

# Mechanism of Adenylate Kinase: Site-Directed Mutagenesis versus X-ray and NMR<sup>†</sup>

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Controversy is an integral part of scientific research and is often a precursor to the truth. However, this lesson has been learned in a very hard way in the case of the structure-function relationship of adenylate kinase (AK),<sup>1</sup> which catalyzes the interconversion between MgATP + AMP and MgADP + ADP. While this small kinase has been considered a "model kinase" and the enzyme-substrate interaction of AK was among the first investigated by X-ray crystallography (Pai et al., 1977) and NMR (McDonald et al., 1975; Fry et al., 1985), the substrate binding sites deduced from the early studies by these two powerful techniques (termed the "X-ray model" and the "NMR model", respectively) were dramatically different as shown in Figure 1. Ironically, both models have had substantial impact on researchers in related fields.

The problems have finally been dealt with since 1987 by the interplay between site-directed mutagenesis, X-ray, and NMR. Although the road to the truth has also been quite rough, a clear picture is evolving (see Figure 12), which turns out to be different from either model shown in Figure 1. Many useful lessons have been learned in the process. The purpose of this review is not only to summarize the current knowledge in the structure-function relationship of adenylate kinase but also to accurately document and critically analyze historical developments in the hope that history will not be repeated. As a concurrent theme, we emphasize the importance of "iterative structure-function studies", which involve interplay (or iteration) between kinetic analysis of site-directed mutant enzymes, structural analysis of the mutant enzymes and their substrate complexes, and further tests of the interpretations by conventional bioorganic and biophysical techniques.

## BACKGROUND

**Primary Structures.** There are five major types of AK: AK1 (from cytosols of mammalian or vertebrate muscles, with c, h, p, and r designating chicken, human, porcine, and rabbit muscles, respectively), AKe (from *Escherichia coli*), AKy (from yeast), AK2 (from mammalian mitochondrial intermembrane space), and AK3 (from mammalian mitochondrial matrix, specific to GTP instead of ATP). There is a major structural difference between AK1 and the other four types of AK: AK1 (194 aa, 21.7 kDa, ≥75% homology between different muscles) is smaller by ca. 30 residues. Although most studies prior to 1987 have been performed on AK1, recent studies have included all types of AK. Direct comparison between the results of different types of AK requires proper alignments of primary sequences and a unified numbering system, which unfortunately have changed from time to time. Figure 2 shows the latest alignment by Schulz (1987), in which the insertion segment is placed between residues 132 and 133 of AK1 on the basis of direct comparison of crystal structures

(Egner et al., 1987). However, this alignment has been further revised by Haase et al. (1989), and the main differences are indicated in Figure 2. Since the alignment is not yet definite, it will be less confusing not to use the family numbering system. Unless otherwise specified, the numbering used in this review represents that of AK1.

**Kinetic and Catalytic Properties.** AK enhances the reaction between AMP and MgATP by a factor of  $>10^{12}$  (Sanders et al., 1989) and is a nearly perfect enzyme ( $k_{cat}/K_m \geq 10^7 \text{ s}^{-1} \text{ M}^{-1}$ ). Both external and internal equilibrium constants are close to unity (Nageswara Rao et al., 1978; Tian et al., 1990). The basic kinetic pattern is random Bi-Bi (Rhoads & Lowenstein, 1968). However, whether the chemical step or the dissociation step is rate-limiting has also been a subject of controversy as discussed in Tian et al. (1990). This issue is indeed critically important in the interpretation of the  $k_{cat}$  of mutant enzymes, as further addressed in a later section.

**How Was the X-ray Model Derived?** Three crystal forms have been obtained for free AK1p: A (pH 6.9–8.0), B (pH 5.7–5.9), and C (pH 4.5–5.4) (Schulz et al., 1973; Sachsenheimer & Schulz, 1977). The structure of crystal form A has been determined at 3-Å resolution (Schulz et al., 1974), and refined to 2.1-Å resolution recently (Dreusicke et al., 1988). The structure of crystal form B has been determined first at 4.7-Å resolution (Sachsenheimer & Schulz, 1977) and later refined to 3.3-Å resolution (Dreusicke & Schulz, 1988), whereas that of crystal form C has not been reported. The structure of carp muscle AK has also been determined at 5.8-Å resolution (Reuner et al., 1988).

The substrate sites in the X-ray model (Figure 1A) were assigned by soaking the crystals (forms A and B of AK1p) with various substrates and substrate analogues (Pai et al., 1977). In retrospect, there are three potential problems in such assignments: (i) The ATP site was assigned to the site occupied by salicylate, which has little structural resemblance to ATP; (ii) perhaps the most puzzling of all is that the site occupied by MnATP was assigned to the AMP site [recently this has been suggested to be probably an artifact by Diedrichs and Schulz (1990)]; and (iii) the measurements of the complexes were restricted to 6-Å resolution because of crystal inactivity. Nevertheless, the X-ray model seemed attractive in that the phosphate groups are surrounded by several highly conserved arginine side chains and that the two nucleotides

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<sup>1</sup> Abbreviations: 1D, one dimensional; 2D, two dimensional; ADP, adenosine 5'-diphosphate; ADPαS, adenosine 5'-O-(1-thiodiphosphate); AK, adenylate kinase; AMP, adenosine 5'-monophosphate; AMPS, adenosine 5'-monothiophosphate; AP<sub>5</sub>A, P<sub>1</sub>,P<sub>5</sub>-bis(5'-adenosyl) penta-phosphate; ATP, adenosine 5'-triphosphate; ATPαS, adenosine 5'-O-(1-thiotriphosphate); CD, circular dichroism; EDTA, ethylenediaminetetraacetate; FTIR, Fourier-transform infrared spectroscopy; Gdn, guanidine; GppNp, guanosine 5'-(β,γ-imidotriphosphate); GTP, guanosine 5'-triphosphate; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser enhanced spectroscopy; TOCSY, total correlated spectroscopy; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; WT, wild type.

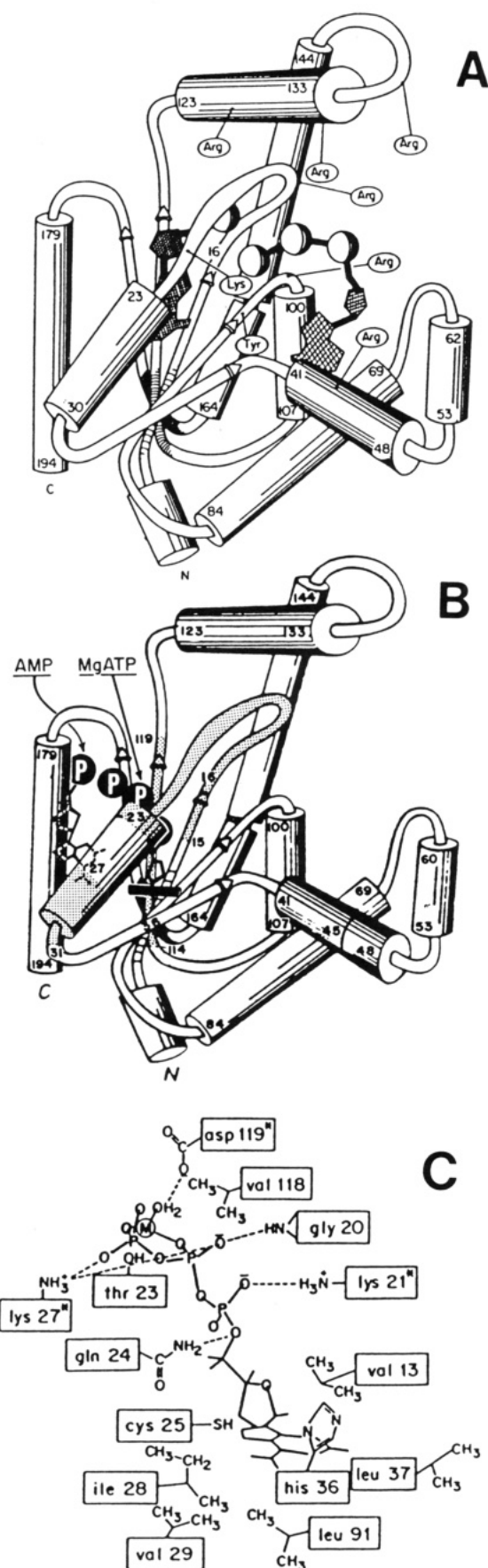


FIGURE 1: Sketches of AK1p and its substrate sites deduced from X-ray crystallography [(A) from Pai et al. (1977)] and from NMR studies [(B) from Fry et al. (1987)]. The protein structure shown is that of crystal form B. The detailed interactions in the NMR model are shown in form C [from Mildvan and Fry (1987)].

AK1p	MEEKLKKSKIIIFVVGPGSGKGTQCEKIVQKYGYTHLSTGDLRR	44 (53)
AK1c	ST...HH.....H.....	44 (53)
AKy	SSEIRMLVIGPPGAGKGTQAPNLQERFHAHLATGDMLE	40 (53)
AKe	MRIILLGAPGAGKGTQAFIMEKYGIPQISTGDMLE	36 (53)
AK1p	AEVSSGSARGKMLSEIMEKQGVLPLETVLDMLRDMVAKVDTSKGLIDGYP	96 (105)
AK1c	.....E...K.QA.....D.....L.A.....	96 (105)
AKy	SGIAGTQLGLEAKKIMDQGLVSDIMVMIKDELNNACKNGFILDGFP	92 (105)
AKe	AAVSGSELGKQAKDMDAGKLVDELVIALVKERI-AQEDCRNGFLLDGFP	87 (105)
[---RKIGOPTLLLYVDAGPETMTKRLKR---GETSGRV---		
AK1p	REVKGEEFERKIGOPTLLLYVDAGPETMTKRLKR---	132 (141)
AK1c	.....K..AP.....K..V.....	132 (141)
AKy	RTIPQAEKLDQMLKEQGTPEKATIELKYDELVARITGRILHPASGSYHK	144 (157)
AKe	RTIPQA---DAM-KEAGINVDYVLEFDPDELIVDRVGRVHAPSGRVHV	135 (157)
[---GRVDDNEETIKRLETTYKATEPYIAFYEKRI---		
AK1p	-----GRVDDNEETIKRLETTYKATEPYIAFYEKRI	169 (209)
AK1c	.....QL...T..E..Q...SY..K.	169 (209)
AKy	IFNPPKEDMKDDVTGEALVQRSDNDADALKRLAAZHAQTEPIVDEYKGTGI	196 (209)
AKe	KFNPKVEGKDDVTGEELTRKDDQETVRKRLVEYHQMTPALIGYYSKEAE	187 (209)
[---YRKVNAEGSVDDVFSQVCTHLDTLK---		
AK1p	-----YRKVNAEGSVDDVFSQVCTHLDTLK	194 (239)
AK1c	.....QL...T..E..Q...SY..K.	193 (238)
AKy	---WAGVDASQPPATVWADIILNKLGN	220 (238)
AKe	AGNTYAKVVDGTPVAEVRADLEKILG	214 (236)

FIGURE 2: Primary sequences of the three different types of AK. Two AK1 sequences are listed since AK1c is the system used in our work and AK1p is the system used in early crystallographic studies. The alignment and the "family numbering" shown in parentheses are according to Schulz (1987). The bracket indicates the main difference in the AK1p alignment suggested by Haase et al. (1989). The sources and references are as follows: AK1p, porcine skeletal muscle cytosolic AK (Heil et al., 1974); AK1c, chicken skeletal muscle cytosolic AK (Kishi et al., 1986); AKy, cytosolic AK from bakers' yeast (Tomasselli et al., 1986); AKe, *E. coli* AK (Brune et al., 1985). It should be noted that AK1c has one additional methionine at the N-terminal (Kishi et al., 1986), which is absent in the enzyme overproduced in *E. coli* (Tanizawa et al., 1987).

are located on the two sides of the "glycine-rich loop" (G-loop) (Dreusicke & Schulz, 1986), also termed the "phosphate-binding loop" (P-loop) (Saraste et al., 1990), which is a common motif found in most (but not all) adenine and guanine nucleotide binding proteins.

**How Was the NMR Model Derived?** The remarkable NMR studies on AK by A. S. Mildvan and collaborators (Fry et al., 1985, 1987; Mildvan & Fry, 1987) led to the "NMR model" shown in Figure 1 (B and C). A key background development in this work is the discovery by Hamada et al. (1979) of a MgATP binding fragment (residues 1–44) and an AMP binding fragment (residues 172–194) of AK1r. These fragments allowed separate studies of both *putative* substrate sites by distance measurements on the basis of paramagnetic relaxations and NOE. Studies were also conducted with the native enzyme to attempt corroboration of the results. Although significantly different from the X-ray model, the NMR model has been broadly cited, and the work has encouraged many other researchers to use peptide fragments for structure–function studies of enzymes.

In retrospect, there are a few serious problems in such NMR studies, even though the techniques were state-of-the-art. (i) Since the crystal structure used for "NMR docking" was only at 3-Å resolution and the distances determined from NMR could also have errors on the order of angstroms, the reliability of the high-resolution structure as shown in Figure 1C is questionable. (ii) Distance measurements require resonance assignments of protein residues. However, the resonance assignments reported in these papers appear to be highly speculative. (iii) Although Fry et al. (1988) have further used 2D NMR, FTIR, and CD to show that the MgATP binding fragment appears to maintain a conformation similar to that in the X-ray structure of intact AK, the specificity of this peptide fragment toward MgATP (relative to AMP) and that of the C-terminal fragment toward AMP (relative to MgATP) reported by Hamada et al. (1979) appear too good to believe. Indeed the same paper also reported a nonapeptide fragment of AK (residues 32–40) with similar binding capability and specificity to the 1–44 fragment. However, we have syn-

Table I: Summary of Steady-State Kinetic Data Obtained in Our Laboratory<sup>a</sup>

	$k_{\text{cat}}$ (s <sup>-1</sup> )	$K_{\text{MgATP}}$ (mM)	$K_{\text{AMP}}$ (mM)	$K_{\text{i(MgATP)}}$ (mM)	$K_{\text{i(AMP)}}$ (mM)	$k_{\text{cat}}/K_{\text{MgATP}}$ (s <sup>-1</sup> M <sup>-1</sup> )	$k_{\text{cat}}/K_{\text{AMP}}$ (s <sup>-1</sup> M <sup>-1</sup> )
WT <sup>b</sup>	650	0.042	0.098	0.16	0.37	$15.5 \times 10^6$	$6.6 \times 10^6$
K21M <sup>b</sup>	0.015 ( $2.3 \times 10^{-5}$ )	0.052	0.044	0.19	0.16	$290 (1.9 \times 10^{-5})$	$340 (5.2 \times 10^{-5})$
C25S <sup>b</sup>	113	0.074	0.099	0.31	0.41	$1.5 \times 10^6$	$1.1 \times 10^6$
K27M <sup>b</sup>	850	0.105	0.13	0.32	0.40	$8.1 \times 10^6$	$6.5 \times 10^6$
H36N <sup>b</sup>	600	0.13	0.52	0.26	1.01	$4.6 \times 10^6$	$1.1 \times 10^6$
H36Q <sup>b</sup>	640	0.54	1.43	0.29	0.78	$1.2 \times 10^6$	$0.45 \times 10^6$
T39A <sup>c</sup>	450	0.047	0.089	0.038	0.073	$9.6 \times 10^6$	$5.1 \times 10^6$
R44M <sup>c</sup>	210	0.048	3.53 (36)	0.11	8.25 (22)	$4.4 \times 10^6$	$5.9 \times 10^4 (8.9 \times 10^{-3})$
D93A <sup>d</sup>	1 ( $1.7 \times 10^{-3}$ )	0.16	0.47	0.11	0.33	$0.6 \times 10^4 (0.4 \times 10^{-3})$	$0.2 \times 10^4 (0.3 \times 10^{-3})$
R97M <sup>e</sup>	22 (0.034)	0.083	2.78 (28)	0.066	2.22 (6)	$0.26 \times 10^6 (0.017)$	$7.9 \times 10^3 (1.2 \times 10^{-3})$
R128A <sup>c</sup>	36	0.65	1.38	1.63	3.48	$5.5 \times 10^4$	$2.6 \times 10^4$
R132M <sup>f</sup>	0.083 ( $1.3 \times 10^{-4}$ )	0.056	0.053	0.18	0.17	$1.5 \times 10^3 (1.0 \times 10^{-4})$	$1.6 \times 10^3 (2.4 \times 10^{-4})$
R138K <sup>g</sup>	0.1 ( $1.5 \times 10^{-4}$ )	0.36	1.43	0.090	0.36	$280 (1.8 \times 10^{-5})$	$70 (1.1 \times 10^{-5})$
R138M <sup>g</sup>	0.049 ( $7.5 \times 10^{-5}$ )	0.40	2.23	0.085	0.47	$120 (7.7 \times 10^{-6})$	$22 (3.3 \times 10^{-6})$
R149M <sup>c</sup>	0.42 ( $6.5 \times 10^{-4}$ )	5.6 (130)	12.4 (130)	0.29	0.64	$75 (4.8 \times 10^{-6})$	$34 (5.1 \times 10^{-6})$

<sup>a</sup> Numbers in parentheses indicate the ratios between the mutant and WT. Only the large changes are indicated. <sup>b</sup> From Tian et al. (1990). <sup>c</sup> From Yan et al. (1990b). <sup>d</sup> From Yan and Tsai (1991). <sup>e</sup> Dahne, unpublished results. <sup>f</sup> Shi, unpublished results. <sup>g</sup> From Yan et al. (1990a).



FIGURE 3: Stereoview of the backbone of the 2.1-Å structure of the crystal form A of AK1p (Dreusicke et al., 1988), along with the side chains of the residues listed in Table I.

thesized this nonapeptide and a larger fragment (residues 20–45) and found that the affinity of both peptides to ATP or MgATP is very weak ( $K_d > 10$  mM) (Wisner, 1987). (iv) Even if these peptides would bind specific nucleotides, they may bind differently from the native AK.

#### SITE-DIRECTED MUTAGENESIS: ITERATIVE STRUCTURE-FUNCTION STUDY

The discrepancy between the X-ray model and the NMR model was an obvious target to be tested by site-directed mutagenesis. The timely cloning of the chicken muscle AK1 gene (Kishi et al., 1986) and its expression in *E. coli* (Tanizawa et al., 1987), coupled with the kindness of A. Nakazawa in making the overproducing strains available to us, allowed us to start the venture. It should be noted that the AKE gene had been cloned 1 year earlier (Brune et al., 1985), and mutagenesis studies of AK from different sources have been performed in several laboratories as described in a later section. This section focuses on the work in our laboratory since our interest was not only in testing the two models but also in using AK as a system to emphasize "iterative structure-function studies" of enzymes.

In our view, the real breakthrough in the understanding of protein functions and the ability to design functional proteins will not come until there is another quantum jump in the resolution of structure-function investigations. This is, of course, the goal of the entire field, but we specifically emphasize two points: (a) It is important not only to characterize the structure of the mutant enzyme and interpret the kinetic data quantitatively but also to design further experiments to test interpretations. These will allow us not only to conclude

that a particular residue is important but also to understand its structural and/or functional roles in *quantitative* and *specific* terms. (b) While the majority of researchers have been searching for regularity, homology, or rules, the next step should be to look for differences within the regularity, or exceptions to the rules. For example, the P-loop is a common motif in nucleotide binding proteins (Dreusicke & Schulz, 1986; Saraste et al., 1990), but does it function differently in different proteins? With these thoughts in mind, our structure-function studies have been performed in three stages:

**Stage 1: Functional Studies.** We first established that the chemical step is partially rate-limiting in the catalysis by AK and that  $k_{\text{cat}}$  is a good approximation (within a factor of ca. 5) of the rate of the chemical step (Tian et al., 1990). This allowed us to interpret the  $k_{\text{cat}}$  data without getting into pre-steady-state kinetics (which has not yet been developed for AK). A full initial velocity analysis was performed by varying the concentrations of both substrates. The turnover number  $k_{\text{cat}}$ , the Michaelis constants  $K_m$  ( $K_{\text{MgATP}}$  and  $K_{\text{AMP}}$ ), and the dissociation constants  $K_{\text{i(MgATP)}}$  and  $K_{\text{i(AMP)}}$  were then calculated from the rate equation for a random Bi-Bi system (Cleland, 1986) and summarized in Table I. For the WT AK, we have shown that the  $K_m$  values determined from such a full analysis agree with the corresponding  $K_m$  values obtained by holding one substrate at saturating concentration, and the  $K_i$  values agree with the dissociation constants  $K_d$  obtained from NMR studies within experimental errors, which justified the random Bi-Bi treatment. The locations of the side chains of the residues mentioned in Table I are shown in Figure 3.

**Stage 2: Structural Studies.** The changes in conformation and/or stability of mutant enzymes are joys to structural

Table II: Free Energy of Unfolding Induced by Gdn-HCl<sup>a</sup>

	$\Delta G_D^{H_2O}$ (kcal/mol)	$C_m$ (M)	$m$ [kcal/(mol·M)]
WT AK1c <sup>b</sup>	3.8	0.79	4.7
K21M <sup>c</sup>	3.5	0.77	4.4
K27M <sup>c</sup>	5.6	1.04	5.4
H36Q <sup>b</sup>	3.1	0.68	4.4
H36N <sup>b</sup>	2.5	0.52	4.5
H36G <sup>b</sup>	very unstable		
porcine AK <sup>b</sup>	3.9		4.8
WT AK1c <sup>d</sup>	4.5	0.81	5.7
T39A <sup>d</sup>	5.1	0.79	6.5
R44M <sup>d</sup>	4.0	0.81	4.9
D93A <sup>d</sup>	4.0	0.71	5.6
R97M <sup>d</sup>	5.1	0.82	6.2
R128A <sup>d</sup>	4.5	0.79	5.6
R132M <sup>d</sup>	5.2	0.88	5.9
R138K <sup>d</sup>	5.3	0.83	6.3
R149M <sup>d</sup>	5.5	0.88	6.1

<sup>a</sup>Calculated from the equation  $\Delta G_D = \Delta G_D^{H_2O} - m[\text{Gdn-HCl}]$ , where  $\Delta G_D$  is the Gibbs free energy change in denaturation at various concentrations of Gdn-HCl,  $\Delta G_D^{H_2O}$  is the Gibbs free energy change extrapolated to zero concentration of Gdn-HCl, and  $m$  is a constant. <sup>b</sup>From Tian et al. (1988). <sup>c</sup>From Tian et al. (1990). <sup>d</sup>Obtained under modified conditions using CD instead of UV (T. Dahnke, unpublished results).

biochemists since these will generate useful structural insights, but they are headaches to mechanistic enzymologists since the kinetic data will be difficult or impossible to interpret. The importance of structural analysis of mutant enzymes has been emphasized previously (Hibler et al., 1987). Indeed, the problems facing enzymologists are more serious than many have realized for two reasons: (i) Even if the complete structure of every mutant enzyme can be determined, there are still two problems: how much perturbation is to be considered as significant, and how to determine if structural perturbations are really related to perturbations in the function. (ii) For a bisubstrate enzyme such as AK, an unperturbed conformation in the free mutant enzyme justifies interpretation of the  $K_d$  of binary complexes only, since a mutant enzyme with unperturbed conformation in the free form could be perturbed in binary or ternary complexes. Proper interpretation of  $K_m$  requires an unperturbed conformation in the binary complex, and proper interpretation of  $k_{cat}$  requires an unperturbed conformation in the ternary complex. On the other hand, a mutant enzyme with perturbed structure in the free form could be induced back to the right conformation by a substrate.

The best strategy to handle these problems is to be as rigorous as possible in experiments and as cautious as possible in interpretations. For each mutant enzyme, we have tried to perform the following experiments as long as possible or necessary: (i) The free energy of unfolding,  $\Delta G_D^{H_2O}$ , which is a measure of conformational stability, is determined from guanidine hydrochloride induced denaturation (Pace, 1986) and listed in Table II. (ii) The conformations of free AK and its complexes with AMP, MgATP, the reaction mixture, and the static mimic of the reaction mixture, MgAP<sub>5</sub>A, have been compared qualitatively by 1D proton NMR. (iii) When quantitative analysis is necessary, 2D NMR was used to identify the aromatic spin systems of free AK and AK + MgAP<sub>5</sub>A (often referred to as the ternary complex in our papers), which then allowed comparison between the chemical shifts of the mutant enzymes and WT, in the free form and in ternary complexes. (iv) The same comparisons were made on the basis of NOE cross-peaks in the NOESY spectra. (v) Whenever a conformational perturbation was observed, the possible causes of the perturbations were interpreted with the aid of the crystal structure of AK1p (Dreusicke et al., 1988).

Since AK does not have disulfide bonds, it may be expected to have a nonrigid structure and to be sensitive to point mutations. However, among the ca. dozen residues investigated by us, only His-36 is important in conformational stability, and only K21M shows significant changes in proton NMR. This is a fortunate point in the structure-function study of AK. An unfortunate point is that the  $\Delta G_D^{H_2O}$  values listed in Table II are smaller than the usual range of 5–15 kcal/mol for globular proteins (Pace, 1990), which can explain the relative instability of AK1. According to Bock et al. (1988), AK1 is significantly more stable.

**Stage 3: Interpretations and Further Tests of the Interpretations.** When perturbations in kinetic parameters are  $\leq 5$ -fold, the mutated residue is considered to be nonessential or unimportant to catalysis. In such cases, we have always examined whether the residue could play any structural role by examining the crystal structure, determining its conformational stability, and comparing its proton NMR properties with the WT AK. When perturbations in  $k_{cat}$ ,  $K_m$ , and/or  $K_i$  are  $\geq 20$ -fold, the mutated residue is considered to play a functional role provided the conformation of the mutant enzyme is not significantly perturbed relative to WT (in the free form and in complexes with substrates). Changes between 5- and 20-fold are in a gray area and should be interpreted in consideration with other evidence.

While the studies up to this point may be quite rigorous, they are not air-tight for three reasons: (i) showing a residue is functionally important does not necessarily specify *how* the particular residue interacts with the substrate; (ii) conformational perturbation (or lack of) is only a relative term, and its relationship to catalysis is uncertain; and (iii) the decreases in  $k_{cat}$  may not necessarily be caused by perturbations in the chemical step. Thus, *whenever possible and justified, additional experiments are then designed to test whether the catalytic properties of mutant enzymes are consistent with the interpretations.*

In the following sections, we address the roles of specific residues. It should be noted that not every mutant enzyme has been brought through all three stages since the whole process has been developed through a period of time. The mutant enzymes which have been studied most rigorously are R44M and D93A.

**His-36 Plays Structural Rather than Functional Roles.** His-36 was our first target since it had been suggested to be related to substrate binding on the basis of three key observations related to the <sup>1</sup>H NMR signal of the C2-H of His-36: (a) it was shifted downfield upon addition of AMP, GTP, ATP, MgATP, or the reaction mixture (McDonald et al., 1975); (b) it was broadened by MnATP (McDonald et al., 1975) and CrATP (Smith & Mildvan, 1982); (c) NOE was clearly observed between this proton and the adenine H<sub>2</sub> of bound MgATP (Smith & Mildvan, 1982; Fry et al., 1985). In the X-ray model, His-36 is located near the phosphate region, which would be compatible with observations a and b but not observation c. In the NMR model, His-36 is located near the adenine ring of ATP, which could be compatible with all three observations.

To our surprise, the kinetic data in Table I show that *His-36 is not essential for catalysis*. Instead, *it is involved in the conformational stabilization of free AK since the  $\Delta G_D^{H_2O}$  decreases in the order WT > H36Q > H36N > H36G* (Table II) (Tian et al., 1988). Examination of the crystal structure in Figure 3 reveals a His-36...Asp-93...Cys-25 "triad", in which the OD1 oxygen atom of Asp-93 is hydrogen-bonded to both His-36 and Cys-25 (Dreusicke et al., 1988). However, such

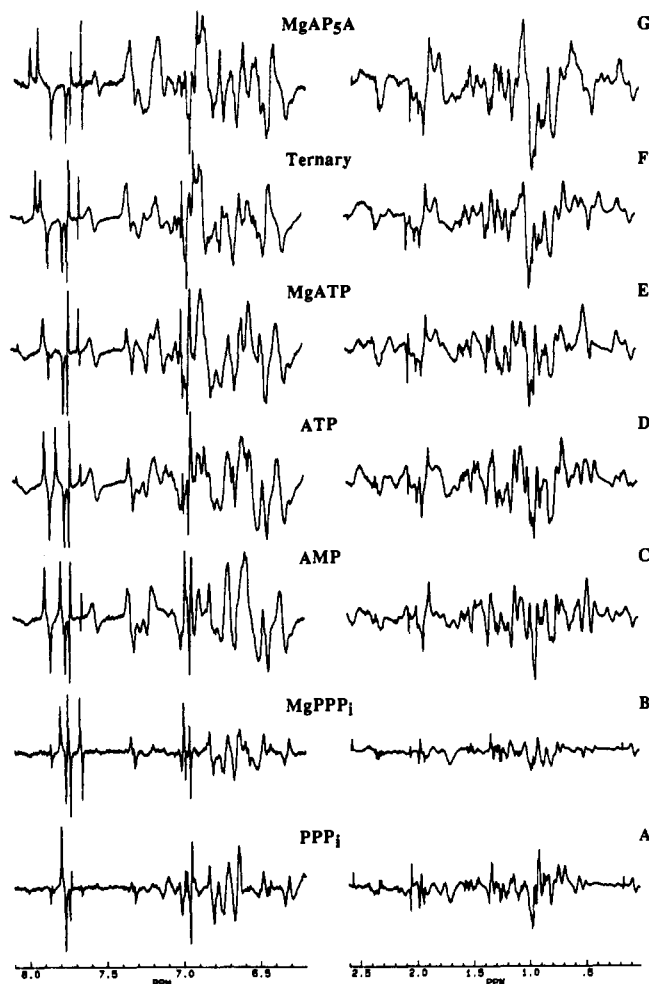


FIGURE 4: Difference proton NMR spectra (at 500 MHz) obtained by subtracting the free AK1c spectra from the spectra of various complexes reported by Sanders et al. (1989).

hydrogen bondings are not essential to conformational stability since the  $\Delta G_D^{\text{H}_2\text{O}}$  of D93A is unchanged. Instead, the role of His-36 in conformational stabilization could lie in its interaction with Cys-25 (of unknown nature) since the reactivity of Cys-25 toward Ellman's reagent increases in the order WT < H36Q < H36N. Indeed, H36G forms a dimer, possibly due to an intermolecular disulfide link involving Cys-25 (Tian et al., 1988).

How, then, could observations a–c related to the  $^1\text{H}$  NMR signal of the C2–H of His-36 be rationalized? The downfield shift (a) should not be considered to be of particular significance since *the changes induced by substrate (or analogue) binding cross the entire spectral region* as shown in Figure 4 (Sanders et al., 1989). The broadening by paramagnetic ions (b) is expectable since Asp-93 is interacting with the divalent metal ion as described in a later section, and His-36 is H-bonded to Asp-93. The potential significance of the NOE (c) will be discussed under Recent NMR Studies.

**Are the "Essential Lysines" Essential?** In most enzymes using metal-ATP (or GTP) as a substrate, there is a P-loop (Saraste et al., 1990) and an "invariant lysine" (Hanks et al., 1988). The latter is characterized by a dramatic loss of activity upon chemical modification and by the ability of the nucleotide to protect against chemical modification (Tagaya et al., 1987). In the case of AK, the invariant lysine is part of the P-loop consisting of residues 15–22: G-X-P-G-X-G-K-G. In the X-ray model of substrate binding (Pai et al., 1977), Lys-21 is interacting with the  $\gamma$ -phosphate of ATP. In the NMR

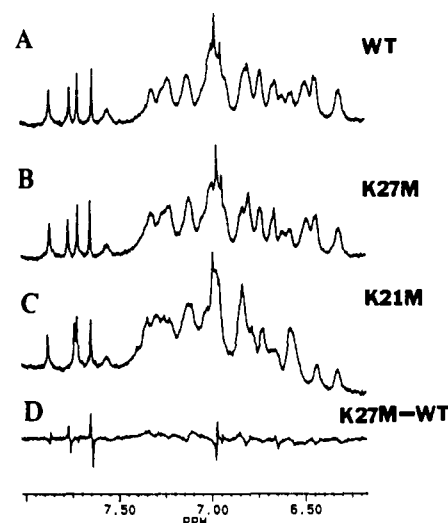


FIGURE 5: Proton NMR spectra (at 500 MHz) showing the aromatic regions of WT (A), K27M (B), K21M (C), and K27M-WT (D). Reproduced from Tian et al. (1990).

model, Lys-21 was suggested to be able to move and interact with the  $\alpha$ -phosphate of ATP, and another lysine, Lys-27, could interact with the  $\gamma$ -phosphate as shown in Figure 1C (Fry et al., 1985; Mildvan & Fry, 1987).

The kinetic data in Table I suggest that Lys-27 is nonessential for catalysis, which provides strong evidence against the NMR model. We then asked whether this residue plays any structural role, and found that the conformational stability of K27M is greater than that of WT by 1.8 kcal/mol (Table II). Interestingly, the proton NMR of K27M is not perturbed (Figure 5A,B). Thus, *K27M has essentially unchanged functional and structural properties but is more stable than WT* (Tian et al., 1990).

The  $k_{\text{cat}}$  of K21M is greatly reduced. However, its proton NMR has been significantly perturbed (Figure 5C). Examination of the crystal structure of AK1p suggests that Lys-21 is likely to form an H-bond with the carbonyl oxygen of Gly-15, and such an H-bonding could be important for stabilization of the loop. These results suggest that *Lys-21 plays a key structural role and that whether it also plays a functional role remains to be established*. Another lesson revealed by this residue is that the  $\Delta G_D^{\text{H}_2\text{O}}$  has not changed despite the significant perturbation in proton NMR. Taken together with the results of K27M, there seems to be *no direct correlation between changes in conformation and changes in conformational stability in site-specific mutant enzymes* (Tian et al., 1990).

**Arg-132, -138, and -149 Are Important in Transition-State Stabilization.** Most of the arginine residues surrounding the negatively charged phosphates in the X-ray model turned out to be very important functional residues. These three arginine residues are grouped together because their main functions appear to be in stabilizing the transition-state structure. The studies of these arginines are illustrated by R138K (Yan et al., 1990a). This is a very conservative mutation, but the kinetic constants are greatly perturbed: 6500-fold decrease in  $k_{\text{cat}}$ , 10–15-fold increases in  $K_m$ , and little change in  $K_i$ . Essentially the same results were obtained for R138M, except a 2-fold greater decrease in  $k_{\text{cat}}$ . As shown in Figure 6A, the proton NMR spectra of WT and R138K are almost superimposable for the free enzymes. This has been corroborated by 2D TOCSY and NOESY spectra. Quantitatively, the aromatic spin systems of both WT and R138K have been identified, and all resonances agree within 0.1 ppm (all but three

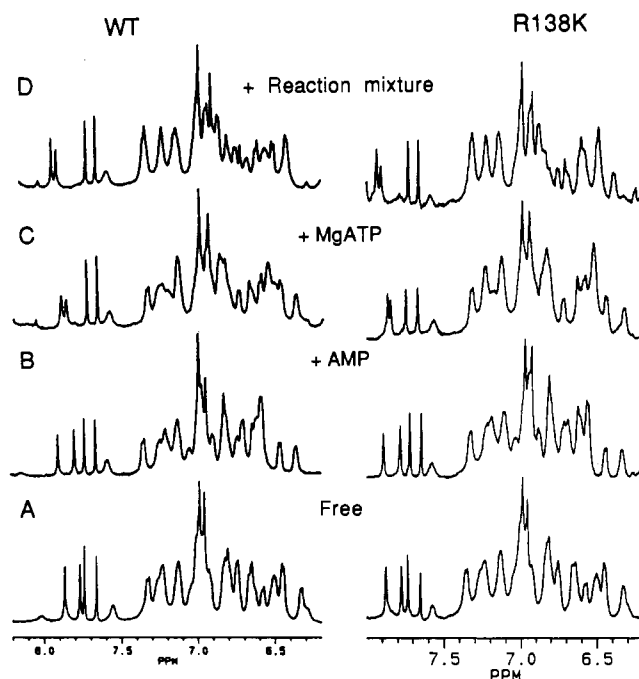


FIGURE 6: Proton NMR spectra (at 500 MHz) of the aromatic protons of WT (left panel) and R138K (right panel). Reproduced from Yan et al. (1990a).

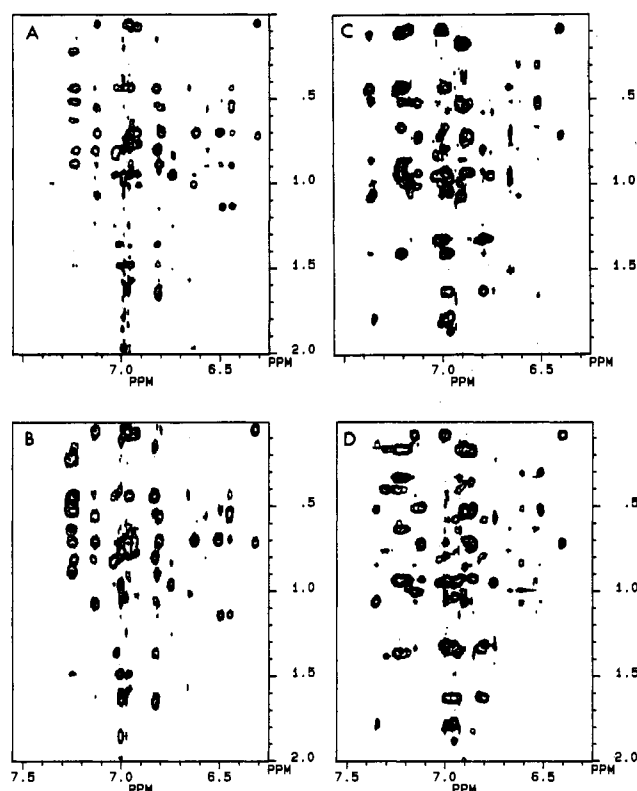


FIGURE 7: Aromatic-aliphatic NOE cross-peaks from the NOESY spectra of WT (A), R138K (B), WT + MgAP<sub>5</sub>A (C), and R138K + MgAP<sub>5</sub>A (D). Spectra A and B were obtained at 600 MHz and spectra C and D at 500 MHz, with a mixing time of 200 ms. Reproduced from Yan et al. (1990a).

resonances agree within 0.02 ppm).

In a previous section, it is stated that substrate binding could induce conformational differences between WT and mutant enzymes. This appears to be the case for R138K, since the spectra in Figure 6B–D indicate small differences between the substrate complexes of WT and R138K. The differences appear to be more significant for the ternary complex (reaction

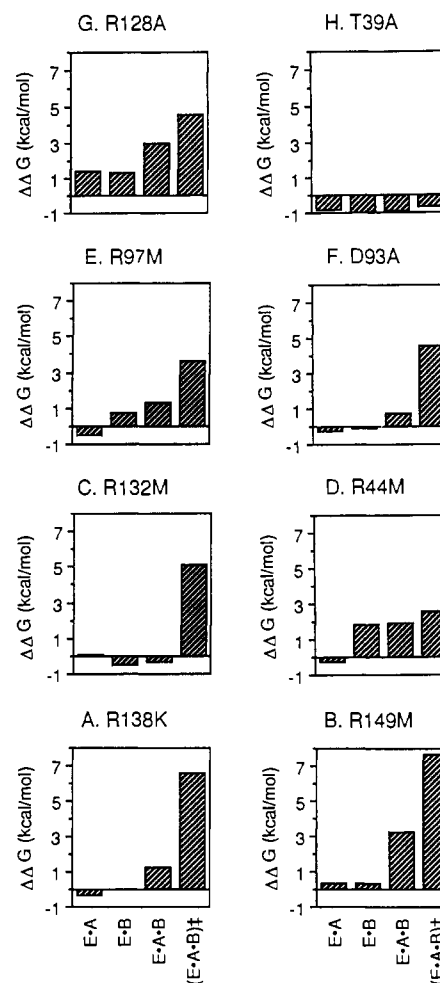


FIGURE 8: Free energy diagrams showing  $\Delta\Delta G$  values calculated from the kinetic data in Table I. A and B represent MgATP and AMP, respectively, and the prime designates a mutant enzyme.  $\Delta\Delta G_{E-A} = RT \ln (K_{iA}'/K_{iA})$ ,  $\Delta\Delta G_{E-B} = RT \ln (K_{iB}'/K_{iB})$ ,  $\Delta\Delta G_{E-A-B} = RT \ln (K_{iA}'K_{iB}'/K_{iA}K_{iB})$ , and  $\Delta\Delta G_{(E-A-B)^\ddagger} = RT \ln [(k_{cat}/K_{iA}K_{iB})/(k_{cat}'/K_{iA}'K_{iB}')]$ .

mixture) than the binary complexes. The question, then, is how significant these changes are quantitatively. To address this question, we performed 2D NMR experiments and identified the aromatic spin systems for the most perturbed complex, the ternary complex, using MgAP<sub>5</sub>A as a static mimic of the reaction mixture. The results indicate that all but six aromatic resonances agree within 0.02 ppm. Comparison of the aromatic-aliphatic NOE cross-peaks, shown in Figure 7, confirms that the conformations of WT and R138K are almost identical, whereas those of WT + MgAP<sub>5</sub>A and R138K + MgAP<sub>5</sub>A are very similar except some small (though notable) differences. Such structural analysis assured that the kinetic data can be interpreted with confidence and led to the conclusion that Arg-138 stabilizes the ternary complexes by ca. 1.5 kcal/mol ( $K_m$  effect) and the transition state by ca. 7 kcal/mol ( $k_{cat}/K_m$  effect), but does not stabilize the binary complexes (no effects on  $K_i$ ). These are illustrated in a diagram in Figure 8A. Since the functional roles of Arg-138 are disrupted by the very conservative change to lysine, this residue should be a strong candidate in interacting with the transferring phosphate at the transition state.

The R132M (Z. Shi, unpublished results) and R149M (Yan et al., 1990b) mutant enzymes have been subjected to essentially the same structural and functional studies, and the results are similar to that of R138K. As shown by the data in Table I and in Figure 8, the effects of Arg-132 and Arg-149 on  $k_{cat}$



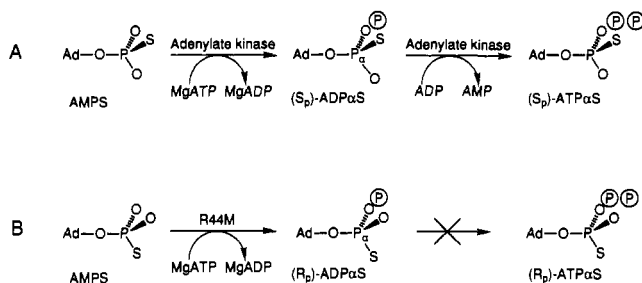


FIGURE 9: Schemes showing the stereospecificity of wild-type adenylate kinase (A) and R44M (B). As implied by the schemes, the orientation of the acceptor oxygen of AMPS should not change, and the reversal in stereospecificity is likely to be due to a rotation in the bridging-O-P bond. Reproduced from Jiang et al. (1991).

are similar to that of Arg-138; however, the effects on  $K_m$  (i.e., stabilization of the ternary complex) increase in the order Arg-132 < Arg-138 < Arg-149. The extent of increases in  $K_m$  is comparable for both substrates. The 130-fold increases in  $K_m$  for R149M are quite noteworthy. None of the three residues seems to interact with binary complexes since  $K_i$  values are not appreciably perturbed.

As shown in Figure 3, the side-chain guanidino groups of Arg-132, Arg-138, and Arg-149 form a small triangle. Judging from the functional significance of these three residues on the basis of the above results, one could postulate that the charges of these residues surround the transferring phosphate and stabilize the monomeric metaphosphate in case of a dissociative mechanism or the pentacoordinate transition state in case of an associative mechanism. The  $\epsilon$ -amino group of Lys-21 could also be part of this positively charged cluster if the side chain of Lys-21 moves toward the cluster upon substrate binding to form a square with the three arginine guanidino groups. Further experiments will be designed to test this postulation in the future.

**Arg-44 and Arg-97 Interact with AMP Specifically.** These two residues differ from the other group of arginine residues in their differential effects toward AMP. As shown by Figure 8D and the data in Table I for R44M [ $K_{i(AMP)}$  and  $K_{AMP}$  increase by 22- and 36-fold, respectively], Arg-44 interacts with AMP specifically starting with the binary complex, and the interaction is not further enhanced at the ternary complex or the transition state. The increase in  $K_i$  has been independently confirmed by NMR measurement of  $K_d$  (Yan et al., 1990b). The data shown in Table I and Figure 8E suggest that Arg-97 also interacts with AMP specifically. The effects of R97M on  $K_i$  and  $K_m$  are somewhat smaller than those of R44M, but the role of Arg-97 is further enhanced at the transition state.

As shown in Figure 1, both Arg-44 and Arg-97 are located near the MgATP site in the X-ray model. Our results suggest that the MgATP site in the X-ray model should be reassigned to the AMP site (Yan et al., 1990b). This conclusion has also been reached by others as described under Recent X-ray Studies and under Related Mutagenesis Studies from Other Groups.

Although the positively charged Arg-44 (and Arg-97) is likely to interact with the negatively charged phosphate group of AMP, we set out to obtain experimental evidence using the phosphorothioate analogue of AMP, AMPS. Using  $^{31}\text{P}$  NMR analysis, we have demonstrated the conversion of AMPS to  $S_p$ -ADPaS (at the AMP site) and its subsequent conversion to  $S_p$ -ATPaS (at the MgATP site) catalyzed by WT AK1c (scheme A of Figure 9), which agree with previous reports on the stereospecificity of AK1r toward AMPS (Sheu & Frey, 1977) and that of AK1r (Eckstein & Goody, 1976), AK1p

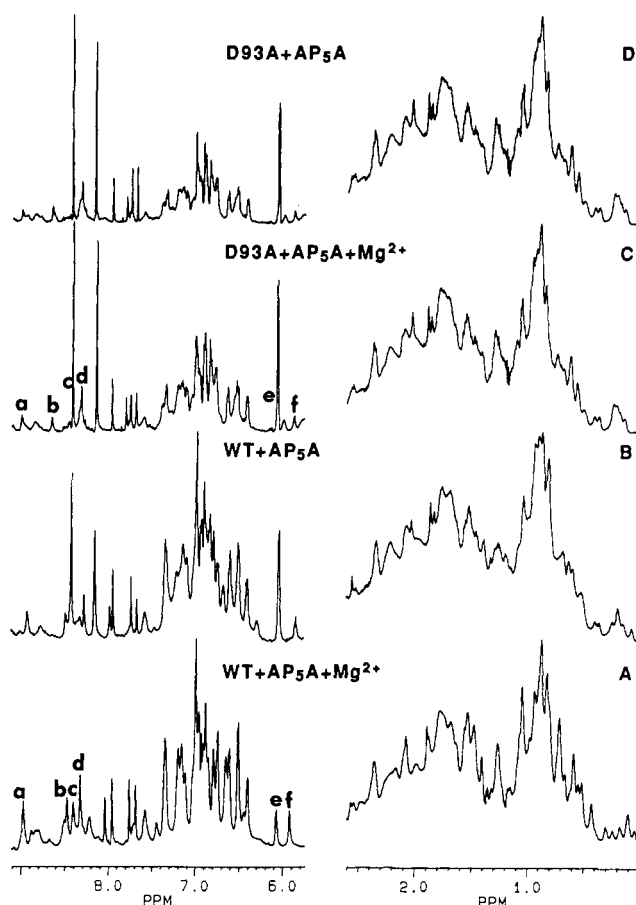


FIGURE 10: Proton NMR spectra (at 500 MHz) showing the effects of  $\text{Mg}^{2+}$  ions to the AP<sub>5</sub>A complexes of WT and D93A. Reproduced from Yan and Tsai (1991).

(Kalbitzer et al., 1983), and AKy (Tomasselli & Noda, 1983) toward ATPaS. Such stereospecificity should arise from restricted orientations of the P-S and P-O bonds at the active site, such that the unnatural sulfur assumes a position "least painful" to the enzyme. By changing one of the residues involved in the interaction with the thiophosphoryl group of AMP, the preferred orientation of the P-S bond could be changed, and the stereospecificity at the AMP site could be perturbed or even reversed. On the other hand, observation of a significant perturbation in stereospecificity can serve as evidence that the mutated residue interacts directly with the pertinent phosphate group (but lack of perturbation is not evidence against such an interaction). Using  $^{31}\text{P}$  NMR, we have demonstrated that the stereospecificity toward AMPS is completely reversed in the reaction catalyzed by R44M (Figure 9B). On the other hand, the stereospecificity is not perturbed at the MgATP site, which is expectable since Arg-44 does not interact with ATP. These results not only provide direct evidence for the interaction between Arg-44 and the phosphoryl group of AMP but also demonstrate that it is possible to manipulate the phosphorus stereospecificity of enzymes by site-directed mutagenesis (Jiang et al., 1991).

**Asp-93 is Critical to  $\text{Mg}^{2+}$  Binding.** Asp-93 interacts with the ternary complex and the transition state, as shown by the results of D93A (Figure 8F). Since this is the only negatively charged residue identified to be functionally important, it was suspected to be interacting with  $\text{Mg}^{2+}$  even though previous magnetic resonance studies suggested that  $\text{Mg}^{2+}$  does not bind to free AK (Mildvan, 1979). As shown in Figure 10, there are notable differences between the proton NMR resonances of the aromatic residues of WT + AP<sub>5</sub>A and WT + MgAP<sub>5</sub>A,

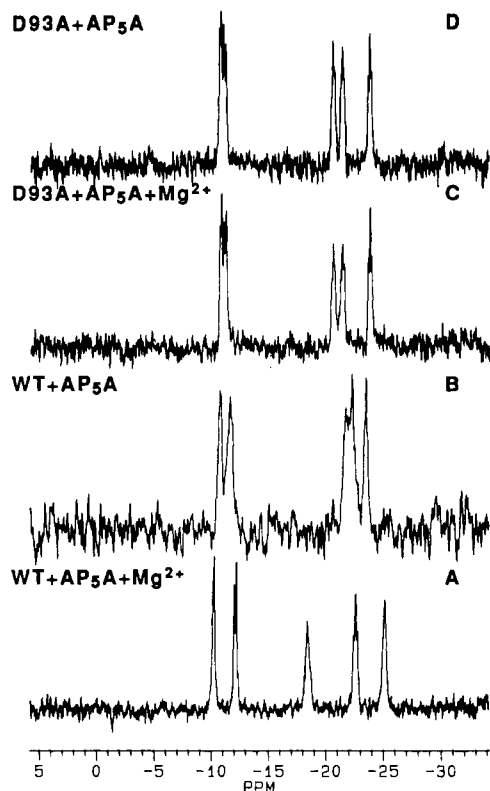


FIGURE 11:  $^{31}\text{P}$  NMR spectra (at 121.5 MHz) showing the effects of  $\text{Mg}^{2+}$  ions to  $\text{AP}_5\text{A}$  bound to WT and D93A. Reproduced from Yan and Tsai (1991).

but there are no such differences between D93A +  $\text{AP}_5\text{A}$  and D93A +  $\text{MgAP}_5\text{A}$ . This suggests that  $\text{Mg}^{2+}$  induces changes in the conformation of WT but not D93A (when complexed with  $\text{AP}_5\text{A}$ ). Furthermore, the  $^{31}\text{P}$  NMR spectra in Figure 11 show that  $\text{Mg}^{2+}$  induces changes in the conformation of  $\text{AP}_5\text{A}$  when complexed to WT (compare spectra A and B) but not D93A (compare spectra C and D). These results suggest that the  $\text{Mg}^{2+}$  ion is unable to exert its effects on the D93A- $\text{AP}_5\text{A}$  complex, while in WT the carboxylate group of Asp-93 binds and fixes the  $\text{Mg}^{2+}$  ion, which in turn orients the phosphates to proper conformations. Further studies with  $^{25}\text{Mg}$  NMR confirmed that  $\text{Mg}^{2+}$  does not bind to free AK and suggested that it binds to D93A +  $\text{AP}_5\text{A}$  with a lower affinity than to WT +  $\text{AP}_5\text{A}$  (Yan & Tsai, 1991).

**Roles of Arg-128 and Thr-39 Are Inconclusive.** 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 (Figure 8G). The proton NMR analyses indicate that the conformation of R128A has been perturbed slightly in free and complexed forms. These results favor possibility a, but cannot rule out possibility b (Yan et al., 1990b).

It is somewhat unfortunate that the results of this mutant are not clear-cut, since Arg-128 is at a strategic position for two issues: in the alignment suggested by Schulz (1987), Arg-128 lines up with a hydrophobic Leu whereas in the alternative alignment (Haase et al., 1989) it lines up with an Arg; in the new X-ray model described in the next section Arg-128 appears to be located close to the adenosine moiety at the ATP site.

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 (Figure 8H). It may not play significant structural roles either since proton NMR analysis indicated little perturbation in the conformation (Yan et al., 1990b). Ironically, Thr-39 has been suggested to be an important residue in recent X-ray studies as described in the following section. It is possible that even though Thr-39 is in proximity 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 AK1c is somewhat different from that depicted in the X-ray structure of  $\text{AKy}\cdot\text{MgAP}_5\text{A}$ .

#### RECENT X-RAY STUDIES

**$\text{AKy}\cdot\text{MgAP}_5\text{A}$  and  $\text{AKe}\cdot\text{MgAP}_5\text{A}$  Complexes.** Simultaneous to our mutagenesis studies, significant progress has also been made by use of X-ray crystallography. A series of papers reporting the structures of cocrystals of AK with a substrate or an inhibitor have been published by G. E. Schulz and co-workers. The first of these papers is on the structure of  $\text{AKy}\cdot\text{MgAP}_5\text{A}$  at 2.6-Å resolution (Egner et al., 1987). This structure, along with the chain fold overlay of the free AK1p (crystal form A), is shown in Figure 12. Although *this structure is turning out to be a landmark in the field*, the authors cautioned that only adenosine B (the right-hand side in Figure 12) may assume a natural binding site in the crystal, and that adenosine-A may occupy a spurious site since the residues in proximity to adenosine-A are not highly homologous among the natural variants of AK. Perhaps an unspoken reason behind this interpretation is that the adenosine-A site in Figure 12 does not correspond to either substrate site in Figure 1A proposed earlier by the same group. The adenosine-B site in Figure 12 does correspond to the  $\text{MgATP}$  site

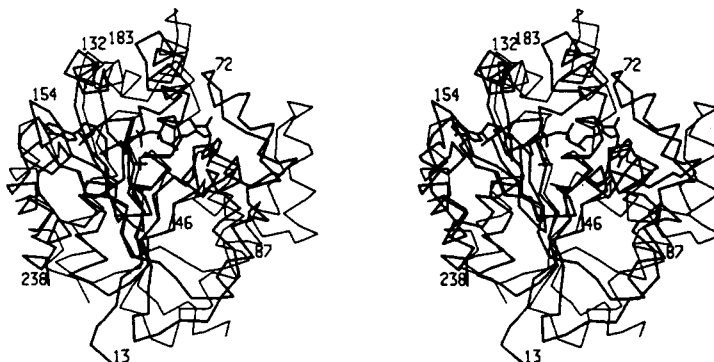


FIGURE 12: Chain fold overlay of free AK1p (thin line) and  $\text{AKy}\cdot\text{MgAP}_5\text{A}$  (thick line). The adenosines at the left- and right-hand sides are the A and B moieties, respectively; (+) indicates the  $\text{Mg}^{2+}$  position. The residue numbers are according to the family numbering system. Reproduced from Egner et al. (1987).



in Figure 1A, but the authors suggested (rightfully) that this should be the AMP site for two reasons: (i)  $Mg^{2+}$  sits between the third and the fourth phosphoryl groups from the adenosine-B side; (ii) the substrate specificity is more stringent for AMP, and the adenosine-B site involves more conserved residues.

The situation was quite perplexing at this point. However, reassignment of the old  $MgATP$  site to the AMP site quickly gained strong support from mutagenesis studies and from other crystallographic studies: the B site (new AMP site) corresponds to the GMP site in the guanylate kinase-GMP complex (Stehle & Schulz, 1990) and to the AMP site in the AK3-AMP complex (Diederichs & Schulz, 1990). Furthermore, after finding that  $AP_5A$  binds to AKe in essentially the same way (Müller & Schulz, 1988), Schulz *et al.* (1990) revised their view and suggested that  $MgAP_5A$  occupies both substrate sites. This view has been strengthened by the observation that the A site (new  $MgATP$  site) corresponds to the GTP site in the p21 *ras* protein-GppNp complex (Vetter *et al.*, 1991). This binding mode is hereby termed the "new X-ray model" for future reference.

**Support from Mutagenesis Results.** Although the AK-inhibitor structure shown in Figure 12 has been available since 1987 (for AKy and AKe, but not AK1), it has not deterred our mutagenesis studies in testing the original X-ray and NMR models for several reasons: (i) the first interpretation by crystallographers was quite perplexing; (ii) the coordinates of these complexes have not been available in a protein data bank; (iii) the A site is surrounded by the "insertion segment" which is absent in AK1; and (iv) our mutagenesis results can quantify the contributions of enzymatic residues at different stages of catalysis as shown in Figure 8, and provide further functional information as in R44M and D93A.

The AMP site in the new X-ray model has been supported by our results of R44M and R97M. The phosphate binding region in the new X-ray model has also been supported by our results of Arg-132, Arg-138, Arg-149 (except for the reservation in the next paragraph), Arg-44 and Arg-97, and the  $Mg^{2+}$  site by the results of D93A. We have not interpreted the functional role of Lys-21 due to conformational changes in K21M, but it is likely to interact with phosphate group(s) on the basis of the crystal structures of AKy- $MgAP_5A$  (Egner *et al.*, 1987) and *ras* p21- $MgGppNp$  complex (Pai *et al.*, 1989). Reinstein *et al.* (1990a) hypothesized that Lys-21 stabilizes the pentacovalent transition state of the transferred phosphoryl group during the catalytic reaction. In summary, *results from our lab and others' generally support the AMP site, the polyphosphate sites, and the  $Mg^{2+}$  site in the new X-ray model.*

Support for the ATP site in the new X-ray model mainly came from NMR studies in Rösch's lab, as described under Recent NMR Studies. The mutagenesis studies from other laboratories, as summarized in the next section, have suggested that the  $MgATP$  site should correspond to the AMP site in the old X-ray model (Figure 1A). As commented later, most of these claims are unfounded, perhaps with the exception of the result of the Y133W mutant of AKe (Liang *et al.*, 1991). So far, we have not yet found a mutant AK1c whose ATP binding is specifically perturbed. *Since the adenosine-A site in the new X-ray model is surrounded by the insertion segment which is absent in AK1, whether the  $MgATP$  site of AK1 is identical with that of AKy and AKe remains to be established.* Although the structure of free AK1 may not be directly compared with the structure of the AKy- $MgAP_5A$  complex, careful examination suggests that the polyphosphate chain in Figure 12 does not pass through the space delineated by the

side chains of the three critical residues, Arg-132, -138, and -149, shown in Figure 3 for AK1. According to Egner *et al.* (1987), Arg-149 is not within 3.5 Å of the bound inhibitor. However, it could move closer at the transition state. Alternatively, Arg-149 could function differently in AKy and in AK1c.

**Substrate-Induced Conformational Changes.** The importance of the induced-fit process in the catalysis by AK is unclear since the ratio  $K_i/K_m$  is only ca. 4 for WT AK1c, which is significantly smaller than that of hexokinase for which binding of glucose enhances its affinity to  $MgATP$  by up to 150-fold (DelaFuente *et al.*, 1970; Viola *et al.*, 1982). However, the NMR results clearly indicate that binding of substrates or inhibitors induces global conformational changes (see Figure 4). The structures of crystal forms B and A have been suggested to represent the structures of the enzyme before and after substrate binding, respectively, since the B form has a more open conformation (Pai *et al.*, 1977; Dreusicke & Schulz, 1988). However, by comparing the crystal structures of free AK1p (form A), AK3-AMP, and AKe- $MgAP_5A$  complexes, Schulz *et al.* (1990) now consider these three structures represent the conformations of free AK, AK-AMP, and AK-AMP- $MgATP$ , respectively, and they together yield a "moving film" of the induced-fit process.

## RECENT NMR STUDIES

Other than assisting the interpretation of the results of site-directed mutagenesis, efforts have also been made in several laboratories to use NMR to locate the substrate sites, or to examine the dynamic and conformational properties of bound substrates. In essence, these studies have the same goal as that of the earlier NMR studies by Mildvan and co-workers, but the recent studies are taking the hard and hopefully right course. There are three major categories in such NMR studies: (a) resonance assignments and dynamic properties of bound substrates or inhibitors; (b) resonance assignments of AK, in the free and complexed forms, and elucidation of tertiary structures in the future; and (c) qualitative and quantitative distance measurements between the nuclei of bound substrates (or inhibitors) and enzymatic residues. Overall, the progress in NMR seems to fall behind that in X-ray crystallography. Although NMR will be a laborious practice, it can provide other mechanistic and structural insights which are not obtainable from X-ray crystallography.

**Do Substrates and Inhibitors Bind to the Wrong Sites?** Although we have so far focused on the location of  $MgATP$  and AMP sites, there are yet other controversial problems in substrate binding: whether ATP also binds to the AMP site, whether AMP also binds to the  $MgATP$  site, and whether  $MgAP_5A$  occupies both sites (the question raised by crystallographers). We will try to summarize the current knowledge without getting into too much detail.

All studies, by kinetics (Rhoads & Lowenstein, 1968; Hamada & Kuby, 1978), NMR (Nageswara Rao *et al.*, 1978), and other means (Reinstein *et al.*, 1990b), agree that the AMP site does not accept a metal-nucleotide complex. This could be because the phosphates on the AMP side are surrounded by arginine residues and leave no room for  $Mg^{2+}$ . While the AMP site can bind uncomplexed AMP and ADP (substrate and product), whether uncomplexed ATP can go to the AMP site is still a controversial issue (Nageswara Rao *et al.*, 1978; Shyy *et al.*, 1987; Glushka *et al.*, 1990; and others). Complementarily, while it has been firmly established that the  $MgATP$  site can also bind uncomplexed ATP, indeed with higher affinity than  $MgATP$  (Sanders *et al.*, 1989; Reinstein *et al.*, 1990b), whether it also binds AMP has been contro-

versal. In our view, *both controversial issues could just be a matter of relative affinity, and the relative affinity may depend on the source of AK and the experimental conditions.* For example, AMP can bind to the MgATP site of AKe with a high enough affinity to be detectable by NMR (Vetter et al., 1990), fluorescence (Reinstein et al., 1990b), and substrate inhibition (Liang et al., 1991). On the other hand, the affinity of AMP for the MgATP site of AK1 is weaker (Rhoads & Lowenstein, 1968; Sanders et al., 1989), and substrate inhibition is not a serious problem. Liang et al. (1991), however, suggested that the substrate inhibition of AKe by AMP could be caused by the formation of an abortive complex rather than competitive inhibition.

Since the proton NMR change induced by MgAP<sub>3</sub>A (Figure 4G) is very similar to that by the reaction mixture (Figure 4F), MgAP<sub>3</sub>A most likely occupies both sites in solution (Sanders et al., 1989). Vetter et al. (1990) reached the same conclusion by comparing the chemical shifts of bound substrates and bound MgAP<sub>3</sub>A. Since the bound MgAP<sub>3</sub>A is in slow exchange on the proton NMR time scale, two sets of adenosine resonances can be resolved and have been assigned, one set to the adenosine corresponding to bound AMP and the other to bound MgATP, for AK1c (Yan et al., 1990a,b), AK1p and AKe (Vetter et al., 1990), and AKy (Vetter et al., 1991). Such assignments have set the stage for identification of specific enzyme-substrate interactions once the protein resonances can be assigned.

**Resonance Assignments of AK.** Most aromatic spin systems have been identified for AK1p (Klaus et al., 1988), AK1c (Yan et al., 1990a), AKe (Bock et al., 1988), and AKy (Vetter et al., 1991). Selective aliphatic spin systems have been identified for AKe by selective deuteration and selective protonation (Bock-Möbius et al., 1991). Sequence-specific assignments for a few aromatic resonances have been suggested by correlating interresidue NOE with the crystal structures, but such assignments should be considered tentative.

The molecular weight of AK1 is right at the cutting edge for total assignment and structural determination by NMR. However, the relative instability of AK1 (but not AKe) makes it difficult for NMR studies, since the enzyme usually denatures irreversibly after ca. 3 days of data accumulation at 30 °C. Lowering the temperature could slow down denaturation but also causes line broadening. Despite such limitations, our initial studies using <sup>15</sup>N- and/or <sup>13</sup>C-labeled AK have demonstrated that total assignment of AK1c is an achievable goal (Yan, 1991), and further work is in progress.

**Substrate-Protein NOE: Evidence for the MgATP Site.** There is still one fact difficult to argue against in the earlier NMR result: the proximity between the H<sub>2</sub> of bound MgATP and the H<sub>2</sub> of His-36 as evidenced by a transferred NOE for AK1p qualitatively (Smith & Mildvan, 1982) and for AK1r quantitatively (Fry et al., 1985). Ironically, we have not been able to detect appreciable NOE between the H<sub>2</sub> of His-36 and either H<sub>2</sub> or H<sub>4</sub> of the adenine of MgATP, AMP, or MgAP<sub>3</sub>A for AK1c (Yan et al., 1990a; H. Yan, unpublished results), and Rösch et al. (1989) reported results similar to ours for AK1p. It should be noted that the reported NOE between His-36 and MgAP<sub>3</sub>A (Rösch et al., 1989) was due to a misassignment in proton resonances, which has been corrected later (Yan et al., 1990a; Vetter et al., 1990, 1991). The dilemma here is that lack of observable NOE is a negative result which cannot prove or disprove something and that Smith and Mildvan (1982) did document the NOE with spectra. Thus, until the ATP site can be established independently for AK1, we are not ready to discount this NOE.

The NMR studies of large variants of AK (AKe and AKy) from Rösch's group, however, have provided convincing support for the MgATP site of the new X-ray model. For AKe, Vetter et al. (1990) observed NOE cross-peaks between the adenine protons of adenosine-A and the side-chain protons of Phe-19 (corresponding to Lys-27 in AK1) and His-134 (in the insertion segment, absent in AK1) in the AKe + MgAP<sub>3</sub>A complex. For AKy, similar cross-peaks were observed for Trp-210 and His-143 (corresponding to Phe-183 in AK1 and His-134 in AKe, respectively) (Vetter et al., 1991). Thus, *the new X-ray model as shown in Figure 12 can best represent the current knowledge of substrate sites for AKy and AKe.* As noted earlier, *more evidence needs to be gathered for the MgATP site of AK1.*

#### RELATED MUTAGENESIS STUDIES FROM OTHER GROUPS

Site-directed mutagenesis studies have also been conducted in other laboratories for AK1c (Tagaya et al., 1989; Matsuura et al., 1989; Yoneya et al., 1990), AK1h (Kim et al., 1989, 1990), and AKe (Gilles et al., 1986; Reinstein et al., 1988, 1989, 1990a,b; Liang et al., 1991). The genes have also been isolated and characterized for AKy (Proba et al., 1987), AK2 (Tanaka et al., 1990), and AK3 (Yamada et al., 1990). Table III summarizes the kinetic data of site-directed mutants reported to date. It is unfortunate that these data have been gathered with different degrees of rigor and interpreted with different degrees of caution (or speculation).

On the positive side, work from other laboratories has provided several significant points. (i) Reassignment of the AMP site in the revised X-ray model has also been supported by the data of R44A AK1h (Kim et al., 1990) and F86W AKe (Liang et al., 1991). (ii) The observations that Y133W AKe showed no effects on kinetics and no fluorescence changes upon nucleotide binding (Liang et al., 1991) provide perhaps *the only accountable evidence against (though insufficient to disprove) the MgATP site of AKe in the new X-ray model* since Tyr-133 is in proximity to the adenine ring of MgATP in this model. (iii) Another interesting observation by Liang et al. (1991) is that the substrate inhibition by AMP in catalysis by AKe is lost in F86W. (iv) Changes at the turning point of the glycine-rich loop, Pro-17 and Gly-18, resulted in substantial increases in  $K_m$  but not in  $k_{cat}$ . Binding studies also indicated increased  $K_d$  values for the P9G, P9L, G10V, and K13Q mutants of AKe (Reinstein et al., 1990a). These suggest that the main function of the loop (except Lys-21 discussed earlier) is in substrate binding. (v) While some of the differences between the kinetic data in Table III and those of the corresponding mutants in Table I could be due to differences in experimental conditions, others could mean that the quantitative contribution by the same catalytic residue varies from species to species. If these could be verified in the future, they could have significant implication on the theories of enzyme evolution.

On the negative side, we have the following reservations: (a) Most, if not all, of the kinetic data reported in these papers are only *apparent*  $k_{cat}$  and  $K_m$ , which were obtained by holding one substrate at a fixed (not necessarily saturating) concentration. As we have pointed out in Yan et al. (1990b), the  $K_{m,app}$  could be significantly smaller than the real  $K_m$  when the  $K_m$  is significantly greater than the dissociation constant, as in the case of R149M. (b) Except for P9G, P9L, G10V, K13Q, and Q28H of AKe (Reinstein et al., 1990a,b), the mutant enzymes have not been characterized for conformational perturbations by NMR or X-ray. Some reports used CD to check possible conformational changes (Kim et al., 1990; Yoneya et al., 1990), but CD is a relatively insensitive

Table III: Summary of Apparent Kinetic Constants Reported by Other Laboratories

	$k_{cat}(MgATP)$ ( $s^{-1}$ )	$k_{cat}(AMP)$ ( $s^{-1}$ )	$K_m(MgATP)$ (mM)	$K_m(AMP)$ (mM)	source
Chicken Muscle AK					
WT	690		0.19	0.19	Tagaya et al. (1989)
P17G	500		1.4	1.4	Tagaya et al. (1989)
P17V	470	360	8.0	4.5	Tagaya et al. (1989)
R128W	120		2.0	6.0	Matsuura et al. (1989)
WT	790	760	0.098	0.084	Yoneya et al. (1990)
L190A <sup>a</sup>	395	380	0.198	0.126	Yoneya et al. (1990)
L190K <sup>a</sup>	290	300	1.07	0.251	Yoneya et al. (1990)
$\Delta 190-193^b$	170		4.1	1.3	Yoneya et al. (1990)
Human Muscle AK					
WT	1100	751	0.1	0.16	Kim et al. (1990)
R44A	164 <sup>c</sup>	167	0.41	6.99	Kim et al. (1990)
R97A	12.8	14.3	0.02	0.34	Kim et al. (1990)
R132A	0.0387	0.0557 <sup>c</sup>	1.84	2.09	Kim et al. (1990)
R138A	0.0116	0.0157	0.13	3.28	Kim et al. (1990)
R149A	0.0957 <sup>c</sup>	0.108	0.79	2.92	Kim et al. (1990)
D119N	646	837	0.06	0.34	Kim et al. (1990)
Y95F	1415		0.567	0.743	Kim et al. (1989)
<i>E. coli</i> AK <sup>d</sup>					
WT	305	301	0.071	0.026	Reinstein et al. (1990a)
P9(17)G	348	430	0.775	0.35	Reinstein et al. (1990a)
P9(17)L	207	270	1.18	0.442	Reinstein et al. (1988)
G10(18)V	75	140	0.53	0.482	Reinstein et al. (1988)
K13(21)Q	0.016	0.017	1.4	0.825	Reinstein et al. (1990a)
Q28(36)H	58.5	93.6	0.011	0.0075	Reinstein et al. (1990b)
R88(97)G	3.4 <sup>c</sup>		0.35	2.2	Reinstein et al. (1989)
P87(96)S	90		0.345	0.3	Gilles et al. (1986)
WT	325	475	0.09	0.05	Liang et al. (1991)
F86(95)W	90	530	0.29	2.1	Liang et al. (1991)
L107(116)Q <sup>e</sup>	45	67	0.54	0.04	Liang et al. (1991)
Y133W	405	510	0.15	0.04	Liang et al. (1991)
Y133C	50	100	0.40	0.18	Liang et al. (1991)

<sup>a</sup>Nine mutants of Leu-190 have been reported, but all other mutants have smaller perturbations than L190K. <sup>b</sup>Eight deletion mutants have been reported. <sup>c</sup>These values are corrected by extrapolating to saturation. <sup>d</sup>The number in parentheses following the residue number indicates the corresponding residue in muscle AK. <sup>e</sup>The conversion of residue numbers is based on Haase et al. (1989).

technique for this purpose. (c) There are serious overinterpretations in many papers. *The overinterpretations are 2-fold: one in accepting and interpreting unreliable data and the other in further proposing binding sites on the basis of the overinterpreted roles of specific residues.*

Illustrations of the overinterpretations can be found in three recent reports which suggested that the location of the aden-

osine moiety of MgATP should correspond to that of AMP in the old X-ray model [i.e., Pai et al. (1977) simply interchanged the positions of the two nucleotide sites] (Kim et al., 1990; Yoneya et al., 1990) or to that of the ATP site in the old NMR model (Liang et al., 1991). Regardless of whether these interpretations are correct, they are *not* supported by the results presented in these papers. The most serious of all is Yoneya et al. (1990), in which the key evidence for their claim is that "the apparent  $K_m$  values of Leu-190 mutants were affected to a greater extent for ATP than for AMP". The kinetic data of the two AK1c mutants (L190K and  $\Delta 190-193$ , listed in Table III) which can support this statement to the greatest extent show that the  $K_m$  of ATP is affected only ca. 3-fold greater than that of AMP. The data of some of their other mutants, such as L190A (also listed in Table III), have even smaller differences or no difference at all between the  $K_m$  of ATP and AMP. Thus, overinterpretations in this paper are demonstrated by not merely accepting the *apparent* kinetic data and interpreting the data without checking potential conformational changes, but also grossly speculating binding sites on the basis of essentially random data.

The argument in Liang et al. (1991) is mainly based on a 6-fold increase in the (apparent)  $K_m$  of ATP but not AMP for the L107Q mutant of AKc. Even if the apparent  $K_m$  is reliable and the conformation does not change in this case, a 6-fold difference corresponds to <1 kcal/mol in energy and should not be interpreted in any significant way, unless it is corroborated by other independent evidence.

Among the mutants of AK1h presented by Kim et al. (1990), R44A and R138A (but not R97A) show differential effects toward the (apparent)  $K_m$  of AMP, which led them to conclude that both Arg-44 and Arg-138 interact with AMP only. This is different from our conclusions that Arg-44 and Arg-97, but not Arg-138, interact with AMP specifically. Even if their kinetic data were reliable and interpretations were justified (neither is true if our results are correct and if the detailed mechanisms are the same between AK1h and AK1c), they can at best reassign the ATP site in the old X-ray model to the AMP site but *not* vice versa, since their paper did not include any positive data on the MgATP site.

One may wonder why chemical modifications would not have been employed to resolve the controversial problems. Indeed, chemical modification has also been employed in the study of AK as in many other enzymes, but it is not a subject for this review. The earlier work has been summarized by Noda (1973). Recently a few residues have been identified to be located near substrate sites: Lys-21 (Tagaya et al., 1987); Tyr-95 (Crivellone et al., 1985); and Leu-115, Cys-25, and probably His-36 (Chuan et al., 1989). Some of these residues have also been shown to be important by site-directed mutagenesis. However, the results of chemical modification have been interpreted with relatively "low resolution". For example, Chuan et al. (1989) concluded that their results are "in agreement with previous conclusions ... based on amino acid sequence, X-ray diffraction, and NMR studies".

#### PAST LESSONS AND FUTURE PERSPECTIVES

Risking the danger of being overcritical to the work of our colleagues, we have tried to illustrate how problems can be unnecessarily complicated by overinterpretations, to document the controversial events since the first crystal structure of free AK had been solved (Schulz et al., 1974), and to convince researchers in related fields that AK should be considered a "model kinase" for the lessons we can learn from mistakes, but not for the convenience in quoting various AK papers to support their causes. It should be noted that the coverage of

this review is not necessarily comprehensive. There are others not addressed in this review, such as a model proposed by Caldwell and Kollman (1988) on the basis of energy-refining of the earlier X-ray and NMR models.

*It is really the interplay between X-ray, NMR, and site-directed mutagenesis that has led to the progress in our understanding of the structure-function relationship of AK.* Ironically, although the two powerful structural techniques have fumbled somewhat in early days, *the new and powerful site-directed mutagenesis is a technique that has been most seriously abused.* The practice of making a few mutant enzymes and quickly drawing major conclusions is widely spread in the literature, including prestigious journals. Aside from overinterpretations, the technique bears many other potential hazards as pointed out by Schimmel (1989, 1990). A real case in our own house is that the R97M mutant enzyme we first obtained showed virtually undetectable activity and undetectable binding of AMP. Being puzzled by these unusually large effects (which could account for >10 kcal/mol), we re-sequenced the AK gene in the mutant strain and found an additional mutation, R149S (T. Dahnke, unpublished results). A human error is unlikely since we have never ordered (or considered to order) an oligonucleotide for an R149S mismatch.

Although the substrate sites have now been nearly established (except the MgATP site of AK1), one should always keep in mind that these provide only static structures in the resting state of the enzyme. In principle, a residue not in direct contact with the substrate in the crystal or solution structure [such as Arg-149] could move in at the transition state and play critical roles in catalysis. On the other hand, a residue in close proximity to a substrate at the resting state (such as Thr-39, which is also a highly conserved residue) may not necessarily play an important role in catalysis. Furthermore, we should be looking for (and trying to understand) different functions between similar structures (such as AK from different sources, or the same structural motif in different proteins). Thus, *the iterative structure-function studies as described in this review can still go a long way before the catalytic mechanism of AK can be understood at the "chemical level", for both the substrates and the enzyme.*

As a concluding remark, we quote an excerpt from the comment by the anonymous reviewer of this paper: "I am sure the authors do not intend to imply that overinterpretation of incomplete data is a problem that is especially characteristic of the adenylate kinase field or of mechanistic studies of enzymes. Owing to the complexities of the systems and phenomena in biochemistry, this has always been a problem for the entire field. Many examples come to mind in the physical and chemical characterization of proteins, the searches for various hypothetical species of nucleic acids, the delineation of metabolic pathways, studies of the regulation of metabolic pathways, the searches for receptors, studies of energy transduction, etc."

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