Ligand–Protein Interactions via Nuclear Magnetic Resonance of Quadrupolar Nuclei

By Charles R. Sanders II and Ming-Daw Tsai

Introduction

A significant number of biologically relevant elements have isotopes that possess nuclear magnetic spin quantum numbers greater than \( \frac{1}{2} \) and thereby possess nuclear quadrupole moments. This results in the powerful quadrupolar relaxation mechanism that normally dominates the relaxation and, therefore, the line shapes and widths of the NMR peaks observed from such nuclei. To a certain extent, quadrupolar NMR (QNMR) is advantageous because only a single relaxation mechanism is effectively operative. However, because of theoretical and experimental difficulties, QNMR is an underdeveloped and underutilized technique in the study of biological systems, particularly in enzymology. Keniry ([19], Vol. 176, this series) deals with \(^2\)H NMR of \(^2\)H-labeled proteins, where the useful information extracted regards the local dynamics of amino acid side chains. This chapter presents the use of QNMR to study ligand–protein or substrate–enzyme interactions. In addition to the possible determination of stoichiometry, binding constants, and exchange rates, QNMR can be a particularly effective method in the study of the motional dynamics (through determination of \( \tau_e \), the effective correlation time) and the electrical environment (through \( \chi \), the quadrupolar coupling constant) of bound ligands. The term QNMR should not be confused with a related physical technique: nuclear quadrupole resonance. Because the studies we are concerned with usually involve motion that is isotropic on the NMR time scale, we are technically concerned with high-resolution (HR) NMR. However, because QNMR lines tend to be rather broad, the practical study of quadrupolar species tends to involve a mixture of HR and "broad-line" NMR techniques.

Some aspects of QNMR have been discussed in a number of recent reviews.\(^{1-6} \) This chapter emphasizes practical aspects and is written spe...
cifically for biochemists with a general knowledge of HR NMR but without previous experience in QNMR.

Fundamental Considerations in Experimental Design

*Sensitivity*

Three factors contribute to the sensitivity problem in QNMR. (1) As shown in Table I, the intrinsic sensitivity of most quadrupolar nuclei is much lower than that for $^1$H. Furthermore, many nuclei have low natural abundance. (2) Linewidths of quadrupolar species are often broad and the amount of sample is often limited in biochemically relevant studies. (3) Because $T_1$ and $T_2$ are usually very short for quadrupolar nuclei, the loss of signal during the "preacquisition delay" (DE; see Experimental Techniques) becomes a detrimental factor to sensitivity. On the other hand, short $T_1$ and $T_2$ allow rapid acquisition, and it is sometimes possible to collect as many as $10^6$ scans within 24 hr.

To attain a rough estimate of the limit of detection of a sample and spectrometer system, it is often advisable to prepare a standard sample of the free ligand (or a structurally similar compound) at the same concentration as that to be used in the experiment of interest. If a signal can be

<table>
<thead>
<tr>
<th>Nucleus</th>
<th>$I$</th>
<th>Natural abundance (%)</th>
<th>Sensitivity (relative to $^1$H)</th>
<th>$Q$ ($10^{-24}$ m$^2$)</th>
<th>Typical $\chi$ range (MHz)</th>
<th>Typical $\delta$ range (ppm)</th>
<th>Typical linewidth for $\tau_c = 0.1$ nsec (Hz)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^6$Li</td>
<td>1</td>
<td>7.4</td>
<td>0.01</td>
<td>0.0007</td>
<td>$&lt;0.01$</td>
<td>5</td>
<td>0.01</td>
</tr>
<tr>
<td>$^7$Li</td>
<td>3/2</td>
<td>92.6</td>
<td>0.29</td>
<td>$-0.03$</td>
<td>0.015–0.15</td>
<td>200</td>
<td>4,200</td>
</tr>
<tr>
<td>$^14$N</td>
<td>1</td>
<td>99.6</td>
<td>0.001</td>
<td>0.016</td>
<td>1–5</td>
<td>200</td>
<td>4,200</td>
</tr>
<tr>
<td>$^17$O</td>
<td>5/2</td>
<td>0.037</td>
<td>0.03</td>
<td>$-0.026$</td>
<td>1–15</td>
<td>700</td>
<td>3,000</td>
</tr>
<tr>
<td>$^3$H*</td>
<td>1</td>
<td>0.015</td>
<td>0.01</td>
<td>0.0027</td>
<td>0.16–0.22</td>
<td>19</td>
<td>19</td>
</tr>
<tr>
<td>$^23$Na</td>
<td>3/2</td>
<td>100</td>
<td>0.1</td>
<td>0.14</td>
<td>0.2–2</td>
<td>20</td>
<td>125</td>
</tr>
<tr>
<td>$^25$Mg</td>
<td>5/2</td>
<td>10</td>
<td>0.003</td>
<td>0.22</td>
<td>1–4</td>
<td>—</td>
<td>120</td>
</tr>
<tr>
<td>$^31$S</td>
<td>3/2</td>
<td>0.76</td>
<td>0.002</td>
<td>$-0.064$</td>
<td>0.5–15</td>
<td>600</td>
<td>7,000</td>
</tr>
<tr>
<td>$^35$Cl</td>
<td>3/2</td>
<td>75</td>
<td>0.005</td>
<td>$-0.079$</td>
<td>1–4</td>
<td>700</td>
<td>500</td>
</tr>
<tr>
<td>$^39$K</td>
<td>3/2</td>
<td>93</td>
<td>0.0005</td>
<td>0.11</td>
<td>—</td>
<td>30</td>
<td>—</td>
</tr>
<tr>
<td>$^40$Ca</td>
<td>7/2</td>
<td>0.15</td>
<td>0.006</td>
<td>$-0.065$</td>
<td>0.5–4</td>
<td>70</td>
<td>29</td>
</tr>
<tr>
<td>$^{60}$Co</td>
<td>7/2</td>
<td>100</td>
<td>0.28</td>
<td>0.40</td>
<td>2–80</td>
<td>18,000</td>
<td>20,000</td>
</tr>
</tbody>
</table>

* Quadrupolar relaxation generally does not dominate in $^6$Li NMR.

* Despite low $Q$, quadrupolar relaxation dominates due to nonspherical symmetry.
detected, the viscosity can be varied using glycerol and decreasing temperature to broaden the signal, until it reaches the limit of detection.

Chemical Shifts and Spin–Spin Coupling

As exemplified in Table I, the chemical shift range of each nucleus is variable. Observed chemical shifts are, of course, potential sources of information. However, the resolution of multiple signals is a problem when chemical shift differences do not exceed linewidths. This factor tends to limit the number of species observable in a single sample.

Because lines are often broad in QNMR, spin–spin coupling is frequently unobservable. Nevertheless, coupling constants are sometimes large enough to affect the spectra through broadening or splitting and must then be taken into account in determining line shape or linewidth (Δν1/2).

Relaxation in the Absence of Chemical Exchange

Longitudinal (T₁) and transverse (T₂) relaxation for I > 1/2 nuclei in the absence of chemical exchange is usually dominated by quadrupole-induced relaxation. Transverse relaxation largely dictates the shape and width of NMR peaks.

Quadrupolar relaxation properties are generally classified in two defined regions: extreme narrowing (where ω₀²τ² ≪ 1) or nonextreme narrowing (where ω₀²τ² ≳ 1), where ω₀ is the angular Larmor frequency of the nuclei.

The rotational correlation time τₑ is related to the molecular tumbling rate; the faster the motion, the smaller the τₑ. This parameter can be roughly estimated for use in predicting the narrowing region using the Stokes–Einstein–Debye equation:

\[ τₑ = \frac{4πηr^2}{3kT} \]  

where k is Boltzmann’s constant, T is in degrees Kelvin, η is solution viscosity (~1 cP for H₂O at 20°), and r is the radius of the molecule. Yguerabide et al. listed the τₑ values calculated from Eq. (1) and determined experimentally for a number of proteins of varying molecular weight. The τₑ value of a protein represents only the upper limit of the τₑ of the bound ligand, because the bound ligand is likely to possess some internal rotational freedom.

7 S.-L. Huang and M.-D. Tsai, Biochemistry 21, 951 (1982).
Relaxation in the Extreme Narrowing Limit. In this region transverse and longitudinal relaxations are both monoexponential decays characterized by single relaxation times related to the observed linewidth as follows:

$$\Delta \nu_{1/2} = \frac{1}{\pi T_2} = \frac{1}{\pi T_1} = \frac{3\pi}{10} \frac{2I + 3}{I(I - 1)} \left(1 + \frac{\eta^2}{3}\right) \left(\frac{e^2 q_{zz} Q}{\hbar}\right)^2 \tau_c$$  (2)

The proportionality between $\tau_c$ and linewidth $\Delta \nu_{1/2}$ is evident; slow-moving species produce large linewidths. Equation (2) has been widely used to deduce $\tau_c$ from the observed linewidth in QNMR.

The term $(e^2 q_{zz} Q/\hbar)$ is the quadrupolar coupling constant ($\chi$) that describes the strength of the interaction of the quadrupole moment with its electric environment. This interaction is the physical basis for the quadrupolar relaxation mechanism. $Q$ is the intrinsic quadrupole moment of the nucleus, $e$ is the charge of the electron, and $q_{zz}$ is the largest component of the electric field gradient interacting with $Q$ and vanishes when electric symmetry around the nucleus becomes very high (e.g., $T_d$ or $O_h$ groups).

Table I shows that $Q$ varies considerably from nucleus to nucleus. In the case of $^6$Li and $^7$Li, $\chi^2$ can be so small that other relaxation mechanisms can actually compete, while for high-$Q$ nuclei $\chi^2$ is often so large that peaks become very broad, even for free ligands (unless $q_{zz}$ is very low due to high symmetry).

The asymmetry parameter $\eta$ varies from 0 to 1 and describes the deviation of the electric field gradient from axial symmetry ($\eta = 0$ for axial symmetry). Because even in the rare instance that $\eta = 1$ the term $1 + \eta^2/3$ is only 1.33, the asymmetry parameter can usually be neglected.

Relaxation Outside of Extreme Narrowing. As molecular motion becomes slower and $\omega_0^2 \tau_c^2$ approaches or exceeds 1, Eq. (2) no longer holds. $T_2$ and $T_1$ become nonequivalent: $T_2$ continues to get smaller (with corresponding broader linewidths), while $T_1$ starts to get larger. In the case of $I = 1$, nuclei lineshapes remain Lorentzian and $T_1$ and $T_2$ are described by Eqs. (3) and (4), respectively$^{12,13}$ [which reduce to Eq. (2) when $\omega_0^2 \tau_c^2 \ll 1$]:

$$\frac{1}{T_1} = \frac{3\pi^2}{100} \frac{2I + 3}{I(I - 1)} \chi^2 \left(1 + \frac{\eta^2}{3}\right) \left(\frac{2\tau_c}{1 + \omega_0^2 \tau_c^2} + \frac{8\tau_c}{1 + 4\omega_0^2 \tau_c^2}\right)$$  (3)


\[
\Delta \nu_{1/2} = \frac{1}{\pi T_2} = \frac{3\pi^2}{100 I^2(2I-1)} \times \left( 2I + \frac{3}{4} I^2 \right) \left( 1 + \frac{\eta^2}{3} \right) \\
\times \left( 3\tau_c + \frac{5\tau_c}{1 + \omega_0^2 \tau_c^2} + \frac{2\tau_c}{1 + 4\omega_0^2 \tau_c^2} \right)
\] (4)

For nuclei with \( I > 1 \), Eqs. (3) and (4) are approximately accurate only when \( \omega_0 \tau_c \approx 1.5 \).\(^{14,15}\)

Above \( \omega_0 \tau_c \approx 1.5 \), relaxations of \( I > 1 \) nuclei become multiexponential, with line shapes being the superposition (thus non-Lorentzian) of contributions from each exponential. Data from such species (e.g., large proteins) can be very difficult to analyze. In solids and membranes \( \tau_c \) becomes even larger, and the "quadrupolar splitting" becomes a measurable and useful parameter in QNMR (see Anisotropic Motion).

Because relaxation is so intimately associated with interpretability and observability, it is often helpful to predict likely behavior using estimated \( \tau_c \), \( \chi \), and \( \omega_0 \) before running difficult and potentially fruitless experiments. Table I lists typical spectral behavior for different nuclei in extreme-narrowing conditions having the same \( \tau_c \).

**Exchange**

Direct observation of a quadrupolar species fully bound to an enzyme is often difficult. In such cases, the only way of getting NMR information about the bound species is to observe the spectra of the free component involved in exchange with the bound species. Some strategies for extracting the needed data are discussed later in this chapter (see Selective Examples of Applications).

**Anisotropic Motion**

As \( \tau_c \) approaches or exceeds \( 1/\chi \), NMR begins to detect orientational anisotropy and peaks broaden and eventually split. Extreme examples are found in the NMR of solids and membranes when QNMR yields multiple resonances separated by "quadrupolar splittings."\(^{16}\) For liquid samples of protein-ligand complexes \( \tau_c \) is usually not large enough to induce quadrupolar splitting (if \( \chi = 10^6-10^8 \) Hz, \( \tau_c \) would have to be at least \( 10^{-6}-10^{-5} \) sec). However, true motional isotropy rarely exists at a local

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level in macromolecular systems, and the $\tau_c$ deduced from Eqs. (2)–(4) should be regarded as "effective" correlation times resulting from a combination of slower, generally isotropic overall tumbling and faster, anisotropic internal motions.

*Determining of $\tau_c$ and/or $\chi$*

Correlation times and $\chi$ provide information of interest to biochemists and chemists. Because $\omega_0$ and $I$ are always known and $\eta$ is usually negligible, relaxation times are straightforward functions of $\tau_c$ and $\chi$ according to Eqs. (2)–(4). If one of these values is known, the other can be determined from the relaxation time or linewidth using algebra (in extreme narrowing) or the Newton-Raphson (or some other) numerical method.\(^{17}\)

The correlation time is strictly a motional parameter and is affected by chemistry, solvation, temperature, etc., only to the extent that these affect molecular motion. On the other hand, $\chi$ is dependent on chemical factors. When the electrical environment of the quadrupolar nuclei is defined by stable covalent bonds, $\chi$ is likely to be constant in a variety of environments provided that no change in bonding occurs. Thus, $\chi$ for a C–D species is likely to be nearly constant free and bound to an enzyme. On the other hand, $\chi$ for $^{39}$K\(^+\) is likely to change on binding to an anion or a protein.

In \(^2\)H NMR studies $\chi$ can be estimated by chemical analogy with similar compounds for which $\chi$ has been determined by independent techniques,\(^{6,13,18,19}\) and can be used to determine changes in $\tau_c$ upon binding. In observing ligands bound to proteins, the simultaneous solution of $\tau_c$ and $\chi$ based on Eqs. (3) and (4) can be accomplished outside of extreme narrowing by measuring both $T_1$ and $T_2$ or by measuring either value at two different magnetic field strengths.

**Experimental Techniques**

*Special Problems in QNMR*

Experimental difficulties in QNMR are sometimes different from those commonly encountered in other HR NMR or solid-state NMR. In this section the nature of some of these problems is described. The discussion includes hardware and software techniques used to minimize these and other problems.


Acoustic Ringing. Acoustic ringing refers to the acoustic waves generated by electromagnetic interaction of the radiofrequency pulse with probe materials. These waves result in spurious rolling in the spectral baseline and can also be manifested as a sharp spike at the beginning of a free induction decay (FID). This phenomenon is present in all NMR experiments but is typically a serious problem in QNMR. In other HR NMR, acoustic ringing can be minimized by employing a sufficiently long preacquisition delay (DE), but in QNMR the length of this DE may cause other problems (see later). Furthermore, in usual HR NMR, signals are often sharp and are easily distinguished from a rolling baseline, and the spectral width is often so small that the entire “ring” is not observed. In QNMR large spectral widths make ringing very apparent and broad peaks may be difficult to differentiate from the baseline rolling, as illustrated previously. Acoustic ringing is also more severe at relatively low observation frequencies, a fact of some importance because many quadrupolar nuclei have low Larmor frequencies due to low magnetogyric ratios (γ). Gerothanasssis recently published an excellent review on the phenomenon of acoustic ringing and how to deal with it.

Need and Consequences of “Hard” Pulsing. Another consequence of the small γ possessed by many nuclei is that, under a given condition, a relatively long pulselength is needed. A long 90° pulse may present difficulties in complex pulse sequences and limits the breadth of spectral excitation. This latter effect is in conflict with the frequent need in QNMR to cover a large spectral range including broad signals. In order to reduce the length of the 90° pulse, higher transmitter power (to produce a “hard” pulse) must be utilized, which worsens acoustic ringing.

Preacquisition Delay (Deadtime). Many spectrometers automatically set DE equal to the dwell time to facilitate phasing. In pulsed FT NMR, the dwell time (DW) is the inverse of the sampling rate that is determined by the spectral width (SW), i.e., DW = 1/(2SW). In a typical 1H NMR experiment at 300 MHz, SW = 3000 Hz and DW = 167 μsec. Because T₁ and T₂ are on the order of 0.1–10 sec (for protons), the data lost during DE are usually negligible. However, in QNMR there are a number of conflicting problems. (1) A large SW is generally needed, resulting in smaller DW (and thus a smaller default value of DE). (2) Acoustic ringing is often more serious (as described previously), which would require a longer DE to minimize. (3) T₁ and T₂ are often so short (e.g., <1 msec) that a significant portion of their signal can be lost during DE. This results in a loss of S/N,
distortion of peaks, and problems in intensity/integration measurement. As a result, the $T_1$ data should be treated with caution, and the integrated signals with different linewidths cannot be compared.

**Hardware Considerations**

**Magnet and Console.** Large superconducting magnets have the advantages of superior sensitivity and stability and result in higher Larmor frequencies. However, because of the broad signals usually encountered in QNMR, resolution and field stability requirements are often less stringent than in typical HR work.

Rapid pulsing and data sampling of a great number of scans require a high dynamic range, which puts high demands on the electronic and computational components of the spectrometer system.

**The Probe.** The performance of the probe is a key variable in QNMR. In QNMR, as for solid-state NMR, high-power probes are frequently used. These probes generally utilize a single horizontal solenoid transmitter/receiver coil which is inherently more sensitive than Helmholz saddle coils. These coils produce a short 90° pulse compared to saddle coils and also handle higher pulse power more efficiently. High-power probes are also built with materials chosen to reduce acoustic ringing (a negative factor in terms of sensitivity).

The disadvantages of high-power probes lie in the difficulty of shimming and achieving high resolution. Furthermore, they often possess no locking or spinning capability. Fortunately, these factors are usually less important in QNMR. Sample change is also laborious because the entire probe has to be removed from the magnet.

High-resolution probes are often quite suitable when linewidths are not too broad, and these probes are a necessity when accurate linewidths are needed in the high-resolution region.

**Software Considerations**

**Pulse Sequences.** When initiating a study, a standard single-pulse experiment can be attempted to evaluate the system at hand (sensitivity, baseline distortion, linewidth, etc.). At this point, DE could be manually adjusted to either reduce acoustic ringing (larger DE) or to increase S/N (smaller DE). Alternatively, one can employ a pulse sequence which will reduce acoustic ringing. Many such pulse sequences have recently become available and have been reviewed by Gerothanassis.

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experience the RIDE (ring-down elimination) sequence\textsuperscript{21,23} worked effectively.

Some pulse sequences involving use of spin echoes have also been developed for QNMR.\textsuperscript{21} When $T_2$ is very short but $T_1$ is much longer, spin-echo techniques should allow the true spectra to be observed and also can increase $S/N$.\textsuperscript{21,24,25} However, when $T_1$ and $T_2$ are both very short (0–100 $\mu$sec), acquisition becomes very difficult due (among other reasons) to the significant relaxation that occurs during the actual pulse sequence. To our knowledge, there is no way around this problem in pulsed FT NMR and in this case CW NMR can actually be advantageous if sensitivity is not a big problem.\textsuperscript{21,26} (CW NMR also eliminates the problems associated with generating adequate pulse power to cover a very broad spectral width.)

Although the sampling rate could be as high as 100 scans per sec, it should be kept in mind that the recycling time needs to be $\geq 3 T_2$ to avoid truncation of the FID (which will cause a regular series of spikes at the bottom of the peak),\textsuperscript{27} and needs to be $\geq 5 T_2$ when the quantitative intensity is of interest.\textsuperscript{28} Becker \textit{et al.}\textsuperscript{29} presented a lucid description of the optimization of pulse repetition times as well as pulse angles.

$T_1/T_2$ Determination. The relative merits of the various $T_1/T_2$ determination methods have been discussed extensively.\textsuperscript{26} These methods can be integrated into the sequences used to minimize acoustic ringing. Data taken for very short $T_1/T_2$ should be interpreted with caution, because these data may deviate from true values due to significant relaxation during pulsing and DE.

Selective Examples of Applications

\textit{Metal Ion Binding to Proteins}

As shown in Table I, many biologically relevant metal ions are quadrupolar nuclei. QNMR has been extensively utilized to study binding of these ions to various proteins, as described by several reviews.\textsuperscript{2,3,23,30} The

most successful study in this category is binding of \( \text{Ca}^{2+} \) to calmodulin (CaM), which is used to illustrate the procedures, problems, and interpretations of the actual experiments.

CaM (\( M_r \), 16,800) has four \( \text{Ca}^{2+} \)-binding sites, numbered I–IV starting from the N-terminus. Various studies have suggested that the four sites can be grouped into two high-affinity sites (III and IV, \( K_a = 10^6–10^7 \, M^{-1} \)) and two lower affinity sites (I and II, \( K_a = 10^2–10^5 \, M^{-1} \)).\(^{31-34}\) The \( ^{45}\text{Ca} \) NMR signals of \( \text{Ca}^{2+} \) fully bound to the high-affinity sites have been observed at 2.8 mM CaM and 4.8 mM \( \text{Ca}^{2+} \), with \( \Delta \nu_{1/2} = 770 \pm 50 \, \text{Hz} \) (\( T_2 = 0.42 \, \text{msec} \)). An inversion–recovery experiment determined \( T_1 = 0.86 \, \text{msec} \). The nonequivalence of \( T_1 \) and \( T_2 \) suggests that the system is not in the extreme narrowing limit and Eq. (2) cannot be applied. Because CaM is a small protein and satisfies \( \omega_0 \tau_e \leq 1.5 \), even if binding is totally rigid, Eqs. (3) and (4) can be used to solve for \( \chi \) (1.1 MHz) and \( \tau_e \) (8.2 nsec) simultaneously from \( T_1 \) and \( T_2 \).\(^{15}\)

Additional information concerning binding of \( \text{Ca}^{2+} \) and \( \text{Mg}^{2+} \) to CaM have been obtained by titration and temperature-dependence experiments, which are illustrated using \( ^{25}\text{Mg} \) NMR.\(^{35}\) Because the affinity of \( \text{Mg}^{2+} \) to CaM is much weaker than that of \( \text{Ca}^{2+} \), it is not possible to observe fully bound \( \text{Mg}^{2+} \). Even if a large excess of CaM can be used to fully bind \( \text{Mg}^{2+} \), the larger quadrupole moment of \( \text{Mg}^{2+} \) could result in a \( \Delta \nu_{1/2} \) too large to be measured at the concentration used. Figure 1 shows \( ^{25}\text{Mg} \) NMR spectra at different ratios of CaM:Ca\( _{2+} \) (which represents CaM when sites III and IV are saturated with \( \text{Ca}^{2+} \) and sites I and II are open for binding \( \text{Mg}^{2+} \)). The plots of \( \Delta \nu_{1/2} \) versus \( \text{[Mg}^{2+}] / \text{(CaM}\cdot\text{Ca}_{2+}] \) are shown in Fig. 2A. All spectra are Lorentzian. as exemplified in spectra I and J (fittings of A and H, respectively). The Lorentzian shape and the successive changes in linewidths rule out the possibility that the observed signal is due to overlap of the signals of free and bound \( \text{Mg}^{2+} \) in \textit{very slow exchange} relative to the NMR time scale (Case A in Table II). There are, however, three other possible interpretations. (1) The observed signal is due to free \( \text{Mg}^{2+} \), which is in \textit{slow exchange} with bound \( \text{Mg}^{2+} \) [Case B(a) in Table II]. The signal of bound \( \text{Mg}^{2+} \) is either too broad to be detected or is well separated from the observed signal of free \( \text{Mg}^{2+} \) so that the signal


remains Lorentzian. The broadening is caused by chemical exchange as shown in Table II. (2) The observed signal is due to the fast exchange average of free and bound Mg\textsuperscript{2+} [Case C(a) in Table II] and the successive broadening is mainly due to the rapid relaxation (large $\Delta\nu_{1/2}$) of bound Mg\textsuperscript{2+}. (3) The system is in the intermediate exchange region (Case D in Table II), and the broadening is caused by chemical exchange and by averaging with bound Mg\textsuperscript{2+}.

A temperature-dependence study can differentiate the above situa-
Fig. 2. Titration curve (A) and temperature curve (B) of Mg$^{2+}$ binding to CaM·Ca$^{2+}$. The titration curve was obtained at 25°. The temperature curve was obtained at [Mg$^{2+}$]/[CaM·Ca$^{2+}$] = 5.24. Circles are experimental points and solid curves are obtained by connecting the calculated points from the iterative fitting. The $\Delta \nu$ values shown include a line broadening of 100 Hz. (Reproduced from Tsai et al. with permission.)

...tions. In the case of slow exchange, increasing temperature will increase the exchange rate (i.e., increase $\tau_{\alpha}$) and cause an increase in the observed $\Delta \nu$ if the increase due to exchange rate is large enough to outweigh the decrease in the intrinsic linewidth (due to increased molecular motion). In the case of fast exchange, the intrinsic linewidths of both free and bound Mg$^{2+}$ decrease due to decreasing $\tau_{\alpha}$, which will result in a decrease in the observed $\Delta \nu_1 \cdot \Delta \nu_2$ upon increasing temperature. Figure 2B shows that the Mg$^{2+}$/CaM·Ca$^{2+}$ system is in slow exchange at 25° and gets into the intermediate region at 30°-50°.

When such a complete set of data can be obtained, the data can be analyzed by 'total line shape analysis.' The details of such analysis have
TABLE II
EFFECTS OF EXCHANGE ON TITRATION EXPERIMENTS AND TEMPERATURE DEPENDENCE OF \( \Delta \nu_{12} \)^a

<table>
<thead>
<tr>
<th>Cases</th>
<th>Titration experiments</th>
<th>Temperature dependence</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Very slow exchange</td>
<td>(a) Bound species not detectable: one Lorentzian signal with successive decrease in intensity, but with constant ( \Delta \nu_{12} )</td>
<td>( \Delta \nu_{12} ) decreases with increasing temperature due to decreasing ( \tau_e )</td>
</tr>
<tr>
<td></td>
<td>(b) Bound species detectable: two signals, or one apparently non-Lorentzian signal, if the two signals overlap</td>
<td></td>
</tr>
<tr>
<td>( \frac{1}{\tau_{ex}} \ll \frac{1}{(T_{1,2})_b} )</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B. Slow exchange</td>
<td>(a) Bound species not detectable: one Lorentzian signal, successively broadened</td>
<td>( \Delta \nu_{12} ) increases with increasing temperature due to decreasing ( \tau_{ex} )</td>
</tr>
<tr>
<td></td>
<td>( \frac{1}{\tau_{ex}} &lt; \frac{1}{(T_{1,2})_b} )</td>
<td></td>
</tr>
<tr>
<td></td>
<td>( \frac{1}{T_{1,2}} = \frac{1}{(T_{1,2})<em>b} + \frac{1}{\tau</em>{ex}} \frac{P_b}{P_f} ) (6)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>or ( \Delta \nu_{12} = \Delta \nu_1 + \frac{1}{\pi \tau_{ex}} \frac{P_b}{P_f} )</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(b) Bound species detectable: two signals, each broadened by exchange, or one apparently non-Lorentzian signal, if the two signals overlap</td>
<td></td>
</tr>
<tr>
<td>C. Fast exchange</td>
<td>(a) Exchange narrowing (( 1/\tau_{ex} \gg \Delta \omega )): one signal.</td>
<td>Same as A</td>
</tr>
<tr>
<td></td>
<td>( \frac{1}{\tau_{ex}} \gg \frac{1}{(T_{1,2})_b} )</td>
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<td></td>
<td>( \frac{1}{T_{1,2}} = \frac{P_f}{(T_{1,2})<em>b} + \frac{P_b}{(T</em>{1,2})_b} ) (7)</td>
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<tr>
<td></td>
<td>(b) Not exchange narrowing (( 1/\tau_{ex} \ll \Delta \omega )): two signals separated by ( \Delta \omega )</td>
<td></td>
</tr>
<tr>
<td>D. Intermediate exchange</td>
<td>In between B and C</td>
<td>Irregular or insensitive</td>
</tr>
<tr>
<td></td>
<td>( \frac{1}{\tau_{ex}} \sim \frac{1}{(T_{1,2})_b} )</td>
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</tr>
</tbody>
</table>

^a \( 1/\tau_{ex} \) is the ligand–protein on/off exchange rate (\( k_{ex} \)), \( b \) and \( f \) designate free and bound states, respectively. \( \Delta \omega \) represents the separation in resonance frequencies of free and bound ligands, and \( P_f \) and \( P_b \) represent fractions of ligand in free and bound states, respectively. Only one ligand site per molecule is assumed as is the relationship \( 1/(T_{1,2})_b = 1/(T_{1,2})_f - 1/(T_{1,2})_l \).
been described elsewhere\textsuperscript{36} and are beyond the scope of this article. The curves in Fig. 2 resulted from iterative fittings, which yielded the following information: $K_a = 2000 \ M^{-1}$ (assuming two equivalent sites), $k_{\text{off}}$ ($= k_{\text{on}}$) = 2300 sec$^{-1}$, and $\Delta \nu_{1/2} = 3.5$ kHz for bound Mg$^{2+}$. In this case, $\tau_c$ and $\chi$ cannot be obtained simultaneously, but if $\tau_c$ of Mg$^{2+}$ is assumed to be the same as $\tau_c$ of Ca$^{2+}$ when bound to CaM, $\chi = 1.6$ MHz can be obtained. The result unequivocally establishes that Mg$^{2+}$ binds to sites III and IV of CaM, as described in detail by Tsai \textit{et al.}\textsuperscript{35}

In many other studies, complete line shape analysis by the above method is not possible because the complete temperature range may not be obtainable, and the protein available for experiments may be limited in quantity and stability. Even if the protein is stable enough for a variable temperature study, the system may remain in slow (or fast) exchange throughout the entire region. The titration data of these systems are frequently analyzed by the "Swift–Connick" equation\textsuperscript{37} [Eq. (5)], which is a combination of Eqs. (6) and (7) described in Table II when $P_I \approx 1$ and $P_b \ll 1$:

$$\frac{1}{T_{1,2}} = \frac{1}{(T_{1,2})_f} + \frac{P_b}{\tau_{\text{ex}} + (T_{1,2})_b}$$  \hspace{1cm} (5)

Equation (5) is applicable for slow, intermediate, and fast exchange regions. It allows a straightforward measurement of $1/((\tau_{\text{ex}} + T_2)$ from the slope of the plot of $(\pi \Delta \nu_{1/2})$ versus $P_b$. Because $P_b \ll 1$ (e.g., 0–5%), it can often be assumed that $P_b = [\text{protein}]/[\text{ligand}]_{\text{total}}$. The main problem in this approach is to determine whether the system is in slow exchange (slope $\approx 1/\tau_{\text{ex}}$ because $\tau_{\text{ex}} \gg T_2$), fast exchange (slope $\approx \pi \Delta \nu_b$ because $T_2 \gg \tau_{\text{ex}}$), or intermediate exchange (slope is not directly interpretable).

\textbf{Halide Ion Binding to Proteins}

The technical problems for halide ion binding are very similar to those of metal ion binding to proteins, but the systems amenable to such studies are more limited. Well-studied proteins include, among others, hemoglobin\textsuperscript{38} and Cl$^{-}$ transport proteins.\textsuperscript{39,40} One advantage of $^{35}$Cl NMR is that fast exchange can be established by the following criterion:\textsuperscript{3}

\textsuperscript{38} E. Chiancone, J.-E. Norne, and S. Forsén, this series, Vol. 76, p. 552.
\[
\frac{(\Delta \nu - \Delta \nu_I)}{(\Delta \nu - \Delta \nu_{I'})} \text{ for } ^{35}\text{Cl} = \left(\frac{\chi \text{ for } ^{35}\text{Cl}}{\chi \text{ for } ^{37}\text{Cl}}\right)^2 = 1.60
\]

where \(\Delta \nu\) and \(\Delta \nu_I\) represent the observed linewidth and the linewidth of free \(\text{Cl}^-\), respectively. In \(\text{Cl}^-\) binding studies, fast exchange is observed in most cases, and the detailed \(^{35}\text{Cl}\) NMR analysis under various conditions (e.g., pH dependence and effect of other ligands) has been used to deduce the number and the environment of \(\text{Cl}^-\) binding sites.

**Substrate Binding to Enzymes**

The most desirable information in this category is the "internal motions of bound substrates." It is widely accepted that enzyme-bound substrates possess a certain extent of internal rotational freedom relative to the enzyme. However, it is difficult to describe the dynamics of bound substrates quantitatively, because a single measurement can yield only the *effective* \(\tau_e\), which may include contributions from several motional components. Indeed, more studies have been done on the dynamics of local segments of proteins (e.g., London [18] and Keniry [19], Vol. 176, this series) than that of bound substrates. The latter studies are often complicated by exchange processes as described previously herein.

In enzyme–substrate binding studies, \(^2\text{H} NMR\) has been used to show that the adenine portion of \(\text{NAD}^-\) bound to lactate dehydrogenase has a longer \(\tau_e\) than the \(\tau_e\) of the pyridine ring,\(^{41}\) and that when linoleic acid is bound to lipoxygenase, the internal motions of the substrate increase at positions away from the carboxylic acid group.\(^{42}\) We describe in detail a binding study of adenylate kinase (AK) using \(^2\text{H} NMR.\(^{43}\)

Chicken muscle AK was titrated with adenylyl(\(\beta,\gamma\)-methylene) diphosphonate (AMPPCP) deuterated on the phosphonate chain and on the adenine ring and followed by measuring the \(^2\text{H} NMR\) linewidth of the single peak that results from the average of the bound and free AMPPCP. Plots of \(\Delta \nu_{\gamma}\) versus \([\text{AMPPCP}]_{\text{bound}}/[\text{AMPPCP}]_{\text{total}}\) were linear, as shown in Fig. 3. The line shapes of the observed peaks were usually Lorentzian, and limited \(T_1\) inversion recovery data taken were always monoexponential. Thus, the data meet "fast-exchange" criteria and the linewidths of the fully bound species can be determined from linear extrapolations to the fraction bound equal to 1.\(^{44}\) The effective \(\tau_e\) values were then calcu-


FIG. 3. High-resolution $^3$H NMR (46.1 MHz) linewidths ($\Delta \nu_{1/2}$ in hertz) of $^3$H-labeled AMPPCP and MgAMPPCP as a function of fractions bound to AK. The experiments were carried out by titrating 1–2 mM AK with small aliquots of AMPPCP or MgAMPPCP ([Mg$^{2+}$/AMPPCP] = 4). Sample conditions: pH 7.0 in $^2$H-depleted H$_2$O with 45 mM HEPES–K$^+$ or imidazole–HCl, 117 mM KCl, 1–8 mM dithiothreitol, and 0.1 mM EDTA in a 10-mm NMR tube (starting volume of 1.75 ml). Spectral conditions: the probe temperature was 10°C; the digital resolution varied from 1 Hz/point for narrow signals to 15 Hz/point for very broad signals; the 90° pulsewidth was 12 $\mu$s. The CYCLOPS pulse sequence was used. The reported $\Delta \nu_{1/2}$ have been corrected for line broadening (1–10 Hz). The fraction of AMPPCP bound to AK was calculated using $K_d$ values of 210 $\mu$M for AMPPCP and 190 $\mu$M for MgAMPPCP. (○) [8-$^3$H]AMPPCP, (○) MgAMPPCD$_2$P, and (●) AMPPCD$_2$P. (From Sanders and Tsai.\textsuperscript{a})

lated from Eq. (4) using values of $\chi$ from structurally related compounds. The contributions of two-bond $^2$H–$^{15}$N and $^2$H–$^{31}$P scalar and dipolar couplings to the observed $\Delta \nu_{1/2}$ were insignificant. The validity of deducing $\tau_c$ from linewidths was further supported by limited $T_1$ experiments.

The $\tau_c$ values obtained indicate that the adenine ring of bound AMPPCP is motionally rigid ($\tau_c = 27$ nsec) and approaches the overall $\tau_c$ of AK. The $\beta$–$\gamma$ region of the phosphonate chain ($\tau_c = 7$ nsec) possesses considerable local mobility, consistent with more qualitative $^{17}$O NMR
This motional freedom is reduced upon binding of Mg$^{2+}$ ($\tau_c = 16$ nsec). The implication of these dynamic properties on the catalytic mechanisms of AK has been discussed.\textsuperscript{43}