Mechanism of Adenylate Kinase. Structural and Functional Demonstration of Arginine-138 as a Key Catalytic Residue That Cannot Be Replaced by Lysine†

Honggao Yan, Zhengtao Shi, and Ming-Daw Tsai*
Department of Chemistry and Ohio State Biochemistry Program, The Ohio State University, Columbus, Ohio 43210
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ABSTRACT: Replacement of the arginine-138 of adenylate kinase (AK) by lysine or methionine resulted in a decrease in k_{cat} by a factor of 10^4, increases in K_m by a factor of 10–20, and relatively little changes in dissociation constants. Proton nuclear magnetic resonance (NMR) studies were then undertaken to obtain structural information for quantitative interpretation of the kinetic data. Since the lysine mutant (R138K) represents a conservative mutation with surprisingly large effects on kinetics, structural studies were focused on the wild type (WT) and R138K. The results and conclusions are summarized as follows: (i) The aromatic spin systems of WT and R138K were assigned from total correlated spectroscopy (TOCSY). Comparison of the chemical shifts of aromatic protons, one-dimensional spectra, TOCSY, and nuclear Overhauser enhanced spectroscopy (NOESY) indicated that the conformation of R138K was almost unperturbed relative to that of WT. Thus Arg-138 is not important for the tertiary structure. (ii) Proton NMR titrations with AMP and MgATP suggested that substrate binding affinities and substrate-induced conformational changes are nearly identical between WT and R138K. Thus arginine-138 should not be involved in stabilizing the first substrate in the binary complex. (iii) Notable differences were observed between the proton NMR spectra of the WT and R138K complexes with the reaction mixture, which agrees with the perturbation in the K_m values of R138K. The differences were analyzed in detail by using a "static reaction mixture"—P^1,P^5-bis(5′-adenosyl)pentaphosphate ($MgAP_5A$). The aromatic spin systems of WT+$MgAP_5A$ and R138K+$MgAP_5A$ were partially assigned from various two-dimensional spectra. The results suggest that the conformational differences between the ternary complexes of WT and R138K are only minor. (iv) Qualitative comparison of the NOESY cross peaks between aliphatic side chains and aromatic protons indicates that the patterns are almost identical between free WT and free R138K. For WT+$MgAP_5A$ and R138K+$MgAP_5A$ the patterns are also very similar, but some small shifts can be observed. These observations reaffirm the conclusions drawn from the quantitative analysis of the aromatic region. (v) The above kinetic and structural results led to the conclusion that Arg-138 stabilizes the ternary complexes by 1.4–1.8 kcal/mol and stabilizes the transition state by at least 7 kcal/mol and that the critical functional role of Arg-138 cannot be replaced by lysine. (vi) Since Arg-138 is distant from the substrate sites proposed from previous NMR studies [Mildvan, A. S., & Fry, D. C. (1987) *Adv. Enzymol. Relat. Areas Mol. Biol. 58*, 241–313], serious revision will be required for this model. (vii) The adenine and $H_{1'}$ protons of bound $MgAP_5A$ were unequivocally assigned from the one-dimensional and NOESY spectra of WT and mutants, which indicated no detectable NOE between histidine-36 and bound $MgAP_5A$ resonances as opposed to a recent report on porcine muscle AK [Rösch, P., Klaus, W., Auer, M., & Goody, R. S. (199) *Biochemistry 28*, 4318–4325].

Although site-directed mutagenesis has now become a common approach in identifying catalytically important residues of enzymes, it has often generated new controversies rather than solved problems. In the case of adenylate kinase (AK),[1] three residues have now been suggested to interact with the γ-phosphoryl group of ATP on the basis of the kinetic and limited structural data of site-specific mutants: Lys-21 (Lys-13 in AKe;[2] Reinstein et al., 1990), Arg-97 (Arg-88 in AKe; Reinstein et al., 1989), and Arg-138 (in human AK; Kim et al., 1989). Unless all three are involved, we are already faced with a new controversy before resolution of the old controversy between the binding sites suggested by NMR (Fry et al., 1985, 1988; Mildvan & Fry, 1987) and X-ray (Pai et al., 1977) studies. The key factors in the uncertainty of site-directed mutagenesis are the effect of point mutation on the structure of the enzyme and the effect of such structural perturbation on the catalytic function. Thus, while it can be concluded that

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[1] Abbreviations: ADP, adenosine 5′-diphosphate; AK, adenylate kinase; AMP, adenosine 5′-monophosphate; AP_5A, P^1,P^5-bis(5′-adenosyl) pentaphosphate; ATP, adenosine 5′-triphosphate; 1D, one dimensional; 2D, two dimensional; DQF-COSY, double quantum filtered correlated spectroscopy; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser enhanced spectroscopy; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; TOCSY, total correlated spectroscopy; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; WT, wild type.

[2] The AK from different sources are abbreviated as follows: AK from chicken muscle, cAK; from porcine muscle, pAK; from rabbit muscle, rAK; from human muscle, hAK; from *Escherichia coli*, AKe; from yeast AKy. All of the muscle AKs are type 1 AK (AK1) and are highly (>80%) homologous (Kishi et al., 1986). AKe and AKy have different sizes and are less homologous (Schulz et al., 1986). Unless otherwise specified, the numbering system used in this paper is the conventional system for AK1. Although 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 that of other AK1's.

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Table I: Summary of Steady-State Kinetic Data Obtained by Varying Concentrations of Both Substrates[a]

	k_{cat} (s^{-1})	$K_{(MgATP)}$ (mM)	$K_{(AMP)}$ (mM)	$K_{i(MgATP)}$ (mM)	$K_{i(AMP)}$ (mM)	$k_{cat}/K_{(MgATP)}$ (s^{-1} M^{-1})	$k_{cat}/K_{(AMP)}$ (s^{-1} M^{-1})
WT[b]	650	0.042	0.098	0.16	0.37	1.55×10^7	0.66×10^7
R138K	0.1 (6500)	0.36	1.43	0.090	0.36	280 (5.5×10^4)	70 (9.4×10^4)
R138M	0.049 (1.3×10^4)	0.40	2.23	0.085	0.47	120 (1.3×10^5)	22 (3.0×10^5)

[a] Numbers of parentheses indicate the ratios between WT and the mutant. [b] From Tian et al. (1990).

a particular residue is not involved in catalysis if the kinetic properties of its mutants are not perturbed, it is difficult to unequivocally identify catalytically important residues and to quantitatively evaluate their contributions to the transition-state stabilization unless it can be demonstrated that the structure of the mutant is not perturbed (Wilde et al., 1988).

Our site-directed mutagenesis studies with chicken muscle AK (cAK)[2] have shown that, contrary to suggestions from previous NMR studies (Fry et al., 1985; Mildvan & Fry, 1987), Lys-27 and His-36 are not important catalytically (Tian et al., 1988, 1990). The catalytic role of the absolutely conserved Lys-21 was suggested to be uncertain despite a large decrease in the k_{cat} of K21M due to significant changes in the proton NMR spectrum of this mutant (Tian et al., 1990). The results from other groups suggested that changes in the residues of the "glycine-rich loop" of *Escherichia coli* AK (AKe) and cAK affected mainly the K_m values (Reinstein et al., 1988; Tagaya et al., 1989). In this paper we report quantitative and rigorous demonstration of Arg-138 as a critically important catalytic residue on the basis of the following studies: (a) Replacement of Arg-138 by Met or even the positively charged Lys resulted in a decrease in k_{cat} by ca. 10^4, and in 10–20-fold increases in K_m. (b) The 1D proton NMR, 2D TOCSY, 2D NOESY spectra, and the aromatic spin systems assigned from these spectra are almost identical between WT and R138K. (c) Proton NMR titrations with AMP and MgATP also suggested that substrate binding affinities and substrate-induced conformational changes are nearly identical between WT and R138K, but notable differences exist between the complexes with the reaction mixture. (d) The binding of $MgAP_5A$ with WT and R138K was investigated in detail by using 2D NMR. The results are also used to evaluate the existing models of the substrate binding sites of AK.

Materials and Methods

Materials. E. coli expression system for chicken AK was kindly provided by Dr. Nakazawa (Tanizawa et al., 1987). Oligonucleotides were synthesized by an Applied Biosystem 381A DNA synthesizer at the Biochemical Instrument Center of The Ohio State University. DNA sequencing and mutagenesis kits were purchased from United States Biochemicals and Amersham, respectively. Reagents and coupling enzymes were obtained from Sigma. Perdeuterated Tris was purchased from MSD Isotopes.

Construction of Mutants and Purification of Enzymes. R138K and R138M mutants were generated by a single degenerate oligonucleotide GACCAGCGGGA(A/T)GGTGGACGAC by using an Amersham mutagenesis kit according to the manual provided by the manufacturer. Since the mutation efficiency was very high, the mutants were selected by DNA sequencing performed according to the booklet accompanying the sequencing kit. Both WT and mutant enzymes were purified and assayed essentially as described by Tian et al. (1988). 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 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 Cleland (1986). The K and K_i values (Michaelis and dissociation constants, respectively) obtained from such analysis are close to the K_m and K_d values, respectively, measured by saturating one substrate (K_m) and by titration studies with NMR (K_d) (Tian et al., 1990; Sanders et al., 1989).

Proton NMR Methods. All NMR experiments were performed on Bruker AM 500 or AM 600 spectrometers. All spectra were referenced to internal sodium 3-(trimethylsilyl)propionate-*2,2,3,3-d*$_4$. Sample preparation and 1D NMR spectroscopy (including titration with MgATP and AMP) were essentially the same as described by Sanders et al. (1989) except for the buffer, which was composed of 20 mM perdeuterated Tris, 65 mM KCl, 2 mM DTT, and 0.5 mM EDTA, pH 7.5–8.0 (pH meter reading without correction for deuterium isotope effects). Titration studies indicated that the spectra are very similar in this pH range except a few pH-sensitive resonances. The samples also contained ca. 4 mM HEPES remaining from the last step of purification. The enzyme concentrations were about 1 mM for 1D NMR experiments and 2.5 mM for 2D NMR experiments. The temperature was 27 °C unless otherwise specified.

Standard pulse sequences and phase cycling were used for all 2D NMR experiments: DQF-COSY (Rance et al., 1983), TOCSY (Bax & Davis, 1985; Rance, 1987), and NOESY (Bodenhausen et al., 1984). The TOCSY spectra were acquired with 40- and 60-ms mixing periods. The mixing time for the NOESY experiments was 200 ms. All spectra were obtained in the phase-sensitive mode with quadrature detection in the $f1$ dimension achieved by time-proportional incrementation (Marion & Wüthrich, 1983). The sweep widths were 10 ppm for free enzymes and 11.5 ppm for enzyme–$MgAP_5A$ complexes. Generally, a 2048 × 256 or 2048 × 512 matrix in time domain was recorded and zero-filled to a 4096 × 1024 matrix prior to multiplication by a shifted sine bell function (SSB1 = SSB2 = 6) and Fourier transformation.

Results and Discussion

Kinetic Properties of R138 Mutants. The steady-state kinetic data of WT, R138K, and R138M are listed in Table I. It is surprising that for R138K, where the charge is conserved, a dramatic decrease in k_{cat} (by a factor of 6.5×10^3) and modest increases in K_m (10–15-fold) occurred. To our knowledge, this is one of the largest effects of a Arg-to-Lys mutation reported in the literature. For R138M in which the positive charge on the side chain has been removed, similar effects have been observed, except that the magnitudes are greater by a factor of ca. 2.

Before these kinetic data can be interpreted quantitatively it is necessary to examine the effect of mutation on the structure of AK. This was achieved by proton NMR studies of the WT AK and the mutants. Since minimal structural perturbation is always desired in quantitative interpretation of the kinetic data of site-specific mutants, the more conservative mutant R138K was chosen for detailed investigation.

Table II: Chemical Shifts of the Aromatic Residues of WT and R138K (pH 7.6)[a,b]

spin system	WT			R138K			possible residue
Fa	6.31	6.51	6.62	6.30	6.51	6.62	
Fb	6.32	6.97	7.11	6.32	6.98	7.13	
Fc	6.82	6.95	7.01	6.82	6.95	7.02	
Fd	6.95	7.20	7.26	6.96	7.27	7.35	[163]
Fe		7.14	7.57		7.14	7.58	
Ya	6.41	6.49		6.45	6.50		[164/189]
Yb	6.44	6.57		6.45	6.58		[164/189]
Yc	6.63	6.76		6.64	6.75		[153/154]
Yd	6.74	7.03		6.74	7.04		
Ye	6.79	7.12		6.79	7.12		
Yf	6.82	7.22		6.82	7.23		
Yg	6.91	6.95		6.92	6.96		
Yh	6.99	7.36		7.00	7.36		[153/154]
Ha	6.99 (H_4)	7.67 (H_2)		7.00 (H_4)	7.69 (H_2)		[H7/H8]
Hb	6.96 (H_4)	7.76 (H_2)		6.97 (H_4)	7.78 (H_2)		[H7/H8]
Hc	7.20 (H_4)	7.78 (H_2)		[7.23 (H_4)]	7.81 (H_2)		H36
Hd	6.66 (H_4)	7.88 (H_2)		6.67 (H_4)	7.89 (H_2)		H30

[a] Assigned from the TOCSY spectra in Figure 2. The assignments in brackets are tentative. The underlined are the resonances which differ by >0.02 ppm between WT and R138K. The systematic shifts of 0.02–0.03 ppm for Ha, Hb, and Hc are likely to be due to a small difference in pH (although both samples were prepared at pH 7.6, the actual pH of R138K is likely to be ca. pH 7.5). [b] Comments on the assignments of histidines: The H_2 and H36 was assigned from site-specific mutants as described by Tian et al. (1988). The H_4 of this residue was assigned from pH titration of WT. The spin system of Hd was assigned from a TOCSY experiment with longer mixing time (60 ms). The spin systems of Ha and Hb were assigned in accordance with the assignments of the $MgAP_5A$ complexes in Table IV (the resonances of two residues are relatively unperturbed by binding of various substrate analogues).

Proton NMR Properties of Free AK and R138K. As shown in Figure 1A (one-dimensional spectra), Figure 2 (TOCSY spectra), and Figure 3 (NOESY spectra), the proton NMR spectra of WT and R138K are almost identical. The 1D spectrum of R138M is also nearly identical with that of WT (spectra not shown). The spin systems of the aromatic protons of WT and R138K were assigned from the TOCSY spectra as shown in Figure 2. The 1D spectrum of WT labeled with the spin systems is shown in Figure 4. The chemical shifts of both WT and R138K are listed in Table II. The only residues whose chemical shifts differ by >0.02 ppm are Fd and Ya (those underlined in Table II). The results indicate that the conformation of R138K is virtually unperturbed relative to that of WT.

The aromatic spin systems of pAK have been assigned by Klaus et al. (1988), and AKe by Bock et al. (1988), but there are significant differences between cAK and pAK: pAK was assigned at pH 6.2 whereas cAK was at pH 7.6; there is one additional Tyr in cAK (Y189); there are two histidines (36 and 189) in pAK and four (7, 8, 30, and 36) in cAK. On the basis of rough comparison of chemical shifts, Fa, Fb, and Fe of cAK may correspond to F^A, F^B, and F^E, respectively, of pAK, and Yb (or Ya), Yc, Yd, Ye, Yf, Yg, and Yh of cAK may correspond to Y^A, Y^B, Y^C, Y^E, Y^F, Y^D, and Y^G, respectively, of pAK. The additional Tyr in cAK, Y189, appears to be either Ya or Yb. Klaus et al. (1988) observed strong NOE for Y^B/Y^G and weak NOE for Y^A/Y^F and F^A/F^C and assigned them to Y153/Y154, Y32/Y34, and F12/F105, respectively, on the basis of the distances in the crystal structure. For both WT and R138K (Figure 3) we observed strong NOE for Fd/Ya, intermediate NOE for Yc/Yh, and weak NOE for Fb/Ye (more interresidue cross peaks can be observed at lower contour levels). While Yc/Yh could correspond to Y153/Y154 (consistent with the assignment of pAK) and Fd/Ya could correspond to F163/Y164 if it is assumed that distances are the shortest between neighboring residues, such assignments should be considered tentative at best since there are many aromatic pairs in the tertiary structure of cAK, and NOE is also sensitive to the mixing time.

Quantitative Interpretation of the Kinetic Data. The above structural information allowed us to attribute the decrease in k_{cat}/K_m to destabilization of the transition state, and to conclude that Arg-138 stabilizes the transition state by 6.9 kcal/mol on the basis of the data of R138K, or 7.6 kcal/mol on the basis of the data of R138M. The actual value could be even higher since the chemical step is only partially (though nearly) rate-limiting (Tian et al., 1990), and since it cannot be totally ruled out that the residual activity of the mutants is due to minute amount of active revertants (Schimmel, 1989). The fact that dissociation constants K_i are relatively constant but K_m increase 10–15-fold suggests that the binding capability

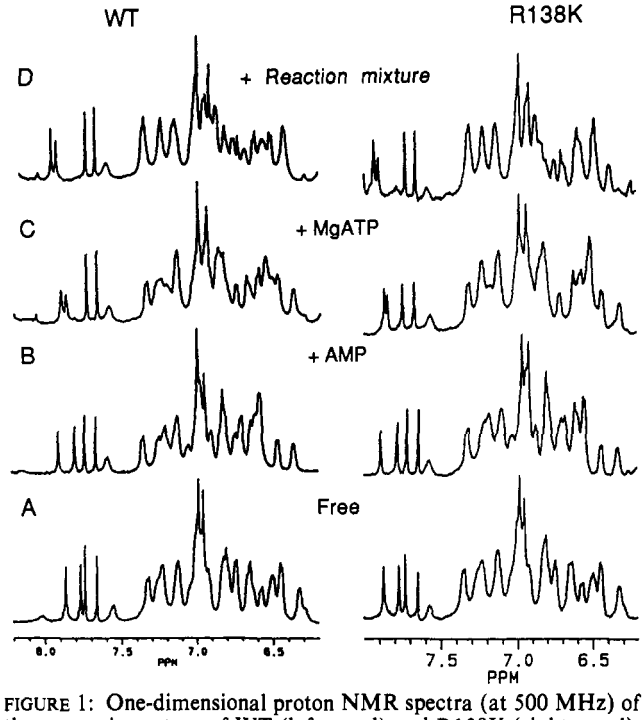

FIGURE 1: One-dimensional proton NMR spectra (at 500 MHz) of the aromatic protons of WT (left panel) and R138K (right panel). The conditions for the right panel are as follows: (A) 1.0 mM free R138K; (B) 1.0 mM R138K + 13 mM AMP; (C) 1.0 mM R138K + 6.5 mM ATP + 13 mM $MgCl_2$; and (D) 1.0 mM R138K + 18 mM ATP + 40 mM $MgCl_2$ + 23 mM AMP. The spectra were obtained at pH 8.0, 27 °C, and processed with 1-Hz expontential line broadening. Since further addition of 13 mM $MgCl_2$ to sample B gave little change to the spectrum, binding of AMP to the MgATP site should be insignificant under the conditions used. The left panel was reproduced from Sanders et al. (1989) under similar conditions.

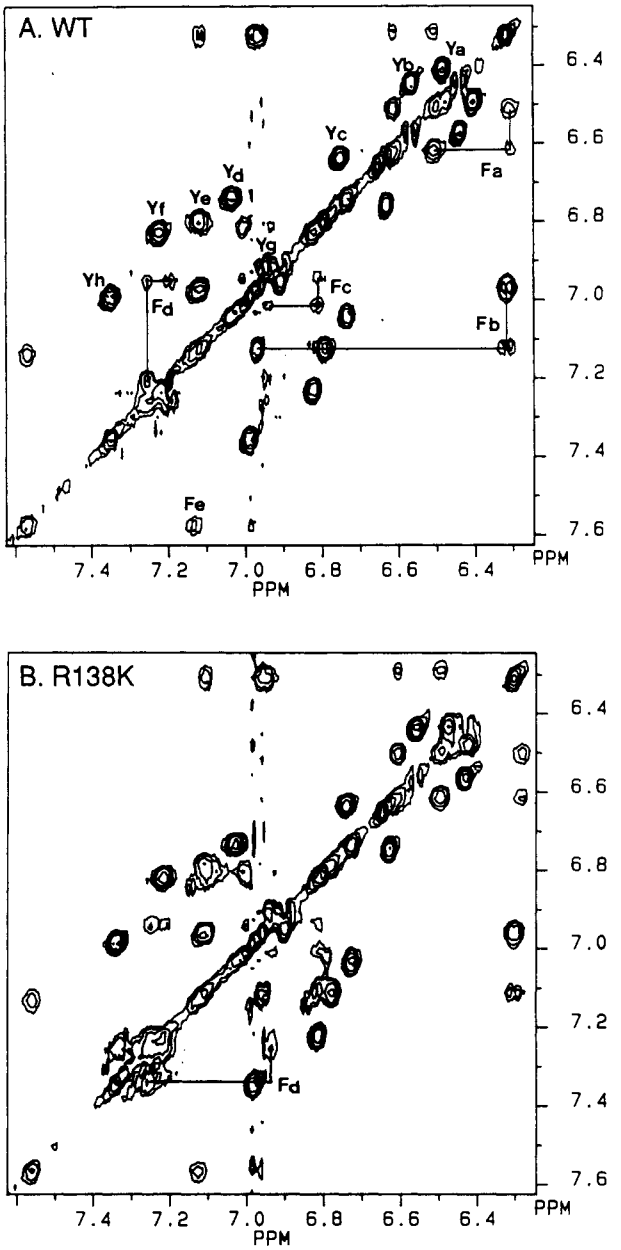

FIGURE 2: TOCSY spectra (at 600 MHz) of the aromatic protons of WT cAK (A) and R138K (B), pH 7.6, 27 °C. The mixing time was 40 ms.

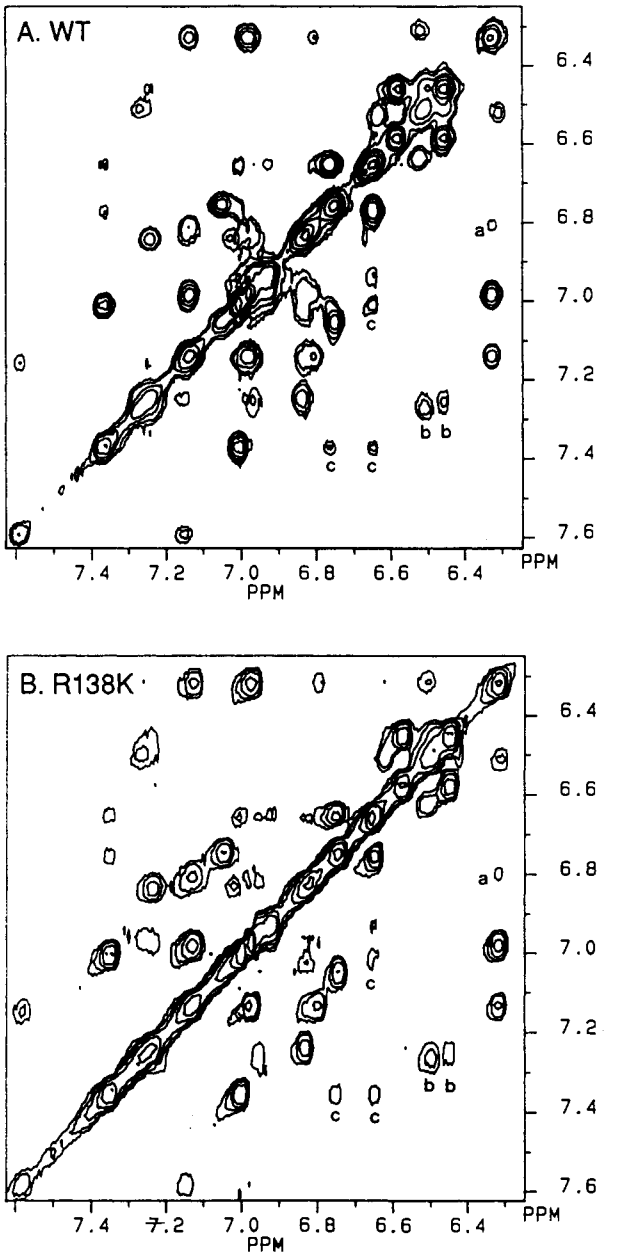

FIGURE 3: NOESY spectra of the aromatic protons of WT cAK (panel A; 500 MHz, pH 7.8) and R138K (panel B; 600 MHz, pH 7.6). Intraresidue cross peaks are not labeled since they can easily be deduced by comparison with the TOCSY spectra in Figure 2. The mixing time was 200 ms. Cross peaks for a, b, and c can be assigned to interresidue NOE for Fb/Ye, Fd/Ya, and Yc/Yh, respectively.

of R138K is unperturbed for the first substrate but slightly perturbed for the second substrate. This would suggest that the free energy level of the binary complex EA or EB (A and B represent MgATP and AMP) is unchanged while that of the ternary complex EAB increases by 1.4–1.8 kcal/mol. The latter could be due to either an unfavorable enzyme–substrate interaction or a somewhat impaired active conformation in the ternary complex of the mutant, as discussed previously for a histidine-36 mutant (Tian et al., 1990). Structurally this would predict that the proton NMR spectra of R138K+AMP and R138K+MgATP are very similar to those of WT+AMP and WT+MgATP, respectively, whereas some differences may occur between WT+reaction mixiture and R138K+reaction mixture.

Structural Verification of Kinetic Data. R138K was titrated with substrates and monitored by proton NMR. The proton NMR spectra of its complexes with AMP, MgATP, and the reaction mixture, in comparison with the corresponding spectra of WT obtained previously (Sanders et al., 1989), are shown in Figure 1 (spectra B, C, and D, respectively). It is clear from the figure that the spectra of R138K+AMP and WT+AMP are essentially identical, and those of R138K+-MgATP and WT+MgATP are only slightly different. The dissociation constants for R138K calculated from such titrations are 0.82 mM for AMP and 0.39 mM for MgATP, which are also close to the values for WT (0.50 and 0.17 mM, respectively) obtained by the same procedure (Sanders et al., 1989). The chemical shifts of H_2, H_8, and $H_{1'}$ of bound nucleotides (listed in Table III) deduced from the titration curves also show only small differences ($\leq$0.05 ppm) between WT and R138K. These results suggest that the conformations of the binary complexes R138K+MgATP and R138K+AMP are also little perturbed relative to WT and verify the prediction for binary complexes on the basis of kinetic data.

As shown in Figure 1D, there are notable, though not substantial, differences between the spectra of WT+reaction mixture and R138K+reaction mixture. This suggests that

Table III: Chemical Shifts of AMP, ATP, and AP_5A in Various Complexes[a]

complex	H_2		H_8		$H_{1'}$	
free AMP	8.26		8.60		6.12	
WT+AMP	8.61		8.58		6.03	
R138K+AMP	8.56		8.60		6.08	
free MgATP	8.29		8.52		6.12	
WT+MgATP	~8.4		~8.4		6.07	
R138K+MgATP	8.35		8.39		6.08	
free $MgAP_5A$	8.19		8.40		6.09	
WT+$MgAP_5A$	8.98 (I)	8.32 (II)	8.47 (I)	8.40 (II)	6.07 (II)	5.93 (I)
R138K+$MgAP_5A$	8.99 (I)	8.31 (II)	[8.38]		6.07 (II)	5.92 (I)
free AP_5A	8.17		8.43		6.07	
WT+AP_5A	8.92 (I)	8.29 (II)	8.50 (I)	8.43 (II)	6.07 (II)	5.88 (I)
R138K+AP_5A						[6.06]

[a] The data for AMP and MgATP were obtained from substrate titration experiments under fast exchange conditions. The data for $MgAP_5A$ and AP_5A were obtained from the spectra directly since the exchange is slow, except for those in brackets which are averaged chemical shifts under intermediate or fast exchange conditions.

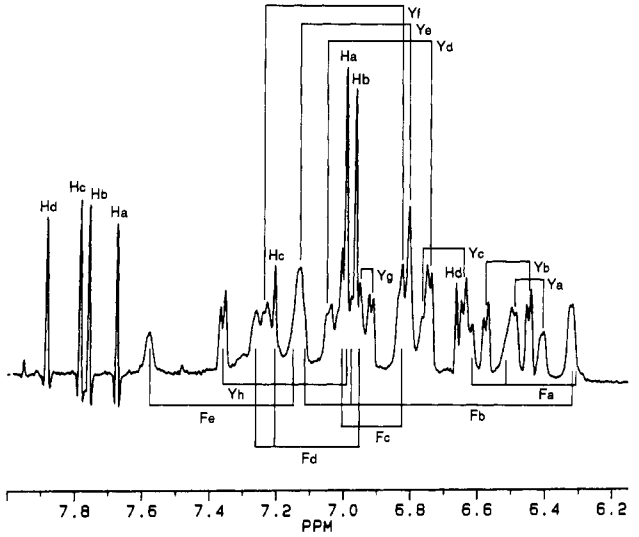

FIGURE 4: One-dimensional proton NMR spectra (at 600 MHz) of the aromatic protons of WT cAK showing the spin systems. This spectrum was obtained at pH 7.6 (same sample used in Figure 2A), 27 °C, and processed with Gaussian multiplication (LB = −10, GB = 0.15).

small structural perturbation does occur at the ternary complex of R138K and again supports the prediction from kinetic data. However, the differences between the spectra of the reaction mixture complexes cannot be analyzed quantitatively since they are a mixture of substrate and product complexes as well as some dead-end complexes (and even the very short-lived transition-state complex), and differences in the ratios of these complexes could contribute to part or even all of the observed differences in the NMR spectra. To overcome these problems, we used a "static reaction mixture", $MgAP_5A$, and compared the spectral properties of WT+$MgAP_5A$ and R138K+-$MgAP_5A$ in detail.

Spectral Properties of $MgAP_5A$ Complexes. $MgAP_5A$ is a well-known high affinity binding inhibitor of AK (Lienhard & Secemski, 1973; Bone et al., 1986; Feldhaus et al., 1975; Hampton et al., 1982; Van Der Lijn et al., 1979; Kuby et al., 1978). These kinetic studies also agree that $MgAP_5A$ is a competitive inhibitor versus both AMP and MgATP. The binding energy of $MgAP_5A$ (11 kcal/mol, calculated from binding or inhibition constants in the above references) is only slightly larger than the sum of that of MgATP and AMP (9.8 kcal/mol) (Sanders et al., 1989). Our proton NMR studies suggested that the $MgAP_5A$ and the reaction mixture induce qualitatively similar changes to the 1D spectrum of WT cAK, though the changes induced by $MgAP_5A$ are slightly more extensive (Sanders et al., 1989). These results suggest that

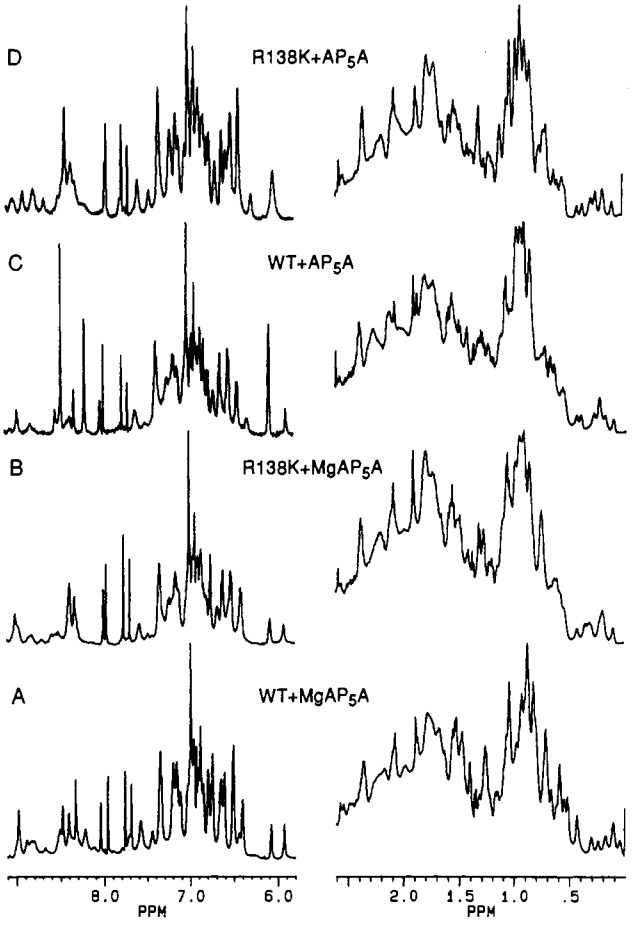

FIGURE 5: One-dimensional proton NMR spectra (at 500 MHz) of the aromatic protons of various AP_5A complexes: (A) 2.5 mM WT + 2.5 mM AP_5A + 5.0 mM $MgCl_2$; (B) 2.5 mM R138K + 2.5 mM AP_5A + 5.0 mM $MgCl_2$; (C) 2.0 mM WT + 4.0 mM AP_5A; and (D) 2.0 mM R138K + 2.5 mM AP_5A (D). The spectra were obtained at pH 7.8, 27 °C, and processed with Gaussian multiplication (LB = −5, GB = 0.1).

$MgAP_5A$ is a bisubstrate analogue, though it could possess a very small transition-state character.

The 1D spectra of WT+$MgAP_5A$, R138K+$MgAP_5A$, WT+AP_5A, and R138K+AP_5A are shown in Figure 5 (spectra A, B, C, and D, respectively). Although the AP_5A resonances look quite different between different spectra in Figure 5, they are caused by differences in exchange rates which will be explained in a later section. For the aromatic protons (6.3–8.1 ppm), it is interesting to note that in the absence of Mg^{2+} the spectra of WT+AP_5A and R138K+-AP_5A are very similar, whereas in the presence of Mg^{2+} the

Table IV: Chemical Shifts of the Aromatic Residues of the $MgAP_5A$ Complexes (pH 7.8)[a,b]

spin system	WT+$MgAP_5A$			R138K+$MgAP_5A$			possible residue
Fa	6.44	6.51	6.65	6.43	6.54	6.67	
Fb	6.40	7.01	7.17	6.40	7.00	7.16	
Fd				6.94	7.19	7.23	
Fe		7.17	7.58		7.16	7.58	
Xa	6.79	6.98		6.80	6.99		
Xb	6.97	7.20		6.99	7.16		
Yb	6.51	6.61		6.51	6.61		
Yd	6.75	7.03		6.75	7.04		
Ye	6.88	7.11		6.87	7.11		
Yf	6.92	7.36		6.90	7.34		
Yh	6.96	7.34		6.95	7.33		
Ha	7.00 (H_4)	7.69 (H_2)		7.00 (H_4)	7.69 (H_2)		[H7/H8]
Hb	6.93 (H_4)	7.76 (H_2)		6.93 (H_4)	7.76 (H_2)		[H7/H8]
Hc	[6.80 (H_4)]	8.04 (H_2)			7.99 (H_2)		H36
Hd	6.74 (H_4)	7.96 (H_2)		6.74 (H_4)	7.96 (H_2)		H30

[a] The underlined are the resonances that differ by more than 0.02 ppm between WT and R138K. The assignments in brackets are tentative. [b] Comments on the assignments of histidines: Ha and Hb were assigned from the cross peaks in the DQF-COSY spectrum of R138K+$MgAP_5A$. The H_4 protons of H30 and H36 were assigned on the basis of weak NOE cross peaks between H_2 and H_4.

differences between WT+$MgAP_5A$ and R138K+$MgAP_5A$ become noticeable to the extent comparable to the differences between the complexes of the reaction mixture. This again suggests that the catalytic role of Arg-138 starts with the true ternary complex. We then performed detailed spectral analysis for both the aromatic spin systems and the resonances of bound $MgAP_5A$, in order to obtain a quantitative (or semiquantitative) view of the chemical shift differences between WT+$MgAP_5A$ and R138K+$MgAP_5A$.

Aromatic Spin Systems of WT+$MgAP_5A$ and R138K+$MgAP_5A$. The assignment of the spin system of the $MgAP_5A$ complexes was less straightforward than that of free AK, which could be the reason why such assignment has not been reported to date. Although the 1D spectra shown in Figure 5 are of very high resolution, they are deceptively simple. From the TOCSY, DQF-COSY, and NOESY spectra three Tyr residues and one Phe residue in both WT+$MgAP_5A$ and R138K+$MgAP_5A$ could not be established and two cross peaks (designated as Xa and Xb) could not be assigned. In other words, at least two aromatic rings could not be observed, possibly due to broadening of the resonances. The resonances assigned are listed in Table IV, with the resonances differing by >0.02 ppm underlined. Presumably the unobservable residues could be involved in binding and could be different between WT+$MgAP_5A$ and R138K+$MgAP_5A$. Other than that, notable differences were observed for Fa, Fd, and H36 (Fd was clearly identifiable in R138K+$MgAP_5A$ but was unobservable in WT+$MgAP_5A$). However, the remaining residues (four phenylalanines, five tyrosines, and three histidines) have almost identical chemical shifts and NOE cross peaks (see below). Therefore, the conformational differences between WT+$MgAP_5A$ and R138K+$MgAP_5A$ are notable, but only minor.

It should be noted that the residues a, b, c, ..., in the $MgAP_5A$ complexes do not necessarily correspond to those in the free enzymes except Fa, Fb, Fe, Ye, Ha, and Hb which appear to be perturbed only slightly by $MgAP_5A$. Other aromatic residues were substantially perturbed by $MgAP_5A$. As shown in the NOESY spectrum of WT+$MgAP_5A$ (Figure 6), the detectable interresidue NOE cross peaks p–t can be tentatively assigned to Fb/Ye (same as in free enzyme), Fa/Xb, Yb/Yf, Fb/Yf, and Fe/Yf, respectively.

Resonances of His-36 and Bound $MgAP_5A$. The H_2 of H36 (Hc) undergoes a relatively large downfield shift upon binding of every substrate and substrate analogue (Sanders et al., 1989) and is thus assigned to the downfield resonance at 8.0 ppm. In the work by Rösch et al. (1989) this resonance was assigned

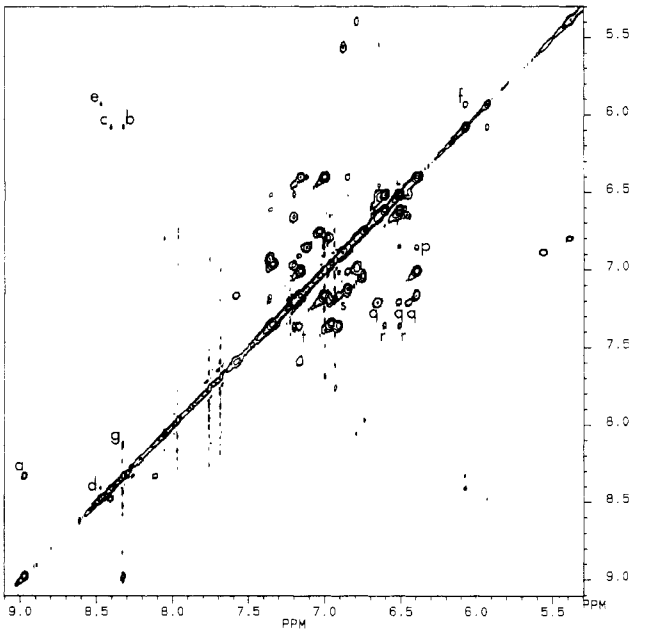

FIGURE 6: NOESY spectrum (at 500 MHz) of WT+$MgAP_5A$ (the same sample used for Figure 5A) at pH 7.8, 27 °C. The mixing time was 200 ms. Cross peaks a–g are related to bound $MgAP_5A$ (exchange or intramolecular NOE), and cross peaks p–t are due to interresidue NOE's, as explained in the text. The NOESY spectrum of WT+AP_5A (without Mg^{2+}) can be found in Tian et al. (1990).

to the H_2(B) of bound $MgAP_5A$, and another resonance further downfield (8.32 ppm) was assigned to the H_2 or H36, which led to assignment of a strong NOE between the H_2(A) of bound $MgAP_5A$ and the H_2 or H36 [actually the cross peak between H_2(A) and H_2(B) of bound $MgAP_5A$] and support of the MgATP site assigned from NMR studies of Fry et al. (1985). Our assignment was confirmed by the following evidence: (i) the 8.0 ppm signal was missing in the H36Q+AP_5A complex (spectrum not shown); (ii) as shown in the 1D spectra of R138K+AP_5A and R138K+$MgAP_5A$ in Figure 5, the resonance at 8.0 ppm is always present when the AP_5A resonances are in fast or intermediate exchange. Thus the strong NOE between H36 and AP_5A and related interpretations reported by Rösch et al. (1989) are no longer valid.[3]

With the assignment of His resonances settled, the NOESY spectrum in Figure 6 allowed us to assign the resonances of bound $MgAP_5A$ as listed in Table III. To avoid confusion with

[3] We have communicated this information with Dr. P. Rösch, and they have reached the same conclusion independently.

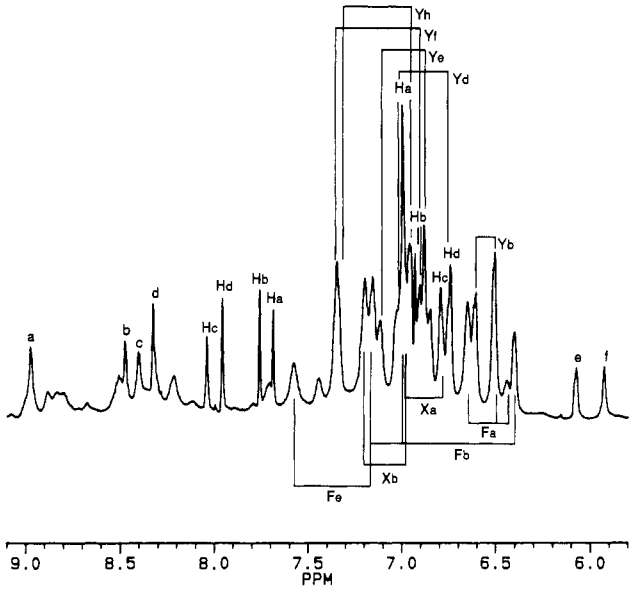

FIGURE 7: One-dimensional proton NMR spectra (at 500 MHz) of the aromatic protons of WT+$MgAP_5A$ (same as Figure 5A) showing the assignments of bound $MgAP_5A$ and partial assignments of the aromatic spin systems. Peaks a–f are due to bound $MgAP_5A$: a, H_2(I); b, H_8(I); c, H_8(II); d, H_2(II); e, $H_{1'}$(II); f, $H_{1'}$(I).

the A-site/B-site designations used by previous crystallographic (Egner et al., 1987) and NMR (Rösch et al., 1989) studies, we used I and II to represent the two sites. The most downfield resonance (8.98 ppm) of $MgAP_5A$ was assigned to H_2(I). The basis of other assignments are as follows: H_2(II), exchange cross peak a with H_2(I); $H_{1'}$(II), NOE cross peak b with H_2(II); H_8(II), NOE cross peak c with $H_{1'}$(II); H_8(I), exchange cross peak d with H_8(II); $H_{1'}$(I), NOE cross peak e with H_8(I), and exchange cross peak f with $H_{1'}$(II). Peak g is likely to be due to NOE between H_2(II) and an amide proton at 8.12 ppm. Since the adenine H_2 of AMP undergoes a large downfield shift (0.3–0.4 ppm) upon binding to cAK as shown in Table III, it is tempting to assign site I as the AMP site. However, we think it is premature to make such an assignment unless additional independent evidence can be obtained. Figure 7 shows the expanded 1D spectrum of R138K+$MgAP_5A$ with labeling of the bound $MgAP_5A$ resonances and the assigned aromatic spin systems.

It can be noted in Figure 5 that the AP_5A resonances are in slow exchange in spectra A and C and in intermediate or fast exchange in spectra B and D. The resonances of AP_5A in spectra B–D have also been assigned from the corresponding NOESY spectra (not shown) of the same samples, and the data are also listed in Table III. The results suggest that, despite the apparent differences in the 1D spectra due to different exchange rates, the H_2, H_8, and $H_{1'}$ of bound AP_5A are all very similar between WT+$MgAP_5A$ and R138K+$MgAP_5A$, and between WT+AP_5A and R138K+AP_5A. This again suggests that the conformational differences between WT+$MgAP_5A$ and R138K+$MgAP_5A$ are only minor.

Spectral Properties in the Aliphatic Region. While all of the above analysis is focused on the aromatic resonances, one reviewer has insightfully pointed out that in Figure 5 a number of aliphatic resonances (particularly those shifted upfield) do change. While we have not yet assigned the aliphatic resonances or spin systems, qualitative comparison of the NOESY cross peaks can address this problem. As shown in Figure 8, the aromatic–aliphatic NOE cross peaks are almost identical between WT (A) and R138K (B), except that some weak peaks show up in one but not the other. The corresponding spectra for WT+$MgAP_5A$ (C) and R138K+$MgAP_5A$ (D)

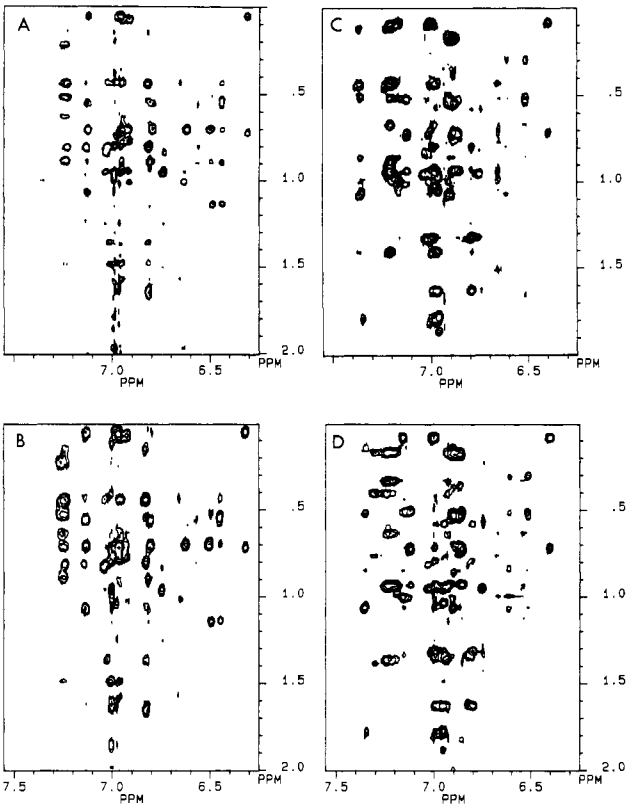

FIGURE 8: Aromatic–aliphatic NOE cross peaks from the NOESY spectra of WT (A), R138K (B), WT+$MgAP_5A$ (C), and R138K+$MgAP_5A$ (D). Spectra A–C came from the experiments for Figure 3, panels A and B, and Figure 6, respectively. Spectrum D (at 500 MHz) came from the same sample used for Figure 5B, and the mixing time for the NOESY experiment was 200 ms.

also have similar patterns, but there are notable (but small) changes in the positions of some of the cross peaks. These results reaffirm the conclusions from the analysis of the aromatic–aromatic NOE cross peaks and aromatic spin system that the conformation of R138K is almost identical with that of WT in the free enzyme, and that there are notable but minor conformational differences in the $MgAP_5A$ complexes.

Evaluation of Existing Binding Site Models. Although our results are still insufficient for proposing detailed substrate sites of AK, they can be used to evaluate the existing models. In the "NMR model" (Fry et al., 1985; Mildvan & Fry, 1987), the substrates are very far away from and unlikely to interact with Arg-138. On the basis of the results in this paper and our earlier studies (Tian et al., 1990), this model would require a major revision. There was still one fact difficult to argue against in the NMR results—the proximity between MgATP and His-36 evidenced by NOE in the cases of porcine and rabbit muscle AK (Smith & Mildvan, 1982; Fry et al., 1985). The only corroboration to this result was the observation of NOE between the H_2 or H36 and the H_2(A) of bound $MgAP_5A$ (but not bound MgATP) for pAK reported by Rösch et al. (1989) but refuted as described above. We have examined this problem for cAK and found no detectable NOE between the H_2 of H36 and the adenine protons of bound $MgAP_5A$ (see Figure 6). This problem requires further investigation.

The results presented in this paper suggest that Arg-138 is a strong candidate to interact with the transferring phosphoryl group. In the refined (2.1-Å) crystal structure of AK, Arg-138 interacts with a sulfate ion which could be a mimic of phosphate (Dreusicke et al., 1988). In the crystal structure of the AKy–$MgAP_5A$ complex (2.6-Å resolution; Egner et al., 1987),

Arg-138 is positioned to interact with a phosphate group close to the adenosine-B site. This is consistent with the notion by Egner et al. (1987) that the adenosine-B site, which occupies the site previously proposed for ATP (Pai et al., 1977), should be the AMP site. Thus our results are more consistent with the results from crystallographic studies than the previous NMR studies. However, the problem is far from being resolved, particularly on the MgATP site (Egner et al., 1987; Müller & Schulz, 1988).

Conslusions. The results described above demonstrate the following important points: (i) Arg-138 is not important for the tertiary structure of cAK on the basis of quantitative proton NMR analysis of the aromatic residues; (ii) Arg-138 is not involved in the binding of the first substrate (AMP or MgATP) on the basis of lack of perturbation in dissociation constants and the proton NMR spectra of the binary complexes; (iii) Arg-138 stabilizes the ground-state ternary complex to a small extent, on the basis of increased K_m and the minor but notable structural perturbation in the R138K complexes with the reaction mixture and with $MgAP_5A$; and (iv) Arg-138 stabilizes the transition state by as much as 7–8 kcal/mol on the basis of the large decreases in the k_{cat}/K_m of R138K and R138M. The critical role of Arg-138 in catalysis is further reflected by the fact that its functions are almost completely abolished by a very conservative mutation to lysine. The results also suggest that the substrate binding model proposed from previous NMR studies (Fry et al., 1985; Mildvan & Fry, 1987) is no longer viable and that the NOE between histidine-36 and $MgAP_5A$ resonances and related interpretations reported previously (Rösch et al., 1989) were due to assignment of the H_2 resonance of histidine-36 to an adenine proton.

Acknowledgments

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