Low-Temperature Solid-State NMR of Proteins

ANDREW E. DEROME* and SUZANNE BOWDEN

The Dyson Perrins Laboratory, South Parks Road, Oxford OX1 3QY, England

Received March 1, 1991

Contents

Andy Derome grew up in Liverpool, in the North of England, and completed his first degree at Cambridge University. His doctoral studies were carried out in the group of Jack Baldwin at Oxford, and after three years trying to synthesize a fungal metabolite a career in NMR spectroscopy came to seem attractive. He was appointed as manager of the NMR facility in the Dyson Perrins Laboratory in 1982, and to the Physical Sciences faculty of Oxford University in 1988. His research interests included studies of both small and large molecule structures by NMR, the development of computational methods for spectral assignment and structure determination, and application of solid-state NMR to biological problems. In the Spring of 1991, subsequent to the submission of this manuscript, he met an untimely death.

Suzi Bowden completed her first degree in chemistry at Oxford this year. This article was written while she was, in Oxford parlance, a "part 2 student" with Andy Derome. The "part 2" is the final stage of the Oxford chemistry course and is roughly equivalent to a Masters program by research; her project involved trying to apply solid-state NMR techniques to the investigation of several enzymes, including isopenicillin-N synthase. Occasionally it proved possible to perform these experiments at low temperature.

I. Introduction

In surveying the literature of solid-state NMR it is striking that the ratio of reviews to original publications is unusually high. We believe that this reflects on the one hand the evident great potential of this technique for the study of chemical and biological systems, and on the other the formidable technical obstacles to be overcome in obtaining useful results. Despite some reluctance to worsen the review/publication ratio still further, we hope in this article to address, in particular, the community of potential users of solid-state NMR in biology, providing a survey of the area that highlights the practical problems and the means by which they may be tackled, and outlining the results obtained to date within the title field. It is not the intent to provide a comprehensive discussion of the physical basis of solid-state NMR, which may readily be obtained from other sources,[1-6] but a brief qualitative discussion is included to set the context. Likewise, some selected experimental techniques are described in section IV, but this is not intended to be a complete survey. Reviews of the technique of solid-state NMR can be found elsewhere.[7,8] There are also prior reviews of the application of solid-state NMR to biological macromolecules,[9,10] peptides,[11] and proteins.[12] In these introductory sections we have in mind in particular those who are quite familiar with NMR experiments in solution, and so we try to emphasize both the similarities and the differences between the two states.

* Deceased. Address all correspondence to Dr. C. M. Dobson, Inorganic Chemistry Laboratory, University of Oxford, South Parks Road, Oxford OX1 3QR, England.

II. Why Solid-State NMR?

It is clear why NMR in general is a useful technique, but since there is a significant cost in experimental difficulty associated with working in the solid state it

0009-2665/91/0791-1307$09.50/0

is appropriate to examine the motivation for doing so. In studying proteins, we often ask questions about static structure, internal dynamics and chemical mode of action; the latter being of course intimately related to the first two. By *choosing* the solid state, we may selectively increase both our control over the system under study and the information available in these areas. We may also find ourself in the solid state more or less inadvertently as a result of other considerations, like for instance when samples cannot be dissolved or when we wish to work at very low temperatures. In this section we outline briefly the benefits of working in the solid state with respect to the structure and dynamics of biological systems.

A. Structure

With regard to structure, it is appropriate to distinguish between the structure of the protein itself and the structure of any associated small molecules such as cofactors, inhibitors, substrates, and intermediates. There are two main sources of structural information in NMR spectra: chemical shifts, which tell us about the environments of individual nuclei, and couplings, which tell us about the relationships between pairs of nuclei. In the solid state, the coupling of interest is the dipolar coupling, which is much larger than the more familiar scalar couplings that are all that remain under motional averaging in solution. In principle, measurements of dipolar couplings can give rather accurate information about interatomic distances, since they are directly related. However, solid-state NMR is unlikely in general to yield a highly detailed overall structure of a protein in the same way as has been demonstrated for solution experiments in recent years,[13] although there have been some studies of the structure of small peptides and structural proteins.[14] What is possible is to measure selected distances, the selection being made by isotopic labeling. A recurring theme in solid-state NMR is the need to use labeling, for reasons of both sensitivity enhancement and selectivity. In this application, for instance, relevant areas of the protein and its substrate might be labeled in order to investigate the interaction between the two. Success of course requires sufficient foreknowledge of the expected interaction.

The second source of structural data is the chemical shift, familiar from solution work. This is most often useful with respect to associated small molecules rather than the protein itself. In the solid there is in principle more information available from chemical shift measurements, because in the absence of molecular motion the shift depends not only on the chemical environment of the nucleus but also on the orientation of that environment with respect to the external field. The "shift" is thus different in different directions, and must be characterized in three dimensions as a 3×3 matrix, the chemical shift tensor. By suitable choice of axis system this matrix can be diagonalized, and the three diagonal elements are then known as the principal values of the shift tensor, often symbolized σ_{11}, σ_{22}, and σ_{33}. These parameters can be obtained directly either from powder patterns observed in static samples or from analysis of sideband intensities in magic-angle spinning spectra,[15,16] but for a detailed interpretation of their meaning it is also necessary to determine the orientation of the shift tensor relative to the molecule.[17] This latter determination requires either measurements in a single crystal or the use of two-dimensional techniques.[18,19] From the qualitative perspective, the advantage of these solid-state measurements is that we have three parameters to work with instead of the single average, or *isotropic*, shift obtained in solution. Changes in the isotropic shift are sometimes found to be localized in only one of the three principal values,[20] and this may cast extra light on the underlying structural change. The difficulty with interpreting such data, as always, is the need for references to compare them with,[21] and as these are much more sparsely available in the solid state than in solution interpretation in a particular case may require the development of suitable model systems. Even in the absence of models, though, the relationship between the three principal values of the shift gives valuable information about the symmetry of the environment of the nucleus concerned.

B. Dynamics

In many ways it is those parts of a protein that change during its action that are of interest, yet unfortunately most structural techniques yield information more easily about those parts that remain the same. Solid-state NMR has some striking advantages in the investigation of dynamically changing systems. Clearly much of the large-scale dynamic behavior of a protein, such as overall molecular rotation and translation, will be absent in the solid state. However, many more localized motions remain, at least at room temperature, and the study of these may thus be conveniently isolated. Presence or absence of motion may be detected in a variety of ways. Complete absence of motion often leads to loss of symmetry, as for instance when monosubstituted phenyl rings show a different ^{13}C shift for each ring carbon. Motion on the appropriate time scale then leads to the same kind of line broadening and averaging effects that are familiar from solution NMR, and these may be analyzed in the usual way. More detailed information, often sufficient to distinguish between subtly different mechanisms of motional averaging, is available from the study of the line shapes of quadrupolar nuclei (usually ^2H), and this technique will be discussed later.

A less helpful manifestation of local motion is that if it occurs on certain time scales it can interfere with aspects of the solid-state technique, such as heteronuclear decoupling, and it can lead to unsatisfactory relaxation behavior in some cases. Interesting time scales in this respect include those related to chemical shifts (typically ranging from hertz to kilohertz), dipolar couplings (10s to 100s kilohertz), quadrupolar couplings (100s to 1000s kilohertz) and the strength of the radio frequency (rf) field employed (10s to ca. 125 kilohertz). In working with samples over a range of temperatures the rates of various internal motions often go in and out of tune with these frequencies, leading to surprising changes in the spectrum obtained. One of the most important considerations in studying biological systems is the lower detection limit imposed by the sensitivity of the NMR experiment, and it is critically important to ensure that signal is not sacrificed by an unfortunate choice of experimental conditions. This problem is often best addressed by experimenting with suitable model systems.

III. Why Low Temperature?

The principle motivation for studying systems at low temperature is to stop things from happening, or at least to slow them down to an extent sufficient to enable them to be investigated by NMR. This is a familiar requirement in the study of enzyme mechanism, in which reduction in the sample temperature in order to stabilize transient intermediates is the basis of cryoenzymology.[22]

Cryoenzymology requires the use of mixed solvent systems like methanol/water or dimethyl sulfoxide/water that can be cooled below 273 K without freezing and is therefore restricted to the study of enzymes that are soluble in such solvents and retain their activity under these conditions. An additional problem, if cryoenzymology is to be used in conjunction with NMR, is that in cold, viscous solvents line widths increase, leading to loss of resolution and sensitivity. The range of temperatures that can be employed is also subject to considerations of solubility and freezing point. A logical step beyond traditional cryoenzymology, then, is to abandon the attempt to work in solution, reduce the temperature much further, and try to use the techniques of solid-state NMR to recover the required information. The same argument could be advanced in favor of the use of low-temperature solid-state NMR for the study of a variety of chemical systems.[23]

Another justification often cited for working at low temperature is the increase in sensitivity that may be obtained, but it is necessary to be careful about the meaning of "sensitivity" when making this claim. Clearly, if some species is observed at low temperature that cannot be observed at room temperature because it is transient, in that sense the sensitivity has been improved. Similarly, if a system suffers from unsatisfactory rates of internal motion that are removed at low temperature, as in the studies of membrane proteins referred to later, then that is also an improvement in sensitivity. As to whether an increase in signal-to-noise ratio can be obtained for a species that exists in equal concentration at high and low temperature and does not suffer from motion problems, that is a much more difficult question. In principle signal strength should be increased on cooling as a result of an improved Boltzmann distribution at equilibrium, and noise should be reduced as a result of less thermal motion of electrons in the receiver coil, but it is necessary to set against this reduction changes in relaxation behavior.

In the case of the cross-polarization experiment usually employed to observe spin-$^1/_2$ nuclei,[24] the optimum repetition rate is determined by the proton longitudinal relaxation time T_1^H. In the solid this relaxation time almost always increases as the temperature is reduced, and this is very likely to outweigh any gains obtained from the two factors mentioned previously. A second consideration is that the rotating-frame relaxation time $T_{1\rho}^H$, which determines the rate of decay of the spin-locked proton transverse magnetization, needs to be long compared with the time taken to achieve cross-polarization. $T_{1\rho}^H$ is affected by motions whose rate is comparable with the rf field strength, and although this is less predictable than the variation in T_1 it is often found in practice that $T_{1\rho}^H$ assumes inconveniently short values at lower temperatures.

A final consideration that will be recognized by practitioners of low-temperature solid-state NMR is that there are a number of rather intangible, but nevertheless real, practical difficulties associated with the experimental technique. Careful experimental setup is essential to obtain the best solid-state spectra, and both electrical and mechanical problems may arise as the sample is cooled. Some of these difficulties are discussed in the following section.

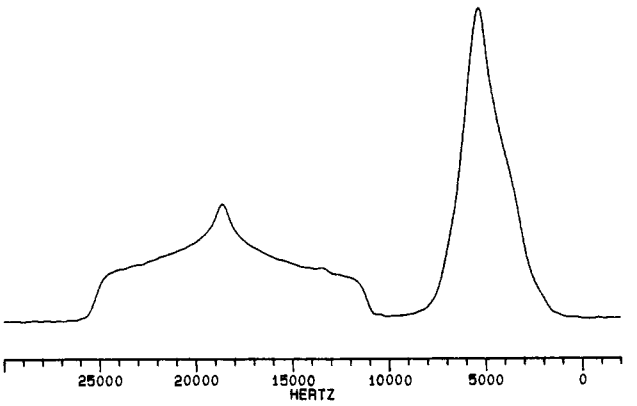


Figure 1. Static ^{13}C powder pattern for glycine (100.6 MHz; cross polarized).

IV. Problems and Solutions

In this section we discuss certain aspects of the technique of solid-state NMR. This is by no means a comprehensive survey, but we have selected some areas likely to be unfamiliar to those who have previously only worked with samples in solution, and a variety of experimental methods that are both useful and practical so as to offer guidance in choice of experiment.

A. Unfamiliar Interactions

The orientation dependence of chemical shifts, known as chemical shift anisotropy, the dipolar coupling and the quadrupole interaction are all large effects, and their impact on spectra obtained in the solid state is considerable. Although, as indicated previously, the existence of these interactions provides a potential source of extra information, it is often desirable either to eliminate or to reduce their effects so as to work back toward a spectrum similar to that obtained in solution. A combination of several techniques is required in order to achieve this.

1. Chemical-Shift Anisotropy

Chemical-shift anisotropy means that if one were to take a single crystal of some substance and measure its NMR spectrum, the shift values observed would vary as the crystal was rotated relative to the static field. In a powdered (but microcrystalline) sample, every orientation is present simultaneously and so the spectrum obtained is a superimposition of the various lines from different crystallites. The resulting powder pattern obviously reflects the range of variation of the shift, and it can in fact be used to evaluate the elements of the shift tensor. However, the total width of this pattern, which depends on the intrinsic anisotropy of the environment of the nucleus concerned and on the static

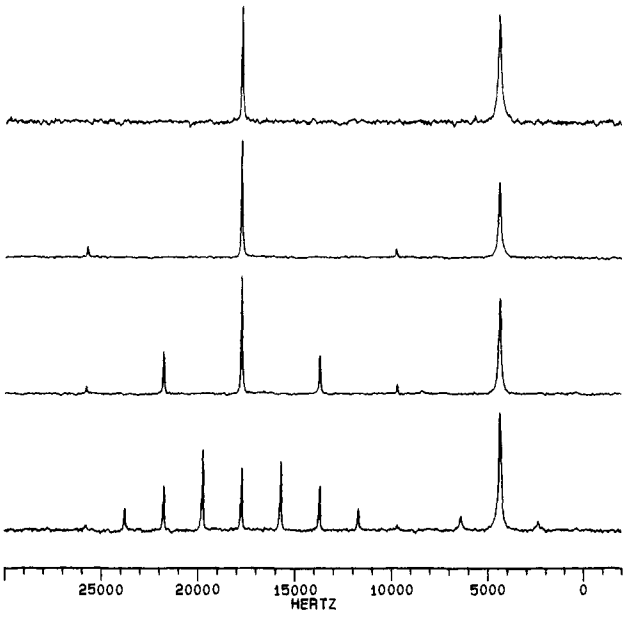


Figure 2. ^{13}C CP-MAS (100.6 MHz) spectra of glycine at various spinning speeds (bottom to top: 2015, 4025, 8000, 12040 Hz).

field, typically ranges up to 10s of kilohertz so that in a real sample with many lines it is impossible to separate the various overlapping patterns. In the static ^{13}C spectrum of glycine (Figure 1) it can be seen that the anisotropy associated with the carbonyl group is much larger than that associated with the methylene, as of course we know from the influence of the carbonyl on the shifts of adjacent nuclei in solution. If the object of the experiment is simply the determination of the isotropic shift, it is thus desirable to remove the effect of the anisotropy, and this can be achieved by magic angle spinning[25,26] (MAS).

The essence of this method is that the motion that was present in the liquid is restored to some degree by rotating the sample. It is clearly impossible to reproduce the completely random reorientation characteristic of a molecule in solution, but for spin $^1/_2$ nuclei it proves sufficient to rotate the sample about an axis set at an angle of 54.7° ($\cos^{-1}(1/\sqrt{3})$) to the static field. The rate of rotation ideally needs to be substantially greater than the width of the powder pattern to be averaged, but even if this condition is not met some averaging is obtained. Figure 2 shows ^{13}C spectra of glycine obtained at various different spinning speeds. From these it can be seen that at slow speed the line breaks up into a series of sidebands, located at multiples of the spinning speed away from the average shift position, and whose intensity distribution reflects the shape of the powder pattern. As the speed increases the sidebands move further apart and more intensity is transferred to the center band, until at very high speeds essentially only the center band remains. The pattern of sidebands obtained at lower speeds can be used to calculate the principal values of the shift tensor.[15,16] It can also be seen that the more anisotropic carbonyl group requires much faster spinning in order to average the anisotropy completely, and Figure 3 demonstrates that the averaging is more difficult at higher field by comparing spectra obtained at 50 and 100 MHz.

It would appear thus far that the faster the spinning the better, and from the point of view of retrieving a single line out of a powder pattern that is indeed true.

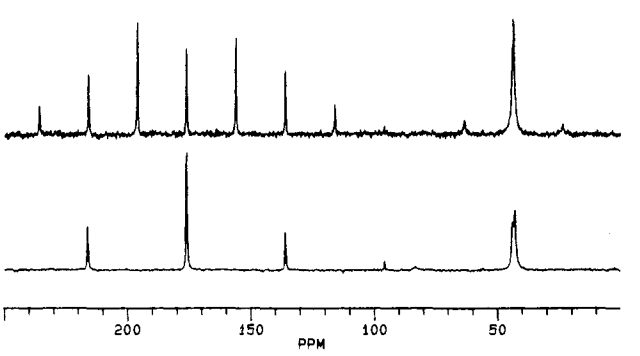


Figure 3. ^{13}C CP-MAS at 50 MHz (bottom) and 100 MHz (top); equal spinning speed (2 kHz). The sidebands appear closer together in the top trace because the spectra are plotted on equal parts per million scales.

However, it will be noted that the top trace in Figure 2 shows a significantly degraded signal-to-noise ratio, even though all experimental conditions apart from spinning speed remain the same. The origin of this problem will be described in section IV.B.1. Very fast spinning can only be obtained on samples of small size (e.g. 4–5 mm diameter), and this may unduly restrict the quantity of material under study. For typical 7-mm diameter samples with a volume ca. 300–400 μL the routine upper limit on the spinning speed is ca. 5 kHz at room temperature, and this limit falls slightly as the temperature is reduced. Recently designed probes extend the limit as far as 8 kHz in 7-mm rotors, but it is necessary to take care that the rotor is made from strong enough material to withstand the stresses involved.

2. *Dipolar Coupling*

The second unfamiliar interaction is the dipolar coupling. This is the direct magnetic interaction between nuclei, analogous to the force one feels on holding two bar magnets next to each other. For an isolated pair of spin $^1/_2$ nuclei the dipolar coupling splits the line into a doublet, and the size of the splitting depends on the internuclear distance and the orientation of the internuclear vector relative to the static field. In solution the molecular reorientation averages the splitting to zero, and the dipolar interaction is detected only since its variation is the principle stimulus to relaxation for spin $^1/_2$ nuclei.[1] Once again in a powdered sample all possible orientations are present, leading to a characteristic line shape due to a superimposition of many doublets with different splittings.[27]

The size of the dipolar coupling is often greater than the range of chemical shifts, and this has a number of important consequences. It means that for abundant nuclei, such as ^1H, the complete spin system is always strongly coupled, with the result that it is not possible to distinguish between individual spins and the system has to be treated as a whole. There is thus only one rate of relaxation for the complete ^1H spin system, and perturbations in the population of any proton propagate almost immediately to the remainder. The homonuclear dipolar coupling could in principle be averaged by MAS, but this would require spinning speeds significantly greater than the magnitude of the couplings, which for ^1H–^1H interactions is not feasible. Multiple-pulse schemes have been proposed that cause line narrowing in homonuclear coupled systems,[28,29] but even

when these are used in conjunction with MAS[30] the line widths presently obtainable are still too large for practical application to biological systems. We can thus exclude solid-state ^1H NMR from further consideration.

For a dilute nucleus, such as ^{13}C, there is no homonuclear dipolar interaction to consider, and the problem with respect to the heteronuclear interaction with ^1H spins is amenable to experimental solution. The ^1H–^{13}C dipolar couplings are very large, especially for directly bonded atoms, but they can be removed by heteronuclear decoupling. It is necessary to irradiate the protons continuously with an rf field of strength significantly greater than the total width of the proton spectrum—in practice fields from 70 to 100 kHz are fairly readily achieved. The main experimental limitation on the field employed is the ability of the NMR probe to withstand the voltages involved without suffering electrical failure, and it may also be necessary to consider the effect of the average power dissipation on the temperature of sensitive samples. However, since acquisition times in solid-state NMR are typically quite short while repetition rates are rather low, this latter point is not normally too restrictive. There is no equivalent at present to the composite pulse decoupling schemes that have been so successful in solution NMR,[31] and because of the magnitude of the interactions involved, and the strong coupling in the ^1H spin system, the problems of devising methods applicable in the solid state are formidable. Thus the decoupling employed is straightforward continuous irradiation at the center of the ^1H resonance range.

The combination of high-speed MAS and strong decoupling of ^1H leads, for nuclei like ^{13}C, ^{15}N, and ^{31}P, to spectra quite similar to those obtained in solution, subject to the considerations discussed in the next two sections.

Determination of heteronuclear dipolar couplings, and of the geometric relationship between the dipolar interaction and the chemical shift anisotropy, can be achieved by a variety of two-dimensional techniques. The earliest experiment, applied in static single crystals and powders, is known as separated local field spectroscopy.[32-35] This employs an evolution period during which heteronuclear dipolar couplings are allowed to evolve, while homonuclear interactions are suppressed by a multipulse decoupling sequence. The result for a single crystal is a spectrum with chemical shifts in the F_2 dimension and the dipolar splitting in F_1, somewhat akin to J spectroscopy in solution. When used in conjunction with MAS the t_1 sampling period needs to be a multiple of the sample rotation period, leading to an experiment designated rotational echo NMR.[36-39]

3. Quadrupolar Effects

Nuclei with spin $> {}^1/_2$ have, in addition to the dipolar interaction described above, a quadrupole which can couple with the electric field gradient at the nucleus. This interaction is usually larger than both the chemical shift and the dipolar coupling (for instance, in the region of 130 kHz for ^2H and several MHz for ^{14}N), and as a result it completely dominates the spectra of quadrupolar nuclei. This may affect the results obtained either directly, when a nucleus such as ^2H or ^{14}N is measured, or indirectly when the observed nucleus interacts with a quadrupolar nucleus. The latter situation is particularly common in protein studies because of course many of the carbons involved are attached to ^{14}N.

The direct consequence for the observation of quadrupolar nuclei is once again a doublet for an oriented single crystal and a characteristic wide static pattern for a powder, since the quadrupolar interaction depends on the orientation of the electric field gradient relative to the static field. In contrast with the dipolar case, in which the coupling is between *pairs* of nuclei, for the quadrupole *each* nucleus contributes a doublet. For the commonly studied ^2H, the quadrupole coupling is vastly greater than the chemical shift range, so that the line shapes obtained reflect only the quadrupolar interaction. This line shape is very sensitive to motion with time scales of the same order as the reciprocal of the quadrupole coupling, and this has been exploited to good effect in the study of protein dynamics as outlined later. The relaxation times for quadrupolar nuclei are also sensitive to motions on interesting time scales, and are almost always very much shorter than those of spin $^1/_2$ nuclei. This makes multiscan signal acquisition more efficient, but also contributes significantly to the width of the lines.

The effect of MAS on quadrupolar powder patterns depends on whether the nucleus has integral (e.g. ^2H, ^{14}N with $I = 1$) or odd half-integral (e.g. ^{23}Na, $I = {}^3/_2$; ^{17}O, $I = {}^5/_2$) spin. For integral spin nuclei, spinning can in principle average the pattern[40] but the speed required for complete averaging would of course be prohibitively high (see also the discussion of effects arising when the quadrupolar coupling is the same order of magnitude as the Larmor frequency in the following paragraph). In practice rather broad lines are still likely to be obtained because of their short T_2s. Thus, on balance, this leads to a loss rather than a gain in information content and hence is seldom employed. For odd half-integral spin nuclei, spinning about a single axis is not sufficient; it is necessary to spin simultaneously about two axes, which sounds impossible but has in fact been achieved.[41,42] However this technique has not yet been applied to systems within the scope of this review.

The indirect effect of the quadrupolar interaction of ^{14}N on ^{13}C spectra is of some significance in the study of peptides and proteins. The heteronuclear dipolar coupling between these nuclei would in principle be averaged by MAS, but the large quadrupole interaction interferes with this process. The net result is either a splitting or a broadening of the resonances of ^{13}C atoms attached to ^{14}N.[43] This is readily apparent in the relative widths of the carbonyl and methylene carbons of glycine in the spectra shown previously. The effect depends on the relative sizes of the ^{14}N quadrupole coupling (3–5 MHz and independent of the static field) and the Larmor frequency (7.2 MHz at a proton frequency of 100 MHz and scaling with the static field), and so it decreases as the static field strength increases.

4. Solid-State Effects

Efficient decoupling and high-speed MAS notwithstanding, solid-state spectra nearly always have broader lines than those obtained in solution, and in addition there are often more lines than would be expected from solution studies. The former problem arises from a whole host of factors, both intrinsic to the sample and

of an experimental nature, and these have been surveyed previously.[44] Many of the contributions to the line width increase with the static field, so, in contrast to solution NMR, increased effective dispersion may not be obtained on increasing the field.

Extra lines typically appear in crystalline powders because of both locking of molecular conformations, as in the example of phenyl rings mentioned earlier, and due to the occurrence of more than one distinct environment in the unit cell. Neither circumstance is easily predictable a priori, and each case must be analyzed as it arises. Motion within the solid sample can average out conformational effects or convert them to a source of line broadening and occurs more often than would perhaps be expected. Aromatic side chains of amino acids, for instance, are often found to be undergoing various kinds of ring-flipping motion even in the crystal.[45] In amorphous samples, which may arise for example when one tries to catch an intermediate by rapidly freezing a solution, individual molecules may find themselves in a wide variety of different environments, leading to line broadening of many parts per million. This does not mean that it is impossible to obtain useful spectra from amorphous materials, but dispersion will not be anywhere near that achieved in solution or in crystalline powders.

B. Sensitivity

Lack of adequate sensitivity is always a problem in NMR spectroscopy, and this is especially true in the solid state where often lines are broad and relaxation properties unfavorable. In the measurement of spin $^1/_2$ nuclei, an essential technique for sensitivity enhancement is cross-polarization of the nucleus of interest using the magnetization of ^{1}H.[24,46,47] This procedure is somewhat similar to the polarization transfer methods that have become well known in solution NMR, but with the difference that it is mediated by the dipolar coupling. The goal is to equilibrate the less-polarized ^{13}C spins with the more strongly polarized (as a result of the higher Larmor frequency) ^{1}H spin system, using the dipolar couplings as the connection. What is required, in essence, is to bring about the situation that already exists *within* the ^{1}H system, in which the strong dipolar couplings immediately propogate population disturbances through the system as mentioned in section IV.A.2. Unfortunately, from the point of view of the *heteronuclear* dipolar interaction the system is normally weakly coupled, since the difference in Larmor frequencies (100s MHz) is much greater than the dipolar coupling.

In cross-polarization this situation is temporarily circumvented, by transferring the ^{1}H magnetization to the transverse plane with a 90° pulse, and then holding it there by continuous irradiation 90° out of phase with the original pulse (spin locking). In this state the ^{1}H spins precess around the rf field instead of the static field, and so they have a kind of "effective Larmor frequency" determined by the magnitude of the rf field. At the same time the ^{13}C spins are also subject to spin locking, and since the precession rates of the two kinds of nuclei depend on the respective rf field strengths, we are free to adjust conditions so that the ^{13}C precession occurs at an equal rate to that of ^{1}H—the Hartmann–Hahn matching condition.[48] Since the ^{13}C gyromagnetic

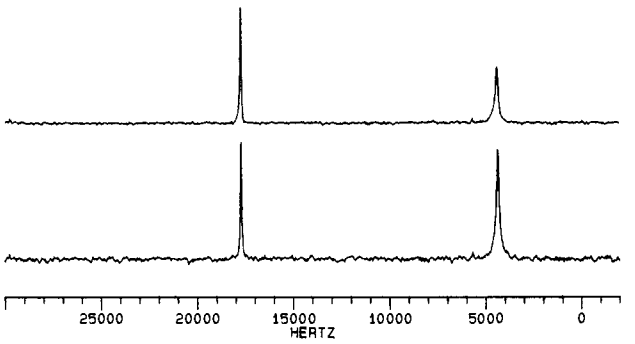


Figure 4. ^{13}C CP-MAS at 12 kHz spin speed; contact time 1 ms (bottom) and 10 ms (top). The intensity of the carbonyl signal varies more than that of the methylene because it experiences smaller dipolar couplings.

ratio is one fourth that of ^{1}H, this requires that the ^{13}C field be 4 times bigger. Under these conditions the nuclei have, in effect, no difference in frequency, and hence they become strongly coupled by the dipolar interaction. Over some ensuing period (the contact time), whose duration depends on the size of the dipolar couplings, the two systems come to equilibrium, and on switching off the ^{13}C field we can measure a signal with roughly 4-fold enhancement in sensitivity. In addition the experiment can be repeated after a time determined by the ^{1}H T_1, which is usually relatively short.

Efficient cross-polarization requires that each carbon is reasonably close to some protons, and in ordinary organic molecules this condition is normally met even for quaternary carbons. If for some reason proton–carbon distances are large, equilibration takes longer and the contact time must be correspondingly increased. This will only be effective so long as the spin-locked ^{1}H magnetization does not decay too much during the contact, which requires that the contact time is less than $T_{1\rho}^{H}$. In practice contact times ranging from 100s microseconds to 10s milliseconds are often appropriate. Long contact times, during which two strong rf fields are being applied to the sample, can lead to electrical or heating problems.

Very fast sample spinning, such as was used for the top trace in Figure 2, can begin to interfere with cross-polarization as the spinning rate approaches the same order of magnitude as the dipolar couplings. Increasing the contact time can recover some of the lost signal in this case too (Figure 4), subject to the same limitations of practicality.

The ultimate question of interest in practical applications of cross-polarization is the minimum quantity of material that can be detected. It is impossible to give a definitive answer to this question because it depends on such a complicated combination of factors such as relaxation, line widths, and field strength. However, as a *very rough* rule of thumb, we find that for ^{13}C observation at 100 MHz *with 100% enrichment* in a single site, it is desirable to have around 1 μM of the species to be detected present. This is not a large quantity from the perspective of small organic molecules, but when one considers the prospects for observing enzyme-bound species, even assuming saturating conditions, the amount of a medium molecular weight protein required becomes uncomfortably large. This is reflected in the fact that solid-state observation of enzyme-bound species has been restricted to cofac-

tors and inhibitors to date. The observation of a true transient intermediate is indeed a challenging objective.

C. Low-Temperature MAS

Low temperature work presents few problems with static samples, as in wide-line ^2H experiments, but the stringent mechanical requirements for magic-angle spinning mean that temperature reduction requires care. Much of the literature material on low-temperature MAS comes from groups that build their own spectrometers and hence employ a variety of solutions to this problem,[23,49] but commercial systems are now available that, in principle at least, permit low-temperature work. In a MAS probe there are typically two gas streams, one for the gas bearings on which the sample floats and one to drive the sample round. Either, or preferably both, of these may be precooled in order to regulate the sample temperature. A prerequisite for success is a source of very dry, CO_2 free gas. If the gas employed is N_2, then the best source in principle is evaporating liquid, but the amount of liquid consumed can be quite large. High-purity nitrogen from cylinders is also satisfactory, but once again the rate of consumption means that long-term operations are difficult to sustain. We have found that a satisfactory alternative is to use a commercial N_2-purifying machine that employs a zeolite-based absorption process to make dry N_2 from air.[50] This has the advantage that it never runs out. For very fast spinning at low temperatures it is necessary to use helium as the drive and bearing gas, and such experiments have been reported in the literature.[51] However, in this case it is essential that the gas does not come into contact with the electrical components of the probe because it has unsatisfactory properties with respect to electrical breakdown. Since most commercial probe designs have no provision for isolating the gas streams from the transmit/receive coil this is not a routine application.

A second consideration in low-temperature work is the behavior of the rotor/cap combination. Various different substances have been proposed for construction of rotors for MAS, including both plastics and ceramic-type materials.[8] It is necessary to balance considerations of mechanical strength and satisfactory thermal expansion/contraction behavior against a requirement that the rotor material does not contribute NMR signals of the nucleus of interest. For ^{13}C and ^{15}N work zirconia or sapphire rotors are satisfactory. In choice of cap, background signals are of less significance since it will be outside the sensitive region of the probe, but it is essential that contraction at lower temperatures does not loosen the fit of the cap into the rotor. In many commercial designs the driving force for sample rotation is transmitted through the cap, so there is a significant stress between cap and rotor. The plastic Kel-F has quite good performance in low-temperature applications, but for complete safety it may be found necessary to glue the cap to the rotor. This is particularly convenient for samples that are initially liquid, since by drilling a small hole in the exact center of the cap it is possible to insert and remove the sample through a plastic cannula. The sample can then either be frozen or dropped directly into the pre-cooled probe, after whatever incubation period is necessary to generate the species of interest. An alternative to gluing the cap is to use caps made from boron nitride, but this material is quite soft and the caps tend to wear out or break after only a few uses.

D. Wide Lines

NMR of static samples, typically employing ^2H observation in protein studies, presents some interesting experimental difficulties. The basic problem is that the spectral range is so large that it is difficult to get uniform excitation and detection of the signal with pulse methods. The problem is particularly acute since the main point of interest in wide-line work is the precise form of the line shape, and distortion of this due to experimental deficiencies detracts considerably from the value of the measurements. So far as excitation is concerned, though some composite pulse schemes have been reported in the literature,[52] the majority of work still simply employs the highest possible rf field strengths, and hence the shortest possible 90° pulse widths. Pulse widths down to 1–2 μs can be achieved reasonably routinely in probes employing a horizontal sample and solenoidal coil, corresponding to field strengths from 125 to 250 kHz, but since the widths of ^2H lines in static samples are of a similar magnitude distortions due to off-resonance effects are still likely.

With regard to detection, problems arise in a number of different ways. It is necessary for the spectrometer receiver to have a flat response over the large frequency range in question, and receivers designed originally for high-resolution work may not have incorporated this as a design goal. Spectrometers oriented specifically toward wide-line work usually address this problem quite well. It is also necessary to be able to sample at a sufficiently high rate, but suitable digitizers are now readily available. A more difficult problem relates to the timescale of the FID in comparison with the time that must elapse after the pulse before it is possible to start measuring the signal. This time is determined by both the recovery rate of the receiver electronics after the pulse is turned off, and by the time taken for mechanical disturbances in the probe, induced by the pulse, to die away. The latter ringdown time is often 10s of microseconds, and unfortunately it is not acceptable to wait this long before starting the measurment, since the most important part of the FID determining the frequency domain line shape occurs early on in the decay.

The solution, universally employed, is to excite a spin echo, known in this case as the quadrupolar echo,[53-55] in order to regenerate the early part of the FID after a sufficient time for ringdown and receiver recovery. The sequence required for quadrupolar nuclei is to follow the initial 90° pulse with a second 90° pulse phase-shifted by 90°. By leaving a few 10s of microseconds between the pulses it is possible to ensure that the echo peak occurs after the spectrometer is ready to start measuring. In practice, sampling is usually started significantly before the peak of the echo, and the resulting FID is adjusted by left-shifting the data until the exact echo peak is at the beginning. This technique substantially improves the detection of wide line shapes, but it is necessary to interpret the results with care in the presence of molecular motion because this may cause the line shape to become dependent on the choice of echo interval.[56]

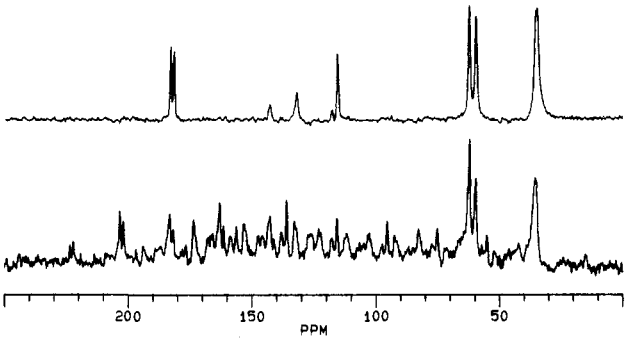


Figure 5. ^{13}C CP-MAS spectra of tryptophan spinning at ca. 2 kHz; direct observation (bottom) and TOSS (top). There is no sensitivity advantage in the TOSS experiment; the upper trace has substantially more scans.

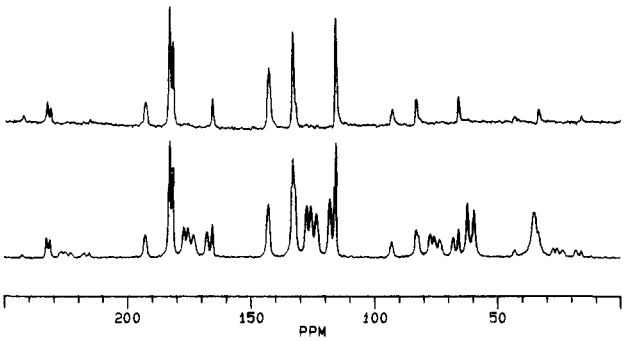


Figure 6. Tryptophan at 5.1 kHz spin speed; all signals (bottom); quarternaries and their sidebands, only (top).

E. Spinning Sidebands

It has been shown previously that in many cases it is not possible to average completely the chemical shift anisotropy by MAS, so spectra retain a considerable contribution from spinning sidebands. The sideband intensities may of course be of interest, but in more complex molecules it can be difficult to distinguish between center bands and sidebands (Figure 5), and if the goal is simply determination of the isotropic shift, dispersion of the signal into the sidebands degrades sensitivity. There are a variety of techniques available to help with this problem. Chemical shift scaling[57,58] uses a multiple-pulse sequence to scale down the effective anisotropy, so that MAS is more effective at transferring intensity to the center band. However, since the ordinary chemical shift differences are reduced as well, discrimination between signals will be worsened unless the line widths also decrease, which is not the case in practice. In addition this method can introduce artefact lines in the spectrum!

Sidebands can be removed from the spectrum by a number of variants of the TOSS (total suppression of sidebands) method,[59-61] but since they are only cancelled by phase alternation there is no enhancement in the strength of the center band. Nevertheless, the experiment is reliable and effective and may be very useful in identifying the center-band positions (Figure 5). The simplest variant of TOSS is restricted to use at quite low spinning speeds (< ca. 2.5 kHz) because otherwise an interval in the pulse sequence related to the spinning speed becomes too short.

F. Finding the Peak

Often we are searching for a single interesting peak in the midst of a host of others. In solution NMR many manipulations can be employed in order to edit out peaks according to some criterion, but the range of operations available in the solid state is much more limited. One straightforward and useful method eliminates resonances from carbons directly attached to ^{1}H by the simple expedient of switching off proton decoupling for a short time, typically <100 μs. The large dipolar coupling between attached nuclei causes the protonated carbon signals to dephase rapidly, leaving only the signals from quaternaries (Figure 6). This dipolar dephasing experiment[62] is generally effective and easy to apply if signals of interest are expected to come from quaternary carbons. The delay before acquisition does cause a significant phase gradient across the spectrum, but not so large that it is difficult to correct.

The principle technique for isolating the signal of interest is, however, isotopic enrichment. In ^{13}C NMR, even with 100% enrichment, signals may not stand out too clearly from a protein background, since, although the protein has 90 times less ^{13}C, it may have 100 times as many atoms contributing to the signal in a particular part of the spectrum. So careful comparison with control experiments is needed. It is obvious that such a control must be set up to be as similar as possible to the real experiment. This means ensuring equivalent concentrations of enzyme and buffers and identical NMR measurement conditions. In MAS experiments it is particularly important to ensure that the spinning speed is stable and the same in each experiment. Finally, the control should not be an incubation in the *absence* of substrate, but rather an incubation with the *unlabeled* substrate. With sufficient care it is possible to isolate peaks by difference spectroscopy, subtracting the control from the real experiment.[63,64] However it must be stressed that even slight deviations in conditions between the two experiments can lead to apparent peaks in the difference spectrum, and because the real peak we are looking for may quite likely be broad and weak, it is easy to be misled into thinking that some such experimental glitch is in fact an interesting signal. Care is needed!

V. Applications

A. Structure

1. Proteins

Structural studies on proteins by solid-state NMR fall into two categories: the gathering of circumstantial evidence for structure by means of the determination of chemical shifts of labeled sites in the protein, and the direct determination of distances and angles using the orientation dependence of the shift anisotropy and the dipolar and quadrupolar couplings. Some of the material referenced in this section does not strictly fall within the scope of the review title, since it was not carried out at low temperature, but it is included to give a realistic perspective on an important field. Examples involving structural relationships between the protein and some other substance are referenced in section V.A.2.

Opella has developed a protocol that aims to describe the structure of peptides and proteins on the basis of

a collection of data from solid state NMR. This has been reviewed previously,[65,66] so only a very brief outline of the idea will be given here. The method is applicable to oriented systems, the orientation arising either because of crystallinity or because the system is subject to mechanical or magnetic constraints. The latter situation is particularly interesting, because sometimes systems that cannot be crystallized can be prepared in an oriented form that still permits the use of solid-state techniques. The structural model that is chosen in this work is to regard the backbone atoms of each amino acid residue in the protein (i.e. the atoms C_α^1–NH–CO–C_α^2) as defining a plane and to then try to determine the orientation of that plane relative to the static field by examining various orientation-dependent NMR parameters. Generally, no single measurement unambiguously determines the orientation, but by combining the restrictions available from several measurements the possibilities may be narrowed down to a unique solution or a few related solutions. Computational methods have been developed to fit calculated NMR parameters against experimental data and to carry out the analysis of the plane orientation.[65,67]

The difficulty with this method lies in the choice of parameters that it is feasible to measure in a complex protein. Without isotopic enrichment the only accessible nucleus is ^{14}N, and in a single crystal of *N*-acetyl-Val-Leu measurements on this nucleus alone have been sufficient to determine the structure.[68] In more complex systems it is necessary to employ enrichment of ^{15}N and ^{13}C both for reasons of assignment and to enable the measurement of more NMR parameters. Unfortunately uniform enrichment, which is reasonably practical for proteins that have expression systems, is not sufficient because it is not possible to resolve fully or assign the spectra. This seriously restricts the applicability of the technique, but in what must have been a heroic series of experiments Opella's group have demonstrated the structure determination of the coat protein of a filamentous bacteriophage in situ in the virus. This was done by preparing a series of samples with ^{15}N enrichment in single amino acids, and with ^{13}C and ^{15}N in selected pairs of amino acids.[68–70] The latter labeling was to enable discrimination between the ^{15}N signals of the same amino acid at different positions in the protein sequence.

Less ambitious structural studies generally home in on an interesting site, such as an amino acid suspected of being involved in some catalytic activity, and try to correlate either the isotropic shift or the anisotropy with changes in conditions. There are a variety of correlations of shift with protein conformation and pH in the literature.[71–73] Unfortunately some interesting groups, such as carboxylic acids, only show small shift changes between protonated and deprotonated forms, which are usually not discernible in the solid. More success has been achieved with ^{15}N shifts, which can vary substantially as a function of hydrogen bonding and protonation.

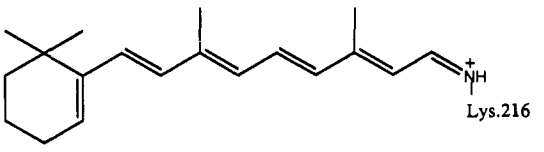
Careful measurements of chemical shift anisotropy have proved of value in determining the conformation of proteins and peptides in membranes, but as these investigations are intimately connected with the dynamic behavior of the protein in the lipid bilayer they are referenced in section V.B.

2. Cofactors and Inhibitors

The most successful area of application of low-temperature solid-state NMR to proteins has been in the study of the interaction between the protein and associated small molecules. In this case any required isotopic enrichment can be carried out synthetically in the nonprotein component of the system, and the generation of high levels of the combined species is assured.

In an extended series of studies, Griffin has used solid-state ^{13}C and ^{15}N NMR to investigate the structure and function of various retinal-containing proteins, and in particular bacteriorhodopsin from the purple membrane of *Halobacterium halobium*. In common with many membrane proteins, bacteriorhodopsin is difficult to study by normal NMR methods because its location in a lipid bilayer gives it unfavourable motional properties.[74] By employing low-temperature and CP-MAS, it has proved possible to circumvent this problem.[75]

Retinal-containing proteins are employed by a number of biological systems that interact with light. In rhodopsin, the visual pigment, absorption of light leads eventually to the perception of vision. In bacteriorhodopsin light absorption causes transmembrane proton transport, leading to a pH gradient that the bacterium employs for ATP synthesis. A curious feature shared by all these proteins is that the cofactor, bound to the protein as a Schiff's base (1), has its optical absorption maximum shifted about 200 nm relative to equivalent simple Schiff's bases—the opsin shift. This gives the bacterial membrane its characteristic purple color. The origin of the opsin shift is of interest both because it may provide indirect evidence for the mechanism of ion transport, and because its variation forms the basis of color vision. Initial studies of bacteriorhodopsin used systematic labeling of the retinal at different sites along the conjugated alkene together with variations in pH and light/dark conditions to build up support for a model of the mechanism of the opsin shift that depends on a subtle combination of ionic influences from groups in the protein and small conformational distortions of the retinal.[74–81,83,85–87] ^{15}N NMR has been used to probe for hydrogen bonding of the Schiff's base nitrogen,[82] and by ^{13}C labeling tyrosine residues in the protein it has been possible to test for the existence of tyrosinates, by first demonstrating that such species can be formed and detected under conditions of high pH, and then demonstrating their absence under normal conditions.[84] In equivalent studies of rhodopsin it has been found that the interaction between the protein and the retinal appears to occur at different locations than in bacteriorhodopsin.[88]



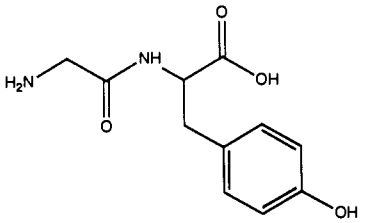
(1)

These studies of rhodopsin illustrate the careful accumulation of evidence by a combination of systematic isotopic labeling, low-temperature CP-MAS, and difference spectroscopy, and they have provided good structural information about retinal and its environment in these enzymes. For the eventual understanding

of the mechanism of ion transport it will be necessary to use the essentially static information from NMR in conjunction with other methods that can follow the fast processes involved.

As has been outlined previously, there are formidable obstacles to the observation of genuine intermediates in enzyme catalyzed reactions, whether by solid-state NMR or cryoenzymology. The kinetic criteria that must be met in order for an intermediate to accumulate, and the requirements for identification of a species as a true intermediate have been reviewed previously,[89,90] but unfortunately these are rarely fulfilled. This is, of course, natural, since the job of the enzyme is to turn over the substrate rather than to pool intermediates.[91,92] Convincing observation of the results of chemical processes on enzymes has thus been restricted to inhibition to date, but in some of these examples interesting structural information has been obtained.

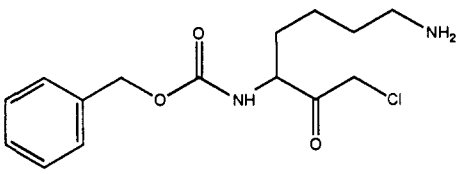
An early attempt to observe an enzyme-bound intermediate by CP-MAS NMR involved the zinc protease carboxypeptidase-A.[93] Catalysis of amide hydrolysis by this enzyme probably involves direct attack by water on the amide carbonyl, promoted by the vicinity of a coordinated zinc atom, but a reasonable alternative that has not been absolutely excluded is nucleophilic catalysis by attack of a residue such as Glu-270. In the latter case one might hope to observe the resulting anhydride intermediate under some conditions. In experiments with the artificial substrate Gly-Tyr (2), which is known to react about 5000 times



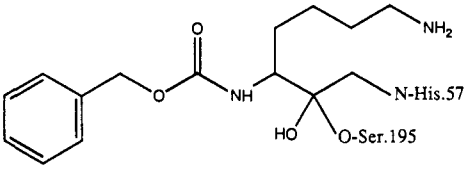
(2)

more slowly than optimum substrates for this enzyme, and whose complex with carboxypeptidase-A has been investigated by crystallography, Scott was able to observe only the hydrolysis products, possibly bound to the enzyme.[93] These experiments employed ^{13}C labeling of the glycine carbonyl and ^{15}N labeling of the tyrosine nitrogen, and the CP-MAS measurements were made on crystalline samples obtained by diffusing the substrate into the protein crystal. In each case only signals attributable to the product (i.e. glycine or tyrosine) were identified; neither the starting dipeptide nor any intermediate were seen.

Scott's group have also studied inhibition of the serine protease trypsin.[94] In this system there is no doubt about the involvement of the enzyme in nucleophilic attack on the substrate. The inhibitor carbobenzyloxylysyl chloromethyl ketone (3) acts by irreversibly attaching itself to the active-site imidazole; the carbonyl of the inhibitor may then form a hemiacetal with the active site serine. An attempt to distinguish between this hemiacetal (4) and the reasonable alternative hydrate was made (in solution) by analysis of isotope shifts on performing the experiment in partially ^{18}O enriched water, but the results obtained were difficult to interpret. In further investigations using crystallography, solid-state NMR was employed to demonstrate that the

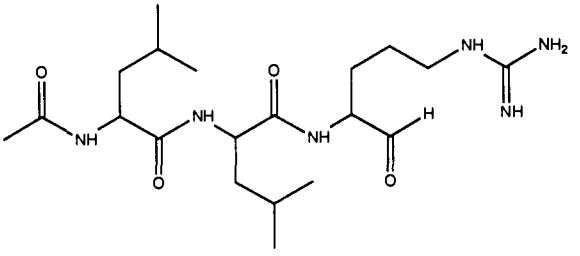


(3)



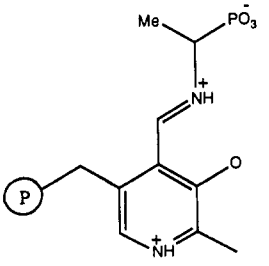
(4)

species obtained in the solid was the same as that obtained in solution. This example was irreversible inhibition, brought about by covalent bonding of the inhibitor to a histidine residue, but in recent work from the same group a species formed by reversible binding of the peptide aldehyde *N*-acetyl-L-leucyl-L-leucyl-arginal (leupeptin, 5) has been observed in the solid state.[95]



(5)

The former studies were not, in fact, performed at low temperature. A system for which low-temperature CP-MAS has proven necessary to stabilize the complex under study is alanine racemase inhibited by alanine phosphonate.[96] In its normal mode of action, alanine racemase employs a pyridoxal phosphate cofactor to bind alanine, as an imine, and catalyze its conversion from L to D form. Alanine phosphonate was demonstrated to form an analogous imine (6) during inhibition

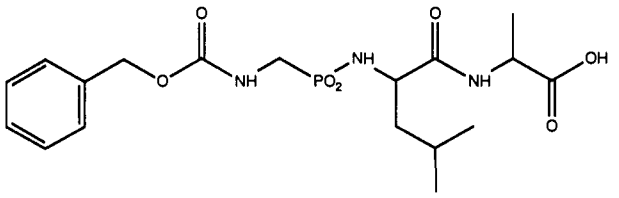


(6)

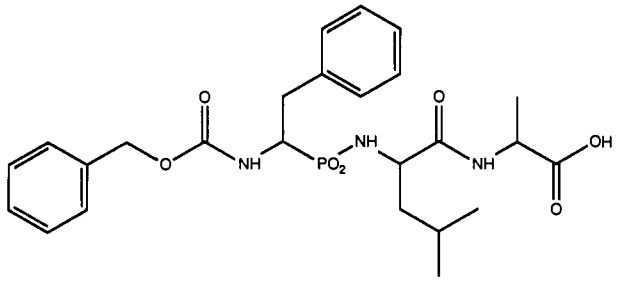
by means of low-temperature solid-state ^{15}N NMR. The protonated imine nitrogen has a very characteristic isotropic shift, and in addition in this study the principal values of the shift tensor were obtained and compared with those of suitable model compounds.

Finally, in a study of another zinc protease, the binding of peptide analogues 7 and 8 to thermolysin has been investigated by measurement of ^{31}P and ^{15}N shift tensor elements and the phosphorus–nitrogen dipolar

coupling.[97] A special technique was employed in order



(7)



(8)

to avoid problems due to the small size of this coupling (ca. 1 kHz), so as to be able to obtain a variant of separated local field spectroscopy even when spinning the sample at a high speed relative to the dipolar interaction. The distances obtained disagreed significantly with prior measurements by X-ray crystallography. It has been observed before that distances obtained from NMR are often less than those obtained by crystallography, and this has been attributed to errors in the NMR measurement resulting from partial averaging of the dipolar coupling by residual molecular motion.[98-100] However in this system, in which heavy atoms were studied at low temperature, this is not an acceptable explanation, and it must be that either the NMR distance is more accurate than that obtained from crystallography, or that there is some unidentified source of error. It is certainly possible in principle for the NMR measurement to be highly accurate, so it will be of interest to see the results of further studies of this kind.

B. Dynamics

All the NMR interactions measured in the solid state are susceptible to modification by molecular motion, and there is little difficulty calculating the line shape expected given a model for the motion and an assumed rate. The problem is obtaining adequate experimental data to enable the often quite subtle changes in line shape involved to be identified reliably. There are many references in this field, and as material prior to 1985 has been reviewed previously[101-103] we restrict our attention to more recent work. These prior reviews also include a detailed discussion of the line shapes obtained when anisotropy, dipolar coupling, and quadrupolar coupling interactions are perturbed by motion.

It is useful to subdivide the dynamics studies according to the interaction involved. For spin $^1/_2$ nuclei the possibilities are to measure either shift anisotropy or dipolar coupling, while for quadrupolar nuclei usually only the quadrupole interaction is accessible. Each of these three interactions presents its own particular advantages and problems. It is also, of course, possible to use relaxation time measurements of various kinds to investigate motion, just as in solution. These experiments differ from those involving anisotropy or couplings in both the frequency and form of motion to which they are sensitive. Spin–lattice relaxation responds to the highest frequency motions of all the interactions under consideration, and in addition it can be brought about by small-amplitude vibrations. In contrast averaging of the various powder patterns requires large displacements of atoms, since they need to experience widely varying orientations in order for averaging to be obtained.

Chemical shift anisotropy requires no special measurement techniques, but the static powder pattern can only be obtained if background signals from the sample are small enough not to interfere. This is easily achieved by labeling in small molecules, but is not likely to be possible in the presence of a protein. In addition, though the effect of motion on the pattern can be calculated given knowledge of the shift tensor, the latter has to be obtained from static model systems. This is in contrast to the coupling interactions which can be calculated a priori. The motions which affect anisotropy powder patterns are rather slow, in the kilohertz range, and the effects on the line shape can sometimes be too small to detect in practical cases. This is especially true for sites with intrinsically small anisotropy, which means, for instance, most carbons except carbonyls and aromatics.

Heteronuclear dipolar coupling between ^{1}H and lower γ nuclei is a large interaction and hence readily accessible for measurement. In protein studies coupling to ^{13}C and ^{15}N are of most interest, in the former case usually restricted to carbons bearing a single proton for reasons of simplicity of interpretation. The quadrupolar interaction is at least 1 order of magnitude larger again, and, subject to the difficulties discussed in section IV.A.3, line shapes are highly sensitive to motions on the appropriate timescale. The rates of motion required in fact approach the threshold of fluid-like behavior, and for this reason ^{2}H line shape measurements have found much application in the study of lipid bilayers, but this is outside the scope of this review.

1. Spin $^1/_2$ Nuclei

As part of the studies of bacteriorhodopsin discussed in section V.A.2, the dynamic behavior of the system has also been investigated.[104] Bacteriorhodopsin ^{13}C labeled in all leucine carbonyls was studied both in the native purple membrane and when reconstituted with extra lipid. In the native membrane the protein is static and gives an ordinary powder pattern in the carbonyl region, and essentially the same result is obtained for the reconstituted membrane at low temperature. As the temperature of the reconstituted system is increased above the phase transition temperature of the lipid, motional averaging of the powder pattern begins. In this state the motion of the protein is not random, but consists of rotational diffusion about an axis perpendicular to the lipid bilayer. Since the principal values of the carbonyl shift tensor were available from the static powder pattern, it was possible to calculate the expected effect of motional averaging by assuming various models for the structure of the membrane protein. By this means it was argued that the observed line shape was not consistent with a protein structure

consisting either of α-helices perpendicular to the lipid bilayer or β-sheets in a variety of orientations. Other structural models consistent with the observations were proposed, but clearly from data of this kind it is not possible to come to a definite conclusion about the structure. Nevertheless, since other techniques had not yielded any structural information whatsoever, this is an interesting approach.

Gramicidin A, a 15-residue ion channel forming membrane peptide, has also been the subject of similar anisotropy studies.[105,106] In this system it is practical to make the peptide synthetically, so several samples were prepared with ^{13}C labels in specific amino acid carbonyls. Initial studies concerned the dynamic behavior of the peptide, and then by measurements of powder patterns over a range of temperatures and sample orientations a structural model was developed. The same system has also been studied by ^{15}N NMR.[107]

The dipolar interaction has been exploited in order to investigate the motion of bacterial cell wall peptidoglycan in the intact bacterium *Aerococcus viridans*.[108] Growth of the organism on a medium containing either ^{15}N ammonium sulfate, or lysine ^{15}N labeled in the α or ϵ nitrogens, enabled the study of both peptide and cross-link sites in the cell wall. The dipolar rotational echo experiment was used to determine the ^{1}H–^{15}N dipolar couplings, and from both these and observations of the chemical shift anisotropy it was deduced that the N–H bond vector was undergoing rapid motion with a root-mean-square deviation of 23° in lyophilized cells and 37° in wet cells. This conclusion is in accord with results from X-ray crystallography and with molecular dynamics calculations.

Other rather qualitative studies using both chemical shift and relaxation data include work on lens cytoplasmic protein,[109] tropomysin,[110] and silk fibroins.[111] It will be noted that the majority of dynamics studies by solid-state NMR have not been made on catalytic systems, but rather on structural proteins, membrane channels, and so on. This reflects the more regular structure of the latter, which makes interpretation of the results more practical.

2. Quadrupolar Nuclei and Combined Studies

The majority of dynamics studies using ^{2}H wide-line spectra involve lipids and hence fall outside the scope of this review. However there have been a few direct applications to proteins, and also some studies in which a protein has been investigated indirectly by means of its interaction with a lipid.[112]

Though it is not strictly a protein study, an interesting starting point that illustrates some of the potential complexity of protein dynamics is an investigation of the behavior of the phenyl ring of phenylalanine in crystalline samples.[45] This material was studied by both ^{13}C and ^{2}H NMR with labels in a variety of sites. It is clearly possible, in principle, to distinguish between three potential states of motion of the phenyl ring: static, constant rotational diffusion, and 180° flips. In practice the ring behavior was found to depend on the way in which the sample was crystallized. In material crystallized from ethanol/water two sets of ring resonances were observed, and there was no evidence for motion. In contrast, material crystallized from water at neutral pH exhibited two classes of molecule, one undergoing rapid ring-flipping motion with a rate of the order of 10^6 Hz and the other apparently static. In crystals of the corresponding hydrochloride there appears to be motion, but on a much longer time scale.

The existence of such complexity in the behavior of a single amino acid indicates the potential difficulties involved in studies of proteins. However in pancreatic phospholipase A_2 it was found in the solid state that ^{2}H labels on residues Phe-5 and Trp-3 gave completely static line shapes.[113] When the same protein was studied in solution it was found that the tryptophan side chain became mobile, while the phenylalanine remained immobile relative to the protein and only showed line shape changes in accord with the overall motion of the protein.

In the coat protein of the bacteriophage M13 it was possible to distinguish two different components in the ^{2}H NMR spectrum, after treatment with D_2O at pH 10 and subsequent isolation of the virus.[114] One component exhibits a quadrupole coupling consistent with a static site, and is attributed to the backbone amide NH's of the protein, whereas the other, which shows a reduced coupling consistent with a more mobile species, is attributed to side chain NH_3's of lysine residues. The intensity ratio of the two components is in accord with this assignment and the known sequence of the protein. When the protein was dispersed in a lipid bilayer, as a possible model of the process of viral infection, the backbone signals showed no temperature dependence while the quadrupole splitting for the lysines varied from 27 kHz at 278 K to 18 kHz at 328 K. This indicates that, not surprisingly, the lysine termini are susceptible to motion within the lipid while the backbone atoms are not. Similar results have been obtained for the bacteriophage fd.[115]

Perhaps the best example to date of the investigation of internal protein dynamics by ^{2}H NMR is the study by Torchia of staphyloccocal nuclease.[116] Since an efficient expression system is available for this enzyme it was possible to prepare it with deuterium labels in a number of interesting amino acids: methionine, proline, phenylalanine, and tyrosine. Measurements were made on crystalline samples over a wide range of temperatures. At low temperatures, below 238 K, all the amino acids studied were found to be static except for methionine, which showed evidence for reorientation of its S–CH_3 bond axis even at 208 K. At higher temperatures a variety of motions were observed; prolines showing puckering of the ring, phenylalanines 180° flips of the phenyl ring with a short correlation time, tyrosines similar flips but on varying time scales, and methionines a variety of complex motions. By working from model systems and calculating lineshapes, quite detailed models of these motions were developed, though this required a number of hypotheses and some of the line shape fits are made with a large number of variable parameters. To justify the use of data obtained in the crystal in order to draw conclusions about the solution behavior of the protein, a comparison was made between the X-ray structure and NOE contacts obtained in solution, and between ^{13}C and ^{15}N shifts in the solid and in solution for protein labeled at Val (^{15}N) and Ala (^{13}C); these comparisons indicated very similar conformations in the two states. The dynamics of the structural protein keratin from mouse epidermis have

also been by a combination of ^{13}C and ^{2}H NMR at various temperatures.[117]

VI. Conclusions

Solid-state NMR at low temperature has proven able to generate information about certain proteins not available in any other way. However, because of the intrinsically low sensitivity of the technique, and the practical difficulties involved, it is necessary to choose quite carefully the kind of system to be studied. It must be available in reasonable quantity and amenable to isotopic enrichment. If intermediates are to be generated and caught, then there must be adequate prior knowledge of the kinetics of their formation and the conditions under which they can be generated in high concentration. Using solid-state NMR to search for a putative intermediate without investigating it first by other means is likely to be a fruitless endeavour, since the scope for failing to observe signals is very high.

There has not yet been an observation of a true, reactive intermediate on an enzyme-catalyzed pathway, but given a system with favorable kinetics we may look forward to such a success in due course. However, when the kinetics favor observation in the solid, they also favor observation in solution, and it is interesting to consider when the choice of the solid state is appropriate. There are two cases: when stabilization of the species to be observed requires such low temperatures that the sample is necessarily solid, and when the mobility of the system of interest in solution is so poor that the line widths obtained are larger than those that can be achieved in the solid. The latter case applies to the membrane proteins, and would also apply to enzymes with high molecular weight, say greater than 50 kD. This is much higher than the upper limit normally specified for protein studies in solution, but it should be remembered that in this case we are considering ^{13}C or ^{15}N observation, not ^{1}H, and that the solid-state line widths are large. At lower molecular weights, if a species can be generated in solution, it is probably best observed in solution.

VII. References

(1) Abragam, A. *Principles of Nuclear Magnetism*; Clarendon Press: Oxford, 1961.
(2) Goldman, M. *Spin Temperature and Nuclear Magnetic Resonance in Solids*; Clarendon Press: Oxford, 1970.
(3) Haeberlen, U. *High Resolution NMR in Solids*; Academic Press: New York, 1976.
(4) Slichter, C. P. *Principles of Magnetic Resonance*; Springer-Verlag: New York, 1980.
(5) Mehring, M. *High Resolution NMR in Solids*; Springer-Verlag: New York, 1983.
(6) Gerstein, B. C.; Dybrowski, C. R. *Transient Techniques in the NMR of Solids*; Academic Press: London, 1985.
(7) Fukushima, E.; Roeder, S. B. W. *Experimental Pulse NMR: a Nuts and Bolts Approach*; Addison-Wesley: Reading 1981.
(8) Fyfe, C. *Solid State NMR for Chemists*; C.F.C. Press: Guelph, 1983.
(9) Saito, H.; Ando, I. *Annu. Rep. NMR Spectrosc.* **1989**, *21*, 209 (Webb, G. A., Ed.).
(10) Opella, S. J. *Ann. Rev. Phys. Chem.* **1982**, *33*, 533.
(11) Opella, S. J.; Gierasch, L. M. *The Peptides* **1985**, *7*, 405 (Gross, E.; Meienhofer, J., Eds.).
(12) Smith, S. O.; Griffin, R. G. *Ann. Rev. Phys. Chem.* **1988**, *39*, 511.
(13) Wüthrich, K. *NMR of Proteins and Nucleic Acids*; Wiley: New York, 1986.
(14) See section V.A.1.
(15) Maricq, M. M.; Waugh, J. S. *J. Chem. Phys.* **1979**, *70*, 3300.
(16) Herzfeld, J.; Berger, A. E. *J. Chem. Phys.* **1980**, *73*, 6021.
(17) Veeman, W. S. *Prog. Nucl. Magn. Reson. Spectrosc.* **1984**, *16*, 193.
(18) Pausak, S.; Pines, A.; Waugh, J. S. *J. Chem. Phys.* **1973**, *59*, 591.
(19) Roberts, J. E.; Harbison, G. S.; Munowitz, M. G.; Herzfeld, J.; Griffin, R. G. *J. Am. Chem. Soc.* **1987**, *109*, 4163.
(20) Facelli, J. C.; Grant, D. M.; Michl, J. *Acc. Chem. Res.* **1987**, *20*, 152.
(21) For tables, see ref 5.
(22) Douzou, P. *Cryobiochemistry: An Introduction*; Academic Press: New York, 1977.
(23) Lyerla, J. R.; Yannoni, C. S. *Acc. Chem. Res.* **1982**, *15*, 208.
(24) Pines, A.; Gibby, M. G.; Waugh, J. S. *J. Chem. Phys.* **1973**, *59*, 569.
(25) Andrew, E. R.; Bradbury, A.; Eades, R. G. *Nature* **1958**, *182*, 1659.
(26) Lowe, I. J. *Phys. Rev. Lett.* **1959**, *2*, 285.
(27) Pake, G. E. *J. Chem. Phys.* **1948**, *16*, 327.
(28) Waugh, J. S.; Huber, L. M.; Haeberlen, U. *Phys. Rev. Lett.* **1968**, *20*, 180.
(29) Mansfield, P. *Prog. Nucl. Magn. Reson. Spectrosc.* **1971**, *8*, 41.
(30) Waugh, J. S.; Haeberlen, U. *Phys. Rev.* **1968**, *175*, 453.
(31) Shaka, A. J.; Keeler, J. *Prog. Nucl. Magn. Reson. Spectrosc.* **1987**, *19*, 47.
(32) Hester, R. K.; Ackerman, J. L.; Neff, B. L.; Waugh, J. S. *Phys. Rev. Lett.* **1976**, *36*, 1081.
(33) Rybaczewski, E. F.; Neff, B. L.; Waugh, J. S.; Sherfinski, J. S. *J. Chem. Phys.* **1977**, *67*, 1231.
(34) Bodenhausen, G.; Stark, R. E.; Ruben, D. J.; Griffin, R. G. *Chem. Phys. Lett.* **1979**, *67*, 424.
(35) Griffin, R. G.; Bodenhausen, G.; Haberkorn, R. A.; Huang, T. H.; Munowitz, M.; Osredkar, R.; Ruben, D. J.; Stark, R. E.; van Willigen, H. *Phil. Trans. R. Soc. London* **1981**, *A299*, 547.
(36) Munowitz, M. G.; Aue, W. P.; Griffin, R. G. *J. Chem. Phys.* **1982**, *77*, 1686.
(37) Munowitz, M. G.; Griffin, R. G. *J. Chem. Phys.* **1983**, *78*, 613.
(38) Munowitz, M. G.; Griffin, R. G.; Bodenhausen, G.; Huang, T. H. *J. Am. Chem. Soc.* **1981**, *103*, 2529.
(39) Munowitz, M. G.; Griffin, R. G. *J. Chem. Phys.* **1982**, *76*, 2848.
(40) Ackerman, J. L.; Eckman, R.; Pines, A. *Chem. Phys.* **1979**, *42*, 423.
(41) Samoson, A.; Pines, A. *Rev. Sci. Instrum.* **1989**, *60*, 3239.
(42) Wu, Y.; Sun, B. Q.; Pines, A.; Samoson, A.; Lippmaa, E. *J. Magn. Reson.* **1990**, *89*, 297.
(43) Hexem, J. G.; Frey, M. H.; Opella, S. J. *J. Chem. Phys.* **1982**, *77*, 3847.
(44) Vanderhart, D. L.; Earl, W. L.; Garroway, A. N. *J. Magn. Reson.* **1981**, *44*, 361.
(45) Frey, M. H.; DiVerdi, J. A.; Opella, S. J. *J. Am. Chem. Soc.* **1985**, *107*, 7311 and references cited therein.
(46) Pines, A.; Gibby, M. G.; Waugh, J. S. *J. Chem. Phys.* **1972**, *56*, 1776.
(47) Pines, A.; Gibby, M. G.; Waugh, J. S. *Chem. Phys. Lett.* **1972**, *15*, 373.
(48) Hartmann, S. R.; Hahn, E. L. *Phys. Rev. B.* **1962**, *128*, 2042.
(49) Reference 8, pp 281–2.
(50) Nitrox NG60, Nitrox Ltd. Basildon, U.K.
(51) Fyfe, C. A.; Mossbruger, H.; Yannoni, C. S. *J. Magn. Reson.* **1979**, *36*, 61.
(52) Raleigh, D. P.; Olejniczak, E. T.; Griffin, R. G. *J. Magn. Reson.* **1989**, *81*, 455.
(53) Davis, J. H.; Jeffrey, K. R.; Bloom, M.; Valic, M. I.; Higgs, T. P. *Chem. Phys. Lett.* **1976**, *42*, 390.
(54) Boden, N.; Hanlon, S. M.; Levine, Y. K.; Mortimer, M. *Mol. Phys.* **1978**, *36*, 519.
(55) Bloom, M.; Davis, J. H.; Valic, M. I. *Can. J. Phys.* **1980**, *58*, 1510.
(56) Spiess, H. W.; Sillescu, H. *J. Magn. Reson.* **1981**, *42*, 381.
(57) Ellet, J. D., Jr.; Waugh, J. S. *J. Chem. Phys.* **1969**, *51*, 2851.
(58) Aue, W. P.; Ruben, D. J.; Griffin, R. G. *J. Chem. Phys.* **1984**, *80*, 1729.
(59) Dixon, W. T.; Schaefer, J.; Sefcik, M. D.; Stejskal, E. O.; McKay, R. A. *J. Magn. Reson.* **1982**, *49*, 341.
(60) Raleigh, D. P.; Olejniczak, E. T.; Vega, S.; Griffin, R. G. *J. Magn. Reson.* **1987**, *72*, 238.
(61) Raleigh, D. P.; Olejniczak, E. T.; Vega, S.; Griffin, R. G. *J. Am. Chem. Soc.* **1984**, *106*, 8302.
(62) Opella, S. J.; Frey, M. H. *J. Am. Chem. Soc.* **1979**, *101*, 5854.
(63) Mollevanger, L. C. P. J.; Kentgens, A. P. M.; Pardoen, J. A.; Courtin, J. M. L.; Veeman, W. S.; Lugtenburg, J.; de Grip, W. J. *Eur. J. Biochem.* **1987**, *163*, 9.
(64) de Groot, H. J. M.; Copié, V.; Smith, S. O.; Allen, P. J.; Winkel, C.; Lugtenburg, J.; Herzfeld, J.; Griffin, R. G. *J. Magn. Reson.* **1988**, *77*, 251.
(65) Opella, S. J.; Stewart, P. L. *Methods Enzymol.* **1989**, *176*, 242.
(66) Opella, S. J.; Stewart, P. L.; Valentine, K. G. *Quart. Rev. Biophys.* **1987**, *19*, 7.
(67) Cross, T. A.; Brenneman, M. T. *J. Chem. Phys.* **1990**, *92*, 1483.

(68) Stewart, P. L.; Valentine, K. G.; Opella, S. J. *J. Magn. Reson.* **1987**, *71*, 45.
(69) Cross, T. A.; Opella, S. J. *J. Am. Chem. Soc.* **1983**, *105*, 306.
(70) Cross, T. A.; Opella, S. J. *J. Mol. Biol.* **1985**, *182*, 367.
(71) Opella, S. J.; Gierasch, L. M. *Peptides: Anal. Synth. Biol.* **1985**, *7*, 405.
(72) Munowitz, M.; Bachovchin, W. W.; Herzfeld, J.; Dobson, C. M.; Griffin, R. G. *J. Am. Chem. Soc.* **1982**, *104*, 1192.
(73) Frey, M. H.; Opella, S. J. *J. Magn. Reson.* **1986**, *66*, 144.
(74) Harbison, G. S.; Herzfeld, J.; Griffin, R. G. *Biochemistry* **1983**, *22*, 1.
(75) Smith, S. O.; Palings, I.; Copié, V.; Raleigh, D. P.; Courtin, J.; Pardoen, J. A.; Lugtenburg, J.; Mathies, R. A.; Griffin, R. G. *Biochemistry* **1987**, *26*, 1606.
(76) Harbison, G. S.; Smith, S. O.; Pardoen, J. A.; Winkel, C.; Lugtenburg, J.; Herzfeld, J.; Mathies, R. A.; Griffin, R. G. *Proc. Natl. Acad. Sci. U.S.A.* **1984**, *81*, 1706.
(77) Harbison, G. S.; Smith, S. O.; Pardoen, J. A.; Courtin, J. M. L.; Lugtenburg, J.; Herzfeld, J.; Mathies, R. A.; Griffin, R. G. *Biochemistry* **1985**, *24*, 6955.
(78) Harbison, G. S.; Mulder, P. P. J.; Pardoen, H.; Lugtenburg, J.; Herzfeld, J.; Griffin, R. G. *J. Am. Chem. Soc.* **1985**, *107*, 4809.
(79) Lugtenburg, J.; Mathies, R. A.; Griffin, R. G.; Herzfeld, J. *TIBS* **1988**, *13*, 388.
(80) Harbison, G. S.; Roberts, J. E.; Herzfeld, J.; Griffin, R. G. *J. Am. Chem. Soc.* **1988**, *110*, 7221.
(81) Smith, S. O.; Courtin, J.; van den Berg, E.; Winkel, C.; Lugtenburg, J.; Herzfeld, J.; Griffin, R. G. *Biochemistry* **1989**, *28*, 237.
(82) de Groot, H. J. M.; Harbison, G. S.; Herzfeld, J.; Griffin, R. G. *Biochemistry* **1989**, *28*, 3346.
(83) Smith, S. O.; de Groot, H. J. M.; Gebhard, R.; Courtin, J. M. L.; Lugtenburg, J.; Herzfeld, J.; Griffin, R. G. *Biochemistry* **1989**, *28*, 8897.
(84) Herzfeld, J.; Das Gupta, S. K.; Farrar, M. R.; Harbison, G. S.; McDermott, A. E.; Pelletier, S. L.; Raleigh, D. P.; Smith, S. O.; Winkel, C.; Lugtenburg, J.; Griffin, R. G. *Biochemistry* **1990**, *29*, 5567.
(85) de Groot, H. J. M.; Smith, S. O.; Courtin, J.; van den Berg, E.; Winkel, C.; Lugtenburg, J.; Griffin, R. G.; Herzfeld, J. *Biochemistry* **1990**, *29*, 6873.
(86) Lugtenburg, J.; Muradin-Szweykowska, M.; Heeremans, C.; Pardoen, J. A.; Harbison, G. S.; Herzfeld, J.; Griffin, R. G.; Smith, S. O.; Mathies, R. A. *J. Am. Chem. Soc.* **1986**, *108*, 3104.
(87) Rodman-Gilson, H. S.; Honig, B. H. *J. Am. Chem. Soc.* **1988**, *110*, 1943.
(88) Smith, S. O.; Palings, I.; Miley, M. E.; Courtin, J.; de Groot, H.; Lugtenburg, J.; Mathies, R. A.; Griffin, R. G. *Biochemistry* **1990**, *29*, 8158.
(89) Malthouse, J. P. G. *Prog. Nucl. Magn. Reson. Spectrosc.* **1986**, *18*, 1.
(90) Anderson, K. S.; Johnson, K. A. *Chem. Rev.* **1990**, *90*, 1131.
(91) Hanson, K.; Rose, I. *Acc. Chem. Res.* **1975**, *8*, 1.
(92) Jencks, W. P. *Mol. Biol. Biochem. Biophys.* **1980**, *32*, 3.
(93) Mackenzie, N. E.; Fagerness, P. E.; Scott, A. I. *J. Chem. Soc., Chem. Commun.* **1985**, 635.
(94) Scott, A. I.; Mackenzie, N. E.; Malthouse, J. P. G.; Primrose, W. U.; Fagerness, P. E.; Brisson, A.; Le, Z. W.; Bode, W.; Carter, C. M.; Yi, J. J. *Tetrahedron* **1986**, *42*, 3269.
(95) Ortiz, C.; Tellier, C.; Williams, H.; Stolowich, N. J.; Scott, A. I. Personal communication.
(96) Copié, V.; Faraci, W. S.; Walsh, C.; Griffin, R. G. *Biochemistry* **1988**, *27*, 4966.
(97) Copié, V.; Kolbert, A. C.; Drewry, D. H.; Bartlett, P. A.; Oas, T. G.; Griffin, R. G. *Biochemistry* **1990**, *29*, 9176.
(98) Stark, R. E.; Jelinski, L. W.; Ruben, D. J.; Torchia, D. A.; Griffin, R. G. *J. Magn. Reson.* **1983**, *55*, 266.
(99) Stoll, M. E.; Vega, A. J.; Vaughan, R. W. *J. Chem. Phys.* **1976**, *65*, 4093.
(100) Roberts, J. E.; Harbison, G. S.; Munowitz, M. G.; Herzfeld, J.; Griffin, R. G. *J. Am. Chem. Soc.* **1987**, *109*, 4163.
(101) Torchia, D. A. *Ann. Rev. Biophys. Bioeng.* **1984**, *13*, 125.
(102) Opella, S. J. *Methods Enzymol.* **1986**, *131*, 327.
(103) Keniry, M. A. *Methods Enzymol.* **1989**, *176*, 376.
(104) Lewis, B. W.; Harbison, G. S.; Herzfeld, J.; Griffin, R. G. *Biochemistry* **1985**, *24*, 4671.
(105) Smith, R.; Thomas, D. E.; Separovic, F.; Atkins, A. R.; Cornell, B. *Biophys. J.* **1989**, *56*, 307.
(106) Smith, R.; Cornell, B. A. *Biophys. J.* **1986**, *49*, 117.
(107) Nicholson, L. K.; LoGrasso, P. V.; Cross, T. A. *J. Am. Chem. Soc.* **1989**, *111*, 400.
(108) Garbow, J. R.; Jacob, G. S.; Stejskal, E. O.; Schaefer, J. *Biochemistry* **1989**, *28*, 1362.
(109) Morgan, C. F.; Schleich, T.; Caines, G. H.; Farnsworth, P. N. *Biochemistry* **1989**, *28*, 5065.
(110) Tuzi, S.; Sakamaki, S.; Ando, I. *J. Mol. Struct.* **1990**, *221*, 289.
(111) Saito, H.; Ishida, M.; Yokoi, M.; Asakura, T. *Macromolecules* **1990**, *23*, 83.
(112) Dufourc, E. J.; Freer, J. H.; Birkbeck, T. H.; Dufourcq, J. *Prog. Colloid Polym. Sci.* **1988**, *76*, 54.
(113) Allegrini, P. R.; van Scharrenburg, G. J. M.; Slotboom, A. J.; de Haas, G. H.; Seelig, J. *Biochemistry* **1985**, *24*, 3268.
(114) Datema, K. P.; Van Boxtel, B. J. H.; Hemminga, M. A. *J. Magn. Reson.* **1988**, *77*, 372.
(115) Valentine, K. G.; Schneider, D. M.; Leo, G. C.; Colnago, L. A.; Opella, S. J. *Biophys. J.* **1986**, *49*, 36.
(116) Sparks, S. W.; Cole, H. B. R.; Torchia, D. A.; Young, P. E. *Chem. Script.* **1989**, *29A*, 31.
(117) Mack, J. W.; Torchia, D. A.; Steinert, P. M. *Biochemistry* **1988**, *27*, 5418.