31P NMR Studies of the Arginine Kinase Reaction

EQUILIBRIUM CONSTANTS AND EXCHANGE RATES AT STOICHIOMETRIC ENZYME CONCENTRATION*

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The arginine kinase reaction, the reversible transfer of the terminal phosphoryl group of ATP to L-arginine, has been investigated by the technique of 31P NMR at catalytic and stoichiometric concentrations of the enzyme. Three of the four substrates, ATP, ADP, and P-arginine produce easily distinguishable resonances in the 31P NMR spectrum, thus permitting a determination of equilibrium constants from the integrated areas of the resonances. From the linewidths, the exchange rates between reactants and products may be evaluated.

At pH 7.25 and a temperature of 12°C, the equilibrium constant at catalytic enzyme concentration: $K_{cat} = [MgADP][P-arginine]/[MgATP][L-arginine]$, is found to be $0.10 \pm 0.02$ and that at stoichiometric enzyme concentration: $K_{stoich} = [E\cdot MgADP][E\cdot P-arginine]/[E\cdot MgATP][E\cdot arginine]$ to be $1.56 \pm 0.5$. Thus, as the enzyme concentration is increased, the production of P-arginine is increasingly favored.

From the NMR line shapes in the presence of excess enzyme, the rate of the single step, the transfer of the phosphoryl group on the surface of the enzyme is found to be $192 \pm 15$ s$^{-1}$ in the forward direction, i.e. from $E\cdot MgATP$, and $164 \pm 15$ s$^{-1}$ in the reverse direction from $E\cdot P$-arginine. At $12^\circ C$ and pH 7.25, the rate of the overall reaction in the forward direction was determined from kinetic measurements to be $19$ s$^{-1}$, an order of magnitude slower than the rate measured by NMR. It can, therefore, be concluded that the interconversion of substrates on the surface of the enzyme is not the rate-determining step in the overall reaction. From the equilibrium constants and other known data the dissociation constant of P-arginine from its enzyme complex can be determined and is found to be $100$ μM.

In this paper, a 31P NMR study is presented of the enzyme-bound substrate complexes of arginine kinase, with special reference to the determination of equilibrium constants of the reaction at stoichiometric concentrations of the enzyme and of exchange rates at equilibrium. Some other aspects of this study related to 31P chemical shifts in the various enzyme-bound complexes will be presented in a forthcoming paper. The 31P-nuclei in the six phosphate groups that occur in three of the four reaction substrates (including the group that transfers back and forth between P-arginine and ATP) produce clearly distinguishable resonances in the NMR spectrum. Thus, the positions, the line shapes, and the integrated areas of the resonances depict the effect of the enzyme on the substrates and consequently allow the determination of some of the parameters associated with the enzyme interaction simply and directly.

EXPERIMENTAL PROCEDURES

Materials—The procedures used for isolation of arginine kinase from lobster muscle and for enzyme assays have been described previously.
The abbreviations used are: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; FT, Fourier transform; CW, continuous wave; FID, free induction decay; rf, radiofrequency.

Preparation of Samples—For the experiments with high enzyme concentrations, arginine kinase normally stored in 10 mM glycine, 1 mM EDTA, 1 mM 2-mercaptoethanol, pH 8.0, was freshly reprecipitated by the addition of (NH₄)₂SO₄ to 70% saturation. The precipitated enzyme was redissolved to a final concentration of 1 mM. After two dialyses against 50 volumes of 50 mM Hepes, 1 mM 2-mercaptoethanol at the desired pH and a final dialysis against 150 mM Hepes, 1 mM 2-mercaptoethanol at the same pH, the enzyme solution was concentrated to about 4 to 5 mM in a B-15 Amicon concentrator. Enzyme concentrations were measured spectrophotometrically using an extinction coefficient of 6.7 at 280 nm for a 1% solution of arginine kinase and a molecular weight of 40,000 (5). Possible contamination of ATP solutions by metal ions is appreciably reduced by extracting with 8-hydroxyquinoline (6). Measurements of 31P-nuclear longitudinal relaxation times, T₁, indicate that this procedure is effective in removing about 80% of the contribution to the 31P relaxation rates of ATP arising from paramagnetic metal contamination at pH 8.0. The values of pH and pD were direct readings on a Radiometer pH meter.

Kinetic Measurements—The rate for the forward reaction of arginine kinase was measured using the coupled enzyme assay with pyruvate kinase and lactate dehydrogenase (7). The reaction was initiated by adding about 1.5 µg of arginine kinase to a reaction mixture containing about 120 mM glycine, 9 mM magnesium acetate, 50 mM potassium acetate, 1 mM sodium phosphoenolpyruvate, 50 µg of lactate dehydrogenase, 50 µg of pyruvate kinase, 0.2 mM NADH, ATP (concentration varied from 2.5 to 5 mM), and L-arginine (concentration varied from 0.9 µM to 0.9 mM) in a total volume of 1 ml. The measurements were made at the same pH, 7.25, and temperature, 12°, as the NMR measurements to facilitate comparison. The reaction was followed by measuring the decrease in absorbance at 340 nm on a Cary 15 spectrophotometer. Maximum velocities were determined from a double reciprocal plot of the velocity versus substrate concentration.

NMR Measurements—The NMR spectra were obtained on a JEOL PS-100 NMR spectrometer operating at 40.3 MHz for 31P resonance and equipped with a field-frequency lock on the deuterium resonance. The sample tubes used (Wilmad #513-1PP) are 10 mm, outer diameter. Typical sample volumes ranged from 1 to 1.5 ml and contained at least 10% D₂O by volume. The spectra were obtained in the FT mode and signal-averaging was performed by a Texas Instruments EC-100 computer dedicated to the spectrometer. Broad band proton decoupling is used in some experiments to decouple α-P of ATP and ADP from the 31P resonances of interest. All the experiments were performed at a temperature of 12° ± 1.5° using a JEOL model JNM-VT-3B variable temperature controller. All chemical shifts are referred to 85% H₃PO₄ as the external reference.

Measurement of Equilibrium Constants—The integrated areas of the 31P NMR resonances arising from the different phosphate groups are proportional to their respective concentrations. Each resonance peak can be integrated by the computer to obtain the areas and the relative concentrations. Thus, the equilibrium constant of the reaction may be determined in a straightforward manner. There are, however, a few precautions to be observed in determining the ratios of concentrations reproducibly by the NMR method, especially in the FT mode, with signal averaging (6). It is important to ascertain that the interval between successive rf pulses is large compared to the T₁ values of all the nuclei of interest. This condition is analogous in CW operation to maintaining the rf power levels and sweep rates sufficiently low so that all resonance peaks are below saturation. In order to improve the accuracy of integration, it is also useful to (a) avoid signals occurring close to the edges of the filters used in detection, (b) use a large number of data points per Hz of the spectrum, (c) introduce negligible line broadening by the sensitivity enhancement procedure (8), and (d) accumulate a sufficient number of scans to obtain a signal to noise ratio of about 10:1. Finally, decoupling rf fields should not be used in these experiments as they produce intensity changes due to nuclear Overhauser effect.
higher pH. The apparent equilibrium constants, $K_{eq}$, for catalytic enzyme concentrations,

$$K_{eq} = \frac{[\text{MgADP}][P-\text{arginine}]}{[\text{MgATP}][\text{L-arginine}]}$$

are found to be $0.10 \pm 0.02$ at pH 7.25 and $0.22 \pm 0.04$ at pH 8.0 at $20^\circ$. No attempt was made to make a quantitative comparison of the two equilibrium constants obtained above as a function of hydrogen ion concentration because of the complications arising from comparing multiple equilibria in H2O and D2O. However, the change in the equilibrium constant is qualitatively in the expected direction. Earlier measurements of $K_{eq}$ for the arginine kinase reaction yielded values of 0.31 (10) and 0.46 (11) at pH 8.0 and $30^\circ$. The spectra in Fig. 2, A and C are obtained for the same samples as those used for Fig. 1, A and B, respectively, with a strong broad band radiofrequency field at the proton resonance which decouples the $\gamma\text{-protons}$ from the $\alpha\text{-P}$ nuclei in both ATP and ADP and produces the sharp resonances shown in the spectra (Fig. 2, A and C) for these groups. A comparison of the spectra recorded at two different pH values (Fig 2, A and C) shows the usual upfield chemical shift upon protonation (9) of the various $^{31}\text{P}$-nuclei in the reaction mixture. These spectra are not suitable for the determination of equilibrium constants as the signals of $\alpha\text{-P}$ nuclei are enhanced by proton decoupling and as further investigation showed, a pulse repetition interval of 10 s is inadequate to ensure complete recovery of the spin system to thermal equilibrium between pulses.

Fig. 2, B and D show the $^{31}\text{P}$ NMR spectrum of the equilibrium mixtures used for Fig. 1, A and B, respectively, to which sufficient amounts of EDTA have been added to remove the Mg$^{2+}$ ions and consequently stop the reaction. The pH was readjusted to the original values. A comparison of Fig. 2A with 2B and of 2C with 2D shows the effects of the metal ion on the $^{31}\text{P}$ resonances. It is apparent that the chelation of Mg$^{2+}$ to ATP and ADP produces a major chemical shift of the $\beta\text{-P}$ of ATP greater than 2 ppm, and considerably smaller shifts for the other $^{31}\text{P}$ nuclei. There is also an increase of about 5 Hz in all three P-P spin-spin coupling constants $i.e.$ the $\alpha\beta$ of ADP and both $\alpha\beta$ and $\beta\gamma$ of ATP, upon the removal of the Mg$^{2+}$ ion. These chemical shifts and coupling constants will be considered in detail in a forthcoming paper. It is not surprising that no significant changes in the linewidths of the signals result from terminating the reaction by the addition of EDTA, since at the low enzyme concentrations used the exchange rate between reactants and products of the reaction is too small to cause significant broadening of the resonances.

1 The assignment of the overlapping resonances of $\gamma\text{-P}$ of ATP and $\beta\text{-P}$ of ADP and those of $\alpha\text{-P}$ of ATP and $\alpha\beta$ of ADP as marked in Fig. 2D is arrived at by noting that the two $^{31}\text{P}$ nuclei in ADP are coupled more strongly, $i.e.$ have a larger spin-spin coupling constant to chemical shift ratio, than any of the pairs in ATP. The signals of ATP therefore resemble an AX pattern for which the intensities of the two signals are equal while those of ADP resemble an AB pattern for which the outer signals are weaker in intensity compared to the inner signals of the doublets.

It may be noted that the signals of $\beta\text{-P}$ of ATP in Fig. 2, B and D are rather broad, particularly at pH 7.25. The broadening arises from the exchange between the small amount of MgATP that persists in equilibrium with free ATP upon the addition of 30 mM EDTA to a solution containing 17 mM Mg$^{2+}$ and 15 mM ATP at pH values of 7.25 and 8.0, respectively. The broadening caused by this exchange is determined by the difference in chemical shifts between the signals of MgATP and ATP which is appreciable only for $\beta\text{-P}$ of ATP and, therefore the signals of $\beta\text{-P}$ are significantly broadened.

**Stoichiometric Enzyme Concentrations**

Experiments similar to the above were performed on reaction mixtures containing the enzyme at concentrations exceeding those of the substrates. On the basis of the known dissociation constants of the enzyme-substrate complexes (2, 3), the concentrations in the samples were chosen so that more than 90% of the substrate molecules are bound to the enzyme. Typical enzyme concentrations in these experiments range from 4 to 5 mM and typical substrate concentrations from 2 to 4 mM. The substrate concentrations are considerably lower than those in Figs. 1 and 2 and therefore, the signal to noise ratio of...
the spectra is usually inferior and the long accumulation times necessary make the experiments relatively laborious.

A 31P NMR spectrum of the equilibrium mixture of the enzyme-bound reactants and products while the reaction is turning over is shown in Fig. 3A. A comparison of Fig. 3A with 2A reveals a number of contrasting features of the high and low enzyme conditions. Figure 3A shows three broad groups of signals approximately in the same regions as the \( \alpha \), \( \beta \), and \( \gamma \)-phosphate resonances of MgATP in Fig. 2A. These signals show little fine structure due to spin-spin coupling. Broadening of the signals in Fig. 3A may be attributed to two possible sources, (a) rapid turnover at high enzyme concentrations producing broadening due to chemical exchange of \( \alpha \)- and \( \beta \)-P of ADP with a \( \alpha \)- and \( \beta \)-P of ATP, respectively, and \( \gamma \)-P of ATP with P of \( \text{P-arginine} \) and (b) the \( \text{31P}-\text{nuclei} \) in the substrates, now fully bound to the enzyme, are not only likely to experience larger dipolar interactions with many more protons on the protein than in the unbound state, but also the correlation times governing the molecular motion are considerably longer in the bound form and both of these factors may cause additional broadening.

The two possible causes of line broadening considered above can be separated by adding EDTA to stop the reaction by removing Mg\(^{2+}\) ions from the substrates, thus eliminating chemical exchange broadening. However, the substrates remain in the fully bound state. A dramatic narrowing of the lines is observed in the \( \text{31P} \) NMR spectrum obtained after adding EDTA to the equilibrium mixture as shown in Fig. 3B. In fact, a comparison of the linewidths of various resonances of the completely bound forms of the substrates (Fig. 3B) and the free substrates (Fig. 2A) reveals at once that binding to the enzyme does not produce a great deal of broadening of the \( \text{31P} \) resonances of the substrates. Furthermore, independent experiments on the binary E-ADP and E-ATP and on the ternary E-MgADP and E-MgATP complexes to be described in a forthcoming paper, show that addition of Mg\(^{2+}\) produced appreciable broadening only for the \( \beta \)-P signal of E-MgATP. Thus the broadening of \( \beta \)-P of ATP, \( \gamma \)-P of ATP, and P-arginine signals in Fig. 3A may be attributed primarily to the chemical exchange of phosphate groups resulting from the rapid turnover by the enzyme.

A comparison of the line positions in Figs. 3B and 2B yields the chemical shifts of the \( \text{31P} \)-nuclei in the different phosphate groups of the substrate caused by binding to the enzyme. The only significant chemical shift (~2 ppm) occurs for the \( \beta \)-P of ADP.

The contrast between the spectra in Fig. 3, A and B which differ only in removal of Mg\(^{2+}\) by chelation with EDTA, strikingly demonstrates the obligatory role of the metal ion in the catalysis of the reaction by the enzyme. It may also be noted that in Fig. 3B no additional resonances other than those for E-ATP, E-ADP, E-P-arginine complexes are observed indicating that there are no intermediates (e.g. an E-P complex) of significant concentration and lifetimes in the reversible transfer of the phosphoryl group during the course of the reaction (10, 11). In the experiments with high enzyme concentration an additional \( \text{31P} \) signal corresponding to P, is observed. This signal grows with time and is attributed to a weak ATPase activity observable at such high concentrations. Whether the weak ATPase activity is an inherent property of the enzyme as in the case of yeast hexokinase (12) and rabbit muscle creatine kinase (13) or a contaminant has not been established for arginine kinase.

**Equilibrium Constant**—In the experiment designed to evaluate the equilibrium constant at stoichiometric concentrations of enzyme (Fig. 3A), designated \( K'_{\text{eq}} \) the initial concentrations of ATP and L-arginine were chosen to be equal. Under these conditions, \( K'_{\text{eq}} \) is given by:

\[
K'_{\text{eq}} = \frac{[E_{\text{MgADP}}][E_{\text{P-Arg}}]}{[E_{\text{MgATP}}][E_{\text{Arg}}]} = \frac{[E_{\text{MgADP}}]^2}{[E_{\text{MgATP}}]^2}
\]

The integrated areas of the isolated \( \beta \)-P signal of E-MgATP and that of the superposed \( \alpha \)-P resonances of E-MgATP and E-MgADP are proportional to \([E_{\text{MgATP}}] + [E_{\text{MgADP}}] \) respectively. From the areas, \([E_{\text{MgATP}}]/[E_{\text{MgADP}}] = 0.8\), leading to \( K'_{\text{eq}} = 1.56 \pm 0.05 \) at \( 12^\circ \), pH 7.25. The stoichiometric equilibrium constant is thus nearly 15 times larger than the catalytic equilibrium constant. This difference clearly arises because the enzyme-bound substrates are components of the equilibrium for the stoichiometric case and the above result shows that under these conditions the production of P-arginine is strongly favored.

The equilibrium constants at catalytic and stoichiometric levels of the enzyme are related by the dissociation constants, \( K_d \), of the reactants and products for binding to the enzyme:

\[
K'_{\text{eq}} = \frac{K_d_{\text{MgATP}} K_{\text{P-arginine}}}{K_d_{\text{MgADP}} K_{\text{arginine}}}
\]

The \( K_{\text{MgADP}} \) and \( K_{\text{MgATP}} \) and the Michaelis constant \( K_{\text{arginine}} \) were determined to be 79 \( \mu \text{M} \), 7.5 \( \mu \text{M} \), and 135 \( \mu \text{M} \), respectively.

\[
K_{\text{MgATP}}, K_{\text{MgADP}} \text{ and } K_{\text{arginine}} \text{ were determined to be 79 } \mu \text{M, 7.5 } \mu \text{M, and 135 } \mu \text{M, respectively}
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\]
Arginine Kinase Equilibrium Constants and Exchange Rates

(2) $K_{\text{eq}}^{\text{P-arginine}}$ has not been reported for this enzyme. Assuming

$K_{\text{eq}}^{\text{MgATP}/\text{MgADP}} = (K_{\text{eq}}^{\text{MgATP}/\text{MgADP}}')$ and $K_{\text{eq}}^{\text{P-arginine}} = K_{\text{eq}}^{\text{arg}}$ using the values of $K_{\text{eq}}$ and $K_{\text{eq}}^{\text{arg}}$ obtained above, $K_{\text{eq}}^{\text{P-arginine}}$ can be estimated to be $100 \mu M$. P-arginine thus binds more tightly to the enzyme than L-arginine, analogous to the case of the creatine kinase system, where the binding constant of P-creatine to the enzyme is twice as great as that for creatine (14).

Exchange Rates—The interconversion of bound substrates on the enzyme obscures all the fine structure due to spin-spin splitting in Fig. 3A. The broad resonance in the low field region results from a partial merging of the $\gamma$-P of ATP and P-arginine signals superimposed over a broadened signal of $\beta$-P of ADP. The nonoverlapping resonance of $\beta$-P of ATP centered at $\sim 19$ ppm is only broadened and not shifted by its exchange with $\beta$-P of ADP since in this case the chemical shift difference between the exchanging $\beta$-P-nuclei is large compared to the inverse of the exchange time. In this limit, the additional linewidth caused by exchange in the resonance of site A is simply related to the residence time ($\tau_A$) in that site:

$$\pi(\Delta\nu_A - \Delta\nu_A') = \tau_A^{-1}$$

where $\Delta\nu_A$ and $\Delta\nu_A'$ are, respectively, the linewidths (full width at half the peak height) in Hz in the presence and absence of the exchange. For $\beta$-P of ATP in Fig. 3B, $B$, and $A$, $\Delta\nu_A$ and $\Delta\nu_A'$ are given by $8.0$ Hz and $69.0$ Hz, respectively, which yield $\tau_A^{-1} = 192 \pm 15$ s$^{-1}$, for the mean reciprocal residence time, i.e., the rate of transfer, of the phosphoryl group at the $\gamma$ position of E-MgATP. Theoretically, $\Delta\nu_A'$ is the linewidth in the absence of exchange but in the presence of MgATP and L-arginine. Since such a spectrum is inherently impossible to obtain it may be estimated either from Fig. 3B (no Mg$^{2+}$) or from the spectrum of the E-MgATP complex which was obtained in an independent experiment. These two measurements of $\Delta\nu_A'$ were equal within an experimental error about $\pm 2$ Hz well within the error of about $\pm 5$ Hz estimated$^2$ above for $\tau_A^{-1}$. In this two site exchange problem the residence time, $\tau_B$, in the E-P-arginine form is related to $\tau_A$ by:

$$\frac{\tau_A}{\tau_B} = \frac{[E\cdot\text{MgATP}]}{[E\cdot\text{MgADP}]}$$

since $[E\cdot\text{P-arginine}] = [E\cdot\text{MgADP}]$ in the present experiment. This gives $\tau_A^{-1} = 154 \pm 15$ s$^{-1}$ for the mean reciprocal lifetime, i.e., the rate of transfer, of the phosphoryl group of the E-P-arginine complex. The linewidth thus affords a method of determining the exchange rate between reactants and products, a parameter which can otherwise be estimated indirectly only by the more laborious technique of isotope exchange rates at equilibrium (16, 17).

With the lifetimes of the complexes given above, the signals of P-arginine and $\gamma$-P of ATP in Fig. 3A with a chemical shift of about $80$ Hz between them are in the intermediate exchange

$^3$It may be noted that with the onset of exchange a concomitant collapse of the multiplet structure of the $\beta$-P resonance of ATP occurs. A rigorous description of the resultant line shape for arbitrary ratios of the exchange rate and coupling constants is somewhat complicated and is unlikely to yield a simple relation like Equation 5 for $\tau_A^{-1}$ particularly when the collapse of the multiplet structure is incomplete. An estimate of the contribution to the linewidth from the collapse of the multiplet structure, by using the procedure of Grunwald et al. (18), shows that it is about $5\%$ of the total linewidth. The approximation made in the calculation may overestimate $\tau_A^{-1}$ and $\tau_B^{-1}$ by $5\%$, which remains within the stated error.

It may be noted that the exchange rate measured from the NMR experiments at stoichiometric enzyme concentrations is solely the interconversion rate of the substrates on the enzyme. Possible contribution to the linewidth of $\beta$-P resonance of bound MgATP (which is the important parameter in this measurement) due to exchange with the small amount of free MgATP (at equilibrium) is negligible since the chemical shift of $\beta$-P resonance of MgATP is practically unaffected by binding to the enzyme.

Kinetic measurements of the maximal velocity of the overall reaction in the forward direction at $12^\circ$ and pH 7.25 made with catalytic quantities of the enzyme yield $19$ s$^{-1}$ which is almost an order of magnitude slower than the interconversion rates determined above. The overall rate of the reaction includes the rate of interconversion and the rate of dissociation of the substrates as well as other possible steps. It can, therefore, be concluded that the interconversion of reactants and products on the enzyme is not the rate-limiting step in the arginine kinase reaction.

**Discussion**

The $^3$P NMR study of the arginine kinase reaction presented in this paper illustrates the usefulness and limitations of this spectroscopic method in studying reactions of phosphorus-containing substrates. The ability to observe distinct resonances for each of the phosphate groups in the substrates allows direct observation of the interaction of enzymes, like the kinases, with the chemical moieties that actually participate in the reaction. Equilibrium constants of the reaction under any desired set of conditions can be obtained in a straightforward manner from the integrated areas of the resonances in the spectrum. As with other spectroscopic methods this procedure permits the determination of the equilibrium constant without any perturbation of the system. The usual analytical methods suffer from the possible inaccuracies arising from a shift in the equilibrium that a change in pH or temperature or any other parameter involved in terminating the reaction itself might produce.

Specifically for the case of the arginine kinase reaction the results show that at about $12^\circ$ and pH 7.25 the equilibrium constant at stoichiometric concentrations of the enzyme is approximately 15 times larger than that at catalytic concentrations of the enzyme indicating that at high enzyme concentrations production of P-arginine is strongly favored. In view of the high concentration of the enzyme in the lobster muscle ($>0.5$ mM) the physiological equilibrium is likely to be governed, at least in part, by enzyme-bound substrates. Consequently, the value of $K_{\text{eq}}$ should be of interest in understanding the equilibrium under physiological conditions.

In addition to the thermodynamic information, kinetic information may sometimes be obtained when the line shapes and positions of the resonances in an equilibrium mixture permit a determination of the exchange rates between reac-
tants and products. The evaluation of the rates of exchange between enzyme-bound substrates when compared to the overall rates of the reaction indicate whether the interconversion of substrates on the enzyme is the rate-determining step in the enzymatic reaction. For lobster arginine kinase, the rate of enzyme-bound substrate interconversion is not the rate-determining step since the rate of exchange of enzyme-bound substrates and products is found to be much faster than the maximal velocity of the overall reaction obtained from kinetic measurements with catalytic amounts of enzyme.

The $^{31}$P resonances of the enzyme-bound substrates are not excessively broadened either by the dipolar interactions with the protons on the enzyme or by the slow tumbling of the bound complexes, and are well within the limits of attainable sensitivity for experimental study. This permits further use of this technique for the characterization of the active site structures under chosen conditions. The chemical shift studies of diamagnetic enzyme-substrate and transition-state analog complexes in a forthcoming paper are examples of this. Experiments at higher operating NMR frequencies are likely to appreciably increase the speed and ease of measurements by this technique both due to higher sensitivity and increased chemical shift between resonances although the linewidth would increase if contributions from anisotropic chemical shift to relaxation becomes significant. Further experiments with paramagnetic metal ions such as Mn$^{2+}$ should yield structural information on enzyme-bound substrates.

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