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Mechanism of Adenylate Kinase. 3. Use of Deuterium NMR To Show Lack of Correlation between Local Substrate Dynamics and Local Binding Energy[1]

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The fact that enzymes utilize binding energy to bring about entropically unfavorable events and thus facilitate catalysis[2] suggests that enzymes are fundamentally involved in the control of substrate motion. We have examined the local motions of AMPPCP bound to adenylate kinase (AK) in order to examine the relationships between local rigidity of a bound substrate, binding energy, and catalysis by AK.

Chicken muscle AK was titrated with AMPPCP[3] deuteriated upon the phosphonate chain and upon the adenine ring[4] and followed by measuring the deuterium NMR line width ($\Delta\nu_{1/2}$) of the single peak which results from the average of the bound and free AMPPCP. Plots of $\Delta\nu_{1/2}$ versus [AMPPCP]$_{bound}$/[AMPPCP]$_{total}$ were linear, as shown in Figure 1. The line shapes of the observed peaks were usually Lorentzian, and limited T_1 inversion recovery data taken were always monoexponential. Upon increasing temperature from 5 °C to 35 °C, the line widths decreased with a magnitude approximately proportional to the decreasing solution viscosity. Thus, our data meet "fast-exchange" criteria, and the line widths of the fully bound species can be determined from linear extrapolations to fraction bound = 1.[5] Indeed very little extrapolation is required for two of the curves. The line widths obtained are listed in Table I. The effective rotational correlation times (τ_c) for isotropic motions were then calculated from the well-known relationship between line width ($1/\pi T_2$) and τ_c.[6-8] The contributions of two-bond ^{2}H–^{14}N and ^{2}H–^{31}P scalar and dipolar couplings to the observed $\Delta\nu_{1/2}$ were insignificant. The validity of the τ_c data determined from such analysis was further supported by the fact that the same τ_c values (within experimental errors) were obtained from T_1 experiments

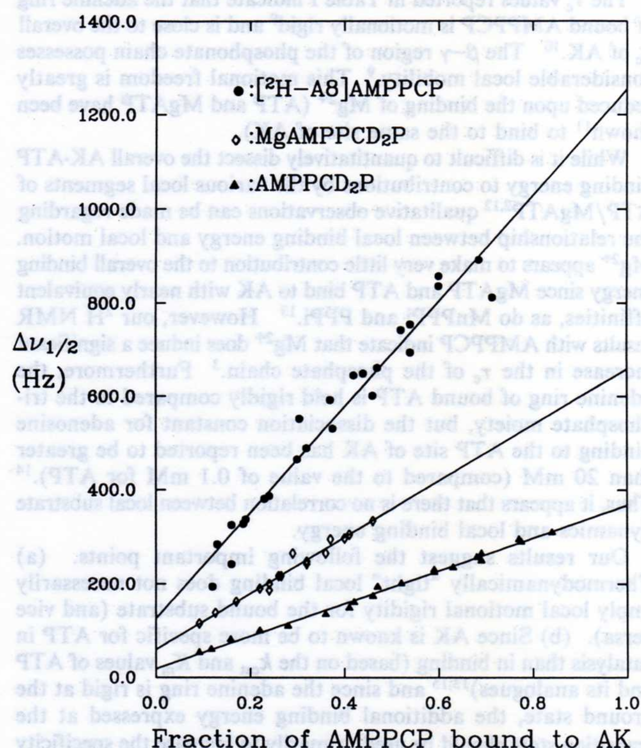

Figure 1. ^{2}H NMR (46.1 MHz) line widths ($\Delta\nu_{1/2}$ in Hz) of ^{2}H-labeled AMPPCP and MgAMPPCP ($[Mg^{2+}]$/[AMPPCP] = 4) as a function of fractions bound to AK, obtained by titrating 1–2 mM AK with the nucleotides. Sample conditions: pH 7.0 in ^{2}H-depleted H_2O with 45 mM Hepes–K^+ or imidazole–HCl, 117 mM KCl, 1–8 mM dithiothreitol, and 0.1 mM EDTA in a 10-mm NMR tube (starting volume of 1.75 ml) at 10 °C. Spectral conditions: digital resolution 1 Hz/point (narrow signals) to 15 Hz/point (very broad signals), 90 °C pulse width 12 μs. The reported $\Delta\nu_{1/2}$ have been corrected for line broadening (1–10 Hz). The fraction of AMPPCP bound to AK was calculated by using K_d values of 210 μM for AMPPCP and 190 μM for MgAMPPCP determined in our lab and elsewhere.[19,25]

(1) Abbreviations: AMPPCP, adenylyl (β,γ-methylene)diphosphonate; AK, adenylate kinase; ATP, adenosine 5′-triphosphate; AMP, adenosine 5′-monophosphate; EDTA, ethylenediamine tetraacetate; Hepes, *N*-(2-hydroxy ethyl)piperazine-*N*-2-ethanesulfonic acid; PPPi, triphosphate; T_1, spin–lattice relaxation time; T_2, spin–spin relaxation time.

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(3) It is assumed that AMPPCP interacts with AK in a motionally and energetically similar manner as ATP, since the observed binding energies (based on K_d) for ATP and AMPPCP are similar (−5.2 and −5.1 kcal/mol, respectively).

(4) [8-^{2}H]AMPPCP was prepared by heating AMPPCP in D_2O at pH$_{obsd}$ = 10 for 3–6 h at 95 °C. AMPPCD$_2$P was synthesized by modified literature procedures.[20,21]

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(7) We assume effectively isotropic motion (on an NMR time scale) since $\tau_c \ll$ [quadrupolar coupling constant]$^{-1}$. We also assume purely quadrupolar relaxation because of the relatively high quadrupolar coupling constants exhibited by deuterated hydrocarbons due to aspherical electronic symmetry and because there are no reasons to expect a competing mechanism. For our calculations we utilized an asymmetry parameter of zero which is quite reasonable due to the axial symmetry of the electric field gradient of the deuterium nuclei in our compounds. Finally, we assume quadrupolar coupling constants of 178 MHz for [8-^{2}H]AMPPCP and 168 MHz for both AMPPCD$_2$P and MgAMPPCD$_2$P. These values were chosen through chemical analogy with [8-^{2}H]AMP[22] and deuteriated malonic acid.[23] This is reasonable since ^{2}H quadrupolar coupling constants are quite invariant with even major covalent electronic perturbations.[23]

(8) Viscosity measurements were also performed (at room temperature) which suggest that the total viscosity change which occurred during the titrations is only ~5%.

Table I. Rotational Correlation Times for ^2H in AK·AMPPCP Complexes

complex	line width (Hz)	τ_c (ns)
AK·AMPPCD$_2$P	367 ± 15	6.5 ± 0.5[a]
AK·MgAMPPCD$_2$P	660 ± 50	16 ± 1[a]
AK·[8-^{2}H]AMPPCP	1250 ± 150	27 ± 4[a]
AK		75[b]

[a] Calculated from line widths as described in ref 6 and 7. [b] Calculated from the Stokes–Einstein equation assuming that AK is a rigid sphere.[10]

at the same magnetic field (46.1 MHz) and from line width measurements at a different magnetic field (78.7 MHz).

The τ_c values reported in Table I indicate that the adenine ring of bound AMPPCP is motionally rigid[9] and is close to the overall τ_c of AK.[10] The β–γ region of the phosphonate chain possesses considerable local mobility.[9] This motional freedom is greatly reduced upon the binding of Mg^{2+} (ATP and MgATP have been shown[11] to bind to the same site of AK).

While it is difficult to quantitatively dissect the overall AK·ATP binding energy to contributions by the various local segments of ATP/MgATP[2,12] qualitative observations can be made regarding the relationship between local binding energy and local motion. Mg^{2+} appears to make very little contribution to the overall binding energy since MgATP and ATP bind to AK with nearly equivalent affinities, as do MnPPPi and PPPi.[13] However, our ^2H NMR results with AMPPCP indicate that Mg^{2+} does induce a significant increase in the τ_c of the phosphate chain.[3] Furthermore, the adenine ring of bound ATP is held rigidly compared to the triphosphate moiety, but the dissociation constant for adenosine binding to the ATP site of AK has been reported to be greater than 20 mM (compared to the value of 0.1 mM for ATP).[14] Thus, it appears that there is no correlation between local substrate dynamics and local binding energy.

Our results suggest the following important points. (a) Thermodynamically "tight" local binding does not necessarily imply local motional rigidity for the bound substrate (and vice versa). (b) Since AK is known to be more specific for ATP in catalysis than in binding (based on the k_{cat} and K_m values of ATP and its analogues)[13,15] and since the adenine ring is rigid at the ground state, the additional binding energy expressed at the transition state should be used primarily to increase the specificity not to rigidize the adenine ring. (c) Binding of Mg^{2+} may serve, among other functions, to immobilize and properly orient the γ-phosphate in preparation for the transition state. (d) Although the relatively large local freedom of the triphosphate moiety of AK·ATP has been revealed qualitatively by ^{31}P NMR (very narrow signals of bound ATP)[16] and ^{17}O NMR (relatively small increases in $\Delta\nu_{1/2}$ upon binding),[17] we have demonstrated that ^2H NMR can provide a comparison of relative local motional freedom in a straightforward way.

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(9) Although the τ_c obtained from ^2H NMR are only effective values ($1/\tau_{c,eff} = 1/\tau_{c,overall} + 1/\tau_{c,internal}$),[24] differences in $\tau_{c,eff}$ determined for the various species can be used to compare the local, internal dynamics of the different groups since $1/\tau_{c,overall}$ can be assumed to be similar for the three species since it is dictated by protein motion.

(10) The calculated value of 75 ns presented in Table I is almost certainly a great overestimation of this value since the calculation does not take the dynamic nature of proteins into account.

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(25) For MgAMPPCP, the points where the fraction bound exceeds 0.5 were not shown since at such low concentrations AMPPCP may not be fully complexed with Mg^{2+}, probably due to competition by other nonspecific binding. As a consequence some of these points fell below the plotted line and were difficult to reproduce.