Applications of nuclear magnetic resonance to determine the structure and interactions of ligands, peptides and enzymes

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Nuclear magnetic resonance (NMR) spectroscopy is emerging as a powerful tool for the study of enzyme structure and function. This article discusses the general principles of NMR and the potential information this technique can provide in the study of enzymes along with its limitations.

Key terms: *enzyme-ligand interactions; nuclear magnetic resonance (NMR); solvent relaxation*

GLOSSARY OF TERMS

A	hyperfine coupling constant	S	total electron spin quantum number
g	electronic 'g' factor	T	absolute temperature
COSY	J-correlated spectroscopy	T_1	longitudinal (spin-lattice) relaxation time
Ho	intensity of the stationary magnetic field	T_2	transverse (spin-spin) relaxation time
$\hbar$	Planck's constant divided by 2π	T_{1M}, T_{2M}	relaxation times for nuclei in the sphere of influence of the paramagnetic species
I	total nuclear spin quantum number		
J	Scalar spin-spin coupling constant	TOCSY	total correlation spectroscopy
K	equilibrium constant	ß	Bohr magneton
k	rate constant	γ	gyromagnetic (magnetogyric) ratio
M_x, M_y, M_z	components of the macroscopic magnetic moment along x, y and z axes, respectively	δ	chemical shift (ppm)
		ε	water proton spin lattice relaxation rate enhancement
NOE	nuclear Overhauser effect (or enhancement)	μ	magnetic moment of an individual spin
NOESY	nuclear Overhauser effect spectroscopy	ν	frequency (Hz)
		τ_c	correlation time
P	angular momentum	τ_e	hyperfine correlation time
q	number of ligands bound in the sphere of influence of the paramagnetic species.	τ_m	lifetime of a nucleus in the coordination sphere of a paramagnetic species.
r	internuclear distance	τ_r	rotational correlation time
RELAY	relayed coherence transfer spectroscopy	τ_s	electron spin relaxation time
		ω_I	nuclear resonance frequency $(2\pi\nu)$
ROESY	closely related rotating frame NOESY	ω_S	electron resonance frequency

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INTRODUCTION

The developments in Nuclear Magnetic Resonance (NMR) and the advances in protein engineering provide powerful means to elucidate the structure-function relationships of substrates, peptides, proteins and, in particular, enzymes. NMR spectroscopy can, in principle, yield detailed information regarding enzyme structure and the structure of the specific ligands which bind to the enzyme. The structure of the ligands at the binding site of enzymes and the structure of enzyme-ligand complexes can also obtained. The tertiary structure of small proteins and peptides can now be determined in solution, independently of diffraction data, by homonuclear two dimensional NMR.

A further improvement to determine the structure of larger proteins has been obtained using heteronuclear three- and four- dimensional NMR. Since the NMR phenomenon is a time dependent phenomenon, kinetic as well as thermodynamic and structural information regarding both enzymes and substrates can be obtained.

The goal of this article is to stimulate the reader to use NMR spectroscopy as a tool, in conjunction with protein modification studies, to obtain detailed information on enzyme active sites and their functions. Although the applications of NMR to such problems are not treated exhaustively, the potential information to be garnered and the limitations of the method are discussed. As the appropriate NMR applications are chosen for the specific peptides, proteins and enzymes in question, additional information can be found in the current literature to assist in their fundamental understanding.

The attraction of NMR is that (again, in principle) one can investigate the magnetic nuclei of each of the atoms within the molecule of the enzyme (^{1}H, ^{13}C, ^{14}N...), of the ligands which bind to the enzyme (^{1}H, ^{19}F, ^{31}P, ^{13}C...), or of the environment of the active-site (solvent H_2O, ^{23}Na, ^{39}K, ^{35}Cl...). Since a large number of enzymes either contain metal ions (metalloenzymes) or require the addition of metal ions for activity (metal -requiring enzymes) a variety of these metal ions can be observed by NMR. These metals include divalent cations (^{25}Mg, ^{43}Ca,

^{59}Co, ^{113}Cd...) and monovalent cations (^{7}Li, ^{23}Na, ^{39}K, ^{205}Tl...). The rapid advance in technology of high-resolution, high-field super-conducting magnets, radio frequency electronics, and computer systems have given birth to several new generations of high-field, high-resolution, multinuclear NMR spectrometers. Such instruments can allow experiments and measurements to be made which were nearly unthinkable only two decades ago. A further attraction of the NMR methodology is that ligand binding, structural changes, and environmental changes at the catalytic site of an enzyme can be studied regardless of whether the enzyme is catalytically active, partially active, or inactive. This ability, currently highly underused can yield important information concerning the function of specific amino acids in ligand (substrate, metal activator, heterotropic modulator...) binding and in the catalytic processes. This chapter will attempt to discuss the utilization of one and two dimensional NMR spectroscopy to determine active site structure and protein structure, respectively. Because of the relative ease in studying metal ion effects, and in the utilization of certain metal ions as probes in the study of enzyme and enzyme-ligand interactions, these applications may appear to be inordinately stressed in this chapter. The basic approaches will be examined, and the limitations discussed. Some general familiarity with the NMR method will be assumed, and general references will be given but, they will not be exhaustive. Theory will be discussed only as deemed necessary to comprehend and to utilize the method in a practical manner. More detailed background can be obtained from the references cited.

NMR yields three general parameters which are useful in obtaining information regarding the structure and dynamics of the system under investigation. The chemical shift (δ) of a resonance which is observed is a function of the magnetic environment of the nuclei being investigated. This property makes NMR spectroscopy a potent tool in the study of molecules and their structure. If all protons within a molecule absorbed radio frequency power at exactly the same frequency, NMR would have little utility.

The phenomenon of a chemical shift arises owing to shielding of the nuclei under examination from the applied magnetic field by the electrons. Thus it is the electronic environment that causes variations in chemical shift. Any factor that will alter the electron density at the nucleus will alter the chemical shift. Shielding of methyl protons is greater than that of methylene protons, and still greater than that of aromatic protons, for example. Thus the resonance of a methylene proton is further upfield than that of a protons on an aromatic system, and methyl proton is furthest upfield. If spectra are obtained on samples that are fully relaxed and additional effects such as Overhauser effects do not occur, the area under the peak for each resonance is directly proportional to the concentration of nuclei. Both the relative and, in some cases, absolute distribution of magnetically non-equivalent nuclei and contaminant levels can be quantitated.

The second parameter is the spin-spin coupling or scalar coupling constant, J_{ij}, that occurs between two nuclei of spin I, I_i and I_j. The term I is the nuclear spin or spin angular momentum which has integer and half integer values (I = 0, $^1/_2$, 1, 3/2 ....). Spin I_i is split into $2nI_j$ + 1 lines by spin j. Spin j is split into $2nI_i$ + 1 lines by spin i. The term n indicates the number of nuclear spins. Thus, if spin i is one ^{13}C (I = $^1/_2$) and spin j is two ^1H's (I = $^1/2$) in a methylene group, then the ^{13}C spectrum has three lines and the ^1H spectrum has two. The magnitude of the coupling constant, J_{ij}, depends upon the interactions between the spins. Since most coupling is observed with nuclei of spin = $^1/_2$, discussion is primarily limited to such cases. Coupling constants depend on the environment of the molecule and the relative orientation or molecular geometry of the nuclei under observation, and therefore are important in structure determination. These coupling constants are independent of the magnetic field. The closer the nuclei are to each other (fewer bonds), the larger the magnitude of the coupling for related molecules. There are certainly cases, however, where three-bond coupling constants are larger than two-bond coupling constants. If the chemical shifts or effective chemical shifts of the coupled nuclei are large

compared to the coupling constant, then the spectral patterns are relatively simple and are considered first-order. When the chemical shifts are of the magnitude of the coupling constant, the spectra become more complex and are called second order. Resolution of coupling is an important spectroscopic technique in structure determination. Spin-spin coupling can be studied by double resonance, spin-decoupling experiments, spectral simulation and by two dimensional correlation spectroscopy (Becker, 1980).

The third and most often neglected of the parameters are the relaxation rates of the nuclei. In fact, in the initial search for a nuclear resonance phenomenon, dynamic processes and line shapes were of primary interest, and coupling constants and chemical shifts observed in liquids came as a surprise. The equations derived to define the motion of the magnetic moment (μ) or magnetization M in the samples were given by Bloch (1946). The motion in the direction of the external magnetic field H_0 is designated as dM, z/dt. In the plane perpendicular to H_0, the x, y plane, the motion of the magnetization vector is designated as dM, x/dt. Magnetization in the x,y plane occurs because of the property of spin of the nuclei. When a sample with a nuclear spin is placed in an external magnetic field, H_0, a torque is placed on the magnetic moment M by H_0 to change the angular momentum, P.

$$\frac{dP}{dt} = - H_0 \times M \qquad (1)$$

Since the spin angular momentum is related to the magnetic moment by the magnetogyric ratio g,

$$M = \gamma P \qquad (2)$$

then

$$\frac{dm}{dt} = -\gamma H_0 M \qquad (3)$$

This expression describes the motion of the magnetic moment or magnetization about the z axis defined as the direction of the H_0

TABLE I

Electron spin quantum numbers and
approximate values of isotropic g factors
and electron spin relaxation times for some
paramagnetic probes

Probe	S	g *	τ_s * (sec)
Nitroxide	1/2	2,0	10^{-8}
Cu(II)	1/2	2,0-2,5	10^{-6}
V(IV)	1/2	2,0	10^{-9}
Ni(II)	1	2,0-2,9	10^{-13}
Co(II)	3/2	2,1-2,8	10^{-13}
Cr(III)	3/2	2,0	10^{-10}
Fe(II)	2	2,1-2,3	10^{-11}
Mn(II)	5/2	2,0	10^{-9}
Fe(III), high spin	5/2	2,0	10^{-10}
Gd(III)	7/2	2,0	10^{-10}

* These are approximate values that may be used in
the Solomon-Bloembergen interactions. For lanthanide
ions, the electron angular momentum j is necessary.
The g factor may not correspond to the true isotropic
factor.

\# The electron spin longitudinal relaxation time is
dependent upon ligands coordinated to the ion, frequency
and temperature. These values are approximate for the
spin in question.

field. At equilibrium the nucleus has a magnetization of M_o. The decay or relaxation of the magnetization in the z axis is characterized by a relaxation rate, $1/T_1$. A change in M_z is accompanied by a transfer of energy between the nuclear spin and other degrees of freedom or the lattice of the surroundings and is hence called the 'longitudinal relaxation rate' or the 'spin-lattice relaxation rate, $1/T_1$. A decay in the transverse components of the magnetization, M_x and M_y, results in an exchange of energy between spins of different nuclei without transfer to the lattice, and is called the 'transverse relaxation rate' or the 'spin-spin relaxation rate', $1/T_2$. In solution studies, both T_1 and T_2 are affected by the exchange of energy between the spin system being studied and the environment. Since energy exchange between spin systems is dependent upon dipolar effects, distances between these dipoles can be calculated if the magnitudes of these effects can be measured. Since these relaxation phenomena are time-dependent, kinetic information such as molecular motion is possible from the studies. More detailed treatments are available (Abragam, 1973; James, 1975).

ENZYME STUDIES

In the study of enzymes it is conceivable that a ^1H spectrum of the enzyme can yield absorption peaks for each of the protons in the molecule. In order to do such an experiment, several problems must first be considered. A proton-free solvent and proton-free buffer are, in general, desired. The solvent of choice which should give a minimum of perturbation of the protein structure is deuterium oxide, ^2H_2O or simply D_2O. To attempt protein NMR studies, there are a variety of NMR methods to suppress solvent signals (suppress the signal from H_2O or DHO), each with their inherent advantages and disadvantages (Turner, 1984). The D_2O commercially available can be obtained with 0.1% protons or less. If a 99.99 atom % D_2O solvent is used, the proton content of the solvent is 11 mM, higher than the possible concentration of most enzymes.

Little information is available concerning the effects of D_2O on protein structure. However, it is common that a solvent isotope effect on enzymatic activity is observed. Reasons for such effects are often difficult to asses (Schowen, 1972). Buffer systems can also be a problem. If a phosphate buffer causes no problem with the enzyme under investigation, it will be ^1H NMR invisible. Otherwise either low concentrations of buffer or no buffer may be used.

The two major problems with this NMR approach are the concentration of enzyme and resolution of the spectra. The signal-to-noise of the spectrum is directly proportional to the concentration of the sample. Many enzymes may not be sufficiently soluble to yield a 1 x 10^{-3} M solution. If 5 mm sample tubes are used, 0.30 ml is a minimum volume to obtain spectra. If a protein of molecular weight 1 x 10^5 is used, this required 30 mg of enzyme for a spectrum. Even if solubility is not a major problem, an increase in concentration increases the viscosity of the sample. In more viscous solutions rapid averaging of the sample no longer occurs and broad absorption lines are observed, which decreases resolution of the spectrum.

In an enzyme of molecular weight approximately 70 000 (an average size protein)

the rotational correlation time, τ_g in aqueous solution may be estimates at 10^{-8} s using the Stokes-Einstein equation, assuming the protein is roughly globular. This enzyme is also expected to contain approximately 600 amino acids. The large number of residues results in a high number of overlapping resonances because of the number of protons present. A 'typical' ^1H spectrum shows a large envelope of overlapping resonance peaks upfield from the water resonance, that results from the aliphatic groups in the protein. An envelope downfield from the water resonance is due to the aromatic groups of the protein. The resonances that appear are usually quite broad as well. The broad lines are caused by dipole-dipole interactions with either the same or with other nuclei. The effect of dipolar interactions on line widths $(1 / \pi T_2)$ is modulated by the rotational correlation time of the group under investigation. An increase in the line widths of resonances in small molecules with decreasing temperature is often seen for the same reason. Regardless of the nature of the dipoles that affect the relaxation, $1/T_2$ is directly proportional to τ_r. If some groups have less restrictive motion in the enzyme they may yield sharper peaks that may protrude from the envelope of broad, overlapping peaks. Although assignments of resonances of free amino acids (Roberts and Jardetzky, 1970) and amino acids in small peptides (Table II) have been made,

TABLE II

Random coil ^1H chemical shifts for the 20 common amino acid residues

Residue	NH	αH	βH	Others	
Gly	8.39	3.97			
Ala	8.25	4.35	1.39		
Val	8.44	4.18	2.13	γCH₃	0.97, 0.94
Ile	8.19	4.23	1.90	γCH₂	1.48, 1.19
				γCH₃	0.95
				δCH₃	0.89
Leu	8.42	4.38	1.65, 1.65	γH	1.64
				δCH₃	0.94, 0.90
Pro (trans)		4.44	2.28, 2.02	γCH₂	2.03, 2.03
				δCH₂	3.68, 3.65
Ser	8.38	4.50	3.88, 3.88		
Thr	8.24	4.35	4.22	γCH₃	1.23
Asp	8.41	4.76	2.84, 2.75		
Glu	8.37	4.29	2.09, 1.97	γCH₂	2.31, 2.28
Lys	8.41	4.36	1.85, 1.76	γCH₂	1.45, 1.45
				γCH₂	1.70, 1.70
				εCH₂	3.02, 3.02
				εNH₃⁺	7.52
Arg	8.27	4.38	1.89, 1.79	γCH₂	1.70, 1.70
				δCH₂	3.32, 3.32
				NH	7.17, 6.62
Asn	8.75	4.75	2.83, 2.75	γNH₂	7.59, 6.91
Gln	8.41	4.37	2.13, 2.01	γCH₂	2.38, 2.38
				δNH₂	6.87, 7.59
Met	8.42	4.52	2.15, 2.01	γCH₂	2.64, 2.64
				εCH₃	2.13
Cys	8.31	4.69	3.28, 2.96		
Trp	8.09	4.70	3.32, 3.19	2H	7.24
				4H	7.65
				5H	7.17
				6H	7.24
				7H	7.50
				NH	10.22
Phe	8.23	4.66	3.22, 2.99	2,6H	7.30
				3,5H	7.39
				4H	7.34
Tyr	8.18	4.60	3.13, 2.92	2,6H	7.15
				3,5H	6.86
His	8.41	4.63	3.26, 3.20	2H	8.12
				4H	7.14

Data measured at pH 7.0, 35°C for residues X in tetrapeptide GGXA.

From K Wütrich (1986) *NMR of Proteins and Nucleic Acids*. Wiley Interscience, New York.

the assignments of resonances which may be observed for an enzyme must be made for specific amino acid residues within the enzyme structure. This can be a severe limitation. Often an approach such as specific amino acid derivatization prior to obtaining the spectrum can help in making assignments. Multi-pulse methods can aid in structure determination. There is detailed information on peptides (Wüthrich, 1986), and nuclear relaxation and Overhauser effects have been successfully used in studies of enzyme-substrate interactions (Mildvan, 1989).

The most useful approach to study enzyme structure by protein NMR with a minimum of perturbation has been the observation of the resonances from histidine. The C-2 and C-5 proton resonances are downfield from the aromatic protons (Markley, 1975). If a limited number of these amino acids are present in the enzyme, they have sufficient molecular motion (to yield 'sharp' lines), and are in different (magnetic) environments in the enzyme, they can yield reasonably sharp, resolved resonances. The classical use of these properties was with the small enzyme RNAase (M_r = 24.500) (Meadow and Jardetzky, 1968); the large enzyme (M_r = 237.000) pyruvate kinase has also been studied (Meshitsuka et al, 1981). The C-2 proton resonance is especially sensitive to the ionization state of the imidazole nitrogens, thus the pK_a for each individual histidine within the native enzyme can be obtained from titration studies. It remains that proper assignments be made for each histidine. The binding of a ligand or metal ion to a specific histidine or histidines could result in a change in the magnetic environment (chemical shift) of the resonance and an alteration in the pK_a. This application of NMR has usefulness in some limited number of enzymes.

It is possible that ^{13}C and ^{14}N studies can also be performed. An increase in the range of chemical shifts for these nuclei enhances spectral dispersion and increases the possibility of resolving more resonances. A major problem with these nuclei is the low abundance (1.1%) and sensitivity (1.6%) for ^{13}C and the low sensitivity (0.1%) for ^{14}N compared to the sensitivity of ^1H (100%). The quadrupolar ^{14}N nucleus also has a nu-

clear spin of 1 which gives substantial line broadening. With enzymes from bacterial systems where it is feasible to consider growing the organism on media or precursors (i.e. amino acids) that are selectively enriched (^{13}C or ^{15}N) (Hunkapiller et al, 1973), some of these studies may become more reasonable. DNA cloning techniques can expand this potential. A detailed review of ^{13}C NMR studies of enzymes has been published (Malthouse, 1986). Structural and dynamics studies of larger proteins have been done with ^{13}C and ^{15}N isotope labels through proton NMR and nuclear Overhauser effect (Redfield et al, 1989).

An alternative approach to looking at the enzyme in an effort to obtain information regarding enzyme structure and the effects of ligand binding on the enzyme is to use a reporter groups on the enzyme. One of the more sensitive groups that can be studied is ^{19}F. The use of this nucleus in enzyme systems has been reviewed (Gerig, 1981). This nucleus is 83% as sensitive as ^1H, has a large range of chemical shifts, is rather sensitive to its magnetic environment, and there are no background resonances of ^{19}F which cause interference. A ^{19}F reporter groups can be incorporated by one of two methods. A specifically fluorinated amino acid (i.e. fluorotyrosine, fluoroalanine) can be added to growth medium and incorporated into the protein (Sykes and Weiner, 1980). Under these conditions one group of amino acids (i.e. tyrosines, alanines) would contain the ^{19}F resonance. Most organisms will not grow on 100% fluorinated amino acids as they are toxic at higher levels. Furthermore, each of the residues is labeled and will exhibit a resonance. In a case where each residue is non-equivalent, assignments for each residue (i.e. each tyrosine) may be necessary. In the particular case of tubulin heterodimer, the principal protein of microtubules, fluorotyrosine can be specifically incorporated as the C-terminal amino acid of the alpha-subunit through the reaction catalyzed by tubulin-tyrosine-ligase (Monasterio et al, 1995).

An alternative to this approach is to covalently label the enzyme at a specific residue with a fluorine-containing reagent. Among the possible reagents one may use

are trifluoroacetic anhydride, trifluoro-acetyliodide, or 3-bromo-1,1,1-trifluoro-propanone. The chemical shift and/or the line width ($1/T_2$) of the ^{19}F label, a 'reporter' for a change in the enzyme structure, must reflect ligand binding and/or catalysis. If the ^{19}F resonance is sensitive to conformational changes in the enzyme then site-specific modification of groups at the active-site will be reflected by changes in the ^{19}F resonance. Ligand binding to modified enzyme may also be monitored by a measure of the spectral parameter (d or $1/T_2$) as a function of ligand concentration. A titration of the spectral parameter versus ligand concentration yields a titration curve that is evidence for ligand binding. A dissociation constant for ligand binding can be determined.

The method of using reporter groups can be expanded with other labels. Most other labels would be less sensitive than fluorine. However, the modification may be more selective or may yield reporter groups that are more sensitive to changes in enzyme structure. ^2H labels or ^{13}C labels can also be incorporated into the protein. A potential strength of using these labels is that the incorporation of ^2H for ^1H or ^{13}C for ^{12}C into the protein will have a very minor, if any, effect on the protein itself. Although reporter groups yield information regarding the environment of the group and not specific structural features of the enzyme, comparative structural changes can be studied by such methods.

The method of photo-chemically induced nuclear polarization (photo CIDNP) which originates from free radical reactions has been developed as a sensitive method to measure structural changes on the surface of proteins (Kaptein, 1982; Berliner, 1989). The method requires a modified spectrometer and a proper light source (laser) to begin to probe surface changes. These changes, when observed, are reflected in changes about aromatic amino acids. This technique has the advantage of high sensitivity, and it yields general conformation information.

STUDIES OF THE LIGANDS

An alternative to measuring aspects of the enzyme and its structure in the study of enzyme-ligand interactions is an investigation of the ligand itself. A general definition of a ligand implies substrates, modifiers, inhibitors and activators including metal ions. The proper studies depend upon the enzyme of interest. There are two potential types of experiment one can perform. In some cases the interaction of a ligand with an enzyme results in the formation of an enzyme-ligand complex such that partial immobilization of a portion of the ligand occurs. A decrease in the mobility of a group (*i.e.* a methyl group) increases the correlation time, the time constant for the process that modulates or interferes with the relaxation process. The rotational correlation time of the methyl group is the rotation time of that group which modulates the dipolar interactions among the methyl protons and results in an increase in $1/T_2$ and $1/T_1$. The $1/T_2$, estimated from the line width of the resonances, is the parameter that is more easily measured. If the effect on $1/T_2$ is sufficiently large and the ligand is in the fast exchange domain (the lifetime, tm, of the ligand in the E-L complex is short compared to the relaxation time of the nucleus, $T_{2,b}$, in the E-L complex) an average line width ($1/T_{2,obs}$) for the bound ligand ($1/T_{2,b}$) and free ligand ($1/T_{2,0}$) is observed.

$$\frac{1}{T_{2,obs}} = \frac{[L_b]}{[L_T]} \left(\frac{1}{T_{2,b}} \right) + \frac{[L_f]}{[L_T]} \left(\frac{1}{T_{2,0}} \right) \quad (4)$$

The observed effect is a mole average effect of bound ligand [L_b] and free ligand [L_f] where the sum of the concentrations of L_b and L_f give the concentration of total ligand, [L_T]. From a determination of the amount of ligand bound (the concentration of enzyme sites if the enzyme is saturated with ligand) and the total amount of ligand present, $1/T_{2,b}$ can be calculated. Values for $1/T_1$ can be handled by similar treatment if $1/T_{1,obs}$ is measured. If the dipolar effect is all intramolecular and the nature of the dipoles is known (*e.g.* ^1H-^1H interactions), the value for the rotational correlation time for that group in the enzyme-ligand complex can be calculated. From a determination of ligand binding, values for [L_b] and [L_f] can be obtained and $1/T_{1,b}$ and $1/T_{2,b}$ calculated.

From the structure of the molecule, the distance r between the dipoles is usually obtained. The distance r is estimated from crystal structure data or from models of such compounds (Mildvan *et al*, 1967).

$$\frac{1}{T_{1,b}} = \frac{3\gamma_I^4\hbar^2}{10r^6} \left(\frac{\tau_c}{1+\omega_I^2\tau_c^2} + \frac{4\tau_c}{1+4\omega_I^2\tau_c^2}\right) \qquad (5)$$

$$\frac{1}{T_{2,b}} = \frac{3\gamma_I^4\hbar^2}{20r^6} \left(3\tau_c + \frac{5\tau_c}{1+\omega_I^2\tau_c^2} + \frac{2\tau_c}{1+4\omega_I^2\tau_c^2}\right) \qquad (6)$$

In these equations, $\hbar$ is Planck's constant/2π and τ_c, the correlation time for the dipolar interactions, is τ_r. The value ω_I is the Larmor frequency in rad sec^{-1}. If such immobilization is detected and calculated for the ligand bound to the native enzyme, then one can determine if immobilization of the same ligand occurs with modified enzyme. Restriction of molecular motion is one possible mechanism of catalytic activation.

Another approach to the study of ligand binding to enzymes is by the use of paramagnetic probes on the enzyme. The use of paramagnetic species to probe ligand interactions is feasible because an unpaired electron is about 657 times more effective than a proton in causing a dipolar effect on relaxation. Several approaches can be utilized to take advantage of these large dipolar effects. Stable nitroxides, many of which are commercially available (*e.g.* from Aldrich Chemical Co. and Merck and Co.) can potentially be covalently attached to the enzyme. These nitroxides include derivatives of iodoacetate, N-ethylmaleimide, and diisopropylfluorophospate that can be used to label reactive groups such as cysteine, histidine, lysine, or reactive serine (Berliner, 1976). Selectivity of labeling and choice of amino acid residue, discussed elsewhere in this text, is necessary. These probes can be monitored by EPR spectroscopy, or their effects on ligands can be studied by NMR. This label can be used as the reference point to study ligand interactions to labeled enzyme.

Alternative paramagnetic species that can be used are metal ions. These metals may either bind to the enzyme or can bind as a metal-substrate complex to the enzyme.

Some of the metal ions that can be used or substituted for the 'physiological' cation are Mn(II), Fe(II), Co(II), Cu(II), Gd(III) or Cr(III). If the enzyme being studied gives the investigator a choice of cations there are distinct advantages to using a few of these cations, particularly Mn(II), as will be shown. A determination of the stoichiometry of the paramagnetic center is necessary. With the nitroxide 'spin label' an integration of the EPR spectrum of labeled enzyme to obtain a spin count can be used. A comparison of the spectrum of the sample with a spectrum of a known spin label can be made. This is often the method of choice. In the case of metal ions the investigator has a variety of techniques available to measure concentration. With tight binding metals, atomic absorption spectroscopy can be used to determine the metal content of the enzyme for any metal ion. Alternatively, metal binding using unstable nuclei can be performed using one of a variety of equilibrium techniques such as equilibrium dialysis, gel permeation, ultrafiltration, ... The Mn(II) cation is almost uniquely suited for EPR studies where a solution spectrum of the free cation can be measured, and it yields a simple six-line spectrum. Upon ligand binding (ligand implying anything from a small molecule such as orthophosphate or ADP to protein) the change in zero field splitting and line broadening results in a 'disappearance' in the spectrum of bound Mn(II). The remaining signal is due to the free Mn(II) and the intensity of the spectrum is directly proportional to the concentration of free Mn(II) (Cohn and Townsend, 1954). Proper binding studies will lead to a determination of the dissociation constant for the label and its stoichiometry per enzyme or enzyme active site.

In most cases the metal ion utilized is either the physiologically important cation activator to elicit catalysis. The paramagnetic center is at the activator site which may be either at, near, or remote from the active-site. Other probes such as the lanthanides (*e.g.* Gd III) may serve as activators in a few cases or as inactive analogs that are competitive with the physiologically relevant cation. The lanthanide metals, in spite of the fact that they are most commonly trivalent, have f

shell electrons which give nearly all of them interesting spectroscopic properties. For many NMR studies the physical properties of Gd(III) make it most useful. The Cr(III) cation which forms exchange inert ligand-metal complexes can also be used as a probe. This metal has found use as a kinetic and an NMR probe by being used as a Cr(III)-nucleotide complex (Cleland and MIldvan, 1979). This metal nucleotide complex is an analog of Mg-nucleotide or Ca-nucleotide complexes that serve as substrates.

The paramagnetic probes, particularly nitroxides, Mn(II), Gd(III) and Cr(III), can have a substantial effect on the longitudinal and the transverse relaxation rates of the nuclei of the ligands that are in close proximity to the paramagnetic center. In the studies of enzyme active-sites by chemical modification, the use of such probes may be of exceptional value. After modification of the enzyme one can first determine if the binding site for the paramagnetic probe is still intact. Equilibrium binding or EPR binding (of Mn(II) can determine if there is any alteration in the stoichiometry or in the dissociation constant for the cation to the modified enzyme. If the cation binding sites remain intact in the enzyme, then ligand binding to the modified enzyme can be studied. The results of a proper series of NMR experiments can describe the alteration in the binding of the ligands to the modified enzyme, the structure of the ligands at the binding site, and their exchange rates. This information can be compared to what is known regarding the structure and dynamics of ligand binding with the native enzyme to determine the effects of modification. Again, these studies can be performed even if the modified enzyme is totally inactive.

The effect of the paramagnetic species on the relaxation rates of the nucleus/nuclei in question must first be quantitated. The choice of nucleus studied is often dictated by the nature of the enzyme, its ligands, and ease of experimentation. For example, if the interaction of ATP to an enzyme-metal complex will be investigated, the ^{31}P nuclei of ATP are probably of most interest and are relatively easy to detect. The ^{1}H nuclei of the ribose portion of ATP yield a complex spectrum with overlapping lines and the

resonances of the individual protons are much more difficult to resolve. Also ^{19}F can be incorporated at the gamma phosphate of ATP or GTP given a competitive inhibitor with respect to the nonfluorinated nucleotide (Monasterio and Timasheff, 1987). The ^{13}C nuclei are of low natural abundance, and in an unenriched sample the experiments would take an inordinately long time.

To quantitate the paramagnetic effect of the probe on the relaxation rate of the nuclei, the relaxation rates are measured in the absence of the paramagnetic species ($1/T_{1,0}$, $1/T_{2,0}$). This may be performed by a measurement of the nuclei in the presence of enzyme but no added metal, a diamagnetic metal (Mg(II), Zn(II), Ca(II)...), or with a reduced nitroxide label. The addition of the paramagnetic species is made by either adding the paramagnetic metal to the analytical sample that contains ligand and apoenzyme, or by adding the enzyme-metal complex to the solution. The procedure of choice depends upon the properties of the enzyme. If the enzyme is a metalloenzyme the latter approach can be used. If the enzyme is metal-requiring, then sufficient apoenzyme is present such that when metal is added most if not all of the metal binds to the enzyme. If a spin-label enzyme is added, since most spin labels are covalently attached to the enzyme, the labeled enzyme is added in increments. The observed relaxation rate ($1/T_{1,obs}$, $1/T_{2,obs}$) is a function of the diamagnetic relaxation rate and the paramagnetic relaxation rate:

$$\frac{1}{T_{1,obs}} = \frac{1}{T_{1p}} + \frac{1}{T_{1,0}} \qquad (7)$$

$$\frac{1}{T_{2,obs}} = \frac{1}{T_{2p}} + \frac{1}{T_{2,0}} \qquad (8)$$

The paramagnetic effect is measured as a function of the concentration of paramagnetic species. If possible, a plot of $1/T_{i,obs}$ vs the concentration of paramagnetic species can be made to show expected linearity in the relaxation rate where i = 1 or 2. The rate

can be normalized for the concentration of the ligand, (L), and for the concentration of the paramagnetic species (p) by the term f where f = (p)/(L). The normalized paramagnetic effects to the relaxation rates are related to the number of ligands (q) which bind to the specific site(s) in the vicinity of the paramagnetic probe, the relaxation time of the nucleus at this site (T_{iM}) and the lifetime of the nucleus of this site (τ_m). In some cases with paramagnetic ions a chemical shift change, Delta omega, is also observed which affects T_2 relaxation.

These effects have been described by Swift and Connick (1962) and by Luz and Meiboom (1964).

$$\frac{1}{fT_{1p}} = \frac{q}{T_{1m} + \tau_m} \qquad (9)$$

$$\frac{1}{fT_{2p}} = \frac{q}{\tau_m} \left[\frac{\frac{1}{T_{2M}}\left(\frac{1}{T_{2m}} + \frac{1}{\tau_M}\right) + \Delta\omega^2}{\left(\frac{1}{T_{2M}} + \frac{1}{\tau_m}\right)^2 + \Delta\omega^2} \right] \qquad (10)$$

If chemical shift changes are negligible or absent, eq.(10) reduces to:

$$\frac{1}{fT_{2p}} = \frac{q}{T_{2m} + \tau_m} \qquad (11)$$

In most such cases $\Delta\omega \approx$ zero, and eq. (11) can be used. The enzyme (enzyme-label) should be corrected for saturation by the ligand. If the K_d for the formation of the E-ligand complex is such that the complex is only partially saturated, then f = (E-label-ligand)/(ligand). In the cases where the label is a metal ion then saturation of the E-M-ligand complex must also occur or be corrected. The formation of binary M-ligand complexes must be minimized or corrected. The value for n, the mole fraction of M in the E-M-ligand complex, can be calculated from known dissociation constants or by a measure of $1/fT_{1p}$ under analogous conditions at three different values of ω_I (Nowak, 1981).

If the values for $1/fT_{1p}$ and $1/fT_{2p}$ can be correctly determined and $\Delta\omega$ for E-M-L is negligible, evaluation of these parameters must be made. If $1/fT_{ip}$ is in fast exchange, $T_{iM} \gg \tau_m$, then:

$$\frac{1}{fT_{1p}} = \frac{q}{T_{IM}} = \frac{1}{T_{IM}} \qquad (12)$$

$$\frac{1}{fT_{2p}} = \frac{q}{T_{2M}} = \frac{1}{T_{2M}} \qquad (13)$$

An evaluation of q, the number of ligands binding at the paramagnetic label site, can either be made by direct binding studies; or, in most cases, q is simply one.

These relationships are somewhat simplified by the assumption that outer sphere effects are negligible. These effects occur when ligands in solution approach the paramagnetic center but do not bind at the normal binding site (which may already be occupied). The time of interaction and the longer dipolar distance for these outer sphere ligands results in a small, usually insignificant, effect. The measured relaxation rates can then be related to the structure of the ligand on the enzyme relative to the paramagnetic center. This information can be obtained from the Solomon-Bloembergen relationships (Solomon, 1955; Bloembergen, 1957). These relationships relate the dipolar (through space) and scalar (through chemical bonds) contributions of the paramagnetic centers to the nuclear relaxation rates:

$$\frac{1}{T_{1M}} = \frac{2S(S+1)\gamma_I^2 g^2 \beta^2}{15r^6} \left(\frac{3\tau_c}{1 + \omega_I^2\tau_c^2} + \frac{7\tau_c}{1 + \omega_S^2\tau_c^2} \right) +$$
$$\frac{2S(S+1)}{3} \left(\frac{A}{\hbar} \right)^2 \left(\frac{\tau_e}{1 + \omega_S^2\tau_e^2} \right) \qquad (14)$$

$$\frac{1}{T_{2M}} = \frac{S(S+1)\gamma_I^2 g^2 \beta^2}{15} \left(4\tau_c + \frac{3\tau_c}{1 + \omega_I^2\tau_c^2} + \frac{13\tau_c}{1 + \omega_S^2\tau_c^2} \right) +$$
$$\frac{S(S+1)}{3} \left(\frac{A}{\hbar} \right)^2 \left(\tau_e + \frac{\tau_e}{1 + \omega_S^2\tau_e^2} \right) \qquad (15)$$

The term S is the electron spin quantum number; γ_I is the magnetogyric (gyromagnetic) ratio of the nuclear spin; g is the electronic 'g' factor; ß is the Bohr magneton; ω_I and ω_S are the Larmor angular precession frequencies for the nuclear and the electron spins respectively ($\omega_S = 57\ \omega_I$); r is the ion (spin-label electron)-nucleus distance; A is the hyperfine coupling constant; ℏ is Planck's constant divided by 2π; and τ_c and τ_r are the correlation times for the dipolar and the scalar interactions, respectively. The first term in each equation describes the dipolar interaction, and the second term describes the scalar interaction. Scalar interactions are caused by electron density from the electron spin at the nucleus under observation. Nuclei on ligands not directly bonded to the probe (metal) ions are expected to have no scalar effect, as is also the case for some nuclei such as ^{1}H. Scalar effects on $1/T_{2M}$ for ^{31}P nuclei in second sphere complexes appear to be significant in some cases, however (Lee and Nowak, 1984).

Upon perusal of the Solomon-Bloembergen equations, it is clear that the correlation time functions for T_{1M} and T_{2M} are different, and therefore their frequency and temperature behavior should differ. For T_{1M}, scalar interactions are very small and can usually be ignored. Values for S, g and τ_s, the electron relaxation time for the paramagnet for a variety of possible paramagnetic probes, are listed in Table I. The τ_c may be an important component of tc in eqs. 14 and 15.

Other constants used in the equation are ß = 9.284 x 10^{-21} erg Gauss^{-1} and ℏ = 1.055 x 10^{-27} erg sec. Although the choice of probes may be dictated by the nature of the enzyme, its chemical, physical, and biophysical properties, Mn(II) would, given a choice, be the probe to be used. This choice is predicated by its ability to substitute for a number of physiological metal ions (Mg(II), Ca(II), Zn(II)...), its labile hydration sphere (τ_m for Mn(II) (H$_2$O)6 ~ 3 x 10^{-8} s), large electron spin quantum number (5/2), normally isotropic behavior, and long electron spin relaxation time, τ_s.

In a study of Mn(II)-^{1}H interactions where g = 2.675 x 10^4 rad Gauss^{-1} sec^{-1}, g = 2 and S 5/2, and hyperfine coupling is negligible, the

equations for $1/T_{1M}$ and $1/T_{2M}$ can be simplified:

$$\frac{1}{T_{1M}} = \frac{2.878 \times 10^{-31}}{r^6} f(\tau_c) \tag{16}$$

$$\frac{1}{T_{2M}} = \frac{1.439 \times 10^{-31}}{r^6} f'(\tau_c) \tag{17}$$

where $f(\tau_c)$ and $f'(\tau_c)$ are the correlation time functions for T_1 and T_2 relaxation respectively. A determination of tc can allow the calculation of r for the ^{1}H to Mn(II) in the E-Mn-ligand complex. The assumption of negligible hyperfine coupling for $1/T_{1M}$ usually holds, but with some nuclei, specifically ^{31}P of phosphates, this is not the case for $1/T_{2M}$.

Several methods may be used to estimate τ_c. Discussions concerning the rigor of such estimates are detailed elsewhere (Dwek, 1973; Mildvan and Gupta, 1978; Nowak, 1981).

With a value for τ_c, r can thus be obtained. Choosing Mn(II) as an example, from a measurement of T_{1M} and an estimate of τ_c, r, in Angstroms, can be calculated:

$$r = X\ [T_{1M} f\ (\tau_c)]^{1/6} \tag{18}$$

T_{1M} comes from direct measurements, and $f(\tau_c)$ is calculated at ω_I after an estimate of τ_c. The value X is a collection of constants giving X = 812 for ^{1}H; 796 for ^{19}F; 601 for ^{31}P; or 512 for ^{13}C. Because of the sixth-power dependence of r on T_{1M}, the absolute value of r is reasonably insensitive to minor errors in the estimation of $f(\tau_c)$ or T_{1M}. On the other hand, small differences in r for different nuclei within the ligand give rise to large differences T_{1M}. Thus the method is very sensitive to small changes in the structure of the ligand on the enzyme. From a determination of the various values of r in the enzyme-label-ligand ternary complex the structure of the ligand relative to label can be determined.

In cases, for example, where a bisubstrate enzyme is apparently labeled at site 2 to

yield inactive enzyme, the effect of this modification on the binding (K_d) and structure of substrate 1 at the catalytic site can be investigated.

In some cases where one nucleus of a ligand is very close to the paramagnetic center compared to other nuclei measured, the relaxation may be so efficient that the nucleus may be in slow exchange ($T_{2M} \ll \tau_m$) ($1/fT_{2p} = 1/\tau_m$). If this is the case, then a temperature-dependence of $1/fT_{2p}$ will give a value for koff and for the energy of activation, E_{act}, for the ligand exchange process. In this case the structure of the ligand at the catalytic site (from $1/T_{1M}$), its exchange rate, and the energy barrier for this exchange process, can be obtained and compared with these parameters for the unmodified enzyme. In the case where the exchange process is simple, and

$$K_d = \frac{k_{off}}{k_{on}} \tag{19}$$

for ligand binding is known, values of k_{on} can also estimated (Monasterio, 1987).

WATER RELAXATION RATE PROCESSES

A rapid and sensitive method of measuring ligand-enzyme interactions, where the enzyme system is appropriate, is to measure the effect of ligand binding on the solvent (^1H of H_2O). This method requires a paramagnetic probe that can affect the longitudinal relaxation rate of the solvent. The probe elicits an effect on the proton longitudinal relaxation rate (PRR or PRE) to give a proton relaxation rate enhancement. If the enhancement effects are sensitive to ligand binding, then studying the environment around the probe can yield important thermodynamic and structural information.

Although a number of probes can be used for these studies, Mn(II) will again be chosen as an example because of its physical-chemical properties and its usefulness in many cases. The interaction of the solvent with the paramagnetic probe increases the relaxation

rates of the ^1H's of H_2O by dipole-dipole interactions as discussed in the previous section. Such studies are usually performed measuring only T_1 values and, as will be seen, lower values of ω_I (15-40 MHz) are preferable to higher frequencies (100-400 MHz). Dedicated, low-resolution pulsed instruments have been designed especially for such studies.

In free solution the interaction of the unpaired electrons of the metal ions or of the nitroxide with ^1H nuclei can be normalized as shown in eq. (9). For metal ions the number of protons is twice the hydration number, q, (2 x 6 for Mn(II); 2 x 8-9 for Gd(III)). Hydration may be less clear for organic nitroxides. In solution at room temperature rapid exchange conditions prevail and the correlation times is often τ_r. For Mn(H_2O)$_6$, τ_r is approximately 2.9 x 10^{-11} s and with τ_s ~ 10^{-9} s and the residence time for Mn-bound water, τ_m ~ 2.9 x 10^{-7} s, τ_c is τ_r. For some metals where τ_s is short (for Fe(II), τ_s ~ 10^{-11} s) τ_c is determined by τ_r and τ_s. When the metal binds to an enzyme, at least two phenomena occur: q decreases, decreasing the value for $1/fT_{1p}$ and τ_r increases. The increase in τ_r, if τ_r modulates relaxation, increases $1/fT_{1p}$. For probes with long τ_s values, an increase in τ_r to the rotational correlation time of the enzyme (~10^{-9} - 10^{-7}s) results in a substantial increase in τ_r to the point where τ_c may be dominated by processes other than τ_r. Thus the $1/fT_{1p}$ for ^1H of H_2O by the paramagnetic species is enhanced by some factor ϵ^*. The observed enhancement can be quantitated by comparing the paramagnetic effect of the label in the presence of enzyme, designated by the asterisk (*) to that in its absence:

$$\epsilon^* = \frac{\left(\dfrac{1}{T_{1p}}\right)^*}{\left(\dfrac{1}{T_{1p}}\right)} = \frac{\left(\dfrac{1}{T_{1,obs}} - \dfrac{1}{T_{1,0}}\right)^*}{\left(\dfrac{1}{T_{1,obs}} - \dfrac{1}{T_{1,0}}\right)} \tag{20}$$

The control used for the denominator term is simply the paramagnetic effect of the probe on the PRR measured in the absence of enzyme.

The observed relaxation rate is the sum of the paramagnetic effects due to free species and bound species:

$$\left(\frac{1}{T_{1p}}\right)^* = \left(\frac{fq}{T_{1M} + \tau_m}\right)_f + \left(\frac{fq}{T_{1M} + \tau_m}\right)_b \quad (21)$$

The term designated subscript f describes the normalization factor f for the concentration of free paramagnetic species ([p] f) and the concentration of water (55.5 M) (f = [p]f/55.5); q, the hydration number for free metal ion (nitroxide); T_{1M} for the 1H of H_2O bound to the free probe; and the lifetime, τ_m of the complexes. The term designated subscript b describes the same parameters for the species which is enzyme bound and is primarily responsible for enhancement. This effect, or the enhancement, can be quantitated by relating the free and bound paramagnetic species $(M)_f$ and $(M)_b$ respectively:

$$\varepsilon^* = \frac{[M]_f}{[M]_T}\,\varepsilon_f + \frac{[M]_b}{[M]_T}\,\varepsilon_b \quad (22)$$

The enhancement of free species, ε_f is defined as unity, and the binary enhancement (of the enzyme-label complex), ε_b is characteristic of the complex being studied. The preceding equation relates the mole fraction of each species. Direct binding studies of the probe can be used to evaluate ε_b. The value for ε_b is a reflection of the environment about the bound probe, and contains information concerning q, τ_m and τ_c for the H_2O at the probe. A comparison of stoichiometry, K_d, and ε_b for metal binding to modified and unmodified enzyme can relate the effect of modification to the environment of the activator. If the stoichiometry of an enzyme-M complex is known or assumed to be 1:1, a titration of ε^* versus [M] can yield K_d and ε_b.

An actual quantitation of q for H_2O in the enzyme-label complex can be obtained by a determination of τ_c for the complex. Several methods to determine τ_c for Mn(II)-H_2O interactions in binary enzyme-Mn complexes have been attempted to evaluate these parameters. Such approaches are not very simple, and the evaluation of additional physical-chemical parameters and several assumptions

are required. Reasonable approximations to these parameters can be made, but the evaluation of the actual hydration number by such studies should be taken with some amount of skepticism (Burton et al, 1979). One important detail obtained from frequency-dependent studies of (1/T_{1p}) is that for enzyme-bound Mn(II), the value for τ_c is usually frequency-dependent, showing that τ_c must be at least partially determined by τ_s in those cases. A fit of the data to equations (9) and (14) would suggest that fT_{1p} is linear with $(\omega_I)^2$ if τ_c is constant. Lower frequency measurements should yield greater paramagnetic effects on T_1.

This approach to the study of ligand interactions with enzymes can also be used when paramagnetic ions bind only to enzyme-ligand complexes (i.e., creatine kinase where Mn(II) binds to creatine kinase ATP but not to creatine kinase). A similar evaluation of the data can be made.

The addition of a ligand (substrate or allosteric modifier) to the over enzyme-label complex can result in a perturbation of one or several parameters which influence relaxation. The bound ligand can change metal binding (K_d and/or n), resulting in a change in $[M]_b$ or can result in changes in q, τ_m or τ_c. A change in τ_c can affect T_{1M}. Regardless of the reason for the perturbation in 1/T_{1p}, a change in ε^* upon addition of ligand may be obtained. Such a titration can result in a determination of the dissociation constant of the ligand from the enzyme complex and a value for the enhancement of the ternary enzyme-label-ligand complex, ε_t. The equation for observed enhancement now becomes:

$$\varepsilon_{obs} = \frac{[M]_f}{[M]_T}\,(1) + \frac{[E\cdot M]}{[M]_T}\,\varepsilon_b + \frac{[E\cdot M \cdot L]}{[M]_r}\,\varepsilon_T \quad (23)$$

In the case of metal complexes where the ligand also competes with the enzyme for metal binding, a term $[M\text{-}L]/[M]T(\varepsilon_a)$, must also be considered. A value for the enhancement of the metal-ligand complex, ε_a, can be evaluated independently. In a general case when all possible equilibria may be present in such a titration experiment, the following complexes, their dissociation

constants, and enhancement values must be considered:

$$K_D = \frac{[E][M]}{[EM]} \varepsilon_b \qquad (24)$$

$$K_1 = \frac{[M][L]}{[ML]} \varepsilon_a \qquad (25)$$

$$K_2 = \frac{[E][M-L]}{[EML]} \varepsilon_T \qquad (26)$$

$$K_3 = \frac{[EM][L]}{[EML]} \varepsilon_T \qquad (27)$$

$$K_A = \frac{[EL][M]}{[EML]} \varepsilon_T \qquad (28)$$

$$K_S = \frac{[E][L]}{[EL]} \qquad (29)$$

From thermodynamics:

$$K_1 K_2 = K_D K_3 = K_A K_S \qquad (30)$$

In a simple case, the addition of a ligand to an enzyme-label which results in a change in ε^* indicates the formation of an enzyme-label-ligand ternary complex. Values for ε_T can be either greater than for ε^* or less than ε_b; no change in ε^* may result from failure of ligand to bind; no change in physical parameters affecting ε^*; or fortuitous changes in $1/T_{1p}$ that result in no observed change. A change in ε^* with concentration of ligand can be either graphically evaluated to yield K_d and ε_T or a fit to the data can be attempted. A computational analysis of ε^* vs [ligand], considering all possible equilibria, has been developed (Reed et al, 1970) and is the more rigorous treatment. An evaluation of K_3 and ε_T reflects any change of enzyme modification on ligand binding and on any environmental change about the probe induced by the ligand, respectively. Such changes can be compared to those observed in the native or non-derivatized enzyme. This

method can be a powerful yet simple tool to evaluate the effects of enzyme modification on ligand binding.

Analogous to PRR measurements, the environments of the catalytic site can also be studied by observing the relaxation ($1/T_2$ from the line width measurements) of inorganic anions which serve as part of the milieu. The interaction of an anion such as Cl⁻ using ³⁵Cl NMR with paramagnetic centers can elicit a paramagnetic effect analogous to effects observed using PRR. On the other hand, the interaction with diamagnetic centers can also give rise to substantial quadrupolar relaxations resulting in T_2 effects. The observation of such an effect will demonstrate that the diamagnetic center, i.e. Zn(II), has access to the solvent. The addition of ligands influences the metal center. Another variation is the use of the more sensitive ¹⁹F nucleus of F⁻ if this ion is innocuous for the enzyme under investigation. The F⁻ interaction with the Cu(II) center of galactose oxidase was studied by relaxation rate measurements (Marwedel et al, 1975). The limitation of ¹⁹F NMR is that F⁻ is an inhibitor for a number of enzymes and may not be a good anion in every case. The study of anion relaxation has important potential in the study of environment effects and ligand effects on the active site environment.

CATION NMR

An alternative to studying the enzyme directly by NMR is to observe a portion of the enzyme, preferably the active-site, where good resolution of the important functional groups can be observed. One example where such a study is possible is in the case of metal utilizing enzymes where the spectra of one of several metals can be observed. These metal ions often play a key role in the catalytic processes. There is a potential for the use of the 'physiologically common' divalent cations ²⁵Mg and ⁴³Ca; a review of the attempted applications contains some examples (Forsén and Lindman, 1981). Most of the studies have been with ⁴³Ca which is somewhat easier to study. The primary drawback in these studies is the very low

sensitivity of these nuclei. The high concentration of these cations necessary to observe the resonances of bound cations are limited by the solubility of the proteins.

Applications of monovalent cations ^{7}Li, ^{23}Na, ^{39}K or ^{205}Tl are also quite possible. Because of much weaker binding of the monovalent cations to enzymes, the effects, including ligand-induced cation perturbations, can also be observed and perhaps quantitated.

A broader application of ^{113}Cd NMR has been made. Although Cd(II) is a toxic metal ion, it has been found to substitute for Zn(II) or Mg(II) in several enzyme systems. Its chemical shift is sensitive to the nature of its ligands, and a change in ligand environment (e.g. addition of substrates) is reflected in a change in chemical shift (Armitage and Otvos, 1982). The nucleus can be used to study chemical exchange between metal sites, multimetal sites, and the interactions of ligands to the enzyme-bound metal. The low sensitivity and low resonance frequency keeps this metal from being routinely used. It can prove to be very useful, however, in the cases where plenty of enzyme that is quite soluble can be obtained.

POLYPEPTIDE AND PROTEIN STRUCTURE

Knowledge of the three-dimensional structure of a polypeptide or protein is a prerequisite to the understanding of its physical, chemical, and biological properties. Since the time that Perutz and Kendrew determined the structure of hemoglobin and myoglobin, more than three decades ago, about 750 non-identical structures of a total number of 2700 has been determined by crystallographic and NMR techniques (Orengo, 1994). The precision with which the NMR structures of small proteins can now be determined approaches that of moderately good X-ray crystal structures. In the protein interior, the structures obtained from the highest quality NMR data can be as precise as all but the very best X-ray structure, whereas the surface residues often appear disordered in solution and hence in the NMR structures derived from solution data. Thus, the main differences between the NMR and X-ray

structures of proteins are in fact usually found on protein surfaces.

In the last few years the significant increase in the number of known three-dimensional structures of small proteins in solution became possible due to advances in NMR technology such as the development of superconducting magnets, Fourier transform spectroscopy, computer control of the instrumentation and new multidimensional NMR techniques developed by Ernst (Ernst et al, 1987), who won the Nobel Prize in 1991.

The basic steps for protein determination from NMR are the following: 1) Assignment of proton resonances signals to individual protons. 2) Determination of proton-proton distance constrains and dihedral angle constrains from NOE's and J couplings, respectively. 3) Calculation of a family of three-dimensional structures on the basis of the distance restrains, supplemented if possible by some torsion-angle restrains derived from coupling constants. 4) Refining of the structures by using geometric constrains and potential energy functions, for instance, with restrained energy minimization and restrained molecular dynamics. We will discuss these steps in some detail.

1. ^{1}H Resonance assignments

The first step is the assignment of the ^{1}H signals to individual protons of the protein under study. It is convenient to start the study with the analysis of the mono-dimensional spectrum in order to know the conditions of the sample, i.e. the presence of impurities, aggregation (millimolar concentrations are normally used), the signal to noise ratio and the presence of some region in the protein without conformation or, in the case of peptides, the presence of conformation. In general well defined and narrow signals indicate the presence of regions exposed to the solvent and without interaction with the rest of the polypeptide chain, except through the peptide bond. The dispersion of the signals frequencies and broader signals, show a crowded spectrum with mutually overlapping lines in the case of a monomer protein where the polypeptide chain has many interactions with the rest of

the structure and the movement is restricted in the region where the proton under observation is located. In table II the chemical shift for protons of natural proteins in the random coil conformation are listed. They fall in several classes as shown in Figure 1 for the ^{1}H- NMR spectra, at 600 (A) and 300 (B) MHz, of the peptide containing the ~ 60 amino acids of the beta tubulin subunit.

The assignment of the total signals from a monodimensional spectra of a polypeptide is not straightforward because when the complexity (length of the polypeptide chain) of the protein increases, the resolution of the spectra diminishes. To increase resolution it is necessary to use two dimensional NMR in order to have a complete assignment of the spectrum. Wüthrich et al (1986) have developed a standard method for the systematic assignment of a NMR spectrum

for proteins. For peptides (5 to 30 residues) the application of this method is easier than for proteins (80 to 130 residues). The assignment method has two steps. The first correspond to the identification of the spin systems for each amino acid. The characteristic spin systems are shown in figure 2. The identification is based on the scalar coupling obtained from the two dimensional experiments COSY (J-correlated spectroscopy), RELAY-COSY (relayed coherence transfer spectroscopy) and TOCSY (total correlation spectroscopy) which are the most common methods. The simplest experiment is COSY in which the off-diagonal cross-peaks arise only between protons connected through J-coupling networks (fig 4). This allows identification of the signals NH-H$_\alpha$, H$_\alpha$-H$_\beta$, etc. from the same residue, because the scalar coupling is interrupted by the
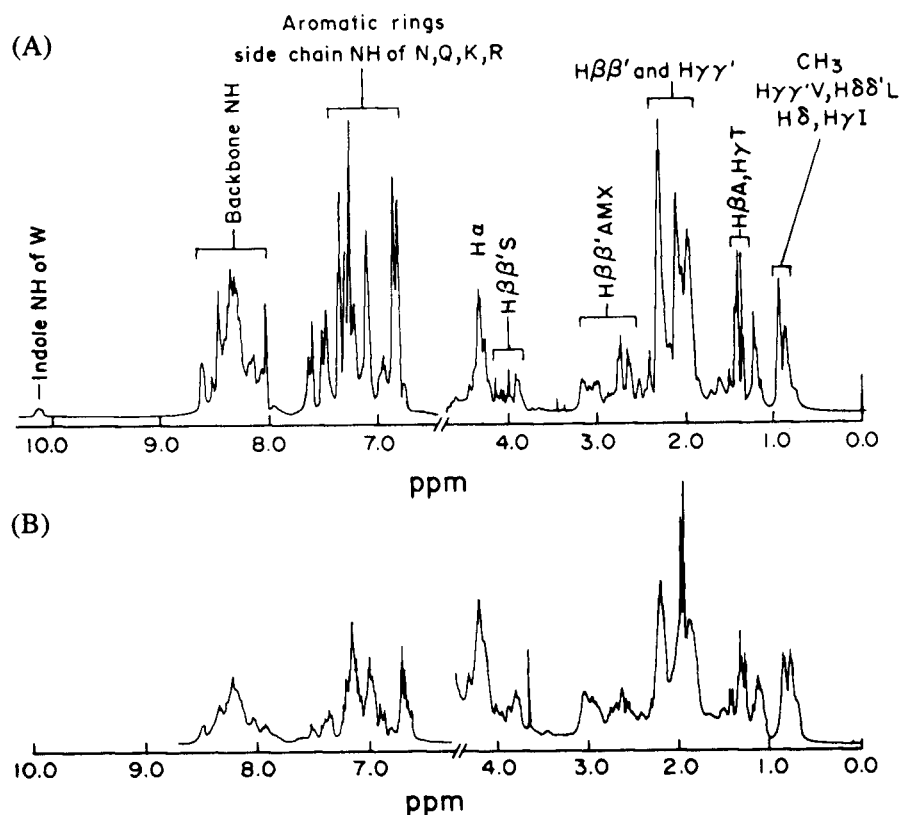

Fig 1. ^{1}H-NMR spectra, at 600 (A) and 300 (B) MHz of the ß-tubulin fusion peptide, RL33ß6 (~ 5 mM), in H$_2$O with 10% D$_2$O containing 50 mM NH$_4$HCO$_3$ at pH 6.2 at 25 °C. This peptide correspond to the carboxy-terminal region of isoform cß2, with the following amino acid sequence:

| 400 | 410 | 420 | 430 | 440 |

*ARIRA*PFLHWYTGEGMDEMEFT*E*NESNMNDL*VS*EYQQYQDA*T*ADEQGEFEEEGEEDEA

In italics, the non-tubulin sequence segment that was inserted by the vector construction is indicated.
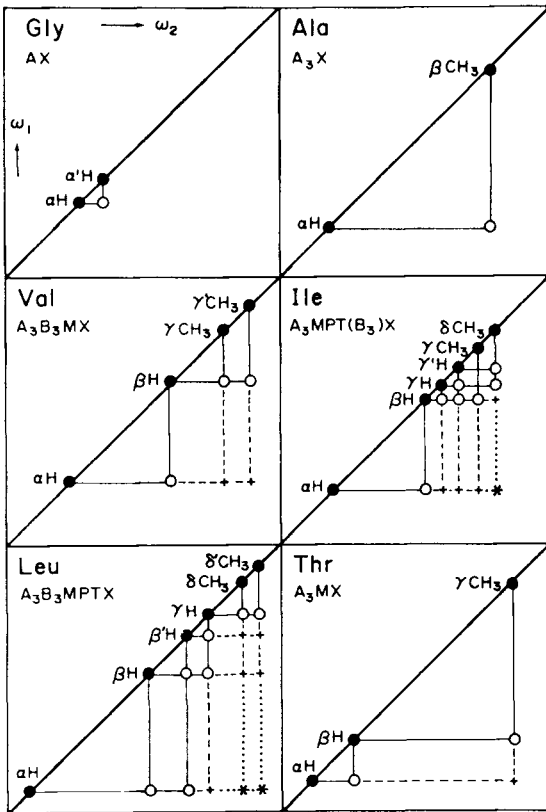
Fig 2. Examples of COSY, RELAYED-COSY and DOUBLE-RELAYED-COSY connectivity diagrams for the spin systems of nonlabile protons in six common amino acid residues. The diagrams show the position of the diagonal peaks (filled circles) and the connectivities of 2D-experiments, COSY (open circles), RELAYED-COSY (crosses and broken lines) and DOUBLE-RELAYED-COSY (stars and dotted lines). Values of the chemical shifts are arbitrary.

distinguish amino acid residues with the same system of spin or amino acids that are repeated in the sequence. These ambiguities are dilucidated with NOESY and ROESY experiments which give distance information.

The second class of two dimensional NMR experiments (2D NOE) cross-peaks connect protons that are spatially at a distance shorter than 5 Å, independently whether they present or not scalar coupling. The information from NOESY and ROESY is similar. In contrast to all other parameters, proton-proton distance measurements by NOE experiments can be directly related to the peptide or protein conformation. The analysis usually starts with a search of the cross-peak patterns belonging to the spin systems of types of amino acids. These are then connected through cross-peak in a two dimensional NOE spectrum between neighboring amino acids in the polypeptide chain. Useful short distances for the
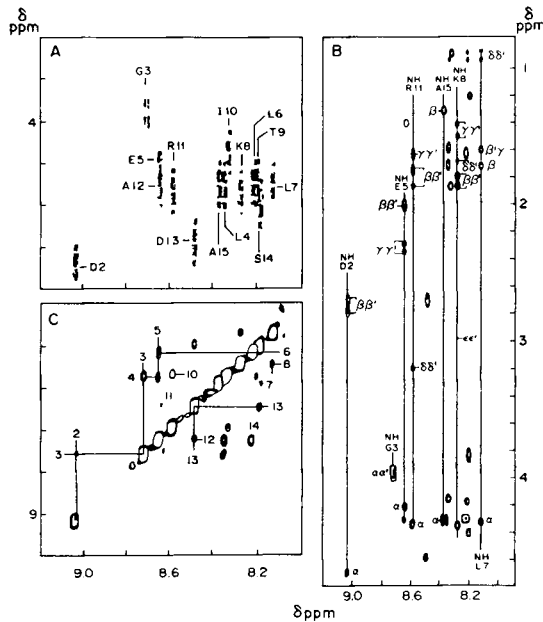

Fig 3. 2D ^{1}H-NMR spectra of the hexadecapeptide CheY-C (1 mM) in H_2O with 10% D_2O (pH 5.0, 5°C). A. Phase-sensitive COSY 'finger print region'. B. TOCSY region. The correlation between the NH proton with the rest of the protons of the spin system for several residues are connected by a solid line. C. NOESY region showing the sequential assignments through the correlations $d_{NN(i, i + 1)}$.

The amino acid sequence of CheY-C is:

1   5   10   15
MDGLELLKTIRADSAY

carbonyl group of the peptide bond. The 2D ^{1}H-NMR spectra of a hexadecapeptide of CheY, a 129-residue protein involved in bacterial chemotaxis are shown in figure 3. Figure 3 A shows the COSY patterns of the cross peaks found in the spectral region between 3.6 to 4.8 ppm and 8.0 to 9.2 ppm (known as the "COSY fingerprint"), that contains the scalar correlation NH-H$_\alpha$. TOCSY gives the correlated signals among all the frequencies that belong to same network of couplings (the same system of spins), because the magnetization is spread through the coupling network. The pattern of signals for the scalar coupling of the different amino acids in the COSY and TOCSY (figure 3B) allows the identification of all the protons that belong to a same residue. In this step it is not possible to

assignment are those observed between H_α of residue i and the NH proton of the next residue ($d_{\alpha Ni,i + 1}$), between the NH protons of adjacent residues ($d_{NNi,i + 1}$), and the H_β proton of residue i and the NH proton of the next residue ($d_{\beta Ni,i + 1}$), see figure 4. From these correlations, the sequential order of the spin systems can be established. The intensity of the signal depends on the structure of the polypeptide chain. Often the sequential assignment procedure is redundant, thus many internal checks are possible. This makes the assignment unambiguous.

2. Distance and dihedral angle constrains

When all the resonances of the NMR spectra are assigned, the data from J couplings and NOE distances are used to infer the conformation of the polypeptide chain. The principal advantage of NOEs is that while all the other spectral parameters are a linear average of the different conformations in equilibrium, NOE has a nonlinear dependence on the interprotonic distance, r; the NOE intensity is directly related to r^{-6}, therefore the short distances are emphasized. This allows the detection and identification of preferential polypeptide conformations, notwithstanding if the preferred conformation is a small fraction. Secondary structure is usually apparent from the strong NOEs used to make the assignments. Stretches of residues in an α-helix have strong NOEs between NH_i - $NH_{i + 1}$ and $C_\beta H_i$ - NH_{i+1} but not between $H_{\alpha i}$ - NH_{i+1}. In ß-strands, adjacent residues give strong NOEs between $H_{\alpha i}$ - NH_{i+1} but not between

NH_i - NH_{i+1}. The relationship between the intensities of the NOEs (NH_i - NH_{i+1}) / ($H_{\alpha i}$ - NH_{i+1}) is much higher in the α-helix than in the ß-strands, because the difference between the sequential distances NH - NH and H_α - NH is amplified by the six power dependence of the NOE with respect to the interprotonic distance. The true identification of the preferred polypeptide structure is only possible with the observation in its structure of specific NOEs between protons of residues non adjacent in the sequence. Thus, the geometry of α-helix gives NOEs of the type (i,i + 3) and (i,i + 4) considering that the helices found in proteins are α-helices and 3_{10} helices. The 3_{10} helix is important because it usually forms the last turn of the C-terminal end of numerous α-helices. In favorable cases, dihedral angle constrains can be obtained from three-bond J couplings (^3J). The value of ^3J is related to the dihedral angle θ of the bond between the atoms to which the protons are bonded. The relationship, based on the Karplus equation, is of the form:

$$^3J = A \cos^2 \theta + B \cos \theta + C$$

For example, the value of $^3JNH\text{-}H_\alpha$ between the NH and the H_α protons gives information about the torsion angle φ:

$$^3J_{NH\text{-}H\alpha} = 6.4 \cos^2 \theta - 1.4 \cos \theta + 1.9$$

For helical regions $^3J_{NH\text{-}H\alpha}$ is small (ca. 4 Hz), while for extended chain conformations such as in ß-sheets the values are larger (9-10 Hz). Usually the large J couplings (8-10 Hz)
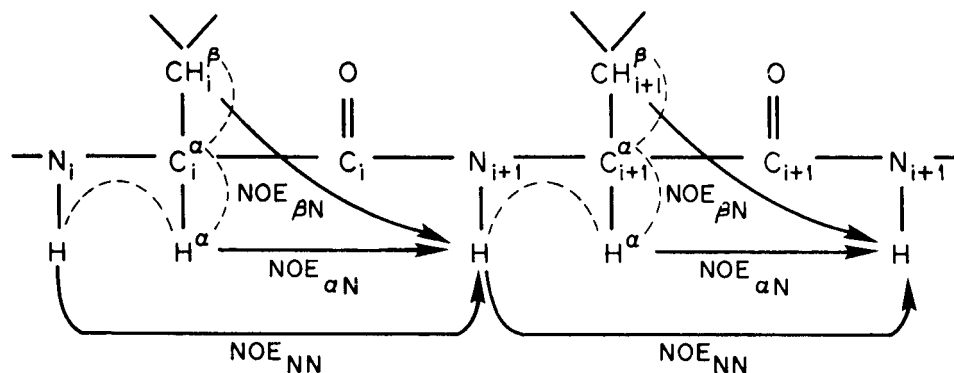

Fig 4. Connectivities within and between residues that are used in sequential assignments of individual protons in peptides and proteins. The solid arrows indicate the sequential NOEs (connectivities through space) and the broken lines the scalar coupling (connectivities through bond).

are the most useful source of information, because J couplings smaller than the line width (5 Hz or larger cannot be reliably measured). The interpretation of the larger J constants in terms of dihedral angles is less ambiguous.

The parameters for the identification of secondary structures, shown in Table III, are summarized below:

α-helix

1. The presence of medium range NOEs, $d_{NN(i,i+2)}$, $d_{\alpha N(i,i+3)}$, $d_{\alpha\beta(i,i+3)}$) and $d_{\alpha N(i,i+4)}$ along consecutive residues of a peptide segment. Likewise, the presence of medium range NOEs i,i+3 or i,i+4 involving protons of lateral chains.

2. The presence of a series of intense NOEs $d_{NN(i,i+1)}$ plus less intense or weak NOEs along a peptide chain segment.

3. Different chemical shifts (δ) for the protons H_α with respect to its δ in reference peptides; negative $\Delta\delta_{H\alpha}$ for a series of consecutive residues.

4. Small coupling constants $^3J_{HN-H\alpha}$ for a segment of the polypeptide chain.

β-sheet

1. The presence of a NOEs network $d_{NN(i, j)}$, $d_{\alpha N(i,j)}$ and $d_{\alpha\alpha(i,j)}$ between the strands of the parallel or antiparallel ß-sheets.

2. The presence of weak $d_{NN(i,i+1)}$ and intense $d_{\alpha N(i,i+1)}$ NOEs.

3. Different chemical shift (δ) for the protons H_α with respect to its d in reference peptides; positive $\Delta\delta_{Ha}$ for a series of consecutive residues.

4. Large coupling constants $^3J_{HN-H\alpha}$ for a segment of the polypeptide chain.

β-Turns

1. The presence of NOE $d_{\alpha N(i,i+2)}$ between the residues 2-4.

2. The presence of intense NOEs $d_{NN(i,i+1)}$ between residues 2-3 and 3-4 for ß-Turn type I and 2-3 for type II.

3. Different chemical shift (δ) for the protons H_α with respect to its δ in reference

TABLE III

NMR parameters for the identification of peptide secondary structure

NMR Parameter	Structure				
	helix		sheet	ß-turn	
	α	3_{10}		I	II
$\Delta\delta_{H\alpha}$	negative (-0.39 ppm)		positive (+0.37 ppm)		negative
$^3J_{NH-H\alpha}$ (Hz)	4	4	9	2, 4 3, 9	2, 4 3, 5
NOE type (Å)					
$d_{\alpha Ni, i+1}$	medium (3.5)	medium (3.4)	strong (2.2)	2,3 medium (3.4)	2,3 strong (2.2)
$d_{NNi,i+1}$	strong (2.8)	strong (2.6)	weak (4.3)	2,3 strong (2.6) 3,4 strong (2.4)	2,3 weak (4.5) 3,4 strong (2.4)
$d_{NNi,i+2}$	weak (4.2)	weak (4.1)	no	2,4 medium (3.8)	2,4 weak (4.3)
$d_{\alpha Ni,i+2}$	weak (4,4)	medium (3.8)	no	2,4 medium (3.6)	2,4 medium (3.3)
$d_{\alpha Ni,i+3}$	medium (3.4)	medium (3.3)	no	1,4 yes (3.1 - 4.2)	1,4 yes (3.8 - 4.7)
$d_{\alpha Ni,i+4}$	weak (4.2)	no	no	no	no
$d_{\alpha\beta i,i+3}$	yes	yes	no	no	no
$d_{lat. chaini,i+3}$	yes	yes	no	–	–
$d_{lat. chaini,i+4}$	yes	–	no	–	–

peptides; negative $\Delta\delta_{H\alpha}$ for consecutive residues of the turn.

4. Normal coupling constants $^3J_{HN-H\alpha}$ (see Table III).

In summary, the presence of NOEs between protons that are close in the covalent structure can define the secondary structure and those NOEs between protons that are distant in the primary structure but close in the space define the tertiary structure. Often preliminary reports on NMR studies of a protein that describe the resonance assignments and the secondary structure are found in the literature. The secondary structures so identified can be used as a starting point for interactive model building of the tertiary structure; however this strategy has been little used as compared to computational structure determination.

3. Structure calculations

Once resonance assignments are available for all protons, the NOESY data are again analyzed, now in terms of structural information. Each off diagonal cross peak indicates that two protons in known locations in the protein sequence are separated by a distance of less than about 5 Å. The measurement of a large number of such cross peaks must thus impose stringent constrains on the protein tertiary fold. By measuring the intensity of the cross peak, a qualitative estimate can be made of the distance between the two protons. These structural constrains are put into computer programs to search for those protein conformations that are compatible with all of the experimental measurements. The most common programs for generation of structures use either a metric matrix distance geometry algorithm or restrained least square minimization in torsion angle space. By repeating the calculations, several structures will be generated that agree with the experimental data. Provided a sufficient number of constrains are used, a family of structures which closely agree will be obtained from many passes.

The structures generated by such procedures are generally of relatively high energy, and merely serve as initial estimates of the protein fold. It is then necessary to

subject these structures to restrained molecular dynamics calculations. This involves the simultaneous solution of the classical equations of motion for all atoms in the system for several hundred picoseconds with the NMR distance constrains incorporated as effective potentials in the total energy function. The power of the method lies in its ability to overcome local energy barriers and reliably locate the global minimum region. In general, the procedure significantly improves the agreement between the structural model and the experimental data. An informative picture of the resulting family of molecules can now be displayed using molecular graphics software. An important feature of NMR-derived structures is that some regions of the protein will be less defined than others. This is a consequence of the non-uniform distribution of NMR constrains within the molecule and reflects the molecular motions taking place in solution.

4. Refinement of NMR structures

There are two crucial questions regarding structures determined by NMR, namely, how unique are they and how accurately have they been determined? It is thus essential to analyze the derived structures and examine the degree of convergence. If the set converges well and all experimental restrains are satisfied, then they can be said to represent a realistic and accurate picture of the solution structure.

A more rigorous assessment of NMR-derived structures can be made from the application of back calculation methods. Back-calculation involves simulating the NOESY spectrum from the calculated molecular structure and using the result to compare with the experimental NOESY spectrum. This process serves to check the quality of the structure and it is also an integral part of the refinement strategy. In the commonly used procedure NOEs are converted into rough upper distance limits in order to allow for the effects of internal motion and diffusion of magnetization signals, as well as experimental uncertainty. The final structures thus fit the upper distance limits rather the true experimental values.

Back-calculation involves using the calculated structure in conjunction with a simple model for the dynamic behavior of the atoms in the molecule in order to simulate its NOESY spectrum. However, the method is currently rather imprecise. Despite this, back-calculation allows an evaluation of how well different parts of the protein structure agree with the input data. By providing a quality measure of the fit of the derived structure, it is analogous to the R-factor used for assessing structures derived using crystallography. The comparison of simulated and measured NOESY spectra allows an estimate of the magnitude and direction of changes to be made to the molecule that might improve the agreement between the spectra. In order to achieve the full benefits of back-calculation, it is necessary to make it an integral part of the strategy for protein structure determination. This would involve a readjustment of the distance restrains used in the structure calculation steps after analyzing the calculated NOESY spectrum. A new structure would be calculated and the process repeated until simulated and measured spectra match. For structure determination on the basis of distance constrains such as distance geometry and restrained molecular dynamics, among others, the software NMRchitect and Discovery from Biosym Technologies can be used.

The validity of the NMR method was established conclusively by determining the tree dimensional structure of the protein "tendamist" independently using NMR and normal X-ray diffraction analysis (Billiter *et al*, 1989).

Three- and four-dimensional heteronuclear NMR spectroscopy offer a way to improve spectral resolution and circumvent problems due to larger line widths that are associated with increasing molecular weight. With these methodology the determination of a high resolution NMR structure of a protein greater than 150 amino acids residues have been possible (for a review see Clore and Gronenborn, 1991).

TRANSFERRED NOE

As we discussed before, NMR spectroscopy is a useful tool to study one of the most important issues in biology which is the interaction of ligands with macromolecules. When part of the macromolecule is in close proximity to a bound ligand, a NOE can be observed in the ligand if the macromolecule's protons are irradiated (James and Cohn, 1974). Concomitant with the developments in two-dimensional NMR and the use of NMR to determine the structure of peptides and proteins in solution, interest in transfer NOE (TRNOE) has emerged (Campbell and Sykes, 1993) The TRNOE is the extension of two dimensional NOE to exchanging systems such as ligand-protein complexes. TRNOE measurements give information on the conformation of the bound ligand. This methodology has been used to study the conformations of nucleotides bound to peptides and proteins (Leanz and Hammes, 1986; Koide *et al*, 1989), binding of peptides to phospholipid bilayers (Milon *et al*, 1990), the codon to anticodon interaction (Clore *et al*, 1984), binding of peptides to enzymes (Meyer *et al*, 1988) and binding of hormones to proteins (Live *et al*, 1987).

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