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

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The fact that enzymes utilize binding energy to bring about entropically unfavorable events and thus facilitate catalysis 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 deuterated upon the phosphate chain and upon the adenine ring 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] free were linear, as shown in Figure 1. The line shapes of the observed peaks were usually Lorentzian, and limited to inversion recovery data taken were always monoeponential. 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. Indeed very little extrapolation is required for two of the curves.

The line widths obtained are listed in Table 1. The effective rotational correlation times (\(\tau_r\)) for isotropic motions were then calculated from the well-known relationship between line width (1/\(\tau_r\)) and \(\tau_r\).

The contributions of two-bond \(^2\text{H}-^{15}\text{N}\) and \(^2\text{H}-^{31}\text{P}\) scalar and dipolar couplings to the observed \(\Delta
\nu_{1/2}\) were insignificant. The validity of the \(\tau_r\) data determined from such analysis was further supported by the fact that the same \(\tau_r\) values (within experimental errors) were obtained from \(T_1\) experiments.

Figure 1. \(^3\text{H}\) NMR (46.1 MHz) line widths (\(\Delta
\nu_{1/2}\)) in Hz of \(^3\text{H}\)-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 \(^3\text{H}\)-depleted H\(_2\)O with 45 mM Hesper-K° or imidazole-HCl, 117 mM KCl, 1-8 mM diethiothreitol, 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.

(7) We assume effectively isotropic motion (on an NMR time scale) since \(\tau_r \ll [\text{quadrupolar coupling constant}]^{-1}\). We also assume purely quadrupolar relaxation because of the relatively high quadrupolar coupling constants exhibited by deuteriated 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-\(^2\text{H}\)]AMPPCP and 168 MHz for both AMPPCP-P and MgAMPPCP-P.
(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>
<thead>
<tr>
<th>complex</th>
<th>line width (Hz)</th>
<th>$\tau_c$ (ns)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AK-AMPPCP₂⁺</td>
<td>367 ± 15</td>
<td>6.5 ± 0.5*</td>
</tr>
<tr>
<td>AK-Mg⁺ AMPPCP₂⁺</td>
<td>660 ± 50</td>
<td>16 ± 1*</td>
</tr>
<tr>
<td>AK-[8-²H] AMPPCP₂⁺</td>
<td>1250 ± 150</td>
<td>27 ± 4*</td>
</tr>
<tr>
<td>AK</td>
<td></td>
<td>75*</td>
</tr>
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* Calculated from line widths as described in ref 6 and 7.

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

The $\tau_c$ values reported in Table I indicate that the adenine ring of bound AMPPCP is motionally rigid and is close to the overall $\tau_c$ of AK. The $\beta$-$\gamma$ region of the phosphonate chain possesses considerable mobility. This motional freedom is greatly reduced upon the binding of Mg²⁺ (ATP and MgATP have been shown 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, qualitative observations can be made regarding the relationship between local binding energy and local motion. Mg²⁺ appears to make very little contribution to the overall binding energy since MgATP and ATP bind to AK with nearly equivalent affinities, as do MnPPi and PPi. However, our $^2$H NMR results with AMPD indicate that Mg²⁺ does induce a significant increase in the $\tau_c$ of the phosphate chain. Furthermore, the adenine ring of bound ATP is held rigidly compared to the triphosphate moiety, but the dissociation constant for adenine 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). 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) 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 Mg²⁺ may serve, among other functions, to immobilize and properly orient the $\gamma$-phosphate in preparation for the transition state. (d) Although

$9$ Although the $\tau_c$ obtained from $^2$H NMR are only effective values $(1/\tau_c)^{n\text{eff}} = (1/\tau_c)^{n\text{cat}} + (1/\tau_c)^{n\text{protein}}$, and differences in $\tau_c^{n\text{eff}}$ determined for the various species can be used to compare the local, internal dynamics of the different groups since $1/\tau_c^{n\text{eff}}$ 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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(13) Price, N. C.; Reed, G. H.; Cohn, M. Biochemistry 1973, 12, 3322–3327.
(23) $\tau_c^{n\text{eff}} = (1/\tau_c)^{n\text{cat}} + (1/\tau_c)^{n\text{protein}}$.