"Site-Directed Ra
in N-terminal do

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ndom Mutagenesis of
main and C-terminus c

Hamada[1)], Hitoshi Takenaka[1)], O
Nagahama[2)], Atsushi Yamamotc
2nd Surgery, [3)] Primate Research In
Kiyotake, Miyazaki-gun, Miyazaki, 88

Lysine residues (K9,
of Human Adenylate Ki

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K21, K27, and K194)
inase (AK1)"

ıka[4)], Hideharu Maruyama[1)],

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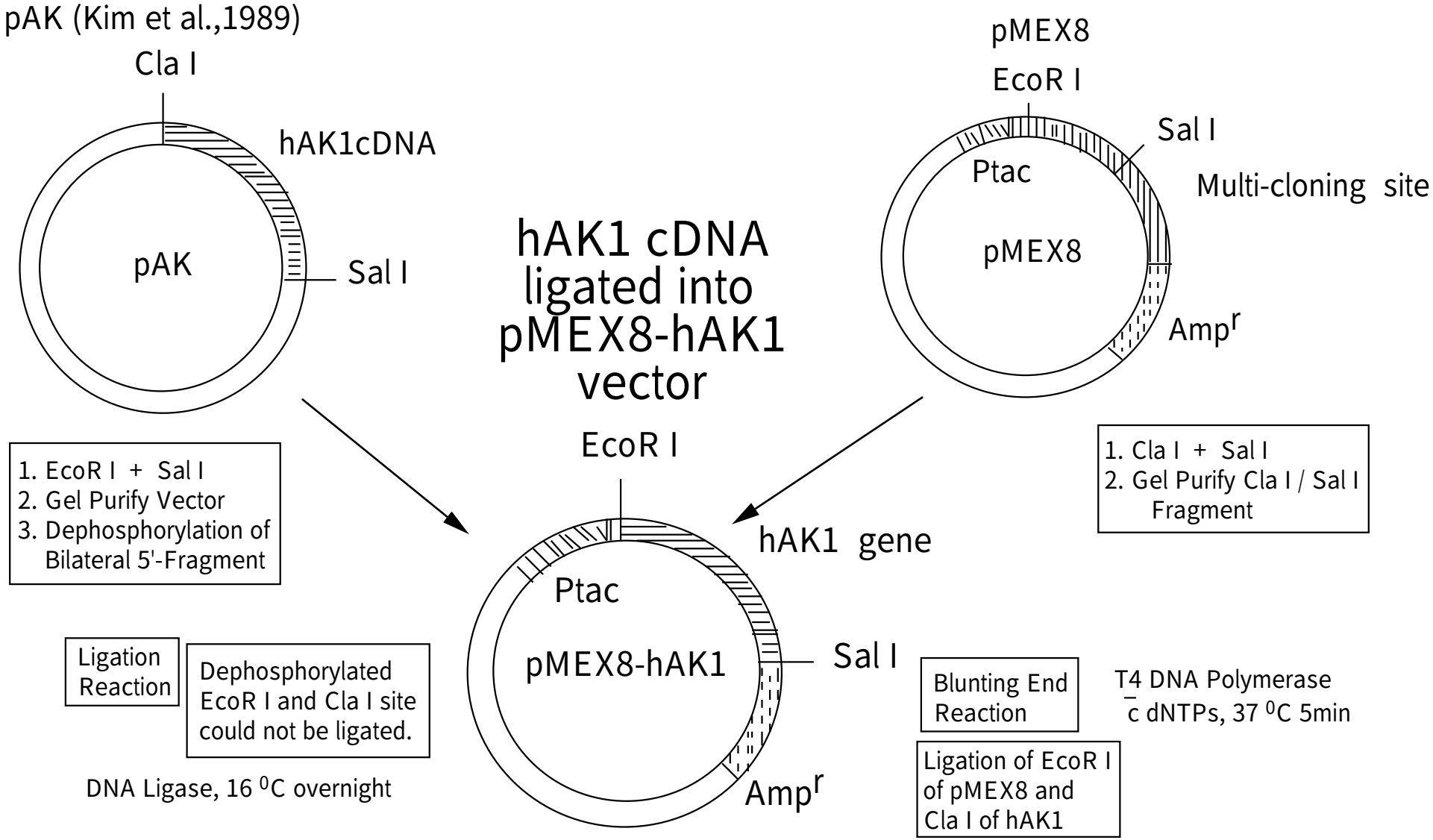
ABS1

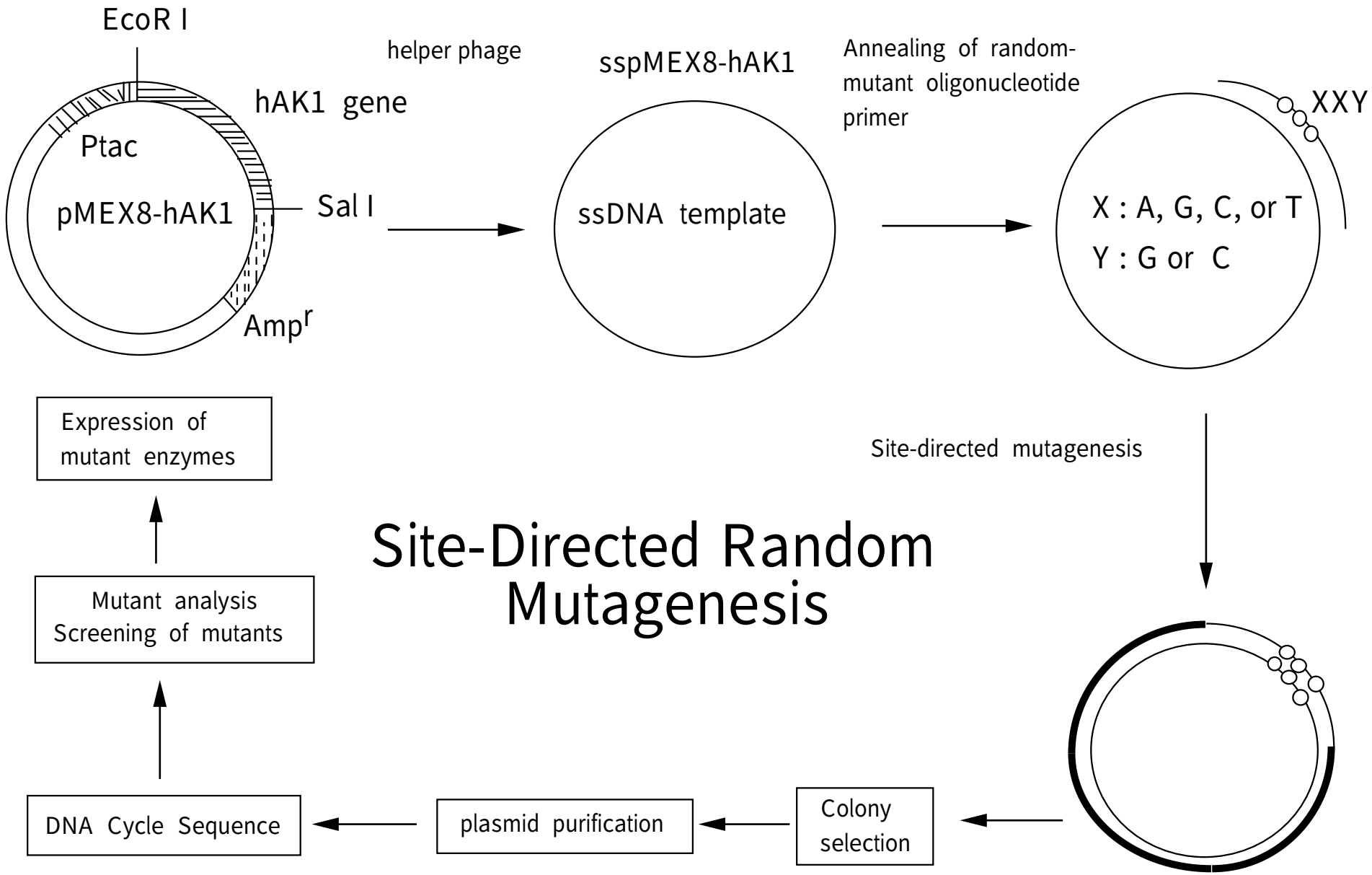
To elucidate the structural factors of substrate
cytosolic adenylate kinase (hAK1 EC 2.7.4.3)
residues to Phe, Pro, Arg, and Ile using pME
by the random site-directed mutagenesis met
gene (Kim, H. J. et al. Protein Engineering 2 (
analysis, (1)from the results of K9F kinetics, v
values with unchanged Kms for $MgATP^{2-}$ and
presumed to play an important role for catalys
decreased k'cat value, this might be played a
reaction. (3)The K27R was assumed to be inv

RACT

e binding and of catalytic mode for human
, we have replaced K9, K21, K27 and K194
X8-hAK1 vector. Mutant enzymes were obtained
:hod with a chemically synthesized artificial hAK1
(5) pp.379-386, 1989). Based on the kinetic
which was shown decreased k'cat and k'cat/Km
l AMP^{2-}, K9 residue in N-terminal domain was
sis. (2)K21P in the glycine rich region revealed
n significant role in transphosphorylation
volved an $MgATP^{2-}$ binding site from markedly

Construction of pMEX8-hAK




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
Colony selection
plasmid purification
DNA Cycle Sequence
Mutant analysis Screening of mutants
Expression of mutant enzymes

decreased k'cat values. (4) K194I mutant de
increasing affinity to $MgATP^{2-}$ substrates. Th
to play a role of catalysis and substrate-bindi
considered to be essential for enzymatic activ
may be interacted with nucleotide having eac

INTROD

Adenylate

$$MgATP^{2-} + AMP^{2-} \rightleftharpoons$$

1. Globular protein consisting of 194 amino a

creased catalytic parameters in spite of
en, K194 residue in C-terminus was suggested
ng and an appropriate affinity to substrates was
vity. It is expected that these key lysil residues
h different mode.

DUCTION

kinase (AK)

$\rightleftarrows$ $MgADP^{-}$ + ADP^{3-}

cid residues

DNA sequence of primers

Lys21

	15	16	17	18	19	20	21	22	23	24	25	26	27	
	Gly	Gly	Pro	Gly	Ser	Gly	Lys	Gly	Thr	Gln	Cys	Glu	Lys	
5'-	GGC	GGC	CCG	GGT	TCT	GGC	**AAA**	GGT	ACC	CAG	TGC	GAG	AAA	-3'
			3'-	CCA	AGA	CCG	**XXY**	CCA	TGG	GTC	ACG	-5'		

Lys27

	21	22	23	24	25	26	27	28	29	30	31	32	33	
	Lys	Gly	Thr	Gln	Cys	Glu	Lys	Ile	Val	Gln	Lys	Tyr	Gly	
5'-	AAA	GGT	ACC	CAG	TGC	GAG	**AAA**	ATC	GTG	CAG	AAA	TAC	GGC	-3'
			3'-	GG	GTC	ACG	CTC	**XXY**	TAG	CAC	G	-5'		

Lys9 5'- C GAA GAT GAT **YXX** AGT CTT CTT AAG C -3'

Lys194 5'- CGA CTA TTA **XXY** CAG AGC GTC C -3'

XXY : codon consisted of random bsase

X: A, G, C, or T base Y : G or C base

Site-Directed Random Mutagenesis

Residue	Effectiveness	Mutants
Lys9	10% (1/10)	TCC (9-F)
Lys21	10% (1/10)	CCG (21-P)
		CGC (27-R)
Lys27	20% (2/10)	GTC (27-V)
Lys194	10% (1/10)	ATA (194-I)

Purification of Mutants

Enzyme	Expression yields	Specific Activity
WTAK	3.70 mg	100 %
Lys9Phe	1.93 mg	0.2 %
Lys21Pro	0.16 mg	11 %
Lys27Arg	0.52 mg	4 %
Lys194Ile	1.88 mg	0.3 %

2. Widely distributed in prokaryotic and eukar
3. Consisting of three isozymes: AK1 in cytos
   space and AK3 in mammalian mitochondri

Studies on Structure and Functi

1. Fluorescence-quenching studies (Hamada
2. X-ray crystallographic studies (Egner et al.
3. NMR studies (McDonald et al., 1975; Smit
   Fry et al., 1985, 1986, 1987, 1988)
4. Chemical modification studies (Yazawa &
   Crivellone et al., 1985; Tagaya et al., 1987
5. Site-directed mutagenesis (Gilles et al., 19

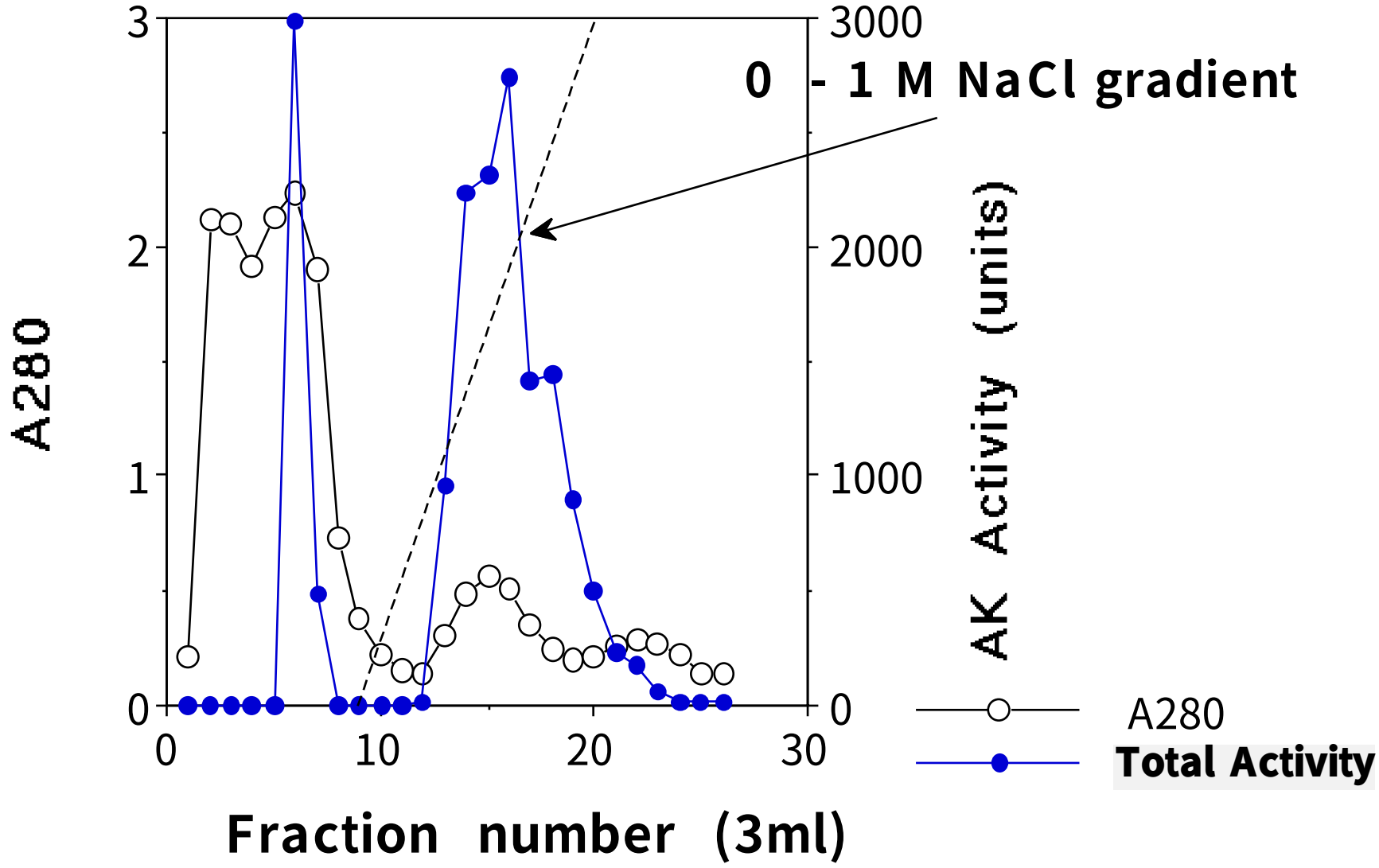
yotic cells
ol, AK2 in mitochondrial intermenbrane
al matrix.

on Correlated Adenylate Kinase

et al., 1979)
., 1987; Dreusicke et al.,1988)
h & Mildvan., 1982; Kalbitzer et al., 1982;

Noda, 1976; Berghauser & Schirmer, 1978;
)
86; Reinstein et al., 1988, 1990; Liang et

Blue Sepharose Column Chromatography



Purification Summary of WTAK expressed in E. coli

			Enzyme activity		Purification	
Fraction	Volume (ml)	Protein (mg)	Total (units)	Specific (units/mg)	Fold	Yield (%)
Cell-free extract	10	36.8	12880	350	1	100
Blue Sepharose	15	8.24	12010	1224	3.6	27.5
Superose 12	4	7.0	11200	1600	5.3	19

al., 1991; Kim et al., 1989, 1990; Tagaya e
al.,1990; Okajima et al., 1991; Taian et al.,

Target Key Res

1) What Function Dose N-terminal dom

♥K9 residue in N-terminal domain is existed i
back- and downward of AK model by Pai et

2) Proposed Role of Lys21

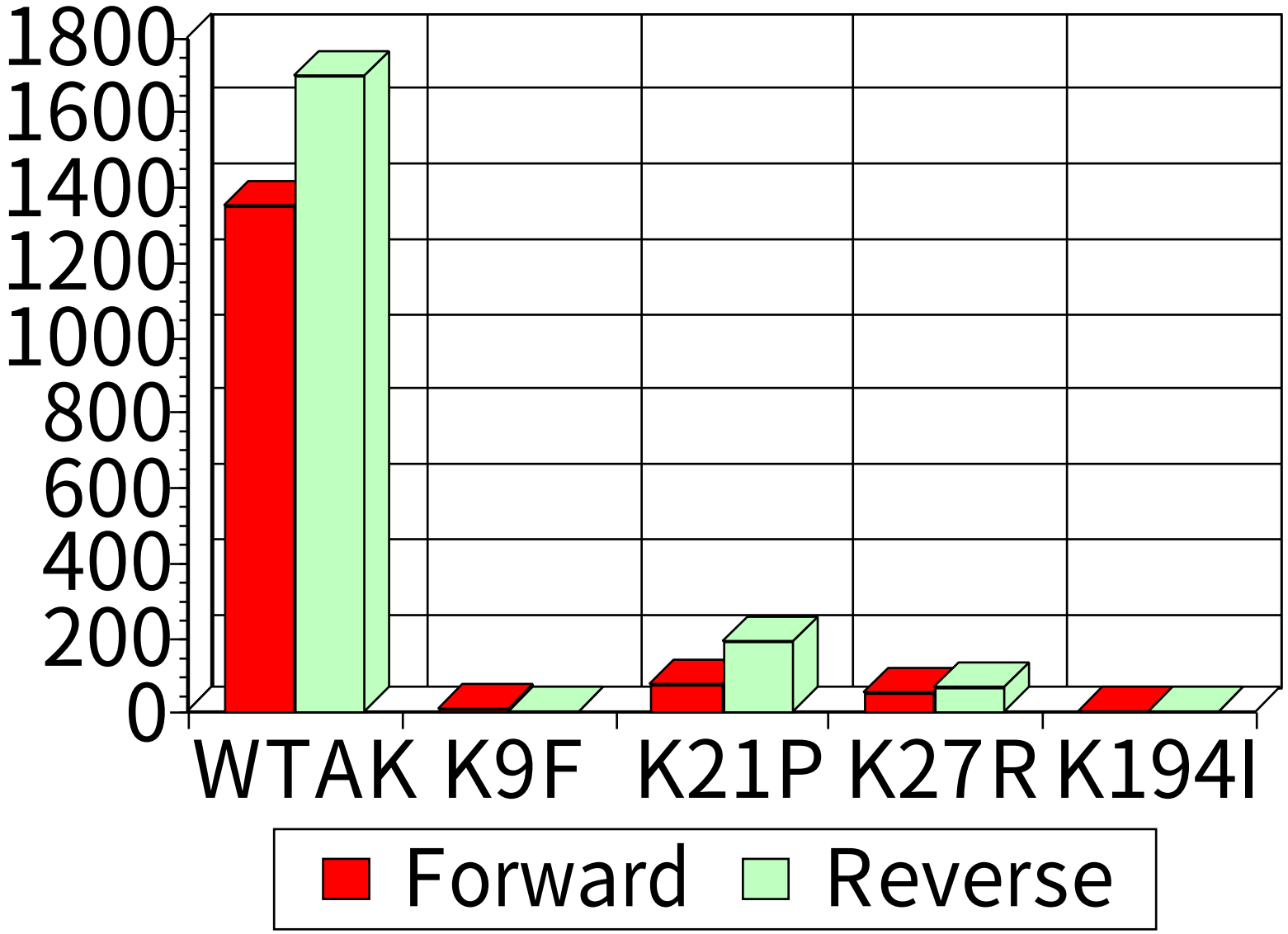
t al., 1989; Matsuura et al., 1989; Yoneya et
,1990; Yan et al., 1990; Yan &Tsai, 1991)

idues of hAK1

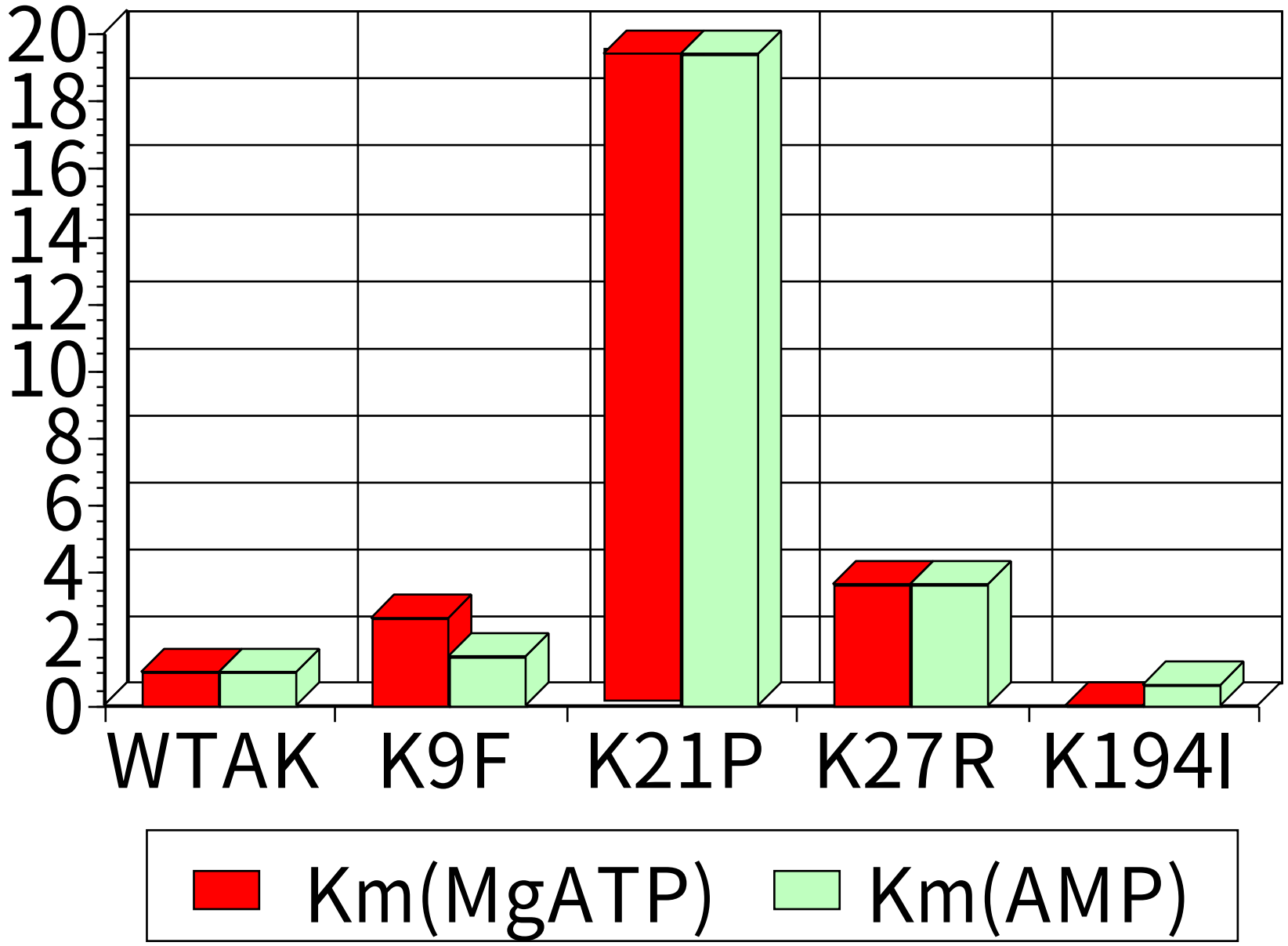
ain have?

n β-sheet after the first α-helices located in
al. (1977)

The specific activities



Comparison of Km values



♦Participation to phosphate-binding glycine-ri
P-G-X-G-K-G from X-ray model by Pai et al.
♦A motif as one of key residues to interact wi
al., 1977)
♦Suggestion to move and interact with α-pho
1985, 1986, Mildvan & Fry, 1987)
♦Lys21 forms hydrogen bonds with both the
AMP (Caldwell & Kollman, 1988)

3) Does Lys27 interact with γ-phosphat

♠Interaction with γ-phosphate of MgATP (Fry

ich loop (Res 15 - 22) in adenylate kinase: G-X-
(1977)
th γ-phosphate of ATP in X-ray model (Pai et

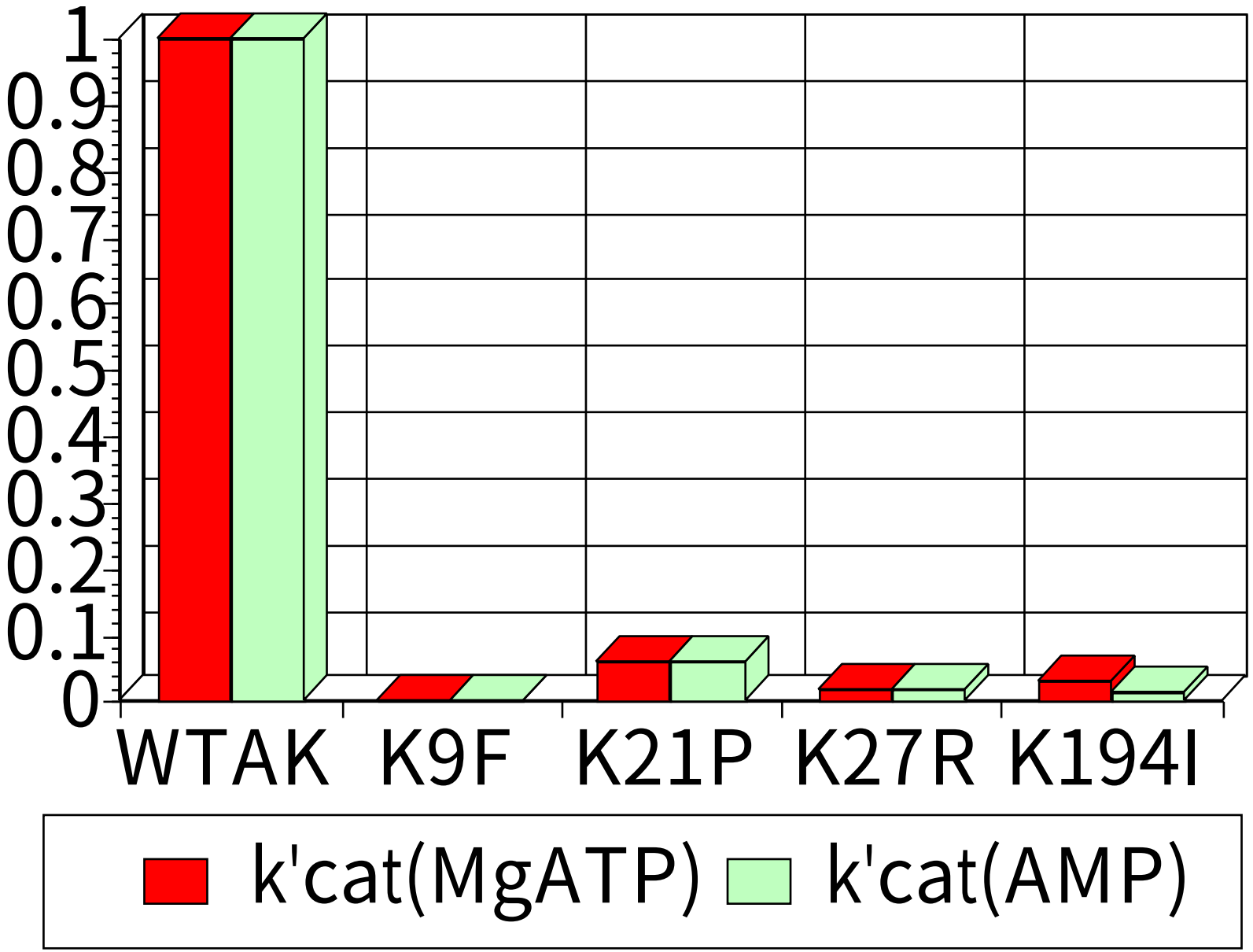
sphate of MgATP in NMR model (Fry et al.,

γ-phosphate of ATP and the α-phosphate of

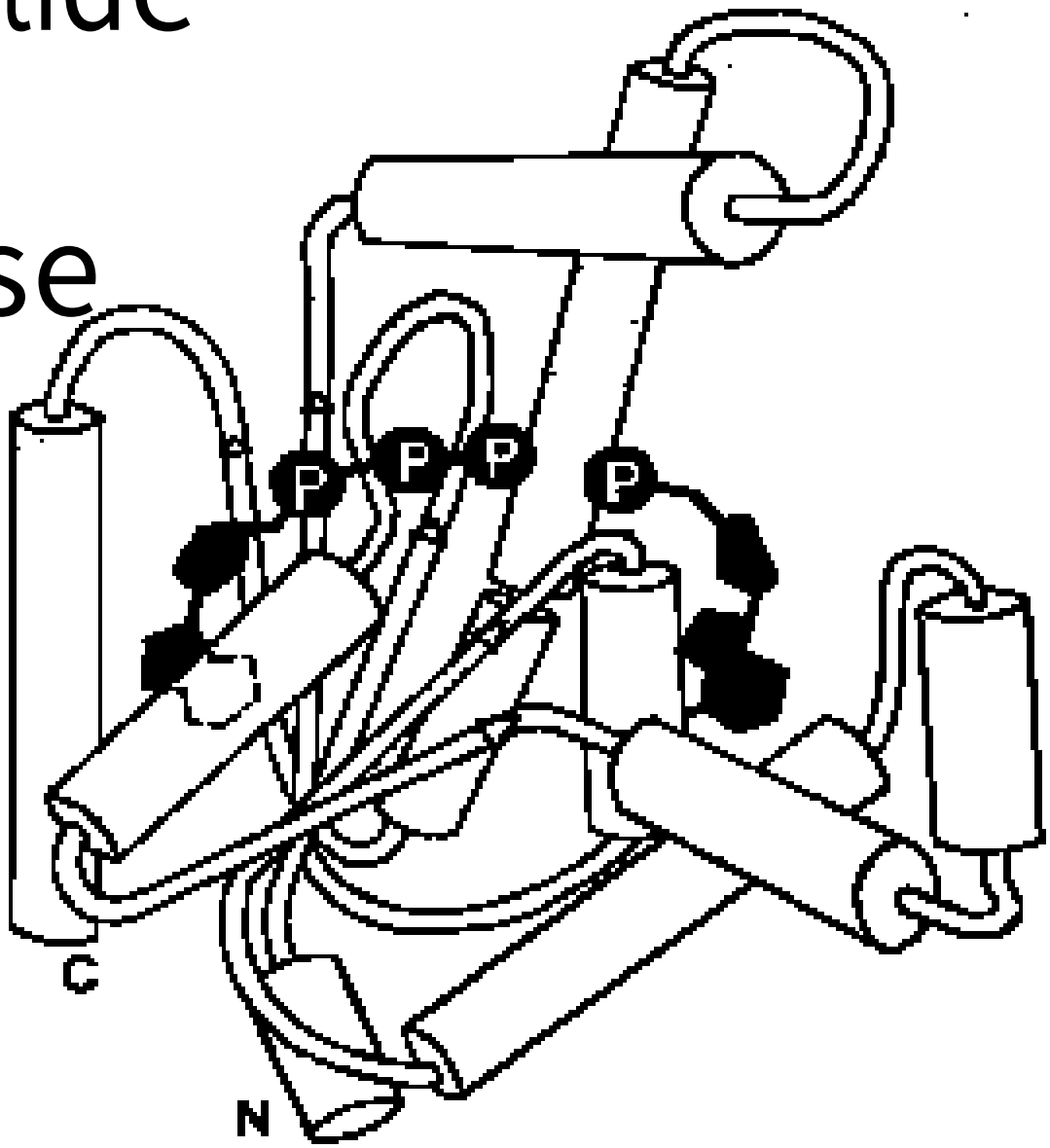
e ?

et al., 1985; Mildvan & Fry, 1987)

Comparison of k'cat values



A drawing of adenine nucleotide binding site of Adenylate kinase



3) Does C-terminus involve in catalysis

- ♣ C-terminal domain is denoted at left side of close to MgATP binding site. (Kim et al., 19
- ♣ K190 in C-terminal domain of chicken AK w than AMP^{2-}.

PLAN

1. Construction of pMEX8-hAK1 vector
2. Elucidation for the structural factors of sub cytosolic adenylate kinase (hAK1 EC 2.7,4
3. Fulfillment of random site-directed mutage

s ?

AK model (Pai et al., 1977) and is shown to be
990)
was presumed to interact with $MgATP^{2-}$ greater

NING

strate binding and of catalytic mode for human
.3)

RES

(1) Various mutants could be quickly and effic
mutagenesis

(2) Based on the kinetic analysis, K9F showec
retained Km, K9 residue in N-terminal dom
role for catalysis.

(3) K21P in the glycine rich region indicated d
significant role in phosphotyl transfer.

ULTS

iently obtained by random site-directed

d decreased k'cat and k'cat/Km values with
ain was presumed to play an important

ecreased k'cat value, might be played a

3. Fulfillment of random site-directed mutage
   random specific oligonucleotide primer
4. Kinetic analysis of mutants at lysine residu

Materials an

Items;

1. Construction of human cytosolic ade
2. Purification of single strand pMEX8-
3. Site-directed random mutagenesis c
4. Transformation and purification of d
5. Screening of mutant by DNA cycle s
6. Expression and purification of WTA
7. Kinetic analysis of forward and reve

nesis at key residue by simple annealing with

es (K9, K21, K27, and K194 residues)

nd Methods

enylate kinase (hAK1) vector
-hAK1 DNA
of hAK1
ouble strand pMEX8-hAK1 plasmid
sequencing
K and mutant
erse reaction of AK

(4)K27R was assumed to be involved MgATF

(5)K194I showed decreased catalytic parame
K194 residue in C-terminus was suggested
binding.

CONCI

Lys9, Lys21, Lys27, and Lys194
interacted with adenine nucleotic

$^{2-}$ binding site.

ters in spite of increasing affinity to $MgATP^{2-}$
d to play a role for catalysis, and substrate-

LUSION

· residues;
de denoting each different mode