"Structural and Functiona
terminal domain of Humai

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INTRODUCTION

Adenylate kinase (AK)

$$MgATP^{2-} + AMP^{2-} \rightleftarrows MgADP^{-} + ADP^{3-}$$

1. Globular protein consisting of 194 amino acid residues
2. Widely distributed in prokaryotic and eukaryotic cells
3. Consisting of three isozymes: AK1 in cytosol, AK2 in mitochondrial intermenbrane space, and AK3 in mammalian mitochondrial matrix.

Studies on Structure and Function Correlated Adenylate Kinase

1. Fluorescence-quenching studies (Hamada et al., 1979)
2. X-ray crystallographic studies (Egner et al., 1987; Dreusicke et al.,1988)
3. NMR studies (McDonald et al., 1975; Smith & Mildvan., 1982; Kalbitzer et al., 1982; Fry et al., 1985, 1986, 1987, 1988)
4. Chemical modification studies (Yazawa & Noda, 1976; Berghauser & Schirmer, 1978; Crivellone et al., 1985; Tagaya et al., 1987)
5. Site-directed mutagenesis (Gilles et al., 1986; Reinstein et al., 1988, 1990; Liang et al., 1991; Kim et al., 1989, 1990; Tagaya et al., 1989; Matsuura et al., 1989; Yoneya et al.,1990; Okajima et al., 1991; Taian et al.,1990; Yan et al., 1990; Yan &Tsai, 1991)

Target Key Residues (*Thr35 and Thr39)*

♠The35, Thr39 residues are conserved between rabbit, calf, human, porcine.
♠The synthetic peptide consisting of residues 1-45 of the rabbit muscle AK (consisting 1-44 residues) has previously shown to bind MgεATP (Hamada et al., 1979).
♠The Thr35 is next to the His36 which had been identified to be located to near substrate site (Chuan et al., 1989).
♠The Thr35 appears to exist in the center of AK and the Thr39 residue is located at the same distance from the phosphate group of MgATP and AMP in Kim's X-ray AK model (1990).
♠The Thr39 residue is very close to the adenine ring of bound AMP (Diederichs et al., 1991).
♠Thr39 is very close to an adenine site (Egner et al., 1987).
♠Thr39 is nonessential either structually or functionally by NMR analysis (Yan et al., 1990).

Materials and Methods

1. pMEX8-hAK1 vector carrying human cytosolic adenylate kinase (hAK1)
2. Purification of single strand pMEX8-hAK1 DNA by helper phage
3. Site-directed random mutagenesis of pMEX8-hAK1
4. Transformation of mutant homoduplex and small LB culture
5. Purification of double strand pMEX8-hAK1 plasmid
6. Screening of mutants by DNA cycle sequencing
7. Expression of mutants by induction of IPTG and large culture (250 ml)
8. Sonication of the harvested E. coli pellets
9. Purification of wild type and mutant enzymes by Blue sepharose chromatography and Superose 12 gel filtration.
10. Steady-state kinetic analysis of mutants.

Primer DNA sequence

T35:5'-GTAGACAGGTGYXXGTAGCCGTATTTC-3' **X; A, G, C, T**

T39:5'-GCAGGTCACCYXXAGACAGGTG-3' **Y; G, C**

l role of Threonine residues
n Cytosolic Adenylate Kinas

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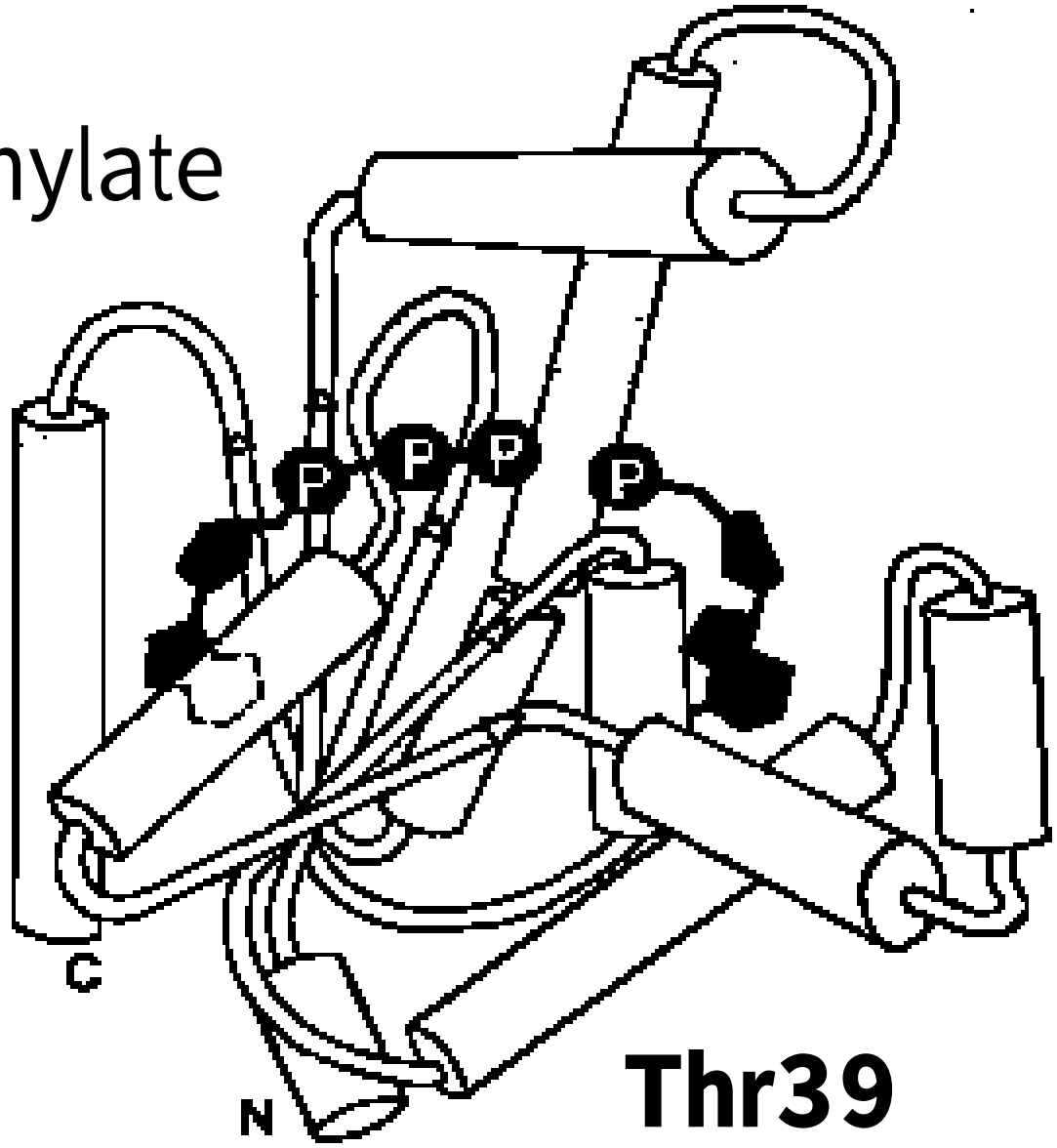
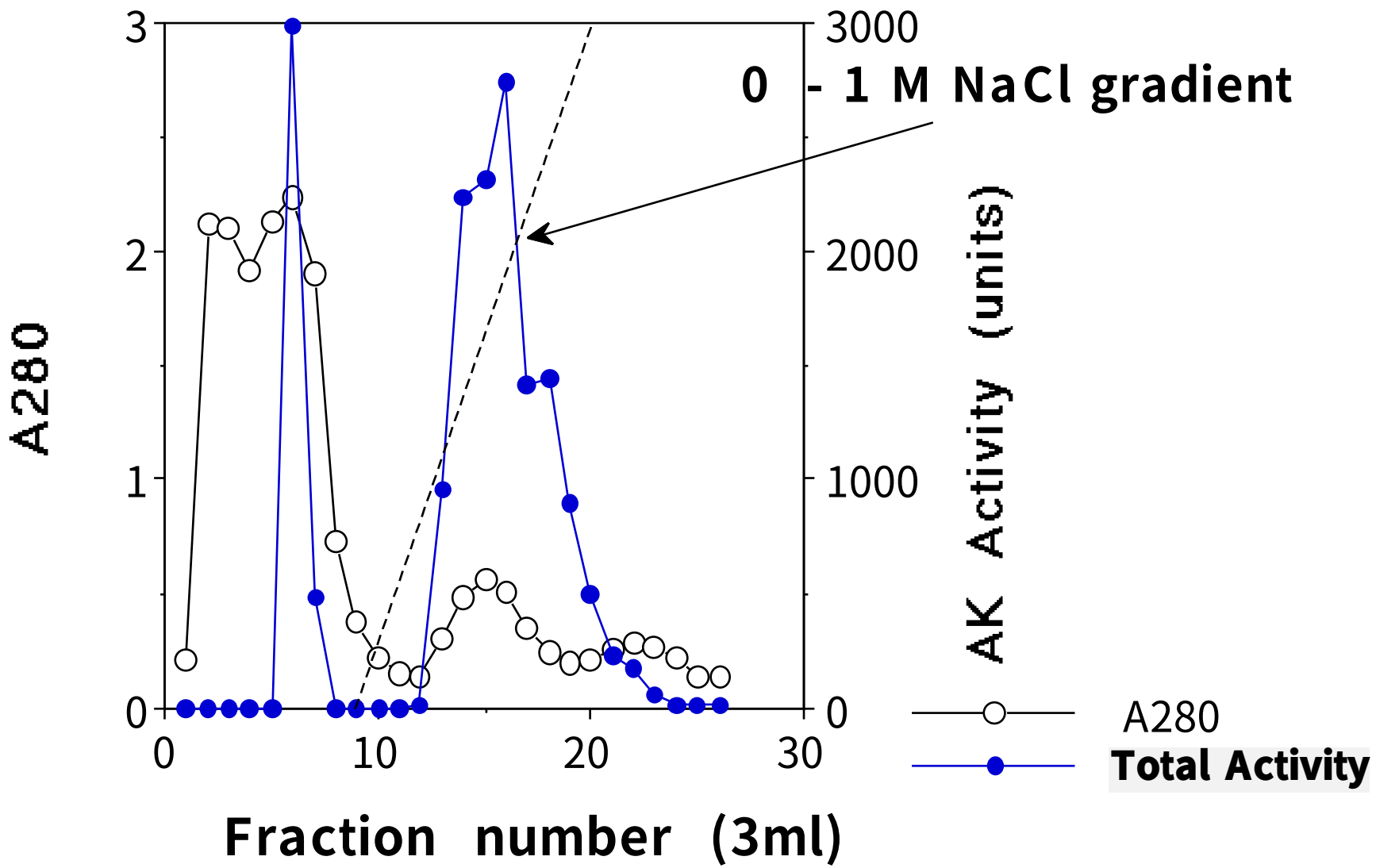
A drawing of adenine nucleotide binding site of Adenylate kinase



Blue Sepharose Column Chromatography



Kinetic results of WTAK and Thr35 mutants

Enzyme	Km ($MgATP^{2-}$) (mM)	Km (AMP^{2-}) (mM)	Vmax ($MgATP^{2-}$) (U/mg)	Vmax (AMP^{2-}) (U/mg)	k'cat (S^{-1})	k'cat/Km ($MgATP^{2-}$) ($S^{-1}\cdot M^{-1}$)	k'cat/Km (AMP^{2-}) ($S^{-1}\cdot M^{-1}$)
WTAK	0.27 (1.00)	0.33 (1.00)	1651 (100%)	1552 (100%)	589 (100%)	2.21×10^{6} (100%)	1.67×10^{6} (100%)
T35P	0.57 (2.14)	0.05 (0.15)	20 (1.21%)	62 (3.98%)	14.5±7.5 (2.6±1.4%)	1.25×10^{4} (0.57%)	4.56×10^{5} (27.34%)
T35Y	0.86 (3.22)	0.46 (1.38)	7 (0.43%)	2 (0.14%)	2±1 (0.28±0.14%)	2.93×10^{3} (0.13%)	1.65×10^{3} (0.10%)

RESULTS

(1)Various mutants of adenylate kinase (AK) could be quickly and efficiently obtained by random site-directed mutagenesis of pMEX8-hAK1.

(2)The Km values of T35P and T35Y for $MgATP^{2-}$ and AMP^{2-} were not so changed (0.15 - 3.22 fold), however, the k'cat values were decreased (0.29% - 2.6%), and the k'cat/Km values were also decreased (0.1% - 27.3%) compared to those of wild type AK (WTAK). In case of the T35P mutant, although the Km value for AMP^{2-} were decreased (0.15-fold), the k'cat and k'cat/Km values for AMP^{2-} were decreased. The Thr35 residue in N-terminal domain was presumed to play an important role in catalysis, and to maintain catalytic efficiencies rather than substrate holding.

(3)In case of T39S, T39P, and T39V, the Km values for AMP^{2-} were strikingly increased (5.58 - 58.53 fold) than those for $MgATP^{2-}$. However, in case of T39F and T39L, the Km values for $MgATP^{2-}$ were not so changed (1.90 - 3.25 fold), but those for AMP^{2-} were decreased (0.34 - 0.3 7 fold) compared to those of WTAK. The k'cat/Km values were all decreased (0.12% - 17.69%) compared to those of WTAK. The Thr39 residue might play a significant role of the interaction with α-phosphate binding of AMP, and was also suggested to involve in phosphoryl transfer.

(T35 and T39) in N-

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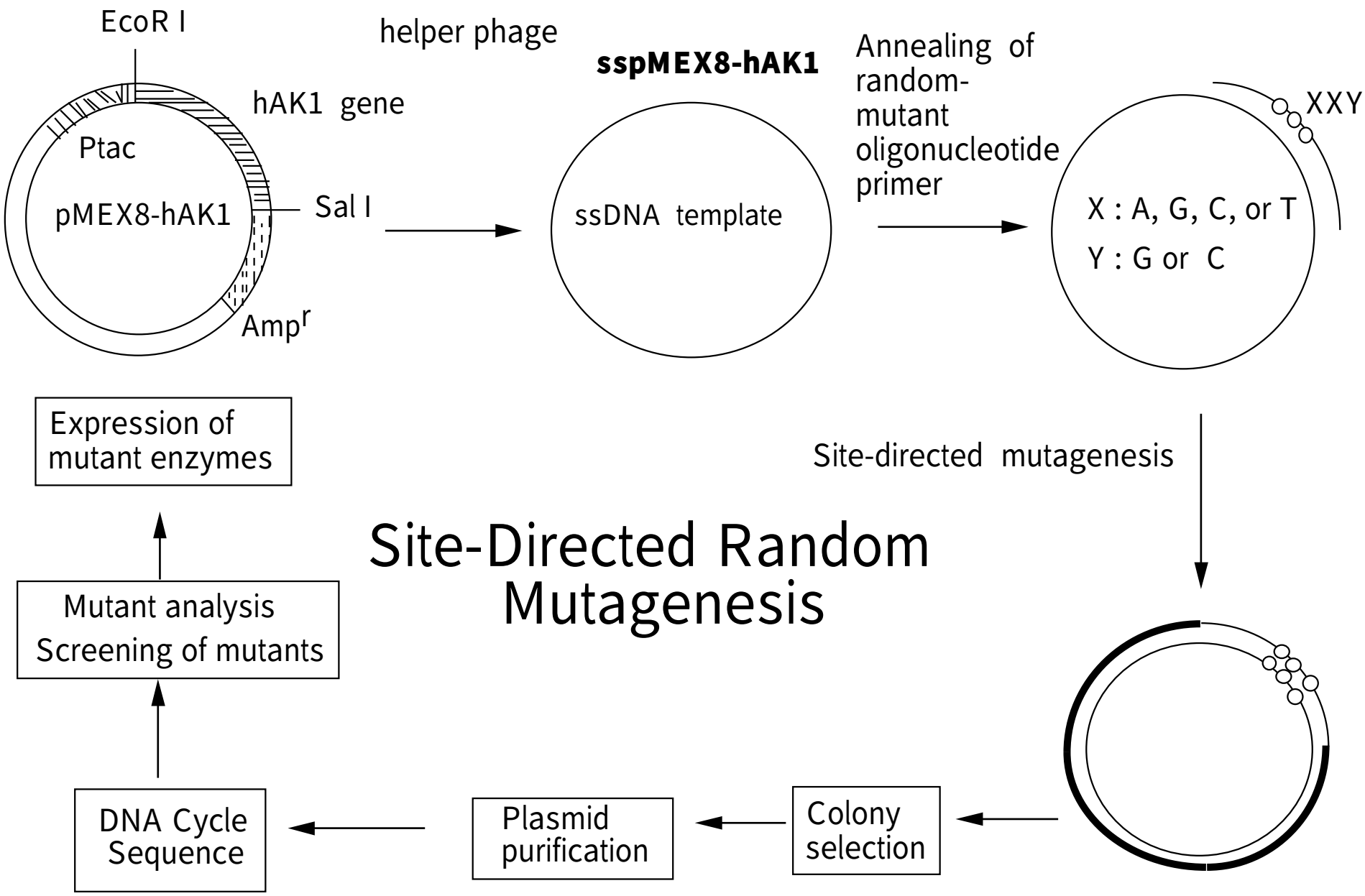

EcoR I
hAK1 gene
Ptac
pMEX8-hAK1
Sal I
Amp^r
helper phage
sspMEX8-hAK1
ssDNA template
Annealing of random-mutant oligonucleotide primer
XXY
X : A, G, C, or T
Y : G or C
Site-directed mutagenesis
Site-Directed Random Mutagenesis
Expression of mutant enzymes
Mutant analysis
Screening of mutants
DNA Cycle Sequence
Plasmid purification
Colony selection

Site-Directed Random Mutagenesis

Residue	Effectiveness	Mutants
Thr35 Residue	20% (2/10)	CCC (35-Pro) TAC (35-Tyr)
Thr39 Residue	31% (5/16)	TTC (39-Phe) TTG (39-Leu) TCC (39-Ser) CCC (39-Pro) GTC (39-Val)

Kinetic Results of WTAK and T39 mutants

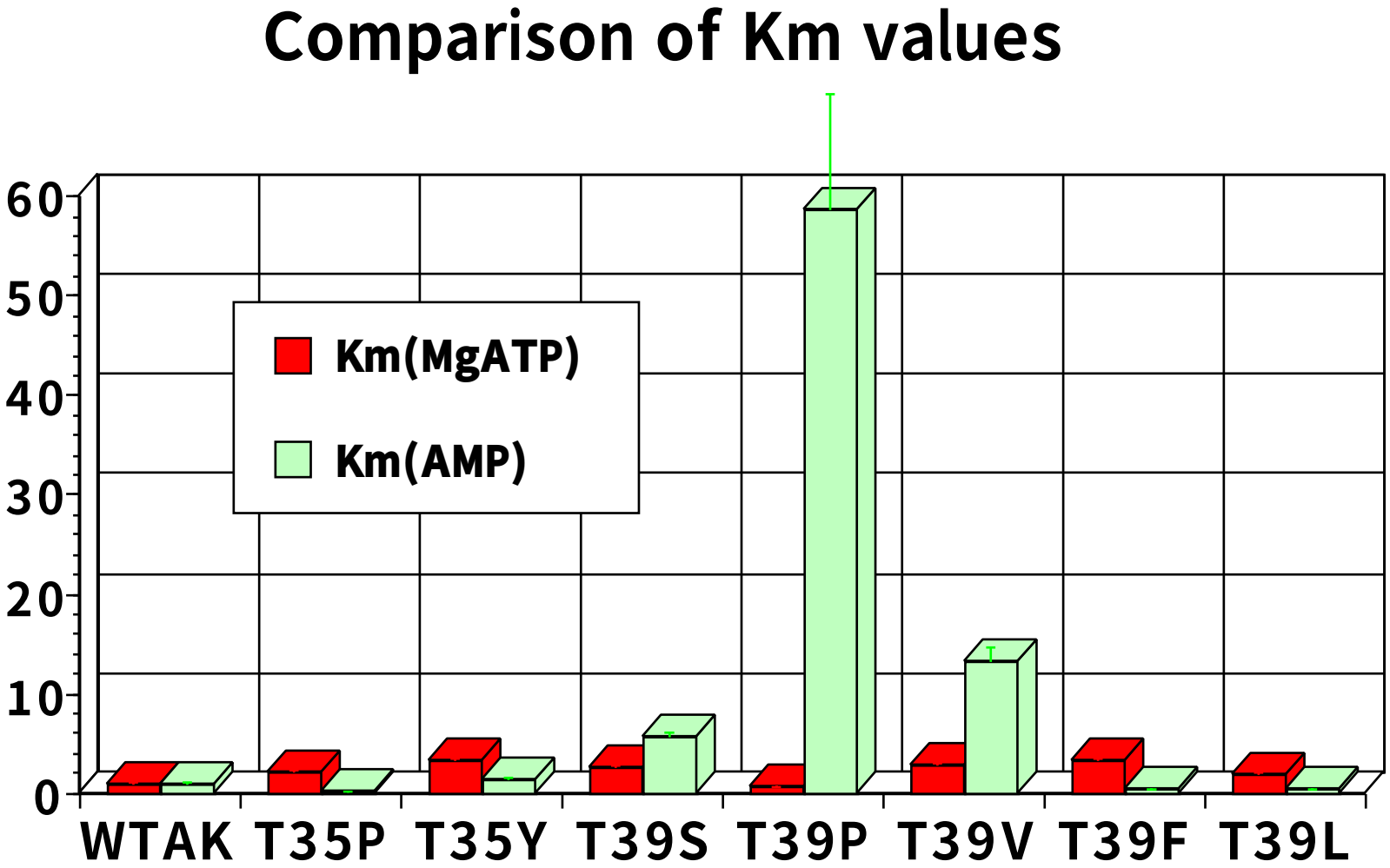
Enzyme	Km ($MgATP^{2-}$) (mM)	Km (AMP^{2-}) (mM)	Vmax ($MgATP^{2-}$) (U/mg)	Vmax (AMP^{2-}) (U/mg)	k'cat (S^{-1})	k'cat/Km ($MgATP^{2-}$) ($S^{-1} \cdot M^{-1}$)	k'cat/Km (AMP^{2-}) ($S^{-1} \cdot M^{-1}$)
WTAK	0.27 (1.00)	0.33 (1.00)	1651 (100%)	1552 (100%)	589 (100%)	2.21×10^{6} (100%)	1.67×10^{6} (100%)
T39S	0.68 (2.52)	1.84 (5.58)	34 (2%)	55 (4%)	16±4 (2.8±0.8%)	1.81×10^{4} (0.82%)	1.07×10^{4} (0.64%)
T39P	0.17 (0.64)	19.45 (58.53)	80 (4.84%)	3137 (202%)	572±547 (103.4±98.6%)	1.67×10^{5} (7.55%)	5.76×10^{4} (3.45%)
T39V	0.73 (2.74)	4.40 (13.26)	801 (48.5%)	2304 (148%)	554±268 (98.3±49.8%)	3.91×10^{5} (17.69%)	1.87×10^{5} (11.20%)
T39F	0.86 (3.25)	0.11 (0.34)	6 (0.38%)	17 (1.10%)	4±2 (0.74±0.36%)	2.57×10^{3} (0.12%)	5.48×10^{4} (3.28%)
T39L	0.51 (1.90)	0.12 (0.37)	19 (1.17%)	36 (2.33%)	10±3 (1.75±0.58%)	1.36×10^{4} (0.62%)	1.06×10^{5} (6.37%)

CONCLUSION

Thr35 and Thr39 residues interacted with adenine nucleotide denoting each different mode

ABSTRACT

To elucidate key residues of substrate binding and of catalytic mode for hum cytosolic adenylate kinase (hAK1 EC 2.7.4.3), we introduced replacement at T35 residue to Tyr, Pro, and also T39 residue to Ser, Pro, Val, Phe, and Leu. Mutant enzymes were obtained by the random site-directed mutagenesis method using an artificial hAK1 gene (Kim, H. J. et al. Protein Engineering 2 (pp.379-386, 1989). From the kinetic analysis, (1)the Km values of T35P, T for $MgATP^{2-}$ and AMP^{2-} were not so changed (0.15 - 3.22 fold), however, th cat values were decreased less (0.29% - 2.6%) compared to those of wild ty AK (WTAK). In case of T35P mutant, the Km value for AMP^{2-} were decrease (0.15-fold), the k'cat values for AMP^{2-} were also decreased. The Thr35 resi in N-terminal domain was presumed to play an important role in catalysis, an to maintain catalytic efficiencies rather than substrate holding. (2)In case o 9S, T39P, and T39V mutants, the Km values for AMP^{2-} were strikingly increased (5.58 - 58.53 fold) than those for $MgATP^{2-}$ compared to those of WTAK. However, in case of T39F, T39L mutants, the Km values for $MgATP^{2}$ were not so changed (1.90 - 3.25 fold), but those for AMP^{2-} were decreased 34 - 0.37 fold) compared to those of WTAK. The k'cat values were all decreased (0.12% - 17.69%) compared to those of WTAK. The Thr39 resid might play a significant role of the interaction with α-phosphate binding of AMP, and was also suggested to involve in phosphoryl transfer.


Comparison of Km values
60
50
40
30
20
10
0
Km(MgATP)
Km(AMP)
WTAK
T35P
T35Y
T39S
T39P
T39V
T39F
T39L

Comparison of k'cat values