Progress in NMR Spectroscopy, Vol. 18, pp. 123–169, 1986.
Printed in Great Britain. All rights reserved.

0079-6565/85/$0.00 + .50
Copyright © 1986. Pergamon Press Ltd.

NMR-STUDIES OF PHOSPHORYL TRANSFERRING ENZYMES

PAUL RÖSCH
Max-Planck-Institut für medizinische Forschung, D-6900 Heidelberg 1, FRG

(*Received* 8 *July* 1985)

CONTENTS

1. INTRODUCTION

The study of enzymes and enzyme/substrate interactions by NMR methods is an explosively growing area in biochemistry and biophysics. NMR methods are especially rewarding in investigations of biological systems which may be tackled by a multinuclear approach, i.e. a combination of observations of several types of nuclei. Examples of such systems are the

phosphotransferring enzymes, which occur almost ubiquitously in living organisms. These phosphotransferases lend themselves to studies by proton-NMR, phosphorus-NMR, and, more recently, carbon-NMR, fluorine-NMR, and magnesium-NMR. The latter methods usually require the use of specifically labeled or unspecifically isotopically enriched compounds. Two reviews on phosphorus NMR studies of enzymes appeared recently.[1,2]

The phosphotransferring systems most widely distributed in nature are the phosphokinases and the nucleoside-triphosphatases, in particular the ATPases. Most known kinases use nucleoside-5'-phosphate Mg^{2+} complexes as donors (or acceptors in the reverse reaction) of the phosphate group. Nucleoside-triphosphates are also the substrates of the triphosphatases, which transfer a phosphate group to a water molecule. Other phosphotransferring enzymes include sugarphosphate-transferases and also complete enzyme systems like the phosphoenolpyruvate dependent phosphotransferase system of microorganisms; in this system each enzyme component is a phosphotransferase in its own right with a phosphorylated enzymic intermediate.

Among the earliest biologically important molecules ever studied by NMR were the substrates of phosphotransferases, in particular the adenosine phosphates; their Mg^{2+} complexes could be investigated even with the low resolution and the low sensivity available in spectrometers more than twenty-five years ago.[3,4] From these experiments it was a long road to the study of chemically modified nucleotides with more sophisticated methods as performed in recent years. These investigations of substrates are always a necessary prerequisite for the interpretation of results obtained with their protein complexes.

The other major aspect of studies of enzyme/substrate interactions is the NMR investigation of the isolated proteins. A low resolution spectrum of RNAase in solution existed as early as 1957.[5] Again, it was far removed from the structural research performed on proteins by the multiple-quantum NMR methods of today. Over the last few years NMR methods have been developed to the point where protein/substrate interactions can be observed at least indirectly even in living tissue, especially in muscle and microorganisms, opening the field of NMR research on enzymes within supramolecular structures.

The present report is restricted to *in vitro* studies of isolated enzyme systems, their substrates, and the respective complexes, though NMR studies of phosphoryl transfer reactions *in vivo* have become standard in recent years. Studies of intact tissue and *in vivo* studies of phosphotransfer systems are overwhelming in number and numerous reviews have appeared elsewhere. Also, proton relaxation enhancement (PRE) studies, which were performed mainly before 1975, are mentioned only briefly, partially because most of these experiments and their interpretation appear somewhat unclear to the author.

2. NMR METHODS

The intention of this Section is to provide a very brief and superficial listing of the set of non-routine NMR experiments which have either been applied to the study of phosphoryltransferring systems or look very promising for their study in the near future.

2.1. *X-Nuclei*

Of the nuclei other than protons, ^{31}P is a natural candidate for investigators studying phosphoryltransfer systems. In general, the resonance lines are easily identified, and at least one of the phosphorus atoms is always close to an active site. The ^{31}P sensitivity is about 6.6% that of protons and its natural abundance is 100%. Sensitivity enhancement by isotopic enrichment is thus excluded for this nucleus. Several standards are in use for chemical shift referencing, so that some caution must prevail when comparison of chemical shifts is desired. Eighty-five percent phosphoric acid is the most common standard, others include methylphosphonate, sodium pyrophosphate, and triethyl phosphate. The latter ones are in many cases used as secondary standards from which chemical shift values are measured and then referenced to phosphoric acid taken as zero. The

chemical shifts studied range from −25 ppm (upfield from phosphoric acid) to 50 ppm, i. e. from the region of the β-phosphate resonance of ATP to the phosphorothioate resonances.

The ^{19}F nucleus is also very attractive for enzyme studies because of its high sensitivity of 83 % that of protons and its natural abundance of 100 %. It is possible with many of the available modern spectrometers to obtain ^{19}F-NMR spectra via the proton measuring channel, although the sensitivity is obviously lower than one gets with a specially tuned circuit. Trifluoroacetic acid is generally used as a standard for chemical shift calibration. The shift range of 60 ppm to 30 ppm includes F-Tyr and F-adenosine. The major drawback of biological ^{19}F-NMR spectroscopy is that neither proteins nor nucleotides contain fluorine, so that these compounds must be modified chemically. Fortunately, the flourine atom resembles sterically an OH group, which is not too uncommon in biological compounds.

To date, ^{17}O-NMR has been applied in biological work only in connection with nucleotides labelled at the phosphate chain. The NMR resonances of ^{17}O are comparatively broad (several hundred Hz) because of the quadrupolar moment of this isotope. Its sensitivity is about 3 % that of protons, its natural abundance 0.037 %. Only isotopically enriched samples are thus suited to be studied by ^{17}O-NMR in biological applications. As a chemical shift standard, $H_2{}^{17}O$ is generally used. A review on various aspects of the use of oxygen isotopes in magnetic resonance in biology has appeared recently.[6]

In a few cases, ^{113}Cd-NMR has been applied to biochemical studies. ^{113}Cd has a sensitivity of 1 % that of protons and a natural abundance of 12 %. Therefore, isotopically enriched samples (typically 96 %) are generally used. As a chemical shift standard, 100 mM $Cd(ClO_4)_2$ is in use. The resonance of the Cd^{2+} ion is observed at about 26 ppm. As environmentalists know, cadmium is in general not the most appropriate divalent ion for physiological use and may be used as a substitute for other divalent ions only in *in vitro* studies. The rationale behind the use of the $^{113}Cd^{2+}$ ion in NMR studies is this isotope having a spin of 1/2 will have narrow linewidths when compared to signals from a quadrupolar nucleus such as ^{25}Mg ($I = 5/2$). In addition, interaction of $^{113}Cd^{2+}$ with phosphate groups may be observed in favourable cases via a resolved J-coupling in ^{31}P-NMR studies.

Sensitivity problems also hamper the study of ^{13}C to some extent, though this nucleus is potentially of great value for biological investigations. Its relative sensitivity is 1.6 % that of protons, but it occurs in only 1 % abundance in nature. Isotopically enriched compounds are very expensive, so that, for example, the production of completely isotopically enriched proteins is in most cases currently out of reach. ^{13}C studies of enriched molecules will no doubt become easier to carry out with the help of proton/carbon and carbon/proton polarization transfer techniques.

2.2. *Two-Dimensional NMR*

Although two-dimensional (2D) NMR methods are currently practiced in many laboratories dealing with macromolecular applications of magnetic resonance spectroscopy, they are still considered non-standard by some investigators. There are a few basic problems inherent in the application of 2D-NMR, especially in cases where one is limited by the total amount of sample, the available sample concentration, the lifetime of the sample or the available spectrometer time. Unfortunately, at least one of these factors applies in most projects. While the kind of detailed information obtainable from 2D-NMR is especially useful in the study of small molecules, these methods can also be applied to yield information on peak assignments even in the larger systems of interest such as those involved in phosphoryltransfer reactions. Though the introduction of the 2D-NMR story was written in Belgium by Jeener, the main part has come from Switzerland, where the theory and experiments were developed by Ernst and coworkers and where Wüthrich and coworkers were the first to show that these experiments could be usefully applied to biological systems.

Basically, a two-dimensional NMR experiment is performed by the application of two or more radio frequency pulses to the sample and subsequent acquisition of the free induction decay as a function of time, FID(t_2). At least two of these pulses are spaced by a time t_1, which is incremented in equal amounts (corresponding to the dwell time in one-dimensional NMR), so that the free

induction decay consists of magnetization which is a function of two time parameters, i.e. a two-dimensional matrix in the time domain $S(t_1, t_2)$ is created. Two-dimensional Fourier transformation is then used to calculate a two-dimensional frequency domain matrix, $S(\omega_1, \omega_2)$. The information content of this matrix depends of course on the type of experiment performed, i.e. on the pulse train applied to the sample. For each type of 2D experiment, complex phase cycling procedures were developed in order to suppress unwanted resonances in the final spectrum and to get the equivalent of quadrature phase detection in ω_1 dimension. (For a concise review dealing with the theoretical aspects of 2D-NMR, see Bax[7]).

A brief phenomenological description of three of the common 2D experiments will now be given.

COSY. The correlated spectroscopy (COSY) experiment is the 'original' 2D experiment. It consists of a sequence of two 90°-pulses spaced by a time t_1:

$$90°\text{–}t_1\text{–}90°\text{–FID}(t_2).$$

Transverse magnetization is created during the first 90° pulse. The various magnetization components precess during the time t_1 with their characteristic precession frequencies, resulting in a frequency labelling of these components. Transfer of magnetization among the different transitions belonging to the same J-correlated spin system is caused by the second 90° pulse.

The final frequency domain matrix $S(\omega_1, \omega_2)$ ideally consists only of a main diagonal, which is equivalent to the one-dimensional spectrum, and off-diagonal peaks at positions $P(\omega_1{}^a, \omega_2{}^b)$ and $P(\omega_1{}^b, \omega_2{}^a)$, where the a and b frequencies are the resonance positions of J-connected transitions in the one-dimensional spectrum (both axes calibrated to the same zero).

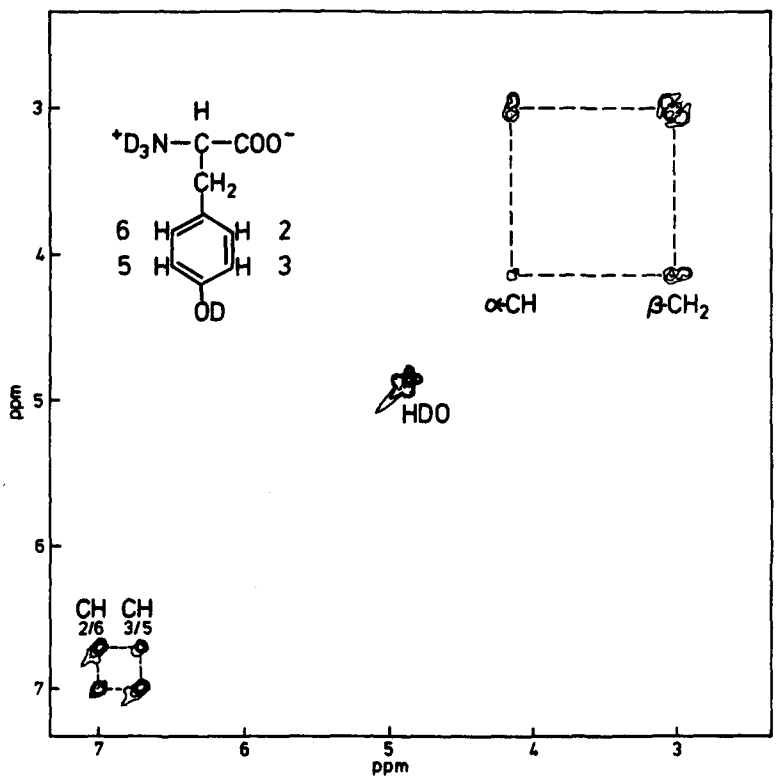


FIG. 1. COSY ^{1}H spectrum of tyrosine.

Figure 1 shows as an example the COSY ^1H spectrum of tyrosine. The J-coupling of the C2/6H and the C3/5H gives rise to the off-diagonal peaks at 6.7 ppm and 7 ppm, the coupling between the α-CH and the β-CH_2 to the off-diagonal peaks at 3 ppm and 4.2 ppm.

NOESY. Whereas the COSY experiment is revealing J-connectivities of nuclear spins, the nuclear Overhauser effect spectroscopy (NOESY) experiment reveals dipolar connections. The basic scheme for the NOESY experiment consists of three 90°-pulses spaced by times t_1 and t_m:

$$90°\text{–}t_1\text{–}90°\text{–}t_m\text{–}90°\text{–FID}(t_2).$$

The first part of the pulse scheme is identical to the COSY experiment. The frequency-labelled components exchange magnetization via cross-relaxation during t_m after the second 90°-pulse. This mixing time t_m thus corresponds roughly to the delay time t_d in the usual nuclear Overhauser effect (NOE) scheme: saturation–t_d–FID(t). The third 90°-pulse serves as a reading pulse.

In essence, the frequency domain matrix then consists of a main diagonal as in the COSY experiment and of off diagonal peaks, which in this case represent dipolar coupled transitions between nuclei which are near to each other.

DQ. Double quantum (DQ) spectroscopy yields basically similar information as COSY. The pulse scheme is an offspring of the INADEQUATE scheme first used in a one-dimensional experiment in ^{13}C spectroscopy:

$$90°\text{–}t\text{–}180°\text{–}t\text{–}90°t_1\text{–}90°\text{-FID}(t_2).$$

t is a time constant equal to $1/4nJ$, where n is an integer (for example $n=1$). The double quantum coherence is excited with the first three pulses, the 90°-pulse creating transverse magnetization, a

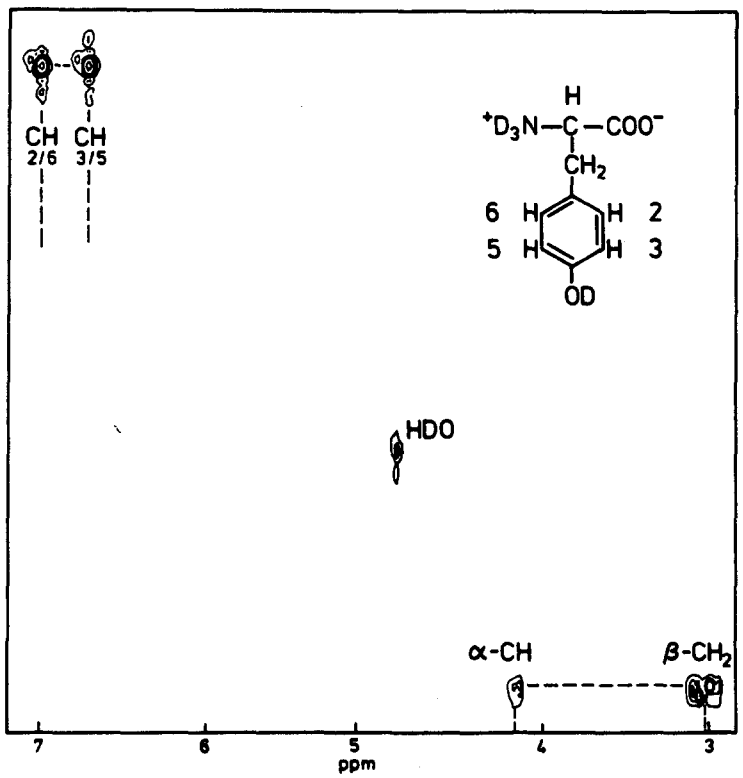


FIG. 2. 2D double quantum ^1H spectrum of tyrosine.

180° refocussing pulse and a 90°-pulse populating all orders of coherence (for ^{13}C this will involve, 0, 1, 2, for ^{13}C–^{13}C pairs). Phase cycling cancels zero and single quantum coherence while retaining double quantum coherence. This is converted back after the evolution period t_1, which has the same significance as in the COSY experiment, to detectable single quantum coherence, i.e. transverse magnetization.

The final two-dimensional frequency-space matrix ideally displays no diagonal resonances. Peaks are observed at positions $P(\omega_1{}^a,\omega'_2{}^a+\omega'_2{}^b)$ and $P(\omega_1{}^b,\omega'_2{}^a+\omega'_2{}^b)$, where the ω' frequencies are calculated as offset from the carrier frequency.

The DQ spectrum of tyrosine as shown in Fig. 2 gives the same kind of information as the COSY spectrum. However, there are cases in which long range couplings can be detected by DQ spectrocopy which are not revealed in COSY spectra. In Fig. 2 the peaks with chemical shifts 6.7 ppm and 7 ppm on the ω_2 axes originate from the C2/6H–C3/5H coupling, the peaks with chemical shifts 3 ppm and 4.2 ppm from the α–CH–β–CH_2 coupling.

2.3. *Light Induced CIDNP*

The effect of chemically induced nuclear magnetic polarization (CIDNP) can be explained briefly by the fact that the rate of return of a radical pair in an excited triplet state back to its singlet ground state (intersystem crossing) is dependent on the nuclear spin state. The reaction pathway from a radical pair in the triplet state back to the singlet ground state necessary for the formation of a molecule is thus nuclear spin state selective. This leads to the nuclear polarization of the product molecule. In biological photo-CIDNP studies a flavin dye is usually used and this is photoexcited to a triplet state by strong light irradiation (normally a laser is employed). Flavin is known to interact with tyrosine, histidine, tryptophan, cysteine, and methionine, the latter two being of minor practical importance because of their vanishingly small photo-CIDNP polarizability. The primary step of the interaction involves probably a proton abstraction in the case of His and Tyr and an electron transfer in the case of Trp. The polarization induced in His and Trp residues enhances the population of the ground state, whereas the C3/5H of Tyr residues are polarized in the direction of population inversion (the Tyr-C2/6H are hardly effected). Thus, absorption type spectra are observed for His and Trp and emission type spectra for Tyr. In proteins, polarization of only those amino acids which are sterically able to interact with the excited dye is expected. A general review on biological applications of this technique is available.[8]

2.4. *The Determination of Rate Constants*

Another interesting subject which may be studied directly by NMR methods is the kinetic aspect of the phosphoryl transfer reaction. In recent years, several different NMR methods have been employed extensively to determine kinetic parameters related to biochemical processes: these include lineshape analysis, magnetization transfer (MT) studies, isotope exchange studies, and two-dimensional exchange spectroscopy. The method relying on a Carr–Purcell pulse sequence[9] has not found extensive applications in biochemistry. The prerequisites for the application of these methods differ from one case to another. Lineshape analysis is restricted to cases where the exchange rate between any two interchanging species is comparable to their inverse chemical shift difference. Magnetization transfer studies are restricted to cases where it is possible to irradiate one of the interchanging species without influencing the others; in addition, spin–lattice relaxation rates must be of a similar order of magnitude as the exchange rates. Studies of exchange using two-dimensional spectroscopy is limited by its comparatively low sensitivity, which makes rather large amounts of sample and fast exchange rates mandatory for these experiments; a definite advantage of the two-dimensional spectroscopy is the observation of different exchange pathways in a single experiment. Most of these experiments benefit from an equilibrium constant of the exchanging species in the order of unity. MT techniques and the two-dimensional experiments demand an equilibrium constant of the exchanging species of the order of unity as an important feature of the reaction under investigation. Isotope exchange studies may, of course, be employed only when the enzyme under

study actually catalyzes an isotope exchange in a reaction initialized with a labelled substrate and this exchange can be followed by a magnetically active probe (which may be either the isotope itself or an active nucleus influenced by the isotopic substitution).

2.4.1. *Lineshape Calculations.* Lineshape calculations as applied to studies of dynamic processes in NMR spectroscopy have been reviewed extensively by Binsch[10] and in a modern classic work by Kaplan and Fraenkel.[11] For a very introductory level account, Sandstroem's book[12] is recommended.

The calculation of the lineshape of an NMR resonance in the general case, i.e. in the presence of J-coupling and chemical exchange, requires the use of the density matrix formalism. Simplifications of the general calculation procedure are strongly dependent on the specific problem under consideration and are far outside the scope of this review. The interested reader should consult the papers of Nageswara Rao and coworkers,[13,14] which deal with specific problems arising in lineshape calculations as applied to phosphoryltransferring systems.

2.4.2. *Isotope Exchange Studies.* The discovery of an influence of isotopic substitution of ^{16}O by the magnetically inert ^{18}O in a phosphoryl group on the ^{31}P chemical shift of this group by Cohn and Hu[15] and Lutz *et al.*[182] opened the field of oxygen isotope exchange studies to magnetic resonance spectroscopy. The chemical shift induced by ^{18}O substitution in a phosphoryl group is approximately 0.03 ppm downfield per ^{18}O, i.e. $P_i{}^{18}O_2{}^{16}O_2$, for example, would be shifted 0.06 ppm downfield from $P_i{}^{16}O_4$ and 0.06 ppm upfield from $P_i{}^{18}O_4$. Two reviews appeared in recent years on ^{18}O exchange studies.[16,17]

The simple theoretical methods developed for the modelling of the time course of this type of isotope exchange lend themselves to the description of other types of label exchange. The general

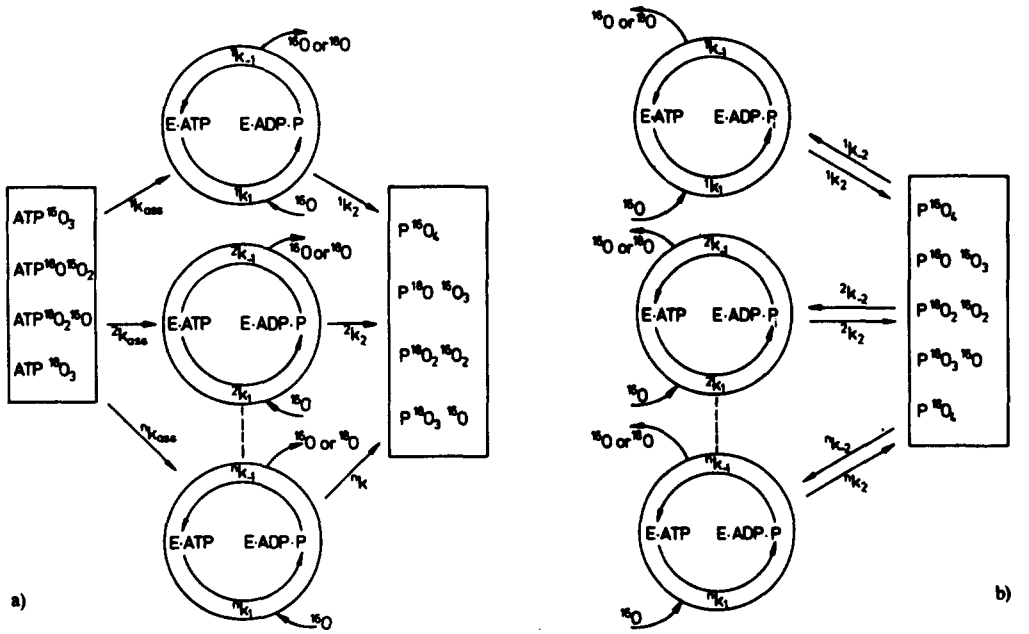


FIG. 3. Exchange of labelled oxygens by hydrolysis of the phosphate ester bond. Left: ^{18}O labelled nucleoside triphosphate is cleaved by an NTPase in the presence of a divalent metal ion at the terminal phosphate. After several repetitions of the bond cleavage step, NTP is released as NDP and inorganic phosphate. The latter one may contain from 0 to 3 labels (^{18}O content of surrounding water not taken into account) Right: Exchange of ^{18}O in inorganic phosphate in the presence of an NTPase and NDP·Me^{2+} by synthesis and subsequent cleavage of NTP.

situation to be described is either the cleavage process of a nucleoside triphosphate NTP(^{18}O) labelled at the γ-positon or the synthesis process of NTP(γ-^{18}O) from NDP and P_i(^{18}O) by a hydrolase. The -PO_2-O-PO_3 group is always cleaved at the terminal bond. The experiments take place in unlabelled H_2O as solvent. This situation is shown in Fig. 3 for both types of experiment. The observed quantity in each case is the number of P_i molecules in the differently labelled states, i.e. $P_i(^{18}O_4)$, $P_i(^{18}O_3\ ^{16}O_1)$, $P_i(^{18}O_2{}^{16}O_2)$, $P_i(^{18}O_1{}^{16}O_3)$ and $Pi(^{16}O_4)$. The theoretical description of the exchange process is, of course, independent of the mode of detection of the P_i concentrations.

The master equation in matrix notation governing each exchange process is under the usual experimental conditions of the form:[18]

$$\mathrm{d}/\mathrm{d}t\ \bar{A}(t) = \bar{\bar{T}}\,\bar{B}(t) - \bar{\bar{D}}\ \bar{A}(t).$$

$\bar{A}(t)$—vector with the concentration of $P_i{}^{18}O_n{}^{16}O_{4-n}$, $n = 0, \ldots, 4$ as elements.
$\bar{B}(t)$—vector with the concentration of $P_i{}^{18}O_n{}^{16}O_{4-n}$ or the corresponding concentration of NTP($\gamma{}^{18}O_n{}^{16}O_{4-n}$), $n = 0, \ldots, 4$, depending on the type of experiment, as elements.
$\bar{\bar{T}}$—transition matrix describing the transitions from the elements of $B(t)$ to the elements of $A(t)$.
$\bar{\bar{D}}$—transition matrix describing the losses of $A(t)$.

Whereas it is trivial to adjust the matrix $\bar{\bar{D}}$ to the experimental situation, it is somewhat more complicated to calculate the transition matrix $\bar{\bar{T}}$ which may be a sum of matrices ${}^i\bar{\bar{T}}$ describing different reaction pathways. The matrices ${}^i\bar{\bar{T}}$ may be decomposed into products of apparent first order rate constants ${}^ik$, which describe the rate of labelled compounds entering the exchange cycle of the i^{th} pathway, a probability function ${}^iP(X = \mathrm{N})$ with expectation value ${}^i\hat{N} = {}^ik_{-1}/{}^ik_2$, and transition matrices ${}^i\bar{\bar{T}}^*(N)$ for exactly N reversals according to:

$${}^i\bar{\bar{T}} = {}^ik\ {}^i\bar{\bar{T}}^*(N)\ {}^iP(X = N).$$

The elements of the matrices ${}^i\bar{\bar{T}}^*$ may be calculated from first principles of probability theory for one reversal of the NTP cleavage step for different types of rotational freedom of the P_i molecule in the enzyme-bound form E·NDP·P_i, resulting in a matrix ${}^i\bar{\bar{T}}^*(1)$. ${}^i\bar{\bar{T}}^*(N)$, the transition matrix for N reversals of the cleavage step, may now be calculated according to Markov's theory by ${}^i\bar{\bar{T}}^*(N) = {}^i\bar{\bar{T}}^*(1)^N$.

The solution of the master equations may be obtained by standard methods.[18]

This general procedure includes many special cases, in particular the ones treated earlier by Hackney,[19] Rösch *et al.*,[20] and Rösch.[21] The calculations by Hackney,[19] for example, are equivalent to the current ones as long as only one pathway is under consideration, $P(X = N)$ is the geometrical distribution, and the ^{18}O content of the solvent is equal to zero throughout the experiment. Attempts to take non-zero ^{18}O concentrations in the solvent into account in a more trivial way[19] led to erroneous results.

Figure 4 gives an example for the ^{31}P-NMR spectra expected as a function of time for a special case. In principle, a data fitting procedure applied to the time dependence of the concentration of the five different P_i species can give the expectation value for the number of reversals, the number of reaction pathways, the degree of mobility of P_i in the bound state, information on the distribution $P(X = N)$, and the apparent first order rate constants ${}^ik$. In practice, the number of free parameters in the data refinement procedure should not exceed three, usually including $\hat{N}$ and k. A data fitting procedure taking into account all five different P_i species simultaneously has also been introduced.[18]

The method of choice for many years for the detection of the different P_i species or the total incorporation of ^{18}O into P_i was mass spectrometry. The major advantage of the NMR detection as compared to the mass spectrometric analysis is the possibility of monitoring the exchange reaction directly in real time, at least in cases where the ^{18}O exchange proceeds rapidly enough so that the level of inorganic phosphate may be chosen to be reasonably high (e.g. 100 mM). In addition, no chemical alterations of the inorganic phosphate are necessary in order to obtain a volatile compound. The major advantage of the mass spectrometric approach is the high sensitivity of this method. It should be mentioned that Boyer and collaborators have played the major role in

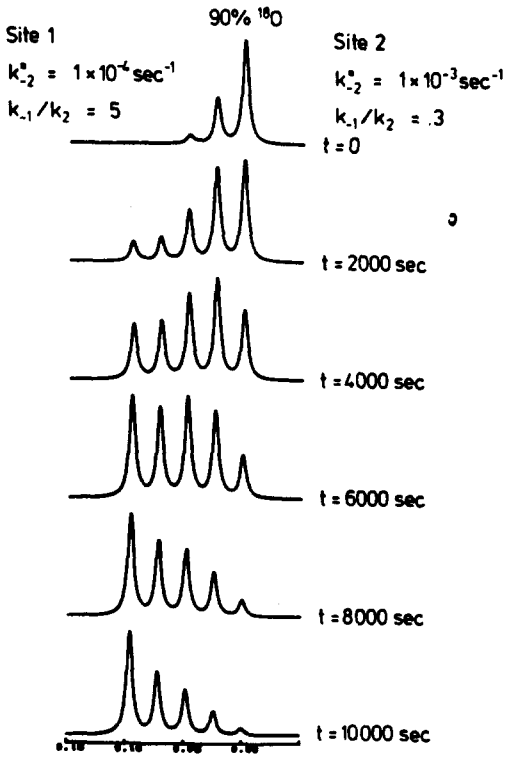


FIG. 4. Simulated ^{31}P spectra of labelled inorganic phosphate as a function of time in the presence of two independent NTPase active sites in the solution with the characteristics given in the figure.

supplying the basic ideas required to advance the ^{18}O exchange method to the current state (Hackney *et al.*[16]).

Another method of indirect detection of magnetically insensitive nuclei like ^{15}N and ^{13}C via their J-coupling to more sensitive nuclei such as ^{1}H and ^{31}P was suggested by Brindle *et al.*[22] based on an idea by Freeman *et al.*[23] The insensitive nuclei were detected by the phase modulation they impose on the coupled active nuclei after a spin echo pulse sequence is applied to the latter ones:

$$90°\text{–}t\text{–}180°\text{–}t\text{–Acquisition.}$$

Simultaneously with the 180° pulse, an 180° pulse (or a 90°–90° composite pulse (Freeman *et al.*[23])) was applied to the insensitive nuclei. This experiment results in a phase modulation of the detected signal according to $\cos(2\pi Jt)$, so that for $t = 1/2J$ an inversion of phase only for those nuclei having J-coupled insensitive partners was observed. In this way, the time behaviour of the concentration of these nuclei may be followed. A practical application of this experiment to the creatine kinase reaction was performed by Brindle *et al.*[24]

2.4.3. *Magnetization Transfer Studies.* Other methods capable of probing phosphoryl transfer kinetics, especially in living systems, are based on transfer of magnetization (MT). These techniques rely on the fact that it is possible to impose by high frequency irradiation a certain level of magnetization on a nucleus at a specific site in chemical exchange with other sites usually well separated in resonance frequencies. The magnetization imposed on the nucleus at the first position is transferred via the T_1 memory of the spins to the second group and can there be detected as a

decrease of the equilibrium intensity of this resonance.[25,26] Usually, a chemical equilibrium situation during the experiments is assumed. This method is applicable whenever the lifetimes of the states are of the same order of magnitude as the spin lattice relaxation times involved. The master equations describing this process are the McConnell equations, which for two sites are given by:[27]

$$\mathrm{d}/\mathrm{d}t\ M_z^a(t) = (M_z^a(0) - M_z^a(t))/T_1^a - M_z^a(t)/t_a + M_z^b/t_b$$
$$\mathrm{d}/\mathrm{d}t\ M_z^b(t) = (M_z^b(0) - M_z^b(t))/T_1^b - M_z^b(t)/t_b + M_z^a/t_b$$

(t_a, t_b: lifetime of state a and b, respectively).

The McConnell equations describe basically an exchange process by modifying the Bloch equations by the addition of an exchange term. The equations may be easily extended to exchange processes involving more than two sites. They can be solved by standard methods. For example, the intensity of resonance I after complete inversion of resonance S by a selective 180° pulse is given as a function of the time t elapsed between the inverting pulse and the observation pulse by:

$$I(t) = I_0 - \{2S_0 k_S/(k_S + k_I)\}\{\exp(-rt) - \exp(-[r + k_i + k_S]t)\}.$$

I_0 and S_0 represent the equilibrium magnetizations of spins I and S, respectively, k_S and k_I are the reciprocal lifetimes of S and I spins in the two site exchange reaction

$$I \underset{k_S}{\overset{k_I}{\rightleftharpoons}} S$$

In contrast to this transient technique, the saturation transfer (ST) experiment is a steady state technique. The steady state is created by constant irradiation of one site. Thus, all time derivatives are zeroed. The intensity of resonance I on irradiation of resonance S for a two site exchange is given by:

$$I = I_0 r/(r + k_I).$$

In both expressions, equal relaxation rates r of the interchanging species are assumed.

So, in order to measure rate constants involved in exchange processes by MT, either the time development of the intensity of a signal after disturbing the connected resonance may be monitored or the steady state value may be determined on permanent saturation of the connected resonance. In the latter method, the spin lattice relaxation times need to be measured.

It should be noted at this point that dynamic NMR studies by lineshape analysis (in the slow exchange case) and by MT techniques require similar prerequisites as far as the ratios of the relaxation rates to the exchange rates are concerned. Thus, an experimental comparison of these two methods, for example applied to the study of on enzyme rate constants, could prove worthwhile.

The situation where MT shows its real power is the determination of rate constants within living tissue. A review on this subject is available.[28]

2.4.4. *Two-Dimensional Experiments.* Jeener *et al.*[29] proposed pulse schemes for two-dimensional exchange spectroscopy. There are at least two possibilities to determine rate constants by two-dimensional NMR experiments: Observation of a set of NOESY spectra or the ACCORDION[30] experiment.

The NOESY pulse sequence consists of three pulses of equal length:

$$90°\text{–}t_1\text{–}90°\text{–}t_m\text{–}90°\text{–Acquisition–}t_{rel}$$

The relevant magnetization transfer takes place during the mixing period t_m. NOESY spectra of samples with interchanging species will show off-diagonal peaks in the spectrum at frequency coordinates (ω_A, ω_B) and (ω_B, ω_A) connecting either two interchanging resonances A and B with chemical shifts ω_A and ω_B on the diagonal. The intensities of these cross-peaks are functions of the mixing time and of the rate constants involved. Given a reaction of the simplest type:

$$I \underset{k_S}{\overset{k_I}{\rightleftharpoons}} S$$

the expression for the cross peak intensities reads:

$$I(t_m) = S_0\{k_S/(k_S+k_I)\}\{\exp(-rt_m) - \exp(-[r+k_I+k_S]t_m)\},$$

where S_0 is the equilibrium magnetization of species S and r the relaxation rate assumed to be equal for to both species.

Thus, a set of NOESY spectra with different mixing times contains information on exchange rates in the time dependence of the cross-peak intensities. The interpretation of the cross-peak intensities is especially simple in such favourable cases when it is possible to keep the mixing time very short compared to the inverse of the involved rate constants, because then only linear effects in the buildup of magnetization need to be considered. In essence, the above equation reduces to

$$I(t_m) = S_0 k_S t_m.$$

The ACCORDION variant of the NOESY pulse sequence may be produced by simultaneously incrementing the mixing time t_m and t_1 during the experiment, where $t_m = nt_1$. Rate constants may be extracted from an ACCORDION experiment by fitting the lineshapes of the diagonal and the off-diagonal peaks to the theoretical lineshape function along the ω_m axes (two site exchange, absorption mode phase sensitive spectra, $\omega_m = 0$ at the chemical shift ω_A or ω_B in the ω_1 domain):

$$I(\omega) = 0.5\{R_1/({R_1}^2+{\omega_m}^2) \pm (2k+R_1)/[(2k+R_i)^2+{\omega_m}^2]\}$$

where R is the relaxation rate and k is the inverse lifetime of the spins in the site under consideration. The + sign is valid for the diagonal peaks, the − sign for the cross peaks.

So far, experiments performed as applications of two-dimensional exchange spectroscopy, especially in the context of phosphotransfer enzymes, are rare.

2.5. *Stereochemistry of the Phosphoryltransfer*

The stereochemistry involved in the mechanism of the phosphoryltransfer may be probed further by using NMR methods.

If the phosphoryltransfer is started with a substrate which is chiral with respect to different oxygen isotopes at the phosphorylgroup to be transferred, then reaction products with two different configurations at phosphorus may be obtained. The transfer of the phosphorylgroup may either involve a retention or an inversion of configuration. The former one is indicative of a phosphoryltransfer with an odd number of intermediates, i.e. an even number of transfer steps, the latter one with an even number of intermediates, i.e. an odd number of transfer steps. The simplest reaction mechanism causing inversion of configuration is thus a direct in-line transfer. Inversion of configuration was so far observed for all kinase reactions. The method of choice for the determination of the configuration of the reaction products is their chemical modification to yield cyclic triesters. These occur in two stereoisomers containing no ^{17}O, depending on the configuration of the starting material. These stereoisomers may be distinguished most easily by ^{31}P-NMR. (The ^{17}O containing compounds are not detected easily by ^{31}P-NMR due to the quadrupolar relaxation effect of this isotope.) For a review see Buchwald *et al.*[31]

Another question which may be answered by positional isotope labelling is the one concerning the temporal succession of the bond breaking and bond making processes during the phosphoryltransfer.

As shown in Fig. 6 these two processes may be distinguished with the aid of specifically ^{18}O labelled compounds under the assumption that a dead end second substrate R″ promotes the bond cleavage step in the dissociative mechanism and the labelled phosphorylgroup is free to rotate in the time scale of the existence of the dissociated compound. Analysis of the equilibrium products in order to distinguish their containing or not containing a bridge ^{18}O is accomplished most easily by ^{31}P-NMR. This procedure was first suggested by Midelfort and Rose.[32] Another method in order to identify the first step of the dissociative reaction pathway involves positional oxygen isotope labelling of the transferred phosphorylgroup. Bond breaking and bond reformation would result in scrambling of the oxygens of -P-O-P(^{16}O,^{17}O,^{18}O) if the phosphorylgroup were free to rotate in the

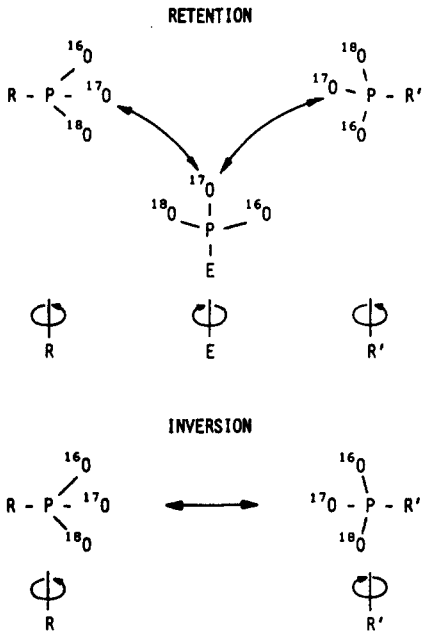


FIG. 5. Retention versus inversion of configuration: Retention is obtained if the phosphoryltransfer is catalyzed via an odd number of covalent enzyme-phosphate intermediates, inversion is obtained if the catalysis proceeds via an even number (including zero) of enzyme-phosphate intermediates.

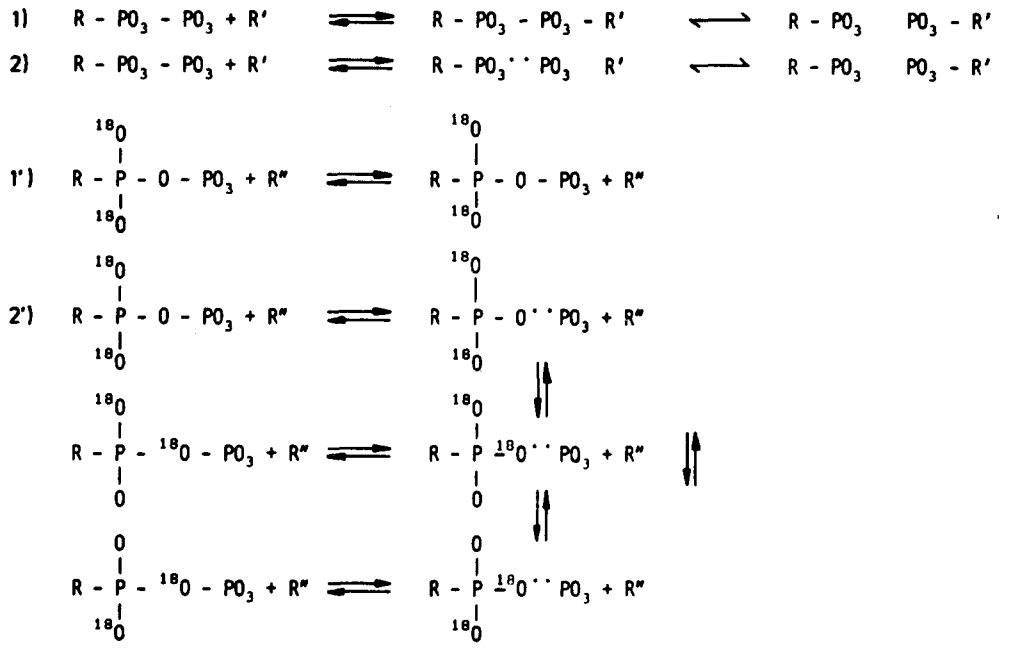


FIG. 6. Upper part: (1) associative mechanism of the phosphoryltransfer: Bond making preceeds bond breaking, (2) dissociative mechanism of the phosphoryltransfer: Bond breaking preceeds bond making. Lower part: equilibrium situation obtained for the two different mechanisms if the reaction is started without ^{18}O label in the terminal phosphate bridge: The associative mechanism yields no ^{18}O in this phosphate bridge, whereas the dissociative mechanism results in a compound with an ^{18}O bridge in two-third of all cases.

dissociated compound.[33] So far, the associative mechanism seems to be the preferred one for phosphokinases.

3. NUCLEOSIDE-5'-PHOSPHATES AND THEIR METAL-COMPLEXES

NMR-studies of nucleotides have profited quite a bit from the progress in nucleotide chemistry, allowing the production of chemically modified compounds in quantities large enough for NMR research. Still, a huge number of experiments have already been performed with the naturally occurring compounds over the last 25 years.

3.1. *Naturally Occurring Compounds*

Proton and phosphorus NMR were much used in studies of nucleotides and metal/nucleotide interactions because of the high sensivity of those nuclei and their natural abundance approaching 100 %.

In fact, proton and phosphorus NMR brought about new data on nucleotide/metal ion interactions mostly before 1980. The conformational feature studied most extensively was the syn vs anti conformation of the glycosidic bond. For those studies, basically four different methods were used: analysis of coupling constants, proton homonuclear Overhauser effects, measurements of proton relaxation times, and analysis of the influence of various chemical groups on the chemical shift values. On one hand, the general feeling about the conformation of nucleotides emerging from the NMR data is that there is no conformation which may be called a stable one even under only marginally varying experimental conditions. Thus, no clear cut quasi static structure is to be expected for these compounds. On the other hand, the anti conformation seems to be the preferred one in most cases. For concise reviews see Davies[34] and Govil and Ramakrishna.[35]

This is in agreement with a study of the syn–anti equilibrium in purine nucleotides performed recently. The ^{1}H and ^{13}C chemical shift values of model syn and anti compounds were compared to the chemical shift values exhibited by nucleotides, and the relative populations determined. It was concluded that about 70 % of a GMP or AMP solution was in the anti conformation.[36]

Though the base moieties of the nucleotides are by no means unimportant for the substrate/enzyme interaction, and the study of the base recognition process is definitely a subject matter which is worth while studying in detail, the more important feature for the energetics and the chemical mechanisms of phosphotransferring enzymes is the conformation of the phosphate chain with and without complexed divalent metal ion.

The nucleotides studied most intensively in this respect are the adenosine-5'-di- and -tri-phosphates, because these are the ones used by most phosphotransferring enzymes as substrates. The general picture emerging mainly from proton and carbon NMR data in the last two decades is as follows: Divalent metal ions bind to the phosphate chains in all known nucleoside phosphates. In a few cases the metal ion is also suspected to bind to the N1 and N7 position of the base. The metal complexation did not appear to establish a clear preference for either syn or anti conformation. It must be suspected, therefore, that the requirement for metal ion complexation before the catalysis of the phosphate–ester bond cleavage bond cleavage by enzymes is mainly for the purpose of stabilization of a (possibly unusual) conformation of the rather flexible phosphate chain and for the redistribution of charges within this chain. Unfortunately, ^{31}P-NMR does not seem to offer the possibility of solving the problem concerning the exact complexation site for metal ions along the phosphate chain.[37]

Recently, the adenosine nucleotide with a phosphate chain consisting of four phosphate groups, AP_4, and its Mg^{2+} complex has been studied by phosphorus NMR. The resonance lines were assigned, the titration behaviour and the stability constant for the primary and the secondary Mg^{2+} complex was determined. Not surprisingly, AP_4 exhibits titration and stability parameters closely similar to ATP. The binding of a second metal ion, resulting in a ternary complex, could be followed.[38]

3.2. *Modified Compounds*

In recent years new methods of nucleotide synthesis and chemical modification of nucleotides opened new ways for the study of nucleoside phosphates by NMR. One of the major targets was to determine where the divalent metal ion is complexed on the phosphate chain. Another major target was to study the stereochemistry of the metal/nucleotide interaction both for the free nucleotide and for the enzyme complexes.

Phosphorothioates of adenosine and guanosine nucleotides, i.e. nucleotides with a sulphur atom substituted for an oxygen atom of the phosphate chain, could be prepared stereospecifically, i.e. the R_P and S_P conformers could be separated (see Fig. 7).

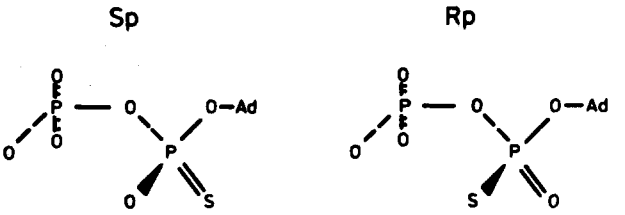


FIG. 7. The two diastereomers of ADP(α-S).

Experiments were performed on adenosine phosphorothioates as well as on guanosine phosphorothioates in order to characterize their phosphorus NMR parameters.[37, 39–41] The R_P and S_P compounds showed a difference in the value of the chemical shift of the resonance of the phosphate group where the substitution took place of about 0.3 ppm.[42]

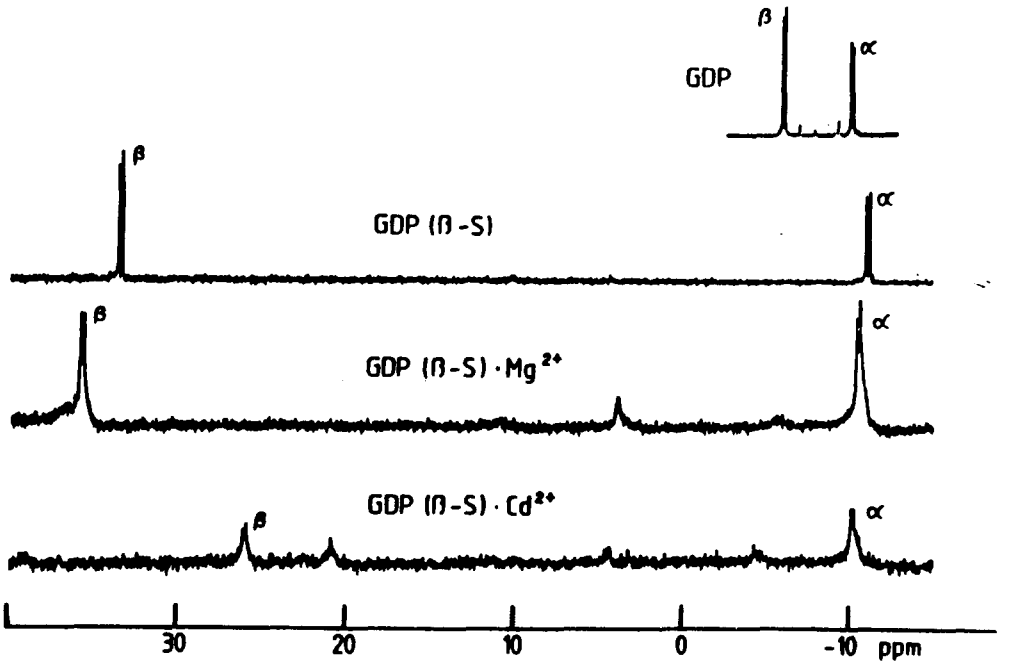


FIG. 8. ^{31}P NMR spectra of (top to bottom): GDP–GDP(β-S)–GDP(β-S)·Mg^{2+}–GDP(β-S)·Cd^{2+} (from: Rösch *et al.*[40]).

For further studies of enzyme-free nucleotide solutions the R_P compound was used in general.

Figure 8 shows as an example a comparison of the phosphorus spectra of guanosine-5′-diphosphate, guanosine-5′-diphosphate (β-S), and the Mg^{2+} and Cd^{2+} complexes of the phosphorothioate compound.

The guanosine compounds exhibited, as expected, virtually the same behaviour as far as their phosphorus NMR parameters were concerned as the corresponding adenosine compounds. Common features of all the nucleotides and metal nucleotide complexes were:

(a) a downfield shift of the resonance of the substituted phosphate group by about 50 ppm;
(b) a decrease of the coupling constant for the PO_3–O–PO_2S bond as compared to the PO_3–O–PO_3 bond by 2 Hz to 10 Hz;
(c) a drop of the pK_a-values by about 1 unit on substitution at the terminal phosphate group;
(d) a downfield shift of the resonance of the substituted phosphate group in the range from 2 ppm to 7 ppm on Mg^{2+} complexation;
(e) a drop of the pK_a-value on Mg^{2+} complexation by approximately 0.5 units for the monophosphates, approximately 1 unit for the diphosphates, and approximately 2 units for the triphosphates;
(f) an upfield shift of the resonance of the substituted phosphate group on Cd^{2+} complexation of about the same magnitude as on Mg^{2+} complexation;
(g) an even more pronounced drop of the pK_a-values on Cd^{2+} complexation than on Mg^{2+} complexation, the values ranging from 2 units for the monophosphates to over 4.5 units for the triphosphates.

The different directions of the chemical shift changes on Mg^{2+} and Cd^{2+} complexation in connection with the observation that these differences were not detected in unmodified nucleotides led to the conclusion that Mg^{2+} coordinates preferentially to the oxygen atom of the phosphorothioate group, whereas Cd^{2+} coordinates preferentially to the sulphur atom. This is not unexpected in light of the fact that Mg^{2+} is known to belong to the 'hard' metal ions of inorganic chemistry, whereas Cd^{2+} belongs to the 'soft' ion group. As a consequence, both metal ion complexes exist in two stereochemically different compounds. Replacing a specific thionucleotide stereoisomer by its counterpart and, at the same time, substituting one kind of metal ion for the other leads to topologically identical objects. An example is given in Fig. 9.

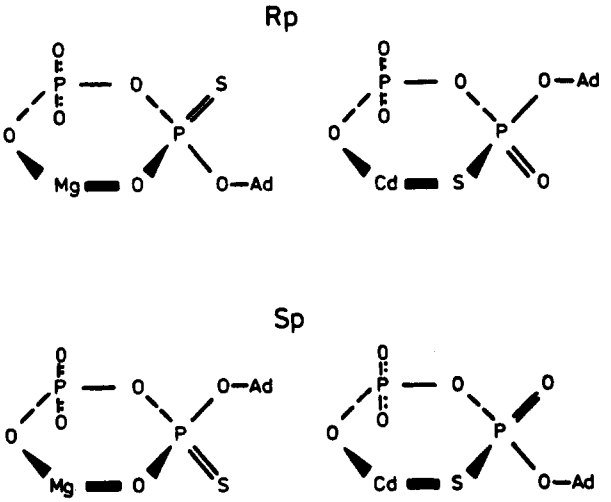


FIG. 9. The Cd^{2+} and Mg^{2+} complexes of the two isomers of ADP(α-S).

Trivially, substitution of sulphur for oxygen at the β-position of ATP changes the ATP/(ADP + P_i) equilibrium. It also influences the equilibrium constants of all reactions of the type:

$$ATP + Y \rightleftharpoons ADP + Y - P.$$

Reactions according to this scheme are catalyzed in the presence of divalent metal ions by phosphokinases. The equilibrium constant for reactions catalyzed by arginine kinase, creatine kinase, pyruvate kinase, and 3-P-glycerate kinase with ATP(β-S) as a substrate were determined by Lerman and Cohn[43] with the aid of ^{31}P-NMR. In the first two cases, the reaction equilibrium was shifted towards the nucleoside diphosphate side by a factor of approximately 60. This result was used in turn to calculate the equilibrium constant of the pyruvate kinase and the 3-P-glycerate kinase reaction with their physiological substrates, which is too far towards the triphosphate side to be measured directly.

The stereospecific substitution of oxygen with sulphur to yield the phosphorothioates of nucleotides in turn opened up the possibility of stereospecific substitution of the ^{17}O or ^{18}O isotope for the ^{16}O isotope, which occurs with an abundance of 99.8 % in nature. The isotopes with atomic mass 16 and 18 are magnetically rather inaccessible, whereas the spin-5/2 isotope ^{17}O is amenable to NMR studies; its sensitivity in a 100 % enriched sample would be about 3 % that of protons—for reasons of availability, the maximum enrichment which may be achieved is currently limited to around 50 %. Recently, ^{17}O NMR studies of adenosine nucleotides with a non-stereospecifically labelled phosphate chain were used in an attempt to localize the Mg^{2+} coordination site. The oxygen resonances of ATP(α-^{17}O), ATP(β-^{17}O), and ATP(γ-^{17}O) were observed in nucleotide metal ion complexes. In a first report on this work it was stated that the oxygen resonance related to the γ-phosphate tends to broaden on Mg^{2+} complexation.[44]

An extension of this work led to the suspicion that the complexation of Mg^{2+} is mainly due to a coordination by the two terminal phosphates of ATP. The argument of site-specific linebroadening of the oxygen resonances on which this result rested was supported by the observation of site-specific linebroadening of the oxygen resonances in the substitution inert ATP·Cr^{3+} complex. The same line of argument indicated a Mg^{2+} coordination in ADP by both phosphates.[45]

Also, a study relating the variation of different physical parameters to the phosphorus NMR parameters of ^{17}O-containing phosphate esters with probable relevance to nucleotide research was undertaken.[46]

Especially designed for the use as NMR probes on enzymes are the 2′-fluoro-adenosine and the 2-fluoro-adenosine derivatives. Both compounds were characterized by ^{19}F-NMR and used for enzyme studies (see below). (Baldo *et al.*[47]; Goody and Rösch, unpublished.)

The general use of ^{15}N labels in biological NMR, including research on nucleosides and nucleotides, was reviewed by Blomberg and Rüterjans. ^{15}N NMR revealed the deprotonation sites of uridine, cytidine, adenosine, and guanosine. To date, the low sensitivity of the ^{15}N nucleus puts severe limits on studies of enzymes and enzyme nucleotide complexes involving observation of this isotope. A similar situation, though less severe, is encountered with ^{13}C NMR. This situation is currently about to change rapidly by the introduction of new polarization transfer techniques, yielding large sensitivity enhancements for magnetically active nuclei coupled to observable protons.

The H8 and H2 of AMP showed a polarization by the CIDNP effect strongly dependent on pH and buffer conditions.[49] The H8 of GMP showed enhancement factors of up to 8.[50] Scheffler and Cohn[51] searched for nucleotide analogues exhibiting higher polarization effects to allow them to carry out feasible CIDNP studies of these compounds and their protein complexes. They could show that the etheno H7 of 1,N^6-ethenoadenosine and 3,N^4-ethenocytidine exhibited about 20-fold polarization enhancements at pH 7.5. This result is noteworthy especially in the light of the fact that no CIDNP effect was observed for pyrimidine nucleotides.[50] The H8 of formycin showed an 8-fold enhancement under the same conditions.

4. PHOSPHORYL TRANSFERASES

4.1. *Adenylate Kinase*

One of the most widely studied kinases is the adenylate kinase (AK, nucleoside-5′-triphosphate : nucleoside-5′-monophosphate phosphotransferase, EC 2.7.4.3). The best known member of this class of enzymes is probably the ATP : AMP phosphotransferase of skeletal muscle (AK_1, myokinase), which is identical to the one located in the cytosol. The reaction catalyzed in the presence of a divalent metal ion, usually Mg^{2+}, is:

$$ATP + AMP \rightleftharpoons 2\ ADP.$$

All known adenylate kinases are rather low molecular weight proteins. Indeed, the human and porcine adenylate kinases exhibit the lowest molecular weight of all kinases known to date (21,700). The adenylate kinases which were subjects of NMR studies so far include the adenylate kinase from carp muscle, from human muscle, from pig muscle, from European baker's yeast, and from American baker's yeast. The crystal structure of porcine muscle adenylate kinase is known,[52] although it was not possible to obtain crystals with bound substrates.[53]

4.1.1. *^{31}P-NMR.* One of the natural starting points in studies of enzyme/nucleotide interactions is the observation of the phosphorus NMR signals of the enzyme-bound reactants.

The AK reaction is extremely well suited to be studied by ^{31}P-NMR methods because all the reactants contain phosphate groups. The symmetric molecule diadenosine pentaphosphate (Ap_5A, a bisubstrate inhibitor supposed to be a kind of transition state analogue) is changed to an asymmetric molecule on binding to porcine adenylate kinase. This is reflected in the fact that two groups of phosphorus resonances of Ap_5A are observed for the free molecule and for its Mg^{2+} complex, whereas five resonances are observed in the enzyme-bound form. This splitting is even more pronounced in the presence of Mg^{2+}, indicating the introduction of assymmetry by the metal ion.[54]

In the course of these phosphorus NMR studies it was claimed that it was possible to distinguish ATP and ATP·Mg^{2+} bound to the two different nucleotide sites at a temperature of 288 K. The same was indicated for ADP. The chemical shifts of the bound substrates were determined under various conditions. It was also possible to determine the on-enzyme reaction rates directly by crude lineshape analysis; the reciprocal lifetime of the AK·AMP·ATP·Mg^{2+} complex was determined to be roughly 700 sec^{-1}, the reciprocal lifetime of the AK·ADP·ADP·Mg^{2+} complex roughly 400 sec^{-1}, at 288 K. This is about one order of magnitude larger than the overall reaction rate, so that the transfer step is not rate limiting (for a recent definition of the expression 'rate limiting step' see Ray[55]). The independently determined on-enzyme equilibrium constant of the reaction was $K_{eq} = [E{\cdot}ADP{\cdot}ADP{\cdot}Mg^{2+}]/[E{\cdot}AMP{\cdot}ATP{\cdot}Mg^{2+}] = 1.5$.

Also, the reason for the influence of Mg^{2+} on the reaction rate—the maximum rate was observed at a Mg^{2+} concentration of $[Mg^{2+}] \approx [ATP] + 1/2[AMP]$—was studied. It was suggested that formation of inert complexes such as AK·AMP·ADP·Mg^{2+} were the reason for the inhibition above optimal Mg^{2+} concentration.[56]

Recently, chemical exchange calculations taking into account spin–spin splittings of the reactants and therefore necessarily based on direct density matrix calculations were applied to the calculation of on-enzyme exchange rates of the AK reaction; the estimated numbers given by Nageswara Rao *et al.*[56] were basically confirmed. New results based on earlier experiments concerned the exchange of the two ADP molecules between the two different sites, which was calculated to be about 100 sec^{-1} in the presence of the optimal Mg^{2+} concentration and about 1500 sec^{-1} in the absence of the divalent metal ion[13]. These resonances could be detected only in samples in which an appreciable amount of P_i and AMP was present, indicating progressed ageing of the sample. It thus was suggested that the signal from bound ADP·Mg^{2+} originates from the abortive complex AK·AMP·ADP·Mg^{2+}.

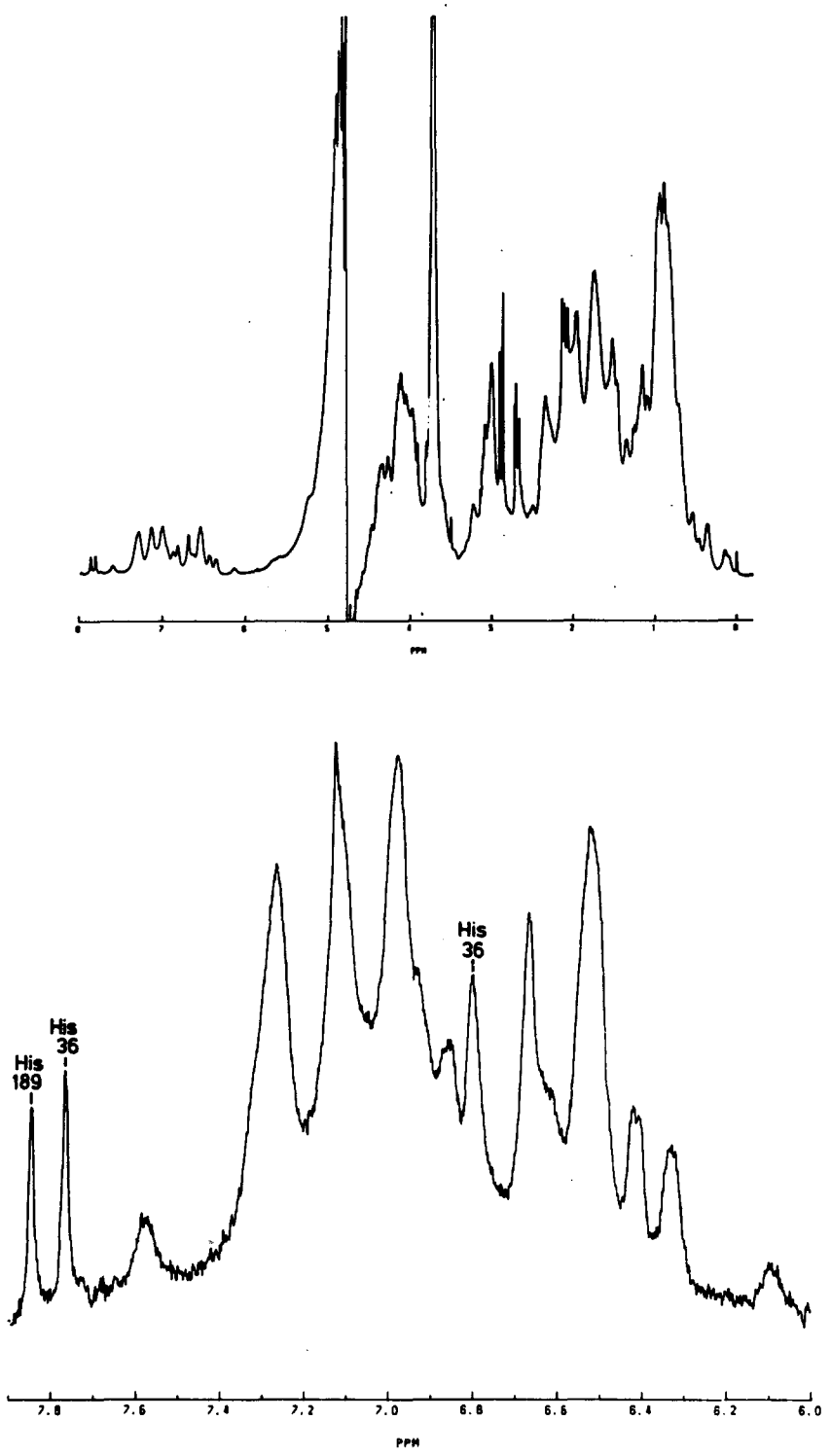


FIG. 10. The ^{1}H-NMR spectrum of porcine adenylate kinase (top: complete spectrum—bottom: aromatic part). Assignments derived from McDonald *et al.*[60] and Kalbitzer *et al.*[61]

The adenylate kinase reaction pathway was mapped for demonstration purposes by ACCORDION spectroscopy of the free interchanging nucleotides.[57] Brown and Ogawa[58] analyzed the AK reaction by magnetization transfer. The overall nucleotide interconversion rates catalyzed by this enzyme were measured for ATP, ADP, and AMP free in solution. A nonequilibrium situation was generated by application of a weak 180° pulse in order to invert a specific resonance (inversion transfer). To complete the kinetic data, an estimation of the on and off rates from the linebroadening effects was performed.

Analysis by ^{31}P-NMR of the reaction products of the adenylate kinase reaction initiated with the two diastereomers of ATP(α-S) as substrates led to the conclusion that the Mg^{2+} complex of the R_P stereoisomer is the one recognized by the enzyme.[42]

The linewidth of three different ATP(^{17}O) species was measured in the presence of varying amounts of porcine AK by Wisner *et al.*[59] From these results a rather non-rigid binding of the phosphate moiety in the absence of Mg^{2+} was deduced.

4.1.2. *^{1}H-NMR.* The first proton NMR experiments on adenylate kinase were performed in 1975 at 220 MHz[60] (a ^{1}H-NMR spectrum of AK at 360 MHz is shown in Fig. 10). These experiments consisted firstly of a comparison of carp adenylate kinase with porcine adenylate kinase. This brought about the assignment of the histidyl C2H resonance of the two histidyl residues contained in the protein, His-36 and His-189, because His-189 is absent in the carp enzyme and present in the porcine enzyme and the resonance for C2H of His-36 is identical in both proteins. Secondly, the pH-behaviour of these two histidines was studied, yielding a very low pK_a value for His-189 (<5.5) and a pK_a of 6.3 for His-36. An apparent pK value of 8.3 was recently measured for His-189, the high value probably being due to the influence of Cys-187 on the His residue.[62] Thirdly, the influence of nucleotide binding was observed to result in a downfield shift of the C2H resonance of His-36. Fourthly, a linebroadening of C2H of His-36 was observed on binding of ATP·Mn. These results brought about the suspicion that His-36 contributes in some way to the active site structure of adenylate kinase.[60]

This view is now supported by new evidence, including that from NMR experiments. The most direct way of monitoring spatial proximities of nuclei by NMR is by observation of nuclear Overhauser effects (NOE). On irradiation of the C2H resonance of His-36 of porcine AK an NOE was observed on the C2H resonance of ATP·Mg^{2+}; this effect could not be observed for the ternary complex of AMP, Mg^{2+}, and AK. All nucleotides were present in excess over the protein in these experiments. In addition, the stable chromium ATP complex was observed to cause a linebroadening effect on C2H of His-36 on binding to porcine AK.[63]

This seems to be rather convincing evidence of a proximity of the purine ring of ATP·Mg^{2+} to His-36, the only point for minor criticism being that in the NOE experiments a threefold excess of nucleotides was used in order to improve the signal to noise ratio, and there is no independent evidence for specific binding of the nucleotide at these concentrations.

In the course of these nucleotide binding studies it became evident that aromatic resonances other than the C2H of His-36 were also shifted on nucleotide binding. This led to an attempt to identify tyrosyl ring spin systems within the aromatic spectral part of AK. A combination of J-decoupling experiments, double-quantum (DQ) experiments, and two-dimensional J-correlated spectroscopy (COSY) brought about the identification of all seven tyrosyl ring spin systems and of two phenylalanyl ring spin systems. The COSY and DQ spectra are shown in Fig. 11 and Fig. 12.[64, 62]

The assignments indicated in Fig. 13 serve currently as a starting point for more detailed studies of the protein, including assignments by nitration of specific tyrosyl residues.

A proton NMR study of human AK, which has 186 out of 193 amino acids in common with the porcine enzyme (including all aromatic residues), was undertaken in order to learn more about transitions between three different states of AK as observed in porcine enzyme crystals on changing pH values from neutral to the acidic range. One form prevailed above pH 7 ('A-form'), one between pH 5.7 and pH 6 ('B-form') and one below pH 5.4 ('C-form'). The B-form was supposed to mimic the 'active' form of AK, i.e. the form where the nucleotides were bound, the A-form the 'inactive'

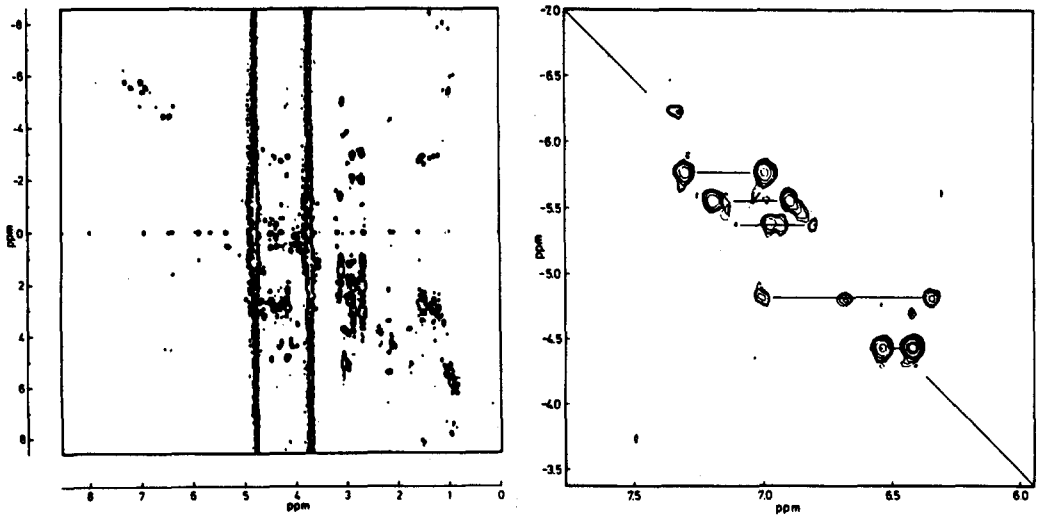


FIG. 11. Left: complete DQ ^{1}H spectrum of porcine adenylate kinase at 360 MHz. Right: aromatic part of the DQ spectrum (connectivities indicated).

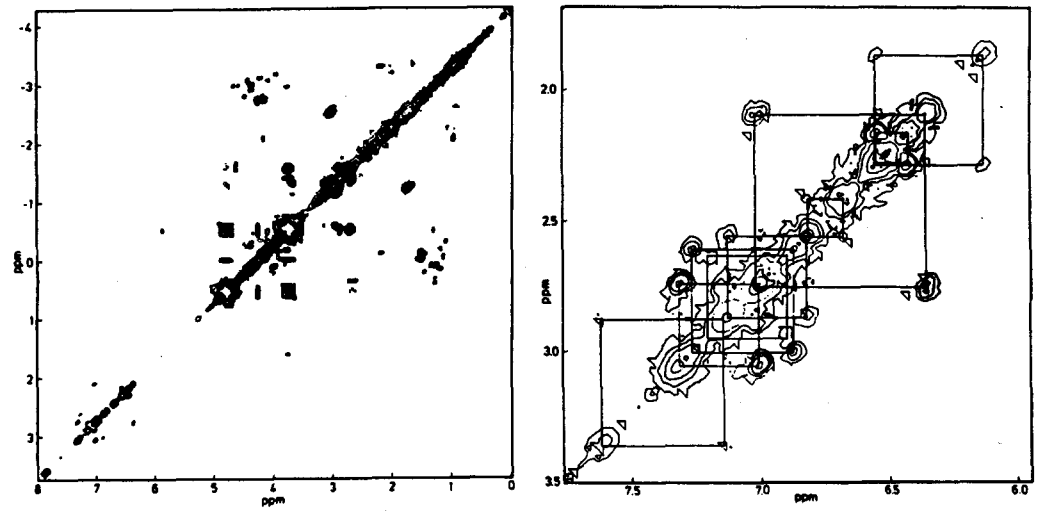


FIG. 12. Left: complete COSY ^{1}H spectrum of porcine AK at 360 MHz. Right: aromatic part of the COSY spectrum (connectivities indicated) (Rösch and Gross[62]).

one.[53, 65] A transition between these two forms can be induced in the porcine enzyme crystal by alterations of the pH value. In solution, no A to B transition could be observed conclusively by ^{1}H-NMR for the human enzyme (neither was this transition observed for the human enzyme variety by crystallography). In the course of these studies it was noted that the two His-C2H proton resonances behave very similarly to the corresponding proton resonances of the porcine enzyme.[61]

New evidence for an important role of His-36 was brought about by a comparison of the aromatic part of the proton NMR spectra of porcine and human AK with American and European baker's yeast AK. The baker's yeast AKs showed a resonance at exactly the position of the C2H resonance of His-36 of the former ones (Fig. 14). In addition, the position and linewidth of this resonance was

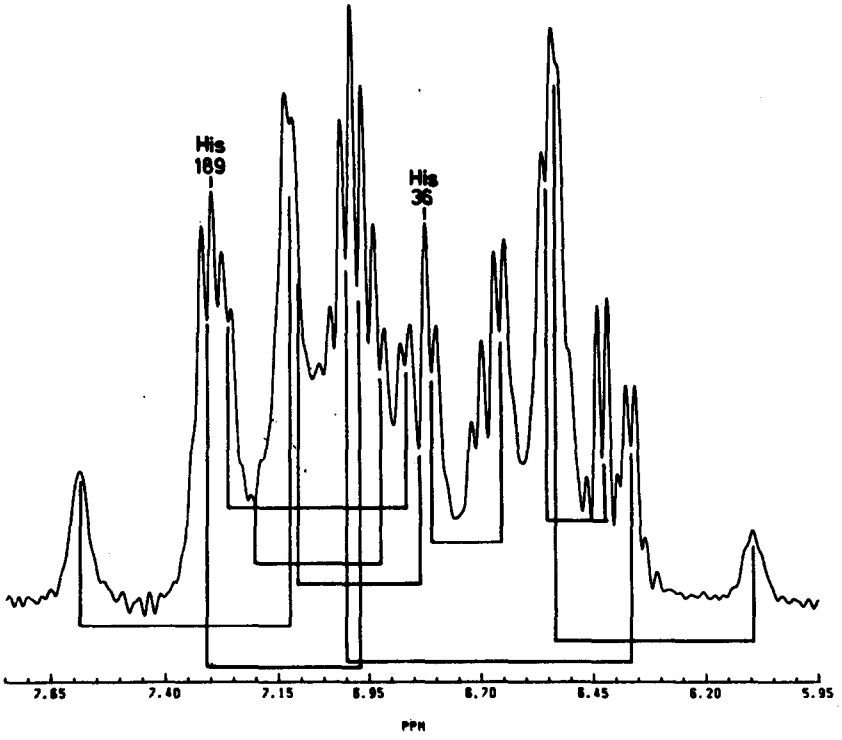


FIG. 13. Identified connectivities (in the ^1H spectrum) for the seven tyrosyl residues of porcine AK and two phenylalanyl residues (Rösch and Gross[62]).

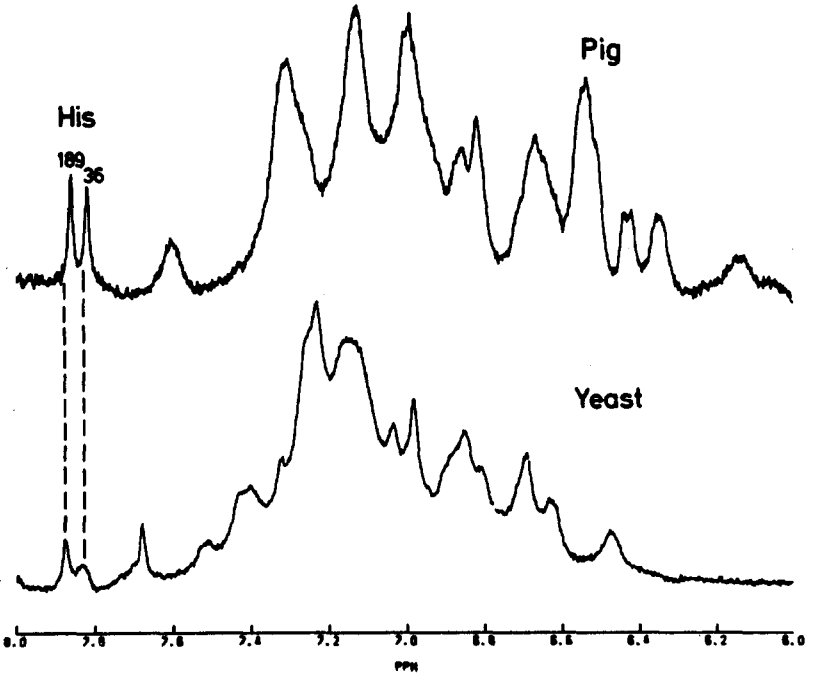


FIG. 14. Comparison of the aromatic part of the proton NMR spectrum of porcine and yeast adenylate kinase at pH 7.4. Two His C2 protons and one Tyr spin system exhibit virtually identical chemical shifts in both proteins (Rösch and Tomasselli, unpublished).

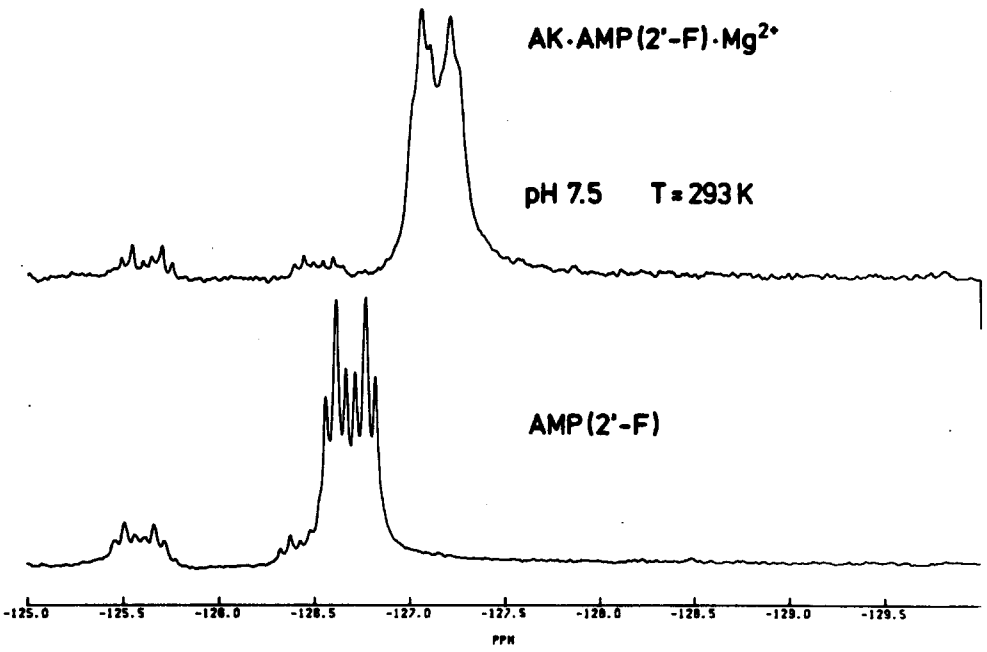


FIG. 15. ^{19}F-NMR spectrum of (2'-F)AMP bound to porcine adenylate kinase (top) as compared to free (2'-F)AMP (bottom) (Goody and Rösch, unpublished).

influenced by nucleotide binding in a way similar to the His-36 resonance of porcine and human AK on substrate binding, so that there seemed to be a histidyl residue with a very similar function in the yeast, the carp, and the porcine (or human) enzyme (Goody, Konrad, Rösch and Tomasselli, unpublished).

First studies of AK with the aid of fluorinated nucleotide analogues (2'-F-adenosine) were very promising (Fig. 15). Fluorine NMR with nucleotides labelled at the ribose ring would open new possibilities as compared to phosphorus NMR, because the ribose ring should be influenced only by its direct environment within the protein, not by any changes in the phosphate chain (Goody and Rösch, unpublished).

4.2. *Creatine Kinase*

Creatine kinase (CK, adenosine-5'-triphosphate : creatine phosphotransferase, EC 2.7.3.2) serves to tap the energy reservoir supplied by phosphocreatine (PCr) in vertebrates via transfer of the phosphate group of phosphocreatine to ADP according to:

$$PCr + ADP \rightleftharpoons Cr + ATP.$$

As in all kinase reactions, a divalent metal ion—physiologically Mg^{2+}—is a mandatory component. All NMR experiments so far performed on CK used the rabbit muscle enzyme. The active form consists of a dimer (one active site per subunit) of total molecular weight 83,000. Recently, a review on general aspects of structure–activity relationships of creatine kinase has appeared.[66]

4.2.1. *^{31}P-NMR.* The substrates PCr, ADP and ATP are all accessible to ^{31}P-NMR spectroscopic methods. Creatine kinase was subjected to the same spectroscopic procedures as adenylate kinase, i.e. the transfer reaction on the enzyme was monitored. Again, the chemical shifts of the bound substrates could be determined.[67]

The quaternary complex CK·ADP·MG^{2+}·creatine·NO_3^- is supposed to form a kind of transition state analogue. Phosphorus NMR spectra of the CK·ADP·Mg^{2+}·creatine complex show an upfield shift of the β-resonance on binding of the NO_3^- ion at 300 K. Cooling of the sample with a CK : ligand ratio of 2 : 1 (i.e. a 1 : 1 ratio of active sites) results in a splitting of the β-resonance into two distinct resonances.[68]

The observation of the CK·ADP·Mg^{2+}·creatine·$HCOO^-$ transition state analogue complex resulted in qualitatively similar results.[67] The splitting of the β-resonance of ADP in the transition state analogue complex as well as in the complex free of planar ions was interpreted as indication of two non-equivalent active sites. On the other hand, similar signal patterns were observed with the monomeric arginine kinase by Nageswara Rao and Cohn[69] and adenylate kinase.[56] In the latter case two resonances were observed and interpreted as being indicative of nucleotide binding to the two subsites in the abortive complexes. Inhomogeneities in the protein preparation or nucleotide also partially bound to denatured protein should possibly be taken into account for an explanation of the results with the arginine and the creatine kinase.

Recently the linebroadening effects on the signals in the phosphorus NMR spectra due to the paramagnetic ions Mn^{2+} and Co^{2+} and due to exchange effects between the metal ion complexes CK·ADP·Me^{2+} and CK·ATP·Me^{2+} and the respective metal free complexes were subjected to an extensive study. Investigations of the linebroadening effects at three different frequencies showed that the linebroadening observed with the Mn^{2+} complexes is basically exchange limited; thus, these complexes may not be used for estimating distances. The same was shown to be true for the NO_3^- transition state analogue complex. In contrast, the corresponding inert Co^{2+} complexes could be used to set limits on the ^{31}P–metal ion distances, which were 0.24 nm–0.46 nm in each case.[70]

In addition to these more structurally oriented results, the overall interconversion rates of the CK catalyzed reaction aroused much interest, especially due to the fact that this reaction is the immediate phosphoryl source ensuring a rather constant ATP level in muscle even during exercise. The kinetic analysis of the CK reaction may be based on a simple two site scheme:

$$PCr \rightleftharpoons MgATP.$$

Boyd *et al.*[57] compared the results obtained using a ^{31}P saturation transfer experiment performed on the creatine kinase catalyzed phosphoryl exchange with those from the ACCORDION experiment performed on the same reaction. They obtained an overall pseudo first order rate constant of about 0.3 sec^{-1} at a temperature of 304 K for the transfer in PCr direction from both experiments. The final conclusion drawn as far as the efficiency of the technique was concerned was that the ST experiment is superior in most cases of interest in ^{31}P-NMR spectroscopy, especially *in vivo*.

Brindle *et al.*[24] also performed an ST experiment to determine the overall rate constant of the CK reaction. They compared the result to the one they got from a ^{15}N label exchange experiment monitored by observation of the ^{31}P spin echo. Both experiments gave very similar pseudo first order rates of approximately 0.4 sec^{-1} at 303 K.

The on-enzyme substrate interconversion rate was estimated from a complete lineshape analysis of the spectrum to be 90 sec^{-1} and the equilibrium constant determined to be $K_{eq} = 1$ at 277 K. The interconversion rate is nearly twenty times faster than the overall reaction rate. Thus the transfer step cannot be the rate limiting factor.[67,14]

An associative reaction mechanism scheme, i.e. a reaction where bond formation to the second substrate is performed prior to bond cleavage, was shown to be applicable to CK. To this end, ATP with three ^{18}O labels at the β-position, one of them in the α–β bridge and two non-bridge labels, was synthesized. This ATP ($^{18}O_3$) was incubated with CK and the competitive inhibitors L-arginine or tauromycin. A dissociative reaction scheme was expected to lead to an on-enzyme bond breakage under these conditions and thus scrambling of the ^{18}O label in the β-position of the observed free ATP($^{18}O_3$) would be observed. The experiment showed no indication of oxygen scrambling. In a control experiment, the cosubstrate creatine was added and the reaction taking place resulted in the expected complete scrambling as detected via two γ-doublets from ATP with an intensity ratio of 2 : 1.[71]

4.2.2. *^{1}H-NMR.* Proton NMR studies of CK are severely restricted by the comparatively high molecular weight of the enzyme (two identical subunits, MW 41,000 per subunit). Nonetheless, early NOE experiments on the CK formate complex (formate is supposed to constitute another transition state analogue) revealed the proximity of a nucleus resonating at 2.6 ppm to the single formate proton. This observation would be consistent with the participation of a lysyl residue in the protein/substrate interaction.[72]

These experiments were supplemented by investigations of the CK·ADP complex. The C2H of the adenine ring experienced a strong NOE caused by irradiation at 0.9 ppm and 1.7 ppm under a variety of circumstances. It was suggested that this might be due to the involvement of an arginyl residue in the binding process.[73]

The measured transfer of polarization by an NOE from CK to ADP in the CK·ADP complex lead to other studies, trying the measurement of the reverse effect, i.e. an NOE from the substrate ADP to the protein. Indeed, irradiation of the C2H and C8H resonances resulted in negative Overhauser effects for several protein resonances in the aliphatic region, including those which were observed to give an NOE on the adenine resonances on irradiation, and several resonances from aromatic amino acids.[74]

The attempt at direct observation of distinct protein resonances resulted in the detection of six imdidazole C2H resonances and one C4H resonance of the sixteen histidyl residues per subunit of CK. THe determination of the p*K*-value of one of the histidines in the presence either of the phosphorylated substrates (p*K* 7.6) as compared to its p*K*-value in the presence of creatine (p*K* 7.0) led to the suspicion that this histidine residue may be the one responsible for an acid/base catalysis of the phosphotransfer. Distances of three histidyl residues from the paramagnetic Cr^{3+} of bound ATP·Cr^{3+} could be estimated to be from 1.2. to 1.4 nm by the linebroadening effect induced by this paramagnetic ligand. In addition, exchange rates were determined directly from the exchange linebroadening for ADP Mg^{2+}, giving an off rate of 350 sec^{-1}.[75]

4.3. *Pyruvate Kinases*

Much attention has also been focussed on the pyruvate kinase (PK, phosphoenolpyruvate : ADP phosphotransferase, EC 2.7.1.40) from rabbit muscle in recent years. PK requires two metal ions for its catalytic activity, one of which is probably complexed directly by the enzyme, the other one by the enzyme-bound nucleotide. In addition, a monovalent activator, usually K^+, is required. PK catalyzes the reaction (in the presence of two divalent metal ions):

$$PEP + ADP \rightleftharpoons Pyr + ATP.$$

The interaction of the two metal ions was studied in the complex PK·Mn^{2+}·ATP·Cr^{3+}. The influence of the paramagnetic ions on the water relaxation rate was determined and their mutual distance estimated to be around 0.5 nm.[76]

The direct observation of proton resonances originating from the protein or phosphorus resonances from the bound substrates is impaired by the high molecular weight of the tetrameric PK (MW 59,000 per subunit). Nevertheless, both types of resonance were investigated. ^{31}P-NMR revealed the on-enzyme equilibrium constant to be about unity. The chemical shifts of the resonances of the nucleotide substrates were quite insensitive to complexation by the enzyme. In contrast, the ^{31}P resonance of PEP proved to be sensitive to the nature of the enzyme/substrate complex. The binding of Mg^{2+} ions to the PK·ATP complex could be monitored via the changes of the chemical shift of the β-resonance of bound ATP, which is always the one influenced most by metal ion complexation. Qualitatively, the apparent dissociation constant of the E·ATP·Mg^{2+} complex is larger than the dissociation constant of the enzyme-free ATP·Mg^{2+} complex, which might be indicative of complexation of Mg^{2+} to a secondary ATP site in fast exchange with the primary one with respect to either the complete ATP·Mg^{2+} complex or Mg^{2+} alone.[77]

The C2H proton resonances of six out of a total of fourteen histidyl residues per subunit could be detected and their p*K* values determined. One of those histidyl residues showed a decrease in p*K* value on formation of the PK·PEP complex.[78]

The stereochemical course of the PK catalyzed reaction was shown to proceed with inversion of configuration of the phosphogroup transferred from ATP(γ-^{16}O, ^{17}O, ^{18}O), in accord with a direct in-line transfer mechanism without a phosphoenzyme intermediate.[79]

The question of whether the transfer mechanism of PK is associative or dissociative was answered by Hassett *et al.*[33] The experiments were performed using a reaction mixture containing ATP(α,β-^{18}O, β-$^{18}O_2$) with a ^{16}O β–γ-bridge oxygen but lacking the cosubstrate pyruvate. No ^{18}O incorporation in the β,γ-bridge was observed. In addition, the experiments were repeated with ATP(γ-^{16}O, ^{17}O, ^{18}O). No scrambling of the γ-phosphoryl oxygens was observed. These experiments thus show no evidence of a dissociative phosphoryltransfer. Also, theoretical arguments were presented in favor of the associative pathway being the more efficient one in terms of transfer rate constants.

A somewhat more exotic approach to the study of active site conformational changes of PK was undertaken by Hutton *et al.*[80] They observed the ^{7}Li relaxation of a LiCl solution in the presence of the Li^+-activated enzyme-Mn^{2+} complex. It was concluded that the monovalent and divalent cation sites increased their mutual distance on PEP binding to about 0.6 nm. In addition, Ash *et al.*[81] deduced the existence of two structures of the PK·Mn^{2+}·Li^+·PEP complex with differing Li^+–Mn^{2+} distances.

4.4. *Arginine Kinase*

Arginine kinase (AgK, phosphoarginine : ADP phosphotransferase, EC 2.7.3.3), an enzyme found in invertebrates where it substitutes for creatine kinase, was also investigated by several NMR methods. The reaction catalyzed by arginine kinase in the presence of a divalent metal ion is:

$$\text{PArg} + \text{ADP} \rightleftharpoons \text{Arg} + \text{ATP}.$$

Again, Nageswara Rao *et al.*[82] used ^{31}P-NMR to observe substrates bound to the American lobster enzyme. The on-enzyme equilibrium constant of the reaction was found to be 0.8. Estimates for the reaction rates from complete lineshape analysis of the exchange broadened resonances resulted in values of 120 sec^{-1} in the direction from ATP to PArg. This is about an order of magnitude faster than the overall reaction rate; therefore, the phosphate transfer step is not rate limiting. Surprisingly, the bound nucleotides showed a resolved J-splitting. The β-resonance of ADP and ADP·Mg^{2+} experienced a large shift on binding to AgK, whereas the α-resonance and resonances from ATP and ATP·Mg^{2+} were hardly shifted. From pH titration studies it was deduced that an amino acid side chain with an apparent pK of about 7.5 is either close to the active site or at least contributes to the active site conformation. An alternative possibility could be the influence of such an amino acid side chain on the active site conformation. Studies of the transition state analogue complex AgK·ADP·Mg^{2+}·Arg·NO_3^- showed a shift of the β-resonance of ADP·Mg^{2+} of 3 ppm towards the position of the β-resonance of ATP·Mg^{2+}, probably indicating a change in the electronic structure towards the one of the reaction products.[82, 69, 14]

4.5. *3-Phosphoglycerate Kinase*

Another kinase studied by NMR methods was the 3-phosphoglycerate kinase (PGK, 1,3-bis-phosphoglycerate : ADP phosphokinase, EC 2.7.2.3), which in the presence of a divalent metal ion catalyzes the interconversion of 3-phosphoglycerate (3-PG) and 1,3-bis-phosphoglycerate (1,3-DPG):

$$\text{3-PG} + \text{ATP} \rightleftharpoons \text{1,3-DPG} + \text{ADP}.$$

So far, all NMR experiments have been performed on the yeast enzyme. PGK from yeast has a molecular weight of 47,000.

The ^{31}P-NMR parameters of the bound substrates have been determined. The on-enzyme equilibrium constant was measured to be about one, which compares to an equilibrium constant for the overall reaction of 3×10^{-4}. Forward and reverse reaction rates on the enzyme were around 100 sec^{-1}. Considering the ratio of concentrations of enzyme-bound substrates [E·Mg^{2+}·ADP·1,3-

DPG]/[E·Mg^{2+}·ATP·3-PG] of 0.8 and the corresponding ratio of free substrates of around 3000, one has to conclude that the phosphotransfer step is not rate limiting. The presence of sulphate ions resulted in a reduction of the interconversion rates and a slight shift of the equilibrium constant of the enzyme-bound substrates to the side of ATP production. The changes of the chemical shifts of the phosphorus resonances of ATP, ATP·Mg^{2+} and ADP on binding to the enzyme did not show significant values, in contrast to those of the phosphorus resonances of ADP·Mg^{2+} (1.1 ppm upfield for the α-phosphate and 1.6 ppm for the β-phosphate). Shifts of approximately 2 ppm were induced in the PG and DPG resonances. Two resonances for the β-phosphate of enzyme-bound ATP in the presence of excess Mg^{2+} were observed, one at the position expected for the metal complex, one at the position of the metal free compound, thus proposedly indicating the presence of a second ATP binding site with a larger dissociation constant for the metal complex.[83]

The equilibrium constant of the PGK reaction with ATP(β-S) as a substrate was also determined by NMR and had a value of 4×10^2.[84]

Despite the molecular weight of PGK being relatively high for ^{1}H-NMR, Tanswell *et al.*[85] attempted the assignment of residues. A few peaks of rather narrow linewidth could be detected in the His-C2H region. The pK_a values of two of these peaks with total intensity three suggested these peaks as representing His-C2H resonances. The corresponding His-C4H resonances could also be identified by making use of the convolution difference technique. The addition of ADP and Mg^{2+} caused shifts of various peaks in the aromatic region. Formation of the PGK·ATP·Gd^{3+} and PGK·ATP·Mn^{2+} complexes resulted in specific paramagnetic broadening of the same resonances. Three of the resonances in the His region were broadened selectively. In order to get more information on the active site structure, the PGK·nucleotide·Eu^{3+} and PGK·nucleotide·Pr^{3+} complexes were investigated. The NMR results suggested differences with the X-ray structure (which is known for the horse enzyme and for the yeast enzyme[86,87]) in several respects concerning mainly the neighbourhood of the phosphates. Cohn[88] has mentioned a few preliminary results on PGK obtained with photochemically induced dynamic nuclear polarization. She and her coworkers used two different flavin dyes in order to photoinduce a CIDNP in yeast PGK, namely N^{10}-carboxyethyllumiflavin and 8-aminoriboflavin. The latter one is known not to induce a polarization in His residues, whereas the former one does, thus yielding a means to separately detect His and Trp by just comparing the effect of the two dyes. In this way, a resonance was assigned to Trp-308 (the only other Trp is expected to be buried in the interior of the protein from X-ray studies and thus inaccessible to the dye), three others to three tyrosyl residues, and four more to His-C2H and His-C4H, respectively. These results provide one more example of the feasibility of studying specific amino acids in large molecules with the photo-CIDNP method.

4.6. *Hexokinase*

Hexokinases (HK, ATP : hexose-6-phosphotransferase, EC 2.7.1.1) catalyze the transfer of the terminal phosphoryl group from ATP to the 6-position of a hexose molecule:

$$\text{ATP} + \text{Hexose} \rightleftharpoons \text{ADP} + \text{Hexose-6-P}.$$

The stereochemistry of the transfer reaction has been studied by ^{31}P-NMR, employing adenosine-5'-(γ-^{16}O,^{17}O,^{18}O) as a substrate. The sugar phosphate product of the yeast HK catalyzed reaction:

$$\text{ATP} + \text{Glucose} \rightleftharpoons \text{ADP} + \text{Glucose-6-P},$$

glucose-6-(^{16}O,^{17}O,^{18}O), was shown to possess inverted chirality as compared to the labelled ATP, consistent with an in line phosphoryl transfer.[79,89]

The stereochemistry of the rat liver glucokinase (GK, ATP : glucose-6-phosphotransferase, EC 2.7.1.2) catalyzed reaction was probed by the same method. The results were in agreement with the stereochemistry observed for the yeast enzyme. This and other evidence led to the conclusion that GK qualifies as a member of the enzyme class of hexokinases and deserves no special entry in the EC.[90]

The determination of distances within the ternary complex formed by yeast hexokinase with ADP·Cr^{3+} and glucose-6-phosphate was tried by Petersen and Gupta.[91] They measured the influence of the paramagnetic nucleotide probe on the relaxation rates of the water protons, the α- and β-anomeric glucose protons, and the single phosphorus nucleus of the free compound in the presence of the complex. From the frequency dependency of the water proton relaxation rate in the presence of the ternary complex a correlation time of 0.7×10^{-9} sec could be calculated. With this value, the Cr^{3+} to phosphorus distance in the complex was estimated to be about 0.7 nm, the metal ion to α-anomeric proton distance to be about 0.9 nm, and the metal ion to β-anomeric proton distance to be around 1 nm.

4.7. *Protein Kinases*

The activity of some proteins is regulated by phosphorylation of a specific amino acid residue within their sequence, usually serine or threonine. The phosphorylation of these residues is in the presence of divalent metal ions catalyzed by protein kinases (ATP : Protein phosphotransferase, EC 2.7.1.37):

$$\text{Protein} + \text{ATP} \rightleftharpoons \text{P-Protein} + \text{ADP}.$$

The messenger activating the protein kinases is usually cyclic AMP (cAMP). Protein kinases play a key role in cellular regulatory mechanisms. Typically, the inactive tetrameric enzymes (R_2C_2) are decomposed into three units on cAMP binding, one dimer/cAMP complex (R_2·cAMP) and two monomeric subunits ($2 \times C$) active in protein phosphorylation. The R_2 dimer is known as regulatory unit, whereas the two C subunits are termed catalytic. Recently, interest in protein kinases increased strongly because it was noted that oncogene products are enzymes of this classification, catalyzing the phosphorylation of tyrosyl residues in the target enzymes.

Armstrong *et al.*[92] investigated different complexes formed by the catalytic subunit of cAMP dependent protein kinase from bovine heart by proton relaxation enhancement (PRE) studies. They found rather large values for the dissociation constant of Mn^{2+} and enzyme ($K > 1$ mM). The dissociation constant was determined to decrease dramatically in the presence of ADP or ATP(β,γ-CH_2). The formation of an E·ATP·Mn^{2+}·Mn^{2+} complex ($K = 19$ μM for both ions) was suggested. There were no indications of binding of an additional metal ion to the E·Co^{3+}·$(NH_3)_4$ATP·Mn^{2+} complex, thus suggesting that only one of the bound metal ions is complexed directly to the enzyme. Titration of an enzyme solution with ADP in the presence of Mn^{2+} showed only one nucleotide binding site. Subsequent kinetic studies indicated an inhibitory effect of the second metal binding site.

The authors of another PRE study of the same enzyme combined with measurements of the ^{31}P relaxation rate of free $Co^{3+}(NH_3)_4$ATP in the presence of the protein kinase claimed that it was possible to derive nine distances from the second bound metal ion (Mn^{2+}) to the bound metallonucleotide. They suggested formation of an enzyme–polyphosphate chain bridge via the second metal ion as a possible mechanism for the inhibition.[93]

The linebroadening induced by Mn^{2+} ions in resonances of a septapeptide containing one serine, which served as a substrate analogue, in the presence of small amounts of catalytic subunits of protein kinase and ATP was measured by Granot *et al.*[94] Addition of peptides functioning as competitive inhibitors caused the linebroadening effect to disappear. Linebroadening effects were not induced by the holoenzyme R_2C_2.

Phosphorylase kinase consists of four different subunits termed α, β, γ, and δ, forming a holoenzyme of the form $(\alpha\beta\gamma\delta)_4$. The target enzyme of phosphorylase kinase is the glycogen phosphorylase. Phosphorylation of the latter enzyme at residue Ser-14 activates glycogen degradation. Phosphorylase kinase, in turn, is activated by phosphorylation of one serine in its α and β subunits catalyzed by the cAMP dependent protein kinase. A mandatory component for the enzymatic activity is the binding of a Ca^{2+} ion to the δ subunit, which is identical to the calcium binding protein calmodulin. The catalytically active site is located in the γ subunit. Phosphorylase kinase may also cause autophosphorylation, which results in an incorporation of up to 20 mol of

phosphate per mol ($\alpha\beta\gamma\delta$) unit. There are, in addition, two moles of phosphate per ($\alpha\beta\gamma\delta$) unit contained in the protein.[95] When the protein is purified in the presence of NaF, which is an inhibitor of phosphoprotein phosphokinase, a total of three or five phosphates per ($\alpha\beta\gamma\delta$) unit is contained in the enzyme. By ^{31}P-NMR it could be shown that all these phosphates form phosphoseryl monoesters. This was concluded from the phosphorus chemical shift values measured with native and denatured protein and the pH dependence of the shift values exhibited by the denatured protein. Unfortunately, resolution and sensitivity of the spectra obtained with the native enzyme were very poor, so that no further conclusions could be drawn.

4.8. *Phosphoenolpyruvate Dependent Phosphotransferase System*

The phosphoenolpyruvate (PEP) dependent phosphotransferase system (PTS, Phosphoenolpyruvate : Sugar Phosphotransferase System) found in anaerobic and facultatively anaerobic microorganisms is a multienzyme system transferring a phosphoryl group from PEP to a carbohydrate. This marking of the carbohydrate is a step performed during the transport through the cell membrane. In the case of *Staphylococcus aureus* this enzyme system consists of four different protein molecules: Enzyme 1 (E I), Heat-stable Protein (HPr), Factor 3 (F III), and Enzyme 2 (E II). The last one is a membrane bound protein, whereas the other three are located in the cytosol. The reaction scheme of the phosphoryl transfer from PEP to a sugar is described by:

$$\begin{aligned} \text{PEP} + \text{E I} &\rightleftharpoons \text{P-E I} + \text{Pyruvate} \\ \text{P-E I} + \text{HPr} &\rightleftharpoons \text{P-HPr} + \text{E I} \\ \text{P-HPr} + \text{F III} &\rightleftharpoons \text{P-F III} + \text{HPr} \\ \text{P-F III} + \text{E II} + \text{Lactose} &\rightleftharpoons \text{F III} + \text{E II} + \text{Lactose-6-phosphate.} \end{aligned}$$

E I and HPr are nonspecific, whereas F III and E II are sugar specific proteins. F III is substituted by a multifunctional protein termed enzyme 3 (E III) for example in *E. coli* and *S. typhimurium*. E III is assumed to have regulatory properties not directly connected with its function in the phosphoryltransfer. The unphosphorylated E III is suspected to stimulate adenylate cyclase activity, whereas this functional property is switched off on phosphorylation of E III. For recent reviews on the PTS see Meadows *et al.*[96] and Reizer *et al.*[97]

The studies concentrated in the first place on HPr, because this is an ideal system from a spectroscopist's point of view. This extremely stable protein has a very low molecular weight of 7650 d and may therefore be studied in atomic detail by ^{1}H-NMR methods.

In a ^{1}H-NMR study Schrecker *et al.*[98] suggested the N1 nucleus of the single histidyl residue (His-15) of the enzyme to be the phosphoryl-carrying site of the *S. aureus* protein. This was confirmed by ^{31}P-NMR as well as ^{1}H-NMR data for model phosphohistidine compounds and the comparison of these data with results from titration experiments with HPr followed by ^{1}H-NMR.[99] Some doubt was shed on the validity of these results,[100] but the question could finally be settled by the study of phosphorylated model peptides in favour of an N1 phosphorylated histidyl residue of P-HPr.[101]

^{1}H-NMR studies intended to probe structural features of the HPr were first performed by Maurer *et al.*[102] They determined pK values for the single histidyl residue and for the three tyrosyl residues of the enzyme. Also, backbone NH exchange has been followed and the heat denaturation of the protein studied. It was inferred from these measurements that the structure of HPr was rather rigid.

These proton NMR studies were extended by the study of tyrosyl residues specifically nitrated within the active protein. In this way all the proton resonances of the aromatic part of the protein spectrum could be assigned to specific residues in the sequence. In addition, it was concluded that the three tyrosyl residues possess a rather distinctly different accessibility to the solvent. This conclusion was based on the different pK values and the different conditions which had to be employed for the nitration procedure. The ring current effect on a valine residue was shown to be caused by one of the tyrosyl rings. A cotitration of the single histidyl residues with the nitrotyrosyl residue exhibiting the lowest pK was observed and a possible interpretation in terms of a hydrogen bond between both residues attempted.[103,104]

The latter observation gave rise to additional studies with HPr proteins from two different sources, namely *B. subtilis* and *S. faecalis*. Again, a cotitration of the low p*K* nitrotyrosyl residue and the active centre His-15, which is present in the *B. subtilis* and *S. faecalis* as well as in the *S. aureus* protein, was detected. The low p*K* tyrosyl residue was identified as Tyr-37 in all three proteins, correcting a previous error.[105]

Dooijewaard *et al.*[100] studied the conformational changes of HPr and P-HPr of the *Escherichia coli* protein (MW 9600), which differs from the *S. aureus* protein in several respects (e.g. the *E. coli* enzyme contains two histidyl residues) and cannot substitute for it in the assay. From their studies of phosphorylated and unphosphorylated HPr they proposed a two state model for the phosphorylation process. This model suggested itself by the conformational changes of aromatic and aliphatic resonances on enzymatic phosphorylation or pH titration. Roossien *et al.*[106] suggested a heterogeneity of the amino acid composition of the *E. coli* protein, because they observed a tyrosyl residue with about half the intensity expected for one tyrosyl residue per protein molecule in the ^{1}H-NMR spectrum. This result was disputed later by Kalbitzer *et al.*[107]

A comparison based on ^{1}H-NMR spectra of HPr from *Staphylococcus aureus*, *Streptococcus lactis*, *Streptococcus faecalis*, *Bacillus subtilis*, and *Escherichia coli* was undertaken recently. The active centre histidyl residues of all HPrs showed a rather low p*K* value (<6.1). It was suggested that all HPrs were phosphorylated at the N1 position of this histidyl residue. The active site histidyl residue of the *S. lactis* protein showed a cotitration with a tyrosyl residue with similar characteristics as the one observed cotitrating with the histidyl residue in the *S. aureus* protein. As expected, the *E. coli* protein spectrum revealed features quite different from the others. The tyrosyl residue suggested to be contained in about 50 % of the *E. coli* protein by Dooijewaard *et al.*[100] could not be detected in these more recent experiments.[107]

Another component of the PTS, Factor 3, was also studied by ^{1}H-NMR methods. The trimeric F III protein specific for galactosides contains four histidyl residues per monomer (MW 11,000 per subunit). It was deduced from the NMR results that one of these histidyl residues is phosphorylated. By isolation and NMR-analysis of a peptide containing the active site histidyl residue it was shown that the phosphorylation site is the N3 position. The interaction of F III with HPr could be followed in the ^{1}H-NMR spectrum, where a shift of the C2H resonances of the active site histidyl residue was observed. A mechanism for the phosphoryl transfer between HPr and F III on the basis of the observed differences of the histidyl p*K* values in the phosphorylated and unphosphorylated form in the two proteins was suggested.[108]

The glucose specific E III, E III^{glc}, of *E. coli* (monomeric, MW 20,000) exhibited a well-resolved ^{1}H-NMR spectrum of the aromatic part, probably due to the absence of Tyr and Trp. A single His residue could be assigned. No dramatic changes of the spectrum were observed on phosphorylation of E III^{glc}. The chemical shift value of His in the denatured phosphoenzyme was close to the one expected for an N3 P-His.[109]

The stereochemical course of the phosphoryl transfer of the PEP : glucose PTS of *E. coli* was investigated starting with (^{16}O, ^{17}O, ^{18}O)PEP. The stereochemistry of the reaction product, methyl a-D-glucopyranoside, was analyzed by ^{31}P-NMR. As Knowles[110] suggested, an inversion of configuration at phosphorus is expected to take place for every in line phosphoryl transfer. The reaction product of the phosphoryl transfer catalyzed by the *E. coli* PTS exhibited inversion of configuration at the phosphoryl group. This, in turn, suggested an odd number of transfer steps between PEP and the product, implying a phosphorylated E II intermediate.[111]

5. PHOSPHORIC ESTER HYDROLASES

Enzymes hydrolyzing phosphoester bonds are also very common in nature. In general, nucleoside triphosphates, usually ATP, are used by organisms as storage compounds for metabolic energy. The stored energy is retrieved by cleavage of the terminal phosphate–phosphate ester bond. This process is performed by hydrolysis through the action of hydrolases, which may be considered as

phosphoryltransferring enzymes, because , in the presence of a divalent metal ion, they transfer a phosphoryl group from a nucleoside triphosphate (NTP) to a water molecule:

$$NTP + H_2O \rightleftharpoons NDP + P_i.$$

5.1. *Myosin*

Myosin is part of the contractile apparatus of muscle. The myosin molecule can be split by limited proteolysis into two different units, heavy meromyosin (HMM) and light meromyosin (LMM).

The HMM unit can be split further into basically two subunits, S1 and S2, of which the S1 part contains the ATPase site and the actin binding site (Fig. 16). The whole myosin molecule has a molecular weight of approximately 5×10^5, whereas the S1 part has one of 1.1×10^4. For a general review on NMR of muscle-related proteins see Ribeiro *et al.*[112]

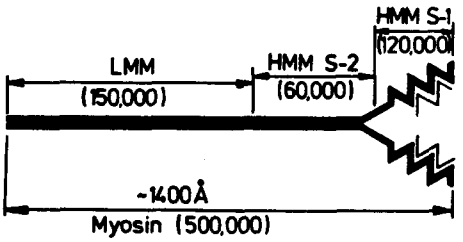


FIG. 16. The myosin molecule and its proteolytic fragments.

5.1.1. *^{31}P- and ^{19}F-NMR.* ^{31}P-NMR was again the method of choice in order to observe nucleotides bound to the S1 part of myosin. Shriver and Sykes[113,114] observed ADP and the non-hydrolyzable ATP analogue ATP(β,γ-NH_2) (AMPPNP) bound to myosin-S1 for the first time. For the β-peak two different resonances of the bound nucleotides around -1.6 ppm were observed. It was concluded that the nucleotide complexes of myosin-S1 existed in two states forming an equilibrium mixture. By observation of the temperature dependence of the population of these two states they were able to calculate thermodynamic parameters for their interconversion. An approximately equal population of both states was observed at 277 K.

Shriver and Sykes[115] also found indications of the validity of this two state model by fluorine-19 NMR. They observed a single ^{19}F signal from a fluorine containing probe attached to a specific sulphydryl group on S1 (SH1). The chemical shift of this signal was temperature dependent. This could be explained by the presence of two limiting states of the molecule in fast exchange. The low temperature state was suggested to be identical with the state of the S1·nucleotide·Mg^{2+} complex. For reviews of the NMR studies and also a consideration of the kinetic implications of these results see Shriver[116] and Wray *et al.*[117]

A ^{31}P-NMR spectrum of myosin showed a signal in the spectral region expected for phosphoserine. It was shown that this signal stems from a phosphorylated light chain (P-LC_2). A claim of functional similarities between the phosphoseryl groups in P-LC_2 and in phosphorylated troponin T was based on rather weak evidence.[118]

Fluorine spectra from the normal Michaelis S1·ADP(2-F) complex were compared to the S1·ADP(2-F) complex established by crosslinking two SH-groups of S1 with *p*-phenylene-dimaleimide. The two complexes showed identical ^{19}F-NMR spectra.[47]

As in Baldo *et al.* (1983)[47], a ^{19}F-NMR spectrum of the S1·ADP(2′-F)·Mg^{2+} complex gave no indication of two different observable states via two different ^{19}F signals at a single temperature (Fig. 17). A study of the temperature dependence of the ^{19}F-NMR spectrum of this complex is in progress. (Goody and Rösch, unpublished).

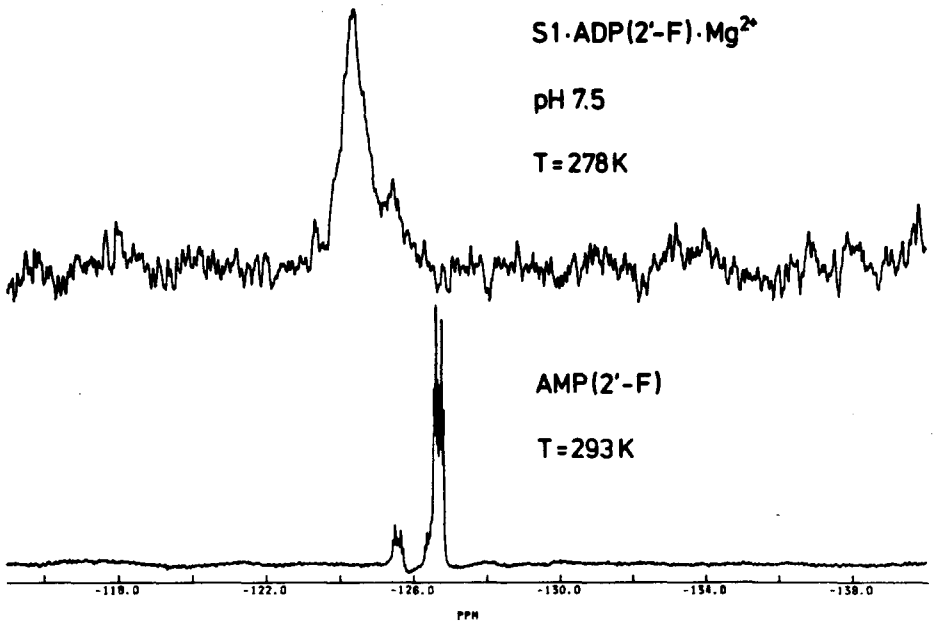


FIG. 17. ^{19}F-NMR spectrum of the myosin S1·(2′-F)ADP·Mg^{2+} complex at pH 7.5 (top) as compared to the ^{19}F-NMR spectrum of (2′-F)AMP free in solution (bottom) (Goody and Rösch, unpublished).

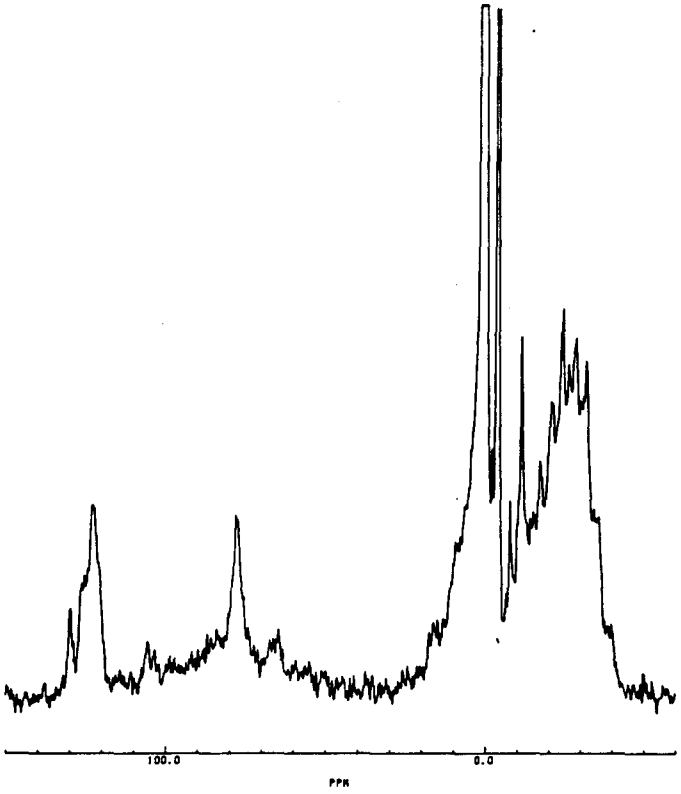


FIG. 18. ^{13}C-NMR spectrum of a 0.2 mM solution of myosin S1. 12ml solution in Tris buffer, spectrum obtained after 10 hrs, linebroadening 30 Hz (Goody and Rösch, unpublished).

Advances in spectrometer design even allowed the direct measurement of ^{13}C spectra of myosin S1 in 0.2 mM solution within a few hours (see Fig. 18).

Myosin S1 kinetics have been determined by the ^{18}O isotope exchange method. Indeed, the myosin molecule has proved to be a popular subject for all kinds of oxygen isotope exchange studies. The ^{31}P experiments of Webb *et al.*[119] yielded values at $T=295$ K and pH 8 of $N>50$ and $k=0.23$ $(\text{msec})^{-1}$, where N and k are defined as described above. Under similar conditions, but at $T=288$ K and pH 7.5, Rösch *et al.*[120] obtained $N=65$, $k=0.123$ $(\text{msec})^{-1}$. At $T=283$ K and pH 7.5 $N=38$ and $k=0.32$ $(\text{msec})^{-1}$ were obtained. The fact that ADP(a-S)(R_p) and ADP(a-S)(S_p) form two sterically different Mg^{2+} complexes was used in order to obtain information about the stereochemistry of ATP binding to myosin S1. To this end, the oxygen isotope exchange catalyzed by the four different complexes S1·ADP·Mg^{2+}, S1·ADP(α-S)(R_p)·Mg^{2+}, S1·ADP(α-S)(S_p)·Mg^{2+}, and S1·ADP(β-S)·Mg^{2+} was analyzed. From the results shown in Table 1 it is clear that the R_p isomer is the one with the correct stereochemistry.

TABLE 1. Results of ^{18}O exchange experiments with ADP(S) as substrates of myosin S1 (parameters defined as in Section 2.4.2)

	k $(M^{-1}sec^{-1})$	N
ADP	0.32	38
ADP(α-S)(R_p)	0.13	40
ADP(α-S)(S_p)	0.013	26
ADP(β-S)	0.025	25

Figure 19 gives an example of an ^{18}O exchange experiment. The number of publications concerning oxygen exchange kinetics of the myosin system with mass spectroscopic detection of the different P_i species is huge and unfortunately can not be included in the present report. In general, the results from similar experiments analyzed by different experimental methods are in good agreement.

5.1.2. *^{1}H-NMR.* Proton NMR studies on myosin, which in all mentioned cases was from rabbit muscle, concentrated on the possibility of detecting flexible parts within the myosin molecule. Differences in flexibility of some units within a macromolecule are on the average expected to give rise to differences in linewidth in their ^{1}H-NMR signals originating from different correlation times.

Highsmith *et al.*[121] compared proton NMR spectra of rabbit muscle myosin and its subunits HMM, LMM, and S1. A few narrow signals at virtually the same position and normalized area were observed in the ^{1}H-NMR spectra of myosin, HMM, and S1, but not in the spectrum of LMM. It was concluded that the myosin molecule contains parts of unusual flexibility, which are mainly located in the S1 subunit. Addition of PP_i or ATP(β,γ-NH_2), both nonhydrolyzable analogues of ATP, did not result in any observable change of the NMR spectrum. Formation of the S1·actin complex resulted in the disappearance of a number of narrow S1-resonances. Dissociation of actin from S1 by ATP analogs resulted in the reappearance of these signals.

Exchange of NH backbone protons was observed to be virtually completed for the S1 molecule in 30 min, which may also be interpreted as indicating a high internal mobility of the protein chain, resulting in contacts of the peptide backbone with the solvent. A comparison of the spectra of S1(A1) and S1(A2), which lacks the N-terminal 41 amino acids as compared to S1(A1), shows high flexibility of the 41 residue N-terminal segment. Signals from this segment disappear on actin binding, thus at first sight suggesting direct participation of this domain,[122] in agreement with Highsmith *et al.*[121]

An unusual amino acid residue, namely α-*N*-trimethylalanine, was detected by ^{1}H-NMR within the 41 residue region in which S1(A1) and S1(A2) differ.[123]

The observation of rather narrow signals in the ^{1}H-NMR spectrum of myosin S1 gave rise to a ^{1}H-NMR study of myosin S1(A1) and S1(A2) in the presence of the paramagnetic ions $Cr(CN)_6^{3-}$,

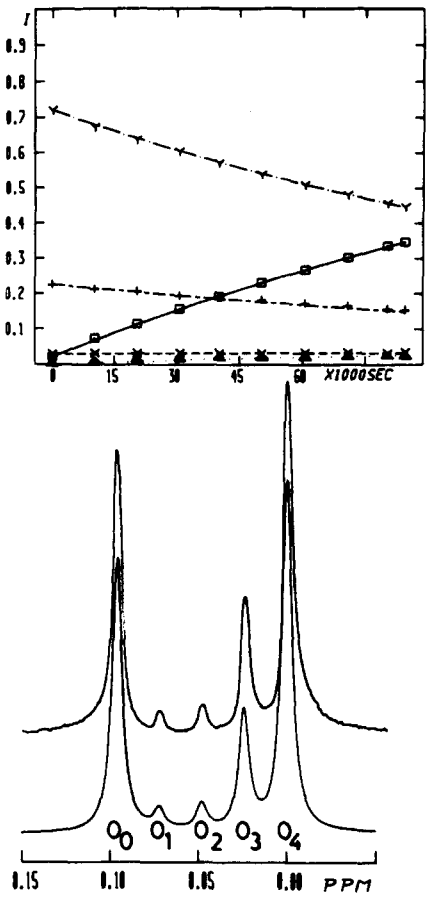


FIG. 19. Top: Time course of the concentration of five different ^{18}O species of inorganic phosphate in a reaction mixture consisting of: 0.2 mM ADP/100 mM PO_4 (^{18}O enrichment 90% at $t=0$) /0.18 mM S1 1 mM $MgCl_2$. Bottom: Spectrum of inorganic phosphate after 23.5 hr reaction time in the reaction mixture as above. Upper spectrum: experiment. Lower spectrum: Computer fit as described in the text (Rösch *et al.*[120]).

$Fe(CN)_6^{3-}$, Mn^{2+}, and a Gd^{3+} salt. Among others, the trimethylalanine resonance of S1(A1) was broadened significantly by the presence of the two anions. By comparison of the different effects induced by different ions conclusions concerning the nature of the surface groups of S1 could be drawn which were necessarily of only very general nature.[124]

In a subsequent paper, Highsmith *et al.*[125] studied the myosin rod as compared to LMM by ^1H-NMR methods. No additional narrow signals arising from flexible parts of the hinge region connecting LMM and S2 could be detected.

Differences in the ^1H-NMR spectra of short and long S2, the latter one being basically short S2 +hinge region, were also observed to be marginal by Stewart and Roberts.[126]

5.2. *Alkaline Phosphatase*

Alkaline phosphatase (AP, orthophosphoric monoester phosphohydrolase, EC 3.13.1) is a metalloenzyme with a maximum activity above pH 7.5 present in a variety of organisms. Several different divalent metal ions may be used to activate the phosphatase. The alkaline phosphatase studied most extensively by NMR methods was the one from *E. coli*, though AP from other sources, including mammals, was available. The reaction catalyzed by this enzyme (dimer, MW 48,000 per

monomer) in the presence of divalent metal ions (usually Zn^{2+} and Mg^{2+}) is the cleavage of phosphoester bonds according to:

$$ROP + E \rightleftharpoons E{\cdot}ROP \rightleftharpoons E{-}P + ROH \rightleftharpoons E{\cdot}P + ROH \rightleftharpoons E + P + ROH,$$

where E·ROP and E·P indicate noncovalent complexes. The phosphorylated enzyme E–P is a phosphoseryl intermediate (Ser-102 in the case of the *E. coli* enzyme). Alcohols, especially amino alcohols, may also serve as acceptor molecules for the phosphate group, although they are rather poor substrates. The rate limiting step for hydrolysis at alkaline pH, where the enzyme exhibits maximum activity, is the E·P dissociation, whereas the E–P cleavage step is rate limiting at low pH.

The physiological role of the alkaline phosphatases within organisms is not fully understood. For a recent review on this group of enzymes see Coleman and Gettins.[127] A review on the multinuclear NMR studies performed on alkaline phosphatase has been given by Coleman *et al.*[128]

The use of multinuclear NMR—i.e. ^{13}C, ^{19}F, ^{31}P, ^{113}Cd—answered the two main questions concerning the number of covalently bound phosphoryl groups per active dimeric unit and the number of metal binding sites. The minimum and maximum number of metal ions required to obtain full catalytic activity of the protein was discussed in length, and some confusion was obviously introduced by comparison of phosphatase prepared by different procedures. It is now generally agreed that there are six divalent metal ions bound per native unit in the fully activated state, four Zn^{2+} and two Mg^{2+} ions, and that there are two phosphorylated sites.

A variety of NMR investigations have probed this enzyme under diverse conditions. The catalyzed reaction suggested immediately ^{31}P-NMR experiments.

The first phosphorus magnetic resonance spectrum of the AP from *E. coli* was reported by Bock and Sheard.[129] They observed two different ^{31}P-resonances, which could be assigned by the pH dependence of their intensities to the covalent and noncovalent enzyme phosphate intermediates E–P and E·P.

Bock and Kowalsky[130] also tried to elucidate the role of the four Zn^{2+} ions bound to the enzyme in its native form $AP(Zn^{2+})_4$. The ^{31}P-NMR spectrum of this native compound was compared to the NMR spectrum of the $AP(Zn^{2+})_2$ prepared from the native form by dialysis and to $AP(Zn^{2+})_2$ prepared by addition of metal to the apoenzyme. The two samples differed only in catalytic activity. Unfortunately, no final conclusions could be drawn.

The early ^{31}P observations by Bock and Sheard were essentially confirmed by Hull *et al.*[131] From linewidth calculations a decay rate from $E{\cdot}P_i$ to $E + P_i$ of less than 25 sec^{-1} was deduced, so that this step would be the rate limiting one.

The large downfield shift of the ^{31}P-resonance of the covalently bound phosphorus was interpreted in terms of a highly strained phosphoryl group by Chlebowski *et al.*[132] They also observed a maximum capacity of the phosphatase $(Zn^{2+})_2$ complex of one phosphoryl group per dimeric unit. The disappearance of the single phosphate peak by paramagnetic linebroadening induced by formation of the Co^{2+} and Mn^{2+} complexes indicated spatial proximity of these ions to the phosphoryl group.

The first NMR studies involving $^{113}Cd^{2+}$ ions (an enriched source was used) proved the establishment of inequivalence of the two sites occupied by metal ions of the AP $(Cd^{2+})_2$ complex on binding of the phosphoryl group. The two ^{113}Cd metal ions exhibited the same chemical shift values of their resonances in the unphosphorylated complex but had two differing ones in the phosphoenzyme.[133] This observation was interpreted later as migration of one metal ion bound to the high affinity site of one subunit to one of the two low affinity sites of the second subunit on phosphorylation of the latter.[134]

Whereas the AP $(Zn^{2+})_2$ complex was observed to be only capable of binding one phosphoryl group,[132] the prevalent mode of binding being either covalent or noncovalent, depending basically on the pH value, the AP $(Zn^{2+})_4(Mg^{2+})_2$ complex was observed of being capable of binding two phosphoryl groups per dimeric unit. This capability does not change on substitution of two Cd^{2+} ions for two Zn^{2+} ions. The second pair of metal ions (Zn^{2+} or Cd^{2+}) necessary for the binding of

the additional phosphoryl group was termed 'structural', because it was suggested to be important for structural stabilization.[135]

These conclusions were confirmed by further investigations of the AP $(Mn^{2+})_2$ and the AP $(Zn^{2+})_2$ complexes by EPR and ^{31}P-NMR. The former complex was capable of binding one phosphoryl group, the latter one two phosphoryl groups, showing the importance of the second pair of metal ions for the phosphoryl binding. Paramagnetic linebroadening of the phosphoryl ^{31}P resonance induced by Mn^{2+} ions was observed in the four ion complex.[136]

The possibilities of ^{13}C-NMR for the characterization of AP were explored by Otvos and Browne[137] and Otvos and Armitage.[138] A ^{13}C label was introduced into AP via histidine labelled at the γ-position and incorporated *in vivo*. Of the ten histidyl residues per subunit, three were proposed to be involved in metal ligation, two different ones to be close to metal sites; four residues were proposed to be close to the surface of the protein, the remaining one to be buried in a solvent inaccessible region. Three of the histidyl residues showed a spin splitting induced by ligation of ^{113}Cd. A detailed analysis of the ^{13}C results in conjunction with ^{113}Cd-NMR studies[139] revealed the detailed mode of occupation of the three metal sites, a high affinity site ('A') and two low affinity sites ('B' and 'C'), in different states of the AP complexes.

These results were extended by two recent publications by Gettins and Colman[134,140] who have further probed the phosphoenzyme intermediates by ^{113}Cd-NMR[134] and ^{31}P-NMR.[140] It was observed that the three different metal sites per monomeric subunit may all be occupied under different conditions by a Cd^{2+} ion. It could be shown that the time necessary for the attainment of an equilibrium situation after certain changes induced in the Cd^{2+} enzyme may be of the order of hours. Gettins and Coleman[141] observed the spin splitting of the phosphorus resonance of the E·P complex induced by the presence of ^{113}Cd, yielding the final proof of the contact of the metal ion with the phosphate. The ^{31}P chemical shift of this complex was sensitive to the type of metal ion—Cd^{2+} or Zn^{2+}—in the complex. This sensitivity was not observed for the phosphorus resonance of the E–P form, neither was a J-coupling between ^{113}Cd and ^{31}P observed, indicating loosening of the metal/phosphate interaction.

The high affinity metal site was also shown by ^{113}Cd-NMR to be the site of Cl^- ion binding in the phosphorylated $AP(Cd^{2+})_6$ and the unphosphorylated $AP(Cd^{2+})_2$.[141]

The *in vivo* incorporation of fluorine labelled tyrosine into AP was used by Sykes *et al.*[142] in order to obtain ^{19}F spectra of fluorotyrosyl AP. Browne and Otvos[143] also obtained ^{19}F-NMR spectra of fluorotyrosyl AP. Hull and Sykes[144] monitored conformational changes of the fluorotyrosyl enzyme induced by phosphate binding via ^{19}F-NMR. They were able to distinguish individual resonances of each of the eleven fluorotyrosyl residues.

One of the first experimental results on oxygen isotope exchange analyzed by phosphorus NMR was on Zn^{2+} and Co^{2+} alkaline phosphatase.[145] Data analysis was carried out by a Monte Carlo method. The complete set of data was reevaluated later by the direct probabilistic calculation and data refinement.[21] The results were in good agreement.

The phosphorylgroup transfer from phenylphosphate(R-$^{16}O^{17}O^{18}O$) to the product 1-phospho($^{16}O^{17}O^{18}O$) propane-1,2-diol was found to proceed via retention of configuration.[146]

Otvos *et al.*[147] determined the interconversion rates of the three possible phosphate environments of the alkaline phosphatase (AP) reaction, i.e. $E+P_i$, $E{\cdot}P_i$, and E-P, using the saturation transfer (ST) technique under the assumption of a three site exchange. The rates were determined for the Zn^{2+} and the Cd^{2+} enzymes.

More recently, the transferase potential of the AP was investigated by Gettins *et al.*[148] The ratio of the initial rates of the transferase reaction to the initial rates of the hydrolase reaction was followed as a function of pH for 1 M Tris and 3 M glycerol as acceptor substrates. In these experiments *p*-nitrophenyl phosphate was used as a donor. The relative transferase activity of the $(Zn^{2+})_4$ enzyme to Tris exhibited a maximum at pH 7.5, whereas the transfer activity to glycerol increased steeply above pH 9 (C2 of glycerol was the preferred transfer site above this pH). The $(Co^{2+})_4$ enzyme showed no detectable transferase activity with the Tris-acceptor. Again, ^{113}Cd-NMR of the phosphorylated $(Cd^{2+})_6$ enzyme showed the A site being the one influenced most by Tris coordination. A minor shift of the A site resonance was also observed on addition of glycerol to

the $(Cd^{2+})_6$ enzyme. The slow exchange conditions prevailing between free P_i and E·P allowed the performance of inversion transfer experiments in order to determine the exchange rate constants for different complexes.

5.3. *Ion Transport ATPases*

The transport of sodium and potassium across cell membranes is an active process associated with cleavage of ATP by an ATPase (ATP phosphohydrolase, EC 3.6.1.3). Two divalent metal ions, presumably at the active site, seem to be required for full catalytic activity. This ATPase forms a phosphorylated intermediate, which could be produced either by addition of Mg^{2+}, Na^+ and ATP or by addition of Mg^{2+} and P_i in the absence of Na^+ to a suspension of the Na,K-ATPase. Similar observations pertain for the Ca-ATPase and the H,K-ATPase.

Fossel *et al.*[149] interpreted a peak in the ^{31}P-NMR spectrum of the Na,K-ATPase in the presence of ATP appearing at 17.4 ppm, close to the β-peak of ATP·Mg^{2+}, as corresponding to the phosphorylated intermediate. The Ca-ATPase of rabbit skeletal muscle exhibited a peak with similar ^{31}P-NMR characteristics under equivalent conditions. All spectra were taken at a low temperature close to 273 K with an ATP-to-Mg^{2+} ratio of 2.5. The same peak was also detected in an experiment involving the Na,K-ATPase without added ATP in the presence of P_i, Mg^{2+}, and ouabain, which was supposed to stabilize the intermediate. Comparison with a phosphorylated model compound suggested an aspartyl residue as a phosphorylated amino acid.

In order to determine structural features of the ATP·ATPase complex, Klevickis and Grisham[150] determined ^{31}P $1/T_1$ relaxation rates of a solution of $Co(NH_3)_4$ATP (both stereoisomers bind to the protein), Mn^{2+}, and catalytic amounts of the Na,K-ATPase from sheep kidney medulla. They interpreted their results in terms of a shorter distance of the β- and γ-phosphorus than the α-phosphorus to the Mn^{2+} ion.

Clore *et al.*[151] determined the nucleotide conformations of bound ATP·Mg^{2+}, ADP·Mg^{2+} and AMP·PNP·Mg^{2+} by observation of the NOE detected for the free substrates in the presence of rabbit sarcoplasmic reticulum (SR) Ca-ATPase. The observed NOE exhibited by the H8 resonance on irradiation of the H1′ resonance could be shown to originate from dipolar interaction in the bound state of the nucleotides. Thus, the conformation of the bound substrates could be determined in principle. The existence of a low affinity nucleotide binding site, which introduced ambiguities in the interpretation of the structural results, could be demonstrated by blocking the catalytic site with fluorescein isothiocyanate. Nevertheless, a conformation close to anti about the glycosidic bond seemed to predominate in both sites.

The ^{18}O exchange reaction catalyzed by gastric H,K-ATPase from hog was the subject of studies performed with the help of ^{31}P-NMR spectroscopy. Formation and subsequent cleavage of an E-PO_3^{2-} intermediate performed in a solution containing labelled HPO_4^{2-} and enzyme is the reason for the ^{18}O exchange catalyzed by the Na,K-ATPase and the Ca-ATPase as detected with the aid of mass spectroscopy. The observation of an H,K-ATPase catalyzed ^{18}O-exchange starting with enzyme and labelled P_i by ^{31}P-NMR confirmed the formation of a covalent form E-PO_3 for this protein also. From the time dependence of the relative concentration of $P^{18}O_4$ the pseudo-first-order rate constant for the cleavage step and N could be calculated under the assumptions implicitly given by Hackney.[19] The ionic requirements for the ^{18}O exchange were similar to those for the ATP cleavage. Substitution of the activating divalent metal ion Mg^{2+} with Ca^{2+} resulted in a 90% decrease of the exchange activity, in accord with a strong decrease of the ATPase activity under the same conditions. The exchange reaction was not inhibited by the Na,K-ATPase inhibitor ouabain, which has no effect on H,K-ATPase hydrolysis activity. The presence of the substituted benzimidazole omeprazole, which has the opposite specificity, inhibited the exchange reaction virtually completely. These experiments excluded a Na,K-ATPase contamination in the preparations as the component causing the exchange reaction. Presence of vanadate also inhibited the reaction strongly. These experiments thus allowed the conclusion that the ATPase reaction catalyzed by H,K-ATPase proceeds via a phosphorylintermediate.[152]

5.4. *Elongation Factor Tu*

The polypeptide elongation factor Tu (EF-Tu) promotes the binding of aminoacyl-tRNA (aa-tRNA) to ribosomes. This process requires the presence of GTP, which is hydrolyzed in the complex EF-Tu·aa-tRNA·GTP·Mg^{2+}. Isolated EF-Tu is also able to bind GDP and GTP and slowly hydrolyze GTP in the presence of divalent metal ions only.

The ^{31}P-NMR spectrum of GTP and GDP bound to EF-Tu from *Thermus thermophilus* in the presence of $MgCl_2$ was studied by Nakano *et al.*[153] They found a large downfield shift of approximately 4 ppm for the β-phosphate resonance of GDP as well as GTP on binding to the factor and an upfield shift for the γ-phosphate resonance of GTP of approximately 1.5 ppm. The α-resonances were hardly affected. The authors suggested this to be indicative for a strong interaction of the GDP and GTP β-phosphates and the γ-phosphate of GTP with EF-Tu. No pH-dependence of the chemical shifts for either complex could be found in the range from pH 5.7 to pH 9.5

The results on the ^{31}P chemical shift values of the EF-Tu·GDP·Mg^{2+} complex agree basically with results by Wittinghofer *et al.*[154] on the same complexes with EF-Tu from *Bacillus stearothermophilus*. They also observed a downfield shift of approximately 4 ppm for the β-phosphate and 0.6 ppm for the α-phosphate of GDP·Mg^{2+} on complexation. The metal free complexes showed a downfield shift of 0.9 ppm (α-phosphate) and 3.3 ppm. The experiments were repeated with GDP(β-S) substituted for GDP. GDP(β-S) showed a downfield shift of 0.6 ppm for the α-phosphate and 3.9 ppm for the β-phosphate in the metal free complex and virtually no shift for the α-phosphate and a downfield shift of 3.8 ppm for the β-phosphate in the metal complex. In other words, the influence of metal ion complexation on the α-phosphate chemical shifts tended to be diminished in the EF–Tu complex, whereas they tended to be unaltered or enhanced for the β-phosphate as compared to the free nucleotide for GDP and GDP(β-S). These results were used together with EPR experimental results in order to suggest a model for the EF-Tu·GDP·Me^{2+} complex in which an enzyme–GDP bridge is formed via the metal ion and the β-phosphate of the nucleotide.

As a side product of neutron diffraction studies, a perdeuterated form of EF-Tu became available (estimated deuteration level: 90%). A ^{1}H-NMR spectrum of this compound showed very narrow resonances due to the loss of J-splittings and the reduction of the spin–spin relaxation rate.[155] Though it is doubtful that the study of unspecifically deuterated proteins adds much new information on the protein itself, it provides a convenient way for proton studies of bound substrates. In addition, it is a first step towards the goal of having some amino acids selectively protonated, which could then be studied directly by proton NMR.

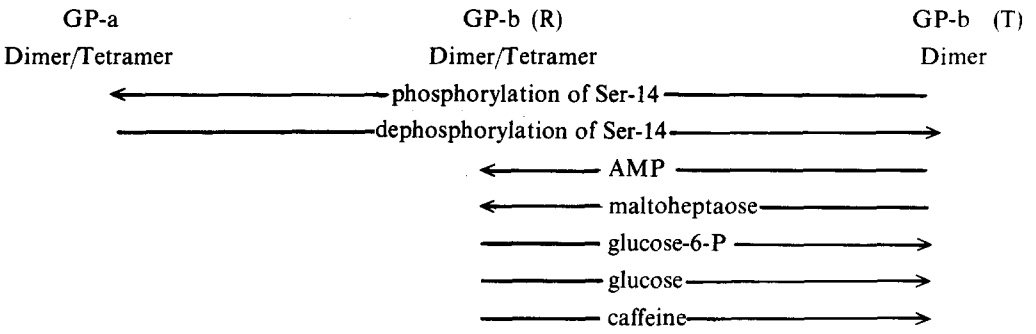
5.5. *Glycogen Phosphorylase*

Glycogen phosphorylase (GP, α-1,4-glucan : orthophosphate glucosyl transferase, EC 2.4.1.1) in its active (R-) form catalyzes the reaction:

$$(\text{Glucose})_n + P_i \rightleftharpoons (\text{Glucose})_{n-1} + \text{Glucose-1-Phosphate}.$$

Although GP is a hexosyltransferase by enzyme classification and physiological function, the reaction in the reverse direction is a hydrolysis. GP as a regulatory enzyme has the property of not only being a catalyzing reaction component, but also being the substrate of a phosphoryltransferring enzyme, namely phosphorylase kinase.

The enzyme is supposed to exist in two structurally different forms, GP-a and GP-b. GP-a exists as an active tetramer or dimer with phosphorylated subunits. Phosphorylation is performed by phosphorylase kinase, which thus functions as a regulatory component. The dimeric form of GP-b (i.e. unphosphorylated GP) may be active (R-) or inactive (T-). All forms may be interconverted by several means, which may be represented in an oversimplified scheme, deduced in part from the experiments described below:



The AMP activation does not depend on the presence of the N-terminal phosphorylation site Ser-14. Activation is only necessary in the mammalian enzyme. It must be emphasized that the tetrameric forms are suspected to occur in concentrated solutions only (e.g. under NMR conditions) and thus be a ghost. Their having a physiological function is doubtful. The concentration of activating and deactivating compounds in living cells is usually in such a range that only GP-a and GP-b (T) are present in relevant amounts.

One pyridoxal-5'-phosphate molecule (Fig. 20) per subunit is a covalently bound mandatory component for the activity of the enzyme. The target of most NMR studies on GP was the clarification of the role the coenzyme plays in the catalytic process. It was deduced from several

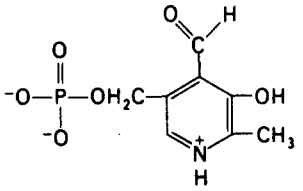


FIG. 20. Pyridoxal-5'phosphate.

reconstitution experiments with coenzyme analogues that the phosphate group of the coenzyme is the group most likely to be involved in catalysis. X-ray experiments also support this view by showing a close proximity of this phosphate group to the catalytic centre. However, the situation is a little more complicated, the X-ray structure showing a few charged aminoacyl residues with a pK-value in the required range in close proximity to the reaction centre. This led to the proposal that His376 is acting as a general acid and the coenzyme as a nucleophile with the C1 carbon of glucose as a target.[156]

For reviews on GP see Fletterick and Madsen[157] and Klein and Helmreich,[158] who have presented a more functionally oriented short review on the role of pyridoxal phosphate in the phosphorylase catalysis. They summarized arguments in favour of the coenzyme phosphate acting as a proton donor/proton acceptor in a general acid/base catalysis. Another possibility would involve a direct phosphate–phosphate interaction, the pyridoxal-phosphate acting as an electrophile attacking the phosphate ester bond.[159]

Most NMR experiments described below were carried out with the rabbit skeletal muscle enzyme.

Busby *et al.*[160] as well as Feldmann and Helmreich[161] have obtained phosphorus spectra of phosphorylase b. Busby *et al.*[160] claimed the observation of two phosphorus resonances of the bound pyridoxal phosphate corresponding to two forms of GP with a lifetime of longer than 1 msec each. The spectra presented by Feldmann and Helmreich[161] did not show any signs of a splitting in two distinct resonances, although the resolution of their spectra was much better. They did not

observe any pH dependence of the signal in the stability range of the enzyme. Addition of both AMP and arsenate, which is an analogue of P_i, resulted in a 2.8 ppm downfield shift of the pyridoxal phosphate resonance, overlapping with the AMP resonance.

Feldmann and Hull[162] continued these experiments, substituting the thioanalogue AMP(S) for AMP as an allosteric effector in order to remove the AMP phosphorus signal from the spectral region of interest. This substitution proved to be most valuable also in many later studies. The downfield shift of the phosphorus resonance of pyridoxal phosphate on formation of the GP·AMP(S)·arsenate complex was confirmed (3.2 ppm). The state of the pyridoxal phosphate corresponding to the unligated GP was termed form I, the state of the pyridoxal phosphate in the AMP(S) arsenate complex termed form III. The GP·AMP(S) complex exhibited two different signals for the pyridoxal resonance, corresponding to two forms of the complex (I and II). The resonance corresponding to form III of the coenzyme was also the dominant one for GP-a obtained by thiophosphorylation of Ser-14. Therefore, form III was identified as the form of the GP coenzyme closely related to the active state of the holoenzyme. It was concluded from the chemical shift values that the transition from the inactive form I to the active form III corresponded to the deprotonation of the phosphate group, resulting in the dianionic state. The dissociation rate for AMP(S) was estimated to be 200 sec^{-1} from linewidth measurements. Later Withers *et al.*[163] showed by ^{31}P-NMR this value to be strongly dependent on the buffer system, as was suspected much earlier from the results of kinetic measurements.[164]

The interactions of AMP(S) and thiophosphoryl residues were investigated by Hoerl *et al.*[165] in native and succinylated GP-a and GP-b. Dissociation of the GP resulting in a monomer–dimer mixture did not affect the pyridoxal phosphate resonance, thus at least not contradicting the proposed location of the cofactor pyridoxal phosphate in a part of the GP molecule farther away from the contact surface between subunits. The binding of AMP(S) at alkaline pH seemed to influence the surroundings of the thiophosphoseryl residue so as to reestablish the conditions around this residue at pH 6.5, which was the optimum for activity. Thus, cooperativity between the two different effectors was suspected. As for the resonance of the apoenzyme, two different signals from the thiophosphoseryl group were observed, depending on the experimental conditions. The lower field signal is observed in the pH range where form I of the coenzyme resonance prevails, the higher field signal in the pH range where form III prevails. The effect of the inhibitor glucose on GP-a reconstituted with the pyridoxal phosphate analogue deoxypyridoxal-methylenephosphonate was a transition from form III of GP-a to form I, as could be expected optimistically. As noted later,[166] the ionic state of this cofactor analogue is reversed as compared to the natural cofactor pyridoxal phosphate in the inactive and the active form.

Withers *et al.*[163] studied the effect of two other inhibitors, caffeine and glucose, on the ^{31}P resonance of the coenzyme pyridoxalphosphate in the holoenzyme. As activator, AMP(S) was used. The AMP(S) dissociation constant as well as the dissociation rate was increased dramatically on addition of either inhibitor, the latter one even more so on simultaneous addition of both, so that exchange conditions intermediate on the NMR time scale between free and bound AMP(S) prevailed. Both inhibitors had also the effect of shifting the equilibrium between the nonactive form I and the active form III of the coenzyme strongly in the direction of the form I.

The structural effects induced by the substrate analogue α-D-glucopyranosyl cyclic 1,2-phosphate were the subject of a study by Withers *et al.*[167] This analogue was shown to be a competitive inhibitor of both GP-a and GP-b. On binding of the cyclic phosphate to the AMP(S) activated enzyme, the dissociation rate constant of the activator as well as its dissociation constant decreased. This effect was also observed with the substrate glucose-1-phosphate. Binding of the inhibitor α-D-glucopyranosyl fluoride on the activated enzyme established intermediate exchange conditions for the AMP(S) resonance, and, at the same time, increased the dissociation constant. As far as the phosphorus resonance of pyridoxal phosphate is concerned, it exhibited the shift value characteristic of the form I on binding glucose, glucosyl fluoride, and the cyclic phosphate; the pyridoxal phosphate resonance could not be observed for the glucose-1-phosphate complex because the two peaks overlapped. The ^{31}P-resonance due to the thiophosphoseryl residue of thiophosphoryl-GP-a exhibited—in the presence of AMP(S)—the low field resonance[165] on addition of glucose and the

high field resonance on addition of glucose-1-phosphate. Addition of the oligosaccharides maltopentaose and maltoheptaose to GP-a or GP-b in the presence of AMP(S) resulted in a transition from form III to form I of the coenzyme.

Pyridoxal-5'-diphospho-1-α-D-glucose was a coenzyme-plus-substrate analogue studied in the GP reconstituted from this compound and the apoenzyme.[159] This analogue is basically the coenzyme connected with one substrate via a pyrophosphate linkage. A very low off rate for AMP(S) was observed with this reconstituted product. Indications were found that the reconstituted complex is able to cleave the glycosidic bond, thus freeing glucose (which could not be observed) or resulting in a covalent enzyme intermediate. The rate of bond cleavage was greatly enhanced by the presence of maltopentaose. As a possible explanation, the transfer of glucose to the oligosaccharide was suggested.

Withers *et al.*[168] also reconstituted an altered holoenzyme with pyridoxal pyrophosphate in order to obtain more information on the catalytic role of the coenzyme. The β-phosphate was shifted downfield, i.e. in the direction of deprotonation, by about 2.2 ppm on binding, whereas the α-phosphate hardly changed position. On addition of AMP(S) to the holoenzyme analogue, the β-phosphate shifted farther downfield, and the P_i concentration increased markedly, indicating an ongoing enzymatically catalyzed cleavage of the pyrophosphate bond. Again, the exchange rate of free and bound AMP(S) is rather low, thus the complex probably resembles the R-state. Addition of the inhibitors glucose and caffeine had virtually no effect on the spectrum, neither had the addition of maltopentaose any influence. GP reconstituted with pyridoxal also showed a very low off rate and dissociation constant for AMP(S). Addition of glucose caused little effects but addition of potassium phosphite resulted in an increase in AMP(S) off rate and dissociation constant. This effect was further enhanced by addition of caffeine. Similar effects were observed with pyrophosphate instead of potassium phosphite. With the pyridoxal reconstituted enzyme it was also possible to study the resonance of the AMP effector, because the pyridoxal phosphate peak was of course missing. The downfield shift observed for the AMP resonance on binding as compared to the upfield shift of the AMP(S) resonance suggested a deprotonation of the nucleotide as the probable cause, for this is the effect deprotonation exerts on the chemical shift of the free nucleotide and its thioanalogue, respectively.

Two glycogen phosphorylases not requiring activation were subsequently subjected to NMR studies. The *E. coli* protein maltodextrin phosphorylase was the subject of ^{31}P-NMR studies by Palm *et al.*[169] Only one resonance attributed to the dianionic form of the cofactor pyridoxin phosphate was observed at pH 6.7 with the same chemical shift (−3.4 ppm) in the presence and absence of the substrate analogue arsenate. Titration studies led to the conclusion that the dianionic state is predominant in the optimal pH range 6.2–6.9. Similar results were obtained by Klein and Helmreich[170] for the potato phosphorylase, thus confirming a similiarity of the active forms of these phosphorylases. The potato enzyme showed a shift of the pyridoxin phosphate resonance on binding of arsenate or glucose of the magnitude generally expected for O–P–O bond angle distortions.

Klein *et al.*[171] investigated the phosphorylase reaction with D-glucal instead of glucose-1-P as a substrate in the presence of α-cyclodextrin. As catalyzing enzymes, GP from potato and GP-b from rabbit muscle were used. Arsenate or phosphate proved to be a mandatory component of the reaction mixture. The reaction mixture could be analyzed by ^{13}C-NMR as containing D-glucal and both the α- and β-anomer of 2-deoxy-D-glucose. 2-deoxy-α-D-glucose-1-phosphate was establised as a reaction product of the GP-a catalyzed reaction with the aid of ^{31}P-NMR and ^{1}H-NMR spectroscopy. The stereochemical course of the reaction was followed by ^{1}H-NMR spectroscopy. The results were interpreted in terms of the enzyme acting via a general acid/base catalysis involving the pyridoxal phosphate. The interaction of the inhibitory heptulose-2-phosphate and GP from potato as well as GP-b from rabbit muscle was also studied by Klein *et al.*[166] As far as mechanistic ^{31}P-NMR studies were concerned, heptulose-2-P had the advantages over other inhibitors of being capable of being protonated in the activity range of GP and not obscuring the coenzyme phosphate resonance. The pyridoxal phosphate dianion in the potato GP was suspected of undergoing partial protonation on heptulose-2-P binding. Also, the cofactor analogue deoxypyridoxal-methylene-

phosphonate monoanion in the active state of rabbit muscle GP possibly shares a proton with bound heptulose-2-P. Both clues were arrived at on the basis of changes of the phosphate chemical shifts. This observation would give conclusive evidence of the coenzyme acting as a general acid/base catalyst. So, at this point, one might be inclined to follow the arguments of the Würzburg group.

The ^{19}F and ^{31}P resonances of GP-b reconstituted with pyridoxal fluorophosphate and AMP(S) activated were examined somewhat closer by Withers *et al.*[172] The phosphorus resonance doublet generated by J-coupling to the fluorine nucleus was assymetric in the sense that the lower field component was much broader than the higher field component. It was vice versa for the fluorine resonance. This effect was shown to be due to the cross terms between the chemical shift anisotropy (CSA) and the dipole–dipole (DD) relaxation terms. The relative contributions of these two terms to the total relaxation mechanism could be calculated to be around 10% for the ^{19}F resonance and around 5% for the ^{31}P resonance, in agreement with the value expected at a field strength of 6.4 T.

5.6. *c-Ha-ras Oncogene Product p21*

The cellular Harvey ras (c-Ha-ras) oncogene product p21 ($p21_c$) (MW 21,000) exhibits GTPase activity in the presence of divalent metal ions. It possibly constitutes a protein kinase. The most interesting feature of this protein is its capacity to transform certain cell strains (NIH 3T3) into tumour cells after codon 12 or codon 59 (or both) of the gene coding for p21 is mutated. Normally, a glycyl residue is at position 12 and an alanyl residue at position 59. Decreased GTPase activity was found after mutation of these positions. Preliminary ^{1}H- and ^{31}P-NMR studies were obtained recently[173] after a large scale preparation method for the authentic viral p21 ($p21_v$) cloned into *E. coli* became available. $p21_v$ contains an arginyl residue at position 12 and a threonyl residue at position 59. p21 was reported to copurify always with one mole GDP per mole of protein.

The aromatic part of the ^{1}H-NMR spectrum of this protein·GDP·Mg^{2+} complex is shown in Fig. 21. The C2H resonances of the imidazole rings of the three histidyl residues of this protein could be identified immediately. One of these resonances underwent a strong downfield shift on removal of Mg^{2+}. A most interesting feature of the ^{31}P-NMR spectrum of this complex is the chemical shift of the β-phosphate resonance of GDP. This resonance is shifted downfield by more than 4 ppm, an

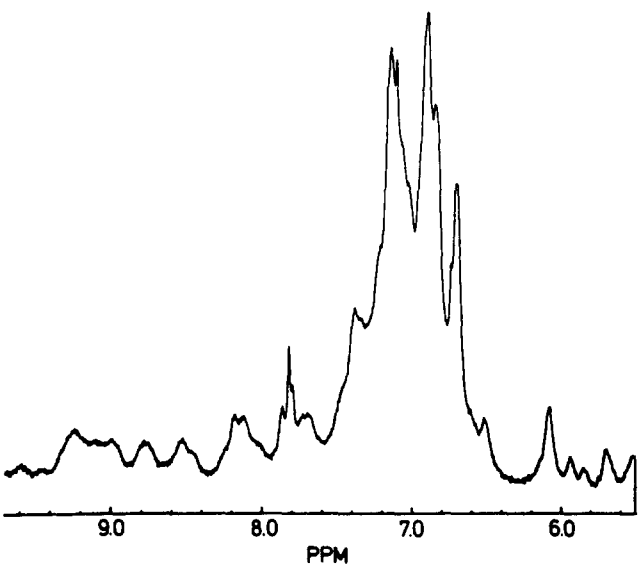


FIG. 21. Aromatic part of the ^{1}H-NMR spectrum of the p21·GDP·Mg^{2+} complex.

effect which was also observed in the ^{31}P-NMR spectrum of the elongation factor Tu·GDP·Mg^{2+} complex[153,154] and the myosin S1·ADP·Mg^{2+} complex.[113,114] It may well be that this suggests a common mode of binding of the nucleotides to these proteins.

TABLE 2. Chemical shift values of the ^{31}P-resonances of the nucleotide diphosphate complexes of EF-Tu, Myosin S1, and $p21_v$ in the presence of Mg^{2+}

	GDP		
	α	β	Ref.
unbound	−9.5	−5.3	40
Myosin S1	−9.4	−1.8	113
EF-Tu	−8.9	−1.5	154
	−9.6	−1.7	153
$p21_v$	−9.6	−1.3	173

6. OTHERS

6.1. *Phosphoglucomutase*

Phosphoglucomutase (PGM, EC 2.7.5.1) catalyzes in its active form the reaction

$$\text{Glucose-1-P} \rightleftharpoons \text{Glucose-6-P}$$

with glucosediphosphate as an enzyme-bound intermediate. The α-anomer is the natural substrate. PGM is activated by phosphorylation of Ser-116. This phosphate group is transferred in an intermediate reaction to the glucose molecule. Divalent metal ions (physiologically Mg^{2+}) are, as always in phosphoryltransfer reactions, mandatory components.

In a multinuclear NMR-study, Rhyu *et al.*[174,175] were able to observe the enzyme-bound form of the three different glucose species by ^{31}P-NMR. To this end they slowed down the reaction by substituting Li^+ for Mg^{2+}, so that conditions allowed detection of single species. The phosphorus resonances of the E-P·Glu-P complexes were assigned using ^{17}O labelled phosphorus, which exhibited broadened resonances because of the quadrupolar moment of the oxygen. With excess Cd^{2+}, two peaks, presumably corresponding to an PGM·$(Cd^{2+})_2$·Glu-P complex, were observed. ^{113}Cd bound to the phosphoenzyme caused a J-splitting of the phosphorus resonance ($J_{Cd-O-P}=16$ Hz) which disappeared on substrate addition. This indicated a direct interaction between the metal ion and the enzyme-bound phosphate group. Chemical shifts of the ^{113}Cd and ^{7}Li resonances of the PGM$(Me)_2$ complex were reported, but no resonances for the supposed secondary metal sites could be detected. An attempt to determine the mobility and solvent accessibility of the enzymic serylphosphate with the aid of relaxation methods led to the suggestion that the phosphogroup is highly solvent accessible in the free phosphoenzyme as well as in the E-P·Li^+ complex but not so in the ternary E-P·G-6-P·Li^+ complex. This result was not unexpected.

6.2. *Phosphoenolpyruvate Carboxykinase*

Phosphoenolpyruvate Carboxykinase (PCK, GTP : oxalacetate carboxylase (transphosphorylating), EC 4.1.1.32) catalyzes, in the presence of divalent metal ions, the reaction

$$\text{P-enolpyruvate} + CO_2 + \text{IDP} \rightleftharpoons \text{oxalacetate} + \text{ITP}$$

where guanosine may substitute for inosine. The molecular weight of this enzyme is about 73,000. Early water proton relaxation enhancement measurents performed by Miller *et al.*[176] with the pig

liver enzyme in connection with EPR measurements suggested an interaction of the substrates PEP and IDP within the first coordination sphere of the enzyme-complexed manganese cation. This result was also obtained by Barns *et al.*[177] with the sheep kidney enzyme. Formation of a binary PCK enzyme complex was observed. Because the IDP bound as a metal complex, PCK was supposed to form a bimetallic complex with IDP. Hebda and Nowak[178] investigated the chicken liver enzyme with the same methods. They also suggested the existence of the PCK·IDP·$(Mn^{2+})_2$ complex. In contrast to the results of the other two groups, they found no indication of a direct interaction between IDP and the enzyme-bound metal ion, whereas a direct interaction between this ion and ITP was suggested.

More recently, Duffy and Nowak[179] measured ^1H and ^{31}P relaxation rates of ligands in the presence of catalytic amounts of chicken liver PCK at two different frequencies. They tried to calculate the Mn^{2+}-proton and Mn^{2+}-phosphorus distance and found that the metal and PEP form an outer sphere complex, in contrast to the earlier work by Miller *et al.*[176] and Barns *et al.*[177] on the pig liver and sheep kidney enzyme.

Lee *et al.*[180] used ^{31}P-NMR as a tool for the observation of the GTP(γ-S) degradation in the presence of PCK in combination with other kinetic methods. They found the reaction rates to be strongly dependent on the nature of the cations used.

6.3. *Phosphoenolpyruvate Synthetase*

The phosphoenolpyruvate synthetase (PEPS, EC 2.7.9.2) catalyzes the reaction:

$$ATP + pyruvate + H_2O \rightleftharpoons AMP + PEP + P_i$$

A similar reaction is catalyzed by the pyruvate, orthophosphate dikinase (POD, EC 2.7.9.1):

$$ATP + pyruvate + P_i \rightleftharpoons AMP + PEP + PP_i$$

Thus, POD as well as PEPS establish an equilibrium situation between PEP and ATP. Both enzymes are found in microorganisms.

For POD as well as PEPS a phosphorylated intermediate is known, whereas a pyrophosphorylated intermediate has been detected only for the POD enzyme. Several rather sophisticated reaction schemes for the phosphoryltransfer pathway of these two enzymes have been proposed. Cook and Knowles[181] determined the stereochemical pathway of phosphoenolpyruvate(^{16}O, ^{17}O, ^{18}O) and ATP(γ-S, β-$\gamma^{17}O$,γ-^{17}O,^{18}O). Stereochemical analysis of the reaction products of PEPS as well as POD with the aid of ^{31}P-NMR showed that the β-phosphate group of ATP reacted with retention of configuraton, whereas the γ-phosphate reacted with inversion. The β-group is thus supposed to be displaced twice, the γ-phosphate only once, thus showing the existence of phospho- as well as of pyrophospho-intermediates. As for the overall pathway of the reaction-sequences, the details are not entirely clear in the light of contradicting results from observations with other methods.

7. CONCLUSIONS

The technique of NMR-spectroscopy has advanced in several respects during the few years past. Unfortunately, the enzymes catalyzing phosphoryltransfers are all in a molecular range preventing structural studies by current 2D-NMR methods in the magnetic field range presently available. On the other hand, the largest of them are still small enough to be subjected to solution NMR methods. The most important advances in the study of phosphoryltransfer systems are definitely the improvement in signal to noise ratio of the NMR spectrometers and the increased simplicity of performing multinuclear experiments on modern spectrometers. These have allowed direct studies of the enzymes by proton NMR and also allowed the direct observation of bound substrates by phosphorus NMR and the direct observation of bound metals by cadmium NMR. In the future it will be not too surprising to see more applications of the use of the magnetically active isotopes of

magnesium, oxygen, and carbon, because these may be incorporated into the reacting complexes without disturbing their chemical environments.

It has also become clear that NMR is not the miraculous method allowing simple elucidation of molecular mechanism, neither is it, at present, a method for determining the complete tertiary structure of proteins in the molecular weight range relevant to the discussion of phosphoryl transfer processes. One of the merits of NMR is that it is a spectroscopic method for probing the reaction centre directly. Of major importance is the fact that changes of the protonation state of phosphorylgroups may be determined easily via the changes in their ^{31}P-NMR chemical shifts. Also, the power of NMR, in particular ^{31}P-NMR, as the only way currently available for mapping the on-enzyme kinetics of substrate interconversion should not be overlooked. This field is probably only in its infancy. The general opinion is that the on-enzyme exchange of the phosphorylgroup is not the rate limiting transfer step. The combination of stereochemical methods with NMR has already yielded valuable information on the classification of phosphoryltransferring enzymes according to their having a phosphoryl-enzyme intermediate. It seems that the transfer of a phosphorylgroup to a non-water molecule always proceeds via a direct in-line mechanism without an intermediate.

In our own group, we are continuing work on the adenylate kinase. This protein is of special interest because it is the smallest of the phosphokinases. In fact, one may expect that this protein is reduced in size to the point where only the mere necessities for the substrate specificity and the phosphoryl transfer are present. Unfortunately, at present it is not even clear where the substrates bind to the enzyme. That question must be answered first, by the combined efforts of NMR-spectroscopists and X-ray crystallographers. Then there is a fair chance that even proton NMR will be able to be used as a tool to study the active site structure of this protein in detail, and there is some hope that we will then begin to understand the phosphoryltransfer mechanism of at least this smallest of enzymes belonging to the kinase category.

REFERENCES

1. M. COHN and B. D. NAGESWARA RAO, *Bull. Magn. Res.* **1**, 38 (1979).
2. B. D. NAGESWARA RAO, in *Biol. Magn. Res.* **5**, 75 (1983).
3. M. COHN, *J. Cell. Comp. Physiol.* **54**, Suppl. 1,17 (1959).
4. M. COHN, and HUGHES, *J. Biol. Chem.* **235**, 11, 3250 (1960).
5. M. SAUNDERS, A. WISHNIA, and J. G. KIRKWOOD, *J. Am. Chem. Soc.* **79**, 3289 (1957).
6. M. -D. TSAI, and K. BRUZIK, in *Biol. Magn. Res.* **5**, 129 (1983).
7. A. BAX, *Two-Dimensional Nuclear Magnetic Resonance in Liquids*, Reidel, Hingham (1981).
8. R. KAPTEIN, in *Biol. Magn. Res.* **4**, 145 (1982).
9. H. S. GUTOWSKY, R. L. VOLD, and I. J. WELLS *J. Chem. Phys.* **43**, 4107 (1965).
10. G. BINSCH, in *Dynamic Nuclear Magnetic Resonance Spectroscopy*, Eds. L. JACKMAN and F. A. COTTON, p. 45, Academic Press, New York, (1975).
11. J. I. KAPLAN, and G. FRAENKEL, *NMR of Chemically Exchanging Systems*, Academic Press, London (1980).
12. J. SANDSTROEM, *Dynamic NMR Spectroscopy*, Academic Press, London (1982).
13. K. V. VASAVADA, J. I. KAPLAN, and B. D. NAGESWARA RAO, *Biochem.* **23**, 961 (1984).
14. K. V. VASAVADA, J. I. KAPLAN, and B. D. NAGESWARA RAO, *J. Magn. Res.* **41**, 467 (1981).
15. M. COHN, and A. HU, *Proc. Nat. Acad. Sci. USA* **75**, 200 (1978).
16. D. HACKNEY, K. E. STEMPEL and P. D. BOYER, *Meth. Enzymol.* **64B**, 60 (1980).
17. R. A. MITCHELL, in *Curr. Top. Bioenerg.* **13**, 203 (1984).
18. P. RÖSCH, *Z. Naturforsch.* **37c**, 1161 (1982).
19. D. HACKNEY, *J. Biol. Chem.* **255**, 5320 (1980).
20. P. RÖSCH, H. R. KALBITZER and R. S. GOODY, *Z. Naturforsch.* **36c**, 534 (1981).
21. P. RÖSCH, *Z. Naturforsch.* **36c**, 539 (1981).
22. K. M. BRINDLE, J. BOYD, I. D. CAMPBELL, R. PORTEOUS, and N. SOFFE, *Biochem. Biophys. Res. Comm.* **109**, 864 (1982).
23. R. FREEMAN, T. H. MARECI, and G. A. MORRIS, *J. Magn. Res.* **42**, 341 (1981).
24. K. M. BRINDLE, R. PORTEOUS, and G. K. RADDA, *Biochem. Biophys. A.* **786**, 18 (1984).
25. S. FORSEN and R. A. HOFFMANN, *J. Chem. Phys.* **39**, 2892 (1963).
26. S. FORSEN and R. A. HOFFMANN, *J. Chem. Phys.* **40**, 1189 (1964).
27. H. M. MCCONNELL, *J. Chem. Phys.* **28**, 430 (1958).
28. J. R. ALGER and R. G. SHULMAN, *Q. Rev. Biophys.* **17**, 83 (1984).

29. J. JEENHER, B. H. MEIER and R. R. ERNST, *J. Chem. Phys.* **71**, 4546 (1979).
30. G. BODENHAUSEN and R. R. ERNST, *J. Am. Chem. Soc.* **104**, 1304 (1982).
31. S. L. BUCHWALD, D. E. HANSEN, A. HASSETT and J. R. KNOWLES, *Meth. Enzymol.* **87**, 279 (1982).
32. C. F. MIDELFORT and I. A. ROSE, *J. Biol. Chem.* **251**, 5881 (1976).
33. A. HASSETT, W. BLAETTLER and J. R. KNOWLES, *Biochem.* **21**, 6335 (1982).
34. D. D. DAVIES, *Prog. Nucl. Magn. Reson. Spectrosc.* Vol.12, 135 (1978).
35. G. GOVIL and H. V. RAMAKRISHNA, in *NMR: Basic Principles and Progress*, Eds. P. DIEHL, E. FLUCK and R. KOSFELD, (1982).
36. R. STOLARSKI, C. -E. HAGBERG, and D. SHUGAR, *Eur. J. Biochem.* **138**, 187 (1984).
37. E. K. JAFFE and M. COHN, *J. Biol. Chem.* **254**, 10839 (1979).
38. W. KLAUS, P. RÖSCH and R. S. GOODY, *Res. Exp. Med.* **185**, 145 (1984).
39. P. RÖSCH, H. R. KALBITZER and R. S. GOODY, *FEBS Lett.* **121**, 211 (1980).
40. P. RÖSCH, R. S. GOODY, and M. ISAKOV, *Phosphorus and Sulfur* **21**, 9 (1984).
41. V. L. PECORARO, J. D. HERMES and W. W. CLELAND, *Biochem.* **23**, 5262 (1984).
42. K. -F. R. SHEU and P. A. FREY, *J. Biol. Chem.* **252**, 4445 (1977).
43. C. LERMAN and M. COHN, *J. Biol. Chem.* **255**, 8756 (1980).
44. M. -D. TSAI, S. L. HUANG, J. F. KOZLOWSKI and C. C. CHANG, *Biochem.* **19**, 3531 (1980).
45. S. L. HUANG and M. -D. TSAI, *Biochem.* **21**, 951 (1982).
46. J. A. GERLT, P. C. DEMOU and M. SHUJAATH, *J. Am. Chem. Soc.* **104**, 2848 (1982).
47. J. H. BALDO, P. E. HANSEN, J. W. SHRIVER and B. D. SYKES, *Can. J. Biochem.* **61**, 115 (1983).
48. F. BLOMBERG and H. RÜTERJANS, *Biol. Magn. Res.* **5**, 21 (1983).
49. R. M. SCHEEK, S. STOB, J. SCHLEICH, N. C. M. ALMA, C. W. HILBERS and R. KAPTEIN, *J. Am. Chem. Soc.* **103**, 5930 (1981).
50. E. F. MCCORD, K. M. MORDEN, A. PARDI, I. TINOCO and S. G. BOXER, *Biochem.* **23**, 1926 (1984).
51. J. E. SCHEFFLER and M. COHN, *J. Magn. Res.* **61**, 550 (1985).
52. G. E. SCHULZ, M. ELZINGA, F. MARX and R. H. SCHIRMER, *Nature* **250**, 120 (1974).
53. E. F. PAI, W. SACHSENHEIMER, R. H. SCHIRMER and G. E. SCHULZ, *J. Mol. Biol.* **114**, 37 (1977).
54. B. D. NAGESWARA RAO and M. COHN, *Proc. Natl. Acad. Sci. USA* **74**, 5355 (1977).
55. W. J. RAY, *Biochemistry* **22**, 4625 (1984).
56. B. D. NAGESWARA RAO, M. COHN and L. NODA, *J. Biol. Chem.* **253**, 4, 1149 (1978).
57. J. BOYD, K. M. BRINDLE, I. D. CAMPBELL and G. K. RADDA, *J. Magn. Res.* **60**, 149 (1984).
58. T. R. BROWN and S. OGAWA, *Proc. Natl. Acad. Sci. USA* **74**, 3627 (1977).
59. D. A. WISNER, C. A. STEGINSKY, Y. -J. SHYY and M. -D. TSAI, *J. Am. Chem. Soc.* **107**, 2815 (1985).
60. G. G. MCDONALD, M. COHN and L. NODA, *J. Biol. Chem.* **250**, 6947 (1975).
61. H. R. KALBITZER, R. MARQUETANT, P. RÖSCH and R. H. SCHIRMER, *Eur. J. Biochem.* **126**, 531 (1982).
62. P. RÖSCH and K. H. GROSS, *J. Mol. Biol.* **182**, 341 (1985).
63. G. M. SMITH and A. S. MILDVAN, *Biochem.* **21**, 6119 (1982).
64. P. RÖSCH and K. H. GROSS, *Z. Naturforsch.* **39c**, 738 (1984).
65. W. SACHSENHEIMER and G. E. SCHULZ, *J. Mol. Biol.* **114**, 23 (1977).
66. G. L. KENYON and G. REED, *Adv. Enz.* **54**, 367 (1983).
67. B. D. NAGESWARA RAO and M. COHN, *J. Biol. Chem.* **256**, 1716 (1981).
68. E. J. MILNER-WHITE and D. S. RYCROFT, *Biochem. J.* **167**, 827 (1977).
69. B. D. NAGESWARA RAO and M. COHN, *J. Biol. Chem.* **252**, 3344 (1977).
70. G. K. JARORI, B. D. RAY and B. D. NAGESWARA RAO, *Biochem.* **24**, 3487 (1985).
71. G. LOWE and B. S. SPROAT, *J. Biol. Chem.* **255**, 3944 (1980).
72. T. L. JAMES and M. COHN, *J. Biol. Chem.* **249**, 2599 (1974).
73. T. L. JAMES *Biochem.* **15**, 4742 (1976).
74. M. VASAK K. NAGAYAMA, K. WÜTHRICH, M. L. MERTENS and J. H. R. KTGI, *Biochem.* **18**, 5050 (1979).
75. P. R. ROSEVEAR, P. DESMEULES, G. L. KENYON and A. S. MILDVAN, *Biochem.* **20**, 6155 (1981).
76. R. J. GUPTA, *J. Biol. Chem.* **252**, 5183 (1977).
77. B. D. NAGESWARA RAO, F. J. KAYNE and M. COHN, *J. Biol. Chem.* **254**, 2689 (1979).
78. S. MESHITSUKA, G. M. SMITH and A. S. MILDVAN, *J. Biol. Chem.* **256**, 4460 (1981).
79. W. A. BLAETTLER and J. R. KNOWLES, *Biochem.* **18**, 3927 (1979).
80. W. C. HUTTON, E. M. STEPHENS and C. M. GRISHAM, *Arch. Biochem. Biophys.* **184**, 166 (1977).
81. D. E. ASH, F. J. KAYNE and G. H. REED, *Arch. Biochem. Biophys.* **190**, 571 (1978).
82. B. D. NAGESWARA RAO, D. H. BUTTLAIRE and M. COHN, *J. Biol. Chem.* **251**, 6981 (1976).
83. B. D. NAGESWARA RAO, M. COHN and R. K. SCOPES, *J. Biol. Chem.* **253**, 8056 (1978).
84. E. K. JAFFE and M. COHN, *J. Biol. Chem.* **255**, 3240 (1980).
85. P. TANSWELL, E. W. WESTHEAD and R. J. P. WILLIAMS, *Eur. J. Biochem.* **63**, 249 (1976).
86. T. N. BRYANT, H. C. WATSON and P. C. WENDELL, *Nature* **247**, 14 (1973).
87. C. C. F. BLAKE and P. R. EVANS, *J. Mol. Biol.* **84**, 585 (1974).
88. M. COHN, *Curr. T. Cell. Reg.* **24**, 1 (1984).
89. G. LOWE, and V. L. POTTER, *Biochem. J.* **199**, 227 (1981).
90. D. POLLARD-KNIGHT, B. V. L. POTTER, P. M. CULLIS, G. LOW and A. CORNISH-BOWDEN, *Biochem. J.* **201**, 421 (1982).

91. R. L. PETERSEN and R. K. GUPTA, *Biophys. J.* **27**, 1 (1979).
92. R. N. ARMSTRONG, H. KONDO, J. GRANOT, E. T. KAISER and A. S. MILDVAN, *Biochem.* **18**, 1230 (1979).
93. J. GRANOT, H. KONDO, R. N. ARMSTRONG, A. S. MILDVAN and E. T. KAISER, *Biochem.* **18**, 2339 (1979).
94. J. GRANOT, A. S. MILDVAN, K. HIYAMA, H. KONDO and E. T. KAISER, *J. Biol. Chem.* **255**, 4569 (1980).
95. M. W. KILIMANN, K. D. SCHNACKERZ and L. M. G. HEILMEYER, JR., *Biochem.* **23**, 112 (1984).
96. MEADOW *et al.* in *Enzymes of Biological Membranes*, Ed. A. MARTONOSI, (2nd edition) Vol. 3, p. 523 (1984).
97. J. REIZER, J. DEUTSCHER, S. SUTRINA, J. THOMPSON and M. H. SAIER, JR., *TIBS* **10**, 32 (1985).
98. O. SCHRECKER, R. STEIN, W. HENGSTENBERG, M. GASSNER and D. STEHLIK, *FEBS Lett.* **51**, 309 (1975).
99. M. GASSNER, D. STEHLIK O. SCHRECKER, W. HENGSTENBERG, W. MAURER and H. RÜTERJANS, *Eur. J. Biochem.* **75**, 287 (1977).
100. G. DOOIJEWAARD, F. F. ROOSSIEN and G. T. ROBILLARD, *Biochem.* **18**, 2996 (1979).
101. H. R. KALBITZER and P. RÖSCH, *Org. Magn. Res.* **17**, 88 (1982).
102. W. MAURER, H. RÜTERJANS, O. SCHRECKER, W. HENGSTENBERG, M. GASSNER and D. STEHLIK, *Eur. J. Biochem.* **75**, 297 (1977).
103. U. SCHMIDT-ADERJAN, P. RÖSCH, R. FRANK, and W. HENGSTENBERG, *Eur. J. Biochem.* **96**, 43 (1979).
104. P. RÖSCH, H. R. KALBITZER, U. SCHMIDT-ADERJAN, and W. HENGSTENBERG, *Biochem.* **20**, 1599 (1981).
105. H. R. KALBITZER, H. P. MUSS, R. ENGELMANN, H. H. KILTZ, K. STUBER and W. HENGSTENBERG, *Biochem.* **24**, 4562 (1985).
106. F. F. ROOSSIEN, G. DOOIJEWAARD and G. T. ROBILLARD, *Biochem.* **18**, 5793 (1979).
107. H. R. KALBITZER, W. HENGSTENBERG, P. RÖSCH, P. MUSS, P. BERNSMANN, R. ENGELMANN, M. DORSCHUG and J. DEUTSCHER, *Biochem.* **21**, 2879 (1982).
108. H. R. KALBITZER, J. DEUTSCHER, W. HENGSTENBERG and P. RÖSCH *Biochem.* **21**, 6178 (1981).
109. M. DÖRSCHUG, R. FRANK, H. R. KALBITZER, W. HENGSTENBERG and J. DEUTSCHER, *Eur. J. Biochem.* **144**, 113 (1984).
110. J. R. KNOWLES, *Annu. Rev. Biochem.* **49**, 877 (1980).
111. G. S. BEGLEY, D. E. HANSEN, G. R. JACOBSON and J. R. KNOWLES, *Biochem.* **21**, 5552 (1982).
112. A. RIBEIRO, J. PARELLO and O. JARDETZKY, *Prog. Biophys. Molec. Biol.* **43**, 95 (1984).
113. J. W. SHRIVER and B. D. SYKES, *Biochem.* **20**, 2004 (1981).
114. J. W. SHRIVER and B. D. SYKES, *Biochem.* **20**, 6357 (1981).
115. J. W. SHRIVER and B. D. SYKES, *Biochem.* **21**, 3022 (1982).
116. J. W. SHRIVER, *TIBS* **9**, 322 (1984).
117. J. WRAY, R. S. GOODY, K. C. HOLMES, (1986), manuscript in preparation.
118. B. KOPPITZ, K. FELDMANN, and L. M. G. HEILMEYER JR., *FEBS Lett.* **117**, 199 (1980).
119. M. R. WEBB, G. G. MCDONALD and D. R. TRETHAM, *J. Biol. Chem.* **253**, 2908 (1978).
120. P. RÖSCH, R. S. GOODY, H. R. KALBITZER and H. ZIMMERMANN, *Arch. Biochem. Biophys.* **211**, 2, 622 (1981).
121. S. HIGHSMITH, K. AKASAKA, M. KONRAD, R. S. GOODY, K. HOLMES, N. Y. WADE-JARDETZKY and O. JARDETZKY, *Biochem.* **18**, 4238 (1979).
122. H. P. PRINCE, H. R. TRAYER, G. D. HENRY, I. P. TRAYER, D. C. DALGARNO, B. A. LEVINE, C. D. CARY, and C. TURNER, *Eur. J. Biochem.* 121, 213 (1981).
123. G. D. HENRY, D. C. DALGARNO, G. MARCUS, M. SCOTT, B. A. LEVINE, and I. P. TRAYER, *FEBS Lett.* **144**, 11 (1982).
124. D. C. DALGARNO, H. P. PRINCE, B. A. LEVINE and I. P. TRAYER, *Biochim. Biophys. Acta* **707**, 81 (1982).
125. S. HIGHSMITH, C. C. WANG, R. ZERO, R. PECORA, and O. JARDETZKY, *Biochem.* **21**, 1192 (1982).
126. M. STEWART and G. C. K. ROBERTS, *FEBS Lett.* **146**, 293 (1982).
127. J. E. COLEMAN and P. GETTINS, *Adv. Enzymol.* **55**, 381 (1983).
128. J. E. COLEMAN, I. M. ARMITAGE, J. F. CHLEBOWSKI, J. D. OTVOS, and A. J. M. SCHOOT UITERKAMP, in *Biological Applications of Magnetic Resonance*, Ed. R. G. SHULMAN, p. 345, Academic Press, New York (1979).
129. J. L. BOCK and B. SHEARD *Biochem. Biophys. Res. Comm.* **66**, 24 (1975).
130. J. L. BOCK and A. KOWALSKY, *Biochim. Biophys. Acta* **526**, 135 (1978).
131. W. E. HULL, S. E. HALFORD, H. GUTFREUND and B. D. SYKES, *Biochem.* **15**, 1547 (1976).
132. J. F. CHLEBOWSKI, I. M. ARMITAGE, P. P. TUSA and J. E. COLEMAN, *J. Biol. Chem.* **251**, 1207 (1976).
133. J. F. CHLEBOWSKI, I. M. ARMITAGE and J. E. COLEMAN, *J. Biol. Chem.* **252**, 7053 (1977).
134. P. GETTINS and J. E. COLEMAN, *J. Biol. Chem.* **258**, 396 (1983).
135. J. D. OTVOS, J. R. ALGER, J. E. COLEMAN and I. M. ARMITAGE, *J. Biol. Chem.* **254**, 1778 (1979).
136. R. E. WEINER, J. F. CHLEBOWSKI, P. H. HAFFNER and J. E. COLEMAN, *J. Biol. Chem.* **254**, 9739 (1979).
137. J. D. OTVOS and D. T. BROWNE, *Biochem.* **19**, 4011 (1980).
138. J. D. OTVOS and I. M. ARMITAGE, *Biochem.* **19**, 4021 (1980).
139. J. D. OTVOS and I. M. ARMITAGE, *Biochem.* **19**, 4031 (1980).
140. P. GETTINS and J. E. COLEMAN, *J. Biol. Chem.* **258**, 408 (1983).
141. P. GETTINS and J. E. COLEMAN, *J. Biol. Chem.* **259**, 11036 (1984).
142. B. D. SYKES, H. I. WEINGARTEN and M. J. SCHLESINGER, *Proc. Natl. Acad. Sci. USA* **71**, 469 (1974).
143. D. T. BROWNE and J. D. OTVOS, *Biochem. Biophys. Res. Comm.* **68**, 907 (1976).
144. W. E. HULL, and B. D. SYKES, *Biochem.* **15**, 1535 (1976).
145. J. L. BOCK and M. COHN, *J. Biol. Chem.* **253**, 4082 (1978).

146. S. R. Jones, L. A. Kindman and J. R. Knowles, *Nature* **257**, 564 (1978).
147. J. D. Otvos, I. M. Armitage, J. F. Chlebowski and J. E. Coleman, *J. Biol. Chem.* **254**, 4707 (1979).
148. P. Gettins, M. Metzler and J. E. Coleman, *J. Biol. Chem.* **260**, 2875 (1985).
149. E. T. Fossel, R. L. Post, D. S. O'Hara, and T. H. Smith, *Biochem.* **20**, 7215 (1981).
150. C. Klevickis and C. M. Grisham, *Biochem.* **21**, 6979 (1982).
151. G. M. Clore, A. M. Gronenborn, C. Mitchinson and N. M. Green, *Eur. J. Biochem.* **128**, 113 (1982).
152. L. D. Faller, and G. A. Elgavish, *Biochem.* **23**, 6584 (1984).
153. A. Nakano, T. Miyazawa, S. Nakamura and Y. Kaziro, *FEBS Lett.* **116**, 72 (1980).
154. A. Wittinghofer, R. S. Goody, P. Rösch and H. R. Kalbitzer, *Eur. J. Biochem.* **124**, 109 (1982).
155. H. R. Kalbitzer, R. Leberman and A. Wittinghofer, *FEBS Lett.* 180, 40 (1985).
156. L. N. Johnson, J. A. Jenkins, K. S. Wilson, E. A. Stura and G. Zanotti, *J. Mol. Biol.* **140**, 565 (1980).
157. R. J. Fletterick and N. B. Madsen, *Annu. Rev. Biochem.* **49**, 31 (1980).
158. H. W. Klein and E. J. M. Helmreich, *Curr. T. Cell.* **26**, 281 (1985).
159. S. G. Withers, N. B. Madsen, B. D. Sykes, M. Tgaki, S. Shimomura, and T. Fukui, *J. Biol. Chem.* **21**, 10759 (1981).
160. S. J. W. Busby, D. G. Gadian, G. K. Radda, R. E. Richards and P. J. Seeley, *FEBS Lett.* **55**, 14 (1975).
161. K. Feldmann and E. J. M. Helmreich, *Biochem.* **15**, 2394 (1976).
162. K. Feldmann and W. E. Hull, *Proc. Natl. Acad. Sci. USA* **74**, 856 (1977).
163. S. G. Withers, B. D. Sykes, N. B. Madsen and P. J. Kasvinsky, *Biochem.* **18**, 5342 (1979).
164. E. Helmreich, M. C. Michaelides and C. F. Cori, *Biochem.* **6**, 3695 (1967).
165. M. Hoerl, K. Feldmann, K. D. Schnackerz and E. J. M. Helmreich, *Biochem.* **18**, 2457 (1979).
166. H. W. Klein, M. J. Im, D. Palm, and E. J. M. Helmreich, *Biochem.* **23**, 5853 (1982).
167. S. G. Withers, N. B. Madsen and B. D. Sykes, *Biochem.* **20**, 1748 (1981).
168. S. G. Withers, N. B. Madsen and B. D. Sykes, *Biochem.* **21**, 6716 (1982).
169. D. Palm, K. -H. Schaechtele, K. Feldmann, and E. J. M. Helmreich, *FEBS Lett.* **101**, 403 (1979).
170. H. W. Klein and E. J. M. Helmreich, *FEBS Lett.* **108**, 209 (1979).
171. H. W. Klein, D. Palm, and E. J. M. Helmreich, *Biochem.* **21**, 6675 (1982).
172. S. G. Withers, N. B. Madsen, and B. D. Sykes, *J. Magn. Res.* **61**, 545 (1985).
173. J. Tucker, G. Sczakiel, R. Leberman, P. Rösch and A. Wittinghofer, manuscript in preparation (1985).
174. G. I. Rhyu, W. J. Ray, Jr. and J. L. Markley, *Biochem.* **23**, 252 (1984).
175. G. I. Rhyu, W. J. Ray, Jr. and J. L. Markley, *Biochem.* **24**, 2536 (1985).
176. R. S. Miller, A. S. Mildvan, H. -C. Chng, R. L. Easterday, H. Maruyama, and M. D. Lane *J. Biol. Chem.* **243**, 6030 (1968).
177. R. J. Barns, D. B. Keech, and W. J. O'Sullivan, *Biochim. Biophys. Acta* **289**, 212 (1972).
178. C. A. Hedba, and T. Nowak, *J. Biol. Chem.* **257**, 5515 (1982).
179. T. H. Duffy, and T. Nowak, *Biochem.* **24**, 1152 (1985).
180. M. H. Lee, R. S. Goody, and T. Nowak, *Biochem.* in press (1985).
181. A. G. Cook and J. R. Knowles, *Biochem.* **24**, 51 (1985).
182. O. Lutz, A. Nolle and D. Staschewski, *Z. Naturforsch. A*, **33**, 380, (1978).