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Mechanism of Adenylate Kinase. Critical Evaluation of the X-ray Model and Assignment of the AMP Site†

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ABSTRACT: The substrate binding sites of adenylate kinase (AK) proposed by X-ray crystallographic studies [Pai, E. F., Sachsenheimer, W., Schirmer, R. H., & Schulz, G. E. (1977) *J. Mol. Biol. 114*, 37-45, and subsequent revisions] were evaluated by site-specific mutagenesis in conjunction with structural analysis by NMR. The residues examined in this report include two near an adenosine site (threonine-39 and arginine-44) and two in the phosphate binding region (arginine-128 and arginine-149). The results and conclusions are summarized as follows: (a) *Although Thr-39 is very close to an adenine site* [Egner, U., Tomasselli, A. G., & Schulz, G. E. (1987) *J. Mol. Biol. 195*, 649-658], *it is nonessential either structurally or functionally.* (b) The R44M mutant enzyme showed significant increases in the Michaelis and dissociation constants of adenosine 5′-monophosphate (AMP) (36- and 22-fold, respectively) while all other kinetic parameters were relatively unperturbed. The proton NMR property of this mutant was unchanged in the free enzyme and only slightly perturbed in the binary complexes with AMP and with MgATP (adenosine 5′-triphosphate), and in the ternary complex with $MgAP_5A$ [P^1,P^5-bis(5′-adenosyl) pentaphosphate]. These results indicate that *Arg-44 interacts specifically with AMP starting at the binary complex, and suggest that the MgATP site proposed by Pai et al. (1977) is likely to be the AMP site.* (c) The kinetic parameters of R149M were dramatically perturbed: k_{cat} decreased by a factor of 1540, K_m increased to 130-fold, and k_{cat}/K_m decreased by a factor of 2×10^5. The structure was also unperturbed in the free enzyme and slightly perturbed in the $MgAP_5A$ complex. These results led to the conclusion that Arg-149 stabilizes the ternary complex by 2.9 kcal/mol and the transition state by 7.3 kcal/mol. Thus, *Arg-149 is also a critically important residue in the catalysis by AK.* (d) The k_{cat}, K_m, and dissociation constant of R128A were all perturbed to a small extent (within factors of 10-20). The proton NMR was also perturbed to a small extent in the free enzyme and in binary and ternary complexes. Thus, the structural and/or functional roles of Arg-128 are minor. (e) One set of AP_5A resonances (the set assigned to site I previously) was assigned to the AMP site on the basis of two findings: (i) the adenine-I H_2 of $MgAP_5A$ and the adenine H_2 of AMP were both shifted downfield (by 0.8 and 0.4 ppm, respectively) upon binding to AK, while the shifts of other adenine protons of AMP, MgATP, and $MgAP_5A$ were all <0.15 ppm upon binding to AK; (ii) the adenine-I H_8 of R44M+$MgAP_5A$ was perturbed by 0.09 ppm relative to that of WT+$MgAP_5A$ while all other adenine protons were unperturbed. (f) The results are used to critically evaluate existing binding site models, including the most recent addition by Kim et al. [Kim, H. J., Nishikawa, S., Tokutomi, Y., Takenaka, H., Hamada, M., Kuby, S. A., & Uesugi, S. (1990) *Biochemistry 29*, 1107-1111].

It is hard to believe that, for an enzyme as small, common, and extensively studied as adenylate kinase (AK),[1,2] little has been firmly established about how it catalyzes the reaction or even just where and how it binds to substrates. Ironically, AK is often considered as a typical kinase or ATP binding protein,

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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; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetate; FID, free induction decay; 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; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; WT, wild type.

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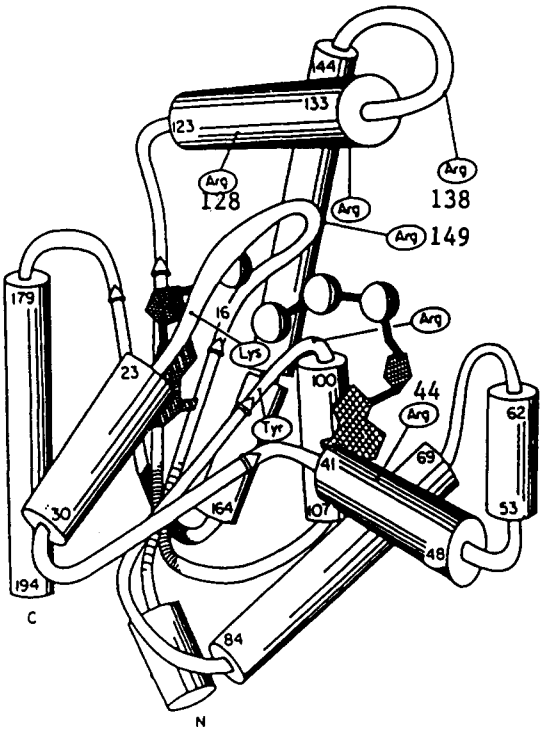

FIGURE 1: Sketch of the crystal structure of porcine muscle AK and the proposed binding sites for ATP and AMP, according to Pai et al. (1977). The ATP site corresponds to the "adenosine-B" site in Egner et al. (1987) and has been reassigned to the AMP site in this work and by others as described in the text.

and the various binding site theories, including a recent addition by Kim et al. (1990), have been presented so forcefully as to create misconception that substrate binding sites have been well resolved.

In summary, two sets of substrate binding sites have been proposed prior to 1988, one based on NMR studies (Fry et al., 1985, 1987, 1988; Mildvan & Fry, 1987) and the other based on X-ray studies (Figure 1) (Pai et al., 1977). These two theories of substrate binding sites are referred to as the "NMR model" and the "X-ray model", respectively, in this paper. Several modifications of these two models have been suggested since 1987. A "theoretical model" was proposed (Caldwell & Kollman, 1988) from energy-refining of the NMR and the X-ray models. Egner et al. (1987) reported the crystal structure of the complex of yeast AK (AKy) with $MgAP_5A$, but they concluded that only "adenosine-B" occupies a natural binding site since adenosine-A occupies a nonhomologous site and is inconsistent with the model of Pai et al. (1977). The adenosine-B site corresponds to the MgATP site of Pai et al. (1977), but Egner et al. (1987) suggested that it should be reassigned to the AMP site on the basis of the position of Mg^{2+} in the $MgAP_5A$ complex. This argument has recently been strengthened by the crystal structures of the guanylate kinase·GMP complex (Stehle & Schultz, 1990; published while this paper was in preparation) and the AK3·AMP complex (Diederichs & Schulz, 1990; communicated by the authors after this paper has been reviewed).

The employment of site-directed mutagenesis can only create new controversies if researchers are too quick to draw conclusions. For example, Lys-21 (Reinstein et al., 1990), Arg-97 (Reinstein et al., 1989), and Arg-138 (Kim et al., 1989) have all been suggested to interact with the γ-phosphoryl group of ATP. While this paper was in preparation, the accelerated publication by Kim et al. (1990) concluded, on the basis of the "apparent" kinetic data of R44A, R97A, R132A, R138A, and R149A mutant enzymes, that "the assigned substrate binding sites of Pai et al. (1977) have been reversed". Although the assignment of the AMP site agrees with the recent crystallographic results noted above and the conclusion of this work, it was premature to place the MgATP site at the AMP site of Pai et al. (1977) since this was not supported by their data. Furthermore, the "apparent K_m" can deviate substantially from real values as demonstrated by the R149M mutant enzyme in this paper.

To avoid any further complication, our approach has been to critically evaluate the potential catalytic roles of the target residues by *both* structural and functional studies of site-specific mutants. Our results have shown that the MgATP site of the NMR model is inconsistent with the results of site-specific mutagenesis and therefore will require serious revision (Tian et al., 1988, 1990). We then turned to the important residues suggested by the X-ray model and demonstrated that Arg-138 is a key catalytic residue that cannot be replaced by a lysine (Yan et al., 1990).

In this paper, we continue to evaluate the X-ray model by examining two residues near the adenosine-B site (Thr-39 and Arg-44) and two near the phosphate binding region (Arg-128 and Arg-149). As shown in Figure 1, Thr-39 is very close to the adenine ring of the MgATP site (the adenosine-B site) whereas Arg-44 is close the phosphate of adenosine-B. Egner et al. (1987) reported "9 contacts" between Thr-39 and adenosine-B in the AKy·$MgAP_5A$ complex. Arg-128 and Arg-149 were both suggested to be the likely residues to interact with phosphates in the crystal structures of muscle AK (Pai et al., 1977; Dreusicke et al., 1988). Arg-128 is conserved in all muscle AKs (Kishi et al., 1986), Thr-39 is conserved in all types of AK except AK3 (replaced by a serine), and Arg-44 and -149 are conserved without exception (Schulz et al., 1986; Schulz, 1987; Egner et al., 1987).

Our structural and functional studies of T39A, R44M, R128A, and R149M suggest that Thr-39 is nonessential in structure or catalysis, Arg-44 is important to the binding of AMP in the binary and the ternary complexes, Arg-128 either is unimportant or is involved in very weak uniform binding, and Arg-149 stabilizes the ternary complex by 2.9 kcal/mol and the transition state by 7.3 kcal/mol. The results have also allowed assignment of one set of adenine proton NMR resonances of $MgAP_5A$ to the AMP site.

Materials and Methods

Materials and Construction of Mutants. These are all the same as described previously (Tian et al., 1988, 1990). The oligonucleotides used for the construction of mutants are as follows: T39A, TCACCTCTCC*G*CTGGGGACCTG; R44M, GACCTGCTC*AT*GGCAGAGGTC; R149M, ATCAAGAAG*ATG*TTGGAGACG; R128A, ATGGTGAAG*NN*GCTGCTGAA (N = any deoxynucleotide).

Purification of Enzymes. T39A and R128A mutant enzymes were purified and assayed essentially as described by Tian et al. (1988). R44M and R149M were purified by the

[2] The AKs 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 *E. coli*, AKe; from yeast, AKy; from mammalian mitochondrial intermembrane space, AK2; from mammalian mitochondrial matrix, AK3. All of the muscle AKs are type 1 AK (AK1), 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 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 other AK1's.

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
T39A	450	0.047	0.089	0.038	0.073	0.96×10^7	0.51×10^7
R128A	36	0.65	1.38	1.63	3.48	5.5×10^4	2.6×10^4
R44M	210	0.048	3.53 (36)	0.11	8.25 (22)	0.44×10^6	5.9×10^4 (8.9×10^{-3})
R149M	0.42 (6.5×10^{-4})	5.6 (130)	12.4 (130)	0.29	0.64	75 (4.8×10^{-6})	34 (5.1×10^{-6})
R138K[c]	0.1 (1.5×10^{-4})	0.36	1.43	0.090	0.36	280 (1.8×10^{-5})	70 (1.1×10^{-5})
R138M[c]	0.049 (7.5×10^{-5})	0.40	2.23	0.085	0.47	120 (7.7×10^{-6})	22 (3.3×10^{-6})

[a] Numbers in parentheses indicate the ratios between the mutant and WT. Only the large changes are indicated. [b] From Tian et al. (1990). [c] From Yan et al. (1990).

same two-column procedure, except that the linear NaCl gradient used for eluting the phosphocellulose column was 0–200 mM for R44M and 50–200 mM for R149M. The purity of each preparation was checked by SDS–PAGE with silver staining on PhastSystem.

Steady-State Kinetics. The kinetic experiments were carried out by measuring ADP formation with pyruvate kinase/lactate 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 (Cleland, 1986):

$$v = \frac{ABV}{K_a K_{ib} + K_b A + K_a B + AB}$$

where v is the reaction rate, the subscripts a and b represent the two substrates MgATP and AMP, respectively, A and B are the concentrations of the corresponding substrates, and V is the maximum velocity. 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. Proton NMR experiments were performed on Bruker AM-500 spectrometers. Chemical shifts are 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 7.8 (pH meter reading without correction for deuterium isotope effects). 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 NOESY experiments (Bodenhausen et al., 1984), with a mixing time of 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 11.5 ppm. A 2048 × 400 matrix in time domain was recorded and zero-filled to a 4096 × 1024 matrix prior to multiplication by a Gaussian function (LB = −3, GB = 0.1) and Fourier transformation.

Results and Discussion

Kinetic Properties of All Mutants. The steady-state kinetic data k_{cat}, K_m, and K_i (dissociation constants), obtained by a full initial velocity analysis varying both substrates (Cleland, 1986), are summarized in Table I. Since we have shown that the chemical step is nearly rate-limiting for WT (Tian et al., 1990), perturbations in k_{cat} should reflect perturbation in the chemical step, if the conformations of the mutants are not perturbed.

Although the perturbations in the k_{cat} and K_m values of R44M and the k_{cat} value of R149M are qualitatively similar to the corresponding data for R44A and R149A, respectively, reported by Kim et al. (1990), there are significant differences between the K_m values of our R149M (130-fold increases for both MgATP and AMP) and their R149A (7.9- and 18.3-fold for MgATP and AMP, respectively). Although the R149M of cAK is not expected to behave exactly the same as the R149A of hAK, the lower K_m values reported by Kim et al. (1990) arise primarily because only partial instead of full kinetic analyses were performed. The apparent K_m ($K_{m,app}$) reported by Kim et al. (1990) is related to the real K_m by the equation ([A] is the concentration of the fixed substrate):

$$K_{m,app}/K_m = \left(1 + \frac{K_{ia}}{[A]}\right) \Big/ \left(1 + \frac{K_a}{[A]}\right)$$

Thus, when K_{ia} is substantially smaller than K_a, the $K_{m,app}$ will be significantly smaller than the real K_m for the varying substrate B. This appears to be the case for R149M but not for R44M. Such analysis suggests that it is important to perform full kinetic analysis rather than to draw conclusions from apparent or even preliminary kinetic data.

Proton NMR Properties of All Mutants. To allow quantitative interpretation of the kinetic data, 1D proton NMR spectra of all mutants were first examined. As shown in Figure 2, the 1D proton NMR spectra of R44M (B) and R149M (C) are almost superimposable with that of WT (A). The spectra of R128A (D) and T39A (E) are also very similar to that of WT, but very small perturbations can be detected. The results suggest that the conformations of the mutant enzymes are unperturbed in R44M and R149M and slightly perturbed in R128A and T39A.

Generally these are adequate control experiments and suggest that the kinetic data of R44M and R149M can be interpreted with confidence whereas those of R128A and T39A should be interpreted with caution. However, in a more rigorous sense, it is important to examine not only the structure of the free enzyme but also that of binary and ternary complexes. Potentially, *a mutant enzyme with perturbed structure in the free form could be induced back to the right conformation by a substrate, and a mutant enzyme with unperturbed conformation in the free form could be perturbed in binary or ternary complexes.* An example of the former is the K48A mutant enzyme (catalytically active) of *Staphylococcal nuclease* (Hibler et al., 1987). The latter has been well demonstrated in R138K (Yan et al., 1990). The proton NMR

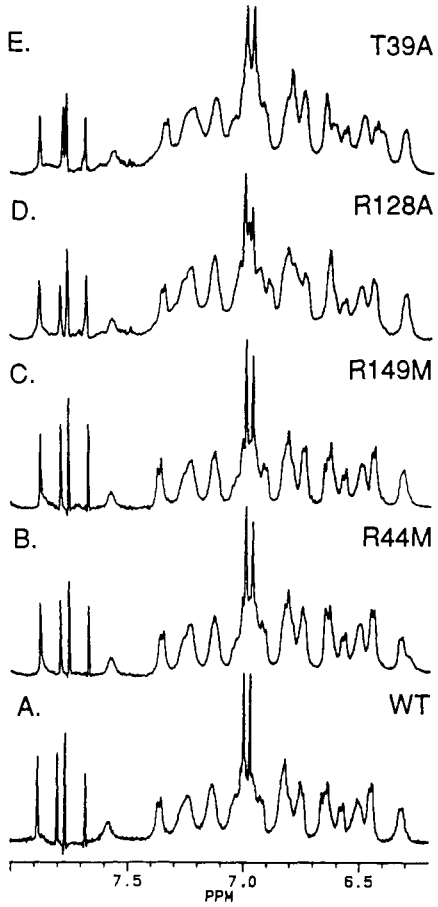

FIGURE 2: One-dimensional proton NMR spectra showing the aromatic regions of WT (A), R44M (B), R149M (C), R128A (D), and T39A (E). The spectra were obtained at pH 7.8, 27 °C, and with Gaussian multiplication (LB = −5, GB = 0.1). The spin systems of WT have been reported in Yan et al. (1990).

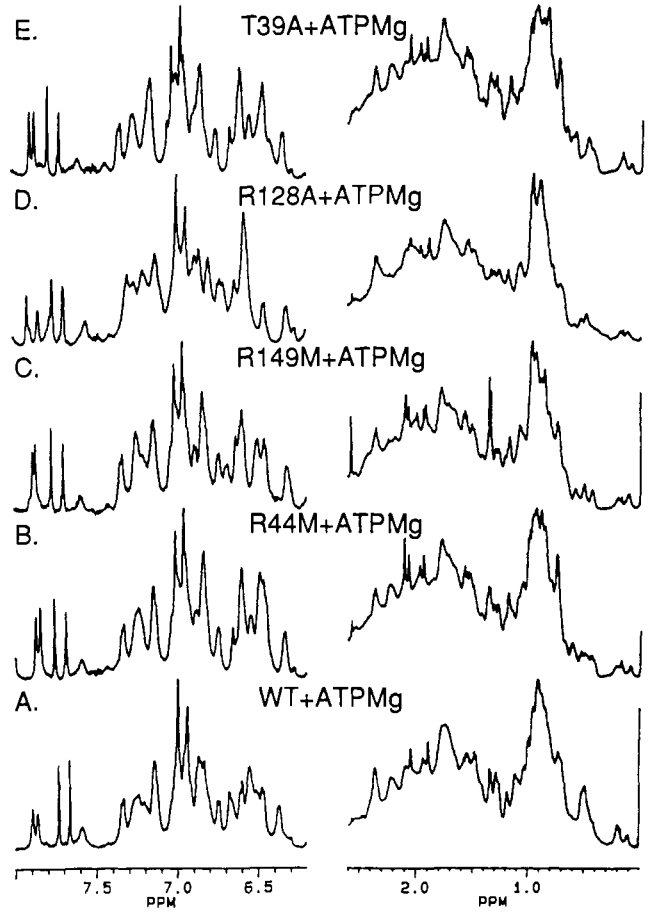

FIGURE 3: One-dimensional proton NMR spectra showing the aromatic and aliphatic regions of the MgATP complexes of WT (A), R44M (B), R149M (C), R128A (D), and T39A (E). The FID were processed with exponential multiplication (LB = 1 Hz). The concentrations (AK/ATP/Mg^{2+}, in millimolar) are 0.4/8/12, 1.0/18/20, 0.8/13/15, 1.0/20/22, and 1.0/20/22 for spectra A, B, C, D, and E, respectively.

spectrum of R138K is almost superimposable with that of WT in the free enzyme, while very small perturbations occur in binary complexes and become more noticeable in ternary complexes. Quantitative analyses by using 2D NMR indicated that the perturtation is due to small changes in chemical shifts, rather than global conformational changes, even in its ternary complex with $MgAP_5A$. Such detailed structural analyses allowed quantitative interpretation of the kinetic data of R138K, and led to the conclusion that Arg-138 stabilizes the ternary complex by 1.4–1.8 kcal/mol and the transition state by >7 kcal/mol.

Figure 3 shows the 1D proton NMR spectra of the binary complexes of the mutant enzymes with MgATP. Qualitatively, there are small perturbations (relative to WT) in every MgATP complex in the regions 6.4–6.7 and 0–0.5 ppm, with the perturbation being the most extensive in R128A+MgATP. Since T39A is essentially unperturbed functionally, the small structural perturbations in the free enzyme and the binary complex should not be related to catalysis and are not further characterized. For the other three mutants, we further characterized the conformational changes of their ternary complexes using the bisubstrate analogue $MgAP_5A$. The MgATP complexes were not analyzed quantitatively since they are not stable enough for prolonged 2D NMR analysis, and their perturbations are less extensive than the corresponding ternary complexes.

Figure 4 shows the 1D proton NMR spectra of the $MgAP_5A$ complexes, which reveal somewhat more extensive perturbations in the regions 6.4–6.8 and 0–0.7 ppm. The NOESY spectra of three of these complexes are shown in Figure 5 (WT+$MgAP_5A$), Figure 6 (R44M+$MgAP_5A$), and Figure 7 (R149M+$MgAP_5A$). Analyses of these spectra have provided useful information in three aspects: (i) The chemical shifts of the aromatic protons of R44M+$MgAP_5A$, R149M+$MgAP_5A$, and R128A+$MgAP_5A$ have been partially assigned from the 1D and NOESY spectra in analogy to the assignment of WT+$MgAP_5A$ (Yan et al., 1990). The results are listed in Table II, where the resonances different from the corresponding resonances of WT+$MgAP_5A$ by >0.02 ppm are underlined and the magnitudes of differences indicated in parentheses. (ii) The patterns of the NOESY cross-peaks can be used to assess whether the conformation has been globally perturbed. The aromatic–aromatic interresidue NOE cross-peaks are labeled as p–t and explained in the legend, whereas the aromatic–aliphatic interresidue NOE cross-peaks are unlabeled and are presented in the upper panel of the figure for qualitative comparison. (iii) The two sets of protons of AP_5A in the ternary complexes, H_2(I), H_8(I), $H_{1'}$(I), H_2(II), H_8(II), and $H_{1'}$(II), have been assigned from 1D and NOESY spectra as described previously for WT+$MgAP_5A$ (Yan et al., 1990). These are labeled as resonances a–f in the 1D spectra and explained in the legend of Figure 4, and the specific chemical shifts are listed in Table III. In the NOESY spectra, the cross-peaks arising from AP_5A are labeled as a–h as explained in the legends.

The interpretations and further studies on the structural and functional roles of each mutant enzyme are described in the following sections.

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

spin system	R44M+$MgAP_5A$			R149M+$MgAP_5A$			R128A+$MgAP_5A$		
Fa	6.41 (−0.03)	6.55 (+0.04)	6.68 (+0.03)	6.42	6.57 (+0.06)	6.68 (+0.03)	6.36 (−0.08)	6.54 (+0.03)	6.69 (+0.04)
Fb	6.41	7.00	7.17	6.40	7.00	7.17	6.40	7.00	7.17
Fe		7.17	7.58		7.15	7.59		7.18	7.59
Xa	6.80	6.97		6.81	6.98		6.80	7.00	
Xb	6.94 (−0.03)	7.22		6.93 (−0.04)	7.23 (+0.03)		6.93 (−0.04)	7.22	
Yb	6.52	6.62		6.51	6.61		6.51	6.61	
Yd	6.76	7.03		6.75	7.04		6.75	7.05	
Ye	6.86	7.12		6.85 (−0.03)	7.10		6.84 (−0.04)	7.12	
Yf	6.92	7.37		6.90	7.36		6.88 (−0.04)	7.38	
Yh	6.96	7.34		6.95	7.36		6.98	7.35	
Ha	6.99	7.67		7.00	7.68		6.99	7.68	
Hb	6.92	7.74		6.92	7.74		6.92	7.75	
H36		8.04			8.00 (−0.04)			8.00 (−0.04)	
H30	[6.76]	7.96		[6.75]	7.96		[6.75]	7.95	

[a] The underlined values are the resonances which differ by >0.02 ppm from the corresponding resonances of WT+$MgAP_5A$, and the magnitudes of the differences are shown in parentheses. The assignments in brackets are tentative. [b] Assignments were made from 1D and 2D spectra and in reference to those of WT+$MgAP_5A$ (Yan et al., 1990). Xa and Xb could be Phe or Tyr.

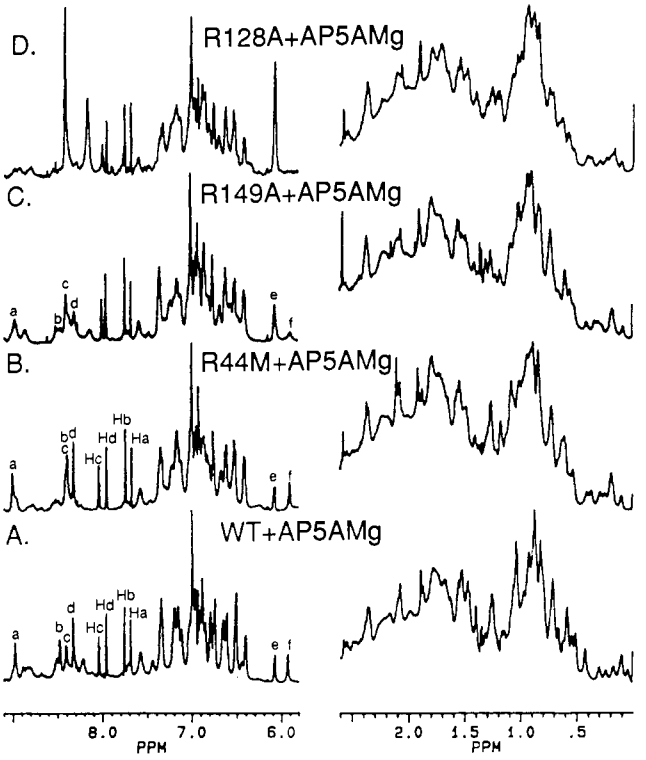

FIGURE 4: One-dimensional proton NMR spectra of the $MgAP_5A$ complexes of WT (A), R44M (B), R149M (C), and R128A (D). Peaks a–f are due to bound $MgAP_5A$: (a) H_2(I); (b) H_8(I); (c) H_8(II); (d) H_2(II); (e) $H_{1'}$(II); (f) $H_{1'}$(I). The FID were processed with Gaussian multiplication (LB = −5, GB = 0.1). The concentrations ($AK/AP_5A/Mg^{2+}$, in millimolar) are 2.5/2.5/5.0, 2.0/2.0/2.5, 2.0/2.5/3, and 1.0/1.5/2 for spectra A, B, C, and D, respectively. It should be noted that the two sets of adenosine signals are in slow exchange in spectra A and B, in intermediate exchange in spectrum C (signals broadened), and in fast exchange in spectrum D. There is also excess $MgAP_5A$ (relative to the enzyme) in spectra C and D. The H_8(free) and $H_{1'}$(free) resonances in spectrum C overlap with H_8(II) and $H_{1'}$(II) resonances, respectively, whereas H_2(free) resonates at 8.19 ppm. Other unlabeled, broad resonances at >8 ppm in all four spectra could be due to amide protons.

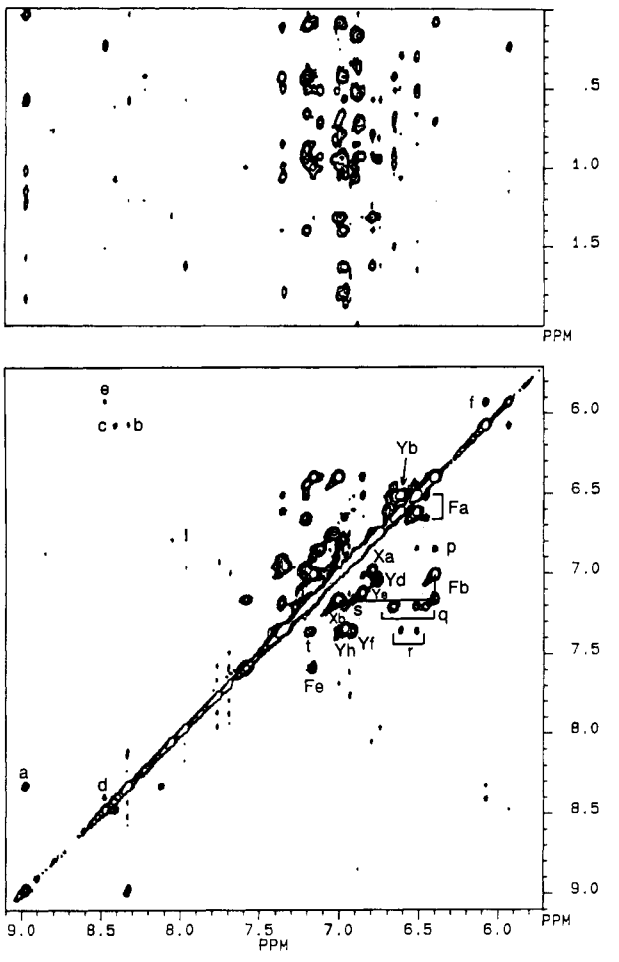

FIGURE 5: NOESY spectrum of WT+$MgAP_5A$ (the same sample used for Figure 4A). The cross-peaks a–f are due to bound $MgAP_5A$: (a) exchange cross-peak between H_2(I) and H_2(II); (b) NOE cross-peak between H_2(II) and $H_{1'}$(II); (c) NOE cross-peak between H_8(II) and $H_{1'}$(II); (d) exchange cross-peak between H_8(I) and H_8(II); (e) NOE cross-peak between H_8(I) and $H_{1'}$(I); (f) exchange cross-peak between $H_{1'}$(I) and $H_{1'}$(II). The cross-peaks p–t have been tentatively assigned to interresidue NOE for Fb/Ye, Fa/Xb, Yb/Yf, Fb/Yf, and Fe/Yf, respectively (Yan et al., 1990).

Thr-39 Is Nonessential for Catalysis. As shown in Table I, the kinetic data of T39A are little perturbed relative to those of WT, except 4–5-fold decreases in K_i. Thus, Thr-39 is not essential for the catalysis by AK, except that it could destabilize the binary complexes slightly. *This result is surprising since in the crystal structure of the AKy·MgAP$_5$A complex, Thr-39 is located very close to adenosine-B and has as many as nine contacts with MgAP$_5$A* (Egner et al., 1987). Possible rationalizations for the discrepancy between the results of functional and structural studies will be discussed in the last section.

The 1D proton NMR spectra of T39A (Figure 2) and its complex with MgATP (Figure 3) are slightly perturbed relative to WT. The perturbations were not further characterized

Table III: Chemical Shifts (ppm) of AP_5A in Various Complexes

complex	H_2		H_8		$H_{1'}$	
free $MgAP_5A$[a]	8.19		8.40		6.09	
WT+$MgAP_5A$[a]	8.98 (I)	8.32 (II)	8.47 (I)	8.40 (II)	6.07 (II)	5.93 (I)
R44M+$MgAP_5A$	9.00 (I)	8.32 (II)	8.39 (I)	8.41 (II)	6.08 (II)	5.90 (I)
R149M+$MgAP_5A$	8.98 (I)	8.31 (II)	8.48 (I)	8.40 (II)	6.07 (II)	5.90 (I)

[a] From Yan et al. (1990).

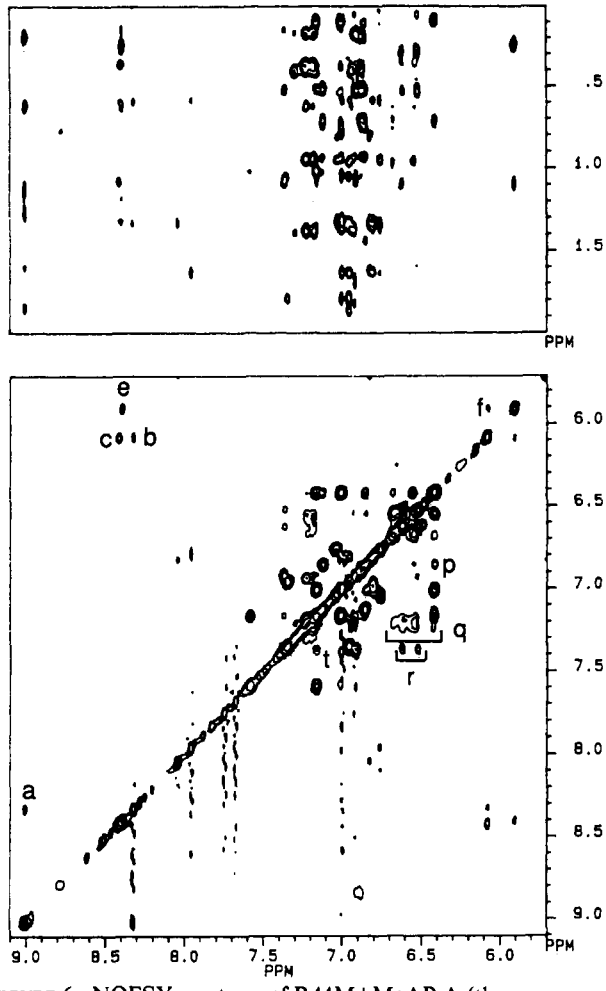

FIGURE 6: NOESY spectrum of R44M+$MgAP_5A$ (the same sample used for Figure 4B). The cross-peaks a–f and p–t are the same as those for WT+$MgAP_5A$ explained in Figure 5, except that peak d is obscured by the diagonal and peak s is not resolved.

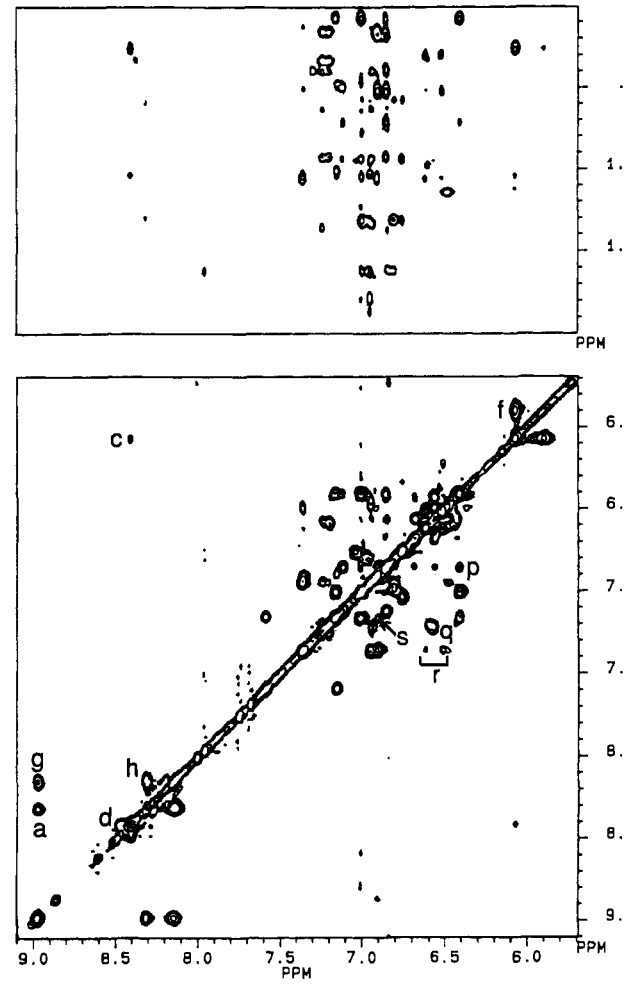

FIGURE 7: NOESY spectrum of R149M+$MgAP_5A$ (the same sample used for Figure 4C). The cross-peaks a–f and p–t are the same as those for WT+$MgAP_5A$ explained in Figure 5, except that the NOE cross-peaks b, e, and t and part of q are not detectable in this spectrum. There are other notable differences in the unlabeled peaks between this spectrum and Figure 5. The additional peaks g and h arise from the exchange of H_2(free) with H_2(I) and H_2(II), respectively. This is not observed for H_8(free) and $H_{1'}$(free) since their resonances overlap with H_8(II) and $H_{1'}$(II), respectively.

since this residue is not functionally essential, but the small perturbations suggest that Thr-39 could play a very minor structural role.

Arg-44 Interacts with AMP Starting with the Binary Complex. The kinetic data of R44M show a very specific effect on the K_m and K_i of AMP, which represents the first example of differential perturbation of the two binding sites among all of the mutant enzymes of cAK we have studied. Since R44M is also the first of our AK mutants to show a significant increase in *both* K_i and K_m, we performed proton NMR titrations to confirm that the binding affinity of R44M to AMP is indeed perturbed for the binary complex. As shown in Figure 8A, two obvious changes indicative of binding of AMP to WT are the downfield shift of the H_2 of AMP and the upfield shift of the most upfield aromatic resonance Fa. Comparison of these shifts between Figure 8A and Figure 8B indicates that the binding affinity of R44M to AMP is much weaker than that of WT to AMP. The data for the WT/AMP titration have been fitted to give two K_d values: 0.5 mM for binding to the AMP site and 4.3 mM for binding to the MgATP site (Sanders et al., 1989). The same fitting could not produce good K_d values for R44M/AMP, possibly because the affinity of AMP to the AMP site is now comparable to the MgATP site.

One might expect to see significant perturbations (relative to WT) in the proton NMR of R44M+AMP but not in R44M+MgATP since K_i is significantly perturbed for AMP but not for MgATP. However, this is not necessarily the case if the perturbation in K_i is due to a change in a very localized interaction. The 1D proton NMR spectra suggest that there are small perturbations in both R44M+MgATP (compare spectra A and B in Figure 3) and R44M+AMP (compare the top spectra in Figure 8A,B). Quantitative analyses of the $MgAP_5A$ complexes suggest that the perturbations are small even in the ternary complex, since the data in Table II show

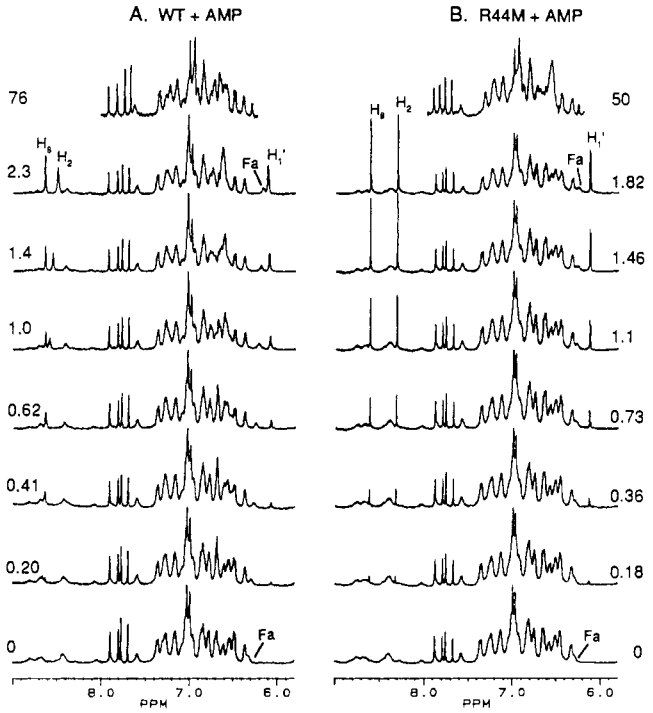

FIGURE 8: Proton NMR titration of WT (A) and R44M (B) with AMP. The starting concentrations of free AK are 1 mM. The ratios [AMP]/[AK] are shown in the figure. Only the spectra in the low ratio range are shown, except that the fully saturated complexes are shown on the top. The labeled peaks H_2, H_8, and $H_{1'}$ are the AMP resonances, whereas Fa is a protein resonance as defined in Table II. The results, but not the spectra, of the WT titration with AMP have been reported in Sanders et al. (1989).

that only two aromatic residues have been perturbed by >0.02 ppm and the largest perturbation is only 0.04 ppm. Direct comparison between the NOESY spectra of R44M+$MgAP_5A$ (Figure 6) and WT+$MgAP_5A$ (Figure 5) indicates that the interresidue NOE cross-peaks between aromatic–aromatic residues (peaks p–t) and between aromatic–aliphatic residues (in the upper panel of the spectra) are very similar in both spectra. Thus, the perturbations in K_d and K_m of AMP appear to be due to a very localized effect.

The above results suggest that the conformation of R44M is not perturbed in the free enzyme and only slightly perturbed in binary and ternary complexes. This permits quantitative interpretation of the kinetic data: Arg-44 stabilizes AMP by ca. 2 kcal/mol, starting with the AK·AMP binary complex. Thus, *the results of R44M suggest that the "adenosine-B" site of Egner et al. (1987), which is also the MgATP site of Pai et al. (1977), is likely to be the AMP site.*

Arg-149 Is Critically Important to Catalysis. The kinetic data of R149M are similar to those of R138K and R138M (also listed in Table I) (Yan et al., 1990) in that K_i values are only slightly perturbed, k_{cat} values decrease, and K_m values increase significantly for both substrates, and k_{cat}/K_m values decrease by as much as 2×10^5. The only difference between R149M and R138K or R138M is that the extent of perturbations for R149M is larger in K_m and smaller in k_{cat}. The increases in K_m values for R149M are among the largest for site-specific mutants of enzymes. The invariance in the K_i values of R149M has been independently verified by proton NMR: titration of R149M with MgATP gave a dissociation constant of 0.26 mM.

The structural property of R149M is also very similar to that of R138K (and R138M). The 1D spectrum of the free enzyme is essentially superimposable with that of WT (Figure 2). The MgATP complex is slightly perturbed (Figure 3). Most intriguingly, both 1D NMR and NOESY spectra of R149M+$MgAP_5A$ are very similar to those of R138K+$MgAP_5A$ (Yan et al., 1990) while differ somewhat with WT+$MgAP_5A$. On the basis of the chemical shift data in Table II and the NOESY spectra (noted in the legend of Figure 7), the differences between R149M+$MgAP_5A$ (or R138K+$MgAP_5A$) and WT+$MgAP_5A$, though still minor, are clearly more extensive than the differences between R44M+$MgAP_5A$ and WT+$MgAP_5A$.

In the case of R138K, we attributed the perturbations in the $MgAP_5A$ complex to the "K_m effect"; i.e., it reflects the increases in the K_m values of the mutant enzyme. The same interpretation can be applied to R149M. Then, why is the perturbation smaller in R44M+$MgAP_5A$ than in R138K+$MgAP_5A$ and R149M+$MgAP_5A$? For R138K and R149M, the K_m values of *both* MgATP and AMP are increased, which suggests that Arg-138 and Arg-149 are involved in the conformational stabilization (possibly only locally) of the ternary complex. Disruption of this interaction resulted in local and minor conformational changes in the ternary complex. On the other hand, for R44M, the K_m of AMP only is perturbed, which suggests that Arg-44 is involved in a localized interaction with AMP as mentioned earlier. Disruption of this interaction thus resulted in minimal structural perturbation.

Such structural analyses allow quantitative interpretation of the kinetic data: *Arg-149 is not important structurally, but is critically important to catalysis by stabilizing the ternary complex by 2.9 kcal/mol* (1.4–1.8 kcal/mol for Arg-138) *and the transition state by 7.3 kcal/mol* (similar to Arg-138). Although we are confident with these analyses and interpretations, *a minor reservation should be kept in mind: the contribution of Arg-149 and Arg-138 to transition-state stabilization could be overestimated due to minor structural perturbations in the ternary complexes of R149M and R138K.*

Roles of Arg-128 Are Minor, if Any. Quantitative interpretation of the structural and functional roles of Arg-128 is difficult since R128A appears to be moderately perturbed in every kinetic parameter (<20-fold in any parameter). Such data can suggest two possibilities: (a) The conformation of R128A is slightly perturbed, which causes nonspecific effects on the kinetic parameters; (b) Arg-128 is involved in weak uniform binding in the catalysis by AK.

The 1D spectrum of R128A has been perturbed slightly relative to that of WT (Figure 2). The perturbation in the binary complex of R128A with MgATP appears to be the most extensive among the four mutants shown in Figure 3. The data in Table II also indicate that the chemical shift changes in the ternary complex are more extensive compared to those of R44M and R149M. These results favor possibility a, but cannot rule out possibility b. In any case, Arg-128 is not critically important to either the structure or the function of AK.

Chemical Shift Assignment of $MgAP_5A$ Complexes. Assignment of the chemical shifts of $MgAP_5A$ resonances in its complex with AK is an important step toward elucidating AK-substrate interactions by NMR (the other important step is sequential assignment of AK residues). In Yan et al. (1990), we have assigned the $MgAP_5A$ resonances (see Table III) on the basis of 1D and 2D NMR and site-specific mutagenesis. However, the two sets of adenosine resonances (designated as I and II) had not been assigned to the two sites.

Deducing binding sites on the basis of chemical shift changes is a useful but sometimes risky approach. Thus, although we have shown that the adenine H_2 of AMP is shifted downfield

by 0.4 ppm upon binding to cAK (shifts of other adenine protons of AMP and MgATP are all <0.15 ppm) and that the $H_2(I)$ of WT+$MgAP_5A$ is shifted downfield by 0.8 ppm (shifts of all other adenine protons of AP_5A are also <0.15 ppm), we did not assign the downfield $H_2(I)$ of WT+$MgAP_5A$ at 8.98 ppm to the AMP site on the basis of this single evidence (Yan et al., 1990). However, when this evidence is coupled with another one from the R44M mutant as described below, the assignment becomes convincing.

As shown in Table III, while the chemical shifts of all other adenine protons for the $MgAP_5A$ complexes of WT, R44M,and R149M are all constant within 0.02 ppm, the $H_8(I)$ proton of R44M+$MgAP_5A$ is shifted upfield by 0.09 ppm. This is a small shift, but it is very clear even visually in both 1D NMR spectra (compare peak b in spectra A and B of Figure 4) and NOESY spectra (compare the position of cross-peak e in Figures 5 and 6). Since the AMP site is specifically perturbed in R44M, and the $H_8(I)$ resonance is the only distinctly shifted resonance among all resolvable resonances of R44M+$MgAP_5A$ (including the resonances of the protein), the results strongly support that $H_8(I)$ is at the AMP site. Thus, *the two independent pieces of evidence together justify the assignment of site I to the AMP site.*

Evaluation of Binding Site Models. The results of R44M strongly support the suggestion by Egner et al. (1987) that the "adenosine-B site" in the AKy·$MgAP_5A$ complex, originally assigned as the MgATP site by Pai et al. (1977), should be the AMP site. The same conclusion has been reached independently by Kim et al. (1990) on the basis of the kinetic data of the R44A mutant enzyme of hAK, by Stehle and Schulz (1990) on the basis of the crystal structure of the guanylate kinase·GMP complex, and by Diederichs and Schulz (1990) on the basis of the crystal structure of the AK3·AMP complex. However, the AMP site should not be considered "solved", since Thr-39, which is located near the adenine moiety of adenosine-B in the AKy·$MgAP_5A$ complex [nine contacts as suggested by Egner et al. (1987)], is not essential to binding or catalysis on the basis of the results of T39A. It is possible that even though Thr-39 is very close to the adenine ring there is no direct interaction. Alternatively, the function of Thr-39 could be replaced by another residue or by a water molecule in the mutant enzyme T39A. Another possibility is that the detailed interaction between AMP and cAK is somewhat different from that depicted in the X-ray structure of AKy·$MgAP_5A$. In any case, further studies are necessary to firmly establish and fine-tune the AMP site, not only in terms of the static structure but also in the functional roles of the residues surrounding the substrate. Assignment of the resonances of bound $MgAP_5A$ represents a significant step toward this direction.

Reassignment of the MgATP site of Pai et al. (1977) to the AMP site should not be taken to suggest that the MgATP site and the AMP site in Pai et al. (1977) are reversed, as suggested by Kim et al. (1990). There are at least two other candidates for the MgATP site: the MgATP site in the NMR model (Fry et al., 1985) and the "adenosine-A" site in the AKy·$MgAP_5A$ complex (Egner et al., 1987) and the AKe·$MgAP_5A$ complex (Müller & Schulz, 1988). Although our previous results (Tian et al., 1988, 1990; Yan et al., 1990) suggest that the NMR model requires serious revision, the NOE between the H_2 of His-36 and the adenine H_2 of ATP (Smith & Mildvan, 1982; Fry et al., 1985) cannot be easily discounted. In regard to the adenosine-A site of $MgAP_5A$, although Egner et al. (1987) originally suggested it to be a spurious site, we have suggested that $MgAP_5A$ is a true bisubstrate analogue since the proton NMR change induced by $MgAP_5A$ is very similar to that induced by the reaction mixture (Sanders et al., 1989; Yan et al., 1990). The same suggestion has been reported by Schulz et al. (1990) and Vetter et al. (1990).

Arg-149 was not listed as one of the residues in contact (<3.5 ± 0.4 Å) with $MgAP_5A$ in the AKy·$MgAP_5A$ complex (Egner et al., 1987). However, the results of R149M indicate that Arg-149 is as important as Arg-138 (Yan et al., 1990) to the catalysis by AK and that its role in catalysis also starts with the ternary complex.

The contrasting facts that Arg-149 was not suggested to be interacting with $MgAP_5A$ directly but was found to be critically important to catalysis whereas Thr-39 was shown to be important in the crystal structure but found to be nonessential by functional studies present another important message: even if the crystal structure of the enzyme·substrate complex has been solved, it is still important to perform functional studies using site-specific mutagenesis since *deducing the functional significance from crystal structures could sometimes be misleading.* More explicitly, *a residue in proximity to a substrate may not necessarily be functioning during catalysis, whereas a functional residue may not always be obvious by examining the crystal structure at the resting state.*

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Registry No. AK, 9013-02-9; AMP, 61-19-8; MgATP, 1476-84-2; $MgAP_5A$, 41708-91-2; L-Arg, 74-79-3.

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Reaction of Ascorbate with Lysine and Protein under Autoxidizing Conditions: Formation of N^{ϵ}-(Carboxymethyl)lysine by Reaction between Lysine and Products of Autoxidation of Ascorbate†

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ABSTRACT: N^{ϵ}-(Carboxymethyl)lysine (CML) has been identified as a product of oxidation of glucose adducts to protein in vitro and has been detected in human tissue proteins and urine [Ahmed, M. U., Thorpe, S. R., & Baynes, J. W. (1986) *J. Biol. Chem. 261*, 4889-4894; Dunn, J. A., Patrick, J. S., Thorpe, S. R., & Baynes, J. W. (1989) *Biochemistry 28*, 9464-9468]. In the present study we show that CML is also formed in reactions between ascorbate and lysine residues in model compounds and protein in vitro. The formation of CML from ascorbate and lysine proceeds spontaneously at physiological pH and temperature under air. Kinetic studies indicate that oxidation of ascorbic acid to dehydroascorbate is required. Threose and N^{ϵ}-threuloselysine, the Amadori adduct of threose to lysine, were identified in the ascorbate reaction mixtures, suggesting that CML was formed by oxidative cleavage of N^{ϵ}-threuloselysine. Support for this mechanism was obtained by identifying CML as a product of reaction between threose and lysine and by analysis of the relative rates of formation of threuloselysine and CML in reactions of ascorbate or threose with lysine. The detection of CML as a product of reaction of ascorbate and threose with lysine suggests that other sugars, in addition to glucose, may be sources of CML in proteins in vivo. The proposed mechanism for formation of CML from ascorbate is an example of autoxidative glycosylation of protein and suggests that CML may also be an indicator of autoxidative glycosylation of proteins in vivo.

The Maillard or browning reaction between reducing sugars and amines is a complex series of reactions that leads eventually to the formation of brown and fluorescent polymeric products, known as melanoidins (Hodge, 1953; Feather & Waller, 1983; Fujimaki et al., 1986; Baynes & Monnier, 1989). The early products of the reaction are Schiff base and ketoamine (Amadori) adducts formed between sugars and amines, while the brown products, which are formed during later stages of the reaction, are not well characterized. Maillard reactions between glucose and amino groups of proteins are thought to contribute to the browning, fluorescence, and cross-linking of protein in vivo, suggesting a mechanism for the development of pathophysiology in diabetes and aging (Fujimaki et al., 1986; Brownlee et al., 1988a,b; Kennedy & Lyons, 1989; Baynes & Monnier, 1989). However, evidence in support of this hypothesis is limited because of the lack of information on the structure of browning products formed by reactions of proteins with glucose either in vitro or in vivo.

In recent studies on the Maillard reaction we have begun to characterize products formed from the model Amadori compound N^{α}-formyl-N^{ϵ}-(1-deoxy-D-fructos-1-yl)-L-lysine

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