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Examination of the relationship of substrate dynamics to enzymic structure, binding energy, and catalysis: NMR studies of adenosine 5'-triphosphate and adenylate kinase

Sanders, Charles Ray, II, Ph.D.

The Ohio State University, 1988
EXAMINATION OF THE RELATIONSHIP OF SUBSTRATE DYNAMICS TO ENZYMIC STRUCTURE, BINDING ENERGY, AND CATALYSIS:
NMR STUDIES OF ADENOSINE 5'-TRIPHOSPHATE AND ADENYLATE KINASE.

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree of Doctor of Philosophy in the Graduate School of The Ohio State University

By

Charles Ray Sanders II, B. S.

The Ohio State University
1988

Dissertation Committee
Ming-Daw Tsai
Marita M. King
James Cowan

Approved By

Advisor
Department of Chemistry
To my wonderful parents on both sides of the family and to my dear wife, Becky.
ACKNOWLEDGEMENTS

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I thank my wife, Becky, for endless patience, encouragement, and support. Finally, I thank my family and friends for their love, encouragement, and prayers and to my Lord Jesus and his Father for all good things.
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"Mechanism of Adenylate Kinase. Use of Deuterium NMR to Probe the Local Mobility of Substrates and the Relationship Between Local Mobility and Local Binding Energy." Charles R. Sanders II and Ming-Daw Tsai, *Biochemistry* (submitted for publication).

Field of Study

Major Field: Chemistry
Emphasis in Biochemistry, Professor M.-D. Tsai, Advisor.
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<tr>
<td>Ado</td>
<td>adenosine</td>
</tr>
<tr>
<td>ADP</td>
<td>adenosine 5’-diphosphate</td>
</tr>
<tr>
<td>AK</td>
<td>adenylate kinase</td>
</tr>
<tr>
<td>AMP</td>
<td>adenosine 5’-monophosphate</td>
</tr>
<tr>
<td>AMPPCP</td>
<td>adenylyl (β,γ-methylene)diphosphonic acid</td>
</tr>
<tr>
<td>AMPPCD₂P</td>
<td>adenylyl (β,γ-dideuteromethylene)diphosphonic acid</td>
</tr>
<tr>
<td>[8-²H]AMPPCP</td>
<td>[8,²H]adenylyl (β,γ-methylene)diphosphonic acid</td>
</tr>
<tr>
<td>Ap₅A</td>
<td>diadenosine 5’-pentaphosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine 5’-triphosphate</td>
</tr>
<tr>
<td>CHES</td>
<td>2-[(cyclohexylamino)ethanesulfonic acid</td>
</tr>
<tr>
<td>2'-dATP</td>
<td>2’-deoxyadenosine 5’-triphosphate</td>
</tr>
<tr>
<td>3'-dATP</td>
<td>3’-deoxyadenosine 5’-triphosphate</td>
</tr>
<tr>
<td>DPM</td>
<td>disintegrations per minute</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediamine tetraacetic acid</td>
</tr>
<tr>
<td>EPR</td>
<td>electron paramagnetic resonance</td>
</tr>
<tr>
<td>FID</td>
<td>free induction decay</td>
</tr>
<tr>
<td>Hepes</td>
<td>N-(2-hydroxyethyl)piperazine-N’-2-ethanesulfonic acid</td>
</tr>
<tr>
<td>Kₐ</td>
<td>association constant</td>
</tr>
<tr>
<td>K_d</td>
<td>dissociation constant</td>
</tr>
<tr>
<td>K_I</td>
<td>kinetic inhibition constant</td>
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<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
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<tr>
<td>NOE</td>
<td>nuclear Overhauser effect</td>
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<tr>
<td>PEG</td>
<td>polyethylene glycol</td>
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<tr>
<td>PEI-cellulose</td>
<td>polyethyleneimine cellulose</td>
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<tr>
<td>PP₁</td>
<td>pyrophosphate</td>
</tr>
<tr>
<td>PPP₁</td>
<td>triphosphate</td>
</tr>
<tr>
<td>T₁</td>
<td>longitudinal relaxation time</td>
</tr>
<tr>
<td>T₂</td>
<td>transverse relaxation time</td>
</tr>
<tr>
<td>TEAB</td>
<td>triethyl ammonium bicarbonate;</td>
</tr>
<tr>
<td>TLC</td>
<td>thin-layer chromatography</td>
</tr>
<tr>
<td>Tris</td>
<td>2-amino-2-(hydroxymethyl)-1,3-propanediol</td>
</tr>
<tr>
<td>TSP</td>
<td>3-(trimethylsilyl)propionic acid</td>
</tr>
<tr>
<td>τ_c</td>
<td>effective correlation time</td>
</tr>
<tr>
<td>τ_i</td>
<td>internal correlation time</td>
</tr>
<tr>
<td>τ_o</td>
<td>overall correlation time</td>
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CHAPTER I
INTRODUCTION

The relationship of the motional dynamics of substrates to enzymic catalysis and binding energy is a fundamental issue in enzymology but has received relatively little experimental attention. It is now well established that enzymes utilize binding energy to facilitate efficient catalysis (Jencks, 1975; Fersht, 1987, 1985). The manner in which binding energy effects catalysis is generally depicted very informatively using free energy-reaction coordinate diagrams and by correlating portions of such diagrams with static "time-frame" structures of enzyme-substrate complexes. An examination of some fundamental principles of enzyme catalysis leads to the suggestion that it is also conceptually helpful to consider that enzymes utilize binding energy to bring about the spatial and temporal control of substrate mobility.

When two reactants are present in a dilute solution the probability of their collision to form a potentially reactive complex is low. Enzymes overcome this difficulty by using ground state binding energy to bring reactants in proximity to each other. As is intuitively apparent, overall substrate mobility is drastically reduced at this point by the fact that the substrate is now located within the matrix of the much more slowly tumbling enzyme molecule.

Reaction probability is also enhanced in ground state binding by the fact that enzymes bind substrates such that they are oriented in the correct manner, both spatially and temporally, with respect to each other. The catalytic enhancement
brought about by such binding (which occurs in both ground and transition states) is
generally considered to be analogous to the rate enhancements observed by organic
chemists for intramolecular reactions relative to corresponding intermolecular
reactions, the exact physical chemical nature of which has been the matter of
considerable debate. Jencks (1975) lists no less than 12 terms and theories proposed
to help explain this effect. A recent contribution by Page (1988) had updated this list
to 21. Possible explanations invoke entropy (Jencks, 1975; Page, 1977), a critical
time/distance postulate (Menger, 1985, 1988), orbital steering (Dalfovo and
Koshland, 1971), substrate anchoring (Reuben, 1971), propinquity (Bruce, 1976)
and stereo-electronic control (Gorenstein, 1988).

Many of these theories have a common but implicit theme: they emphasize the
role which the enzymic control of substrate motion plays in catalysis. In this case, not
only overall substrate mobility is relevant and is reduced, but the local segmental
mobilities of portions of a substrate are likely to be catalytically significant and
affected as well. A large portion of this paper is dedicated to the examination of such
"local mobility" and its relationship to binding energy and catalysis. Only a few
previous experimental examinations of such relationships have appeared. Fischer and
Jardetsky (1965) qualitatively determined the local mobilities of portions of
albumin-bound penicillin and were able to establish via accompanying binding studies
that a correlation does exist between the immobilization of various parts of penicillin
and the interaction energies of those parts. Nowak and Milidvan (1973) examined
portions of phosphoenol pyruvate analogs bound to pyruvate kinase and observed
greater immobilization in the phosphate (reaction center) region than in portions
spatially removed from the phosphate. In addition, Cohn and her coworkers (Cohn,
1975) in extensive EPR and proton relaxation rate studies very qualitatively observed
that the active sites of kinases are progressively immobilized as various portions of transition state mimicking complexes are successively bound. A similar observation was made by Leyh et. al. (1985) based on EPR studies of transition state-like complexes of creatine kinase.

In this dissertation the local mobilities of AMPPCP (an ATP analogue, see Figure 1) and MgAMPPCP free in solution and bound to the enzyme adenylate kinase are examined. Adenylate kinase catalyzes the direct transfer (Richard and Frey, 1978) of the γ-phosphate of MgATP (bound at the ATP or MgATP specific site) to the phosphate of AMP (bound at its specific site) with a rate enhancement of at least $10^{11}$ relative to the uncatalyzed reaction. It is probable that a significant portion of this rate increase is brought about by the utilization of binding energy and the spatial control of the substrate in conjunction with transition state stabilization as none of the "chemical mechanisms" (general acid/base, coenzyme assistance, etc.) have ever been implicated to be involved in its reaction mechanism except for the non-enzymic electrophilic role of Mg$^{+2}$. A reasonable, but still largely hypothetical mechanism for the reaction is
depicted in Figure 2. Furthermore, structures for the AK-MgATP complex have been proposed (Caldwell and Kollman, 1988; Milidvan and Fry, 1987; Fry et. al., 1988, 1985; Dreusicke et. al., 1988; Pal et. al.,1977) and are under further scrutiny by site-specific mutagenic studies (Tian et. al., 1988; 1989b). Thus, if substrate dynamics are determined, they can be correlated with tentative structural models. Binding studies using $^1$H NMR have also been completed which allow us to determine which portions of ATP/MgATP interact with AK to provide the binding energy which drives ground state binding. The necessary thermodynamic data is therefore available for examination of the relationship between substrate dynamics and binding energy. These binding studies also, coincidentally, yielded significant information regarding substrate-induced conformational change in the AK molecule. The results of these binding studies are detailed in Chapter III.

We examined the local mobilities of specifically deuterated AMPPCP and MgAMPPCP free in solution and bound to AK by determination of free and bound $^2$H NMR relaxation rates. This required that we “work through” two significant difficulties. First, while $^2$H NMR has previously been utilized in studies of protein-ligand interactions, the actual experimental approaches taken have been rather limited in scope. Chapter IV is largely dedicated to the systematic examination and establishment of high resolution $^2$H NMR approaches which can be used in systems such as ours to determine intrinsic free and bound relaxation rates. Most significantly, we have characterized lineshape behavior in systems undergoing exchange processes which are “intermediate” with respect to the difference between bound and free relaxation rates and show that NMR data analysis from systems in this exchange region need not always be particularly difficult. The second problem lies in obtaining meaningful information on the nature of substrate dynamics from such NMR
relaxation rates. Significant portions of the Chapter V are therefore dedicated to the use of approximate methods to extract information on local substrate mobility for AMPPCP free in solution and bound to AK.

Having establishing the local motions of AMPPCP and MgAMPPCP free in solution and bound to AK and assuming these to be similar to the dynamics of ATP and MgATP, the relationship of substrate dynamics to enzyme structure, binding energy, and catalysis for adenylate kinase and MgATP are examined in Chapter VI.
Figure 2. Possible reaction mechanism of AK. This mechanism assumes phosphoryl transfer goes through an "associative" (SN$_2$-type) transition state. A-E are lysine or arginine side chains. Please note that two extra hydrogen bonds are present in the transition state which are not present in ground states—this demonstrates "transition-state binding".
CHAPTER II
MATERIALS AND METHODS

Materials. Chemicals used in the synthesis of AMPPCD$_2$P, 99.999% MgCl$_2$·6H$_2$O, D$_2$O, deuterium-depleted H$_2$O, and the sodium salt of [2,2,3,3-d$_4$]TSP were purchased from Aldrich. AMPPCP, AMP, ATP, and the biochemicals used in the binding studies were purchased from Sigma. [2-3$^3$H]ATP was obtained from Amersham and perdeuterated Trizma base from MSD Isotopes. Chelex-100 was purchased from Bio-Rad, Scinti-Verse E scintillation cocktail from Fisher, and PEI-cellulose (Machery-Nagel PEI/UV$_{254}$, 0.1 mm pre-coated) from Brinkmann. Ion-exchange resins were obtained from Pharmacia or Bio-Rad, PEG 20,000 from Fluka, silica gel TLC plates from E.M. Sciences, and puratronic grade Mg(NO$_3$)$_2$·6H$_2$O from Alfa.

*E. coli* JM103 harboring pKK-cAK1-1 (containing the cloned and overproduced chicken muscle AK gene, Kishl et. al., 1986) which was the source of the AK used in this study was a generous gift from Professor Atsushi Nakazawa and Dr. Fumio Kishl at Yamaguchi University. The enzyme was purified as described previously (Tian et. al., 1988; Tanizawa et. al., 1987).

Preparation of AK and Substrate Solutions for Proton NMR. AK samples in a D$_2$O buffer solution were prepared as follows. Following purification, the enzyme AK is usually ca. 2 mg/ml in 5 mM Imadazole-HCl, 0.1 mM EDTA, 1 mM DTT, pH 6.9. KCl was added to the AK solution to a concentration of 15-30 mM and the solution was lyophilized. It was observed that the AK concentration needs to be $\geq$ 2
mg/mL for AK to lyophilize without denaturing. The dried enzyme was dissolved to a concentration of 20 mg/mL in 1 mM potassium Hapes, 65 mM KCl, 0.1 mM EDTA, 1 mM DTT, pH 7.8 and dialyzed against 4 L of this same buffer. Following dialysis exactly 1.2 mL of the enzyme was placed into a 15 mL pear-shaped flask along with a small number of beads of Chelex-100 to sequester paramagnetic impurities followed by lyophilization, redissolution in D$_2$O, and relyophilization. Immediately before $^1$H NMR experiments exactly 1.2 mL of 75 mM perdeuterated Tris-DCI, pH = 8.0 in D$_2$O was added to the dried enzyme, still in the pear-shaped flask and to this was added DTT to a total concentration of 1.5 mM along with a small amount of [2,2,3,3-$_d^4$]TSP as an internal reference. The resulting solution was then passed through a D$_2$O-rinsed Gelman "Acrodisk" (No. 4192) to remove insoluble AK and two 0.4 - 0.5 mL portions of the resulting solution were transferred to two 5 mm NMR tubes. The solution composition was 0.2 - 0.9 mM AK in 75 mM perdeuterated Tris-DCI, 65 mM KCl, 1.5 mM DTT, 1 mM K$^+$ Hapes, 0.1 mM EDTA, pH = 8.0. The exact AK concentration was determined by measuring peak integrals of individual AK peaks (C2-H of histidine residues) relative to those arising from ligands added to a known concentration.

Ligand stock solutions (ATP, AMP, etc.) were made by dissolution of the ligand into D$_2$O, addition of a few grains of dried chelex and adjustment of the pH to the appropriate value (7.5 or 8.0). In some instances these solutions were lyophilized to remove residual HDO followed by redissolution and readjustment (when necessary) of the pH. Nucleoside and nucleotide concentrations were determined by their absorbancies at 259 nm while inorganic ligand concentrations were estimated based on their dry weights. In the case of the ATP used for the MgATP titration of AK further purification of the ATP was accomplished by ion-exchange chromatography using DEAE Sephadex A-25 equilibrated with TEAB. ATP was applied as a basic aqueous solution and
eluted with a linear gradient of water to 0.65 M TEAB. Fractions containing ATP were identified by their absorbance at 259 nm and TLC on silica gel and the ATP was pooled and processed to a K+ salt in a manner analogous to that used (Reynolds et. al., 1983) to produce the Na+ salt of ATP. Stock solutions of adenosine (> 50 mM) had to be warmed on a heating block at 90 °C in small test tubes equipped with a condensor. In this manner solubility was maintained with no change in the concentration occurring or adenosine breakdown even over a period of a number of hours, as determined by UV absorbancy, TLC, and proton NMR.

Proton NMR Methods. ¹H NMR monitored binding studies were performed by titrating AK or peptide samples and taking the proton NMR spectrum at each point. All experiments were performed on a Bruker AM-500 spectrometer using an alternating phase pulse sequence with HDO peak suppression being accomplished by selective pre-irradiation. The spectral width was usually 6000 Hz with 16 or 32K data points resulting in a spectral resolution of 0.3 - 0.8 Hz/pt. The total pulse repetition rate was > 3 sec.

Spectra were referenced either to external TSP or to the internal TSP peak for the first point in a titration. This was done because high concentrations of nucleotides induced concentration-dependent shifts in the reference peak, presumably due to non-specific interactions between the nucleotides and TSP.

Proton NMR Titration Experiments and Binding Analysis. Proton NMR spectra of AK samples contained a number of easily followed individual resonances many of which shift upon substrate/ligand binding. Complete titration experiments were carried out in all cases (by titrating the peptide or enzyme solution with the ligand and taking the 500 MHz ¹H NMR spectrum at each point), except in the case of AK-MgAP₅A and AK-equilibrium mixture where only one point with excess ligands
(more than enough to saturate the enzyme) was taken. Typically 200-500 transients were taken at each point. The observed peaks appeared at their population weighted-averaged (between free and bound positions) chemical shifts characteristic of fast exchange (or nearly so) and could therefore be used to monitor the concentration dependence of ligand-AK association. It should be noted that two of the four histidine C2-1H peaks (peaks 3 and 4 in Figure 4) were insensitive to ligand binding but would be perturbed by significant variations in the pH (Tian et. al., 1988). In the few instances where significant shifts in these peaks were observed, the histidine resonances (pH sensitive) were not used in the binding analysis. For individual peaks, plots of the free ligand concentration vs. the observed chemical shift were fitted to 1:1 and, occasionally, 2:1 binding equations.

For our titrations there are three experimental variables, $E_1$ (total AK concentration), $L_1$ (total substrate or analogue concentration), and $v$ (the observed resonance frequency). If 1:1 binding stoichiometry pertains the observed resonance position at a point during titration is:

$$v = \frac{(E \cdot V_f + EL \cdot v_b)}{E_1}$$  \hspace{1cm} (1)

where $E$ and $EL$ are free and complexed AK concentrations, and $v$, $V_f$, and $v_b$ are the observed, free and complexed resonance frequencies of the AK peak in question. Using mass balance equations for $E_1$ and $L_1$ equation 1 can be rewritten:

$$v = \frac{[E \cdot V_f + (E_1 - E) \cdot v_b]}{E_1}$$  \hspace{1cm} (2)

Using the mass balance equations and the expression for the dissociation constant:

$$K_d = \frac{E \cdot L}{EL}$$  \hspace{1cm} (3)
E can be explicitly solved:

\[ E = \frac{E_t - L_t - K_d + [(E_t - L_t - K_d)^2 + 4 \cdot K_d \cdot E_t]^{1/2}}{2} \]  

Equation 4 is substituted into equation 2 and the titration data points in \( E_t, L_t, \) and \( n \) are fit to the resulting equation to yield the parameters \( K, v_f, \) and \( v_b. \) A non-linear least squares/simplex fitting program (MINSQ, Micromath Software) was utilized for the iterative fitting. The calculation of dissociation constants is therefore a 3-dimensional analysis. In depicting these fits (Figure 5) we present approximate 2-D fits where \( E_t \) is held constant rather than attempting true 3-D representations.

In the case of AMP, data were fit to a 2:1 sequential model as well. The model strictly applies only to the following sequence:

\[
\begin{array}{c}
\text{E + L} \quad \xrightarrow{K_{a,1}} \quad \text{EL} \quad \xrightarrow{K_{a,2}} \quad \text{LE} \\
\quad \xleftarrow{K'_{a,1}} \quad \text{LE} \quad \xleftarrow{K'_{a,2}} \quad \text{E} \quad \xrightarrow{K_{a,1}} \quad \text{LE} \quad \xleftarrow{K_{a,2}} \quad \text{EL}
\end{array}
\]  

where \( K_{a,1} \) and \( K_{a,2} \) are association constants and \( EL \) and \( LE \) represent enzyme-substrate complexes where the substrate is bound at different sites in the two complexes. The model also approximately applies to:

\[
\begin{array}{c}
\text{E + L} \quad \xrightarrow{K_{a,1}} \quad \text{EL} \quad \xrightarrow{K_{a,2}} \quad \text{LE} \\
\quad \xleftarrow{K'_{a,1}} \quad \text{LE} \quad \xleftarrow{K'_{a,2}} \quad \text{E} \quad \xrightarrow{K_{a,1}} \quad \text{LE} \quad \xleftarrow{K_{a,2}} \quad \text{EL}
\end{array}
\]  

provided that \( K_{a,1} \gg K'_{a,1}. \) In these cases:
\[ v = \frac{(E \cdot v_f + EL \cdot v_b + LEL \cdot v_c)}{E_t} \]  \hspace{1cm} (7)

where \( LEL \) is the concentration of the ternary complex and \( v_c \) is the resonance frequency of a particular peak in that complex. Using the mass balance equations along with expressions for \( K_{a,1} \) and \( K_{a,2} \), equation (7) is recast:

\[ v = \frac{(v_f + K_{a,1} \cdot L \cdot v_b + K_{a,1} \cdot K_{a,2} \cdot L^2 \cdot v_c)}{E_t} \]  \hspace{1cm} (8)

where \( L \) is the free substrate/analogue concentration. We were unable to derive an explicit solution for \( L \) for this model. However, if, under the titration conditions, \((L + EL) \gg LEL\) then the explicit solution for \( L \) from the case of 1:1 binding provides an excellent approximation for the value of \( L \) in the 2:1 case. From the 1:1 mass balance equations and \( K_a \) we find:

\[ L = \frac{\{K_{a,1} \cdot (L_t \cdot E_t) - 1 + ((1 + K_{a,1} \cdot (E_t - L_t))^2 + 4 \cdot K_{a,1} \cdot L_t)^{1/2}\}}{(2 \cdot K_{a,1})} \]  \hspace{1cm} (9)

This equation is inserted into Equation 7 and the resulting equation is used to fit experimental data in \( L, E \) and \( v \) to yield parameters \( K_{a,1}, K_{a,2}, v_f, v_b, \) and \( v_c \). In general, it was found that either \( K_{a,1} \) or \( K_{a,2} \) needed to be set as a constant for the iterative fitting to converge on reasonable values for the other parameters.

Peaks from the nucleotides were also observed to shift as they titrated the enzyme. The free and bound frequencies for individual peaks were calculated using the following equation (see Appendix A):
\[ \frac{L_1 - L}{L_1} \cdot (V_b - V_f) + V_f \]

into which Equation 9 has been substituted for L. In the case of the nucleotide peaks no attempt was made to extract \( K_a \) from titration data; rather, \( K_a \) was set to a predetermined value and the data is then fit to obtain estimates of \( V_f \) and \( V_b \).

**Determination of Dissociation Constants for AMPPCP and MgAMPPCP Using Proton NMR.** Binding constants for AMPPCP and MgAMPPCP were determined using the \(^1\)H NMR titration method as described above except that the temperature was maintained at 10 °C and the \( D_2O \) buffer system contained 6 mM K\(^+\)Hepes, 65 mM KCl, 1 mM DTT, 0.1 mM EDTA, pH = 7.0.

**TLC Assay of the AK Reaction.** In this study we tested AMP and adenosine as inhibitors of AK (competitive with MgATP). Because high levels of either compound might also inhibit the coupling enzymes present in the standard spectrophotometric assay (Tian et al., 1988) we used a direct assay using PEI-cellulose and radioactive ATP derived from the assays described by Pederson and Catterall (1979) and Tomasselli and Noda (1979). Reaction mixtures contained the appropriate concentrations of Mg\(^{2+}\), ATP, AMP and (sometimes) adenosine in 75 mM triethanolamine-HCl, 65 mM KCl, 0.5 mM DTT, and 0.1 mM EDTA, pH 8.0. Reactions took place in 0.6 mL microcentrifuge tubes partially submerged in a 30 °C water bath. 2 μCi of \([2,3^3]H\)ATP was added to the solution and the reaction was initiated by adding enough stock AK (dissolved in the above buffer plus 10 mM DTT and 1 mg/mL bovine serum albumin) to convert 5 - 10% of the MgATP present to MgADP over a period of 5 minutes. The total reaction volume was 0.4 mL. Aliquots (usually 50 μL) of the
reaction mixtures were removed at 0, 1.5, 3.0, and 4.5 minutes after starting the reaction and immediately quenched by mixing the aliquots with 5μL 0.8 M EDTA, pH 8-9 in microcentrifuge tubes which were then labelled and frozen in liquid nitrogen until TLC were run. When the kinetic runs were finished the tubes containing the "time point" mixtures were thawed. 2 μL of an AMP/ADP/ATP mixture (7/7/5 mM in 50 mM EDTA, pH 8) mixture was spotted 2 cm from the end of a PEI-cellulose TLC plate and act as "tracers" permitting visualization of the nucleotide positions under ultraviolet light. Upon this dried spot was spotted 2 X 5μL portions of the quenched assay mixture for a single time point. This was, of course, repeated for each of the time points for all of the reactions run. TLC plates were then developed with 1 M LiCl such that the solvent front travelled about 15 cm. ADP and ATP spots were identified under ultraviolet light and cut out and placed face up and flat on the bottom of scintillation vials and the ³H DPM were measured after addition of scintillation cocktail. By measuring the DPM for [2-³H]ATP (reactant) and [2-³H]ADP (product) at each of the four time points taken for a single reaction the normalized (to all of the other reactions run in a single experiment with a single AK stock solution) reaction rate could be determined:

\[
\frac{[\Delta \text{DPM/min. (of ADP)} \cdot \mu\text{Moles MgATP initially present}]}{[\text{total DPM (ATP + ADP)} \cdot \mu\text{L of AK stock used}]} = \frac{\mu\text{Moles MgATP converted}}{[\text{min} \cdot \mu\text{L AK stock}]}
\]

No reverse reaction was ever observed to have taken place when assays were run according to these procedures, as judged by the lack of appearance of radioactivity in the AMP spot.
Synthesis of [8-2H]AMPPCP The procedures are outlined in Figure 3. AMPPCP was dissolved in D2O containing 10 mM CHES, the pH was raised to 10 and the sample was dried by rotary evaporation. The nucleotide was redissolved in D2O to a concentration of 0.1 g/mL and the pH was then readjusted to ca. 10. This solution was transferred to a 13 x 100 mm test tube which was heated on a heating block for 4 h at 95 °C. The product was a ca. 1:1 mixture of AMP and AMPPCP with the level of deuteration at the adenine C-8 position being 90% as observed by 1H NMR. [8-2H]AMPPCP was purified by ion exchange chromatography using DEAE Sephadex A-25 equilibrated with TEAB. The nucleotides were applied as a basic aqueous solution and eluted with a linear gradient of water to 0.65 M TEAB. Fractions containing the nucleotides were identified by their absorbance at 259 nm and TLC on silica gel and the AMPPCP was pooled and processed to a K+ salt in a manner analogous to that used (Reynolds et. al., 1983) to produce the Na+ salt of ATP. The [8-2H]AMPPCP was judged to be pure by 1H NMR, and TLC on silica gel. 31P NMR sometimes indicated the presence of 2 impurities, both 10 % or less which were methylene diphosphonic acid and (probably) inorganic phosphate. The silica gel TLC solvent system used in all experiments was isobutyric acid/conc. NH4OH/H2O (66/1/33) (Pharmacia, 1984).

Synthesis of AMPPCD2P. The procedures are also outlined in Figure 3. Tetraisopropyl-[2H2]methylene diphosphonate was synthesized (20-40% yield) in a dry box under argon by the method of Roy (1966) using CD2Br2 and trisopropyl phosphite which had been distilled and dried with molecular sieves. Stringent anhydrous conditions were used to prevent exchange of deuterium with protons. This ester was observed to be ca. 90% deuterated by fast atom bombardment mass spectroscopy and was converted to [2H2]methylene diphosphonic acid with no further
Figure 3. Syntheses of [8-²H]AMPPCP and AMPPCD₂P.
loss of deuteration (as observed by $^1$H NMR) by transesterification with trimethylsilylexyl iodide (Olah and Nurang, 1982) followed by water hydrolysis. The original HCl ester hydrolysis described by Roy (1966) was found to result in H-D exchange. The product was partially purified by ion exchange methods and converted to its dithiobutylammonium-dipyridinium salt (Myers et al., 1965) and reacted with adenosine phosphomorpholidate in dry pyridine (Moffatt and Khorana, 1961) to produce AMPPCD$_2^P$ (40-60% yield) which was purified and processed as described for [6-$^2$H]AMPPCP and judged to be pure by silica gel TLC and $^1$H NMR and >90% pure by $^{31}$P NMR (methylene diphosphonic acid was sometimes observed to be present at a low level).

**Preparation of Nucleotide Stock Solutions for Deuterim NMR.**

For $^2$H NMR use, deuterated AMPPCP or was dissolved in $^2$H-depleted H$_2$O and a few beads of Chelex-100 were added followed by pH adjustment to 7.0. The AMPPCP concentration was determined by measuring absorbance at 259 nm and was stored at 65 °C between NMR experiments. Stock solution concentrations were 50 - 250 mM. These stock solutions were used both to make 6 mM deuterated nucleotide solutions at pH 7.0 and 10.0 for titration by Mg$^{2+}$ and to titrate AK solutions. The concentration of Mg$^{2+}$ in stock Mg(NO$_3$)$_2$ solutions used in AMPPCP titrations was determined by volumetric titration with EDTA (Schenk et. al., 1977). During the titrations volumetric additions of Mg(NO$_3$)$_2$ to deuterated AMPPCP or of the deuterated nucleotides to AK solutions were made directly into 10 mm NMR tubes using plastic-tipped micropipettors. In pH 10 titrations of AMPPCP by Mg$^{2+}$ the solution was not buffered and the pH was readjusted to 10 after every second or third addition of Mg(NO$_3$)$_2$. 
Preparation of AK Samples for Deuterium NMR. The purified enzyme, as noted earlier, was originally a ca. 2 mg/mL solution in 5 mM Imidazole-HCl, 1 mM DTT, 0.1 mM EDTA, pH 6.9. For $^2$H NMR studies the enzyme was usually transferred to dialysis tubing and concentrated by placing the tubing directly onto dry PEG 20,000 or into a buffered PEG syrup to a concentration of > 4 mg/mL. A certain amount of low molecular weight PEG was found to enter the dialysis tubing which was observed in the $^2$H NMR spectra. This was found to have no effect on $^2$H NMR results but makes this method of concentration unsuitable for $^1$H NMR work. Following dialysis the concentration of the enzyme was determined by measuring absorbance at 280 nm and assuming $\varepsilon_{280}(0.1\%) = 0.5$. Designating this approximate concentration "Y" mg/mL the enzyme was then dialyzed against $Y/45 \times 10$ mM K$^+$ Hapes or Imidazole-HCl, $Y/45 \times 150$ mM KCl, 1 mM mercaptoethanol, 0.2 mM DTT, pH 7.0 and then lyophilized. At the time of the NMR experiment 90 mg of this preparation was dissolved in 1.91 mL of 40 mM K$^+$ Hapes or Imidazole-HCl, 0.1 mM EDTA, pH 7.0, in $^2$H-depleted H$_2$O. Extra DTT was added at this point and at various times during the NMR experiment. Following centrifugation for 10-15 min at 8000 x g, 1.7-1.85 mL of this preparation was placed into a 10 mm NMR tube and was ready for titration. By calculation, the solution contains 1.6 mM AK, 117 mM KCl, 45 mM K$^+$ Hapes or Imidazole-HCl, 0.1 mM EDTA, 1-2 mM DTT, pH 7.0. Such solutions were used in all of the $^2$H NMR studies involving AK described in this dissertation. The actual AK concentration was determined for calculational purposes using the coupled assay system and assuming that at 30 °C the enzyme exhibits an apparent specific activity (at 2 mM ATP and AMP) of 1500 U/mg (Tian et al., 1988) and was found to vary from 1-1.5 mM from preparation to preparation.
Deuterium NMR Methods. ²H NMR was usually run on a Bruker MSL-300 spectrometer operating at 46.1 MHz although some spectra were also obtained at 77.8 MHz on a Bruker AM-500 spectrometer. Free induction decays were acquired while running "unlocked". Shimming to < 3 Hz inhomogeneity was routinely achieved by "shimming on the FID" from an aqueous [²H₆]acetone standard. Inhomogeneity could be monitored during the actual titrations by measuring the linewidth of the HDO peak, which has a natural linewidth of <1 Hz. Samples containing AK were not spun. Temperature control was maintained with a Bruker variable temperature unit and a flow of cold nitrogen gas.

Spectra used for T₂ determination from linewidths were acquired using 90° pulses (12 µsec) and pulse sequences CYCLOPS or RIDE (when acoustic ringing was observed to be a problem, Gerothanassis, 1987). The spectral resolution was 0.2-15 Hz/point (the higher values when very broad nucleotide peaks were observed) and was always more than sufficient to allow accurate definition of lineshapes. The spectral width was always at least 10 X the linewidth of the broadest peak present. In all cases pulse sequence repetition rates were greater than 4 X T₁ and 5 X T₂ of the deuterated nucleotide, again sufficient to allow quantitative spectra. In the case of studies at 77.8 MHz, an alternating phase pulse sequence was utilized and the FID was left-shifted following acquisition to reduce the affects of acoustic ringing. FIDs were zero-filled. The FID was further processed by exponential multiplication. The T₁ and T₂ of HDO present in all samples are much longer than that of deuterated AMPPCP partially bound to AK. Thus, while the dwell time during which the FID was sampled was sufficiently long to allow full decay of the AMPPCP component of the FID (whose T₁ and T₂ are < 20 msec) the HDO component was truncated. This resulted in distortion of the "wings" of the HDO peak. This was a serious problem since the HDO resonance is
superimposed upon the much broader AMPPCP resonances (see Figure 14). This problem was dealt with, when encountered, by further apodization. The FID was subjected to a weighted sine bell function (weighting constant of 2). This artificially forced the tail of the FID (containing only the HDO component and noise) to zero while not affecting the early portion of the FID (containing all of the quickly decaying AMPPCP resonance) and thereby eliminated spectral distortion. \( T_2 \) were determined using a Bruker program GLINFIT which allows the experimenter to fit a peak to a Lorentzian function and then calculates the linewidth at half maximal height for the fit. \( T_2 \) is determined from the simple relationship (Martin et. al., 1980):

\[
\Delta v_{1/2} = \frac{1}{\pi T_2}
\]

(12)

\( T_1 \) were determined by the inversion recovery method (Martin et. al., 1980). 5-13 variable delay points were acquired and delay versus intensity data were fit to a single exponential equation using a Bruker non-linear fitting routine. The pulse repetition rate was in all cases > 5 X \( T_1 \).

**Viscosity Studies.** Relative viscosities were determined for solutions analogous to those used in \(^2\)H NMR titrations. An Ostwald viscometer was submerged (up to 1.5 cm from its top) in water contained in a 1 L graduated cylinder. A thermometer was used to determine the temperature of the water which was maintained at room temperature (23 °C). Flow times were determined for various solutions in the viscometer and densities were determined for the corresponding solutions by weighing them in a 2 mL pycnometer. Relative viscosities were calculated (using water as a reference) using the following equation (McKie and Brandts, 1972):
relative viscosity = \frac{\text{density of solution} \times \text{flow time of solution}}{\text{density of water} \times \text{flow time of water}} \quad (13)

In some cases the temperature was found to drift during the course of the experiments in which case relative viscosities determined at slightly different temperatures were normalized using the differences in absolute viscosities of water at different temperatures (Weast and Astle, 1980) as a normalization factor.

**NMR Lineshape Simulations.** NMR lineshapes for a simple two-site exchange system were simulated by use of a variant of the modified Bloch equation for two-site exchange (Sutherland, 1971). For a given set of parameters, intensities were calculated over a frequency range of at least 10 times the maximal possible calculated linewidth (i.e., 1000 Hz) with resolution sufficient to adequately define the simulated spectral lines.
CHAPTER III
PROTON NMR STUDIES OF THE BINDING OF SUBSTRATES TO ADENYLATED KINASE

Binding Studies. One of the primary goals of our studies was to analyze the energetics of MgATP-AK interactions. Specifically, we wished to determine which parts of MgATP interact favorably with AK, thereby providing the energy needed to drive MgATP association with AK. The theory utilized in making such an analysis (Jencks, 1981) requires the determination of the association constants ($K_a=1/K_d$) for the binding of both MgATP and its various components (adenosine, etc.) to AK. As these constants are not available in the literature we attempted their determination using equilibrium dialysis, UV difference spectrometry, and enzyme kinetics. While limited success was accomplished by the kinetic method, fundamental technical and practical difficulties with these techniques induced us to attempt utilization of $^1$H NMR as a means of measuring enzyme-substrate affinity.

While AK, at 22 kDa is approaching the upper molecular weight limit for effective study by proton NMR, its 500 MHz spectrum (Figure 4A) displays a number of well-resolved or easily observed individual resonances. Upon complexation with MgATP (or its components) the majority of these peaks are observed to undergo shifts in frequency. Spectra of various saturated AK complexes are presented in Figure 4B-H. In many cases the large spectral rearrangements which occur during point by point titrations of AK are so large that peaks become "lost in the shuffle" and thus cannot be
Figure 4. 500 MHz $^1$H NMR spectra of native AK and its complexes with substrates and analogues at pH 8.0 and 30 °C after processing the FID with 1 Hz of exponential line broadening. (A) 0.86 mM AK; (B) 0.4 mM AK + 7.8 mM PPPI; (C) 0.25 mM AK + 19.4 mM PPP$_1$ + 21.3 mM Mg$^{2+}$; (D) 0.74 mM AK + 4.5 mM AMP; (E) 0.5 mM AK + 9 mM ATP; (F) 0.4 mM AK + 8 mM ATP + 12 mM Mg$^{2+}$; (G) 0.37 mM AK + 9.3 mM AMP + 7.2 mM ATP + 10.7 mM Mg$^{2+}$; (H) 0.22 mM AK + 1 mM MgAP$_5$A. All solutions also contain 75 mM d$_{11}$-Tris, 65 mM KCl, 1.5 mM DTT, 1 mM K$^+$Hepes and 0.1 mM EDTA. NMR procedures and sample preparation are described in Chapter II.
Figure 4.
followed by NMR throughout the duration of the titration and used in binding analysis. Fortunately, however, there are a number of peaks which can be followed throughout the titrations and these are labelled 1-18 in Figure 4. Of these a number do not shift significantly during titration or are too broad to permit accurate measurement of the resonance frequency (e.g. peak 6) leaving us with a number of peaks which are readily followed and which do shift significantly. As described in Chapter II the titrating substrate/ligand concentration dependence of the shifts observed ($\Delta \delta$) were fit to 1:1 and sometimes 2:1 binding models to yield association constants (1 for each peak followed).

Titration data for MgATP, ATP, MgPPP$_i$, PPP$_i$, and MgPP$_i$ generally gave satisfactory fits to a 1:1 binding model. Typical fits are shown for MgATP in Figure 5 and the dissociation constants determined are reported in Table 1. The binding analysis involving adenosine, AMP, and Mg$^{+2}$ was not as straightforward.

The adenosine titration was ceased at 30 mM due to precipitation of adenosine at that point. In the 0 to 30 mM range, the peaks which changed exhibited shifts with linear dependence upon adenosine concentration. This suggests that we were only in the linear (Initial) portion of the hyperbolic binding isotherm which would indicate that $K_d$ is $> 30$ mM. This possibility was examined by kinetic studies of adenosine as a competitive inhibitor against both AMP and MgATP. We were barely able to detect (at the solubility limited concentrations of the nucleoside and using the imprecise TLC assay method) that adenosine is a very poor inhibitor with $K_i$ of approximately 40 and 70 mM against AMP and MgATP, respectively. Thus, the NMR result is consistent with kinetics.

AK was titrated with AMP up to 50 mM. The titration curves generally gave good fits to a 1:1 model with the average $K_d$ of 0.7 mM being determined as exemplified in
Figure 5. Examples of curves from titrations of AK by MgATP (A) and AMP (B) as followed by 500 MHz proton NMR at pH 8.0 and 30 °C. The data in (B) were fitted to both 1:1 (dashed curves) and 2:1 (solid curves) models while for MgATP the curves shown are for 1:1 fits.
Table 1. Dissociation Constants for AK-Ligand Complexes Determined by \(^1\text{H} \text{NMR}\).^a

<table>
<thead>
<tr>
<th>Substrate/Analogue</th>
<th>no. of titration points</th>
<th>range of concentration (mM)</th>
<th>no. of K(_d) determined(^b)</th>
<th>(K_{d,\text{avg.}}) (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMP (AMP site)</td>
<td>13</td>
<td>0-13</td>
<td>8</td>
<td>0.50 ± 0.25^c</td>
</tr>
<tr>
<td>AMP (MgATP site)</td>
<td></td>
<td></td>
<td></td>
<td>4.3 ± 2^d</td>
</tr>
<tr>
<td>MgATP</td>
<td>7</td>
<td>0-2.65^e</td>
<td>6</td>
<td>0.17 ± 0.05</td>
</tr>
<tr>
<td>ATP</td>
<td>7</td>
<td>0-2.9^e</td>
<td>6</td>
<td>0.44 ± 0.040</td>
</tr>
<tr>
<td>Adenosine</td>
<td>8</td>
<td>0-2.7</td>
<td>1</td>
<td>70 ± 30^f</td>
</tr>
<tr>
<td>Adenosine</td>
<td></td>
<td></td>
<td></td>
<td>40 ± 20^g</td>
</tr>
<tr>
<td>PPP(_i)</td>
<td>9</td>
<td>0-14</td>
<td>6</td>
<td>0.96 ± 0.4</td>
</tr>
<tr>
<td>MgPPP(_i)</td>
<td>11</td>
<td>0-1.9</td>
<td>6</td>
<td>1.67 ± 0.5</td>
</tr>
<tr>
<td>MgPP(_i)</td>
<td>6</td>
<td>0-6.9</td>
<td>5</td>
<td>5.5 ± 3</td>
</tr>
<tr>
<td>Mg(^{+2})</td>
<td>7</td>
<td>0-560</td>
<td>0</td>
<td>&gt;500</td>
</tr>
<tr>
<td>AMPPCP(^h)</td>
<td>9</td>
<td>0-8.0</td>
<td>5</td>
<td>0.22 ±0.1</td>
</tr>
<tr>
<td>MgAMPPCP(^h)</td>
<td>8</td>
<td>0-7.9</td>
<td>4</td>
<td>0.23 ±0.13</td>
</tr>
</tbody>
</table>

^a At pH 8.0 and 30 °C. ^b This the number of K\(_d\) determined (for different resonances) which were used to actually calculate K\(_d,\text{avg.}\). ^c This is the K\(_d,1\) determined by combination of NMR and kinetic methods as described in the text. ^d Determined by kinetic inhibition experiment (Figure 7). ^e For both ATP and MgATP points were taken above 5 mM. At such high concentrations points seemed to deviate from strict 1:1 behavior and were therefore not used in the calculation of dissociation constants. ^f K\(_d\) determined by NMR and kinetic methods for adenosine binding to the MgATP site. ^g K\(_d\) of adenosine inhibition against AMP determined by kinetics. ^h Titrations performed at pH 7.0 and 10 °C.
Figure 5. However, we were concerned about whether a 1:1 model strictly applies. A $K_d$ of 0.7 mM is somewhat larger than that determined from steady-state kinetics (0.37 mM) (Tian et al., 1989a) and subsequent analysis of the meaning of the observed spectral changes required that we take into account the possibility that AMP may also bind to the MgATP site. We therefore tested AMP as a competitive inhibitor (with MgATP) of AK and found that it has a $K_i$ of 4.3 mM (see Figure 6) which is almost certainly its dissociation constant for binding to the MgATP site ($K_{d,2}$) when the AMP site is saturated. Converting this value to an association constant and holding it as a fixed variable the $^1\text{H}$ NMR titration curves were fit to a 2:1 model. We found the data fit as well (sometimes better) to a 2:1 model as to a 1:1 model (see examples in Figure 5) and an average $K_{a,1}$ of 2000 M$^{-1}$ ($K_{d,1} = 0.5$ mM) being determined which is within experimental error of the kinetically derived value (Tian et al., 1989a).

Our observation that AMP binds weakly (i.e. $K_i = 4.3$ mM, probably equals $K_d$) to the ATP site is actually of considerable importance. Mildvan and his coworkers (Fry et al., 1987; Mildvan and Fry, 1987) described experiments where 0.3 mM AK was saturated with 10-25 mM AMP and then titrated with very low concentrations (< 50 μM) of Cr(III)AMPPCP which is an inhibitor of AK, competitive with MgATP with a $K_i$ of 0.39 mM. During the titration the $^1\text{H}$ and $^{31}\text{P}$ relaxation rates of AMP were measured. Cr(III) is paramagnetic and thus relaxes the protons or phosphorus nuclei of any nearby AMP in a distance dependent manner (the closer the more induced relaxation). In the interpretation of these studies Mildvan assumed that AMP could not bind to the MgATP site and that all the CrAMPPCP present, at such low concentrations, would therefore be complexed at the MgATP site of AK. Based on the relaxation observed for the proton and phosphorus nuclei of AMP, Mildvan proposed the distance
Figure 6. Double reciprocal plot demonstrating AMP to be an inhibitor of AK competitive with MgATP. A slope replot (inset) was used to calculate the inhibition constant (K_i). TLC assays were run at 30 °C and pH 8.0 as described in Chapter II. Each point shown is the average of two data points actually taken. Free [Mg^{2+}] was maintained at 1 mM throughout the experiment as described by Morrison (1979). The free AMP concentrations have been corrected for the small amount of MgAMP present while the MgATP concentrations have been corrected for the small amount of ATP present.
between AMP and MgATP sites and the orientation of the AMP molecule with respect to the Cr³⁺ ion of the CrAMPPCP complex. Furthermore, they also saturated AK with 5-10 mM AMP and selectively irradiated the protons of AMP and measured internuclear NOE enhancement of the intensity of AK resonances. Based on highly tentative proton NMR resonance assignments of AK peaks and again assuming that AMP does not bind to the MgATP site they combined the apparent results of this experiment with the results of the CrAMPPCP experiment and earlier studies of the MgATP site to generate a high resolution description of the structure of the AK-AMP-MgATP complex and even used this structure to propose an ångström-resolution description of the reaction coordinate and transition state for the AK reaction. While their approach certainly has the appearance of elegance, all of their conclusions with regard to the AMP site may be unreliable as we observe that under the conditions in which they ran their experiments AMP probably occupied a large fraction of the MgATP sites available (according to the K_d we determined). In other words, in their experiments AMP was present at high enough concentrations both to prevent CrAMPPCP from ever binding to the MgATP site and to insure that NOE observed between AMP and AK to arise from AMP bound to either the AMP site or the MgATP site. In all fairness, it should be noted that they did run some "controls" with respect to the CrAMPPCP study and that their studies were performed with rabbit muscle AK rather than the chicken muscle enzyme. Thsu, our criticism of their work must be deemed tentative.

Titration of AK by MgCl₂ up to a concentration of 0.5 M induced a number of changes in the spectrum of AK (Figure 7). The detailed analysis of shifts in resonance frequency observed showed a linear dependence on the metal ion concentration
Figure 7. 500 MHz proton NMR spectra and difference spectra with adenosine and Mg\(^{2+}\) present at pH 8.0 and 30 °C. Aromatic and aliphatic regions of AK are shown for (A), uncomplexed AK (0.86 mM); (B) 0.2 mM AK in the presence of 560 mM MgCl\(_2\); (C) ca. 0.3 mM AK in the presence of 27 mM adenosine; (D) difference spectrum where free AK spectrum was subtracted from (B); (E) difference spectrum when free AK spectrum is subtracted from (C). Additional information on the generation of difference spectra can be found in the caption of Figure 8.
suggesting that Mg$^{+2}$ interacts with AK extremely weakly in the absence of nucleotide ($K_d > 0.5$ M). This conclusion is supported by both EPR studies using Mn$^{+2}$ (Price et al., 1973) and theoretical calculations (Caldwell and Kollman, 1988).

The dissociation constants for the interaction of the substates and derivatives are listed in Table I along with some details of the individual titrations. The changes in resonant frequency of the AK peaks followed due to substrate/ligand complexation are listed in Table 2.

The binding constants determined were the very ones needed for the analysis of binding energy and are discussed at length in Chapter VI. In addition to these constants we found that these studies yielded information regarding substrate-induced conformational changes in the AK molecule. This information is very qualitative and is gleaned from examining the spectra in Figure 4 and comparison of the signs and magnitudes of the shifts of various peaks upon ligand binding detailed in Table 2. In addition, by subtracting (using Bruker software) the uncomplexed AK spectrum from the spectra of the various saturated complexes difference spectra are obtained (Figure 8) which are also useful in the qualitative comparison of spectra.

**Binding of PPPI, MgPPPI, and Adenosine to AK Induce Very Small Conformational Changes.** Although both PPPI and MgPPPI bind to AK with fairly high affinities ($K_d \leq 10x$ relative to ATP), they induce rather minor changes in the proton NMR of AK (Figures 4 and 8). Thus, it can be concluded that binding of PPPI or MgPPPI does not induce as large a global conformational change of AK as do the substrates.

A similar situation seems to hold true for the binding of adenosine to either site as well. While the titration of AK by adenosine had to be stopped well short of the point
### Table 2. Changes in Chemical Shifts upon Ligand binding to AK at pH 8.0 and 30 °C.

| Peak No. | 
|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|
|          | AM(a)    | MgATP    | ATP      | ternary  | MgADP/A | MgPPP    | PPP      | MgPPP    | AMP(d)   | AMP(d)   | Mg2+ 2o | Azo     |
|          | (ppm)    | (ppm)    | (ppm)    | (ppm)    | (ppm)    | (ppm)    | (ppm)    | (ppm)    | (ppm)    | (ppm)    | (ppm)   | (ppm)   | (ppm)   | (ppm)   |
| 1        | 7.888    | 0.038    | 0.058    | 0.030    | 0.082    | 0.092    | 0.00     | 0.00     | 0.00     | 0.040    | 0.040   | 0.00    | 0.00    | 0.005   |
| 2        | 7.770    | 0.034    | 0.130    | 0.060    | 0.144    | 0.230    | 0.038    | 0.020    | 0.00     | 0.028    | 0.028   | 0.00    | 0.00    | 0.000   |
| 3        | 7.593    | 0.034    | 0.034    | 0.040    | 0.040    | 0.00     | 0.00     | 0.00     | 0.00     | 0.048    | 0.048   | 0.00    | 0.00    | 0.000   |
| 4        | 7.228    | 0.030    | 0.020    | 0.030    | 0.020    | 0.00     | 0.00     | 0.00     | 0.010    | 0.064    | 0.041   | 0.01    | 0.01    | 0.018   |
| 5        | 7.128    | 0.00     | 0.010    | 0.004    | 0.020    | >00      | 0.004    | 0.020    | 0.00     | 0.00     | 0.000   | 0.000   | 0.00    | 0.00    |
| 6        | 6.858    | -0.010   | -0.020   | -0.020   | -0.040   | -0.040   | -0.010   | -0.00    | 0.00     | -0.018   | -0.018  | 0.01    | -0.01   |
| 7        | 6.808    | 0.034    | >0.0     | 0.038    | 7        | 7        | 0.024    | 0.034    | 0.018    | 0.028    | 0.041   | 0.00    | 0.00    |
| 8        | 6.434    | 0.020    | 0.004    | 0.014    | 0.060    | 0.060    | 0.00     | 0.00     | 0.00     | 0.020    | 0.012   | 0.00    | 0.00    |
| 9        | 6.293    | 0.038    | 0.030    | 0.038    | 0.199    | 0.090    | -0.012   | -0.008   | 0.019    | 0.030    | 0.048   | 0.00    | 0.00    |
| 10       | 6.094    | -0.220   | >0.7     | -0.212   | 7        | -0.400   | 0.008    | -0.080   | 0.010    | -0.350   | 0.01    | -0.04   |
| 11       | 2.060    | 0.99     | -0.020   | 0.00     | -0.020   | 0.00     | 0.00     | 0.00     | 0.00     | 0.00     | 0.01    | -0.22   |
| 12       | 1.862    | 0.008    | -0.040   | -0.032   | -0.030   | -0.010   | -0.014   | -0.008   | 0.014    | 0.01     | 0.02    | 0.02    |
| 13       | 1.642    | -0.062   | -0.044   | -0.044   | -0.044   | 0.00     | 0.00     | 0.00     | 0.00     | -0.034   | -0.072  | 0.012   |
| 14       | 1.328    | -0.32    | -0.028   | -0.030   | -0.04    | 0.00     | 0.00     | 0.00     | 0.00     | 0.00     | 0.01    |
| 15       | 0.967    | 0.032    | 0.032    | 0.028    | 0.05     | 0.04     | 0.008    | 0.006    | 0.00     | 0.01     |

*aSee Figure 8 for peak labeling.
*bPeaks 3, 4, and 8 did not undergo any shifts upon the binding of any ligands.
*cShifts calculated assuming 1:1 stoichiometry holds.
*dShifts calculated assuming 2:2 stoichiometry where AMP1 and AMP2 are the shifts calculated for the 1:1 and 2:1 complexes, respectively.
*eShifts reported for Mg2+ and adenosine are the shifts observed in strating AK in the presence of these ligands to 860 mM and 27 mM, respectively.
Figure 8. Difference spectra of the various AK complexes. The spectra depicted in Figure 4 (B-H) were each subtracted by the uncomplexed wild type AK spectrum (the zero point of titration in each specific set). Aromatic regions of the difference spectra are approximately normalized with respect to each other based on the intensity of the largest histidine C-2 proton difference peak present in each difference spectrum. The aliphatic region are also normalized to the histidine peak except that in this picture the absolute intensity of this region has been reduced 1/4 relative to the aromatic region (the actual difference spectra of this region are 4 X in intensity that depicted relative to the aromatic region).
of saturation, at its maximal observed concentration (27 mM) it should be occupying about 1/4 of the available AMP and MgATP sites. Yet, fairly small changes in the spectrum of the enzyme were observed (Figure 7) relative to AMP, MgATP, and AMP. In addition, the shifts induced by adenosine (see Table 2 - even when multiplied by four) when added to the shifts induced by MgPPP, do not come anywhere near to the number or magnitude of shifts induced by MgATP. Therefore, it appears that conformational changes in AK induced by MgATP and (probably) AMP require both adenosine and phosphate portions to be present. Our results do not indicate whether or not they also need to be connected.

**Substrate-Induced Conformational Changes are Global.** The results of Figures 4 and 8 and Table 2 suggest that many of the substrate-induced changes in the NMR spectrum of AK are related to global structural effects, not primarily to specific interaction of the substrate with the residues giving rise to the peaks. This conclusion is based on the fact that most peaks do shift upon substrate binding while most residues of AK are presumably not specifically involved in binding. The global spectral rearrangements cannot be accounted for solely by the ring-current effect of the adenine nucleotides. Interesting calculations by Kalbitzer et. al. (1982) indicate that the ring current effect on the spectrum of AK upon binding of a single adenine nucleotide should induce significant shifts (> 0.03 ppm) for a maximum of 17% of the resonances (35% for MgAP5A). The analysis of the shifts of individual peaks (Table 2) as well as examination of the difference spectra indicate that a much larger percentage actually shift upon substrate binding. The global nature of the shifts we observed suggests an explanation for why His 36 was previously thought to be involved in catalysis on the basis of substrate induced shifts in the proton NMR spectrum (Noda, 1973; McDonald et. al., 1975) while our site-specific mutagenesis results
demonstrate that this residue is not involved directly in catalysis (including binding) (Tian et al., 1988). The early studies were performed at lower magnetic fields. Thus, in those studies, the C2-H of His-36 was one of only few resolved resonances which could be observed and monitored upon binding of the substrate. Our results suggest that caution must be exercised when interpreting the meaning of substrate-induced shifts in well-resolved resonances, particularly when there are few other resonances which can be monitored.

Our results are consistent with both X-ray crystallographic results (Pal et al., 1977; Anderson et al., 1979) and solution X-ray scattering experiments (Dumas and Janin, 1983) which quantitatively show that most kinases do indeed undergo a global conformational change upon binding of one or both of the substrates.

One of our goals will be to shed light upon the nature of the observed conformational changes. Substrates could induce global structural changes as perceived qualitatively by NMR in one of two ways. First, enzymes exist in a conformational equilibrium between multiple conformers. NMR generally "sees" the weighted averaged conformation. The substrate may only bind to one of these conformers and thereby shifts the conformational equilibrium to induce a new weighted averaged conformation which is actually observed by NMR. Secondly, the substrate may bind to the enzyme and induce a change in enzyme structure to a conformer not present in the conformational equilibria of the free enzyme. While it is difficult to discern between these possibilities, both of which may partially occur, our results tend to favor the latter interpretation, at least in explaining the nature of the change actually observed by proton NMR. Titration of AK by up to 560 mM MgCl₂ resulted in fairly minor changes in the spectrum of the enzyme (Figure 7, particularly note the aliphatic region). Of these changes detailed analysis (Table 2)
reveals that the shifts observed do not generally correlate with those induced by the substrates or other ligands. It is also interesting to note that peaks 3 and 4 did shift significantly (no other species affected these peaks). Resonances 3 and 4 are surface histidines (of the 4 histidines of AK only 1 of them, His 36- peak 2, is internal). Based on these facts and the previous observation that Mg$^{+2}$ does not appear to specifically bind to AK we conclude that the shifts induced by Mg$^{+2}$ are largely due to nonspecific interaction of the metal ion with surface residues of AK, not to a global conformational change, and certainly not to a conformational change related to that relevant to substrate binding. Thus, any perturbation (even a small one, say 5%) in the pre-existing conformational equilibrium which might well be expected by raising the ionic strength by 1.2 seems to be unrelated to the changes we observed upon nucleotide binding. Variation of pH from <6 to 9 was also observed to have relatively small effect on the spectrum (Kalbitzer et. al., 1982; Sanders and Tsai, unpublished results; Rösch and Gross, 1985), except of course, for the histidine peaks. Thus, while changes in pH and ionic strength probably do perturb the conformational equilibrium of free AK (also seen in different crystal forms of AK, Sachsenheimer and Schulz, 1977), they do not result in large spectral changes or in changes which correlate with those observed upon substrate binding. This analysis suggests (we emphasize suggests) that the large shifts seen upon substrate binding are probably related to the substrate inducing a structural change to a new conformation, not to an induced shift in the position of a preexistent conformational equilibrium.

AMP and MgATP Induce Similar Conformational Changes. Another important point suggested by the data in Table 2 and the difference spectra of Figure 8 results from the observation that AMP, ATP and MgATP induce shifts in the same direction for a majority of peaks, despite the fact that AMP possesses a binding site
distinct from that of ATP and MgATP. This observation is made regardless of whether a 1:1 or 2:1 stoichiometry holds for AMP binding: since the calculated shifts for 1:1 binding of AMP are similar to those calculated for the binding of a first AMP molecule in a 2:1 binding process. To borrow a term In vogue among those quantum mechanically inclined, the changes caused by binding of either substrate are nearly degenerate. AK possesses a bi-bi random kinetic mechanism (Rhoads and Lowensten, 1968; Hamada and Kuby, 1978) but does exhibit some positive synergism (binding of either substrate enhances binding of the other by a factor of 3-4)(Hamada et. al., 1979; Tian et. al., 1989a).

Qualitatively, the degree of most shifts observed (Table 2) follow the order AMP = ATP ≤ MgATP < equilibrium mixture. Thus, it appears that AMP, ATP and MgATP induce very similar conformational changes in the AK structure upon binding to AK. Binding of the equilibrium mixture results in shifts in the same direction as those induced by the AMP or MgATP, but usually in a larger shift. While these data are qualitative in terms of explicit application to structural changes, it suggests that formation of a ternary complex (equilibrium mixture) induces additional conformational changes which are the continuation or completion of the conformational change initiated by formation of either binary complex. A schematic of the proposed process is depicted in Figure 9.

The existence and importance of the conformational changes we observed using proton NMR were confirmed by kinetic studies in our lab completed by Gaochao Tian (Tian, 1988; Tian et. al., 1989a,b). In these studies it was observed that wild type AK undergoes a kinetically significant, viscosity-dependent conformational change upon the binding of either AMP or MgATP. While it was observed that this conformational change is not very important in the catalytic efficiency of the enzyme,
Figure 9. Schematic of the conformational states of adenylate kinase suggested by our $^1$H NMR results.
it is conceivable that at some point in the evolution of AK the development of the conformational change was an extremely important step in the development of its efficiency but has since been superceded or outmoded by other developments in the mechanism.

There are, however, a small number of peaks for which AMP or MgATP induce shifts in different directions, or where one causes a shift but the other does not. A possible explanation is that such resonances are shifted due to local specific interactions of the corresponding AK residues with the substrate. Another possibility is that such resonances represent residues located in a local environment which is extremely NMR sensitive to minor conformational changes. Peak number 13, for example, undergoes a large upfield shift upon binding of AMP or ATP but seems to undergo a slight downfield shift upon the binding of MgATP.

![Figure 10. Structure of AP5A.](image)

**Does MgAP5A Bind to Both Substrate Sites?** Our proton NMR studies also permitted a very old problem of AK research to be addressed. MgAP5A (Figure 10) is a well known tight binding inhibitor of AK (Lienhard and Secemski, 1973; Bone et al.,
1986; Feldhaus et al., 1975; Hampton et al., 1982; Van Der Lijn et al., 1979; Kuby et al., 1978). It is unknown whether it acts as a transition state analogue in which binding energy is optimized by transition state-like interactions with AK or whether it binds to both sites with the overall binding energy benefitting from simultaneous ground state-like interactions of MgAP5A with both the AMP and MgATP sites. Recent crystallographic studies have led to the suggestion that neither may be true. The crystal structure of the yeast AK (AKy)-MgAP5A complex has been solved to 2.6 Å resolution (Egner et al., 1988). The authors of this work concluded that in this complex one of the adenosine moieties (adenosine-B) occupies the site previously proposed for ATP (Pal et al., 1977), but they reassigned this site as being the actual AMP site. The other adenosine moiety of AP5A (adenosine-A) occupies a site not previously associated with either AMP or MgATP and which is in an inhomologous region of the primary sequence and was therefore assigned to be a spurious site with regard to the normal substrate binding topology of AK. Essentially the same behavior has been observed in the AP5A complex with E. coli AK (AKE) (Muller and Schutz, 1988). The authors concluded that "one can safely assume that it is the inhibitor binding site in solution".

The above crystallographically derived conclusions are at variance with our NMR results as well as a number of kinetic studies. A comparison of the difference spectra for the AK-ternary and for the AK-MgAP5A complexes (Figure 8) shows remarkable similarity qualitatively and probably quantitatively (the method of difference spectra normalization is probably accurate to only about 25% - see caption for Figure 8). This strongly suggests that MgAP5A simultaneously occupies both AMP and MgATP sites, or at least induces the same conformational change as the equilibrium mixture does. In addition, while we were unable to assign the peaks for bound MgAP5A, the
pattern observed is in reasonable agreement with those determined for both bound AMP and bound MgATP. A number of kinetic studies (see references in the preceding paragraph) all agree that MgAP5A is a competitive inhibitor versus both AMP and MgATP. Therefore, MgAP5A apparently does bind to both AMP and MgATP sites in solution. Rosch et al. (1988) also suggested that the binding sites for AP5A proposed by Egner et al. (1987) are inconsistent with their NMR results using porcine muscle AK.

Since the crystal structure of the AK-MgAP5A complex is unlikely to be wrong (and even more unlikely to be wrong for both AKε and AKγ), a possible explanation is that MgAP5A binds differently in solution (to both substrate sites) from that in the crystal, or that muscle AK behaves differently from AKε and AKγ. This problem is further complicated by the controversy in the substrate binding sites (see discussions in Tian et al., 1988; 1989b). An encouraging sign is that site-specific mutagenesis (Reinstein et. al., 1988; Tian et. al., 1988; Tian et. al., 1989b) and theoretical methods (Caldwell and Kollman,1988) are now being applied to the problem previously probed primarily with NMR and X-ray crystallography.

Is MgAP5A a Transition-State Analogue? By "transition state analogue" we do not mean that analogue structure appears is visually similar to the true transition state- in the case of MgAP5A it certainly is not (see Figure 2). What is relevant is whether the enzyme perceives the analogue to be similar, therefore binding the analogue with both ground state interactions plus transition state binding. The difference spectra (Figure 8) probably cannot address the question of whether MgAP5A is a transition state analogue or simply a multi-substrate analogue. While the spectra is nearly the same as the ternary complex (NMR will see only the "ground state" of this complex, not the fleeting transition state) It is not clear that a
conformational change should necessarily occur in going from the ternary complex into the transition state. However, the inhibition constant of MgAP₅A (ca. 10 nM) can be used to calculate a binding energy for its association with AK of 11 kcal/mol. The binding energies for AMP and MgATP can be calculated from the dissociation constants reported in Table 1 and add up to 9.8 kcal/mol, nearly that of MgAP₅A. If MgAP₅A is a true transition state analogue it would be expected to benefit from a large amount of transition-state binding energy in addition to the ground state binding energies of the substrates. The fact that AK catalyzes phosphoryl transfer by a factor of at least 10¹¹ relative to the uncatalyzed reaction (see Footnote 1) indicates that this additional "transition state binding energy could be as large as 15 kcal/mol. Energy diagrams which should help explain this phenomena are shown in Figure 11 while one of the ways in which "transition state binding" could occur on the molecular level is shown back in Figure 2. This extra amount of energy expression is not observed. In addition, when MgAP₅A binds to AK it only loses 1 degree of translational entropy which is energetically less costly than the 2 degrees lost when MgATP and AMP bind individually which could account for some of the slight difference in the total binding energies for the two substrates and MgAP₅A. Therefore, while arguments based on binding energies are not without their pitfalls (Jencks, 1981), it appears that MgAP₅A is simply a bi-substate analogue, not a transition state mimic and suggests that arguments regarding the reaction coordinate distance for phosphoryl transfer in AK based on this analogue are not realistic.

Binding of AMPPCP/MgAMPPCP to AK. We also used the ᵃH NMR method to determine the Kᵣ of AK complexes with AMPPCP and MgAMPPCP under the conditions eventually used in the deuterium NMR studies of AK (pH 7.0 and 10 ° C, see Chapters
By selectively binding only transition-state structures AK lowers the transition state by 10 kCal/mol or more.

\[ \Delta G^* \] are observed binding energies calculated from association constants.

If MgAP$_6$A is merely a bi-substrate analogue:

\[ \Delta G^*_{\text{MgAP}_6\text{A}} = \Delta G^*_\text{AMP} + \Delta G^*_\text{MgATP} \]

If MgAP$_6$A is a true transition-state analogue:

\[ \Delta G^*_{\text{MgAP}_6\text{A}} = \Delta G^*_\text{AMP} + \Delta G^*_\text{MgATP} + \Delta G^*_{\text{TS}} \]

Figure 11. Analysis of the Possible Energetics of AK-MgAP$_6$A Association.
IV and V). Knowledge of these constants will facilitate subsequent $^2$H NMR analysis. In addition, comparison of dissociation constants and the chemical shifts of nucleotide resonances for the AK-AMPPCP and AK-ATP complexes will allow examination of the suitability of AMPPCP as an analogue of ATP. The dissociation constants for the interactions of AMPPCP and MgAMPPCP with AK were determined under conditions approximating those used in the $^2$H NMR studies (pH 7.0 and 10 °C). These conditions were not optimal for $^1$H NMR titrations. Fewer individual peaks were resolved and spectral linewidths tended to be broader than those encountered under the conditions of the previous study. In addition, at pH 7 the chemical shifts of the histidine C2-H and C4-H peaks are highly sensitive to minor perturbations in pH (Tian et al., 1988) and could not be used to monitor binding. Nevertheless, for both AMPPCP and MgAMPPCP, several pH insensitive, resolved peaks were observed and followed throughout the course of the titrations which covered a range of AMPPCP and MgAMPPCP concentrations of 0 to 8 mM. Data appeared to be hyperbolic and were found to give satisfactory fits to a 1:1 binding equation (see Chapter II). For AMPPCP, data from the five peaks which were followed yielded an average dissociation constant of 0.22 ± 0.1 mM. In the case of MgAMPPCP (where the Mg$^+2$ to AMPPCP ratio was maintained at 4 during the titration) four peaks were followed which yielded an average dissociation constant of 0.23 ± 0.13 mM. This value is in agreement with the competitive (with MgATP) kinetic inhibition constant for MgAMPPCP reported by Fry et al. (1987) of 0.19 mM.

Summary. In addition to establishing a number of dissociation constants for MgATP and its derivatives the use of proton NMR in binding studies fortuitously shed light upon two important questions with respect the mechanisms of AK.
CHAPTER IV
DEUTERIUM NMR RELAXATION STUDIES OF DEUTERATED AMPPCP FREE IN SOLUTION AND BOUND TO AK

Perspective. Having determined the binding constants for the association of AK with MgATP and its various derivatives we now turn our attention to the determination of the local motional properties of ATP and MgATP. This was accomplished by examining the motion of the adenine ring and phosphonate chain of AMPPCP, an ATP analogue in which the oxygen atom normally bridging the $\beta-\gamma$ phosphates has been replaced by a methylene group (see Figure 1). As an extended multisegmental molecule it is quite possible that in addition to the overall molecular tumbling, the different parts of MgAMPPCP or MgATP (adenine ring, phosphate chain, ribose link) may also be involved in their own distinct local motions.

The measurement of NMR relaxation rates is a powerful tool for studying the motional dynamics of biomolecules. Information on dynamics can be gleaned from the transverse and longitudinal relaxation rates ($1/T_2$ and $1/T_1$, respectively) and from NOE measurements. NOE are not relevant in our studies. As described in Chapter II, $1/T_2$ are experimentally determined by measurement of the NMR peak linewidth while $1/T_1$ are measured using a pulse sequence. "NMR relaxation" refers to the return to an equilibrium population of nuclear spins states following perturbation of this population by application of a radiofrequency pulse. A description of this phenomena and an explanation of the differences between transverse and longitudinal relaxation can be found in nearly any elementary discussion of NMR theory.
The basic format we used in moving from experimental measurement towards a definition of molecular motion is described below.

1. Measure the apparent relaxation rates of the nuclei of interest under a particular set of experimental conditions. Sometimes the relaxation rate of the species of interest can be measured directly. However, as shall be described, we were unable to directly observe pure complexes of AMPPCP with Mg$^{+2}$ and/or AK. Therefore, relaxation rates were measured as AMPPCP was titrated with Mg$^{+2}$ or as AK was titrated with AMPPCP.

2. From the measurements made in (1) extract the intrinsic relaxation rates for the pure species of interest. In our studies this involved determination of the true relaxation rates for AMPPCP complexed with Mg$^{+2}$ and AK from titration curves. This analysis can be complicated by various factors, most notably the effect of chemical and physical exchange processes which are not fast on the NMR time scale.

3. Quantitatively determine the relative importance of the various relaxation mechanisms which are effecting NMR relaxation on the species of interest. For deuterium NMR this problem is trivial as the nuclei relax by the quadrupolar relaxation pathway which is so efficient that other mechanisms cannot compete. The physical description of the relaxation process is expressed (for quadrupole-induced relaxation):

$$\frac{1}{T_2} = \frac{1}{40} \pi^2 (e^2 q Q/h)^2 (1+\eta^2) [9\varphi(\omega = 0) + 15\varphi(\omega) + 6\varphi(2\omega)]$$ (14)

$$\frac{1}{T_1} = \frac{3}{20} \pi^2 (e^2 q Q/h)^2 (1+\eta^2) [\varphi(\omega) + 4\varphi(2\omega)]$$ (15)
The specific meaning of the various terms of this equation will be discussed later. For these equations, as for all other relaxation mechanisms, these expressions can be thought of as having two components. The first portion of the equations (bold type) is the "coupling constant portion". When an excited nucleus relaxes it must lose energy through some sort of pathway. Quadrupolar relaxation occurs when the nuclei (a quadrupole) couples with its electronic environment. When this coupling occurs relaxation is very efficient and the coupling constant describes this degree of efficiency. The second portion of these equations is what one might refer to as the "motion function" and is actually a function of the spectral density functions \( \phi(\omega) \). Quadrupolar coupling leading to relaxation is mediated by motion. These spectral density functions are obtained by taking the Fourier transform of the correlation function. This latter function is a description of the actual types of motion involving the nuclei of interest. The spectral density functions are therefore unique to a particular motional environment and contain correlation times for each particular type of motion present. For example, the simplest spectral density function is for isotropic reorientation where the only motion involving the nucleus is the completely random tumbling of the molecule containing the nucleus. In this case:

\[
\phi(\omega) = \frac{2\tau_c}{1 + \omega^2 \tau_c^2} \quad (16)
\]

where \( \omega \) is the angular larmor frequency and \( \tau_c \) is the correlation time for the random tumbling.

It is probably worthwhile to attempt a definition of the "correlation time". For deuterium it is the rotational motion of C-D bond vectors which mediates relaxation. If an ensemble of randomly reorienting C-D bond vectors exists at time \( T \), at a very
short time after $T, T + \Delta t$, the orientations of the C-D vectors will have changed, but it is still a simple matter to identify which of the new set of vectors correspond to which of the original ensemble- correlation has not been lost. However, at a much longer time such correlation with the original set is impossible: correlation has been lost. The decay half-time for this loss of correlation is the correlation time. Moreover, the inverse of the correlation time is a rate constant which, as will be seen, can be approximately used to determine the rate of the particular motion involved in the reorientation of a single C-D vector.

While the above definition is by no means entirely satisfactory, it probably does capture the "essence" of the correlation time. From this definition two facts can be inferred. First, faster (higher frequency) motions will reduce the correlation time. Secondly, large amplitude motions, such as full rotations, will also reduce the correlation time.

This chapter is devoted to a description of how the above steps 1-3 were accomplished for deuterated AMPPCP in its various complexes with $\text{Mg}^{2+}$ and AK while the next chapter is devoted to the fourth step: the actual motional analysis.

Suitability of Using AMPPCP as a Model for ATP. AMPPCP was chosen for use as a model for ATP for two reasons. First, it can be used in studies with AK in the presence and absence of $\text{Mg}^{2+}$ and/or AMP without being hydrolyzed. Secondly, deuterated AMPPCP provides a relatively facile route to the acquisition of information regarding the dynamics of both the adenine ring and the $\beta-\gamma$ phosphate regions through determination of $^2\text{H}$ NMR relaxation rates. Unlike $^{31}\text{P}$ ($I = 1/2$), the relaxation of deuterium, as a quadrupole ($I=1$), is dominated by a single relaxation mechanism (Boere and Kidd, 1982). The other potentially useful quadrupolar nuclei, $^{25}\text{Mg}$ ($I = 5/2$), $^{17}\text{O}$ ($I = 5/2$), and $^{14}\text{N}$ ($I = 1$) all have large quadrupole coupling constants.
resulting in such broad linewidths that observation of spectra becomes difficult under dilute and slow motion conditions; they also have coupling constants which are much more environment-dependent than those of $^2$H. In addition, the $I > 1$ nuclear spin of $^{25}$Mg and $^{17}$O dictates that relaxation behaviour becomes multieponential when the condition of the extreme narrowing limit (fast motion: $\omega^2\tau_c^2 << 1$, where $\omega$ is the angular larmor frequency and $\tau_c$ is the effective correlation time) is not met (Forsen et. al., 1987). Despite the above difficulties it should be noted that significant work on the motional dynamics of protein bound ATP has been reported for both $^{31}$P (Brauer and Sykes, 1981a, b) and $^{17}$O NMR (Wisner et. al., 1985).

While there are minor structural differences between ATP and AMPPCP, these differences do not appear to be significant for the purposes of this study. However, the possible differences do merit scrutiny. With respect to studies of substrate dynamics Carerel et. al. (1975) have rightly observed that "caution must be used before data obtained with analogues can be extrapolated to the true substrate". The $pK_{a,4}$ for ATP is approximately 7 (Ramirez and Marecek, 1980) while that for AMPPCP is ~8 (Vogel and Bridger, 1982; Schilsaliefd et. al., 1982) resulting in a net charge of ~ -3.5 and ~ -3 at pH 7 for ATP and AMPPCP, respectively. Complexation with Mg$^{2+}$ lowers the $pK_{a,4}$ for both compounds such that at pH 7 both possess a net charge of -2 (see above references). AMPPCP and ATP exhibit very similar affinities for Mg$^{2+}$ (Yount et. al., 1971; Dawson et. al., 1986) and are known to weakly complex a second Mg$^{2+}$ (Vogel and Bridger, 1982; Bishop et. al., 1981). Despite differences in the P-C-P and P-O-P bond angles, 117° and 130° respectively, the $P\beta-P\gamma$ distance for the two compounds remains nearly identical due to compensating bond length differences (Yount, 1975; De La Matter et. al., 1973). Most importantly, we observed that AMPPCP and MgAMPPCP bind to AK at pH 7 at 10 °C with fairly similar affinities ($K_d$
= 0.22±0.1 and 0.23±0.13 mM, respectively) as those of ATP and MgATP at 30 °C and pH 8 (K_d = 0.044±0.04 and 0.17±0.05 mM, respectively—see Table 1). The similarity in structures, ionization behaviour, affinity for Mg^{2+}, and binding affinities for AK suggests that AMPPCP is indeed a very suitable ATP model for the present dynamic and energetic study.

Effect of Mg^{2+} on the Relaxation Rates of AMPPCP. ²H NMR relaxation times were determined for [8-²H]AMPPCP and AMPPCD₂P as they were titrated with Mg^{2+} at both pH 7.0 and pH 10.0. Titrations were performed at concentrations of AMPPCP where self-association should be negligible (Tribolet and Sigel, 1988; Lam and Kotowycz, 1977). The pH 10 experiments were terminated at [Mg] : [AMPPCP] = 2.0 at which point Mg(OH)₂ began to form and precipitate. Although T₁ = T₂ is justified in nonenzymatic studies (due to α²τ₀² being << 1), T₁ were measured at each point and were, in the case of non-enzymic studies, preferred to T₂ determined from linewidths. Linewidths were narrow enough (10-25 Hz) to be significantly and spuriously influenced by field inhomogeneity, particularly since spectra were acquired while running unlocked. The data obtained are presented in Figure 12 in the form of relaxation rates. Chemical shift changes during these titrations were negligible. The quality of data for [8-²H]AMPPCP is somewhat lower than that for AMPPCD₂P because of the relative difficulty of obtaining sufficient signal to noise due to broader linewidths and only one ²H being present in the ring deuterated compound. For this reason some of the data for [8-²H]AMPPCP in Figure 12B were supplemented with T₂ obtained from linewidths (which had been corrected for field inhomogeneity). The data in Figure 12 were then used to calculate the intrinsic relaxation rates (1/T₁) and the effective correlation times (τ₀) of [8-²H]AMPPCP and AMPPCD₂P and the corresponding Mg^{2+} complexes (see Appendix A). The results
Figure 12. Variation of the $^2$H NMR longitudinal relaxation rate at 23 ± 1 °C as a function of the ratio $[\text{Mg}^{2+}]$/[AMPPCP]. Titrations were performed by adding Mg(NO$_3$)$_2$ to 6 mM solutions of deuterated AMPPCP at pH 10.0 (A) and pH 7.0 (B). The pH 10.0 solution was unbuffered while the pH 7.0 solution contained 25 mM K$^+$Hepes.
listed in Table 3 show that at both pH 7 and pH 10 the phosphonate chain of free AMPPCP possesses rotational freedom relative to the adenine ring, and that binding of Mg$^{2+}$ immobilizes the phosphonate chain. Detailed procedures, assumptions, justifications, and controls for the derivation of the data in Table 3 are described below.

The exchange rate for Mg$^{2+}$ on and off of ATP$^{-4}$ is 1000-2000 s$^{-1}$ (Vasavada et al., 1984) and is probably similar for AMPPCP$^{-4}$ present at pH 10 as the association constants of Mg$^{2+}$ for ATP$^{-4}$ and AMPPCP$^{-4}$ are similar (Yount et al., 1971; Dawson et al., 1986). This rate is likely to be even higher at pH 7.0 where AMPPCP possesses a charge of -3. The exchange rate greatly exceeds differences in resonance frequency and relaxation rate differences between chelated and unchelated [$^8$-H]AMPPCP and AMPPCD$_2$P. The observed relaxation rates are therefore "fast-exchange" rates which are the population weighted average of chelated and unchelated AMPPCP (McLaughlin and Leigh, 1973):

$$\frac{1}{T_{obs}} = \frac{f_A}{T_A} + \frac{f_{AM}}{T_{AM}} \quad (17)$$

where $f_A$ and $f_{AM}$ are fractions of observed species A in environments A and AM, respectively, and T are corresponding intrinsic relaxation times for those species ($T_1$ or $T_2$). A and AM are the free and 1:1 complexed forms of the nucleotide (additional terms must be added to this equation if higher ordered complexes are present).

The change in observed relaxation rates for AMPPCD$_2$P are nearly linear over a range of [Mg$^{2+}$]/[AMPPCP] of 0 to 1. This is consistent with the initial, tight, association of a Mg$^{2+}$ ion with AMPPCP. The fact that the observed relaxation rates continue to change above Mg:AMPPCP = 1 suggests that a second Mg$^{2+}$ is binding to
Table 3. Intrinsic Relaxation Rates and Effective Correlation Times for Free AMPPCP at 23 °C.

<table>
<thead>
<tr>
<th>pH</th>
<th>Species</th>
<th>(1/T_1) (s(^{-1}))</th>
<th>(\tau_c) (nsec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>([8-^2\text{H}]\text{AMPPCP})</td>
<td>65 ± 6</td>
<td>0.14 ± 0.015</td>
</tr>
<tr>
<td>10</td>
<td>Mg[8-2H]AMPPCP</td>
<td>75 ± 7.5</td>
<td>0.16 ± 0.02</td>
</tr>
<tr>
<td>10</td>
<td>AMPPCD(_2)P</td>
<td>30 ± 2</td>
<td>0.072 ± 0.01</td>
</tr>
<tr>
<td>10</td>
<td>MgAMPPCD(_2)P</td>
<td>63 ± 5</td>
<td>0.15 ± 0.02</td>
</tr>
<tr>
<td>7</td>
<td>([8-^2\text{H}]\text{AMPPCP})</td>
<td>67 ± 5</td>
<td>0.14 ± 0.01</td>
</tr>
<tr>
<td>7</td>
<td>Mg[8-2H]AMPPCP</td>
<td>76 ± 10</td>
<td>0.165±0.035</td>
</tr>
<tr>
<td>7</td>
<td>AMPPCD(_2)P</td>
<td>36 ± 4</td>
<td>0.084 ± 0.01</td>
</tr>
<tr>
<td>7</td>
<td>MgAMPPCD(_2)P</td>
<td>70 ± 5</td>
<td>0.16 ± 0.02</td>
</tr>
</tbody>
</table>
AMPPCP. The curvature of the plots (most evident in the pH 7.0 case) indicates that
the association of the second Mg$^{2+}$ is considerably weaker than that of the first. This is
consistent with the 2:1 stoichiometry (light/weak) previously observed qualitatively
for AMPPCP (Vogel and Bridger, 1982) and quantitatively for ATP (Bishop et al.
1981; Mohan and Richnitz, 1974; Frey et al. 1972). The existence of this complex
probably explains why many MgATP utilizing enzymes are inhibited at [Mg]/[ATP]
greater than 1 (Paićzewski et al., 1988; Chrisman et al. 1984; Thomely and
Willison, 1974; Rhoads and Lowenstein, 1968; Ito et al., 1980; Noda, 1958).

For [8-2H]AMPPCP intrinsic relaxation rates for free and 1:1 complexes (listed
in Table 3) were estimated by visual linear fit of the initial portion of the titration
data and extrapolated to [Mg$^{2+}$]/[AMPPCP] = 1. The data for AMPPCD$_2$P were fit to a
2:1 sequential binding model (see Appendix A) containing variable parameters $K_{d,1}$
and $K_{d,2}$ (dissociation constants for high and low affinity Mg$^{2+}$, respectively) and
intrinsic relaxation rates for free, 1:1, and 2:1 complexes. Attempts to fit the data
allowing all five parameters to vary gave nonsensical parameters. Therefore, $K_{d,1}$
was held invariant at its independently determined values, 0.1 mM at pH 7, and 0.025
mM at pH 10 (Yount et al., 1971) and the other parameters were allowed to vary.
The intrinsic relaxation rates thus determined for free and the 1:1 complex are
reported in Table 3. Values of $K_{d,2}$ and the relaxation rate of the 2:1 complex thus
calculated possess very large uncertainty and thus are not listed.

For deuterium, under effectively isotropic conditions equations 14, 15, and 16
can be combined to yield (Allegrini et al., 1985; Abragam, 1961):

\[
\frac{1}{T_2} = \frac{2\tau_c}{8\tau_c} \left( \frac{2\pi^2/20}{e^2qQ/h} \frac{(1 + \eta^2)}{1 + \omega^2\tau_c^2} + \frac{2\pi^2/20}{e^2qQ/h} \right) \tag{18}
\]
where $e^2Q/h$ is the quadrupolar coupling constant, $\omega$ is the angular Larmor frequency, $\tau_c$ is the effective rotational correlation time and $\eta$ is the asymmetry parameter. The C-D bond is the dominant factor in determining the electric field gradient and thus the quadrupolar coupling constant. Due to the non-spherical symmetry of this bond, the quadrupolar coupling constants for deuterium are sufficiently high to dictate that quadrupolar relaxation is effectively the only relaxation mechanism. Quadrupolar coupling constants for deuterated hydrocarbons are fairly invariant and change significantly only with major covalent changes in the vicinity of the C-D bond (Mantsch et al., 1977; Brevard and Kintzinger, 1978). Thus, in the metal ion titrations of deuterated AMPPCP as well as in titrations of AK by AMPPCP, quadrupolar coupling constants can be assumed to be invariant during the course of the titrations.

The asymmetry parameter, $\eta$, is equal to zero for axially symmetric electric field gradients and has a maximal value of 1 for maximal deviation from axial symmetry. As the C-D bond is axially symmetric we assume $\eta = 0$. This assumption is supported by tabulations of experimentally determined values of $\eta$ for deuterated hydrocarbons (Mantsch et al., 1977).
Since neither the asymmetry parameter nor the quadrupolar coupling constant varies significantly in our experiments, variations in intrinsic relaxation rates are due to differences in the rotational correlation times of the different species.

In order to calculate correlation times it was necessary to obtain values of the quadrupolar coupling constants. A value of 178 kHz was utilized for $[8^{-2}H]$ AMPPCP which is the value determined for $[8^{-2}H]$AMP (Tsang et. al., 1987) by solid-state NMR methods. We attempted to determine the constant for AMPPCD$_2$P by taking the solid state NMR spectrum of AMPPCD$_2$P. The $^2$H NMR powder pattern (Figure 13) is not the "owl's face" pattern expected for a truly immobile solid sample but suggest that even in the solid state, a significant portion of the AMPPCP molecules in the sample possess mobility in the phosphonate chain resulting in a narrowing some of the quadrupolar splitting. Nevertheless, the maximal splitting seen in the powder pattern (Figure 13) was 165 kHz which is close to that determined (Derbyshire et. al. 1969) for chemically analogous $[2H_2]$malonic acid (168 kHz). We therefore employed this latter value in all calculations.

The above approach was justified by frequency variation experiments done under non-extreme narrowing conditions (see next sections). We therefore calculated effective correlation times using Equation 18 and list their values in Table 3.

The correlation times determined reflect the rotational motion of the C-D bond axis (Huntress, 1970). They should be considered "effective" correlation times in that these molecules are not undergoing solely isotropic reorientation. This matter will be discussed at length in the next chapter.

Because correlation times are usually viscosity dependent regardless of the type of associated motion (London, 1980; Boere and Kidd, 1982), we examined the possibility that our results could have been complicated by viscosity changes.
Figure 13. 46.1 MHz solid state deuterium NMR spectrum of powdered AMPPCD$_2$P obtained at ambient (23 ± 2°C) temperature using a Bruker MSL-300 and a horizontal "high power" probe. Spectrum was produced after 160,000 scans and after application of 1 kHz of exponential linebroadening to the FID (to average out some of the noise). The spectral resolution is 1120 Hz/point. A quadrupolar echo pulse sequence was utilized: $180^\circ_x$-D1-$90^\circ_x$-D2-$90^\circ_y$-D3-Acquire, where D are delays (4.6, 20, and 25 µsec, respectively).
occurring during the course of the titrations. Viscosity studies were undertaken at both pH 10 and pH 7 using solutions identical to those used in the NMR experiments, except that ATP was substituted for AMPPCP (for economic reasons). It was found that viscosity changes during the titrations were < 3%, which lies within the experimental uncertainty of our measurements. Thus, the effective correlation times can be used for direct comparison of the dynamics of AMPPCP and MgAMPPCP in solution.

Determination of Intrinsic Linewidths and Correlation Times of Deuterated AMPPCP and Bound to AK. We wished to determine the intrinsic linewidths of the various AK-deuterated nucleotide complexes (recall, $\Delta \nu_{1/2} = 1/T_2$). The fully bound species could not be directly observed because at 1.25 mM AK concentrations of AMPPCP would have to be < 0.5 mM in order for them to be > 90% bound to AK. The observation of broad lines from such dilute species by $^2$H NMR is difficult, at best, due to sensitivity and instrumental limitations. Thus, AK was titrated with the various deuterated species in the hope of obtaining titration curves which could be fit to yield the intrinsic linewidths of the fully bound nucleotides.

AK was titrated with AMPPCD$_2$P, MgAMPPCD$_2$P, and [8-$^2$H]AMPPCP, using nucleotide concentrations of 0.5 mM to 15 mM. A single titration generally took 20-25 hours to complete. During this time, no loss of AK activity was ever observed and no hydrolysis of nucleotides was ever found to have occurred as judged by silica gel TLC. At least two titrations were performed for each deuterated species to insure the reproducibility of the data.

In all cases the deuterated nucleotides gave rise to a single, usually Lorentzian peak. Examples of spectra are shown in Figure 14. Spectra were fit to a Lorentzian function (see Chapter II) from which linewidths at half maximal height were determined. Instances in which lineshapes were not Lorentzian were found to be due to
acoustic ringing or insufficient signal to noise and were not reproducible. Line broadening due to $^{2}\text{H}^{31}\text{P}$ or $^{2}\text{H}^{14}\text{N}$ two-bond coupling was determined to be negligible based on the $^{1}\text{H}-X$ coupling constants observed for the corresponding protonated compounds. The phosphonate methylene protons of AMPPCP couple with the neighboring phosphorus nuclei with a coupling constant of 21 Hz while no coupling is observed between adenine nitrogens and the A-8 protons. Since $^{2}\text{H}-X$ coupling constants are approximately 1/6 of the corresponding $^{1}\text{H}-X$ constants (Mantsch et. al., 1977) the effect of scalar coupling on linewidth is negligible.

Data for AMPPCD$_2$P, [8-$^{2}\text{H}$]AMPPCP, and MgAMPPCP$_2$P are presented in Figure 15 in a linearized form of a 1:1 binding model (see Appendix B). The fits shown in the figure were performed while holding $K_d$ at fixed values (0.22 mM for AMPPCP and 0.23 mM for MgAMPPCP) determined by $^{1}\text{H}$ NMR as described in the previous chapter. Fits where $K_d$ were also allowed to vary yielded dissociation constants which were within experimental errors of those determined independently by $^{1}\text{H}$ NMR. The intrinsic linewidths of the fully bound AMPPCP obtained from the fits in Figure 15 are listed in Table 4. These data were then used to calculate the effective correlation times ($\tau_c$) of the corresponding complexes as described for free AMPPCP using Equations 12 and 19. The results are also listed in Table 4. It should be noted that the above results were obtained from linewidth measurements ($T_2$) since $T_1$ was found to be of very limited value in the AK-deuterated nucleotide studies. This is because the steeply falling $T_1$ vs. $\tau_c$ curve changes directions and begins to slowly rise beyond $\omega^2\tau_c^2 = 1$ (beyond the extreme narrowing limit) (Schramm and Oldfield, 1983). In our system the correlation times of free and bound nucleotides lie just before and after this change of direction and are therefore nearly equivalent (within a factor of 2). Beyond $\omega^2\tau_c^2 = 1$, $T_1$ also becomes rather insensitive to large variations.
Figure 14. Examples of 46.1 MHz $^2$H NMR spectra of deuterated AMPPCP at 10 °C and pH 7.0. Spectrum A is [8-$^2$H]AMPPCP 40% bound to AK and spectrum B is MgAMPPCD$_2$P 45% bound to AK, with 10 and 6 Hz of additional linebroadening for A and B, respectively. The narrow upfield resonance of spectrum A is the natural abundance deuterium signal of a small amount of PEG present in the enzyme sample while the narrow downfield component of spectrum B is the residual HDO peak. The HDO peak in spectrum A and the PEG peak of spectrum B were artifically removed from the spectra for cosmetic reasons. Additional information on the solution conditions are found in Chapter II.
Figure 15. Variation of the 46.7 MHz ²H NMR linewidths (Δν₁/₂) of deuterated AMPPCP and MgAMPPCP (4:1 molar ratio of Mg²⁺ to AMPPCP) during titrations of AK at pH 7.0 and 10 °C by the nucleotide. Linewidths have been corrected for the linebroadening used in processing FIDs and have not been corrected for field inhomogeneity, which was < 10 Hz. Data fitting is described in the text and Appendix B.
Table 4. Intrinsic Linewidths and Effective Correlation Times for AK • AMPPCP Complexes at 10 °C and pH 7.0.

<table>
<thead>
<tr>
<th>Complex</th>
<th>Intrinsic Linewidth</th>
<th>$\tau_c$ (ns)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AK • AMPPCD$_2$P</td>
<td>371 ± 5</td>
<td>6.8 ± 1.2</td>
</tr>
<tr>
<td>AK • MgAMPPCD$_2$P</td>
<td>645 ± 55</td>
<td>15 ± 2</td>
</tr>
<tr>
<td>AK • [8.2H]AMPPCP</td>
<td>1280 ± 120</td>
<td>28 ± 3</td>
</tr>
<tr>
<td>AK • Mg[8.2H]AMPPCP</td>
<td>~1280$^a$</td>
<td>~28</td>
</tr>
</tbody>
</table>

$^a$This data was not obtained from a full titration experiment. Instead, we found that addition of 1 equivalent of Mg$^{2+}$ to AK-[8.2H]AMPPCP at fraction bound = 0.45 caused no detectable effect on the linewidth. On the basis of this observation and the conclusion that the adenine ring of bound AMPPCP is fully rigidized by AK, AK-Mg[8.2H]AMPPCP should have the same intrinsic linewidth as AK-[8.2H]AMPPCP.
In the correlation time. This is not apparent from most plots describing this effect in the literature because $T_1$ vs $\tau_c$ plots are usually drawn with $\tau_c$ scaled in orders of magnitude (see, for example Schramm and Oldfield, 1983). For example, in the extreme narrowing region where the correlation time dependence is high, $T_1$ changes from ca. 20 msec to 10 msec as $\tau_c$ changes from 0.1 to 0.2 nsec; outside of extreme narrowing $T_1$ only changes from 3 to 3.2 msec as $\tau_c$ varies from ca. 1 to 5 nsec.

A priori, the validity of the above approach taken to determine intrinsic linewidths (and thus the transverse relaxation rates $1/T_2$) would seem to be dependant upon two assumptions. First, viscosity changes during the titration must be negligible or the linewidths will change, in part, due to changes in viscosity, not solely due to changes in the fraction bound. Secondly, fast exchange NMR conditions, where the observed peaks represents the population-weighted average between free and bound species present (see Equation 17), is required. The validity of both assumptions with respect to our system were investigated and the necessity of the second assumption was examined as described in the following sections.

**Control: Effect of Viscosity Changes.** The $^2$H NMR titrations described above typically involved the titration of a 1-2 mM solution of AK with 75 mM nucleotide over a concentration range of 0 to 20 mM. In order to estimate the amount of viscosity change which could occur during such titrations solutions of 1-2 mM chymotrypsin (27 kDa) and lysozyme (14 kDa) were titrated with 75 mM ATP from 0 to 20 mM and the solution viscosities were measured (at pH 7 and 22 °C). These titrations allowed estimation of the change in viscosity of AK under conditions similar to those in our NMR titrations as lysozyme is somewhat smaller than AK (22 kDa) and chymotrypsin is somewhat larger. In both cases the total change in relative viscosity was < 5% during the titrations. Assuming that similar results would be obtained at 10
65

°C for AK this demonstrated that the viscosity changes occurring during the 2H NMR
titrations were negligible.

Validity of the Fast Exchange Assumption: Analysis of the System.
Chemical and physical exchange processes can affect NMR spectra in different ways. In
the AK-nucleotide system the relevant exchange rate is that for the association and
dissociation of a single nucleotide on and off of the enzyme:

\[
\frac{1}{k_{ex}} = \frac{1}{k_{on} \cdot AK_{total}} + \frac{1}{k_{off}}
\]  

(20)

This exchange rate could manifest itself in two ways with regard to our spectra. The
first relates to the difference in resonance frequency between fully bound and free
nucleotides and the exchange rate. If \( k_{ex} \gg (v_{\text{bound}} - v_{\text{free}}) \) (where \( v \) are, in this
case, resonance frequencies) then fast exchange with respect to chemical shifts
pertains and the peaks of free and bound species will coalesce in terms of frequency to
a population weighted averaged frequency (Martin et. al., 1980).

For our system shifts in resonant frequency can be estimated from the 1H NMR
titrations since 2H NMR chemical shifts are equivalent to 1H shifts for otherwise
identical deuterated and protonated compounds (Mantsch et. al., 1977). In our 1H
NMR titrations it was observed that the adenine C-8 proton of AMPPCP and MgAMPPCP
shifts less than 0.2 ppm upon binding to AK while the methylene protons of AMPPCP
and MgAMPPCP shift less than 0.1 ppm and 0.6 ppm, respectively. At 46.1 MHz (the
2H NMR frequency used) this indicates that the maximal difference in frequency for
the deuterium in free and bound nucleotides is only 28 Hz. As will be apparent in the
following sections, the exchange rates for AMPPCP and MgAMPPCP on and off of AK
certainly exceed this value by well over an order of magnitude and the condition of fast exchange with respect to chemical shift differences is easily met.

For our system, the other possible manifestation of exchange relates to the differences in the relaxation rates of free and bound nucleotides. If:

\[(1/T_2, \text{bound} - 1/T_2, \text{free}) \ll k_{\text{ex}} \quad \text{(fast exchange)} \quad (21)\]

then (when fast exchange with respect to chemical shifts also holds) a single Lorentzian peak will be observed which possesses a linewidth which is the population weighted average between bound and free linewidths. On the other hand, if:

\[(1/T_2, \text{bound} - 1/T_2, \text{free}) \gg k_{\text{ex}} \quad \text{(slow exchange)} \quad (22)\]

then the observed spectra will appear as the non-Lorentzian superposition of a broad peak from the bound nucleotide and a narrow peak due to the free nucleotide. Exchange rates which fall between these two extremes fall into the "intermediate exchange" region. Similar arguments apply for $T_1$ although in our system $T_1$ changes very little upon binding of the nucleotide to AK and fast exchange in terms of $T_1$ can easily be met (see later section).

Validity of the Fast Exchange Assumption: Theoretical Analysis. The subject of intermediate exchange with respect to differences in relaxation rates has received scant attention. The well-known Swift-Connick equation (Swift and Connick, 1962; Luz and Meiboom, 1964; Dwek, 1973) has received much use, but is limited to situations where the bound species is quite dilute relative to the free species and is of limited utility. The multi-exponential behavior of $T_1$ and $T_2$ relaxation for
Intermediate exchange has also been examined (Schotland and Leigh, 1983; Lambert and Keepers, 1980; Jen, 1978). However, no description of the sort of lineshapes to be expected for species undergoing this type of intermediate exchange has appeared. We have therefore simulated spectra (see Methods) for the case where a bound species with linewidth of 1000 Hz is in exchange with free species with a bound linewidth of 20 Hz. Under this condition \( \frac{1}{T_2, \text{bound}} - \frac{1}{T_2, \text{free}} = 3140 \text{ Hz} \). It was assumed that there is no difference in the chemical shifts of bound and free species. The results of these simulations are shown in Figure 16. The 75% bound case permits a good view of what happens as the exchange rate is varied from slow to fast conditions. At very slow exchange, the broad component and the sharp (free) component are both observed. As the exchange rate is increased the broad component becomes narrower and the narrow component becomes broader. Even at the fairly slow rate of 500 Hz, the two components have become similar enough such that the non-computational eye may not be able to discern that the peak is non-Lorentzian; indeed, at the signal-to-noise ratio at which quadrupolar NMR spectra are typically obtained it may be impossible to discern by any method such deviations from Lorentzianity. This problem becomes even more pronounced at lower fractions bound where it may be difficult to observe the broad component, even in a very slow exchange situation. Therefore, the analysis of a single lineshape may be of little help in establishing rate of exchange processes when the exchange effect is due to the relationship between the exchange rate and differences in relaxation rates.

Another approach to obtaining information regarding exchange rates is to independently vary one of the parameters of the general two-site equation (Sutherland, 1971) experimentally to obtain a series of spectra which are then subjected to a multivariate analysis to yield the non-variable parameters.
Figure 16. Simulated lineshapes showing the effects of exchange upon the spectra of two exchanging species having the same intrinsic chemical shifts but differing in intrinsic relaxation rates. The broad species (relative population = 0.75) possesses an intrinsic linewidth of 1000 Hz while the narrow species (relative population = 0.25) possesses an intrinsic linewidth of 20 Hz. From Equation 12 it can thus be determined that the difference in transverse relaxation rates between the two species is 3140 Hz. Additional details of the simulations are found in Chapter II. The top 5 spectra are normalized in terms of total peak area with respect to each other while the total area of the $k = 100$ and $k = 1$ spectra have total areas 1/2 and 1/4 those of the top five, respectively. In addition close inspection will reveal that we have cut the top of the sharp component of the $k = 1$ spectrum short of its maximal value.
Figure 16.
(Drakenberg et. al., 1983; Tsai et. al., 1987). The most simple independently variable parameters are the relative populations of the observed species. We did not attempt to analyze our data by this method, but herein present what might be regarded as a simplified, but very useful cousin of the above.

The apparent linewidths of the data shown in Figure 16 and additional simulations at different values of fraction bound were measured by hand and plotted as a function of the fraction bound (Figure 17). Fast exchange data \( (k_{ex} = 500,000) \) yield a straight line as expected, while the intermediate exchange data \( (k_{ex} = 6000 \) and 3000) are only slightly curved. This suggests that even if exchange is not fast, a fairly good estimate of the intrinsic linewidth of the fully bound species \( (\text{fraction bound} = 1) \) can be obtained provided a significant number of points are taken in the fraction bound > 0.5 region. On the other hand, the curves in Figure 17 also suggest that the Swift-Connick approach, which extrapolates the initial portion of the curve to fraction bound = 1, could result in significant underestimation of the intrinsic linewidth of the fully bound species if the fast exchange condition is not met rigorously.

The linearity of the data for the three deuterated AMPPCP titrations (Figure 15) suggests that exchange of the nucleotides on and off of AK is fast with respect to differences in relaxation rates of free and bound species. Furthermore, the fact that most titrations were carried out beyond fraction bound = 0.5 assures that even if the fast exchange condition is not rigorously met, the results listed in Table 4 should be very close to true values.

Validity of the Fast Exchange Assumption: Temperature Variation. Additional experiments were undertaken to verify that the deuterium NMR data is in the fast-exchange region. Spectra were taken of \([8-^2H]\text{AMPPCP}\) and MgAMPPCD_{2}P,
Figure 17. Simulated titration curves for 1:1 enzyme-ligand binding where the observed NMR linewidth ($\Delta v_{1/2}$) is complicated by chemical exchange. The apparent linewidths at half maximal intensity are plotted against the fraction of the total ligand which is in the bound environment. The parameters used in spectral simulations are identical to those described in the caption of Figure 16 except, of course, that the relative populations were varied.
20 and 40% bound to AK, respectively, at various temperatures. The simplest model for the relationship between $\tau_c$ and temperature is the Stokes-Einstein equation (Boere and Kidd, 1980):

$$\tau_c = 4\pi R^3 \eta / 3kT$$  \hspace{1cm} (23)

where $R$ is the molecular radius, $\eta$ is the absolute viscosity, $k$ is Boltzmann's constant and $T$ is the absolute temperature. While this equation is derived for rigid, isotropic spheres, it does give one a "feel" for how temperature and viscosity should affect the data. In fast exchange, increasing the temperature will result in a decrease in the intrinsic correlation times of both bound and free species and therefore results in a reduced observed linewidth. If exchange is intermediate or slow, the narrowing caused by decreasing $\tau_c$ will be offset by apparent line broadening as the rate of exchange increases as suggested by Figure 17. This may manifest itself as an actual linebroadening of the spectral peak with increasing temperature or will at least reduce the amount of line-narrowing due to reductions in correlation times. Variable temperature data taken for MgAMPPCD$_2$P and [8-2H]AMPPCP are shown in Figure 18 and in both cases displays significant line-narrowing as the temperature is raised. The amount of narrowing expected in a fast exchange situation was estimated using the intrinsic correlation times for free and bound nucleotides (Tables 3 and 4) and assuming a Stokes-Einstein viscosity and temperature dependence for these correlation times. The temperature dependence of viscosity was taken to be that displayed by water (Weast and Astle, 1980). The lines drawn in Figure 18 represent the theoretical slopes determined in this manner. The observed peaks actually narrowed more than predicted. This result suggests that fast exchange conditions are
Figure 18. Influence of temperature upon the 46.7 MHz $^2$H NMR linewidth ($\Delta v_{1/2}$) of deuterated AMPPCP partially bound to AK at pH 7.0. Experimental data points are shown. The lines depicted demonstrate the theoretical slope predicted for the data if fast exchange conditions hold for our system. See Chapter II for additional description of solution and NMR conditions.
met in our system. The additional narrowing beyond that predicted for fast exchange could be due to a number of factors, such as the non-absolute applicability of the Stokes-Einstein equation to our system. [8-2H]AMPPCP has a much larger difference between AK-bound and free relaxation rates than AMPPCD₂P and, since [8-2H]AMPPCP was found to be in fast exchange, AMPPCD₂P must therefore be in this limit as well.

Validity of the Fast Exchange Assumption: Frequency Variation. Equation 19 predicts that 1/T₂ (and thus the linewidth) of the bound species is frequency dependent, since the correlation times for the bound nucleotides are all well out of the extreme narrowing region (i.e., \( \omega^2 \tau_c^2 \geq 1 \)). The linewidths of bound AMPPCD₂P, MgAMPPCD₂P, and [8-2H]AMPPCP roughly determined (Figure 19) from titrations at 78.7 MHz yield correlation times (7.7±2.1, 14±3, and 26 ±5 nsec, respectively) which are in agreement, within experimental error, with those determined at 46.7 MHz (see Table 4). This suggests that the linewidths in Table 4 are true values, justifies the fast exchange assumption, and supports the suitability of the quadrupolar coupling constants utilized (see earlier section).

Reliability of the Approach used to Determine \( \tau_0 \) of Bound Nucleotides. Previous studies involving the binding of ²H-labelled ligands to proteins (Fenske and Cushley, 1984; Gerig and Hammond, 1984; Khaled et. al., 1982; Viswanathan and Cushley, 1981; Neurohr et. al., 1980; Zens et. al., 1976; Oster et. al., 1975; Glasel et. al., 1973; Gerig and Rimerman, 1972) have generally been based upon either direct observation of linewidths from covalently or tightly bound species, or the Swift-Connick equation with the assumption of fast-exchange conditions. In this discussion "exchange" refers to the relationship of the ligand on-off rate to differences in the 1/T₂ between free and bound species. Direct observation of
Figure 19. Variation of the 78.7 MHz $^2$H NMR linewidth of deuterated AMPPCP at pH 7.0 and 10 °C as a function of the fraction of the total AMPPCP which is bound to AK. Data were fit visually. The X axis intercept for AMPPCD$_2$P was estimated based on figure 15 and the fact that at fraction bound = 0 (free AMPPCP) extreme narrowing conditions hold and the linewidths are not frequency dependent.
the bound species cannot be achieved in many systems due to the broad signal of the bound species and/or the inability to observe an often very dilute species under the conditions required to achieve >95% bound ligand. In our experiments, observation of a defined spectral line from deuterated AMPPCP 85% bound to 1.2 mM AK became routine, but a 95% bound case was deemed hopeless. In the other approach, the Swift-Connick equation (Swift and Connick, 1962; Luz and Melboom, 1964):

\[
\frac{1}{T_2} = \frac{1}{T_{2,\text{free}}} + \frac{f_b}{(T_{2,\text{bound}} + \tau_{\text{ex}})}
\]

(24)

(where \(\tau_{\text{ex}}\) is the inverse of the exchange rate and \(f_b\) is the fraction of the observed species in a bound environment) requires that the bound species be dilute relative to the free species. The problem with use of this equation is that unless the exchange rate is accurately known from an independent source or is known to be \(>> 1/T_{2,\text{bound}}\) there is no way to extract \(T_{2,\text{bound}}\) using the equation. A plot of linewidth vs. fraction bound always give a line with a slope of \(1/\pi(T_{2,\text{bound}} + \tau_{\text{ex}})\). Also, because only a small part of the fraction bound range is utilized, extrapolation to the linewidth at fraction bound = 1 will necessarily contain a large uncertainty. When the exchange rate is not known independently it is not always easy to establish that fast exchange conditions hold. Temperature variation studies are not straightforward because (as described previously) as temperature is raised the exchange rate increases but the correlation time decreases with these two effects having opposite influences on the observed linewidth which may be difficult to weight.

Exchange Rates of AK-Nucleotide Complexes. The rates of exchange of AMPPCP and MgAMPPCP (as well as ATP/MgATP) on and off of AK have not been reported previously, though lower limits to these rates can be established by \(^{1}\text{H NMR}\).
to be > 100 sec$. We found that while individual lineshapes in deuterium NMR were not very informative in discerning the exchange situation, examination of the titration curves (Figure 15) in light of simulated curves (Figure 17) provided effective, though empirical, evidence for fast exchange in the various cases examined. This was verified by temperature and frequency variation experiments. In fact, even if non-linearity had been observed, the simulated curves suggest that an accurate determination of the bound linewidths would still have been accomplished in the cases where data points were taken in the fraction bound > 0.6 region. Exchange rates might also have been estimated from such curves. As it stands, lower limits to the exchange rate can be determined. Figure 17 suggests that "fast exchange" conditions are effectively met whenever the exchange rate is at least 2 times the difference in free and bound exchange rates. Since $(1/T_2,\text{bound} - 1/T_2,\text{free}) = 3500$ Hz for AMPPCP or MgAMPPCP can be obtained from the data in Figure 15 and Table 4, a lower limit of 7000 Hz can be established at pH 7 and 10$^\circ$C. Because MgATP and ATP have similar dissociation constants this rate probably applies to their exchange on and off of AK as well. Such limits are in the same order of magnitude as the limits from another rough estimation. A dissociation rate of 10 s$^{-1}$ has been estimated for MgAp$_5$A (Kalbitzer et al., 1982), a tight binding (but not a slow binding) inhibitor of AK. This value can be used to estimate off rates for MgAMPPCP and AMPPCP. Assuming that these compounds have about the same (diffusion controlled) on rates as MgAp$_5$A, using the inhibition constant of MgAp$_5$A, 10 nM (Van Der Lijnt et al., 1979; Tian and Tsai, unpublished results) and the relationship $K_d = k_{off}/k_{on}$ a dissociation rate of ~20,000 sec$^{-1}$ is calculated for both AMPPCP and MgAMPPCP. However, since it was kinetically observed that a substrate-induced conformational change is partially rate-
limiting (Tian et. al., 1989a), the actual exchange rates are more likely to be on the low side of the fast-exchange range; i.e., a few kHz.

Summary. In this chapter we have rigorously determined the intrinsic deuterium NMR relaxation rates for AMPPCP in its complexes with AK and/or Mg\textsuperscript{2+}. In the next chapter we use these relaxation rates to analyze the dynamics of the various complexes of AMPPCP. This will require that we move beyond the "isotropic motion" assumption used for sake of convenience in this chapter.
CHAPTER V
DETERMINATION OF THE DYNAMICS OF AMPPCP FREE IN SOLUTION AND COMPLEXED WITH Mg²⁺ AND/OR AK

Perspective. In the previous chapter we determined the ²H NMR relaxation rates for AMPPCP complexes in which AMPPCP had been labelled with deuterium upon the adenine ring and upon the phosphonate chain. To risk redundancy, the general forms of the ²H NMR relaxation equations are:

\[
\frac{1}{T_2} = \frac{1}{40} \pi^2 (\frac{e^2 Q}{h})^2 (1 + \eta^2) [9 \phi(0^\circ) + 15 \phi(\omega) + 6 \phi(2\omega)] \tag{25}
\]

\[
\frac{1}{T_1} = \left(\frac{3}{20}\right) \pi^2 (\frac{e^2 Q}{h})^2 (1 + \eta^2) [\phi(\omega) + 4 \phi(2\omega)] \tag{26}
\]

In the last chapter a thorough analysis of the actual experimental measurements and the coupling constant portions of these equations were made. As noted \(\phi(\omega)\), the “spectral density” equations are functions describing the different types of motion being undergone and includes correlation times for the various motions involved. In the last chapter we used the spectral density function for simple “isotropic” motion to calculate effective correlation times (Tables 3 and 4). “Isotropic” strictly means that the entire free AMPPCP molecule undergoes random tumbling with no internal degrees of motional freedom (no local segmental motions) or, (when bound to AK) that AMPPCP is totally immobilized by AK which is itself undergoing isotropic reorientation and therefore takes the AMPPCP “along for the ride”. If either of these
situations pertains then the "effective" correlation time is indeed the true correlation time for the one type of motion present. If, however, internal mobility is present in addition to overall tumbling, the correlation time will be complicated and reduced by this internal mobility and is therefore an effective time. When different effective correlation times are observed for different parts of the same molecule, the differences in correlation times necessarily reflect differences in internal motions since the presence of only truly isotropic motion would result in both parts of a single molecule having identical correlation times. Therefore, the reduced correlation time observed for free AMPPCD$_2$P (Table 3) relative to [8$^2$H]AMPPCP indicates that the phosphonate chain possesses more local mobility than that of the adenine ring. For AMPPCP bound to AK effective correlation times (Table 4) indicate that the phosphonate chain of AK-AMPPCP possesses more mobility than the phosphonate chain of AK-MgAMPPCP which, in turn, possesses more mobility than the adenine ring of AK-AMPPCP or AK-MgAMPPCP. In the case of free MgAMPPCP the amount of local mobility possessed by the chain and the ring, if any, is approximately equal, at least in terms of affecting relaxation.

The above results are so qualitative that they are probably little better than being merely suggestive. We would like to be able to assess the types of internal motions present as well as their rates in order to assess their enzymic significance. To do this we must move to more exact spectral density functions which include individual correlation times for both overall motions and internal motions. A number of spectral density equations for various models of motion have been reported (Heatley, 1986; London, 1980). To employ these in the calculation of correlation times (and hence, rates of motion) one must know that the motions actually present correspond to those upon which the particular spectral density function is based.
example, one common model applies to a situation where a single internal rotation is superimposed upon overall molecular tumbling (such as a methyl group rotating within a slowly tumbling protein). In addition, a "model-free" approach has been presented (Upari and Szabo, 1982a,b) which has received considerable usage in biomolecular studies. Unfortunately, this latter approach cannot be applied when internal motions fall outside of the extreme narrowing limit ($\omega^2 \tau I^2 > 1$, where $\omega$ is the angular larmor frequency and $\tau I$ is the internal correlation time) as is the case in our studies involving AK.

We therefore have a problem. AMPPCP is a large "strung out" molecule and can possess a complicated set of local internal motions. We don't know exactly what these motions are, and, even if we did, might find that the appropriate $\phi(\omega)$ is not available in the literature and is beyond our derivational capabilities. We shall therefore attempt to roughly define the motions present based on work described by others in the literature. With these rough definitions we can choose an existing spectral density equation which approximately applies to the defined motions and then calculate the correlation times for the motions from the equations and the experimentally determined relaxation rates. While this approach will only bring us to a point well short of providing a quantitative description of the motional dynamics of AMPPCP it may provide a sufficiently good qualitative estimation to permit the examination of the relationship of substrate dynamics to enzyme structure, binding energy, and catalysis. Our analysis shall commence by reexamining the effective correlation times already reported (Tables 3 and 4) to see if there might be some really useful information which can be gleaned from them.

First, however, it is extremely important to note that for all of the different spectral density functions which can be used the overall correlation time establishes a
lower limit to the timeframe of processes which will affect relaxation. Internal motions which are significantly (more than an order of magnitude) slower than overall molecular tumbling will not influence relaxation behaviour. The longest $\tau_c$ encountered in this study are 0.16 nsec (for free AMPPCP) and 29 nsec (for an AK bound species). Thus our study can yield information on motions occurring at rates of approximately $10^8$ and $10^8$ sec$^{-1}$ and faster (for free and bound species, respectively; rates are based on the inverse of the correlation times minus 1 order of magnitude). Slower motions may well be present which are not capable of effecting relaxation and are therefore "unobservable" in our experiments but which can be inferred from results found in the literature.

**Dynamics of Free AMPPCP and MgAMPPCP.** The effective correlation times for free deuterated AMPPCP and MgAMPPCP are reported in Table 3. It can be seen that the effective correlation time for the adenine ring is significantly longer than that of the phosphate chain of unchelated AMPPCP. The inclusion of Mg$^{+2}$ results in a decrease in $\tau_c$ for both the adenine ring and the phosphonate chain with the $\tau_c$ of the chain increasing to the point where it is nearly equal to that of the ring. These results suggest that the phosphonate chain possesses local mobility relative to the adenine ring in the absence of Mg$^{+2}$ and that the presence of the metal ion freezes out most or all of this local mobility.

The fact that the correlation times for the ring and the chain converge upon complexation of AMPPCP by Mg$^{+2}$ suggests that the time converged to (0.15 - 0.16 nsec) is approximately the $\tau_{overall}$ for MgAMPPCP and that the $\tau_c$ observed for the adenine ring of free AMPPCP (0.14 nsec) is approximately the overall correlation time of AMPPCP which increases slightly when Mg$^{+2}$ binds due to the increase in molecular volume. This interpretation does have independent support. The local
mobility of the adenine ring of ATP is probably limited to two significant motions. First, the adenine ring of AMP is known to flip between two main conformers: syn and anti with respect to the ribose "plane" (Saenger, 1984). ATP probably behaves in a similar manner (Millner and Anderson, 1975). Secondly, while in each conformer the ring is probably involved in low amplitude (< 45°) deviations around the two average syn and anti glycosidic angles (Millner and Anderson, 1975). The rate for flipping from one (syn or anti) conformer to the other and then back again for adenosine occurs with an inverse rate constant of 5 nsec (Hemmes et. al., 1974). This is an order of magnitude larger than the overall correlation time and should therefore not affect the relaxation rate. It is reasonable that this rate of isomerization will be even slower for ATP due to steric hindrance between the adenine ring and the phosphate chain. The low amplitude associated with the second type of internal motion suggests that it will also not affect the relaxation rate (and thus, the effective correlation time). The affect of such internal mobility on relaxation rates has been examined theoretically by London and Avitable (1978; London, 1980) who observed that when the amplitude of partial rotation is < 40° it has very little effect on relaxation rates, particularly in a case such as ours where the rate of internal motion is similar to that of the overall motion. Thus, we believe that the values of 0.14 nsec for [8-2H]AMPPCP and 0.15 nsec for Mg[8-2H]AMPPCP are, approximately, the τoverall for the free and chelated forms of AMPPCP in solution.

The above conclusion is related to a very important assumption made in our studies. We assume that total motions of the β-γ methylene C-D vectors are indicative of the motion of the entire β-γ phosphonate region of AMPPCP. If the methylene group can undergo significant internal rotation independent of the β and γ phosphates, conclusions regarding the dynamics of the β-γ region will be confused by the presence
and observation of this extra motion. Because the freezing out of the motion of the phosphonate chain by Mg$^{+2}$ was fully "reported" by the C-D vectors with no residual mobility being evidenced this assumption is upheld, at least for motions occurring with correlation times $< 0.2$ nsec.

While the local mobility observed for the phosphonate chain is apparently completely frozen out by Mg$^{+2}$, the nature and significance of the original local mobility begs examination. In a very general sense, the motional freedom of the phosphate chain is expected to be fairly high. The phosphate backbone of RNA and DNA helices is known to be rather flexible (McCain, 1987) and NADH is thought to rapidly interconvert between two "closed" forms and an "open" form requiring reorientation of the diphosphate portion (Saenger et. al. 1977). In calculations involving diphosphoric acid Ewig and Van Wazer (1988) determined that staggered and eclipsed forms of diphosphoric acid differ in energy by 2.3 kcal/mol and thus free rotation of the phosphates would be expected at room temperature. This is supported by $^{17}$O NMR results of Gerlt et. al. (1983) who observed a very broad $^{17}$O signal linewidth from the bridging oxygen, ca. 4 times relative to the signal from the nonbridging oxygens. Although differences in quadrupolar coupling constants (Kintzinger, 1983) might account for part of the difference, this strongly suggests that the phosphates on each end of the $^{17}$O bridge are undergoing faster motion (rotation around the P-O$_{bridge}$) relative to the P--O--P backbone. Thus, a certain amount of free rotation around all of the chain of bonds from the ribose C'-4 to the end of the phosphate chain is expected. This finds further literature support in $^{17}$O NMR studies of specifically labelled ATP (Wisner et. al., 1985; Haung and Tsai, 1982) in which $\alpha$-, $\beta$-, and $\gamma$-$^{17}$O labelled (free) ATP gave relative $^{17}$O NMR linewidths of 13:10:7, consistent with increased mobility (due to an increased number of effecting rotations) in going from the $\alpha$-
phosphate to the terminal phosphate (again partial contribution by differences in quadrupolar coupling constant cannot be ruled out). It was also observed (Huang and Tsai, 1982) that the addition of Mg\(^{2+}\) resulted in an approximate doubling of the linewidths for the \(\beta\)– and \(\gamma\)-labelled phosphates, consistent with the approximate doubling in the effective correlation time we observed for the phosphonate portion of AMPPCP upon chelation.

Assuming, therefore, that the motion resulting in the reduced \(\tau_c\) for free AMPPCD\(_2\)P lies in multiple full or partial rotations around bonds along the chain extending from the C'-4 ribose to the \(\beta\)–\(\gamma\) methylene carbon, the question remains as to the rates of these motions and their possible enzymological significance. Although the precise dissection of the exact motions and their rates from our data is impossible, the data can be interpreted using a highly simplified model in order to get a "ballpark" idea of the rates of rotation which could account for the results.

The model chosen was originally employed for use in studies of the local motion of extended hydrocarbon chains and is approximate and subject to assumptions (Kuo et al., 1979). Assuming that the internal mobility possessed by the \(\beta\)–\(\gamma\) methylene group is solely due to independent rotations occurring at equivalent rates around the N (i.e., 6) bonds in the chain, a spectral density function equivalent to Equation 18 pertains

\[
\tau_c = 1 / \left(\tau_0^{-1} + N(3\tau_i)^{-1}\right)
\]

From the overall correlation time (\(\tau_0\)) established for AMPPCP (0.14 nsec) and the observed correlation time of AMPPCD\(_2\)P (0.072 nsec) an internal correlation time (\(\tau_i\)) of 0.3 nsec is thus obtained. For isotropic species the rotational correlation time
can be described (Boere and Kidd, 1982) as the length of time needed for 1 rad (57°) of molecular rotation. Using this as a rough approximation, it can be determined that rotation around each of the bonds of the chain would occur at a rate of $\sim 5 \times 10^6 \text{s}^{-1}$. This suggests that the internal mobility, while certainly being more complex than that of the model, is both rapid and quite possibly significant from the standpoint of enzymatic reactions. This is further elaborated in the next chapter.

In the case of MgAMPPCP no such internal mobility is observed. The metal ion of MgATP is believed to be coordinated primarily with the $\beta$- and $\gamma$-phosphoanions with a lesser amount of interaction with the $\alpha$-phosphate (Huang and Tsai, 1982; Pecoraro et. al., 1984; Takeuchi et. al., 1988). Our results indicate that full or partial rotations which may occur around any of the chain bonds from the C'-4 to the methylene must be slow relative to overall molecular tumbling of MgAMPPCP. The results of the next section suggests that some rotational motion is indeed present, but, as predicted, is too slow to be observed for free MgAMPPCP.

The dynamics we determined for free AMPPCP and MgAMPPCP are summarized in Figure 20.

**Dynamics of AMPPCP and MgAMPPCP bound to AK.** The effective correlation times $\tau_c$ determined for the various AMPPCP complexes with AK are reported in Table 3. The $\tau_c$ for AK-AMPPCD$_2$P is significantly shorter than that of AK-[$\beta,2^H$]AMPPCP, suggesting that the phosphonate chain of the complex possesses considerable local mobility relative to adenine ring. The addition of Mg$^{2+}$ to this complex reduces this local mobility substantially while not affecting that of the adenine ring. These results are reminiscent of the situation for AMPPCP free in solution.
Rotations along terminal portion of chain occur rather freely ($> 10^6$ sec$^{-1}$).

Limited rotation is probably allowed. (See section on AK bound MgAMPPCP.)

Ring can undergo slow ($< 2 \times 10^6$ sec$^{-1}$) 180$^\circ$ flips between syn and anti positions as well as low amplitude partial rotations.

Free MgAMPPCP (anti conformer)

Overall tumbling is slightly reduced due to increased molecular volume.

Terminal portion of chain is immobilized by the metal ion.

Limited rotation is probably allowed. (See section on AK bound MgAMPPCP.)

Figure 20. Dynamics of free AMPPCP.
The adenine ring appears to be totally immobilized. Using the Stokes-Einstein equation (Equation 23) an estimated viscosity, and the volume of AK determined by Pavlov and Fedorov, (1983), the correlation time for AK at 10 °C is calculated to be 12 nsec. The $\tau_o$ of 26 nsec determined for [8-2H]AMPPCP bound to AK is considerably longer. This is actually in accord with a number of studies (Anderson et. al. 1970; Yguerabide et. al., 1970; Hunkapiller et. al., 1973; Cocco et. al., 1978) which report the experimentally determined correlation times for proteins of approximately 20 kDa to be 15-30 nsec: 2 to 3 times that predicted using the Stokes-Einstein equation. Work in Mildvan’s lab (Fry et. al., 1985; Mildvan and Fry, 1987) suggests that the adenine ring of MgATP bound to AK possesses a discrete conformation relative to the ribose, consistent with immobilization.

$^{17}$O NMR studies completed in our lab (Wisner et. al., 1985) in which $^{17}$O NMR spectra were obtained for Swift-Connick type titrations of $\alpha$-, $\beta$-, and $\gamma$-$^{17}$O labelled ATP with AK may shed some light upon the motions involved in the local mobility observed for the phosphonate chains of AK-bound AMPPCP and MgAMPPCP. The relative $^{17}$O NMR linewidths for the $\alpha$-, $\beta$-, and $\gamma$-phosphate labelled ATP partially bound to AK are 10/8.5/5.3. Thus, the terminal phosphate seems to possess more mobility than the $\beta$ phosphate which, in turn, possesses more mobility than the $\alpha$ phosphate. These results can readily be explained in terms of a simple model in which hindered or partial rotations can occur around any of the chain of bonds extending from the ribose C-4 to the terminal of the chain. The motion of the phosphoanions is influenced only by rotations along the chain up to the phosphate in question. Thus, the $\gamma$-phosphate is influenced by more of these rotations than the others and thus possesses a reduced correlation time while the $\alpha$-phosphate is influenced the least.
If the improbable, but instructive, assumption is made that free rotation can occur along any of the bonds forming the ribose C'-4 to β-γ methylene (for AMPPCP) or along bonds from the ribose to the α-phosphate (for MgAMPPCP), we can again use the approximate model (Equation 27) with $\tau_0$ set at 25 nsec and $\tau_c$ set at experimentally determined values, and calculate internal rotation rates of $8 \times 10^6$ and $4 \times 10^8$ sec$^{-1}$ for the 6 "free" bonds of bound AMPPCP and for the 3 (or 4, depending on the structure of the metal ion complex.) "free" bonds of bound MgAMPPCP, respectively. While the actual motions involved are probably a number of hindered, partial rotations, these calculations suggest that the local mobility of the β-γ region of bound AMPPCP, though considerably reduced relative to that of free AMPPCP, is substantial from an enzymic standpoint. The β-γ region of the phosphonate chain of AK-bound MgAMPPCP also seems to possess a certain amount of local mobility, but much less than that of AK-AMPPCP, probably possessing fewer modes of motional freedom and decreased rates for these modes. This local mobility was not observed in our study of free MgAMPPCP because it apparently occurs much slower than overall MgAMPPCP tumbling.

The dynamics of AMPPCP and MgAMPPCP bound to the MgATP site of AK are summarized in Figure 21.

**Summary.** Based on the above rather oversimplified analysis a general description of the dynamics of AK-AMPPCP binding can be outlined for motions occurring on the nsec or faster scale as depicted in Figure 20 and 21. The adenine ring of free AMPPCP is essentially fixed in a particular (usually anti) conformation while the phosphonate chain is flexible and undergoes fast, large amplitude motions unless Mg$^{2+}$ is present in which case these local motions are drastically reduced. Upon binding to the MgAMPPCP site of AK, the adenine ring is anchored to the AK molecule.
AMPPCP Bound To AK

Rotations around bonds all along the phosphate chain are permitted but are much slower than for free AMPPCP due to "sticky" active site.

MgAMPPCP Bound To AK

Mg\(^{2+}\) immobilizes terminal of the phosphonate chain.

Figure 21. Dynamics of AMPPCP Bound to AK.
and cannot "thrash about" and can probably no longer undergo slow syn-anti isomerization. The local mobility of the phosphonate chain is substantially reduced by AK relative to free AMPPCP, but is still considerable in terms of both rate and amplitude. The presence of Mg$^{+2}$ in the AK-AMPPCP complex reduces the local mobility of the β-γ region to a level which is probably approximately that of free MgAMPPCP but which is still of fairly large amplitude and rate.

In our studies the "significance", or lack thereof, of a particular motion is ultimately judged by its relationship to the fundamental principles of enzymic catalysis. We examine such relationships in the next chapter.
CHAPTER VI
RELATIONSHIP OF MgATP DYNAMICS TO ADENYLATE KINASE
STRUCTURE, BINDING ENERGY AND CATALYSIS

We have already discussed why AMPPCP and MgAMPPCP are excellent models for ATP and MgATP in these studies (see Chapter IV). Having now established the qualitative motional properties of AMPPCP we therefore assume these to be the dynamics of ATP and MgATP in the corresponding environments (free in solution and bound to AK) and proceed to examine the relationship of these dynamics to basic principles and events of enzymic catalysis.

Relationship of Free Substrate Dynamics to Kinase Binding. The observed internal mobility of the phosphonate chain of AMPPCP thus appears to be fast (possibly $\sim 5 \times 10^8 \text{ s}^{-1}$ as derived in the previous chapter) relative to the rate of collision of ATP with AK at mM concentrations (since $k_{on}$ is diffusion-limited as described in Tian et al., 1989a, $k_{on} \times [AK] = 10^5 \text{ s}^{-1}$). Therefore, when AK encounters AMPPCP, the phosphonate chain probably undergoes many conformational changes in a single "meeting". In the instance of MgAMPPCP, the number of conformers is significantly reduced before encountering AK. We emphasize that our results do not shed light on processes occurring on timescales longer than a nsec. Recall, it was determined that AMPPCP bound to AK possesses less mobility in the phosphonate chain than the free nucleotide. The question therefore arises as to the process by which AK encounters the mobile free AMPPCP and succeeds in reducing some of its local mobility. When confronted with the plethora of AMPPCP conformations AK may
selectively bind only certain conformers or may bind all conformers and then induce a reduction in the conformational population. Gaochao Tian of our lab (Tian et al., 1988; Tian and Tsai, 1989) determined that AK can bind both \( \Delta \) and \( \Lambda \) "screw-sense" isomers of \( \beta-\gamma \)-coordinated, substitution inert CrATP with similar affinity and also allows a conformational isomerization from the \( \Delta \) isomer to the \( \Lambda \) (catalytically active) isomer which is consistent with the latter interpretation. This will be discussed further in a later section.

It is interesting to note that AK recognizes and binds Mg\(^{2+}\) complexed and uncomplexed forms of AMPPCP and also ATP with nearly equivalent affinity (see Tables 1 and 5) despite significant differences in their local dynamics. This observation can also be made for ATP and MgATP binding to other isozymes of AK (Ito et al., 1980), 3-phosphoglycerate kinase (Wiksell et al., 1982; Chapman et al., 1977), creatine kinase (O'Sullivan and Cohn, 1966; Brevet et al., 1975) and hexokinase (Rijksen and Staal, 1976). However it is not possible to directly comment on the importance (i.e., unimportance) of free substrate dynamics to association since MgATP and ATP are chemically distinct.

**Relationship of Local Substrate Dynamics to Enzyme Structure.** The observed dynamics for ATP bound to AK must have a fundamental molecular and energetic basis. Three models now exist for the location of the MgATP site of cytosolic AK: an X-ray diffraction derived model (Dreusicke et al., 1988; Pal et al., 1977); an NMR based model (Mildvan and Fry, 1987; Fry et al., 1988, 1985) and a third model arising from molecular mechanics calculations (Caldwell and Kollman, 1988). The reliability of these models are under scrutiny by site-specific mutagenesis studies (Tian et al., 1988; 1989a; 1989b). However, all three models have two features in common of importance to the present discussion. First, the phosphate chain of MgATP
is thought to interact (presumably by hydrogen or ionic bonding) with multiple lysines and/or arginines. Secondly, the adenine ring is located at least partially within a hydophobic cleft. From these models and our results we observe that despite the fact that AK forms multiple hydrogen bonds with the phosphate chain, this chain, for both ATP and MgATP maintains considerable local mobility within the enzyme matrix while hydrophobic interactions are probably responsible for locking in the adenine ring very effectively.

The local mobility retained by the phosphate chain could be accounted for by three possible effects. First, hydrogen or ionic bonds may break and reform many times during an AK-MgATP encounter. A similar phenomenon has recently been proposed for dihydrofolate reductase (Searle et al., 1988) and might also help account (along with possible entropy effects) for the observed binding energy of MgATP being only 6 kcal/mol, somewhat lower than might be expected in a binding situation where multiple highly specific (long lifetime) hydrogen bonds exist between counterions (Fersht et al., 1986; Bartlett and Marlowe, 1987). During the time where one or more of the hydrogen bonds is broken a portion of the phosphonate chain would be temporarily free to move around. The second possible explanation is that the intrinsic flexibility of the terminal amino groups of lysine and arginine side chains may allow the entire phosphate-side chain complex to move around in a coordinated fashion (a sort of "multiple tethering" effect). Finally, the mobility could be explained on the basis of the type of energetics involved. The Debye-Huckel potential energy function for electrostatic interactions has an $r^{-1}$ dependency (where $r$ is the interionic distance), while the potential energy for dipole-dipole/Van der Waals interactions (such as those expected between the adenine ring and hydrophobic or aromatic amino acids) has an $r^{-6}$ dependency (Levine, 1983). The $r^{-1}$ dependence of ionic
Interactions is spatially promiscuous allowing favorable attraction to occur over a much wider range of distances than for dipole-dipole interactions. Thus, the phosphonate chain could move around a lot relative to the basic side chains of the binding site without compromising the energetics. This possibility is supported by affinity labelling studies (Yagami et al., 1988) in which adenosine di-, tri-, and tetraphosphopyridoxal all specifically label lysine 21. For the adenine ring any favorable dipole-dipole attraction with the hydrophobic cleft requires close, immobile interactions due to the $r^{-6}$ dependency.

**Relationship of Local Substrate Dynamics to Local Binding Energy.**

In Chapter III, we reported the binding constants for MgATP, ATP, and various portions of these molecules with the MgATP site of AK. From these binding constants, binding energies can be calculated:

\[
\Delta G^o = -RT \ln K_a
\]

These binding energies are reported in Table 5. It is tempting to suppose that the overall binding constant for a protein and a multisegmental molecule such as MgATP will be the sum of the observed binding energies for the individual segments with the protein. Jencks has warned (1981) against this oversimplification and suggests more accurate ways to approach the question of additive contributions to binding. Applying his theory to our system:

\[
\Delta G^o_{\text{MgATP}} = \Delta G^I_{\text{Ado}} + \Delta G^I_{\text{MgPPP}} + \Delta G^{\text{conn}}.
\]

where $\Delta G^{\text{conn}}$ is a "connectivity" energy (containing miscellaneous energetic contributions which are due to the connection of Ado and MgPPP) and $\Delta G^I$ are "intrinsic" binding energies. In the present case we prefer to use the term "local"
Table 5. Observed Binding Energies for Ligands to AK\textsuperscript{a}.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>( \Delta G^\circ ) (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MgATP</td>
<td>-5.2 ± 0.2</td>
</tr>
<tr>
<td>Mg\textsuperscript{2+}-dATP</td>
<td>-5.2\textsuperscript{b}</td>
</tr>
<tr>
<td>Mg\textsuperscript{3+}-dATP</td>
<td>-5.2\textsuperscript{b}</td>
</tr>
<tr>
<td>ATP</td>
<td>-6.3 ± 0.9</td>
</tr>
<tr>
<td>MgAMPPCP</td>
<td>-5.1 ± 0.4</td>
</tr>
<tr>
<td>AMPPCP</td>
<td>-5.0 ± 0.5</td>
</tr>
<tr>
<td>Adenosine</td>
<td>-1.6 ± 0.2</td>
</tr>
<tr>
<td>MgPPP\textsubscript{1}</td>
<td>-3.8 ± 0.2</td>
</tr>
<tr>
<td>PPP\textsubscript{1}</td>
<td>-4.2 ± 0.4</td>
</tr>
<tr>
<td>MgPPP\textsubscript{1}</td>
<td>-3.1 ± 0.4</td>
</tr>
<tr>
<td>Mg(H\textsubscript{2}O)\textsubscript{8}\textsuperscript{+2}</td>
<td>~0</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Calculated from dissociation constants presented in Table I.

\textsuperscript{b}Based on the fact that \( K_m \) for Mg\textsuperscript{2+}-dATP and Mg\textsuperscript{3+}-dATP are approximately equal to that of MgATP (L. Brice, unpublished results).
binding energies since "intrinsic" binding energies have come to be associated with the maximal binding energy which is expressed in the transition state of enzyme-substrate complexes while our studies deal only with the ground state complex. Our derivation of Equation 29 (see Appendix C) suggests that $\Delta G^i$ are, in our case, the local interaction energy differences between Ado or MgPPP$_i$ interacting with water and with the AK active site but does not include translational entropy. In other words, local binding energies represent the amount of energy local interaction of one portion of MgATP provides to drive overall MgATP binding. Jencks suggests (1981) that local binding energies are readily estimated from experimentally observed binding energies. For example, again applying his equation to our system:

$$\Delta G^I_{\text{Ado}} = \Delta G^0_{\text{MgATP}} - \Delta G^0_{\text{MgPPP}_i} \tag{30}$$

$$\Delta G^I_{\text{MgPPP}_i} = \Delta G^0_{\text{MgATP}} - \Delta G^0_{\text{Ado}} \tag{31}$$

The local binding energies were calculated from observed binding energies using such equations and are reported in Table 6. It is worth noting that for MgATP-AK interaction the observed binding energies for the various parts of MgATP are approximately additive, indicative of a small "connectivity" term for ground state binding.

Our results in Table 6 demonstrate that most of the binding energy which drives overall MgATP binding derives from interactions of the triphosphate moiety with AK. Adenosine supplies considerably less (with the 2' and 3' OH groups supplying none) and Mg$^{+2}$ makes a negligible contribution. This is in complete accord with calculated
Table 6. Local Binding Energies for Portions of MgAMPPCP and MgATP.

<table>
<thead>
<tr>
<th>Segment</th>
<th>$\Delta G^\dagger$ (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenosine</td>
<td>-1.4 ± 0.4</td>
</tr>
<tr>
<td>2'-OH</td>
<td>-0</td>
</tr>
<tr>
<td>3'-OH</td>
<td>-0</td>
</tr>
<tr>
<td>MgPPP$_1$</td>
<td>-3.6 ± 0.4</td>
</tr>
<tr>
<td>MgPPCP$_1$</td>
<td>-3.5 ± 0.7</td>
</tr>
<tr>
<td>PPP$_1$</td>
<td>-4.7 ± 0.13</td>
</tr>
</tbody>
</table>
interaction energies (Caldwell and Kollman, 1988) for the various segments of MgATP with AK using two of the existing binding site models as well as previous binding/kinetic studies (Hiratsuka, 1983; Kappler et. al., 1982; Hamada et. al., 1979; Price et. al., 1973).

The deuterium NMR results suggest that the adenine ring of both ATP and MgATP is rigidly locked into place by AK. Nevertheless, the local binding energy observed for the interaction is quite weak. The phosphonate chain of ATP is fairly mobile at the MgATP site, yet the local binding energy is quite favorable. Mg\(^{2+}\) reduces this local mobility considerably without significantly perturbing the thermodynamics of the system. Based on these observations we conclude that there is no generalizable correlation between local binding energy and local mobility. The observation of tight local binding energetically does not necessarily imply tight local binding, motionally (and vice versa). Conversely, the observation of local mobility for one segment of a substrate cannot be taken to imply that the local binding energy for that portion of the substrate is weak (and vice versa). These points are significant limitations to the interpretation of data related to either the motional dynamics of biological systems or to examination of the detailed energetics of protein-ligand interactions.

The lack of a general correlation between local binding energies and dynamics is probably due to two facts. First of all, observed local dynamics can be somewhat incidental. For example, the adenine ring is large and intrinsically rigid as part of ATP. It is probable that it would appear to be rigidly bound even if it was sticking out into solvent (local binding energy of 0) with the ribose/phosphonate segments being rigidly anchored within the AK matrix. Secondly, the lack of a correlation is due to the fundamental complexity of energetics in biological systems. As mentioned earlier, different forms of attraction have different spatial dependencies. In addition,
enthalpically favorable interactions tend to favor decreased mobility (formation of bonds) while entropy tends to favor increased motion. Solvation further complicates the already complicated nature of the problem.

Relationship of Local Substrate Dynamics to Catalysis. Most kinases utilize MgATP as substrate but the precise role of Mg$^{2+}$ in catalysis is not exactly known, though it is generally thought to be involved as an electrophile. Our results suggest that one additional role Mg$^{2+}$ may play is that of reducing the mobility of the phosphate chain in the ground state in preparation for efficient phosphoryl transfer. It is significant to note that the enzyme itself does not contribute to such local immobilization at the ground state. Instead this sequestration occurs before the complex gets to the active site when Mg(H$_2$O)$_6^{2+}$ reacts to form a complex with ATP in an entropically favorable process (Tu and Heller, 1974). X-ray diffraction studies on hexokinase (Steltz et al., 1982) to which ATP had been bound in the absence of metal ions showed "disorder" in the phosphate chain region, suggesting that the mobility we observed for AMPPCP and AK may reflect a general property of ATP binding for kinases. The fact that many other metal ions, which probably also immobilize the phosphate chain, do not always bring about optimal catalysis suggests that this is probably not the only role it plays (O'Sullivan and Noda, 1968; Morrison and Cleland, 1983).

A second significant point is that despite the rigidization of the phosphonate moiety induced by Mg$^{2+}$, the ground state AK-MgAMPPCP complex seems to possess a certain amount of mobility in the $\beta,\gamma$-phosphate region. This suggests that the triphosphate site of AK is not restrictively constrained to allow a fit of only a single isomer or conformer of MgATP in the AK-MgATP complex. This fully supports the "tolerance" of AK to other metal ions (Noda, 1973) and to different isomers of
Cr(III)ATP (Dunaway-Mariano and Ciecland, 1980) in terms of ground state binding. In addition, it also suggests that AK could actually allow these isomers to interconvert at the active site as a way to minimize nonproductive binding. The results also suggest that the enzyme may enhance further immobilization of the terminal phosphate at the transition state. Alternatively, it is possible that there may be multiple spatially distinct pathways that the reaction could pass through with different possible ground state conformers leading to these reaction pathways. This latter possibility is supported by at least one (albeit weak) experimental observation. AK is capable of slowly catalyzing the formation of Mg-adenosine tetraphosphate from MgATP and ADP (Kupriyanov et al., 1986)- a reaction which, while presumably using the same enzymic machinery as the normal AK reaction, probably possesses a transition state spatially distinct (within the enzymic coordinate frame of reference) from that of the usual reaction.

The role of the adenosine in catalysis is somewhat of a mystery. We observed that it does not seem to play a significant role in ground state binding. There is a modest increase in the expression of binding energy for the adenine ring as it goes through the transition state as evidenced by the fact that ($V_{\text{max}}/K_m$)$_{\text{MgATP}}$/$V_{\text{max}}/K_m$ for AK and alternate nucleotide triphosphates are larger than the corresponding $K_m$,$\text{MgNTP}/K_m$,$\text{MgATP}$ (O'Sullivan and Noda, 1968). However, it is not clear that this transition-state expression of binding energy is the only catalytically important process involving the adenine ring or how this binding energy is actually coupled to the catalytic process. We observed it to be essential for inducing conformational change in the enzyme since PPP$_i$ or MgPPP$_i$ induce very small conformational changes, but substrate-induced conformational changes may be relatively unimportant in the catalysis of AK (Tian et al., 1989a). It is, however, needed for optimal phosphoryl
transfer to occur. Using the TLC assay system (see Chapter II) we determined that 
MgPPPi as a substrate possesses a $V_{max}$ $10^{-4}$-$10^{-5}$ that of MgATP (Figure 22).
While $K_m$ was not accurately determined, if it is assumed to be within an order of 
magnitude of its dissociation constant (Table 1) the $V_{max}/K_m$ for MgPPPi is < $10^{-5}$ 
that of MgATP. In addition, using 5'-deoxyadenosine and MgPPPi it was determined 
that optimal catalysis also requires the adenosine to be attached to the triphosphate, 
since 5'-deoxyadenosine did not enhance the activity appreciably as judged using the 
TLC assay system. In light of these observations, our conclusion that the adenine ring 
is rigid in the ground state does suggest one possible role. The spatially permissible or 
flexible interactions responsible for the local binding of the triphosphate region may 
be incapable of properly localizing the γ-phosphate as needed for efficient catalysis.
The adenine ring may play the role, mediated through the linking ribose, of an anchor 
for one end of the triphosphate chain which would substantially reduce the number of 
binding modes for the triphosphate chain. This "anchoring" is similar to the 
previously known "anchor effect" (Jencks, 1975) but may not necessarily be 
ergetically costly or significant.
Figure 22. Lineweaver-Burke plot for Mg$^{2+}$-triphosphate as a substrate for adenylate kinase at pH 8.0 and 30 °C. The TLC assay system described in Chapter II was utilized, except that $^{14}$C-AMP was used (instead of $^3$H-ATP) and no ATP was present. Mg$^{2+}$ was maintained at a 10 % excess relative to the triphosphate present. The rate is in terms of $^{14}$C-AMP converted into $^{14}$C-ADP. The AMP concentration was 1 mM.
FOOTNOTES

1. The $k_{cat}/K_m$ for AK at 30 °C is $6.5 \times 10^6$ M·sec⁻¹ (Tian et al. 1989a) which is the pseudo-first order rate constant for the diffusion of one substrate onto a complex of AK with the other substrate, phosphoryl transfer, and then diffusion off of the enzyme. To estimate the uncatalyzed reaction rate we incubated pH 8.0 buffered solutions of 1 mM $^{14}$C-AMP, 1 mM ATP, and 2 mM MgCl₂ at 50 °C for 24 hours. At the end of the incubations, no production of $^{14}$C-ADP could be detected as judged by the TLC assay described in Chapter II which has a 1% limit of detection. (A control was run using $^3$H-ATP which indicated that phosphate hydrolysis was insignificant and not a complicating factor in this experiment.) This indicates that the maximal rate at which phosphoryl transfer could have occurred (and escape detection) can be described by a pseudo-first order rate constant of $1.2 \times 10^{-4}$. Thus, the 50 °C uncatalyzed reaction is a minimum of $5 \times 10^{10}$ slower than the 30 °C reaction catalyzed by AK. We have also observed that between 10 and 40 °C the $k_{cat}/K_m$ for AK approximately doubles with every 10 °C increase in temperature. Assuming this trend extends on up to 50 °C, the AK catalyzed reaction rate would then have a rate constant of approximately $2.7 \times 10^8$ which would raise the minimum catalytic enhancement factor to $2 \times 10^{11}$.

2. The observed binding energy is calculated from the association constant ($1/K_d$) using the equation: Binding energy = $-RT\ln K_a$ (Equation 32), where $R$ is the gas constant and $T$ is the absolute temperature.

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3. Their simulations explicitly apply only for $^{13}$C dipolar relaxation. However, due to a similar spectral density dependence, these results can be used (qualitatively) for quadrupolar relaxation rates.
APPENDIX A

Data Fitting For Deuterium NMR: Titration of a Deuterated Ligand by a Metal Ion where 2:1 Metal to Ligand Stoichiometry holds and where the $^2\text{H}$ NMR Relaxation Rate of the Ligand is followed.

The relevant process is:

$$K_A \quad J$$
$$L + M \rightleftharpoons LM + M \rightleftharpoons MLM \quad (33)$$

where $K_A$ and $J$ are association constants. For this situation:

$$ML \quad MLM$$
$$R - R_f = \frac{ML}{L_1} (R_b - R_f) + \frac{MLM}{L_1} (R_c - R_f) \quad (34)$$

where $R$, $R_f$, $R_b$, and $R_c$ are the observed, Intrinsic free, Intrinsic 1:1, and Intrinsic 2:1 relaxation rates for the ligand/ligand complexes. $L_1$, $ML$, and $MLM$ are the total, 1:1 complexed, and 2:1 complexed ligand concentrations, respectively. Also,

$$ML$$
$$K_A = \frac{ML}{M \cdot L} \quad (35)$$

$$MLM$$
$$J = \frac{MLM}{ML \cdot M} \quad (36)$$

where $M$ and $L$ are free metal ion and ligand concentrations. This can lead to:
and from (36) and (37):

\[ MLM = J \cdot K_a \cdot M^2 \]  

(38)

(37) and (38) are substituted back into (34) to yield:

\[
R = \frac{(K_a \cdot M \cdot L + J \cdot K \cdot L \cdot M^2) \cdot (R_b - R_f) + (J \cdot K_a \cdot L \cdot M^2) \cdot (R_c - R_f)}{L_t} + R_f
\]  

(39)

Now, combining 37 and 38 with the mass balance equation,

\[ L_t = L + ML + MLM \]  

(40)

yields:

\[
L = \frac{L_t}{1 + K_a \cdot M + J \cdot K_a \cdot M^2}
\]  

(41)

which is substituted back into (39) to give:

\[
R = \frac{L_t \cdot K_a \cdot M \cdot ((1 + J \cdot M) \cdot (R_b - R_f) + J \cdot M \cdot (R_c - R_f))}{L_t \cdot (J \cdot K_a \cdot M^2 + K_a \cdot M + 1)} + R_f
\]  

(42)

An explicit solution for \( M \) (free metal ion) is not available and the approximation \( L = L_t \) is often poor. A much better approximation is \( L = L_t - ML \). This approximation is equivalent to assuming only 1:1 stoichiometry holds. In this case,
By combining these equations it is readily demonstrated that:

\[ K_a \cdot M^2 + (1 + K_a \cdot (L_t - M_t)) - M_t = 0 \]  

which yields a solution for \( M \):

\[
M = \frac{(K_a \cdot (M_t - L_t) - 1 + ((1 + K_a \cdot (L_t - M_t))^2 + 4 \cdot K_a \cdot M_t)^{1/2}}{2 \cdot K_a}
\]

This solution for \( M \) is (for our data), an excellent approximation and so (47) can be substituted into (42).

The resulting equation (not shown- too big!) expresses the observed relaxation rate as a function of experimental variable \( L_t \) and \( M_t \) and parameters \( K_a, J, R_f, R_b, \) and \( R_c \). Data fits are therefore three-dimensional and difficult to depict in a meaningful manner without resorting to surface plots. The results reported in Table 3 are the result of such 3-dimensional fitting. In our titrations \( L_t \) varied during titrations and from titration to titration by < 25 % and if \( L_t \) is assumed to be constant at 6 mM the problem is reduced to two dimensions. The data fit shown in Figure 23 is the result of this approximate approach.
Figure 23. Influence of Mg$^{2+}$ upon the longitudinal relaxation rates of AMPPCP at pH 7.0 (A) and pH 10.0 (B) as solutions of the nucleotides were titrated with MgCl$_2$. The nucleotide solutions were 5-7 mM. The fits shown are approximate (2-dimensional) fits to a 2:1 binding model as described in Appendix A. Additional details of the experiment as found in Chapters II and IV.
APPENDIX B

Data fitting for Deuterium NMR: 1:1 Stoichiometry where Protein is Titrated with a Deuterated Ligand and the Weighted Average $^2$H NMR Linewidth is Observed.

The fraction of ligand bound to protein is:

$$\frac{v \cdot v_f}{v_b \cdot v_f} = \frac{E_L}{L_t} \quad (48)$$

Where $E_L$ and $L_t$ are bound and total ligand concentrations and $v$, $v_f$ and $v_b$ are the observed, free and bound linewidths, respectively. Rearranging (48):

$$v = \frac{E_L}{L_t} \left( v_b - v_f \right) + v_f \quad (49)$$

A-2 is the equation used in figure 5. We must solve for $E_L$:

$$E_t = E + E_L \quad (50)$$

$$L_t = L + E_L \quad (51)$$

$$K_d = \frac{L \cdot E}{E_L} \quad (52)$$

Where $E$ and $L$ are free concentrations of protein and ligand, $E_t$ and $L_t$ are the corresponding total concentrations, and $K_d$ is the dissociation constant.

From (51): 110
\( EL = L_1 \cdot L \) \hspace{1cm} (53)

(53) is substituted into (49) to give:

\[
L_1 \cdot L
\]

\[
\nu = \frac{(V_b - V_f) + V_f}{L_1}
\]

(54)

Combining (50), (51), and (52) algebraically:

\[ L^2 + L (K_d + E_t - L_1) - K_d \cdot L_1 = 0 \] \hspace{1cm} (55)

which yields a solution for \( L \):

\[
L = \frac{[(L_1 - E_t - K_d) + ((K_d + E_t - L_1)^2 + 4 \cdot K_d \cdot L_1)^{1/2}]/2}{2}
\]

(56)

which is substituted into (54). The resulting equation can be used to fit experimental data for \( \nu \), \( E_t \), and \( L_1 \) to obtain parameters \( K_d \), \( V_b \), and \( V_f \). \((L_1-L)/L_1\) is, of course, the fraction bound (see Figure 15).
APPENDIX C
Explanation of the Origins of the Equations used to Determine Local Binding Energies

In 1981 Jencks stated (Jencks, 1981) that for the binding of a multisegmental molecule AB to an enzyme the observed binding energy could be broken down:

\[ \Delta G_{AB}^0 = \Delta G_A^I + \Delta G_B^I + \Delta G_C \]  \hspace{1cm} (57)

where \( \Delta G_{AB}^0 \) is the observed binding energy for AB, \( \Delta G_A^I \) and \( \Delta G_B^I \) are the "intrinsic", or "local" binding energies of segments A and B as a part of AB, and \( \Delta G_C \) is the "connectivity" energy. Furthermore, Jencks suggested that \( \Delta G_I \) could be readily estimated from experimentally determined binding energies for A, B and AB:

\[ \Delta G_A^I = \Delta G_{AB}^0 - \Delta G_B^0 \]  \hspace{1cm} (58)

\[ \Delta G_B^I = \Delta G_{AB}^0 - \Delta G_A^0 \]  \hspace{1cm} (59)

Jencks did not explain where these equations came from and the definitions given in the paper are a bit confusing. To illuminate the nature of these equations and their terms we have undertaken their derivation. We prefer to do this using our own system as the test case. MgATP is a multisegmental molecule and we have chosen to regard "A" as the adenosine portion and "B" as the MgPPP moeity. His theory apparently centers on the use of thermodynamic cycles (Equation 60):
In this scheme all of the $\Delta G$ are for the various processes as one goes in a clockwise direction around the cycles(s). If the $\Delta G^0$ had been measured in our studies then a direct quantitative analysis of the cycles would be possible. As it stands, we must assume that $\Delta G_{\text{Ado}}^0 = \Delta G_{\text{Ado}}$ and that $\Delta G_{\text{MgPPPl}}^0 = \Delta G_{\text{MgPPPl}}^{0'}$ which allows us to reduce the scheme to:
Since the total free energy change in making one round of thermodynamic cycle is zero:

\[ \Delta G^c + \Delta G_{MgATP} - \Delta G^c - \Delta G_{Ado} - \Delta G_{MgPPPi} = 0 \]  

which can lead to:

\[ \Delta G_{MgATP} = \Delta G_{Ado} + \Delta G_{MgPPPi} + \Delta G^c - \Delta G^c \]  

At this point please note that \((\Delta G^c - \Delta G^c)\) is the difference in equilibrium free energy for the connection of adenosine and MgPPPi in solution versus that at the active site and can include such terms as conformational changes on the enzyme when they are connected, the release of steric compression energy, or the translational entropy of the free "reactants". Each of the above \(\Delta G\) can be broken down:

\[ \Delta G = \Delta H - T(\Delta S_{\text{translational}} + \Delta S_{\text{other types}}) \]

Now, the point of the equations we are trying to derive is to determine how much energy local interactions of a part of the substrate (i.e. adenosine or MgPPPi) with the enzyme contribute to help drive the overall binding of the substrate. Once
bound to an enzyme "local" interactions (which are the ones which are variable by evolution or by man) include all of the terms in Equation (64) except for the translational entropy term: once the substrate is bound it automatically loses its one degree of translational entropy. Thus translational entropy has nothing to do with the "local binding energy" and so we remove it from the $\Delta G^0$ for Ado and MgPPPi and throw it in with the connectivity energies which we are treating as a "miscellaneous" or "junk" terms and which are redesignated $\Delta G^C$. This allows us to rewrite Equation (63) into a form formally the same as Equation (57):

$$\Delta G_{AB}^0 = \Delta G_{\text{Ado}}^I + \Delta G_{\text{MgPPPi}}^I + \Delta G^C$$  \hspace{1cm} (65)

These $\Delta G^I$ reflect the difference in local interaction energies for parts of the MgATP in water versus those at the active site, e.g.:

$$\Delta G_{\text{Ado}}^I = G_{\text{at active site}} - G_{\text{in aqueous solution}}$$  \hspace{1cm} (66)

Now, the other problem we wished to address here is the origin of Equations (58) and (59).

Jencks argued that translational entropy really varies very little from molecule to molecule. If this is so then:

$$T \Delta S_{\text{Ado}} = T \Delta S_{\text{MgPPPi}} = T \Delta S_{\text{MgATP}}$$  \hspace{1cm} (67)

where, in this case, $S$ is the translational entropy lost upon bind to a macromolecule. Thus, when one subtracts:

$$\Delta G_{\text{MgATP}}^0 - \Delta G_{\text{MgPPPi}}^0$$  \hspace{1cm} (68)

or

$$\Delta G_{\text{MgATP}}^0 - \Delta G_{\text{Ado}}^0$$  \hspace{1cm} (69)
translational entropy terms drop out and it is equivalent to subtracting local binding energies.

What is the identity of the energy left over when the local binding energy of one part of a substrate is subtracted from the local binding energy of the whole substrate? - The local binding energy of the missing part. Hence:

\[ \Delta G_{\text{MgATP}^0} - \Delta G_{\text{Ado}^0} = \Delta G_{\text{MgATP}^1} - \Delta G_{\text{Ado}^1} = \Delta G_{\text{MgPPi}^1} \]  
(70)

This author feels somewhat uneasy about this theory. The derivation is not the usual straightforward type of algebraic derivation and may not take into account such energetically relevant processes as desolvation of the substrate or the active site upon binding. Nevertheless, this theory is the best available which is readily accessible to the average biochemist and is, as hopefully demonstrated, extremely usable based on straightforward binding experiments.
REFERENCES


