EXPLORING THE NUCLEOTIDE-BINDING SITES IN ADENYLATE KINASE BY SITE-DIRECTED MUTAGENESIS

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CONTENTS

List of Abbreviations

AK	adenylate kinase
ATP	adenosine 5′-triphosphate
ADP	adenosine 5′-diphosphate
AMP	adenosine 5′-monophosphate
dAMP	2′-deoxyadenosine 5′-monophosphate
GMP	guanosine 5′-monophosphate
IMP	inosine 5′-monophosphate
CMP	cytidine 5′-monophosphate
UMP	uridine 5′-monophosphate
AP_5A	adenosine pentaphosphoadenosine
IPTG	isopropyl β-D-thiogalactopyranoside
Tris	tris(hydroxymethyl)aminomethane
EDTA	ethylenediaminetetraacetic acid
SDS	sodium dodecyl sulfate
CD	circular dichroism
PCR	polymerase chain reaction

Mutant enzyme of adenylate kinase:

For example "Leu66-Ala mutant enzyme" denotes a mutant enzyme replaced Leu66 by Ala.

Chapter I

General Introduction

GENERAL INTRODUCTION

Adenylate kinase (myokinase, ATP:AMP phosphotransferase; EC 2.7.4.3) catalyzes the reversible phosphoryl transfer between MgATP and AMP, producing MgADP and ADP, as shown by the following equation:

$$MgATP + AMP \rightleftharpoons MgADP + ADP$$

These adenine nucleotides play pivotal roles in acquiring, transferring, and storing chemical energies in living systems. Adenine as well guanine nucleotides are also important as modulators of a number of allosteric enzymes. Consequently, adenylate kinase is indispensable for essentially all organisms, maintaining an equilibrium of cellular concentrations of the three nucleotides depending on the required energy level. The enzyme is particularly abundant in tissues with high energy turnover such as muscle (1). Genetic and biochemical studies of certain *adk* mutants of *Escherichia coli* (2,3) suggested that adenylate kinase may also be involved in the phospholipid synthesis through the formation of a complex with the membranebound *sn*-glycerol-3-phosphate acyltransferase.

Since adenylate kinase exists as a monomeric protein with relatively small size (molecular weight, 21,000-26,000), the enzyme has been studied extensively as a model for nucleotide-binding proteins. So far, the complete amino acid sequence has been determined for the enzymes from various sources. In mammalian tissues, three isozymes, designated AK1, AK2, and AK3,

have been found, purified, and characterized. AK1 is localized in the cytoplasm of skeletal muscle, brain, and erythrocytes, while AK2, the major isozyme in liver and kidney, is localized in the intermembrane space of mitochondria, and AK3, using GTP rather than ATP as the phosphoryl donor, is localized in the mitochondrial matrix. The enzymes from *E. coli* (AKe) and yeast (AKy) also have been purified and characterized. AK1 and the other types of AK are classified into small and large variants, respectively, on the basis of the distinct structural difference between the two variants; AK1 lacks a 30 amino acid residue sequence at about the middle of the polypeptide chain (Fig. 1) (4). Although the overall sequence homology between the small and large variants is rather low (27%), those within each group are significantly high (89% in the small variant and 41% in the large variant). In addition, the conserved residues are unevenly distributed in the entire sequence, forming six highly homologous regions (Fig. 1). These six regions correspond to the polypeptide segments constituting the active-site cleft in the enzyme molecule (5). Thus, the polypeptides of both the small and large variants fold into the three-dimensional structure in an essentially identical manner as revealed by numerous x-ray studies.

The adenylate kinase reaction proceeds with a rapid equilibrium random Bi-Bi mechanism, in which the enzyme binds the two nucleotide substrates without any preferential order. Steady-state kinetic analysis suggested that the rate-limiting step of the reaction is the chemical exchange between the two ternary complexes (E-MgATP-AMP and E-MgADP-ADP) (6,7). The enzyme shows high specificity for nucleoside monophosphate and

relatively broad specificity for nucleoside triphosphate (1). These findings indicate that the enzyme has two distinct binding sites for individual substrates and that these sites should recognize asymmetrically the adenine ring of either ATP or AMP.

The x-ray crystallographic study of adenylate kinase was initiated by Schulz and his co-workers with the porcine muscle AK1 (8). They then compared two crystal forms obtained at pH 6.9 (A, active form) and at pH 5.8 (B, inactive form)(9). A particularly large conformational difference between the two forms was found in the glycine-rich segment of amino acid residues 15-23 (10). Such a pH-dependent conformational movement in the limited region was assumed to correlate with the catalytic mechanism of the enzyme. The three dimensional structure of the A form was finally refined to 2.1 Å resolution (11). Meanwhile, the three dimensional structures of yeast cytosolic enzyme (AKy) (12) and *E. coli* enzyme (AKe) (13) were also determined, showing virtually the same structure with AK1 except for the 30-residue insertion in the large variants. However, X-ray analysis for the enzyme complexed with any substrates, aiming at the identification of the substrate-binding sites, has long been unsuccessful due to the extreme instability of the crystals soaked with substrates (14).

Instead of using the true substrates, the x-ray crystallographic studies employed an efficient inhibitor for the enzyme, adenosine pentaphosphoadenosine (AP_5A), also called "bi-substrate analogue", in the soaking experiments, and proposed a model for the substrate-binding sites as shown in Fig. 2A (14). On the other hand, Mildvan and his co-workers proposed a quite different model (Fig. 2B) based on the results of NMR studies

using an ATP-binding fragment (residue 1-45 of the rabbit muscle AK1) and an AMP-binding fragment (residue 172-194) (15). Hence, it began a long history of controversy on the substrate-binding sites in adenylate kinase. The discrepancy was further augmented by the proposal of another model based on the structure of AKy complexed with the bi-substrate analogue at 2.6 Å resolution (12).

Aside from the above x-ray and NMR studies, the location of the substrate-binding sites has also been investigated by affinity labeling and site-directed mutagenesis. Tagaya *et al.* (16) and Yagami *et al.* (17) showed that all of a series of the affinity labeling reagents specific for ATP-binding proteins, adenosine di-, tri-, and tetraphosphopyridoxals, modify the same lysyl residue, Lys21, contained in the glycine-rich region of the rabbit muscle AK1, and that the enzyme inactivation caused by the modification is retarded most effectively by the addition of ATP but not by the addition of AMP. These observations were interpreted by the location of the ϵ-amino group of Lys21 close to the γ-phosphate of bound MgATP. It was also suggested that the glycine-rich loop containing Lys21 has higher mobility than the other part of the enzyme molecule and that the conformational flexibility of this loop is important for catalytic activity of adenylate kinase. To examine this possibility, Gly15, Pro17, or Gly20 of the recombinant chicken muscle enzyme, all of which are highly conserved in the loop region and probably afford the flexibility to the loop (18), was replaced by other residues by site-directed mutagenesis. The mutant enzymes showed markedly low affinities for both AMP and MgATP, whereas their V_{max} values were comparable to that of the wild-type enzyme. Therefore, it

was concluded that the glycine-rich region in adenylate kinase is important for binding of both substrates (19,20). Similar results were also reported by Reinstein *et al.* (21,22) with the Pro9-Gly or Leu, Gly10-Val, and Lys13-Gln mutant enzymes of AKe. Thus, these studies did not provide the conclusive answer as to either the x-ray model (Fig. 2A) or the NMR model (Fig. 2B) represents the location of the real substrate-binding sites.

To inspect residues in the C-terminal region that has been suggested to interact with the adenine ring of bound AMP by NMR studies, Yoneya *et al.* (23) prepared a series of the chicken muscle mutant enzymes successively truncated in the C-terminal region. A sudden increase in K_m values for substrates, in particular for MgATP, was observed upon removal of Leu190 and residues therefrom. All the mutant enzymes, in which Leu190 was replaced by a variety of other amino acid residues, had substantially low V_{max} values and decreased thermostabilities. Their apparent K_m values for MgATP also changed, whereas those for AMP were affected to a lesser extent. Hydrophobicity of the amino acid residues at position 190 was found to positively correlate with the k_{cat}/K_m values for MgATP. Based on these results, they suggested that the C-terminal residues, particularly those around Leu190, are present in a hydrophobic region which may be involved in binding of MgATP rather than AMP.

The unequivocal and ultimate identification of the substrate-binding sites in adenylate kinase is prerequisite not only to elucidate the catalytic mechanism of adenylate kinase but also to understand the interaction between nucleotides and proteins in molecular detail. The present investigation has been undertaken to shed light on the binding sites in adenylate

kinase for substrate nucleotides, in particular for AMP, by site-directed mutagenesis of the recombinant chicken muscle AK1 and enzymological analysis of the mutant proteins. In the following chapter (Chapter II), the role of Leu66 in the asymmetric recognition of substrates is described and evidence is presented that the hydrophobic region containing Leu66 is involved in binding of AMP directly or indirectly. In Chapter III, a polymerase chain reaction (PCR)-based technique has been applied to introduce random mutation at Val67 and Gln101, both of which are involved in the presumed AMP-binding hydrophobic region. In Chapter IV, I have attempted to change the substrate specificities of adenylate kinase on the basis of sequence comparison with UMP/CMP:ATP kinase (24, 25) and the results described in Chapters II and III. Finally, in Chapter V, these results are summarized and discussed comprehensively.

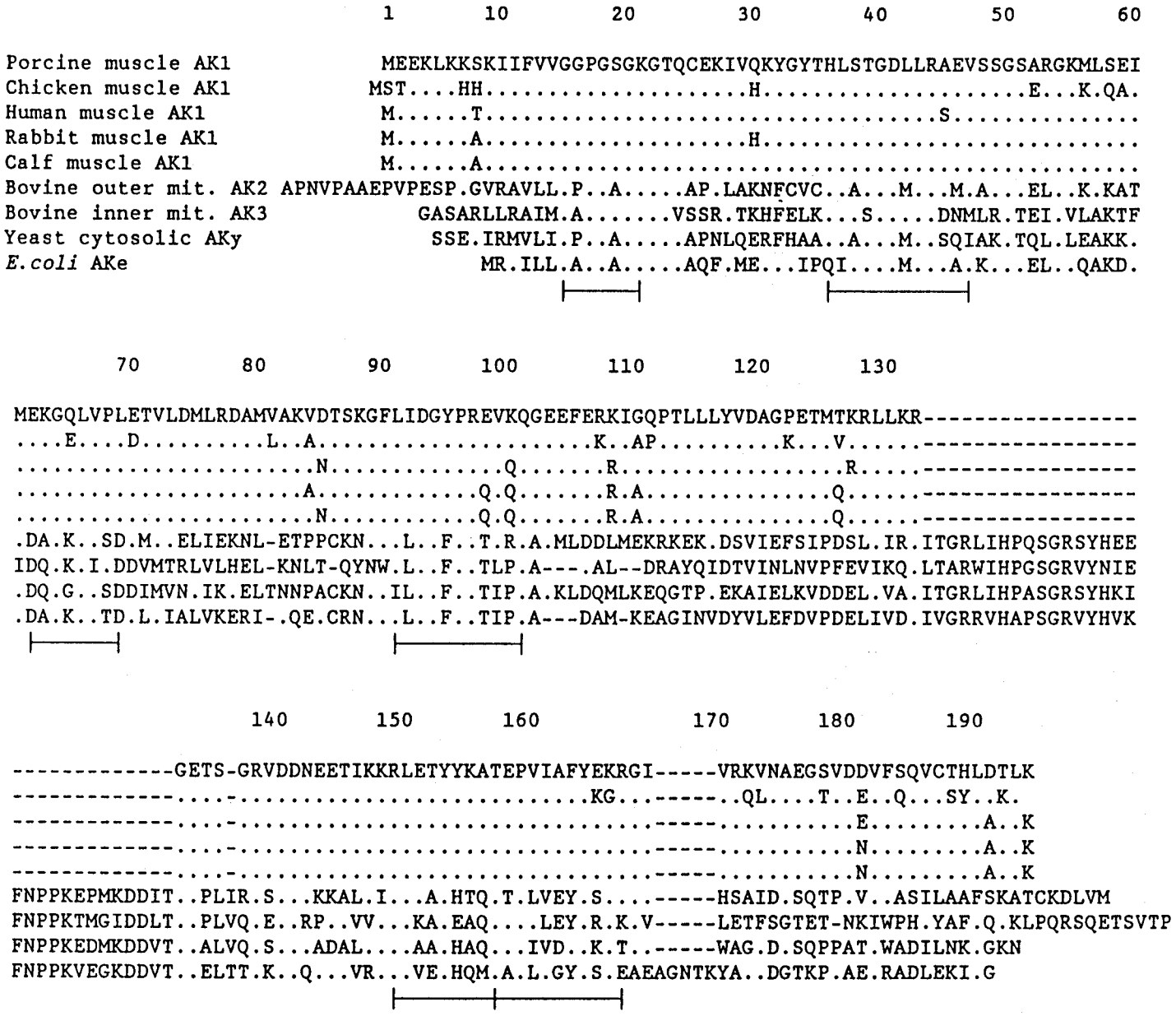


Fig. 1. **Comparison of amino acid sequences of adenylate kinases.** The alignments are according to Schulz *et al.* (5). Six highly homologous regions are indicated. The first Met of the recombinant chicken muscle enzyme is removed when expressed in *E. coli* (18). References for these sequences are: the porcine muscle AK1 [Heil *et al.* (26)], the human muscle AK1 [von Zabern *et al.* (27)], the rabbit muscle AK1 [Kuby *et al.* (28)], the calf muscle AK1 [Kuby *et al.* (28)], the chicken muscle AK1 [Kishi *et al.* (29)], the bovine outer mitochondrial AK2 [Frank *et al.* (30)], the bovine inner mitochondrial AK3 [Tomasselli *et al.* (31)], the yeast cytosolic AKy [Tomasselli *et al.* (32)], and the *E. coli* AKe [Brune *et al.* (33)].

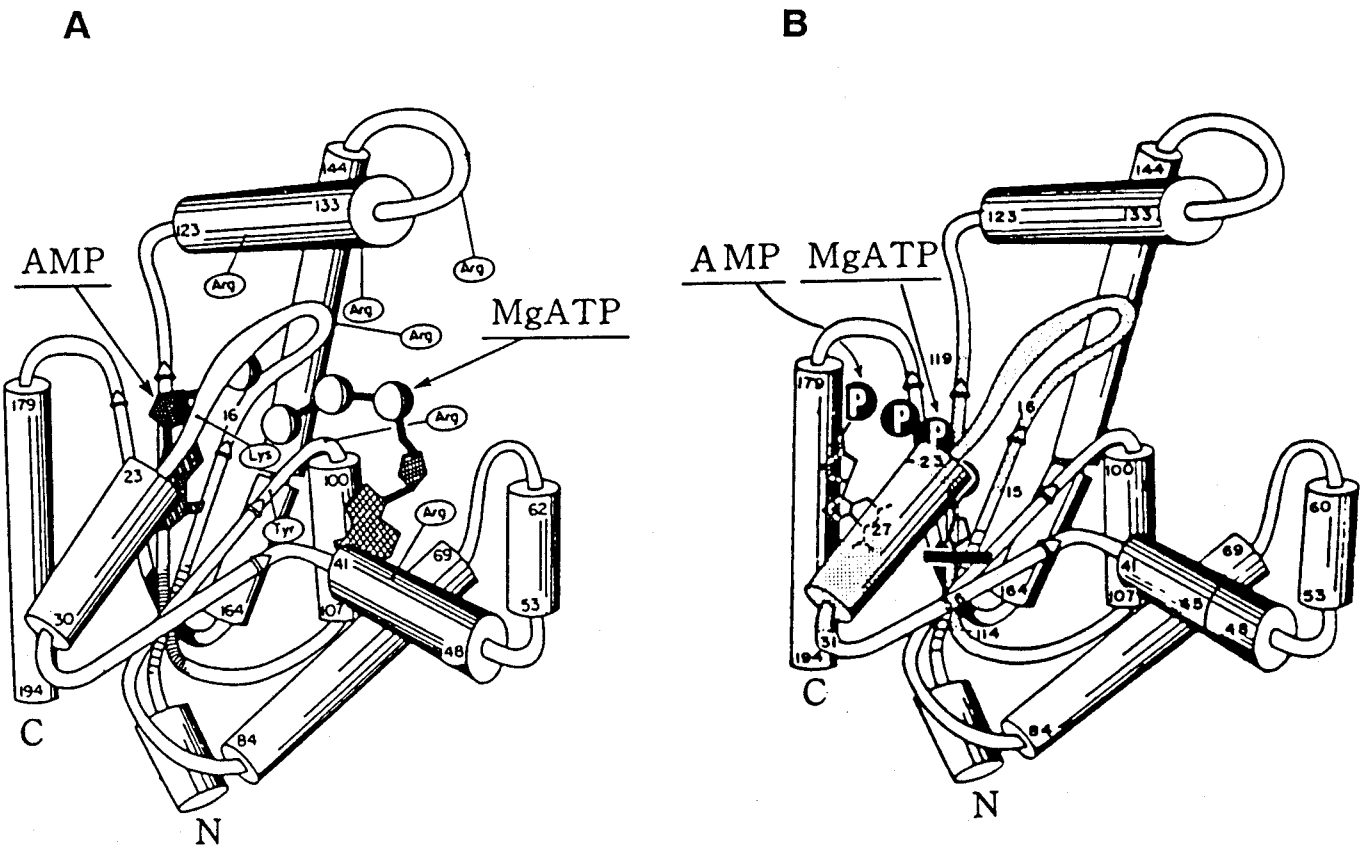


Fig. 2. **Schematic representation of the stereo structure of porcine muscle AK1 and the binding sites for ATP and AMP proposed by x-ray (A; Ref. 14) and NMR (B; Ref.15) studies.** Helices are shown by cylinders and the five strands of plated sheets by allows. It should be noted that Schulz *et al.* (4) later suggested that only the phosphate-binding region in model A is conceivable. Egner *et al.* (12) also reconfirmed the phosphate-binding region but suggested that the adenosine site of ATP in model A is more likely to be that of AMP, on the basis of the crystal structure of AKy and AP_5A complex.

REFERENCES

1. Noda, L. (1973) in *The Enzymes* (Boyer, P. D., 3rd ed) Vol. 8, pp. 279-305, Academic Press, New York
2. Hasse, G. H. W., Brune, M., Reinstein, J., Pai, E. F., Pingoud, A., and Wittinghofer, A. (1989) *J. Mol. Biol.* **207**, 151-162
3. Goelz, S. E. and Cronan, J. E., Jr (1982) *Biochemistry* **21**, 189-195
4. Schulz, G. E., Schiltz, E., Tomasselli,A. G., Frank, R., Brune, M., Wittengofer, A., and Schirmer, R. H. (1986) *Eur. J. Biochem.* **161**, 127-132
5. Schulz, G. E. (1987) *Cold Spring Harbor Symp. Quant. Biol.*, **52**, 429-439
6. Su, S. and Russell, P. J. (1968) *J. Biol. Chem.* **243** 3826-3833
7. Hamada, M. and Kuby, S. A. (1978) *Arch. Biochem. Biophys.* **190**, 772-792
8. Sachsenheimer, W. and Schulz, G. E. (1977) *J. Mol. Biol.* **114**, 23-36
9. Dreusicke, D. and Schulz, G. E. (1988) *J. Mol. Biol.* **203**, 1021-1028
10. Walker, J. E., Saraste, M., Runswick, M. J., and Gay, N. J. (1982) *EMBO J.* **1**, 945-941
11. Dreusicke, D. P., Karplus, A., and Schulz, G. E. (1988) *J. Mol. Biol.* **199**, 359-371
12. Egner, U., Tomasselli, A. G., and Schulz, G. E. (1987) *J. Mol. Biol.* **195**, 649-658
13. Müller, C. W. and Schulz, G. E. (1988) *J. Mol. Biol.* **202**, 909-912
14. Pai, E. F.,Sachsenheimer, W., and Schirmer, R. H. (1977) *J. Mol. Biol.* **114**, 37-45
15. Fry, D. C., Kuby, S. A., and Mildvan, A. S. (1987) *Biochemistry* **26**, 1645-1655
16. Tagaya, M., Yagami, T., and Fukui, T. (1987) *J. Biol. Chem.* **262**, 8257-8261
17. Yagami, T., Tagaya, M., and Fukui, T. (1988) *FEBS Lett.* **229**, 261-264
18. Tanizawa, Y., Kishi, F., Kaneko, T., and Nakazawa, A. (1987) *J. Biochem. (Tokyo)* **101**, 1289-1296
19. Tagaya, M., Yagami, T., Noumi, T., Futai, M., Kishi, F., Nakazawa, A., and Fukui, T. (1989) *J. Biol. Chem.* **264**, 990-994

20. Yoneya, T., Tagaya, M., Kishi, F., Nakazawa, A., and Fukui, T. (1989) *J. Biochem. (Tokyo)* **105**, 158-160
21. Reinstein, J., Brune, M., and Wittinghofer, A. (1988) *Biochemistry* **27**, 4712-4720
22. Reinstein, J., Schlichting, I., and Wittinghofer, A. (1990) *Biochemistry* **29**, 7451-7459
23. Yoneya, T., Okajima, T., Tagaya, M., Tanizawa, K., and Fukui, T. (1990) *J. Biol. Chem.* **265**, 21488-21493
24. Liljelund, P., Sanni, A., Friesen, J. D., and Lacroute, F. (1989) *Biochem. Biophys. Res. Commun.* **165**, 464-473
25. Wiesmüller, L., Noegel, A. A., Bârzu, O., Gerish, G., and Schleicher, M. (1990) *J. Biol. Chem.* **265**, 6339-6345
26. Heil, A., Muller, G., Noda, L. H., Pinder, T., Schirmer, R. H., and von Zabern, I. (1974) *Eur. J. Biochem.* **43**, 131-144
27. von Zabern, I., Wittmann-Liebold, B., Untucht-Grau, R., Schirmer, R. H., and Pai, E. F. (1976) *Eur. J. Biochem.* **68**, 281-290
28. Kuby, S. A., Palmieri, R. H., Frischat, A., Fischer, A. H., Wu, L. H., Maland, L., and Manship, M. (1984) *Biochemistry* **23**, 2393-2399
29. Kishi, F., Maruyama, M., Tanizawa, Y., and Nakazawa, A. (1986) *J. Biol. Chem.* **261**, 2942-2945
30. Frank, R., Trosin, M., Tomasselli, A. G., Noda, L., Krauth-Siegel, R. L., and Schirmer, R. H. (1986) *Eur. J. Biochem.* **154**, 205-211
31. Tomasselli, A. G., Frank, R., and Schiltz, E. (1986) *FEBS Lett.* **202**, 303-308
32. Tomasselli, A. G., Mast, E., Janes, W., and Schiltz, E. (1986) *Eur. J. Biochem.* **155**, 111-119
33. Brune, M., Schumann, R., and Wittinghofer, A. (1985) *Nucleic Acids Res.* **13**, 7139-7151

Chapter II

Role of Leucine 66 in the Asymmetric Recognition of Substrates

INTRODUCTION

Adenylate kinase (EC 2.7.4.3) is a small monomeric enzyme that catalyzes the reversible phosphoryl transfer between MgATP and AMP. The enzyme occurs in a wide variety of organisms and is important in maintaining a high energy level in tissues such as muscle. The complete amino acid sequences determined for the enzymes from various sources show considerable similarities with each other (1), and their three-dimensional structures are virtually the same (2-5). The enzyme has distinct binding sites for the two nucleotide substrates. However, their locations in the three-dimensional structure are still inconclusive, despite extensive efforts for the determination by NMR (6,7) and x-ray diffraction studies (4,8).

Previous studies in this laboratory demonstrated that Lys21 located in the glycine-rich flexible loop of adenylate kinase was specifically modified by affinity labeling with adenosine di-, tri-, and tetraphosphopyridoxals, suggesting that the γ-phosphate group of the bound MgATP is close to Lys21 (9,10). A recent site-directed mutagenesis study of the recombinant chicken muscle enzyme has also suggested that Leu190 in the C-terminal helix plays an important role in the binding of MgATP probably through a hydrophobic interaction with the adenine ring of MgATP (11).

Aiming at an unequivocal identification of the substrate-binding sites of adenylate kinase, in particular the binding site for the adenine ring of AMP, I have chosen Leu66 as a target residue for site-directed mutagenesis. Leu66 is invariant in all the enzymes sequenced to date (1), and yet its conservation has

been unexplained functionally. In addition, a hydrophobic region including Leu75 of the yeast cytosolic enzyme (corresponding to Leu66 of the enzymes from other sources) has been proposed as the putative AMP-binding site by x-ray diffraction studies of the crystals complexed with a bi-substrate analog, adenosine pentaphosphoadenosine (4). Various Leu66 mutant enzymes of the chicken muscle adenylate kinase have been prepared and analyzed for their catalytic and spectral properties. The possible role of Leu66 in binding of the adenine ring of AMP and its involvement in the positive cooperativity of substrate binding are discussed in this chapter.

EXPERIMENTAL PROCEDURES

Materials——Nucleoside mono- and triphosphates were obtained from Seikagaku Kogyo or Sigma. A site-directed mutagenesis kit, Mutan-G, was purchased from Takara Biochemicals. All other reagents were of the highest purity commercially available.

Site-directed Mutagenesis——Replacement of Leu66 by Ala, Gly, Gln, Val or Trp was accomplished by oligonucleotide-directed mutagenesis of the *Eco*RI-*Hin*dIII fragment (encoding chicken adenylate kinase) excised from the pKK-cAK1-1 (12) and subcloned into the M13tv19 vector. Oligonucleotide primers used were synthesized with an Applied Biosystems DNA synthesizer model 381. Their sequences are summarized in Table I. Mutagenesis was carried out by the method of Kramer *et al.* (13). The entire region of the enzyme gene including the mutated site was confirmed by DNA sequencing (14) of the *Eco*RI-*Hin*dIII fragments subcloned into M13mp18 and M13mp19 vectors. The *Eco*RI-*Hin*dIII fragments were recloned into pKK223-3, and the resultant plasmids were transformed into *E. coli* JM109 as described by Maniatis *et al.* (15).

Purification of Wild-type and Leu66 Mutant Enzymes——Wild-type and Leu66 mutant enzymes expressed in *E. coli* JM109 were purified to homogeneity from the soluble fraction of cell lysates essentially as described by Tanizawa *et al.* (12). The purity of the enzyme preparations was checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (16).

Steady State Kinetic Analysis——The enzyme activity was determined in the standard assay mixture as described previously

(17). Steady state kinetic parameters were obtained by systematic variation of the concentrations of both AMP and MgATP (18) in the assay mixture consisting of 87 mM triethanolamine-HCl buffer (pH 7.0), 10 mM $MgCl_2$, 100 mM KCl, 1 mM phosphoenol-pyruvate, 0.16 mM NADH, 5 units of pyruvate kinase, and 20 units of lactate dehydrogenase. After preequilibration at 25 °C for about 2 min, an appropriate amount of adenylate kinase was added to the assay mixture, and the reaction was carried out at 25 °C and monitored by the decrease in absorbance at 340 nm.

CD Measurements——CD spectra were measured at 25 °C in 30 mM Tris-HCl buffer, pH 7.5, containing 0.1 M NaCl, 1 mM EDTA, and 0.1 mM dithiothreitol with a Jasco spectropolarimeter model J-600. The CD instrument was calibrated with ammonium (+)-10-camphorsulfonate, $\Delta\epsilon$ =+2.37 $M^{-1}cm^{-1}$ at 290.5 nm. In the calculation of the mean residue ellipticity, [θ], the mean residue weight was taken to be 111 for the enzyme protein. The CD spectra were obtained at a protein concentration of 0.3 mg/ml in a 2.0-cm light pass length cell in the wavelength region above 250 nm and at a protein concentration of 0.1 mg/ml in a 0.1-cm light pass length cell in the wavelength region below 250 nm.

Fluorescence Measurements——Fluorescence spectra of the Leu66-Trp mutant enzyme (1 μM) were measured at 25 °C with a Hitachi spectrofluorometer model F-4010. The fluorescence emission due to Trp66 was recorded upon excitation at 295 nm.

Thermal Stability——Thermal stabilities of the wild-type and Leu66 mutant enzymes were analyzed by monitoring the protein unfolding process by heat, following the changes in ellipticity at 222 nm. All measurements were carried out in 5 mM sodium phosphate buffer, pH 7.2, containing 0.1 M NaCl, 1 mM EDTA, and 1

mM dithiothreitol at a protein concentration of 0.1 mg/ml. The temperature was raised at a constant rate of 1 °C/min through a thermostatic cell holder and was measured directly in the cuvette with a Rikagaku Kogyo thermometer model DP-500. Reversibility of the unfolding process was examined by measuring the ellipticity after gradual lowering of the temperature to 25 °C.

RESULTS

Purification of Leu66 Mutant Enzymes——The substitutions of Ala, Gly, Val, Gln, and Trp for Leu66 of the recombinant chicken muscle enzyme were done by site-directed mutagenesis with synthetic oligonucleotide primers (Table I). The expression levels of the Leu66 mutant proteins obtained were as high as that of the wild-type enzyme (about 10%) as judged from polyacrylamide gel electrophoresis of the cell lysate (Fig. 1). Although it was previously observed that some of adenylate kinase mutant proteins were recovered in the pellet of cell lysate (11,17,19), all the Leu66 mutant enzymes prepared in this study were found in the soluble fraction as was the wild-type enzyme. Thus, the Leu66 mutant enzymes were readily purified to homogeneity (Fig. 1) essentially according to the procedure reported for the purification of the recombinant wild-type enzyme (12). An averaged final yield in purification of the mutant proteins was about 5 mg from 200-ml cultures of each clone.

Physical Characterization of Mutant Enzymes——To examine whether single mutations at Leu66 had affected deleteriously the overall conformation of the enzyme protein, CD spectra of the Leu66 mutant enzymes were measured (Fig. 2). Their spectra, after correction at the same protein concentration, overlapped completely with that of the wild-type enzyme in the entire wavelength region from 200 to 320 nm. This suggests that conformational changes of the protein caused by the replacement of Leu66 with other residues are very subtle, if any. It is also noteworthy that the CD spectrum of the Leu66-Trp mutant protein

is identical with those of the wild-type and other mutant proteins in the region for the ellipticity of aromatic amino acid residues (260-300 nm); the chicken wild-type enzyme contains no tryptophanyl residue. Thus, the single newly introduced tryptophanyl residue in the Leu66-Trp mutant protein exhibits no molecular ellipticity, in contrast with that in the Leu190-Trp mutant protein reported previously (11), which is located in the hydrophobic protein interior and appears to be restricted in its movement, showing some ellipticity.

Conformational stabilities of the wild-type and Leu66 mutant proteins were analyzed by monitoring the thermal unfolding process, following the changes in ellipticity at 222 nm. Thermodynamic parameters for the unfolding process calculated according to the method of Pace *et al.* (20) are summarized in Table II. Although reversibilities of the unfolding process were rather low (30-50%) with both the wild-type and mutant proteins under the experimental conditions employed, it is evident that the mutation at position 66 had little effect on the conformational stability of adenylate kinase, as judged from the small changes in these thermodynamic parameters. Contribution of Leu66 for stability of the enzyme protein is probably negligible, and conformational changes derived from the mutation may be confined to a local region around Leu66.

Steady State Kinetic Parameters——All the Leu66 mutant enzymes showed significantly lowered but detectable activities when measured under the standard assay conditions (Table III), indicating that Leu66 is catalytically dispensable. To evaluate kinetically the decreased activities of the Leu66 mutant enzymes, I have performed steady state kinetic analysis using the method

reported for two-substrate enzyme systems (18,21). Assuming that the chicken enzyme catalyzes the reaction in a random quasi-equilibrium Bi-Bi mechanism as do other mammalian adenylate kinases (12), the steady state reaction mechanism is described as shown in Scheme I, where K_{m1} and K_{m4} represent the Michaelis constants for AMP at zero and infinite concentrations of the other substrate MgATP, respectively, and K_{m2} and K_{m3} are those for MgATP at zero and infinite concentrations of AMP, respectively. If the phosphoryl transfer is the rate limiting step in the forward reaction and all the other equilibria are adjusted rapidly, then the initial reaction rate (v) is given by equations (i) and (ii) using these Michaelis constants and the maximum rate (V_{max}) obtained at infinite concentrations of both AMP and MgATP (21).

$$v = \frac{V_{max}}{1 + \frac{K_{m4}}{[AMP]} + \frac{K_{m3}}{[MgATP]} + \frac{K_{m2}K_{m4}}{[AMP][MgATP]}} \qquad \text{(i)}$$

$$K_{m1}K_{m3} = K_{m2}K_{m4} \qquad \text{(ii)}$$

By plotting the reciprocals of initial reaction rates measured by systematic variation of the concentrations of both AMP and MgATP (see "EXPERIMENTAL PROCEDURES") against the reciprocals of concentrations of either substrate, steady state kinetic parameters were obtained for the wild-type and Leu66 mutant enzymes as summarized in Table III. In the cases where the activity was significantly inhibited at high substrate concentrations as observed with some mutant enzymes (see below), only linear portions of the double-reciprocal plots were taken

for calculation of the kinetic parameters. The data in Table III clearly show that the Michaelis constants for AMP (K_{m1} and K_{m4}) increased markedly by replacement of Leu66 by other residues, while those for MgATP (K_{m2} and K_{m3}) remained in the same order of magnitude. The maximum rates V_{max} of the mutant enzymes were also only 7-34% of that of the wild-type enzyme. These results support a view that Leu66 is involved in binding of AMP directly or indirectly.

It is known that adenylate kinase has a highly specific binding site for AMP or ADP and a less specific one for MgATP or MgADP (22). In fact, the chicken wild-type enzyme also binds AMP to the MgATP-binding site as revealed by the notable inhibition by AMP at a concentration above 1 mM in the presence of fixed concentrations of MgATP (0.2-5 mM) (data not shown). However, the inhibition by AMP was undetectable with Leu66 mutant enzymes except for the Leu66-Trp mutant. Furthermore, MgATP above 2 mM, which is uninhibitory to the wild-type enzyme, in the presence of fixed concentrations of AMP (0.5-5 mM) also inhibited considerably the Leu66 mutant enzymes except for the Leu66-Gly mutant (Fig. 3). These observations lead to a suggestion that the AMP-binding site in the mutant enzymes has been deteriorated to a less specific one with a weakened affinity even for AMP; the MgATP-binding site, *vice versa*, has gained an elevated specificity. Thus, Leu66 may play a role in the asymmetric recognition of the adenine ring of AMP from that of MgATP. Leu66 also appears to be important for the positive cooperativity of substrate binding as will be discussed later.

Quenching of Leu66-Trp Fluorescence by Substrates——The absence of tryptophanyl residues in the vertebrate cytosolic

adenylate kinases (the small variants) makes it possible to study spectroscopically the environment surrounding a sole tryptophanyl residue newly introduced by site-directed mutagenesis. The Leu66-Trp mutant emitted the expected fluorescence around 350 nm upon excitation at 295 nm (Fig. 4A), where the wild-type enzyme exhibits no fluorescence (11). When the Leu66-Trp mutant protein was denatured in the presence of 4 M guanidine hydrochloride, the fluorescence intensity decreased to about a half of that in the native state, showing that the tryptophanyl residue is fully exposed to solvent. However, a red shift of the fluorescence maximum, which is often observed when tryptophans in hydrophobic surroundings are exposed to solvent by denaturation of proteins, did not take place. It is, therefore, suggested that the tryptophanyl residue at position 66 may be in a solvent accessible environment to some extent. This is consistent with the observation described above that the side chain of Trp66 has high mobility without showing molecular ellipticity.

The addition of substrates at low concentrations resulted in marked quenching of the tryptophanyl fluorescence, AMP being more effective than MgATP (Fig. 4B). Since the fluorescence of the denatured Leu66-Trp mutant protein was not influenced at all by the presence of substrates, the quenching is attributable to the binding of substrates with the native Leu66-Trp protein. The dependence of the extent of quenching on both substrate concentrations was studied to determine the binding constants K_d for AMP and MgATP. However, the Leu66-Trp mutant enzyme appeared to bind MgATP also to the AMP-binding site and AMP also to the MgATP-binding site as suggested from the significant inhibition at high concentrations of the two substrates. Thus, taking into

account of the existence of two independent binding sites for both substrates, the fluorescence quenching was analyzed by equation (iii) (23), where ΔF, ΔF_{max}, and F_0 represent the extent of quenching at a given concentration of the ligand [L] (either AMP or MgATP), the maximum quenching at an infinite concentration of the ligand, and the fluorescence intensity in the absence of the ligand, respectively, and the suffixes 1 and 2 denote the parameters for the two binding sites.

$$\Delta F/F_0 = (\Delta F_{max,1}/F_0)\{[L]/([L]+K_{d,1})\} + (\Delta F_{max,2}/F_0)\{[L]/([L]+K_{d,2})\} \quad \text{(iii)}$$

The Scatchard-type plots of $\Delta F/F_0$ against $\Delta F/F_0 \cdot 1/[L]$ (Fig. 5) were subjected to curve analysis with a computer program, dissolving into two linear functions, the slopes of which are equal to $K_{d,1}$ and $K_{d,2}$ and the intercepts on the ordinate are equal to $\Delta F_{max,1}/F_0$ and $\Delta F_{max,2}/F_0$. With MgATP as a ligand, the best fit of the curve yielded 1.54 and 0.17 mM for $K_{d,1}$ and $K_{d,2}$, respectively, and 0.87 and 0.13 for $\Delta F_{max,1}/F_0$ and $\Delta F_{max,2}/F_0$, respectively. In contrast, with AMP as a ligand, most of the data points were fitted to one linear function, and only a few points at high concentrations of AMP deviated from that. Therefore, the curve was analyzed based on an assumption that the $K_{d,2}$ value for AMP of the Leu66-Trp mutant enzyme is close to 4.3 mM, which was reported as an inhibition constant for AMP at the MgATP-binding site of the chicken muscle wild-type enzyme (24), and with $\Delta F_{max,2}/F_0 = 0.13$ obtained from the quenching by MgATP. Hence, a $K_{d,1}$ value of 0.44 mM and a $\Delta F_{max,1}/F_0$ value of 0.81 were obtained. These results are interpreted to show that 80-90% of

the quenching of Trp66 fluorescence is derived from the binding of either substrate to the AMP-binding site, and the rest is due to the binding of either substrate to the MgATP-binding site. Although the binding constant $K_{d,2}$ for MgATP is comparable with the Michaelis constant K_{m2} for MgATP of the Leu66-Trp mutant determined by the steady state kinetic analysis (Table III), there is a notable discrepancy between the binding constant $K_{d,1}$ and Michaelis constant K_{m1} for AMP. A likely explanation for this discrepancy is that the rapid equilibrium random mechanism may be inapplicable to the Leu66-Trp mutant enzyme. Nevertheless, the marked fluorescence quenching due to the binding at the AMP-binding site may be attributable to a direct interaction of the bound nucleotides with the indole ring of Trp66, whereas the small quenching due to the binding at the MgATP-binding site may be a result of indirect conformational changes around Trp66 elicited upon binding of substrate at that site.

Substrate Specificities——The results described so far show that the replacement of Leu66 by other residues impaired kinetically the binding properties for substrates, in particular for AMP. To study whether the replacement also affected the phosphoryl acceptor specificity at the AMP-binding site, several nucleoside monophosphates including dAMP, GMP, IMP, CMP, and UMP (1 mM) were examined in the assay solution containing 1 mM MgATP as the phosphoryl donor, following the rates of ADP formation. The relative activities of the Leu66 mutant enzymes (except for the Leu66-Trp mutant) in the reaction with the MgATP-CMP pair did not vary significantly from that of the wild-type enzyme, but the relative activities of the mutants (except for the Leu66-Gly

mutant) in the reaction with the MgATP-dAMP pair were considerably different from that of the wild-type enzyme (Table IV). Other nucleoside monophosphates, such as GMP, IMP, and UMP, served as a phosphoryl acceptor neither for the wild-type nor for the Leu66 mutant enzymes. Thus, the phosphoryl acceptor specificity was also affected by the mutation at Leu66, though only in the reaction with the MgATP-dAMP pair.

DISCUSSION

Taking advantage of the spatial coordinates of the porcine muscle adenylate kinase available in the protein data bank (3), hydrophobic regions which may interact with the adenine ring of the enzyme-bound AMP were searched by a computer graphics and a region containing Leu43, Met61, Leu66, Val67 (Fig. 6), Val47, and Val72 was found. These residues coincide with those that have been pointed out to be involved in the interaction with one of the two adenine rings of pentaphosphoadenosine bound to the yeast enzyme crystals (4). Furthermore, all these residues are highly conservative in adenylate kinase sequences reported so far (1). As a first step to elucidate the role of this hydrophobic region in the function of adenylate kinase, Leu66 have been selected as a target residue for site-directed mutagenesis and replaced by Ala, Gly, Val, Gln, and Trp, which have side chains with hydrophobic properties different from that of Leu. Amino acids having completely different side chains (*e.g.*, charged and hydrophilic groups) were excluded from the replaced residues to avoid destructive influences on the total hydrophobicity of this region and on the overall conformation of the enzyme protein possibly exerted by substitution with such residues.

Based on the results described herein, it is proposed that the AMP-binding site in adenylate kinase is located close to Leu66 and this residue interacts directly or indirectly with the adenine ring of the bound AMP. Although the significant activities found with all the Leu66 mutant enzymes clearly show that Leu66 is nonessential for catalysis, its replacement by

other residues leads to a marked distortion of substrate binding properties, more pronouncedly for AMP than for MgATP. The marked quenching of Trp66 fluorescence by either substrate is strong evidence for the direct interaction of the indole ring with an adenine ring. The proposed location of the AMP-binding site is fully consistent with that most recently reported in the three-dimensional structure of the complex between the bovine mitochondrial enzyme and AMP (25), in which Leu63 (corresponding to Leu66 of the small variants) is one of the residues contacting with the adenine ring of AMP (distances below 4 Å).

Most of the Leu66 mutant enzymes not only show perturbed K_m values for AMP but also are inhibited by high concentrations of MgATP, indicating that MgATP also binds to the AMP-binding site originally specific for AMP in the wild-type enzyme. In contrast, the MgATP-binding site is less specific for nucleotide substrates; the wild-type enzyme and the Leu66-Trp mutant enzyme as well are inhibited by high concentrations of AMP. Thus, to analyze the reaction mechanism including these inhibitions, Scheme I described above should be modified as shown in Scheme II, where K_{12}, K_{13}, and K_{14} are the Michaelis constants for AMP of the indicated states of the enzyme, and K_{22}, K_{23}, and K_{24} are those for MgATP. Similarly, equations (i) and (ii) given for the initial reaction rate (v) also should be rewritten to equations (iv) and (v).

$$v = \frac{V_{max}}{1+\frac{K_{m4}}{[AMP]}\ 1+\frac{K_{m2}}{K_{22}}+\frac{K_{m3}}{[MgATP]}\ 1+\frac{K_{m1}}{K_{12}}+\frac{[AMP]K_{m3}}{[MgATP]K_{14}}+\frac{[MgATP]K_{m4}}{[AMP]K_{24}}+\frac{K_{m1}K_{m3}}{[AMP][MgATP]}} \qquad \text{(iv)}$$

$$K_{12}K_{13} = K_{m1}K_{14},\ K_{22}K_{23} = K_{m2}K_{24},\ K_{m1}K_{m3} = K_{m2}K_{m4} \qquad \text{(v)}$$

The kinetic data at high substrate concentrations obtained here were insufficient to calculate all these K_m values, but it is possible to give qualitative interpretations for kinetics of the substrate inhibition according to equations (iv) and (v). For example, the significant inhibition by MgATP of most Leu66 mutant enzymes is most likely ascribed to its binding to the AMP-binding site, and therefore their K_{m4}/K_{24} values would be larger than that of the wild-type enzyme. In contrast, the wild-type enzyme would have a K_{m3}/K_{14} value larger than the K_{m4}/K_{24} value because of the detectable inhibition by AMP. Since K_{m3} and K_{m4} of the wild-type enzyme are almost equal (Table III) and K_{14} is around 4.3 mM (24), K_{24} should be far above 5 mM, in agreement with no inhibition by MgATP below 5 mM. The NMR study (24) also indicated that more than one molecule of MgATP are bound with the wild-type enzyme at a concentration of MgATP higher than 5 mM. On the other hand, the absence of AMP inhibition in most Leu66 mutant enzymes may be explained by their markedly large K_{m1} values, resulting in a very small formation of the AMP-enzyme binary complex, which is the enzyme species responsible for the AMP inhibition. Only the wild-type and Leu66-Trp mutant enzymes, both having K_{m1} values comparable to the K_{m2} values, are inhibited by AMP through its binding to the MgATP site. The idea that the inhibition by MgATP of most Leu66 mutant enzymes is caused through its competitive binding to the AMP site is also supported by the significant fluorescence quenching by MgATP of the Leu66-Trp mutant. The binding constant $K_{d,1}$ for MgATP (1.54 mM) determined from the quenching analysis would correspond to the Michaelis constant K_{24} measurable in the inhibition by MgATP, the value of which would be comparable with that of K_{m4} (1.0 mM,

Table III). Taken altogether, it is suggested that Leu66 plays a role in the asymmetric recognition of the adenine ring of AMP from that of MgATP.

Steady state kinetic analysis of the wild-type and Leu66 mutant enzymes reveals another interesting aspect of the catalytic properties of adenylate kinase, *i.e.* the cooperativity of substrate binding (26). As shown in Table III, the Michaelis constants K_{m3} and K_{m4} for MgATP and AMP, respectively, are smaller than K_{m2} and K_{m4}, respectively, in the wild-type enzyme. Thus, the affinities for the second substrate increase after binding of the first substrate (either AMP or MgATP). This positive effect is probably mediated by allosteric conformational changes induced upon binding of either substrate. If a simplified factor for the cooperativity is expressed by β ($= K_{m2}/K_{m3} = K_{m1}/K_{m4}$, *cf.* equation (ii); $\beta > 1.0$, positively cooperative; $\beta = 1.0$, uncooperative; $\beta < 1.0$, negatively cooperative), the wild-type, Leu66-Ala, Leu66-Val, and Leu66-Trp mutant enzymes have β values of larger than 1.0 (Table III). However, the β values of Leu66-Gly and Leu66-Gln mutant enzymes are lower than 1.0, indicating that these two mutant enzymes show negative cooperativity in binding of substrates. On the basis of the nature of residues substituted for Leu66, it appears likely that hydrophobicity of the residue at position 66 positively correlates with the extent of allosteric transitions induced by the substrate binding. The segment of residue 50 through residue 77 (including Leu66) has been postulated to move considerably toward the protein interior upon binding of AMP (25,27). Replacement of Leu66 by less hydrophobic residues may retard such conformational changes required for showing the positive cooperativity.

Although Leu66 is one of the clustered hydrophobic residues (Fig. 6), it is situated in a solvent accessible environment in the absence of substrate and has a relatively high "B factor" in the x-ray diffraction data (3), showing its rather free movement in the crystal structure. This is consistent with the present results that the indole ring of Leu66-Trp mutant enzyme shows no molecular ellipticity and its fluorescence maximum is not blue-shifted as is generally observed for Trp residues in a fully hydrophobic environment. Presumably, the region of the clustered hydrophobic residues including Leu66 is somewhat open to solvent before binding of AMP but moves toward the protein interior upon binding of AMP with this region, covering up the active site cleft to protect against water.

All the Leu66 mutant enzymes showed substantially decreased maximum rates V_{max} (Table III). This indicates that Leu66 also participates indirectly in the catalytic process. Assuming that the location of the adenine ring of AMP bound with the Leu66 mutant enzymes is slightly different from that in the wild-type enzyme, the phosphate group of AMP that accepts the γ-phosphate group from the bound MgATP would be also located at the position slightly different from that in the wild-type enzyme. This incorrect positioning of the AMP phosphate could be a reason for the decreased activity of the Leu66 mutant enzymes. Supported by this suggestion is that the phosphoryl acceptor specificity is affected by mutation at Leu66 to such an extent that even the absence of a hydroxyl group in the ribose ring of AMP can be discriminated (Table IV). Alternatively, the mutation at Leu66 may affect the catalytic efficiency indirectly through gross conformational alteration.

TABLE I

Sequences of synthetic primers for site-directed mutagenesis

Sequence[a]	Corresponding mutant
63 66 69 -Lys-Gly-Glu-Leu-Val-Pro-Leu- AAG GGC GAG CTG GTT CCC CTG	(wild-type)
5'-AAG GGC GAG G**C**G GTT CCC CT-3'	Leu66-Ala
5'-AAG GGC GAG G**G**G GTT CCC C-3'	Leu66-Gly
5'-GGC GAG *C AG GTT CCC-3'	Leu66-Gln
5'-AG GGC GAG *G TG GTT CCC-3'	Leu66-Val
5'-AG GGC GAG T**G**G GTT CCC CT-3'	Leu66-Trp

[a]The mutated codons are underlined and mismatched bases are shown by asterisks.

TABLE II

Thermal unfolding parameters for wild-type and Leu66 mutant adenylate kinases[a]

Enzyme	T_m	ΔH	ΔS
	°C	*kcal/mol*	*kcal/deg/mol*
Wild-type	60.2	73.3	0.220
Leu66-Ala	60.0	73.3	0.220
Leu66-Gly	54.7	70.0	0.214
Leu66-Gln	59.2	66.6	0.200
Leu66-Val	56.5	67.6	0.205
Leu66-Trp	53.4	70.4	0.216

[a]Thermodynamic parameters for the thermal unfolding process were determined by monitoring the changes in ellipticity [θ] at 222 nm as described under "*Experimental Procedures*". T_m is the temperature at the midpoint of the unfolding transition. The thermal transition curve was analyzed by assuming the two-state approximation of N (native) $\rightleftharpoons$ D (unfolded), and the equilibrium constant of unfolding (K_D = [D]/[N]) was determined from the equation $K_D = f_D/(1-f_D)$, where f_D is the fraction of the unfolded molecule at each temperature. Enthalpy and entropy changes (ΔH and ΔS) at T_m were determined from the van't Hoff plot.

TABLE III

Kinetic parameters for wild-type and Leu66 mutant adenylate kinases

Enzyme	Specific activity	K_{m1}	K_{m2}	K_{m3}	K_{m4}	β^a	V_{max}
	units/mg	*mM*	*mM*	*mM*	*mM*		*units/mg*
wild-type	1900	0.42	0.31	0.12	0.16	2.5	2500
Leu66-Ala	20	11.2	0.84	0.33	4.8	2.4	180
Leu66-Gly	28	5.9	0.44	0.62	8.3	0.7	244
Leu66-Gln	33	6.1	0.33	0.52	9.5	0.6	379
Leu66-Val	70	8.3	0.68	0.50	6.1	1.4	840
Leu66-Trp	83	2.0	0.23	0.12	1.0	1.9	202

[a]The simplified cooperativity factor calculated according to $\beta = K_{m2}/K_{m3} = K_{m1}/K_{m4}$, see text for details.

TABLE IV

Phosphoryl acceptor specificity of wild-type and Leu66 mutant adenylate kinases[a]

Enzyme	Phosphoryl acceptor		
	AMP	dAMP	CMP
Wild-type	(100)	9.1	0.30
Leu66-Ala	(100)	1.1	0.50
Leu66-Gly	(100)	1.0	0.29
Leu66-Gln	(100)	0.8	0.48
Leu66-Val	(100)	0.3	0.27
Leu66-Trp	(100)	4.6	<0.04

[a]The enzyme was assayed at 25 °C in 87 mM triethanolamine-HCl (pH 7.0) containing 10 mM $MgCl_2$, 100 mM KCl, 1 mM ATP, 1 mM mononucleotide indicated, 1 mM phosphoenol-pyruvate, 0.16 mM NADH, 5 units of pyruvate kinase, and 20 units of lactate dehydrogenase. The activity of each enzyme in the MgATP-AMP pair was taken as 100.

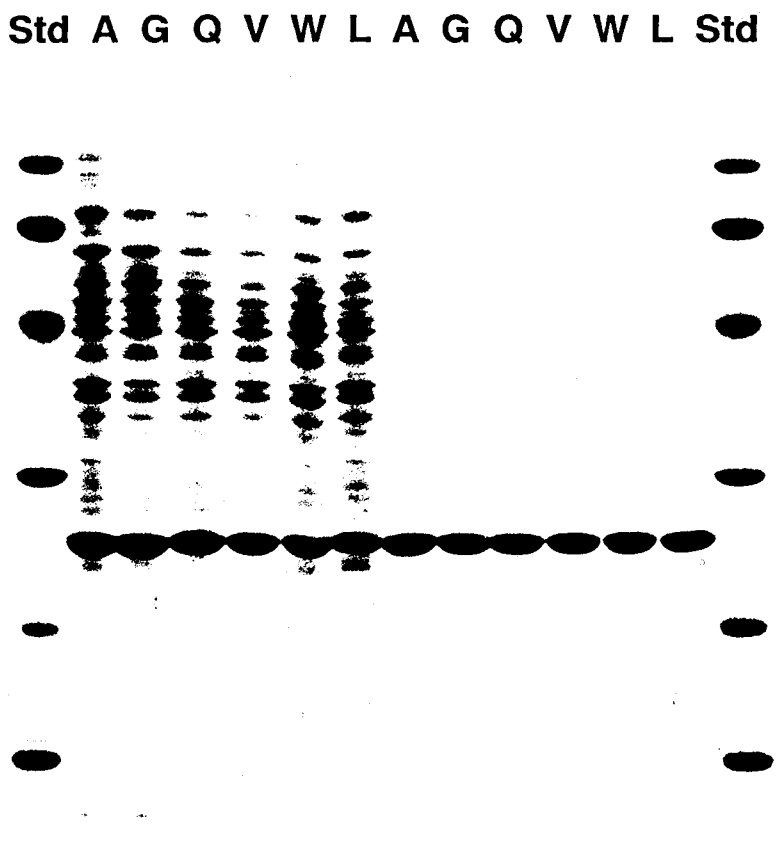


FIG. 1. **Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the recombinant wild-type and Leu66 mutant adenylate kinases.** The supernatants from recombinant cell lysates (left six lanes, about 20 μg total protein in each lane) and the purified enzymes (right six lanes, about 5 μg protein in each lane) were analyzed with a 12.5% polyacrylamide gel. The wild-type and mutant enzymes are shown by one letter code for the residue at position 66. Standard proteins (Std) are phosphorylase b (M_r 94,000), bovine serum albumin (M_r 67,000), egg albumin (M_r 43,000), carbonic anhydrase (M_r 30,000), trypsin inhibitor (M_r 20,100), and α-lactalbumin (M_r 14,400) (from top to bottom).

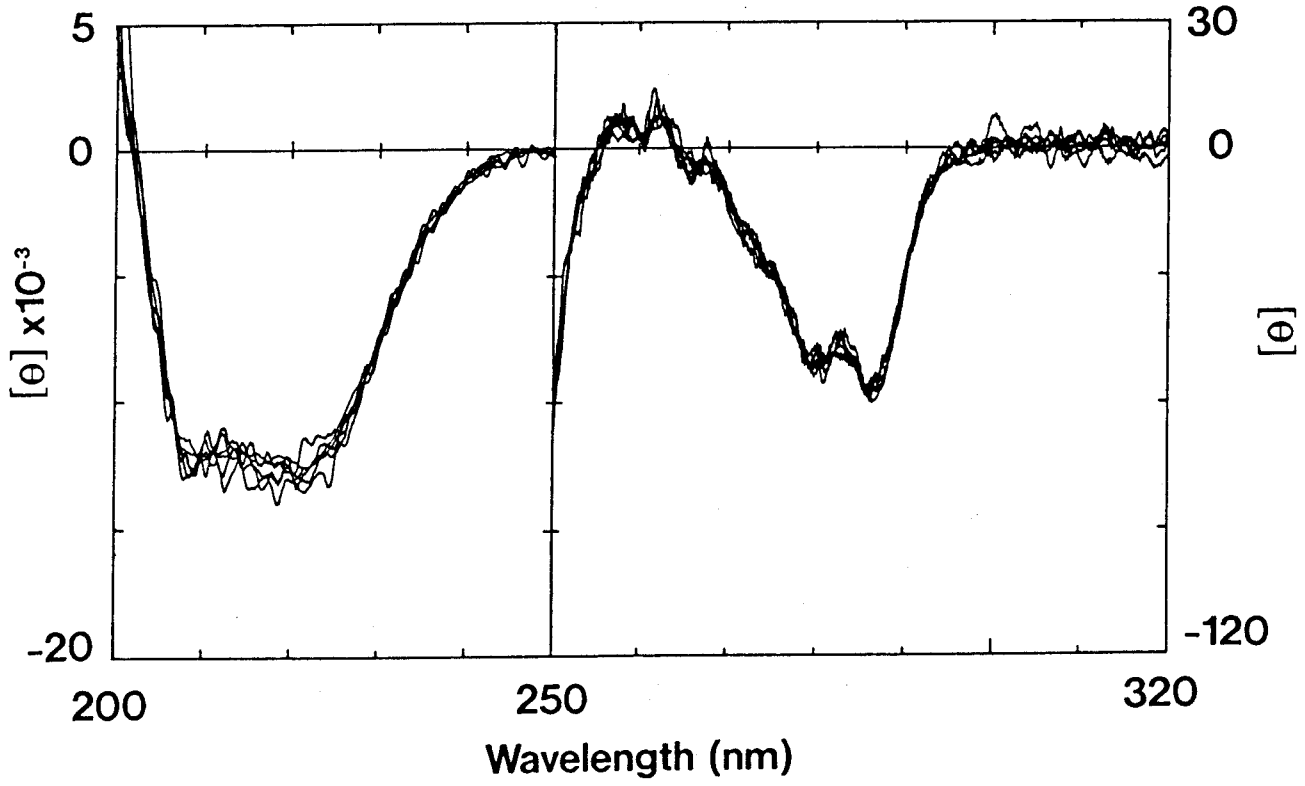


FIG. 2. CD spectra of the wild-type and Leu66 mutant adenylate kinases. The CD spectra were measured at a protein concentration of 0.3 mg/ml in the region between 250 and 320 nm and at 0.1 mg/ml in the region below 250 nm. The spectra of the wild-type and all Leu66 mutant enzymes are shown without respective designation due to their nearly complete overlaps.

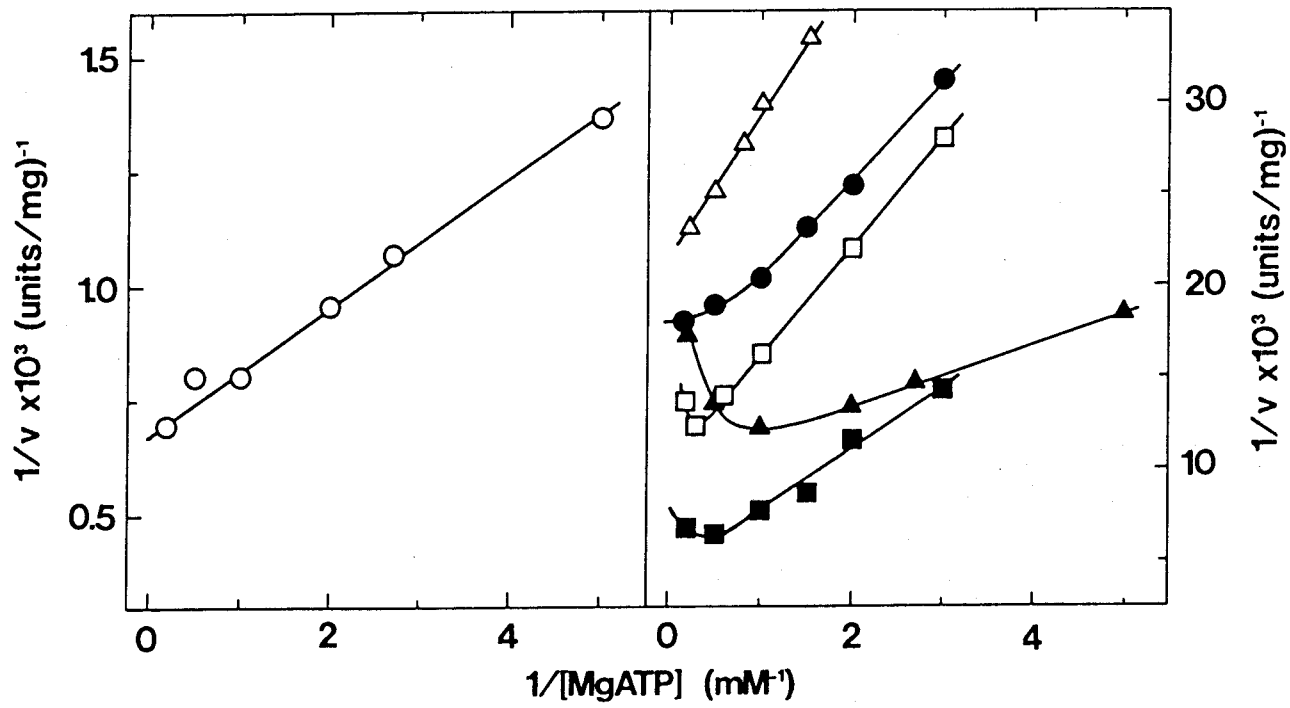


FIG. 3. **Steady state kinetic analysis for the wild-type and Leu66 mutant adenylate kinases with various concentrations of ATP and fixed concentrations of AMP.** The figure shows only exemplified data of the reaction rates (v) determined at a series of fixed concentrations of AMP; for the wild-type enzyme at 0.2 mM AMP (○), for Leu66-Ala mutant enzyme at 2.5 mM AMP (□), for Leu66-Gly mutant enzyme at 2.0 mM AMP (△), for Leu66-Gln mutant enzyme at 2.0 mM AMP (●), for Leu66-Val mutant enzyme at 2.0 mM AMP (■), and for Leu66-Trp mutant enzyme at 1.0 mM AMP (▲).

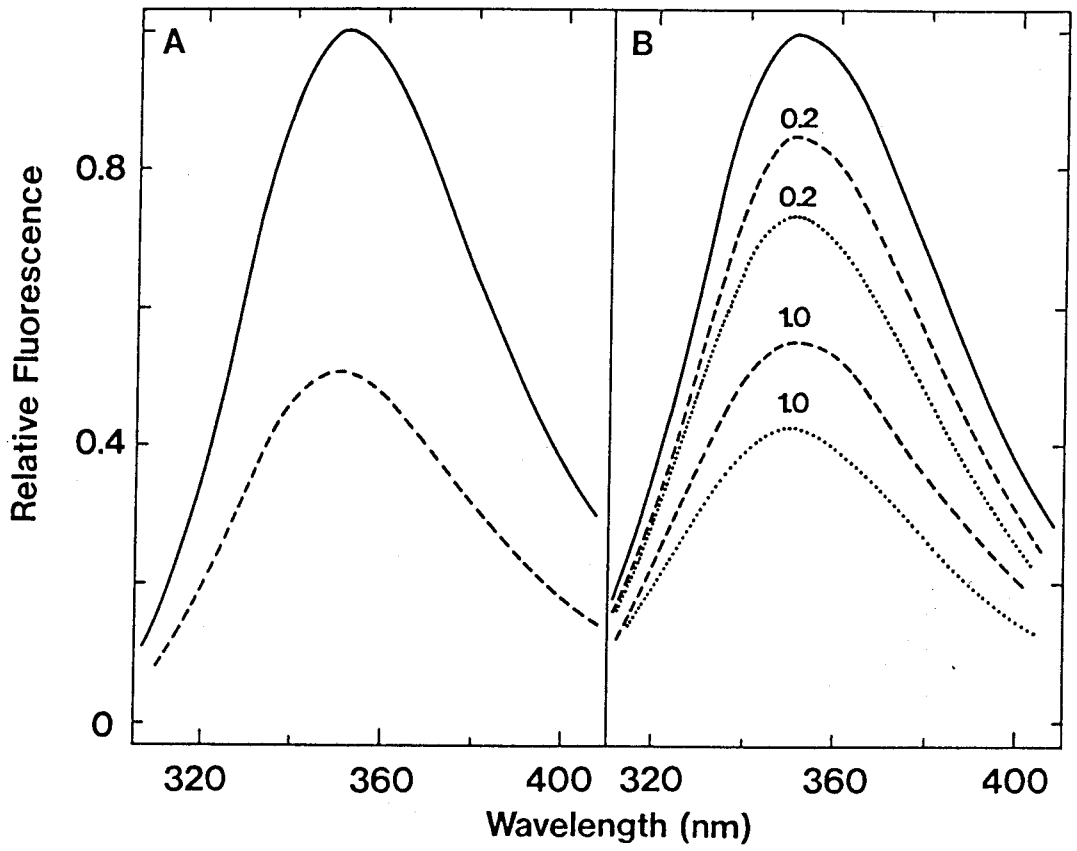


FIG. 4. **Fluorescence emission spectrum of the Leu66-Trp mutant enzyme (*A*) and its quenching by AMP and MgATP (*B*).** The excitation wavelength was 295 nm, and the protein concentration was 1 μM in 30 mM Tris-HCl (pH 7.5), 0.1 M NaCl, 1 mM EDTA, and 0.1 mM dithiothreitol. *A*, (——), emission spectrum of the Leu66-Trp mutant enzyme in the native state recorded at 25 °C; (----), the spectrum in the presence of 4 M guanidine hydrochloride. *B*, (——), emission spectrum of the Leu66-Trp mutant enzyme in the absence of substrate; (·····), the spectra in the presence of indicated concentrations (mM) of AMP; (----), the spectra in the presence of indicated concentrations (mM) of MgATP.

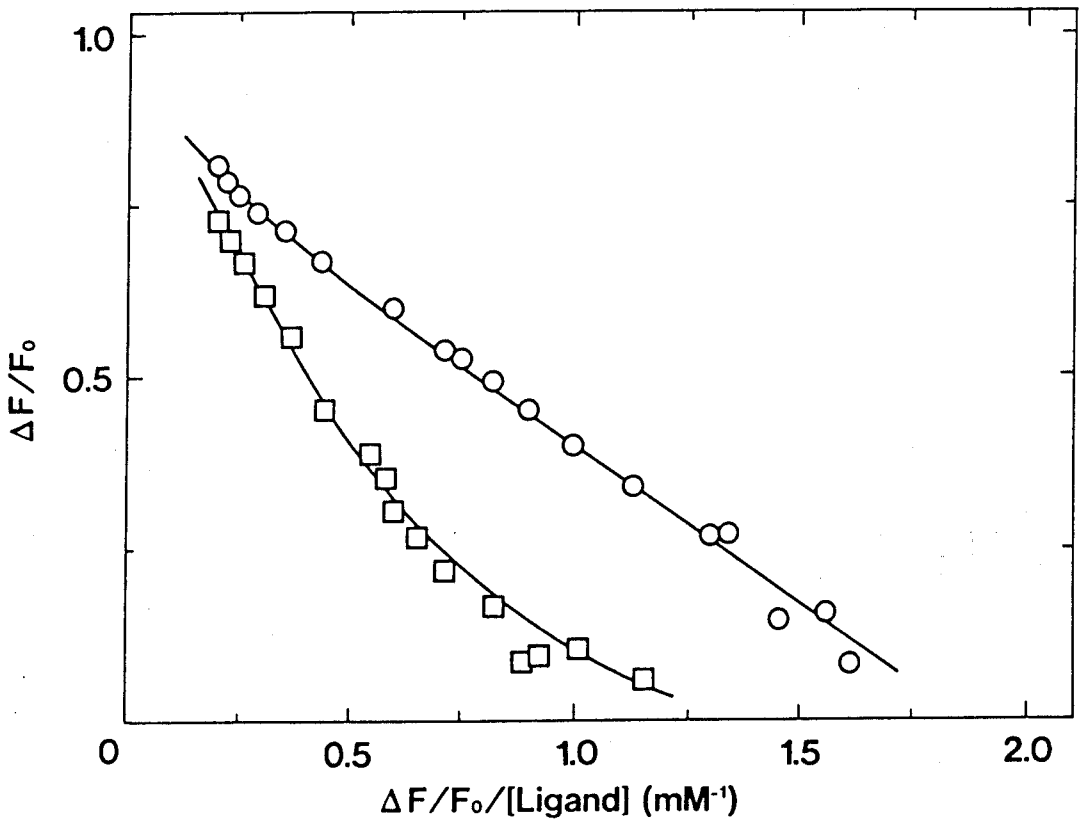


FIG. 5. **Scatchard-type plots for fluorescence quenching of Leu66-Trp mutant enzyme by substrates.** The fluorescence quenching by various concentrations of AMP (○) and MgATP (□) was measured as described in the text and Fig. 4. The curves are the least-square best fits for the data.

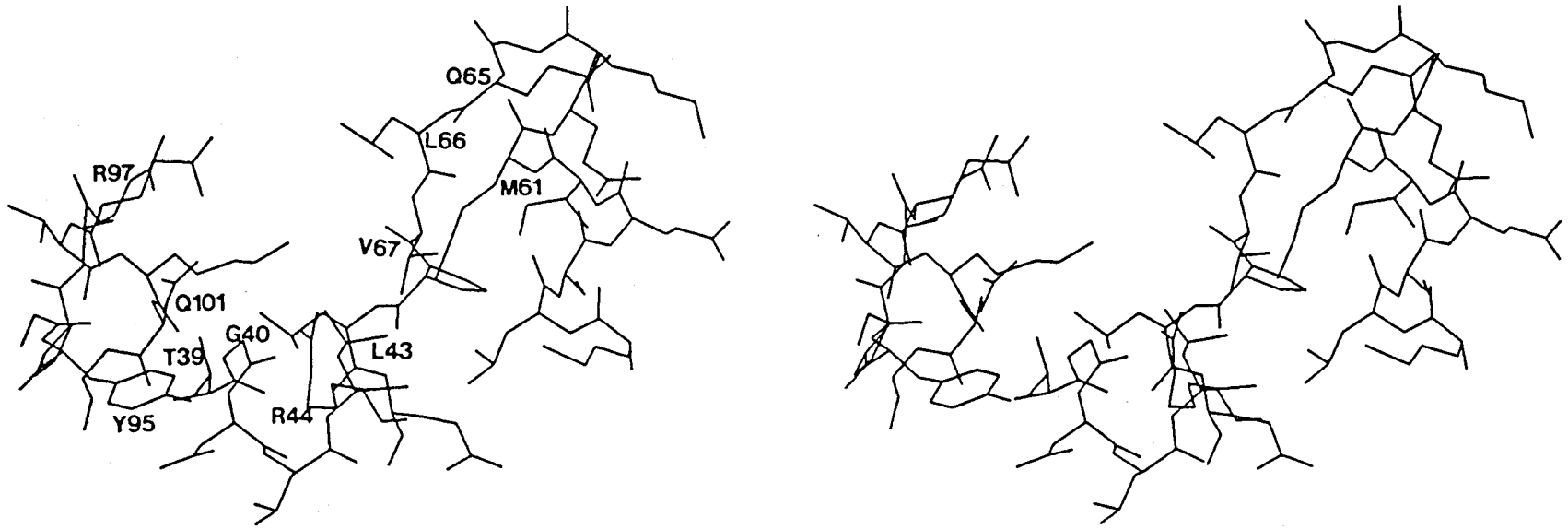
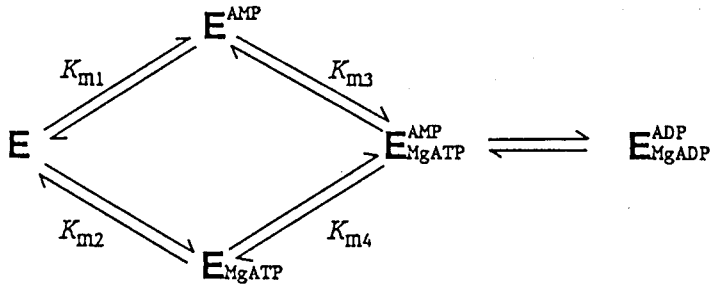
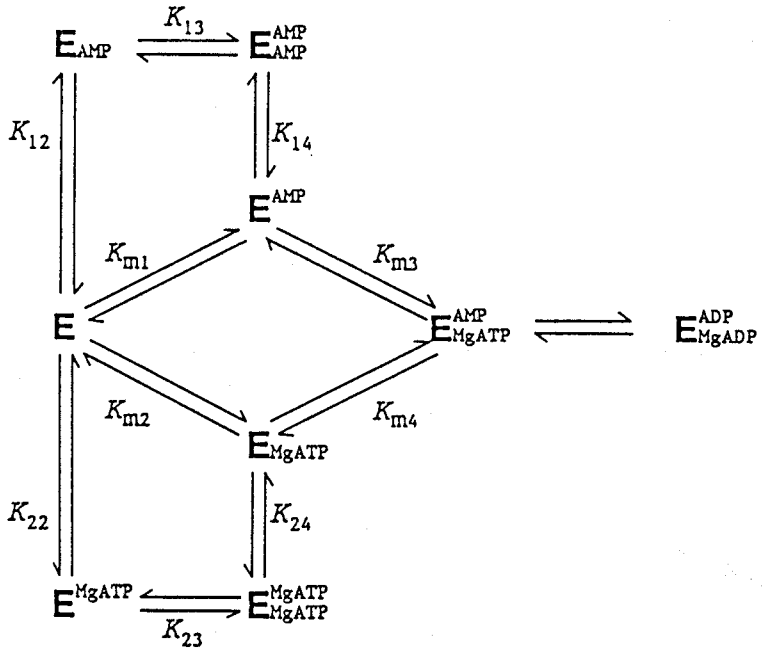


FIG. 6. Stereoscopic view of the local hydrophobic region involving Leu66 and other nearby residues of the porcine muscle adenylate kinase. The figure was drawn by a computer graphics using the spatial coordinates registered in the protein data bank (3).

Scheme I:



Scheme II:



REFERENCES

1. Schulz, G. E., Schiltz, E., Tomasselli, A. G., Frank,R.,Brune, M., Wittinghofer, A., and Schirmer, R. H. (1986) *Eur. J. Biochem.* **161**, 127-132
2. Sachsenheimer, W., and Schulz, G. E. (1977) *J. Mol. Biol.* **114**, 23-36
3. Dreusicke, D., Karplus, P. A., and Schulz, G. E. (1988) *J. Mol. Biol.* **199**, 359-371
4. Egner, U., Tomasselli, A. G., and Schulz, G. E. (1987) *J. Mol. Biol.* **195**, 649-658
5. Müller, C. W., and Schulz, G. E. (1988) *J. Mol. Biol.* **202**, 909-912
6. Fry, D. C., Kuby, S. A., and Mildvan, A. S. (1985) *Biochemistry* **24**, 4680-4694
7. Fry, D. C., Kuby, S. A., and Mildvan, A. S. (1987) *Biochemistry* **26**, 1645-1655
8. Pai, E. F., Sachsenheimer, W., Schirmer, R. H., and Schulz, G. E. (1977) *J. Mol. Biol.* **114**, 37-45
9. Tagaya, M., Yagami, T., and Fukui, T. (1987) *J. Biol. Chem.* **262**, 8257-8261
10. Yagami, T., Tagaya, M., and Fukui, T. (1988) *FEBS Lett.* **229**, 261-264
11. Yoneya, T., Okajima, T., Tagaya, M., Tanizawa, K., and Fukui, T. (1990) *J. Biol. Chem.* **265**, 21488-21493
12. Tanizawa, Y., Kishi, F., Kaneko, T., and Nakazawa, A. (1987) *J. Biochem. (Tokyo)* **101**, 1289-1296

13. Kramer, W., Drutsa, V., Jansen, H.-W., Kramer, B., Pflugfelder, M., and Fritz, H.-J. (1984) *Nucleic Acids Res.* 12, 9441-9456

14. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A.* 74, 5463-5467

15. Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

16. Laemmli, U. K. (1970) *Nature* 227, 680-685

17. Tagaya, M., Yagami, T., Noumi, T., Futai, M., Kishi, F., Nakazawa, A., and Fukui, T. (1989) *J. Biol. Chem.* 264, 990-994

18. Florini, J. R., and Vestling, C. S. (1957) *Biochim. Biophys. Acta* 25, 575-578

19. Yoneya, T., Tagaya, M., Kishi, F., Nakazawa, A., and Fukui, T. (1989) *J. Biochem. (Tokyo)* 105, 158-160

20. Pace, C. N., Shirley, B. A., and Thomson, J. A. (1989) in *Protein Structure: A Practical Approach* (Creighton, T. E., ed) pp. 311-330, IRL Press, Oxford

21. Hamada, M., and Kuby, S. A. (1978) *Arch. Biochem. Biophys.* 190, 772-792

22. Noda, L. (1973) in *The Enzymes* (Boyer, P. D., ed) Vol. 8, pp. 279-305, Academic Press, New York

23. Westphal, U. (1969) in *Methods in Enzymology* (Clayton, R. B., ed) Vol. 15, pp.761-796, Academic Press, New York

24. Sanders, C. R., II, Tian, G., and Tsai, M.-D. (1989) *Biochemistry* 28, 9028-9043

25. Diederichs, K., and Schulz, G. E. (1990) *Biochemistry* **29**, 8138-8144

26. Tian, G., Yan, H., Jiang, R.-T., Kishi, F., Nakazawa, A., and Tsai, M.-D. (1990) *Biochemistry* **29**, 4296-4304

27. Schulz, G. E., Müller, C. W., and Diederichs, K. (1990) *J. Mol. Biol.* **213**, 627-630

Chapter III

Site-Directed Random Mutagenesis

of AMP-Binding Residues

INTRODUCTION

Although several attempts to improve catalytic functions and stabilities of enzyme proteins by site-directed mutagenesis have been recently succeeded (1), no general strategy is yet available as to which residue in a particular protein should be selected for the target of mutagenesis and by which residue it should be replaced to achieve the purposed improvement. Even if the three-dimensional structure has already been elucidated, it is also very difficult to predict the change in its structure and function resulting from the replacement of a residue by others. One feasible approach to obtain mutant proteins with desirably changed properties is the use of phenotypic selection of *E. coli* cells transformed with plasmids containing a randomly mutagenized gene. If one or a few residues with or without certain roles in that protein are changed randomly into the other kind of nineteen amino acid residues and the resultant plasmids are introduced into an *E. coli* mutant strain lacking the corresponding gene required for its growth under normal conditions, a transformant producing a functional protein can be easily selected by the growth under otherwise lethal conditions. Functionally allowable amino acid residues at the mutated site also can be deduced from DNA sequences of the isolated clones. Lim and Sauer (2) introduced random mutations site-specifically to the hydrophobic core positions in Cro repressor of λphage to characterize residues necessary for its functional folding (2). Similar methods have also been developed for *in vitro* mimicking the molecular evolution of proteins but by random (site-nonspecific)

mutagenesis using chemical mutagens.

The recent invention of polymerase chain reaction (PCR) makes it possible to perform random mutagenesis site-specifically (3); a specific residue (or region) in a protein, if the gene for which has already been cloned, can be replaced arbitrarily by other residues with appropriately designed PCR primers. This method, hereinafter designated "site-directed random mutagenesis", is also useful to obtain a variety of mutant proteins in a single round of mutagenic procedure. Without the universal principle for the structure-function relationship of proteins, one needs as many as possible mutant proteins to avoid hastily drawing of an incorrect conclusion based on the results from only a single mutant protein.

As described in Chapter II (4), Leu66 in the recombinant chicken muscle adenylate kinase has been suggested to play an important role in the symmetric recognition of substrates, in particular in binding of AMP. To further probe and characterize AMP-binding residues in adenylate kinase, the PCR-based site-directed random mutagenesis has been applied to Val67 and Gln101, the former of which is included in a local hydrophobic region together with Met61, Leu66, and Val72, probably participating in binding of the adenine ring of AMP (4,5). According to the three-dimensional structure (5,6), the side chain of Gln101 is positioned face to face with Leu66 and Val67 and within a hydrogen bond-forming distance from the adenine ring N1 of the bound AMP. Enzymatically active mutant proteins with random mutation at Val67 or Gln101 have been selected by transformation of an *E. coli* mutant strain that is temperature sensitive with regard to its inherent adenylate kinase.

EXPERIMENTAL PROCEDURES

Materials——Nucleoside mono- and triphosphates were obtained from Seikagaku Kogyo or Sigma; lactate dehydrogenase (rabbit muscle) and pyruvate kinase (rabbit muscle) were from Oriental Yeast; the recombinant *Taq* DNA polymerase (AmpliTaq™) and M13 tv19 and mp18 RF DNAs were from Takara Shuzo; and the expression vector pKK223-3 was from Pharmacia. Oligonucleotide primers for mutagenesis and DNA sequencing were synthesized with an Applied Biosystems DNA synthesizer model 381. All other reagents were of the highest purity commercially available.

Random Mutagenesis by PCR——Random mutagenesis at Val67 and Gln101 of the chicken muscle adenylate kinase was carried out by PCR-based amplification of the entire coding region in two parts, the amino terminal half (fragments I and III) containing the mutated site and the remaining carboxy terminal half (fragments II and IV) (see Figs. 1 and 2). Synthetic primers for amplification of these fragments by PCR are summarized in Table I; A2 and A5 primers contained A,G,C,T-mixed bases at the first and second positions and a G,C-mixed base at the third position of the corresponding codons, and others contained appropriate restriction sites. PCR was performed in a 100-μl solution containing 10 mM Tris-HCl (pH 8.9), 1.5 mM $MgCl_2$, 80 mM KCl, 0.5 mg/ml bovine serum albumin, 0.1% sodium cholate, 0.1% Triton X-100, 0.1 mM each deoxynucleotides (dATP, dGTP, dCTP, and dTTP), 1 μM each two primers (A1 and A2 for fragment I, A3 and A4 for fragment II, A1 and A5 for fragment III, and A6 and A7 for fragment IV), about 4 ng of the cDNA in the M13tv19 phage (4, 7),

and 5 units of *Taq* DNA polymerase with a program consisting of the cycles 1st-30th (94°C for 1 min, 60°C for 3 min, and 72°C for 3 min) and the last cycle (94°C for 1 min, 60°C for 3 min, and 72°C for 20 min) in a thermal cyclic reactor (Hoei Science Co., Ltd., Tokyo, Model TC-100). The PCR products were purified twice by chloroform/phenol extraction, digested with restriction enzymes, and ligated into the phage vector M13mp18 to examine the DNA sequences of each fragment by the dideoxy chain termination method (8); no undesired mutation was found in the amplified regions except for the codons for Val67 and Gln101. The *Eco*RI-*Hin*dIII fragments from the resultant plasmids pMH67 (Fig. 1) and pMH101 (Fig. 2) were inserted into the expression vector pKK223-3 to form pKH67 and pKH101, respectively. Finally, the *Eco*RI-*Xba*I fragment from pMR67 (a mixture of mutated plasmids isolated from transfected *E. coli* culture, but not from individual plaques) and the *Eco*RI-*Nsp*(7524)V fragment from pMR101 (also a mixture of mutated plasmids) were ligated with pKH67 and pKH101 to produce pKK-R67AK and pKK-R101AK, respectively.

Screening of Adenylate Kinase Mutant——The expression plasmids thus constructed were transformed into *E. coli* JM109, and the colonies expressing adenylate kinase proteins were screened immunochemically with a rabbit antiserum raised against the purified chicken muscle enzyme (wild type) as described (9). An anti-rabbit-IgG goat antibody conjugated with horseradish peroxidase (Bio-Rad) was used as the second antibody. The enzymatically active adenylate kinase mutant was selected by transformation with pKK-R67AK and pKK-R101AK of *E. coli* CV2 (*HfrC, plsA2, glpD3, glpR^{c}2, phoA8, tonA22, T2^{R}, rel-1,* (λ)), which is a temperature sensitive mutant with regard to adenylate

kinase (10). In a preliminary experiment, the *E. coli* mutant was shown to grow at 45°C within 24 h upon transformation with pKK-cAK1-1 carrying the wild-type adenylate kinase cDNA (11), but not with the vector pKK223-3 alone. Thus, enzymically active adenylate kinase mutants at Val67 and Gln101 were readily isolated on the basis of the growth at 45°C of *E.coli* CV2.

The entire coding region for adenylate kinase in the plasmids purified from appeared colonies was directly sequenced with synthetic oligonucleotide primers, B1 and B2 (Table I), to identify the codons at Val67 and Gln101 and also to confirm the absence of base changes in the other region that might occur during PCR due to the incomplete fidelity of *Taq* DNA polymerase.

Enzyme Purification——Plasmids isolated from the *E. coli* CV2 clone cells were re-transformed into *E. coli* JM109, and all mutant enzymes were expressed by induction with 0.5 mM IPTG. The mutant enzymes recovered from the soluble fraction of cell lysates were purified to homogeneity essentially as described by Tanizawa *et al.* (11). The enzymes recovered from the pellet of cell lysates were purified by acid extraction and gel-filtration as reported previously (12). The purity of the enzyme preparations was checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (13).

Enzyme Assay and Steady State Kinetic Analysis——The enzyme activity was determined in the standard assay mixture as reported previously (12). Steady state kinetic parameters were obtained by systematic variation of the concentrations of both AMP and MgATP (14) in the assay mixture consisting of 87 mM triethanolamine-HCl buffer (pH 7.0), 10 mM $MgCl_2$, 100 mM KCl, 1 mM phosphoenolpyruvate, 0.16 mM NADH, 5 units of pyruvate kinase,

and 20 units of lactate dehydrogenase. After preequilibration at 25 °C for about 2 min, an appropriate amount of adenylate kinase was added to the assay mixture, and the reaction was carried out at 25 °C and monitored by the decrease in absorbance at 340 nm.

CD Measurements——CD spectra were measured at 25 °C in 30 mM Tris-HCl buffer, pH 7.5, containing 0.1 M NaCl, 1 mM EDTA, and 0.1 mM dithiothreitol with a Jasco spectropolarimeter model J-600, which was calibrated with ammonium (+)-10-camphorsulfonate (4). The other conditions for CD measurement are the same as described in Chapter II.

Fluorescence Measurements——Fluorescence spectrum of the purified Gln101-Trp mutant enzyme (2 μM) was measured at 25 °C with a Hitachi spectrofluorometer model F-4010. The fluorescence emission due to the single Trp at position 101 was recorded upon excitation at 295 nm.

Thermal Stability——Thermal stabilities of the wild-type and mutant enzymes were analyzed by monitoring the protein unfolding process by heat, following the changes in ellipticity at 222 nm, and that of the Gln101-Trp mutant protein was done by following the changes in the tryptophanyl fluorescence emitted at 340nm by excitation at 295 nm. All measurements were carried out in 5 mM sodium phosphate buffer, pH 7.0, 1 mM EDTA, and 1 mM dithiothreitol. Protein concentrations were 0.1 mg/ml and 2 μM in measurements by fluorescence and CD, respectively. The temperature was raised at a constant rate of 1 °C/min through a thermostatic cell holder and was measured directly in the cuvette with a Rikagaku Kogyo thermometer model DP-500. Reversibility of the unfolding process was examined by measuring the ellipticity or fluorescence after gradual lowering of the temperature to 25 °C.

RESULTS

PCR-Based Random Mutagenesis at Val67 and Gln101——When the plasmids randomly mutated at Val67 by PCR (Fig. 1) were transformed into *E. coli* JM109 competent cells, about 70% of the recombinant colonies were found to produce the chicken muscle adenylate kinase proteins by an immunochemical assay of the clone cells lysed *in situ* using the rabbit antiserum (9). Twenty three positive clones were chosen arbitrarily and analyzed for their DNA sequences. As shown in Table II, a total of eleven mutant enzymes were obtained, in which Ala (number of clones, 1), Ile (4), Pro (3), Ser (1), Thr (2), Cys (5), Asn (1), Gln (3), His (1), Arg (1), and Glu (1) were substituted for Val67. It is noteworthy that none of them had the codons for Val (wild type) at position 67, although the reason was unknown. The Val67-His mutant had also a silent mutation at another site (not shown).

Similarly, by the site-directed random mutagenesis at Gln101, about 60% of the appearing colonies were positive in the immunoblotting, producing adenylate kinase proteins. However, only a total of four mutant enzymes [replaced by Thr (no. of clones, 1), Pro (2), Leu (2), and Trp (2) for Gln101] were obtained by sequencing of 7 positive clones (Table II). To isolate active mutant enzymes, the plasmids with random mutation at Gln101 were directly introduced into the temperature sensitive *E. coli* CV2 competent cells. By overnight incubation at 45 °C on an LB plate containing 50 μg/ml ampicillin, colonies with two different sizes were formed. Eight of relatively large colonies were chosen, and the DNA sequences around the codon for residue

101 were analyzed; all clones had CAG at position 101 coding for Gln as in the wild-type enzyme. By sequencing the plasmids from 15 relatively small colonies, another two mutants carrying His (no. of clones, 12) or Met (1) at position 101 were obtained as well as 2 wild-type clones (Gln) (Table II).

The codons for residues 67 and 101 in these mutant enzymes obtained are also summarized in Table II. As expected from the sequences of primers A2 and A5, the first and second bases in both codons contained either one of all four bases (G, A, T, or C), and the third base either one of G or C. The codon usages appeared unbiased, indicating that the PCR-based random mutation worked successfully.

Curing of Temperature Sensitive E. coli CV2 and Enzyme Activity of Mutant Proteins——The plasmids coding for Val67 and Gln101 mutant enzymes were isolated and re-transformed into *E. coli* CV2 to examine whether the expressed mutant enzymes restore the temperature sensitivity of the host strain by their enzyme activities. By 24-hr incubation at 45 °C on an agar plate containing 50 μg/ml ampicillin, most of the Val67 mutant enzymes significantly assisted the growth of *E. coli* CV2 at that temperature as the wild-type enzyme, whereas only two of Gln101 mutants slightly helped the growth (Table II).

All the mutant enzymes were then expressed in *E. coli* JM109 by the addition of 0.5 mM IPTG, and crude lysates were prepared. Their adenylate kinase activities were measured under the standard assay conditions containing 1 mM each of MgATP and AMP (Table II). The expression levels of these mutant enzymes were found to differ considerably when analyzed by SDS-polyacrylamide gel electrophoresis (data not shown). Although a slight but

detectable activity is contained inherently in the host JM109 cells, the crude lysates showed various levels of adenylate kinase activity, suggesting the mutant enzymes have considerably different specific activities. It appears that at position 67 hydrophobic residues with an aliphatic side chain or hydrophilic residues with a hydroxyl or thiol group provide specific activity as high as the wild-type enzyme. On the other hand, at position 101 only the His mutant shows considerable activity. These levels of specific activity of the mutant enzymes in crude lysates correspond well to the extents of the growth of temperature sensitive *E. coli* CV2 harboring the mutant plasmids. Thus, the acceptability of residues at position 67 is wide, while that at position 101 is limited only to Gln (wild type), His, or Met.

Steady State Kinetic Parameters of Some Purified Mutant Enzymes——To analyze kinetic properties of some of the Val67 and Gln101 mutant enzymes in detail, Val67-Ala, -Ile, -Thr, and -Glu, and Gln101-His and -Trp mutant enzymes were purified from the soluble fractions of cell lysates, and Val67-Arg, Gln101-Met, and Gln101-Leu mutant enzymes were purified from the pellet. As expected from the specific activities in crude lysates, the purified Val67-Thr and Val67-Ile mutant enzymes had significantly high specific activities, whereas the other purified Val67 and Gln101 mutant enzymes had much lowered specific activities when measured under the standard assay conditions (Table III). The steady state kinetic parameters of these mutant enzymes were analyzed according to the random quasi-equilibrium Bi-Bi mechanism using variable concentrations of both MgATP and AMP (4,14) and calculated with a computer program (15) as summarized

in Table III, where K_{m1} and K_{m4} represent the Michaelis constants for AMP at zero and infinite concentrations of the other substrate MgATP, respectively, and K_{m2} and K_{m3} are those for MgATP at zero and infinite concentrations of AMP, respectively.

When compared with the values of the wild-type enzyme, the Michaelis constants for AMP increased 2.8~17-fold in K_{m1} and 3.6~82-fold in K_{m4} with most Val67 mutant enzymes, while those for MgATP, K_{m2} and K_{m3}, virtually unchanged. Exceptionally, Val67-Ala had even lower Michaelis constants for MgATP and AMP than the wild-type enzyme in K_{m3} and K_{m4}, although those in K_{m1} and K_{m2} were comparable with those of the wild type. The V_{max} values of Val67-Ile and Val67-Thr significantly increased as compared with the wild-type enzyme. The increase in V_{max} has been observed in previous site-directed mutagenesis studies of adenylate kinase (16), and compensates the increase in Michaelis constants leading to the high specific activities as exemplified with Val67-Ile and Val67-Thr (Table III). Only the Val67-Arg mutant enzyme that did not restore the temperature sensitivity of *E. coli* CV2 showed a very low V_{max}, corresponding with its low specific activity.

With Gln101 mutant enzymes, the Michaelis constants for AMP also increased dramatically in both K_{m1} and K_{m4}, those for MgATP remaining in the same order of magnitude as the wild-type enzyme. The Gln101-Leu and Gln101-Trp mutant enzymes, both being unable in restoring the temperature sensitivity of *E. coli* CV2, showed very low V_{max} values. These results are essentially identical with those described in Chapter II (4) and support a view that Val67 and Gln101 are involved in binding of AMP together with Leu66.

The wild-type enzyme shows positive cooperativity in

substrate binding ($\beta = K_{m2}/K_{m3} = K_{m1}/K_{m4} > 1.0$), probably mediated by allosteric conformational changes induced upon binding of either substrate (4). Among Val67 mutant enzymes, Val67-Glu and Val67-Arg showed negative cooperativity ($\beta < 1.0$), suggesting that charged residues at position 67 are unfavorable for the positive cooperativity in substrate binding. All mutant enzymes at position 101 also had β values less than 1.0, indicating that Gln101 is essential for the positive cooperativity. A considerable conformational change might have been elicited by replacement of Gln101 by other residues as detected by CD measurement (see below).

Substrate Specificities——Replacement of Val67 or Gln101 by other residues influenced the Michaelis constants of the enzyme particularly for AMP as described above. Adenylate kinase catalyzes the phosphoryl transfer from MgATP not only to AMP but also to other nucleoside monophosphates, though to a lesser extent. Thus, the substrate specificities of the mutant enzymes for dAMP, CMP, and UMP (1 mM) were studied in the assay mixture containing 1 mM MgATP as a phosphoryl donor, following the rates of ADP formation. As shown in Table IV, relative activity in the reaction with the MgATP-dAMP pair increased considerably in the Val67-Ala mutant enzyme, although all other mutant enzymes apparently showed lower activities for dAMP than the wild-type enzyme. The kinetic parameters of the reaction with the MgATP-dAMP pair were then measured for the wild-type, Val67-Ala, and Val67-Ile mutant enzymes assuming the same random Bi-Bi mechanism as the MgATP-AMP pair. The Val67-Ala and Val67-Ile mutant enzymes exhibited the maximum and minimum relative activities, respectively, for the MgATP-dAMP pair. Specific activities and

k_{cat}/K_m values for dAMP of both mutant enzymes varied to about 1.8- and 0.1-fold of the wild-type, respectively (Tables V and VI). The kinetic parameters (Table V) show that the variation of the specific activities and k_{cat}/K_m values among the three enzymes resulted from changes in both the K_m and V_{max} values. The Val67-Ile mutant and wild-type enzymes showed remarkably decreased V_{max} values for the MgATP-dAMP pair (2.5 and 36 % of those for the MgATP-AMP pair, respectively), but the Val67-Ala mutant enzyme had comparable V_{max} values in the two substrate pairs. In addition, the cooperativity factor in the MgATP-dAMP pair decreased in the wild-type and Val67-Ala mutant enzymes as compared with AMP as a phosphoryl acceptor. Therefore, these results suggest that the interaction between the 2'-OH group of AMP and the enzyme seems to be important for the induced fitting. The Michaelis constants for MgATP (K_{m2}) obtained with the MgATP-dAMP pair have to agree with those obtained with the MgATP-AMP pair. This point fits well for the wild-type and Val67-Ala mutant enzymes, but does not for the Val67-Ile mutant enzyme due to the K_{m2} value obtained with the MgATP-dAMP pair 2.5-fold higher than that obtained with the MgATP-AMP pair. The rapid equilibrium random mechanism may be inapplicable to the Val67-Ile mutant enzyme. Interestingly, these findings show that the replacement of Val67 to a more bulky Ile by only a methylene group or to a less bulky Ala affects the recognition of sugar moiety and the cooperativity in substrate binding. When CMP or UMP was used as a phosphoryl acceptor, the relative activities also varied in the Val67 mutant enzymes, but not so drastically.

The Gln101 mutant enzymes showed markedly higher relative activities for UMP than the Val67 mutant and wild-type enzymes

(Table IV). The apparent k_{cat}/K_m values for CMP and UMP were then measured with 2 mM MgATP for the Gln101 mutant enzymes except for Gln101-Trp having undetectably low activities. As shown in Table VI, all the Gln101 mutant enzymes showed extremely low k_{cat}/K_m values for AMP as compared with the wild-type enzyme, but the Gln101-Met and Gln101-His mutant enzymes had about 3-fold larger values for UMP. The values for CMP are also significant in the Gln101 mutant enzymes when compared with those for AMP. These findings suggest that Gln101 plays a role in the recognition of base moieties of nucleoside monophosphates.

CD Spectra——All the mutant enzymes showed almost the same CD spectra as the wild-type enzyme in the region between 200 and 250 nm mainly due to the secondary structures (Figs. 2 and 3). In the region between 250 and 320 nm related to the circumstances of aromatic residues, the Val67 mutant enzymes except Val67-Arg showed almost the same spectra as the wild-type enzyme. The shapes of CD spectra of Val67-Arg and all the Gln101 mutant enzymes were similar to that of the wild-type enzyme, but the ellipticity values were lower than that of the wild-type enzyme (Figs. 2 and 3). Since Gln101 is in contact with aromatic residues, Tyr95 and Phe105, in the three-dimensional structure (5), the mutation at Gln101 might have resulted in a conformational change leading to the decrease in ellipticity in that region.

The Gln101-Trp mutant enzyme showed a CD spectrum different from those of the other Gln101 mutant enzymes, especially in the region between 250 and 320 nm. Presumably, the newly introduced tryptophanyl residue at position 101 is restricted in its movement affecting the CD spectrum, together with the

perturbation of other nearby aromatic residues. Since the vertebrate cytosolic adenylate kinases (the small variants) contain no tryptophanyl residue, the fluorescence emission spectrum due to the newly introduced Trp101 reflects the environment surrounding the sole tryptophanyl residue. Fig. 4 shows the fluorescence emission spectra of Gln101-Trp in the native and denatured state. Compared with the denatured state in 4 M guanidine-HCl, the peak in the spectrum of the native enzyme was blue-shifted from 350 nm to 340 nm and its maximum intensity increased about 1.7-fold. This shows that the movement of the tryptophanyl residue is restricted due to its presence in a hydrophobic region as has been observed with that of the Leu190-Trp enzyme (17), but not with that of Leu66-Trp (Chapter II).

Thermal Stabilities——Conformational stabilities of the wild-type, Val67, and Gln101 mutant enzymes were analyzed by monitoring the thermal unfolding process following the changes in ellipticity at 222 nm, except for Gln101-Trp, the stability of which was measured by monitoring the change in fluorescence at 340 nm due to the tryptophanyl residue at position 101. The T_m value obtained from the fluorescence measurement is therefore an apparent value. Reversibilities of the unfolding process were rather low (about 50 %) in all enzymes. Fig. 5 shows the relationship between the T_m values thus determined and the specific activities of the purified enzymes. It also includes the results as to whether the mutant enzymes restore the growth of transformed *E. coli* CV2 at 45 °C or not. Thermal stabilities (T_m) of Val67-Ile, Val67-Ala, Val67-Glu, and Gln101-His were comparable to that of the wild-type enzyme and those of the others were lower at most by 9 °C than that of the wild-type

enzyme, suggesting that neither Val67 nor Gln101 is important for the thermal stability of the enzyme. Therefore, it appears likely that the temperature sensitivity of *E. coli* CV2 is restored more effectively by the expressed enzyme activity (and probably its expression level) than the thermal stability.

DISCUSSION

Both Val67 and Gln101 are highly conserved in adenylate kinases from various sources. Functional reasons for the conservation are as yet unclear, but the results of the present study have shown that at least at position 67 various amino acid residues other than Val are allowed to occupy without significant loss of enzymic functions. On the basis of the restoration of the temperature sensitivity of *E. coli* CV2, the measured kinetic parameters, and the cooperativity in substrate binding (Tables II and III) of the Val67 mutant enzymes, hydrophobic residues with an aliphatic side chain or hydrophilic residues with a hydroxy or thiol group can be accommodated at position 67. This result is somewhat inconsistent with the previous studies with the Leu66 mutant enzymes (4), in which hydrophobic interactions of Leu66 and nearby residues with the adenine ring of AMP are thought to be important for AMP binding. However, since Thr, Ser, and Cys are less hydrophilic than charged residues (18,19), and since their side chains may rotate around the $C\alpha$-$C\beta$ to orient toward the solvent, the hydrophobic interactions by the region are probably still maintained. Therefore, it appears likely that Val67 participates in the AMP binding in cooperation with Leu66, Met61, and Val72 constituting the hydrophobic region. In accordance with this idea, the replacement of Val67 by a charged residue, Glu or Arg, results in the marked perturbation of enzymic properties (see Table III) presumably through significant distortion of the hydrophobicity of this region.

Schulz *et al.* (3) proposed a model from x-ray analysis of

the complex between bovine mitochondrial AK3 and AMP, in which the NE2 nitrogen atom of Gln101 is hydrogen-bonded to N1 of the adenine ring of AMP. This is supported by the present results that the replacement of Gln101 causes remarkable increases in Michaelis constants for AMP (Table III). Random mutagenesis at residue 101 revealed that only Gln and His (and also Met, though the activity is much retarded), being able to form hydrogen bonds (20), fulfill the functional requirement for residue 101. Furthermore, the three-dimensional structure of porcine cytosolic AK1 shows that Gln101 is almost buried in a protein interior, as also suggested from the fluorescence spectrum of Gln101-Trp. To accommodate a residue inside a protein without structural constraints, molecular volumes of the side chain must be an important factor; those of Gln, His, and Met are 143.9, 153.2, and 162.9 $Å^3$ (21), respectively, which are similar with each other as compared to Thr, Pro, Leu, and Trp, having molecular volumes of 116.1, 122.7, 166.7, and 227.8 $Å^3$, respectively. The idea that Gln101 is playing a role mainly by forming a hydrogen bond with the adenine ring of AMP is also supported by the general concept that a definitive molecular distance and direction are needed for forming a hydrogen bond between two atoms, but not for making a hydrophobic interaction.

It is noteworthy that some mutant enzymes, Val67-Ile, Val67-Thr, and Gln101-His, show larger V_{max} values than the wild-type enzyme. This suggests that these two residues participate indirectly in the catalytic process. Assuming that the location of the adenine ring of bound AMP in the ternary complex of the two mutant enzymes is slightly different from that in the wild-type enzyme, the phosphate group of AMP that accepts the

γ-phosphate group from the bound MgATP would be also located at the position slightly different from that in the wild-type enzyme. This positioning of the AMP phosphate could be a reason for the increased activity of the two Val67 and Gln101 mutant enzymes. Alternatively, the mutation at Val67 and Gln101, as well as Leu66 (4), may affect the catalytic efficiency indirectly through gross conformational alteration.

In the model of AK3-AMP proposed by Schulz *et al.* (6), a hydrogen bond is formed between the ribose 2'-hydroxyl group of bound AMP and the O atom of Lys62 (corresponding to Glu65 in the chicken cytosolic enzyme). The necessity of this hydrogen bond is supported by the fact that the enzymes from various sources show only subtle activity for dAMP as a phosphoryl acceptor (4, 22, see also Table IV). Presumably, the absence of the hydrogen bond prevents the tight binding of the ribose ring and thus causes steric hindrance to nearby residues such as Leu66 and Val67. The replacement of Val67 by a bulkier residue, Ile, yields a mutant enzyme with a high Michaelis constant (K_{ml}) for dAMP, and that by a smaller one, Ala, yields a mutant enzyme with a low K_{ml} (Table V). The Val67-Ala mutant enzyme also has a V_{max} value for dAMP larger than that for AMP, whereas the wild-type and Val67-Ile enzymes have smaller V_{max} values for dAMP. The transition state ternary complex of Val67-Ala with MgATP and dAMP may be stabler than those of the wild type and Val67-Ile due to the absence of the steric hindrance.

The two Gln101 mutant enzymes, Gln101-His and Gln101-Met, have higher activity for UMP than the wild-type enzyme in terms of k_{cat}/K_m values (Table VI). The increase in k_{cat}/K_m values appears to be resulted from the increase in k_{cat} but not from the

decrease in K_m (data not shown). Therefore, it may be possible in future to convert adenylate kinase into uridylate kinase by site-directed mutagenesis of residues interacting with the nucleotide base including Gln101.

Haase *et al.* (23) identified the Pro87-Ser mutation in the *E. coli* adenylate kinase of the temperature sensitive strain CV2. The Pro87-Ser mutation decreases the thermostability of the enzyme, changing the strain lethal at a high growth temperature. Thus, by using such an *E. coli* mutant as a recipient of plasmids encoding adenylate kinase mutant proteins, one may expect to obtain transformants expressing the mutant enzymes that have acquired high thermostability. However, mutant enzymes with various levels of activity but similar thermostabilities have been obtained in the present study. It is suggested that the temperature sensitivity of CV2 can be restored either by constitutive expression of a very active enzyme or by overexpression of a less active enzyme.

After all, the present study demonstrates that the PCR-based site-directed random mutagenesis is very useful to obtain a comprehensive set of various mutant proteins. The target residue for mutagenesis is not necessarily to be a single site, but may well be multiple; mutations could be introduced randomly into the contiguous four residues in the glycine-rich region of adenylate kinase (Okajima *et al.*, unpublished data). Although proper screening methods are needed, the site-directed random mutagenesis is considered to be one of powerful methods to alter functions or to improve stabilities of proteins.

Table I

Sequence of synthetic primers for random mutagenesis and DNA sequencing

Primer	Sequence
	0 1
	EcoR I Met Ser-
Template	5'-GTTGTAAAACGACGGCCAGT GAATTC CC ATG TCG-
	3'-CAACATTTTGCTGCCGGTCA CTTAAG GG TAC AGC-
A1	5' GTAAAACGACGGCCAGT 3'
	Xba I
A2	3' GAC GTT CGG TAG TAC CTC TTC CCG CTC GAC XXY GGA GAT CTG TGC CC 5'
	*** * * *
	55 56 57 58 59 60 61 62 63 64 65 66 67 68 69 70 71 72 73 74
	-Lys Lys Leu Gln Ala Ile Met Glu Lys Gly Glu Leu Val Pro Leu Asp Thr Val Leu Asp-
Template	5' -AAG AAG CTG CAA GCC ATC ATG GAG AAG GGC GAG CTG GTT CCC CTG GAC ACG GTG CTG GAC-
	3' -TTC TTC GAC GTT CGG TAG TAC CTC TTC CCG CTC GAC CAA GGG GAC CTG TGC CAC GAC CTG-
	64 65 66 67 68 69 70 71 72 73 74 75 76 77
	-Gly Glu Leu Val Pro Leu Asp Thr Val Leu Asp Met Leu Arg-
Template	5' -GGC GAG CTG GTT CCC CTG GAC ACG GTG CTG GAC ATG CTG CGG-
	3' -CCG CTC GAC CAA GGG GAC CTG TGC CAC GAC CTG TAC GAC GCC-
	* *
A3	5' CTG GTT CCT CTA GAC ACG GTG CTG GAC ATG 3'
	Xba I
A4	3' T GCC GGGCCCCTAGG AGATCA CAGCTG GACGTC CGTACG TTC 5'
	*
A7	3' ACT GCC GGGCCCCTACC AGATCA CAGCTG GACGTC CGTACG TT 5'
	** **
	193
	-Leu Ter
Template	5' -CTG TGA CGG CCCGGGGATCC TCTAGA GTCGAC CTGCAG GCATGC AAGCTT GGC-
	3' -GAC ACT GCC GGGCCCCTAGG AGATCT CAGCTG GACGTC CGTACG TTCGAA CCG-
	Sma I *Bam*HI *Xba* I *Sal* I *Pst* I *Sph* I *Hin*dIII
	Nsp V *Eco*R I
A5	3' CTG CCG ATG GGA GCG ATA CAC TTC XXY CCT CTC CTC AAG CTT AAG TTC TAA CGG 5'
	*** * ***
	91 92 93 94 95 96 97 98 99 100 101 102 103 104 105 106 107 108 109 110 111 112
	-Leu Ile Asp Gly Tyr Pro Arg Glu Val Lys Gln Gly Glu Glu Phe Glu Lys Lys Ile Ala Pro Pro-
Template	5' -CTC ATT GAC GGC TAC CCT CGC GAG GTG AAG CAG GGA GAG GAG TTT GAA AAG AAG ATT GCC CCC CCC-
	3' -GAG TAA CTG CCG ATG GGA GCG ATA CAC TTC GTG CCT CTC CTC AAA CTT TTC TTC TAA CGG GGG GGG-
	99 100 101 102 103 104 105 106 107 108 109 110 111 112 113 114 115
	-Val Lys Gln Gly Glu Glu Phe Glu Lys Lys Ile Ala Pro Pro Thr Leu Leu-
Template	5' -GTG AAG CAG GGA GAG GAG TTT GAA AAG AAG ATT GCC CCC CCC ACG CTG CTG-
	3' -CAC TTC GTG CCT CTC CTC AAA CTT TTC TTC TAA CGG GGG GGG TGC GAC GAC-
	** * *
A6	5' G CAG GGA GTC GAC TTC GAA AAG AAG ATT GCC CCC CCC ACG 3'
	Sal I *Nsp* V
B1	5' AGCGGATAACAATTTCAC 3'
B2	5' CAGCGTGGGGGGGGCAAT 3'

A1~6 are oligonucleotides with PCR primers, together with their templates numbering by residues number in a protein. B1 and B2 are sequence primers. X and Y represent a mixture of A, G, C, and T, and that of G and C, respectively. The sites recognized by restriction enzymes are underlined and mismatched bases are shown by asterisks.

Table II

Mutant enzymes obtained by random mutagenesis

Enzyme	Codon at Random Site	Specific Activity[a] of Crude Lysate	Growth of *E.coli*[b] CV2 at 45°C
		units/mg	
Val67-Ala	GCC	178	+++
Val67-Ile	ATC	306	+++
Val67-Ser	TCC	117	+++
Val67-Thr	ACC	396	++
Val67-Cys	TGC	187	+++
Val67-Pro	CCC	7.6	+++
Val67-Asn	AAC	32.2	+++
Val67-Gln	CAG	33.3	++
Val67-Glu	GAG	5.9	+++
Val67-His	CAC	66.2	+++
Val67-Arg	CGG	0.7	-
Gln101-Leu	CTC	1.1	-
Gln101-Met	ATG	1.6	+
Gln101-Thr	ACG	1.4	-
Gln101-Pro	CCC	1.5	-
Gln101-Trp	TGG	1.0	-
Gln101-Gln	CAG	242	+++
Gln101-His	CAC	31.4	+
wild type	—	329	+++
vector	—	1.5	-

[a]Specific activity of crude lysate was measured when the mutant enzymes were expressed in *E.coli* JM109 cell. [b]When the wild-type and mutant enzymes were expressed with *E.coli* CV2 as host, growth of the cell was examined by incubating at 45°C for 24hr on LB plate containing 50 μg/ml ampicillin.

Table III

Kinetic parameters for wild-type, Val67, and Gln101 mutant adenylate kinases

Enzyme	Specific activity	K_{m1}	K_{m2}	K_{m3}	K_{m4}	β^a	V_{max}
	units/mg	*mM*	*mM*	*mM*	*mM*		*units/mg*
wild type	1900	0.42	0.28	0.11	0.17	2.5	2620
Val67-Ala	913	0.39	0.21	0.035	0.064	6.1	1070
Val67-Ile	2260	1.21	0.31	0.19	0.73	1.7	4700
Val67-Thr	2040	1.17	0.36	0.19	0.62	1.9	4080
Val67-Glu	85.7	7.29	0.31	0.60	14.0	0.52	1730
Val67-Arg	11.0	7.18	0.48	0.63	9.36	0.78	166
Gln101-Met	79.5	3.01	0.21	0.77	11.3	0.46	1170
Gln101-His	314	1.70	0.15	0.71	8.10	0.29	3660
Gln101-Leu	2.7	5.19	0.36	0.84	12.2	0.41	55.0
Gln101-Trp	0.97	1.28	0.21	0.49	2.95	0.55	4.0

[a]The simplified cooperativity factor calculated according to $\beta = K_{m2}/K_{m3} = K_{m1}/K_{m4}$, see text for details.

Table IV

Phosphoryl acceptor specificity of wild-type, Val67, and Gln101 mutant adenylate kinases[a]

Enzyme	AMP	dAMP	CMP	UMP
wild type	(100)	9.1	0.30	0.008
Val67-Ala	(100)	34.6	1.37	0.048
Val67-Ile	(100)	0.74	0.015	0
Val67-Thr	(100)	1.31	0.82	0.002
Val67-Glu	(100)	1.05	0.048	0.023
Val67-Arg	(100)	2.77	0.128	0.025
Gln101-Met	(100)	0.27	0.10	0.30
Gln101-His	(100)	0.34	0.11	0.14
Gln101-Leu	(100)	4.2	0.51	1.7
Gln101-Trp	(100)	1.7	0.66	9.51

[a]The enzyme was assayed at 25 °C in 87 mM triethanolamine-HCl (pH 7.0) containing 10 mM $MgCl_2$, 100 mM KCl, 1 mM ATP, 1 mM mononucleotide indicated, 1 mM phosphoenolpyruvate, 0.16 mM NADH, 20 units of pyruvate kinase, and 20 units of lactate dehydrogenase. The activity of each enzyme in the MgATP-AMP pair was taken as 100.

Table V

Kinetic parameters in phosphoryl transfer from MgATP to dAMP for wild-type, Val67-Ala, and Val67-Ile mutant adenylate kinases

Enzyme	Specific activity	K_{m1}	K_{m2}	K_{m3}	K_{m4}	β^a	V_{max}
	units/mg	*mM*	*mM*	*mM*	*mM*		*units/mg*
wild type	173	2.89	0.35	0.36	2.94	1.0	941
Val67-Ala	316	1.51	0.23	0.33	2.20	0.69	1253
Val67-Ile	16.7	8.29	0.77	0.25	2.67	3.1	116

[a]The simplified cooperativity factor calculated according to $\beta = K_{m2}/K_{m3} = K_{m1}/K_{m4}$, see text for details.

Table VI

Kinetic efficiency for dAMP, UMP, CMP, and AMP in wild-type, Val67, and Gln101 mutant adenylate kinases

Enzyme	k_{cat}/K_m (1/s/mM)			
	dAMP	UMP	CMP	AMP
wild type	115 0.054[a]	ND 1.73[a]	ND 4.28x10^3[a]	5.52x10^3
Val67-Ala	204	ND	ND	5.99x10^3
Val67-Ile	15.6	ND	ND	2.31x10^3
Gln101-Met	ND	0.163[a]	0.058[a]	34.5[a]
Gln101-His	ND	0.172[a]	0.306[a]	138[b]
Gln101-Leu	ND	0.019[a]	0.012[a]	1.33[a]

[a]An apparent value in 2 mM MgATP. [b] An apparent value in 1 mM MgATP.

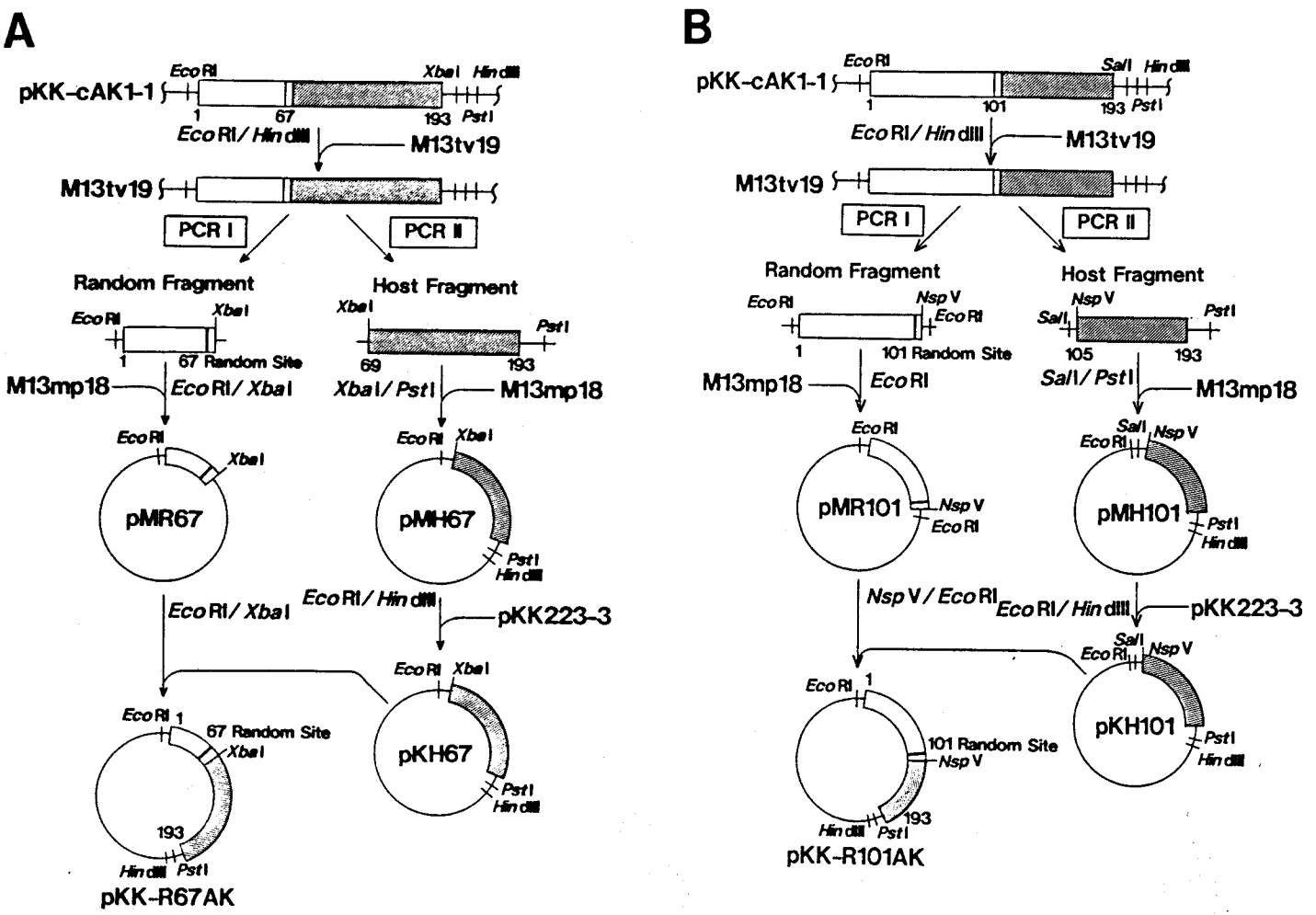


Fig. 1. Construction scheme for the expression plasmid of adenylate kinase containing random mutation at the position 67(A) and 101(B). Only relevant restriction sites are indicated, and lengths (sizes) of the DNAs are shown arbitrarily. Restriction fragments of the open reading frame are shown by bars and painted differently for easier understanding of the construction strategy; lines are the region from vector DNAs. See text for details.

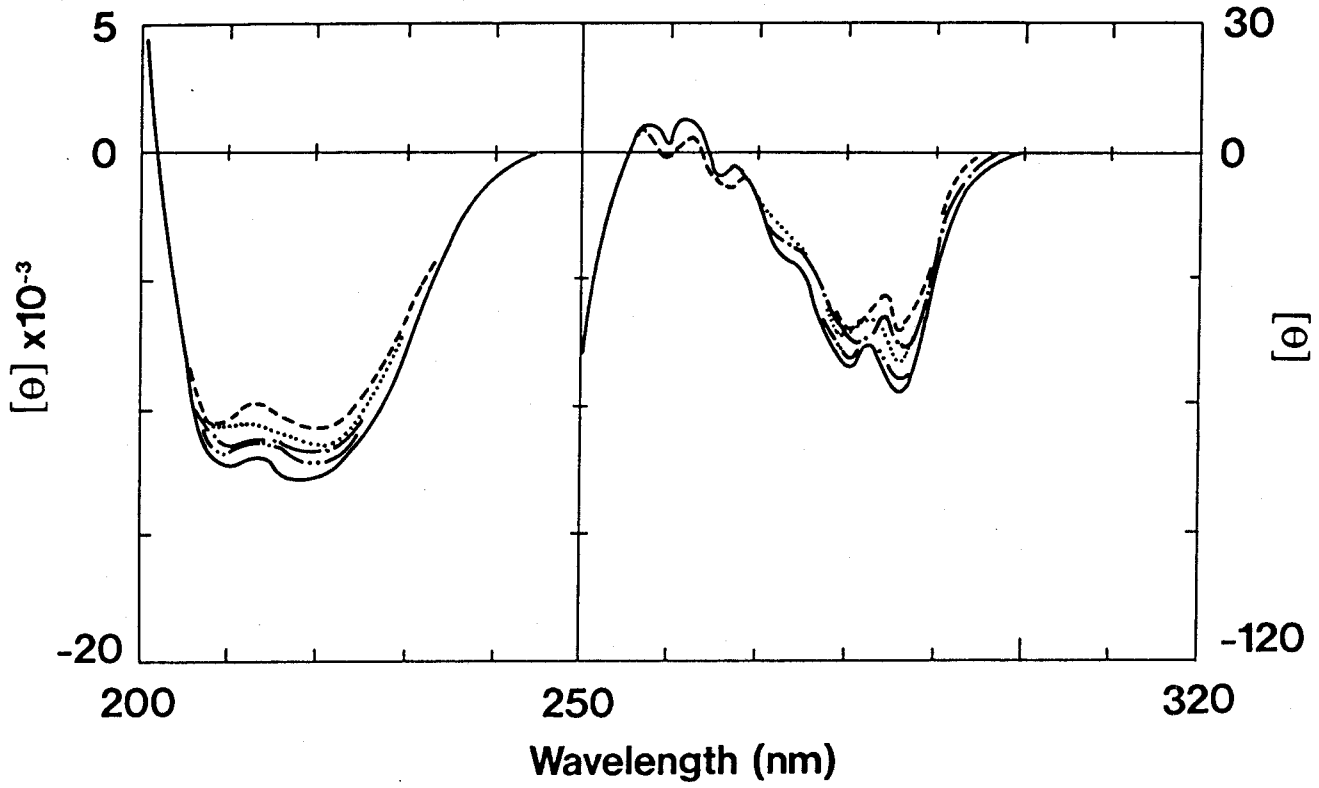


FIG. 2. **CD spectra of the wild-type and Val67 mutant adenylate kinases.** The CD spectra were measured at a protein concentration of 0.3 mg/ml in the region between 250 and 320 nm and at 0.1 mg/ml in the region below 250 nm :(——), wild-type and Val67-Ala mutant enzyme; (·····), Val67-Ile mutant; (-·-·-), Val67-Thr mutant; (-·-), Val67-Glu mutant; (----), Val67-Arg mutant.

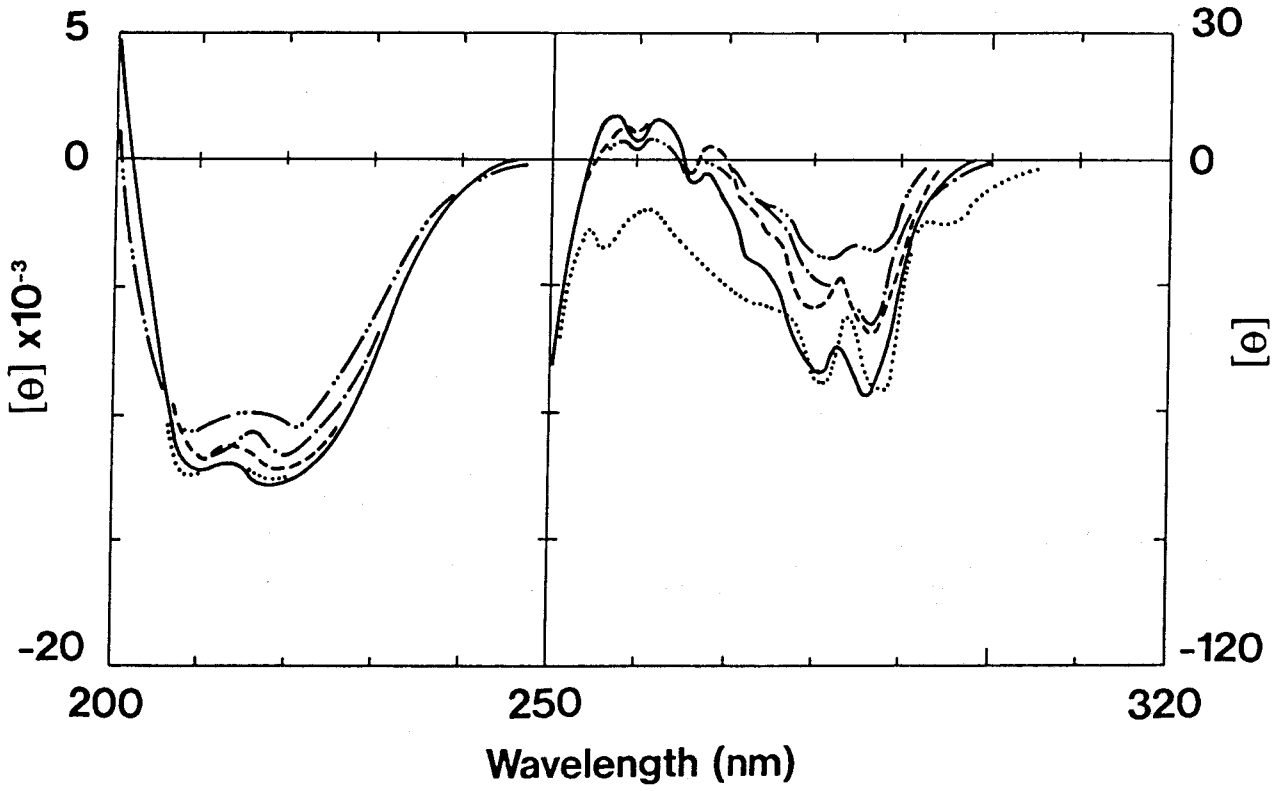


FIG. 3. **CD spectra of the wild-type and Gln101 mutant adenylate kinases.** The CD spectra were measured at a protein concentration of 0.3 mg/ml in the region between 250 and 320 nm and at 0.1 mg/ml in the region below 250 nm: (——), wild-type enzyme; (-·-), Gln101-His mutant; (----), Gln101-Met mutant; (-··-), Gln101-Leu mutant; (·····), Gln101-Trp mutant.

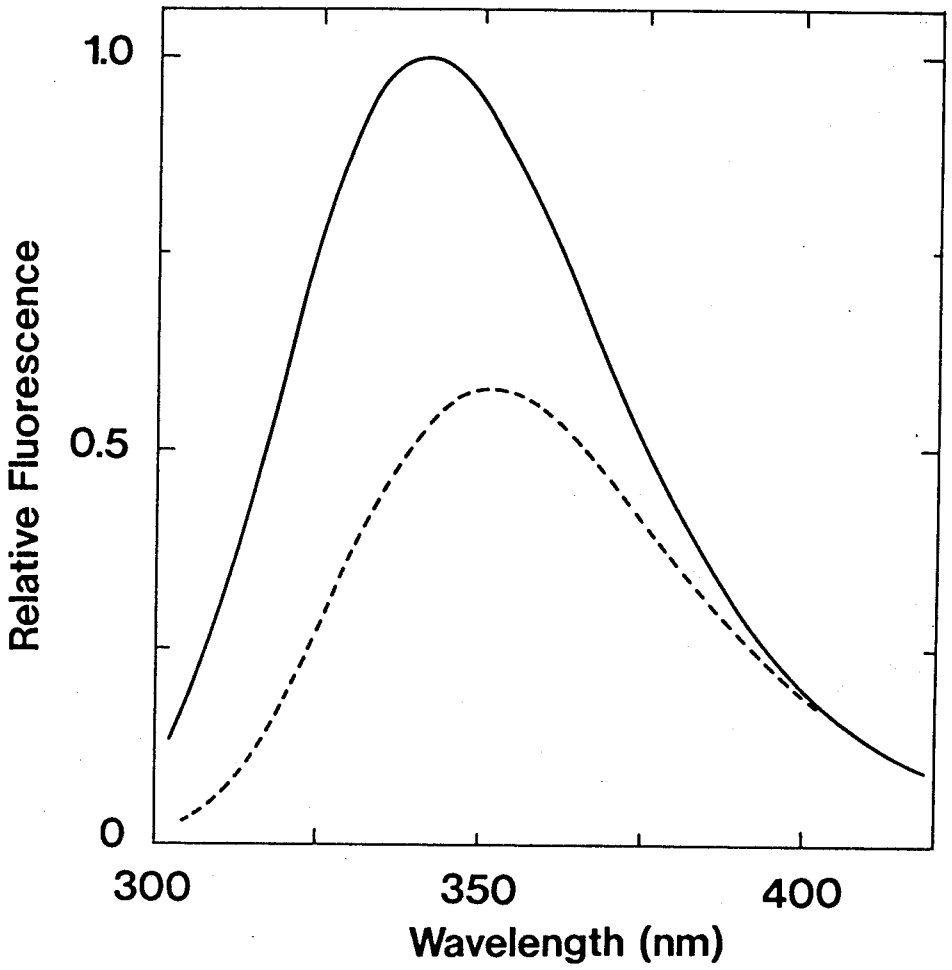


FIG. 4. **Fluorescence emission spectrum of the Gln101-Trp mutant enzyme.** The excitation wavelength was 295 nm, and the protein concentration was 1 μM in 30 mM Tris-HCl (pH 7.5), 0.1 M NaCl, 1 mM EDTA, and 0.1 mM dithiothreitol. (——), emission spectrum of the Leu66Trp mutant enzyme in the native state recorded at 25 °C; (----), the spectrum in the presence of 4 M guanidine hydrochloride.

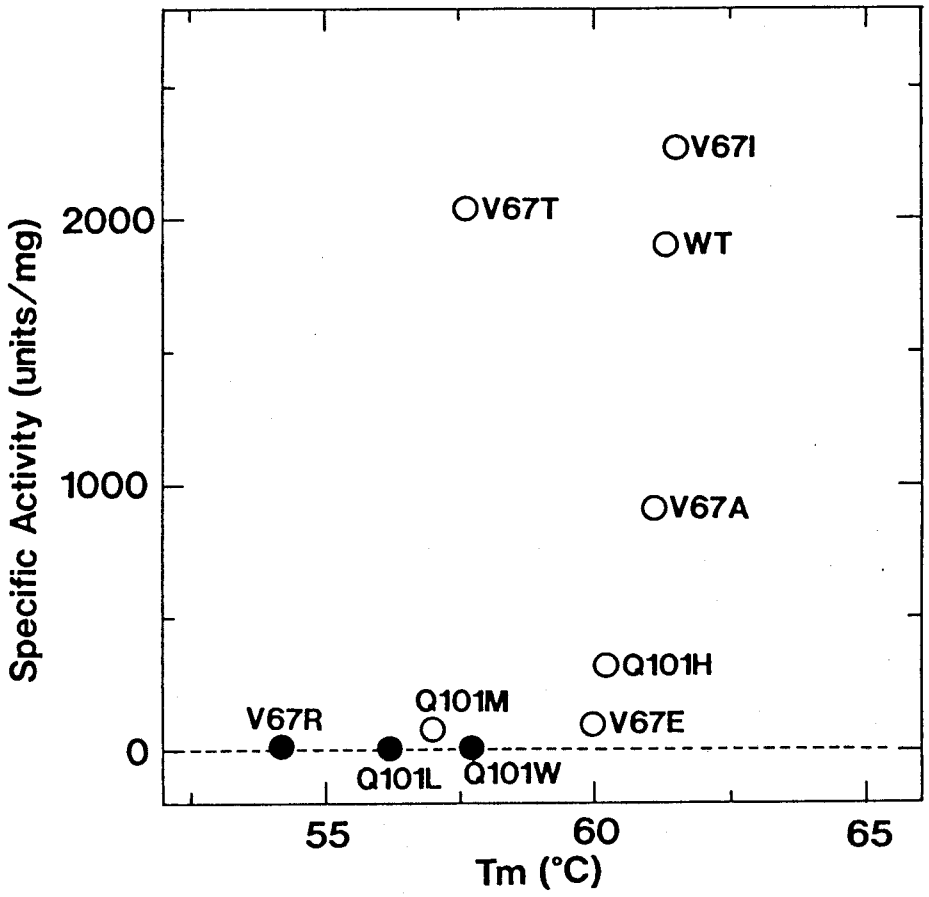


Fig. 5. Dependence of growth of *E.coli* CV2 on Tm and specific activity of the wild-type, Val67 mutant, and Gln101 mutant enzymes. Tm is the temperature at the midpoint of the unfolding transition in the respective enzyme and specific activity was measured in the purified enzyme. When the wild-type and mutant enzymes were expressed with *E.coli* CV2 as host, growth of the cell was examined by incubating at 45°C for 24hr on LB plate containing 50 μg/ml ampicillin. Open circle, growable at 45 °C ;closed circles, lethal at 45 °C.

REFERENCES

1. Johson, K. A. and Benkovic, S. J. (1990) *The Enzymes* (Boyer, P. D., 3rd ed) Vol.19, pp. 159-211, Academic Press, New York
2. Lim, W. A. and Sauer, R. T. (1989) *Nature* **339**, 31-36
3. Mullis, K. B. and Faloona, F. A. (1987) *Methods Enzymol.* **155**, 335-350
4. Okajima, T., Tanizawa, K., Yoneya, T., and Fukui, T. (1990) *J. Biol. Chem.* **18**, 1142-1147
5. Dreusicke, D. and Schulz, G. E. (1988) *J. Mol. Biol.* **203**, 1021-1028
6. Schulz, G. E., Müller, C. W., and Diederichs, K. (1990) *J. Mol. Biol.* **213**, 627-630
7. Kishi, F., Maruyama, M., Tanizawa, Y., and Nakazawa, A. (1986) *J. Biol. Chem.* **261**, 2942-2945
8. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 5463-5467
9. Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
10. Ray, T. K., Cronan, J. E., Jr, Mavis, R. D. and Vagelos, R. V. (1970) *J. Biol. Chem* **245**, 6442-6448
11. Tanizawa, Y., Kishi, F., Kaneko, T., and Nakazawa, A. (1987) *J. Biochem. (Tokyo)* **101**, 1289-1296
12. Tagaya, M., Yagami, T., Noumi, T., Futai, M., Kishi, F., Nakazawa, A., and Fukui, T. (1989) *J. Biol. Chem.* **264**, 990-994

13. Laemmli, U. K. (1970) *Nature* **227**, 680-685

14. Florini, J. R., and Vestling, C. S. (1957) *Biochim. Biophys. Acta* **25**, 575-578

15. Cleland, W. W. (1979) *Methods Enzymol.* **63**, 103-138

16. Tsai, M.-D. and Yan, H. (1991) *Biochemistry* **30**, 6806-6818

17. Yoneya, T., Tagaya, M., Kishi, F., Nakazawa, A., and Fukui, T. (1989) *J. Biochem. (Tokyo)* **105**, 158-160

18. Wolfenden, R., Anderson, L., Cullis, P. M., and Southgate, C. C. B. (1981) *Biochemistry* **20**, 849-855

19. Sweet, R. M. and Eisenberg, D.(1983) *J. Mol. Biol.* **171**, 479-488

20. Gregoret, L. M., Rader, S. D., Fletterick, R. J., and Cohen, F. E. (1991) *Protein* **9**, 99-107

21. Zamyatinn, A. A. (1972) *Prog. Biophys. Mol. Biol.* **24**, 107-123

22. Noda, L. (1973) in *The Enzymes* (Boyer, P. D., 3rd ed) Vol. 8, pp. 279-305, Academic Press, New York

23. Hasse, G. H. W., Brune, M., Reinstein, J., Pai, E. F., Pingoud, A., and Wittinghofer, A. (1989) *J. Mol. Biol.* **207**, 151-162

CHAPTER IV

A Challenge to
Changing the Substrate Specificity
for Nucleoside Monophosphate

INTRODUCTION

Adenylate kinase has two distinct substrate-binding sites, a low specific one for nucleoside triphosphates and a high specific one for nucleoside monophosphates (1). As compared to the high activity with the MgATP-AMP pair, the wild-type enzyme possesses 5.7, 0.008, and 9.1% activities with the MgATP-CMP, MgATP-UMP, and MgATP-dAMP pairs, respectively, and does not use GMP and IMP as a phosphoryl acceptor for MgATP (Chapter III) (2). Recent studies by x-ray crystallography (3,4), NMR (5,6), and site-directed mutagenesis (2,7,8) have established the outline of the AMP-binding site. Although some residues involved in the binding of AMP have been identified, the molecular mechanism recognizing AMP remains unclear.

In Chapter III, it was shown that Gln101 plays an important role in the AMP binding and that, by replacing Gln101 by Met or His, the activity toward UMP increases, though it may be due to indirect effects. This result suggested that even the high specificity for AMP of adenylate kinase can also be engineered. Hence, changing the substrate specificity for nucleoside monophosphate of adenylate kinase has been challenged in this chapter. The sequence of UMP/CMP kinase, which has been isolated from several sources (9,10) and catalyzes the reversible transphosphorylation specifically with pyrimidine nucleoside monophosphates CMP and UMP as a phosphoryl acceptor, has been used as a natural reference to decide target residues to be mutated. The cDNAs for UMP/CMP kinase of a yeast (*Saccharomyces cerevisiae*) and of a slime mold (*Dictyostelium discoideum*) have

been cloned, and their deduced amino acid sequences have been reported (11,12). The sequences were found to be highly homologous with those of vertebrate cytosolic adenylate kinases (11,12). This finding suggests that the substrate specificity of adenylate kinase may be altered to that of UMP/CMP kinase by substitution of the functional residue(s) variable between the two enzymes. By such sequence comparison, Thr39-Ala, Leu66-Ile, and Gln101-Asn have been planned for mutagenesis. These mutations have been introduced singly, doubly, and triply, and the resultant mutant proteins have been purified and characterized. In the protein engineering studies, the conversion of substrate specificities is one of the goals. Such studies would also provide important information for understanding the molecular mechanism of AMP recognition in adenylate kinase.

EXPERIMENTAL PROCEDURES

Materials——Nucleoside mono- and triphosphates were obtained from Seikagaku Kogyo or Sigma; lactate dehydrogenase (rabbit muscle) and pyruvate kinase (rabbit muscle) from Oriental Yeast; plasmids M13tv19 and pUC118 from Takara Shuzo; a plasmid pKK223-3 from Pharmacia; and a recombinant *Vent* DNA polymerase from New England Biolabs. All other reagents were of the highest purity commercially available.

Preparation of Mutant Enzymes——Replacement of Thr39 by Ala was accomplished by two steps of PCR (13,14). The first PCR was performed in two separate tubes with two pairs of oligonucleotide primers, A1 and A2, and A3 and A4 (Table I), which were synthesized with an Applied Biosystems DNA synthesizer model 381. An *Eco*RI-*Hin*dIII fragment (encoding chicken muscle adenylate kinase) excised from pKK-cAK1-1 (15) and inserted into M13tv19 phage vector was used as a template. The second PCR was performed with oligonucleotides A1 and A4 as primers and the two products purified electrophoretically from the first PCR as templates. The product of the second PCR was digested with *Eco*RI and *Pst*I and subcloned into pUC118. After the entire region amplified by PCR was sequenced by the dideoxy chain termination method (16) to examine the result of PCR, the 0.7-kbp *Eco*RI-*Hin*dIII fragment was inserted into the expression plasmid, pKK223-3.

Single mutation of Leu66-Ile and double mutation of Thr39-Ala/Leu66-Ile were introduced by amplifying with PCR a 240-bp fragment corresponding to an amino terminal region of the enzyme.

The one-step PCR for the single mutation was performed with oligonucleotides A1 and A5 (Table I) as primers, and the two-step PCR for the double mutation was with oligonucleotides A1 and A2, and A3 and A5 in the same manner as that for Thr39-Ala. Both PCR products were subcloned into pUC118 at *Eco*RI and *Xba*I sites to confirm DNA sequence of the entire region amplified by PCR, and then inserted into pKH67 (Chapter III) at the same sites.

Triple mutation (Thr39-Ala/Leu66-Ile/Gln101-Asn) was introduced by amplifying a 370-bp fragment with oligonucleotides A2 and A6 (Table I) as primers and with the expression plasmid for the double mutant (Thr39-Ala/Leu66-Ile) as a template. The PCR product was inserted into pKH101 by the same method as described in Chapter III.

The resultant plasmids were transformed into *E. coli* JM109 competent cells as described by Maniatis *et al.* (17). The mutant enzymes, Thr39-Ala, Leu66-Ile, and Thr39-Ala/Leu66-Ile, were purified to homogeneity from the soluble fraction of cell lysates essentially as described by Tanizawa *et al.* (15), and the triple mutant enzyme, Thr39-Ala/Leu66-Ile/Gln101-Asn, was from the pellet of cell lysates by acid extraction and gel-filtration as described previously (18). All PCR was performed in a 100-µl solution containing 20 mM Tris-HCl (pH 8.8), 10mM $(NH_4)_2SO_4$, 10mM KCl, 2 mM $MgCl_2$, 0.1 mg/ml bovine serm albumin, 0.1% Triton X-100, 0.2 mM each of deoxynucleotides (dATP, dGTP, dCTP, and dTTP), 1 µM each of two primers, about 4 ng of the template DNA, and 2 units of the *vent* DNA polymerase with a program consisting of 1-30 cycles (94 °C for 1 min, 60 °C for 3 min, and 72 °C for 3 min) and the last cycle (72 °C for 20 min) in a thermal cyclic reactor (Hoei Science Co., Ltd., Tokyo, model TC-100).

Steady State Kinetic Analysis——The enzyme activity was determined in the standard assay mixture as described previously (18). Steady state kinetic parameters were obtained with variable concentrations of AMP, CMP or UMP as a phosphoryl acceptor, and of MgATP as the donor. The assay mixture was consisted of 87 mM triethanolamine-HCl buffer (pH 7.0), 10 mM $MgCl_2$, 100 mM KCl, 1 mM phosphoenolpyruvate, 0.16 mM NADH, 5 units of pyruvate kinase, and 20 units of lactate dehydrogenase. When nucleoside monophosphates other than AMP were used as a substrate, 20 units of pyruvate kinase was added in the assay mixture.

CD Measurements——CD spectra were measured at 25 °C in 30 mM Tris-HCl buffer, pH 7.5, containing 0.1 M NaCl, 1 mM EDTA, and 0.1 mM dithiothreitol with a Jasco spectropolarimeter model J-600. The conditions for CD measurements have been described in the previous chapters.

RESULTS

Sequence Comparison with UMP/CMP Kinase and Decision of Target Sites——Fig. 1 shows the sequence alignment comparing the chicken muscle adenylate kinase (AK1), the yeast cytosolic adenylate kinase (AKy), and the yeast and slime mold UMP/CMP kinases. Although the yeast cytosolic AKy has a large insertion of 30 residues (19), the three other enzymes are consisted of a similar number of amino acid residues. The sequence comparison reveals a high degree of homology among these proteins. The overall homology between the chicken muscle AK1 and either one of the two UMP/CMP kinases is about 42 %, which is even higher than that (32 %) between the chicken muscle AK1 and the yeast cytosolic AKy except for the insertion. These findings strongly suggest that the two UMP/CMP kinases fold into a conformation very similar to that of adenylate kinase. Their specificities for nucleoside monophosphate are different, but the mechanisms of phosphoryl transfer are probably the same. Therefore, it is assumed that the difference in substrate specificity is derived from the difference(s) in the residues proposed as AMP-binding residues in adenylate kinase in the previous studies (see Chapters II and III). By surveying residues to be close to the adenine ring of the bound AMP (4) in adenylate kinase and to be different in the two UMP/CMP kinases, Thr39 and Leu66 have been selected as target residues, which are replaced by Ala and Ile, respectively, in both UMP/CMP kinases (Fig. 1). Although Gln101 is replaced by Asn only in the yeast UMP/CMP kinase (Fig. 1), it also has been selected as a target residue, since Gln101 may play

a role not only in binding of AMP by the hydrogen bond but also indirectly in recognition of the adenine ring as described in Chapter III. Based on these analyses, two single mutants (Thr39-Ala and Leu66-Ile), one double mutant (Thr39-Ala/Leu66-Ile), and one triple mutant (Thr39-Ala/Leu66-Ile/Gln101-Asn) have been prepared to compare the substrate specificities with that of the wild-type enzyme.

CD Spectra of Mutant Enzymes——CD spectra of Thr39-Ala, Leu66-Ile, and Thr39-Ala/Leu66-Ile were identical with that of the wild-type enzyme, overlapping completely each other in the entire wavelength region from 200 to 320 nm (Fig. 2). This suggests that conformational changes of the protein caused by the replacements are very subtle, if any. However, the triple mutant, Thr39-Ala/Leu66-Ile/Gln101-Asn, shows a CD spectrum different from the wild type (Fig. 2), suggesting that significant conformational changes occurred by the triple mutation. Particularly, the mutation of Gln101 into Asn might have produced the conformational changes as has been observed with some other single Gln101 mutant enzymes (Chapter III).

Kinetic Parameters for MgATP and AMP——The purified Thr39-Ala and Leu66-Ile mutant enzymes show a high specific activity with AMP comparable to the wild type (Table II). In contrast, the double mutant enzyme has a much lowered activity, and the triple one is almost inactive (in the 10^{-5} order of the wild-type enzyme activity). Determination of the steady state kinetic parameters according to the random Bi-Bi mechanism (see Chapter II) indicates that the Thr39-Ala mutant enzyme possesses the kinetic parameters very similar to those of the wild type (Table II), suggesting that Thr39 is directly involved neither in

catalytic process nor in substrate binding. Thr39-Ala/Leu66-Ile showed very high K_m values for AMP and a moderately decreased V_{max} value. The triple mutant, Thr39-Ala/Leu66-Ile/Gln101-Asn, also had high K_m values for AMP and a very minute V_{max} value. Since K_m values for MgATP and AMP of the double and triple mutant enzymes are similar and their V_{max} values are different in the 10^{-4} order of magnitude, the additional Gln101-Asn mutation caused the main damage in the catalytic efficiency of the double mutant. In accordance with the significant conformational changes observed in the CD spectrum of the triple mutant enzyme (Fig. 2), the replacement of Gln101 into Asn is probably deleterious for both structure and function of the enzyme, as also described in Chapter III.

Substrate Specificities of Mutant Enzymes——To examine whether these mutations have actually altered the substrate specificity for nucleoside monophosphate, phosphoryl transfer activities were measured in the assay mixture containing dAMP, CMP, UMP, IMP, or GMP (1 mM) as the acceptor and MgATP (1 mM) as the donor. GMP and IMP did not serve as a phosphoryl acceptor for all the wild-type (2) and mutant enzymes. With the other nucleoside monophosphates, the specific activities and the relative values to those with AMP are summarized in Table III. All the mutant enzymes showed lower specific activities for AMP and dAMP than the wild type. Because the additional replacement of Leu66 into Ile for Thr39-Ala reduced the activity with AMP more significantly than that with CMP, the specific activities with CMP of Thr39-Ala and Thr39-Ala/Leu66-Ile were enhanced by 11.6- and 3.3-fold, respectively, as compared with that of the wild type. Thus, the relative activity with CMP of Thr39-

Ala/Leu66-Ile increased from that of Thr39-Ala. When UMP was used as a phosphoryl acceptor, Thr39-Ala showed an 8.5-fold higher specific activity of the wild type. The triple mutant enzyme, Thr39-Ala/Leu66-Ile/Gln101-Asn had a considerable activity only with UMP.

Steady State Kinetic Analysis Using CMP or UMP as a Phosphoryl Acceptor——The steady state kinetic analysis was performed using CMP (1~6.6 mM) or UMP (4~20 mM) as a phosphoryl acceptor and a fixed concentration (2 mM) of MgATP as the donor. Maximal velocities and the apparent K_m values were calculated from the double reciprocal plot of the initial rate (v) against concentrations of the nucleoside monophosphate. Table IV summarizes the apparent K_m values for CMP, UMP, and AMP, the V_{max} values, and the corresponding k_{cat}/K_m values of the mutant and wild-type enzymes. As was clear from its specific activity, the Leu66-Ile mutant enzyme showed kinetic parameters for AMP, CMP, and UMP comparable to those of the wild type, except for the K_m for AMP, showing the replacement of Leu66 by Ile insignificantly affects the substrate specificity. The triple mutant, Thr39-Ala/Leu66-Ile/Gln101-Asn, again showed markedly low k_{cat}/K_m values for the three substrates. Thr39-Ala and Thr39-Ala/Leu66-Ile exhibited 17- and 5-fold higher k_{cat}/K_m values for CMP than the wild type, respectively. Table IV also shows that the enhancement of k_{cat}/K_m values for CMP is due both to the decrease in K_m values and to the increase in V_{max} values of the two mutant enzymes and that the difference in k_{cat}/K_m values between both mutant enzymes results from the difference in their V_{max} values. The V_{max} value of the Thr39-Ala mutant enzyme is comparable to that reported for UMP/CMP kinase, although its K_m value for CMP

is much higher than that of UMP/CMP kinase (12). It should be pointed out that the two mutant enzymes, Thr39-Ala and Thr39-Ala/Leu66-Ile, show considerably lower k_{cat}/K_m values for UMP than for CMP, although the natural UMP/CMP kinase uses UMP and CMP as a substrate with an almost equal efficiency (12).

DISCUSSION

Schulz *et al.* (4) suggested from x-ray crystallography of the AK3-AMP complex that the O atom of Ser36 (corresponding to Thr39 in the chicken muscle AK1) is located within the distance of 3.5 Å from the N7 atom of the bound AMP and that their positionings are in good hydrogen-bond-forming geometries. However, the present results show that the mutation of Thr39 into Ala, which does not form a hydrogen bond, produces only a subtle change in kinetic parameters, and therefore suggest that the side chain of Thr39 is probably not hydrogen-bonded with the adenine ring of the bound AMP. A similar result has been reported by Yan *et al.* (6); Thr39 might be merely located at the position close to the adenine ring of the bound AMP. The finding that Thr39-Ala uses not only AMP but also CMP significantly as a phosphoryl acceptor suggests that Thr39 may prevent CMP from binding with the enzyme. In other words, Thr39 may be important for the high specificity for AMP of the enzyme. If one assumes that the wild-type enzyme binds the pyrimidine nucleoside monophosphate CMP at the AMP-binding site, the pyrimidine six membered ring would be positioned at the region occupied by the purine five membered ring, and the size difference between the rings of substrates should cause an unfavorable steric hindrance. On the other hand, Thr39-Ala, in which hydrogen or ionic bonds are not newly formed but only the volume of the side chain is reduced, shows increased k_{cat}/K_m values for pyrimidine nucleoside monophosphates, maintaining the value for AMP comparable to that of the wild type. In view of such a steric effect, the increased activity

for CMP of the mutant enzyme can be explained by assuming that the pyrimidine ring is well accommodated in the space provided by the mutation of Thr39 into Ala. This assumption is also supported by the 0.4-fold decrease in K_m values for CMP of Thr39-Ala and Thr39-Ala/Leu66-Ile (Table IV). Clarke *et al.* (20) showed that the substrate specificity of lactate dehydrogenase specific for pyruvate can be altered to that specific for oxaloacetate by the replacement of Thr246 into Gly, removing the bulky side chain at the active site.

The double (Thr39-Ala/Leu66-Ile) and triple (Thr39-Ala/Leu66-Ile/Gln101-Asn) mutant enzymes have been constructed, expecting that adenylate kinase is changed to be similar to the yeast and slime mold UMP/CMP kinases, respectively. Substitution of Gln101 causes a considerable increase in a K_m value for AMP as described in Chapter III. Therefore, the triple mutant enzyme is expected to show a selectively reduced activity for AMP with high activities for CMP and UMP. However, this mutation seemed to induce conformational changes that lead to a marked decrease in activities for both AMP and CMP with a slight activity for UMP. Gln101 is relatively buried in the protein interior, and accordingly the shorter side chain of Asn101 in the triple mutant enzyme may interact unfavorably with nearby residues. Thus, it is premature to conclude from the present results as to whether the mutation Gln101-Asn alone affects the recognition of CMP or UMP. The mutation Leu66-Ile was effective for reducing the K_m value for AMP about 20-fold in both the single and double mutants. However, this mutation did not reduce the K_m for CMP. Therefore, the mutation Leu66-Ile makes the enzyme relatively specific to UMP, although the double mutant enzyme shows lower

V_{max} values for UMP and CMP than Thr39-Ala. The double mutant enzyme may well be considered as an intermediate to UMP/CMP kinase.

To achieve the complete conversion of adenylate kinase to UMP/CMP kinase, the decrease in K_m values for CMP and UMP by two orders of magnitude is needed, while maintaining the high V_{max} value. The following two methods are conceivable: 1) Based on the sequence comparison with the natural UMP/CMP kinases, chimeric enzymes are constructed through successive and multiple site-directed mutagenesis or cassette mutagenesis. 2) Apart from the sequences of UMP/CMP kinases, the random mutations are introduced site-specifically. For example, the mutations Gln101-Met and Gln101-His may be taken as candidates for increasing the k_{cat}/K_m values for UMP in 3 times of that of the wild type, together with for reducing the affinity for AMP (see Chapter III).

Table I

Sequences of synthetic primers for PCR

Primer	Sequence

```
                                          0  1
                                  EcoR I  Met Ser-
Template   5'-GTTGTAAAACGACGGCCAGT GAATTC CC ATG TCG-
           3'-CAACATTTTGCTGCCGGTCA CTTAAG GG TAC AGC-
      A1     5' GTAAAACGACGGCCAGT 3'

      A2                          3' GA GTG GAG AGG CGA CCC CTG GAC 5'
                                                  *
            29  30  31  32  33  34  35  36  37  38  39  40  41  42  43  44  45  46
           Val His Lys Tyr Gly Tyr Thr His Leu Ser Thr Gly Asp Leu Leu Arg Ala Glu
Template   5'-GTG CAC AAG TAT GGG TAC ACT CAC CTC TCC ACT GGG GAC CTG CTC CGG GCA GAG
           3'-CAC GTG TTC ATA CCC ATG TGA GTG GAG AGG TGA CCC CTG GAC GAG GCC CGT CTC
                                                  *
      A3                          5' CT CAC CTC TCC GCT GGG GAC CTG 3'

      A4           3' GCC GGGCCCCTAGG AGATCA CAGCTG GACGTC CGTACG TTC 5'
                                        *
            193
           -Leu Ter
Template   5' -CTG TGA CGG CCCGGGGATCC TCTAGA GTCGAC CTGCAG GCATGC AAGCTT GGC-
           3' -GAC ACT GCC GGGCCCCTAGG AGATCT CAGCTG GACGTC CGTACG TTCGAA CCG-
                        Sma I BamHI  Xba I  Sal I  Pst I  Sph I HindIII

                                                                    Xba I
      A5                    3' CGG TAG TAC CTC TTC CCG CTC GAC TAT GGA GAT CTG TGC CA 5'
                                                          * *   *   *
            55  56  57  58  59  60  61  62  63  64  65  66  67  68  69  70  71  72  73  74
           -Lys Lys Leu Gln Ala Ile Met Glu Lys Gly Glu Leu Val Pro Leu Asp Thr Val Leu Asp-
Template   5' -AAG AAG CTG CAA GCC ATC ATG GAG AAG GGC GAG CTG GTT CCC CTG GAC ACG GTG CTG GAC-
           3' -TTC TTC GAC GTT CGG TAG TAC CTC TTC CCG CTC GAC CAA GGG GAC CTG TGC CAC GAC CTG-

                                                                Nsp V EcoR I
      A6               3' CCG ATG GGA GCG CTC CAC TTC TTG CCT CTC CTC AAG CTT AAG TTC TAA 5'
                                                    * *           *     ***
            91  92  93  94  95  96  97  98  99 100 101 102 103 104 105 106 107 108 109 110 111 112
           -Leu Ile Asp Gly Tyr Pro Arg Glu Val Lys Gln Gly Glu Glu Phe Glu Lys Lys Ile Ala Pro Pro-
Template   5' -CTC ATT GAC GGC TAC CCT CGC GAG GTG AAG CAG GGA GAG GAG TTT GAA AAG AAG ATT GCC CCC CCC-
           3' -GAG TAA CTG CCG ATG GGA GCG CTC CAC TTC GTC CCT CTC CTC AAA CTT TTC TTC TAA CGG GGG GGG-
```

Oligonucleotides A 1~6 are used as PCR primers and their templates DNA are also indicated with residues number in a protein. The sites recognized by restriction enzymes are underlined and mismatched bases are shown by asterisks.

Table II

Kinetic parameters for wild-type, Thr39-Ala, Thr39-Ala/Leu66-Ile, and Thr39-Ala/Leu66-Ile/Gln101-Asn mutant enzymes

Enzyme	Specific activity	K_{m1}	K_{m2}	K_{m3}	K_{m4}	β^a	V_{max}
	units/mg	*mM*	*mM*	*mM*	*mM*		*units/mg*
wild-type	1900	0.42	0.28	0.11	0.17	2.5	2620
Thr39-Ala	1550	0.32	0.19	0.16	0.28	1.1	2350
Thr39-Ala/Leu66-Ile	117	1.88	0.16	0.67	7.65	0.25	1210
Thr39-Ala/Leu66-Ile /Gln101-Asn	0.077	1.76	0.21	0.97	8.10	0.22	0.92

[a]The simplified cooperativity factor calculated according to $\beta = K_{m2}/K_{m3} = K_{m1}/K_{m4}$, see text for details.

Table III

Phosphoryl acceptor specificities of wild-type and mutant enzymes[a]

Enzyme	AMP	dAMP	CMP	UMP
wild type	1900	173	0.57	0.15
	(100)	(9.1)	(0.30)	(0.008)
Thr39-Ala	1550	31.4	66.0	1.28
	(100)	(2.0)	(4.2)	(0.083)
Leu66-Ile	718	2.02	3.42	0.02
	(100)	(0.28)	(0.48)	(0.003)
Thr39-Ala/Leu66-Ile	117	0.27	18.6	0.16
	(100)	(0.23)	(15.9)	(0.14)
Thr39-Ala/Leu66-Ile	0.077	0.006	0.075	0.208
/Gln101-Asn	(100)	(7.7)	(97.4)	(270)

[a]The enzyme was assayed at 25 °C in 87 mM triethanolamine-HCl (pH 7.0) containing 10 mM $MgCl_2$, 100 mM KCl, 1 mM ATP, 1 mM nucleoside monophosphate indicated, 1 mM phosphoenolpyruvate, 0.16 mM NADH, 20 units of pyruvate kinase, and 20 units of lactate dehydrogenase. Specific activities were indicated in units/mg in the upper columns and relative activities were shown in parenthesis on the lower columns where the activity of each enzyme with the MgATP-AMP pair was taken as 100.

Table IV

Kinetic parameters for AMP, CMP, and UMP in the wild-type, Thr39-Ala, Thr39-Ala/Leu66-Ile, and Thr39-Ala/Leu66-Ile/Gln101-Asn mutant enzymes[a]

	UMP			CMP			AMP		
	K_m	V_{max}	k_{cat}/K_m	K_m	V_{max}	k_{cat}/K_m	K_m	V_{max}	k_{cat}/K_m
	mM	units/mg	1/s/mM	mM	units/mg	1/s/mM	mM	units/mg	1/s/mM
wild type	33.4	5.00	0.0536	20.9	101	1.73	0.17	2030	4.28×10^3
Thr39-Ala	34.9	49	0.503	7.7	643	29.9	0.30	2240	2.67×10^3
Leu66-Ile	78	1.6	0.0074	35	145	1.5	3.00	3530	4.22×10^3
Thr39-Ala/Leu66-Ile	11.0	3.38	0.110	8.75	210	8.60	4.97	809	58.3
Thr39-Ala/Leu66-Ile /Gln101-Asn	24.7	6.0	0.087	13.4	1.38	0.037	5.94	0.61	0.037

[a]Apparent values were obtained with 2 mM MgATP.

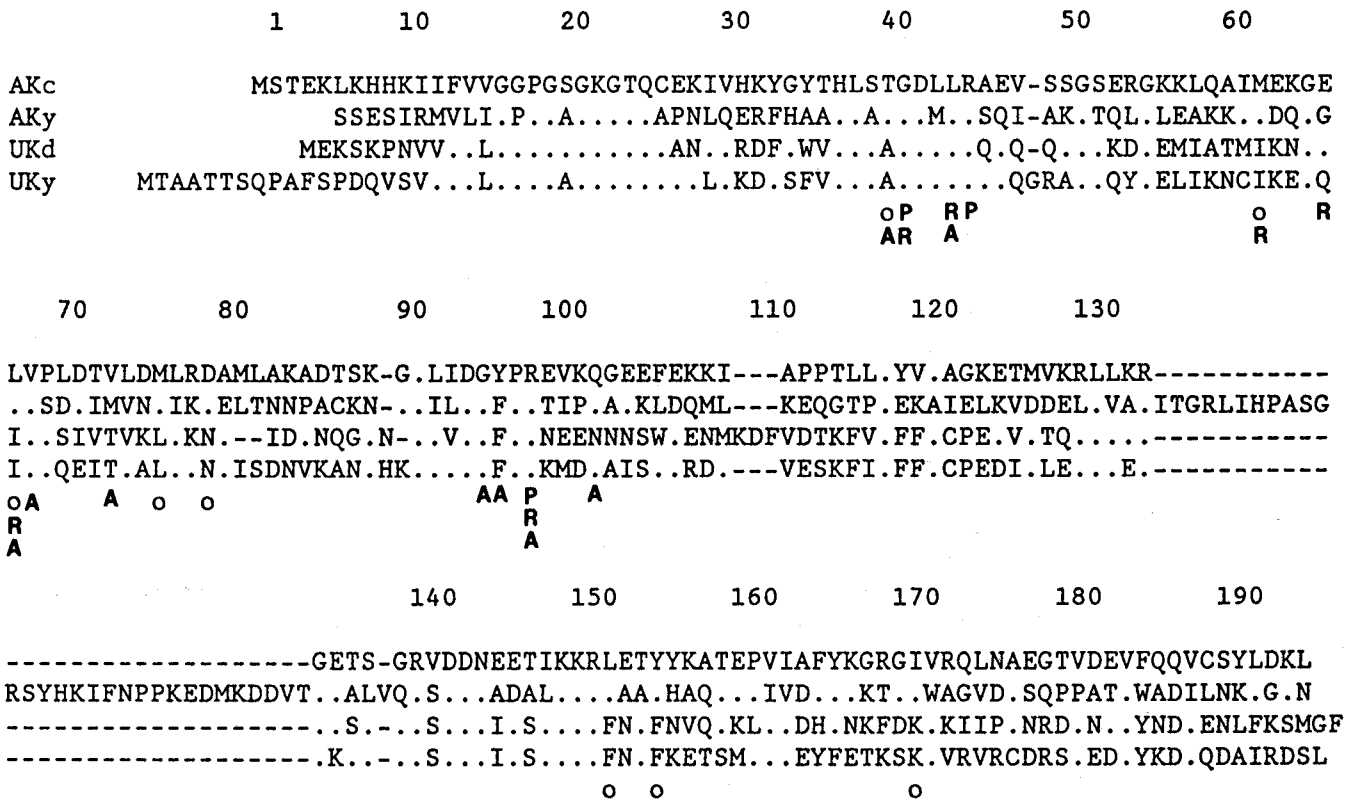


Fig. 1. **Sequence comparison of adenylate kinase with UMP/CMP kinase.** The alignment was done by modifying that of Schulz (19), Liljelund *et al.* (11), and Wiesmüller *et al.* (12). AKc, chicken muscle adenylate kinase (21); AKy, yeast cytosolic adenylate kinase (22); UKd, *D. discoideum* UMP/CMP kinase (12); UKy, *S. cerevisiae* UMP/CMP kinase (11). (o), residues conserved in two adenylate kinases and two UMP/CMP kinases but not in adenylate and UMP/CMP kinases. (P), (R), and (A) represent the residues assumed to be interacting with the phosphate, ribose, and adenine ring, respectively (4).

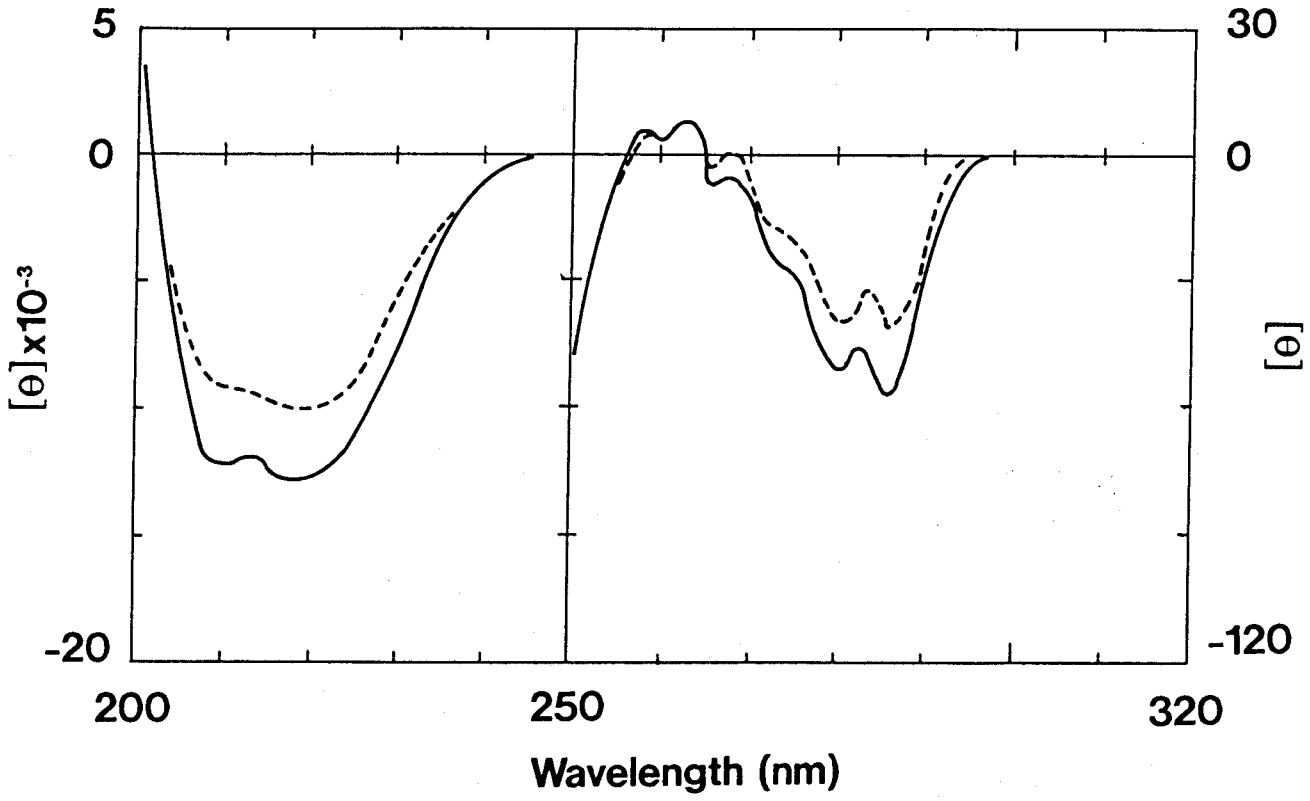


Fig. 2. CD spectra of the wild-type and mutant adenylate kinases. The CD spectra were measured at a protein concentration of 0.3 mg/ml in the region between 250 and 320 nm and at 0.1 mg/ml in the region below 250 nm. Solid lines indicate the CD spectra of the wild-type, Thr39-Ala, Leu66-Ile, and Thr39-Ala/Leu66-Ile mutant enzymes and the broken line is that of Thr39-Ala/Leu66-Ile/Gln101-Asn.

REFERENCES

1. Noda, L. (1973) in *The Enzymes* (Boyer, P. D., 3rd ed) Vol. 8, pp. 279-305, Academic Press, New York
2. Okajima, T., Tanizawa, K., Yoneya, T., and Fukui, T. (1990) *J. Biol. Chem.* **18**, 1142-1147
3. Egner, U., Tomasselli, A. G. and Schulz, G. E. (1987) *J. Mol. Biol.* **195**, 649-658
4. Schulz, G. E., Müller, C. W., and Diederichs, K. (1990) *J. Mol. Biol.* **213**, 627-630
5. Reinstein, J., Vetter, I. R., Schlichting, I., Rosch, P., Wittinghofer, A., and Goody, R. S. (1990) *Biochemistry* **29**, 7440-7450
6. Yan, H., Dahnke, T., Zhou, B., Nakazawa, A., and Tsai, M.-D. (1991) *Biochemistry* **29**, 10956-10964
7. Kim, H., Nishikawa, S., Tokutomi, Y., Tanaka, H., Hamada, M., Kuby, S. A. and Uesugi, S. (1990) *Biochemistry* **29**, 1107-1111
8. Liang, P., Phillips, G. N., Jr., and Glaser, M. (1991) *Proteins: Struc., Funct., Genet.* **9**, 28-36
9. Maness, P. and Orengo, A. (1975) *Biochemistry* **14**, 1484-1489
10. Okada, H. and Suzuki, J. (1990) *Biochem. Int.* **21**, 677-684
11. Liljelund, P., Sanni, A., Friesen, J. D., and Lacroute, F. (1989) *Biochem. Biophys. Res. Commun.* **165**, 464-473
12. Wiesmüller, L., Noegel, A. A., Bârzu, O., Gerish, G., and Schleicher, M. (1990) *J. Biol. Chem.* **265**, 6339-6345
13. Mullis, K. B. and Faloona, F. A. (1987) *Methods Enzymol.* **155**, 335-350
14. Higuchi, R. (1990) *PCR protocols* (Innis, M. A., Gelfand, D. H., Sninsky, J. J., and White, T. J., ed) pp. 177-183, Academic Press, San Diego
15. Tanizawa, Y., Kishi, F., Kaneko, T., and Nakazawa, A. (1987) *J. Biochem. (Tokyo)* **101**, 1289-1296
16. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 5463-5467

17. Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
18. Tagaya, M., Yagami, T., and Fukui, T. (1987) *J. Biol. Chem.* **262**, 8257-8261
19. Schulz, G. E. (1987) *Cold Spring Harbor Symp. Quant. Biol.*, **52**, 429-439
20. Clarke, A. R., Atkinson, T., and Holbrook, J. J. (1989) *Trends Biochem. Sci.* **14**, 145-148
21. Kishi, F., Maruyama, M., Tanizawa, Y., and Nakazawa, A. (1986) *J. Biol. Chem.* **261**, 2942-2945
22. Tomasselli, A. G., Mast, E., Janes, W., and Schiltz, E. (1986) *Eur. J. Biochem.* **155**, 111-119

Chapter V

Comprehensive Discussion

COMPREHENSIVE DISCUSSION

Recently the crystal structure of the complex between bovine mitochondrial AK3 and AMP has been further refined into 1.85 Å resolution by Diederichs *et al.* (1) and the interaction between the side chain of the enzyme and the bound AMP has been illustrated (Fig. 1). Substitutions in the residues at the AMP binding site were observed between the chicken muscle AK1 and the bovine mitochondrial AK3 as follows; Thr39 (AK1)-Ser36 (AK3), Met61-Ile58, Glu65-Lys62, Val67-Ile64, and Val72-Met69. Only in Ser39 of the above residues, the hydrogen bond is formed between its side chain and the adenine ring of bound AMP (1), and β-hydroxy group is also conserved in Thr39, the corresponding residue in AK1. Side chains of the other residues are not involved in the AMP binding through the hydrogen bond. Although there is such a difference between the amino acid sequences of the chicken muscle AK1 and AK3 (2), it is virtually possible that the structure of the AK3-AMP complex applies to the AK1. Their results give important findings for understanding the mechanism of base recognition in the AK1. Therefore, the results of the present studies are discussed based on the detailed three-dimensional structure of the AK3-AMP complex (Fig. 1) (1).

As shown in Fig. 1, the adenine ring of bound AMP is sandwiched between the side chain of Leu63 (Leu66) (residues in parenthesis denote those corresponding in the chicken muscle enzyme) and Ile64 (Val67) from the solvent side. Furthermore, hydrophobic residues such as Leu40 (Leu43), Ile58 (Met61), and Met69 (Val72) are located near the bound AMP. It is suggested

that these residues are cooperative as a hydrophobic region involved in separating AMP from the solvent and forming the hydrophobic interaction (3) to the adenine ring. In Chapters II and III, it was shown that replacements of Leu66 and Val67 of the chicken muscle adenylate kinase affect the V_{max} value through gross conformational changes (4). The locations of Leu66 and Val67 suggest that the two residues are necessary for determining the position of the adenine ring. Fluorescence emission spectra due to Trp66 in the Leu66-Trp mutant enzyme showed that its intensity is remarkably reduced by the addition of AMP without shifting its maximal wavelength from 350 nm, which indicates that the indole ring remain accessible to the solvent (Chapter II). It is inconsistent with the proposed hydrophobic effect for Leu66. However the fluorescence property is considered to be reasonable from the fact that the side chain of Leu63 (Leu66) complex covers the adenine ring and that the side chain is in contact with both the adenine ring of the bound AMP and the solvent. It is reported that the distance between the CD2 atom of Leu63 (Leu66) and the N3 atom of the bound AMP (Figs. 1 and 2) is only 3.58 Å (1), which seems responsible for the marked quenching of Trp66 fluorescence by addition of AMP. It is assumed from the activities for dAMP in the wild-type, Val67-Ala, and Val67-Ile mutant enzymes that a larger side chain at position 67 produces steric hindrance to the ribose and reduces the activity when dAMP is used as a substrate (Chapter II). This assumption is also supported by the close contact of the side chain of Ile64 (Val67) to the ribose (Fig. 1). Leu66 and Val67 might be regarded as a "hydrophobic cap" for the adenine ring and ribose. RNase T1 is the guanine-specific ribonuclease and the

complex between 2'-GMP and the enzyme has been analyzed by x-ray crystallography (5). In this complex the guanine ring is found to be completely shielded by Tyr42 and Tyr45 from the solvent. The roles of Leu66 and Val67 seem to resemble those of Tyr42 and Tyr45.

Gln96 (Gln101) in the AK3-AMP complex is bound to the adenine ring through two hydrogen bonds between the NE2 atom and the N1 atom, and between the OE atom and the N10 atom (Figs. 1 and 2) (1). It is suggested that this residue plays an important role in the recognition of the adenine ring. Random mutagenesis studies of Gln101 in AK1 (chapter III) revealed that only Gln, His, and Met were functionally acceptable at position 101 to maintain the enzymatic activity. In the Gln101-His mutant enzyme, which has a relatively higher activity than the Gln101-Met mutant enzyme, the His residue can also form binary hydrogen bonds on its nitrogen atoms. It is interesting to note that the functionally acceptable exchange between His and Gln occurs in the *E. coli* AKe and the chicken muscle AK1. His36 is conserved in almost all adenylate kinase sequences, which is regarded to show that it is important or even essential for the catalytic reaction of adenylate kinase, whereas only the residue at the corresponding position 28 of AKe is replaced by Gln. Replacement of His36 by Gln, Asn, and Gly in AK1 (6) and that of Gln28 by His in AKe (7) demonstrated that His36 or Gln28 is nonessential for the catalytic activity. Dreusicke *et al.* (8) and Tian *et al.* (6) suggested that His36 contributes to the induced fit through interaction with Asp93. The distances of Gln28 (NE2) to Asp84 (OD1) in AKe (4.1 Å) and of His36 (N3) to (O1) in the porcine muscle AK1 (3.0 Å) are comparable (Fig. 2), although these

interactions are not required for catalytic activity (7).

It is likely that the remarkable decrease in V_{max} values observed in the several Gln101 mutant enzymes is caused by the structural perturbation. As shown in Fig.1, the position of Arg92 seems to be mainly affected by substitution of Gln101. Arg92 (Arg97) in the AK3-AMP complex is located at the position close to the side chain of Gln96 and tightly binds both the adenine ring and the α-phosphate of bound AMP through several hydrogen bonds (1). The replacement of Arg97 by Ala in the human muscle AK1 (9), that by Met in the chicken muscle AK1 (10), and that by Gly in the *E. coli* AKe (11) all reduced their V_{max} values to about 0.01-fold. Kim *et al.* (9) suggested that Arg97 contributes to the stabilization of the transition state due to its interaction with the α-phosphate of the bound AMP. Similarly, the Gln101-Leu and Gln101-Trp mutant enzymes showed very low V_{max} values (Chapter III). Therefore, it appears that one of the roles of Gln101 is to keep Arg97 at an effective location for the catalytic action.

Chapter IV showed that the mutation Thr39-Ala does not affect the kinetic parameters (12). However, the AK3-AMP complex structure reveals that the OG atom of Ser36 (Thr39) is hydrogen-bonded with the N7 atom of the adenine ring in a 2.92 Å distance (Fig. 2)(1). The kinetic properties of the Thr39-Ala mutant enzyme are inconsistent with the crystallographic results. As described in Chapter IV, it is suggested that Thr39 is located near the bound AMP, but without interacting with the nucleotide. However, based on the recent x-ray results (1), it can be considered as follows: In the N7 atom of the adenine ring, two more hydrogen bonds other than that with the OG atom of Ser39 are

also formed with the NH2 atom of Arg92 and the N atom of Gly37, although the latter dose not have good geometries for hydrogen bonding (1,13). When the hydrogen bond between the OG atom and the N7 atom is not formed, for example by replacement of Thr39 into Ala, the disappearance could be compensated by the other two hydrogen bonds. As shown in Chapter IV, at least Thr39 seems to be employed for providing a higher specificity for AMP to the AMP binding site by preventing the binding of CMP or UMP. The side chain of Ser36 is located at the position where the steric hindrance to the O4 atom of UMP or the N4 atom of CMP are significant (Figs. 1 and 2).

The results obtained in the present studies are compatible with the x-ray structure of the AK3-AMP complex (1). Fig. 3 shows the schematic drawings of the interaction between Thr39, Leu66, Val67 and Gln101 of AK1 and AMP predicted from the present studies and the X-ray studies of the AK3-AMP complex. The roles of the residues studied here are summarized as follows: Leu66 and Val67 interact the adenine ring through the hydrophobic interaction and are assumed to protect the adenine ring from the solvent. In particular, Val67 is also located close to the ribose and fixes its position. Gln101 recognizes the adenine ring through two hydrogen bonds and keeps the position of catalytically important Arg97. Thr39 also recognizes the adenine ring through a hydrogen bond, but this bond is not necessary for the binding of AMP. It appears that Thr39 is important for preventing the binding of pyrimidine nucleotides such as UMP and CMP.

By comparing the three-dimensional structures of the free AK1 (porcine muscle), the AKe-AP_5A complex, and the AK3-AMP

complex, Schulz *et al.* (14) and Diederichs (15) proposed an idea that remarkably large conformational changes occur during the reaction process of adenylate kinases (Fig. 4). Three domains of MAIN (residues 1-32, 69-123, and 163-215 in AK3), AMPbd (residues 33-68), and INSERT (residues 124-162) move in close each other to seal the active site "deep cleft" apparent in the free enzyme by binding of substrates. Their movements are estimated to be in distances of as large as 7~30 Å. However, the "trigger" for such large conformational changes is unclear at present. Nevertheless, the large conformational changes induced by substrate binding must be closely related with the allosteric cooperativity of substrates binding. Chapters II and III showed that several mutant enzymes, in which a residue is replaced by another residue with a lower hydrophobicity, possess negative cooperativities in substrates binding (4). Therefore, the hydrophobic interactions between the adenine ring of AMP and the hydrophobic region containing Leu66 and Val67 may be a "trigger" causing large conformational changes. At least in the early stage of substrate binding, the hydrophobic interaction may be an effective force, since hydrogen-bonding or charge interactions are weaker in hydrophilic environments than in hydrophobic environments. This view is also supported by the fact that the interaction between AK1 and the adenine ring of AMP is stronger than that between AK1 and the phosphate of AMP at the AMP-binding site (16).

Finally, the location of the AMP-binding site has been conclusively shown by the results of the present studies combined with those of other studies using x-ray crystallography (1,14,15, 17), NMR (7,12), and site-directed mutagenesis (4,10,18). On the

other hand, the location of the MgATP-binding site in the small variants of adenylate kinase has been proposed by site-directed mutagenesis studies of Yoneya *et al.* (19) and of Kim *et al.* (9) (Fig. 4). For the large variants AKy and AKe, recent X-ray crystallographic (17,20) and NMR (21,22) studies with the AKe-AP_5A and AKy-AP_5A complexes showed that the one site (site B) binding an adenine ring of AP_5A corresponds to the AMP-binding site and the other site (site A) is assumed to be the MgATP-binding site. This model is supported by the two facts that the binding energy of $MgAP_5A$ is comparable with the sum of those of AMP and MgATP (17) and that the GTP-binding site in the guanine nucleotide-binding domain of a *ras* protein p21 is located at a similar position to the proposed MgATP site in adenylate kinase (23). However, no crystals of small variants complexed with neither AP_5A or MgATP have been obtained. In addition, as compared with the AMP-binding site, only a few residues at site A interact with the adenine of the bound AP_5A, most of which are contained in the 30-residue insertion (2). Although the MgATP-binding site still remains unclear, this site is included probably in the MAIN domain (residues 5-32, 69-123, 163-218 in AK3) containing site A of AP_5A and the C-terminal region examined by Yoneya *et al.* (19).

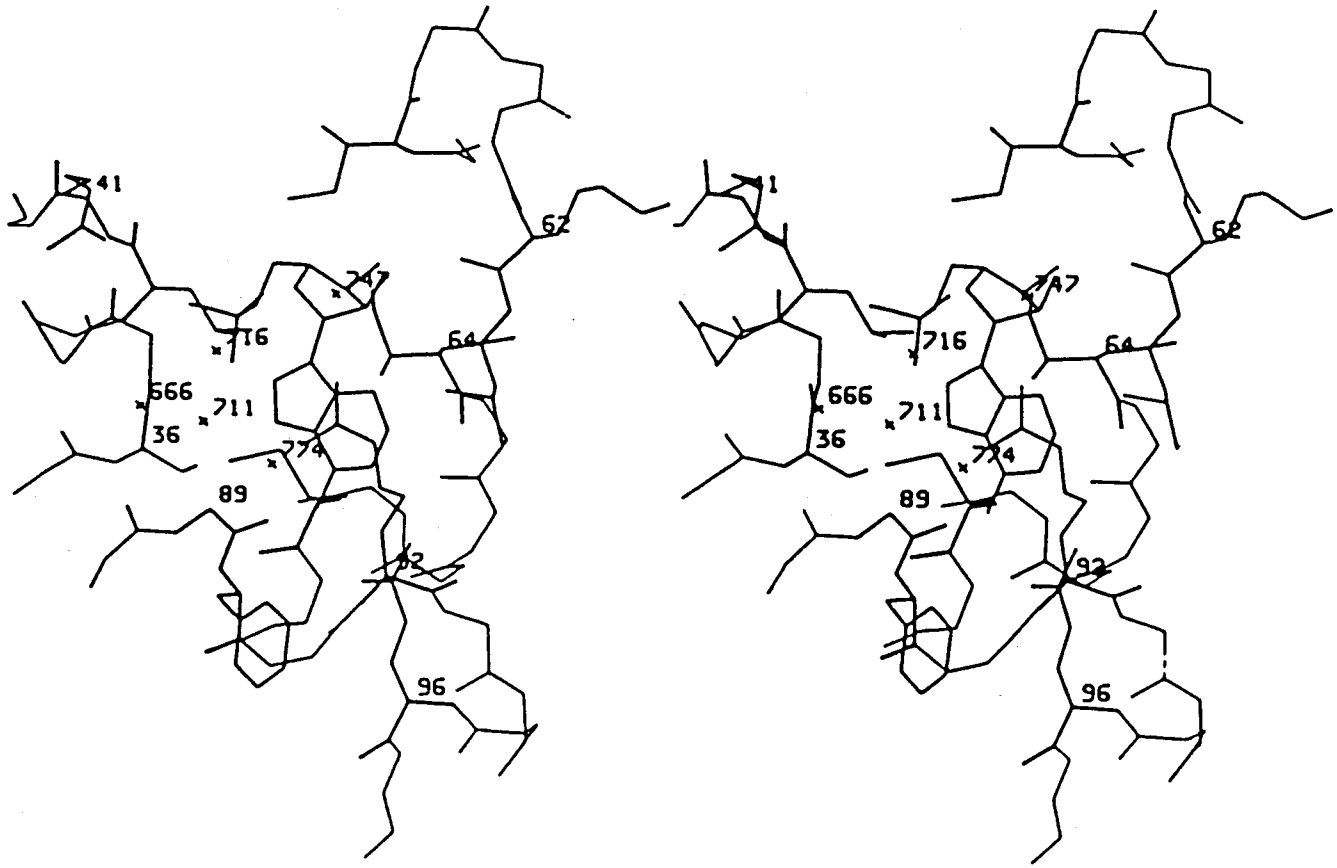
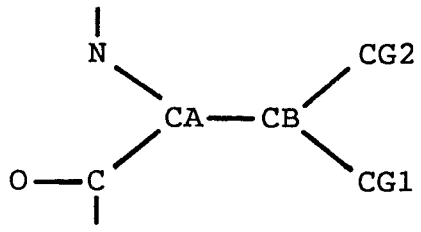


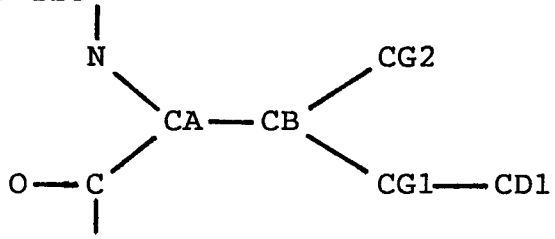
Fig. 1. **Stereoview of the environment of bound AMP in AK3-AMP complex (1).** All residues that have contacts with AMP are indicated, but the other residues are omitted. Several CA-atoms and bound water molecules are labeled.

A. Amino acid residues

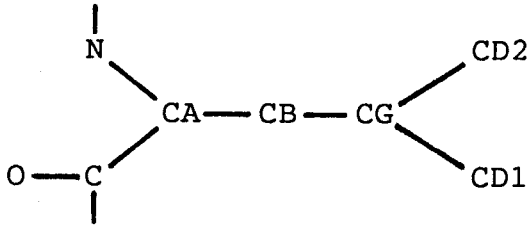
1. Val



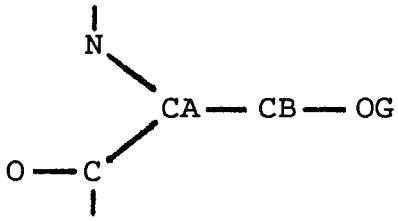
2. Ile



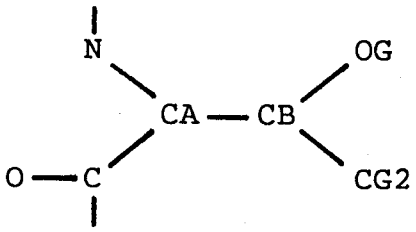
3. Leu



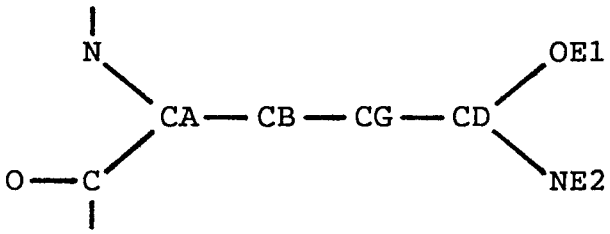
4. Ser



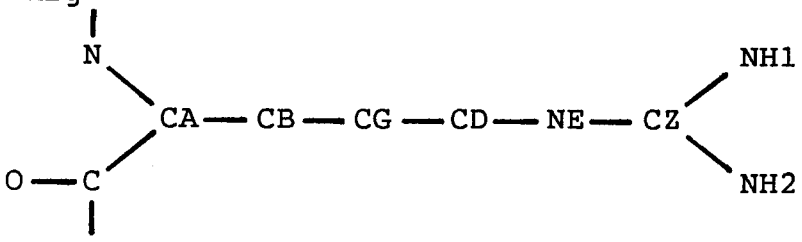
4. Thr



5. Gln



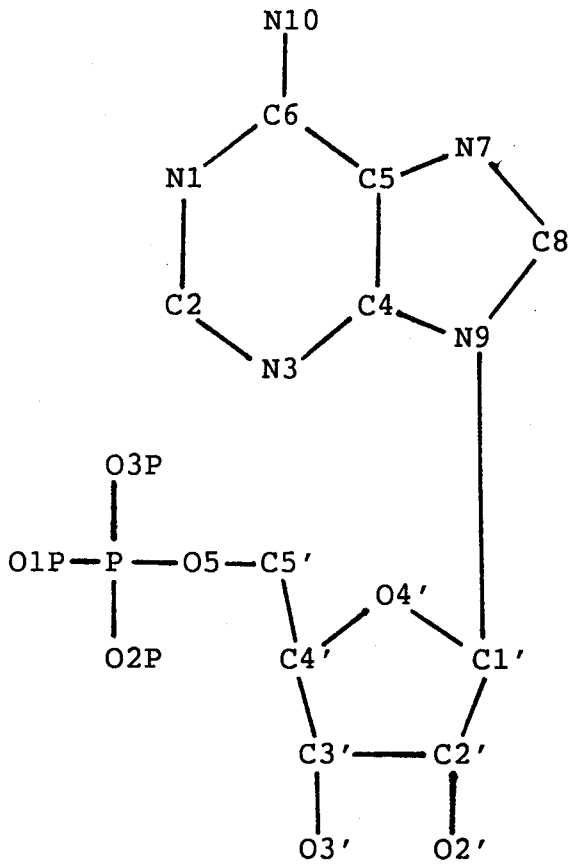
5. Arg



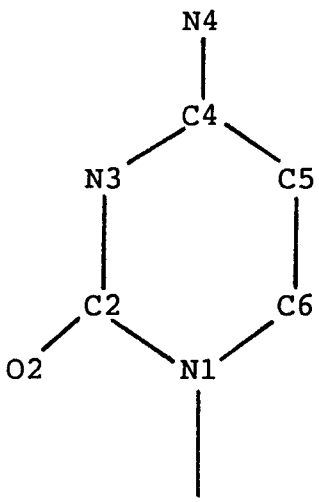
(Continued)

B. Nucleotides

1. AMP



2. CMP



3. UMP

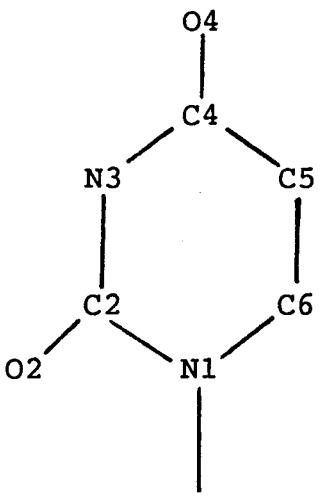


Fig. 2. Abbreviations of atoms in amino acid residues (*A*) and nucleotides (*B*).

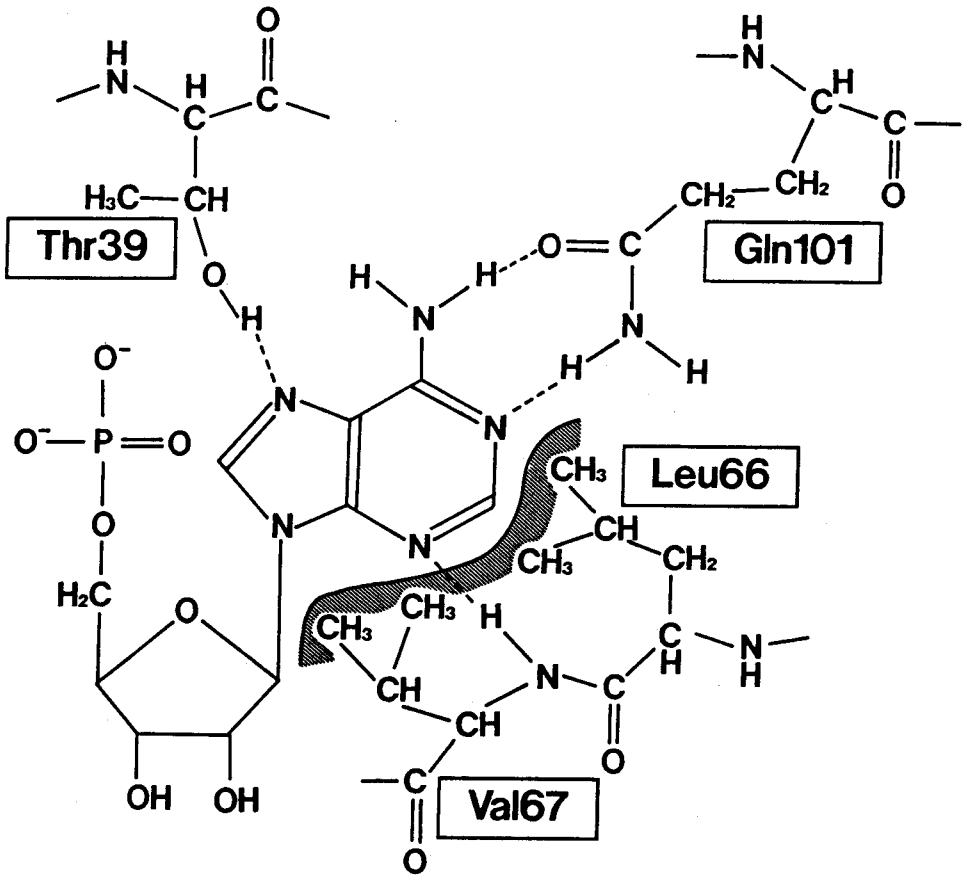


Fig. 3. **Schematic drawing of the predicted interaction between chicken muscle AK1 and AMP.** Broken lines indicate hydrogen bonds between the enzyme and AMP and shaded region corresponds to hydrophobic region contributing to hydrophobic interaction between them.

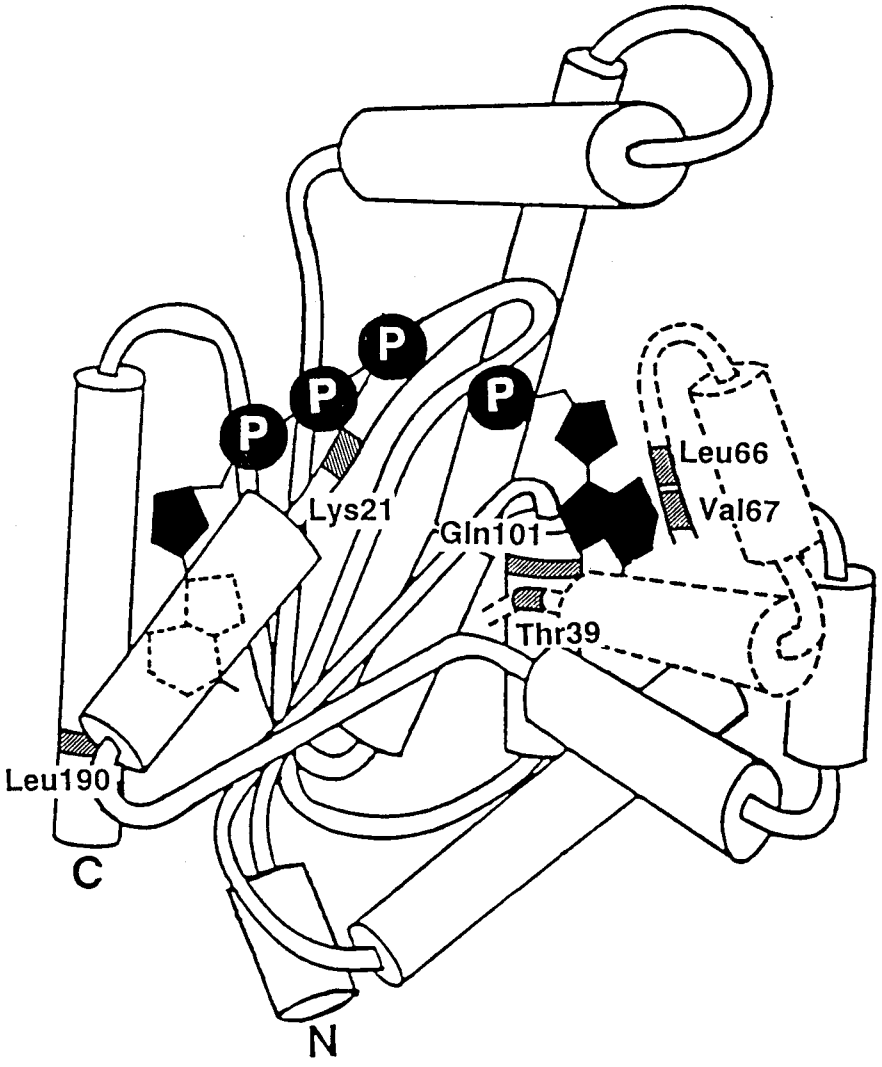


Fig. 4. **Schematic drawing of the proposed MgATP and AMP binding sites in adenylate kinase.** Large conformational changes predicted from X-ray crystallographic studies (1,14,15) are indicated by broken lines. Several important residues concerning the present studies are also indicated.

REFERENCES

1. Diederichs, K. and Schulz, G. E. (1991) *J. Mol. Biol* **217**, 541-549
2. Schulz, G. E. (1987) *Cold Spring Harbor Symp. Quant. Biol.* **52**, 429-439
3. Kauzmann, W. (1959) *Adv. Protein Chem.* **14**, 1-63
4. Okajima, T., Tanizawa, K., Yoneya, T., and Fukui, T. (1990) *J. Biol. Chem.* **18**, 1142-1147
5. Sugio, S., Amisaki, T., Ohishi, H., Tomioka, K., Heinenmann, U., and Saenger, W. (1985) *FEBS Lett.* **181**, 129-132
6. Tian, G., Sanders, C. R., Kishi, F., Nakazawa, A., and Tsai, M. -D. (1988) *Biochemistry* **27**, 5544-5552
7. Reinstein, J., Vetter, I., Schlichting, I., Rösch, P., wittinghofer, A., and Goody, R. S. (1990) *Biochemistry* **29**, 7440-7450
8. Dreusicke, D. and Schulz, G. E. (1988) *J. Mol. Biol.* **203**, 1021-1028
9. Kim, H., Nishikawa, S., Tokutomi, Y., Tanaka, H., Hamada, M., Kuby, S. A., and Uesugi, S. (1990) *Biochemistry* **29**, 1107-1111
10. Tsai, M.-D. and Yan, H. (1991) *Biochemistry* **30**, 6806-6818
11. Reinstein, J., Gilles, A. -M., Rose, T., Wittinbhofer, A., St. Girons, I., Bârzu, O., Surewicz, W. K., and Mantsch, H. (1989) *J. Biol. Chem.* **264**, 8107-8112
12. Yan, H., Dahnke, T., Zhou, B., Nakazawa, A., and Tsai, M.-D. (1991) *Biochemistry* **29**, 10956-10964

13. Baker, E. N., and Hubbard, R. E. (1984) *Prog. Biophys. Molec. Biol.* **44**, 97-179

14. Schulz, G. E., Müller, C. W., and Diederichs, K. (1990) *J. Mol. Biol.* **213**, 627-630

15. Diederichs, K., and Schulz, G. E. (1990) *Biochemistry* **29**, 8138-8144

16. Sanders, C. R., II, Tian, G., and Tsai, M. -D. (1989) *Biochemistry* **28**, 9028-9043

17. Egner, U., Tomasselli, A. G. and Schulz, G. E. (1987) *J. Mol. Biol.* **195**, 649-658

18. Liang, P., Phillips, G. N., Jr., and Glaser, M. (1991) *Proteins: Struc., Funct., Genet.* **9**, 28-36

19. Yoneya, T., Okajima, T., Tagaya, M., Tanizawa, K., and Fukui, T. (1990) *J. Biol. Chem.* **265**, 21488-21493

20. Muller, C. W. and Schulz, G. E. (1988) *J. Mol. Biol.* **202**, 909-912

21. Vetter, I. R., Reinstein, J., and Rosch, P. (1990) *Biochemistry* **29**, 7459-7467

22. Vetter, I. R., Konrad, M., and Rosch, P. (1991) *Biochemistry* **29**, 8138-8144

23. Pai, E. F., Kabsch, W., Krengel, U., Holmes, K. C., John, J., and Wittinghofer, A. (1989) *Nature* **341**, 209-214

SUMMARY

Role of leucine 66 in the asymmetric recognition of substrates

Adenylate kinase has two distinct binding sites for nucleotide substrates, MgATP and AMP. To identify the location of the site that specifically interacts with the adenine ring of AMP, Leu66 of the recombinant chicken muscle enzyme have been replaced by Ala, Gly, Val, Gln, and Trp by site-directed mutagenesis. All the purified Leu66 mutant enzymes exhibited an essentially identical circular dichroism spectrum and had thermal stabilities similar to the wild-type enzyme. Steady state kinetic analysis showed that the Leu66 mutant enzymes have significantly decreased V_{max} values and markedly large K_m values only for AMP. These results show that the binding site for the adenine ring of AMP in adenylate kinase is presumably located close to Leu66, which is invariant in all the enzymes so far sequenced. Significant inhibition of activities of the mutant enzymes and quenching of the Trp66 fluorescence by substrates suggest that, in some Leu66 mutant enzymes, MgATP also binds to the AMP-binding site. Thus, Leu66 of adenylate kinase might play a role in the asymmetric recognition of the adenine ring of AMP from that of MgATP. Furthermore, the hydrophobicity of the residue at position 66 appears to be important for the positive cooperativity of substrate binding.

Site-directed random mutagenesis of AMP-binding residues

The two highly conservative residues, Val67 and Gln101, in adenylate kinase are located in the hydrophobic region putatively involved in binding of the adenine ring of AMP. Using the recombinant chicken muscle enzyme cDNA as a template, random mutagenesis have been performed based on the DNA polymerase chain reaction (PCR) to replace them with various other amino acid residues. The synthetic oligonucleotide primers for PCR contained A,G,C,T-mixed bases in the corresponding codons for Val67 and Gln101. The amplified fragments were ligated with the expression plasmid pKK223-3, and the expressed mutant proteins were identified by immunoblotting using the antiserum against the wild-type enzyme. Enzymatically active mutant proteins were also screened by the growth at 45 °C of the temperature sensitive *Escherichia coli* mutant for adenylate kinase. The substituting amino acid residue was finally identified by DNA sequencing. It was found that at position 67 various other amino acid residues than Val are allowable to restore the growth at 45 °C of the temperature sensitive *E. coli* strain CV2. In contrast, only Gln, His, and Met could be the residue at position 101. These results are compatible with the ideas that Val67 contributes to the AMP binding through hydrophobic interactions and Gln101 by forming a hydrogen bond with the adenine ring. The Val67 and Gln101 mutant enzymes exhibited markedly high K_m values for AMP, but the K_m values for MgATP were comparable to those of the wild-type enzyme. Furthermore, Val67-Ala showed about 2.5-fold increase in a k_{cat}/K_m value for dAMP, and Gln101-His and Gln101-Met showed about 3-fold increase in the value for UMP, as compared to those of the wild-type enzyme.

A challenge to changing the substrate specificity for nucleoside monophosphate

Adenylate kinase shows 42% sequence homology with UMP/CMP kinase. Thr39, Leu66, and Gln101 in adenylate kinase, all of which are proposed to be located at the AMP-binding site, are replaced by Ala, Ile, and Asn, respectively, in UMP/CMP kinase. Based on this sequence comparison, changing the substrate specificity of adenylate kinase to a UMP/CMP specific one has been challenged by PCR-based introduction of single to triple mutations site-specifically. Two single mutants (Thr39-Ala and Leu66-Ile), one double mutant (Thr39-Ala/Leu66-Ile), and one triple mutant (Thr39-Ala/Leu66-Ile/Gln101-Asn) were prepared. The purified mutant enzymes other than the triple mutant exhibited CD spectra essentially identical with that of the wild-type enzyme, but the triple mutant enzyme showed a different spectrum, suggesting considerable conformational changes. Steady state kinetic analysis showed that Thr39-Ala had kinetic parameters for AMP comparable with those of the wild type. The double and triple mutant enzymes had markedly increased K_m values only for AMP and decreased V_{max} values. Thr39-Ala and Thr39-Ala/Leu66-Ile had 17- and 5-fold higher k_{cat}/K_m values for CMP than the wild type. The increases are ascribed to both the increase in V_{max} and the decrease in K_m. The V_{max} value for CMP of Thr39-Ala is similar to the reported value for UMP/CMP kinase. These results suggest that Thr39 is not directly involved in binding of AMP, but prevents the binding of CMP at the AMP-binding site. By engineering the residues around the AMP-binding site, the total conversion of substrate specificity of adenylate kinase to a UMP/CMP specific one would be hopeful without losing its high catalytic activity.

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List of Publications

(1) Papers Related to the Present Study:

Role of Leucine 66 in the Asymmetric Recognition of Substrates in Chicken Muscle Adenylate Kinase
Okajima, T., Tanizawa, K., Yoneya, T., and Fukui, T. (1991) *J. Biol. Chem.* **266**, 11442-11447

Site-Directed Random Mutagenesis of AMP-Binding Residues in Chicken Muscle Adenylate Kinase
Okajima, T., Tanizawa, K., and Fukui, T. (1992) *J. Biol. Chem.*, to be submitted.

(2) Other Published Papers:

The Role of Leu-190 in the Function and Stability of Adenylate Kinase
Yoneya, T., Okajima, T., Tagaya, M., Tanizawa, K., and Fukui, T. (1990) *J. Biol. Chem.* **265**, 21488-21493

Chemical Modification of Tryptophan Residues and Stability Changes in Proteins
Okajima, T., Kawata, Y., and Hamaguchi, K. (1990) *Biochemistry* **29**, 9168-9175