

Mechanism of Adenylate Kinase. Histidine-36 Is Not Directly Involved in Catalysis, but Protects Cysteine-25 and Stabilizes the Tertiary Structure[†]

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ABSTRACT: Several previous reports on muscle adenylate kinase (AK) have suggested that histidine-36 (His-36) is located in the binding site of adenosine 5'-triphosphate (ATP) and is involved in catalysis. We have tested the role of His-36 using site-specific mutagenesis on chicken muscle AK expressed in *Escherichia coli*. Three mutant proteins (H36Q, H36N, and H36G) were obtained by substituting His-36 with glutamine, asparagine, and glycine, respectively. Steady-state kinetic studies showed that the mutants have similar kinetic properties to those of the wild-type (WT) AK, which suggested that His-36 is not directly involved in catalysis. However, His-36 is likely to interact with or protect cysteine-25 (Cys-25) on the basis of the following evidence: The crystal structure of porcine muscle AK revealed a close proximity between His-36 and Cys-25; the mutants were unstable during purification (the order of stability was WT > H36Q > H36N > H36G); the H36G mutant readily dimerized; the sulfhydryl groups of mutants became more reactive (WT < H36Q < H36N) toward 5,5'-dithiobis(2-nitrobenzoic acid). Furthermore, His-36 was found to stabilize the tertiary structure of AK on the basis of guanidine hydrochloride induced denaturation studies, which showed that the conformational stability decreases in the order WT > H36Q > H36N. Three models are proposed to explain the structural roles of His-36: (i) His-36 forms a charge-transfer complex with Cys-25; (ii) His-36 forms a hydrogen bond (N3-H...S-H) with Cys-25; and (iii) His-36 protects Cys-25 by steric shielding and stabilizes the tertiary structure via H-bonding with Asp-93. ¹H NMR experiments were also performed as a function of pH, and the pK_a of His-36 was determined as 6.3. Two other histidines showed pK_a = 6.5, and the fourth histidine showed nontitrating behavior. The chemical shifts and pK_a of the histidines in H36Q and H36N are the same as those in the wild-type AK within experimental error.

Adenylate kinase (AK)¹ (EC 2.7.4.3) catalyzes the interconversion between AMP, ADP, and ATP: MgATP + AMP ⇌ MgADP + ADP (Noda, 1973). The enzyme from mammalian muscle (unless otherwise specified, our discussion will

be confined to muscle AK) is one of the smallest ($M_r \approx 21\,700$) and most extensively studied kinases. The crystal structure

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¹ Abbreviations: ADP, adenosine 5'-diphosphate; AK, adenylate kinase; AMP, adenosine 5'-monophosphate; ATP, adenosine 5'-triphosphate; dCTPαS, 2'-deoxycytidine 5'-O-(1-thiotriphosphate); DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol; EDTA, ethylenediaminetetraacetate; Gdn-HCl, guanidine hydrochloride; Hepes, N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid; IPTG, isopropyl β-D-thiogalactopyranoside; MOPS, 3-(N-morpholino)propanesulfonic acid; NMR, nuclear magnetic resonance; PAGE, polyacrylamide gel electrophoresis; PEP, phosphoenolpyruvate; SDS, sodium dodecyl sulfate; TNB, thionitrobenzoic acid; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; TSP, 3-(trimethylsilyl)propionic acid; UV, ultraviolet.

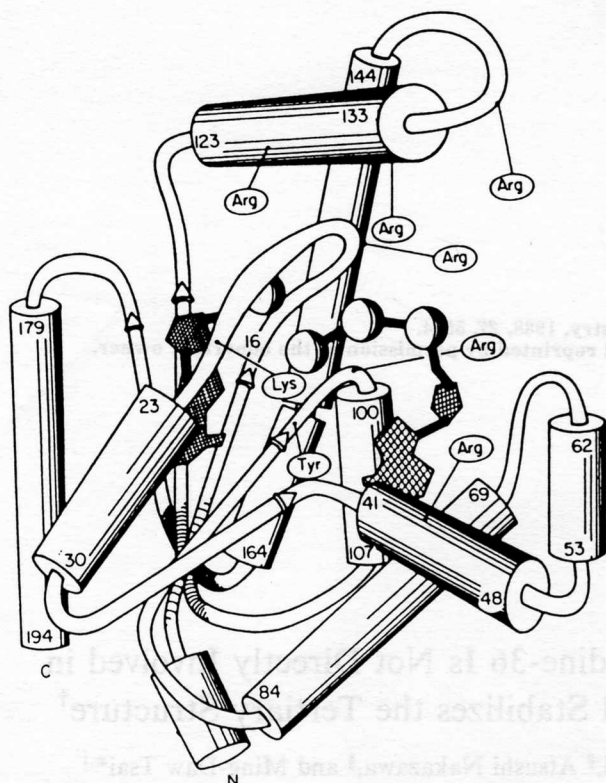


FIGURE 1: Sketch of the crystal structure of porcine muscle AK and the proposed binding sites for ATP and AMP. Helices are represented by cylinders and the five strands of the pleated sheet by arrows. Reproduced with permission from Pai et al., (1977). Copyright 1977 Academic Press, Inc. It should be noted that Schulz et al. (1986) suggested later that only the region for binding the phosphate moieties is firm and that Egner et al. (1987) reaffirmed the phosphate binding region but suggested that the adenosine site of ATP in this structure is more likely to be the AMP site, on the basis of the crystal structure of a yeast AK inhibitor complex.

of porcine muscle AK has been solved by X-ray crystallography at 3-Å resolution (Schulz et al., 1974; Sachsenheimer & Schulz, 1977). Extensive kinetic and NMR studies have demonstrated that the active site of AK consists of a "MgATP site" (which binds MgATP, MgADP, ATP, and ADP) and an "AMP site" (which binds AMP and ADP) [for a summary of references, see Shyy et al. (1987)]. However, the exact location of the two sites has been a controversial issue despite a decade of extensive biophysical studies summarized below.

Since the AKs from animal muscles are highly homologous (Kuby et al., 1984; Kishi et al., 1986), most structural studies have assumed that the information on one muscle enzyme is applicable to another. The histidine residues have received much attention since they are readily observable by ^1H NMR, and a histidine has been implicated in the reaction mechanism as early as 1970 (Schirmer et al., 1970). The AK from calf, porcine, rabbit, and chicken² muscles have one (His-36), two (His-36 and His-189), three (His-30, His-36, and His-189), and four (His-7, His-8, His-30, and His-36) histidine residues, respectively. The His-36 is thus conserved in all muscle AKs. It is also conserved in other types of AKs (Schulz et al., 1986),

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

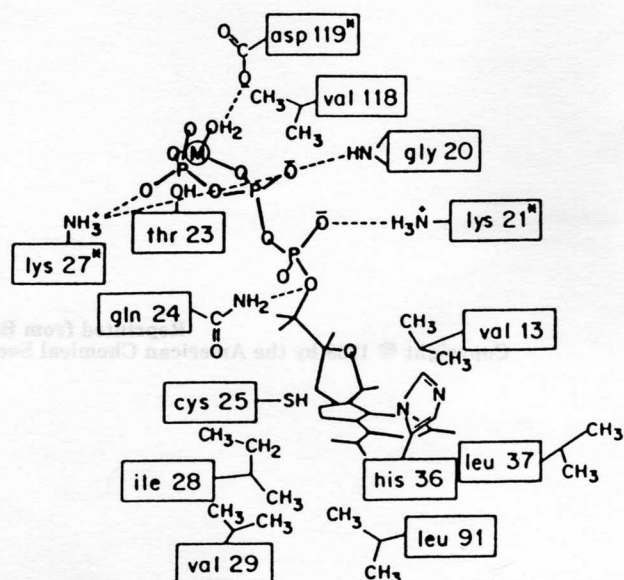


FIGURE 2: Amino acids of AK in proximity to, and which may interact with, bound metal ATP, proposed on the basis of ^1H NMR studies (Fry et al., 1985). The residues marked with an asterisk must move in order to interact as shown. Reproduced with permission from Mildvan and Fry (1987). Copyright 1987 John Wiley and Sons, Inc.

except that it is replaced by glutamine in the AK from *Escherichia coli* (Brune et al., 1985). The ^1H NMR signal of the C2-H of His-36 has been shown to be shifted downfield upon addition of AMP, GTP, ATP, MgATP, or AMP + MgATP (Cohn et al., 1972; McDonald et al., 1975) and to be broadened by MnATP (McDonald et al., 1975) and CrATP (Smith & Mildvan, 1982). Some of these results have been interpreted to "unequivocally and directly demonstrate the involvement of a histidine in the catalytic mechanism" (Noda, 1973) and have been used to conclude that "the active site is in the cleft of the molecule described in the crystal structure with the phosphate groups of ATP and AMP lying close to His-36" (McDonald et al., 1975). These interpretations were later supported by X-ray structural analysis of porcine AK inhibitor complexes that suggests that ATP binds with its triphosphate moiety close to His-36 and with the adenine moiety in a hydrophobic pocket located between helices 69-84 and 100-107 (Figure 1) (Pai et al., 1977).

The above model of the metal-ATP binding site and the proximity of His-36 to the phosphate groups of ATP and AMP were later refuted by detailed NMR studies of Mildvan and co-workers (Smith & Mildvan, 1982; Fry et al., 1985, 1987; Mildvan & Fry, 1987). Taking advantage of a tryptic fragment of rabbit muscle AK (residues 1-44) (Hamada et al., 1979), these authors were able to measure various distances between specific atoms of the substrate and the peptide fragment (or the enzyme) on the basis of paramagnetic relaxation (using CrATP) and the nuclear Overhauser effect (using MgATP). According to the model derived from such distance measurements (Figure 2), His-36 is part of a hydrophobic pocket surrounding the adenine ring (NOE requires 3 ± 1 Å between C2-H of His-36 and adenine H2 of bound MgATP) and is quite distant from the triphosphate moiety (paramagnetic relaxation effect requires 13 ± 1 Å between C2-H of His-36 and Cr^{3+} of bound CrATP).

The availability of rich data in the literature, the well-defined yet controversial problems, the known crystal structure, and the small size of the enzyme make AK an ideal system for quantitative structure-function studies by use of site-specific mutagenesis [for recent reviews, see Gerlt (1987), Knowles (1987), and Shaw (1987)]. This became possible

after the cDNA for chicken muscle AK had been cloned (Kishi et al., 1986) and expressed in *E. coli* (Tanizawa et al., 1987).² Due to the past interest in the functional role of His-36 described above, we chose this residue as the focus of our first study. Our results in this paper indicate that His-36 is *not* directly involved in catalysis, but stabilizes the tertiary structure of AK and partially protects a cysteine residue, most likely Cys-25. The pK_as of histidine residues in the wild-type AK and its mutants have also been determined by ¹H NMR.

MATERIALS AND METHODS

Materials. The oligonucleotides directing the mutations of His-36 to Gln and to Gly, d(TGGGTACACA-CAGCTCTCCACT) and d(TGGGTACACT-GGCCTCTCCACT), respectively, were purchased from SYN-TEK and used without further purification. The oligonucleotide directing the mutation of His-36 to Asn, d(GGGTACTACTAACCTCT), was synthesized by using 381A DNA synthesizer (Applied Biosystems) and purified by using 10% PAGE. Recombinant plasmid pKK-cAK1-1, containing the coding sequence for chicken muscle adenylate kinase, was available from previous work (Tanizawa et al., 1987). The reagents and enzymes for mutagenesis were purchased from Amersham. The reagents and enzymes (pyruvate kinase and lactic dehydrogenase) for kinetic studies were purchased from Sigma. Polyethylene glycol 20000 was purchased from Fluka Chemie AG. Phosphocellulose P-11 and Sephadex G-100 were obtained from Whatman and Sigma, respectively. Gdn-HCl was purchased from Sigma and used without further purification. DTNB and TSP-*d*₄ were obtained from Aldrich. Perdeuterated Tris was obtained from Merck and IPTG from Calbiochem. All other chemicals were of reagent grade.

Construction of Mutant Strains. The RF fragment of wild-type cAK gene was cut out of pKK-cAK1-1 (Tanizawa et al., 1987) with *EcoRI* and *HindIII* and purified by using 5% polyacrylamide gel electrophoresis. This fragment was then ligated into M13mp19 (which had been digested with *EcoRI* and *HindIII*) to give M13mp19-cAK1-1. The site-specific mutagenesis was then carried out according to the method described by Eckstein and co-workers (Taylor et al., 1985a,b). This method uses dCTPαS instead of dCTP during the priming and extension. After the mutant strand was closed with T4 DNA ligase, single-stranded nonmutant DNA molecules were removed by filtration through nitrocellulose filters. The nonmutant DNA strand in the heteroduplex DNA was then nicked with *NciI* and digested with exonuclease III. After the digestion was estimated to have proceeded over the mutation site, this strand was repolymerized to give homoduplex mutant DNA. The efficiency of this procedure was about 95%. Therefore, the selection of mutants was achieved by DNA sequencing of two or three mutant plaques without colony hybridization. The entire gene was sequenced to ensure that no undesirable mutations had occurred. This was carried out by ligation of the mutant gene fragments into both M13mp18 and M13mp19 and sequencing from both ends of the gene. The mutant gene fragments were religated back into pKK 223-3 plasmid and transformed into *E. coli* JM103 as described for the expression of the WT chicken AK (Tanizawa et al., 1987). Finally, the expression of the mutant enzymes was checked by SDS-PAGE analysis of the cell-free extract of a small-scale culture of *E. coli* JM103 harboring each mutant recombinant plasmid. The level of expression of the mutants was similar to that of the WT.

Purification of WT and Mutant Adenylate Kinase. The WT and mutant AK proteins were purified according to the

procedure described previously (Tanizawa et al., 1987) with minor modifications. Two hundred milliliters of a culture of *E. coli* JM103 harboring a mutant recombinant plasmid was used to inoculate 12 × 1 L of Luria broth containing 200 mg/L ampicillin. After shaking vigorously at 37 °C for 1 h, 1 mM IPTG was added, and shaking was continued for 8 h (the absorbance at 600 nm reached 2–3). The cells were harvested by centrifugation at 8000g for 10 min, resuspended in a Tris buffer (20 mM Tris-HCl and 0.14 M NaCl, pH 7.5), and centrifuged again at 8000g for 10 min. The cells were then sonicated in about 200 mL of Tris buffer (30 mM Tris-HCl, 1 mM DTT, and 1 mM EDTA, pH 7.5). The sonication was conducted at about 100 W (Sonic Dismembrator, Model 300) for 30 s × 6 times. The temperature was controlled by an ice bath and kept below 12 °C during sonication. The supernatant containing the enzyme was collected by centrifugation at 25000g for 30 min and either stored at –70 °C (after dropping into liquid nitrogen) or loaded directly onto a phosphocellulose P-11 column (70 g of dry resin, 5.5 × 30 cm). The column had been preequilibrated with buffer A (30 mM Tris-HCl, 50 mM NaCl, 1 mM DTT, and 1 mM EDTA, pH 7.5). After the sample had been loaded, the column was eluted with buffer A until A₂₈₀ of the eluate was the same as that of the eluent (ca. 500 mL of buffer A, 2 h). The column was then eluted with a gradient of 300 mL of buffer A and 300 mL of buffer B (same as buffer A except 0.5 M NaCl) and further chased with 200 mL of buffer B. The fractions containing the AK activity were collected and concentrated by dialyzing in polyethylene glycol 20000. Chromatography of this concentrated enzyme on a Sephadex G-100 column (2.8 × 100 cm), preequilibrated with an imidazole buffer (5 mM imidazole hydrochloride, 1 mM DTT, and 1 mM EDTA, pH 6.9) and eluted with the same buffer, resulted in two well-resolved peaks. The first peak contained a few bands with different molecular weight, and the second peak contained the purified enzyme, as shown by SDS-PAGE analysis. The purified enzyme (ca. 400 mg for WT AK) was concentrated with polyethylene glycol 20000, dialyzed against 5 mM ammonium acetate (containing 1 mM β-mercaptoethanol or DTT and 1 mM EDTA, pH 6.5), lyophilized, and stored under argon at –20 °C. The protein concentration was estimated by using the extinction coefficient ε_{280nm} = 0.51 (mg/mL)^{–1} cm^{–1} determined by the method of Lowry (Lowry et al., 1951).

Steady-State Kinetics. The steady-state kinetic constants were obtained by use of the pyruvate kinase-lactic dehydrogenase coupling system (Rhoads & Lowenstein, 1968). The assays were carried out in 1 mL of 75 mM Tris-HCl, pH 8.0, containing 65 mM KCl, 1 mM DTT, 50 μg each of the coupling enzymes, 1 mM PEP, 0.2 mM NADH, 5 mM MgCl₂, 0.02 μg of WT or mutant AK, and suitable amounts of ATP and AMP. The reaction mixture had been preincubated at 30 °C for 10 min in a cuvette prior to addition of AK. The temperature was controlled by a circulating cooling water bath. The K_m and V_{max} were obtained from Lineweaver-Burk plots.

DTNB Reaction. The double-distilled water used in these experiments was thoroughly degassed by boiling for 1 h. The WT AK (ca. 5 mg/mL) was first dialyzed 4 times against a Tris buffer (7 mM Tris-HCl, 10 mM KCl, and 1 mM EDTA, pH 8.0). The buffer had been degassed by bubbling argon through for 2 h at 4 °C, and the double-distilled water used to prepare the buffer had been boiled for 1 h. The concentration of the enzyme was then estimated spectrophotometrically. Excess DTNB (ca. 200×) was then added to an aliquot of each enzyme, and the released TNB concentration was

determined at 412 nm by using an extinction coefficient of $13600 \text{ M}^{-1} \text{ cm}^{-1}$ (Ellman, 1958). The ratio of the sulfhydryl groups per enzyme molecule was found to be 1.9, and more extensive dialysis of the enzymes against the argon-degassed buffer prior to the reaction did not change this ratio. SDS-PAGE analysis showed that no dimerization occurred. Addition of DTT to the dialyzed DTNB-modified AK resulted in the release of 1.8 equiv of TNB based on A_{412} .

To compare the reactivity of WT and mutants, the reaction was carried out at 4°C , smaller amounts of DTNB were used to slow down the reaction, and the concentrations of DTNB and the enzyme were 0.03 and 0.01 mM, respectively. The time course of the reaction was monitored spectrophotometrically at 412 nm.

Gdn-HCl-Induced Denaturation. The denaturation of the proteins by Gdn-HCl was followed by UV difference spectroscopy (Herskovits, 1967; Donovan, 1973). A stock solution of Gdn-HCl was first prepared in double-distilled water, and its concentration was determined by measuring the refractive index (Nozaki, 1972). Solid MOPS and DTT were added to final concentrations of 75 and 1 mM, respectively, and the pH was adjusted to 7.0 with 6 N NaOH. The volume of the NaOH solution delivered was recorded and used to correct the final concentration of the stock Gdn-HCl solution. A concentrated enzyme stock solution (ca. 30 mg/mL) was prepared by dialyzing the enzyme against 75 mM MOPS buffer containing 1 mM DTT, pH 7.0, followed by centrifugation at $15000g$ for 15 min to remove precipitate. The difference spectra were obtained on a Kontron 820 UV-visible spectrophotometer at 25°C using tandem cells. A base line was obtained before a difference spectrum was taken. The typical difference spectrum has two negative peaks at 280 and 288 nm. The peak at 288 nm was used to follow the extent of unfolding of the enzymes.

pH Titration Using 500 MHz ^1H NMR. A lyophilized AK-salt mixture (20–70 mg) was dissolved into 1.25 mM Hepes buffer containing 75 mM KCl, 0.5 mM DTT, 0.05 mM EDTA, pH 8.0 and dialyzed against the same buffer. After dialysis, n mL of the resulting solution was lyophilized followed by redissolution in $n/2$ mL of 45 mM perdeuterated Tris, pH 8.0 in 99.996% D_2O . To this solution were added microliter amounts of 0.2 M DTT to bring the DTT concentration to 2 mM, and a minute amount of $[2,2,3,3\text{-}^2\text{H}_4]\text{TSP}$ (sodium salt) was added as an internal reference. The sample was centrifuged and transferred to a 5-mm NMR tube, and a spectrum was taken to ensure proper sample purity. Following this, the sample volume was doubled by adding an equal volume of a solution containing 150 mM KCl, 1 mM DTT, and 1 mM EDTA in D_2O , and pH titration was commenced.

Thus, at the start of titration AK was 0.5–2.0 mM in 1–1.5 mL of a deuterated pH 8.0 buffer containing 22.5 mM perdeuterated Tris, 1.25 mM Hepes, 150 mM KCl, 0.5 mM EDTA, and 1.0 mM DTT. pH was measured and recorded with no isotopic correction by using a Fisher Accumet Model 825 MP pH meter equipped with a calomel reference combination electrode. Between each titration point, the electrode was soaked in 6 M Gdn-HCl, recalibrated, and soaked in D_2O . The sample was removed from the NMR tube by using a syringe equipped with a flexible Teflon "needle" and placed in a 1.5-mL plastic vial in which pH was checked and then adjusted by adding microliter amounts of 0.3–1.25 M K_2CO_3 or DCOOD in D_2O .

^1H NMR spectra were recorded at ambient temperature ($22 \pm 1^\circ \text{C}$) by using a Bruker AM 500 NMR spectrometer utilizing phase-cycled 90° pulses and quadrature detection.

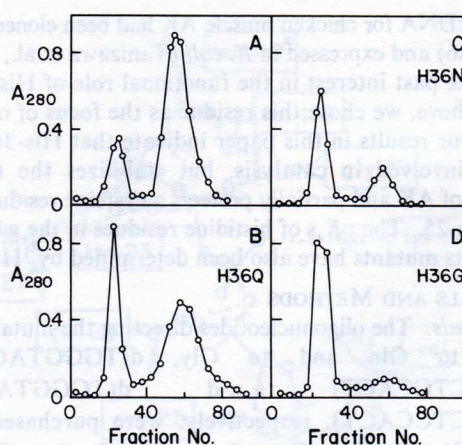


FIGURE 3: Chromatographic profiles of the Sephadex G-100 column in the purification of WT AK (A), H36Q (B), H36N (C), and H36G (D). The peaks to the right represent purified enzymes.

The sweep width was usually 6500 Hz, and 16K or 32K data points were taken. Solvent suppression was sometimes utilized and accomplished by presaturation. Spectra were typically processed by using 0.5–1 Hz of line broadening and exponential multiplication. Chemical shifts were recorded relative to internal TSP.

Computer Modeling of AK. The X-ray structure of porcine AK (Schulz et al., 1974; Sachsenheimer & Schulz, 1977) was obtained from the Brookhaven Protein Database (file number 2ADK). Modeling was performed by using an Evans and Sutherland PS300 terminal and Chemical Design Ltd. CHEMX software.

RESULTS

Preparation and Stability of AK and His-36 Mutants. We have prepared the following mutants of chicken muscle AK: His-36 \rightarrow Gln (H36Q), His-36 \rightarrow Asn (H36N), and His-36 \rightarrow Gly (H36G). In the H36Q and H36N mutants the side chain amide nitrogen may occupy the same position as the N3 and N1, respectively, of His-36 (Lowe et al., 1985). Thus, these mutants will allow us to test whether the aromaticity of the imidazole ring or either of the nitrogen atoms of His-36 are involved in the catalytic function (including substrate binding).

The detailed procedures for site-specific mutagenesis and protein purification are described under Materials and Methods. One noteworthy point is that, although the level of AK expression was very similar for the WT and the mutants (on the basis of the SDS-PAGE analysis of cell-free extracts), the mutants were less stable during purification. Figure 3 shows the chromatographic profiles of the Sephadex G-100 column in the final step of purification. The peak to the right represents purified AK, which decreases in its intensity in the order WT > H36Q > H36N > H36G. The peak to the left, which increases correspondingly, consisted of a few bands of higher molecular weight proteins as shown by SDS-PAGE analysis, which were not further characterized.

Furthermore, we found that the purified H36G was very unstable and readily dimerized. Figure 4 shows the SDS-PAGE analysis of monomeric H36G in the cell-free extract (lane 2) and the pure dimeric H36G (lane 3). Since the dimer is not dissociated under denaturing condition, it should be a covalent dimer and is likely to involve Cys-25. However, we have not further investigated the latter point since the roles of Cys-25 will be probed by Cys-25 mutants in the future.

Kinetic Properties. As shown in Table I, the k_{cat} of H36Q and H36N was almost unchanged relative to that of the WT.

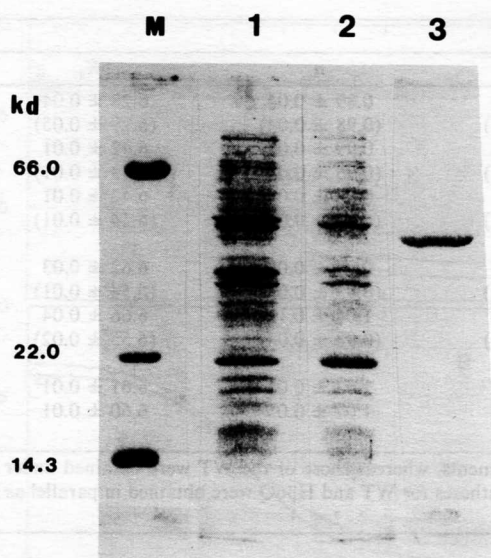


FIGURE 4: SDS-PAGE analysis of H36G (in 2% SDS). Lane M consists of molecular weight markers. Lanes 1 and 2 are the cell-free extracts under uninduced and induced (with IPTG) conditions, respectively. The heavy band near 22.0 kDa represents monomeric H36G. Lane 3 represents dimerized H36G.

If the rate of the chemical step is similar to that of the off-step as reported by Brown and Ogawa (1977), our results indicate that neither step is affected by the substitution of His-36. If the chemical step is faster than the off-step by an order of magnitude (Nageswara Rao et al., 1978), our results suggest that the His-36 mutants did not affect the rate of the chemical step by more than an order of magnitude. The K_m values increase in all cases. However, the increases ($\leq 10\times$) are not large enough to suggest a direct involvement of His-36 in the binding of MgATP or AMP. Even the dimeric H36G showed only an $8\times$ decrease in k_{cat} and a $2\times$ increase in K_m s. The monomeric H36G was too unstable for complete kinetic analysis, but its kinetic properties were very similar to those of WT AK. The most reasonable interpretation of these results is that His-36 is *not* directly involved in the catalysis of AK.

Protection of Cys-25 by His-36. The above results on the stability and kinetics of His-36 mutants suggested that His-36 could play a structural rather than catalytic role in the function of AK. Examination of the crystal structure of porcine muscle AK revealed that His-36 is in close proximity to Cys-25, as shown in Figure 5. This suggested that His-36 could "protect" Cys-25 from being involved in the formation of dimer or other

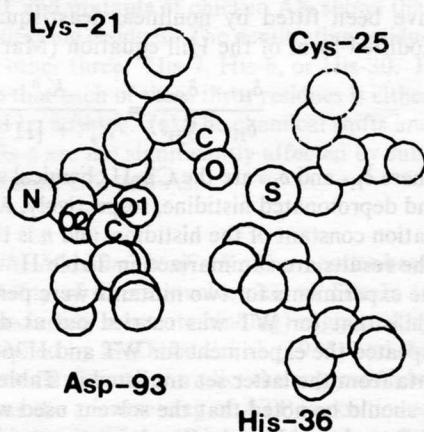


FIGURE 5: Spatial relationship between His-36, Cys-25, Lys-21, and Asp-93 revealed by the 3.0-Å crystal structure of porcine muscle AK. It should be noted that the C and N atoms of the imidazole ring are not distinguishable. Distances between γ -S of Cys-25 and the atoms of the imidazole ring are 3.7, 3.5, 3.3, 3.1, and 3.2 Å. Other distances are 2.7 Å, γ -S of Cys-25 to carbonyl O of Lys-21; 4.9 Å, to O1 of Asp-93; >5 Å, to O2 of Asp-93; 2.2 Å, ϵ -N of Lys-21 to O2 of Asp-93; and 4.2 Å, to O1 of Asp-93.

Table I: Summary of the Kinetic and Unfolding Properties of AK and Mutants

property	WT	H36Q	H36N	H36G ^a	porcine AK
kinetics					
k_{cat} (s^{-1})	650	680	595	81	
K_m (MgATP) (μ M)	35	210	78	91	
K_m (AMP) (μ M)	92	980	410	170	
unfolding					
$\Delta G_D^{H_2O}$ (kcal/mol)	3.8	3.1	2.5	very	3.9
m (kcal/mol M)	4.7	4.4	4.5	unstable	4.8

^a As explained in the text, the kinetic data listed for H36G are for its dimer. The monomeric H36G was too unstable for complete kinetic and unfolding studies.

undesirable events. This was further supported by the experiments described below that compared the reactivity of the sulfhydryl groups of AK and its mutants toward DTNB (Ellman's reagent).

We first demonstrated that DTNB did not induce appreciable intermolecular or intramolecular disulfide formation as follows. The WT AK, after reacting with DTNB, was dialyzed extensively. The SDS-PAGE analysis showed only one band corresponding to monomeric AK. The result was consistent with that of Kress and Noda (1967), who showed that rapid addition of an excess of DTNB to the porcine muscle AK resulted in the formation of a derivative that was a monomer. Treatment of this dialyzed derivative with DTT resulted in the release of 1.8 equiv of thionitrobenzoic acid (TNB)/mol of enzyme. Similar results were obtained for H36Q and H36N.

The reaction of DTNB with WT AK, H36Q, and H36N was then followed by monitoring the formation of TNB spectrophotometrically. The time course of the reaction (Figure 6) showed that the rate increased in the order WT $<$ H36Q $<$ H36N. Thus, our results suggest that His-36 partially protects at least one of the two cysteine groups. Since the crystal structure indicates that Cys-25 is in close proximity to His-36 while Cys-187 is 13 Å away, we conclude that only Cys-25 is likely to be protected by or to interact with His-36.

Stabilization of the Tertiary Structure by His-36. Having shown that His-36 can partially protect Cys-25, we then asked why such an interaction is necessary. Like His-36, Cys-25 is not involved in catalysis. The conclusion is based on previous chemical modification studies (Noda, 1973) and is consistent with the observation that the kinetic property of the H36G dimer is not very different from that of the WT monomer. Why is it necessary to have such a conserved (within muscle

Table II: Chemical Shifts and pK_a of Histidine Residues^a

enzyme	residue	δ_{H^+} (ppm)	δ_{H^0} (ppm)	n	pK_a
WT	His-36	8.81 ± 0.02	7.76 ± 0.01	0.89 ± 0.04	6.23 ± 0.04
		(8.78 ± 0.02)	(7.77 ± 0.01)	(0.98 ± 0.04)	(6.29 ± 0.05)
	His-b	8.71 ± 0.01	7.72 ± 0.01	0.99 ± 0.03	6.42 ± 0.01
	(8.70 ± 0.01)	(7.71 ± 0.01)	(0.95 ± 0.03)	(6.53 ± 0.01)	
His-c	8.66 ± 0.01	7.64 ± 0.01	0.98 ± 0.02	6.42 ± 0.01	
	(8.65 ± 0.01)	(7.62 ± 0.01)	(0.98 ± 0.03)	(6.54 ± 0.01)	
His-d			7.89		
			7.70 ± 0.03	0.98 ± 0.09	6.62 ± 0.03
H36Q	His-b	8.69 ± 0.02	7.70 ± 0.01	0.93 ± 0.04	6.54 ± 0.01
	(8.71 ± 0.01)	(7.70 ± 0.01)	(0.93 ± 0.04)	(6.54 ± 0.01)	
His-c	8.63 ± 0.03	7.61 ± 0.04	1.04 ± 0.13	6.66 ± 0.04	
	(8.65 ± 0.01)	(7.62 ± 0.01)	(0.98 ± 0.04)	(6.55 ± 0.02)	
His-d			7.86		
			7.70 ± 0.01	1.03 ± 0.03	6.61 ± 0.01
H36N	His-b	8.69 ± 0.01	7.70 ± 0.01	1.07 ± 0.05	6.60 ± 0.01
	His-c	8.64 ± 0.01	7.62 ± 0.01		
His-d			7.86		
			7.62 ± 0.01		

^aThe nonparenthesized data of H36Q and H36N were obtained in parallel experiments, whereas those of the WT were obtained under slightly different conditions. These represent the results shown in Figure 8. The data in parentheses for WT and H36Q were obtained in parallel as another set of experiments.

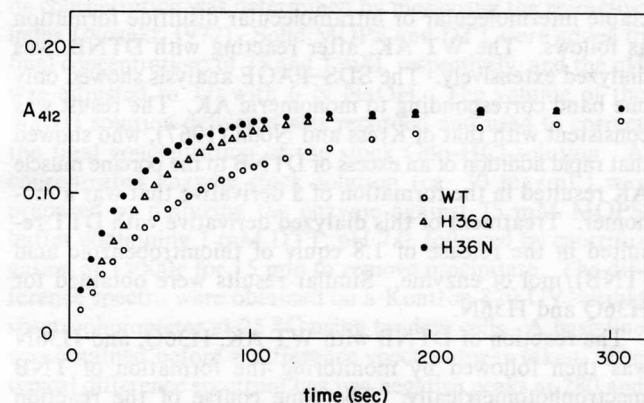


FIGURE 6: Time courses of the reaction of DTNB with WT (O), H36Q (Δ), and H36N (\bullet) based on the release of TNB monitored by the absorbance at 412 nm.

AKs) yet functionally nonessential His...Cys pair?

Although the exact nature of the His-36...Cys-25 interaction is not yet certain (see Discussion), it may stabilize the tertiary structure of AK by holding the helix 23-30 and the strand 32-40 in place as shown in Figure 1. Examination of the detailed structure on a computer revealed that there are no other side chains in this region which may serve a similar function. If this is the case, disruption of the interaction will not only expose Cys-25 but also make the enzyme conformationally unstable. The latter prediction is supported by the following experiments.

Figure 7 shows the denaturation curve of WT, H36Q, and H36N induced by guanidine hydrochloride (Gdn-HCl). The Gibbs free energy change of unfolding was determined from the denaturation curve by use of the equation (Pace, 1986)

$$\Delta G_D = \Delta G_D^{H_2O} - m[\text{Gdn-HCl}]$$

where ΔG_D is the Gibbs free energy change at various concentrations of Gdn-HCl, $\Delta G_D^{H_2O}$ is the Gibbs free energy change at zero concentration of Gdn-HCl, and m is a constant. Plots of ΔG_D as a function of [Gdn-HCl] gave $\Delta G_D^{H_2O}$ and m values as shown in Table I. The results show that the conformational stability (with regard to Gdn-HCl-induced unfolding) decreases in the order WT > H36Q > H36N. It is interesting to note that porcine muscle AK behaves similarly to chicken muscle AK in Gdn-HCl-induced denaturation, even though there are 29 different amino acids (85% homology) between the two natural mutants. The relative invariance of the slope m suggests that the nature of interaction between the enzyme and Gdn-HCl has not been altered in the mutants.

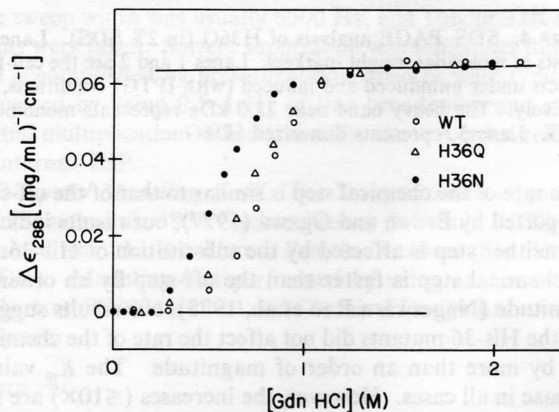


FIGURE 7: Gdn-HCl-induced denaturation curves of WT (O), H36Q (Δ), and H36N (\bullet) monitored by UV difference absorbance at 288 nm.

pH Titration of the ¹H NMR of Histidine Residues. Since the focus of this paper is His-36, we also report the pH dependence of the C2-H of the histidine residues of WT AK and its mutants. This is the first step toward our long-term effort to assign more ¹H NMR resonances of AK and to address the problem of conformational changes in site-specific mutants. The histidine residues in the AK from carp muscle (His-36) and porcine muscle (His-36 and His-189) have been assigned previously by McDonald et al. (1975) and those in the AK from rabbit muscle (His-30, His-36, and His-189) by Fry et al. (1985). Our results are shown in Figure 8, where the data have been fitted by nonlinear least-squares analysis to a modified form of the Hill equation (Markley, 1974)

$$\frac{\delta_{H^+} - \delta_{\text{obsd}}}{\delta_{H^+} - \delta_{H^0}} = \frac{K_a^n}{K_a^n + [H^+]^n}$$

where δ_{H^+} and δ_{H^0} are the C2-H chemical shifts of protonated and deprotonated histidine, respectively, K_a is the acid dissociation constant of the histidine, and n is the Hill coefficient. The results are summarized in Table II. Since in Figure 8 the experiments for two mutants were performed in parallel while that for WT was carried out at different times, we repeated the experiment for WT and H36Q in parallel. The data from the latter set are listed in Table II in parentheses. It should be noted that the solvent used was D₂O instead of H₂O, and the pH and pK_a values were those measured directly in D₂O, without corrections.

The results in Figure 8 and Table II reveal the following noteworthy points. (a) In Figure 8A the His-a was assigned

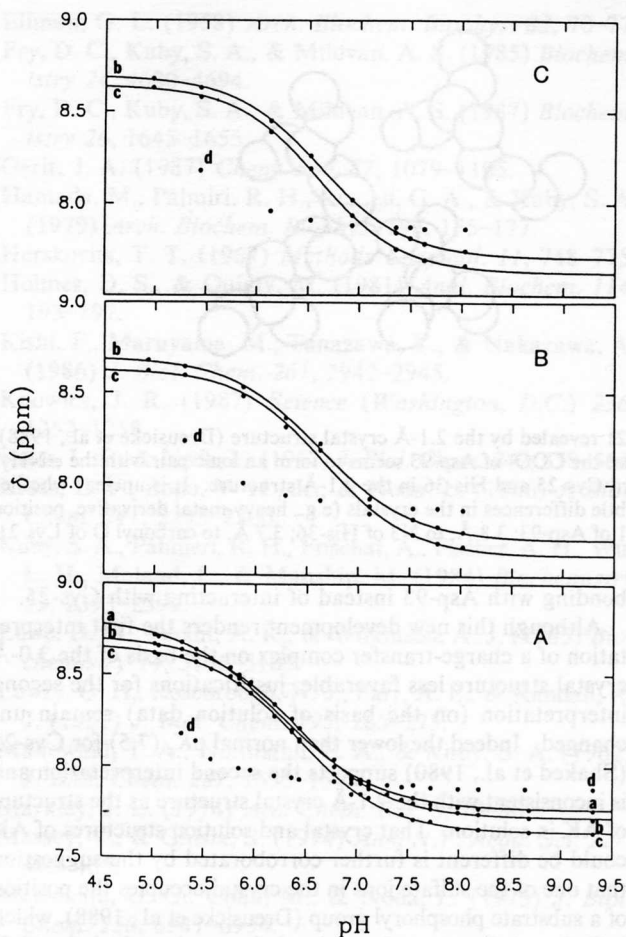


FIGURE 8: Titration curves of the C2-H chemical shifts of the histidine residues in WT (A), H36Q (B), and H36N (C). The detailed conditions are described under Materials and Methods, and the data are summarized in Table II. The pH was a direct reading of the D_2O solution without correction.

to His-36 by assuming that histidines b-d have similar chemical shifts in the WT and the mutants. The pK_a of His-36 is lower than that of His-b and His-c by 0.2 pH unit, which is consistent with the observation of Fry et al. (1985) that the pK_a of His-36 is lower than that of His-30 by 0.2 pH unit in rabbit muscle AK. (b) As in the case of AK from porcine muscle (McDonald et al., 1975) and rabbit muscle (Fry et al., 1985), there is one nontitrating (or abnormally low pK_a) histidine. However, in the two previous cases the nontitrating histidine is His-189, which is absent in chicken AK. Comparison between WT and mutants of chicken AK shows that His-36 is one of the titrating residues. The nontitrating residue could be one of the other three: His-7, His-8, or His-30. It is interesting to note that each of these three residues is either preceded or followed by a lysine. (c) The chemical shifts and pK_a of His-b and His-c are not significantly affected by substitutions of His-36 with Gln and Asn.

DISCUSSION

Implications on the Mechanism of AK. Our results have ruled out the possibility of direct involvement of His-36 in catalytic function (including substrate binding) suggested by Noda (1973) and implied by McDonald et al. (1975) and Pai et al. (1977). The results do not contradict the suggestion by Fry et al. (1985) that the adenine ring of ATP is positioned within a hydrophobic pocket formed by the side chains of Ile-28, Val-29, Leu-91, Leu-37, and His-36, but it suggests that His-36 is not critical for this hydrophobic pocket. Work is in progress in this laboratory to identify other residues that

are crucial for catalysis and to critically test the X-ray model of Pai et al. (1977) and the NMR model of Fry et al. (1985).

Nature of the His-36...Cys-25 Interaction. Although such an interaction has been suggested on the basis of the protection of a sulfhydryl residue by His-36 and the close proximity of His-36 and Cys-25, the nature of this interaction remains to be established. As shown in Figure 5, the crystal structure showed that the sulfur atom of Cys-25 lies above the imidazole ring of His-36, with similar distances to the five atoms of the ring. Such a structure suggests a possible "charge-transfer complex" between the free SH group and the imidazole ring. Although such a thiol-imidazole ring charge-transfer complex has not been reported previously in protein structures, the formation of a charge-transfer complex between thiol and enzyme-bound FAD has been well demonstrated (Massey & Ghisla, 1974; Arscott et al., 1981; Shames et al., 1986). In protein structures, it has been noted that disulfide linkages and aromatic rings often lie near each other (Morgan et al., 1978; Morgan & McAdon, 1980), and a potential role of disulfide-aromatic ring complexes in protein folding and protein conformation has been suggested (Swadesh et al., 1987).

An alternative interpretation of the His-36...Cys-25 interaction is H-bonding. Although this is not favored by the crystal structure, the local conformation of the side chains in solution could be different from that in crystals. Furthermore, the crystal structure was determined for porcine muscle AK and contained sulfate ions, while the experiments in this work used chicken muscle AK in the absence of sulfate ions. The observations that the stability of AK decreases in the order WT > H36Q > H36N and the reactivity of the thiol group increases in the order WT < H36Q < H36N < H36G support H-bonding and argue against a charge-transfer complex. The charge-transfer model would predict drastic differences between WT and the mutants and similar behavior between the mutants. The side-chain nitrogen atom of glutamine and asparagine have been suggested to mimic the N3 and N1, respectively, of histidine (Lowe et al., 1985), and the orders of stability and reactivity suggest that the N3 of His-36 could be involved in interacting with Cys-25 via a H-bonding. The assignment of N3 is consistent with the fact that in the only AK (from *E. coli*) where His-36 is not conserved, it is replaced by a Gln (Brune et al., 1985). The H-bonding, if it exists, is more likely to be N-H...S-H than N...H-S since the latter would predict a decreased nucleophilicity when the H-bonding is disrupted. The former is also supported by the abnormally low pK_a (7.5) for Cys-25 (Shaked et al., 1980). Although previous ^{13}C NMR studies suggested that the predominant protonation site of the histidine ring at high pH is the N1 position (Reynolds et al., 1973), a different behavior for the His-36 of AK is not unlikely.

It should be noted that the sulfur atom of Cys-25 is also close to the carbonyl oxygen of Lys-21, as shown in Figure 5. This suggests a possible H-bonding, S-H...O=C, between Cys-25 and Lys-21. Thus, there could be a H-bonding pair involving His-36, Cys-25, and Lys-21: N-H...S-H...O=C. Future studies will be needed to determine whether the Cys-25...Lys-21 hydrogen bonding does exist and to elucidate the nature of the His-36...Cys-25 interaction. The above discussions should not be considered as definitive answers.

Potential Roles of His-36 in the Structure of AK. Our results showed that disruption of the His-36...Cys-25 interaction made AK more prone to Gdn-HCl-induced denaturation. This supports the prediction on the basis of the crystal structure that His-36 is likely to be involved in holding the strand 32-40 in place.

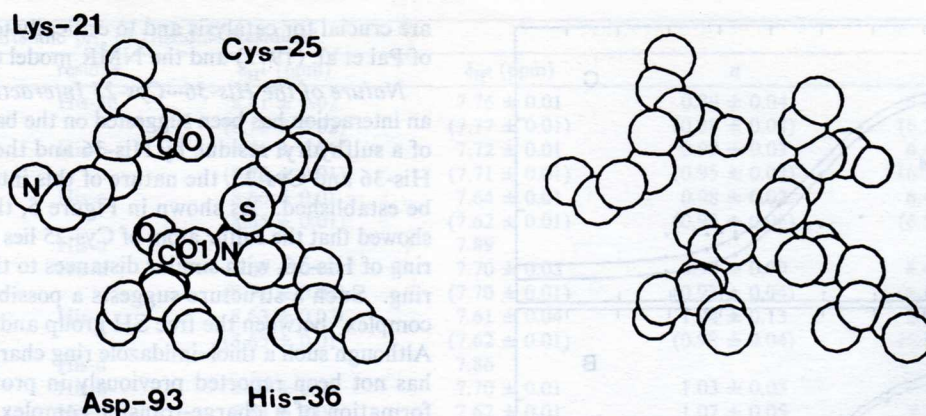


FIGURE 9: Spatial relationship between His-36, Cys-25, Asp-93, and Lys-21 revealed by the 2.1-Å crystal structure (Dreusicke et al., 1988). Compared to the 3.0-Å structure shown in Figure 5, the largest change is that the COO⁻ of Asp-93 seems to form an ionic pair with the ε-NH₃⁺ group of Lys-21 in the 3.0-Å structure but moves by more than 2 Å toward Cys-25 and His-36 in the 2.1-Å structure. It is unclear whether the differences between Figure 5 and 9 could be partially contributed by subtle differences in the crystals (e.g., heavy metal derivative, position of sulfate ions, etc.). The pertinent distances are 2.7 Å, γ-S of Cys-25 to O1 of Asp-93; 3.8 Å, to N3 of His-36; 3.7 Å, to carbonyl O of Lys-21; 3.0 Å, N3 of His-36 to O1 of Asp-93; and 3.6 Å, to O2 of Asp-93.

The His-36...Cys-25 interaction may also be involved in determining the crystal form of AK. Sachsenheimer and Schulz (1977) reported two different conformational forms (A and B) of AK. One of the differences between the two forms lies in the position of the helix 23-30. Since form A and form B were obtained at pH 6.9 and 5.8, respectively, it is likely that protonation-deprotonation of His-36 may change the nature of the interaction or the exact location of the His-36...Cys-25 pair and result in the two different crystal conformations.

Potential Roles of Cysteine Residues. Just as histidine residues have received substantial attention in NMR studies due to their distinct chemical shifts, the cysteine residues of AK have been a target of extensive chemical modification studies due to the chemical reactivity of sulfhydryl groups (Mahowald et al., 1962; Kress et al., 1966; Kress & Noda, 1967; Price et al., 1975; Crivellone et al., 1985). These studies have established that modification of Cys-25 can be protected by ATP or MgATP, that Cys-25 is more reactive than Cys-187 in the absence of substrates, and that substitution of SH by small SSR groups (e.g., R = CH₃) has relatively little effect on the enzymatic activity of AK while larger modifying groups have a larger effect due to a steric factor. These results, the NMR model (Fry et al., 1985), and our results concerning His-36 taken together support the view that the His-36...Cys-25 pair is located near the MgATP site but is not involved in catalysis. Future site-specific mutagenesis of Cys-25 will allow us to quantitatively address the role of Cys-25 in dimerization and in the tertiary structure of AK and to determine the relationship between Cys-25 and its surrounding residues, Lys-21 and His-36.

Additional Information from Refined Crystal Structure. After this paper had been submitted and reviewed, a refined structure of porcine AK at 2.1-Å resolution appeared (Dreusicke et al., 1988). On the basis of this improved structure, the authors suggested that both the SH of Cys-25 and the N3 of His-36 are hydrogen-bonded to (the same) one of the oxygen atoms of the side-chain COO⁻ of Asp-93. Our examination of the structure (Figure 9) (file number 3ADK in the Brookhaven Protein Database) showed that the SH group of Cys-25 is also shielded by the imidazole ring of His-36, but the sulfur atoms does not lie exactly on the top of the ring as shown in the structure in Figure 5. Thus, a third interpretation of our results can be proposed on the basis of the refined structure: His-36 partially protects Cys-25 simply by steric effect, and His-36 stabilizes the tertiary structure by H-

bonding with Asp-93 instead of interacting with Cys-25.

Although this new development renders the first interpretation of a charge-transfer complex on the basis of the 3.0-Å crystal structure less favorable, justifications for the second interpretation (on the basis of solution data) remain unchanged. Indeed the lower than normal pK_a (7.5) for Cys-25 (Shaked et al., 1980) supports the second interpretation and is inconsistent with the 2.1-Å crystal structure as the structure of AK in solution. That crystal and solution structures of AK could be different is further corroborated by the suggestion that one of the sulfate ions in the crystal occupies the position of a substrate phosphoryl group (Dreusicke et al., 1988), which is likely to induce a conformational change since the induced-fit model seems to operate in the catalysis of AK (Pai et al., 1977). It may also be speculated that the fine structure in the His-36, Cys-25, and Asp-93 region is involved in the conformational change upon substrate binding and that the increased K_m values in the mutants reflect compromises in the induced-fit mechanism.

ACKNOWLEDGMENTS

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Registry No. AK, 9013-02-9; AMP, 61-19-8; His, 71-00-1; Cys, 52-90-4; MgATP, 1476-84-2; glutamine, 56-85-9; asparagine, 70-47-3; glycine, 56-40-6.

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