- スライド一覧
  - Lysine residues in human adenylate kinase are essential for interaction with adenine nucleotides as found by site-directed random mutagenesis
    - Takanori Ayabe
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    - Department of Hygiene, The Second Department of Surgery, Miyazaki Medical College
    - Porcine Adenylate Kinase
    - ADENYLATE KINASE ISOENZYME 1 (EC 2.7.4.3) (ATP-AMP TRANSPHOSPHORYLASE)
    - DE   (AK1) (MYOKINASE).
    - OS   SUS SCROFA (PIG).
    - DR   PDB; 3ADK; 16-APR-88.
    - [ENTRY / RASMOL / 3D IMAGE / HSSP ENTRY / SCOP]
    - DR   SWISS-PROT; P00571; KAD1_PIG.
    - KW   TRANSFERASE; KINASE; ATP-BINDING; ACETYLATION; 3D-STRUCTURE.
  - ヒトアデニル酸キナーゼ(AK1)
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    - 1) 194アミノ酸から構成されるリン酸転移酵素である。
    - 2) 分子量21,700の球状蛋白質である。
    - 3) 2つのアデニンヌクレオチド基質結合サイトをもつ。 (MgATPサイトとAMPサイト)
    - 4) ３つのアイソザイムが存在する(AK1, Ak2, AK3)。 AK1は細胞質に存在し、骨格筋、脳、赤血球にみられる。 AK2は、ミトコンドリア膜間スペースに、AK3は、ミトコンドリ
    - アマトリックスに存在し、肝臓、腎臓にみられる。

- 5)赤血球内AK1欠損症による溶血性貧血を引き起こす遺伝性疾患 (Miwa et al., 1983)や、また、AK1の点変異による溶血性貧血 (Arg128からTrpへの置換)(Matsuura et al., 1989)などが報告され
- ている。

Established sources of members of the adenylate kinase family

Species	Source	Characte-rization	Established Sequence	Reference
Human	cytosol	1972	1976	von Zabern et al.
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Bovine	mitochondria	1970	1986	Tommasselli et al.
Porcine	cytosol	1970	1974	Heil et al.
Porcine	mitochondria	1960	-	
Porcine	mitochondria	1961	-	
Rat	cytosol	1970	-	
Rat	mitochondria	1968	-	
Rabbit	cytosol	1957	1984	Kuby et al.
Chicken	cytosol	1986	1986	Kishi et al.
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Yeast	cytosol	1967	1986	Tomasselli et al.
Yeast	mitochondria	1986	-	
Escherichia coli		1973	1985	Brune et al.

アデニル酸キナーゼの構造と機能解析の歴史的背景

1. X-ray crystallographic study
(Schulz et al., 1974, Pai et al., 1977, Egner et al, 1987,
Schulz et al., 1990)

- 2. NMR study
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- 3. Kinetic analysis of peptide fragment (Kuby et al., 1978)
- 4. Site-directed mutagenesis
  (Kim et al., 1990, Yoneya et al., 1990, Yan & Tsai, 1991)

AK1のアミノ酸一次構造の比較

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- Rabbit (Kuby et al., 1984)
- Calf (Kuby et al., 1984)
- Porcine (Heil et al., 1974)
- Chicken (Kishi et al., 1986)

本研究の目的

- 1)ヒト骨格筋AKとATPの相互作用を調べるために、哺乳類動物間
- で保存性の高いリジン残基７つ(K9, K21, K27, K31, K63,
- K131, K194)を選 び、ヒトアデニル酸キナーゼ人工合成遺伝子
- を用いて、部位特異的変異導入を行う。
-
- 2)アミノ酸の置換の方法は、標的リジン残基に対し、プライマーの
- デザインを工夫し、変異体をランダムに複数作製し、複数の変異
- 体を短時間で蛋白質発現、及び、精製する系を確立する。
-
- 3)野生型酵素と変異型酵素の性質を、酵素反応速度論的に解析し、
- AK構造モデルにおいて、リジン残基を置換し得た変異型と野生
- 型酵素の基質との相互作用を考察する試みを行う。

DNA sequence of hAK1 gene
(Kim et al., 1989)

Construction of pMEX8-hAK1

DNA sequence of primer

- Lys9 5'-CGAAGATGATYXXAGTCTTCTTAAGC-3'
- Lys21 5'-GCACTGGGTACCYXXGCCAGAACC-3'
- Lys27 5'-GCACGATYXXCTCGCACTGGG -3'
- Lys31 5'-GTGTAGCCGTAYXXCTGCACGATTTTC-3'
- Lys63 5'-CCAGCTGACCYXXTTCCATGATTTC-3'
- Lys131 5'-GTTTCGCCGCGYXXCAGCAGGCG-3'
- Lys194 5'-CGAAGATGATYXXAGTCTTCTTAAGC-3'

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

Site-directed mutagenesis

Screening of mutant pMEX8-hAK1

1. Transformation with homoduplex DNA and TG1 competent cells
2. Culture on LB-plate with ampicillin (50μg/ml)
3. Small culture of LB medium (overnight)
4. Plasmid purification (Sephaglass, alkaline method)
5. DNA Cycle Sequence by PCR

Reaction mixture

- AmpliTaq DNA Polymerase : Taq DNA Polymerase = 9:1 (2U/2μl)
- double-stranded template DNA (2.8 μg)
- Fluorescent isothiocyanate-labeled sequence primer (2 pmol)

Termination Mixes (ddATP, ddGTP, ddCTP, ddTTP)

PCR Condition

1 cycle 95℃ for 5 sec

- 20 cycle 95℃ for 30 sec
- 53℃ for 30 sec
- 72℃ for 60 sec
- 20 cycle 95℃ for 30 sec
- 72℃ for 60 sec

Results: DNA sequence of Lys9-mutants
Results: DNA sequence of Lys21- and Lys63- mutants
Results: DNA sequence of Lys27-mutants
Results: DNA sequence of Lys31-mutants
Results: DNA sequence of Lys131-mutants
Results: DNA sequence of Lys194-mutants
Results of site-directed mutagenesis
Protein expression and purification of wild-type and mutant AKs

1. Transformation with plasmid DNA and TG1 cells
2. Small culture of the cells in LB medium (5 ml) overnight
3. Culture of the medium in 250 ml for 1 hr
4. Addition of isopropyl-β-D-thio-galactopyranoside (IPTG) (a final concentration of 1 mM)
5. Culture the medium for 16 hr
6. Centrifugation of the medium (5,000 X g for 20 min)
7. Disruption of the pelltet of E.coli cells in standard buffer (10ml) (Ultrasonicator, 20kHz, 20W, 3min)
8. Centrifugation of the homogenate (12,000 X g for 20 min)
9. Blue sepharose CL-6B column chromatography
10. 12.5% SDS-PAGE and concentration
11. Gel filtration (Superose 12)
12. 12.5% SDS-PAGE
13. Measurement of the concentration of protein (Lowry method)
14. Kinetic analysis of forward reaction of AK

- Blue Sepharose Chromatography
  - Column: Blue Sepharose CL-6B (φ1 X 5 cm)
  - Standard buffer: 20mM Tris-HCl, 1 mM EDTA,
  - 0.1 mM dithiothreitol, pH 7.4
  - Gradient: 0 - 1 M NaCl
  - Velocity: 0.5 ml/min
  - Fraction size: 3 ml
- Superose 12 Column Chromatography
  - Column: Superose 12 (φ1 X 30 cm)
  - Imidazole buffer: 5mM imidazol-HCl, 1 mM EDTA,
  - 0.1 mM dithiothreitol, pH 6.9
  - Velocity: 0.5 ml/min
  - Fraction size: 2 ml
  -
- Results of protein purification
- AK assay
  -
  - Forward Reaction
  - <Reaction Mixture (1ml) of the ADP formation reaction>
    - 75mM Triethanolamine HCl (pH 7.5)
    - 120mM KCl, 0.2mM NADH
    - 0.3mM Phosphoenolpyruvate, 2mM $MgSO_4$
    - 2mM ATP, (2, 1, 0.5, 0.33, 0.25) mM AMP
    - 0.3 mg/ml Bovine serum albumine
    - 15U Lactate dehydrogenase, 6U Pyruvate kinase
    - AK sample
  -
  - AK, Mg^{2+}
  - ↓

ATP + AMP ⇄ 2 ADP

Pyruvate Kinase (PK)

2 ADP + 2 Phosphoenolpyruvate (PEP) ⇄ 2 ATP + 2 Pyruvate

Lactate Dehydrogenase (LDH)

2 Pyruvate + 2 NADH + 2H⁺ ⇄ 2 L-Lactate + 2 NAD⁺

↑

observe reduction of NADH at 340 nm

double reciprocal plot

Kinetic results of Lys-mutants

Residue	mutant	Km MgATP	Km AMP	kcat/Km MgATP	kcat/Km AMP
Lys9	K9P	−	−	−−	−
	K9F			−−−	−−−
	K9L		+	−−−	−−−−
	K9T	++	++	−−	−−−

Lys21	K21P	+++	++	−−
Lys27	K27R			−−
	K27L	+		−−
	K27I	+		−−−
Lys31	K31F	++		−−
	K31I	−		−−
	K31S	+	++++	−−−
Lys63	K63F	+	−	−−−
Lys131	K131A	+++		−−−
	K131F			−−−
Lys194	K194S	++		−
	K194I	−	−	−−

- K194L ＋ － －
- K194P － －－－ －－ －－
- K194N －－ －－ －－
- K194V － ＋＋＋＋ －－ －－ －－－－
-
-
- Kmの減少 Km ＜ 1.0 － kcat 10％ － 100％ －
- Kmの増加 1.0 ＜ Km ＜ 5.0 空欄 1％ － 10％ －－
- 5.0 ＜ Km ＜ 10.0 ＋ 0.10％ － 1％ －－－
- 10.0 ＜ Km ＜ 15.0 ＋＋ ＜ 0.10％ －－－－
- 15.0 ＜ Km ＜ 20.0 ＋＋＋
- 20.0 ＜ Km ＜ 25.0 ＋＋＋＋

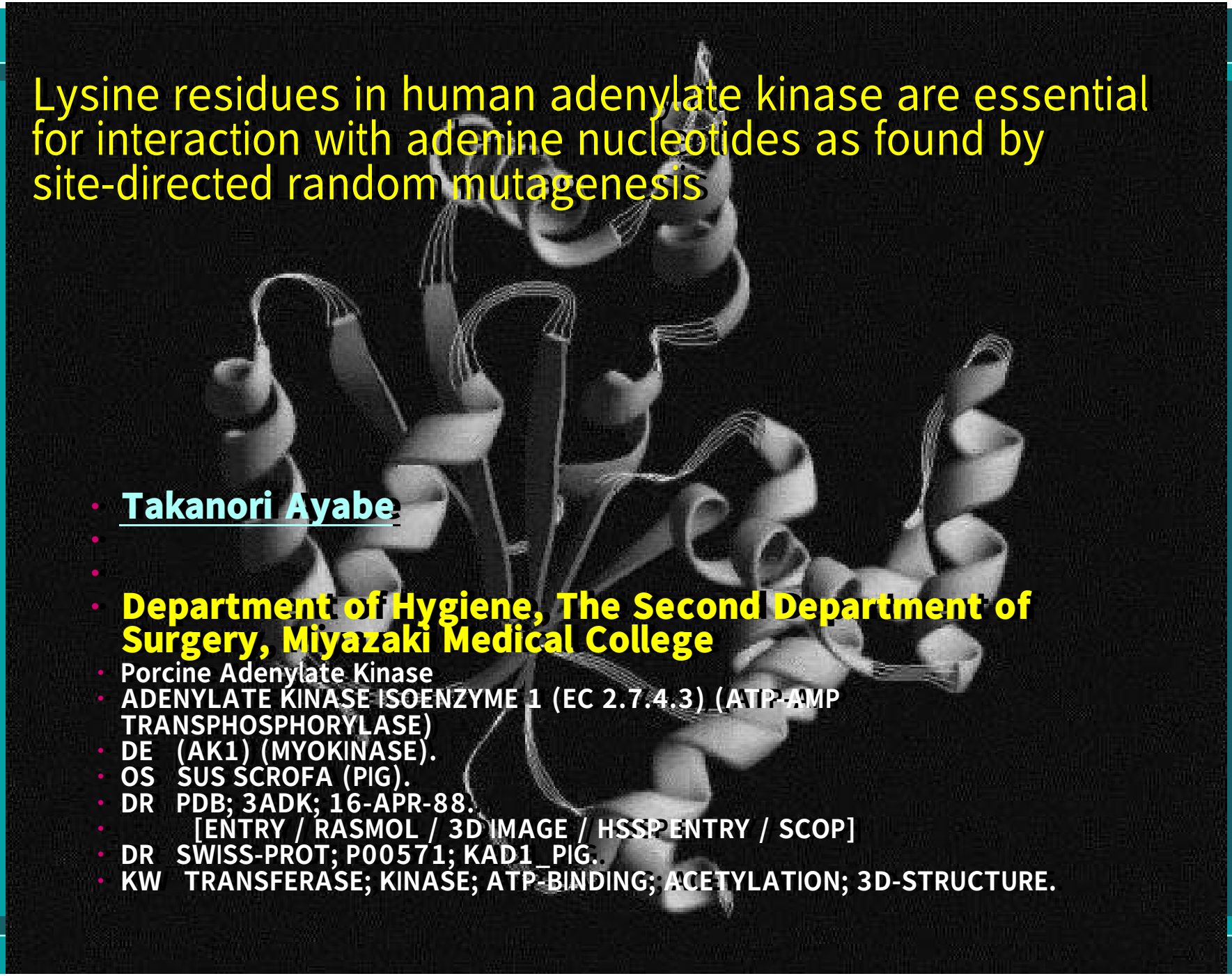
まとめ

-
- (1)ヒトAKをコードするcDNAを用い、比較的容易に短時間に複数の変異体を得る大腸菌発現系を確立した。
-
- (2)リジン残基(K9, K21, K27, K31, K63, K131, K194)に対して、ランダムに部位特異的変異導入を行い、26種類の変異型酵素を得た。
-

スライド1

- (3)変異型酵素の酵素キネテックス解析により、基質(ATP, AMP)親和性及び触媒作用の増加や減少が観察され、リジン残基は、酵素活性に必須なアミノ酸残基であることが示唆された。

Lysine residues in human adenylate kinase are essential for interaction with adenine nucleotides as found by site-directed random mutagenesis



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ヒトアデニル酸キナーゼ(AK1)

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

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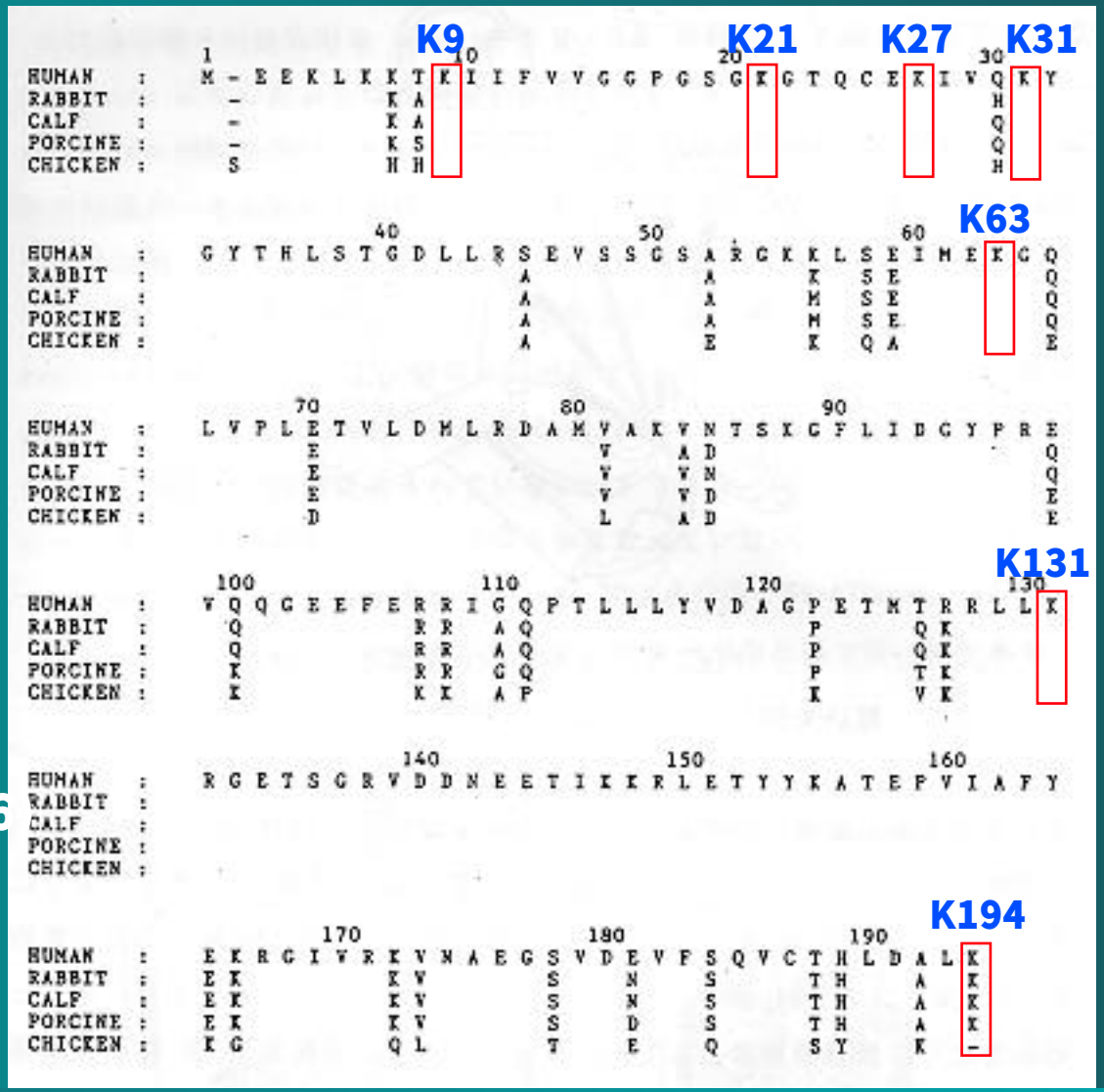

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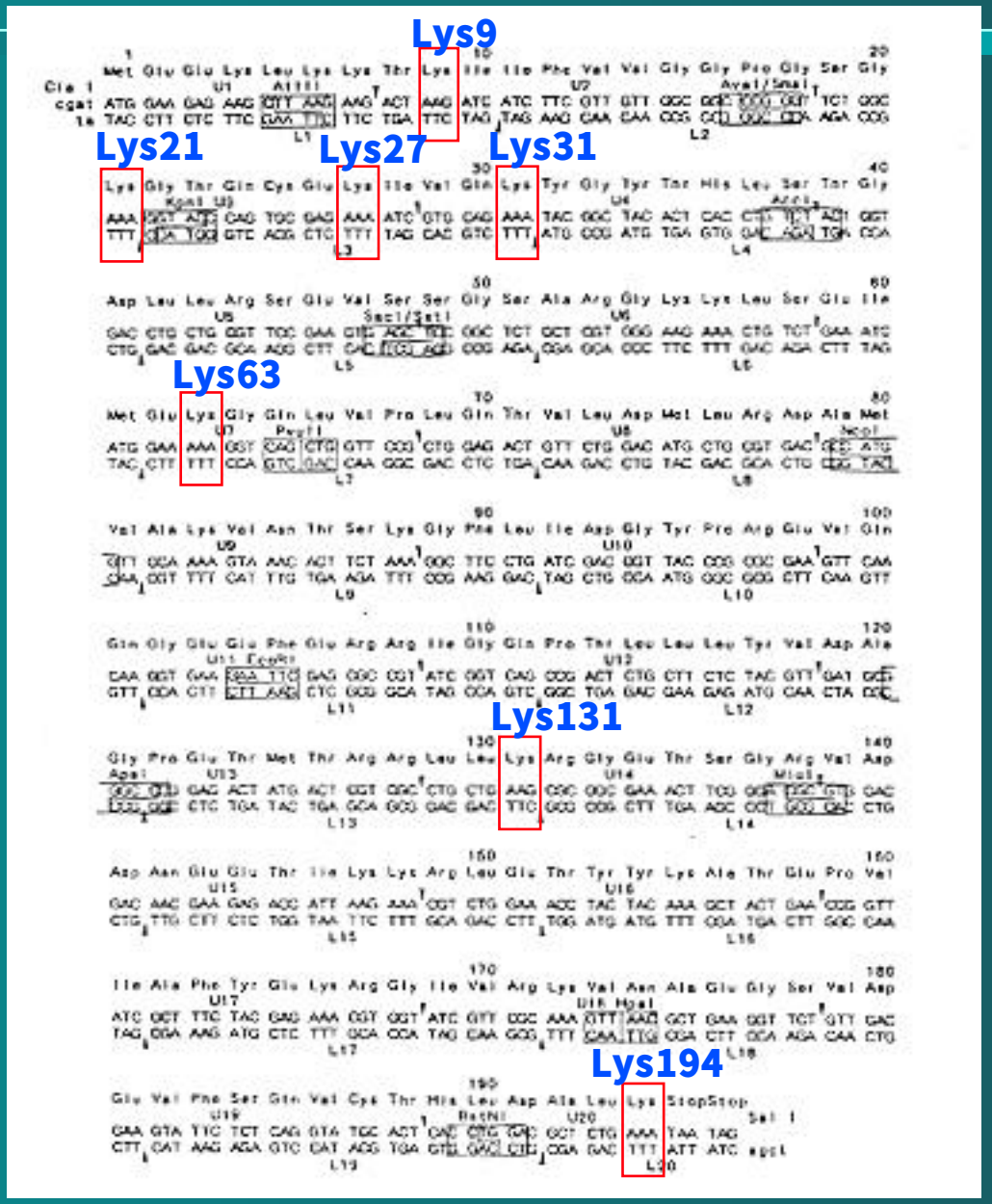
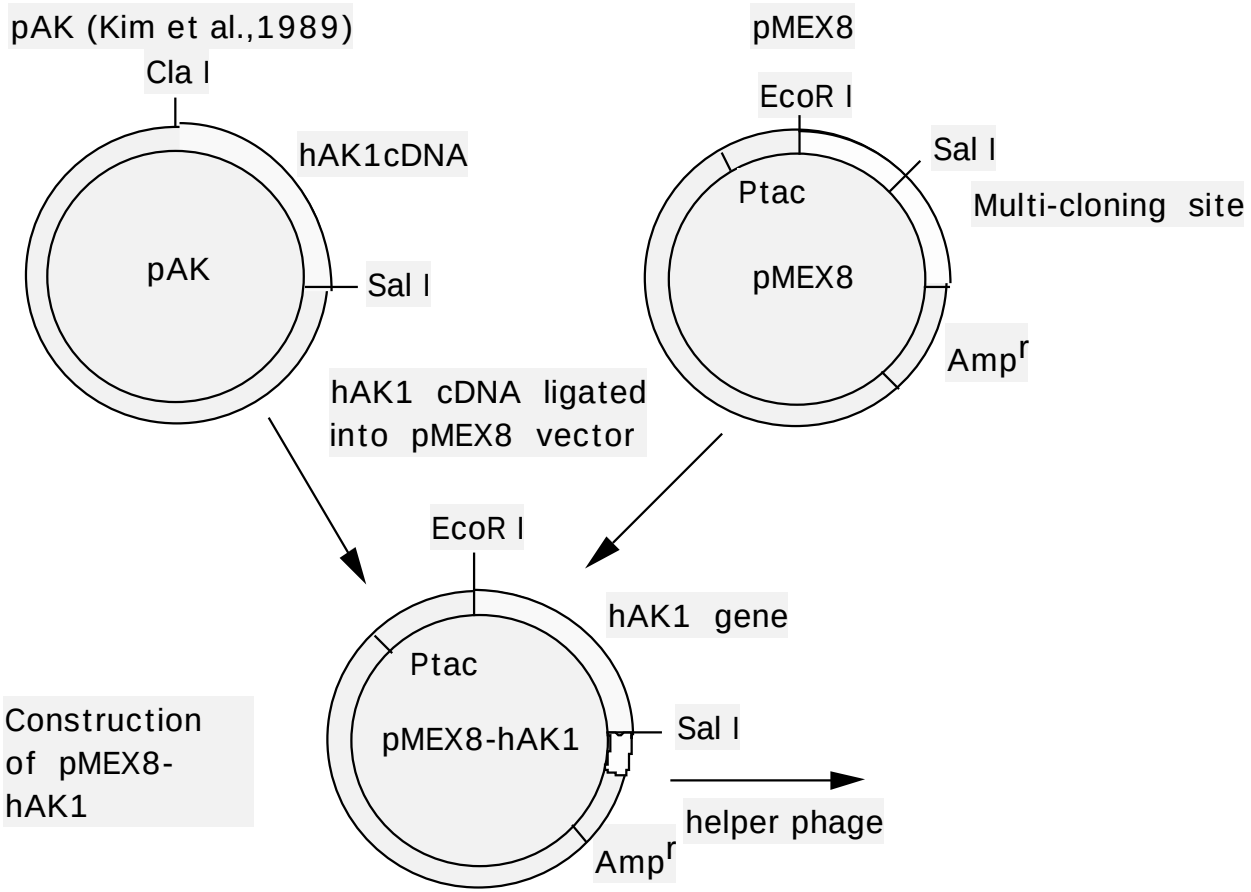


Construction of pMEX8-hAK1



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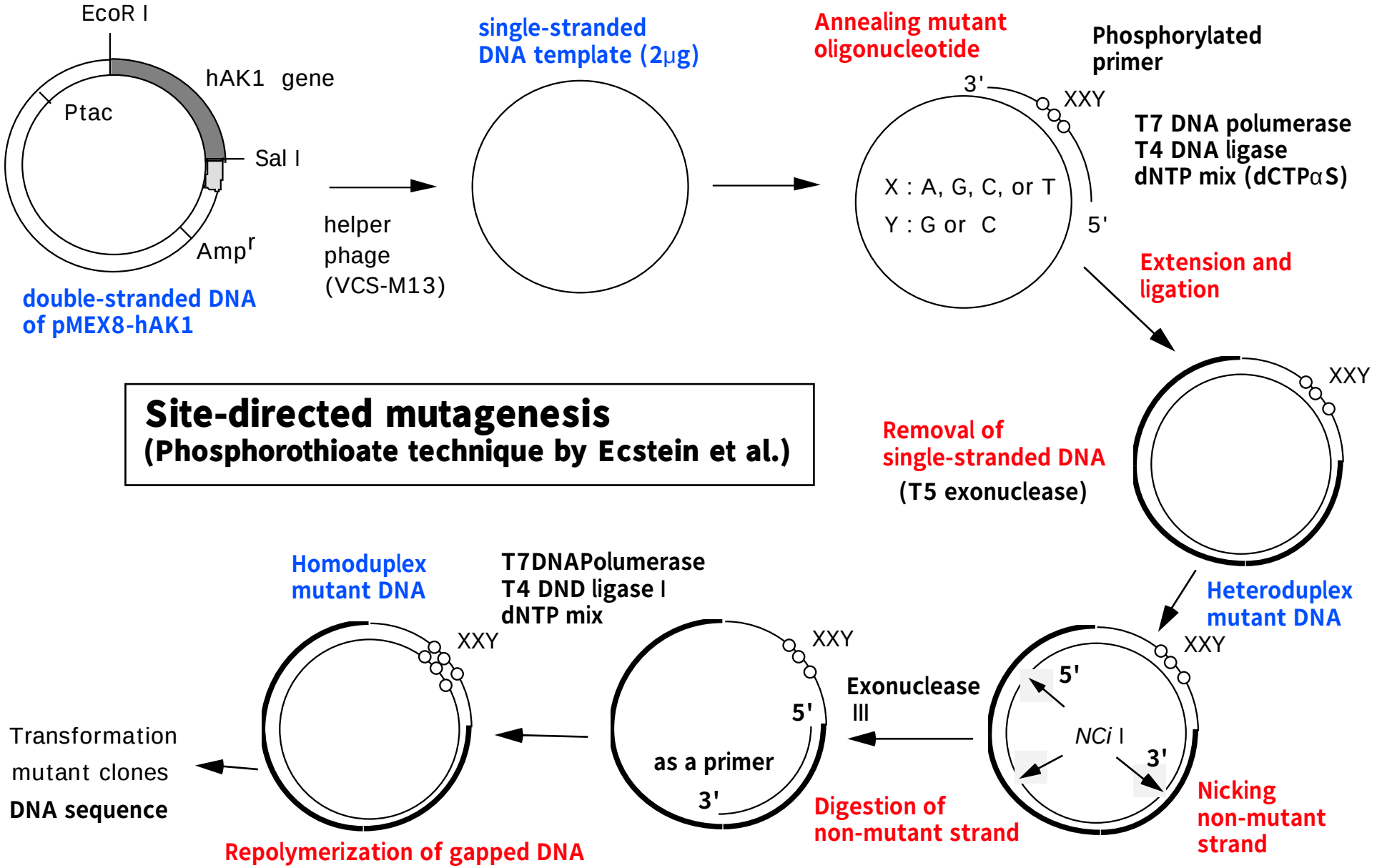
X: A, G, C, T Y: G, C


EcoR I
hAK1 gene
Ptac
Sal I
Amp^r
double-stranded DNA
of pMEX8-hAK1
helper
phage
(VCS-M13)
single-stranded
DNA template (2μg)
Annealing mutant
oligonucleotide
Phosphorylated
primer
3'
XXY
X : A, G, C, or T
Y : G or C
5'
T7 DNA polumerase
T4 DNA ligase
dNTP mix (dCTPαS)
Extension and
ligation
XXY
Site-directed mutagenesis
(Phosphorothioate technique by Ecstein et al.)
Removal of
single-stranded DNA
(T5 exonuclease)
Heteroduplex
mutant DNA
XXY
5'
NCi I
3'
Nicking
non-mutant
strand
Exonuclease
III
Digestion of
non-mutant strand
XXY
5'
as a primer
3'
T7DNAPolumerase
T4 DND ligase I
dNTP mix
Homoduplex
mutant DNA
XXY
Repolymerization of gapped DNA
Transformation
mutant clones
DNA sequence

Screening of mutant pMEX8-hAK1

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- 3. Small culture of LB medium (overnight)
- 4. Plasmid purification (Sephaglass, alkaline method)
- 5. DNA Cycle Sequence by PCR
-
- Reaction mixture
  - AmpliTaq DNA Polymerase : Taq DNA Polymerase = 9:1 (2U/2μl)
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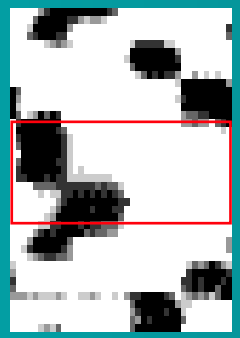
  - Fluorescent isothiocyanate-labeled sequence primer (2 pmol)
- Termination Mixes (ddATP, ddGTP, ddCTP, ddTTP)
- PCR Condition
- 1 cycle 95℃ for 5 sec
- 20 cycle 95℃ for 30 sec
- 53℃ for 30 sec
- 72℃ for 60 sec
- 20 cycle 95℃ for 30 sec
- 72℃ for 60 sec

Results: DNA sequence of Lys9-mutants

WTAK

A G C T



A C T A A G A T C

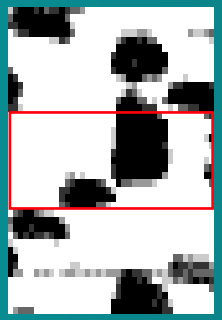
Thr8

Lys9

Ile10

K9P

A G C T



A C T C C G A T C

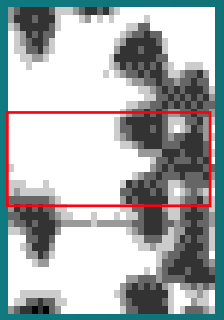
Thr8

Pro9

Ile10

K9L

A G C T



A C T C T C A T C

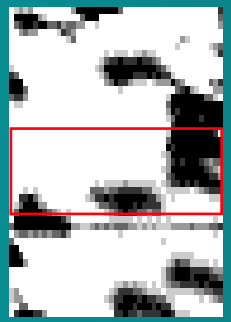
Thr8

Leu9

Ile10

K9F

A G C T



A C T T T C A T C

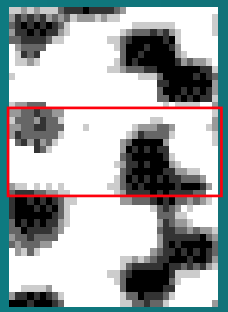
Thr8

Phe9

Ile10

K9T

A G C T



A C T A C C A T C

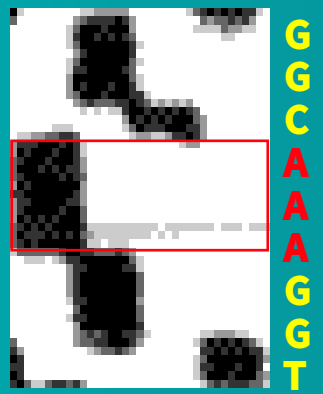
Thr8

Thr9

Ile10

Results: DNA sequence of Lys21- and Lys63- mutants

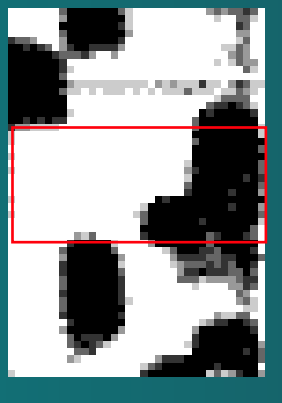
WTAK



K21P

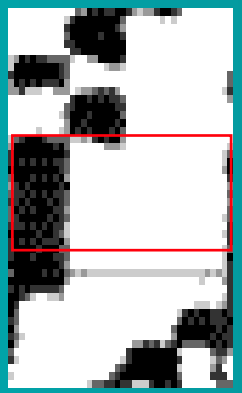
WTAK

K63F

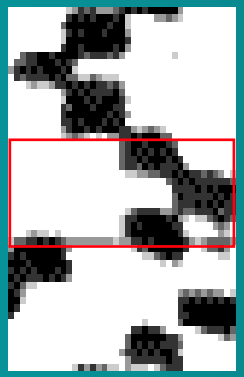


Results: DNA sequence of Lys27-mutants

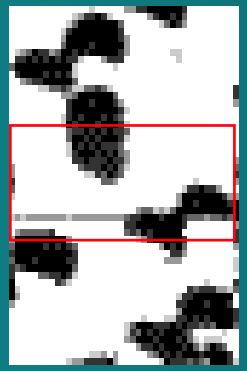
WTAK



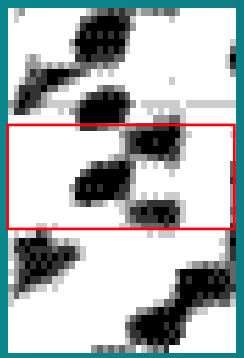
K27L



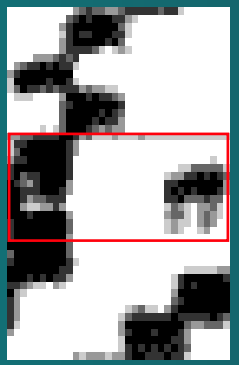
K27V



K27R

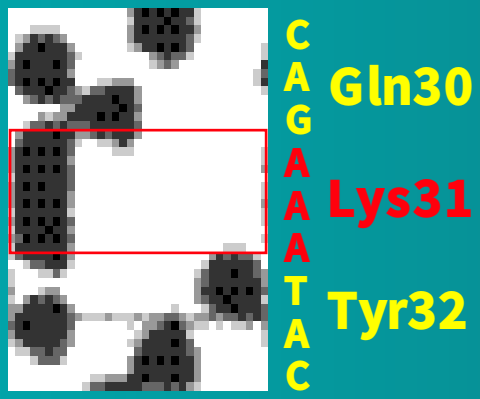


K27I

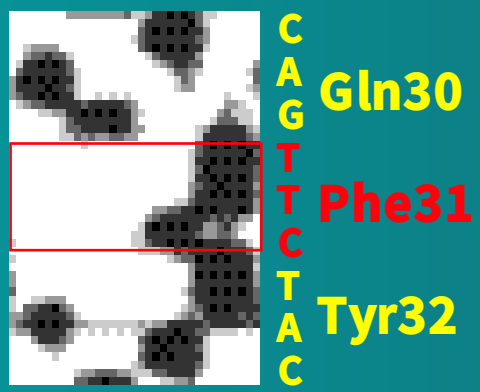


Results: DNA sequence of Lys31-mutants

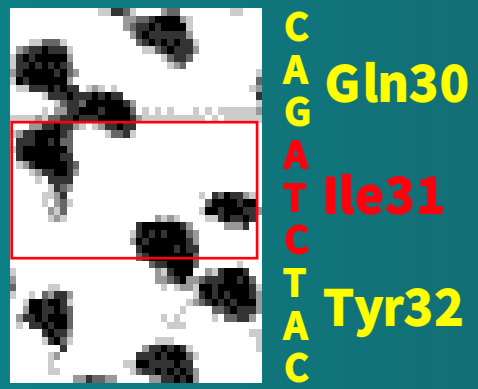
WTAK



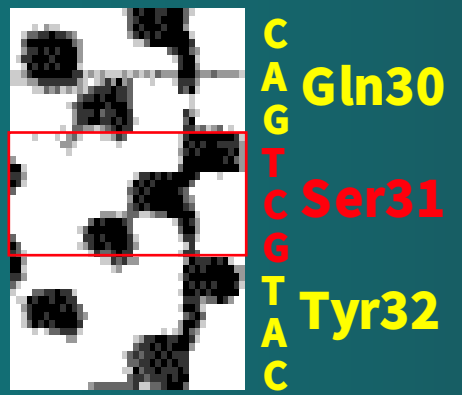
K31F



K31I



K31S

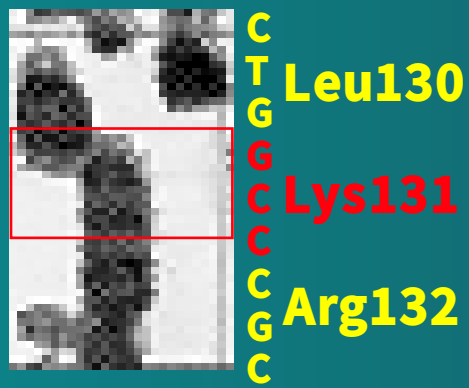


Results: DNA sequence of Lys131-mutants

WTAK

K131F

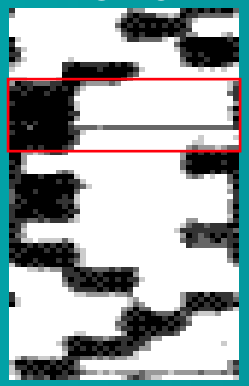
K131A



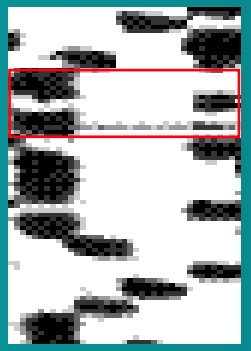
K131P

Results: DNA sequence of Lys194-mutants

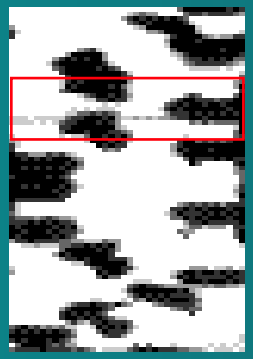
WTAK



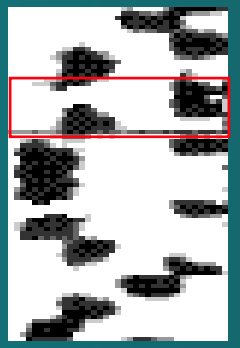
K194I



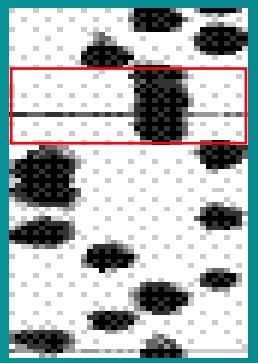
K194V



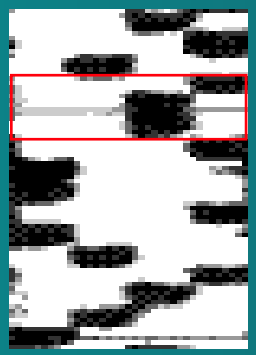
K194L



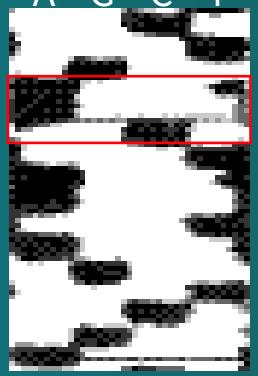
K194P



K194S



K194N



Results of site-directed mutagenesis

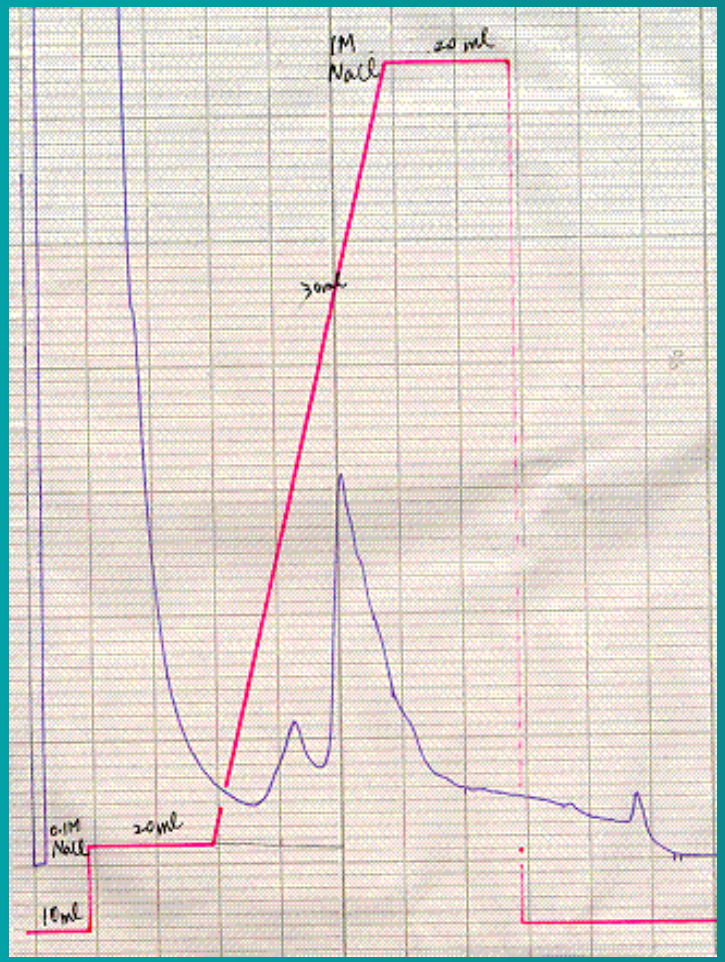
Target Residue	Mutagenesis Efficiency		Mutants
Lys9 (AAG)	33.3%	(5/15) [a]	K9P(CCG) K9F(TTC) K9L(CTC)×2 K9T(ACC)
Lys21(AAA)	10%	(1/10)	K21P(CCG)
Lys27(AAA)	33.3%	(4/12)	K27L(CTC) K27V(GTC) K27R(CGC) K27I(ATA)
Lys31(AAA)	26.7%	(4/15)	K31I(ATC) K31S(TCG) K31F(TTC)×2
Lys63(AAA)	8.3%	(1/12)	K63F(TTC)
Lys131(AAG)	30%	(3/10)	K131A(GCC) K131F(TTC) K131P(CCC)
Lys194(AAA)	40%	(8/20)	K194S(TCC)×3 K194N(AAC) K194V(GTG) K194I(ATA) K194P(CCC) K194L(TTG)

[a] numbers in parenthesis represent ratio of the confirmed mutants/ screening number

Protein expression and purification of wild-type and mutant AKs

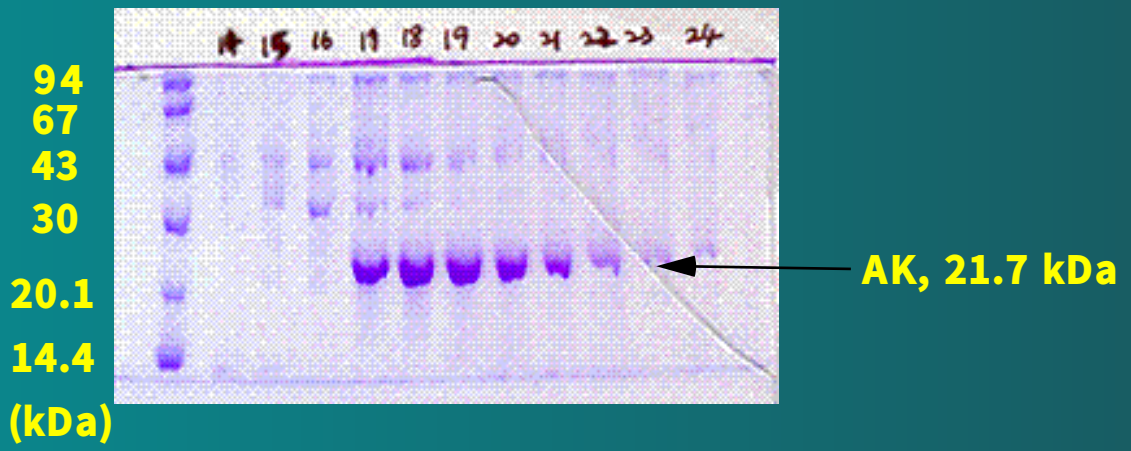
1. Transformation with plasmid DNA and TG1 cells
2. Small culture of the cells in LB medium (5 ml) overnight
3. Culture of the medium in 250 ml for 1 hr
4. Addition of isopropyl-β-D-thio-galactopyranoside (IPTG) (a final concentration of 1 mM)
5. Culture the medium for 16 hr
6. Centrifugation of the medium (5,000 X g for 20 min)
7. Disruption of the pelltet of E.coli cells in standard buffer (10ml) (Ultrasonicator, 20kHz, 20W, 3min)
8. Centrifugation of the homogenate (12,000 X g for 20 min)
9. Blue sepharose CL-6B column chromatography
10. 12.5% SDS-PAGE and concentration
11. Gel filtration (Superose 12)
12. 12.5% SDS-PAGE
13. Measurement of the concentration of protein (Lowry method)
14. Kinetic analysis of forward reaction of AK

Blue Sepharose Chromatography

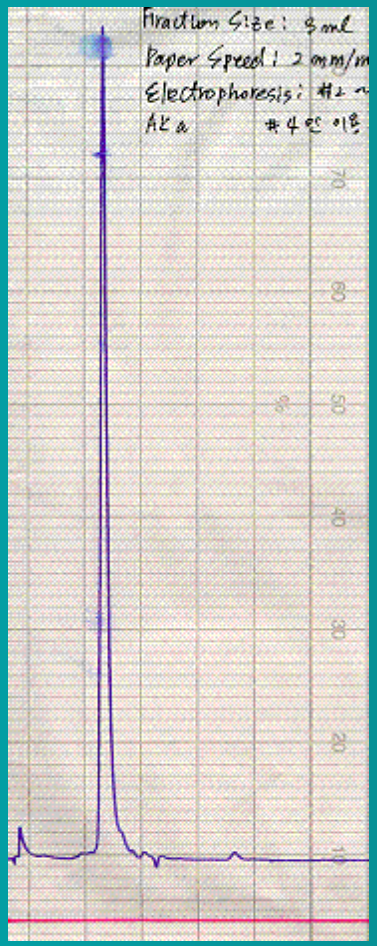


- Column: Blue Sepharose CL-6B (ϕ1 X 5 cm)
- Standard buffer: 20mM Tris-HCl, 1 mM EDTA, 0.1 mM dithiothreitol, pH 7.4
- Gradient: 0 - 1 M NaCl
- Velocity: 0.5 ml/min
- Fraction size: 3 ml

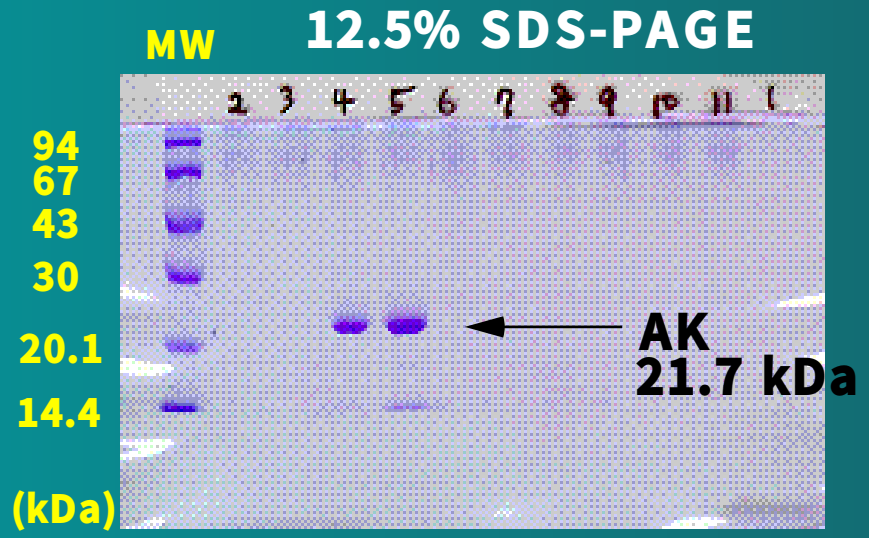
MW 12.5% SDS-PAGE



Superose 12 Column Chromatography



- Column: Superose 12 (ϕ1 X 30 cm)
- Imidazole buffer: 5mM imidazol-HCl, 1 mM EDTA, 0.1 mM dithiothreitol, pH 6.9
- Velocity: 0.5 ml/min
- Fraction size: 2 ml



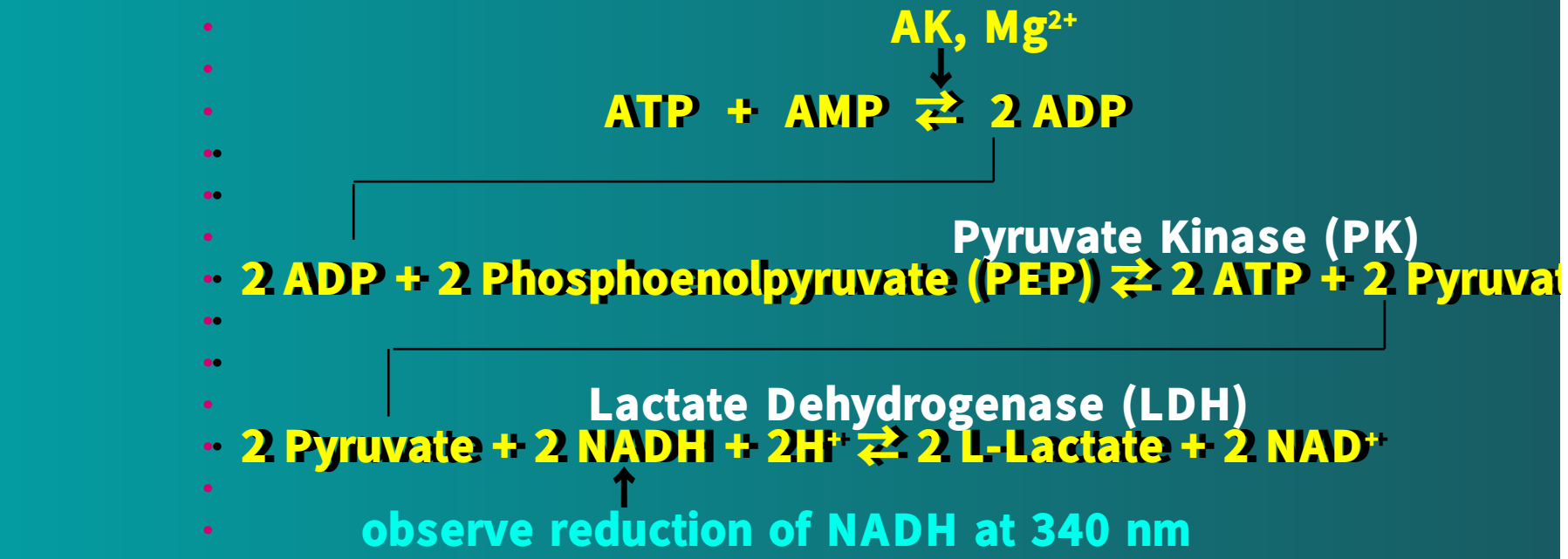
Results of protein purification

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	-
		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	-
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

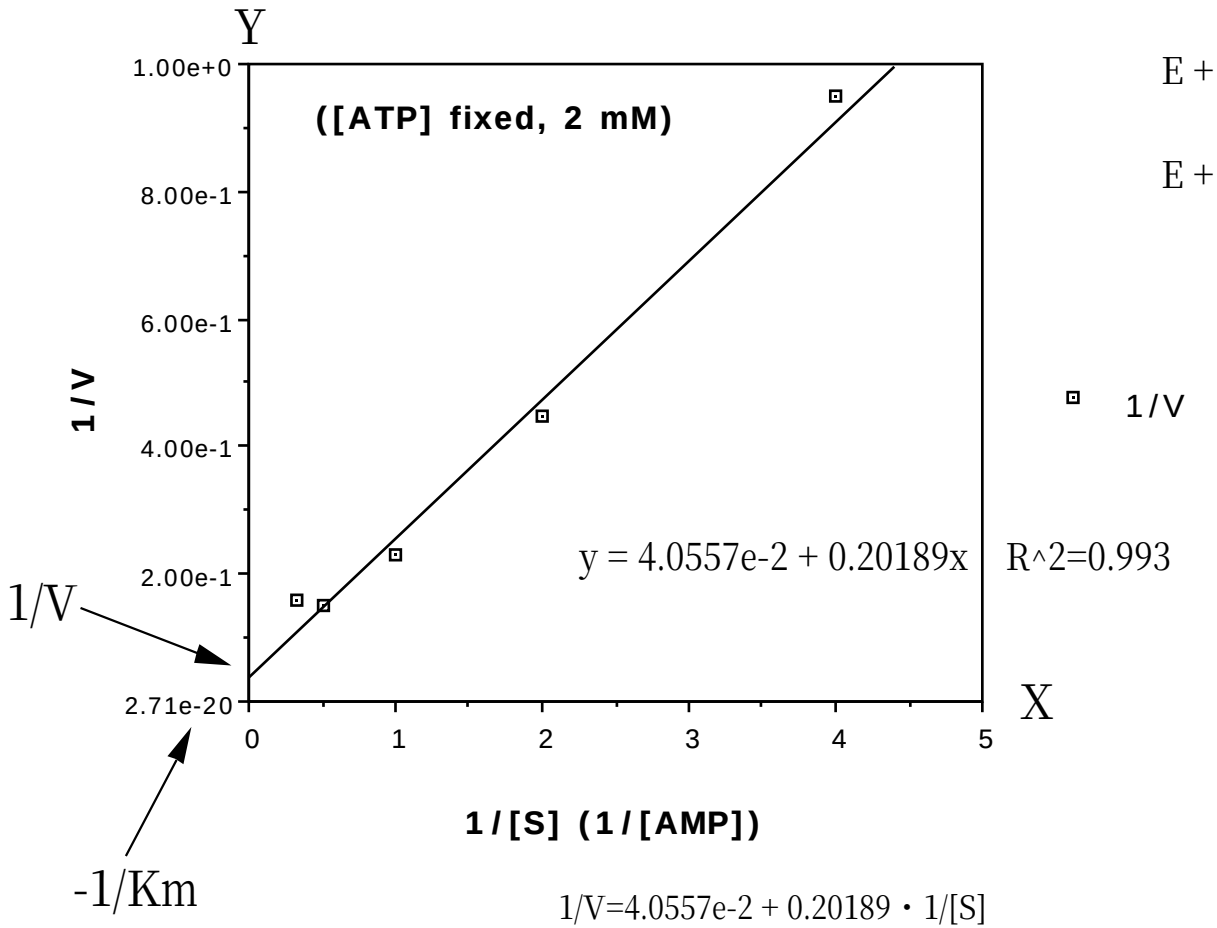
- [a] numbers in parenthesis represent ratio of the confirmed mutants/ screening number
- [b] numbers in parenthesis represent % of wild-type AK; the yield of wild-type AK was 6.90 mg.
- [c] represents % of wild-type AK. Each mutant was assayed in a forward reaction at a fixed concentration of 1mM of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

AK assay

- Forward Reaction
- <Reaction Mixture (1ml) of the ADP formation reaction>
  - 75mM Triethanolamine HCl (pH 7.5)
  - 120mM KCl, 0.2mM NADH
  - 0.3mM Phosphoenolpyruvate, 2mM $MgSO_4$
  - 2mM ATP, (2, 1, 0.5, 0.33, 0.25) mM AMP
  - 0.3 mg/ml Bovine serum albumine
  - 15U Lactate dehydrogenase, 6U Pyruvate kinase
  - AK sample



K194P (Lineweaver-Burk Plot)



1/V=4.0557e-2 + 0.20189・1/[S]

X軸との交点：-1/Km
Y軸との交点：1/V

$$E + S \xrightarrow{k+1} ES$$

$$E + S \xleftarrow{k-1} ES$$

$$ES \xrightarrow{k2} E + P$$

$$v = \frac{V[S]}{Km + [S]}$$

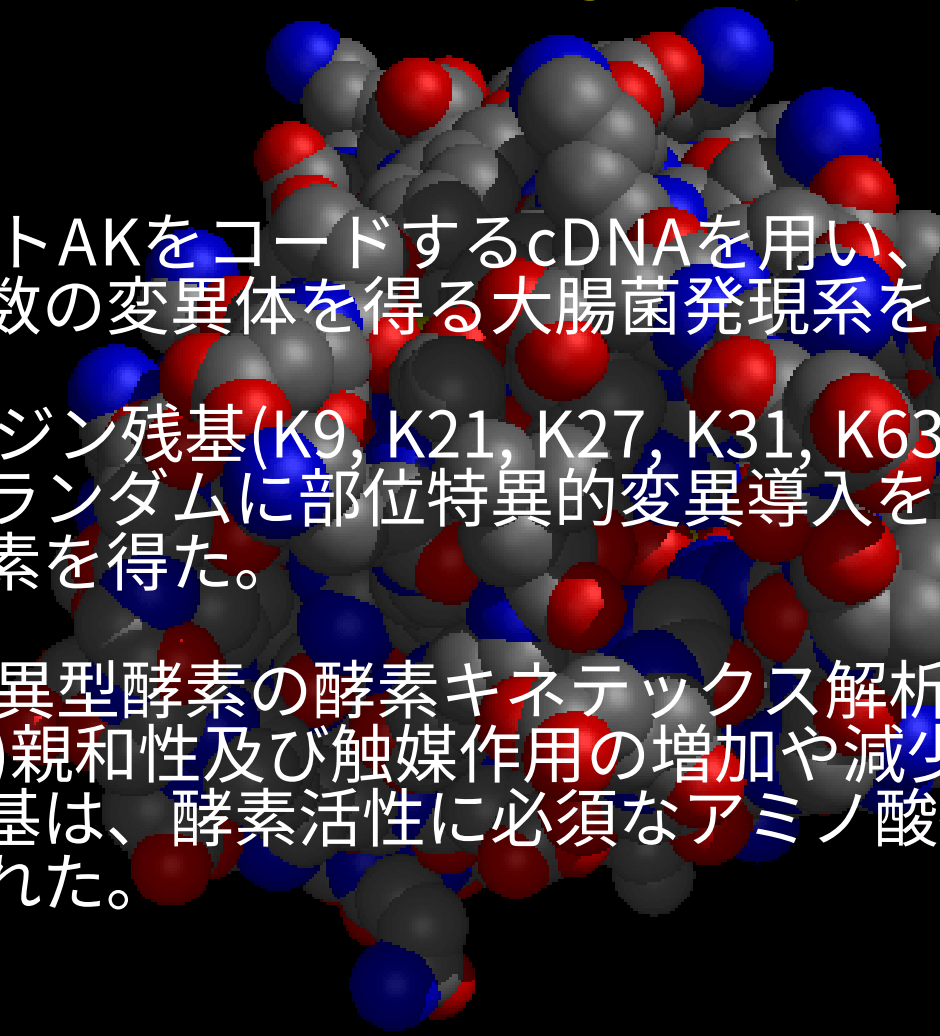
$$Km = \frac{k-1 + k2}{k+1}$$

Kinetic results of Lys-mutants

Residue	mutant	Km MgATP	Km AMP	kcat	kcat/Km MgATP	kcat/Km AMP
Lys9	K9P	−	−	−−	−	−
	K9F			−−−	−−−	−−−
	K9L		+	−−−	−−−	−−−−
	K9T	++	++	−−	−−−	−−−
Lys21	K21P	+++	++	−−	−−−	−−−
Lys27	K27R			−−	−−−	−−
	K27L	+		−−	−−−	−−
	K27I	+		−−−	−−−−	−−−
Lys31	K31F	++		−−	−−−−	−−−
	K31I	−		−−	−−	−−
	K31S	+	++++	−−−	−−−	−−−−
Lys63	K63F	+	−	−−−	−−−−	−−
Lys131	K131A	+++		−−−	−−−−	−−−
	K131F			−−−	−−−	−−−
Lys194	K194S	++		−	−−	−
	K194I	−	−	−−	−	−−
	K194L	+	−	−	−−	
	K194P		−	−−−	−−−	−−
	K194N			−−	−−	−−
	K194V	−	++++	−−	−−	−−−−

Kmの減少	Km ＜ 1.0	−	kcat	10 % ＞ − ＞ 100 %
Kmの増加	1.0 ＜ Km ＜ 5.0	空欄		1 % ＞ − ＞ 10 %
	5.0 ＜ Km ＜ 10.0	+		0.10 % ＞ − ＞ 1 %
	10.0 ＜ Km ＜ 15.0	++		＜ 0.10 %
	15.0 ＜ Km ＜ 20.0	+++		
	20.0 ＜ Km ＜ 25.0	++++		

まとめ



- (1)ヒトAKをコードするcDNAを用い、比較的容易に短時間に複数の変異体を得る大腸菌発現系を確立した。
- (2)リジン残基(K9, K21, K27, K31, K63, K131, K194)に対して、ランダムに部位特異的変異導入を行い、26種類の変異型酵素を得た。
- (3)変異型酵素の酵素キネテックス解析により、基質(ATP, AMP)親和性及び触媒作用の増加や減少が観察され、リジン残基は、酵素活性に必須なアミノ酸残基であることが示唆された。