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The Steady-State Kinetics of the Enzyme Reaction Tested by Site-Directed Mutagenesis of Hydrophobic Residues (Val, Leu, and Cys) in the C-Terminal α-Helix of Human Adenylate Kinase[1]

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To elucidate whether the C-terminal region in human adenylate kinase participates in the interaction with the substrate ($MgATP^{2-}$ and/or AMP^{2-}), hydrophobic residues (Val182, Val186, Cys187, Leu190, and Leu193) were substituted by site-directed mutagenesis and the steady-state kinetics of fifteen mutants were analyzed. A change in the hydrophobic residues in the C-terminal domain affects the affinity for substrates (K_m), that is, not only for $MgATP^{2-}$ but also for AMP^{2-}, and the catalytic efficiency (k_{cat}). The results obtained have led to the following conclusions: (i) Val182 may interact with both $MgATP^{2-}$ and AMP^{2-} substrates, but to a greater extent with $MgATP^{2-}$, and play a role in catalysis. (ii) Val186 appears to play a functional role in catalysis by interacting with both $MgATP^{2-}$ and AMP^{2-} to nearly the same extent. (iii) Cys187 appears to play a functional role in catalysis. (iv) Leu190 appears to interact with both $MgATP^{2-}$ and AMP^{2-} substrates but to a greater extent with AMP^{2-}. (v) Leu193 appears to interact with both $MgATP^{2-}$ and AMP^{2-} but to a greater extent with AMP^{2-}. The activity of all mutants decreased due to the change in substrate-affinity. The closer the residue is located to the C-terminal end, the more its mutation affects not only $MgATP^{2-}$ but also AMP^{2-} substrate binding. The hydrophobic alterations disrupt hydrophobic interactions with substrates and that might destabilize the conformation of the active site. The more C-terminal part of the α-helix appears to interact with AMP, as if it has swung out and rotated to cover the adenine moieties. The C-terminal α-helix of human adenylate kinase appears to be essential for the interaction with adenine substrates by swinging out during catalysis.

Key words: human adenylate kinase, hydrophobicity, C-terminal α-helix.

Adenylate kinase (AK) [EC 2.7.4.3] is a ubiquitous enzyme that catalyzes the reaction $MgATP^{2-} + AMP^{2-} \rightleftarrows MgADP^{-} + ADP^{3-}$. There are two distinct nucleotide-binding sites, one for $MgATP^{2-}$ or $MgADP^{-}$, and the other for AMP^{2-} or ADP^{3-} (*1*). In vertebrates, three isozymes have been characterized (*2–4*): AK1, which is present in the cytosol, AK2, which is localized in the intermembrane space of mitochondria, and AK3, which occurs in the mitochondrial matrix. The three isozymes are important for the homeostasis of the adenine nucleotide metabolism.

The primary structures of various AK isozymes have been determined, and the deduced tertiary structures have been used to identify the two substrate binding sites. These studies include X-ray crystallography of porcine AK (*5*, *6*) and yeast AK (*7*), NMR studies of isolated rabbit muscle AK (*8–10*), and site-directed mutagenesis studies on chicken AK (*11–16*) and human AK (hAK1) (*17*, *18*). A comparison of the structures with and without substrates revealed large domain movements during catalysis (*19*, *20*). Predictions based on the X-ray crystallographic structures suggest that the AMP binding domain undergoes a movement of 8 Å upon AMP binding and the ATP binding domain moves up to 30 Å upon the binding of ATP (*19*, *21*).

However, the catalytic and substrate-binding sites have not been precisely determined. The AK structural model proposed by X-ray crystallography and NMR studies can be used to evaluate the steady-state kinetics, test interactions with substrates, and modify specific amino acid residues by site-directed mutagenesis. In studies of human AK, the conserved arginine residues (at positions 44, 97, 132, and 138) and lysine residues (at positions 9, 21, 27, 31, 63, 131, and 194) were mutated. The results obtained suggested

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Abbreviations: ADP, adenosine 5′-diphosphate; AK, adenylate kinase; AMP, adenosine 5′-monophosphate; ATP adenosine 5′-triphosphate; EDTA, ethylenediaminetetraacetate; 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.

that these residues are essential for catalytic activity (*17*, *18*). Loss of the positively charged ε-amino group of a lysine residue the decreased catalytic efficiency, and the positively charged lysine residues should interact with the negatively charged γ- or α-phosphates of $MgATP^{2-}$ and AMP^{2-} (*18*). However, a detailed analysis of the $MgATP^{2-}$ and the AMP^{2-} sites in the AK structural model remains to be done.

In the present study we evaluated the flanking C-terminal domain of AK by substituting hydrophobic residues conserved in mammalian AK that have not been examined for their interactions with adenine nucleotide substrates. We previously reported that Lys194 at the C-terminus of human AK interacts with both $MgATP^{2-}$ and AMP^{2-} for substrate binding (*18*), even though the C-terminal region is located in the $MgATP^{2-}$ binding site (*17*). To elucidate the participation of the C-terminal domain in the interaction with $MgATP^{2-}$ and AMP^{2-}, we selected hydrophobic residues that might affect the affinity of the adenine nucleotides. We mutated Val182, Val186, Cys187, Leu190, and Leu193 residues by site-directed mutagenesis and the various mutants produced were analyzed by steady-state kinetics.

EXPERIMENTAL PROCEDURES

Materials—The plasmid pMEX8-hAK1 (*22*) was used for random site-directed mutagenesis. The bacterial strain JM109 was purchased from TaKaRa Shuzo (Tokyo). 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 (Tokyo). Adenine nucleoside mono- and triphosphates, AMP, ATP, and nicotinamide adenine dinucleotide (as its reduced form, NADH) were purchased from Oriental Yeast (Tokyo). Pyruvate kinase (PK), phosphoenolpyruvate (PEP), and lactate dehydrogenase (LDH) were from Sigma Chemicals (St. Louis, MO, USA). All other reagents were of analytical grade and purchased from either Wako Pure Chemicals (Osaka) or Nacalai Tesque (Kyoto).

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, 0.25% K_2HPO_4 containing 50 μg/ml of ampicillin) at 37°C overnight until the absorbance at 600 nm was approximately 0.5. Helper phages (VCS-M13, Stratagene, La Jolla, CA, USA) were added to the culture medium at a multiplicity of infection between 10 and 20 (phage:cell ratio between 10:1 and 20:1) with 25 μg/ml of kanamycin. The cells were cultured at 37°C with vigorous aeration overnight and centrifuged at 3,000 ×*g* for 20 min. A solution of polyethyleneglycol 6000 and 2.5 M NaCl was added to the supernatant, and the mixture was allowed to stand at room temperature for 15 min. The solution was centrifuged solution at 5,000 ×*g* for 5 min, and the pellet was resuspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0), and the single methyl sulfoxide stranded DNA of pMEX8-hAK1 was extracted in TE-saturated phenol and chloroform according to the manufacturer's recommendations.

Site-Directed Mutagenesis of Human Adenylate Kinase—The antisense primers were 5′-CCTGAGAGAAYXXTTCG-TCAAC-3′ for Val182 residue, 5′-GCAGGAGAGTGCAYXX-CTGAGAGAATACTTC-3′ for Val186, 5′-CCAGGTGAGTY-XXTACCTGAGAG-3′ for Cys187, 5′-TCAGAGCTCYXXGT-GAGTGCATA-3′ for Leu190, and 5′-CGTCGACTATTATT-TYXXAGCGTCCAGGTG-3′ for Leu193. The primers were synthesized with a DNA synthesizer (Applied Biosystems, Model 394) and phosphorylated with T_4 Polynucleotide Kinase (WAKO, Tokyo). The underlined letters, YXX, represent the target codons for site-directed mutagenesis, where X is either A, G, C, or T, and Y is either G or C. Site-directed mutagenesis was carried out with a combination of the Sculptor™ *in vitro* Mutagenesis Kit using these site-specific primers annealed to the template single strand DNA of pMEX8-hAK1. This method was based on the phosphorothioate technique (*23–25*), that is, dCTP was used instead of dCTPαS during both the annealing and extension reaction of the constructed oligonucleotide.

Screening of Mutants by DNA 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 spread on an LB plate containing 50 μg/ml of ampicillin. Single clones were cultured overnight in 10 ml of LB medium. Double-stranded DNA from the plasmid was purified according to the manufacturer's instructions (Flex Prep Purification Kit, Pharmacia Biotech, Tokyo). The mutant plasmid DNA was sequenced by the dideoxy method (*26*). Sequencing primers labeled with fluorescent isothiocyanate were from Japan Bioservices, Tokyo. The DNA sequence of the forward primer was 5′-TGGAATTGTGAGCGGATAAC-3′ and that of the reverse primer 5′-AAAATCTTCTCTCAT-CCGCC-3′. The polymerase chain reaction (PCR) was performed with an AmpliCycle™ Sequencing kit (Perkin Elmer, Branchburg, NJ, USA) using a DNA Thermal Cycler (Model PJ-480, Perkin Elmer Cetus) according to the following partially modified protocol: To enhance Taq DNA Polymerase activity, AmpliTaq™ DNA Polymerase solution and Taq DNA Polymerase (Promega, Madison, WI, USA) were mixed at a ratio of 9:1 as a cycling mixture. Eight microliters of master mixture was made by mixing 2.8 μg of double-stranded DNA, 1 μl of dimethylsulfoxide (Sigma Chemical, St. Louis, MO, USA), 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 Perfect Match Enhancer (Stratagene, La Jolla, CA, USA) were added to the cooled master mixture. PCR conditions were: (i) initial denaturation at 95°C for 5 min, followed by (ii) 20 cycles at 95°C for 30 s, 53°C for 30 s, and 72°C for 60 s, and (iii) 20 cycles at 95°C for 30 s and 72°C for 60 s. The PCR products were denatured in 95% formamide. Electrophoresis of the PCR product was performed by an autosequencer (Shimadzu, DSQ1, Kyoto). 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 Wild Type AK 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 of ampicillin overnight at 37°C, and transferred into 250 ml of LB medium. After 1 h culture, isopropyl-β-D-thio-galactopyranoside (IPTG) was

added at a final concentration of 1 mM, and growth in the medium was continued for 16 h under the same conditions. *E. coli* cells were centrifuged at 5,000 ×*g* for 20 min, and the pellet was suspended in 10 ml of standard buffer [20 mM Tris-HCl, 1 mM EDTA, 0.1 mM dithiothreitol (DTT), pH 7.4]. All chromatographic steps were carried out at 4°C in a chromatochamber. The suspension of *E. coli* cells was disrupted with an ultrasonicator (Model 250 sonifier, Branson Ultrasonics, Danbury, CT, USA) at 20 kHz and 20 W for 3 min on ice. The homogenate was centrifuged at 12,000 ×*g* for 20 min at 4°C. The supernatant was subjected to affinity chromatography on Blue Sepharose CL-6B (ϕ 1×5 cm), which was equilibrated with the standard buffer and eluted with a NaCl gradient (0 to 1 M NaCl) in standard buffer at a flow rate of 0.5 ml/min. AK protein was detected by 12.5% polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate (SDS-PAGE), and the presence of a single band was confirmed (*27*). The AK fraction was concentrated by centrifugation with a Centriplus-10 device (Amicon, Tokyo). The concentrated AK (0.5 ml) sample was loaded onto a Superose 12 column (ϕ 1 × 30 cm) and eluted with imidazole buffer (5 mM imidazole-HCl, 1 mM EDTA, 0.1 mM DTT, pH 6.9) at a flow rate of 0.5 ml/min. The collected fractions were evaluated by 12.5% SDS-PAGE and the presence of a single band of AK protein was confirmed. The concentration of protein was determined by the method of Lowry *et al.* (*28*).

Kinetic Analysis of the Forward Reaction of Adenylate Kinase—Enzyme activity was assayed in the forward direction by adding various amounts of $MgSO_4$, ATP, and AMP to the standard reaction mixture. 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). We previously described (*29*) the coupled enzyme assay in which NADH is the substrate and pyruvate kinase and lactate dehydrogenase are present to monitor ADP formation at 25°C.

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 various concentrations of $MgATP^{2-}$ and AMP^{2-} as follows: five combinations selected from 0.05, 0.1, 0.2, 0.5, 1.0, 2.0, 3.0, 5.0 mM $MgATP^{2-}$ and a fixed concentration of 2 mM AMP^{2-} for the determination of the apparent Michaelis constant (K_m) for $MgATP^{2-}$. Five concentration combinations selected from 0.05, 0.1, 0.2, 0.5, 1.0, 2.0, 3.0, 5.0 mM AMP^{2-} and a fixed concentration of 2 mM $MgATP^{2-}$ were used for 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 the desired concentrations. The AK sample was diluted in buffer (5 mM imidazole-HCl, 1 mM EDTA, 0.1 mM DTT, 1% BSA, pH 7.4). The K_m and V_{max} values were estimated using double-reciprocal plots (*30*), and k_{cat} was calculated by dividing V_{max} by the total amount of enzyme (Et) present in the reaction mixture. One unit of enzyme activity is defined as the amount of enzyme that produces 1 μmol of ADP in one minute. The concentrations of adenine nucleotides and NADH were determined spectrophotometrically using millimolar extinction coefficients of 15.4 and 6.22, respectively.

RESULTS

Site-Directed Mutagenesis and Purification of Mutant Adenylate Kinases—The results of site-directed random mutagenesis of pMEX8-hAK1 are summarized in Table I. Nineteen mutants at five target residues were constructed with backward and forward sequencing primers. For the Leu193-mutant series, the TAG mutation at position 193 was confirmed, that is, the deletion of residues 193-194 produced the unique L193Stop mutant. The mutant pMEX8-hAK1 plasmids were expressed, and the various AK enzymes produced were purified to homogeneity by column chromatography using Blue Sepharose and Superose 12. These AK mutants possessed the same chromatographic elution patterns as the wild type enzyme. The induced monomer protein migrated as expected for a protein with a molecular weight of 22,000. However, four mutants, Val186Asn, Leu190Pro, Leu190Thr, and Leu190Asn could not be purified after disruption of the expressed cells due to their insolubility in the standard buffer described in "EXPERIMENTAL PROCEDURES." This probably resulted from changes in solubility due to the specific mutations in the C-terminal domain. All other mutant enzymes migrated as single bands with the same mobility as that of wild type AK on 12.5% SDS-PAGE (data not shown).

Kinetic Parameters of Mutant Adenylate Kinases—Steady-state kinetic data for wild type AK and each mutant in the forward reaction are shown in Table II. The K_m, k_{cat}, and k_{cat}/K_m values obtained for each mutant were compared to the wild-type AK and expressed as the mutant/WTAK ratio (see Table II).

(1) Properties of the Val182 mutants: The K_m values of Val182Ala for $MgATP^{2-}$ and AMP^{2-} were markedly increased (by 11-fold and 12-fold) compared to wild-type AK. The k_{cat} value for this mutant was drastically decreased (to 0.1% of the control.) The k_{cat}/K_m ratios for the two nucleotide substrates were dramatically decreased (to 6.2×10^{-3} and 5.7×10^{-3}% of the control, respectively.) The K_m values for Val182Gly showed a 7.4-fold increase for $MgATP^{2-}$ and a 1.9- fold increase for AMP^{2-}; the k_{cat} value was reduced to 0.7% of the control. The K_m values for Val182Ser were ele-

TABLE I. **Results of site-directed random mutagenesis.**

Target Residue	Mutants		Protein yield (mg)
Val182 (GTA)	V182A	(GCG)	14.2
	V182G	(GGG)	2.9
	V182S	(AGC)	9.3
Val186 (GTA)	V186S	(TCC)	1.6
	V186G	(GGG)	0.8
	V186N	(GAC)	insoluble
Cys187 (ACG)	C187V	(GTC)	15.9
Leu190 (CTG)	L190A	(GCG)	0.9
	L190S	(TCG)	1.2
	L190P	(CCC)	insoluble
	L190T	(ACG)	insoluble
	L190N	(AAC)	insoluble
Leu193 (CTG)	L193I	(ATC)	5.2
	L193Q	(CAG)	10.4
	L193P	(CCC)	5.8
	L193S	(TCG)	1.7
	L193F	(TTC)	12.6
	L193R	(CGC)	8.0
	L193Stop	(TAG)	7.3

TABLE II. **Summary of kinetic parameters of wild-type hAK1 (WTAK) and muant hAK1.**

Enzyme	K_m ($MgATP^{2-}$)		K_m (AMP^{2-})		k_{cat}	Relative value	k_{cat}/K_m ($MgATP^{2-}$)		k_{cat}/K_m (AMP^{2-})	
	(mM)	(fold)	(mM)	(fold)	(s^{-1})	(%)		(%)		(%)
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]
V182A	3.01	(11.1)	3.92	(11.9)	0.38	(0.1)	1.3×10^{2}	(6.2×10^{-3})	97	(5.7×10^{-3})
V182G	1.99	(7.4)	0.64	(1.9)	4	(0.7)	2.0×10^{5}	(9.5×10^{-2})	6.3×10^{5}	(0.4)
V182S	0.53	(2.0)	0.49	(1.5)	3	(0.5)	5.7×10^{5}	(0.3)	6.1×10^{5}	(0.4)
V186S	1.90	(7.0)	2.48	(7.5)	5.5	(1.0)	2.9×10^{5}	(0.1)	2.2×10^{5}	(0.1)
V186G	0.34	(1.3)	0.64	(1.9)	7	(1.2)	2.1×10^{4}	(1.0)	1.1×10^{4}	(0.6)
C187V	0.17	(0.6)	0.62	(1.9)	1	(0.2)	5.9×10^{5}	(0.3)	1.6×10^{5}	(9.4×10^{-2})
L190A	0.74	(2.7)	1.76	(5.3)	9	(1.6)	1.2×10^{4}	(0.6)	5.1×10^{5}	(0.3)
L190S	6.99	(25.9)	27.15	(82.3)	3	(0.5)	4.3×10^{2}	(2.0×10^{-2})	1.1×10^{2}	(6.5×10^{-3})
L193I	2.11	(7.8)	10.60	(32.1)	4	(0.7)	1.9×10^{5}	(9.0×10^{-2})	3.8×10^{2}	(2.2×10^{-2})
L193Q	2.50	(9.3)	2.45	(7.4)	2.5	(0.4)	1.0×10^{5}	(4.8×10^{-2})	1.0×10^{5}	(5.9×10^{-2})
L193P	0.73	(2.7)	0.03	(0.1)	77	(13.5)	1.1×10^{5}	(5.2)	2.6×10^{6}	(152.9)
L193S	0.42	(1.6)	0.04	(0.1)	36.5	(6.4)	8.7×10^{4}	(4.1)	9.1×10^{5}	(53.5)
L193F	0.11	(0.4)	1.07	(3.2)	12.5	(2.2)	1.1×10^{5}	(5.2)	1.2×10^{4}	(0.7)
L193R	0.27	(1.0)	1.27	(3.8)	9	(1.6)	3.3×10^{4}	(1.6)	7.1×10^{5}	(0.4)
L193Stop	0.43	(1.6)	3.74	(11.3)	4.5	(0.8)	1.0×10^{4}	(0.5)	1.2×10^{5}	(7.1×10^{-2})

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

vated by 2.0- and 1.5-fold for $MgATP^{2-}$ and AMP^{2-}, respectively; the k_{cat} value was profoundly decreased (to 0.5% of control). In general, the three V182 mutants suppressed the interaction with both the $MgATP^{2-}$ and the AMP^{2-} binding sites, but to a lesser extent with AMP^{2-}. The catalytic efficiency (kc) for each was very low (*i.e.*, the enzymatic activity was barely detectable.)

(2) Properties of the V186 mutants: The K_m values for Val186Ser increased by 7.0- and 7.5-fold for $MgATP^{2-}$ and AMP^{2-}, respectively; the k_{cat} value decreased to 1.0% of the control. The K_m values for Val186Gly were elevated by 1.3- to 1.9-fold for $MgATP^{2-}$ and AMP^{2-}, respectively while the k_{cat} value decreased to 1.2% of control. These results strongly suggest that Val186-mutants interact with both the $MgATP^{2-}$ and AMP^{2-} binding sites to nearly the same extent and that both have a profound effect on catalytic efficiency.

(3) Properties of the Cys187 mutant: The K_m values for Cys187Val were decreased slightly for $MgATP^{2-}$ (by 0.6-fold) and increased slightly (by 1.9-fold) for AMP^{2-}, compared to those for wild type AK. However, the k_{cat} value was greatly decreased (to 0.2% of control). These results strongly suggest that the Cys187Val mutant has a relatively large effect on catalytic efficiency, but little effect on substrate binding.

(4) Properties of the Leu190 mutants: The K_m values for Leu190Ala were increased for both substrates, but to a lesser extent for $MgATP^{2-}$ than for AMP^{2-} (*i.e.*, 2.7-fold *vs.* 5.3-fold, respectively); the k_{cat} decreased to a value only 1.6% that of the control. The Leu190Ser mutant showed markedly increased K_m values for both $MgATP^{2-}$ (26-fold) and AMP^{2-} (82-fold); the k_{cat} value was decreased to 0.5% of the control and the k_{cat}/K_m ratios were decreased to 2.0×10^{-2}% for $MgATP^{2-}$ and to 6.5×10^{-3}% for AMP^{2-}, relative to the wild type. The Leu190Ala and Leu190Ser mutants both affected the affinity for $MgATP^{2-}$ and AMP^{2-}, but the increase in K_m was much greater for AMP^{2-} in the case of Leu190Ser (82-fold) than for the Leu190Ala mutant (5.3-fold). The effects on K_m were greater for Leu190Ser for both the AMP^{2-} and $MgATP^{2-}$ binding sites. The effects of $MgATP^{2-}$ on K_m were greater for Leu190Ser (26-fold) than for Leu190Ala (2.7-fold), a pattern consistent with the results for AMP^{2-}. It appears, therefore, that residue Leu190 is essential for substrate binding.

(5) Properties of the Leu193 mutants: The K_m values for Leu193Ile increased by 7.8-fold for $MgATP^{2-}$ and 32-fold for AMP^{2-}; the k_{cat} value decreased to 0.7% of the control. Thus, the Leu193Ile mutant strongly affects the affinity for AMP^{2-} and to a lesser extent the affinity for $MgATP^{2-}$. The K_m values of Leu193Gln showed an increase for $MgATP^{2-}$. The K_m values for Leu193Gln increased by 9.3-fold for $MgATP^{2-}$ and by 7.4-fold for ATP^{2-}; the k_{cat} value was decreased to 0.4% of the control. There was a large decrease in the k_{cat}/K_m ratio for both substrates (to 4.8×10^{-2}% for $MgATP^{2-}$ and 5.9×10^{-2}% for AMP^{2-}). The Leu193Gln mutant affects the affinity for both substrates and decreases the catalytic efficiency profoundly. The K_m values for the Leu193Pro and Leu193Ser mutants showed a small elevation (2.7-fold and 1.6-fold for $MgATP^{2-}$, respectively) and a decrease to 0.1-fold for AMP^{2-} for both mutants; the k_{cat} values were decreased to 13.5 and 6.4% of the control for Leu193Pro and Leu193Ser, respectively. The k_{cat}/K_m ratio for Leu193Pro for AMP^{2-} was increased to 153% of the control, while that for Leu193Ser was decreased, yielding a catalytic efficiency for AMP^{2-} of 54% of the control; the values of k_{cat}/K_m ($MgATP^{2-}$) were nearly identical (see Table II) for both mutants. The K_m values of Leu193Phe and Leu193Arg for $MgATP^{2-}$ were largely unchanged (0.4- to 1.0-fold), while that for AMP^{2-} was marginally increased (3.2- to 3.8-fold); the k_{cat} values for these two mutants were 2.2 and 1.6% of the control. In the case of Leu193Stop, the K_m value showed a moderate increase of 11-fold for AMP^{2-} but only a 1.6-fold increase for $MgATP^{2-}$. The k_{cat} values decreased to 2.2 and 1.6% of control for Leu193Phe and Leu193Ser, respectively. The deletion mutation (Leu193-Stop), in which residues 193 and 194 are deleted from the C-terminal domain, showed an increase in K_m that was much greater for AMP^{2-} than for $MgATP^{2-}$ (11-fold *vs.* 1.6-fold, respectively). A similar result was obtained in the case of the Leu193Ile mutant, in which the values of K_m for AMP^{2-} and $MgATP^{2-}$ were increased by 32- and 7.8-fold, respectively (see Table II). The above results on seven Leu-193 mutations strongly suggest that these mutants interact with both substrate binding sites but more strongly with AMP^{2-} than with $MgATP^{2-}$.

DISCUSSION

In studies of human adenylate kinase (hAK1), site-directed mutagenesis has been performed only for the arginine and lysine residues at various locations (*17*, *18*). In this study, we evaluated the role of the C-terminal domain of human AK1 in enzymatic function by evaluating the interaction of the nucleotide substrates ($MgATP^{2-}$ and AMP^{2-}) with this region. We selected hydrophobic residues Val182, Val186, Cys187, Leu190, and Leu193, because these residues are well conserved in mammalian species (*31*) and located in the adenine ring of $MgATP^{2-}$. We performed site-directed mutagenesis and characterized the mutants by steady-state kinetics studies. We obtained 15 mutants that could be analyzed further from the total of 19 mutants constructed. Four mutants (Val186Asn, Leu190Phe, Leu190Thr, and Leu190Asn) could not be solubilized during the purification step. These changes in solubility presumably result from structural alterations to the native conformation of the expressed enzyme protein. By changing the side chain of the target residue, the biological structure-activity relationship of enzyme might be altered to some extent. The kinetic results for the target residue being changed usually show alterations in either K_m or k_{cat} values relative to the native enzyme with the side chain remaining unchanged. Steady-state kinetic analysis has been used to explain enzymatic function by considering changes observed in substrate-affinity, substrate-dissociation, and catalytic efficiency relative to the wild-type enzyme. To compare the steady-state kinetics of wild-type AK (hAK1) with those of the various mutants obtained would provide valuable information on structural and/or functional features of the enzyme. Results obtained from the study of these mutants should provide insight for more refined analyses of wild-type AK, such as by X-ray crystallography or NMR spectroscopy.

In a previous study of human AK1, the replacement of the arginines at positions 44, 97, 132, and 138 with alanine resulted in decreased catalytic efficiency (*17*). The loss of the positively charged guanidinium groups of the arginine residues inhibited catalytic efficiency, and the arginine residue was suggested to be essential for catalysis by interacting with the negatively charged phosphates of the two adenine nucleotide substrates. Site-directed mutagenesis of the lysine residues at positions 9, 21, 27, 31, 63, 131, and 194 resulted in decreased catalytic efficiency for each mutant (*18*). The loss of the positively charged ϵ-amino groups of lysine residues inhibited catalytic efficiency, and these seven lysine residues are suggested to be essential for catalysis by interacting with the negative charges of the phosphate groups on the two adenine nucleotide substrates. However, a detailed analysis of the C-terminal domain of human adenylate kinase has not yet been reported. In this study five residues (Val182, Val186, Cys187, Leu190, and Leu193) were targeted for mutation in the C-terminal domain. These residues are depicted on the left side of the wild type hAK1 model (*18*). X-ray crystallographic studies indicate that the AK protein has ten α-helices and five β-strands (*32*), and that the active center cleft opens to some extent onto the substrate binding site (*18*). A convincing detailed proposal for the nucleotide-binding sites and reaction geometry by X-ray analyses of crystalline complexes between the enzyme and sub-strates or substrate analogs (inhibitor) [p^1, p^5-di(adenosine-5′-)pentaphosphate] has not yet been presented. In the report, the ATP binding site was on the right side and the AMP site was on the left side; however, this has been revised by the *in vitro* mutagenesis studies of Kim *et al.* (*17*), that is, the ATP site is on the left side, and AMP site is on the right side. The direction of the side chains of these hydrophobic residues has not been known by the X-ray crystallographic studies, even though the C-terminal α-helix has been noted on the left side of the X-ray–deduced AK model and close to the adenine ring of $MgATP^{2-}$. Adenylate kinase is predicted to undergo large domain movements upon substrate binding (*19*). The adenosine binding center is generated by a hydrophobic pocket consisting of a β-sheet structure, the loop of residues 16–22, and the α-helices between the segments of residues 23–30 and 179–194 (*5*, *6*).

Our results for the steady-state kinetics analysis of mutants for five residues in the C-terminal domain indicate that Val182 might be located close to the $MgATP^{2-}$ site, compared to the Val186, Cys 187, Leu190, and Leu193 residues (*18*). It appears that the greater the interaction with the C-terminal domain, the more the K_m values are increased for AMP^{2-}, and to a lesser extent for $MgATP^{2-}$. From the kinetic results using Val182-mutants, Val182Ala increased the K_m values for both substrates and decreased the k_{cat} values. However, using the Val182Ser mutant, the change in the side chain from a methyl to a hydroxyl group had no effect on the K_m value, but decreased the k_{cat} value. From these two results, the Val182 residue appears to play an important role in catalysis by interacting with both substrates. The decreases in both hydrophobicity and catalytic efficiency affect the substrate-binding and phosphoryltransfer reaction in solution. From the kinetic results of 193-mutants, Leu193Pro (a bending alteration at the C-terminal end) and Leu193Ser (a change in the side chain from a methyl to a hydroxyl group) affect the AMP binding site more than the ATP site, and decrease the K_m value for AMP^{2-}. On the other hand, the results using the Leu-193Arg mutant show that a change from a nonpolar to a polar group has little effect on the K_m value for either substrate, but decreases the k_{cat} values. The above two mutation results show that not only a loss of hydrophobicity but also a C-terminal structural change or positively polar change affect both substrates. Leu193Stop, a deletion mutant lacking residues 193–194, results in a shortened C-terminal-domain. This mutant AK enzyme shows an 11-fold increase in K_m for AMP^{2-} and very little change in the K_m for $MgATP^{2-}$; the k_{cat} decreased substantially relative to the control (Table II). The Leu193Stop mutant affects enzymatic activity and might affect the affinity for AMP^{2-}, as well. Val182, Val186, Cys187, Leu190, and Leu193 contribute to the hydrophobicity in the C-terminal domain. Mutations in this region lead to a loss of hydrophobicity, which leads to an increase in K_m and decreased enzyme activity by changing the conformation of the C-terminal domain. These alterations affect the α-helices of residues 23–30 and residues131–139, which might change the K_m values for the two substrates. The flanking C-terminal region appears to interact with one or both substrates, as suggested by the results of Ayabe *et al.* (*18*) who showed that C-terminal mutations at Lys194 affect not only the affinity for $MgATP^{2-}$ but for AMP^{2-}, as well. In the present study, the steady-state kinetics results obtained using various mutant

species of hAK1 have led to the following conclusions: (i) Val182 likely plays a role in the substrate-binding of both $MgATP^{2-}$ and AMP^{2-}, but the interaction is stronger for $MgATP^{2-}$, and plays a role in catalysis; (ii) Val186 appears to play a functional role in catalysis by interacting with both $MgATP^{2-}$ and AMP^{2-} to nearly the same extent; (iii) Cys187 appears to play a functional role in catalysis; (iv) Leu190 appears to interact with both $MgATP^{2-}$ and AMP^{2-}, but to a greater extent with AMP^{2-}; (v) Leu193 appears to interact not only with $MgATP^{2-}$ but also with AMP^{2-} but to a greater extent with AMP^{2-}. The closer the residue is located to the C-terminal end, the more its mutation affects not only $MgATP^{2-}$ but also AMP^{2-} substrate binding. In chicken AK1, the results of mutations in the C-terminal showed that the K_m values for Leu190 mutants are affected to a greater extent for $MgATP^{2-}$ than for AMP^{2-} (*14*). Previous studies of the C-terminal region of chicken AK (*14*) suggested that the Leu190 residue is not catalytically indispensable, is located inside the protein and is one of the residues in a hydrophobic region that may be involved in ATP binding. The kinetic data for the two chicken AK1 mutants (L190K and a deletion mutant lacking positions 190–193) are fully in accord with our results on human AK1. The C-terminal domain may be involved in the interaction with $MgATP^{2-}$ (*18*).

In the present study, the mutation of hydrophobic residues in the C-terminal domain resulted in decreased or unchanged affinities for the two substrates and reduced catalytic efficiency. The loss of hydrophobicity in the C-terminal domain may lead to reduced binding of $MgATP^{2-}$ and AMP^{2-}, because this domain may align the phosphate groups in the two substrates to the proper conformation required for catalysis. C-terminal mutations decrease the hydrophobicity, affecting $MgATP^{2-}$, binding, an effect that might have indirectly affect the AMP^{2-} binding site. Another possibility is that the mutation affects the region encompassing residues, 16–23 and the α-helix at residues, 131–139, resulting in changes in the binding of $MgATP^{2-}$ and AMP^{2-} and the phosphoryl transfer reaction. The C-terminal α-helix of human adenylate kinase appears to interact not only with $MgATP^{2-}$ but also with AMP^{2-}, and we propose a new model for the enzymic reaction of human adenylate kinase in the enzyme reaction. It is a swing-out model of the C-terminal α-helix from the left side to the center part of the enzyme during the enzyme reaction; that is, the helix rotates to cover the adenine moieties ($MgATP^{2-}$ and AMP^{2-} substrates) (*18*). We predict that movement of the C-terminal α-helix covers and stabilizes both substrates during the enzymatic reaction in solution. Based on the X-ray crystallographic structures, the AMP binding domain is predicted to undergo a movement of 8 Å upon AMP binding and the ATP binding domain moves up to 30 Å upon ATP binding (*19*, *21*). Circular dichroism and optical rotational dichroism studies must also be performed to understand better the changes resulting from these mutations. In a previous study using our human AK mutants (*17*), the circular dichroism spectra of several mutants (Arg44Ala, Arg97Ala, Arg132Ala, Arg138Ala, and Arg149Ala) were unchanged compared with that of wild-type AK, and no secondary structural changes in these mutants upon amino acid replacement could be found. In the future, detailed analyses of our human AK1 mutants should provide a conformational model that can be used to clarify the structure-function relationships of this enzyme. The important role of the flanking C-terminal domain of adenylate kinase in catalysis may be further elucidated by X-ray crystallographic studies using substrate analogs and/or by NMR spectroscopy studies in solution. Such studies should advance our understanding of the catalytic mechanism of this important kinase.

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