of residual dipolar couplings. Unfortunately this can also cause substantial line broadening resulting from residual ^1H–^1H dipolar couplings, thus reducing the effective resolution. Again the resolving power of high field systems becomes very important. As shown in Fig. 8, band-selective ^1H homonuclear decoupling provides further improvement of resolution and sensitivity [13, 14]. Just as in the case of the cryogenic probes, this is not an alternative to increasing the field strength but rather a complementary technique that can be used at any field strength to improve spectral resolution and sensitivity.

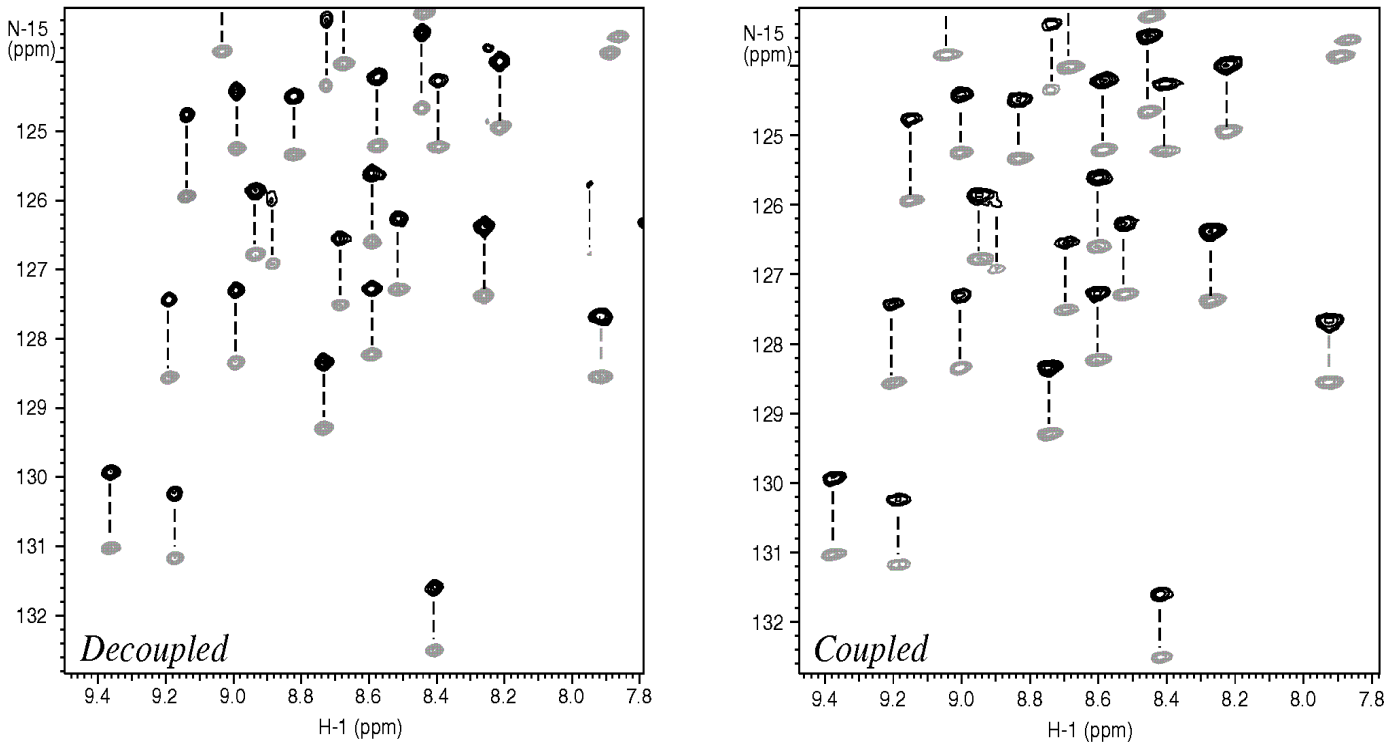

Fig. 8. Measurement of residual dipolar $^{15}N–^{1}H$ couplings in a 101 residue protein, $^{6}F1^{1}F2$ module of the gelatin binding domain of fibronectin, dissolved in dilute liquid crystalline media (bicelles). The spectra were recorded at 900 MHz with (left spectrum) and without (right spectrum) homonuclear $^{1}H–^{1}H$ decoupling. The negative peaks are in gray. The sample is courtesy of Prof. I. D. Campbell, J. Werner, and A. Pickford, Oxford University, UK.

Conclusions

Increasing the magnetic field strength continues to improve the sensitivity and resolution in NMR spectra. Reducing the spectral overlap allows one to study ever larger and larger biomolecular systems such as proteins and DNAs. The extreme resolving power allows detection of tiny perturbations allowing studies of weak molecular interactions and detection of binding sites of potential drugs to proteins and DNAs. On the other hand, at extreme magnetic field strength the behavior of molecules becomes highly unusual. New information that is not easily accessible at lower magnetic fields can be obtained and employed for structure elucidation of large biologically important molecules, such as proteins, DNAs and RNAs. The orientation effects of molecules in a strong magnetic field cause variation of parameters, such as chemical shifts and spin-spin coupling constants, which used to be considered as field independent. NMR is entering a new stage. It becomes very different at these ultimate magnetic field strengths.

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