

## Mechanism of Adenylate Kinase. Demonstration of a Functional Relationship between Aspartate 93 and $Mg^{2+}$ by Site-Directed Mutagenesis and Proton, Phosphorus-31, and Magnesium-25 NMR<sup>†</sup>

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**ABSTRACT:** Earlier magnetic resonance studies suggested no direct interaction between  $Mg^{2+}$  ions and adenylate kinase (AK) in the AK·MgATP (adenosine 5'-triphosphate) complex. However, recent NMR studies concluded that the carboxylate of aspartate 119 accepts a hydrogen bond from a water ligand of the bound  $Mg^{2+}$  ion in the muscle AK·MgATP complex [Fry, D. C., Kuby, S. A., & Mildvan, A. S. (1985) *Biochemistry* 24, 4680–4694]. On the other hand, in the 2.6-Å crystal structure of the yeast AK·MgAP<sub>5</sub>A [*P*<sup>1</sup>,*P*<sup>5</sup>-bis(5'-adenosyl)pentaphosphate] complex, the  $Mg^{2+}$  ion is in proximity to aspartate 93 [Egner, U., Tomasselli, A. G., & Schulz, G. E. (1987) *J. Mol. Biol.* 195, 649–658]. Substitution of Asp-93 with alanine resulted in no change in dissociation constants, 4-fold increases in  $K_m$ , and a 650-fold decrease in  $k_{cat}$ . Notable changes have been observed in the chemical shifts of the aromatic protons of histidine 36 and a few other aromatic residues. However, the results of detailed analyses of the free enzymes and the AK·MgAP<sub>5</sub>A complexes by one- and two-dimensional NMR suggested that the changes are due to localized perturbations. Thus it is concluded that Asp-93 stabilizes the transition state by ca. 3.9 kcal/mol. The next question is *how*. Since proton NMR results indicated that binding of  $Mg^{2+}$  to the AK·AP<sub>5</sub>A complex induces some changes in the proton NMR signals of WT but not those of D93A, the functional role of Asp-93 should be in binding to  $Mg^{2+}$ . We then asked whether disruption of the interaction between  $Mg^{2+}$  and Asp-93 affects the interaction between  $Mg^{2+}$  and the nucleotide at the active site of AK. Analysis by <sup>31</sup>P NMR suggested that  $Mg^{2+}$  orients the conformation of the polyphosphate chain of bound AP<sub>5</sub>A in WT but not in D93A. These results raised the question of whether  $Mg^{2+}$  could bind to D93A-nucleotide complexes, which was then probed by <sup>25</sup>Mg NMR. The results suggest that  $Mg^{2+}$  does bind to the D93A·AP<sub>5</sub>A complex, but possibly only weakly. The weaker affinity of  $Mg^{2+}$  was also confirmed by kinetic analysis. However, the low activity of D93A cannot be restored by higher concentrations of  $Mg^{2+}$  ions. Thus Asp-93 plays critical roles in the binding and function of  $Mg^{2+}$  ions during the catalysis by AK. Substitution of Asp-93 with alanine also lowered the  $pK_a$  of His-36, which supports the proximity between Asp-93 and His-36. However, disruption of the interaction between Asp-93 and His-36 did not affect the conformational stability of the enzyme.

Although  $Mg^{2+}$  is required in the catalysis by adenylate kinase (AK),<sup>1,2</sup> the specific roles of  $Mg^{2+}$  and its interaction with the enzyme have not been established. Early magnetic relaxation studies have led to the classification of the enzymes requiring metal ions in catalysis into three types of coordination schemes: substrate bridge (E–S–M), metal bridge (E–M–S or E–M–S), and enzyme bridge (S–E–M) (Mildvan & Cohn, 1970). Muscle AK was classified in the first category since there was no evidence for direct binding of  $Mg^{2+}$  to AK (Mildvan & Cohn, 1970; Mildvan, 1979; and references therein). However, the metal ion has been suggested to be close to His-36 on the basis of paramagnetic relaxation effects between the C2-H of His-36 and MnATP (McDonald et al., 1975) or CrATP (Smith & Mildvan, 1982). Later an aspartate residue has been suggested to be interacting with  $Mg^{2+}$ , directly or indirectly, on the basis of structural studies: in the "NMR model" proposed by Mildvan and co-workers, Asp-119 has been suggested to accept an H-bond from a water ligand

of the bound  $Mg^{2+}$  ion in the muscle AK·MgATP complex (Fry et al., 1985; Mildvan & Fry, 1987); in the 2.6-Å crystal structure of the yeast AK·MgAP<sub>5</sub>A complex, Asp-93 has been shown to be in proximity to the  $Mg^{2+}$  ion, but it was unclear whether  $Mg^{2+}$  binds directly to the carboxyl group or via one or more water molecules (Egner et al., 1987; Dreusicke et al., 1988).

It has been shown that substitution of Asp-119 with an asparagine residue resulted in little perturbations in the kinetic parameters (Kim et al., 1990). This result disfavors the NMR

<sup>1</sup> Abbreviations: ADP, adenosine 5'-diphosphate; AK, adenylate kinase; AMP, adenosine 5'-monophosphate; AP<sub>5</sub>A, *P*<sup>1</sup>,*P*<sup>5</sup>-bis(5'-adenosyl)pentaphosphate; ATP, adenosine 5'-triphosphate; 1D, one-dimensional; 2D, two-dimensional; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetate; FID, free induction decay; GDP, guanosine 5'-diphosphate; GTP, guanosine 5'-triphosphate; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser enhanced spectroscopy; PAGE, polyacrylamide gel electrophoresis. SDS, sodium dodecyl sulfate; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; WT, wild-type.

<sup>2</sup> All of the muscle AKs are type-1 AK, with  $M_r$  21 700, and are >80% homologous (Kishi et al., 1986). Other types of AK have different sizes and are less homologous (Schulz, 1987). Unless otherwise specified, the numbering system used in this paper is the conventional system for AK1. Although chicken muscle AK (cAK) has one additional residue near the N-terminal (Kishi et al., 1986), the Met-1 residue is absent in the cAK expressed in *E. coli* (Tanizawa et al., 1987). This makes numbering of cAK consistent with other AK1.

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model but does not disprove it since asparagine can also accept an H-bond. The results of other site-directed mutagenesis studies also concluded that the NMR model requires serious revisions (Tian et al., 1988, 1990; Yan et al., 1990a,b). In this paper we report that substitution of Asp-93 by alanine resulted in a significant decrease in  $k_{cat}$  and in the inability of  $Mg^{2+}$  to orient the phosphate groups of bound  $MgAP_5A$ . Our system is chicken muscle  $AK^2$  overproduced in *Escherichia coli* (Kishi et al., 1986; Tanizawa et al., 1987). The results are consistent with the X-ray structure of the yeast  $AK \cdot MgAP_5A$  complex and suggest that Asp-93 plays critical roles in the catalytic function of  $Mg^{2+}$  during the catalysis by AK.

#### MATERIALS AND METHODS

**Materials.** The *E. coli* expression system for chicken AK was kindly provided by Dr. Nakazawa (Tanizawa et al., 1987). The oligonucleotide used for the construction of D93A AK, TTCTCATTGCCGGCTACCCT, was obtained from the Biochemical Instrument Center of The Ohio State University. Mutagenesis and DNA sequencing kits were purchased from United States Biochemicals and Amersham, respectively. Reagents and coupling enzymes for kinetics were obtained from Sigma. Perdeuterated Tris was purchased from MSD Isotopes.  $^{25}MgO$  (98 atom %  $^{25}Mg$ ) was obtained from Oak Ridge National Laboratory, and a stock solution of 0.25 M  $^{25}Mg^{2+}$  was prepared by dissolving the oxide in perchloric acid followed by adjustment to pH 7 with NaOH and determination of the exact concentration by atomic absorption.

**Site-Specific Mutagenesis and Purification of the Mutant Enzyme.** The D93A mutant enzyme was constructed by the phosphorothioate analogue method of Taylor et al. (1985a,b) with an Amersham mutagenesis kit. Since the mutation efficiency was very high, the mutant was selected by DNA sequencing according to Sanger's dideoxy method using a DNA sequencing kit from United States Biochemicals. The mutant enzyme was purified and assayed essentially as described by Tian et al. (1988), except that a 50–300 mM linear NaCl gradient was used for eluting the phosphocellulose column. The purity of each preparation was checked by SDS-PAGE with silver staining on a PhastSystem.

**Steady-State Kinetics.** The kinetic experiments were carried out at 30 °C and pH 8.0 by measuring ADP formation with pyruvate kinase/lactic dehydrogenase as the coupling system (Rhoads & Lowenstein, 1968). The details have been described previously (Tian et al., 1988). The kinetic parameters were obtained by varying both  $MgATP$  and AMP concentrations and the data analyzed according to the equation of Cleland (1986). The  $K$  and  $K_i$  values (Michaelis and dissociation constants, respectively) obtained from such analysis are close to the  $K_m$  values (measured by saturating one substrate) and the  $K_d$  values (determined by titration studies with NMR), respectively (Sanders et al., 1989; Tian et al., 1990).

**$^1H$  NMR Methods.** All  $^1H$  NMR experiments were performed on Bruker AM-500 spectrometers. One- and two-dimensional proton NMR experiments were obtained at 25 °C, pH 7.8, as described previously (Yan et al., 1990b). For pH titration experiments, the pH was adjusted by addition of  $^2HCl$  and  $NaO^2H$ , and all pH measurements were made at room temperature. The  $pK_a$ s of histidine residues were obtained by nonlinear least-squares fitting of the chemical shifts of C2-H resonances as a function of pH according to a modified form of the Hill equation (Markley, 1974), with the Hill coefficient held at 1. It should be noted that the pH values were direct pH meter readings, and the  $pK_a$ s obtained have not been corrected for the deuterium isotope effect.

Table I: Comparison of Kinetic, Binding, and Conformational Properties between WT and D93A<sup>a</sup>

parameter	unit	WT	D93A
Steady-State Kinetics			
$k_{cat}$	(s <sup>-1</sup> )	650	1
$K_{(MgATP)}$	(mM)	0.042	0.16
$K_{(AMP)}$	(mM)	0.098	0.47
$k_{cat}/K_{(MgATP)}$	(s <sup>-1</sup> M <sup>-1</sup> )	$1.55 \times 10^7$	$6 \times 10^3$
$k_{cat}/K_{(AMP)}$	(s <sup>-1</sup> M <sup>-1</sup> )	$0.66 \times 10^7$	$2 \times 10^3$
$K_{i(MgATP)}$	(mM)	0.16	0.11
$K_{i(AMP)}$	(mM)	0.37	0.33
Titration Experiments with NMR			
$K_{d(ATP)}$	(mM)	0.044	0.12
$K_{d(MgATP)}$	(mM)	0.17	0.37
$K_{d(AMP)}$	(mM)	0.50	0.25
Conformational Stability <sup>b</sup>			
$\Delta G_d^{H_2O}$	(kcal/mol)	4.5	4.0
$m$	(kcal/mol M)	5.7	5.6
Chemical Shifts of Bound $AP_5A^c$			
$H_2$ , with $Mg^{2+}$		8.98(I), 8.32(II)	8.99(I), 8.31(II)
no $Mg^{2+}$		8.92(I), 8.29(II)	8.99(I), 8.31(II)
$H_8$ , with $Mg^{2+}$		8.47(I), 8.40(II)	8.65(I), 8.45(II)
no $Mg^{2+}$		8.50(I), 8.43(II)	8.65(I), d
$H_{1'}$ , with $Mg^{2+}$		6.07(II), 5.93(I)	6.06(II), 5.89(I)
no $Mg^{2+}$		6.07(II), 5.88(I)	6.08(II), 5.88(I)

<sup>a</sup>The kinetic data for WT are from Tian et al. (1990), the  $K_d$  values for WT from Sanders et al. (1989), and the chemical shift data for WT from Yan et al. (1990a). <sup>b</sup>Determined by monitoring guanidine-HCl-induced unfolding with circular dichroism (T. Dahnke, unpublished results). The  $\Delta G_d^{H_2O}$  and  $m$  values were calculated as described in Pace (1986). <sup>c</sup>The sites I and II of the  $MgAP_5A$  complexes have been assigned to the AMP and the  $MgATP$  sites, respectively (Yan et al., 1990b). <sup>d</sup>This resonance is not resolved, possibly overlapped with the free  $H_8$  (8.42 ppm).

**$^{31}P$  NMR Methods.**  $^{31}P$  NMR spectra were recorded at 121.496 MHz and 25 °C on a Bruker MSL-300 NMR spectrometer. Samples were in 10-mm NMR tubes and contained 1 mM AK and 1 mM  $AP_5A$  in a 75 mM Tris-HCl buffer (pH 8.0) containing 65 mM KCl, 1 mM DTT, 0.5 mM EDTA, 20%  $^2H_2O$ , and the specified concentration of  $MgCl_2$ . A 70° pulse and a 5-s relaxation delay were used in data acquisition. All spectra were broad-band proton-decoupled with the WALTZ sequence. The spectral width was 40 ppm, and 8K data points were recorded for each spectrum in the quadrature detection mode. A 10-Hz line broadening was applied before Fourier transformation. All chemical shifts were referenced to external 85%  $H_3PO_4$  at 25 °C.

**$^{25}Mg$  NMR Methods.**  $^{25}Mg$  NMR spectra were acquired at 18.374 MHz without sample spinning on a Bruker MSL-300 spectrometer. The samples were in the same buffer as for  $^{31}P$  NMR experiments. The experiments proceeded by titrating 2.2 mL of 1 mM  $^{25}Mg^{2+}$  solution (prepared from the 0.25 M stock solution) with AK (6.3 mM) and/or  $AP_5A$  (63 mM) stock solutions. The RIDE pulse sequence was used to reduce the effects of acoustic ringing (Ellis, 1983). The 90° pulse was 30 ms, the spectral width was 2000 Hz, the acquisition time was 256 ms, and the number of transients was 200 800. A Lorentzian line broadening of 1 or 15 Hz and two left shifts were applied to the time domain data prior to Fourier transformation.

#### RESULTS AND DISCUSSION

**Kinetic Properties of D93A.** The steady-state kinetic parameters of D93A, along with those of WT, are listed in Table I. The data were obtained by a full initial velocity analysis varying both substrates (Cleland, 1986). As shown in Table I, replacement of Asp-93 with an alanine residue resulted in virtually no changes in  $K_i$  and small increases (ca. 4-fold) in

#### $Mg^{2+}$ Binding to Adenylate Kinase

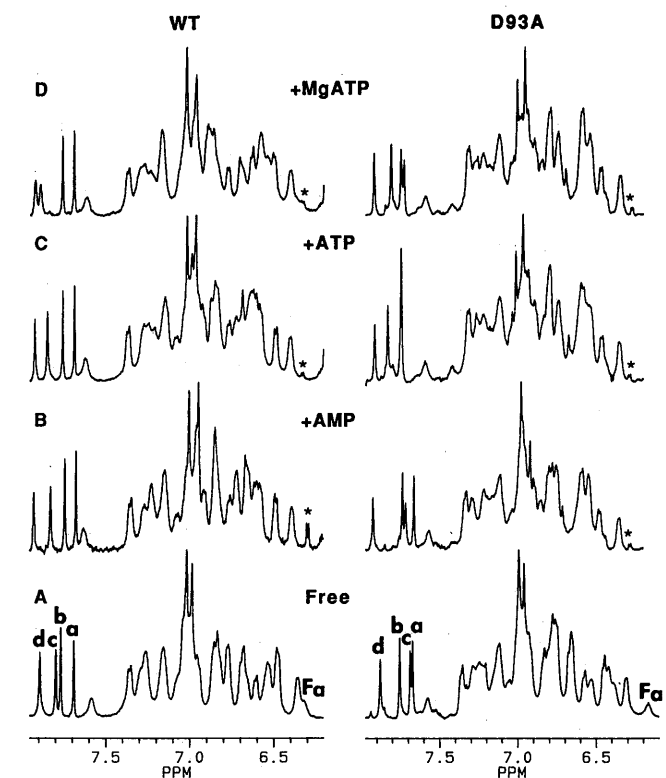


FIGURE 1: One-dimensional proton NMR spectra of the aromatic protons of WT (left panel) and D93A (right panel). The spectra of WT (the left panel) are reproduced from Sanders et al. (1989), except that the spectrum of the AMP complex shown is with larger excess of AMP (ca. 20 mM). The conditions for the right panel are as follows: (A) 0.9 mM D93A; (B) 0.9 mM D93A + 13.5 mM AMP; (C) 1.0 mM D93A + 12.4 mM ATP; (D) 1.5 mM D93A + 12.4 mM ATP + 15.0 mM  $MgCl_2$ . The FIDs were processed with 1-Hz exponential line broadening. The resonance Fa has been shifted upfield to outside of the spectral range in all complexes. Peaks c and d have been assigned to His-36 and His-30, respectively (Yan et al., 1990a). The sharp doublets at 6.3 ppm, labeled with “\*”, arise from the  $^{13}C$  satellites of the C1'-H of the excess nucleotides.

$K_m$  but a large decrease in  $k_{cat}$  (650-fold). Since the kinetic mechanism of AK is random Bi Bi (Rhoads & Lowenstein, 1968) and the chemical step is nearly rate-limiting for WT (Tian et al., 1990),  $K_i$  and  $K_m$  are close to the true dissociation constants of the binary and ternary complexes, respectively. As suggested and demonstrated in our previous studies (Yan et al., 1990a,b), it is necessary to perform structural analysis of the mutant enzyme in the free form and in binary and ternary complexes in order to interpret the kinetic data properly.

**$^1H$  NMR Analysis of Free D93A.** The one-dimensional proton NMR spectra of free D93A and free WT are shown in Figure 1A. Two notable differences are the upfield shifts of the ring C2-H of His-36 (peak c) and the most upfield aromatic resonance (peak Fa). Smaller shifts in other resonances can also be observed. In order to determine whether the chemical shift changes of the two resonances reflect a local structural perturbation or global conformational changes, we performed 2D experiments and assigned the chemical shifts of the aromatic spin systems of D93A in analogy to the previous assignment of WT (Yan et al., 1990a). Analysis of the NOESY spectrum of D93A (Figure 2) indicates that the patterns of the aromatic-aromatic and aromatic-aliphatic interresidue NOEs are similar but not identical to those of WT (Yan et al., 1990a). In the aromatic region the three sets of NOE cross-peaks observed for WT are also present in D93A (peaks a, b, and c in Figure 2). The latter exhibits additional

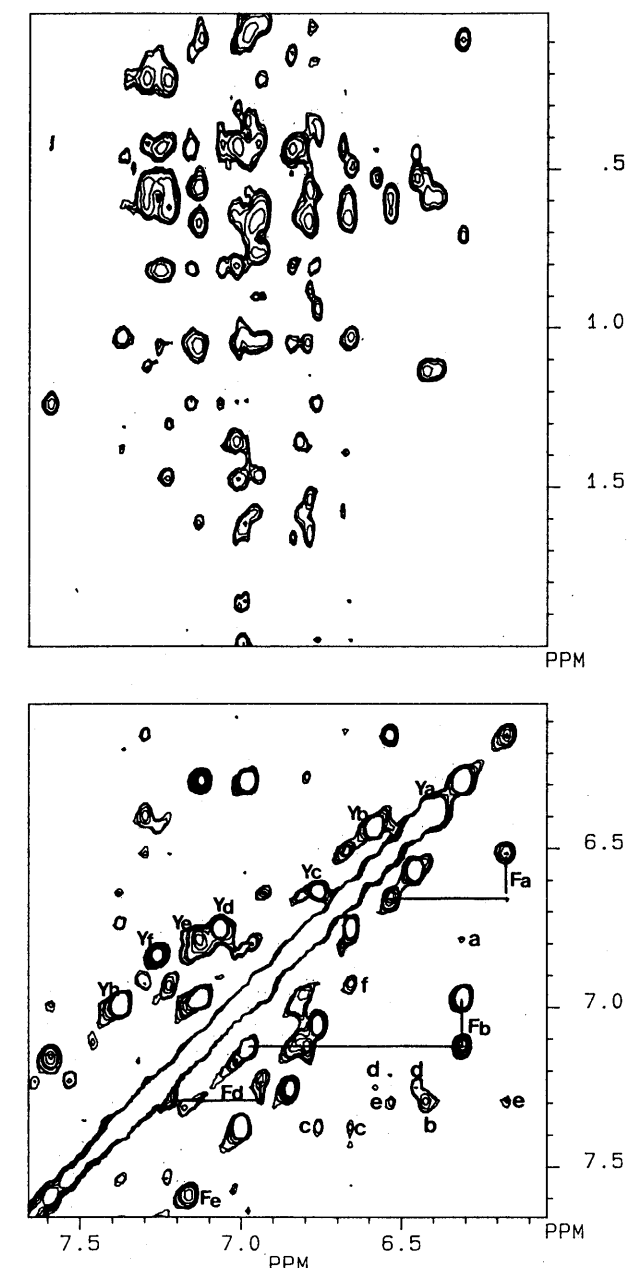


FIGURE 2: NOESY spectrum of free D93A. Cross-peaks a, b, and c have also been observed in WT (Yan et al., 1990a) and can be assigned tentatively to the interresidue NOEs of Fb/Ye, Fd/Ya, and Yc/Yh, respectively. Cross-peaks d, e, and f can be tentatively assigned to Yb/Yf, Fa/Fd, and Fd/Yc, respectively.

NOE cross-peaks, but this does not necessarily suggest a change in conformation since it could be simply due to a better sensitivity in the experiment. Quantitatively, the chemical shifts of the aromatic spin systems of D93A show appreciable changes in only three residues: Fa (6.17, 6.53, and 6.68 ppm, compared to 6.31, 6.51, and 6.62 ppm in WT), Ya (6.38 and 6.42 ppm, compared to 6.41 and 6.49 ppm in WT), and His-36 [7.24 (tentative) and 7.70, compared to 7.20 and 7.78 in WT]. Thus, although there are substantial perturbations in the 1D spectrum of D93A, the effects appear to be largely localized, and the largest change is only -0.14 ppm.

There is no obvious explanation to the relatively large perturbation in Fa, except to say that the aromatic resonances of this residue have been very sensitive to site-specific mutations and to binding of substrates or inhibitors (Sanders et al., 1989; Yan et al., 1990a,b). The perturbation in His-36 is rationalized in the following section.

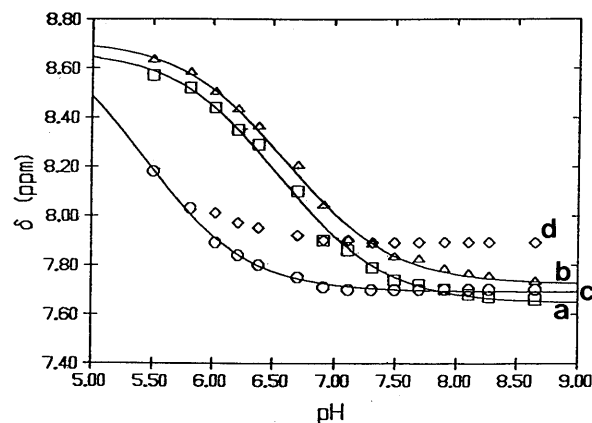


FIGURE 3: Titration curves of the C2-H chemical shifts of the histidine residues in D93A. Curves a–d correspond to His-a, His-b, His-c and His-d, respectively, in Figure 1 and in Yan et al. (1990a) (His-c and His-d correspond to H36 and H30, respectively) but correspond to c, b, a, and d, respectively, in Tian et al. (1988).

**Relationship between Asp-93 and His-36.** The perturbation in His-36 can be attributed to direct interactions between the carboxylate of Asp-93 and the imidazole ring of His-36: in the 2.1-Å crystal structure of porcine muscle AK (at neutral pH), one of the carboxylate oxygen of Asp-93 (OD1) receives an H-bond from the N3-H of His-36 (Dreusicke et al., 1988). This interpretation is further supported by the perturbation in the  $pK_a$  of His-36. Figure 3 shows the nonlinear least-squares fittings of the pH titration data of the histidine residues in D93A. For His-a and His-b, the  $pK_a$  values (6.55 and 6.60, respectively) are close to the corresponding values of WT (6.42 for both) and the  $\delta_{H^+}$  and  $\delta_{H^-}$  values (chemical shifts of the protonated and unprotonated species, respectively) are essentially unchanged. The  $pK_a$  of His-30 (curve d) is also unusually low (not measurable) as in the case of WT. The  $pK_a$  of His-36 is normal in WT (6.23) but becomes significantly lower in D93A (<5.5 as estimated from the incomplete curve c in Figure 3), which can be accounted for by stabilization of the positive charge of protonated His-36 by Asp-93 in WT. According to Zimmerman and Cramer (1988), the carboxylate of a syn-oriented Asp-His couple (which appears to be the case for AK on the basis of the crystal structure) can increase the  $pK_a$  of the His by as much as 1.5 pH unit.

We have previously shown that substitution of His-36 by Gln, Asn, or Gly resulted in decreases in the conformational stability of AK and suggested that His-36 is involved in conformational stabilization by its interaction with Cys-25 and/or Asp-93 (Tian et al., 1988). In the crystal structure of porcine muscle AK, the same oxygen of Asp-93 (OD1) receiving an H-bond from His-36 is also H-bonded to the thiol group of Cys-25 (Derusicke et al., 1988). Thus one might expect the D93A mutant to be very unstable. However, the data in Table I show that the free energy of unfolding,  $\Delta G_D^{H_2O}$ , is identical between D93A and WT within experimental errors. This observation is quite unexpected and suggests that the instability of His-36 mutants is likely to be due to exposure of the thiol group of Cys-25 rather than disruption of the interaction between His-36 and Asp-93.

**$^1H$  NMR Analysis of Binary Complexes.** In order to assess the effects of substituting Asp-93 with Ala and the accompanying perturbations in H36 and Fa on the binding of substrates, titration experiments were performed by monitoring the  $^1H$  chemical shifts of AK while varying the concentrations of AMP, ATP, or MgATP. The spectra of the final points of titration are shown in Figure 1 (B, C, and D, respectively). The dissociation constants  $K_d$  obtained by nonlinear least

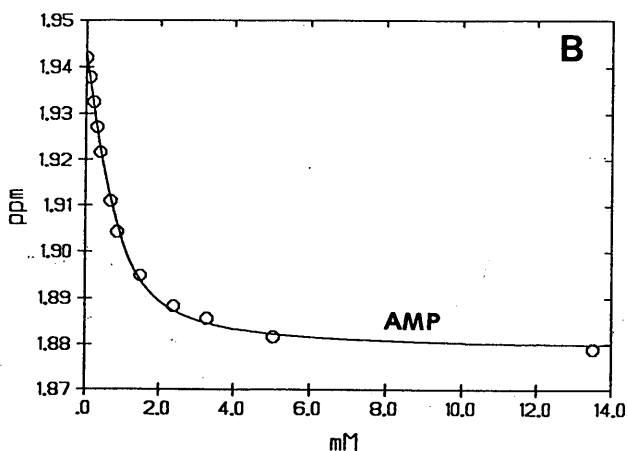
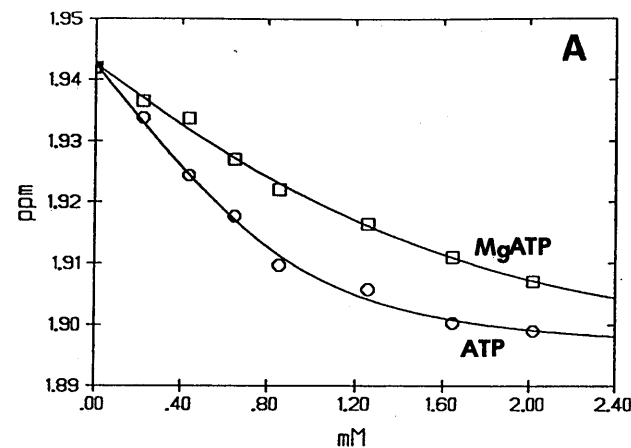


FIGURE 4: Chemical shift curves from titrations of D93A with MgATP and ATP (A) and AMP (B). Solid lines represent 1:1 nonlinear least-squares fittings of the titration data. The chemical shifts on the y-axes are those of the most upfield  $CH_3$  resonance of methionines.

squares fitting of the titration data are listed in Table I, and representative fitting curves are shown in Figure 4. As shown by the data in Table I, the  $K_d$  values of the binary complexes of D93A are close to the corresponding values of WT, which agrees with the lack of perturbation in the  $K_i$  values obtained from steady-state kinetics.

The chemical shifts of the adenine  $H_2$ ,  $H_8$ , and adenosine  $H_1$  protons of bound nucleotides have been deduced from the titration studies. The differences between the chemical shifts of the nucleotides bound to WT (Yan et al., 1990a) and D93A are  $\leq 0.1$  ppm for all binary complexes. Thus both dissociation constants and chemical shifts of bound nucleotides suggest that the binary complexes of D93A have very similar properties relative to the corresponding binary complexes of WT. However, careful examination of the 1D spectra revealed substantial differences, particularly between WT+AMP and D93A+AMP (Figure 1B). Since perturbations in the ternary complex is even more extensive, we proceeded to analyze the ternary complex in detail as described in the next section.

**$^1H$  NMR Analysis of the D93A+MgAP<sub>5</sub>A Complex.** The ternary complex was characterized by use of a bisubstrate analogue, MgAP<sub>5</sub>A, and the 1D proton NMR spectra are shown in Figure 5 (A and C). Since appreciable differences exist between the 1D spectra of WT+MgAP<sub>5</sub>A and D93A+MgAP<sub>5</sub>A, we pursued quantitative (or semi-quantitative) comparison using NOESY spectra and aromatic spin systems.

The aromatic spin systems, assigned from 1D and NOESY spectra and from comparison with the previous assignments of WT+MgAP<sub>5</sub>A (Yan et al., 1990a), show appreciable

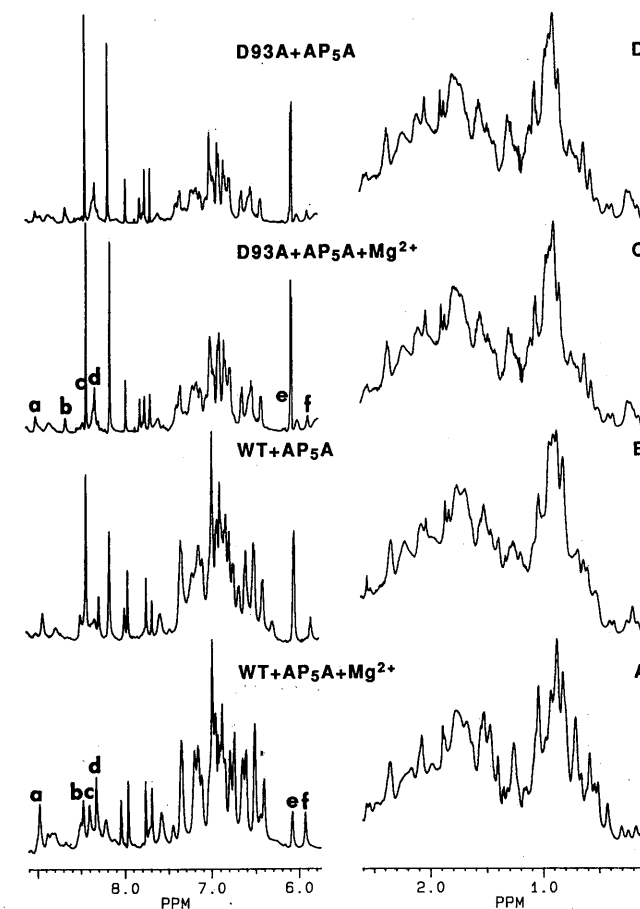


FIGURE 5: One-dimensional proton NMR spectra of various AP<sub>5</sub>A complexes: (A) 2.5 mM WT + 2.5 mM AP<sub>5</sub>A + 5.0 mM MgCl<sub>2</sub>; (B) 2.0 mM WT + 4.0 mM AP<sub>5</sub>A; (C) 1.7 mM D93A + 4 mM AP<sub>5</sub>A + 5 mM MgCl<sub>2</sub>; (D) 1.7 mM D93A + 4 mM AP<sub>5</sub>A. The FIDs were processed with Gaussian multiplication (LB = -5, GB = 0.1). Spectra A and B are reproduced from Yan et al. (1990a). Peaks a–f are due to bound MgAP<sub>5</sub>A: a, H<sub>2</sub>(I); b, H<sub>8</sub>(I); c, H<sub>8</sub>(II); d, H<sub>2</sub>(II); e, H<sub>1</sub>(II); f, H<sub>1</sub>(I). The three sharp and intense resonances in spectra B–D arise from excess AP<sub>5</sub>A.

perturbations in only four residues: Fa (5.99, 6.55, and 6.76 ppm, which are shifted by -0.45, +0.04, and +0.11 ppm, respectively), Xb (6.89 and 7.20, shifted by -0.08 ppm for the first resonance), Ye (6.82 and 7.10, shifted by -0.06 ppm for the first resonance), and H36 (7.80 for H<sub>2</sub>, shifted by -0.24 ppm; unresolved for H<sub>4</sub>). These data suggest that while the perturbations in the chemical shifts of Fa and His-36 become more significant (relative to the perturbations in the free enzyme), the changes in other resonances are minimal. Analysis of the NOESY spectrum of D93A+MgAP<sub>5</sub>A (not shown) suggests that it retains the three major sets of the aromatic-aromatic interresidue NOEs observed in the WT+MgAP<sub>5</sub>A complex (cross-peaks p, q, and r in the Figure 6 of Yan et al., 1990a) and that the NOE patterns in the aromatic-aliphatic regions are qualitatively similar between the WT and the mutant complexes. These results taken together suggest that the two complexes have similar conformations and that the structural perturbation in the MgAP<sub>5</sub>A complex (as a static mimic of the ternary complex) is also localized. Thus the large decrease in  $k_{cat}$  can be attributed to stabilization of the transition state by the carboxylate of Asp-93, by ca. 3.9 kcal/mol.

The chemical shifts of the adenine  $H_2$ ,  $H_8$ , and adenosine  $H_1$  protons of bound AP<sub>5</sub>A have also been assigned and listed in Table I. There are notable differences (0.18 ppm in the presence of Mg<sup>2+</sup>, 0.15 ppm in the absence of Mg<sup>2+</sup>) between D93A and WT in the  $H_8$  resonances of the adenosine I (at

the AMP site, Yan et al., 1990b). The significance of such differences is unclear.

**Asp-93 Mediates the Binding of Mg<sup>2+</sup>.** After demonstrating the catalytic significance of Asp-93 by kinetic and  $^1H$  NMR studies, the question of *how* Asp-93 participates in catalysis follows. Careful examination of the 1D proton NMR spectra revealed that the spectra of D93A+ATP (Figure 1C) and D93A+MgATP (Figure 1D) are very similar and that the spectra of D93A+AP<sub>5</sub>A (Figure 5D) and D93A+MgAP<sub>5</sub>A (Figure 5C) are essentially identical. These are in contrast to the case of WT, where notable differences exist between the complexes with and without Mg<sup>2+</sup>, particularly in the region of 6.4–6.8 ppm for WT+ATP (Figure 1C) and WT+MgATP (Figure 1D) and the regions of 6.3–6.8 ppm and 0.5–2.1 ppm for WT+AP<sub>5</sub>A (Figure 5B) and WT+MgAP<sub>5</sub>A (Figure 5A). The chemical shifts of bound AP<sub>5</sub>A listed in Table I also indicate nearly identical values between D93A+AP<sub>5</sub>A and D93A+MgAP<sub>5</sub>A but detectable differences between WT+AP<sub>5</sub>A and WT+MgAP<sub>5</sub>A. These results suggest that Asp-93 is interacting (possibly directly) with the bound Mg<sup>2+</sup> ion in the AK+MgAP<sub>5</sub>A complex. This interpretation is consistent with the crystallographic observation that Asp-93 is close to the bound Mg<sup>2+</sup> ion in the yeast AK-MgAP<sub>5</sub>A complex (Egner et al., 1987).

The next question, then, is whether (and how) disruption of the interaction between Mg<sup>2+</sup> and Asp-93 affects binding of Mg<sup>2+</sup> to the phosphate groups of AP<sub>5</sub>A. This question was probed by examining the effects of Mg<sup>2+</sup> on the  $^31P$  NMR signals of AP<sub>5</sub>A bound to WT and D93A, as shown in Figure 6. The results for WT are very similar to those of porcine muscle AK reported previously (Nageswara Rao & Cohn, 1977): in the absence of Mg<sup>2+</sup> ions, the NMR spectrum reveals two groups of resonances (at -10.8 and -11.7 and at -21.6, -22.3, and -23.5 ppm); upon addition of Mg<sup>2+</sup> ions, all five resonances become distinct and well resolved (at -10.4, -12.2, -18.5, -22.7, and -25.1 ppm). The spectrum of D93A+AP<sub>5</sub>A is similar to that of WT+AP<sub>5</sub>A (-10.9, -11.1, -20.6, -21.4, and -23.8 ppm). However, addition of Mg<sup>2+</sup> ions to the D93A+AP<sub>5</sub>A solution caused virtually no changes in the NMR spectrum. This is surprising since even in the absence of AK, Mg<sup>2+</sup> induces ca. 1 ppm shifts in the  $^31P$  resonances of AP<sub>5</sub>A (spectra not shown; also Nageswara Rao & Cohn, 1977).

The large differences in chemical shifts in the WT+MgAP<sub>5</sub>A complex relative to the WT+AP<sub>5</sub>A complex could be due to asymmetric binding of the Mg<sup>2+</sup> ion and the resultant conformational changes in the polyphosphate chain of AP<sub>5</sub>A. The inability of Mg<sup>2+</sup> ions to induce changes in the spectrum of the D93A+AP<sub>5</sub>A complex suggests that Asp-93 is required for proper interaction of Mg<sup>2+</sup> with the phosphate groups.

**Can Mg<sup>2+</sup> Bind to the D93A-AP<sub>5</sub>A Complex?** The observation that Mg<sup>2+</sup> ions do not induce any detectable change in either  $^1H$  or  $^31P$  NMR spectra of D93A+AP<sub>5</sub>A led us to suspect whether Mg<sup>2+</sup> ions can bind to the complex at all, even though there is no obvious reason that the binding of Mg<sup>2+</sup> ions with AP<sub>5</sub>A at the active site should be prohibited by substituting Asp-93 with Ala. This problem was probed by  $^{25}Mg$  NMR, and the spectra are shown in Figure 7. The  $^{25}Mg$  nucleus is quadrupolar with  $I = 5/2$ , and the detailed theories in using  $^{25}Mg$  NMR to study Mg<sup>2+</sup> binding to proteins are quite complicated as described previously (Tsai et al., 1987; Sanders & Tsai, 1989). In short, the line width ( $\Delta\nu$ ) is determined by a number of factors: exchange rate, binding constant, relaxation times, quadrupolar coupling constants, and rotational correlation times (of both free and bound forms

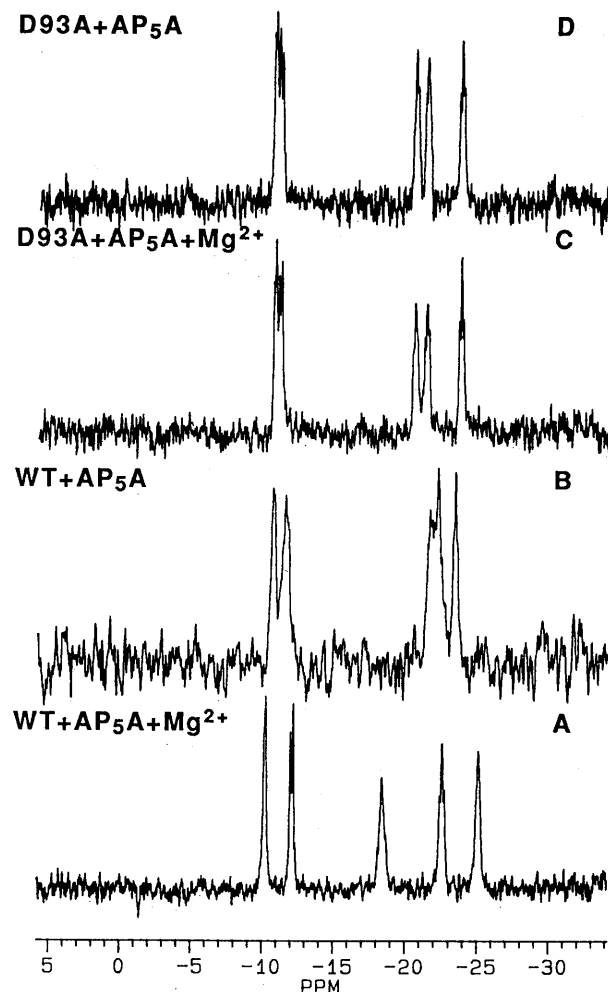


FIGURE 6:  $^{31}\text{P}$  NMR spectra of various  $\text{AP}_5\text{A}$  complexes: (A) 1 mM WT + 1 mM  $\text{AP}_5\text{A}$  + 2 mM  $\text{MgCl}_2$ ; (B) 1 mM WT + 1 mM  $\text{AP}_5\text{A}$ ; (C) 1 mM D93A + 1 mM  $\text{AP}_5\text{A}$  + 2 mM  $\text{MgCl}_2$ ; (D) 1 mM D93A + 1 mM  $\text{AP}_5\text{A}$ . Spectrum C remained unchanged upon further addition of 2 mM  $\text{MgCl}_2$ .

for the latter three factors). The system of AK does not permit complete analysis due to limited stability of the enzyme. However, useful information can be obtained by comparing the behavior of WT and D93A, as described below.

Comparisons between spectra A and B, and between spectra C and D of Figure 7, indicate that addition of the enzyme causes no changes in the line width of the signal within experimental errors. This confirms that the free AK does not bind  $\text{Mg}^{2+}$  and also ensures that the viscosity change due to addition of AK does not induce any appreciable line broadening under our experimental conditions. Comparisons between spectra B and C, and between spectra D and E, indicate that the  $^{25}\text{Mg}$  signal is broadened significantly upon binding to  $\text{WT}+\text{AP}_5\text{A}$ . Comparison between spectra E and F suggests that the overall line-broadening effect is similar between  $\text{WT}+\text{AP}_5\text{A}$  and free  $\text{AP}_5\text{A}$ , which is unusual but not unreasonable since the line width is dependent on several factors as mentioned earlier. With these information in place, we can then examine the behavior of D93A (spectra G to L). Comparison between spectra A and G indicates that 5% of D93A+ $\text{AP}_5\text{A}$  has virtually no detectable effect on the line width of  $\text{Mg}^{2+}$ , which is apparently different from the case of WT where the  $\Delta\nu$  is doubled under the same condition (spectrum C). However, comparison of spectrum A with spectra H, I, and J suggests that the signal is indeed broadened (the  $\Delta\nu$  is doubled) by 40–50% of D93A+ $\text{AP}_5\text{A}$ . The excessive broadening in spectrum K is due to the excess  $\text{AP}_5\text{A}$ , as in-

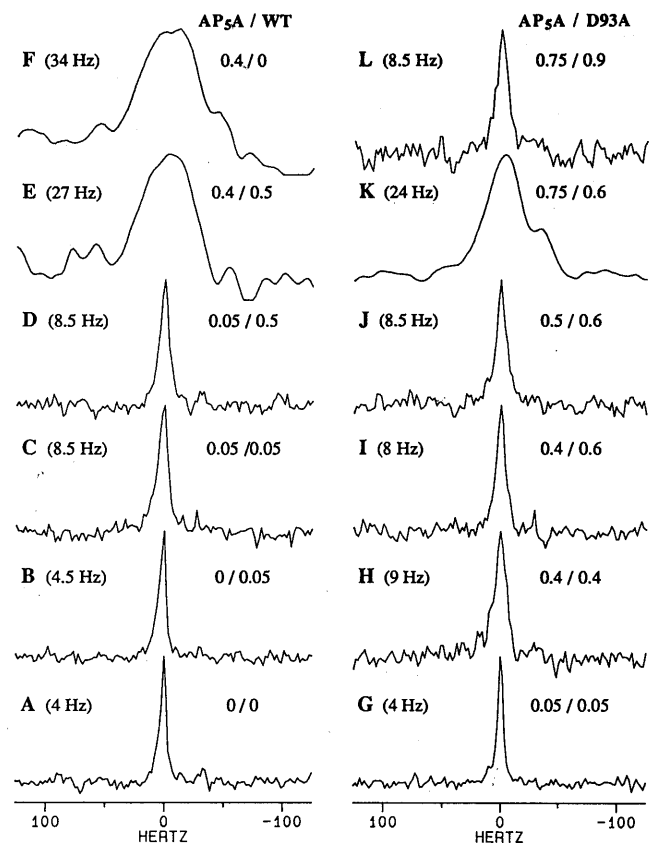


FIGURE 7:  $^{25}\text{Mg}$  NMR spectra of 1 mM  $\text{Mg}^{2+}$  titrated with  $\text{AP}_5\text{A}/\text{WT}$  (left panel) and with  $\text{AP}_5\text{A}/\text{D93A}$  (right panel). The concentrations (in mM) of  $\text{AP}_5\text{A}/\text{WT}$  or  $\text{D93A}/\text{AP}_5\text{A}$  are indicated in each spectrum. The FIDs were processed with 1-Hz exponential multiplication except E, F, and K, which were processed with 15 Hz. The numbers in parentheses are the line widths obtained from line fitting and after correcting for the artificial line broadening.

indicated by the sharpening of signal in spectrum L upon addition of more enzyme to sample K. These results suggest that  $\text{Mg}^{2+}$  ions do bind to the D93A+ $\text{AP}_5\text{A}$  complex, but the binding results in substantially smaller degree of line broadening. If both systems are under the fast exchange and exchange narrowing conditions (which are the most likely conditions judging from all factors), the smaller extent of line broadening in D93A could be due to the combination of several factors: lower affinity, higher rotational freedom, and smaller quadrupolar coupling constant, all of which could result from the lack of coordination between  $\text{Mg}^{2+}$  and Asp-93. If the WT system is in slow or intermediate exchange, the small extent of line broadening in D93A could be due to increased exchange rates in the D93A system, which is also consistent with weaker binding (lower affinity).

**Can the Activity of D93A Be Restored by Higher  $[\text{Mg}^{2+}]$ ?** Since the result of  $^{25}\text{Mg}$  NMR suggests a lower affinity of  $\text{Mg}^{2+}$  ions toward the D93A+ $\text{AP}_5\text{A}$  complex, one may ask whether the activity of D93A can be restored by higher concentrations of  $\text{Mg}^{2+}$  ions. There was a physical evidence against such a possibility: as stated in the legend of Figure 6, further addition of  $\text{Mg}^{2+}$  to the spectrum C of Figure 6 did not induce any obvious change. To provide further support to the physical studies, we determined the specific activities of WT and D93A as a function of  $[\text{Mg}^{2+}]$ . As shown in Figure 8, the activity of WT reaches maximum at 1 mM  $[\text{Mg}^{2+}]$  and then decreases to a ca. 50% level. Such a behavior has been well known for AK (Noda, 1958), but the exact nature is not fully understood. For D93A, there appears to be no " $\text{Mg}^{2+}$  inhibition", and the reason is again unclear. However, it is

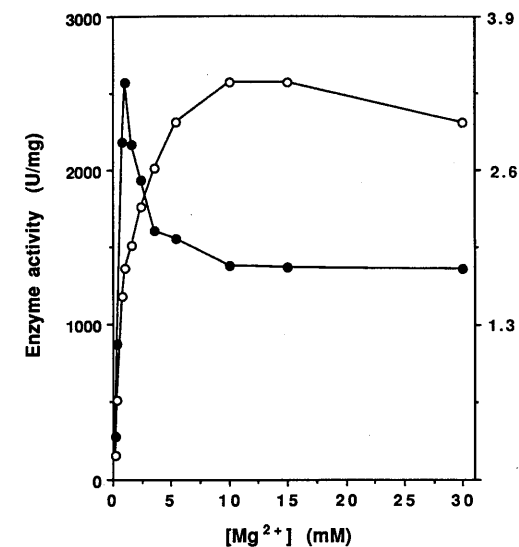


FIGURE 8: Specific activities of WT (closed circles, left y-axis) and D93A (open circles, right y-axis) as a function of  $[\text{Mg}^{2+}]$ . The experiments were performed under our normal assay condition, with ATP and AMP held at 1 and 2 mM, respectively.

quite obvious that excess  $\text{Mg}^{2+}$  ions cannot restore the activity of D93A. The smaller increases in the beginning part of the titration curve for D93A (relative to that of WT) also supports lower affinity of  $\text{Mg}^{2+}$  toward the D93A+ nucleotide complex.

**The Roles of Asp-93 and  $\text{Mg}^{2+}$  Ions in Catalysis.** All of our results suggest that removal of the carboxylate of D93A causes a modest decrease in the affinity of  $\text{Mg}^{2+}$  and a large decrease in  $k_{\text{cat}}$ , but the former is not the direct cause of the latter. The decrease in binding affinity is consistent with the involvement of Asp-93 in the binding of  $\text{Mg}^{2+}$  (when ATP or  $\text{AP}_5\text{A}$  is also bound). The decrease in  $k_{\text{cat}}$  should be due to an increase in the free energy level of the transition state, which is further elaborated as follows.

$\text{Mg}^{2+}$  ions could play both chemical and structural roles in the catalysis by kinases. The possible chemical roles are to shield the charge of the  $\gamma$ -phosphoryl group from the attacking nucleophile, and to enhance the cleavage of the  $\text{P}_\gamma\text{-O}$  bond by electrophilic effects. The structural role is to orient the phosphate chains in proper conformation. The  $^{31}\text{P}$  NMR results specifically suggest that in the absence of the carboxylate of Asp-93 the  $\text{Mg}^{2+}$  ion is unable to orient the phosphate chain of bound  $\text{AP}_5\text{A}$ . Thus, Asp-93 is required for fixing the  $\text{Mg}^{2+}$  ion at the proper position, which in turn leads to proper orientation of the phosphate groups. The structural role of  $\text{Mg}^{2+}$  is clearly impaired in D93A. Since D93A still achieves a rate enhancement of ca.  $10^9$  and the reaction still requires  $\text{Mg}^{2+}$  ions, the chemical roles of  $\text{Mg}^{2+}$  ions may not have been greatly impaired in the catalysis by D93A.

An interesting and important question to ask is when does  $\text{Mg}^{2+}$  come into play during the catalytic cycle. The results from previous studies and the present  $^{25}\text{Mg}$  NMR experiments clearly show that free AK does not bind  $\text{Mg}^{2+}$  ions. Whether Asp-93 interacts with  $\text{Mg}^{2+}$  in the binary complex with  $\text{MgATP}$  has not been explicitly addressed. However, the fact that AK binds uncomplexed ATP with 4-fold higher affinity than  $\text{MgATP}$  (Sanders et al., 1989) but it shows higher affinity to  $\text{MgAP}_5\text{A}$  than to uncomplexed  $\text{AP}_5\text{A}$  (Reinstein et al., 1990) suggests that the function of Asp-93 in binding the  $\text{Mg}^{2+}$  ion is more significant in the ternary complex than the binary complex. Thus, the interaction between AK and the bound  $\text{Mg}^{2+}$  ion is more important at the later stage of the catalytic cycle and is possibly most important at the transition state. Such interpretations can explain the previous observations that

AK can bind different metal-ATP complexes nonproductively but is fairly specific to  $\text{MgATP}$  in catalysis (Dunaway-Mariano & Cleland, 1980; Cohn, 1982). However, the interaction between  $\text{Mg}^{2+}$  and Asp-93 at the transition state is not an absolute requirement and accounts for only ca. 3.9 kcal/mol.

Using time-resolved X-ray crystallography, Schlichting et al. (1990) have observed that in the complex between the *ras* p21 protein and  $\text{MgGTP}$ , the  $\text{Mg}^{2+}$  ion is coordinated to the  $\beta$ - and  $\gamma$ -phosphate groups of the nucleotide and interacts with Asp-57 through a water bridge. In the p21+ $\text{MgGDP}$  complex, the  $\text{Mg}^{2+}$  ion accepts only one ligand from the nucleotide (the  $\beta$ -phosphoryl group) but is coordinated directly to Asp-57. On the basis of the paramagnetic effects on the  $^{31}\text{P}$  NMR relaxation rates of bound nucleotides, Ray et al. (1988) have also shown that in porcine muscle AK the metal ion  $\text{Co}^{2+}$  is within first coordination sphere distances to the  $\beta$ -phosphate and  $\gamma$ -phosphate of bound  $\text{CoATP}$  or  $\text{CoGTP}$  and to the  $\beta$ -phosphate of bound  $\text{CoGDP}$ . Although  $\text{Mg}^{2+}$  also interacts with residues other than Asp-57 in the p21 protein, which could be different in the case of AK, it is possible that  $\text{Mg}^{2+}$  may function in a similar mechanism in the two proteins, with the Asp-93 of AK corresponding to the Asp-57 of the *ras* p21 protein. It will be interesting to see whether the functional relationship between  $\text{Mg}^{2+}$  and Asp-93 as demonstrated for AK can also be demonstrated in the *ras* p21 protein.

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