Paramagnetic Probes in Magnetic Resonance Studies of Phosphoryl Transfer Enzymes

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In living systems, nucleoside triphosphates are the chief repository of chemical energy and mediate the transfer of energy from one metabolic process to another. Approximately 100 known enzymes function as catalysts for phosphoryl transfer reactions of the type: $ATP^{4-} + XH \rightleftarrows ADP^{3-} + XPO_3^{2-} + H^+$, including the formation of ester, phosphoamidate, carboxylic anhydride, phosphoric anhydride, and enol phosphate bonds.[3]

Studies of the mechanism of phosphoryl transfer reactions by methods other than kinetic ones have been hampered in the past by the lack of an observable parameter characteristic of the various enzyme–substrate complexes which are formed in the course of the reaction. The fact that all enzymatic reactions of nucleoside triphosphates require a divalent metal ion for activation and that the paramagnetic manganous ion can always serve as an activator encouraged the use of magnetic resonance spectroscopic techniques with this paramagnetic probe for the investigation of phosphoryl transferring enzyme systems.[4] More recently,[5] the studies have been extended to another paramagnetic probe, a stable nitroxide free radical which has been covalently bound to creatine kinase (ATP:creatine phosphotransferase).

Some of the questions we could hopefully answer from the various types of magnetic resonance measurements with paramagnetic probes concern: (1) binding constants and number of binding sites for metal ions and substrates; (2) the environment of the metal ion; (3) the structures and configurations at the active site of various enzyme–metal–substrate complexes and the disposition of the two substrates with respect to each other on the surface of the enzyme; (4) the mobility at the active site and ligand exchange rates of these complexes, *i.e.*, the dynamic properties of the active complexes; (5) the role of the metal ion in enzyme catalysis. This Account will focus on the results from nuclear relaxation and epr studies with paramagnetic probes which bear on the above questions for phosphoryl transfer enzymes.

Studies of enzyme–substrate interactions by nmr can be conducted on diamagnetic systems where diamagnetic shielding and dipolar proton–proton interactions may manifest themselves in changes of chemical shifts and of relaxation rates.[6] However, relative to effects with paramagnetic ions[7] the diamagnetic interactions are weak and the observation of their effects in dilute solutions is not always feasible. Since the discovery that proton longitudinal relaxation rates of water due to paramagnetic ions are very much enhanced in the presence of biological macromolecules, including nucleic acids[8] and proteins,[9] nuclear magnetic relaxation has become a valuable addition to the arsenal of spectroscopic methods available in biochemical investigation.

The unique features of nmr relaxation compared to other relaxation methods are that (1) the energy required to perturb the nuclear Zeeman levels is very small compared to kT and the chemical equilibrium is unperturbed; (2) the nuclei of individual atoms in the system are observed in solution and the information acquired pertains to both their static and dynamic properties; and (3) the method is nondestructive and the amount of material needed is not excessive (no more than 0.1 ml of a 10^{-4} M solution of the macromolecule for pulsed nmr experiments of proton relaxation rates of water). The advantages of nmr and epr spectroscopy compared to optical spectroscopy are the ease of assignment of a given absorption to individual spins in the system and the interpretability of line shapes in terms of molecular motions based on well-established theory.

Another important property of the paramagnetic probes is their amenability to study by electron paramagnetic resonance. This technique has been used to determine equilibrium concentrations of unbound manganese(II) in enzyme-containing solutions,[10,11] to detect conformational changes induced by metal ions and substrates as reflected in the epr spectrum of spin-labeled creatine kinase, and to estimate the distance between the spin label and Mn(II).[5] Manganese epr work in progress appears promising for studying the structure of Mn(II)–enzyme and Mn(II)–enzyme–substrate complexes.[12]

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(3) Abbreviations used are: ATP, adenosine 5′-triphosphate; ADP, adenosine 5′-diphosphate; AMP, adenosine 5′-phosphate.
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Magnetic Resonance Methods

Nuclear Relaxation in Solutions of Paramagnetic Ions. After perturbation of the thermal equilibrium of a system of nuclear spins, the return to equilibrium occurs with a characteristic time constant called the relaxation time. The time-dependent terms in the Hamiltonian which describes the system of nuclear spins in the liquid are expressed as functions of randomly fluctuating local magnetic fields. These fields induce transitions among the energy levels of the spin system, thereby leading to relaxation. Longitudinal spin relaxation, *i.e.*, relaxation along the external magnetic field, is caused by those terms in the Fourier spectrum of the randomly fluctuating field which contain the nuclear Larmor frequency, ω_I. The magnitude of the time-dependent terms is determined by the intensity of the electron–nuclear interaction and by the rate at which the interaction is interrupted. The correlation time, τ, characteristic of a molecular process responsible for the interruption of the interaction determines the extent to which any particular process contributes to the relaxation. Thus, from analysis of nuclear relaxation rates important information may be obtained in solution regarding (1) dynamic processes from the correlation times and (2) structural properties from the intensity of the interaction. The first category may include the correlation time for molecular rotation, τ_r, the *rate* of chemical exchange, $1/\tau_M$, and the electron spin relaxation time, τ_s, and the second reflects the degree of electron delocalization in the system and the distance between interacting spins.

The relevant relaxation parameter of a given nucleus in a ligand undergoing chemical exchange between a paramagnetic and a diamagnetic environment in solution is the quantity $1/T_{1p}$, the paramagnetic contribution to the nuclear relaxation rate. It is defined as the difference between the observed relaxation rate, $1/T_1$, in the presence of paramagnetic ions and the relaxation rate $1/T^0_1$, observed in a similar solution but in the absence of paramagnetic ions. The relaxation rate $1/T_{1p}$ is given by[13-15]

$$1/T_{1p} = P_M/(T_{1M} + \tau_M) \quad (1)$$

where T_{1M} is the longitudinal relaxation time of a nucleus in the paramagnetic complex; τ_M is the mean residence time of the ligand in the complex; and $P_M = n[M]/[L]$, n being the number of equivalent nuclei in the complex; [M] is the ion concentration, and [L] is the formal concentration of the ligand nuclei under consideration (*e.g.*, 2×55.5 for water protons in aqueous solution). The pseudo-first-order rate constant for chemical exchange, $1/\tau_M$, is expected to obey Eyring's relation, $1/\tau_M = (kT/h)\exp[(-\Delta H^{\ddagger}/RT) + (\Delta S^{\ddagger}/R)]$.

The value of T_{1M} is determined by the interactions between the nuclear spin and the electronic spin of the paramagnetic ion and is given by the Solomon–Bloembergen equation[16]

$$1/T_{1M} = Df_1(\tau_c)/r^6 + 2H\tau_{e_2}/(1 + \omega_s^2\tau_{e_2}^2) \quad (2)$$

where $f_1(\tau_c) = 3\tau_{c_1}/(1 + \omega_I^2\tau_{c_1}^2) + 7\tau_{c_2}/(1 + \omega_s^2\tau_{c_2}^2)$. The first term is due to the dipolar interaction; the second term, arising from the electron–nuclear contact interaction, is usually negligible due to its functional form. In eq 2, r is the length of the ion–nucleus internuclear vector, ω_I and ω_s are the nuclear and electronic Larmor frequencies, respectively, $D = {}^2/_{15}S\cdot(S + 1)\gamma_I^2g^2\beta^2$, where S is the resultant electronic spin angular momentum (in $\hbar$ units), γ_I is the nuclear magnetogyric ratio, g is the electronic g factor, β is the Bohr magneton, and $H = {}^1/_3S(S + 1)(2\pi)^2(A/h)^2$, where A/h is the hyperfine coupling constant (in hertz). The correlation times are given by eq 3, where

$$1/\tau_{c_1} = 1/\tau_r + 1/\tau_{e_1} \quad (3a)$$

$$1/\tau_{c_2} = 1/\tau_r + 1/\tau_{e_2} \quad (3b)$$

$$1/\tau_{e_1} = 1/T_{1e} + 1/\tau_M \quad (3c)$$

$$1/\tau_{e_2} = 1/T_{2e} + 1/\tau_M \quad (3d)$$

τ_r is the characteristic time for random reorientation of $\vec{r}$, and T_{1e} and T_{2e} are the longitudinal and transverse relaxation times of the electronic spin, respectively. Equations 3a and 3b are the usually assumed forms of the dipolar correlation times. It is unclear, however, whether the Solomon–Bloembergen equation is strictly applicable when $1/\tau_M > 1/\tau_r$, $1/T_{1e}$, $1/T_{2e}$, and τ_M is the dominant correlation time; in such cases it may be more appropriate to use the expressions for relaxation due to translational diffusion.[17] It is immediately obvious from eq 2 that if $1/T_{1M}$ could be experimentally evaluated from T_1 measurements, very important information on the structure (r) and dynamics (τ_c) of the complex could be obtained.

The function $f_1(\tau_c)$ at three different frequencies is illustrated in Figure 1. The maximum occurs at $\tau_c = 1/\omega_I$, at which point $f_1(\tau_c) = 3/(2\omega_I)$. The term in ω_s is important only at very short correlation times (10^{-11} sec) where $\tau_c \approx 1/\omega_s$; *e.g.*, in solutions of $Mn(H_2O)_6^{2+}$, $\tau_c = \tau_r$, the rotational correlation time, which is of the order of 10^{-11} sec. In this case the term in ω_s gives rise to a frequency dependence of $1/T_{1p}$ and to a temperature dependence since τ_r obeys a simple exponential law of the form $\tau_r = \tau_r^0\exp(E_r/RT)$. An enhancement in $1/T_{1p}$ is expected when the mobility of the paramagnetic ion is reduced, *i.e.*, τ_r increases, due to binding to a macromolecule. In this case the dipolar interaction is modulated by longer correlation times, and the consequent large increase in $f_1(\tau_c)$ more than compensates the decrease due to loss of

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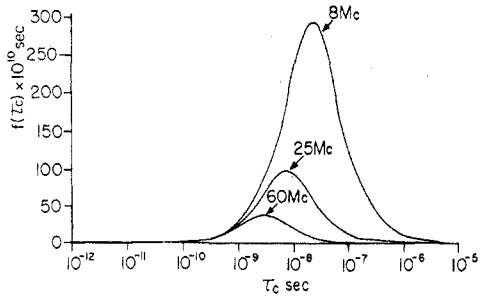

Figure 1. A plot of $f_1(\tau_c)$, as a function of the correlation time at three proton resonance frequencies: 8, 25, and 60 MHz. It is assumed that $\tau_{c1} = \tau_{c2}$ in $f_1(\tau_c)$ (*cf.* eq 2).

water molecules of the first coordination sphere in the process of binding. The frequency dependence as well as temperature dependence of relaxation rates is informative in evaluating τ_c and discriminating among the relaxation mechanisms.[18] Although the data thus far obtained with macromolecular systems have been successfully accommodated within the framework of existing theories developed for small molecules, it is not certain that these theories are applicable without modification to the systems of interest.

Epr Spectroscopy of Paramagnetic Probes. The aquo complex of Mn(II) in solution gives rise to a characteristic epr spectrum. However, the epr spectra for manganous complexes, including Mn(II) bound to macromolecules, are often greatly broadened, leading to an epr spectrum of very low amplitude.[10,19] In those cases where the contribution of the complexes to the spectral amplitude is negligible, the relatively sharp and intense spectrum of $Mn(H_2O)_6^{2+}$ can serve as a measure of its concentration and thus dissociation constants of binary complexes can be determined. Moreover, dissociation constants for other ions can be determined by competition with Mn(II).[20]

In those macromolecular systems where an epr spectrum can be observed, the line shape, which depends on the transverse electron spin relaxation time, yields information regarding the rotational motion at the site of the paramagnetic label.[21] Thus it is possible to monitor the effects of diamagnetic enzyme and of substrates, activators, or inhibitors on the mobility at the active site. For bound Mn(II) ions, it is possible, in addition, to estimate the zero-field splitting and draw conclusions concerning the distortion from cubic symmetry induced in the complex by addition of substrate.[12,19]

The possibility of detecting and quantitating the interaction between two paramagnetic probes on the same enzyme molecule has been realized and has provided additional structural parameters.[5,22] In the case of the interaction of a nitroxide free radical with manganese(II) (both rigidly bound to an enzyme), broadening, peak shifts, and amplitude changes in the epr spectrum of the former may be observed depending upon the mutual orientation of the spins. Under certain conditions an apparent decrease of the spectral amplitude results with no observable broadening. A comparison with theoretically calculated spectra allows the determination of the distance between the two spins.[22]

Properties of Paramagnetic Probes

There are two types of paramagnetic probes available for interaction with enzymes: paramagnetic metal ions and stable free radicals. The choice depends, of course, on the problem to be solved. We will briefly describe their properties of interest.

Paramagnetic Ions. Most of the divalent metal ions of the first transition series and the trivalent lanthanides are paramagnetic. Many enzymes require divalent metal ions for activity; some enzymes require or contain transition metal ions (*e.g.*, manganese, iron, copper) which consequently may serve as natural paramagnetic probes. For enzymes activated by nonparamagnetic ions (*e.g.*, magnesium and calcium) a suitable paramagnetic substitute has to be found. The paramagnetic ion of choice is one which is an activator of the enzyme. For example, in many enzymatic reactions manganese acts in a way similar to that of magnesium, the natural activator, and in fact, in most of the nuclear relaxation studies in enzyme systems, Mn(II) has been used as the paramagnetic probe.[23]

The properties of Mn(II) that render it particularly suitable as a probe are the following. (a) Relatively *long electron relaxation time* ($T_{1e} = 1 \times 10^{-8}$ sec) obtains.[24] One consequence is that the epr spectrum is observable. Furthermore, nuclear relaxation of water protons is determined by the rotational correlation time of the aqua ion (see eq 2) which is much shorter than T_{1e}.[13] However, for ions bound to an enzyme the rotational correlation time is relatively long and the enhancement of the relaxation rate is large since the dominant correlation time for nuclear relaxation is now either the electron spin relaxation time, the rate of chemical exchange, or a combination of the two.[25] If the electron relaxation time T_{1e} were very short (*e.g.*, Co(II)), the effect on the relaxation rate upon binding of the ion to a macromolecule will be small since the dominant correlation time would be T_{1e} in both the bound and unbound forms. (b) A *high electronic spin* ($S = {}^5/_2$) is present. Since the relaxation rate $1/T_{1M}$ is proportional to $S(S + 1)$, the effectiveness of Mn(II) in causing nuclear relaxation is high and thus low concentrations produce easily measurable effects. (c) A *labile hydration sphere* (mean residence time of water 2.7×10^{-8} sec at

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300°K)[26] is present. Conditions of fast exchange ($\tau_M < T_{1M}$, see eq 1) usually prevail, leading to complete averaging of the bound and unbound ligands. Thus, the relaxation properties of the ligands in the coordination sphere can be evaluated. (d) *Binding properties* and ionic radius (0.80 Å) are sufficiently close to those of Mg(II) (ionic radius 0.65 Å) so that it forms similar complexes with nucleotide substrates and many enzymes. Many of these properties of Mn(II) can be ascribed to its half-filled d shell (an S-state ion). (e) Manganese–ligand distances determined from magnetic resonance data agree well with those from X-ray crystallographic data.[23]

The determination of binding constants and number of binding sites in binary metal–enzyme complexes from the analysis of paramagnetic ion effects is fairly straightforward. Equation 1 will contain two terms due to the free and bound forms of the ion, respectively; the relaxation due to the free ions can be determined in separate experiments. From the observed $1/T_{1p}$ values at different enzyme concentrations, the relaxation rate due to the bound form, $(1/T_{1p})_b$, and the binding constant may be evaluated.[11,27] With easily dissociable probes the complexity of the problem increases severalfold when substrates are introduced, since there are now four metal ion containing species in equilibrium: the fully hydrated ion, the substrate–metal complex, the enzyme–metal complex, and the ternary enzyme–metal–substrate complex. In this case many titrations are required and the analysis of the data requires the use of a computer to solve the simultaneous equilibria.[28] Other shortcomings of Mn(II) as a probe are (1) spin $^5/_2$ makes epr difficult to interpret and (2) investigations are limited to those enzyme complexes which contain metal ions.

For calcium, with an ionic radius of 0.99 Å, a paramagnetic probe has yet to be found. In this case one may resort to using lanthanides among which Gd(III), with properties similar to those mentioned for Mn(II), "qualifies"; however, it may not serve as an activator since it is trivalent. The possibility of using Gd(III) as a paramagnetic probe has recently been illustrated in a study of water proton relaxation and epr in solutions of Gd(III) and bovine serum albumin.[29]

Spin Labels. Stable organic free radicals, mainly nitroxides, can be incorporated into reagents capable of reacting with specific amino acid residues on the enzyme.[21] Substrates may also be chemically modified to contain the nitroxide group.[30] The advantages of using spin-labeled enzymes are the following. (a) Epr spectra may be measured with high sensitivity and are interpretable in terms of rotational motion since the spin is $^1/_2$. (b) A *covalently bound* paramagnetic center on the enzyme is available for nuclear relaxation studies, thus eliminating the complexity of several simultaneous equilibria in systems with dissociable paramagnetic probes. (c) The investigations may be extended to complexes and enzyme systems which do not contain metal ions or contain diamagnetic metal ions, either activators or inhibitors. Metal ion specificity, for example, may be monitored by epr or nuclear relaxation rates. (d) The dipolar interaction between paramagnetic ions and the spin label may be detected by effects on the spin-label epr spectrum.[5]

Free radicals are in general less effective than paramagnetic ions in causing nuclear relaxation. Since the electronic spin is $^1/_2$, $S(S+1)$ is only $^3/_4$ compared with $^{35}/_4$ for Mn(II) and $^{63}/_4$ for Gd(III). While spin labels are introduced with a given functional group as target, in some cases it is not possible to label only one specific amino acid residue. Furthermore, they cannot always be located sufficiently close to the active site of the enzyme and to the nuclei of interest, thereby further reducing their effect. Finally, spin labeling of proteins suffers from the same disadvantage as other kinds of protein modification, namely that it is often difficult to assess the extent of distortion introduced by the label itself.

Nuclei Suitable for Studies with Paramagnetic Probes

Protons ($I = ^1/_2$). Protons are, of course, the most suitable nuclei for nmr study of macromolecular systems. In particular, water protons with their very high concentration and strong interaction with paramagnetic ions are most widely used in nuclear relaxation studies of enzymes since in this case relaxation times can be directly measured by pulse techniques. Most substrates in enzymatic reactions contain protons, observable by continuous-wave nmr spectroscopy, and the effects of paramagnetic probes in a number of enzyme systems have been investigated.[23]

Fluorine-19 ($I = ^1/_2$). This nucleus is second only to the proton with respect to its sensitivity in nmr spectroscopy. The use of ^{19}F nmr in studies of enzymes has been rather limited since fluorine-containing compounds are not normally substrates of enzymatic reactions. However the fluoride ion and fluorine-substituted substrate analogs and inhibitors can be used as probes.

Phosphorus-31 ($I = ^1/_2$). ^{31}P is one of the very important nuclei for nmr studies of enzymes since substrates for so many different types of enzymatic reactions are phosphorus-containing compounds. Unfortunately its usefulness is limited by low sensitivity: approximately 7% compared to protons.

Thallium-205 ($I = ^1/_2$). It has recently been found that Tl(I) is a suitable probe for studying the binding of monovalent ions to enzymes.[31] *In vitro*, Tl^+ is a good substitute for K^+ in enzymatic reactions for which K^+ is obligatory.[32]

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Phosphoryl Transferring Enzymes

The detectability of the complex formation by changes in proton relaxation rates of water depends on both the binding constant and the magnitude of the difference in relaxation rates among the various species. For those phosphoryl transferring systems which have been studied in some detail, including the kinase reactions, *i.e.*

$$ATP^{4-} + XH \overset{M^{2+}}{\rightleftharpoons} ADP^{3-} + XPO_3^{2-} + H^+$$

where X is creatine,[33-35] arginine,[36] adenylate,[28,37] or pyruvate,[20,25,38] respectively, the water proton relaxation rate enhancement relative to $Mn(H_2O)_6^{2+}$ has been at least one order of magnitude. For yeast hexokinase,[4] where X is glucose and 3-phosphoglycerate kinase,[4] the observed changes have been considerably smaller, and it has not been ascertained whether this is a consequence of weak binding or low enhancements, or both.

In the initial studies of enzyme–metal–substrate interactions by proton relaxation rate of water with Mn(II) as probe, two patterns of behavior were noted.[9] In one class of metal-activated enzyme reactions (type II), a binary E–Mn complex with a highly enhanced proton relaxation rate is observed, and the enhancement decreases in the ternary enzyme–Mn–substrate complexes. In another class (type I), very little proton relaxation rate enhancement is observed in the binary complex, and only when nucleotide, Mn, and enzyme are simultaneously present in solution does a considerable enhancement ensue. Ternary enzyme–metal–substrate complexes of nonnucleotide substrates are not observed with type I enzymes by the criterion of enhancement. On the basis of this empirical classification, it was suggested that the coordination scheme associated with type II is a metal bridge ternary complex, E–M–S, and that associated with type I is a nucleotide bridge ternary complex, E–S–M.[4,23] Subsequently it was found that this criterion, based on limited measurements of the enhancement at a single temperature and frequency, should be applied with caution since the enhancement may vary with frequency or temperature. However, other types of measurements on pyruvate kinase (type II) and creatine kinase (type I) which will be discussed (*vide infra*) have confirmed the original assignment of coordination schemes.

More complex patterns of behavior have been observed with metal-activated enzymes which catalyze two-step reactions, and it would appear that M–E–nucleotide–M structures must be considered. For example, pyruvate carboxylase,[39] which is the first naturally occurring manganese metalloenzyme found, catalyzes reactions 4 and 5. Only substrates of the

$$Mn\text{–}E\text{–}biotin + ATP^{4-} + HCO_3^- \overset{Mg^{2+},\ acetyl\text{–}CoA}{\rightleftharpoons} ADP^{3-} + HPO_4^{2-} + Mn\text{–}E\text{–}biotin\text{–}CO_2 \quad (4)$$

$$Mn\text{–}E\text{–}biotin\text{–}CO_2 + CH_3COCO_2^- \rightleftharpoons Mn\text{–}E\text{–}biotin + {}^-O_2CCOCH_2CO_2^- + H^+ \quad (5)$$

second step interact with enzyme-bound Mn(II), as evidenced by values of water proton relaxation rate enhancement (type I behavior). The first step which involves phosphoryl transfer from ATP requires an added divalent metal ion. Another enzyme catalyzing a two-step reaction, phosphoenolpyruvate synthetase (eq 6 and 7), appears to require two metal ions,[40] one

$$ATP^{4-} + E \overset{M^{2+}}{\rightleftharpoons} E\text{–}PO_3^{2-} + AMP^{2-} + HPO_4^{2-} + 2H^+ \quad (6)$$

$$E\text{–}PO_3^{2-} + CH_3COCO_2^- \overset{M^{2+}}{\rightleftharpoons} CH_2{=}C(CO_2^-)OPO_3^{2-} + E \quad (7)$$

metal ion bound to the nucleotide and another bound to the enzyme as evidenced from proton relaxation rate of water and from the requirement for a divalent metal ion for reaction 7.

A large number of metal-activated enzymes have been surveyed for the pattern of the water proton relaxation rate enhancements of the binary and ternary complexes at one temperature and frequency.[23] The data have been used to determine binding constants. Recently the methods of computing the binding constants have been revised.[28] In general, a comparison of the binding constants determined by water proton relaxation rate and epr measurements with those derived from kinetic studies of the enzymatic reactions indicates that the complexes observed by magnetic resonance are the kinetically active species.

The two phosphoryl transferring enzymes of rabbit muscle, pyruvate kinase[20,25,31,38,41] (type II) and creatine kinase[42] (type I), were more fully investigated. For pyruvate kinase, in addition to water proton relaxation rate measurements for binary[20,25] and ternary[38,41] Mn(II) complexes, the relaxation rate of other nuclei due to Mn(II) have been measured in the appropriate enzyme complexes, in particular, ^{19}F in a reaction product[41] and ^{205}Tl as a substitute for the obligatory monovalent ion.[31] For creatine kinase, two paramagnetic probes have been used, Mn(II) and a stable nitroxide free radical [*N*-(1-oxyl-2,2,5,5-tetramethyl-3-pyrrolidinyl)iodacetamide].[5] The effect of both probes on the relaxation rates of water protons and of substrate molecules have been investigated for enzyme complexes.[42]

Rabbit Muscle Pyruvate Kinase. Pyruvate kinase

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catalyzes the phosphorylation of ADP by phosphenolpyruvate (eq 8). A divalent and a monovalent ion are

$$ADP^{3-} + CH_2{=}C(CO_2^-)OPO_3^{2-} + H^+ \underset{}{\overset{Mg^{2+},\,K^+}{\rightleftharpoons}} ATP^{4-} + CH_3COCO_2^- \quad (8)$$

obligatory for activity.[43] The stoichiometry and dissociation constant of the binary pyruvate kinase–Mn(II) complex have been determined over the limited temperature range (5–37°) of enzyme stability by monitoring the amount of Mn(II) free in solution from the intensity of its epr spectrum.[25] It has been found that there are four Mn(II) ions bound per enzyme molecule, corresponding to the number of subunits of this enzyme.[44] An equilibrium between at least two conformations of the enzyme, as reflected in the binding properties, has also been observed.[25]

The proton relaxation rate of water in solutions of Mn(II) is very much enhanced in the presence of pyruvate kinase, approximately 25-fold at 24 MHz at room temperature. The phenomenon may be reversed by magnesium which competes for the manganese site as anticipated from the fact that both can serve as activators of the enzymatic reaction.[20] A study of the water proton relaxation rates of the binary pyruvate kinase–manganese(II) complex at different frequencies (8.13–60.0 MHz) as a function of temperature has established the physical mechanism of the enhancement. Contrary to the expected behavior (*cf.* Figure 1) the relaxation rates at 8.13 MHz are lower than those at 24.3 and 40.0 MHz. (*cf.* Figure 2). From this finding, it is concluded that the correlation time for the interaction of water protons with the spin of the bound Mn(II), τ_c, as well as $f_1(\tau_c)$ (*cf.* eq 2), is a function of frequency and that the frequency-dependent contribution to τ_c must be identified as the electron-spin relaxation time, T_{1e}, since it is the only possible frequency-dependent correlation time.[13] Unlike the fully hydrated ion, where the correlation time for the dipolar interaction is the tumbling time of the complex, τ_r (*cf.* eq 3a), for Mn(II) bound to the enzyme it is the sum of the electron relaxation time T_{1e} and the mean residence time of a water molecule τ_M. After evaluation of T_{1M} the number of water molecules, n, remaining in the first coordination sphere of bound Mn(II) was estimated (eq 1) to be 3 when r is set equal to 2.9 Å.[25] Assuming that Mn(II) retains its normal coordination number of six this means that the ion is attached to three points on the enzyme and is likely to induce a conformational change. Such conformational change has indeed been detected by protein difference spectroscopy.[45]

A decrease in the proton relaxation rate of water upon addition of a substrate S to the binary E–M complex, where S may be ATP, ADP, pyruvate, or phosphoenol-

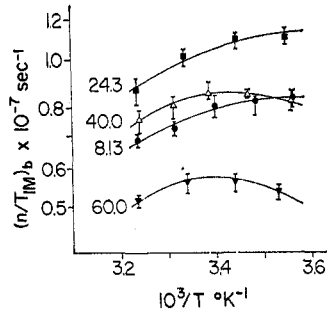

Figure 2. The proton relaxation rate of water (multiplied by the number of water molecules) in the coordination sphere of Mn(II) bound to pyruvate kinase as a function of the reciprocal absolute temperature at four resonance frequencies (in MHz).[25]

pyruvate, gives direct evidence for the formation of ternary complexes.[38] For the phosphoenolpyruvate complex $1/T_{1p}$ is reduced by one order of magnitude; unless all the water in the first coordination sphere has been displaced, the correlation time for this complex is significantly shorter than for the binary complex.

In the presence of potassium ions, the dissociation constant for Mn(II) in the ternary enzyme–metal–phosphoenolpyruvate complex, as monitored by the water proton relaxation rate, is smaller by almost two orders of magnitude than that of the binary enzyme–metal complex. When potassium is substituted by the inert (nonactivating) tetramethylammonium ion, the binding of Mn(II) in the ternary complex is not tightened, strongly suggesting a direct role for monovalent ion activators in the ternary complex.[46] The elucidation of the function of the monovalent ion has been approached by monitoring the nmr spectrum of monovalent thallium-205, which can serve as the obligatory monovalent ion activator.[31,32] From the ^{205}Tl relaxation rates the Tl–Mn distance in the pyruvate kinase–Mn(II) and the pyruvate–Mn(II)–phosphoenolpyruvate complexes has been estimated to be 8.2 and 4.9 Å, respectively.[47] The difference implies the existence of a substrate-induced conformational change.

The Fluorokinase Reaction.[48] Pyruvate kinase also catalyzes the phosphorylation of fluoride ion in reaction

$$ATP^{4-} + F^- \underset{HCO_3^-}{\overset{Mg^{2+},\,K^+}{\rightleftharpoons}} FPO_3^{2-} + ADP^{3-} \quad (9)$$

9. The same low value of $1/T_{1p}$ for water protons of the ternary E–Mn–FPO_3 as for E–Mn–phosphoenolpyruvate (type II behavior) suggested a metal-bridge complex in this case also.[41] Direct verification was achieved from the enhanced effect of enzyme-bound Mn(II) on $1/T_{1p}$ and $1/T_{2p}$ of the fluorine nucleus of FPO_3^{2-} in contrast to the deenhanced effect on the nucleus of F^- as shown in Figure 3. The transverse relaxation rate, $1/T_{2p}$, is defined by a relation similar to eq 1, and $1/T_{2M}$ is given[49] by eq 10, where $f_2(\tau_c)$ =

(43) F. D. Boyer, H. A. Lardy, and P. H. Phillips, *J. Biol. Chem.*, **211**, 237 (1942).
(44) M. A. Steinmetz and W. C. Deal, Jr., *Biochemistry*, **5**, 1399 (1966).
(45) F. J. Kayne and C. H. Suelter, *J. Amer. Chem. Soc.*, **87**, 897 (1965).
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(48) A. Tietz and S. Ochoa, *Arch. Biochem. Biophys.*, **78**, 477 (1958).
(49) R. E. Connick and D. Fiat, *J. Chem. Phys.*, **44**, 4103 (1966), and references cited therein.

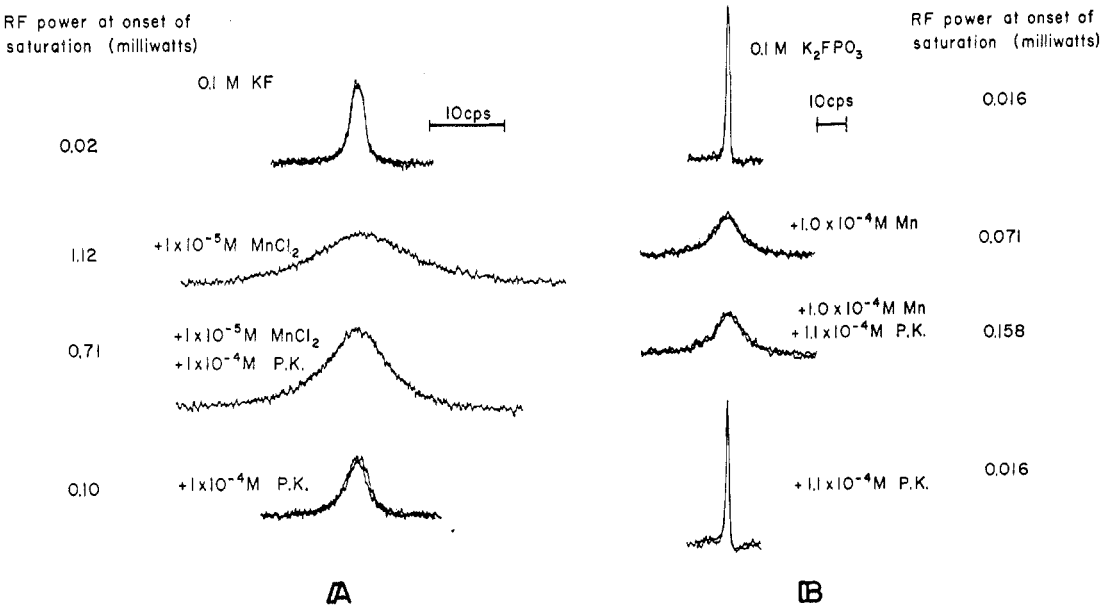


Figure 3. Effect of Mn(II) and pyruvate kinase on the resonance line width and resonance saturation properties of the nmr spectrum of ^{19}F for fluoride ion (A) and fluorophosphate ion (B); the B spectra represent the high-field component of the ^{19}F doublet. The measurements were done at 27° and pH 7.5.[41]

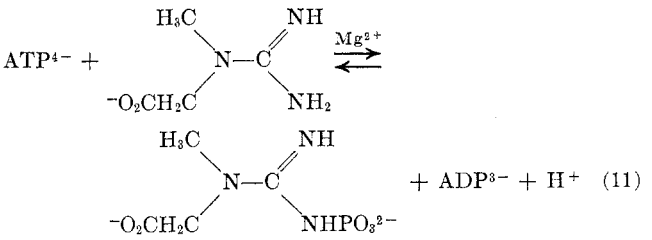
$$1/T_{2M} = Df_2(\tau_c)/(2r^6) + H[\tau_{e_1} + \tau_{e_2}/(1 + \omega_s^2\tau_{e_2}^2)] \quad (10)$$

$4\tau_{c_1} + 3\tau_{c_1}/(1 + \omega_I^2\tau_{c_1}^2) + 13\tau_{c_2}/(1 + \omega_s^2\tau_{c_2}^2)$. It will be noted that the addition of Mn(II) to a solution of KF (Mn:F ratio 1:10,000) increases greatly both the ^{19}F line width ($\propto 1/T_2$) and the saturating power[50] ($\propto 1/T_1T_2$). The concentration of Mn(II) required to show a similar effect on the ^{19}F spectrum of K_2FPO_3 is one order of magnitude greater, indicating that the Mn(II) is more distant from the fluorine in the latter case. Upon addition of enzyme, for the KF system, the ^{19}F line width narrows and the saturating power decreases, in contrast to the fluorophosphate system where the effects are in the opposite sense (*cf.* Figure 3). It may be concluded that enzyme and fluoride ion compete for Mn(II) but that enzyme, fluorophosphate, and Mn(II) form a ternary complex.[41]

From the radiofrequency powers required for saturation and the line widths, T_{1p} values could be calculated.[50] The estimated Mn–F distances for the Mn–fluorophosphate complexes ranged from 3.0 to 5.8 Å (eq 2, with $\tau_{c_2} = \tau_{c_1}$ of water protons).[41] Coordination through oxygen of the phosphoryl group can be inferred from these distances. By following the fluorine nmr, it was established that the substrate phosphoenolpyruvate competes for the same binding site on E–Mn as fluorophosphate. Quantitatively, the competition was in agreement with the relative affinity of the two substrates determined by substrate kinetics and by water proton relaxation rates. It may be concluded that phosphoenolpyruvate, the normal substrate, forms the same type of metal-bridge complex as E–Mn–FPO_3.[41]

(50) See ref 23 for the details on the technique and methods of calculation.

Creatine Kinase. A reversible phosphoryl transfer from ATP to creatine is catalyzed by this enzyme.[51]



Manganese Effects. The water proton relaxation rate due to the Mn(II)–creatine kinase complex is enhanced by a factor of 1.5 relative to $Mn(H_2O)_6^{2+}$.[9] The enhancement increases, however, to 16 and 20, respectively, for the ternary Mn(II)–ATP–enzyme and Mn(II)–ADP–enzyme complexes (type I behavior).[28,33] Moreover, the epr spectrum of the Mn(II)–ADP–enzyme complex is almost unchanged from that of Mn(II)–ADP, indicating that the environment of the Mn(II) has not altered perceptibly in the presence of enzyme. From these results it has been concluded that for creatine kinase the most probable configuration of the ternary complex is enzyme–substrate–metal, rather than enzyme–metal–substrate as found for pyruvate kinase.[9]

Substitution of different nucleotides in the ternary complexes changes the observed enhancements very markedly in the same order as their reaction velocities.[34] Recently, more precise calculations from the data for the ternary complexes of various nucleoside diphosphates have indicated that the differences in their dissociation constant may be greater than the changes in the proton relaxation rate enhancement. The sug-

(51) S. A. Kuby and E. A. Noltmann in "The Enzymes," P. D. Boyer, H. Lardy, and K. Myrback, Ed., 2nd ed, Vol. 6, Academic Press, New York, N.Y., 1962, p 515.

Table I

Internuclear Distances[a] in the "Active" Complex of Creatine Kinase

Mn^{2+}–ADP(H_2)	6.0[b]	NO··–Mn^{2+}	8.0[d]
Mn^{2+}–ADP(H_8)	3.4[b]	NO··–creatine (CH_3)	9.5[e]
Mn^{2+}–ADP(P_α)	3.0[b]	NO··–creatine (CH_2)	9.3[e]
Mn^{2+}–ADP(P_β)	3.0[b]	NO··–ADP(H_2)	7.9[e]
Mn^{2+}–creatine (CH_3)	10.3[c]	NO··–ADP(H_8)	7.3[e]
Mn^{2+}–creatine (CH_2)	9.8[c]	NO··–ADP(H'_1)	7.9[e]

[a] In ångströms; from ref 54. [b] Determined in the binary Mn–ADP complex. [c] Determined both in the quaternary enzyme–creatine–Mn–ADP complex and in the equilibrium mixture (mostly MnATP and creatine). [d] Determined in the quaternary spin-labeled enzyme–creatine–Mn–ADP complex. [e] Determined in the quaternary spin-labeled enzyme–creatine–Mg–ADP complex.

gested interpretation that these changes correspond to conformational changes at the active site induced by substrate was confirmed by chemical reactivity studies on the cysteine residues of the protein in the complexes.[52]

Investigations of the temperature and frequency dependence of $1/T_{1p}$ for the water protons in the enzyme–Mn–ADP complex led to the finding that τ_{c_1} (eq 2 and 3a) itself was frequency dependent and therefore must contain a major contribution from the electron spin relaxation time.[53] As in the binary Mn–pyruvate kinase complex, the enhancement in this case is also due to the fact that the rotational motion becomes so slow that the electron spin relaxation becomes a significant process in modulating the dipolar interaction (*vide supra*). It must be concluded that the correlation time of the complexes is not a measure of the rotational motion at the active site of the complex but some other faster motion which effects the electron spin relaxation of Mn(II).

In an attempt to map the positions of the substrates at the active site, distance calculations were made based on the dipolar interaction of the paramagnetic probes with the nuclei of the substrates.[54] The effect of Mn(II) on $1/T_{1p}$ and $1/T_{2p}$ of the protons and ^{31}P of ATP and ADP have been determined and the distances from Mn(II) in these binary complexes have been calculated (eq 2). Upon addition of the enzyme, no change was observed. Since the conditions of the experiment are necessarily such that the nucleotide is approximately 1000 times in excess of Mn(II) and enzyme concentration is limited, the fraction of nucleotide which is in the ternary complex relative to other species was too small to affect the observed average signal unless the distances had been considerably shortened. However, since the Mn–nucleotide bound to the enzyme is the only paramagnetic species which interacts strongly with the protons of bound creatine, this

(52) W. J. O'Sullivan, H. Diefenbach, and M. Cohn, *Biochemistry*, 5, 2666 (1966).
(53) M. Cohn and H. Diefenbach, to be published.
(54) J. S. Leigh, Jr., Ph.D. Dissertation, University of Pennsylvania, 1971.

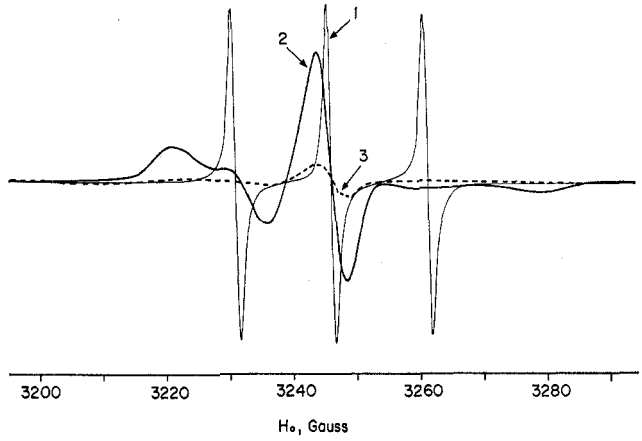


Figure 4. A comparison of epr spectra of the nitroxide spin label under various conditions: (1) unbound free radical; (2) covalently bound to creatine kinase; (3) $Mn^{II}ADP$ added to spin-labeled enzyme. Spectra 2 and 3 were recorded at 12.5-fold higher amplification than spectrum 1.[5] It should be noted that the separation of the outer peaks for the unbound free radical is ~31 G, for the "immobilized" spin-labeled enzyme ~58 G, and for the solid powder spectrum ~64 G.[21]

effect could easily be observed and the Mn–proton (creatine) distances could be evaluated. For the calculation, the value of τ_c was determined from the ratio of T_{1M}/T_{2M} (*cf.* eq 2 and 10) since $\omega_I\tau_c > 1$ and the hyperfine term in T_{2M} is negligible. The calculated distances are summarized in Table I. It should be noted that the distance from Mn(II) to the protons of creatine is the same in the abortive MnADP–creatine complex and in the equilibrium mixture containing mainly MnATP and creatine. These results imply that the metal ion is not coordinated to the phosphoryl group to be transferred.[54]

Spin-Label Effects. The epr spectrum of the enzyme labeled with a nitroxide free radical by specific reaction with one essential sulfhydryl group on each of the two subunits shows that the spin labels are highly immobilized at the active sites.[5] In spite of the fact that the spin-labeled enzyme is enzymatically inactive, it nevertheless binds metal nucleotide substrates with approximately the same binding constants as the unmodified enzyme. Direct evidence for a conformational change at the active site due to binding of metal nucleotide is afforded by the epr spectral change indicating further immobilization; in the absence of metal ion, very little change is observed. Changes of even greater magnitude among the various spin-labeled enzyme complexes are reflected in the water proton relaxation rate due to the covalently bound free radical. For example, the molar relaxivities of enzyme, enzyme–ADP, and enzyme–metal–ADP increased by factors of approximately 6, 10, and 20, respectively, relative to the unbound spin label. The geometry at the active site has been further delineated by obtaining a distance of 8 ± 2 Å between Mn(II) and the nitroxide group in the Mn–ADP complex.[5] The data for this calculation were derived from the dramatic effect of the bound Mn(II) on the epr spectrum of the bound spin label shown in Figure 4.

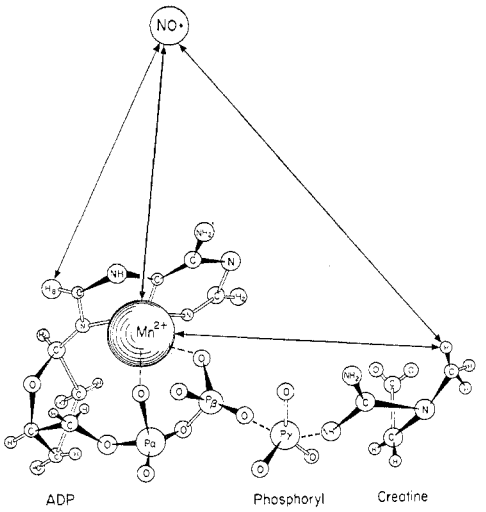


Figure 5. Perspective drawing of a model of the "active" complex in the creatine kinase reaction constructed on the basis of distances (see Table I) estimated from magnetic resonance measurements.[54]

With the paramagnetic probe covalently bound to the sulfhydryl group at the active site, it was possible to calculate distances to the protons in both nucleotide substrates, creatine and phosphocreatine, using T_1 and T_2 measurements. The value of τ_{c_1} needed for the calculation could be estimated from the ratio of T_1/T_2 or alternatively from the sum of $1/T_{1e}$ and $1/\tau_r$ (eq 3a); $1/T_{1e}$ could be estimated from the epr spectrum and $1/\tau_r$ from a Stokes law calculation for the tumbling time of the whole complex. The values thus obtained for nitroxide–proton distances are listed in Table I. A tentative model which accommodates the distances (see Table I) calculated from the binary Mn–ADP complex, the Mn–creatine distances in the native enzyme complexes, and the nitroxide–proton distances in the spin-labeled enzyme complexes has been constructed and is illustrated in Figure 5.[54]

Conclusions

Nuclear magnetic relaxation and epr studies of enzymes utilizing paramagnetic probes provide important information regarding the configuration and dynamics at the active site. A variety of paramagnetic probes and suitable nuclei exist and properties of the system in solution inaccessible by other techniques can be described. With the establishment of accurate procedures for analysis of data on ternary complexes and the understanding of the mechanism of relaxation rates due to bound paramagnetic ions it seems that the way is open for the use of nmr relaxation methods as both analytical and structural tools in enzymology.

We wish to express our appreciation to the able and enthusiastic participants in the development of the concepts described in this review: K. M. Berman, A. Danchin, H. Diefenbach, F. J. Kayne, J. S. Leigh, Jr., A. McLaughlin, A. S. Mildvan, W. J. O'Sullivan, J. E. Pearson, G. H. Reed, and J. S. Taylor. Financial support from the National Science Foundation and the National Institutes of Health is gratefully acknowledged.