^{31}P NMR Studies of Enzymatic Reactions

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I. INTRODUCTION

Phosphate compounds are ubiquitous in living cells. Almost all of the chemical energy derived from metabolic processes is stored in the form of adenosine triphosphate (ATP)[2] or other nucleoside triphosphates. Subsequently these triphosphates are utilized in all energy-requiring functions of the organism such as the biosynthesis of macromolecules, mechanical work, and transport of metabolites. In addition, the phosphorylation of neutral molecules such as glucose to form negatively charged molecules such as glucose-6-phosphate, ensures that the metabolite will be retained inside the cell. Thus, for example, every reaction in the universal glycolytic pathway of glucose metabolism involves phosphorylated substrates and products. Lastly, phosphorylated compounds, including many nucleotides and particularly 3′,5′ cyclic adenosine and guanosine monophosphates, are important regulators of metabolism.

Phosphorus has a single natural isotope ^{31}P that has a nuclear spin and a substantial magnetic moment (ca 40% of ^{1}H). Among the isotopes of atoms that are commonly present in biological molecules (H, C, N, O, P, and S), ^{31}P combines a unique set of properties that make it a useful probe to study these molecules by nmr spectroscopy. These properties are: (a) 100% abundance with a magnetic moment (compare with C, O, and S for which the abundant isotopes ^{12}C, ^{16}O, and ^{32}S have zero spin and are therefore nonmagnetic); (b) the spin of ^{31}P is ½, and the nmr spectra are thus free from quadrupole broadening that might complicate the information available from the spectra (nuclei with spin > ½, e.g., ^{14}N, spin = 1, possess an electric quadrupole moment that tends to broaden nmr lines due to interactions with fluctuating electric-field gradients at the site of the nucleus in the molecule); (c) ^{31}P nmr spectra of specific biological molecules can usually be observed in solution without interference from solvent, unlike the serious complications that may arise in observing ^{1}H spectra in H_2O solutions (1). Furthermore, in complexes with macromolecules such as enzymes, the protons of interest, unlike phosphorus, are usually obscured by the many protons in the spectrum arising from the macromolecule. At the same magnetic field ^{31}P nmr signals occur at a frequency 40% of that for ^{1}H and are obtained with a sensitivity of ca7% of that of ^{1}H.

The possibility of readily distinguishing and assigning each phosphorus atom of ATP and of ADP as well as the state of ionization of their respective terminal phosphates by ^{31}P NMR was recognized and demonstrated in 1960 (2). Shortly thereafter (3) it was shown that the metal chelates of ATP, which are generally the active species in enzymatic reactions, could be distinguished from the unliganded species by a significant change in chemical shift of the β-^{31}P of ATP. T_2 relaxation rates of ^{31}P resonances of ATP were affected by very low concentrations of paramagnetic ions, in a specific metal-dependent pattern.

With the advent of higher-frequency instruments and

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[2]The following abbreviations are used in this paper: ATP, ADP, AMP are adenosine-5′-tri-, di-, and monophosphate, respectively; CMP, cytosine-5′-monophosphate; UMP, uridine monophosphate; P_i, inorganic orthophosphate; PP_i inorganic pyrophosphate; P-, phospho; NAD, NADH, oxidized and reduced forms of nicotinamide adenine dinucleotide; NADP, NADPH, oxidized and reduced forms of nicotinamide adenine phosphate; EDTA, ethylenediaminetetraacetate.

Fourier transform nmr spectrometers, sensitivity was sufficiently increased so that ^{31}P nmr studies of enzyme systems became feasible. Not all such studies will be covered in this review, but rather a sufficiently diverse sample to illustrate the types of information relevant to enzyme structure and function that may be derived from ^{31}P nmr spectroscopy. In considering the mechanism of an enzymatic reaction, it is first necessary to establish what the reactants and products are. Examples of the straightforward use of ^{31}P nmr to identify the products of enzymatic reactions easily and directly will be presented. The more subtle characterization of the anionic species, metal chelate species, anomeric or stereoisomeric form of phosphate substrates, products, or effectors that interact with the enzyme will be discussed in some detail.

The parameters measured from nfmr studies are (1) chemical shifts (δ), (2) indirect spin-spin coupling constants (J), (3) areas enclosed by the resonances, (4) line widths (or spin-spin relaxation times T_2), and (5) spin-lattice relaxation times (T_1). The chemical shift of a nucleus represents change in its resonance frequency for a given value of the external magnetic field, due to shielding from its electronic environment; δ is defined relative to the resonance of 85% H_3PO_4. The magnetic interaction between nuclear spins mediated by the electronic environment is observable in high-resolution nmr as fine structure in the resonance. From the fine structure the strength of this interaction, represented by J, may be deduced. In studies pertaining to enzyme-substrate interactions of the kind mentioned above, in which nmr spectra of substrate molecules are observed in their enzyme-bound complexes, δ and J may depend on the environment of the substrate on the enzyme. Measurements of δ and J in suitably chosen complexes are thus potentially capable of providing information on, e.g., stoichiometry, state of metal chelation, the ionization state of the substrate, and possibly the conformational changes in the substrate produced by interaction with the enzyme.

The application of ^{31}P nmr as a nonperturbing method for determining equilibrium constants between phosphorus-containing compounds (both for the overall reaction and the interconversion step between enzyme-bound species from the areas of the resonances) will be described. The line width of a resonance is primarily determined by factors that govern the lifetime of the spin states. In particular if the nucleus changes its environment, and therefore chemical shift, by rate processes like $E + S \rightleftharpoons E \bullet S$ or $E + P \rightleftharpoons E \bullet P$, the line width changes may allow the measurement of the rate constants in these processes. The effects observed on the nmr line widths may be more dramatic if the particular nucleus is present in an itinerant group as in an equilibrium mixture of a phosphoryl transfer reaction catalyzed by a kinase. The rates or limits of rates of dissociation of enzyme-substrate complexes and rates of product formation at equilibrium have been evaluated from ^{31}P line widths. A theoretical consideration of nmr kinetics (T_1,T_2) in terms of enzyme kinetics has been presented (4). Lastly, the disposition of substrates at the active sites of a considerable number of enzymes has been mapped based on distances between paramagnetic probes and phosphorus atoms of the substrate(s) estimated from the paramagnetic effect on ^{31}P relaxation rates (T_1 and T_2). In many investigations of this type, the ^{31}P data were supplemented by, or were supplementary to, ^{1}H or ^{13}C nmr relaxation data or to data obtained by other methods.

Isotopic substitution of the nuclei attached to the resonating nucleus produces characteristic and small but detectable changes in the resonance frequency, e.g., ^{31}P resonance frequencies in $^{31}P^{16}O_4^-$ and $^{31}P^{16}O_3{}^{18}O^-$ differ by ca 0.02 ppm (5). The changes produced by more than one substitution are approximately additive. The nmr spectrum may thus be used to determine the concentrations of the different isotopically substituted species as in mass spectroscopy. By using the isotopic substitution as a label in a reaction with sequential steps, it will then be feasible to design experiments to investigate intermediates in the reaction.

II. THEORETICAL AND EXPERIMENTAL CONSIDERATIONS

A. Theoretical

The chemical shift δ and the spin-spin coupling constant J are the two spectral parameters that may be measured from the different peak positions in the high-resolution nmr spectrum. If there is a change in chemical shift (or in any other nmr parameter) due to the formation of a complex with enzyme or with metal ion, the stoichiometry can be deduced for fast-exchange conditions from the variation of the chemical shift (or the relevant parameter) with concentration of one component in the complex. If the chemical shift of a nucleus in a particular moiety on the substrate changes with pH, a comparison of δ vs pH of the enzyme-bound substrate with that in free solution is expected to shed light on the environment at the binding site on the enzyme. It might even be tempting to speculate, on the basis of such a comparison, which ionic species might be the true substrate of the reaction, particularly when the pH dependence disappears in the bound state. Due caution must be exercised in doing this, however, since

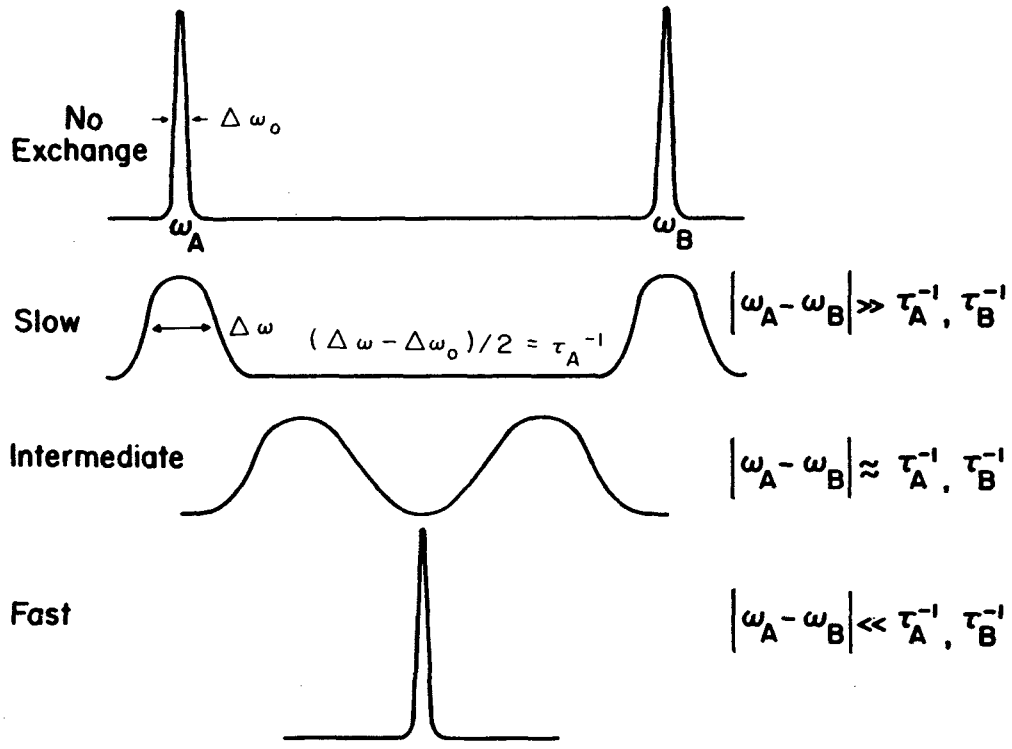


Figure 1. Effect of chemical exchange on line shape and resonance position.

the titration may be limited in *p*H range in the bound state and furthermore, may be governed by entirely different factors in the two cases being compared.

In general, when a chemical shift is observed in an *E•S* complex for a nucleus in the substrate, the shift may be due to both a change in environment and a change produced in the structure (or conformation) of the substrate induced by the enzyme. If a separation of these effects is made, it will be of considerable value to the understanding of the enzyme mechanism if the observed shift can be interpreted in terms of structural changes. The same argument applies to spin-spin coupling constants, changes in which are likely to be entirely due to conformational changes produced in the *E•S* complex. In this respect ^{31}P nmr suffers from a serious drawback in that no reliable theory applicable to molecules of biological interest is available either for δ or J that would allow meaningful correlations between δ and J measurements and molecular structure. The theoretical difficulties associated with δ and J of ^{31}P compounds were discussed by Letcher and Van Wazer (6, 7). Some empirical correlations of chemical shifts with bond angles and torsional angles have been made by Gorenstein and Kar (8). Since phosphorus compounds of biological interest are invariably phosphate derivatives, the four tetrahedrally arranged oxygens shield the ^{31}P nucleus from the environment so that shifts are small. If an O is replaced by S to form phosphorothioates, then the ^{31}P shift of the analogous compound is shifted downfield by 40-50 ppm (9).

We shall now briefly consider the effect of chemical exchange on the line shape of a resonance. Detailed theories for the calculation of these line shapes applicable to simple spin systems are available (10). However, since the systems of interest here are relatively complex, we shall only consider a few limiting conditions for which the line shapes can be qualitatively understood and the relevant rates be quantitated. If ω_A *and* ω_B are the two resonance frequencies and $\Delta\omega_o(A)$ and $\Delta\omega_o(B)$ are respectively the corresponding line widths in the absence of exchange in a two-site exchange process, three limiting conditions are of interest, viz., (See Figure 1)

$$\begin{aligned} &|\omega_A - \omega_B| >> \tau_A^{-1}, \tau_B^{-1} && \text{(slow exchange)} \\ &|\omega_A - \omega_B| \cong \tau_A^{-1}, \tau_B^{-1} && \text{(intermediate exchange)} \\ &|\omega_A - \omega_B| << \tau_A^{-1}, \tau_B^{-1} && \text{(fast exchange)} \end{aligned} \quad (1)$$

where τ_A and τ_B are the lifetimes in the two states, when exchange takes place (11). Under slow exchange the two resonances would still be centered around ω_A and ω_B, but are broadened to $\Delta\omega(A)$ and $\Delta\omega(B)$ respectively, such that

$$\begin{aligned} \Delta\omega(A) - \Delta\omega_o(A) &= \tau_A^{-1} \\ \Delta\omega(B) - \Delta\omega_o(B) &= \tau_B^{-1} \end{aligned} \quad (2)$$

Under fast exchange conditions a single resonance is observed at an intermediate frequency (ω) with intermediate line width ($\Delta\omega$) given by

$$\begin{aligned} \omega &= \rho_A \omega_A + \rho_B \omega_B \\ \Delta\omega &= \rho_A \Delta\omega_o(A) + \rho_B \Delta\omega_o(B) \end{aligned} \quad (3)$$

where ρ_A *and* ρ_B are the fractional populations of the ex-

changing species. If the intermediate exchange condition obtains, both frequency shift and line broadening result. It must be noted that the above is a simplified description of the exchange effects and does not include for example, effects of spin-spin coupling on the line shape. However, for the purpose of understanding the results considered in this paper, the description is adequate.

Relaxation measurements on ^{31}P nuclei in the presence of paramagnetic metal ions, usually replacing the obligatory divalent cation Mg^{2+} in enzymatic systems involving phosphoryl transfer and nucleotidyl transfer reactions, have been used to gather structural information on the geometry of the active site. The details of the theory underlying paramagnetic effects on spin relaxation in solutions and assumptions and limitations involved, are too vast to be described in this article. Several authors have reviewed different aspects of this problem earlier (12-15). We shall only consider some of the results pertaining to ^{31}P nmr studies to illustrate the overall potentiality of the technique in deriving biochemically relevant information in these enzymatic systems.

In practically all cases where paramagnetic probes were used in ^{31}P nmr studies, the following assumptions seem to be implicit. (i) Solomon-Bloembergen equations are valid for the paramagnetic contribution to spin-lattice (T_{1M}^{-1}) and spin-spin relaxation (T_{2M}^{-1}) rates. (ii) Contributions to the observed relaxation rates of the nucleus of interest from other mechanisms (referred to as diamagnetic effects) or due to the ligand nuclei diffusing through the paramagnetic environment without being bound (referred to as outer sphere contributions) are either neglected or accounted for by suitable control measurements. (iii) Only a small fraction (ca 0.01-1%) of the ligand complexes are bound by the paramagnetic probe. (iv) The exchange times relevant to the different complexes undergoing exchange (τ_M) are small compared to T_{1M} and T_{2M}. $\omega_S^2\tau_{2e}^2 >> 1$, where ω_S is the epr frequency and τ_{2e} is the spin-spin relaxation time of the ion; further the isotropic part of the hyperfine interaction between electron and nucleus, A, is small enough such that this interaction does not contribute to T_{1M} significantly. The anisotropic part of the hyperfine interaction is indistinguishable from the through-space dipolar interaction between the electron spin and the nucleus and should also be small in order to ensure that the latter is the exclusive source of T_{1M}. Under these assumptions, the distance r between the ligand nucleus and the paramagnetic probe may be written as

$$r = C\left[T_{1M}\frac{\tau_c^2}{1+\omega_I^2\tau_c^2}\right]^{1/6} \qquad (4)$$

where C is a constant that includes the gyromagnetic ratio of the nucleus and the magnetic moment of the paramagnetic ion, ω_I is the nuclear Larmor frequency, and τ_c is the correlation time modulating the dipolar interaction. τ_c includes the reorientation of the enzyme-substrate-metal complex, the electron relaxation time in that complex, and the exchange time between different complexes. T_{2M}^{-1} cannot be simplified to this form unless additional assumptions are invoked requiring both the shift in the resonance frequency and A to be negligible. These requirements are quite stringent. If T_{1M} and τ_c are reliably estimated, r may be evaluated. Because of the exponent 1/6 in Eq (4), inaccuracies in the estimation of T_{1M} and τ_c are fortunately scaled down in the determination of r.

In the study of paramagnetic effects on substrate nuclei bound to enzyme, it is the measurement of T_{1M} in different EMS complexes that is of primary interest. The many experimental and theoretical considerations required for ensuring the validity of Eq (4) are discussed by McLaughlin et al (16) and Mildvan and Gupta (15). A necessary condition is a reliable estimation of τ_c, which may be made from measuring T_{1M} as a function of frequency or from the ratio of T_{1M}/T_{2M}; the former is likely to be more reliable because of the stringent conditions required for simplifying the equations for T_{2M}. It may be seen from Eq (4) that if the values of T_{1M} are reliably estimated for different nuclei in a complex, the ratios of the distances of these nuclei from the paramagnetic ion do not depend on τ_c and are, therefore, independent of inaccuracies in τ_c measurement provided a single correlation time is appropriate for all the nuclei.

B. Experimental

The design of experiments that would minimize ambiguities in the interpretation of data from ^{31}P nmr studies of enzymatic reactions often requires optimization of parameters from both nmr and biochemical points of view. The constraints placed upon achieving such an optimization due to factors such as availability of appropriate nmr facilities or required quantity of enzymes of high purity perhaps contribute to the fact that when more than one research group investigates a problem even the experimental results are often at variance.

Commercial nmr spectrometers available for ^{31}P nmr range in their operating frequencies from ca 24 MHz to ca 146 MHz. A few other instruments operating at higher frequencies up to 240 MHz are becoming available. From the consideration of sensitivity and for measuring small chemical shifts, higher operating frequencies are desirable. However it is not infrequent that anisotropy of chemical shift contributes to relaxation of

^{31}P nuclei in some compounds (17). The line width due to chemical shift anisotropy increases as the square of the frequency, and depending on the fractional contribution of this mechanism to the overall ^{31}P line widths, part or all of the advantage of choosing a higher operating frequency may be lost by the increased line widths (18). In some cases the increased line width could obscure fine structure in the resonances due to spin-spin couplings or small chemical shifts.

Concentration of the compound of interest is one of the most important parameters for the purpose of optimization. Quite often it may be necessary to work at low concentrations of 1m*M* due to limitations from factors such as solubility, unavailability of sufficient material, or artifacts arising from higher concentrations that would make the results misleading or irrelevant. From the point of view of nmr it is better to work at high concentrations that would increase the signal to noise ratio per unit time utilized for signal averaging. The time factor may be especially important if the sample changes with time or if there are processes taking place that alter the particular aspect under investigation. At the highest operating frequencies available for ^{31}P nmr, it is presently feasible to work with concentrations in the vicinity of 1m*M* that require accumulations ranging from 10 minutes for sharp resonances to a few hours for broad resonances.

In studies of enzymatic reactions where the determination of nmr parameters of enzyme-bound complexes is usually the ultimate goal (whether it is achieved by directly observing enzyme-bound complexes themselves or by deducing the changes caused in the parameters of substrates in free solution due to the presence of the enzyme in the sample), a knowledge of the dissociation constants and exchange rates pertinent to the complexes being studied becomes crucial. In addition, the purity of the enzyme and in some cases the past history of the particular enzyme preparation become important in the reproducibility of the results.

In experiments dealing directly with line widths or those in which small chemical shifts that are comparable to line widths are to be measured (e.g., the ^{18}O isotope shifts of phosphate resonances), especially in compounds like ATP or other phosphorus compounds that carry substantial negative charges in the vicinity of the ^{31}P nucleus in the molecule, it is of paramount importance to remove paramagnetic cationic impurities from the sample by suitable extraction procedures (19) or by the addition of chelating agents when such an addition does not interfere with the experiment. Paramagnetic metal ions at concentrations 10^{-5} times that of ATP cause significant broadening of the ^{31}P resonances of ATP. Impurities at this level may sometimes arise from the glass of the sample tube itself.

III. EXPERIMENTAL STUDIES OF ENZYME SYSTEMS

A. Identification of Products

In a number of enzymatic reactions involving phosphates, as in nonenzymatic reactions, the most direct method of identifying and quantitating the chemical species produced has been to use ^{31}P nmr spectroscopy as an analytical tool. For the phosphocarrier protein HPr, ^{31}P nmr (20) confirmed ^{1}H nmr in identifying the phosphorylated protein (a histidine phosphorylated at the N1 position) enzymatically produced from P-enolpyruvate as the donor in the phosphotransferase system of *Staphylococcus aureus.* In contrast, the chemically synthesized product is phosphorylated at N3 of the histidine of the protein. The assignment was made on the basis of chemical shift and *p*H titration curves of model compounds, N1- and N3-P-histidine. Another example is the identification of the products of the hydrolysis of phospholipids in human serum high-density lipoprotein-3 catalyzed by α-phospholipase A2 (21). The two diastereoisomers of adenosine-5′-O-(1-thiotriphosphate) and also of the corresponding diphosphate, which are epimeric at P_α, can be distinguished by their respective chemical shifts (9, 22). It was thus demonstrated (22) that the phosphorylation of adenosine-5′-phosphorothioate catalyzed by adenylate kinase is stereospecific, leading to the formation of diastereoisomer A of adenosine-5′-O-(1-thiodiphosphate). The identification of the ^{31}P chemical shift of the diastereoisomeric product uridine-5′-(1-thiodiphosphate)-glucose A has been used to establish stereochemical inversion at P_α during the course of the reaction of uridine-5′-(1-thiotriphosphate) B with glucose-1-phosphate catalyzed by UDP-glucose pyrophosphorylase (23).

B. Covalently Bound Phosphates

The interpretation of ^{31}P nmr parameters is simplified when the phosphate of interest is covalently bound to the enzyme, since a multiplicity of exchanging species is avoided. When dissociable phosphates are bound to the enzyme, then the interpretation of chemical shifts, line widths, or paramagnetic effects on relaxation rates is often complicated by equilibria between free and bound species without complete averaging as well as by the possibility of more than one binding site.

Six enzyme systems have been investigated by ^{31}P nmr where phosphates or derivatives thereof are covalently bound to the enzyme. In two cases, alkaline phosphatase of *Escherichia coli* and rabbit muscle phosphoglucomutase, the phosphorylated enzymes in the form

of serine phosphate esters located at the active site are intermediates in the respective reaction pathways. The first enzyme is a dimeric zinc metalloprotein of molecular weight 100,000 that catalyzes the hydrolysis of phosphate monoesters and other phosphate compounds. The second enzyme, a monomer of molecular weight 67,000, requires a divalent metal ion for activity and catalyzes the interconversion of glucose-1-phosphate and glucose-6-phosphate. The ^{31}P chemical shift of the model compound for these protein phosphates, serine phosphate, is -0.4 and -4.0 ppm for the mono- and dianion respectively (pK_a = 5.8) (24). For E-P (phosphoglucomutase) the ^{31}P shift is the same as that of the dianionic form of free serine phosphate, but unlike the latter, the E-P chemical shift does not vary with *p*H in the range of 5.4 to 8.6 (25). On the other hand, the chemical shift of the serine phosphate residue of the E-P form of alkaline phosphatase is unusually low, -8.5 ppm, and is also independent of *p*H (26-28). The unexpected value of the ^{31}P shift in alkaline phosphatase has been ascribed to a strained configuration of the phosphate (26) and to a narrowing of the O-P-O angle by an estimated 2° (27). The absence of a *p*H dependence of the serine phosphate for both enzymes is a fairly common characteristic of enzyme-bound phosphates (covalently or ionically bound) as will become abundantly clear in the course of this review.

A number of enzymes interact with the coenzyme pyridoxal phosphate (pyridoxal-P) by covalent bond formation of a Schiff base (aldimine) with the ϵ-amino group of a specific lysine on the protein. Two such enzymes have been examined by ^{31}P nmr, aspartate transaminase (29) and glycogen phosphorylase (30-33). Pyridoxal phosphate binds covalently at the active site of aspartate transaminase, and the Schiff base formed participates in the reaction with the amino acid to yield the corresponding keto acid and pyridoxamine phosphate. The chemical shift in enzyme-bound forms of both compounds is insensitive to *p*H while for the compounds free in solution, δ changes between the mono- and dianion by 3.7 ppm. The ^{31}P shift of the covalently bound pyridoxal-P on this enzyme yields little information since it is insensitive to the binding of substrate (glutamate) or inhibitors (2-methyl aspartate or 2-oxoglutarate).

There are two enzymes investigated by ^{31}P that can exist in phosphorylated or dephosphorylated forms at a regulatory site rather than at the catalytic site of the enzyme. Glutamine synthetase from *Escherichia coli,* a dodecameric enzyme that catalyzes the reaction of L-glutamate, ammonia, and ATP to form L-glutamine and ADP, is regulated by adenylylation of a particular tyrosyl residue in each subunit to form a phosphodiester. The ^{31}P chemical shift of this phosphodiester (34) is 4.0 ppm, and the line width is ca 22 Hz. A line width of ca 23 Hz was calculated using a value of 2×10^{-7} s for τ_R, and the dipolar interaction with two protons of the tyrosyl $-CH_2OP$ and the single P-OH were considered as the dominant contribution to relaxation. In a later section calculations of distance from measurements of the ^{31}P relaxation due to the paramagnetic Mn(II) bound to the enzyme will be described.

The enzyme glycogen phosphorylase *a* is unique in that it has two different types of covalently bound phosphates involved in the regulation of its activity. Both the active tetrameric form *a* (subunit 96,000 daltons) and the dimeric form *b,* which is inactive without added effectors, contain one pyridoxal-5′-phosphate per subunit covalently bound in the form of a Schiff base to the ϵ-amino group of a particular lysine. Removal of pyridoxal-P inactivates the enzyme. Furthermore, the inactive dimer, phosphorylase *b,* is converted to the active *a* form by phosphorylation of one particular serine residue per subunit. The enzyme has been investigated by ^{31}P nmr by two groups, the Oxford group (30, 33) and the Wurzburg group (31, 32). The work has largely centered on pyridoxal-P since its role is not yet understood. Two features of the ^{31}P resonance of the bound pyridoxal-P have been studied, the ionic species and *p*H dependence and the effect of binding ligands at other sites of the enzyme on the chemical shift and line shape. Busby et al (30) found that each phosphate subunit of phosphorylase *b* gave rise to two signals that were not rapidly interconvertible. The signals were ascribed to two conformational states of the protein. Their line widths increased with increasing frequency. The resonances could be resolved at 36.4 MHz, and the two peaks were assigned to di- and monoanion peaks respectively. This assignment would require that protonation could only occur on one of two slowly interconverting forms. Addition of the activator adenosine monophosphate (AMP) shifts the peak mostly to the monoanion position; any dianion present would be obscured by the AMP resonance. The inhibitor glucose-6-P shifts pyridoxal-P mainly to the dianion position. However, there is no relation between the active forms and the chemical shift since in phosphorylase *a,* which is an active form resulting from phosphorylation of serine, the shift corresponds to the dianion shift, but in the active form of phosphorylase *b,* activated by AMP, the pyridoxal-P shift corresponds to the monoanionic form. The underlying assumption that the state of ionization of phosphates is the single variable that causes a change in the chemical shift is highly questionable.

The complexities of this system are explored in the papers of Feldman and collaborators (31, 32). A detailed study of *p*H effects on the chemical shift of pyridoxal-P and its Schiff base, free in solution and bound to rabbit

muscle glycogen phosphorylase in the presence of various effectors, led to the following conclusions. 1) Pyridoxal-P exists in three forms when bound to phosphorylase *b*. 2) Form I, $\delta = 0$ ppm, *p*H independent, is observed for phosphorylase *b* with no substrate analog present and is ascribed to the fully protonated form. 3) Form II, observed in the presence of arsenate (substrate analog) or the anions SO_4^{2-} and Cl^-, $\delta = -0.37$ ppm, below *p*H 7, has the same *p*H dependence as the model Schiff base. 4) Form III, $\delta = -3.81$ ppm, is observed for the active form of phosphorylase *b*, i.e., in the presence of AMP or its thio analog, AMPS, and for the active phosphorylase *a*; this shift corresponds to that of the dianionic form of the model Schiff base. The discrepancy between the results of the two groups of investigators has no obvious explanation although some of the experimental data of the second group are easier to interpret because of the use of the thioanalog of AMP to avoid overlap of the resonances from AMP and pyridoxal-P.

The last example of a covalent enzyme-phosphate is that of an enzyme inactivated by reaction with an inhibitor, diisopropylfluorophosphate, to form a phosphate bond with the active site serine residue. The proteolytic enzyme α-chymotrypsin (a monomer of 25,000 molecular weight), which normally forms an acyl intermediate from a peptide substrate, forms an inhibited phosphate triester derivative (DIP) by reaction of the active site serine-195 with diisopropylfluorophosphate. Gorenstein and Findlay (35) observed two ^{31}P peaks for DIP-chymotrypsin at ca 0 and -2.1 ppm respectively. The authors ascribed the peaks to two interconvertible forms of the enzyme since they reported that the *p*H-dependent ratio of the two peaks was reversible. However, Bock (36) and Markley (personal communication) both ascribe the downfield peak (ca -2 ppm) to an artifact; the former investigator removed this peak upon dialysis of the enzyme, while the latter observed it only with aged enzyme. Reeck et al (37) report observation of one peak, the upfield one, at +0.52 ppm at *p*H 8.0. The peak observed by Bock (36) at 0.3 ppm, *p*H 7.2, was shifted to 3.3 ppm upon denaturation of the enzyme with 5*M* guanidine HCl.

Reeck et al (37) addressed the question of the difference in active site structures between the enzyme and its almost inactive precursor, chymotrypsinogen, which differ by a short peptide, a difference too subtle to be discerned in X-ray analysis. The DIP derivatives of the two proteins were compared. The ^{31}P chemical shift of the zymogen was 2.46 ppm with a line width of 3 Hz, and under the same conditions the shift for DIP-α-chymotrypsin was 0.52 ppm and the line width was 2 to 3 times larger. The known structural difference between the two resides in the inability of the NH group of glycine-193 to form a hydrogen bond to the phosphate of DIP in chymotrypsinogen; in DIP-α-chymotrypsin, NH groups of serine-195 and glycine-193 form hydrogen bonds to phosphate. It is not clear whether the downfield shift (ca 2 ppm) is due solely to hydrogen bond formation.

C. Interaction of Substrates, Inhibitors, and Effectors with Enzymes

In this section we shall cite examples of the change in nmr parameters (δ, J, T_2, etc) of phosphorus-containing substrates, inhibitors, or effectors brought about by the presence of various types of enzymes. Of the large class of enzymes that catalyze the hydrolytic cleavage of phosphate compounds, two will be considered, alkaline phosphatase *(Escherichia coli)*, which acts on monosubstituted phosphates, and ribonuclease A (bovine pancreas), which acts on the diester phosphates of ribonucleic acid and analogous model compounds. A second group of enzymes, the kinases, which transfer the terminal phosphoryl group of ATP to various acceptors including arginine, creatine, AMP, 3-P-glycerate, and pyruvate, will be discussed. The last group of enzymes to be discussed are those that have phosphate substrates but that do not involve transfer of phosphoryl groups but rather hydrogen transfer, glucosyl transfer, or other reactions.

1. Hydrolytic Enzymes

Alkaline phosphatase was investigated in many laboratories between 1973 and 1978, and although there are many areas of agreement in the ^{31}P nmr studies, there are serious disagreements both in experimental results and interpretation. The native enzyme is a dimeric zinc metalloprotein that binds inorganic phosphate and forms a phosphorylated enzyme E-P (serine phosphate) from any phosphate substrate or the product P_i. As discussed above, it has been found (26-28) that E-P that is stable at low *p*H and is unobservable at *p*H >6.5, has a most unusual chemical shift, -8.5 ppm, considerably downfield from any phosphorylated amino acid. Several lines of evidence have been presented for the assignment of E-P. 1) The Cd form of E-P does not dephosphorylate, and the peak intensity of the Cd form at -8.5 ppm is not diminished even at *p*H 7.1 (26, 27); for native Zn-enzyme intensities of the -8.5 ppm peak and of another peak at -3.5 ppm ($E \bullet P_i$) vary inversely with *p*H (26-28), consistent with known interconversion of E-P to $E \bullet P_i$. 2) No multiplet structure is observed in E-P, but proton decoupling narrows the line for both ZnE (26) and

CdE (28) as expected for serine phosphate. The large shift of serine phosphate in alkaline phosphatase could be explained by a 2° narrowing of the O-P-O angle (27), which might arise (26) from a hydrogen bond acting in concert with a covalent bond to serine to mimic a strained cyclic ester. In the apoenzyme (no metal ion) the covalently bound ^{31}P is observed at -6.5 ppm (26, 28).

It is in the noncovalent binding of P_i, particularly the stoichiometry of P_i binding, that there are some contradictory reports. Csopak and Drakenberg (38) were the first to observe the binding of P_i at *p*H 8 to apoenzyme and the Zn_2 enzyme.

Subsequently two groups of investigators (27, 39) found 1.5-2.0 P/dimer, but another group found only 1 P/dimer (28, 40). Also the latter group finds negative cooperativity in binding of P_i (40) as evidenced not only from ^{31}P nmr but from ^{113}Cd, which yields one peak for two Cd^{2+} in unliganded E but two resonances in the dimer containing one P; only one active site can be phosphorylated. Some of the discrepancies may result from the mode of preparation of the enzyme as indicated by Hull et al (27) and more recently by Bock and Kowalsky (39). The latter investigators showed that a form of the enzyme with a Zn content of 2.4 Zn/E that had been obtained by partial removal of Zn by dialysis from a native preparation (4.9 Zn/E), retained 100% enzymatic activity, bound 1.4 P/E, and had one bound (E•P) resonance peak, -3.5 ppm at *p*H 7.8. On the other hand, a form of the enzyme containing 2.4 Zn/E that had been reconstituted from apoenzyme was only 10% active and had a broadened resonance peak at -6.0 ppm, *p*H 7.5. Hull et al (27) had also concluded that the "purging" procedure yielded a different conformational form of E•P at alkaline *p*H since the high-activity form had a resonance at -3.5 ppm and the "purged" low-activity form had a resonance at -4.2 ppm.

All investigators agree that paramagnetic metalloenzymes make E-P and E•P unobservable [Co^{2+} (26-28, 38), Mn^{2+} (28, 38, 41)] and that the metal ion is consequently close to the bound P. Estimates of the distance vary from <3.3Å (26), which places the metal in the first coordination sphere, to 7.3Å (41), which would place it in an outer coordination sphere. The distance of Mn to P in the E-inhibitor complex (*p*-amino benzylphosphate) was estimated to be 7.7Å (28). However, all distance calculations have many assumptions and are consequently approximate. Since excess P_i with the paramagnetic metalloenzymes always appears as a sharp line, it is concluded that the rate of exchange between free and bound P_i is slow (26-28, 38). Hull et al (27) analyzed in detail the line widths of the various E•P_i complexes and of an enzyme-inhibitor complex (2-hydroxy-5-nitrobenzylphosphonate) and compared the derived rates with known kinetic constants. All investigators agree that the dissociation of E•P_i is a rate limiting step in the reaction.

In conclusion, the studies on alkaline phosphatase illustrate both the usefulness of ^{31}P nmr and difficulties encountered due to poorly understood, subtle conformational changes in the enzyme. The most serious area of disagreement resulting from the ^{31}P studies of alkaline phosphatase is the stoichiometry both of metal binding and of phosphate binding to the dimeric enzyme. The discrepancy may be due in part to the previous history of the enzyme, and hopefully, future experiments will resolve the apparent contradictions.

The ^{31}P nmr studies of ribonuclease have focused on the *p*H dependence of chemical shifts in free and bound inhibitors with the objective of determining the ionic form of the enzyme-inhibitor species. Earlier work had not led to a consensus. On the basis of pioneering work on ^{1}H nmr titration data and the *p*H-binding constant curve, Meadows et al (42) concluded that the mononucleotides are bound in the dianionic form to a diprotonated active site. On the basis of the same binding curve taken in conjunction with kinetic relaxation studies, Anderson et al (43) concluded that the mononucleotides are bound as monoanions to a monoprotonated active site. From ^{31}P nmr data, Gorenstein et al (44) claim that both mono- and dianionic forms of the mononucleotide inhibitors bind to ribonuclease. Haar et al (45) found that the *p*H titration curves of the mononucleotide-enzyme complexes tested, including 2′-AMP, 3′-AMP, the isomeric cytidine phosphates (2′, 3′, 5′), and modified forms 2′-CMP oxide and 2′-deoxy-3′-CMP, as well as 2′-AMP and 3′-UMP could only be analyzed in terms of two *p*K values except for 2′-CMP, which could be explained with one *p*K, and 5′-CMP where the two *p*K values were too close to be resolved. Gorenstein et al (44) also found that two *p*K values were needed to explain the *p*H titration curves of all the mononucleotides that they investigated, 2′-,3′-, and 5′-CMP and 3′-UMP. The primary difference between the two groups is that Haar et al (45) proposed that the second *p*K derived from ^{31}P data is due to the protonation of histidine-12 (mistakenly referred to as 119), because similar *p*K values are derived from ^{1}H nmr titrations. The data of Gorenstein et al (44) yield different *p*K values, and they claim greater accuracy for their method, namely determining chemical shifts of inhibitor-enzyme complexes by titration and extrapolation under fast exchange conditions rather than the direct method with a single 1:1 complex. The direct method assumes complete complex formation at all *p*H values, and the criticism of the values of Haar et al (45) resides in the lower association constants at extremes of the *p*H range. On the other hand, acceptance of the calculations (44) of the four microscopic ionization constants, i.e., $K_{pH(EH)}$, $K_{pH(E)}$,

$K_{EH(pH)}$, and $K_{EH(p)}$ from the macroscopically observed pK values, rests on the assumption that only the ionization state of the phosphate determines its ^{31}P chemical shift. Before the controversial aspects of the interpretation of these data can be resolved, the data from the different laboratories must be in agreement. It is obvious that the ^{31}P nmr experiments on the two hydrolytic enzymes, alkaline phosphatase and ribonuclease, have answered several important questions but have opened up several new ones.

2. *Phosphoryl Transfer Enzymes*

A substantial portion of the ^{31}P nmr work on phosphoryl transfer enzymes has been performed on enzyme-bound substrate complexes. The enzymes studied are arginine kinase from American lobster tail muscle (46, 47), rabbit muscle creatine kinase (48), carp and porcine muscle adenylate kinase (49, 50), rabbit muscle pyruvate kinase (51), and 3-phosphoglycerate kinase from yeast (52). All these reactions are of the type

$$ATP + S \rightleftharpoons ADP + SP \qquad (5)$$

where S is the second substrate, and all require a divalent cation normally Mg^{2+} as an obligatory component; pyruvate kinase requires the presence of a monovalent cation K^+ in addition to Mg^{2+}. In the experiments on enzyme-bound substrates, the enzyme concentrations chosen (3 to 5 m*M*) were in sufficient excess of those of the substrates (2 to 4 m*M*) so that 80 to 90% of the substrate is in the enzyme-bound species if the relevant dissociation constants are below ca 200μM. This condition is easily met for the substrates of the enzymes listed above except for some of the substrates of creatine kinase and pyruvate kinase. The results of these experiments include measurements of nmr spectral parameters, chemical shifts, and spin-spin coupling constants involving the ^{31}P nuclei in the substrates, and equilibrium and kinetic parameters pertaining to the interconversion of reactants and products on the surface of the enzyme. We consider first the results on individual enzymes and later make a comparison of some of the results for different enzymes.

For arginine kinase (53), S in Eq (5) is arginine. Figure 2A shows the ^{31}P nmr spectrum of an equilibrium mixture of the arginine kinase reaction established with catalytic quantities of enzyme (46). The signals of the six phosphate groups contained in the reactants and products are labeled. The γ-P (ATP) signal is a doublet due to spin-spin coupling with the ^{31}P nucleus in β-P (ATP). The α-P (ATP) is also a doublet due to coupling with β-P (ATP). The β-P (ATP) signal is a 1:2:1 triplet because of simultaneous spin-spin coupling with α-P (ATP) and γ-P (ATP) with coupling constants that are indistinguishable. The β-P (ADP) and α-P (MgADP) signals are also doublets due to their mutual spin-spin coupling. The α-P signals of ATP and ADP are both rather broad due to a small spin-spin coupling of the ^{31}P nucleus in α-P with the two 5′ protons. From the areas under the different signals in Figure 2A, the catalytic equilibrium constant

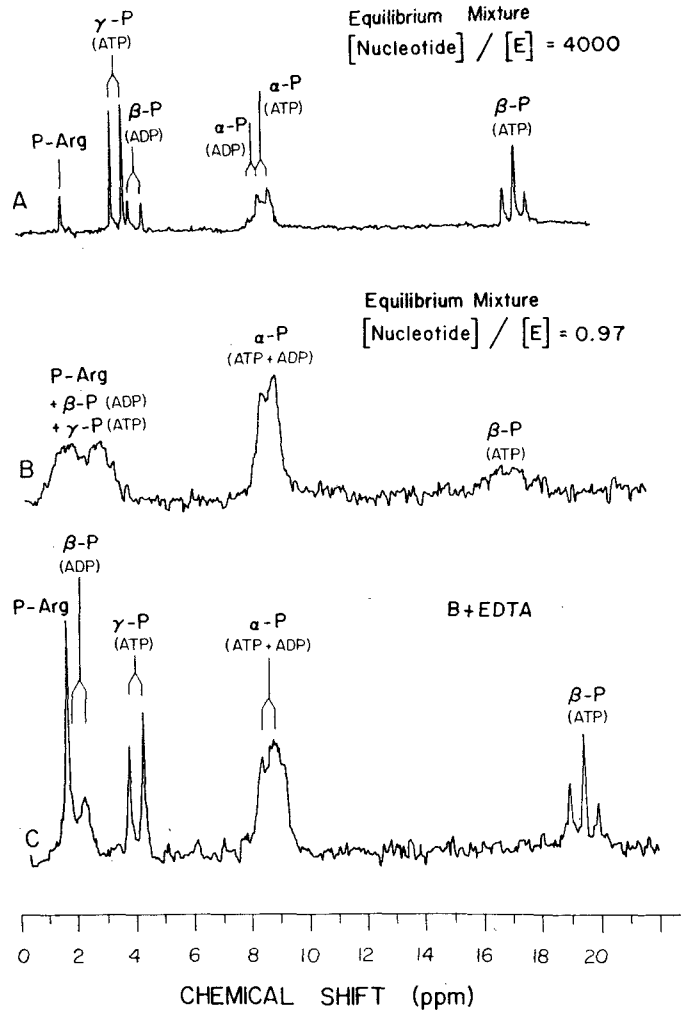


Figure 2. ^{31}P nmr spectra of the arginine kinase reaction (pH = 7.25; T = 12 °C): A) equilibrium mixture of overall reaction, catalytic concentration of enzyme; B) equilibrium mixture of enzyme-bound substrates and products; C) same as B with EDTA added to chelate Mg^{2+} and stop reaction, no chemical exchange.

$$K_{eq} = \frac{[P_1]\,[P_2]}{[S_1]\,[S_2]}$$

may be readily evaluated. At pH 7.2 and T = 12 °C, K_{eq} = 0.1 for arginine kinase.

The spectrum in Figure 2B is obtained by setting up an equilibrium mixture of the reactants and products of arginine kinase such that all the substrates are bound

to the enzyme. It can be readily shown that most of the line broadening in Figure 2B is caused by the participation of almost all the components of the system in the reaction most of the time. By adding EDTA to the sample of Figure 2B to sequester the Mg^{2+} from the enzyme-bound complexes, the reaction was stopped. It is clear from Figure 2C that binding to the enzyme in the absence of reaction does not cause excessive broadening of ^{31}P signals. It may also be seen from Figure 2C that the ^{31}P chemical shifts of the different phosphate groups, with the exception of β-P (ADP), remain essentially unaltered by binding of the substrates to the enzyme. The position of β-P (ATP) at a higher field than in Figure 2B is due to the removal of Mg^{2+}; the same phenomenon occurs for free ATP. Furthermore the equilibrium of the reaction of enzyme-bound substrates appears to favor P-arginine much more than is the case for catalytic levels of the enzyme. (Compare Figure 2B with 2A.)

The three limiting conditions of exchange defined in Figure 1 readily explain the line shapes in Figure 2B. The α-P (ATP) $\rightleftarrows$ α-P (ADP) exchange is in the fast-exchange limit due to the small chemical shift between the signals, and a single resonance is obtained for both these signals. The β-P (ATP) $\rightleftarrows$ β-P (ADP) exchange is in the slow-exchange limit because of the large chemical shift between the respective signals. The γ-P (ATP) $\rightleftarrows$ P-(P-arginine) falls in the intermediate-exchange condition accompanied by both a line broadening and a frequency shift.

From the line width of the β-P (ATP) signal in Figure 2B relative to that in Figure 2C, the lifetime of the E•MgATP-arginine complex in the reaction can be readily determined (cf Eq 2). Furthermore from the areas under the superposed α-P signals of ATP and ADP and the isolated β-P (ATP) signals, the ratio of the concentrations [E•MgATP-arginine] and [E•MgADP•P-arginine] may be obtained. Thus the equilibrium constants and exchange rates associated exclusively with the interconversion step of the reaction $E{\bullet}S_1{\bullet}S^2 \rightleftarrows E{\bullet}P_1{\bullet}P_2$ are obtained from the experiment in a rather straightforward manner.

The ability to isolate and monitor exclusively the step of interconversion of the reactants and products on the surface of the enzyme is one of the attractive features of the ^{31}P nmr of enzyme-bound substrates of these reactions. It must be noted, however, that the accuracy in the determination of the different parameters, equilibrium constants, and exchange rates is primarily governed by the feasibility of having the substrates present only in the active complexes, e.g., in the case of Figure 2B all enzyme-bound complexes should be in either of the two forms E•MgATP•arginine or E•MgADP•P-arginine. Departure from this condition leads to the presence of a substantial fraction of enzyme-bound complexes that do not undergo the reaction. In such cases the interconversion rates determined from the spectra may only be taken as lower limits of the rates.

A comparison of the interconversion rates with the overall rates of the reaction obtained under the same conditions, by enzymatic assay, allows one to conclude whether the interconversion step is the rate-limiting step for the reaction. The equilibrium constants of the enzyme-bound reactants and products are of physiological relevance in situations where the enzyme and substrate concentrations are comparable. For the specific case of arginine kinase the equilibrium constant

$$K'_{eq} = \frac{[E{\bullet}MgADP{\bullet}P\text{-arginine}]}{[E{\bullet}MgATP{\bullet}arginine]} \cong 1.2$$

is an order of magnitude different from K_{eq}. The interconversion rates in either direction are an order of magnitude faster than the overall rate of the reaction, indicating that the interconversion is not the rate-determinating step of the arginine kinase reaction.

The *p*H dependence of the chemical shift of β-P of enzyme-bound MgADP (47) was compared to that of free MgADP, which has a pK_a of ca 6.0. However, the pK_a of β-P (MgADP) bound to arginine kinase is ca 7.5. Furthermore, the variation of chemical shift with *p*H is opposite in direction to that in free MgADP. This variation suggested the possibility that the *p*H dependence of the chemical shift of MgADP bound to the enzyme may actually be due to the titration of one of the amino acid residues on the protein in the proximity of the β-P of bound MgADP. The pK_a of the *p*H profile of the enzymatic activity of arginine kinase is about ca 7.35. The amino acid residue in the proximity of β-P (MgADP), which governs the apparent pK_a of this group on the enzyme, may have a crucial role in the activity of the enzyme.

Experiments have also been performed on the transition state analog complexes E•MgADP•NO_3^-•arginine of arginine kinase (47). It was shown that when all the components of the above complex are present the chemical shift of β-P (MgADP) in the complex is intermediate between those of E•MgADP and E•MgATP.

The ^{31}P nmr results of enzyme-bound substrates of creatine kinase (54) are broadly similar to those on arginine kinase (48). The only significant chemical shift occurs for the β-P of MgADP, the equilibrium on the enzyme favors greater phosphocreatine production compared to the overall reaction, the interconversion rates of enzyme-bound reactants and products are not rate limiting. (See summary of chemical shifts presented in Table I.) Arginine kinase is considered to be the inverte-

Table 1. ^{31}P Chemical Shifts of P-enolpyruvate and its Complexes with Pyruvate Kinase at *p*H 8.0.

Added Components	δ (ppm)
None	0.9
Pyruvate kinase	0.2
Pyruvate kinase + K^+	0.2
Pyruvate kinase + K^+ + Mg^{2+}	−2.1
Pyruvate kinase + K^+ + ADP	−3.1
Pyruvate kinase + K^+ + ADP + Mg^{2+} (equilibrium mixture)	−3.6

brate analog of creatine kinase and the similar behavior pattern is consistent with this idea. However the chemical shift of the β-P of MgADP bound to creatine kinase is independent of *p*H in the range 6.0 to 9.0, in contrast with the result for arginine kinase discussed earlier.

The chemical shift of the β-P (MgADP) in the transition state analog complex increased in the direction of MgATP in experiments with creatine kinase in the same manner as for arginine kinase. The change in chemical shift was observed with nitrate as well as formate ions. Furthermore, the β-P (MgADP) resonance shows a splitting in the broad resonance indicating a conformational heterogeneity of the bound nucleotide at the active site (55). This effect was observed more clearly for creatine kinase than for arginine kinase.

In the case of creatine kinase, the dissociation constants of the different substrates from their enzyme-bound complexes are rather large, leading to the presence of substantial concentrations of substrate molecules not present in the enzyme-bound form in these experiments. The analysis of the experiments, although similar, is not as straightforward as in the case of arginine kinase.

The adenylate kinase reaction ATP + AMP $\overset{Mg^{2+}}{\rightleftharpoons}$ 2ADP (56) is particularly well suited for study by ^{31}P nmr since all four substrates contain ^{31}P nuclei. Of particular interest in the case of this reaction was the attempt to establish the distinction, if any, between the acceptor and donor ADP molecules that are converted to ATP and AMP respectively in the reaction and to pinpoint the possible role of Mg^{2+} in effecting such a distinction (49). The presence of two identical ADP molecules as substrates provides an additional chemical exchange process that is unique to this enzyme. This exchange between the acceptor and donor ADP molecules (see Figure 3) is unrelated to the chemical exchanges occurring during the adenylate kinase reaction, but should be considered in any detailed interpretation of the spectra.

The key experiment that provided the clue for the possible distinction between the donor and acceptor ADP is illustrated in Figure 3. The ^{31}P nmr spectrum of an equilibrium mixture of enzyme-bound substrates of porcine adenylate kinase is obtained after ca 4 h of nmr signal averaging as shown in Figure 3A. In contrast to the arginine kinase experiments, the spectrum in Figure 3A is difficult to explain, particularly since no resonances are seen either in the region where AMP resonates (ca −4 ppm) or in the region for β-P of MgATP (ca 19.0 ppm). Note the presence of a small P_i peak at ca −1.5 ppm indicating that some ATP hydrolysis occurred during the signal accumulation.

Attempts to obtain a signal to noise ratio better than that in Figure 3A by signal averaging for ca 12 h on the same sample yielded the spectrum shown in Figure 3B which appears quite different from that in Figure 3A. The P_i resonance is considerably enhanced, there is an AMP resonance, and there are now two resonances in the region of the β-P resonance of ADP (4-7 ppm) instead of one at 6.5 ppm as in Figure 3A. The appearance of the spectrum in Figure 3B may be explained by noting that as the irreversible ATP hydrolysis proceeds concomitantly with the adenylate kinase reaction, the concentration of ATP is progressively depleted while the concentrations of AMP and P_i increase. The resonance of β-P (MgADP) is in slow exchange with that of β-P of MgATP in the adenylate kinase reaction as in the arginine kinase reaction. As the ATP is progressively depleted a greater fraction of MgADP would be found in abortive enzyme-bound complexes, e.g., E•AMP•MgADP complexes. The MgADP resonance is then effectively narrowed and becomes observable.

The above explanation implies that in Figure 3A the resonance of MgADP is too broad to be observed because of chemical exchange in the presence of sufficient amounts of ATP in the sample. To verify this interpretation, a small quantity of ATP was introduced into the sample of Figure 3B and the ^{31}P nmr spectrum was recorded again. In the resulting spectrum, shown in Figure 3C, the MgADP resonance is significantly broadened. There should then be an appreciable concentration of ATP in the sample of Figure 3C although no resonance is observable in the vicinity of 19 ppm. Apparently, this resonance is still too broad to be observable when turnover is occurring. However, addition of a sufficient quantity of EDTA to the sample of Figure 3C to remove Mg^{2+} from the reaction complexes, stops the reaction. The spectrum of the resulting sample shown in Figure 3D does exhibit an unmistakable β-P (ATP) resonance.

It may also be seen from Figure 3D that the two β-P (ADP) signals of Figure 3B merge into a single peak. This result, along with several other experiments, was used to show that a single resonance is obtained for the two β-P (ADP) groups due to fast exchange between the identical ADP molecules at the acceptor and donor sites on the enzyme. The rate of this exchange is severely reduced in the presence of Mg^{2+} leading to two distinct resonances in Figure 3B. The evidence strongly suggests that MgADP does not bind at the donor ADP site. Furthermore, independent experiments on ATP bound to the enzyme in the presence of varying concentrations of Mg^{2+} show that while ATP or MgATP bind at the ATP site, only metal-free ATP binds at the AMP site. It has also been shown that the optimal Mg^{2+} concentration for the reaction is $[Mg^{2+}] = [ATP] + \frac{1}{2}[ADP]$, i.e., one metal ion per reaction complex. It is quite evident that in addition to facilitating catalysis of the reaction as in other kinases, the Mg^{2+} provides the distinction between acceptor and donor ADP molecules on the enzyme.

The equilibrium constant K_{eq} for the adenylate kinase reaction at catalytic enzyme concentrations determined from ^{31}P nmr is ca 0.4 at 4 °C and *p*H = 7.0; K'_{eq} = [E•ADP•MgADP]/[E•AMP•MgATP] is estimated to be ca 1.5. The equilibrium is thus appreciably shifted towards ADP in the enzyme-bound species. The interconversion rates in either direction are an order of magnitude faster than the overall rates of the reaction and are, therefore, not rate limiting.

Diadenosine pentaphosphate ($A_{P_5}A$, a symmetrical molecule) is a potent inhibitor of the porcine adenylate kinase reaction. The distinction between the acceptor and donor ADP bound to adenylate kinase established in the experiments described above, suggested that in the enzyme-inhibitor complex $A_{P_5}A$ may not be symmetrical. This asymmetry is clearly depicted in the ^{31}P nmr spectrum of the E•$A_{P_5}A$ complex compared with the spectrum of $A_{P_5}A$ or Mg•$A_{P_5}A$ free in solution (see Figures 4A, 4B, and 4C). In the spectrum of the E•$A_{P_5}A$ complex (Figure 4C) the resonances of 1-P and 5-P (ca 11 ppm) are resolved and those of the three middle phosphate groups 2-P, 3-P, and 4-P are also distinct from each other. The resonance of 3-P is in the middle of the three upfield resonances. The resonances of 2-P and 4-P, on either side of the 3-P resonance, are broadened because 2-P and 4-P can interchange when $A_{P_5}A$ dissociates from the E•$A_{P_5}A$ and binds again. This exchange preserves the identity of 3-P. Addition of Mg^{2+} to the sample of Figure 4C highly accentuates the asymmetry of the enzyme-bound $A_{P_5}A$ in the E•Mg$A_{P_5}A$ complex (Figure 4D). It has been shown (57) that only one metal ion binds readily to an $A_{P_5}A$ molecule. All five phosphates in this complex resonate at clearly distinct

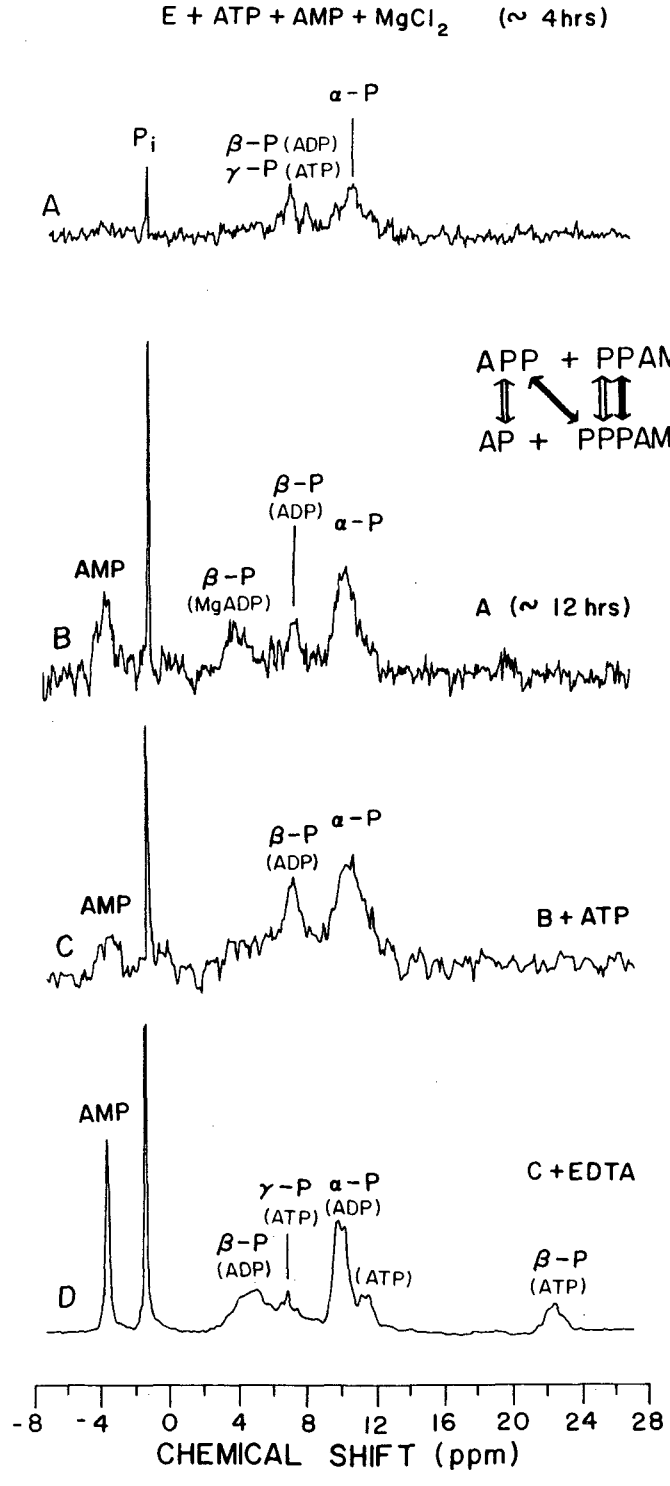


Figure 3. ^{31}P nmr spectra of an equilibrium mixture of substrates and products fully bound to adenylate kinase (porcine; T = 15 °C; *p*H = 7.0): A) after 4 h of accumulation; B) same after 12 h; separate resonances for each ADP bound to enzyme; C) same as B with addition of exogenous ATP; D) same as C with EDTA added to stop reaction as in Figure 2C.

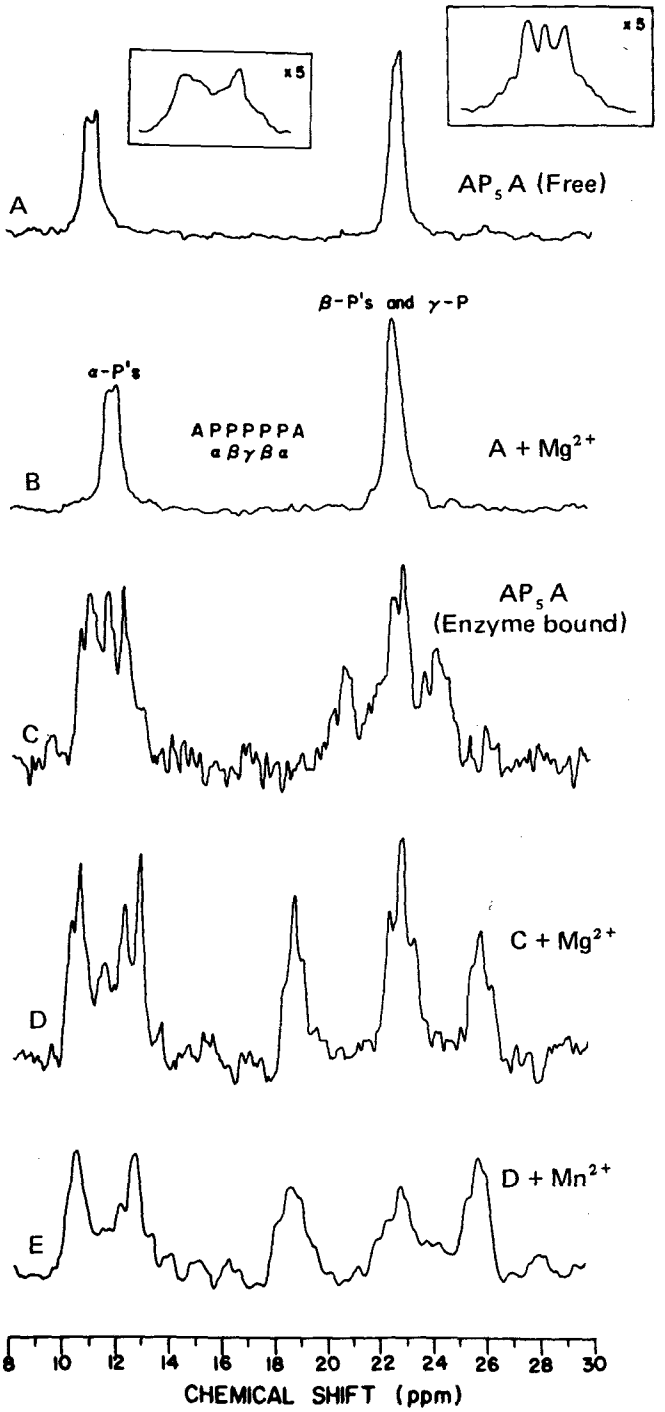


Figure 4. ^{31}P nmr spectra of Ap_5A and $MgAp_5A$ free and bound to adenylate kinase (porcine; T = 4 °C; *p*H = 8.0): A) free Ap_5A; B) free $MgAp_5A$; C) Ap_5A bound to adenylate kinase; D) $MgAp_5A$ bound to adenylate kinase; E) same as D with Mn(II) added (1:50 :: Mn:Mg).

positions. The 2-P and 4-P resonances are no longer significantly broadened compared to the 3-P resonance. Since as mentioned earlier the enzyme has a preferred site for metal-bound nucleotides, the exchange of 2-P and 4-P (and 1-P and 5-P) in the E•$MgA_{P_5}A$ complex cannot proceed with the same ease as before the addition of Mg. This exchange is now slower because an additional step has been added; after $MgA_{P_5}A$ dissociates from the enzyme, the metal ion may shift from one side of $A_{P_5}A$ to the other before $MgA_{P_5}A$ reassociates with the enzyme again. Attempts to assign 2-P and 4-P unambiguously by the addition of Mn^{2+} to the sample of Figure 4D (see Figure 4E) have not yielded definitive results. The chemical shift of ca 7 ppm between the 2-P and 4-P resonances in Figure 4D is one of the largest ^{31}P chemical shifts observed thus far for enzyme-bound substrates (noncovalent) and is comparable to the shifts observed in alkaline phosphatase (26).

Of the various kinases studied thus far adenylate kinase is the only enzyme that is found to produce a significant change in the spectral parameters associated with ATP in different enzyme-bound complexes. The two ^{31}P spin-spin coupling constants (α-P and γ-P to β-P), which are normally equal (ca 20 Hz) differ by ca 4 Hz in the E•ATP complex. Furthermore the β-P resonance in the E•MgATP complex is shifted downfield by ca 1.5 ppm.

Brown and Ogawa (58) performed a saturation transfer experiment on equilibrium mixtures of the adenylate kinase reaction to determine some of the rate constants. In these experiments the magnetization of the superposed α-P resonance of ADP and ATP is inverted by a selective 180° pulse, and the transfer of this saturation to the AMP resonance was monitored at various intervals after the inversion (see Figure 5). The rate of this transfer of saturation depends on the rates of interconversion of ADP and AMP as well as the rates of dissociation of AMP and ADP from their enzyme-bound complexes. The analysis of these data along with line width and K'_{eq} measurements allows the evaluation of all the rate constants. The rates obtained by this method agree with the results of bound substrate experiments, but K'_{eq} values do not. The technique of saturation transfer has the potential of being very useful in assignment of resonances as well as measurement of rate constants.

The enzyme 3-phosphoglycerate kinase (59) has an equilibrium constant strongly in favor of formation of ATP and 3-P-glycerate from ADP and 1,3-bis-P-glycerate, $K_{eq} \cong 3 \times 10^{-4}$. ^{31}P studies of the equilibrium mixture of this reaction with enzyme-bound substrates and products indicates $K'_{eq} \cong 1$. The ^{31}P experiments also provide distinct evidence for ATP binding to the enzyme at two sites; at one of the sites, probably the noncatalytic site, Mg^{2+} binds to the E•ATP complex much more weakly than to the other. The resonances of ATP or MgATP are not shifted significantly by binding to the

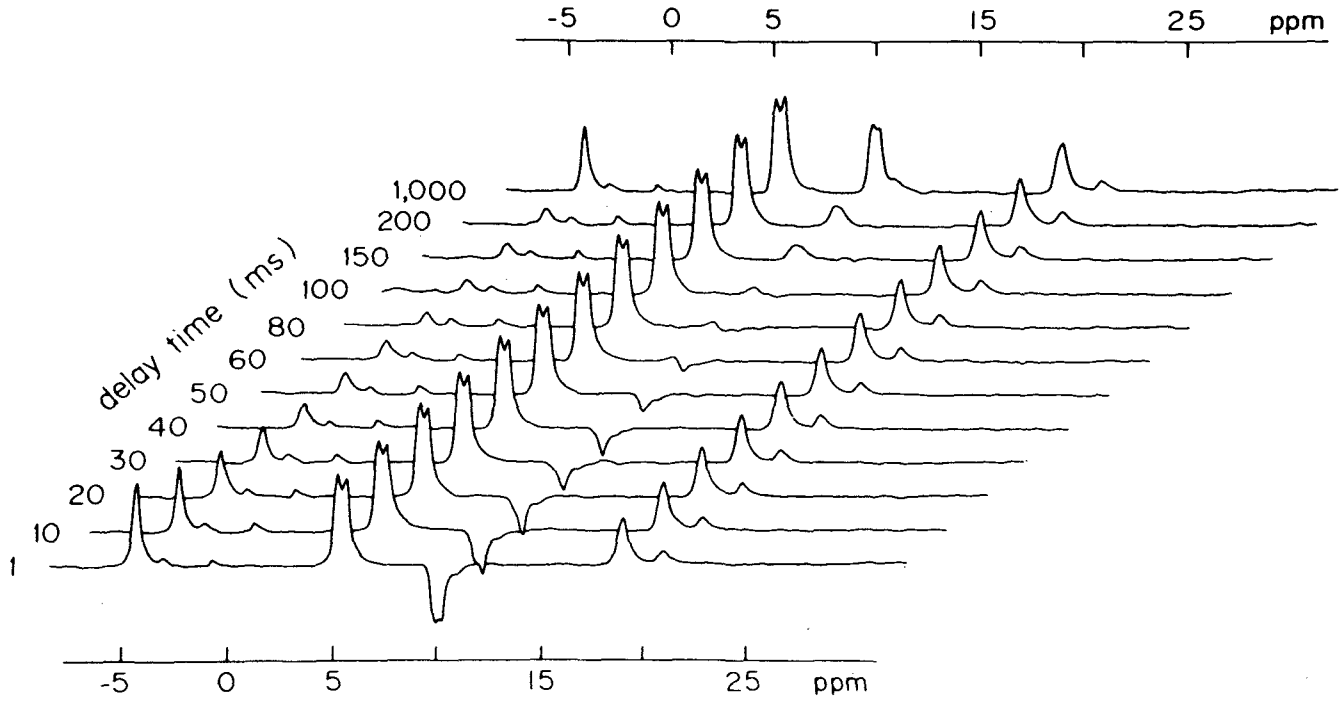


Figure 5. An inversion transfer sequence for the α-^{31}P of ADP and ATP in the adenylate kinase reaction.

enzyme. Significant upfield chemical shifts from free MgADP were observed for the β-P in the E•MgADP complex. Of the kinases studied thus far, 3-P-glycerate kinase is the only enzyme that shows an upfield chemical shift of the bound nucleotide ^{31}P resonances. Chemical shifts (ca 1.5 to 2 ppm) were also observed between the free and enzyme-bound resonances of 3-P-glycerate and 1,3-bis-P-glycerate. Sulfate ion has a pronounced effect on the ^{31}P resonances of the equilibrium mixture of bound substrates (60, 61). The chemical shift of the resonance from 3-P-glycerate changes back to its value off the enzyme, the equilibrium is shifted towards ATP, and the interconversion rate is reduced.

Among the four substrates of pyruvate kinase (62), ATP, ADP, and pyruvate bind the enzyme rather poorly; their dissociation constants are in the millimolar range. P-enolpyruvate binds the enzyme very tightly with a dissociation constant of 50 μM. With enzyme concentrations in the range of 4-5 mM and substrate concentrations in the range 2-4 mM, appreciable fractions of the substrate are not in the enzyme-bound form. The analysis of ^{31}P nmr results obtained on this reaction is subject to the limitations arising from this condition. In spite of this limitation, an appreciable amount of qualitative information is obtained from the study.

At catalytic concentrations of the enzyme the pyruvate kinase reaction has an equilibrium constant

$$K_{eq} = \frac{[\text{ADP}]\,[\text{P-enolpyruvate}]}{[\text{ATP}]\,[\text{pyruvate}]} \cong 3 \times 10^{-4}$$

strongly in favor of ATP. A striking result of the ^{31}P nmr experiments on bound substrates was that an equilibrium mixture with enzyme concentrations in excess of the substrate concentrations yielded an equilibrium constant $K'_{eq} \cong 1$.

The equilibrium constant of enzyme-bound substrates and products was also studied for another reaction catalyzed by pyruvate kinase (63): ATP + glycolate $\rightleftarrows$ ADP + P-glycolate. For this reaction $K_{eq} \geq$ ca 50 in favor of ADP and P-glycolate. Once again $K'_{eq} \cong 1$. Thus for two reactions catalyzed by pyruvate kinase for which the values K_{eq} differ by ca 10^5, both values of K'_{eq} are about unity. Furthermore, for all the kinases studied thus far K'_{eq} is ca 1 regardless of whether K_{eq} is strongly in favor of one side of the reaction (as in 3-P-glycerate kinase and pyruvate kinase) or only moderately so (as in arginine kinase, creatine kinase, and adenylate kinase). A value of K'_{eq} of unity implies that the free-energy change in the interconversion step is very small so that the overall free-energy change in the reaction occurs in the remaining steps of the reaction, probably the association and dissociation steps. Enzyme systems other than kinases exhibit similar behavior (64, 65). Whether this result proves to be a general feature in enzyme catalysis awaits more extensive data.

The phosphate groups of ATP and ADP do not show any significant chemical shift upon binding the enzyme. The largest chemical shift upon binding the enzyme oc-

curs for the ^{31}P resonance of P-enolpyruvate. As shown in Table 2, the change in δ from the dianionic form of P-enolpyruvate in solution increases progressively as each subsite of the active site is progressively occupied. However, δ is not affected by the obligatory monovalent ion K^+, but is strongly affected by Mg^{2+} although Mg^{2+} is not directly coordinated to P-enolpyruvate.

The titration of the chemical shift of β-P of enzyme-bound ATP with Mg^{2+} shows that more than two Mg^{2+} equivalents are required for saturation, indicating a metal binding site on the enzyme in addition to that on ATP at the catalytic site (66-68) and possibly a second bound ATP that has a much weaker affinity for Mg^{2+} (69). No effects that may be attributed to the obligatory K^+ ion were observed on the ^{31}P chemical shifts or line widths. Gupta and Mildvan (68) performed ^{31}P experiments on the pyruvate kinase system with ca 1.5 m*M* enzyme-site concentration and equimolar ATP or ADP concentrations. These experiments were interpreted as evidence for a second metal binding site on the enzyme in addition to that on the nucleotide.

Summary of Chemical Shifts: The chemical shifts of ATP and ADP in enzyme-bound complexes of different kinases are summarized in Table 1. ATP chemical shifts are practically unaffected by binding to the enzymes with one exception, viz, β-P of MgATP bound to adenylate kinase. β-P (ADP) is the only nucleotide resonance that shifts on all enzymes except pyruvate kinase. While there is a low field shift in most cases, an upfield shift occurs on 3-P-glycerate kinase. Thus the ^{31}P chemical shifts of nucleotide-kinase complexes do not reveal a systematic change that might signify gross similarities in the environment or conformation (or both) of the phosphate chain in these complexes. The ^{31}P-^{31}P spin-spin coupling constants in the enzyme-bound complexes of ATP and ADP are equal to those of the nucleotides free in solution within experimental error, with the exception of ATP in its complex with adenylate kinase for which a change of ca 25% is observed.

3. Other Enzymes

In this section enzymatic reactions with phosphate-containing substrates are included, but the phosphate groups are not cleaved or transferred in these reactions. For example, in hydrogen transfer reactions catalyzed by dehydrogenases, several aspects of the interaction of the pyrophosphate moiety of the oxidized and reduced forms of the coenzymes NAD or NADP and of the additional 2′-phosphate of the latter have been investigated. For dogfish lactate dehydrogenase (70) where NAD is known from X-ray crystallography to interact with an arginine residue of the enzyme, the ^{31}P resonance of the bound coenzyme shifts downfield ca 1.9 ppm from the free coenzyme position, but for lobster muscle glyceraldehyde-3-phosphate-dehydrogenase in which a lysine residue is involved in the interaction, the ^{31}P resonance of the bound coenzyme shifts upfield. The lactate dehydrogenase complexes with reduced and oxidized coenzymes respectively yield similar shifts and line shapes, which is rather surprising since the binding constant for NADH is a thousandfold greater than for NAD. At high enzyme concentrations, two peaks are observed in spectra of NADH; one in slow exchange between bound and free forms and the other in fast exchange. The latter persists at low enzyme concentrations. The existence of two aggregated forms of the enzyme is invoked by the author to explain all the observed shifts of NADH. Aggregation can be a problem at high enzyme concentration, but can also be checked independently should interpretation of the nmr results necessitate it. Aggregation is apparently not a problem with the glyceraldehyde-3-phosphate-dehydrogenase where both free NAD and NADH are in slow exchange with the bound forms independent of enzyme concentration.

Some interesting results have been found in the interaction of NADP and NADPH with *Lactobacillus casei* dihydrofolate reductase (71). More recently (72) the interaction of dihydrofolate reductase with 2′-AMP (the P-ad-

Table 2. ^{31}P Chemical Shifts of Free and Enzyme-Bound Nucleotides at *p*H 7.0 (ppm from 85% H_3PO_4).

Enzyme	Mg ADP		Mg ATP		
	α-P	β-P	α-P	β-P	γ-P
None (−Mg)	10.8	7.5	11.0	21.8	7.3
(+Mg)	9.9	5.9	10.8	19.2	5.6
Arginine kinase	11.0	3.3	11.0	19.4	5.6
Creatine kinase	11.0	3.8	10.9	19.0	5.4
Adenylate kinase	10.2	3.5*, 6.7	10.7	17.8	6.1
Phosphoglycerate kinase	11.0	7.5	11.0	19.4	6.0
Pyruvate kinase	10.0	5.7	10.9	19.2	5.5

*Second ADP (Mg) visible under conditions of slow exchange

Table 3. ^{31}P Chemical Shifts of Oxidized and Reduced Coenzyme NADP and NADPH in the Dihydrofolate Complexes (Reference, δ = 0. 50 m*M* KH_2PO_4, *p*H 8.0).

Species	δ (ppm)		
	2′-P	Pyrophosphate	
NADPH	−0.47	13.78*	
E-NADPH	−2.66	13.94	16.47
NADP	0.22	14.15	14.47
E-NADP	−2.72	14.32	16.23

*The two ^{31}P resonances of the pyrophosphate moiety have the same chemical shift.

enosyl moiety of NADP) has also been investigated. The chemical shift data are summarized in Table 3. Two sets of signals are observed for enzyme plus NADPH because of slow exchange. The 2′-P shift in the enzyme complex is independent of *p*H in the range between 4.5 and 7.5 and has a value of 1.7 ppm downfield from the free dianionic species. The pyrophosphate shifts and $-J_{PP}$ of bound NADPH are also independent of *p*H. In addition to the asymmetry introduced in the chemical shifts of the two ^{31}P peaks of the pyrophosphate moiety of NADPH upon binding to enzyme (see Table 3), an asymmetry is introduced in the ^{31}P-^{1}H coupling constants as well.

The bound form of 2′-AMP is in fast exchange with the free form. Furthermore the *p*H dependence of the shift indicates that both mono- and dianionic forms can bind although the latter binds 16 times more strongly. The pK_a is lowered from 6.0 to 4.8 upon binding. The similarity of the 2′-P shift in the bound forms of NADP and NADPH suggests binding at the same site and the same state of ionization. The increased downfield shift of 2′-P in the coenzymes (ca 1.5 ppm) over that in bound 2′-AMP indicates environments are different. The authors suggest that it may be a change in P-O-P angle, yet a thousandfold increase in binding between NADP and NADPH is not accompanied by a change in 2′-P environment as reflected in chemical shift.

For two other enzymes, glycogen phosphorylase discussed earlier and triose phosphate isomerase, which catalyzes the reversible isomerization of dihydroxyacetone-P to glyceraldehyde-3-P, the question of the chemical species that interacts with the enzyme was addressed. With glycogen phosphorylase *b* for the effector glucose-6-P, which exists in solution as 40% α-anomer and 60% β-anomer each with a characteristic ^{31}P resonance, it was found that only the α-anomer resonance was affected in the presence of enzyme. Thus, earlier determinations of binding constants using total glucose-6-P concentrations were in error. For triose phosphate isomerase, the question raised was whether the hydrated form of dihydroxyacetone-P is a substrate or inhibitor of the enzyme (73). The answer is neither; only the ^{31}P resonance of the keto form broadens (from 1 to 14.6 Hz) upon binding and also shifts downfield 0.3 ppm. The keto and hydrated forms are in slow exchange, and the dissociation constant of the hydrated form is at least ten times that of the keto form. The bound and free species of the keto form of dihydroxyacetone-P are in fast exchange (74), and the pK_a values are about the same. The line width depends on *p*H, and the authors conclude that the rate being detected, $k \cong 2X10^3 s^{-1}$ is the rate of ionization. The inhibitor glycerol-3-P behaves like the substrate, but the inhibitor 2-P-glycolate behaves differently since it is in slow exchange and its chemical shift is not *p*H dependent.

A further investigation of the binding of the inhibitor 2-P-glycolate (75) revealed that the chemical shift is the same as that of the dianionic form of the inhibitor free in solution and does not vary between *p*H 5.5 and 8.5; pK_a (free) = 6.4. Since the ^{13}C resonance of carbon-1 of bound 2-P-glycolate showed no change between *p*H 7.4 and 8.5 (although the free form shows a small effect at *p*H 6.5, pK_a = 3.5), the authors conclude that the bound form is neither P^{2-} nor COO^-P^- but a trianion and suggest that the enzyme is protonated in the binding process. The conclusion is once again based on the questionable assumption that the chemical shift in the bound form is identical to that of the corresponding ionic species in the unbound form.

D. Detection of Intermediates: Oxygen-18 Shift of ^{31}P Resonance

The recent finding of an observable shift of the ^{31}P resonance when ^{18}O is substituted for the normally bonded ^{16}O (5, 76) led to the initiation of new types of mechanistic investigations using ^{31}P nmr. As pointed out by Cohn and Hu (76), the resolution of all five ^{31}P resonances in a randomized sample of 50% ^{18}O-phosphate (five species: $^{18}O_{0,1,2,3,4}$) not only yields the ratio of $^{18}O/^{16}O$, but the ^{18}O-labeled phosphate can serve as a tracer in following reactions involving phosphate groups. Obviously the fate of phosphate oxygen can always be followed and the fate of phosphorus in all cases except under those unusual conditions when the phosphate oxygen exchanges with solvent water. Consequently it is now possible with ^{31}P nmr to follow labeled phosphorus or labeled oxygen continuously as reactions proceed.

The magnitude of the isotopic shift is small (0.0206 ppm per ^{18}O for the dianion of inorganic orthophos-

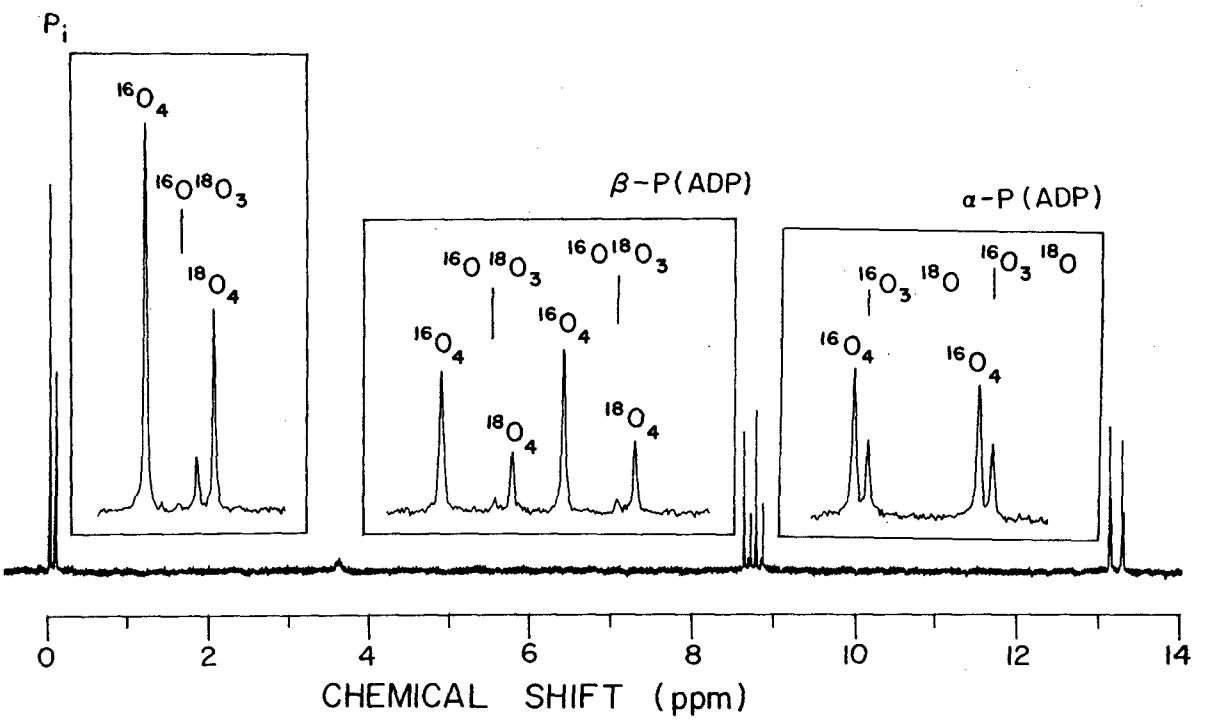
Table 4. Effect of ^{18}O on the ^{31}P Chemical Shifts of ATP.

P of ATP	Oxygen Position	$\Delta\delta$ (ppm) per ^{18}O atom
α	α-β bridge	0.017
β	α-β bridge	0.017
	nonbridge	0.028
	β-γ bridge	0.016
γ	β-γ bridge	0.016
	nonbridge	0.023

phate) and varies depending on the chemical environment of the particular oxygen. For example, as shown in the compilation of Table 4, the isotopic shift produced by the bridge oxygens on the β-P of the polyphosphate chain of ATP (^{18}O) is much smaller than that produced by the nonbridge oxygens (77). The ability to resolve these small shifts for ^{18}O may be a limitation on the usefulness of this probe. The shift between the $^{16}O_4$ and $^{18}O_4$ species can be resolved easily at 24.3 MHz (5). At high frequency (145.7 MHz) there is no difficulty in resolving $^{31}P^{18}O_4$ from $^{31}P^{18}O_3{}^{16}O$, permitting direct quantitation from peak heights but such direct quantitation is not possible at 24.3 MHz. Successful resolution and quantitation was effected at 40.5 MHz (78) by the use of a Curve Resolver set for Lorentzian curves. In this same investigation (78), the nmr method for determining rate constants of a phosphate (^{18}O)-$H_2{}^{16}O$ exchange reaction was found to be in good agreement with the conventional noncontinuous mass spectrometric analytical method for following the kinetics of the reaction. It should be pointed out that narrow lines are essential for the resolution of ^{31}P-^{18}O shifts. Paramagnetic impurities must be sedulously avoided or eliminated by chelating agents. If line broadening due to chemical exchange during the course of the reaction being investigated obscures the isotopic shift, then the reaction can no longer be followed continuously; useful spectra can only be obtained by stopping the reaction at each time point.

Several types of enzymatic reactions involving phosphates have been probed with the isotopic (^{18}O) shift of ^{31}P including an ADP-P_i exchange reaction catalyzed by polynucleotide phosphorylase (5), an ATP-PP_i exchange reaction catalyzed by valyl tRNA synthetase (78), an ATP-ADP scrambling reaction catalyzed by pyruvate kinase (80), and phosphate (^{18}O)-H_2O exchange catalyzed by inorganic pyrophosphatase (5), myosin ATPase (80), alkaline phosphatase (81), acid phosphatase (78), and fructose-1,6-diphosphatase (83). Information may be derived from two parameters: 1) the change of ^{18}O content with time, which yields the rate of the reaction, and 2) the distribution of the ^{18}O among the various phosphate (^{18}O) species at any one point in time. The latter yields the site of bond cleavage or may establish the existence of an undetectable intermediate or

Figure 6. ^{31}P nmr spectrum of the equilibrium mixture of the ADP-P_i exchange in the polynucleotide phosphorylase reaction. Initial species ADP (^{16}O) and P_i (93.4% ^{18}O); ADP:P_i::3:2.



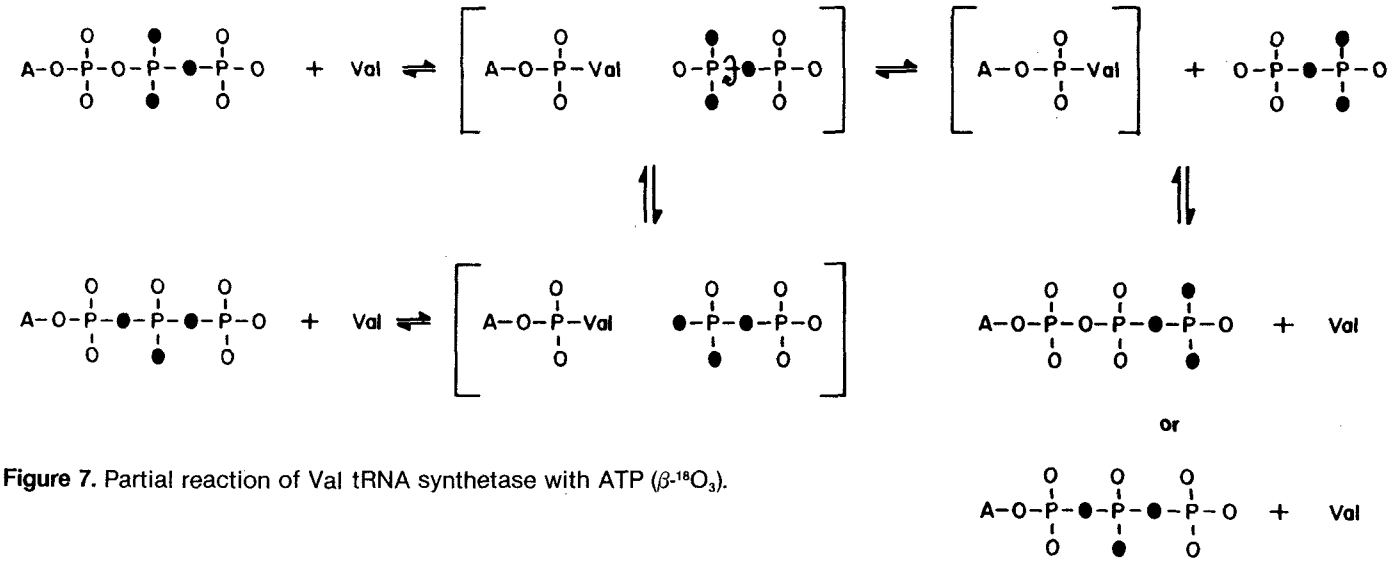


Figure 7. Partial reaction of Val tRNA synthetase with ATP (β-$^{18}O_3$).

establishes the ratio of rates of intermediate steps in the overall reaction under observation.

The catalysis of a phosphate (^{18}O)-$H_2{}^{16}O$ exchange reaction by alkaline phosphatase, inorganic pyrophosphatase, myosin ATPase, and fructose-1,6-diphosphatase had been previously established. The catalysis of the exchange reaction by human prostatic acid phosphatase was established only recently with the ^{31}P nmr method (78). The question of ^{18}O species distribution in the PO_4 ($^{18}O_{4,3,2,1,0}$) as the reaction progresses was addressed in all the studies. It has been shown by Eargle et al (83) by mass spectrometric analysis of phosphate (^{18}O) derivatives at each time point in the alkaline-phosphatase-catalyzed reaction that the distribution of ^{18}O species was random. It can be inferred that the rate of dissociation k_{-1} of P_i from the enzyme is rapid compared to k_2, the rate of formation of phosphorylated enzyme E-P, k_2 as shown in the equation

$$E + P \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E{\bullet}P \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E\text{-}P$$

This result was confirmed by $^{31}P(^{18}O)$ nmr (81) for the native Zn-enzyme, but the Co-enzyme exhibited a different nonrandom pattern. In the latter case (81), the ratio of k_2/k_{-1} is 3 ± 0.5. An extreme case was observed by Webb et al (80) with myosin subfragment 1, where complete exchange of bound P_i occurs before release of P_i from the enzyme so that from the initial $P^{18}O_4$, the only product observed is $P^{16}O_4$, and no mixed $^{16}O^{18}O$ species are observed during the course of the exchange. On the other hand, the acid phosphatase (78), like the native alkaline phosphatase, yields a random distribution of products. The fructose-1,6-diphosphatase (82), like the Co-alkaline phosphatase, is intermediate yielding a non random distribution of ^{18}O species with $k_2/k_{-1} = 2$ for the Mn(II)-activated reaction, and the ratio is 1.4 when Mg(II) is the activator. In all cases, the rate of the P_i-H_2O oxygen exchange was also determined.

The study of the exchange reaction between P_i ($^{18}O_4$) and ADP (^{16}O) catalyzed by polynucleotide phosphorylase (76) exemplifies the use of the ^{18}O shift to label phosphorus. The rate of the exchange reaction can be determined and the appearance of an α-P($^{16}O_3{}^{18}O$)- and a β-P($^{18}O_4$)-ADP species as shown in Figure 6 proves that the site of bond cleavage occurs between the α-P and the α-β bridge O (product A-O-P-^{18}O-P-$^{18}O_3$).

Valyl tRNA synthetase is known to catalyze the partial ATP-PP_i exchange reaction in the presence of valine. The fate of γ-labeled ATP (three nonbridge ^{18}O atoms) and of β-labeled ATP (two nonbridge, one β-γ bridge ^{18}O) in the presence of valine and the adenyl transferring enzyme were investigated as a function of time. The overall rate of appearance of the ^{18}O from γ-phosphate to the β-phosphate is a function of the rate of formation of the valyl-AMP intermediate and enzyme-bound PP_i, the dissociation of PP_i from the enzyme to form symmetrical PP (β- and γ-P equivalent), and the reversal of these steps as indicated in Figure 7. The appearance of ^{18}O from γ-P (^{18}O) in the α-β bridge position confirms the site of cleavage of ATP between the α-P and α-β bridge oxygen which had previously been established in the overall reaction. When the β-P-labeled ATP ($^{18}O_3$, no ^{18}O in α-β bridge) was followed under the same conditions, the rate of approach of ^{18}O to its equilibrium value at the α-β bridge was about 20% greater than for the γ-P oxygen. With the β-P($^{18}O_3$)-labeled ATP as substrate, not only is there a contribution to the α-β bridge position from the dissociation and reversal of the unbound symmetrical PP_i but also from the rotation of the nonbridge ^{18}O of the enzyme-bound PP_i to the bridge positive, i.e., scrambling.

An experiment with pyruvate kinase, a phosphoryl-transferring enzyme with β-P(^{18}O, no ^{18}O in the β-γ bridge)-labeled ATP (79) showed that scrambling occurred in the presence of the substrate pyruvate or an inhibitor, oxalate, and was eliminated by phosphoenolpyruvate. The authors favor a metaphosphate intermediate as the explanation of this phenomenon rather than a phosphorylated enzyme. Nmr spectroscopy is currently being applied (84) to the type of ^{18}O scrambling experiment introduced by Midelfort and Rose (85) to detect intermediates, i.e., bond cleavage of ATP by ^{18}O scrambling in ATP in a partial reaction catalyzed by enzymes such as glutamine synthetase, where no other partial reactions can be detected because the ADP formed does not dissociate from the enzyme.

E. Active Site Structures

We shall now consider a few examples in which ^{31}P relaxation measurements in the presence of paramagnetic metal ions were used to deduce structural information about the active site. Very often the paramagnetic relaxation data are collected for more than one nucleus on the substrate, and distance measurements are grouped together to build a model that permits conclusions that are biochemically significant. One of the initial applications of this method was to predict a possible structure of the creatine kinase active site (16, 86). Measurements of distances of Mn(II) from protons of creatine in the E•MnATP•creatine complex indicated that these distances are nearly the same as in the abortive E•MnADP•creatine•NO_3 transition state analog complex. This led to the conclusion that in the equilibrium mixture on the enzyme the Mn(II) ion may be chelated to the α- and β-phosphate groups of ATP.

A major part of the work in recent years on deriving structural information on active sites of enzymes using paramagnetic probes was contributed by Mildvan and co-workers. In all cases studied by this group, distances of different nuclei of the substrates were calculated on the basis of changes in their relaxation parameters in the presence of enzyme at concentrations much smaller than those of the substrates, which necessitates extrapolation to the enzyme-bound form and validating the condition of fast exchange under the experimental conditions used.

1. Pyruvate Kinase

Rabbit muscle pyruvate kinase was studied at some length using Mn(II), Co(II), and CrATP as paramagnetic probes. This enzyme represents a complicated example since there are two divalent cations per active complex. Distances between the paramagnetic atom and selected protons and phosphorus nuclei of ATP and of P-enolpyruvate and carbon atoms and protons of pyruvate (87-92) were calculated. From these distances it was concluded that the phosphoryl groups of ATP and P-enolpyruvate are located in the second coordination sphere of the enzyme-bound divalent cation. The role of this metal ion (in addition to that chelated to the nucleotide) for the activation of the pyruvate kinase reaction is unclear.

2. DNA Polymerase I

Thymidine-5′-triphosphate (dTTP) bound to DNA polymerase I from *Escherichia coli* was studied using Mn(II) as a paramagnetic probe (93); ^{31}P and ^{1}H relaxation rate measurements were made. A significant conclusion made by the authors was that direct coordination of Mn(II) occurs only with γ-P of dTTP on the enzyme, whereas coordination to α-P, β-P, and γ-P occurs for Mn(II)-dTTP complex free in solution. This feature indicates a possible distinction in the role of the divalent cation between a nucleotidyl transfer and phosphoryl transfer (94). The enzyme-bound Mn•dATP complex was also studied. It was suggested that the conformation of the enzyme-bound Mn•dTTP is similar to that required for Watson-Crick pairing.

3. (Na^+ + K^+) ATPase

Membrane-bound (Na^+ + K^+)-activated ATPase from sheep kidney was investigated. With Mn(II) as the paramagnetic probe (95), epr, proton, and ^{205}Tl relaxation effects were used in conjunction. It was suggested that in the presence of Na^+ the phosphate monoanion interacts with enzyme-bound Mn(II) but that in the presence of K^+ the phosphate dianion interacts. Thus protonation of an enzyme-bound phosphate would convert a K^+-binding site to Na^+-binding site. The interaction of an analog, methyl phosphonate, with enzyme-bound-Mn(II) complex was shown to lead to a second-sphere complex.

4. Phosphoglucomutase

To determine the distance between the bound metal ion and the active-site serine phosphate of phosphoglucomutase from rabbit muscle, ^{31}P nmr relaxation rates were measured (25, 96). Paramagnetic effects on ^{31}P line width (T_2) and spin-lattice relaxation (T_1) due to Ni(II) were used to deduce a distance (4-6 Å) suggesting a second sphere interaction between enzyme-bound

Ni^{2+} and ^{31}P. Ni(II) was used because of its weak paramagnetism compared to Mn(II) since it was felt that a direct coordination between Mn(II) and enzymic phosphate would not be observable. The ^{31}P spectrum of the serine phosphate at the active site of the phosphorylated enzyme has been discussed in the section on enzymes with covalently bound phosphates.

5. *Inorganic Pyrophosphatase*

Recently Hamm and Cooperman (97) made a detailed ^{1}H and ^{31}P study of P_i binding to yeast inorganic pyrophosphatase. Two phosphate sites per subunit with very different affinities (dissociation constants 0.24 m*M* and 18 m*M*) were detected. The enzyme also requires two divalent cations with disparate affinities per subunit. Using Mn(II) as a paramagnetic probe it was shown that the weaker Mn(II) site is far from both phosphate sites, which are in close physical proximity. The tighter Mn(II) site is at a distance consistent with second sphere coordination with the weaker phosphate group.

6. *Glutamine Synthetase*

The ^{31}P spectrum of the AMP-tyrosine moiety on this enzyme was discussed earlier in the section on enzymes with covalently bound phosphates. Two metal ions are required per subunit, one bound directly to the enzyme and another bound to the nucleotide in the reaction. Villafranca et al (34) used paramagnetic effects on ^{13}C and ^{31}P nmr signals from (2-^{13}C) AMP-adenylylated enzyme to measure the paramagnetic effects due to bound Mn(II) ions. Analysis of the data led them to deduce the spatial relationship of the two Mn(II) ions with respect to the regulatory, covalently bound AMP. Furthermore, distances between the adenylyl group and bound Co(II) ions were established utilizing Co(II)-induced quenching of the fluorescence emission spectrum of the ε-ATP-adenylylated enzyme. The pictorial representation of the spatial relationships derived by these two methods are shown in Figure 8. The results are in good agreement.

7. *Other Enzymes*

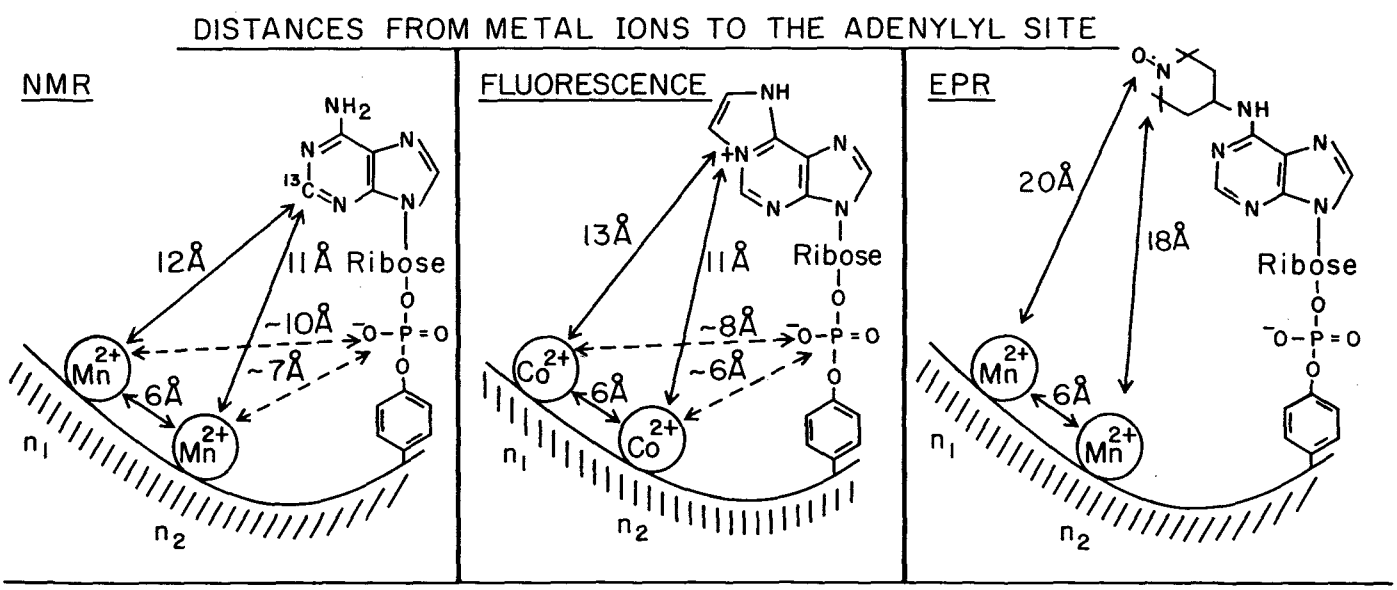
Some of the other enzymatic systems studied by the method of paramagnetic probes an ^{31}P nmr include (a) enolase (98), (b) RNA polymerase (99), (c) fructose diphosphatase (100), (d) transcarboxylase (101), and (e) alkaline phosphatase (26, 28, 41).

(a) In the case of enolase (which catalyzes the reversible hydration of P-enolpyruvate to 2-phospho-D-glycerate), ternary complexes of enolase-Mn(II) with P-enolpyruvate, an active analog of the reaction (CH_2-P-enolpyruvate) and three inactive analogs (D-P-lactate, L-P-lactate, and P-glycolate) were studied to show that Mn(II)-^{31}P distances of 5.5 Å to 6.2 Å are consistent with a second sphere coordination.

(b) For RNA polymerase the effects of Mn(II)-enzyme on adenylyl (3′-5′) uridine (ApU) bound at the initiation site and on ATP bound at the elongation site were used to conclude that the activating divalent cation binds tightly at the elongation site.

(c) ^{31}P relaxation studies were used to detect a fruc-

Figure 8. Spatial relations between the metal ion sites and the adenylyl groups of glutamine synthetase.



tose-diphosphatase-Mn(II)-fructose-1-phosphate bridge complex.

(d) In transcarboxylase paramagnetic effects on ^{31}P relaxation and ^{1}H relaxation were used to obtain information on the conformation of enzyme-bound propionyl coenzyme A.

(e) The results on alkaline phosphatase were briefly described in the section on hydrolytic enzymes.

IV. CONCLUDING REMARKS

It was known for nearly two decades that many biological molecules possess characteristic ^{31}P nmr spectra, and some of these spectra are sensitive to important parameters like *p*H, metal chelation, and so on, making ^{31}P nmr a useful technique for studying these systems. However, the instrumentation required for performing experiments that can provide biochemical information of significance became available only during the past four or five years. A large volume of research work was published in the past few years, and more papers are appearing every month. The present article covers some examples of the diverse aspects of enzymatic reactions studied by this technique and is not meant to be an exhaustive survey of all the work done in this area. It is also beyond the scope of this review to discuss the mechanistic implication of the nmr results.

The volume of the research being done with ^{31}P nmr and the variety and depth of information being extracted on biochemical systems testifies to the promise the method holds. The information obtained on enzymatic reactions includes changes in environment at the active site, chemical identification of substrates and products, dissociation constants, exchange rates, interconversion rates, and active site structural parameters. It may be noted, however, that this information is not usually obtained in a routine manner and often requires careful and subtle analysis of data, keeping in mind the physical principles of nmr. This requirement is especially stringent in the analysis of dynamical features of nmr spectra, line widths, and relaxation effects that are central to the determination of active site structures by paramagnetic effects, for example. Every analysis of these effects is contingent upon the validity of certain assumptions about correlation times, exchange times, dissociation constants, and so on. Inadequate examination of the consequence of these assumptions often leads to structural data of questionable value and consequently tenuous biochemical conclusions. Added to this is the fact that biochemical preparations vary in purity, leading to slightly different results (even by the same group) each time the experiments are performed. The above uncertainties in analysis and experiment do contribute to some of the discrepancies and disagreements in the literature.

One of the problems for which ^{31}P nmr promised to be the most appropriate technique is the question of the role of the cations in biochemical reactions of ATP, in particular the divalent cations in phosphoryl-transfer and nucleotidyl-transfer reactions. In spite of the data collected by substituting activating paramagnetic cations (Mn(II), Co(II), etc) and in several other experiments on diamagnetic enzyme-bound complexes of substrates, equilibrium mixtures, and transition-state analogs, the answer in a number of reactions remains elusive. The availability of thiophosphate analogs of nucleotides (102) may shed fresh light on the issue. Correlation of the stereospecificity of the α or β-S diastereomer with different metal activators (103) with the ^{31}P nmr spectra of enzyme-bound thioanalogs of ATP may well provide a definitive answer.

The present state of the art in nmr instrumentation produces what may be referred to as the "1-m*M* barrier" for ^{31}P nmr studies. Few experiments are feasible at concentrations appreciably lower than 1 m*M*. This is perhaps the single, most serious limitation in using the technique more widely in biochemistry. The second limitation related to ^{31}P nmr, especially in relation to some of the work described in this article, is the unavailability of a reliable theory to relate the chemical shifts and spin-spin coupling constants to structural features of the phosphorus-containing biological molecules. It has been frequently pointed out in the course of this review, for example, that a value of the chemical shift for an enzyme-bound substrate cannot be used to specify the anionic species since many shifts arising from unknown perturbations are far downfield in enzyme-bound substrates from the lowest shift attainable for the substrate free in solution. Progress in overcoming these two limitations would undoubtedly enhance the utility of this already powerful technique by orders of magnitude.

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