Essential Lysine Residues in the N-Terminal and the C-Terminal Domain of Human Adenylate Kinase Interact with Adenine Nucleotides As Found by Site-Directed Random Mutagenesis[†,¶,#]

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ABSTRACT: To elucidate the minimum requirement of amino acid residues for the active center in human adenylate kinase (hAK1), we carried out random site-directed mutagenesis of key lysine residues (K9, K21, K27, K31, K63, K131, and K194), which were conserved in mammalian AK1 species, with the pMEX8-hAK1 plasmid [Ayabe, T., et al. (1996) *Biochem. Mol. Biol. Int. 38*, 373−381]. Twenty different mutants were obtained and analyzed by steady-state kinetics, and all mutants showed activity loss by K_m and/or k_{cat} effects on $MgATP^{2-}$, AMP^{2-}, or both. The results have led to the following conclusions. (1) Lys9 would appear to interact with both $MgATP^{2-}$ and AMP^{2-} but to a larger extent than with AMP^{2-}. (2) Lys21 is likely to play a role in substrate binding of both $MgATP^{2-}$ and AMP^{2-} but more strongly affects $MgATP^{2-}$. (3) Lys27 and Lys131 would appear to play a functional role in catalysis by interacting strongly with $MgATP^{2-}$. (4) Lys31 would appear to interact with $MgATP^{2-}$ and AMP^{2-} at the $MgATP^{2-}$ site. (5) Lys63 would be more likely to interact with $MgATP^{2-}$ than with AMP^{2-}. (6) Lys194 in the flanking C-terminal domain would appear to interact not only with $MgATP^{2-}$ but also with AMP^{2-} at the $MgATP^{2-}$ site by stabilizing substrate binding. The loss of the positively charged ϵ-amino group of lysine affects both the affinity for the substrate and the catalytic efficiency. Hence, hydrophilic lysine residues in hAK1 would appear to be essential for substrate−enzyme interaction with the coordination of some arginine residues, reported previously [Kim, H. J., et al. (1990) *Biochemistry 29*, 1107−1111].

Adenylate kinase (AK)[1] (EC 2.7.4.3) is a ubiquitous enzyme, abundant in the cytoplasm, which catalyzes the reaction $MgATP^{2-} + AMP^{2-} \rightleftarrows MgADP^{-} + ADP^{3-}$. There are two distinct nucleotide-binding sites, one for metal-chelated $MgATP^{2-}$ or $MgADP^{-}$ and the other for unchelated AMP^{2-} or ADP^{3-} (Noda, 1973; Hamada et al., 1979). AK contributes to homeostasis of the adenine nucleotide composition in the cell (Atkinston, 1977). In vertebrates, three isozymes (AK1, AK2, and AK3) have been characterized (Noda, 1973; Khoo & Russel, 1972; Tomasselli et al., 1979). AK1 is present in the cytosol; AK2 is found in the intermembrane space of the mitochondria; and AK3 exists in the mitochondrial matrix. In human genetic disorders, an aberrant AK isozyme has been specifically found in the serum of Duchenne dystrophic patients (Hamada et al., 1985). An AK1 deficiency associated with a hemolytic anemia has been reported (Miwa et al., 1983; Matsuura et al., 1989; Toren et al., 1994), and the replacement of Arg128 with Trp in AK1 was considered to be the cause of the enzyme deficiency. The same mutant prepared by site-directed mutagenesis resulted in reduced catalytic activity and decreased solubility (Matsuura et al., 1989). On the basis of these findings, it seems reasonable to study the properties of various mutant AKs in order to better understand disorders due to such mutant enzymes.

AK has ten α-helices and five β-strands (Schulz et al., 1974). The primary structures of some isoenzymes of AK have been well identified, but their tertiary structures have not been completely elucidated (Schulz et al., 1986). Extensive attempts have been made to identify the substrate binding sites of AK: by X-ray crystallography of porcine AKs (Sachsenheimer & Schulz, 1977; Dreusicke et al., 1988) and yeast AK (Egner et al., 1987), by NMR studies of

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[1] Abbreviations: ADP, adenosine 5′-diphosphate; AK, adenylate kinase; AKc, chicken adenylate kinase; AMP, adenosine 5′-monophosphate; ATP, adenosine 5′-triphosphate; EDTA, ethylenediaminetetraacetic acid; hAK1, human adenylate kinase 1; NADH, nicotinamide adenine dinucleotide; NMR, nuclear magnetic resonance; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; WTAK, wild-type adenylate kinase.

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isolated rabbit muscle AK (Fry et al., 1985, 1987, 1988) and the synthetic $MgATP^{2-}$ binding fragments of AK (Hamada et al., 1979), and by site-directed mutagenesis studies of chicken AK (AKc) (Tagaya et al., 1989; Yan et al., 1990; Tian et al., 1990; Yoneya et al., 1990; Yan & Tsai, 1991; Okajima et al., 1991) and human AK (hAK1) (Kim et al., 1990). However, the active site for catalysis and important residues for substrate binding have not been exactly determined. To elucidate the AK structural model proposed by X-ray crystallography and NMR studies, interaction with substrates and specific residues should be tested by site-directed mutagenesis and steady-state kinetics. In hAK1, the conserved arginine residues (R44, R97, R132, and R138) were substituted with alanine and the ATP site in the original X-ray model was reassigned to the AMP site; these arginine residues were determined to be essential for catalytic activity (Kim et al., 1990). The reassignment of the AMP site in the revised X-ray model has also been suggested by NMR data (Smith & Mildvan, 1982) and by the data for the F86W mutant of AKe (Liang et al., 1991). However, a detailed analysis of the $MgATP^{2-}$ site and the AMP^{2-} site in an AK structural model remains to be accomplished.

In the present study, first, to evaluate a mutant form of hAK1 as a model of enzyme deficiency disorders and, second, to elucidate the minimum requirement of amino acid residues at the active center in hAK1, as succeeding analysis of Arg mutants in hAK1 (Kim et al., 1990), we substituted the lysine residues conserved in mammalian AK, which were suggested to be essential for interaction with the substrate by NMR and X-ray diffraction analyses (Fry et al., 1985, 1988; Dreusicke et al., 1988; Reinstein et al., 1990; Tian et al., 1990; Byeon et al., 1995). As reported previously, Lys21, Lys27, and Lys194 were suggested to interact with the substrates (Hamada et al., 1979). We replaced lysine because the aliphatic positively charged and hydrophilic basic amino acids would be largely changed compared to those from the other amino acid of a neutral, hydrophobic, and noncharged side chain. To define a structural AK model previously proposed by Kim et al. (1990), we constructed the pMEX8-hAK1 plasmid (Ayabe et al., 1996). We partially modified the method for random site-directed mutagenesis based on the phosphorothioate technique (Taylor et al., 1985; Nakamaye et al., 1986; Sayers et al., 1992). To elucidate the function of the positive ϵ-amino groups of the hydrophilic lysine residues, we mutated Lys9, Lys21, Lys27, and Lys31 in the head domain, Lys63 and Lys131 in the middle region, and Lys194 in the tail domain of hAK1. These mutants were analyzed by steady-state kinetics.

MATERIALS AND METHODS

Materials. The plasmid pMEX8-hAK1 (Ayabe et al., 1996) was used for random site-directed mutagenesis. The bacterial strain JM109 was purchased from TaKaRa Shuzo, Co., Ltd. (Tokyo, Japan). TG1 and a Sculptor *in vitro* Mutagenesis Kit were from Amersham Life Science (Buckinghamshire, England). The Blue Sepharose CL-6B column, Superose 12 (HR 10/30), and the fast protein liquid chromatography system were purchased from Pharmacia Biotech, Inc. (Tokyo, Japan). Adenine nucleoside mono- and triphosphates, AMP, ATP, and nicotinamide adenine dinucleotide (its reduced form, NADH) were purchased from Oriental Yeast Co. (Tokyo, Japan). Pyruvate kinase (PK), phosphoenolpyruvate (PEP), and lactate dehydrogenase (LDH) were from the Sigma Chemicals (St. Louis, MO). All other reagents were of analytical grade and were purchased from either Wako Pure Chemicals (Osaka, Japan) or Nacalai Tesque (Kyoto, Japan).

Purification of Single-Strand pMEX8-hAK1 DNA. A single colony of JM109/pMEX8-hAK1 (JM109 transformed with pMEX8-hAK1) was cultured in 10 mL of TYP medium (1.6% Tryptone, 1.6% yeast extract, 0.5% NaCl, and 0.25% K_2HPO_4) containing 50 μg/mL ampicillin at 37 °C overnight until the A_{600} was approximately 0.5. Helper phages (VCS-M13, Stratagene, La Jolla, CA) were added to the culture medium at a multiplicity of infection between 10 and 20 (a phage/cell ratio between 10/1 and 20/1) with 25 μg/mL kanamycin. The medium was cultured at 37 °C with vigorous aeration overnight and was centrifuged at 3000*g* for 20 min. To the supernatant was added a solution of polyethylene glycol 6000 and 2.5 M NaCl. The mixture was allowed to stand at room temperature for 15 min. After centrifugation of the solution, the pellet was resuspended with TE buffer (10 mM Tris-HCl and 1 mM EDTA at pH 8.0), and single-stranded DNA of pMEX8-hAK1 was extracted with TE-saturated phenol and chloroform according to the manufacturer's recommendations.

Random Site-Directed Mutagenesis of hAK1. The antisense primers were 5′-CGAAGATGATYXXAGTCTTCTTAAGC-3′ for Lys9, 5′-GCACTGGGTACCYXXGCCAGAACC-3′ for Lys21, 5′- GCACGATYXXCTCGCACTGGG −3′ for Lys27, 5′-GTGTAGCCGTAYXXCTGCACGATTTTC-3′ for Lys31, 5′-CCAGCTGACCYXXTTCCATGATTTC-3′ for Lys63, 5′-GTTTCGCCGCGYXXCAGCAGGCG-3′ for Lys131, and 5′-CGAAGATGATYXXAGTCTTCTTAAGC-3′ for Lys194. These primers were synthesized with a DNA synthesizer (Applied Biosystems, model 394) and phosphorylated with T4 polynucleotide kinase (Wako Co., Tokyo, Japan). The underlined letters, YXX, represent a target codon for random mutagenesis, where X was either A, G, C, or T, while Y was either G or C. Site-directed mutagenesis was carried out with a combination of the Sculptor *in vitro* Mutagenesis Kit (Amersham Life Science) using these site-specific primers annealed to the template single-strand DNA of pMEX8-hAK1. This method was based on the phosphorothioate technique (Taylor et al., 1985; Nakamaye et al., 1986; Sayers et al., 1992); that is, dCTP was used instead of dCTPαS during both the annealing and extension reaction of the randomly constructed oligonucleotides.

Screening of Mutants by DNA Cycle Sequencing. A homoduplex mutant DNA constructed by site-directed mutagenesis was transformed with competent cells (TG1) prepared according to the directions in the mutagenesis kit and was spread on an LB plate containing 50 μg/mL ampicillin. Single clones were cultured in 10 mL of LB medium overnight. A double-stranded DNA of the plasmid was purified according to the manufacturer's instructions (Flex Prep Purification Kit, Pharmacia Biotech, Inc.). The mutant plasmid DNA was sequenced by the dideoxy method (Sanger et al., 1977). Sequencing primers labeled with fluorescent isothiocyanate were the product of Japan Bioservices. The DNA sequence of the forward primer was 5′-TGGAATTGTGAGCGGATAAC-3′, and that of the reverse primer was 5′-AAAATCTTCTCTCATCCGCC-3′. Polymerase chain reaction (PCR) was performed with the AmpliCycle Sequencing kit (Perkin-Elmer, Branchburg,

NJ) with a DNA Thermal Cycler (model PJ-480, Perkin-Elmer Cetus) according to the partially modified protocol described as follows. To enhance Taq DNA polymerase activity, an AmpliTaq DNA Polymerase solution and Taq DNA polymerase (Promega, Madison, WI) were mixed in a ratio of 9/1 as a cycling mixture. Eight microliters of a master mixture was made by mixing 2.8 μg of double-stranded DNA, 1 μL of dimethyl sulfoxide (Sigma Chemical Co.), and 2 pmol of the FITC-labeled sequence primer. This mixture was incubated at 95 °C for 10 min and immediately cooled on ice. Two microliters of the cycling mixture and 0.2 unit of a Perfect Match Enhancer (Stratagene) were added to the cooled master mixture. PCR conditions were initial denaturation at 95 °C for 5 min, 20 cycles at 95 °C for 30 s, 53 °C for 30 s, and 72 °C for 60 s, and 20 cycles at 95 °C for 30 s and 72 °C for 60 s. PCR products were denatured by 95% formamide. Electrophoresis of the PCR product was performed with an autosequencer (Shimadzu, DSQ1, Kyoto, Japan). DNA sequencing of the mutant plasmid was performed with forward and backward sequencing primers to avoid undesirable mutations in the entire hAK1 gene.

Expression and Purification of WTAK and Mutant AK. JM109/pMEX8-hAK1 (wild-type AK) and TG1/pMEX8/ mutant hAK1 were separately cultured in 10 mL of LB medium containing 50 μg/mL ampicillin overnight at 37 °C and were transferred into 250 mL of LB medium. After the mixture was cultured for 1 h, isopropyl β-D-thiogalactopyranoside (IPTG) was added at a final concentration of 1 mM, and growth in the medium was continued for 16 h under the same conditions. *Escherichia coli* cells were centrifuged at 5000*g* for 20 min, and the pellet was suspended in 10 mL of a standard buffer [20 mM Tris-HCl, 1 mM EDTA, and 0.1 mM dithiothreitol (DTT) at pH 7.4]. All chromatographic steps were carried out at 4 °C in the chromatochamber. The suspension of *E. coli* cells was disrupted with an ultrasonicator (model 250 sonifier, Branson Ultrasonics Co., Danbury, CT) at 20 kHz and 20 W for 3 min on ice. The homogenate was centrifuged at 12000*g* for 20 min at 4 °C. The supernatant was subjected to affinity chromatography on Blue Sepharose CL-6B (ø of 1 × 5 cm) which had been equilibrated with the standard buffer and eluted with a NaCl gradient (0 to 1 M NaCl in the standard buffer) at a velocity of 0.5 mL/min. AK protein was determined by 12.5% polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS−PAGE) to confirm a single AK protein (Sambrook et al., 1989), and the AK fraction was concentrated by centrifuging with Centriplus-10 (Amicon, Inc., Tokyo, Japan). The concentrated AK (0.5 mL) sample was loaded onto a Superose 12 column (ø of 1 × 30 cm) and eluted with imidazole buffer (5 mM imidazole hydrochloride, 1 mM EDTA, and 0.1 mM DTT at pH 6.9) at a velocity of 0.5 mL/min. Each fraction was also determined by 12.5% SDS−PAGE to confirm a single band of AK protein.

Kinetic Analysis of the Forward Reaction of AK. Enzyme activity was assayed in the forward direction by adding various amounts of $MgSO_4$, ATP, and AMP. The initial velocity of the forward reaction was measured by observing the absorbance change at 340 nm with a Cary 2290 spectrophotometer (Varian, Mulgrave, Australia) [for NADH, in a coupled enzyme assay in the presence of pyruvate kinase and lactate dehydrogenase to monitor ADP formation at 25 °C as previously described (Hamada & Kuby, 1978)]. The forward reaction mixture in a total of 1 mL contained 75 mM triethanolamine hydrochloride (pH 7.4), 120 mM KCl, 0.2 mM NADH, 0.3 mM PEP, 0.3 mg/mL bovine serum albumin (BSA), 10 units of LDH, 5 units of PK, and 1.0 mM $MgSO_4$, and the various concentrations of $MgATP^{2-}$ and AMP^{2-} were as follows. Five combinations selected from 0.05, 0.1, 0.2, 0.5, 1.0, 2.0, 3.0, and 5.0 mM $MgATP^{2-}$ and a fixed concentration of 2 mM AMP^{2-} were used in the determination of the apparent Michaelis constants (K_m) for $MgATP^{2-}$. Five combinations selected from 0.05, 0.1, 0.2, 0.5, 1.0, 2.0, 3.0, and 5.0 mM AMP^{2-} and a fixed concentration of 2.0 mM $MgATP^{2-}$ were used in the determination of the apparent K_m for AMP^{2-}. The reaction was initiated by the addition of 10 μL of the recombinant hAK1 solution diluted to desired concentrations. The AK sample was diluted with a buffer (5 mM imidazole hydrochloride, 1 mM EDTA, 0.1 mM DTT, and 1% BSA at pH 7.4). K_m and V_{max} were estimated using a double-reciprocal plot (Lineweaver & Burk, 1934), and k_{cat} was calculated by dividing V_{max} by the total amount of enzyme (E_t) present in the reaction mixture. One unit of enzyme activity is defined as the amount of enzyme that produces 1 μmol of ADP in 1 min.

Others. The concentration of the protein was determined by the method of Lowry et al. (1951). Concentrations of adenine nucleotides and NADH were spectrophotometrically determined by using millimolar extinction coefficients of 15.4 and 6.22, respectively.

RESULTS

Site-Directed Mutagenesis of Lys Residues and Purification of WTAK and Mutant AKs

The results of site-directed random mutagenesis of pMEX8-hAK1 are summarized in Table 1. Twenty-six mutants at seven target residues were determined with backward and forward sequencing primers, and the efficiency of the random mutagenesis was averaged to 27.7% (26 mutants/94 colonies). For the K27I and K194I (ATA) mutant, the third position of codon 27 and 194 was replaced by A, although the third base was set as G or C in the annealing primer. This could be due to either contamination of A during primer synthesis by the DNA synthesizer or an unexpected point mutation during mutagenesis. The mutant pMEX8-hAK1 plasmid was expressed, and the mutant AK enzymes were purified to homogeneity by column chromatography using Blue Sepharose and Superose 12. These enzymes possessed basically the same chromatographic elution patterns as WTAK. However, both K27V and K131P mutants could not be purified after disruption of the expressed cells due to their insolubility in the standard buffer described in Materials and Methods. All other mutant enzymes showed single bands with the same mobility as that of WTAK on 12.5% SDS−PAGE (data not shown). The expressed yields and the specific activities of the mutants were low compared to those of WTAK (Table 1).

Kinetic Parameters of Mutant AKs

Steady-state kinetic data for WTAK and each mutant in the forward reaction are shown in Table 2.

(1) Properties of K9 Mutants. The K_m values for K9P were slightly decreased (0.2-fold) for $MgATP^{2-}$ and AMP^{2-} compared to those of WTAK; however, the decrease in k_{cat}

Table 1: Results of Site-Directed Random Mutagenesis

target residue	mutagenesis efficiency (%)	mutants	protein yield (mg)	AK activity (% of wild-type AK)
Lys9 (AAG)	33.3 (5/15)[a]	K9P (CCG)	1.60 (23%)[b]	1.2[c]
		K9F (TTC)	1.93 (28%)	0.5
		K9L (CTC) × 2	0.92 (13%)	8.0
		K9T (ACC)	0.31 (4%)	40.4
Lys21 (AAA)	10 (1/10)	K21P (CCG)	0.16 (2%)	5.1
Lys27 (AAA)	33.3 (4/12)	K27L (CTC)	1.60 (23%)	4.2
		K27V (GTC)	insoluble	inactive
		K27R (CGC)	0.52 (8%)	2.3
		K27I (ATA)	5.44 (79%)	0.4
Lys31 (AAA)	26.7 (4/15)	K31I (ATC)	2.03 (29%)	0.7
		K31S (TCG)	1.88 (27%)	0.5
		K31F (TTC) × 2	1.69 (24%)	6.9
Lys63 (AAA)	8.3 (1/12)	K63F (TTC)	0.94 (14%)	0.7
Lys131 (AAG)	30 (3/10)	K131A (GCC)	2.07 (30%)	1.9
		K131F (TTC)	3.95 (57%)	1.3
		K131P (CCC)	insoluble	inactive
Lys194 (AAA)	40 (8/20)	K194S (TCC) × 3	1.04 (15%)	12.6
		K194N (AAC)	0.89 (13%)	4.3
		K194V (GTG)	1.85 (27%)	1.7
		K194I (ATA)	1.88 (27%)	0.3
		K194P (CCC)	5.26 (76%)	0.2
		K194L (TTG)	1.26 (18%)	1.0

[a] Numbers in parentheses represent the ratio of the confirmed mutants/screening number. [b] Numbers in parentheses represent the percent of wild-type AK; the yield of wild-type AK was 6.90 mg. [c] Represents the percent of wild-type AK. Each mutant was assayed in a forward reaction at a fixed concentration of 1 mM for MgATP and AMP.

Table 2: Summary of Kinetic Parameters of Wild-Type hAK1 (WTAK) and Mutant hAK1

enzyme	K_m(MgATP^{2-}) (mM) (fold)	K_m(AMP^{2-}) (mM) (fold)	k_{cat} (s^{-1})	relative value (%)	k_{cat}/K_m(MgATP^{2-}) (%)	k_{cat}/K_m(AMP^{2-}) (%)
WTAK	0.27 (1.0)[a]	0.33 (1.0)[a]	571	100.0[b]	2.1×10^6 (100.0)[b]	1.7×10^6 (100.0)[b]
K9P	0.05 (0.2)	0.05 (0.2)	53	9.3	1.1×10^6 (52.4)	1.1×10^6 (64.7)
K9F	0.69 (2.6)	0.49 (1.5)	1.5	0.3	2.2×10^3 (0.1)	3.1×10^3 (0.2)
K9L	0.63 (2.3)	2.26 (6.8)	2.5	0.4	4.0×10^3 (0.2)	1.1×10^3 (6.5×10^{-2})
K9T	3.08 (11.4)	3.50 (10.6)	9.0	1.6	2.9×10^3 (0.1)	2.6×10^3 (0.2)
K21P	5.25 (19.4)	4.55 (13.8)	28.5	5.0	5.4×10^3 (0.3)	6.3×10^3 (0.4)
K27R	0.98 (3.6)	0.41 (1.2)	9.5	1.7	9.7×10^3 (0.5)	2.3×10^4 (1.4)
K27L	1.62 (6.0)	0.49 (1.5)	11.5	2.0	7.1×10^3 (0.3)	2.3×10^4 (1.4)
K27I	2.41 (8.9)	0.37 (1.1)	3.0	0.5	1.2×10^3 (5.7×10^{-2})	8.1×10^3 (0.5)
K31F	3.28 (12.1)	0.78 (2.4)	6.5	1.1	2.0×10^3 (9.5×10^{-2})	8.3×10^3 (0.5)
K31I	0.25 (0.9)	0.45 (1.4)	11	1.9	4.4×10^4 (2.1)	2.4×10^4 (1.4)
K31S	0.97 (3.6)	7.79 (23.6)	3.0	0.5	3.1×10^3 (0.1)	3.9×10^2 (2.3×10^{-2})
K63F	2.20 (8.1)	0.26 (0.8)	4.5	0.8	2.0×10^3 (9.5×10^{-2})	1.7×10^4 (1.0)
K131A	4.56 (16.9)	0.46 (1.4)	2.5	0.4	5.5×10^2 (2.6×10^{-2})	5.4×10^3 (0.3)
K131F	0.86 (3.2)	0.52 (1.6)	2.5	0.4	2.9×10^3 (0.1)	4.8×10^3 (0.3)
K194S	3.92 (14.5)	1.11 (3.4)	213	37.3	5.4×10^4 (2.6)	1.9×10^5 (11.2)
K194I	0.05 (0.2)	0.20 (0.6)	13.5	2.4	2.7×10^5 (12.9)	6.8×10^4 (4.0)
K194L	2.59 (9.6)	0.02 (0.1)	78	13.7	3.0×10^4 (1.4)	3.9×10^6 (229.4)
K194P	0.78 (2.9)	0.05 (0.2)	4.5	0.8	5.8×10^3 (0.3)	9.0×10^4 (5.3)
K194N	0.80 (3.0)	0.54 (1.6)	21	3.7	2.6×10^4 (1.2)	3.9×10^4 (2.3)
K194V	0.25 (0.9)	6.86 (20.8)	8	1.4	3.2×10^4 (1.5)	1.2×10^3 (7.1×10^{-2})

[a] Numbers in parentheses for K_m values indicate the relative change compared to that of the wild-type AK (the mutant/WTAK ratio). [b] For the calculation of the k_{cat} value, a molecular weight of 21 700 was employed, and numbers in parentheses for k_{cat} and k_{cat}/K_m values represent the percent of WTAK.

was comparable (9.3%) among the other K9 mutants. The k_{cat} of K9F was decreased to 0.3% with an unchanged K_m for both substrates. With K9L, there was a 6.8-fold increase in K_m for AMP^{2-}, k_{cat} was decreased to 0.4%, and the k_{cat}/K_m value for AMP^{2-} was dramatically decreased to 6.5×10^{-2}%. The K_m values of K9T showed more than 10-fold increases for MgATP^{2-} and AMP^{2-}, and the k_{cat} was reduced to 1.6%. These results strongly suggest that K9 mutants interact with both MgATP^{2-} and AMP^{2-} and have a larger effect on AMP^{2-} than on MgATP^{2-}.

(2) Properties of K21 Mutants. The K_m values of K21P for MgATP^{2-} and AMP^{2-} were markedly increased, 19.4- and 13.8-fold, respectively. The k_{cat} value was decreased to 5.0%. The results for K21P suggested interaction with both MgATP^{2-} and AMP^{2-}; however, the mutant more strongly affected MgATP^{2-}.

(3) Properties of K27 Mutants. The K_m values for AMP^{2-} of K27R, K27L, and K27I mutants were not changed (1.1−1.5-fold), while those for MgATP^{2-} were moderately increased (3.6−8.9-fold). The k_{cat} values were decreased to greater than 2.0%, and the k_{cat}/K_m values were significantly decreased. The K27 mutants appeared to interact with MgATP^{2-} to a greater extent than with AMP^{2-} and showed correspondingly decreased catalytic efficiency. It is surprising that for K27R, although the positive charge was conserved, a decrease in k_{cat} (to 1.7%) was observed despite

an unchanged K_m.

(4) Properties of K31 Mutants. The K_m values of K31I were unchanged for $MgATP^{2-}$ and AMP^{2-} (0.9- and 1.4-fold, respectively), while the k_{cat} value decreased to 1.9%. K31F showed an increased K_m for $MgATP^{2-}$ (12.1-fold), and the k_{cat}/K_m values for $MgATP^{2-}$ were decreased to 9.5 $\times$ 10^{-2}% In contrast, the K_m value of K31S dramatically increased (23.6-fold) for AMP^{2-}, and the k_{cat}/K_m for AMP^{2-} showed a large decrease to 2.3 $\times$ 10^{-2}%. K31I did not significantly affect the affinity for either substrate, although K31F strongly affected $MgATP^{2-}$ and K31S greatly affected AMP^{2-}. K31 mutants appeared to interact with $MgATP^{2-}$ and AMP^{2-} and to be essential for catalysis.

(5) Properties of K63 Mutants. The K_m values of K63F increased 8.1-fold for $MgATP^{2-}$ and were unchanged (0.8-fold) for AMP^{2-}. The k_{cat} decreased to 0.8%, and the k_{cat}/K_m value for $MgATP^{2-}$ was decreased to 9.5 $\times$ 10^{-2}%. The K63 mutant interacted with $MgATP^{2-}$ and showed decreased catalytic efficiency.

(6) Properties of K131 Mutants. Results for K131A showed a dramatic increase in K_m for $MgATP^{2-}$ (16.9-fold), a decrease by 0.4% for k_{cat}, and a large decrease in k_{cat}/K_m for $MgATP^{2-}$ (2.6 $\times$ 10^{-2}%). The K_m of K131F showed a small change (3.2-fold for $MgATP^{2-}$ and 1.6-fold for AMP^{2-}), but the k_{cat} values were decreased to 0.4%. K131 mutants were suggested to interact greatly with $MgATP^{2-}$ in catalysis.

(7) Properties of K194 Mutants. The K_m value of K194S for $MgATP^{2-}$ was moderately increased (14.5-fold), while that for AMP^{2-} was not changed (3.4-fold); the k_{cat} was not significantly changed to 37.3%. In the case of K194L, the K_m moderately increased 9.6-fold for $MgATP^{2-}$ but decreased 0.1-fold for AMP^{2-}, and the k_{cat} slightly decreased to 13.7%. The k_{cat}/K_m of K194L for $MgATP^{2-}$ was markedly decreased to 1.4%; however, that for AMP^{2-} displayed a strikingly increased catalytic efficiency for AMP^{2-} (229.4%). On the other hand, despite the fact that the K194V mutant was similar to K194L, the K_m value of K194V was greatly increased (20.8-fold) for AMP^{2-} and unchanged (0.9-fold) for $MgATP^{2-}$. The k_{cat} decreased to 1.4%. The k_{cat}/K_m of K194V for AMP^{2-} was markedly decreased (7.1 $\times$ 10^{-2}%) in spite of a small decrease in catalytic efficiency for $MgATP^{2-}$ (1.5%). K194I showed a small decrease of 0.2-fold in K_m values for $MgATP^{2-}$ and 0.6-fold for AMP^{2-}, and the k_{cat} was decreased to 2.4%. K194P showed the largest decrease (0.8%) in k_{cat} among all the K194 mutants. K194 mutants interacted not only with $MgATP^{2-}$ but also with AMP^{2-}.

DISCUSSION

X-ray structural studies indicate that AK protein has ten α-helices and five β-strands (Schulz et al., 1974) and that the active center cleft opens to some extent (Figure 1). In a previous study of hAK1, replacement of the arginine residues at R44, R97, R132, and R138 with alanine resulted in decreased catalytic efficiency (Kim et al., 1990). The loss of the positively charged guanidino groups of the arginine residues would inhibit catalytic efficiency, and the arginine residue was suggested to be essential for catalysis because it interacted with the negative charge of the phosphates of adenine nucleotides. In hAK1, site-directed mutagenesis has not been performed except for Arg residues. In the present

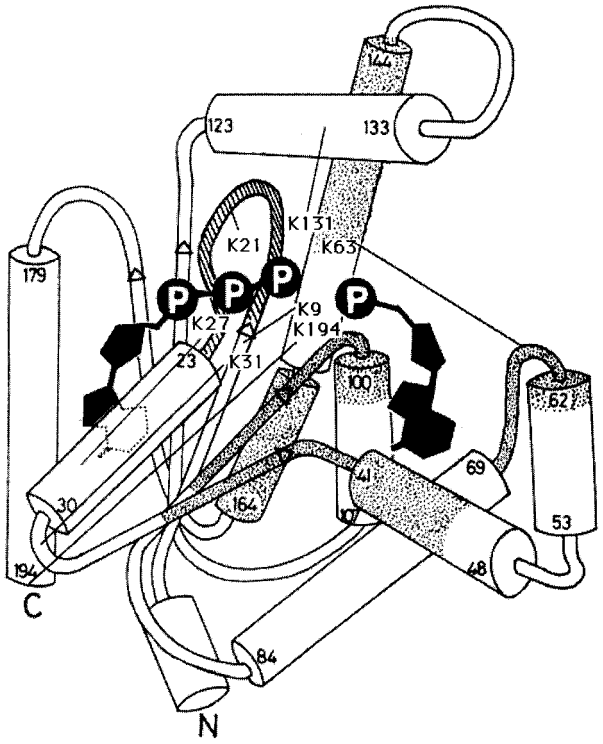

FIGURE 1: Side chains of Lys9, Lys21, Lys27, Lys31, Lys63, Lys131, and Lys194 are depicted in a proposed model of the binding sites for AMP and MgATP in adenylate kinase by Kim et al. (1990). These lysine residues could take part in a phosphoryl transfer reaction between $MgATP^{2-}$ and AMP^{2-}, in cooperation with the other active site residues, such as arginine residues (Kim et al., 1990).

study, to elucidate the minimum requirement for amino acid residues at the active center in hAK1, and to elucidate an interaction with substrates and specific residues, we substituted the positively charged ϵ-amino groups of Lys9, Lys21, Lys27, Lys31, Lys63, Lys131, and Lys194 residues by random site-directed mutagenesis. These lysine residues were well conserved in mammalian species. At first, we partially modified the site-directed mutagenesis strategy based on the phosphorothioate technique (Taylor et al., 1985; Nakamaye et al., 1986; Sayers et al., 1992). In anticipation of random mutations, the antisense primers were synthesized to contain a three-base-mismatched codon of YXX (Y = G or C; X = A, G, C, or T) for a target amino acid, and the constructed mutant homoduplex DNA was annealed to the single-strand DNA of pMEX8-hAK1. Twenty-two different mutant pMEX8-hAK1 plasmids were confirmed by screening from 94 colonies, and the efficiency of our site-specific mutagenesis averaged 23.4% (22/94). We have not analyzed the secondary structures of all mutants with circular dichroism spectra because these lysine residues might not be directly involved in the secondary structures; as reported in a previous paper, in the case of replacement of arginine with alanine, very large differences were not displayed (Kim et al., 1990). However, as a control of the Lys mutant, circular dichroism studies should be performed soon on several kinds of Lys mutants. K27V and K131P mutants could not be analyzed because they were insoluble in the standard buffer described in Materials and Methods, which might be due to the helical change as observed in other proteins. The conformation or hydrophobicity of these insoluble mutants might have been altered upon folding; if the three-dimensional structure of these mutants changed, such a

structural change could be detected by changes of spectra from optical rotational dichroism (ORD) studies.

In human genetic disorders associated with nonspherocytic hemolytic anemia, an AK1 deficiency in erythrocytes has been reported (Miwa et al., 1983; Matsuura et al., 1989; Toren et al., 1994). The nucleotide sequence of the patient's AK1 gene resulted in a replacement of Arg128 with Trp, and the mutant AK expressed *in vitro* showed reduced catalytic activity as well as decreased solubility in AKc (Matsuura et al., 1989). A new mutant enzyme constructed by site-directed mutagenesis may be useful for studying the pathophysiological mechanism of disorders due to a mutant enzyme derived from a point mutation. The change in affinity for substrates and/or the decreased catalytic efficiency of Lys mutants to various degrees, together with a decreased solubility, could contribute to the hemolytic anemia derived from the AK1 mutant.

We have made the following assumptions on the basis of the results of steady-state kinetics of each Lys mutant. K9 mutants affected the affinity for both $MgATP^{2-}$ and AMP^{2-} but more strongly interacted with AMP^{2-}, which decreased catalytic efficiency. Lys9 would be essential for catalysis by interacting with $MgATP^{2-}$ and AMP^{2-}. The glycine-rich loop plays an important role in catalysis (Fry et al., 1988; Mildvan & Fry, 1987; Reinstein et al., 1988). K21P could make the flexible P-loop bend in any direction, which could affect the substrate-binding site, reducing the affinity for both substrates and decreasing the k_{cat} value. The Lys21 residue would be essential for catalysis and substrate binding. Lys21 was proposed to be close to the MgATP binding site (Fry et al., 1985), and the ϵ-amino group was suggested to be close to the subsite for the γ-phosphate of ATP (Tagaya et al., 1987). According to an NMR study of K21M in AKc, Lys21 may play a key structural role (Tian et al., 1990). On the basis of NMR analyses of K21R and K21A, Lys21 would orient the triphosphate chain of MgATP to the proper conformation required for catalysis (Byeon et al., 1995). K27 mutants showed moderately decreased affinity for $MgATP^{2-}$ and decreased catalytic efficiency. From our data, in fact, Lys27 would appear to play a functional role in catalysis at the $MgATP^{2-}$ site. The K27R mutant, in spite of the change of an ϵ-amino group to a guanidino group retaining a positive charge, showed a largely reduced k_{cat}. The catalytic function of Lys27 could not be replaced by a conservative residue arginine, and the ϵ-amino group was presumed to be necessary for phosphoryl binding and transfer. On the basis of NMR studies of K27M in AKc (Tian et al., 1990), Lys27 was nonessential for either catalysis (including substrate binding) or structure; however, the discrepancies between this report and our data might be due in part to the difference in experimental conditions using different species and types of mutations in the side chains. The roles of these same mutations might differ between AKc and hAK1. This should be tested using the K27M mutant in hAK1. A study of K31 mutants revealed that K31F showed a decreased affinity for $MgATP^{2-}$ but K31S showed reduced affinity for AMP^{2-}. Lys31 was proposed to be at or near bound $MgATP^{2-}$ (Fry et al., 1988); however, the result affected both $MgATP^{2-}$ and AMP^{2-}. When the enzyme forms a ternary complex by substrate binding, the side chain of Lys31 at the $MgATP^{2-}$ site might permit the AMP^{2-} substrate to interact with the phosphate of AMP^{2-} for catalysis.

Lys63 is denoted on the right site close to the AMP^{2-} site (Figure 1). K63F showed a greatly reduced affinity for $MgATP^{2-}$ and decreased catalytic efficiency. By photoaffinity labeling analysis, the amino acid domain (Gly64−Arg77) was proposed to constitute the neighborhood identifiable with AMP binding during binary-to-ternary complex formation (Pai et al., 1992). When two substrates ($MgATP^{2-}$ and AMP^{2-}) bind with the enzyme and form a ternary complex, some conformational change could occur which allows the side chain of Lys63 access to the $MgATP^{2-}$ substrate. The 120−133 helical region exists at the top of the AK model (Figure 1). K131 mutants showed moderately decreased affinity for $MgATP^{2-}$ and decreased catalytic efficiency. On the basis of kinetic analyses of R132A in hAK1 (Kim et al., 1990), Arg132 next to Lys131 would appear to be essential for catalysis by interacting strongly with $MgATP^{2-}$ and only partially with AMP^{2-}. According to NMR studies of R132M in AKc, Arg132 was important for transition-state stabilization (Yan & Tsai, 1991). The 131−141 segment was observed to move in substrate holding, which correlates especially with ATP binding (Schulz et al., 1990). Lys131 would be essential for catalysis by interacting with $MgATP^{2-}$.

The flanking α-helices of the C-terminal domain are denoted on the left side of the proposed AK model, and Lys194 is in the tail section (Figure 1). AKc consists of 193 amino acid residues (Kishi et al., 1986), and hAK1 consists of an additional lysine at the 194 position. The Leu190 residue in AKc appears to participate in components of the MgATP-binding domain (Yoneya et al., 1990). In hAK1, K194S showed a moderately decreased affinity for $MgATP^{2-}$. However, similar mutations in K194V and K194L affected the affinity for $MgATP^{2-}$ or AMP^{2-} differently and decreased catalytic efficiency. Different hydrophobic changes such as valine and leucine at position 194 may affect the binding of $MgATP^{2-}$ and AMP^{2-} substrates, and the mutants may cause a conformational change of the C-terminal domain by altering its hydrophobicity. The C-terminal domain might appear to move and to access and interact with the AMP^{2-} substrate, forming a ternary complex. Lys194 would appear to interact not only with $MgATP^{2-}$ but also with AMP^{2-}, possibly indirectly, and thus, the flanking α-helices of the C-terminal domain would be essential for catalysis by stabilizing the transition state and holding the substrates and Lys194 could not be replaced by other amino acids.

In a brief summary, steady-state kinetic studies above have led to the following conclusions. (i) Lys9 would be essential for catalysis by interacting with both $MgATP^{2-}$ and AMP^{2-} but by affecting AMP^{2-} more strongly. (ii) Lys21 would be essential for catalysis by interacting with both $MgATP^{2-}$ and AMP^{2-} but by affecting $MgATP^{2-}$ more strongly. (iii) Lys27 and Lys131 would appear to play a functional role in catalysis by interacting strongly with $MgATP^{2-}$. (iv) Lys31 would appear to interact with $MgATP^{2-}$ and AMP^{2-} at the $MgATP^{2-}$ site and be essential for catalysis. (v) Lys63 is likely to interact to a much greater extent with $MgATP^{2-}$. (vi) Lys194 in the flanking α-helices of the C-terminal domain would interact not only with $MgATP^{2-}$ but also with AMP^{2-} at the $MgATP^{2-}$ site by holding both substrates. The loss of the ϵ-amino group of lysine (Lys9, Lys21, Lys27, Lys31, Lys63, Lys131, and Lys194) resulted in decreased or unchanged affinity for the substrates and reduced catalytic

efficiency. The positive charge of lysine is not replaced in nature and may interact with the negatively charged phosphates of $MgATP^{2-}$ and AMP^{2-}, which in turn may orient the phosphate chains of the two substrates to the proper conformation required for catalysis. We would predict insertion of important lysine residues as shown in a new AK structural model (Figure 1). These lysine residues would take part in a phosphoryl transfer reaction between $MgATP^{2-}$ and AMP^{2-}, in cooperation with the other active site residues, as in the case of arginine residues (Kim et al., 1990). The AK structural model proposed by X-ray crystallographic studies is obtained in crystal with substrate-inhibitory analogs; the structural data from NMR studies are confirmed in solution. The new model derived from the present data on steady-state kinetics (Figure 1) might represent a more dynamic and physiological model in solution than X-ray crystallographic and NMR studies indicate. It is expected that these discrepancies will be resolved in the near future. To elucidate the structures of ternary complexes of substrates with mutant AK, NMR studies or detailed X-ray crystallographic studies would be required, and circular dichroism and ORD studies must be performed on the structural changes resulting from our Lys mutants. Our future efforts will be directed toward other key residues required for physiological function or to define the interaction with substrates and critical amino acid residues in the AK molecule.

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