"Site-Directed Mutagenesis and Functional Analysis of the Active-Site Residues (K21, K27, and T39) of Human Adenylate Kinase (AK1)"

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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)

1) Proposed Role of Lys21

- Participation to phosphate-binding glycine-rich loop (Res 15 - 22) in adenylate kinase: G-X-P-G-X-G-K-G from X-ray model (Pai et al.,1977)
- A motif as one of key residues to interact with γ-phosphate of ATP in X-ray model (Pai et al., 1977)
- Suggestion to move and interact with α-phosphate of ATP in NMR model (Fry et al.,1987, Mildvan & Fry, 1987)
- Formation of hydrogen bonds with both the γ-phosphate of ATP and the α-phospahte of AMP (Caldwell & Kollman, 1988)

2) Does Lys27 interact with phosphate ?

♠ **Interaction with γ-phosphate of MgATP**

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

3) Does Thr39 interact with adenine moiety ?

- Same distance from the phosphate groups of MgATP and AMP in X-ray model (Pai et al., 1977)
- Proximity to adenine moiety in NMR model (Egner et al., 1987)
- Proximity to adenine ring of AMP without direct interaction in NMR studies (Yan et al., 1990)

PLANNING

1. Construction of pMEX8-hAK1 vector

2. Elucidation for the structural factors of substrate
   binding and of catalytic mode for human cytosolic adenylate kinase (hAK1 EC 2.7,4.3)

3. Fulfillment of random site-directed mutagenesis at key residue by simple annealing with random specific oligonucleotide
   primer

Materials and Methods

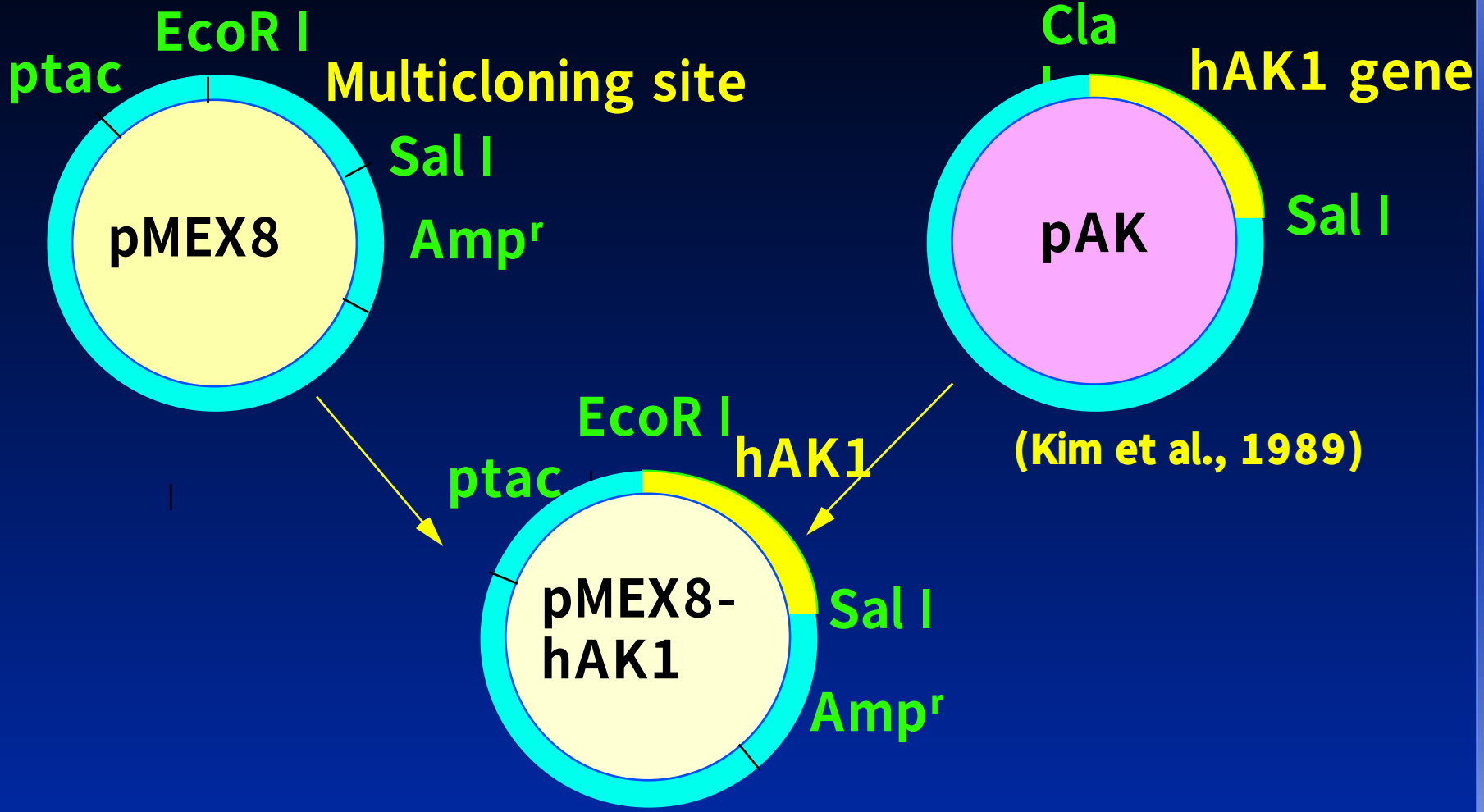
1. Construction of human cytosolic adenylate kinase (hAK1) vector
2. Purification of single strand pMEX8-hAK1 DNA
3. Site-directed random mutagenesis of hAK1
4. Transformation and purification of double strand pMEX8-hAK1 plasmid
5. Screening of mutant by DNA cycle sequencing
6. Expression and purification of WTAK and mutant
7. Kinetic analysis of forward and reverse reaction of AK

Construction of pMEX8-hAK1 vector

1. JM101/pAK [JM101 transformed with plasmid pAK ligated with human cytosolic adenylate kinase cDNA] (Kim et al., 1990)

2. Plasmid pMEX8 vector from Funakoshi Co., Ltd. (Tokyo)

Construction of pMEX8-hAK1



Advantage of pMEX8-hAK1 vector utilization

1. Single strand DNA with the aid of helper phage
2. Potent protein expression with IPTG induction (isopropyl-β-D-thiogalactopyranoside)
3. Ampicillin resistant gene inserted
4. Direct DNA cycle sequence with double strand DNA
5. Site-directed mutagenesis

DNA sequence of primers

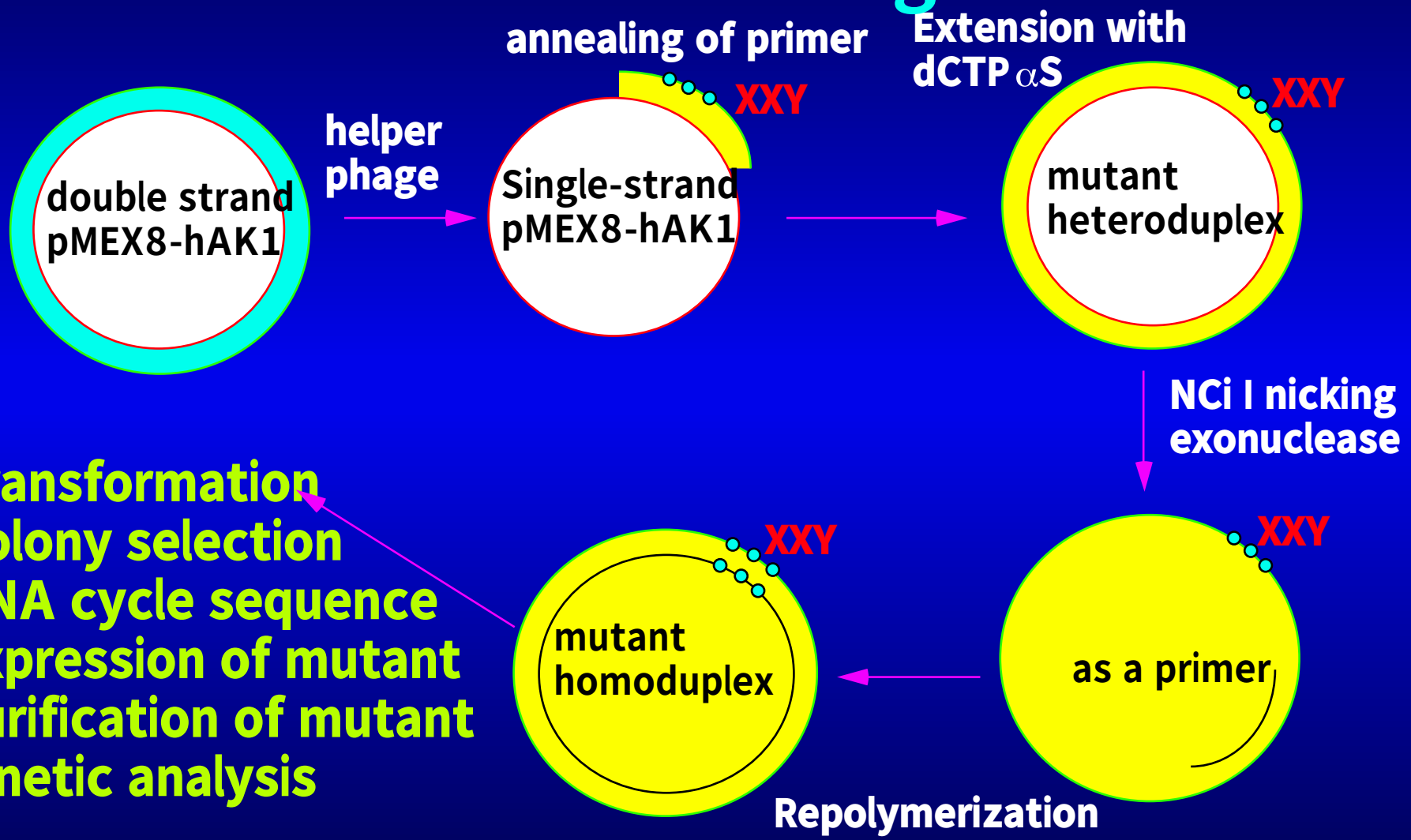
XXY codon (X; A,G,C,or T: Y; G or C)

A1			3'-	CCA	AGA	CCG	XXY	CCA	TGG	GTC	ACG	-5'	
	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'
	CCG	CCG	GGC	CCA	AGA	CCG	TTT	CCA	TGG	GTC	ACG	CTC	TTT

A2			3'-	GG	GTC	ACG	CTC	XXY	TAG	CAC	G	-5'	
	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'
	TTT	CCA	TGG	GTC	ACG	CTC	TTT	TAG	CAC	GTC	TTT	ATG	CCG

A3			3'-	GTG	GAC	AGA	XXY	CCA	CTG	GAC	G	-5'	
	33	34	35	36	37	38	39	40	41	42	43	44	45
	Gly	Tyr	Thr	His	Leu	Ser	Thr	Gly	Asp	Leu	Leu	Arg	Ser
5'-	GGC	TAC	ACT	CAC	CTG	TCT	ACT	GGT	GAC	CTG	CTG	CGT	TCC -3'
	CCG	ATG	TGA	GTG	GAC	AGA	TGA	CCA	CTG	GAC	GAC	GCA	AGG

Site-Directed Random Mutagenesis



1. Transformation
2. Colony selection
3. DNA cycle sequence
4. Expression of mutant
5. Purification of mutant
6. Kinetic analysis

Screening of Mutants

1. Reaction mixture for site-directred random mutagenesis transformed with Competent TG1 cells [TG1{K12, △(lac-pro), supE, thi, hsdD5/F'traD36,proA+B+, laclq, lacZ△ M15
2. Selection of single mutant colony on LB ampicillin plates
3. Inoculation of each single mutant clone into small scale of LB medium (5 ml)
4. Purification of double strand DNA of mutant strain
5. DNA cycle sequence of mutant DNA

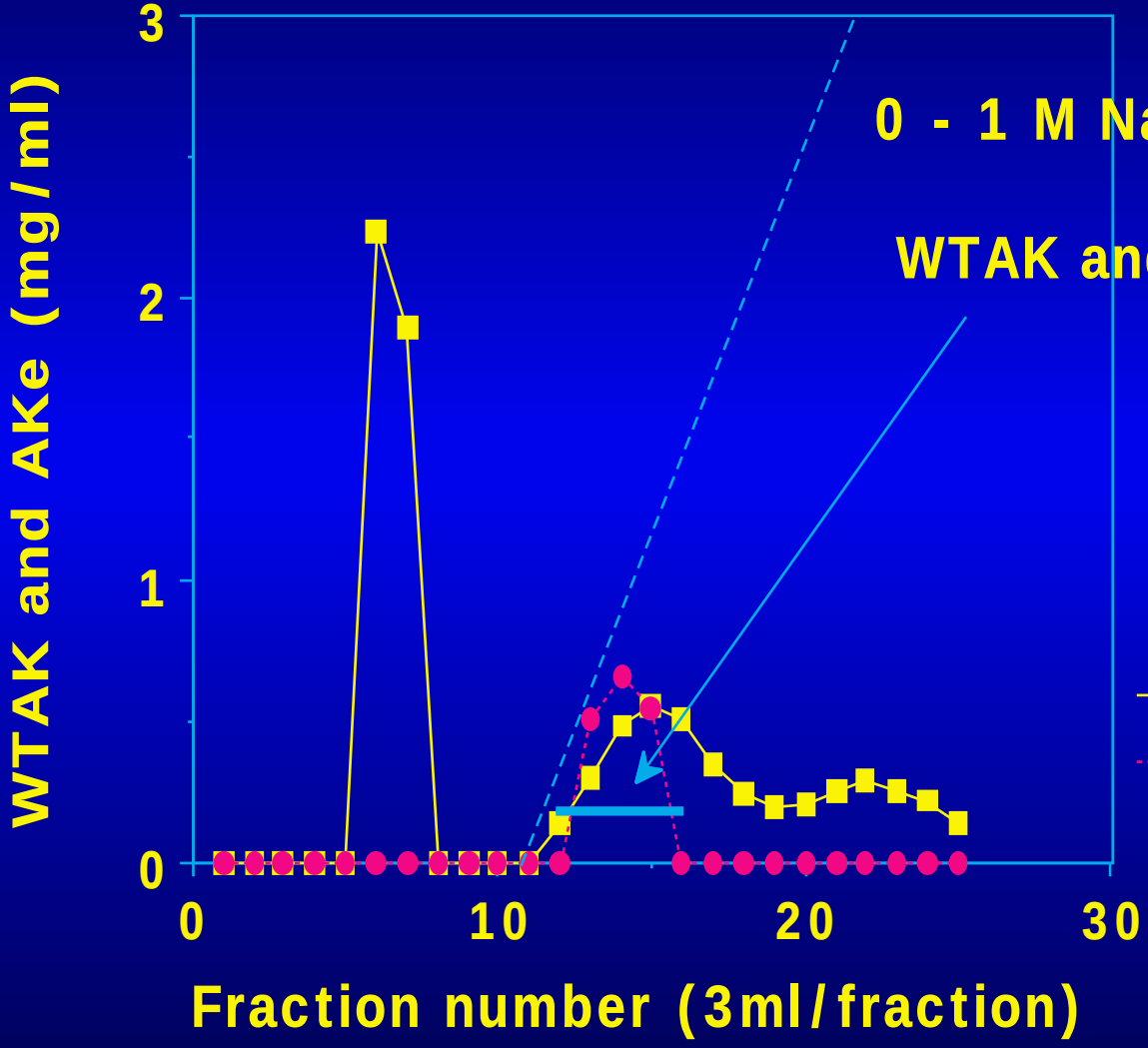
Expression of WTAK and Mutants

1. Small culture (LB 5ml containing 50 mg/ml of ampicillin) for 12 hr
2. Middle scale culture of LB medium (250ml) for 8 hr
3. IPTG induction (at 1 mM isopropyl-b-D-thiogalactopyranoside)
4. Culture for 16 hr

Purification of WTAK and Mutants

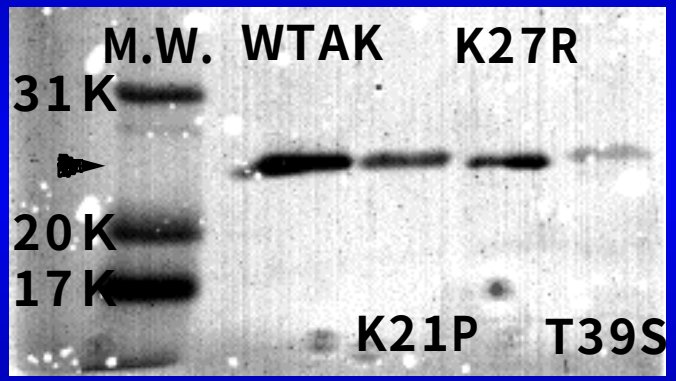
1. Collecting cells by centrifugation.
   (at 8,000 X g for 10 min at 4 ^{0}C)
2. Pellet suspended in standard buffer.
   (20 mM Tris-HCl, 1 mM EDTA, 0.1 mM DTT, pH 7.4)
3. Cell disruption by soinication.
   (at 20 kHz, 20 W for 3 min at 3 ^{0}C using sonicator)
4. Cell debris removed by centrifugation
   at 12,000 X g for 20 min at 4 ^{0}C.
5. Supernatant obtained, stored at -70 ^{0}C after freezing in liquid nitrogen prior to use.
6. Blue Sepharose column chromatography.
7. Protein elution, checked by SDS-PAGE staining by Quick CBB.
8. AK eluate loaded onto Superose 12 column gel filtration.
9. Protein eluate, checked by SDS-PAGE staining by Quick CBB.

Blue Sepharose Column Chromatography



0 - 1 M NaCl gradient

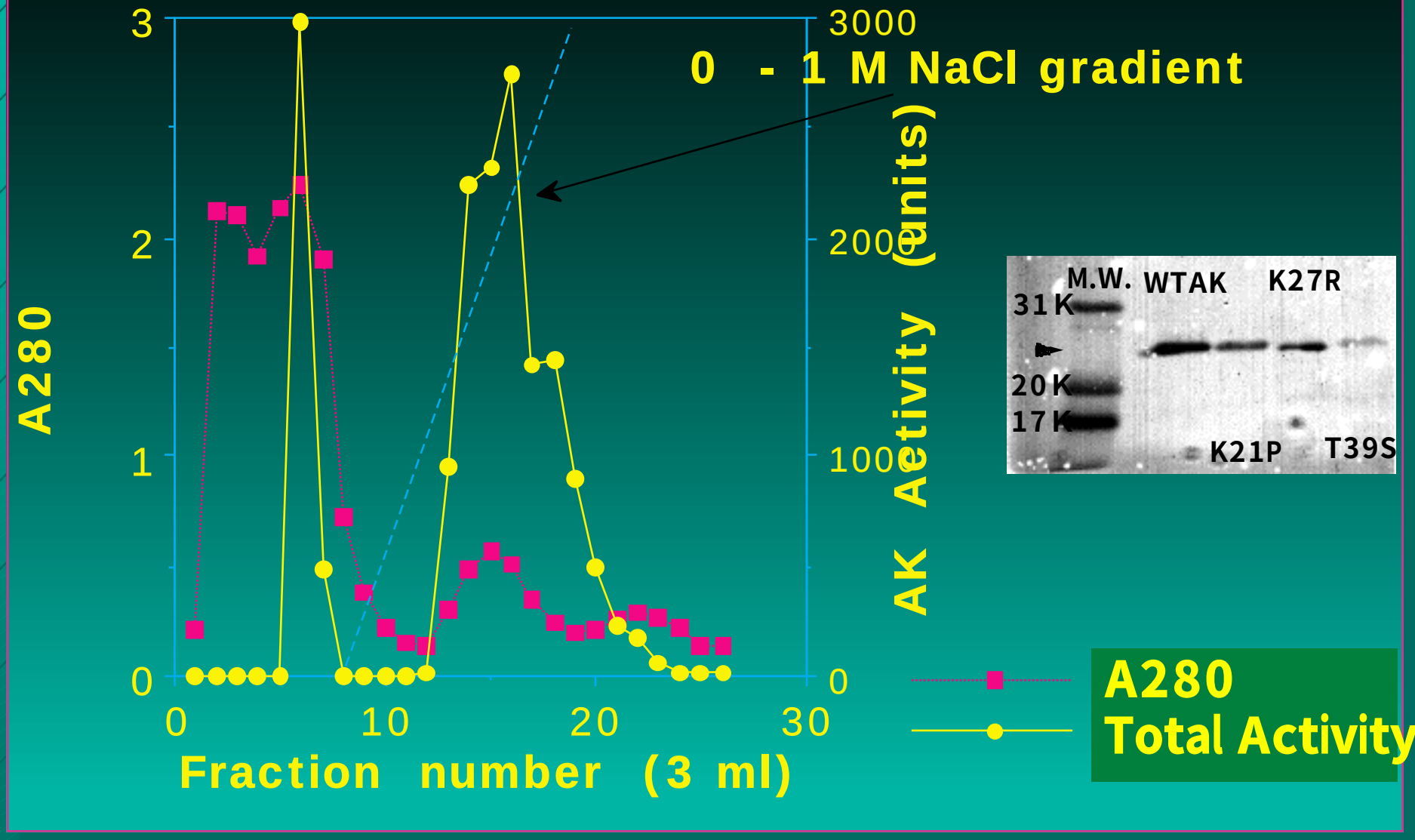
WTAK and AKe Eluate



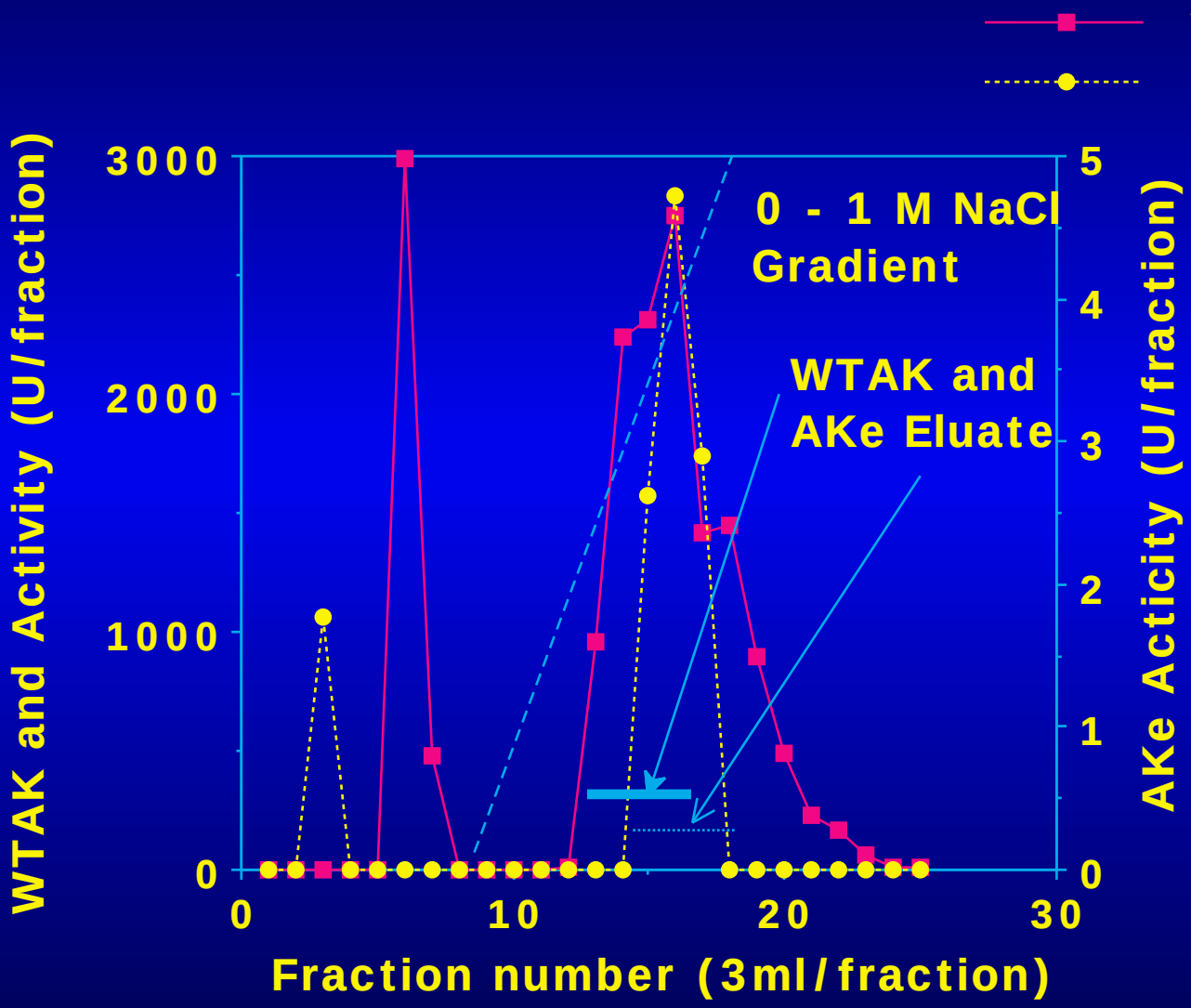
WTAK

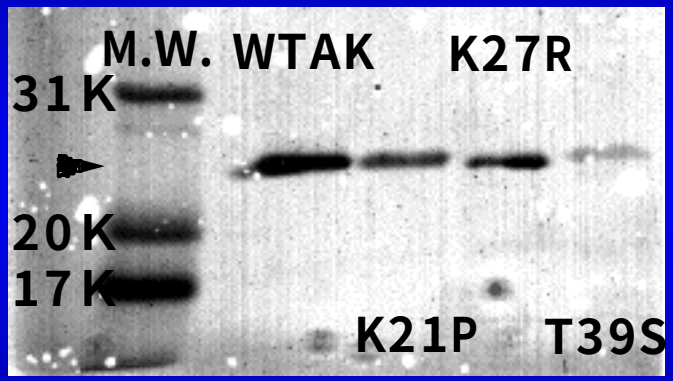
AKe

Blue Sepharose Column Chromatography

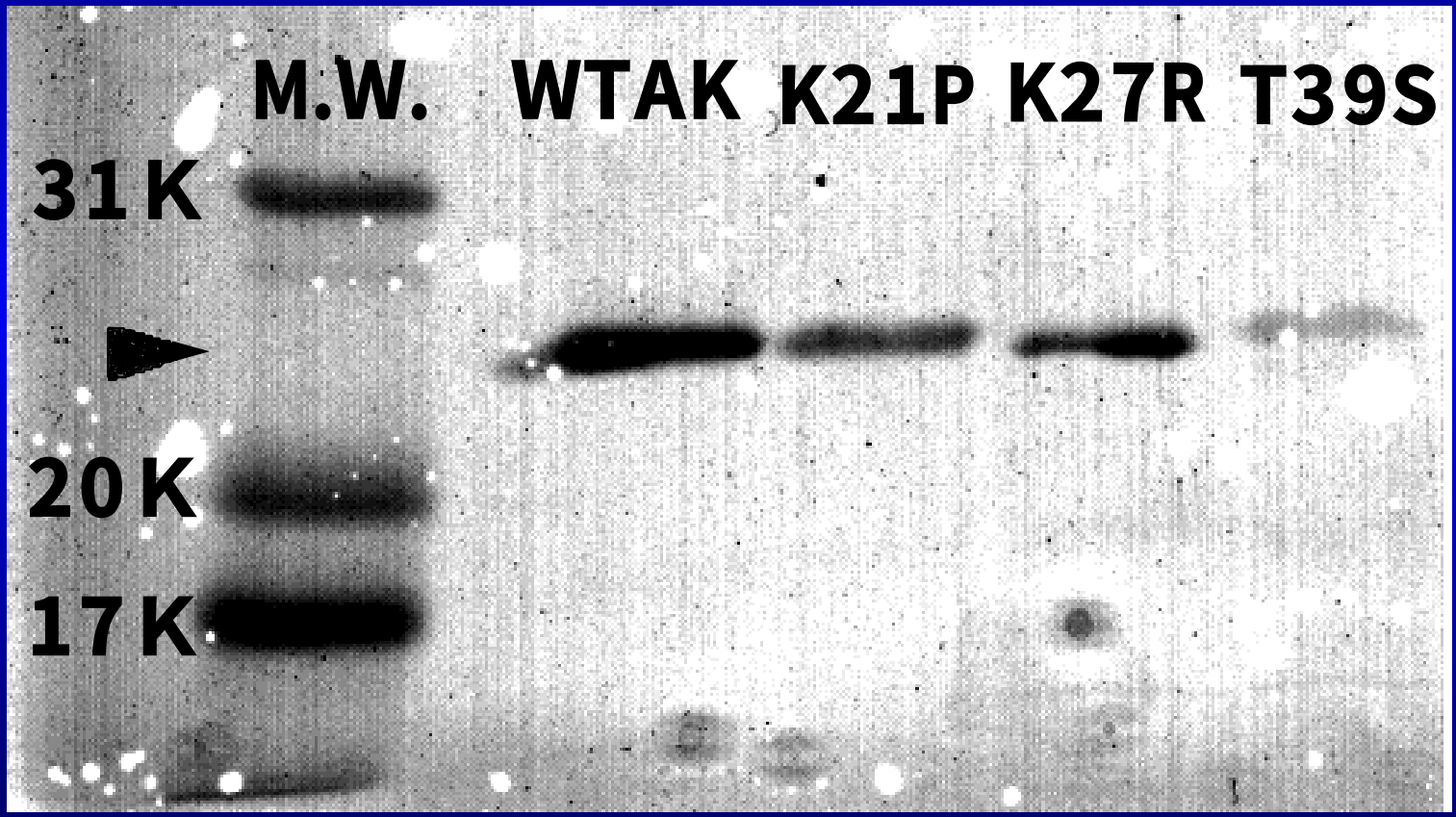


Blue Sepharose Column Chromatography





12.5% SDS-PAGE



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

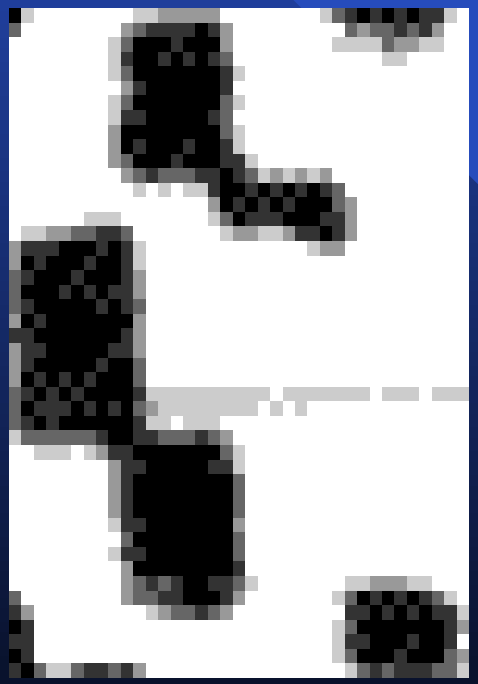
Purification summary of WTAK and AKe

Purification Step		TG1(pMEX8hAK1) WTAK	TG1(without plasmid) AKe
Culture medium	($O.D._{600}$)	2.19	1.94
E.coli wet pellet	(g)	5.0	4.1
Cell-free extract	Protein (mg/ml)	3.68	4.13
	Activity(U/mg)	350	2.3
Blue sepharose	Protein (mg/ml)	0.55	0.11
	Total Protein (mg)	8.24	0.97
	Activity (U/mg)	1224	10.5

Results of DNA sequence (WTAK vs K21P)

WTAK

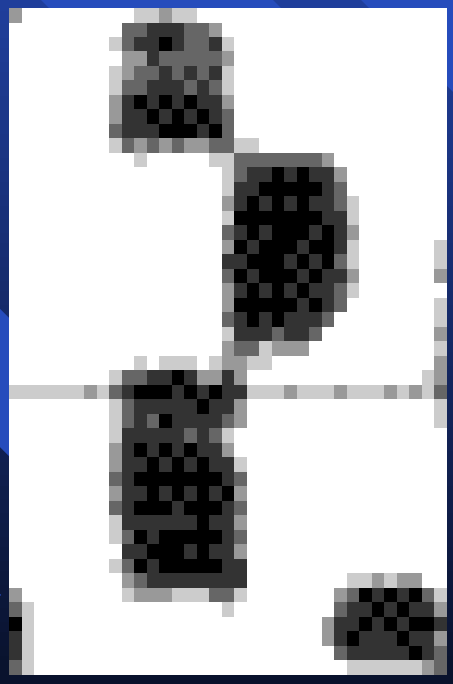
A G C T



G		
G	Gly20	Gly20
C		
A		
A	Lys21	Pro21
A		
G		
G	Gly22	Gly22
T		

K21P

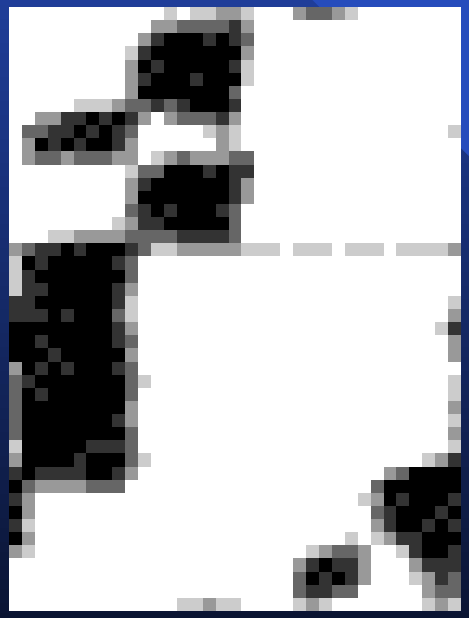
A G C T



Results of DNA sequence (WTAK vs K27R)

WTAK

A G C T



G A G A A A A T C

Glu26

Lys27

Ile28

K27R

A G C T

G A G C G C A T C

Glu26

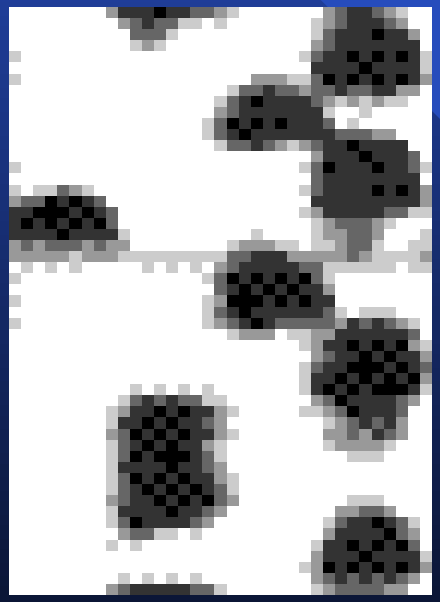
Arg27

Ile28

Results of DNA sequence (WTAK vs T39S)

WTAK

A G C T



T C T A C T G G T

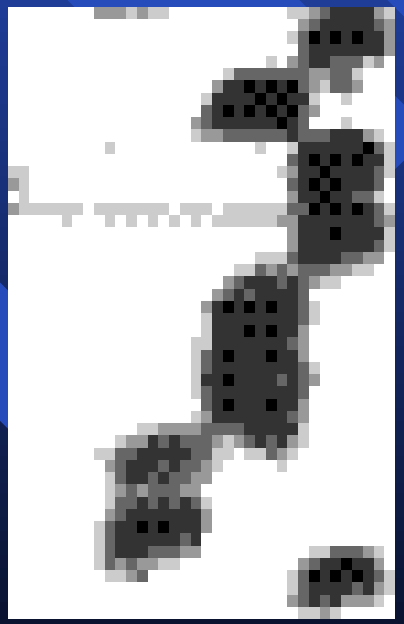
Ser38

Thr39

Gly40

T39S

A G C T



Ser38

Ser39

Gly40

T C T T C C G G T

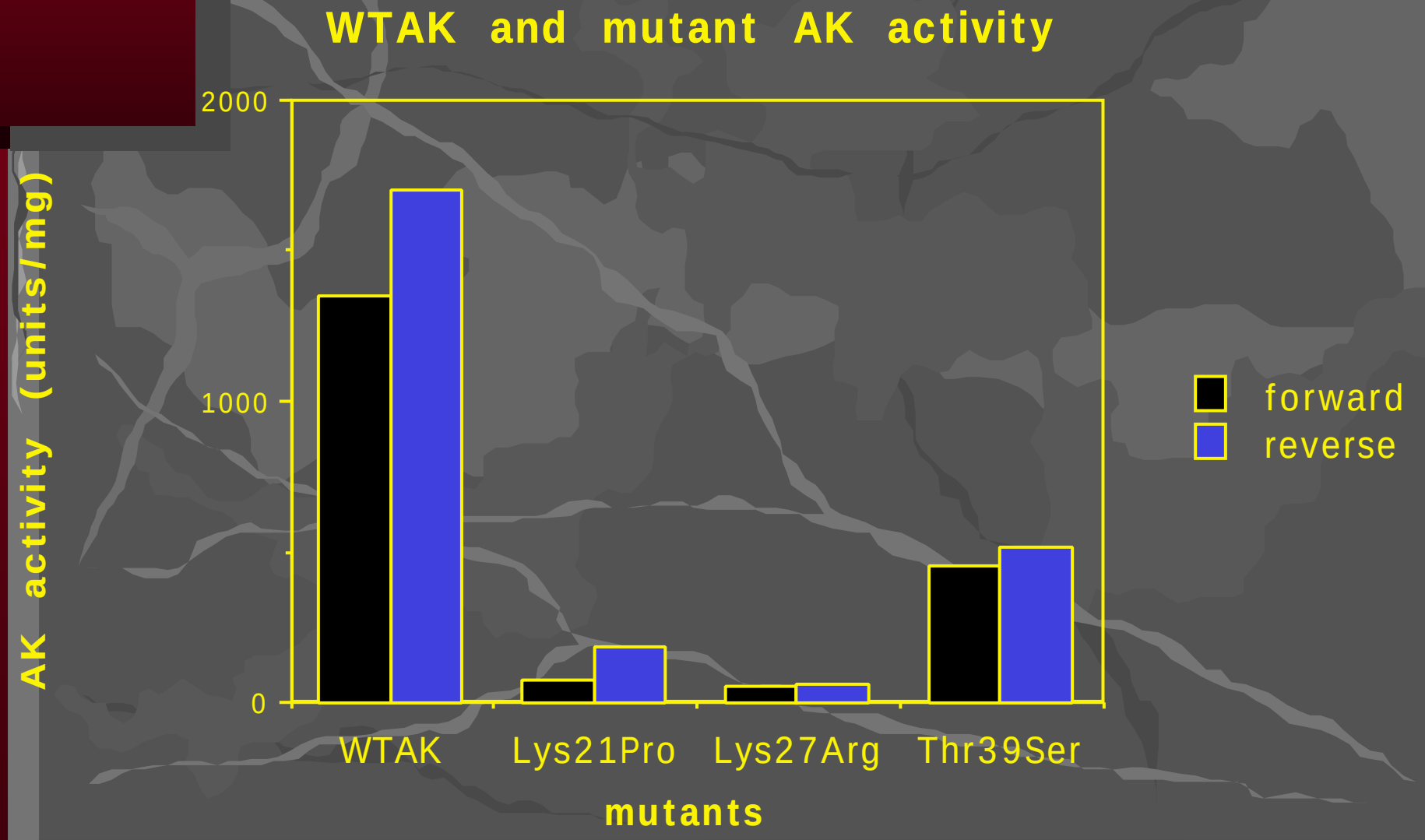
Site-Directed Random Mutagenesis

Residue	Effectiveness	Mutants
Lys21	10 %	CCG (21-P)
Lys27	20 %	CGC (27-R) GTC (27-V)
Thr39	30 %	TCC (39-S) CCC (39-P) GTC (39-V)

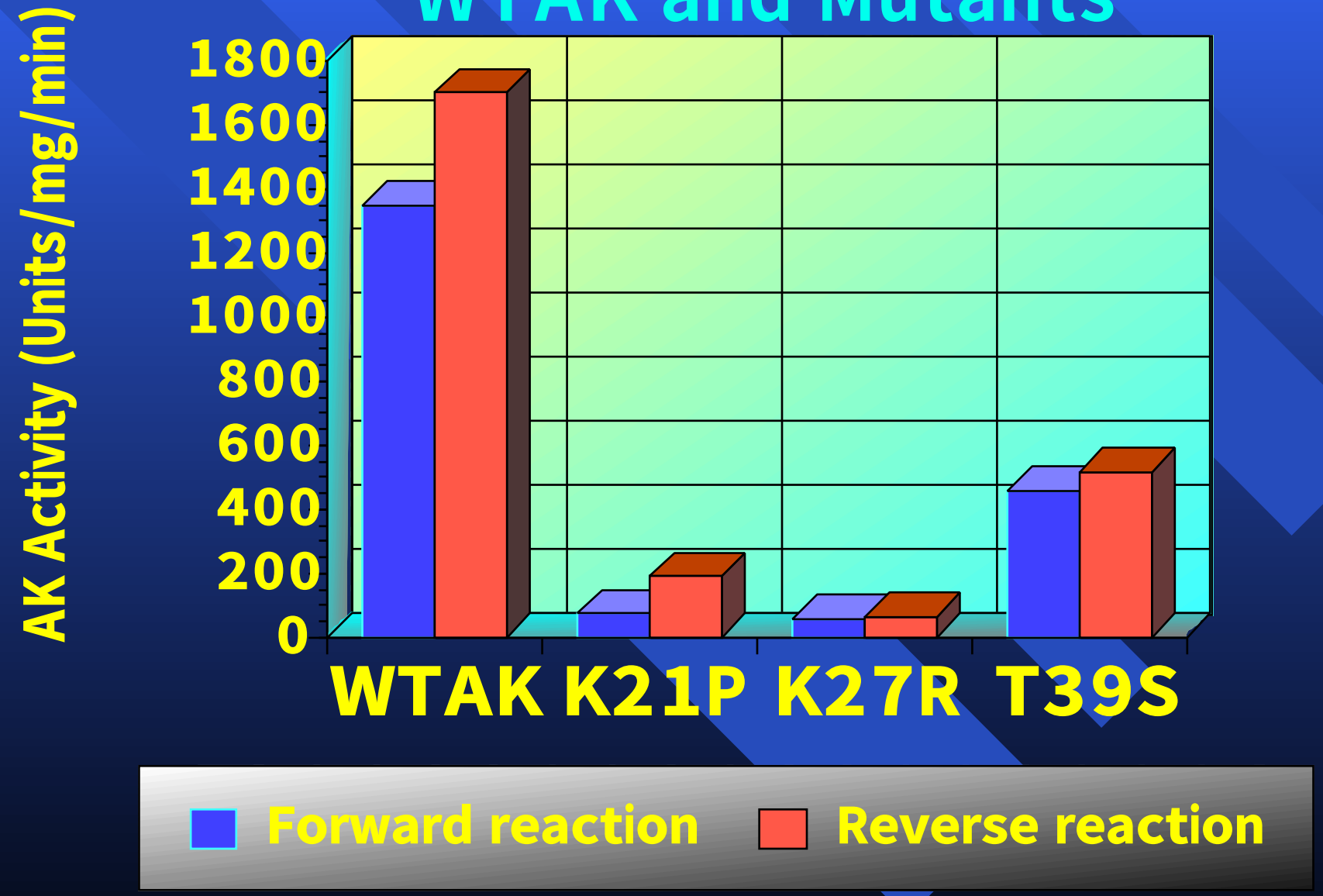
Purification of Mutants

	Expression yields	Specific Activity
WTAK	3.7 mg	100 %
K21P	0.16 mg	2 %
K27R	0.52 mg	7 %
K39S	1.4 mg	20 %

The Specific Activity



The Specific Activities of WTAK and Mutants



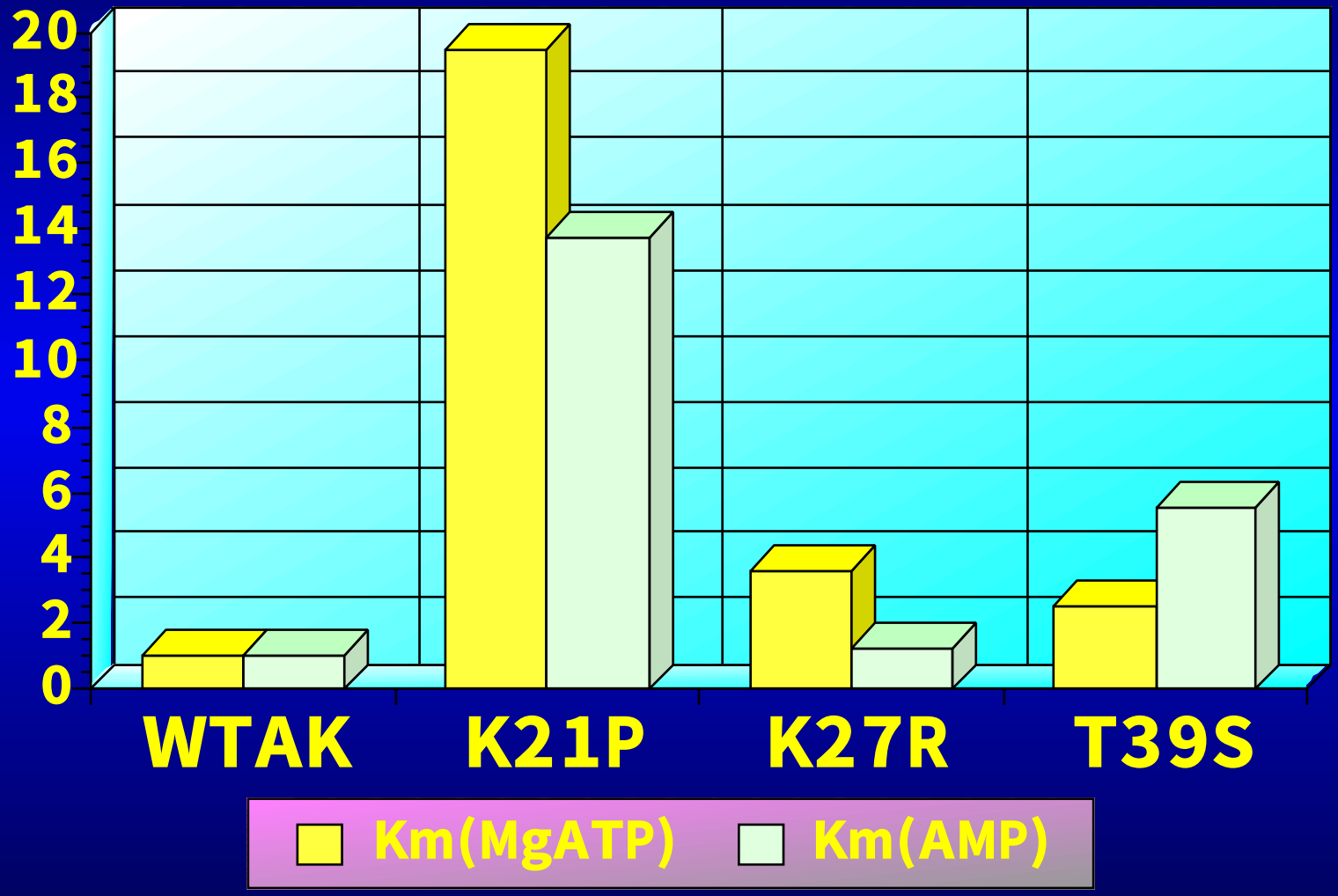
Kinetic parameters of WTAK and mutants

Enzyme	Km($MgATP^{2-}$) (mM)	Vmax (U/mg)	k'cat (S^{-1})	k'cat/Km($MgATP^{2-}$) ($S^{-1} \cdot M^{-1}$)
WTAK	0.27(1.00)	1651(100%)	589(100%)	2.21×10^6(100%)
Lys21Pro	5.25(19.44)	99(6%)	35(5.94%)	6.71×10^3(0.30%)
Lys27Arg	0.98(3.63)	34(2%)	12(2.04%)	1.24×10^4(0.56%)
Thr39Ser	0.68(2.52)	34(2%)	12(2.04%)	1.81×10^4(0.82%)

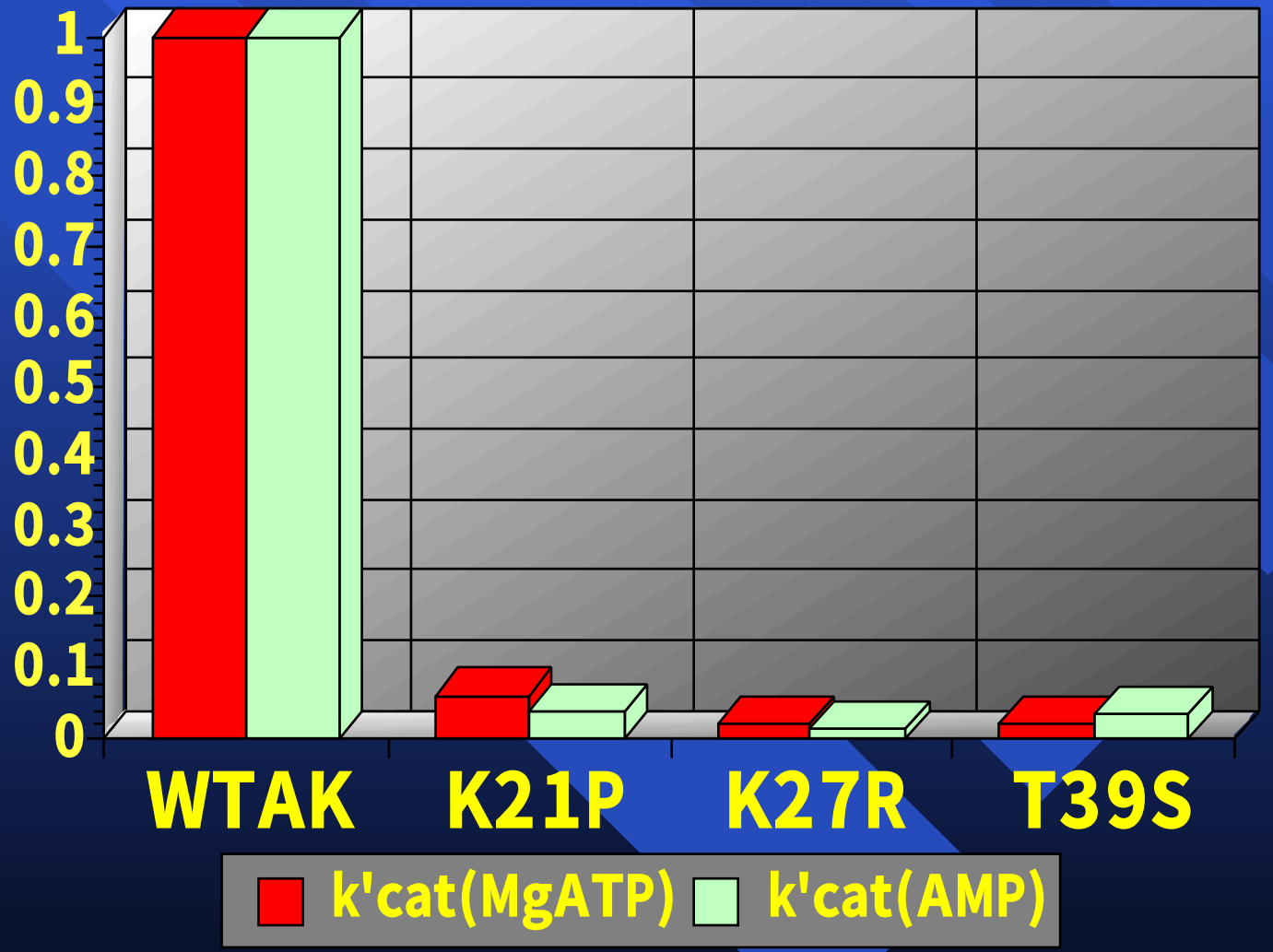
Kinetic parameters of WTAK and mutants

	Km(AMP^{2-}) (mM)	Vmax (U/mg)	k'cat (S^{-1})	k'cat/Km(AMP^{2-}) ($S^{-1}\cdot M^{-1}$)
WTAK	0.33(1.00)	1552(100%)	554(100%)	1.67×10^{6}(100%)
Lys21Pro	4.55(13.79)	62(4%)	22(3.97%)	4.89×10^{3}(0.29%)
Lys27Arg	0.41(1.24)	19(1.2%)	7(1.26%)	1.66×10^{4}(0.99%)
Thr39Ser	1.84(5.58)	55(4%)	20(3.61%)	1.07×10^{4}(0.64%)

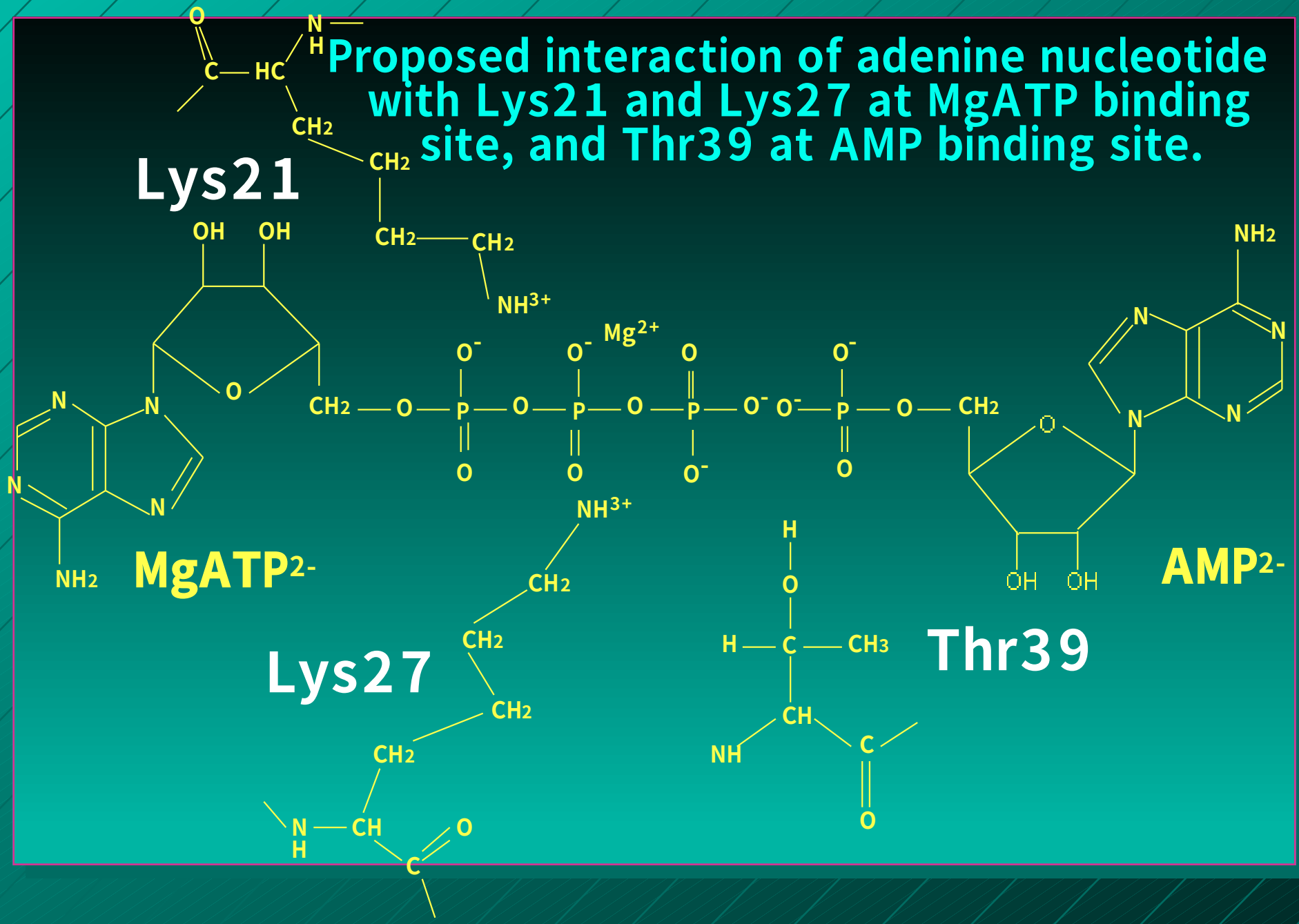
Comparison of Km values



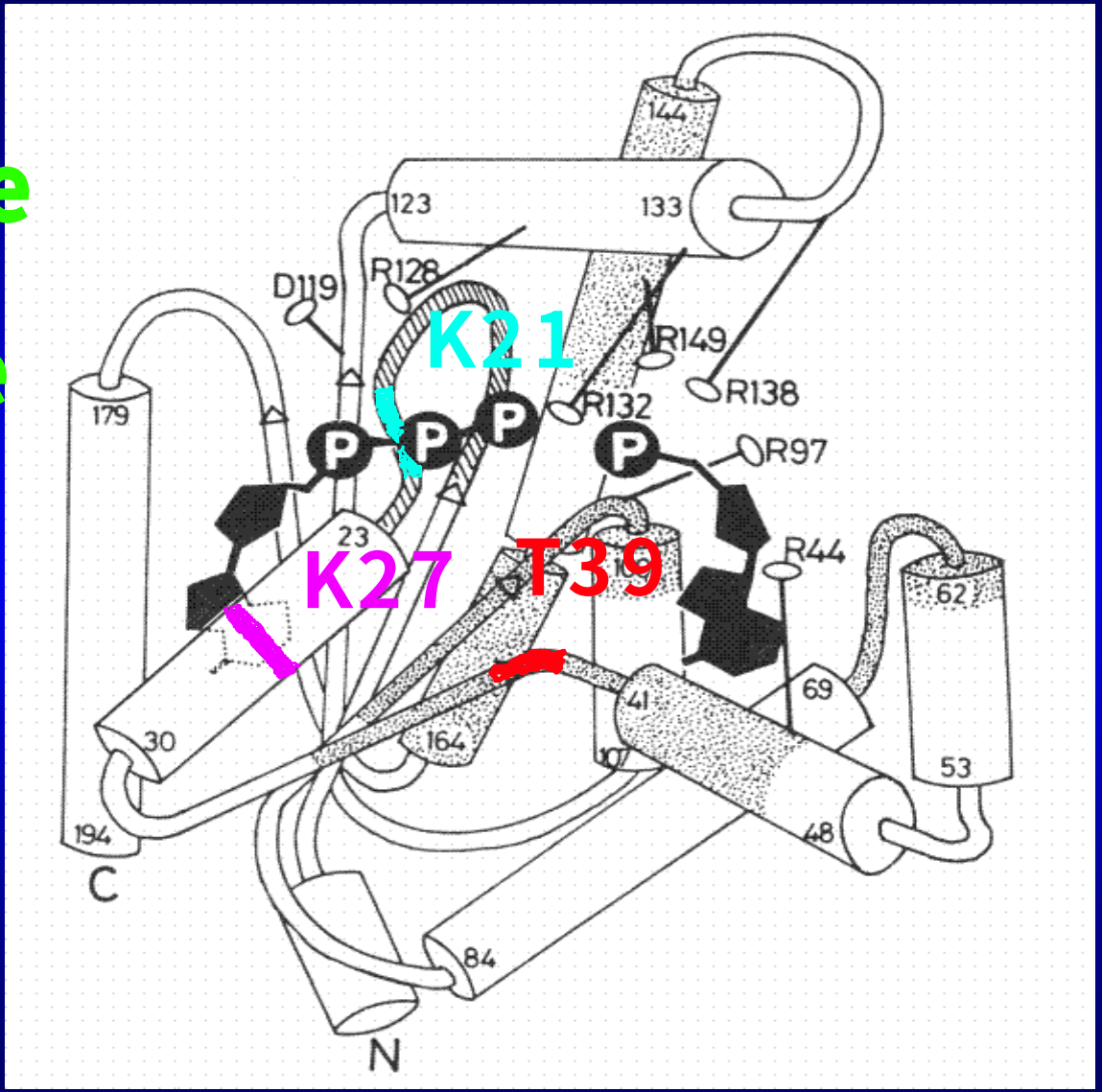
Comparison of k'cat values



Proposed interaction of adenine nucleotide with Lys21 and Lys27 at MgATP binding site, and Thr39 at AMP binding site.



A drawing of adeninenucleotide binding site of Adenylate kinase



RESULTS

Based on the kinetic analysis,

(1) K21P in the glycine rich region: decreased k'cat value, might be played an significant role in phospho-transferation reaction.

(2) K27R, assumed to be involved $MgATP^{2-}$ binding site.

(3) T39S, presumed to be essential for catalysis at AMP^{2-} binding site.

CONCLUSION

Lys21, Lys27, Thr39 residues;
interacted with adenine nucleotide having its different responsibilities

ABSTRACT

To elucidate the structural factors of substrate binding a of catalytic mode for human cytosolic adenylate kinase (hAK1 EC 2.7,4.3), we have replaced K21, K27 and T39 using pMEX8-hAK1 vector with Pro, Arg and Ser, respectively. Mutant enzymes were obtained by the random site-directed mutagenesis method with a chemically synthesized artificial hAK1 gene (Kim, H. J. et al. Protein Engineering 2(5) pp.379-386, 1989). Based the kinetic analysis, K21p in the glycine rich region gane decreased k'cat value, this might be played an significant role in phospho-transferation reaction. The K27R was assumed to be involved an MgATP2- binding site, and T395 was presumed to be essential for catalysis at an close AMP2- binding site. It is expected these residues may be interacted with nucleotide having each different role.