Mechanism of Adenylate Kinase. Are the Essential Lysines Essential?†

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ABSTRACT: Using site-specific mutagenesis, we have probed the structural and functional roles of lysine-21 and lysine-27 of adenylate kinase (AK) from chicken muscle expressed in *Escherichia coli*. The two residues were chosen since according to the nuclear magnetic resonance (NMR) model [Mildvan, A. S., & Fry, D. C. (1987) *Adv. Enzymol. 58*, 241–313], they are located near the α- and the γ-phosphates, respectively, of adenosine 5′-triphosphate (ATP) in the AK–MgATP complex. In addition, a lysine residue (Lys-21 in the case of AK) along with a glycine-rich loop is considered "essential" in the catalysis of kinases and other nucleotide binding proteins. The Lys-27 to methionine (K27M) mutant showed only slight increases in k_{cat} and K_m, but a substantial increase (1.8 kcal/mol) in the free energy of unfolding, relative to the WT AK. For proper interpretation of the steady-state kinetic data, viscosity-dependent kinetics was used to show that the chemical step is partially rate-limiting in the catalysis of AK. Computer modeling suggested that the folded form of K27M could gain stability (relative to the wild type) via hydrophobic interactions of Met-27 with Val-179 and Phe-183 and/or formation of a charge-transfer complex between Met-27 and Phe-183. The latter was supported by an upfield shift of the methyl protons of Met-27 in ^{1}H NMR. Other than this, the ^{1}H NMR spectrum of K27M is very similar to that of WT, suggesting little perturbation in the global or even local conformations. These results suggest that *Lys-27 is nonessential in either catalysis (including substrate binding) or structure*, and that *a gain in conformational stability does not require a global conformational change*. Substitution of Lys-21 by methionine, on the other hand, resulted in a dramatic decrease in k_{cat} corresponding to as much as 7 kcal/mol of transition-state binding energy, while causing little change in the conformational stability. However, there are substantial, and possibly global, conformational changes in K21M, as evidenced by dramatic differences between the ^{1}H NMR spectra of K21M and WT. Examination of the 2.1-Å crystal structure of porcine muscle AK [Dreusicke, D., Karplus, P. A., & Schulz, G. E. (1988) *J. Mol. Biol. 199*, 359–371] suggested that the amino group of Lys-21 is within H-bonding distance (2.84 Å) of the carbonyl oxygen of Gly-15. Such a hydrogen bonding could stabilize the glycine-rich loop. Since the loop is a key feature of the tertiary structure, its disruption or destabilization in K21M could result in global conformational changes (but does not necessarily change the overall conformational stability). These results suggest that *Lys-21 plays a key structural role, and that whether it is catalytically essential remains to be established*. In addition, there seems to be *no direct correlation between changes in conformation and changes in conformational stability in site-specific mutants*. The results also strongly echo the warning [Wilde, J. A., Bolton, P. H., Dell'Acqua, M., Hibler, D. W., Pourmotabbed, T., & Gerlt, J. A. (1988) *Biochemistry 27*, 4127–4132] against straightforward interpretation of kinetic data without considering potential structural changes in site-specific mutants. Furthermore, we have addressed a few other issues related to the catalytic capability of AK, and the possible involvement of His-36 in substrate-induced conformational changes of AK.

In most enzymes using metal–ATP[1] (or GTP) as a substrate, there is a "glycine-rich loop" and an "invariant lysine". The glycine-rich loop has been shown to be involved in nucleotide binding in some cases (Wierenga & Hol, 1983; Gay & Walker, 1983; Moller & Amons, 1985) and has also been suggested to have the function of enabling conformational changes (Fry et al., 1986) since it also exists in non-nucleotide binding proteins. The invariant lysine is usually highly conserved among the same family of enzymes and is characterized by a dramatic loss of activity upon chemical modification and by the ability of the nucleotide to protect against chemical modification. In the protein kinase family, for example, both the glycine-rich loop and the invariant lysine are conserved in the subdomain 1 and subdomain 2, respectively, among the 64 sequences known (Hanks et al., 1988).

In the case of adenylate kinase (AK), the "essential lysine" is part of the "glycine-rich loop" consisting of residues 15–22:

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[1] Abbreviations: ADP, adenosine 5′-diphosphate; AK, adenylate kinase; AMP, adenosine 5′-monophosphate; ATP, adenosine 5′-triphosphate; CD, circular dichroism; dAMP, 2′-deoxyadenosine 5′-monophosphate; dATP, 2′-deoxyadenosine 5′-triphosphate; dCTP, 2′-deoxycytidine 5′-triphosphate; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetate; Gdn-HCl, guanidine hydrochloride; GTP, guanosine 5′-triphosphate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; $NADP^+$, β-nicotinamide adenine dinucleotide phosphate; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser enhancement spectroscopy; PAGE, polyacrylamide gel electrophoresis. SDS, sodium dodecyl sulfate; TLC, thin-layer chromatography; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; UV, ultraviolet.

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G-X-P-G-X-G-K-G. This sequence has been conserved among all five different types of AK (Schulz et al., 1986). The involvement of this loop in conformational change has been demonstrated by the two different crystal forms of porcine muscle AK (Sachsenheimer & Schulz, 1977; Pai et al., 1977). It has been shown that AK was inactivated when Lys-21 was modified by adenosine di-, tri-, and tetraphosphopyridoxals, and that these modifications were partially protected by ADP, ATP, and MgATP, but not by AMP (Tagaya et al., 1987; Yagami et al., 1988).

Despite the potential significance of Lys-21 on the basis of sequence homology and chemical modifications, its role in the catalysis of AK has been controversial. The crystal structure of porcine muscle AK suggests that the side-chain amino group of Lys-21 binds to a sulfate ion (which could be a mimic of a phosphate group of ATP) (Dreusicke & Schulz, 1986; Dreusicke et al., 1988). In the "X-ray model" of substrate binding (Pai et al., 1977), Lys-21 is interacting with the γ-phosphate of ATP. The chemical modification results of Tagaya et al. (1987) and Yagami et al. (1988) support this viewpoint. In the "NMR model", Lys-21 was suggested to be able to move and interact with the α-phosphate of ATP, and another lysine, Lys-27, could interact with the γ-phosphate (Fry et al., 1985, 1986; Mildvan & Fry, 1987). Recently molecular mechanics studies suggested that Lys-21 forms hydrogen bonds with *both* the γ-phosphate of ATP and the α-phosphate of AMP (Caldwell & Kollman, 1988). In this paper we use site-directed mutagenesis to probe the structural and functional roles of these two lysine residues of chicken muscle AK expressed in *E. coli*[2] (Tanizawa et al., 1987).

In order to properly interpret the steady-state kinetic data of mutants, it is also necessary to show that the chemical step is rate-limiting or at least partially rate-limiting, otherwise changes in k_{cat} may not reflect the catalytic role of the mutated residue. Although the kinetic properties of AK have been studied extensively and a random mechanism has been firmly established (Rhoads & Lowenstein, 1968), whether the chemical step or the physical step(s) is rate-limiting is again controversial. For the rabbit muscle AK isotope-exchange experiments suggested on and off steps as rate-limiting steps (Rhoads & Lowenstein, 1968) whereas NMR studies (Kantor et al., 1984) and initial velocity analysis (Hamada & Kuby, 1978) suggested that the chemical step is the rate-limiting step. For the porcine muscle AK ^{31}P NMR line-shape analysis suggested the off step as the rate-limiting step (Vasavada et al., 1984) whereas ^{31}P NMR inversion transfer experiments suggested that the chemical step is partially rate-limiting (Brown & Ogawa, 1977).

These controversial results prompted us to examine whether the chemical step is rate-limiting for the chicken muscle AK, using the viscosity variation method and His-36 mutants. Although this method is sometimes complicated by the effects of viscosity on binding or conformational steps (Kurz et al., 1987; Grissom & Cleland, 1988; Gates & Northrop, 1988), it has been successfully applied to elucidate the rate-limiting step(s) of at least 10 different enzymes [see Blacklow et al. (1988) and references therein]. The kinetic data from viscosity-dependence studies have led us to address a few other issues related to the catalytic capability of AK, and the possible involvement of His-36 in substrate-induced conformational changes of AK. During the preparation and revision of this paper two reports on site-directed mutagenesis of other residues of the glycine-rich loop of AK appeared (Reinstein et al., 1988; Tagaya et al., 1989).

Materials and Methods

Materials. The oligonucleotides directing the mutation of Lys-27 to Met, d(ATGCGAGA*T*GATTGTGC) for K27M and d(CTCAGGGA*T*GGGGACGC) for K21M, were obtained from the Biochemical Instrument Center of The Ohio State University and used without further purification. The reagents and enzymes for mutagenesis and [α-^{35}S]dCTP used for sequencing were purchased from Amersham. The reagents and enzymes (pyruvate kinase and lactic dehydrogenase) used for kinetic assays of AK were purchased from Sigma. Polyethylene glycol 20000 was the product of Fluka Chemie AG. Phosphocellulose P-11 and Sephadex G-100 were obtained from Whatman and Sigma, respectively. All other chemicals were of reagent grade. [2-^{3}H]ATP was obtained from Amersham. All other chemicals were of reagent grade.

Construction of Mutant Enzymes. The construction of mutant strains, expression, and purification were achieved according to the procedure described previously (Tian et al., 1988). The dialysis in polyethylene glycol 20000 was omitted in the purification of protein samples for NMR analysis. All the purified mutant enzymes were homogeneous on SDS–PAGE.

Due to the functional importance of the K21M mutants, we have resubcloned the gene of this mutant back to M13mp19 and reconfirmed the mutation.

Steady-State Kinetics. All the measurements were carried out at 30 °C and pH 8.0 in a standard Tris-HCl buffer (0.075 M Tris-HCl, 0.065 M KCl, and 0.001 M DTT). The steady-state kinetic constants were obtained by use of the pyruvate kinase–lactic dehydrogenase coupling system (Rhoads & Lowenstein, 1968). The detailed procedures have been described previously (Tian et al., 1988). The "initial velocity patterns" were obtained by varying both substrates, and K and K_i values were calculated from eq 11. In the experiments to test ATPase activity, the same condition was used except that AMP was omitted. No ATP hydrolysis was detectable beyond the limit of 0.005 unit/mg. The reverse reaction (formation of AMP/ATP) was carried out using hexokinase/glucose 6-phosphate dehydrogenase as the coupling system. The reaction mixture consists of the same buffer as used in the forward reaction, 1 mM glucose, 0.2 mM $NADP^+$, 100 units of hexokinase, 50 units of glucose 6-phosphate dehydrogenase, and varying concentrations of $MgCl_2$ and ADP at a constant ratio of 1:2.

Viscosity-Dependent Kinetics. A concentrated sucrose stock solution was prepared in double-distilled (deionized) water (50%, w/v) and diluted to desired concentrations (10, 20, 30, and 40%). These sucrose solutions were then used to make the reaction buffers for the viscosity studies by addition of the solid buffer components to the final concentrations: 75 mM Tris-HCl, 65 mM KCl, and 1 mM DTT. Then pH was adjusted with 6 M HCl to 8.0. The bottles of the buffers were tightly sealed with Parafilm and stored at 4 °C before usage. The relative viscosity (η_{rel}) of these buffers were then determined as described in Sanders et al. (1989).

The kinetic assays were performed as described above, except that sufficient coupling enzymes were used to ensure that

[2] In the N-terminal region the second amino acid residue, Glu, of the porcine, rabbit, calf, and human muscle AK changes to a pair of residues Ser-Thr in the chicken muscle AK (Kishi et al., 1986). However, the Met-1 residue is absent in the chicken AK expressed in *E. coli* (Tanizawa et al., 1987). To avoid unneccessary confusion, we number the amino acid sequence of chicken AK starting from Ser such that the homologous residues will have the same numbers among all muscle AKs. This is different from the numbering used in Kishi et al. (1986), which started with Met-1 and resulted in a shift of one relative to other muscle enzymes.

the observed changes in rates were not due to viscosity effects to the coupling enzymes. When dAMP and/or dATP were used as substrates, we have ensured that the coupling enzymes were sufficient by observing no increases in the velocity when additional coupling enzymes were added. The viscosity studies using glycerol were performed similarly.

Determination of the Internal Equilibrium Constant K_{int}. The procedure described here was adapted from Stackhouse et al. (1985) for the determination of K_{int} for pyruvate kinase. The reaction buffer was the same as that used for steady-state kinetics (pH 8.0). The following stock solutions were prepared with the reaction buffer: 4 mM (ca. 90 mg/mL) AK, 0.1 mM ADP, and 0.05 mM $MgCl_2$. To each of a series of 0.5-mL microcentrifuge tubes, 5 μL each of ADP and $MgCl_2$ stock solutions and 2 μL of [2-^{3}H]ATP (1.0 mCi/mL) were added. Then varying amounts of the AK stock solution and the reaction buffer were added to bring the final volume to 50 μL (so that the final concentrations of ADP and $MgCl_2$ were 0.01 and 0.005 mM, respectively). The reaction mixtures were then incubated at 30 °C for 10 min, and the reaction was stopped by addition of 10 μL of 1.0 M EDTA (pH 8.5), followed by heating in a boiling water bath for 5 min and analysis by TLC as described below.

To a polyethylenimine–cellulose plate (Polygram Cel 300, Brinkmann) was spotted 2 μL of a cold carrier solution (5 mM ATP, 7 mM ADP, and 10 mM AMP in 50 mM EDTA, pH 8.0), followed with 5 μL of the reaction mixture on top of the cold carrier. Then the plate was air-dried and developed in 1 M LiCl. The nucleotide spots were identified under a UV lamp (short wavelength), cut off, placed in a counting vial (with the gel facing up), covered with 10 mL of scintillation cocktail, and counted in a scintillation counter (Beckman SL 3801). The data were corrected for the background activity obtained from parallel control experiments with the enzyme omitted.

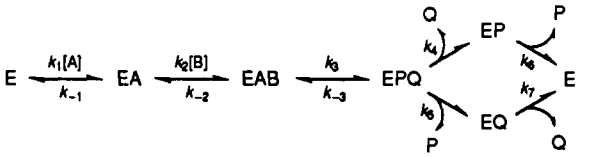
Since the reaction was started with ADP (i.e., initial concentrations of AMP and ATP were zero except the trace amount of [^{3}H]ATP), the equilibrium concentrations of AMP and ATP should be the same. If dead-end complexes are ignored, K_{int} = [E·MgADP·ADP]/[E·MgATP·AMP] = [ADP]/2[ATP]. Therefore, plotting $^1/_2$[ADP]/[ATP] vs [E] gives K_{int} as the plateau value.

^{1}H NMR Analysis. A WT or mutant AK solution (ca. 0.5 mL, 20–30 mg/mL) was prepared in a HEPES buffer (1 mM HEPES, 65 mM KCl, 0.5 mM EDTA, and 0.5 mM DTT, pH 8.0). The enzyme solution was then placed in a small dialyzing tubing and thoroughly dialyzed against the same buffer. After dialysis, the enzyme solution with known volume (usually 0.5 mL) was transferred to a small pear-shaped flask and lyophilized. About 1 mL of 99.8% D_2O was then added to redissolve the enzyme and lyophilization was conducted again to remove the exchangeable protons. The resulting dry enzyme was redissolved in 75 mM perdeuteriated Tris-DCl buffer, pH 8.0 (uncorrected), in 0.5 mL of 99.996% D_2O. Proton NMR spectra were recorded at 24 °C on a Bruker AM 500 NMR spectrometer.

Computer Modeling of WT and Mutant AK. The X-ray crystallographic structure of porcine AK at 2.1-Å resolution (Dreusicke et al., 1988) was obtained from the Brookhaven Protein Database (file number 3ADK). Modeling was performed by using an Evans and Sutherland PS300 terminal, VAX 11/750 FPA computer, and CHEMX (Chemical Design Ltd.).

Conformational energy minimization of K27M was carried out according to the force field method of Allinger (1976) by using Columbia University's MacroModel. This method is a static modeling, which does not take into consideration dynamic contributions to the structure. During the minimization process, the conformational adjustments were made only to the newly generated side chain of Met-27 while the rest of the molecule was kept stationary. In addition, the modeling was carried out in vacuo. Because of the various potential uncertainties that may have been introduced in the modeling procedure, the result obtained should be viewed as only being qualitative. By use of the CHEMX program, the side chain of Lys-27 in the crystal structure of porcine muscle AK was first replaced with that of Met. The surrounding residues (within 15 Å from Lys-27) along with Met-27 were then "cut off" from the parent structure, and the resulting structural segment was analyzed by energy minimization algorithms using MacroModel. To avoid reaching a local minimum, a grid search for the conformation with lowest energy among those generated by dihedral angle changes in 30° increments was first performed. Then the conformation of lowest energy was used as starting points for the energy minimization. In addition, several other conformations of next higher energy generated in the search with 30° increments of the dihedral angles were also energy-minimized, and the resulting conformations were compared with that obtained by using the conformation with lowest energy. An identical conformation was obtained by either of the above procedures.

Scheme I



Viscosity Effect in a Random Bi Bi System. The viscosity-dependent kinetic equations have previously been derived for single-substrate systems (Loo & Erman, 1977; Nakatani & Dunford, 1979; Brouwer & Kirsch, 1982) and for a Theorell–Chance system (Gates & Northrop, 1988), but not for a random bi bi system. The kinetic scheme for a random bi bi system with a saturating substrate A (MgATP in this case) can be expressed by Scheme I, where A, B, P, and Q represent MgATP, AMP, MgADP, and ADP, respectively. The kinetic expression for $1/k_{cat}$ of Scheme I can be derived from Cleland's net rate method (Cleland, 1975), as shown in eq 1:

$$\frac{1}{k_{cat}} = \frac{1}{k_3} + \frac{1}{k_4^\circ + k_5^\circ} + \frac{1}{K_3(k_4^\circ + k_5^\circ)} + \frac{k_4^\circ}{k_6^\circ(k_4^\circ + k_5^\circ)} + \frac{k_5^\circ}{k_7^\circ(k_4^\circ + k_5^\circ)} \quad (1)$$

where k°'s are rate constants of viscosity sensitive steps at $\eta_{rel} = 1$, and $K_3 = k_3/k_{-3}$. Since $k^\circ = k/\eta_{rel}$ (Kramers, 1940), eq 1 can be expressed as

$$1/k_{cat} = a + b\eta_{rel} \quad (2)$$

where

$$a = 1/k_3 \quad (3)$$

$$b = \left[1 + \frac{1}{K_3} + \frac{k_4}{k_6} + \frac{k_5}{k_7}\right]\frac{1}{k_4 + k_5} \quad (4)$$

Similarly

$$K_m/k_{cat} = a + b\eta_{rel} \quad (5)$$

in which

$$a = 1/k_3K_2 \quad (6)$$

$$b = \frac{1}{k_2} + \frac{1}{K_2K_3(k_4 + k_5)} \quad (7)$$

According to eqs 2 and 5, *both $1/k_{cat}$ and K_m/k_{cat} are linear functions of the relative viscosity with positive ordinate intercepts.*

The normalized equation is

$$\frac{(k_{cat}/K_m)^\circ}{(k_{cat}/K_m)} = a + b\eta_{rel} \quad (8)$$

in which $^\circ$ designates the condition when $\eta_{rel} = 1$, and

$$a = \frac{1}{1 + k_3/k_{-2} + k_{-3}/(k_4 + k_5)} \quad (9)$$

$$b = \frac{1}{1 + k_{-2}/k_3 + k_{-2}/K_3(k_4 + k_5)} + \frac{1}{1 + (k_4 + k_5)/k_{-3} + K_3(k_4 + k_5)/k_{-2}} \quad (10)$$

Again, *this is a linear equation with a positive ordinate intercept.* The values of a and b in eq 8 can vary between 0 and 1 depending on the relative rates of the diffusional and chemical steps of the reaction. Two limiting situations are as follows. *When both $k_3 \ll k_{-2}$ and $k_{-3} \ll k_4 + k_5$ (i.e., if the chemical step is fully rate-limiting), $a = 1$ and $b = 0$; when both $k_3 \gg k_{-2}$ and $k_{-3} \gg k_4 + k_5$ (i.e., if the reaction is fully diffusion-controlled), then $a = 0$ and $b = 1$.*

Results and Discussion

Conformational Stability of Lys-21 and Lys-27 Mutants. Both mutants are stable and can be purified in quantity. However, the K27M mutant appears more stable than the WT as revealed by the guanidine hydrochloride (Gdn-HCl) induced denaturation. The concentration (C_m) of Gdn-HCl at the midpoint of unfolding, and the Gibbs free energy of unfolding, $\Delta G_D^{H_2O}$ (Pace, 1986; Tian et al., 1988) are listed in Table I. The results suggest that substitution of Lys-27 by Met either stabilizes the folded state or destabilizes the unfolded state by as much as 1.8 kcal/mol. In a later section structural evidence is presented to explain this change in stability.

Kinetic Properties of Lys-21 and Lys-27 Mutants. As shown in Table II, the k_{cat} of K27M was almost unchanged relative to that of the WT, whereas the k_{cat} of K21M decreased by a factor of 4.3×10^4. The K_m of both mutants changed only slightly. Similar kinetic data were also obtained by a full initial velocity analysis (varying the concentrations of both substrates), as shown in Table III. The quantitative values of K and K_i were calculated according to the following equation (Cleland, 1986):

$$v = \frac{ABV}{K_aK_{ib} + K_bA + K_aB + AB} \quad (11)$$

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 data in Table III indicate that the dissociation constants K_i (for binary complex) and K (for ternary complex, equivalent to K_m obtained by holding one of the substrates at saturating level) were only slightly perturbed in K21M and K27M.

Rate-Limiting Steps and Other Catalytic Properties of AK. Lack of perturbation in k_{cat} may not necessarily suggest lack of perturbation in the chemical step unless the chemical step is rate-limiting. Viscosity-dependent kinetics was used to identify rate-limiting steps. The viscosity-dependent kinetic equations for a random bi bi system are derived in Materials and Methods. Although the theoretical basis relies entirely on a possibly oversimplified relationship of Kramers (1940), it is qualitatively true that the chemical step should not be perturbed by viscosity changes unless the viscogen interacts nonspecifically with some of the residues involved in stabilizing the transition state of the substrates or interacts with the enzyme nonspecifically to alter the global conformation. Thus, a zero slope for the plot of eq 8, if observed, will suggest that the chemical step is fully rate-limiting. On the other hand, a nonzero slope, if observed, will require proper control experiments to exclude the possibility of nonspecific effects caused by the viscogen before it can be used to suggest that some viscosity-sensitive physical steps (diffusion, conformational change, etc.) are rate-limiting.

Figure 1 (A–C) shows the plots of eqs 2, 5, and 8 for WT AK, with sucrose as the viscogen. The specific values of k_{cat} and $K_{m(AMP)}$ are listed in Table IV. It can be noted that the y intercepts of all three plots in Figure 1 are negative, which suggests that the data cannot be properly described by eqs 2, 5, and 8. The possible implications of this behavior will be discussed in the last section. However, nonspecific interaction of the viscogen with active site residues should not be primarily

Table I: Free Energy of Unfolding Induced by Gdn-HCl[a]

	$\Delta G_D^{H_2O}$ (kcal/mol)	C_m (M)	m (kcal/mol·M)
WT	3.8	0.79	4.7
K21M	3.5	0.77	4.4
K27M	5.6	1.04	5.4

[a] Calculated from the equation $\Delta G_D = \Delta G_D^{H_2O} - m$[Gdn-HCl], where ΔG_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.

Table II: Summary of Steady-State Kinetic Data[a]

	k_{cat} (s^{-1})	$K_{m(MgATP)}$ (mM)	$K_{m(AMP)}$ (mM)	$k_{cat}/K_{m(MgATP)}$ ($\times 10^7$ M^{-1} s^{-1})	$k_{cat}/K_{m(AMP)}$ ($\times 10^7$ M^{-1} s^{-1})
WT	650	0.035	0.092	1.9	0.7
K21M	0.015 $(4.3 \times 10^4)^b$	0.050	0.040	0.000030 $(6.3 \times 10^4)^b$	0.000038 $(1.8 \times 10^4)^b$
K27M	850	0.072	0.094	1.2	0.9

[a] Obtained by saturating with one of the substrates. [b] Numbers in parentheses represent the ratio WT/K21M.

Table III: Summary of Steady-State Kinetic Data[a]

	$K_{(MgATP)}$ (mM)	$K_{(AMP)}$ (mM)	$K_{i(MgATP)}$ (mM)	$K_{i(AMP)}$ (mM)	K_i/K
WT	0.042	0.098	0.16	0.37	3.8
K21M	0.052	0.044	0.19	0.16	3.6
K27M	0.105	0.13	0.32	0.40	3.1
C25S[b]	0.074	0.099	0.31	0.41	4.2
H36N	0.13	0.52	0.26	1.01	2.0
H36Q	0.54	1.43	0.29	0.78	0.54

[a] Obtained by varying concentrations of both substrates. [b] This mutant was designed to test the structural and functional roles of Cys-25, but was found to have relatively little perturbed kinetic constants: k_{cat} = 113 s^{-1}; $K_{m(MgATP)}$ = 0.073 mM.

Table IV: Steady-State Kinetic Constants of AK and Its Mutants at Various Relative Viscosity

		WT AK		H36N		H36Q	
η_{rel}	sucrose (%)	k_{cat} (s^{-1})	$K_{m(AMP)}$ (mM)	k_{cat} (s^{-1})	$K_{m(AMP)}$ (mM)	k_{cat} (s^{-1})	$K_{m(AMP)}$ (mM)
1.00	0	610	0.094	600	0.39	640	0.98
1.33	10	370	0.096	480	0.34	380	0.71
1.77	20	240	0.10	340	0.32	340	0.64
2.50	30	160	0.10	230	0.30	240	0.62
3.90	40	95	0.10	150	0.28	200	0.60

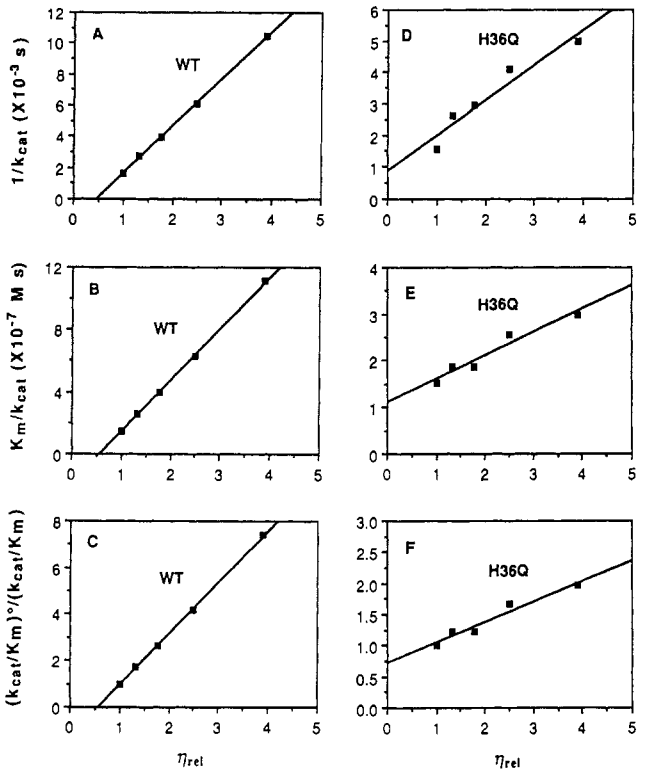


FIGURE 1: Plots of $1/k_{cat}$, K_m/k_{cat}, and $(k_{cat}/K_m)°/(k_{cat}/K_m)$ vs relative viscosity for WT AK (A–C) and H36Q (D–F). The K_m values in this figure are $K_{m(AMP)}$.

Table V: Summary of Steady-State Kinetic Data on Substrate Analogues

substrates	k_{cat} (s^{-1})	k_{cat} (rel)	$K_{m(AMP)}$ (mM)	k_{cat}/K_m (s^{-1} M^{-1})	(k_{cat}/K_m) (rel)
I. ATP + AMP	610	1.0	0.094	6.5×10^6	1.0
II. dATP + AMP	400	0.66	0.072	5.6×10^6	0.86
III. ATP + dAMP	110	0.18	0.45	0.25×10^6	0.038
IV. dATP + dAMP	28	0.046	0.19	0.15×10^6	0.023

responsible for the nonzero slope or the negative y intercepts on the basis of three sets of experiments: (i) Essentially the same plots were obtained with a different viscogen, 10, 20, 30, and 40% glycerol (η_{rel} = 1.24, 1.61, 2.15, and 2.93, respectively); (ii) Positive y intercepts were observed for H36Q with regular substrates; (iii) Zero slopes were observed for WT AK with slow-reacting substrate analogues dAMP and/or dATP. These results were then used to conclude that the chemical step is partially rate-limiting in the catalysis of AK as described below.

The viscosity-dependent kinetic data of the mutant H36Q, which was shown to have k_{cat} unchanged, and K_m increased by 6× (MgATP) and 10× (AMP) relative to WT (Tian et al., 1988), are plotted in Figure 1 (D–F) and also listed in Table IV. Unlike the behavior of WT, the y intercepts for H36Q are normal (positive). The nonzero slopes suggest that the reaction catalyzed by H36Q is at least partially controlled by a step other than k_3, most likely the diffusion step(s). Since the slope in Figure 1F is only 0.3 (<1), the diffusion step should not be fully rate-limiting. The rate-limiting steps in the catalysis by H36Q should include at least a viscosity-independent step. The most likely candidate of this is the chemical step, but a viscosity-independent conformational step cannot be completely ruled out. Since the kinetic constants of H36Q and WT are very similar, the chemical step is also likely to be partially rate-limiting in the WT AK.

The viscosity dependence of dAMP and/or dATP are shown in Figure 2, and the kinetic data are listed in Table V. The chemical step should be fully rate-limiting for substrate systems

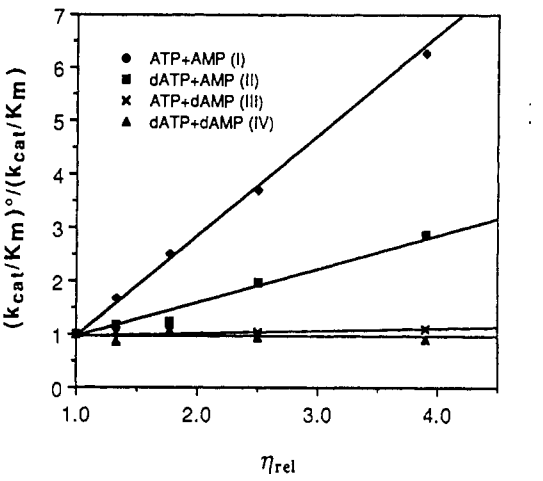


FIGURE 2: Plots of $(k_{cat}/K_m)°/(k_{cat}/K_m)$ vs relative viscosity for WT AK with substrate systems I–IV. The K_m values in this figure are $K_{m(AMP)}$ or $K_{m(dAMP)}$.

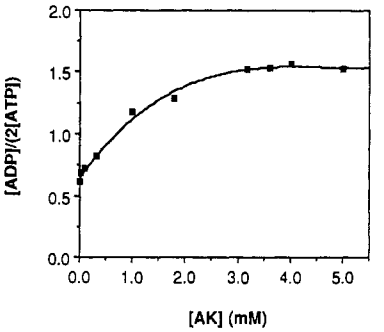


FIGURE 3: Determination of K_{int} by plotting [ADP]/(2[ATP]) vs [AK]. When the plateau is reached all the nucleotides are completely bound, thus the ratio (1.5) represents the internal equilibrium constant. The ratio extrapolated to [AK] = 0 represents the external equilibrium constant (0.6).

III (ATP + dAMP) and IV (dATP + dAMP) on the basis of viscosity-independent slopes. Since the k_{cat} of system III is decreased by only 5× relative to system I, the chemical step should be partially rate-limiting for natural substrates.

The observation that the catalysis of AK is likely to be jointly controlled by diffusion and the chemical step suggested that AK should be a nearly "perfect enzyme" (Albery & Knowles, 1976). We then further examined a few properties pertaining to the catalytic capability of AK. The catalytic efficiency of AK is $>10^{12}$× relative to uncatalyzed reaction between AMP and MgATP within the limit of detection (Sanders, 1988). Unlike other kinases such as hexokinase, AK showed no detectable ATPase activity in the absence of AMP ($<3 \times 10^{-6}$ k_{cat}, also true for H36Q). As shown in Figure 3, the isotope trapping experiments (Stackhouse et al., 1985) indicated that both internal and external equilibrium constants (1.5 and 0.6, respectively) are close to unity. The k_{cat} of the reverse reaction (830 s^{-1}) is comparable to that of the forward reaction.

Lys-27 Is Nonessential For Catalysis. Since the chemical step of the WT AK is at least partially rate-limiting as described above, the chemical step of K27M could not have been perturbed greatly. This was further confirmed by use of slow-reacting substrates dAMP/MgATP. Since the chemical step of WT AK is rate-limiting with this substrate system, the difference in k_{cat} between WT and K27M can therefore be attributed to a perturbation in the chemical step. Only small differences were observed between the kinetic properties of WT and K27M (k_{cat} 110 and 140 s^{-1}, respectively; $K_{m(dAMP)}$ 0.45 and 0.37 mM, respectively) with this substrate system.

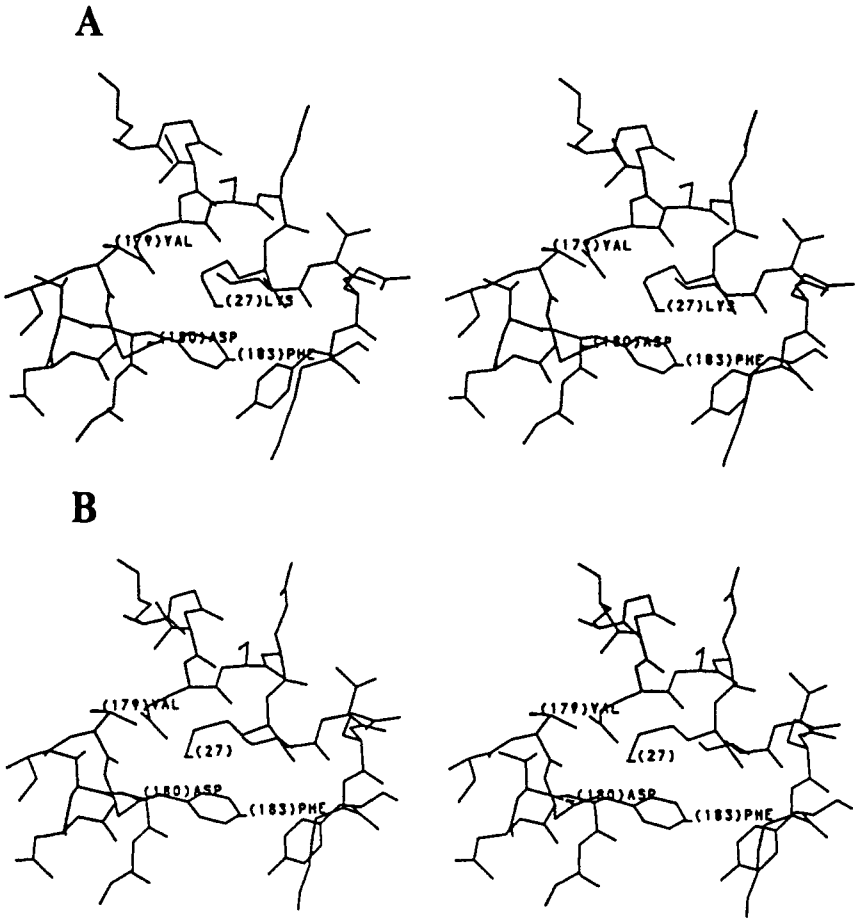


FIGURE 4: (A) Stereoview of the environment of Lys-27, according to the 2.1-Å crystal structure (Dreusicke et al., 1988) obtained from the Brookhaven Protein Database. (B) Stereoview of the environment of Met-27 obtained by substituting Lys-27 in the 2.1-Å crystal structure with Met followed by conformational energy minimization.

Thus, the results suggest that Lys-27 is not involved in catalysis.

Possible Structural Basis for the Stability of K27M. Examination of the 2.1-Å crystal structure of porcine muscle AK (Dreusicke et al., 1988) revealed a potential side-chain interaction between Lys-27 and Asp-180 (Figure 4A), which could be holding the helix 23–30 and the C-terminal helix 179–194 in place. Since this ionic pair is quite exposed to solvent, its disruption may cost little energy. On the other hand, replacement of Lys-27 with Met could enhance hydrophobic interactions between Phe-183, Met-27, and Val-179. Furthermore, the sulfur atom of Met-27 could potentially form a charge-transfer complex with the aromatic ring of Phe-183. It has been noted that disulfide linkages and aromatic rings are often located together in tertiary structures of proteins (Morgan et al., 1978; Morgan & McAdon, 1980).

The above interpretation was supported by two experiments. First, substitution of Lys-27 by Met in the crystal structure followed by energy minimization led to a structure in which the sulfur atom of Met-27 is located above the benzene ring of Phe-183 in roughly even distances (3.7–5.1 Å) to the six ring carbon atoms, as shown in Figure 4B. Secondly, proton NMR spectra (Figure 5) show that the additional singlet of the *S*-methyl in K27M resonates at 1.58 ppm (peak e in spectrum B and C), significantly upfield from the 2.0 ppm region of the WT AK (spectrum A). This is most likely due to the aromatic ring current effect of Phe-183.

It is also important to note that the difference spectrum in Figure 5C indicates only small differences between WT and K27M. Thus, the additional conformational stability does not affect the overall conformation or catalytic property of the enzyme significantly. This should allow us to make unstable mutants more stable in the future by changing Lys-27 to Met in addition to the mutation at the site of interest. This will be very useful since the conformational stability of WT AK is low. The typical $\Delta G_D^{H_2O}$ values for other proteins lie in the range of 5–15 kcal/mol (Pfeil, 1981; Creighton, 1983), while for AK it is <4 kcal/mol.

Possible Structural Roles of Lys-21. As cautioned by Hibler et al. (1987), it is important to examine possible conformational changes before the perturbation in kinetic properties can be attributed to catalysis. Although the relatively unperturbed conformational stability of this mutant could argue against a large conformational change, we found that K21M showed a dramatic change in proton NMR (Figure 5D). The spectra have been reproduced at least twice with samples prepared independently. The results suggest that the conformation of K21M has been perturbed globally. Examination of the 2.1-Å crystal structure of porcine muscle AK (Dreusicke et al., 1988) revealed that the nitrogen atom of the side chain of Lys-21 is within hydrogen-bonding distance (2.84 Å) to the carbonyl oxygen of Gly-15 at the other side of the loop (Figure 6), which could be responsible for holding the loop in its proper conformation. This H bond seems to be the only one within the loop, and the loop is intimately associated with other strands via a number of aromatic–aromatic interactions (e.g., Phe-12···Tyr-95···Tyr-164), as also shown in Figure 6. These structural features could explain the dramatic changes in the ^{1}H NMR spectrum of K21M. That the conformational change is global is further supported by detectable changes in the circular dichroism spectra (data not shown). Thus it is concluded that Lys-21 plays a key structural role

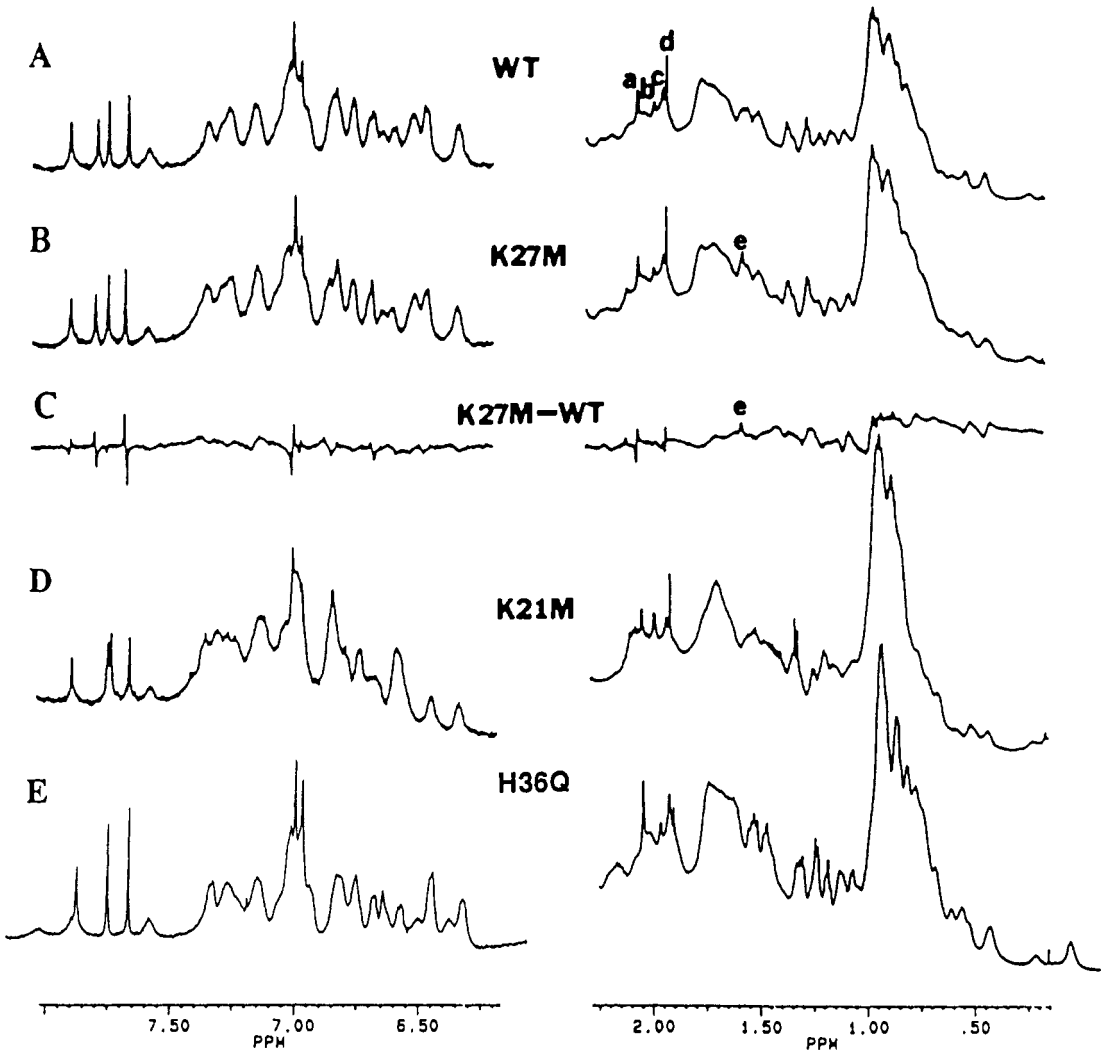


FIGURE 5: Proton NMR spectra of aromatic (left panel) and aliphatic (right panel) regions of WT (A), K27M (B), K27M–WT (C), K21M (D) and H36Q (E). The spectra were obtained at 24 °C and pH 8.0 in 0.075 M perdeuteriated Tris, 0.065 M KCl, 0.5 mM EDTA, and 0.5 mM DTT. All spectra were processed with 1.0-Hz line broadening and exponential multiplication. In spectrum A peaks a–c can be assigned to *S*-methyl groups of the four methionine residues (the very sharp peak d is more likely to arise from buffer). In the spectrum of K27M (B) there is an additional resonance (e) at 1.58 ppm, which becomes quite obvious in the difference spectrum (C).

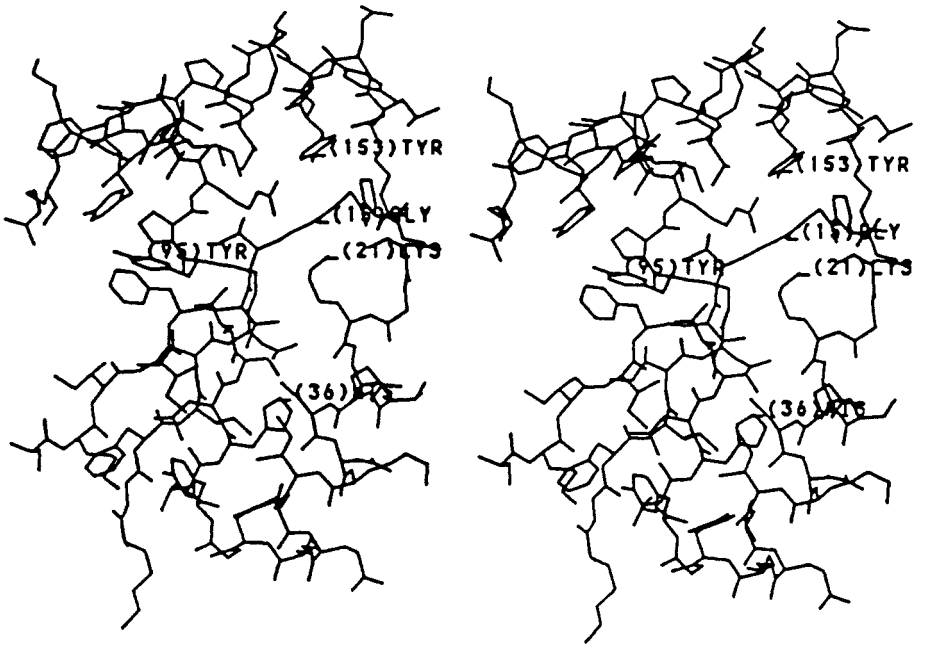


FIGURE 6: Stereoview of the environment of Lys-21, according to the 2.1-Å crystal structure (Dreusicke et al., 1988) obtained from the Brookhaven Protein Database.

by stabilizing the loop directly and the global conformation indirectly.

The Catalytic Role of Lys-21 is Uncertain. The involvement of Lys-21 in structure does not preclude it from playing catalytic roles, particularly since the substrate binding parameters of K21M (Tables II and III) have not been significantly perturbed. Whether this is true requires further investigation. Site-directed mutagenesis of the invariant lysine in several protein kinases has also resulted in loss of protein kinase activity [Hanks et al. (1988) and references therein], but no structural analyses were available for these systems. Changes of Lys-155 in the β-subunit of the F_1-ATPase from *E. coli* to Gln and Glu were found to have only a small effect on the rate of ATP hydrolysis, while the binding affinity of MgATP (but not MgADP) was greatly weakened (Parsonage et al., 1988). The authors suggested that Lys-155 is involved in substrate binding by interacting with the γ-phosphate of bound ATP. This is clearly different from AK where k_{cat} but not K_m are affected. Thus, the "invariant lysine" in kinases and other nucleotide binding proteins appears to be "essential" in all systems studied, but its specific functions vary.

Possible Roles of His-36 in the Conformational Change of AK. The negative y intercepts in the viscosity plots of WT (Figure 1, A–C) could be caused by at least three possible reasons. The nonspecific effect of the viscogen and the failure of Kramers' relationship are less likely on the basis of the control experiments described earlier. Another possible reason is that a viscosity-sensitive conformational step is partially rate-limiting in WT AK, as has been suggested for aminoglycoside nucleotidyltransferase 2″-I (Gates & Northrop, 1988). If this is the case, the normal behavior of H36Q supports our previous suggestion that His-36 is involved in the conformational change of AK (Tian et al., 1988). Since H36N behaves inbetween WT and H36Q (data in Table IV; plots not shown), the interaction should occur via the N1 atom of His-36.

To further substantiate this interpretation, we obtained complete steady-state kinetic data according to eq 11 for WT, H36N, and H36Q (last two rows of Table III). The results indicate that the K_i (dissociation constant) values vary to a small extent only (<3×) whereas the K (Michaelis constant) values vary more significantly (≤14×). The ratio K_i/K is 3.8 for WT, which indicates the existence of substrate synergism. The ratio remained relatively constant for three other mutants: 3.6, 3.1, and 4.2 for K21M, K27M, and C25S, respectively. However, it decreased to 2.0 and 0.54 for H36N and H36Q, respectively, which suggests lack of substrate synergism in H36Q, in agreement with the involvement of His-36 in the induced-fit mechanism. Additional kinetic data and discussion can be found in Tian (1989).

Structurally, it has been shown that two different crystal forms of AK (suggested to represent free AK and an AK–substrate complex) can be interconverted by protonation and deprotonation of His-36 (Dreusicke & Schulz, 1988), which strongly supports the above interpretation of the kinetic data. In proton NMR, we have shown that the C2 H of His-36 is shifted downfield by all substrates and analogues studied, with the magnitudes of shift following the order PPP_i < $MgPPP_i$ < AMP < ATP < MgATP < reaction mixture < $MgAP_5A$ (Sanders et al., 1989). Such a pattern is more consistent with a conformational effect than local interaction with a substrate suggested previously (McDonald et al., 1975; Fry et al., 1985). However, the two could be related. The proton NMR spectra are very similar between WT and H36Q (Figure 5E), and between WT + AP_5A and H36Q + AP_5A. Figure 7 shows

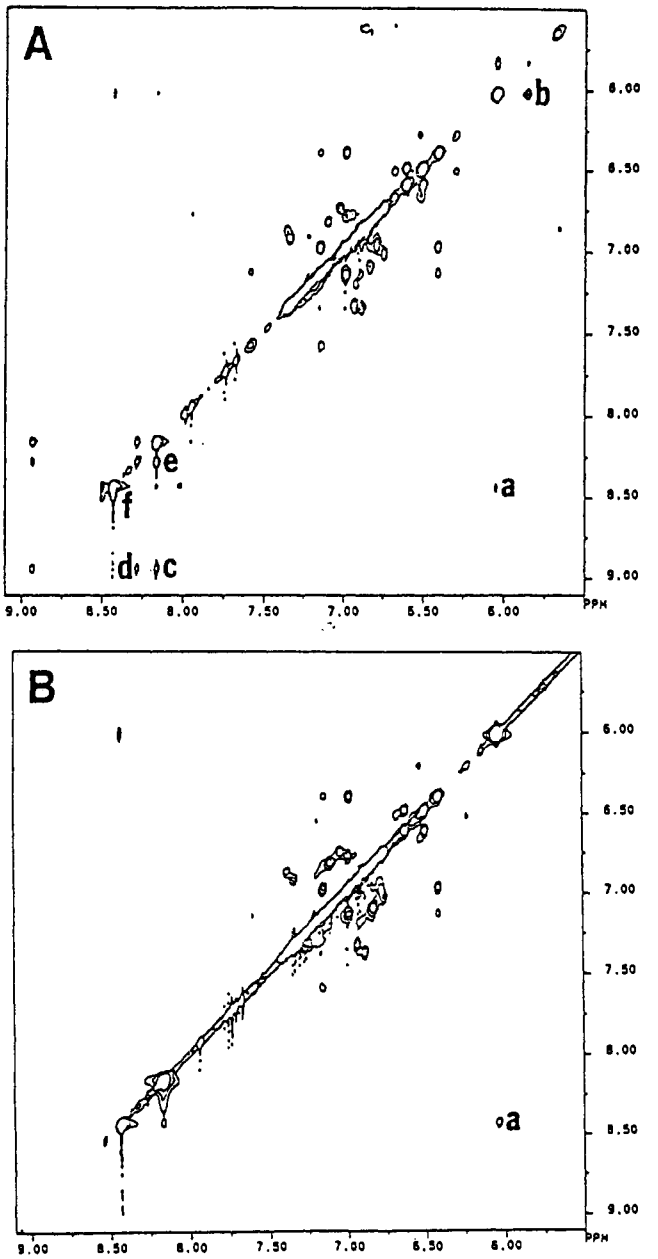


FIGURE 7: Phase-sensitive NOESY spectra of the aromatic region of WT (2 mM) + AP_5A (4 mM) (A) and H36Q (1.5 mM) + AP_5A (2 mM) (B). Experimental conditions: 20 mM perdeuteriated Tris in D_2O, 65 mM KCl, 2 mM DTT, pH 7.8 (uncorrected). Mixing time 150 ms, unsymmetrized. The unlabeled cross peaks are due to protein residues, which are almost identical between A and B. The cross peaks a–e are tentatively assigned as follows (AP_5A–A and AP_5A–B designate the two nonequivalent sets of signals due to binding to the two sites. When not specified, it could be either.): (a) NOE between C1′H and C8H of bound AP_5A (since the observed NOE is negative it is unlikely to be due to free AP_5A); (b) exchange of C1′H between free and bound AP_5A, possibly overlapping with exchange between bound AP_5A–A and AP_5A–B; (c) exchange of C2H between free AP_5A and bound AP_5A–A; (d) exchange of C2H between bound AP_5A–A and bound AP_5A–B; (e) exchange of C2H between free and bound AP_5A–B; (f) exchange of C8H between free and bound AP_5A, possibly overlapping with exchange between bound AP_5A–A and AP_5A–B. The exchange peaks are present in (A) but absent in (B) because free and bound AP_5A are in slow exchange in the WT complex, but in fast exchange in the H36Q complex.

the NOESY cross peaks in the aromatic region of WT + AP_5A and H36Q + AP_5A, which are almost identical. Thus H36Q is not conformationally perturbed globally and is able to bind substrates, to exist in proper conformations, and to catalyze the reaction. The only imperfection for H36Q is that it has higher K_m values, which could be due to an impaired

induced-fit mechanism postulated above. The latter could in turn arise from unfavorable interaction of H36Q with substrate(s), which would be in consistence with the observation of NOE between His-36 and adenine H-2 of bound ATP (Fry et al., 1985).

Conclusion. We have shown two extreme cases of structural perturbation by site-directed mutagenesis: minimal changes in K27M and maximal changes in K21M. In addition, the results of two mutants also demonstrate that there is no straightforward correlation between perturbations in conformation and in stability: K27M is substantially more stable than WT but its proton NMR is almost unperturbed, whereas K21M is unchanged in conformational stability but its proton NMR is greatly perturbed. Having shown that the chemical step is partially (and nearly) rate-limiting using viscosity-dependent kinetics and His-36 mutants, we conclude that Lys-27 is nonessential for catalysis on the basis of unperturbed kinetic properties, which is inconsistent with the previous suggestion that it could interact with the γ-phosphate of ATP (Fry et al., 1985; Mildvan & Fry, 1987). On the other hand, the possible catalytic role of Lys-21 remains uncertain. Finally, we have addressed a few other issues related to the catalytic capability of AK, and the possible involvement of His-36 in substrate-induced conformational changes of AK.

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