

## Mechanism of Adenylate Kinase. 1. Use of $^{17}\text{O}$ NMR To Study the Binding Properties of Substrates<sup>1</sup>

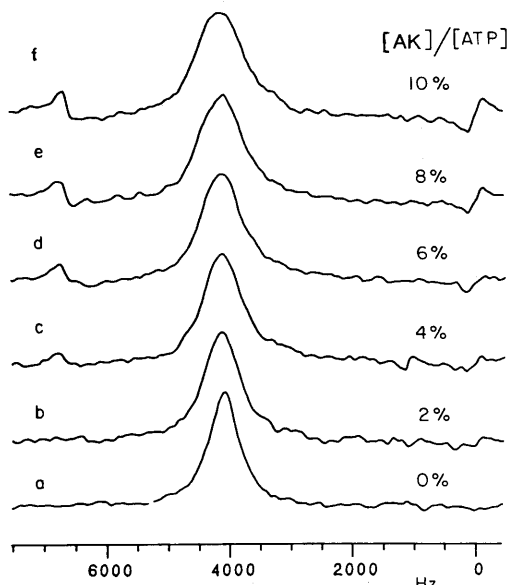
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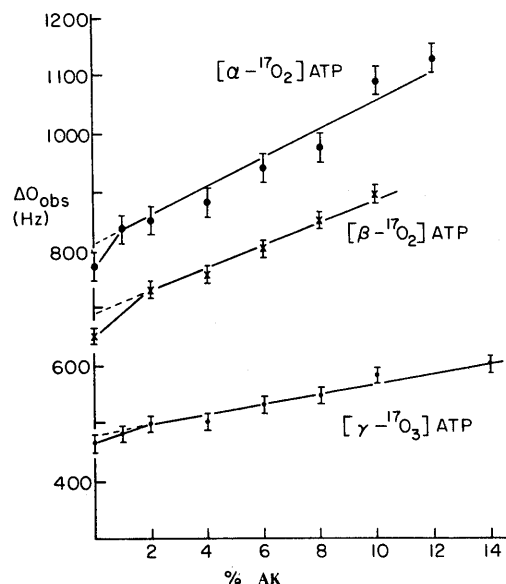
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Despite the ubiquity of oxygen-containing functional groups (e.g., phosphoryl, carboxyl, and hydroxyl groups) in biological systems,  $^{17}\text{O}$  NMR has not been used to study the binding and motional properties of enzyme-substrate complexes. On the other hand, binding of small ligands to proteins has been investigated by the NMR properties of other quadrupolar nuclei such as  $^{43}\text{Ca}$ ,<sup>2</sup>  $^{79}\text{Br}$ ,  $^{81}\text{Br}$ ,<sup>3</sup>  $^{35}\text{Cl}$ ,<sup>4</sup>  $^2\text{H}$ ,<sup>5</sup> etc.

Adenylate kinase (AK) provides a good system to test the applicability of  $^{17}\text{O}$  NMR in enzyme-substrate interactions. The enzyme is small ( $M_r \approx 21\,000$ ), yet consists of two distinct sites: the MgATP site binds ADP, ATP, MgADP, and MgATP, whereas the AMP site binds AMP and ADP.<sup>6</sup> The dissociation constants are in the order of  $10^{-4}$ – $10^{-5}$  M.<sup>6b,c</sup>



**Figure 1.**  $^{17}\text{O}$  NMR spectra (40.7 MHz) of  $[\beta\text{-}^{17}\text{O}_2]\text{ATP}$  in the presence of various concentrations of AK. Sample conditions: (a) 7.0  $\mu\text{mol}$  of  $[\beta\text{-}^{17}\text{O}_2]\text{ATP}$  in 0.20 mL of 100 mM Hepes buffer, pH 7.9, containing 14  $\mu\text{mol}$  of EDTA; (b–f) addition of 2.94 mg of AK (0.14  $\mu\text{mol}$ ) in 0.132 mL of 100 mM Hepes buffer (pH 7.9).  $^{17}\text{O}$ -depleted water (10%  $^{17}\text{O}$  relative to natural abundance) was used in all cases. Spectral parameters: spectral width 25 000 Hz, acquisition time 41 ms, acquisition delay 20 ms, receiver gate 40  $\mu\text{s}$ . The  $T_1$  inversion-recovery program was used to suppress the solvent signal ( $180^\circ = 51 \mu\text{s}$ ,  $90^\circ = 25.5 \mu\text{s}$ , recovery time 4.5 ms). Temperature was 20  $^\circ\text{C}$ , line broadening 100 Hz, no. of transients 12 000–25 000. The instrument and probe have been described elsewhere.<sup>19</sup>



**Figure 2.** Dependence of  $^{17}\text{O}$  NMR line widths,  $\Delta\text{O}$ , on the ratio  $P = [\text{AK}]/[\text{nucleotide}]$ . The conditions and spectral parameters are similar to Figure 1. The  $^{17}\text{O}$ -labeled nucleotides were available from our previous work,<sup>19</sup> with all  $^{17}\text{O}$  label at nonbridging positions of the phosphate groups. The  $\Delta\text{O}$  values have not been corrected for exponential multiplication (100 Hz) and field inhomogeneity (20 Hz).

The  $^{17}\text{O}$  line width ( $\Delta\text{O}$ ) in the extreme narrowing limit ( $\omega^2\tau_c^2 \ll 1$ ) can be expressed by<sup>7</sup>

$$\Delta\text{O} = \frac{1}{\pi T_2} = \frac{1}{\pi T_1} = \frac{12\pi}{125} \left( 1 + \frac{\eta^2}{3} \right) \left( \frac{e^2qQ}{h} \right)^2 \tau_c \quad (1)$$

where  $\omega$  is the angular frequency of  $^{17}\text{O}$ ,  $\tau_c$  is the rotational correlation time, and  $\eta$  and  $e^2qQ/h$  are the asymmetry parameter and the quadrupolar coupling constant, respectively, of the  $^{17}\text{O}$  nucleus. When a small percentage ( $P$ ) of an  $^{17}\text{O}$ -labeled nucleotide is bound to an enzyme, the observed line width is given by<sup>8–10</sup>

(1) This work was supported by research Grant GM 29041 from NIH. M.-D.T. is an Alfred P. Sloan Fellow, 1983–1985. Abbreviations: ADP, adenosine 5'-diphosphate; AK, adenylate kinase; AMP, adenosine 5'-monophosphate; ATP, adenosine 5'-triphosphate; EDTA, ethylenediaminetetraacetate; GTP, guanosine 5'-triphosphate; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*-2-ethanesulfonic acid; PPP, triphosphate;  $T_1$ , spin-lattice relaxation time;  $T_2$ , spin-spin relaxation time.

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$$\Delta O = \frac{1}{\pi T_{2,\text{obsd}}} = \frac{1}{\pi} \left( \frac{1}{T_{2f}} + \frac{P}{T_{2b} + \tau} \right) \quad (2)$$

Depending on the relative values of the lifetime of the bound nucleotide ( $\tau$ ) and the transverse relaxation times of free and bound nucleotides ( $T_{2f}$  and  $T_{2b}$ , respectively), plots of  $\Delta O$  vs.  $P$  may allow calculation of  $T_{2b}$  or  $\tau$ .

Figure 1 shows the  $^{17}\text{O}$  NMR signal of  $[\beta\text{-}^{17}\text{O}_2]\text{ATP}$  titrated with small percentages of AK purified from porcine muscle.<sup>11</sup> The successive line broadening is consistent with eq 2, whereas the Lorentzian line shapes suggest that the broadening is not due to chemical exchange and that the condition  $\omega^2\tau_c^2 \ll 1$  is met.<sup>8</sup> Figure 2 shows the plots of  $\Delta O$  vs.  $P$  for  $[\gamma\text{-}^{17}\text{O}_3]\text{ATP}$ ,  $[\beta\text{-}^{17}\text{O}_2]\text{ATP}$ , and  $[\alpha\text{-}^{17}\text{O}_2]\text{ATP}$ . In all cases the  $\Delta O$  increases upon the first addition of AK due to an increased viscosity. With further addition of the enzyme solution, the change in viscosity is less significant,<sup>12</sup> yet the signals of all three samples of ATP (without  $\text{Mg}^{2+}$ ) show an approximately linear increase in  $\Delta O$ . The result of  $[\gamma\text{-}^{17}\text{O}_3]\text{ATP}$  has been confirmed by titrating an enzyme solution with a negligible volume of ATP solutions, which rules out the viscosity change as the predominant factor in causing the line broadening. At the end of each experiment, neither hydrolysis of ATP nor deactivation of AK was detectable.

The slopes of the plots of the  $\Delta O$  of ATP represent  $1/\pi(T_{2b} + \tau)$ . The fact that the slopes are different for  $\alpha$ -,  $\beta$ -, and  $\gamma$ - $^{17}\text{O}$  of ATP suggests that  $T_{2b}$  instead of  $\tau$  is the predominant factor in causing the line broadening; i.e.,  $\tau \ll T_{2b}$ . The slopes are 2500, 1950, and 900 Hz for  $\alpha$ -,  $\beta$ -, and  $\gamma$ - $^{17}\text{O}$  of ATP, respectively. The  $\Delta O_f$ , obtained from the signals of free ATP after correcting for 100 Hz of line broadening and 20 Hz of inhomogeneity, is 690, 570, and 355 Hz for  $\alpha$ -,  $\beta$ -, and  $\gamma$ - $^{17}\text{O}$  of ATP, respectively. Although the slopes may be considered as  $\Delta O_b$ , it is more accurate to obtain  $\Delta O_b$  by extrapolating the linear curves in Figure 2 to 100% AK, particularly when  $\Delta O_f$  is not negligible compared to  $\Delta O_b$ . The  $\Delta O_b$  values thus obtained (after correction) are 3200, 2520, and 1250 Hz for  $\alpha$ -,  $\beta$ - and  $\gamma$ - $^{17}\text{O}$  of ATP, respectively.

If it is assumed that the differences between  $\Delta O_f$  and  $\Delta O_b$  are mainly due to changes in  $\tau_c$ , the results suggest the  $\tau_c$  of the  $^{17}\text{O}$  of ATP increases by only a factor of 3–5 upon binding to AK. The values could be as high as 10–20 if there is a decrease in  $e^2qQ/h$  due to H-bonding or other reasons.<sup>13,14</sup> Since the increase in  $\tau_c$  should be in the order of  $10^2$  if binding is rigid,<sup>15</sup> the triphosphate moiety of ATP most likely has appreciable internal rotational freedom in the AK–ATP binary complex. This is consistent with the previous finding that the  $^{31}\text{P}$  NMR properties of ATP without ( $\text{Mg}^{2+}$ ) change only slightly upon binding with AK<sup>6a</sup> and suggests that the phosphate moiety of bound ATP may interact only weakly with the enzyme in the absence of  $\text{Mg}^{2+}$ .<sup>16</sup>

A major concern in the use of  $^{17}\text{O}$  NMR in macromolecular systems is that the simple eq 1 is no longer valid when  $\omega\tau_c \gg$

1.<sup>8,10b,17</sup> This seems to be less of a problem for small enzymes such as AK. The upper limit of  $\omega\tau_c$  is ca. 1 at 40 MHz if the substrate assumes the same  $\tau_c$  as the enzyme molecule (15–20 ns).<sup>18</sup> In many cases,<sup>3</sup> such as the one described in this paper, the bound substrate can assume a certain degree of rotational freedom (relative to the enzyme) and justifies the use of eq 1. The requirement that is hard to meet seems to be the “fast exchange limit” ( $\tau \ll T_{2b}$ ). Although it is the case for ATP, we have found that the  $\Delta O$  of  $[\gamma\text{-}^{17}\text{O}_3]\text{MgATP}$  and  $[\text{O}_3]\text{AMP}$  are less sensitive to additions of AK, which suggests that the latter two cases are probably in the “slow exchange limit” due to a long  $\tau$ , a short  $T_{2b}$ , a large chemical shift change,<sup>8,9</sup> or a combination of these.

(9) As pointed out in ref 8, another requirement for eq 2 to be valid is that the difference in chemical shift between the free and the bound state ( $\Delta\delta$ ) is less than  $1/T_{2b}$ . The  $^{17}\text{O}$  chemical shifts of nucleotides rarely change by  $>50$  ppm.<sup>10</sup>

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(12) A small volume of ATP solution was used in order to minimize changes in viscosity. According to the sample conditions described in Figure 1, the concentrations of AK at 2% and 10% (relative to ATP) were 0.42 and 0.82 mM, respectively. Thus, the viscosity change should show a “quantum increase” at 2% AK and become negligible later.

(13) The nuclear quadrupolar coupling constant of  $^{17}\text{O}$  could decrease for two possible reasons. The first is hydrogen bonding between the phosphate moiety of ATP and the active site residues. A model for the H-bonding effect is that the  $e^2qQ/h$  value for  $\text{H}_2^{17}\text{O}$  is 10.17 MHz in the gas phase and 6.525 MHz in ice.<sup>14a</sup> The effect of H-bonding on the quadrupolar coupling constant of  $^{17}\text{O}$  has been studied in detail.<sup>14b</sup> The second possible reason is that the charges on the phosphate of ATP are localized (to  $\text{O}=\text{P}-\text{O}^-$ ) due to ionic interactions with positive residues at the active site and that only the signal due to  $\text{P}=\text{O}$  is being observed (the other,  $\text{P}-\text{O}^-$ , may not be observable due to much shorter relaxation times). A model for this is  $(\text{PhO})_2\text{PO}$ , where the  $e^2qQ/h$  values are 3.825 MHz for  $\text{P}=\text{O}$  and 9.176 MHz for  $\text{P}-\text{O}-\text{Ph}$  (average of three values<sup>14c</sup>). The  $e^2qQ/h$  values for  $\text{KH}_2\text{PO}_4$  are 4.85–5.96 MHz.<sup>14d</sup>

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(16) The dissociation constants of the AK complexes with ATP, GTP, and PPP (without  $\text{Mg}^{2+}$ ) are 35, 1200, and 240  $\mu\text{M}$ , respectively.<sup>6c</sup> This suggests that the adenine moiety is important in binding. The triphosphate also binds to AK, but it is not known whether its binding is limited to the ATP site or it crosses both AMP and ATP sites.

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