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Rate Constants Determined by Nuclear Magnetic Resonance

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Fast kinetic methods are used to measure reactions that take place in less time than required to mix the reagents manually and to measure the reaction by usual methods, like UV–visible spectrophotometry and fluorescence. The best known of them are rapid-mixing and relaxation methods, which are used for reactions with half-times in the millisecond and microsecond ranges, respectively. The picosecond range is usually measured with electrical field and ultrasonic waves (A. Cornish-Bowden, 1976, Principles of Enzyme Kinetics, pp. 164–167, Butterworths, London). Normally these very fast rates occur when a ligand binds to or dissociates from a protein. When the binding is mediated only by the diffusion, the lower limit of the association rate constant (k_{on}) should not exceed the value of a diffusion-controlled reaction (around 10^{10} M^{-1} s^{-1}). Therefore, the values most frequently found for these rate constants, for example, in the association of a substrate with an enzyme, are in the range 10^6 to 10^9 M^{-1} s^{-1} (M. Eigen and G. G. Hammes, 1963, *Adv. Enzymol.* **25,** 1–38). The values for the dissociation rate constants (k_{off}) for these reactions, which depend on the equilibrium constant for the enzyme–substrate complex interaction, are in the range 10^1 to 10^5 s^{-1}, most often between 10^3 and 10^4 s^{-1} (A. Fersht, 1999, Structure and Mechanism in Protein Science, pp. 164–165, Freeman, New York). If the equilibrium constant is known, and the value of k_{off} is determined by nuclear magnetic resonance (NMR), as described in this chapter, the value of k_{on} can be calculated; this should not exceed the value of diffusion rate in the media in which the reaction is performed.

Key Words: nuclear magnetic resonance; exchange; kinetic; rate constants; nuclear relaxation rates.

This article describes the application of NMR to measure exchange of a ligand (dead-end inhibitor or substrate) between two populations by following a specified nucleus (^{1}H, ^{19}F, or ^{31}P). The measurement of rate constants is discussed in terms of the modification of the resonance frequency, chemical shift, or relaxation rates when the ligand forms a binary complex with a protein at equilibrium. The experimental procedure is given with some detail only when the relaxation rates constants are measured. A short introduction is provided to the principles of nuclear magnetic resonance required for understanding the base of the measurement.

NMR spectra are characterized by chemical shifts (δ), spin–spin splitting or the coupling constant (J) and the relaxation rates of the nuclei, and the spin lattice (T_1) and the spin–spin (T_2) relaxation times. Bloch (1) derived the equations to define the motion of the magnetic moment (μ) or magnetization M in the samples. The motion in the direction of the external magnetic field B_0 is designated dM_z/dt. In the plane perpendicular to B_0, the xy plane, the motion of the magnetization vector is designated dM_x/dt. Magnetization in the xy 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 B_0, a torque is placed on the magnetic moment M by B_0 to change the angular momentum P:

$$\frac{dP}{dt} = -B_0 \cdot M. \qquad [1]$$

As the spin angular momentum is related to the magnetic moment by the magnetogyric ratio γ, it follows that

$$M = \gamma P; \qquad [2]$$

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then

$$\frac{dM}{dt} = -\gamma B_0 M. \qquad [3]$$

This expression describes the motion of the magnetic moment or magnetization about the z axis, defined as the direction of the B_0 field. At equilibrium the nucleus has a magnetization of M_0. 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 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 exchange of energy between the spin systems, which depends on dipolar effects. The relaxation can be regarded as being due to fluctuating magnetic fields of surrounding magnetic dipoles, each fluctuation being characterized by a correlation time τ_c. As these relaxation phenomena depend on time their study can lead to kinetic information such as molecular motion. More detailed treatments are available (2–5).

When a nucleus whose resonance is being observed exists in two environments the observed spectrum depends on the rate of its passage between the two environments. Observation of two resonance peaks for such a nucleus implies slow exchange between the two environments, whereas a single peak implies fast exchange; intermediate cases can also be found. From an NMR point of view, Jardetzky and Roberts (4) define a time scale for the nuclear precession frequency that is related to the exchange rate of the nuclei between two or more environments; this is affected by conformational flexibility, chemical reactions, formation of intermolecular complexes, and other events. Thus three regions of exchange can be defined–fast, intermediate, and slow–that depend on the NMR parameter used to measure the rate of exchange. If the chemical shift is used and the nucleus exchanges between two environments with shifts δ_A and δ_B, the exchange is considered slow if $k \ll |\delta_A - \delta_B|$, where k is the rate of exchange, intermediate if $k \approx |\delta_A - \delta_B|$, and fast if $k \gg |\delta_A - \delta_B|$. The values of $|\delta_A - \delta_B|$ for intermediate exchange are in the approximate range 10 to 1000 ms when the nucleus measured is ^{1}H. Slow, intermediate, and fast exchange can be similarly defined on the coupling-constant time scale according to whether k is greater than, approximately equal to, or less than $|J_A - J_B|$, respectively. In this case intermediate exchange is in the ranges 0.1 to 1 s for ^{1}H and 1 to 100 ms for ^{31}P and ^{19}F. On the relaxation time scale, slow, intermediate, and fast exchange is defined according to whether k is greater than, approximately equal to, or less than or $|1/T_{1A} - 1/T_{1B}|$ or $|1/T_{2A} - 1/T_{2B}|$. Intermediate exchange for the difference in relaxation rates has an approximate range 0.1 to 100 ms. As mentioned above, dissociation rate constants for enzyme–substrate complexes are usually between 10^3 and 10^4 s^{-1}, the range for intermediate exchange.

The NMR theory behind the ligand–enzyme interaction has been very well developed by Redfield (6) on the basis of the view that enzymes catalyze nuclear spin relaxation and related processes. Theoretical methods have been developed for calculating the rate of exchange of ligand bound to a macromolecule that induces a chemical shift with respect to the ligand free in solution. The procedure is to fit a theoretical curve to the experimental spectrum measured at a specific temperature, changing the values of the residence time (τ) of the ligand (5). This method is not discussed here.

To measure enzyme–ligand interactions we should consider only the resonance spectra of the ligand, the general term embracing substrates, modifiers, inhibitors, and activators, including metal ions. The appropriate studies depend on the enzyme of interest. There are two types of experiment one can perform. In some cases the interaction of a ligand with an enzyme results in formation of an enzyme–ligand complex, resulting in partial immobilization of the ligand. Decreased mobility of a group (e.g., 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 the group that modulates the dipolar interactions among the methyl protons and results in an increase in $1/T_2$ and $1/T_1$. Of these two parameters, $1/T_2$, estimated from the linewidth of the resonances, is the easier to measure. If the effect on $1/T_2$ is sufficiently large and the ligand is in the domain of fast exchange (the lifetime, τ_m, of the ligand in the E–L complex is short compared with the relaxation time of the nucleus, $T_{2,b}$, in the E–L complex), an average linewidth ($1/T_{2,obs}$) for the bound ligand ($1/T_{2,b}$) and free ligand ($1/T_{2,0}$) is observed:

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

Here the total ligand concentration L_t is the sum of the concentrations L_b and L_f of the bound and free ligand, respectively. 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 allows $1/T_{2,b}$ to be calculated. Values for $1/T_1$ can be handled similarly if $1/T_{1,obs}$ is measured.

Another approach to the study of ligand binding to enzymes is to apply paramagnetic probes to the enzyme. This 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 used to take advantage of these large dipolar effects. Many stable nitroxides are commercially available that can potentially be covalently attached to the enzyme. These include derivatives of iodoacetate, *N*-ethylmaleimide, and diisopropyl fluorophosphate that can be used to label reactive groups such as cysteine, histidine, lysine, or reactive serine (7). Selectivity of labeling and choice of amino acid residue 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 with labeled enzyme.

Alternative paramagnetic species that can be used are metal ions, which may either bind to the enzyme directly or form a metal–substrate complex that binds to the enzyme. Some of the ions that can be used or substituted for the "physiological" cation are Mn^{2+}, Fe^{2+}, Co^{2+}, Cu^{2+}, Gd^{3+}, and Cr^{3+}. If the enzyme being studied allows a choice of cations there are distinct advantages to using certain of these cations, particularly Mn^{2+}, as will be shown. Determination of the stoichiometry of the paramagnetic center is necessary. 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 with one of a variety of equilibrium techniques such as equilibrium dialysis, gel permeation, and ultrafiltration. The cation Mn^{2+} 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. On ligand binding (the term *ligand* here 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^{2+}. The remaining signal is due to the free Mn^{2+} and the intensity of the spectrum is directly proportional to the concentration of free Mn^{2+} (8). The Scatchard plot for the binding of Mn^{2+} to GTPγF is shown in Fig. 1. The straight line shows one class of binding site with a stoichiometry of one molecule of cation bound per molecule of nucleotide and a dissociation constant of 1.64×10^{-4} M. Therefore, proper binding studies will lead to determination of the dissociation constant for the label and its stoichiometry per enzyme or enzyme active site.

In most cases the metal ion used is the physiologically important activator for 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^{3+}) may serve as activators in a few cases or as inactive analogs that are competitive with the physiologically relevant cation (9). The lanthanide metals, despite the fact that they are most commonly trivalent, have *f*-shell electrons that give nearly all of them interesting spectroscopic properties. In NMR the physical properties of Gd^{3+} usually make it the most useful. The Cr^{3+} cation, which forms exchange inert ligand–metal complexes, can also be used as a probe. This metal in the form of Cr^{3+}–nucleotide complexes has found use both as a kinetic probe and as an NMR probe (10), the metal nucleotide complex being an analog of Mg^{2+}–nucleotide or Ca^{2+}–nucleotide complexes that serve as substrates.

The paramagnetic probes, particularly nitroxides, Mn^{2+}, Gd^{3+}, and Cr^{3+}, can have a substantial effect on the longitudinal and transverse relaxation rates of the nuclei of the ligands that are in close proximity to the paramagnetic center. Such probes may be exceptionally useful for studying enzyme active sites by chemical modification. 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^{2+} can determine if there is any alteration in

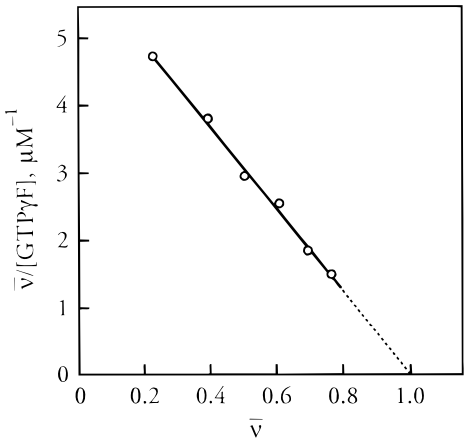

FIG. 1. Scatchard plot for the binding of Mn^{2+} to GTPγF. Binding of Mn^{2+} to the nucleotide was measured by ESR in a Varian E-line spectrometer equipped with a Varian E-257 variable accessory, under the following conditions: time constant 0.25 s, receiver gain 1.6×10^3, microwave power 10 mW, modulation frequency 100 kHz, microwave frequency 9.515 GHz. Solutions containing 50 mM 4-morpholineethanesulfonic acid (MES), pH 7.0, 3.4 M glycerol, and 0.1 mM $MnCl_2$ were tritrated with GTPγF at 25°C. The binding parameters were $K_d = 1.64 \times 10^{-4}$ M and $n = 1.0$.

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 with what is known about the structure and dynamics of ligand 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 or nuclei in question must first be quantified. 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 with an enzyme–metal complex is to be investigated the ^{31}P nuclei of ATP would probably be 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 γ-phosphate of ATP or GTP to give a competitive inhibitor with respect to the nonfluorinated nucleotide (11).

To quantify 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 measurement of the nuclei in the presence of enzyme but no added metal, a diamagnetic metal (Mg^{2+}, Zn^{2+}, or Ca^{2+}), or a reduced nitroxide label. Addition of the paramagnetic species is made either by 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 on 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 ($1/T_{1,\mathrm{obs}}$, $1/T_{2,\mathrm{obs}}$) is a function of the diamagnetic relaxation rate and the paramagnetic relaxation rate:

$$\frac{1}{T_{1,\mathrm{obs}}} = \frac{1}{T_{1\mathrm{p}}} + \frac{1}{T_{1,0}}, \qquad [5]$$

$$\frac{1}{T_{2,\mathrm{obs}}} = \frac{1}{T_{2\mathrm{p}}} + \frac{1}{T_{2,0}}. \qquad [6]$$

The paramagnetic effect is measured as a function of the concentration of paramagnetic species. If possible, a plot of $1/T_{1,2\mathrm{obs}}$ against the concentration of paramagnetic species can be made to show expected linearity in the relaxation rate. The rate can be normalized for the concentrations L and p of the ligand and the paramagnetic species respectively by the term $f = p/L$. The normalized paramagnetic effects to the relaxation rates are related to the number of ligands (q) that bind to the specific site(s) in the vicinity of the paramagnetic probe, the relaxation time of the nucleus at this site ($T_{1,2\mathrm{M}}$), and the lifetime of the nucleus of this site. In some cases with paramagnetic ions a chemical shift change, $\Delta\omega$, is also observed that affects T_2 relaxation.

These effects have been described by Swift and Connick (12) and by Luz and Meiboom (13):

$$\frac{1}{fT_{1\mathrm{p}}} = \frac{q}{T_{1\mathrm{M}} + \tau_{\mathrm{m}}}, \qquad [7]$$

$$\frac{1}{fT_{2\mathrm{p}}} = \frac{q}{\tau_{\mathrm{m}}}\left[\frac{(1/T_{2\mathrm{M}})(1/T_{2\mathrm{M}} + 1/\tau_{\mathrm{m}}) + \Delta\omega^2}{(1/T_{2\mathrm{M}} + 1/\tau_{\mathrm{m}})^2 + \Delta\omega^2}\right]. \qquad [8]$$

If chemical shift changes are negligible or absent, Eq. [8] reduces to

$$\frac{1}{fT_{2\mathrm{p}}} = \frac{q}{T_{2\mathrm{M}} + \tau_{\mathrm{m}}}. \qquad [9]$$

In most such cases $\Delta\omega \approx 0$, and Eq. [9] can be used. The enzyme (enzyme label) should be corrected for saturation by the ligand. If the dissociation constant for the formation of the E–ligand complex is such that the complex is only partially saturated, then f = (E-label–ligand)/(ligand). When the label is a metal ion, 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 measuring $1/fT_{1,2\mathrm{p}}$ under analogous conditions at three different values of ω_{I} (14).

If the values for $1/1/fT_{1\mathrm{p}}$ and $1/fT_{2\mathrm{p}}$ can be correctly determined and $\Delta\omega$ for E–M–L is negligible, these parameters must be evaluated. If $1/fT_{2\mathrm{p}}$ is in slow or intermediate exchange where $\tau_{\mathrm{m}} \gg T_{1,2\mathrm{M}}$, then:

$$\frac{1}{f\tau_{1,2\mathrm{M}}} = \frac{q}{\tau_{\mathrm{m}}}. \qquad [10]$$

An evaluation of q, the number of ligands binding at the paramagnetic label site, can be made by direct binding studies; in most cases $q = 1$.

These relationships are somewhat simplified by the assumption that outer-sphere effects are negligible. Such 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 result in a small and usually insignificant effect.

In some cases where one nucleus of a ligand is very close to the paramagnetic center compared with other nuclei measured, the relaxation may be so efficient that the nucleus is in slow exchange ($T_{2M} \ll \tau_m$ and $1/fT_{2p} = 1/\tau_m$). If this is the case then a temperature dependence of $1/fT_{2p}$ will give a value for k_{off} and the energy of activation E_{act} for the ligand exchange process. In the case where the exchange process is simple, and

$$K_d = \frac{k_{on}}{k_{off}} \qquad [11]$$

for ligand binding is known, values of k_{on} can also be estimated (15).

EXPERIMENTAL PROCEDURE

1. Affinity Properties of the Paramagnetic Metal for the Enzyme or for the Substrate

As mentioned before, the affinity of the metal and the substrate should be determined. The equilibrium constant of spin-labeled substrates and cations like Mn^{2+}, at room temperature, and Gd^{3+}, at -196°C, are determined by ESR. Figure 1 shows an example where the dissociation constant of the complex Mn–GTPγF was determined by ESR. In this experiment the diminution in area of the free Mn^{2+} peaks was measured at different concentrations of the nucleotide, keeping the total concentration of Mn^{2+} constant. From these data the free and bound cation was determined and the variables for a Scatchard plot were calculated. If the substrate is spin-labeled the same protocol can be used, the titration being made here with the enzyme. Also with the spin-labeled analog the K_I value can be determined by competitive inhibition steady-state kinetic studies. Agreement between these values indicates that the binding moiety of the analog was not modified by the spin-label modification. If there is more than one site for the binding of the metal or the analog, and their affinities are different by at least two order of magnitude, it is advisable to work with concentrations calculated to saturate the higher-affinity site only.

2. Stoichiometry and Binding Properties of the Substrate to the Binary Complex Enzyme–Metal or Spin-Label Substrate

This can be done through traditional binding method (16). An NMR method following the relaxation rate of water to determine the formation of the ternary complex, when the binary complex between enzyme and paramagnetic probe is titrated with the substrate, has been described (17) These experiments can be done with the same NMR spectrometer and the same sample.

3. NMR Sample

Around 0.4 mL of a sample, in a tube of diameter 5 mm, should contain enzyme at a concentration in the range 10^{-4} to 10^{-6} M and substrate at a concentration greater than 1×10^{-3} M, if ^1H or ^{19}F is the nucleus to be measured. The volume should be increased to 1.2 mL and the diameter of the tube to 10 mm when ^{31}P samples are used due to its lower relative sensitivity (18). The experimental buffer, prepared with the best water available (nanopure grade), should be appropriate to keep the protein stable for the duration of the experiment. Ten percent D_2O must be included in the buffer to keep the magnet lock. Paramagnetic contamination must be eliminated using Chelex 100 (Bio-Rad) packed in a Pasteur pipet to avoid dilution of the sample. Modern Fourier-transform NMR instruments are equipped with software to calculate the relaxation times. To determine the longitudinal relaxation time, T_1, the method of inversion recovery should be used. When the stability of the magnet is appropriate and the field is homogeneous, T_2 can be calculated from the width of the signal peak at half-height (Δ) according to the relationship given by Pople *et al.* (19): $T_2 = 1/\pi\Delta$. However, to be rigorous the spin–echo method, usually implemented in spectrometers, should be used.

4. Paramagnetic Effect on the Longitudinal (T_1) and Transverse (T_2) Relaxation Times

To measure the paramagnetic effect, during the residence of the substrate in the enzyme binary complex, labeled with a nitroxide or in the presence of a paramagnetic cation, both relaxation times, T_{1obs} and T_{2obs}, should be corrected by the relaxation times T_{1o} and T_{2o}, determined in the presence of native enzyme or with a diamagnetic cation (e.g. Mg^{2+}), according to the equation

$$\frac{1}{T_{1,2p}} = \frac{1}{T_{1,2obs}} - \frac{1}{T_{1,2o}}. \qquad [12]$$

5. Contribution of the Residence Time τ_m to the Paramagnetic Relaxation Time and Determination of the Dissociation Rate Constant

As discussed in detail by Mildvan and co-workers (18, 20), the effect of temperature on $T_{1,2obs}$ of the nuclei under observation is used to determine whether the predominant contribution comes from $T_{1,2p}$ or τ_m (see Eq. [9]). The effect of temperature on the paramagnetic contribution to the relaxation rates ($1/fT_{1p}$ and $1/fT_{2p}$) in the complex tubulin–Mn–GTPγF is shown in Fig. 2. The Arrhenius activation energy calculated from the slopes of the lines are 8.2 and 26.1 kcal/mol for longitudinal and transverse relaxation rates, respectively (15). These exceed 7 kcal/mol, the limit for exchange (21), and indicate that both processes are dominated by τ_m and $k_{off} = 1/\tau_m$, and so the dissociation rate constant can be determined directly from the transverse relaxation rate value. To determine the order of the reaction the dependence of the relaxation rate with respect to the concentration of the substrate should be measured. The lack of dependence indicates that the rate value corresponds to the dissociation of the substrate from the enzyme. A lower limit for k_{on} can also be calculated by means of Eq. [10]. The activation thermodynamic parameters $\Delta H‡$ and $-T\Delta S‡$ for k_{off} can be determined from these experiments, using absolute reaction rate theory.

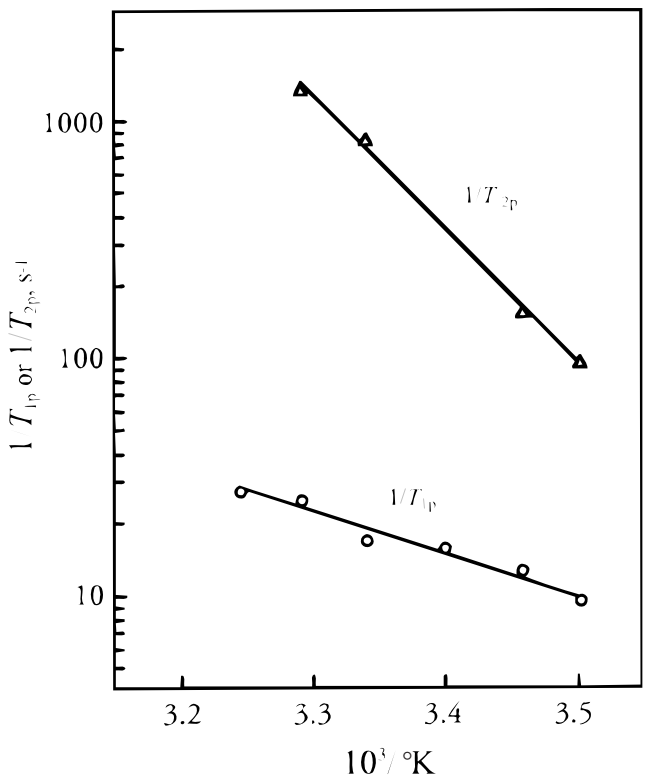

FIG. 2. Arrhenius plot for the temperature dependence of the paramagnetic contribution to the relaxation rates of ^{19}F in the complex colchicine–tubulin–Mn(II)–GTPγF. The mixture in a volume of 0.5 mL contained 50 mM MES, pH 7.0, 3.4 M glycerol, 0.3 mM colchicine, 0.18 mM tubulin, and 0.8 mM GTPγF. The relaxation times were measured as described in Ref. (15). Reprinted with permission from (15). Copyright 1987 American Chemical Society.

CONCLUDING REMARKS

The NMR method for measuring rate constants in an equilibrium reaction of a ligand such as a substrate or dead-end inhibitor with a protein has some advantages over better known methods such as stopped flow (with a lower limit of 1 ms) and temperature relaxation (5 μs). NMR is a noninvasive method that requires a low concentration of protein (10^{-4} to 10^{-6} M) in a volume of 0.3 to 0.5 mL. The ligand (normally an analog of the substrate) is used in high concentrations (around 1 mM for ^1H or ^{19}F), as in steady-state kinetic experiments, because the change in the NMR parameter (δ, J, or $1/T_{1,2}$) depends on a nucleus in the substrate. On the other hand, all the thermodynamic parameters (ΔG^0, ΔH^0, and $T\Delta S^0$) can be determined with the same sample when the experimental measurements are made at different temperatures. Temperature studies also allow determination of the value of the dissociation rate constant (k_{off}) if it is equal to $1/fT_2$, or a lower limit for this rate constant otherwise. NMR spectrometers that allow all the parameters to be obtained automatically are now widely available.

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