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Mutagenetic, stereochemical, and kinetic analyses of the structure and function of adenylate kinase

Tian, Gaochao, Ph.D.
The Ohio State University, 1989

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MUTAGENETIC, STEROEOCHEMICAL, AND KINETIC ANALYSES OF THE
STRUCTURE AND FUNCTION OF ADENYLA TE KINASE

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of the Ohio State University

By

Gaochao Tian, B. S.

* * * * *

The Ohio State University
1988

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Copyright
1989
Gaociao Tian
TO MY FATHER AND MOTHER
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ABBREVIATION AND SYMBOLS

Abbreviations and symbols used in this dissertation are given below:

ADP adenosine 5'-diphosphate
AK adenylate kinase
AMP adenosine 5'-monophosphate
Ap₅A P¹.P⁵-di(adenosine-5')pentaphosphate
ATP adenosine 5'-triphosphate
ATPase adenosine 5'-triphosphatase
ATPβS adenosine 5'-(2-thiotriphosphate)
BSA bovine serum albumin
CD circular dichroism
CTP cytidine 5'-triphosphate
dAMP 2'-deoxyadenosine 5'-monophosphate
dATP 2'-deoxyadenosine 5'-triphosphate
ddATP 2', 3'-dideoxyadenosine 5'-triphosphate
ddCTP 2', 3'-dideoxycytidine 5'-triphosphate
ddGTP 2', 3'-dideoxyguanosine.5'-triphosphate
ddTTP 2', 3'-dideoxythymidine 5'-triphosphate
dCTP 2'-deoxycytidine 5'-triphosphate
dCTPαS 2'-deoxycytidine 5'-O-(1-thiotriphosphate)
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<th>Full Form</th>
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<tbody>
<tr>
<td>dGTP</td>
<td>2'-deoxyguanosine 5'-triphosphate</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>dpm</td>
<td>disintegration per minute</td>
</tr>
<tr>
<td>DTE</td>
<td>dithioerythritol</td>
</tr>
<tr>
<td>DTNB</td>
<td>5,5'-dithiobis(2-nitrobenzoic acid)</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>dTTP</td>
<td>2'-deoxythymidine 5'-triphosphate</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetate</td>
</tr>
<tr>
<td>EM</td>
<td>energy minimization</td>
</tr>
<tr>
<td>EPR</td>
<td>electron paramagnetic resonance</td>
</tr>
<tr>
<td>GDH</td>
<td>glucose 6-phosphate dehydrogenase</td>
</tr>
<tr>
<td>Gdn-HCl</td>
<td>guanidine hydrochloride</td>
</tr>
<tr>
<td>GTP</td>
<td>guanosine 5'-triphosphate</td>
</tr>
<tr>
<td>HEPES</td>
<td>N-(2-hydroxyethyl) piperazine-N'-2-ethanesulfonic acid</td>
</tr>
<tr>
<td>HK</td>
<td>hexokinase</td>
</tr>
<tr>
<td>HPLC</td>
<td>high pressure liquid chromatography</td>
</tr>
<tr>
<td>IMP</td>
<td>inosine 5'-monophosphate</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropyl β-D-thiogalactopyranoside</td>
</tr>
<tr>
<td>ITP</td>
<td>inosine 5'-triphosphate</td>
</tr>
<tr>
<td>LDH</td>
<td>lactic dehydrogenase</td>
</tr>
<tr>
<td>MES</td>
<td>2-(N-morpholino)-ethanesulfonic acid</td>
</tr>
<tr>
<td>MOPS</td>
<td>3-(N-morpholino)propanesulfonic acid</td>
</tr>
<tr>
<td>NAD</td>
<td>β-nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NADH</td>
<td>β-nicotinamide adenine dinucleotide, reduced form</td>
</tr>
<tr>
<td>NADP</td>
<td>β-nicotinamide adenine dinucleotide phosphate</td>
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<tr>
<th>Acronym</th>
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<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>NOE</td>
<td>nuclear Overhauser effect</td>
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<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>pAP</td>
<td>porcine muscle adenylate kinase</td>
</tr>
<tr>
<td>PEG</td>
<td>polyethylene glycol</td>
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<tr>
<td>PEP</td>
<td>phosphoenolpyruvate</td>
</tr>
<tr>
<td>PK</td>
<td>pyruvate kinase</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>TEAB</td>
<td>triethylammonium bicarbonate</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N',N'-tetramethylethylenediamine</td>
</tr>
<tr>
<td>TLC</td>
<td>thin layer chromatography</td>
</tr>
<tr>
<td>TNB</td>
<td>thionitrobenzoic acid</td>
</tr>
<tr>
<td>Tris</td>
<td>2-amino-2-(hydroxymethyl)-1,3-propanediol</td>
</tr>
<tr>
<td>Trizma</td>
<td>tris(hydroxymethyl)aminoethane</td>
</tr>
<tr>
<td>TTP</td>
<td>thymidine 5'-triphosphate</td>
</tr>
<tr>
<td>2D</td>
<td>two-dimensional</td>
</tr>
<tr>
<td>UMP</td>
<td>uridine 5'-'monophosphate</td>
</tr>
<tr>
<td>UTP</td>
<td>uridine 5'-'triphasphate</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
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CHAPTER I

INTRODUCTION

During the past decade, genetic engineering has emerged as a powerful new experimental tool not only for molecular biologists but also for protein chemists (Perry & Weltzel, 1984; Hecht et al., 1986; Mitchinson & Baldwin; Matthews et al., 1987) and mechanistic enzymologists (Gerlt, 1987; Knowles, 1987; Shaw, 1987; Fersht, 1988). It provides easy means to synthesize de novo new proteins or enzymes, such that many questions in protein chemistry or enzymology can be answered by changing the amino acid residues in question. Construction of in vitro mutations can be achieved by introduction of insertion, deletion, or substitution (Shortle et al., 1981; Botstein & Shortle, 1985) into genes. Site-specific mutagenesis (oligonucleotide-directed base substitution), one of the many methods in genetic engineering, has been used mostly for mechanistic studies by enzymologists (Fersht et al., 1984; Ackers & Smith, 1985; Carter, 1986; Knowles, 1987). This dissertation describes the application of this technique and the use of other biochemical and physical methods, in the investigation of the mechanism of adenylate kinase, an extensively studied yet controversial enzyme. The work in this dissertation are divided into three subjects concerning the mechanism of adenylate
kinase, which are (a) testing current active site models, (b) identifying the rate-limiting step, and (c) answering how adenylate kinase deals with the inactive screw sense isomer of the substrate MgATP.

A. GENERAL BACKGROUND

The biochemical reaction of transfer of the terminal phosphate between adenine nucleotides in the presence of Mg(II):

\[
\text{MgATP} + \text{AMP} \rightleftharpoons \text{MgADP} + \text{ADP}
\]

plays a central role in maintaining in both cytosol and mitochondria the phosphate potential close to the value permitting optimal efficiency of oxidative phosphorylation (Stucki, 1980; Stucki et al., 1984; Veuthey & Stucki, 1987). This important reaction is catalyzed by adenylate kinase (AK) (Noda, 1973). The enzyme is ubiquitous, existing in a broad spectrum of organisms, from bacteria to mammals, and especially abundant in cells where the turnover of energy from adenine nucleotides is great (Noda, 1973).

In vertebrates, three isozymes (AK1, AK2, and AK3) have been characterized, each of which has distinct distribution in tissues and cellular compartments. AK1 exists in the cytosol of skeletal muscle, brain, and erythrocyte (Khoo & Russell, 1972; Noda, 1973), while AK2 is present in the mitochondrial intermembrane space of liver, kidney, spleen, and heart (Khoo, & Russell, 1972). AK3 is found in the mitochondrial matrix of liver and heart (Tomasselli et al., 1979). Among the adenylate kinase
family, AK1 and AK3 are loosely connected family members (Suminami et al., 1988), while AK2 (214 residues) resembles closely to yeast adenylate kinase (238 residues) and E. coli adenylate kinase (214 residues) and they are classified as a subgroup (Schulz et al., 1986). Compared to the subgroup of AK2, AK1 is smaller (194 residues) and it has a 30-residue gap in the middle of the amino acid sequence (Schulz et al., 1986). Based on the gene analysis of the AK isozymes, it has been proposed that the AK1 was evolved from AK2 by deletion of one or more exon(s) from the middle region of the AK2 gene or by alterations in the splicing positions of the AK2 gene (Suminami et al., 1988).

Among the three AK isozymes, AK1 is the most extensively studied one because of its easy availability and small size (Mr = 21,700 daltons). The kinetic (Rhoads & Lowenstein, 1968) and chemical mechanisms (Richard & Frey, 1978) have been well characterized for AK from rabbit muscle and porcine muscle, respectively, and its tertiary structure (porcine muscle AK) is available at 2.1 Å resolution (Dreusicke et al., 1988). Different substrate binding models have been proposed on the basis of X-ray crystallographic (Pai et al., 1977) and NMR (Mildvan & Fry, 1987) analyses of porcine muscle AK and by theoretical molecular mechanics calculations (Caldwell & Kollman, 1988). Recently, the gene of chicken muscle AK has been cloned (Kishi et al., 1986) and expressed in E. coli (Tanizawa et al., 1987). Thus, chicken muscle AK has been chosen to apply the method of site-specific mutagenesis in this dissertation work. Because AK from chicken, porcine, and rabbit muscles are ≥ 85% homologous (Kishi et al, 1986), previous and current results from these
systems are compared directly and, unless otherwise specified, the discussion throughout this dissertation will be confined to muscle AK.

B. REACTION MECHANISM OF ADENYLATE KINASE

A random Bi-Bi kinetic mechanism for the AK-catalyzed reaction has been established based on the experiments of isotope exchange at equilibrium (Rhoads & Lowenstein, 1968). Therefore, either substrate can bind to its binding site in the absence of binding of the other substrate at the other site. Once both substrates are bound, the phosphoryl transfer reaction can then take place in an associative fashion (Richard et al., 1978; Richard & Frey, 1978) (Figure 1). It has been proposed by Dunaway-Mariano and Cleland (1980b) that the pentacoordinate transition state is broken down first by fission of the O-P bond between β and γ-phosphate groups of MgATP (Figure 1), so that the immediate product is Mg(II)(H2O)4(ADP)2 in which Mg(II) is still coordinated with both ADP molecules.

The stereospecificity of adenylate kinase has been well defined based on the studies using the screw sense isomers of substitution-inert complex of Cr(III)(H2O)4ATP (Dunaway-Mariano & Cleland, 1980b) and Phosphorothioate analogues of ATP (Tomasselli & Noda, 1983). These studies have established that only the Δ screw sense isomer of bidentate Mg(II)ATP can react as substrate.

Although much of the focus now has moved onto the structure-function relationship, for example, to define the active site structure, many
Figure 1. Proposed Reaction mechanism of adenylate kinase. Mg(II) ion is coordinated with both of β and α phosphate groups of ATP at both ground and transition states.
problems concerning the kinetic and chemical mechanisms remain to be resolved. Resolution of these problems would not only enhance our understanding of the mechanisms but also facilitate the ongoing structure-function relationship studies. Two major problems of interest are as follows. First, although it is known that AK only uses the $\Delta$ screw sense isomer of $\beta,\gamma$-bidentate Mg(II)ATP, it is not clear how adenylate kinase deals with the inactive $\Delta$ screw sense isomer, which exists inevitably in solution or in cells. Specifically, it is not known whether the enzyme can bind the inactive isomer and if it does so whether it can allow the bound inactive isomer to undergo epimerization. To approach these problems, we have used purified screw sense isomers of $\alpha, \beta$-bidentate complex of Cr(III)(H$_2$O)$_4$ATP as stereospecific probes to show the affinities of AK towards the inactive and active isomers and the epimerization of the screw sense isomers in the absence and presence of AK. The results will be discussed in details in Chapter V.

Secondly, rather extensive qualitative and quantitative studies have been carried out previously to identify the rate-limiting step(s) of the AK reaction, but the results are controversial and no agreement has been reached. In attempting to solve this problem, we have chosen the viscosity variation method, which has not been used for such purpose on AK before, to test whether the reaction is diffusion-controlled, or to what extent the reaction is diffusion-controlled. In the viscosity variation studies, which will be discussed in depth in Chapter IV, we found that the rate of a conformational change may partially account for the overall reaction rate.
C. ENZYM-E-SUBSTRATE INTERACTIONS

Adenylate kinase has two substrate binding sites based on extensive kinetic (Noda, 1973; Rhoads & Lowenstein, 1968; Hanada & Kuby, 1978), binding (Hanada et al., 1979), and NMR studies (Nageswara Rao & Cohn, 1978; Nageswara Rao et al, 1984). One of the substrate binding sites has been called "MgATP site" and the other "AMP site". The "MgATP site" can bind MgATP and MgADP as functional substrates. It can also bind ATP and ADP in the absence of Mg(II) ion, although a divalent metal ion is obligatory for the reaction (Noda, 1973).

The specificity of MgATP binding site is moderate, since it can also bind substrate analogues, such as 2'-dATP, 3'-dATP, GTP, ITP, UTP, and CTP, with comparably high affinity and retain substantial catalytic activity on these analogues (Callaghan & Weber, 1959; Markland & Wadkins, 1966; O'Sullivan & Noda, 1968; Criss et al., 1970; Sapico et al., 1972; Noda, 1973). In contrast, the specificity of AMP site, which binds ADP or AMP as functional substrates, is far more rigorous; the substitution of AMP by dAMP, or another monophosphate nucleotide leads to a greater decrease in $k_{cat}/K_m$ than a corresponding substitution by dATP or another triphosphate nucleotide for ATP (Su & Russell, 1967; Secrist et al., 1972). Specifically, ITP can serve as a substrate in the presence of AMP, while IMP with ATP gives no reaction (Secrist et al., 1972).

Although previous studies as discussed above have indicated that AK has an "MgATP site" of moderate specificity and an "AMP site" of rigorous specificity, the location of the two binding sites in the enzyme remains being a mystery even after about two decades of intense investigations. A
variety of methods or techniques including sophisticated ones such as X-ray crystallography, NMR, and molecular mechanics calculation have been utilized and a wealth of information about the active site structure has been produced. On the basis of these studies, three models of the substrate binding sites have been proposed. Unfortunately, the conclusions are rather contradictory.

The X-ray crystallographic structure of adenylate kinase was obtained in 1974 by Schulz and co-workers (Schulz et al., 1974) on porcine muscle enzyme at 3 Å resolution in crystal form A at pH 7.7 (Figure 2A) and has been refined to a resolution of 2.1 Å (Figure 2B) (Dreusicke et al., 1988). A second conformation of the same enzyme was solved at 4.7 Å resolution in crystal form B at pH 5.8 and a induced-fit mechanism was thus proposed based on the observations of the two conformations (Sachsenheimer & Schulz, 1977). On the basis of comparative studies (Frank et al., 1984), a rough active center of AK was proposed as shown in Figure 3. Schulz and co-workers (Pai et al., 1977) were also able to conduct substrate and substrate analogue binding studies and the first model (the X-ray model) of the two binding sites was proposed (see Chapter III for more details). Based on this active site model, AMP and ATP bind with their terminal phosphate being positioned in proximity to His-36. Recently, the crystal structure of yeast adenylate kinase has also been solved and its complex of the inhibitor Ap5A has been investigated at 2.6 Å resolution (Egner et al., 1987). Because of the structural homology between the yeast and porcine AK, the results from the studies on the yeast enzyme were used to reinforce their previous
Figure 2. Stereo view of the backbone of porcine muscle adenylate kinase at 3 Å resolution (A) and 2.1 Å resolution (B).
Figure 3. A sketch representation of porcine muscle AK structure using cylinders for helices and arrows for β-sheets taken from Frank et al. (1984). Shaded areas indicate the regions of high homology between all AK's, which are believed to constitute the substrate binding sites.
conclusions on porcine enzyme with certain revisions. This X-ray model has however been challenged by Mildvan and collaborators (Smith & Mildvan, 1982; Rosevear et al., 1983; Fry et al., 1985; 1986a; 1986b; 1987; Mildvan & Fry, 1987) using NMR techniques such as paramagnetic relaxation (Smith & Mildvan, 1982; Rosevear et al., 1983) and NOE (Fry et al., 1985; 1986a; 1986b; 1987). The second model (the NMR model) of the substrate binding sites has thus emerged based on these NMR studies (Mildvan & Fry, 1987). In this NMR model, His-36 is not located near the phosphate of AMP or ATP. Rather it lies at the bottom of a "hydrophobic pocket" which functions to bind the adenosine moiety of ATP (more details will be given in Chapter III). Recently, a third model (the molecular mechanics model) has been proposed based on a theoretical study by using molecular mechanics calculations (Caldwell & Kollman, 1988). This model resembles the X-ray model in the phosphate binding region while it also accommodates some of the NMR results. It is still under debate which of these models represents the true situation or is closer to the reality.

The rich information and well defined problems on this enzyme make it a good basis for further investigations by using the method of site-specific mutagenesis, which has recently been demonstrated to be useful in the investigation of the relationship between structure and function (Knowles, 1987; Fersht, 1988).

As the first step in a long term of defining the active sites by use of the method of site-specific mutagenesis, we have focused on only three residues, His-36, Lys-21, and Lys-27, which have previously been suggested as important catalytic groups (Noda, 1973; Mildvan & Fry, 1987). Our results (reported in Chapter III) have modified the old view of
the suggested roles of these residues and generated new insights into the structure and function of AK. However, the results cannot yet allow proposal of a new model.
CHAPTER II

GENERAL EXPERIMENTAL PROCEDURES

A. MATERIALS

The oligonucleotides directing the mutations of His-36 to Gln and to Gly, d(TGGGTACACACACGCTCTCTCCACT) (the mismatched bases are indicated by an underscore) and d(TGGGTACACTGGCCTCTCCACT), respectively, were purchased from SYN-TEK and used without further purification. The oligonucleotide directing the mutation of His-36 to Asn, d(GGGTACACTAACCTCT), and the oligonucleotide directing the mutation of Lys-27 to Met, d(ATGCGAGATGATTGTGC), were synthesized by using 381A DNA synthesizer (Applied Biosystems) and purified by using 10% PAGE. The oligonucleotide directing the mutation of Lys-21 to Met, d(CTCAGGGATGGGGACGC), was purchased from Biochemical Instrument Center of the Ohio State University, and used without further purification. Recombinant plasmid pKK-cAK1-2, containing the coding sequence for chicken muscle adenylate kinase, was available from previous work (Tanizawa, et al., 1987). The reagents and enzymes for mutagenesis
and [α-35S] dCTP for sequencing were purchased from Amersham. The Geneclean used for isolation of DNA fragments was purchased from Bio 101.

Polyethylene glycol 20,000 was the product of Fluka Chemie AG. Phosphocellulose P-11 and Sephadex G-100 were obtained from Whatman and Sigma, respectively.

The reagents and enzymes (pyruvate kinase, lactic dehydrogenase, hexokinase, and glucose-6-phosphate dehydrogenase) for kinetic analysis of AK were purchased from Sigma. Sucrose used for viscosity variation studies and DTNB for chemical modification of AK were obtained from Aldrich. Ultrapure guanidine used for protein denaturation studies was purchased from Sigma and used without further purification. [2-3H] ATP used for isotope trapping experiments was the product of Amersham.

CrCl3 used for the synthesis of the inert-substitution complexes of CrATP was the product of Aldrich. HPLC columns were purchased from Beckman. HPLC-grade methanol was purchased from Fischer.

All other chemicals were of reagent grade.

B. BIOLOGICAL METHODS

Since in this dissertation a rather large number of buffers, solutions, and media are involved, particularly in the molecular biology experiments, the concentrations of most of the buffers, solutions, and media used for experiments of molecular biology, and some of the buffers and reagents used for enzyme purification and kinetic experiments are not described in the text, but rather, they are given in the Appendix.
1. SITE-SPECIFIC MUTAGENESIS

Preparation of Competent Cells. The procedure described by Kushner (1978) was followed with minor modifications. A single colony from a glucose-minimal plate culture of *E. coli* (TG1 for mutagenesis, TG1 or JM109 for DNA sequencing, and JM103 for expression) was picked up and cultured overnight in 3 ml of Luria broth (L-broth) at 37 °C. 1 ml of this culture was then inoculated into 200 ml of SOB medium. The culture was allowed to proceed gently at 37 °C and stopped when OD$_{550}$ reached 0.6 - 0.7 (about four to five hours). After being chilled on ice for about 10 minutes, the cells were collected by centrifugation and suspended in 70 ml of ice-cold TFBI buffer. After being incubated at 0 °C for 10 minutes, the cells in TFBI buffer were re-pelleted by centrifugation and then suspended in 16 ml of ice-cold TFBII buffer. The suspension was divided within 15 minutes into 1.5 ml microcentrifuge tubes (0.2 ml each), and frozen immediately in dry ice-ethanol bath (about -70 °C). The frozen competent cells were kept below -65 °C. At this temperature, the cells can be stored for several months without losing competency significantly.

Preparation of Oligonucleotides Directing Mutations. The synthetic oligonucleotides used in this dissertation work were from three sources. Those purchased from SYN-TEK were used successfully without further purification. Those from Biochemical Instrument Center of the Ohio State University were also used successfully without further purification but Sephadex G-25 gel filtration was carried out for desalting. The oligonucleotides synthesized with 381A DNA synthesizer (Applied Biosystems) were further purified by using 10% PAGE after being
desalted.

The oligonucleotides purchased from Biochemical Instrument Center or synthesized with 381A DNA synthesizer were carried in ammonium hydroxide (37%). After being incubated at 55 °C in a tightly sealed small screw capped vial for 12 hours to deblock, an oligonucleotide sample was transferred to microcentrifuge tubes and dried down in a Savant Speed Vac Condenser. Then the dried oligonucleotide was suspended in 0.01M TEAB buffer (pH 7.0 - 7.5) and loaded onto a Sephadex G-25 column (1X15 cm, pre-equilibrated with the TEAB buffer). Fractions were collected in 1.5 microcentrifuge tubes and those containing the oligonucleotide were located by measuring OD_{260}, and combined and dried in the Speed Vac. The salt-free oligonucleotide was then dissolved in distilled H_{2}O and the concentration was determined spectrophotometrically by using ε_{260} = 0.05 (μg/ml)^{-1} cm^{-1} (Maniatis et al., 1982). The oligonucleotides were stored at -20 °C before usage. The 10% PAGE procedure for further purification will be described in a later section.

Preparation of Single Stranded DNA of M13mp19-cAK1-2. A single TG1 colony was picked up from a glucose/minimal medium plate and grown overnight at 37 °C with shaking in 3 ml of 2XTY Medium. One drop of this culture was added to 20 ml of fresh 2XTY Medium, and shaken at 37 °C for 3 hours. 0.1 ml of this fresh culture was then placed in a culture tube, to which 1 ml of 2XTY Medium was added and a single phage plaque was inoculated. This medium was then shaken at 37 °C for 4 hours. The phage supernatant was collected after the cells were pelleted, and it was then inoculated into 100 ml of 2XTY Medium containing 1 ml of an overnight TG1 culture. The inoculated culture was shaken at 37 °C for 5
hours and the cells were removed by centrifugation. To the supernatant 0.2 volume of PEG/NaCl solution was added and the mixture was kept at 4 °C for 1 hour. The phage particles were then pelleted by centrifugation and dissolved in 0.5 ml of TE buffer. To the phage solution 0.5 volume of TE-saturated phenol was added. After being vortexed for 2 minutes, the extraction mixture was allowed to stand for ca. 10 minutes, followed by repeating the vortexing and standing procedure one more time. The aqueous layer from the phenol extraction was placed in a new microcentrifuge tube and extracted with diethyl ether three times and with chloroform twice. Finally, ethanol precipitation (see below) was performed to obtain pure and concentrated single stranded DNA molecules.

Concentration by Ethanol Precipitation. The most widely used method for concentrating DNA is precipitation with ethanol (Maniatis et al., 1982). The precipitated DNA, which forms at low temperature (-20 °C or less) in the presence of moderate concentrations of monovalent cations, can be recovered by centrifugation and redissolved in an appropriate buffer at desired concentration. The technique is rapid and is quantitative.

To the aqueous layer after the phenol extraction, 0.1 volume of 3 M NaOAc (pH 5.0) and 2.5 volumes of ethanol were added, and the contents were mixed well and stored at -20 °C overnight to allow the precipitation to form. Then the mixture was centrifuged to precipitate the DNA molecules (15 minutes in a Eppendorf centrifuge at 12,000g is sufficient). The supernatant was discarded and the pellet was washed with ca. 1 ml of cold (-20 °C) 75% ethanol once. After being dried, the pellet was dissolved in TE buffer or water and stored at -20 °C for later use. The concentration was determined spectrophotometrically by using $\varepsilon_{260} = 0.025 \, (\mu g/ml)^{-1}$.
In vitro Mutagenesis (Eckstein's Method). There are several methods available for making a mutant. The one used in this work was based on Eckstein's method (Taylor et al., 1985a; 1985b) which is of the highest efficiency (usually $\geq 90\%$) among the currently used methods, and it will be described briefly below. Detailed procedure is given by the instruction booklet of Amersham.

First of all, the salt-free oligonucleotide (see Preparation of Oligonucleotides Directing Mutations) was phosphorylated at its 5'-end. This was achieved by using T4 polynucleotide kinase in the presence of ATP. The phosphorylated oligonucleotide was then mixed with the single stranded template DNA (see Preparation of Single Stranded DNA of M13mp19-cAK1-2) and they were annealed together in a 70 °C water bath for 3 min, followed by incubating at 37 °C for 30 min and then on ice. The mutant strand was synthesized with Klenow fragment in the presence of a nucleotide mixture containing dCTP$\alpha$S (dATP, dGTP, dTTP, and dCTP$\alpha$S). After the polymerization, the free ends of the mutant strand were joined together by T4 DNA ligase (Note that since dCTP$\alpha$S was used in place of dCTP, whenever a C residue appeared in the mutant strand, its 5' side phosphate diester would be sulphur-substituted). Then the reaction mixture was filtrated through a nitrocellulose filter, which effectively removed single strand DNA molecules. To the heteroduplex DNA molecules which had survived the filtration, NcoI was added. Since NcoI is a restriction enzyme which can not digest DNA strands which contain sulphur-substituted C residues in the recognition site, it only introduces nicks in the WT strand. By taking the advantage of such nicks, the WT
strand was digested away with Exonuclease III. After the digestion was estimated to have proceeded over the mutation site, the WT strand was repolymerized (this time no dCTPαS was added) and religated to give a homoduplex mutant DNA. Then a mutant recombinant phage was selected directly by DNA sequencing (see below), and the mutant gene fragment was cut out and subcloned (see Subcloning) into the vector pKK 223-2 for expression. The entire procedure for the in vitro mutagenesis (His-36 to Asn as an example) was summarized in Figure 4.

2. DNA SEQUENCING

Preparation of Single Stranded Mutant Recombinant M13 DNA. Prepare an overnight culture of TG1 (or JM109) cell culture (3 ml in 2XTY medium). To 20 ml of 2XTY medium add 0.2 ml of this culture. Divide this mixture in 1.5 ml aliquots into culture tubes. Inoculate each of the tubes with a mutant M13mp19 plaque. Shake at 37 °C for 5.5 to 6 hours. Transfer each of the 1.5 ml cultures to a microcentrifuge tube and spin it at 15000 RPM in a microcentrifuge for 5 min. Transfer the supernatant to a fresh microcentrifuge tube containing 0.25 ml of PEG/NaCl solution. Mix by vortexing, and stand at 4 °C for 1 hour. Centrifuge for 5 min and aspirate the supernatant away. Centrifuge for 3 min and aspirate again to remove the residual PEG/NaCl solution. To the pellet add 0.1 ml of TE buffer. Vortex to dissolve the pellet. Add 50 ml of TE-saturated phenol solution and vortex for 2 minutes. Stand for 15 min and vortex for 2 minutes again. Stand for 5 minutes. Centrifuge for 3 minutes and transfer the top layer to a fresh microcentrifuge tube. Add 10
Figure 4. Scheme of site-specific mutagenesis for chicken adenylate kinase. The mutation from His to Asn at position 36 is used as an example. Pt or Ptac stands for tac promoter, Amp' for ampicillin resistant gene, ds for double strand, and ss for single strand. Detailed experimental procedures are described in the text.
Figure 4
Figure 4 (continued)

- Extension in the presence of dATP, dTTP, dGTP, and dCTP, and ligation with T4 ligase
- 

- After identifying the mutant, digest with EcoRI and HindIII

- Transformation of E.coli JM103
μl of 3 M NaOAc (pH 5.0) and 250 μl of pure ethanol. Put in a freezer (-20 °C) overnight. Centrifuge at 4 °C for 5 minutes and aspirate to remove the supernatant. Wash the pellet with cold 75% ethanol (-20 °C) and leave to dry (or dry by applying vacuum). Dissolve in 15 μl of water and store at -20 °C. The concentration of the preparation is usually ≥ 1 μg/μl

**Sequencing Reactions.** Sequencing reactions using Sequenase and [α-35S] dATP was carried out according to the protocol provided by United States Biochemical Corporation, which is derived from Sanger's chain terminating method (Sanger et al, 1977). First, 7 μl of the above prepared DNA template was mixed in a microcentrifuge tube with 1 μl of the universal primer or MIDPRIM1 (for sequencing the second half of the gene) and 2 μl of the Sequencing Buffer (SB). The primer and the template were then annealed together by heating the mixture for 2 minutes in a heat block (Vanlab) at 65 °C, followed by leaving the tube in the heat block with the power being turned off until the temperature dropped below 35 °C (ca. 30 minutes were required). To the annealed mixture, 1 μl of 0.1 M DTT, 2 μl of each of Labeling Mixture (LM), [α-35S] dATP, and diluted sequenase solution (?units) were added, and the mixture was allowed to stand at room temperature (24 °C) for ca. 10 minutes. Meanwhile, to each of four 0.5 ml microcentrifuge tubes labeled G, A, T, C was added 2.5 μl of each of the terminating solutions of ddGTP, ddATP, ddTTP, and ddCTP, respectively. Then 3.5 μl of the labeling reaction solution, which had been allowed to stand at room temperature for 10 minutes, was added to each of the four

---

1 The MIDPRIM is an oligonucleotide of 17 nucleotides, the sequence of which is 5'-CACCTCCAAGGGTTTCC-3' which is complementary to that of the cAK gene from position 268-284.
tubes labeled G, A, T, and C to start the termination reactions. After being incubated at 37 °C, the termination reactions were stopped by adding to each of the four tubes 4 μl of the stop solution containing 95% formamide, 20 mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol FF. The terminated reaction mixtures were either stored frozen at -20 °C or loaded on the pre-prepared polyacrylamide sequencing gel. After the electrophoresis was finished, the gel was dried in a gel drier under vacuum, and then autoradiography was carried out by tightly lining a X-ray film onto the top face of the dried sequencing gel. After ca. one or two days (the length of exposing time depending on the age of the isotope used) of exposing, the film was developed and fixed, and the mutation site was identified directly by reading the gel ladders. An example of the sequencing result is shown in Figure 5.

3. SUBCLONING

Preparation of Double Stranded (RF) M13mp19-cAK1-mutant Gene. A fresh phage plate was made by transfection of the mutant phage into TG1 cells (see Transfection). A single plaque was picked up and inoculated into 1.5 ml of TG1 / 2XTY medium (1% of TG1). After being shaken for 6 hours, the cells were removed by centrifugation, and the supernatant was inoculated into 20 ml of TG1 / 2XTY medium. The culture was allowed to proceed for 15 hours and then the cells were harvested by centrifugation. Lysis by alkali (Birnboim & Doly, 1979) was conducted to obtain the RF form of the recombinant M13 DNA, which will be described.
PLEASE NOTE:

Page(s) not included with original material and unavailable from author or university. Filmed as received.
here briefly. The cell pellet was resuspended in 0.4 ml of Lysis Solution I (LSI) and transferred to a 2.0 ml microcentrifuge tube. After being allowed to stand at room temperature for 5 minutes, 0.8 ml of LSII was added, and the mixture was incubated in an ice bath for 10 minutes. 0.6 ml of an ice-cold solution of 5 M KOAc (pH 4.8) was then added to the tube. After being mixed by inverting the tube sharply several times, the contents were incubated for 10 minutes at room temperature. The cell DNA and bacterial debris were removed by centrifugation on a microcentrifuge at 15,000 RPM for 30 minutes. The supernatant (ca. 0.7 ml) was transferred to a fresh 2.0 ml microcentrifuge tube, and then 0.6 volume (ca. 0.4 ml) of isopropanol was added. The contents were mixed well and allowed to stand at room temperature for 15 minutes. The DNA was recovered by centrifugation on the microcentrifuge at 15,000 RPM for 30 minutes at room temperature. The pellet was washed with 70% ethanol at room temperature and redissolved in 0.15 ml of TE buffer. Contaminative RNA molecules were removed by RNase digestion and the pure DNA was then obtained by phenol extraction, followed by ethanol precipitation twice.

Subcloning into pKK-223-3 Expression Vector. The mutant gene fragment was cut out with EcoRI and HindIII from the recombinant M13 RF DNA and ligated into pKK-223-3 which had been digested with the same restriction enzymes and 5'-dephosphorylated, as described below.

First, the recombinant M13 RF fragment and pKK-223-3 were both digested with EcoRI and HindIII according to the following procedure. In a microcentrifuge tube the components in the amounts as indicated in Table 1 were mixed. Then the mixtures were incubated in a 37 °C incubator for 6 to Table 1. Compositions of the digest reaction mixtures for digesting pKK-
Table 1. Compositions of the digest reaction mixtures for digesting pKK-223-3 and a recombinant M13mp19 RF DNA.

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>pKK-223-3 (5 μg/μl)^a</td>
<td>4 μl</td>
</tr>
<tr>
<td>RF mutant M13 (1 μg/μl)^a</td>
<td>20 μl</td>
</tr>
<tr>
<td>HindIII (20 units/μl)^a</td>
<td>1 μl</td>
</tr>
<tr>
<td>HindIII (20 units/μl)^a</td>
<td>1 μl</td>
</tr>
<tr>
<td>EcoRI (20 units/μl)^a</td>
<td>1 μl</td>
</tr>
<tr>
<td>EcoRI (20 units/μl)^a</td>
<td>1 μl</td>
</tr>
<tr>
<td>5X High salt buffer</td>
<td>20 μl</td>
</tr>
<tr>
<td>5X High salt buffer</td>
<td>20 μl</td>
</tr>
<tr>
<td>H2O</td>
<td>74 μl</td>
</tr>
<tr>
<td>H2O</td>
<td>58 μl</td>
</tr>
<tr>
<td>Total</td>
<td>100 μl</td>
</tr>
</tbody>
</table>

^a The concentration in the parenthesis was that of the stock solution.

Table 2. Composition of the ligation mixture.

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>RF mutant gene fragment (0.03 pmol/μl)</td>
<td>2 μl</td>
</tr>
<tr>
<td>Larger fragment of pKK-223-3 (0.02 pmol/μl)</td>
<td>1 μl</td>
</tr>
<tr>
<td>5X Ligase buffer</td>
<td>4 μl</td>
</tr>
<tr>
<td>H2O</td>
<td>12 μl</td>
</tr>
<tr>
<td>T4 ligase (350 units/μl)</td>
<td>1 μl</td>
</tr>
</tbody>
</table>
7 hours. The larger fragment of pKK-223-3 and the smaller fragment of the mutant M13mp19 were isolated by using Gene-Clean (see MATERIALS) after separation by an agarose (1%) gel electrophoresis. Then the mutant gene fragment was mixed with the larger fragment of pKK-223-3 in the presence of T4 ligase according to Table 2, and the ligation reaction was allowed to proceed either at 4 °C overnight or at 37 °C for 3 hours.

4. TRANSFECTION AND TRANSFORMATION

**Transfection.** 0.2 ml of stored frozen competent cells were thawed on ice and then incubated on ice for 15 minutes. To the thawed competent cells, 1 μg of the phage DNA was added. The mixture was then allowed to be further incubated on ice for 30 to 40 minutes before being transferred to a glass tube. The cells were then heat-shocked in the glass tube at 42 °C for 90 seconds and placed on ice again for 5 minutes. Then to the cells were added 40 μl of 100 mM IPTG, 40 μl of 2% x-gal solution (in dimethylformamide), and 200 μl of a log phase E. coli cell culture (6-7 hours of a liquid culture would be sufficient) of the same type of cell line as that of the competent cells. Then 3 ml of molten H-top agar was added into the glass tube and the contents was immediately poured onto a pre-warmed (37 °C) H-plate. Once the agar solidified, it was placed in a 37 °C incubator overnight for phage plaques to form.

**Transformation.** The procedure for transformation of an E. coli strain with a plasmid was the same as that for transfection except for that after the heat shock, adding IPTG, x-gal, and log phase cells was omitted.
but instead, 1 ml of L-broth was added and the mixture was shaken gently at 37 °C for ca. 60 minutes. In addition, L-broth agar plate containing 0.1 ampicillin instead of H-plate was used for the final plating-out.

C. BIOCHEMICAL METHODS

1. ENZYME PURIFICATION

Expression in *E. coli* and Preparation of Cell-free Extract. The WT and mutant AK proteins were purified according to the procedure described previously (Tanizawa et al., 1987) with minor modifications. First, a single colony of *E. coli* JM103 harboring a WT or a mutant recombinant plasmid was obtained by plating-out from a stab storage of the strain on glucose/minimum plate containing 0.1 mg/ml of ampicillin. Then this single colony was used to make a 200 ml L-broth culture containing 0.1 mg/ml of ampicillin (overnight). This culture was in turn used to inoculate 12X 1 liter of the L-broth medium (containing 0.1 mg/ml of ampicillin). After shaking vigorously for 1 hour at 37 °C, 1 mM IPTG was added and shaking was allowed to proceed for 8 more hours (the absorbance at 600 nm reached 2 to 3). The cells were harvested by centrifugation at 8,000g for 10 minutes, and resuspended in a Suspension Buffer (SB) and centrifuged again to collect the cells. After being resuspended in about 200 ml of a Sonication Buffer (SNB), the cells were broken by sonication 6 times at about 100 watts (sonic Dismembrator, Model 300), 30 seconds for each time with about 30 seconds after each sonication. The temperature was controlled by an ice bath and kept below
12 °C. The cell debris were removed by centrifugation at 25,000g for 30 minutes. The supernatant containing the enzyme was either stored at -70 °C (after dropping directly into liquid nitrogen to freeze it) or loaded directly onto a phosphocellulose P-11 column (see the next section).

**Phosphocellulose P-11 Cation Exchange Chromatography.**

The precycling of phosphocellulose P-11 resins was carried out according to the following procedure. 70 grams of the dry resins of phosphocellulose were suspended in 1,750 ml of 0.5 N NaOH and allowed to stand for 5 min (but not more than 5 min). Then the resins were washed with water at 4 °C extensively until pH drops to < 10. Then the resins were suspended in 1,750 ml of 0.5 N HCl, allowed to stand for 5 min, and washed at 4 °C until pH increased to > 3. The resins were transferred to 1,400 ml of 0.15 M Tris-HCl (pH 7.5) and titrated with 1 M Trizma base until pH increased to 7.5. The resins after such treatment was then packed in the column (5.5 x 30 cm) and equilibrated at 4 °C (all of the following procedures were conducted at this temperature) with the Column Buffer A (CBA) (6 liters first without DTT, then 2 liters with 1 mM DTT). Then the supernatant from the sonication was loaded onto the column. After the sample had run into the resins, the column was washed with about 1 liter of CBA until $A_{280}$ of the eluate reached that of the eluent. The column was then eluted with a gradient of 300 ml of CBA to 300 ml of Column Buffer B (CBB) and further chased with 200 ml of CBB. A elution profile is shown in Figure 6. The fractions containing the AK activity were pooled and concentrated by dialyzing in PEG 20,000.

**Sephadex G-100 Gel Filtration.** The Sephadex G-100 column was prepared as follows. 30 grams of dry Sephadex G-100 resins were
Figure 6. Phosphocellulose cation exchange column chromatography of the mutant H36N. The cell-free extract was applied to a phosphocellulose cation exchange column equilibrated with Column Buffer A (30 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.1 mM DTT, and 30 mM NaCl). The enzyme was eluted by using a gradient in salt from 30 mM NaCl to 500 mM NaCl (Column Buffer B).
suspended in water containing 0.01% of chlorohexidine diacetate hydrate for 72 hours at room temperature. Then the resins were packed in a column (2.8 X 100 cm) and equilibrated with the Column Buffer C (CBC) at 4 °C. The AK fractions from P-11 column after PEG concentration were then loaded onto this column and eluted with the same buffer as for column equilibrium. A typical elution profile was shown in Figure 7. For further treatment see Long Term Storage of Enzyme Proteins. The enzyme purified in this way shows a single band with a molecular weight ca. 22000 daltons on SDS gel electrophoresis (Figure 8).

HPLC Method. A poly(propyl aspartateamide) hydrophobic interaction HPLC column (4.6 X 200 mm; Nest Group, Southboro, MA) was first regenerated with Methanol. Then it was washed with water and then washed with the elution buffer (1.0 M ammonium sulfate and 0.1 M potassium phosphate, pH 6.4). Porcine muscle AK purified according to Shyy et al. (1987) was dissolved in 1 M ammonium sulfate and injected in the column (ca. 10 mg in one injection) through a 1 ml sample injection loop, and eluted with the equilibrating buffer. The retention time for the enzyme is about 25 min (Figure 9).

Long Term Storage of Enzyme Proteins. The purified AK was further treated as follows. For NMR experiments, the fractions from the G-100 column containing AK activity were collected and dialyzed against the Dialyzing Buffer (DB) at 4 °C overnight. Then the enzyme was lyophilized and stored at -70 °C. For other purposes, the enzyme fractions were first concentrated with PEG 20,000. Then the enzyme was dialyzed in DB, lyophilized, and stored at -70 °C. The enzyme can be stored in this way for more than six months without losing any activity.
Figure 7. Sephadex G-100 gel filtration chromatography of the mutant enzyme H36N. The from P-11 column after PEG concentration were then loaded onto this column and eluted with the column equilibrating buffer (Column Buffer C: 5 mM imidazole-HCl (pH 6.9), 1 mM EDTA, and 0.1 mM DTT).
Figure 8. SDS-PAGE of purified H36N protein. The right lane is a molecular weight marker lane containing, from the top to the bottom, bovine serum albumin ($M_r = 67000$), chicken adenylate kinase ($M_r = 21700$), and lysozyme ($M_r = 14300$). The left lane is the purified H36N protein (after G-100 column chromatography).
Figure 9. HPLC of porcine adenylate kinase. A poly(propyl aspartateamide) hydrophobic interaction HPLC column (4.6 X 200 mm; Nest Group, Southboro, MA) was washed with water and then equilibrated with the elution buffer (1.0 M ammonium sulfate and 0.1 M potassium phosphate, pH 6.4). The enzyme was dissolved in 1 M ammonium sulfate and injected in the column (ca. 10 mg in one injection) through a 1 ml sample injection loop, and eluted with the equilibrating buffer.
2. ENZYME ASSAY AND STEADY STATE KINETICS

The enzyme assay and initial rate studies of AK were achieved by using coupling enzymes. Pyruvate kinase (PK) and lactate dehydrogenase (LDH) were used in the forward reaction (Rhoads & Lowenstein, 1968), whereas hexokinase (HK) and glucose 6-phosphate dehydrogenase (GDH) were used in the backward reaction (Hanada & Kuby, 1978). These two coupled enzyme reactions are summarized in Figure 10.

There are two advantages to use the coupled enzyme assay systems. First, the irreversibility of the AK-catalyzed reaction is maintained by the excessive presence of the coupled enzymes. Secondly, Appearance or disappearance of NAD can be conveniently and accurately followed spectrophotometrically (at 340 nm).

All the AK reactions in this dissertation work were carried out in the same Reaction Buffer (RB) for consistency, except for the viscosity variation studies in which a viscogen (sucrose, in this case) was incorporated into the reaction buffer to raise the viscosity.

Enzyme Assay. The enzyme assay was always carried out in the forward direction of the AK reaction. To a 1.5 ml cuvette (quartz), 0.1 ml of each of Reaction Solution 1 (RS1) and RS2 was added. Then 10 units of each of PK and LDH (in 3.2 M ammonium solution, from Sigma) was added into the cuvette. RB then was added to bring the total volume to (1.0 - x ) ml. The reaction was started by adding x ml of the AK solution to be assayed after the reaction mixture was incubated for about 10 min at 30 °C. The temperature was maintained by a circulating cooling water bath connected to the cuvette holder water jacket. The time dependent
A. Forward reaction.

\[ \text{MgATP} + \text{AMP} \xrightarrow{\text{AK}} \text{MgADP} + \text{ADP} \]

\[ \text{PK} \xrightarrow{\text{PEG}} \text{pyruvate} \xrightarrow{\text{LDH}} \text{lactate} \]

\[ \text{H}^+ + \text{NADH} \xrightarrow{} \text{NAD}^+ \]

B. Backward reaction.

\[ \text{MgADP} + \text{ADP} \xrightarrow{\text{AK}} \text{MgATP} + \text{AMP} \]

\[ \text{HK} \xrightarrow{\text{Glucose}} \text{glucose 6-phosphate} \xrightarrow{\text{GDH}} \text{6-phosphogluconolactone} \]

\[ \text{NAD}^+ \xrightarrow{} \text{NADH} + \text{H}^+ \]

Figure 10. Schemes for coupled enzyme reactions for enzyme assay and steady state kinetics of AK.
absorption change was obtained on a Kontron 820 UV-visible Spectrophotometer. The activity of the AK solution was then calculated according to the following equation

\[
\text{Activity/ml} = \frac{(0.5 \times \Delta A_{340})}{(6.22 \times \text{ml})} \text{(units/ml) } (2.1)
\]

in which, \( \Delta A_{340} \) is the absorbance change per minute at 340 nm.

**Steady State Kinetics.** For the forward reaction, the following procedure was carried out. To a 1.5 ml cuvette, 0.1 ml of each of RS3 and RS2 was added, followed by adding 10 µl of a mixture of PK and LDH (PLM). Then certain amount of each of ATP and AMP stock solutions was added to obtain desired concentrations of the two substrates. Then, RB was added to bring the total volume to 0.990 ml, and the content of the cuvette was incubated at 30 °C for 10 min. The reaction was again started by adding 0.010 ml of an AK solution. After the contents of the cuvette were mixed by covering the cuvette with a piece of parafilm and inverting it 4 or 5 times, the decrease in \( A_{340} \) was followed. Usually, the concentrations of ATP and AMP stock solutions were both made to about 5 mM. (the exact concentrations of which were determined spectrophotometrically by using \( \varepsilon_{260} = 15400 \text{ M}^{-1}\text{cm}^{-1} \) after the solutions were made), and the concentration of the WT AK solution was adjusted to 2 µg / ml containing 1 mg/ml BSA and 1 mM DTT in RB. This AK solution was made by diluting a stock solution of about 1 mg/ml (the exact concentration of which was determined spectrophotometrically by using \( \varepsilon_{280} = 0.51 \text{ (mg / ml)}^{-1} \) (Tian et al., 1988). The backward reaction was conducted similarly. The difference was that for all the reactions with different concentrations of ADP, the
concentration ratio of the metal ion Mg(II) over the substrate ADP was kept the same. MgCl₂ and ADP were dissolved together to make a MgADP stock solution in which the content of Mg(II) over that of ADP was 0.5. The reaction mixture was prepared by mixing in a 1.5 ml cuvette 0.1 ml of RS4, 10 µl of a mixture of HK and GDH (HGM), and certain amount of the MgADP stock solution. After the total volume was adjusted with RB to 0.990 ml, 10 µl of the AK solution was then added to start the reaction. The increase rather than decrease in $A_{340}$ was followed and used for calculation of the reaction rate.

The reaction rate was calculated as follows. For the forward reaction,

$$v = \frac{(0.5 \times \Delta A_{340})}{6.22} \text{ (units)} \quad (2.2)$$

But for the backward reaction,

$$v = \frac{\Delta A_{340}}{6.22} \text{ (units)} \quad (2.3)$$

3. Gdn-HCl-INDUCED UNFOLDING OF ENZYME PROTEINS

Preparation of Buffer and Enzyme Sample. Unfolding of enzyme proteins was carried out in 0.075 M MOPS (pH 7.0) buffer with various concentrations of Gdn-HCl.

First, a concentrated stock solution (ca. 5 M, by weighing) of Gdn-HCl was prepared in double distilled water. The more accurate
concentration of the stock solution was determined by measuring the refractive index and calculated by the following equation (Nozaki, 1972):

\[ C = 57.147(\Delta N) + 38.68(\Delta N)^2 + 91.6(\Delta N)^3 \]  

(2.4)

where \( C \) is the Gdn-HCl concentration and \( \Delta N \) is the refractive index difference between the Gdn-HCl solution and pure water. To this Gdn-HCl solution, solid MOPS and DTT were added to final concentrations of 75 mM and 1 mM, respectively, and pH was adjusted to 7.0 with 6 N NaOH. The volume of the NaOH solution delivered was recorded and used to correct the final concentration of the stock Gdn-HCl solution. A concentrated enzyme stock solution was prepared by dialyzing the enzyme against the MOPS buffer (without Gdn-HCl). The precipitate, if exists, was removed by centrifugation at 15,000g for 15 minutes.

**UV-difference Spectroscopy.** Difference spectra (Herskovits, 1967; Donovan, 1973) by comparing the spectrum of the enzyme in the absence of Gdn-HCl with that in the presence of the salt of different concentrations were obtained on a Kontron 820 UV-visible Spectrophotometer at 25 °C by using tandem cells.

First, a baseline was obtained as follows. The denaturant (Gdn-HCl solution of known concentration, made by diluting the stock solution with the MOPS buffer) was added in compartment A of the reference cell and in compartment B of the sample cell and the MOPS buffer was added in compartment B of the reference cell and compartment A of the sample cell. A spectrum baseline was then obtained from 260 nm to 330 nm, in the range which the Gdn-HCl is transparent. Then the solutions in compartment
B of both cells were pipetted out and both of the compartments were rinsed with distilled water and dried with air. In compartment B of the sample cell, an enzyme solution (ca. 0.8 mg/ml, made by diluting the enzyme stock solution with the MOPS buffer and the denaturant solution such that the concentration of Gdn-HCl was exactly the same as it was in this compartment for making the baseline) was added, but in the compartment B of the reference cell, MOPS buffer was added. After about 10 minutes or longer when the denaturation reaction reached equilibrium, the difference spectrum was taken in the same range of wavelength as for the baseline. A typical difference spectrum had two negative peaks at 280 nm and 288 nm, respectively (Figure 11), and the peak at 288 nm (the left peak) was used to follow the denaturation process.

4. DTNB REACTION WITH ENZYME PROTEINS

The double distilled water was first thoroughly degassed by boiling for 1 h. Then a Tris buffer (DRB) made with the boiling degassed water was further degassed by bubbling argon through for 2 h at 4 °C. This buffer was then used for preparing the stock solutions of DTNB, ligands, and the enzyme (WT or mutant, ca. 5 mg/ml, by extensive dialyzing). The reaction of an enzyme with DTNB was carried out by mixing at 4 °C in a cuvette, and the time course was followed spectrophotometrically at 412 nm (Ellman, 1958). Protection of the enzyme by various ligands was carried out under the same conditions.
Figure 11. The UV-difference spectrum of unfolded chicken adenylate kinase induced by Gdn-HCl. The unfolding reaction was carried out in a MOPS buffer (0.075 M MOPS (pH 7.0), 1 mM DTT, and 2.15 M Gdn-HCl) at 25°C.
5. VISCOSITY VARIATION OF KINETIC PROPERTIES

Preparation of the Reaction Buffers with Sucrose. First, a concentrated sucrose stock solution was made in double distilled (deionized) water (50%, w/v). Then this stock solution was diluted to desired concentrations (10, 20, 30, and 40%). These sucrose solutions were then used to make the reaction buffers for the viscosity studies by addition of the solid buffer components to the final concentrations: 0.075 M Tris-HCl, 0.065 M KCl, and 0.001 M DTT. Then pH is adjusted with concentrated HCl (6 M) to 8.0. The bottles containing the buffers were tightly sealed with parafilm and stored at 4 °C.

Determination of the Relative Viscosities of the Reaction Buffer Solutions. First of all, the density of a reaction buffer solution was determined by weighing a known volume of the solution by using a small volumetric flask (2 ml). Ostwald viscometer (Figure 12) was then used to determine the viscosity of the reaction buffer (Daniels et al., 1970; Atkins, 1982). About 5-10 ml of a solution was placed in the viscometer such that slightly more than half of the lower reservoir was filled. After the solution was equilibrated with the room temperature (24 °C), part of the solution was drawn into the upper reservoir until the level of the solution was higher than the top mark of the reservoir. Then the solution was allowed to flow down back into the lower reservoir. The time required for the solution to flow from the top mark to the bottom one of the upper reservoir was recorded by using a stop watch, which was then used for calculation of the relative viscosity of the solution according to the following equation (Atkins, 1982):
Figure 12. Ostwald viscometer. The arrows indicate the marks' positions on the top reservoir. The flow time from the top mark to the bottom mark determines the drain time $t_{\text{drain}}$, the length of which depends on the viscosity of the solution being measured, and therefore it is used for calculating the relative viscosity (see text for more details).
where $\eta_{rel}$ is the relative viscosity, $\eta$ is the viscosity, $t_{\text{drain}}$ is the time required for the solution to drain from the top mark to the bottom mark of the upper reservoir of the viscometer, and $\rho$ is the solution density, which is obtained by weighing the solution of known volume as described above. The star * is designated for the standard solution (the reaction buffer without sucrose).

**Enzyme Reactions in the Buffers Containing Sucrose.** The enzyme reactions were carried out in the same way as described in Steady State Kinetics, except that the temperature for the viscosity studies was kept constant at 24 °C rather than at 30 °C, because the relative viscosities of the reaction buffers were determined at 24 °C.

6. **DETERMINATION OF $K_{int}$**

$K_{int}$ is designated for internal equilibrium constant of an enzyme reaction, which is the ratio of concentration of the central enzyme-substrate complex over that of the central enzyme-product complex. For example, $K_{int} = [ES]/[EP]$ for single substrate and single product reactions, and $K_{int} = [EAB]/[EPQ]$ for two substrate and two product reactions. The procedure described here was adapted from Benner's method (Stackhouse et al., 1985). The reaction buffer was the same as that used for steady state
kinetics. The concentrations of the stock solutions were 4 mM (ca. 90 mg/ml), 0.1 mM, and 0.05 mM for cAK, ADP, and MgCl₂, respectively. To each of a series of 0.5 ml microcentrifuge tubes, 5 μl of each of ADP and MgCl₂ stock solutions and 2 μl of [2-³H] ATP (1.0 mCi/ml) was added to each of the tubes. Then 38 μl of an enzyme solution of varying concentration was added to each of the tubes to bring the final volume to 50 μl (so that ADP and MgCl₂ concentrations were 0.01 mM and 0.005 mM, respectively). All of the reaction mixtures were then incubated at 30°C for ca. 10 minutes, and by the end, the reaction was stopped by adding 10 μl of 1.0 M EDTA (pH 8.5). 5 minutes later, the mixture was heated up to 100 °C in a boiling water bath for 5 min. Then the reaction mixtures were either stored at -20 °C for later analysis or analyzed immediately for the concentrations of the substrates and products by TLC method, as described below.

To a PEI cellulose plate (Polygram Cel 300, Brinkmann), 2 μl of a cold carrier solution (5 mM ATP, 7 mM ADP, and 10 mM AMP in 50 mM EDTA, pH 8.0) was spotted, followed by spotting 5 μl of the reaction mixture on the top of the spotted cold carrier. Then the plate was dried in air and developed in 1 M LiCl. The nucleotide spots were identified under a UV lamp (short wave length) and cut out. Each of the spots was then put in a counting vial with the spot facing up and it was covered with 10 ml of scintillation counting cocktail. The vials were then put in a scintillation counter (Beckman SL 3801) to be counted for ³H dpm. A parallel control experiment was also carried out to correct the error from the background of counting.
When the substrates were bound to the active sites of the enzyme, they were bound in either of the two forms: [MgATP:AMP] or [MgADP:ADP] (dead end complexes were ignored). Therefore in any cases, the ratio of \([\text{Mg(II)}/\text{nucleotides}]\) is 0.5. When the substrate were all bond, \([\text{MgATP}] = [\text{AMP}]\) and \([\text{MgADP}] = [\text{ADP}]\), and therefore, \(K_{\text{int}} = \frac{[\text{E-MgADP-ADP}]/[\text{E-MgATP-AMP}]}{(1/2)[\text{ADP}]}/[\text{ATP}]\). As a result, plotting \((1/2)[\text{ADP}]/[\text{ATP}]\) vs. \([\text{E}]\) gives \(K_{\text{int}}\) as the plateau value of \((1/2)[\text{ADP}]_{\text{total}}/[\text{ATP}]\).

7. EPIMERIZATION OF SCREW-SENSE ISOMERS OF Cr(III)(H₂O)₄ATP

**Synthesis and Separation of Isomers of Cr(III)(H₂O)₄ATP.**
The screw-sense isomers of \(\beta, \gamma\)-bidentate Cr(III)(H₂O)₄ATP (CrATP) were synthesized and separated according to the procedures established previously (Dunaway-Mariano & Cleland, 1980a; Gruys et al., 1986), and they will be only briefly described here. With stirring 10 ml of 20 mM ATP (pH 3.0) was mixed with 10 ml of 20 mM CrCl₃ (pH 3.0). The mixture was then rapidly brought to 80 °C and kept at this temperature for 10 minutes in boiling water. Then the reaction was terminated by pouring the mixture into a beaker which had been chilled and kept in a salt-ice bath. The products were separated by use of a C-18 reversed phase preparative HPLC column (10 X 250 mm, Beckman). The mobile phase for separation was 0.01 M methanesulfonic acid, pH 2.5, and the elution was followed with an ISCO UV-visual detector at 256 nm. Four peaks were clearly separable on this column and collected separately in glass tubes which were being held in
ice. The isolated isomers were kept at 4 °C and they were stable at this temperature as a dilute solution for about a week. Just before being used, they were concentrated with a rotary evaporator at about 45 °C (within about 30 minutes).

Epimerization Reactions of CrATP Isomers. Before an epimerization reaction was started, the stock solution of a CrATP isomer, which had been concentrated with a rotary evaporator (see above), was adjusted to ca. pH 5.0 with NaOH (pH of the solution before the adjustment was usually below 2.5). All the reactions were carried out in 0.075 M MES buffer (pH 6.1) containing 0.065 M KCl and 0.001 M DTE. No effort was made to duplicate the pH of the reaction mixture strictly because of the difficulty in adjusting pH to an accurate value. It was therefore allowed to vary from one reaction to another within the range of 5.8 to 6.1, depending on the amount of the added stock isomer solution. The exact pH value was then determined after the reaction was completed.

For non-enzymic epimerization reactions, certain amount of an isomer was added to the MES buffer to the final concentration 0.100 mM. At various time intervals, an aliquot (usually 20 μl) of the reaction mixture was withdrawn and directly injected into an analytical C-18 reversed phase HPLC column (4.6 X 250 mm, Beckman) which had been equilibrated with 0.01 M methanesulfonic acid, pH 2.5. The time course of the reaction was then followed by the intensity of the elution profile of the starting or a newly produced isomer. For enzymic epimerization reactions, the enzyme was first dialyzed against the reaction buffer extensively, and added to the reaction buffer to ca. 0.65 mM. A reaction was again started by adding certain amount of an isomer to the reaction buffer (containing the enzyme)
to 0.100 mM. At different time intervals, an aliquot (100 µl) of the reaction mixture was withdrawn and mixed with 900 µl of the 0.01 M methanesulfonic acid elution buffer containing 90% of ammonium sulfate. After vortexing for ca. 1 min, the precipitated enzyme was removed by filtration through either a microfilter (ACRO LC13) or a glass pipet with a glass wool plug in the tip. 50 to 100 µl of the filtrate was injected into the analytical column. Either the peak height or the area was used to follow the time course of the reaction. About 10 minutes or more were allowed in between injections for the reaction components to fully elute out.

Inhibition of pAK by CrATP. The steady state kinetic studies were carried out by using the coupled enzyme as described in Steady State Kinetics. The reactions were carried out in the 0.075 M MES buffer (pH 5.9) containing 0.065 M KCl and 0.001 M DTE, at 25 °C. The enzyme had been dialyzed against the MES buffer before use. The pH of the stock solutions of isomers of CrATP had been adjusted to ca. 5 and kept at 0 °C. Adding of an isomer to a reaction solution was conducted just before the reaction was triggered by adding the enzyme, so that the epimerization and conformational isomerization of the isomer were minimal.

D. PHYSICAL METHODS

1. ELECTROPHORESIS

Electrophoresis is widely used in molecular biology and protein chemistry for purposes of separation, identification, and purification. Two types of electrophoresis which are most widely used are agarose gel
electrophoresis and polyacrylamide gel electrophoresis (PAGE). The methods of electrophoresis concerned in this dissertation include 1% agarose gel electrophoresis for separation, identification, and purification of DNA fragments, SDS-PAGE for identification of proteins, and 6% PAGE for DNA sequencing.

1% Agarose Gel Electrophoresis. Powder agarose was added to a measured quantity of electrophoresis buffer (either TBE or TAE buffer) to make the final concentration of agarose 1%. The resulting slurry was then heated in a boiling-water bath or in a microwave oven until the agarose dissolved. After the solution was cooled to ca. 50 °C, ethidium bromide (from a stock solution of 10 mg/ml in water, stored at 4 °C in a light-proof bottle) was added to a final concentration of 0.5 µg/ml. A mold for casting the gel was made by sealing the edges of a clean, dry, glass plate (or a plastic plate provided commercially with the electrophoresis tank) with autoclaved tape. Then the warm agarose solution was poured into the mold and the comb was immediately clamped into position near one end of the gel. After the gel was completely set, the comb and tape were carefully removed and the gel plate was mounted in the electrophoresis tank. Enough electrophoresis buffer containing 0.5 µg/ml ethidium bromide was then added to cover the gel to a depth of ca. 1 mm. DNA samples were mixed with 5X concentrated loading buffer (5% SDS, 25% glycerol, 0.025% bromophenol blue, and 0.025% xylene cyanol) and slowly loaded into the slots of the submerged gel. The electrophoresis was then run at constant current at ca. 70 mA. After running, the DNA bands were visualized directly under a short UV lamp.
Table 3. SDS-PAGE for analysis of proteins.

<table>
<thead>
<tr>
<th></th>
<th>Running gel (12.5%)</th>
<th></th>
<th>Stacking gel (5%)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>45% acrylamide (w/v) and 1.2% bis-acrylamide (w/v)</td>
<td>5.5 ml</td>
<td>45% acrylamide (w/v) and 1.2% bis-acrylamide (w/v)</td>
<td>1.1 ml</td>
<td></td>
</tr>
<tr>
<td>0.6 M Tris-HCl (pH 8.8)</td>
<td>12.5 ml</td>
<td>0.5 M Tris-HCl (pH 6.8)</td>
<td>2.5 ml</td>
<td></td>
</tr>
<tr>
<td>H₂O</td>
<td>1.8 ml</td>
<td>H₂O</td>
<td>6.3 ml</td>
<td></td>
</tr>
<tr>
<td>10% SDS (w/v)</td>
<td>0.2 ml</td>
<td>10% SDS (w/v)</td>
<td>0.1 ml</td>
<td></td>
</tr>
<tr>
<td>Ammonium persulfate</td>
<td>5.0 mg</td>
<td>Ammonium persulfate</td>
<td>2.5 mg</td>
<td></td>
</tr>
</tbody>
</table>
SDS-PAGE. First, the running gel and stacking gel solutions were prepared by mixing the components as listed in Table 3. Then both of the solutions were degassed by applying vacuum. Then to the running gel solution 20 μl of TEMED was added while the solution was being stirred to mix. The mixture was immediately added from the top opening into the glass plate slab (16 X 16 cm sealed with 1 mm spacers) until the surface of the gel solution in the slab was ca. 2 cm from the top edge. Then ca. 1 cm (in height) water was added over the gel solution surface slowly with a syringe, which would prevent the gel from forming uneven surface during congealing. After the congealation (ca. 20 minutes) was completed (when a sharp interface between the gel and water was noticeable), the water was poured out, and the stacking gel solution, after being mixed with 10 μl of TEMED, was added to fullness, and the comb was slided in immediately (the teeth of the comb should be ca. 1 cm above the running gel surface). The comb was taken out carefully after the stacking gel was solidified (heating at ca. 100 °C if necessary), and the gel slab was then set into the electrophoresis apparatus, and the electrophoresis buffer (SDS-PAGE EB) was added in both the top and bottom tanks. The samples (15 μg for each sample) were dissolved in ca. 15 μl of SDS-PAGE Sample Buffer (SDS-PAGE SB) and loaded with a syringe into the wells which had been casted previously with the comb, and the running was then conducted at a constant current of 20 to 30 mA without any delay. After running (the bromophenol blue had migrated to the bottom edge), the gel was stained with a Staining Solution (SS) for ca. 30 minutes and destained in 7% acetic acid with warming for 5 hours.
6% PAGE for DNA Sequencing. 63 grams of urea was dissolved in 64.5 ml of water and 15 ml of 10X TBE buffer. To this solution was added 22.5 ml of a stock solution (stored at 4 °C in dark when not used) of 38\% (w/v) acrylamide and 2\% (w/v) bis-acrylamide. The resulting volume of the above urea-acrylamide solution is about 150 ml. After being thoroughly mixed, the solution was then degassed by a vacuum line or water aspirator for 15 minutes. To 20 ml of the solution, 235 \( \mu l \) of 30\% ammonium persulfate and 47 \( \mu l \) of TEMED were added. Then the mixture was immediately poured into a casting tray and the pre-clamped glass plate slab (38 x 45 cm) was set and hold firmly in the tray, which allowed the bottom edge of the slab to be sealed in seconds. To the rest of the urea-acrylamide solution, 280 \( \mu l \) of the 30\% ammonium persulfate and 61 \( \mu l \) of TEMED were added. After being mixed by stirring, the solution was added into the slab which had been sealed at the bottom slowly with a large syringe (100 ml in capacity). Then the comb was slided in. About 30 minutes were needed for congealation. The gel slab was then set into the electrophoresis apparatus and the running buffer (TBE buffer) was added. After the comb was taken out some urea was accumulated in the wells, which should be blown out with a glass pipette before loading samples. The samples of DNA sequencing reaction products in the stop solution were then loaded into the wells with a micropipetter equipped with a thinly taped tip. Then the electrophoresis was carried out at a constant voltage of about 1800 V (for optimal results, the running voltage should be so adjusted that the gel temperature is about 55 °C). After running, the slab glass plates were thoroughly washed with soap, rinsed with tap water and then distilled water, and stored dry free from dusts.
2. CIRCULAR DICHROISM SPECTROPOLARIMETRY

An enzyme (WT cAK or mutant) sample was prepared by dialyzing the enzyme against a Tris-HCl buffer (pH 8.0, 20 mM Tris-HCl, 65 mM KCl, 1 mM EDTA, and 1 mM DTT) and the concentration was adjusted to 1-3 mg/ml in the same buffer. After the precipitate was removed by centrifugation, the sample was placed in a cylindrical cuvette (10 mm in width, 2.8 ml in capacity), by delivering the solution through a glass pipet. The cuvette containing the enzyme sample was put in the detection chamber and incubated at 24 °C for about 10 minutes. The scanning was then commenced. The spectral parameters were as follows. Scan speed = 100 nm/minute; scan number = 32; sensitivity = 5 mV/minute; wavelength expansion = 10 nm/cm; chart speed = 10 cm/minute. Whenever the spectrum was taken in the far-UV range (below 220 nm), the light path in the cell chamber was purged with nitrogen gas.

3. ^1^H NMR SPECTROMETRY

A NMR sample was prepared as follows. About 20 mg of enzyme was dissolved in 0.5 ml of Hepes buffer (pH 7.8, 5 mM Hepes, 65 mM KCl, 1 mM DTT, and 1 mM EDTA) and dialyzed against 4 litters of the same buffer at 4 °C overnight. After the precipitate was removed by centrifugation, the enzyme solution was lyophilized. Exchangable protons of the enzyme were then substituted with deuterium by lyophilization of the enzyme in 98.8% D_2O twice and in 99.96% D_2O once. Then the solid
enzyme sample was redissolved in 0.5 ml of perdeuteriated Tris-HCl buffer (75 mM, pH 8.0), prepared by diluting the stock perdeuteriated Tris solution in either 99.96 or 99.996% D₂O, followed by titration with 1 M DCl or KOD to pH 8.0 without isotopic correction by using a Fisher Accumet Model 825 MP pH meter equipped with a calomel reference combination electrode. After being centrifuged to remove precipitate, the sample was transferred to a 5-mm NMR sample tube with a long tip glass pipet and kept at 0 °C before a spectrum was recorded.

¹H NMR spectral analyses were performed at 24 °C on a Bruker AM 500 NMR spectrometer utilizing phase-cycled 45° pulses and quadrature detection. The sweep width was usually 5000 Hz, and 16K or 32K data points were taken. Solvent suppression was utilized and accomplished by presaturation. Spectra were typically processed by using 0.5-1 Hz of line broadening and exponential multiplication, and sometimes, resolution enhancement was performed by applying a Gaussian function multiplication with -0.5 to -1 Hz line broadening.

E. COMPUTER GRAPHICS MODELLING

The X-ray crystallographic structure of porcine AK at 2.1 Å resolution (Dreusicke et al., 1988) was obtained from the Brookhaven Protein Database (file number 3ADK). Modeling was performed by using an Evans and Sutherland PS300 terminal, Vax 11/750 computer, and Chemical Design Ltd. CHEMX.

Conformational energy minimization of K27M was carried out according to the force-field method of Allinger (1976) by using Columbia
University MacroModel. First, by taking the advantage of a Chemical Design Ltd. CHEMX, the side chain of Lys-27 in the porcine muscle crystal structure was replaced with that of Met. The surrounding residues (within 15 Å from Lys-27) along with the Met-27 were then "cut" off from the whole structure, and the resulting structural segment was analyzed by energy minimization (EM) algorithms through use of MacroModel (Columbia University). During the minimization process, only the non-bonding energy contribution to the potential energy of the system by changing the dihedral angles of the newly "generated" Met-27 side chain was considered. To avoid reaching a local minimum, the minimum perturbation method (Shih et al., 1985) for searching for the lowest energy conformation among those generated by dihedral angle changes in 10°-30° increment was performed before the above energy minimization procedure was carried out. Then the conformation of lowest energy was used as the basis for the further EM calculation. To further reduce the chance of sliding into a local minimum, several other conformations of next higher energies generated in the above search with 10°-30° increments of the dihedral changes were also used for the energy minimization, and the resulting conformations were compared with that obtained by using the conformation with lowest energy. An identical conformation was obtained by the above procedure.
CHAPTER III

SITE-SPECIFIC MUTAGENESIS

A. INTRODUCTION

As has been discussed in Chapter I, three active site models of adenylate kinase have been proposed based on the studies using methods of X-ray crystallography (X-ray model) (Pai et al., 1977; Sachsenheimer & Schulz, 1977; Egner et al., 1987), NMR spectroscopy (NMR model) (Fry et al., 1987; Mildvan & Fry, 1987), and molecular mechanics calculations (molecular mechanics model) (Caldwell & Kollman, 1988). However, the conclusions derived from these studies are contradictory, particularly between the X-ray and NMR models. Figures 13 and 14 show the sketch presentations of AK structure and active site (composed of two binding sites, "MgATP site" and "AMP site") of X-ray model (Pai et al., 1977) and NMR model (the binding of AMP is not shown; Fry et al., 1986a), respectively. In the NMR model, the "MgATP site" is found to be located near the place where the "AMP site" of X-ray model is located. Many residues have been suggested by these models as active site groups, among which histidine-36 (His-36), lysine-21 (Lys-21), and lysine-27 (Lys-27) have received much attention. Again the views of these residues in the
Figure 13. Sketch of the crystal structure of porcine muscle AK and the proposed binding sites for ATP and AMP (Pai et al., 1977). Helices are represented by cylinders and the five strands of pleated sheet by arrows. It should be noted that Schulz et al. (1986) suggested later that only the region for binding the phosphate moieties is firm and that Egner et al. (1987) reconfirmed the phosphate binding region but suggested that the adenosine site of ATP in this structure is more likely to be the AMP site, on the basis of the crystal structure of a complex of yeast AK and the inhibitor, MgAp$_5$A.
Figure 14. Sketch of the crystal structure of porcine muscle adenylate kinase and the proposed binding site for ATP based on the NMR model (Fry et al.; 1986a). The black areas indicate the new positions of the regions formed upon substrate binding.
function of adenylate kinase based on these models are different and controversial.

In early 1970’s, Cohn and co-workers (Cohn et al., 1972; McDonald et al., 1975) showed that the \( ^1 \)H NMR signal of the C2-H of His-36 was shifted downfield upon addition of AMP, GTP, ATP, MgATP, or AMP + MgATP and broadened by MnATP (McDonald et al., 1975). The results led to the conclusion that His-36 is directly involved in the catalytic mechanism (Noda, 1973). The results were also used to conclude that the phosphate groups of ATP and AMP lie near His-36 (McDonald et al., 1975). These interpretations were later supported by Schulz and co-workers (Pai et al., 1977) based on the X-ray structural analysis of porcine adenylate kinase inhibitor complexes, which suggests that ATP binds with its triphosphate moiety close to His-36 and with the adenine moiety in a hydrophobic pocket located between helices 69-84 and 100-107 (Figure 13). However, the above model (X-ray model) of the "MgATP site" and the proximity of His-36 to the phosphate groups of ATP and AMP were later challenged by detailed NMR studies of Mildvan and co-workers (Smith & Mildvan, 1982; Rosevear et al., 1983; Fry et al., 1985; 1986a; 1986b; 1987; Mildvan & Fry, 1987). Since a tryptic fragment of rabbit muscle adenylate kinase (residues 1-44) was shown to bind ATP with comparable affinity to that of the enzyme (Hanada et al., 1979), it was used by Midvan and co-workers to measure various distances between specific atoms of the substrate and the peptide fragment (or the enzyme) on the basis of paramagnetic relaxation (using CrATP) and the nuclear Overhauser effect (using MgATP). According to the model derived from such studies (the NMR model) (Figure 15) His-36 is part of a hydrophobic pocket surrounding the
Figure 15. Amino acid residues of adenylate kinase in proximity to, and which may interact with, bound metal-ATP, proposed on the basis of $^1$H NMR studies (Mildvan & Fry, 1987) The residues marked with an asterisk must move in order to interact as shown.
adenine ring (NOE requires $3 \pm 1 \text{ Å}$ between C2-H of His-36 and adenine H2 of bound MgATP) and is quite distant from the triphosphate moiety (paramagnetic relaxation effect requires $13 \pm 1 \text{ Å}$ between C2-H of His-36 and Cr(III) of bound CrATP).

The second important residue suggested to be involved in the active site of adenylate kinase is Lys-21. Many ATP (or GTP)-utilization enzymes have a "glycine rich loop" with an "invariant lysine". This "glycine rich loop" is highly conserved among the same family of enzymes. For example, in the protein kinase family, it is conserved among all of the 64 enzymes of known sequences (Hanks et al., 1988). In the muscle adenylate kinase, this "invariant lysine" is Lys-21, and the common "glycine rich loop" has the sequence G-X-P-G-X-G-L-G, which is conserved among not only the muscle type but also yeast and E. coli AK's (Schulz et al., 1985). In the structure of the porcine adenylate kinase, this loop has been suggested to function as an anion hole for binding the negatively charged phosphate groups of the substrates (Dreusicke & Schulz, 1986), and in the yeast adenylate kinase-Ap5A inhibitor complex the $\beta,\alpha$-phosphate groups of ATP have been shown to be involved in interaction with this loop (Egner et al., 1987). In addition, the involvement of this loop in conformational change has been demonstrated by two different crystal forms of porcine muscle adenylate kinase (Pai et al., 1977; Sachsenheimer & Schulz, 1977).

Affinity labeling studies have shown that adenylate kinase is inactivated when the "invariant" Lys-21 is modified by adenosine di-, tri-, and tetraphosphopyridoxals, and these modifications are partially protected by all adenine nucleotides except AMP (Tagaya et al., 1987; Yagami et al., 1988).
Despite the potential significance of the "invariant" Lys-21 in the mechanism of adenylate kinase, the functional role of this residue is not yet completely in hand. In the X-ray model, Lys-21 is implied to interact with γ-phosphate of bound ATP (Figure 14) (Pai et al., 1977), whereas in the NMR model it is suggested to be able to move and interact with α-phosphate of bound ATP (Figure 15) (Mildvan & Fry, 1987). The affinity labeling results are not sufficient to define the interaction between Lys-21 and the substrates because all of the three affinity modifiers with different lengths of phosphate chain can modify the same lysine (Yagamia et al., 1988).

Unlike His-36 and Lys-21, which have long been indicated as active site groups by both of the X-ray and NMR active site models, Lys-27 is suggested only recently by the NMR studies (Mildvan & Fry, 1987) as a possible catalytic group. As depicted in Figure 15, Lys-27 is suggested to be able to interact with γ and/or β-phosphates of MgATP if it moves into the proper position.

Because these three residues, His-36, Lys-21, and Lys-27, are potentially important active site groups as discussed above, we have chosen them as the first targets for structure-function analyses utilizing the method of site-specific mutagenesis. By the use of recently developed Eckstein's method (Taylor et al., 1985a; 1985b), His-36 has been changed to Gln (H36Q), Asn (H36N), and Gly (H36G), and both of Lys-21 and Lys-27 have been replaced by Met (K21M and K27M, respectively).

Our results suggest the following. His-36 is not important in catalysis nor directly involved in substrate binding. However it stabilizes the tertiary structure of AK possibly through hydrogen bonding between
N3-H of its imidazole side group and a carboxyl oxygen of Asp-93. The imidazole ring also partially protects Cys-25 from being modified by the thiol reagent DTNB. Lys-27 is not involved in the catalytic function either based on the observation of unchanged $k_{cat}$ and $K_m$ in K27M. However, its replacement by methionine increased the stability of the tertiary structure. On the other hand, K21M has a considerably low $k_{cat}$ but similar $K_m$ and conformational stability to those of WT, suggesting the potential involvement of Lys-21 in catalysis. However, structural changes in K21M were also observable by proton NMR analysis, and therefore, the catalytic involvement of Lys-21 can not be yet ascertained.

B. RESULTS

1. HISTIDINE-36

Preparation and Stability of AK and His-36 Mutants. Three His-36 mutants of adenylate kinase have been constructed; they are His-36 to Gln (H36Q), to Asn (H36N), and to Gly (H36G). In the H36Q and H36N mutants, the side chain amide nitrogen of Gln-36 and Asn-36 may occupy the same position as N3 and N1, respectively, of His-36 imidazole group (Lowe et al., 1985) (see Figure 16 for a structure comparison). Thus, these mutants will allow the test of involvement of the aromaticity or either of the nitrogen atoms of imidazole ring of His-36 in the catalytic and structural functions.

The detailed procedures for site-specific mutagenesis and protein purification are described in Chapter II. During the purification of mutants,
Figure 16. The approximate equivalence in position of the nitrogen atoms of asparagine and glutamine with the corresponding nitrogen atoms of the histidine imidazole ring. To a simple approximation, the amide groups of Asn and Gln has been considered to occupy analogous positions of the N1 and N3 groups of the imidazole (Lowe et al, 1985).
Figure 17. Chromatographic profiles of the Sephadex G-100 column in the purification of WT AK (A), H36Q (B), H36N (C), and H36G (D). The peaks to the right represent purified enzymes.
Table 4. Summary of the kinetic and unfolding properties of WT and His-36 mutants.

<table>
<thead>
<tr>
<th>property</th>
<th>WT</th>
<th>H36Q</th>
<th>H36N</th>
<th>H36G*</th>
<th>pAKb</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>kinetics</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$k_{cat}$ ($s^{-1}$)</td>
<td>650</td>
<td>680</td>
<td>595</td>
<td>81</td>
<td></td>
</tr>
<tr>
<td>$K_m$($M_e$ATP) (mM)</td>
<td>0.035</td>
<td>0.210</td>
<td>0.078</td>
<td>0.091</td>
<td></td>
</tr>
<tr>
<td>$K_m$($AMP$) (mM)</td>
<td>0.092</td>
<td>0.980</td>
<td>0.410</td>
<td>0.170</td>
<td></td>
</tr>
<tr>
<td><strong>unfolding</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\Delta G_D H_2 O$ (kcal/mol)</td>
<td>3.8</td>
<td>3.1</td>
<td>2.5</td>
<td>very</td>
<td>3.9</td>
</tr>
<tr>
<td>$C_m$ c</td>
<td>0.79</td>
<td>0.68</td>
<td>0.52</td>
<td>unstable</td>
<td>0.78</td>
</tr>
<tr>
<td>$m$ (kcal/mol M)</td>
<td>4.7</td>
<td>4.4</td>
<td>4.5</td>
<td>4.8</td>
<td></td>
</tr>
</tbody>
</table>

* As explained in the text, the kinetic data listed are for its dimer. The monomeric H36G was too unstable for complete kinetic and unfolding studies.

b pAK is designated for porcine muscle adenylate kinase.

c $C_m$ represents the [Gdn-HCl] concentration at which half of the enzyme molecules are in the unfolded state, and it is obtained by inspection of the denaturation curves (Figure 21).
it was noticed that although the level of the expression for the wild type (WT) and for the mutants was similar (on the basis of the SDS-PAGE analysis of cell-free extracts), the yields of purification for the mutants were lower than that for WT. The enzymes were produced in E. coli, and purified by ion exchange chromatography (phosphocellulose P-11) and gel filtration (Sephadex G-100). Figure 17 shows the chromatographic profiles of the Sephadex G-100 column in the final step of purification. The peak to the right represents purified adenylate kinase, which decreases in its intensity in the order WT > H36Q > H36N > H36G. The peak to the left, which increases correspondingly, consisted of a few bands of higher molecular weight proteins as shown by SDS-PAGE analysis, which were not further characterized. The yields of purification, started with the same amount of culture (12 L), were about 400 mg for WT, 120 mg for H36Q, 60 mg for H36N, and 5 mg for H36G. Because the expression level was similar for WT and for the mutants, we ascribe the reduced yield to the reduced stability of the mutants.

Furthermore, we found that the purified H36G was very unstable and readily dimerized. Figure 18 shows the SDS-PAGE analysis of monomeric H36G in the cell-free extract (lane 2) and the purified dimeric H36G (lane 3). Since the dimer does not dissociate under denaturing condition, the connecting linkage is considered to be covalent.

**Kinetic Properties.** Steady state kinetic constants $k_{cat}$ and $K_m$ were determined from the Lineweaver-Burk plot (Lineweaver & Buck, 1934) by saturating one substrate while varying the other. As shown in Table 4, the $k_{cat}$ of H36Q or H36N was found not significantly different from that of the WT. The monomeric H36G was very unstable and the data
Figure 18. SDS-PAGE analysis of H36G (in 2% SDS). Lane M consists of molecular weight markers. Lane 1 and 2 are the cell-free extracts under uninduced and induced (with IPTG) conditions, respectively. The heavy band near 22.0 kDa represents monomeric H36G. Lane 3 represents dimeric H36G.
were not readily duplicated. Thus only the data for the dimeric form were reported. However, the specific activity of the monomeric H36G was similar to that of WT. Even the dimeric H36G still retained more than 10% activity of the WT AK.

Quantitative interpretation of any steady-state kinetic data requires the quantitative knowledge of the kinetic mechanism. If the mechanism is fast equilibrium, the change in $k_{\text{cat}}$ can be directly related to the perturbation to the chemical step. On the other hand, if the mechanism is far from fast equilibrium (for example, the substrates are sticky), no direct correlation between $k_{\text{cat}}$ and the rate of the catalytic step can be made. Quantitative studies on AK have been carried out previously by several groups (see chapter IV for more details). The two representative results have been reported by Brown and Ogawa (Brown & Ogawa, 1977), in which the rate of the chemical step is similar to that of the off-step, and by Nageswara Rao and co-workers (Nageswara Rao et al., 1978; Vasavada et al., 1984), in which the rate of the chemical step is faster than the overall rate by a factor of 10. No studies have reported that the chemical step is more than 10 times faster than the overall rate. Therefore, our results suggest that mutations at His-36 mutants did not reduce the rate of the chemical step by more than an order of magnitude. In Chapter IV, we will present evidence that suggests that the overall rate is partly controlled by the chemical step. Therefore, in any case, His-36 can not be considered as a catalytically important group. The $K_m$ values of mutants increase in all cases. However, the increases ($\leq 10\times$) are not large enough to suggest a direct involvement of His-36 in the binding of MgATP or AMP. Even the dimeric H36G showed only an 8x decrease in $k_{\text{cat}}$ and a 2x increase in
K_m's. Thus, the most reasonable interpretation of the results is that His-36 is not involved in the catalysis of AK, contradicting the previous view of the roles of this residue.

Protection of Cys-25 by His-36. The above results on the stability and kinetics of His-36 suggested that His-36 may play a structural rather than catalytic role in the function of AK. Inspection of the crystal structure of the refined porcine muscle AK at 2.1 Å resolution (Dreusicke et al., 1988) revealed that His-36 is in close proximity to the side chains of two residues, Cys-25 and Asp-93, as shown in Figure 19. These residues may interact with one another to assist in holding the α-helix 23-30, the β-sheets 33-38 and 87-97 in place. Since His-36 is located in proximity to Cys-25, it could "protect" Cys-25 from being involved in the formation of dimer or other undesirable events. This point was supported by the experiments described below that compared the reactivity of the sulfhydryl groups of AK and the His-36 mutants toward DTNB (Ellman's reagent).

First of all, the possibility of formation of intra or intermolecular disulfide bonds in the enzyme in DTNB reactions was ruled out. The WT AK, after reacting with DTNB, was dialyzed extensively. The SDS-PAGE analysis showed only one band corresponding to monomeric AK. The result was consistent with that of Kress and Noda (1967), who showed that rapid addition of DTNB to the porcine muscle AK resulted in the formation of only a monomeric derivative. Treatment with excessive DTT of this dialyzed WT AK resulted in the release of ca. 1.8 equivalent of thionitrobenzoic acid (TNB)/mol of enzyme. Similar results were obtained for H36Q and H36N.
Figure 19. Spatial relationship between His-36, Cys-25, Asp-93, and Lys-21 revealed by the 2.1 Å crystal structure of porcine muscle AK (Dreusicke et al., 1988). The pertinent distances are 3.0 Å, N3 of His-36 to O1 of Asp-93; 3.6 Å, to O2 of Asp-93; 2.7 Å, γ-S of Cys-25 to O1 of Asp-93; and 3.8 Å, to N3 of His-36.
Figure 20. Time courses of the reaction of DTNB with WT (o), H36Q (△), and H36N (o) based on the release of TNB monitored by the absorbance at 412 nm.
The reaction of DTNB with WT AK, H36Q, and H36N was then followed spectrophotometrically. The time course of the reaction (Figure 20) showed that the rate increased in the order WT < H36Q < H36N. Thus the results suggest that at least one of the two cysteine sulfhydryl groups is partially protected by the His-36 side chain. Since the crystal structure indicates that Cys-25 is in close proximity (3.2 Å from N3 of His-36) (Dreusicke et al., 1988) while Cys-187 is 13 Å away, we conclude that it is Cys-25 which is protected by His-36.

Stabilization of the Tertiary Structure by His-36. The other residue which is also in proximity to His-36 is Asp-93 (Figure 19). One of the oxygen of the side chain COO\(^-\) of Asp-93 has been suggested to be hydrogen-bonded to one of the nitrogen atoms of His-36, possibly the N3 nitrogen (Dreusicke et al, 1988). If this is true, breakage of this hydrogen-bonding will be expected to reduce the structural stability of the enzyme. This was indeed supported by the experiments described below, in which the structural stability of WT toward salt-denaturation was compared with that of the mutants. If it is the N3 nitrogen of the His-36 imidazole ring which is involved in the suggested hydrogen-bonding with an Asp-93 carboxyl oxygen atom, it may be predicted that the structural stability should decrease in the order WT > H36Q > H36N.

Figure 21 shows the denaturation curve of WT, H36Q, and H36N induced by guanidine hydrochloride (Gdn-HCl). The Gibbs free energy change of unfolding was determined from the denaturation curve by use of the equation (Pace, 1986):

\[
\Delta G_D = \Delta G_D^{H_2O} - m [\text{Gdn-HCl}]
\]  

(3.1)
Figure 21. Gdn-HCl-induced denaturation curves of WT (○), H36Q (△), and H36N (○) monitored by UV difference absorbance at 288 nm.
Figure 22. Plots of \( \Delta G_D \) as a function of \([\text{Gdn-HCl}]\) according to eq. 3.1 for WT and His-36 mutants.
where $\Delta G_D$ is the Gibbs free energy change at various concentrations of Gdn-HCl, $\Delta G_D^{H_2O}$ is the Gibbs free energy change at zero concentration of Gdn-HCl, and $m$ is a constant. Plots of $\Delta G_D$ as a function of $[\text{Gdn-HCl}]$ is shown in Figure 22, which gives $\Delta G_D^{H_2O}$ and $m$ values as summarized in Table 4. As predicted, the results show that the conformational stability (in the Gdn-HCl-induced unfolding) decreases in the order WT > H36Q > H36N. It is interesting to note that porcine muscle AK behaves identically to chicken muscle AK in Gdn-HCl-induced denaturation, even though there are 29 different amino acids (85% homology) between the two natural mutants. The relative invariance of the slope $m$ suggests that the nature of interaction between the enzyme and Gdn-HCl has not been altered in the mutants.

**NMR and CD Studies.** NMR spectrometry and CD spectropolarimetry have been used to analyze the structures of WT and mutants. Figures 23 and 24 show, respectively, the NMR and CD spectra of WT and H36Q. Overall, no significant spectral changes were detected by both methods though some minor changes were noticeable.

### 2. LYSINE-21 AND LYSINE-27

**Preparation and Stability of the Mutants.** Methionine was chosen for substitution of both Lys-21 and Lys-27 since the side chain of methionine is reasonably isosteric with that of lysine (Chothia, 1975), and allows specific test of the functions of the charge and the hydrogen bonding capability possessed by the side-chain of lysine. Preparation of the mutants is described in Chapter II. Due to the functionally important
Figure 23. Aromatic (A) and aliphatic (B) regions of 500 $^1$H NMR spectra of WT (top) and H36Q (bottom) at 24 °C in $\text{D}_2\text{O}$ containing 0.075 M Tris-HCl (pH 8.0), 0.065 M KCl, 0.001 M EDTA, and 0.001 M DTT.
Figure 23
Figure 24. CD spectra of WT (A), H36Q (B), and H36Q-WT (C).
implications of the K21M mutant as described in a later section, we have re-subcloned the gene of this mutant back into M13mp19 and reconfirmed the mutation.

Both lysine mutants are stable and can be purified in quantity. The Gdn-HCl-induced denaturation was used to define the stability of WT and mutants and the denaturation curves are shown in Figure 25. The concentration \( C_m \) of Gdn-HCl at the mid-point of unfolding transition, and the Gibbs free energy of unfolding, \( \Delta G_{pH2O} \), calculated from Figure 26 (Pace, 1986), are listed in Table 5. The results suggest that while the stability of K21M is comparable to that of the WT, replacement of Lys-27 by Met results in increase in the stability of the enzyme structure by as much as 1.8 kcal/mol. Possible explanation for the increased stability of K27M is given below.

**Possible Structural Basis for the Stability of K27M.**

Examination of the 2.1 Å crystal structure of porcine muscle AK (Dreusicke et al., 1988) revealed a potential side-chain interaction between Lys-27 and Asp-180 (Figure 27A). This interaction is located between the helix 23-30 and the C-terminal helix 179-194 and can contribute to holding the helices in place. It is puzzling why disruption of this energetically favorable interaction could lead to increased conformational stability.

Attempting to provide a rational explanation, we noticed that the Lys-27···Asp-180 ionic pair is surrounded by at least two hydrophobic residues, Val-179 and Phe-183, as also shown in Figure 27A. All of the three residues are conserved between the porcine muscle AK used for the X-ray structural determination and the chicken muscle AK used in our mutagenesis studies. The replacement of Lys-27 with Met could enhance
Figure 25. Gdn-HCl-induced denaturation curves of WT, K21M, and K27M monitored by UV difference absorbance at 288 nm.
Figure 26. Plots of $\Delta G_D$ as a function of [Gdn-HCl] according to eq. 3.1 for WT and K21M and K27M.
Table 5. Free energy of unfolding of WT, K21M, and K27M induced by Gdn-HCl.

<table>
<thead>
<tr>
<th>enzyme</th>
<th>$\Delta G_{D}^{H2O}$ (kcal/mol)</th>
<th>$C_m$ (M)</th>
<th>$m$ (kcal/mol M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>3.8</td>
<td>0.79</td>
<td>4.7</td>
</tr>
<tr>
<td>K21M</td>
<td>3.5</td>
<td>0.78</td>
<td>3.8</td>
</tr>
<tr>
<td>K27M</td>
<td>5.6</td>
<td>1.04</td>
<td>5.4</td>
</tr>
</tbody>
</table>
Figure 27. Stereo computer graphics representations of the structure of Lys-27 and the surrounding residues (A) and the structural segment of Met-27 and the surrounding residues (B) after conformational energy minimization.
Table 6. Summary of steady state kinetic data obtained by partial initial velocity analysis (saturating one of the substrates).

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>K21M</th>
<th>K27M</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_{cat}$ (s$^{-1}$)</td>
<td>650</td>
<td>0.015</td>
<td>(4.3×10$^4$)$^a$</td>
</tr>
<tr>
<td>$K_m$(MgATP) (mM)</td>
<td>0.035</td>
<td>0.050</td>
<td>0.072</td>
</tr>
<tr>
<td>$K_m$(AMP) (mM)</td>
<td>0.092</td>
<td>0.040</td>
<td>0.094</td>
</tr>
<tr>
<td>$k_{cat}/K_m$(MgATP) $\times 10^{-7}$ M$^{-1}$s$^{-1}$</td>
<td>1.9</td>
<td>0.000030 (6.3×10$^4$)$^a$</td>
<td>1.2</td>
</tr>
<tr>
<td>$k_{cat}/K_m$(AMP) $\times 10^{-7}$ M$^{-1}$s$^{-1}$</td>
<td>0.7</td>
<td>0.000038 (1.8×10$^4$)$^a$</td>
<td>0.9</td>
</tr>
</tbody>
</table>

$^a$ Numbers in parentheses represent the ratio WT/K21M.
hydrophobic interactions between Phe-183 and Val-179.

In protein structures, it has been noted that disulfide linkages and aromatic rings often lie near each other (Morgan et al., 1978; Morgan & McAdon, 1980), and a potential role of disulfide-aromatic ring complexes formed mainly due to hydrophobic interactions in protein folding and protein conformation has been suggested by Scheraga and co-workers (Swadesh et al., 1987). The following evidence suggests that the sulfur of Met-27 is located above the benzene ring of Phe-183 in the K27M mutant. Such a sulfur-aromatic complex may also contribute to the increased stability of K27M.

First, by using a Chemical Design Ltd. CHEMX, we were able to replace the side chain of Lys-27 with that of Met in the porcine muscle crystal structure. Then a minimum perturbation approach according to Karplus and co-workers (Shih et al., 1985) through use of MacroModel (see Chapter II for more details) was carried out to analyze the structure of K27M. This method analyzes only static conformations, which does not take into consideration the dynamic contributions of the structure. During the minimization process, the conformational adjustments were only introduced to the newly generated side chain of Met-27 while the rest of the molecule was kept stationary. In addition, the performance of the modelling was carried out in vacuo. Because of the above potential uncertainties which may have been introduced by the modelling procedure, the result obtained should be only viewed as qualitative. Nevertheless, it led to a conformation in which the sulfur atom of Met-27 was located above the benzene ring of Phe-183 at a distance of 3.7-5.1 Å from the six ring carbon atoms, as shown in Figure 27B. Secondly, one-dimensional $^1$H NMR
spectra (Figure 28) show that in the 2.0 ppm region of the WT AK (Figure 28A) there are four singlets (peaks a-d). The peaks a-c can be assigned to S-methyl groups of methione residues (there are four methionines in chicken AK; Kishi et al., 1986; Tanizawa et al., 1987) while the very sharp peak d arises more likely from buffer. In the spectrum of K27M (Figure 28B) there is an additional singlet at 1.58 ppm (resonance e), which becomes quite obvious in the difference spectrum (Figure 28D). This new singlet signal has similar lineshape and area to those of other methionine signals, and thus it should be due to the S-Me of the new methionine. The upfield shift of this signal, relative to the S-methyl resonances of other methionine residues, is most likely due to the aromatic ring current effect, and strongly suggests the proximity and possible hydrophobic interaction between Met-27 and Phe-183. This interaction, along with the hydrophobic interaction between Met-27 and Val-179, could overcome the loss of the hydrogen bonding between Lys-27 and Asp-180 and results in the increased conformational stability of K27M.

The little difference between the $^1H$ NMR spectra of WT and K27M (Figure 28), as revealed by the difference spectrum (Figure 28D), is remarkable and it suggests that no significant tertiary structural variation has occurred.

Kinetic Properties. As shown in Table 6, the $k_{cat}$ of K27M was almost unchanged relative to that of WT and the $K_m$ of the mutant was neither significantly different. Since the chemical step is likely being partially rate-limiting (see Chapter IV) and at most not faster than the overall rate by more than 10 times (Nageswara Rao et al., 1978; Vasavada et al., 1984), the rate of the chemical step of K27M should not have been
Figure 28. Aromatic (to the left) and aliphatic (to the right) region of 500 \textsuperscript{1}H NMR spectra of WT (A), K27M (B), K21M (C), and K27M-WT (D) at 24 °C in D\textsubscript{2}O containing 0.075 M Tris-HCl (pH 8.0), 0.065 M KCl, 0.001 M EDTA, and 0.001 M DTT.
Figure 28
perturbed significantly (< 10x). Using a slowly reacting substrate pair: MgATP/dAMP, we have further confirmed that the chemical step of K27M has not been perturbed significantly. As shown in Chapter IV, the chemical step is rate-limiting with this substrate pair, and therefore a difference in $k_{cat}$ between WT and K27M can be attributed to a perturbation in the chemical step. As expected, only small differences were observed between the kinetic properties of WT and K27M ($k_{cat}$ 112 and 145 s$^{-1}$, respectively; $K_m(dAMP)$ 0.45 and 0.37 mM, respectively). Thus, the kinetic results show that Lys-27 is not involved in catalysis and the conclusion is at variance with the NMR model, in which Lys-27 can move and interact with the γ-phosphate of bound ATP (Mildvan & Fry, 1987).

On the other hand, although the $K_m$'s of K21M is similar to those of WT, the $k_{cat}$ of K21M is substantially reduced (by a factor of $4.3 \times 10^4$; Table 6). When $k_{cat}$ is used for a calculation, the mutation removed as high as 6.7 kcal/mol of the transition state stabilization energy, whereas, because of the similar $K_m$ values, the ground state should not have been perturbed significantly.

Similar kinetic data (Table 7) on binding properties were also obtained by a full initial velocity analysis (varying the concentrations of both substrates). The quantitative values of $K$ and $K_I$ were calculated according to eq. 3.2 (Cleland, 1986)

$$v = \frac{ABV}{K_p K_{ib} + K_b A + K_A B + AB}$$

(3.2)
where \( v \) is the reaction rate, the subscripts \( a \) and \( b \) represent the two substrates \( \text{MgATP} \) and \( \text{AMP} \), respectively, \( A \) and \( B \) are the concentrations of the corresponding substrates, and \( V \) is the maximum velocity. The data in Table 7 indicate that the dissociation constants \( K_i \) (for binary complex) and \( K \) (for ternary complex, equivalent to \( K_m \) obtained by holding one of the substrates at saturating level) were only slightly perturbed in \( K_{21M} \) and \( K_{27M} \). The straightforward interpretation of such kinetic results is that while Lys-27 is not important in catalysis, Lys-21 stabilizes the transition state of the reaction.

The 6.7 kcal/mol energy change based on the decrease in the \( k_{cat} \) of \( K_{21M} \) stretches the upper limit of documented charged hydrogen bonds (Fersht et al., 1985). The magnitude of this binding energy contributed by Lys-21 could account for as many as two hydrogen bonds. However, as cautioned by Hibler et al. (1987), it is important to examine possible conformational changes before attempting to make mechanistic interpretations of the perturbations in kinetic properties. Although the relatively unperturbed conformational stability of this mutant could argue against a large conformational change, we found that this was not the case, as discussed in the next section.

Possible Structural Changes of \( K_{21M} \). In contrast to \( K_{27M} \) where conformational stability was significantly improved but the conformation was little perturbed, the change in the \( ^{1}H \) NMR spectrum of \( K_{21M} \) (Figure 28C) was dramatic even though the \( \Delta G_d^{H_2O} \) was hardly affected. The spectrum has been reproduced twice by using identically prepared samples. The results suggest that the tertiary structure of \( K_{21M} \) may have been perturbed. Since Lys-21 is part of the "flexible loop"
Table 7. Summary of steady state kinetic data by full initial velocity analysis (varying both substrates).

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>K21M</th>
<th>K27M</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_{M_{ATP}}$ (mM)</td>
<td>0.042</td>
<td>0.052</td>
<td>0.105</td>
</tr>
<tr>
<td>$K_{AMP}$ (mM)</td>
<td>0.098</td>
<td>0.044</td>
<td>0.130</td>
</tr>
<tr>
<td>$K_{iM_{ATP}}$ (mM)</td>
<td>0.160</td>
<td>0.188</td>
<td>0.320</td>
</tr>
<tr>
<td>$K_{iAMP}$ (mM)</td>
<td>0.370</td>
<td>0.160</td>
<td>0.403</td>
</tr>
<tr>
<td>$K_i/K$</td>
<td>3.8</td>
<td>3.6</td>
<td>3.1</td>
</tr>
</tbody>
</table>
(Dreusicke et al., 1988), mutation at this site is likely to cause changes in local, or even global conformational changes.

C. DISCUSSION

1. IMPLICATION ON THE MECHANISM OF AK

Three amino acid residues of chicken muscle AK, His-36, Lys-27, and Lys-21, have been chosen for analysis by site-specific mutagenesis. The involvement of His-36 in the catalytic function (including substrate binding) has been previously suggested by Noda (1973), and implied by McDonald et al. (1975) and Pai et al. (1977). Our results have ruled out the direct involvement of His-36 in the catalytic function of AK, although the results do not contradict the suggestion by the NMR model of substrate binding by Fry et al. (1985), in which the adenine ring of ATP is positioned in a hydrophobic pocket formed by the side chains of Ile-28, Val-29, Leu-91, Leu-37, and His-36. However, our results do suggest that His-36 is not critical for this hydrophobic pocket if it binds the adenine moiety of ATP. In the NMR model, Lys-27 has also been suggested to be able to move and interact with γ and/or β-phosphates of bound ATP (Mildvan & Fry, 1987) and thus important for catalysis, which is however at variance with our results. Lys-21 has been suggested to be able to move and interact with the α-phosphate of bound ATP by the NMR model (Mildvan & Fry, 1987), while it has been implied to interact with γ-phosphate of ATP by the X-ray model (Pai et al., 1977). The latter is supported by the recent molecular mechanics calculations (Caldwell &
Kollman, 1988). Our mutagenetic results, ignorant of the structural changes in K21M, are consistent with the X-ray and molecular mechanics models.

2. POTENTIAL STRUCTURAL ROLES OF THE MUTATED RESIDUES

Decreased Stability of His-36 Mutants. Figure 19 shows the spatial environment of His-36 based on the X-ray structure of AK at 2.1 Å resolution (Dreusicke et al., 1988). It has been suggested on the basis of this structure that one of the nitrogen atoms of the His-36 imidazole is hydrogen-bonded to one of the oxygen atoms of the side-chain COO' of Asp-93. Our examination of the structure (file number 3ADK in the Brookhaven Protein Database) showed that the thiol group of Cys-25 is also shielded by the imidazole ring of His-36, although the sulfur atom does not lie exactly on the top of the ring. Thus this spatial arrangement is consistent with the interpretation of our experimental results that His-36 protects Cys-25 from chemical modification and interacts through a hydrogen bond with Asp-93 to stabilize the tertiary structure. Since the stability decreases in the order WT > H36G > H36N, it is concluded that, it is the N3 nitrogen of the imidazole of His-36 that is involved in the formation of the H-bond.

A H-bonding nature of the interaction between His-36 and Asp-93 is quite certain, as revealed by the X-ray structure in which the N3 of His-36 and the carboxyl oxygen of Asp-93 are in a reasonable H-bonding distance (2.7 Å). However, it is unknown whether there are also any energetically
favorable interactions between His-36 and Cys-25. Although it has been suggested that thiol could form charge-transfer complex with aromatics as in the case of some FAD-utilizing enzymes (Massy & Ghisla, 1974; Arscott et al., 1981; Shames et al., 1986), such interaction does not necessarily exist between His-36 and Cys-25 in the case of AK, particularly because the X-ray structure indicates that the sulfur of Cys-25 thiol is not located above the imidazole ring of His-36. The structure also shows that the formation of a H-bond between the two residues is unlikely. Therefore, it is reasonable that His-36 is merely protecting the thiol of Cys-25 by a steric effect due to the bulkiness of the ring.

While it is understandable for the enzyme to have the interaction between His-36 and Asp-93 since it provides stabilization energy for the tertiary structure, it is not clear however why His-36 should protect Cys-25, particularly in the case of AK where no catalytic roles have been found for Cys-25. In fact, it has been suggested that Cys-25 is neither important for catalysis nor for substrate binding (Noda, 1973). A possible interpretation is that Cys-25 may form a H-bond also with Asp-93, as suggested by Dreusicke et al. (1988), and protection of Cys-25 thus protects this H-bond.

**Improved Stability of Lys-27.** It is quite unusual that the $\Delta G_{D}^{H2O}$ of K27M increases, rather than decreases, by 1.8 kcal/mol relative to the WT, while the catalytic properties remain almost the same. It is also noted that the conformational stability of AK is very low. For most proteins, the stabilization energy that distinguishes the folded and unfolded states is in the range of 5 to 15 kcal/mol (Pfeil, 1981; Creighton, 1983), while for AK it is $< 4$ kcal/mol under our experimental conditions. Because
of the low stability, it is sometimes difficult to purify an AK mutant if the mutation further lowers the stability by even a small amount, as in the case of H36G, and even WT AK readily precipitates in the sample chamber of differential scanning calorimeter upon increasing temperature (Tian & Tsai, unpublished observations). Since K27M is more stable than WT (5.4 kcal/mol compared to 3.8 kcal/mol) while no notable differences in catalytic function exist between the two, it may be worthwhile to substitute K27M for WT in future mutagenetic studies so as to alleviate the stability problem.

It is also interesting to know the nature of the increased stability of K27M. It has been noted that disulfide linkages and aromatic rings are often located together in tertiary structures of protein (Morgan et al., 1978; Morgan & McAdon, 1980). In attempting to understand this phenomenon, Scheraga and co-workers (Swadesh et al., 1987) have recently studied the quenching property of Tyr fluorescence by DTT and suggested a possible static interaction between DTT and Tyr with a rather large hydrophobic character. In the present study, the side chain of Met-27 in the K27M mutant was also found in close proximity to an aromatic residue (Phe-183), and it is not unlikely that the increased stability is partly contributed to by a possible hydrophobic interaction between the two residues. Further, in the tertiary structure of WT, Lys-27 is almost "sandwiched" in between the two hydrophobic residues Phe-183 and Val-179 (Figure 27) and such close arrangement of hydrophobic and hydrophilic residues is energetically unfavorable. However, methionine is a rather hydrophobic amino acid, the hydrophobicity of which is close to that of Val in both the Nozaki-Tanford solvent transfer scales (Nozaki & Tanford, 1971) and in the Sweet-
Eisenberg optimal matching hydrophobicity (OMH) scales (Sweet & Eisenberg, 1983). Therefore, substitution for Lys-27 by a methionine would not only introduce favorable hydrophobic interactions between Met-27, Phe-183, and Val-179 but also remove unfavorable contacts between the Lys-21 and Phe-183 and Val-179. In addition, since the energy minimization procedure led to a structure in which the sulfur atom of Met-27 is located above the benzene ring of Phe-183, the contribution for the increased stability by formation of a charge-transfer complex between the sulfur atom of Met-27 and the benzyl ring of Phe-183 should not be ignored.

Possible Structural Roles of Lys-21. Unlike Lys-27, which is located in a relatively hydrophobic environment, Lys-21 is surrounded by more hydrophilic residues and it possibly forms a H-bond with carbonyl oxygen of Gly-15 (2.84 Å), as shown by the crystal structure (Figure 29). It should be noted that Lys-21 is at one end of the flexible loop (from residue 15 to 22) while Gly-15 is at the other. Therefore, formation of a H-bond between the two residues may hold the loop in certain proper conformation. Since this H-bond may be the only one within the loop which is flexible in nature (Sachsenheimer & Schulz, 1977), breakage of this linkage would bring about conformational changes to the entire loop, and in turn the global conformation may be also affected. This is supported by the observation of changes in the $^1$H NMR spectrum of K21M (Figure 28C). Thus, our results suggest that Lys-21 plays critical roles in maintaining the conformation of AK, most likely in the region of the flexible loop.
3. POTENTIAL FUNCTIONAL ROLES OF THE MUTATED RESIDUES

Possible Catalytic Roles of Lys-21. Conformational changes in K21M has been discussed above, and a structural role of Lys-21 has been suggested. However, the structural changes do not preclude Lys-21 in playing also catalytic roles. A structural change is potentially but not necessarily a cause for changes in catalysis. For example, substantial changes have also been observed in the proton NMR spectra of the P9L and G10V mutants of *E. coli* AK (corresponding to P17L and G18V, respectively, of chicken muscle AK) in the same flexible loop which Lys-21 belongs to. The kinetic properties of these two mutants, however, were only moderately perturbed (*k*_cat/_K*_m decreased by 10-30x) (Reinstein et al., 1988). In addition, proteins are dynamic systems and functional conformations are expected to form upon substrate binding thanks to the induced-fit mechanism (Koshland, 1958; 1959; Jencks, 1975; Wolfenden, 1987; Herschlag, 1988). Since it will be a long venture before these problems can be answered, we attempt to interpret the kinetic data of K21M below assuming structural changes are unrelated to catalysis and the conclusion should not be considered to be final.

The NMR model (Mildvan and Fry, 1987) and the X-ray model (Pai et al., 1977) predict the involvement of α-phosphate and γ-phosphate of ATP in the interaction with Lys-21, respectively, but both models deal with ground state instead of transition state structures. The NMR model did suggest that Lys-21 must move in order to interact, which may imply that Lys-21 is involved in transition state. Our results suggest that Lys-21 is
Figure 29. Stereo computer graphic representation of the structural environment around Lys-21 of porcine muscle adenylate kinase (Dreusicke et al., 1988).
likely to stabilize the transition state rather than the ground state via a hydrogen bonding between charged groups, most likely between the ε-amino group of Lys-21 and γ-phosphate of ATP. This is supported by the large loss in transition state binding energy in K21M (6.7 kcal/mol). Since the chemical mechanism is associative (Richard & Frey, 1978), in which a negative charge develops to the γ-phosphate of ATP in the transition state. Neutralization of a newly generated charge is energetically favorable since it may occur without the desolvation of the charge and "save" the otherwise necessary desolvation energy.

An alternative interpretation of the transition-state binding energy of 6.7 kcal/mol involving Lys-21 is that this residue is involved in two hydrogen bonds at the transition state, one with the γ-phosphate of ATP and the other with the α-phosphate of AMP. This possibility is supported by the recent results of molecular mechanics calculations (Caldwell and Kollman, 1988). In this work the X-ray and NMR models were first energy-refined separately. A third model called NEWMOD was then created which contained features of both models and further energy-refined. In this ground state NEWMOD structure Lys-21, Arg-97, and Arg-149 interact strongly with substrates, with Lys-21 forming hydrogen bonds with both the γ-phosphate of ATP and the α-phosphate of AMP. The authors then modelled the "reaction" by creating a "bond" between the phosphate oxygen of AMP and the γ-phosphorus atom of ATP, at distances of 4.0, 3.0, and 2.0 Å, followed by further energy-refining. The results showed that Lys-21 interact strongly with substrates throughout the path. However, interaction of Lys-21 with the α-phosphate of AMP in the transition state would reduce the nucleophilicity of the α-phosphate of AMP and thus part of the
binding energy would have to be traded off for the reduction in nucleophilicity of the attacking group.

**Potential Functional Roles of His-36.** Our results have ruled out the direct involvement of His-36 in catalysis. However, considering the increased $K_m$ values for both substrates the involvement of this residue in a induced-fit mechanism of this enzyme is suspected, particularly, His-36, Cys-25, and Asp-93 are located in a region where conformational changes occurring upon substrate binding have been suggested (Sachsenheimer & Schulz, 1977). Our results of a viscosity study on WT and His-36 mutants are consistent with this speculation, which will be described in Chapter IV.

4. CATALYSIS AND STABILITY

Since the advent of the site-specific mutagenesis, the method has been employed not only for structure-function relationship studies of enzymatic reactions (Gerlt, 1987; Knowles, 1987; Shaw, 1987; Fersht, 1988) but for design of protein stability for both theoretical and industrial purposes (Perry & Wetzel, 1984; Wells & Powers, 1986; Hecht et al., 1986; Mitchinson & Baldwin, 1986; Pantoliano et al., 1987; Matthews et al., 1987).

More than a decade ago, Albery and Knowles (1976) proposed three mechanisms, the uniform binding, the differential binding, and catalysis of individual steps, by which enzymes evolve to perfection where no further improvement in the catalytic efficiency can be achieved. These evolution mechanisms have been consistently demonstrated by the elegant mutagenetic studies by Fersht and co-workers (Ho & Fersht, 1986;
Leatherbarrow & Fersht, 1987). On the other hand, mutagenetic studies on protein stability indicate that proteins are much less stable than they can be. For example, T4 lysozyme (Perry & Wetzel, 1984) and dihydrofolate reductase (Villafranca et al., 1983) have been made more stable by introduction of disulfide bonds. Introduction of mutations that increase stability of secondary structural units such as α-helices (Hecht et al., 1986; Mitchinson & Baldwin, 1986; Matthews et al., 1987) can also improve the stability of tertiary structure considerably. Thus, it appears that while the catalytic function of most enzymes has evolved to perfection where the catalytic efficiency can no longer be improved (Albery and Knowles, 1976; 1977), the stability of proteins is not at the stage where no improvement can be made. In fact, for most proteins the folded state is only marginally stable than that of unfolded state (5-15 kcal/mol for the stabilization of folded state; Pfeil, 1981; Creighton, 1983).

Two possible reasonings of the low stability of proteins are that the stabilities of most proteins have not evolved to and are therefore still in the process toward perfection and alternatively, the low stability is a required property of proteins and it should neither be reduced nor enhanced. The latter reasoning is favored considering that a certain rate of turnover of proteins can be used for metabolic control of physiological reactions.

In the case of AK, the stabilization energy between the folded and unfolded states is only 3.7 kcal/mol, smaller than that for most of other enzymes. If the stability parameter of a protein is an indication of the lifetime of that protein in cells, the very low stability of chicken muscle AK may simply reflect the requirement by the cells of a rather fast turnover of this enzyme. It is interesting to note that the stability of porcine muscle AK
is almost identical to that of chicken muscle AK even though there are 29
different residues between the two enzymes (Tian et al., 1988), which may
suggest that the stability of AK may have been well adjusted to the
requirement by evolution pressure.
CHAPTER IV

RATE-LIMITING STEPS OF AK REACTION

A. INTRODUCTION

A random Bi-Bi kinetic mechanism of adenylate kinase was first proposed by Rhoads and Lowenstein (1968). By using rabbit muscle adenylate kinase and measuring isotope exchange rates at equilibrium, they were able to exclude ordered Bi-Bi and ping-pong mechanisms and to conclude that the kinetic mechanism for the rabbit muscle adenylate kinase is random Bi-Bi (Scheme I. E, A, B, P, and Q represent, respectively, the enzyme, MgATP, AMP, MgADP, and ADP). Studies on the kinetic mechanism have also been carried out on adenylate kinase of different sources (Muscle, erythrocyte, yeast, etc.), by using a variety of kinetic
methods such as initial velocity analysis (Hanada & Kuby, 1978), substate (Brownson & Spencer, 1972) and product inhibition (Khoo & Russell, 1970; Brownson & Spencer, 1972), and isotope exchange at equilibrium (Markland & Wadkins, 1966; Su & Russell, 1968). All of these studies suggested a random Bi-Bi mechanism, except one which suggests an ordered Bi-Bi for liver AK (Markland & Wadkins, 1966). However, as pointed out by Noda (1973), this ordered mechanism is yet to be confirmed. Quantitative or semi-quantitative information about the mechanism have also been obtained from the above studies, and from quantitative NMR analysis (Brown & Ogawa, 1977; Nageswara et al., 1978). The kinetic results obtained from the previous studies are summarized in Table 8.

While it appears that the random Bi-Bi mechanism has been firmly established, at least for muscle AK, conclusions on the rate-limiting step(s) have been controversial. Among the nine experiments on AK from different sources (Table 8), six suggest that the rate-limiting step is the chemical step, two suggest that the chemical step is not rate-limiting, and one suggests that the chemical step is partially rate-limiting (see Table 8). Controversial results have also been found even for AK from the same sources. For example, for rabbit muscle AK, opposite conclusions have been reached from isotope exchange and NMR studies, and for porcine muscle AK, different results have been obtained from two types 31P NMR experiments. Therefore, the question of which step is rate-limiting for muscle adenylate kinase remains unresolved. For chicken muscle adenylate kinase, which has been used in our mutagenetic studies as described in Chapter III, no quantitative results regarding the rate-limiting step have been previously reported.
Table 8. Summary of kinetic information of adenylate kinase.

<table>
<thead>
<tr>
<th>Source of enzyme</th>
<th>Kinetic mechanism</th>
<th>Rate limiting step</th>
<th>Methods and Reference*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit muscle</td>
<td>random Bi-Bi</td>
<td>on and off steps</td>
<td>isotope exchange (1)</td>
</tr>
<tr>
<td>Rabbit muscle</td>
<td>random Bi-Bi</td>
<td>chemical step</td>
<td>initial velocity analysis (2)</td>
</tr>
<tr>
<td>Rabbit muscle</td>
<td>chemical step</td>
<td></td>
<td>2D NMR (3)</td>
</tr>
<tr>
<td>Porcine muscle</td>
<td>off step</td>
<td></td>
<td>31P NMR lineshape analysis (4, 5)</td>
</tr>
<tr>
<td>Porcine muscle</td>
<td>chemical step</td>
<td></td>
<td>31P NMR inversion transfer (partially) (6)</td>
</tr>
<tr>
<td>Calf muscle</td>
<td>random Bi-Bi</td>
<td>chemical step</td>
<td>initial velocity analysis (2)</td>
</tr>
<tr>
<td>Calf liver</td>
<td>random Bi-Bi</td>
<td>chemical step</td>
<td>initial velocity analysis (2)</td>
</tr>
<tr>
<td>Liver</td>
<td>ordered Bi-Bi</td>
<td></td>
<td>isotope exchange (7)</td>
</tr>
<tr>
<td>Human erythrocyte</td>
<td>random Bi-Bi</td>
<td>chemical step</td>
<td>initial velocity analysis and product inhibition (8)</td>
</tr>
<tr>
<td>Bakers' yeast</td>
<td>random Bi-Bi</td>
<td>chemical step</td>
<td>initial velocity analysis and inhibition (9)</td>
</tr>
<tr>
<td>Bakers' yeast</td>
<td>random Bi-Bi</td>
<td>chemical step</td>
<td>isotope exchange (10)</td>
</tr>
</tbody>
</table>


bAs pointed out by Noda (1973), this ordered mechanism is yet to be confirmed.
Resolution of the above question is thus important in quantitative interpretation of the steady state kinetic data from site-specific mutagenesis studies. For example, if the rate-limiting step is the chemical step in WT, any perturbations in the $k_{\text{cat}}$ of mutants can be directly attributed to perturbations to the chemical step and no perturbations indicate the non-importance in catalysis of the studied residues. If on the other hand, the catalysis is diffusion-controlled in WT, lack of changes in $k_{\text{cat}}$ of mutants do not indicate the absence of perturbations to the chemical step, and means other than the steady state kinetic method should be used.

The second-order rate constant for diffusion-controlled enzymatic reaction is predicted to be in the range of $10^8-10^{10}$ M$^{-1}$ s$^{-1}$ (Hammes and Schimmel, 1970). The $k_{\text{cat}}/K_m$ (MgATP) for chicken muscle AK is $2 \times 10^7$ M$^{-1}$ s$^{-1}$, which is somewhat lower than the predicted diffusion limit. However, this does not indicate whether the AK reaction is diffusion-controlled or the chemical step is rate-limiting. The observed second-order rate constant of a diffusion-controlled enzymatic reaction can be considerably lower than the upper limit of $10^8-10^{10}$ M$^{-1}$ s$^{-1}$, if the substrate only binds to a minor form of the enzyme or the enzyme must find a rare form of the substrate (Blacklow et al., 1988).

Many methods are available for elucidation of rate-limiting steps in enzyme catalysis. Among them the method of determining the isotope partition (Rose et al. 1974) has been frequently used particularly for the enzyme reactions of random mechanism. The viscosity variation method, which is in principle designed for detecting the contribution of diffusion step to the overall reaction rate, can also provide information about the chemical step in favorable circumstances, and it has been applied to many
enzymes, such as phosphorylase b (Damjanovich et al., 1972), horseradish peroxidase (Dunford and Hewson, 1977), chymotrypsin (Brouwer and Kirsch, 1982), β-lactamase (Hardy and Kirsch, 1984), cytochrome c peroxidase (Loo and Erman, 1977), carbonic anhydrase (Hasinoff, 1984; Pocker and Janjic, 1987), invertase (Monsan and Combes, 1984), acetylcholinesterase (Bazelyansky et al., 1986), adenosine deaminase (Kurz et al., 1987), and triosephosphate isomerase (Blacklow et al., 1988).

In this chapter we report the use of viscosity variation kinetics to probe the rate-limiting steps in the catalysis of chicken muscle AK. This method is sometimes complicated by the effects of viscosity on binding or conformational steps (Kurz et al., 1987; Grissom and Cleland, 1988; Gates and Northrop, 1988), use of site-specific mutants have allowed us to take the advantage of such effects and identify a viscosity-sensitive, rate-limiting conformational step.

The mutants used in this study are the His-36 mutants. Our recent results (Tian et al., 1988) suggest that His-36 is not directly involved in catalysis, but stabilizes the tertiary structure. However, we observed increased $K_m$ values in the mutants, particularly in H36Q (see Chapter II). The increases in $K_m$ values were not interpreted as an indication of direct involvement of His-36 in substrate binding based on their relatively small sizes (ca. 10x) and the occurrence to both substrates. However, we speculated that the increased $K_m$ values could reflect compromises in the conformational change in the H36Q mutant of AK (Chapter III; Tian et al., 1988). The potential role of His-36 in conformational changes has also been suggested earlier on the basis of the comparison of the crystal structures of different crystal forms of AK (Sachsenheimer & Schulz,
The perturbation of the C2-H resonance of His-36 in the proton NMR of AK by both substrates (McDonald et al., 1975; Sanders et al., 1989) are consistent with the involvement of His-36 in conformational changes, although the NMR result may also be interpreted to suggest interaction of His-36 with the phosphate groups of substrates (McDonald et al., 1975). Our results in this paper suggest that His-36 is involved, whether directly or indirectly, in conformational changes induced by binding of AMP or MgATP.

The viscosity variation method can be successfully used only when non-specific viscogenic effects on the enzyme activity do not exist. Conventionally this possibility is ruled out by using slowly reacting substrate analogues, with which reaction rates are slow enough that the chemical step is completely rate-limiting (Brouwer & Kirsch, 1982) and thus any viscogenic effects on the chemical step, if exist, can be magnified. Other ways including using mutants with small $k_{cat}$ (Blacklow et al., 1988), observing unaffected binding constants (Gates and Northrop, 1988), utilizing more than one viscogens (Grissom and Cleland, 1988), etc. are also practised sometimes. In the present work, a series of slowly reacting substrate analogues were used for this purpose.

B. THEORY

Viscosity Effect in a Random Bi-Bi system. The viscosity-dependent kinetic equations have previously been derived for single substrate systems (Loo and Erman, 1977; Nakatani and Dunford, 1979; Brouwer and Kirsch, 1982) and for a Theorell-Chance system (Gates and
Northrop, 1988), but not for a random Bi-Bi system. The kinetic scheme for a random Bi-Bi system with a saturating substrate A (MgATP in this case) can be expressed by Scheme II, where A, B, P, and Q represent MgATP, AMP, MgADP, and ADP, respectively.

\[
\begin{align*}
E &\xrightarrow{k_1[A]} EA &\xrightarrow{k_2[B]} EAB &\xrightarrow{k_3} EPQ &\xrightarrow{k_4} P \xrightarrow{k_6} Q
\end{align*}
\]

Scheme II. Reaction mechanism of random Bi-Bi when the substrate A is at saturating level.

Take the advantage of Cleland's net rate method (Cleland, 1975), the reaction rate constant \(1/k_{\text{cat}}\) can be expressed as

\[
\frac{1}{k_{\text{cat}}} = \frac{1}{k_3} + \frac{1}{k_4+k_5} + \frac{1}{k_6(k_4+k_5)} + \frac{k_4^0}{k_7(k_4+k_5)} + \frac{k_5^0}{k_7(k_4+k_5)} \quad (4.1)
\]

where \(k^0\)'s are rate constants of viscosity sensitive steps. Since \(k^0 = k / \eta_{\text{rel}}\) (Kramers, 1940), in which \(k\) is the rate constant in the absence of viscogen and \(\eta_{\text{rel}}\) is the relative viscosity of reaction medium, eq. 4.1 can be expressed as
\[ \frac{1}{k_{\text{cat}}} = a + b \eta_{\text{rel}} \]  

(4.2)

where

\[ a = \frac{1}{k_3} \]  

(4.3)

and

\[ b = \left[ 1 + \frac{1}{K_3} + \frac{k_4}{k_8} + \frac{k_5}{k_7} \right] \frac{1}{k_4 + k_5} \]  

(4.4)

Similarly,

\[ \frac{K_m}{k_{\text{cat}}} = a + b \eta_{\text{rel}} \]  

(4.5)

in which

\[ a = \frac{1}{k_3 K_2} \]  

(4.6)

and
According to eq. 4.2 and eq. 4.5, both of $1/k_{cat}$ and $K_m / k_{cat}$ are linear functions of the relative viscosity with a positive ordinate intercept.

The normalized equation is

$$\frac{\left(k_{cat}/K_m\right) \eta_{rel} = 1}{\left(k_{cat}/K_m\right)} = a + b \eta_{rel}$$

(4.8)

in which

$$a = \frac{1}{1 + k_3/k_2 + k_3/(k_4 + k_5)}$$

(4.9)

and

$$b = \frac{1}{1 + k_2/K_3 + k_2/K_3(k_4 + k_5)} + \frac{1}{1 + (k_4 + k_5)/k_3 + K_3(k_4 + k_5)/k_2}$$

(4.10)

Again, this is a linear equation with a positive ordinate intercept. The values of $a$ and $b$ in eq. 4.8 can vary between 0 and 1 depending on the relative rates of the diffusional and chemical steps of the reaction. Two limiting situations are as follows. When both $k_3 \ll k_2$ and $k_3 \ll k_4 + k_5$ (i.e., if the chemical step is fully rate-limiting), $a = 1$ and $b = 0$; when
both $k_3 \gg k_2$ and $k_3 \gg k_4 + k_5$ (i.e., if the reaction is fully diffusion controlled), then $a = 0$ and $b = 1$.

D. RESULTS

The Chemical Step is Not Fully Rate-limiting. As has been discussed in THEORY, if the chemical step is rate-limiting, the slope of eq. 4.8 should be zero. The viscosity variation data for WT AK are listed in Table 9, and plotted according to equations 4.2, 4.5, and 4.8 (Figure 30). As shown in Figure 30C, the slope is non-zero, which suggests that the chemical step of WT AK is not completely rate-limiting provided that it can be shown that the viscogen sucrose does not affect the rate of chemical step. We have noticed that the ordinate intercepts of all the three plots for WT AK are negative, which cannot be interpreted according to the viscosity theory assuming the diffusional step is the only viscosity-sensitive step. Thus, the possibility of nonspecific effects of the viscogen on the chemical step as the cause of the observed non-zero slope has to be considered. A series of slowly reacting substrate analogues have thus been used in the viscosity studies, and the possibility of nonspecific viscogenic effects has been ruled out as described below. The results also show that the chemical step is not fully but partially rate-limiting.

The Chemical Step Is not Affected by Sucrose. To show that the possibility of nonspecific viscogenic effects do not exists, we have carried out the viscosity variation procedure on the following substrate pairs: ATP + AMP (I), dATP + AMP (II), ATP + dAMP (III), and dATP + dAMP (IV). The rate constants of these substrate pairs were determined
Table 9. Data of viscosity variation of the steady state kinetic constants of WT AK and His-36 mutants.

<table>
<thead>
<tr>
<th>η_{rel} (sucrose%)</th>
<th>k_{cat} (s^{-1})</th>
<th>K_m (AMP) (mM)</th>
<th>k_{cat} / K_m (M^{-1}s^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>WT AK</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.00 (0%)</td>
<td>610</td>
<td>0.094</td>
<td>6.5 X 10^6</td>
</tr>
<tr>
<td>1.33 (10%)</td>
<td>370</td>
<td>0.096</td>
<td>3.8 X 10^6</td>
</tr>
<tr>
<td>1.77 (20%)</td>
<td>250</td>
<td>0.100</td>
<td>2.5 X 10^6</td>
</tr>
<tr>
<td>2.50 (30%)</td>
<td>160</td>
<td>0.100</td>
<td>1.6 X 10^6</td>
</tr>
<tr>
<td>3.90 (40%)</td>
<td>95</td>
<td>0.100</td>
<td>0.9 X 10^6</td>
</tr>
<tr>
<td><strong>H36N</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.00 (0%)</td>
<td>600</td>
<td>0.390</td>
<td>1.6 X 10^6</td>
</tr>
<tr>
<td>1.33 (10%)</td>
<td>480</td>
<td>0.340</td>
<td>1.4 X 10^6</td>
</tr>
<tr>
<td>1.77 (20%)</td>
<td>340</td>
<td>0.320</td>
<td>1.1 X 10^6</td>
</tr>
<tr>
<td>2.50 (30%)</td>
<td>230</td>
<td>0.300</td>
<td>0.8 X 10^6</td>
</tr>
<tr>
<td>3.90 (40%)</td>
<td>150</td>
<td>0.280</td>
<td>0.5 X 10^6</td>
</tr>
<tr>
<td><strong>H36Q</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.00 (0%)</td>
<td>640</td>
<td>0.980</td>
<td>6.5 X 10^5</td>
</tr>
<tr>
<td>1.33 (10%)</td>
<td>380</td>
<td>0.710</td>
<td>5.4 X 10^5</td>
</tr>
<tr>
<td>1.77 (20%)</td>
<td>340</td>
<td>0.640</td>
<td>5.3 X 10^5</td>
</tr>
<tr>
<td>2.50 (30%)</td>
<td>240</td>
<td>0.630</td>
<td>3.9 X 10^5</td>
</tr>
<tr>
<td>3.90 (40%)</td>
<td>200</td>
<td>0.600</td>
<td>3.3 X 10^5</td>
</tr>
</tbody>
</table>
Figure 30. Plots of kinetic constants of WT vs. relative viscosity $\eta_{\text{rel}}$ according eq. 2, 5, and 8. The reactions were conducted at pH 8.0, 24 °C. The units for $1/k_{\text{cat}}$, $K_m/k_{\text{cat}}$, and $R(k_{\text{cat}}/K_m)/(k_{\text{cat}}/K_m)_{\text{rel}=1}/(k_{\text{cat}}/K_m)$ are $10^{-3}$ s, $10^{-7}$ M s, and 1, respectively.
both in the absence and presence of viscogen. In the absence of viscogen, both $k_{\text{cat}}$ and $k_{\text{cat}}/K_m$ decrease in the order $I > II > III > IV$ as shown in Table 10. Since the decreases in $k_{\text{cat}}/K_m$ mainly result from the reduction in $k_{\text{cat}}$ when slow substrate pairs are used, we conclude that the rate of chemical step is reduced by using the slowly reacting substrates. In the presence of viscogen, the slope of the plot of $(k_{\text{cat}}/K_m)_{\text{rel}} = 1/(k_{\text{cat}}/K_m)$ vs. $\eta_{\text{rel}}$ using these substrate pairs decreases also in the order $I > II > III > IV$, as shown in Figure 31. The results suggest that the viscosity effects becomes smaller upon using more slowly reacting substrates. When the substrate pairs ATP + dAMP (III) ($k_{\text{cat}}$ decreased by 5.5x and $k_{\text{cat}}/K_m$ by 26x) or dATP + dAMP (IV) ($k_{\text{cat}}$ decreased by 22x and $k_{\text{cat}}/K_m$ by 43x) is used, the slope of the plot of $(k_{\text{cat}}/K_m)_{\text{rel}} = 1/(k_{\text{cat}}/K_m)$ vs. $\eta_{\text{rel}}$ becomes zero (viscosity-independent), where the chemical step becomes rate-limiting. Therefore, we have ruled out the possibility of nonspecific viscogenic effects on the chemical step of the enzyme reaction, which in turn confirms that the chemical step is not fully rate-limiting shown by the non-zero slope of the eq. 4.8 (Figure 30C).

The Chemical Step Is Partially Rate-limiting. The same experimental data also suggest that the chemical step is at least partially rate-limiting. The substrate system III (ATP + dAMP) showed only 5x decrease in $k_{\text{cat}}$ relative to the natural substrates (ATP + AMP) and became viscosity-independent. The difference between substrate systems III and IV can be attributed to the difference between ATP and dATP, which only causes a 4x decrease in $k_{\text{cat}}$ and a $< 2x$ decrease in $k_{\text{cat}}/K_m$. Since the system III is viscosity-independent (i.e., the chemical step is rate-
Table 10. Summary of steady state kinetic data on substrate analogues.

<table>
<thead>
<tr>
<th>Substrates</th>
<th>$k_{\text{cat}}$ (s⁻¹)</th>
<th>$(k_{\text{cat}})^o/k_{\text{cat}}$</th>
<th>$K_m$(AMP) (mM)</th>
<th>$k_{\text{cat}}/K_m$</th>
<th>$(k_{\text{cat}}/K_m)^o/(k_{\text{cat}}/K_m)$</th>
</tr>
</thead>
<tbody>
<tr>
<td>I ATP+AMP</td>
<td>610</td>
<td>1.0</td>
<td>0.094</td>
<td>6.5 x 10⁻⁶</td>
<td>1.0</td>
</tr>
<tr>
<td>II dATP+AMP</td>
<td>400</td>
<td>1.5</td>
<td>0.072</td>
<td>5.6 x 10⁻⁶</td>
<td>1.2</td>
</tr>
<tr>
<td>III ATP+dAMP</td>
<td>110</td>
<td>5.5</td>
<td>0.450</td>
<td>0.25 x 10⁻⁶</td>
<td>26</td>
</tr>
<tr>
<td>IV dATP+dAMP</td>
<td>28</td>
<td>22</td>
<td>0.190</td>
<td>0.15 x 10⁻⁶</td>
<td>43</td>
</tr>
</tbody>
</table>

*° represents that of the nature substrate pair: ATP + AMP.*
Figure 31. Plots of $R(k_{cat}/K_m) = (k_{cat}/K_m) \eta_{rel} = 1/(k_{cat}/K_m)$ vs. $\eta_{rel}$ for WT with substrate systems I-IV.
limiting), such a difference should be due to a slightly higher transition state energy of the chemical step of IV relative to that of III. The same small difference in the transition state energy of the chemical step is expected to occur between systems I and II. Such a small difference should not perturb the viscosity dependence if the chemical step is not a partially rate-limiting step. As shown in Table 10, the $k_{cat}$ of the substrate pair II is only slightly decreased (significantly smaller than the differences between III and IV) from the normal substrate pair I, which is consistent with the conclusion in the proceeding section that the chemical step is not the rate-limiting step. However, the small perturbation in the chemical step is sufficient to cause a decrease in the viscosity dependence, as shown in Figure 31. The results suggest that the chemical step of WT AK is also partially rate-limiting when the natural substrates are used.

A Comformational Step Is Partially Rate-limiting. After showing that there does not exist non-specific viscogenic effects on the chemical step and that the chemical step is not fully but partially rate-limiting, then we ask why the y-intercepts in all three plots of Figure 30 (WT AK) are negative. Such abnormal behavior cannot be properly described by equations 4.2, 4.5, and 4.8. A possible explanation is that a viscosity-sensitive conformational change step is at least partially rate-limiting. To examine this possibility, we chose to test the mutant H36Q which we speculated on the basis of increased $K_m$ values (Table 11) that a conformational step may have been impaired (Tian et al., 1988). The mutant H36N is intriguing because it has $K_m$ values in between WT and H36Q (Table 11) and thus it is also used for the test. The viscosity-dependent kinetic data on H36Q and H36N are listed also in Table 9 and
Table 11. Steady State Kinetic Data and Dissociation Constants From Previous Work

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$k_{cat}^a$ (s$^{-1}$)</th>
<th>$K_m$ (mM)$^a$</th>
<th>$k_{cat}/K_m$ (x10$^{-6}$ M$^{-1}$s$^{-1}$)</th>
<th>$K_d$ (mM)$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MgATP</td>
<td>AMP</td>
<td>MgATP</td>
<td>AMP</td>
</tr>
<tr>
<td>WT</td>
<td>650</td>
<td>0.035</td>
<td>0.092</td>
<td>1.9</td>
</tr>
<tr>
<td>H36N</td>
<td>600</td>
<td>0.078</td>
<td>0.41</td>
<td>7.6</td>
</tr>
<tr>
<td>H36Q</td>
<td>680</td>
<td>0.21</td>
<td>0.98</td>
<td>3.2</td>
</tr>
</tbody>
</table>

$^a$From Tian et al. (1988). $^b$From proton NMR studies (Sanders and Tsai, 1989a).
Figure 32. Plots of kinetic constants of H36N vs. relative viscosity $\eta_{rel}$ according eq. 2, 5, and 8. The reactions were conducted at pH 8.0, 24 °C. The units for $1/k_{cat}$, $K_m/k_{cat}$, and $R(k_{cat}/K_m)(= (k_{cat}/K_m)\eta_{rel}^{-1}/(k_{cat}/K_m))$ are $10^{-3}$ s, $10^{-7}$ M s, and 1, respectively.
Figure 33. Plots of kinetic constants of H36Q vs. relative viscosity $\eta_{rel}$ according eq. 2, 5, and 8. The reactions were conducted at pH 8.0, 24 °C. The units for $1/k_{cat}$, $K_m/k_{cat}$, and $R(k_{cat}/K_m)$ = $k_{cat}/K_m$)$\eta_{rel}=1/(k_{cat}/K_m)$) are $10^{-3}$ s, $10^{-6}$ M s, and 1, respectively.
plotted in Figure 32 (H36N) and Figure 33 (H36Q). The y-intercepts in Figure 33 are normal (positive), and the behavior of H36N (Figure 32) is in between WT and H36Q. The extent of viscosity dependence (i.e. the slopes) also follows the order WT > H36N > H36Q. These results suggest that a component of the viscosity dependence, most likely contributed by the conformational step in WT, is absent in H36Q. This conclusion is supported by the experiments which show that substrate synergism in WT is removed in H36Q and that substrate binding of WT is affected by the variation of viscosity as reported in the next two sections.

Substrate Synergism and the Conformational Change. The previous kinetic data on WT and His-36 mutants (Table 11) and the present results shown in Figures 29, 31, and 32 suggest the existence of a partially rate-limiting conformational change and indicate a successive removal of such a conformational change by substituting His-36 with Asn and Gln. Since the side-chain nitrogen atom of Asn and Gln has been suggested to mimic the N1 and N3, respectively, of histidine (Lowe et al., 1985), the results suggest that the His-36 of AK is likely to be involved in the conformational change via the N1 atom.

The above interpretation is consistent with the increasing $K_m$ values in the order WT < H36N < H36Q as shown in Table 11 (Tian et al., 1988). However, the effect on $K_m$ values of the mutants could also be caused by impaired binding capability of the enzyme, rather than impaired conformational change. To differentiate the two possibilities, we determined both dissociation constant $K$ (for ternary complexes, equivalent to $K_m$), and $K_i$ (for binary complexes, equivalent to $K_d$) by a full initial velocity analysis (varying both substrates). The initial velocity patterns for
WT and the two mutants are shown in Figure 34. It is important to note that the crossover point lies above, near, and below the horizontal axis in the plots of WT, H36N, and H36Q, respectively. This suggests that the ratio $K_i / K$ is above, near and below zero for WT, H36N, and H36Q, respectively (Cleland, 1986).

The quantitative $K$ and $K_i$ values (Table 12) were calculated from eq. 4.11:

$$v = \frac{ABV}{K_a K_{ib} + K_b A + K_b B + AB}$$

(4.11)

where the subscripts $a$ and $b$ represent the two substrates MgATP and AMP, respectively, $A$ and $B$ are the concentrations of the corresponding substrates, and $V$ is the maximum velocity. The results (first three rows of Table 12) indicate that the $K_i$ values vary to a small extent only ($< 3x$) whereas the $K$ values vary more significantly ($\leq 14x$). The ratio $K_i / K$ is 3.8 for WT, which indicate the existence of substrate synergism. The ratio remained relatively constant for two other mutants: 3.6 for K21M and 3.1 for K27M (Chapter III). However, it decreased to 1.9 and 0.6 for H36N and H36Q (Table 12), respectively, which suggests lack of substrate synergism in H36Q. These results suggest that the capability of H36Q to bind the first substrate is not impaired but the binding affinity to the second substrate is lowered, which supports the involvement of the N1 of His-36 in the conformational change induced by binding of the first substrate.
Table 12. $K_i$ and $K$ values of AK and its mutants showing the extent of substrate synergism and the effects of viscosity.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$\eta_{rel}$</th>
<th>$K_{MgATP}$ (mM)</th>
<th>$K_{AMP}$ (mM)</th>
<th>$K_{i(MgATP)}$ (mM)</th>
<th>$K_{i(AMP)}$ (mM)</th>
<th>$K_i/K$</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT$^a$</td>
<td>1.0</td>
<td>0.042</td>
<td>0.098</td>
<td>0.16</td>
<td>0.37</td>
<td>3.8</td>
</tr>
<tr>
<td>H36N$^a$</td>
<td>1.0</td>
<td>0.13</td>
<td>0.52</td>
<td>0.26</td>
<td>1.0</td>
<td>1.9</td>
</tr>
<tr>
<td>H36Q$^a$</td>
<td>1.0</td>
<td>0.54</td>
<td>1.43</td>
<td>0.29</td>
<td>0.78</td>
<td>0.55</td>
</tr>
<tr>
<td>WT$^b$</td>
<td>1.3</td>
<td>0.033</td>
<td>0.10</td>
<td>0.045</td>
<td>0.15</td>
<td>1.4</td>
</tr>
<tr>
<td></td>
<td>1.8</td>
<td>0.032</td>
<td>0.092</td>
<td>0.042</td>
<td>0.11</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>0.028</td>
<td>0.10</td>
<td>0.026</td>
<td>0.10</td>
<td>1.0</td>
</tr>
<tr>
<td>H36N$^c$</td>
<td>3.9</td>
<td>0.17</td>
<td>0.30</td>
<td>0.063</td>
<td>0.11</td>
<td>0.38</td>
</tr>
<tr>
<td>H36Q$^c$</td>
<td>3.9</td>
<td>0.12</td>
<td>0.58</td>
<td>0.11</td>
<td>0.53</td>
<td>0.92</td>
</tr>
</tbody>
</table>

$^a$From Figure 34. $^b$From Figure 35. $^c$From Figure 36.
Figure 34. Initial velocity patterns for WT (A), H36N (B), and H36Q (C), in the absence of viscogen.
It should be noted that the $K$ and $K_i$ values listed in Table 12 are strictly valid only if the mechanism is rapid equilibrium random (Cleland, 1986). Comparison between the data in the first row of Table 11 and Table 12 indicates that $K_i = K_d$, and $K = K_m$ within a factor of 2. Thus although the mechanism is not strictly rapid equilibrium, it is close to rapid equilibrium (which supports the conclusion from the previous section that the chemical step is partially rate-limiting).

**Viscosity Effects on the Substrate Synergism.** The existence of substrate synergism is consistent with the substrate induced-fit mechanism and it is strongly supported by the observation of viscosity effects on the dissociation constant $K$ and $K_i$.

The initial velocity patterns of WT AK at different viscosities showed that increasing viscosity causes the $K_i$ of WT but not the mutants to decrease significantly. The kinetic constants obtained from this set of experiments are plotted in Figure 35 and also listed in Table 12 (rows 4-6). At 40% sucrose the $K_i$ of WT becomes smaller and the initial velocity pattern looks quite parallel, as shown in Figure 36A. In contrast, the initial velocity pattern for H36Q at 40% sucrose (Figure 36C) is only slightly different from that at 0% sucrose (Figure 34C), and the $K_i$ decreases from 0.29 to 0.11 mM for MgATP and from 0.78 to 0.53 mM for AMP, which are small compared to the changes in WT. Even at 30% sucrose, the $K_i$ of WT decreases from 0.16 to 0.026 mM for MgATP and from 0.37 to 0.10 mM for AMP. The difference between WT and H36Q may be attributed to a change in the equilibrium of the conformational step, since viscosity in principle does not affect an equilibrium of the formation of a simple collision complex (Hardy & Kirsch, 1984). The behavior of H36N is again
Figure 35. Viscosity effect on $K_i$ and $K$. The data were normalized by dividing $K_i$ or $K$ by $K_i$ or $K$ at $\eta_{rel} = 1$. 
Figure 36. Initial velocity patterns for WT (A), H36N (B), and H36Q (C), in the presence of 40% sucrose (relative viscosity = 3.9).
intermediate between WT and H36Q. Thus, the results suggest that viscosity affects $K_i$ by shifting the equilibrium of a conformational step in the overall binding process. It is interesting to note from the data in Table 12 that increasing the viscosity has caused the ratio $K_i/K$ to approach 1 for both WT and H36Q. This suggests that increasing viscosity removes the substrate synergism of WT AK by enhancing binding of the first substrate.

The Catalysis Is Partially Diffusion-controlled. The above results indicate that a conformational step which is partially rate-limiting in WT has been removed in H36Q. Therefore, for the mutant H36Q, the partial rate-limiting by the diffusion step may accounts for the observed slope (0.3) (Figure 32C). A slope of 0.3 indicates that the diffusion step is less rate-limiting than the chemical step (1 for a full control by the diffusional step, and 0 for a full control by the chemical step). Since WT and H36Q have similar $k_{cat}$ values, the WT catalyzed reaction is also likely partially limited by the diffusion step.

Is AK a Perfect Catalyst? Since the catalysis is partially diffusion controlled and other rate-limiting steps have similar free energy barrier to the diffusion step, AK should be a nearly "perfect enzyme" (Albery and Knowles, 1976). We then further examined a few properties pertaining to the catalytic capability of AK. The catalytic efficiency of AK is $> 10^{12}$x relative to uncatalyzed reaction between AMP and MgATP within the limit of detection (Sanders and Tsai, 1989b), which is approaching the upper limit of enzyme catalysis. Unlike other kinases such as hexokinase, AK showed no detectable ATPase activity in the absence of AMP ($< 3 \times 10^{-6} k_{cat}$, also true for H36Q). As shown in Figure 37, the isotope trapping experiments (Stackhouse et al., 1985) indicated that both internal and
Figure 37. Determination of $K_{int}$ by plotting $[\text{ADP}]/2[\text{ATP}]$ versus $[\text{AK}]$. When the plateau is reached all the nucleotides are completely bound, thus the ratio (1.5) represents the internal equilibrium constant. The ratio extrapolated to $[\text{AK}] = 0$ represents the external equilibrium constant (0.6).
external equilibrium constants (1.5 and 0.6, respectively) are close to one. The \( k_{cat} \) of the reverse reaction (830 s\(^{-1}\)) is comparable to that of the forward reaction. The significance of some of these aspects are elaborated in Discussion.

**D. DISCUSSION**

**Effects of Viscosity on Conformational Changes.** It has been recognized by many researchers that conformational changes involving intramolecular isomerization and other functionally important motions could be viscosity-dependent (Pocker & Janjic, 1987; Faukenfelder & Wolynes, 1985; Perutz et al., 1987; Crissom & Cleland, 1988; Gates & Northrop, 1988). If the isomerization of the two alleged conformational states involves fluctuation of a large portion of the protein in a diffusion-like motion (Karplus & McCammon, 1983), an increase in solvent friction induced by viscogenic cosolute may retard the rate of this process.

Our results suggest the existence of a conformational change induced by binding of the first substrate to the WT AK. It should be noted that viscosity affects both the rates (reflected in the viscosity dependence of \( k_{cat} \)) and the equilibrium (reflected in the viscosity dependence of \( K_1 \)) of this particular conformational step. The equilibrium of substrate binding (the first step, viscosity insensitive) including a conformational change (the second step, viscosity sensitive) can be described by:

\[
E_1 + S \xrightleftharpoons{K_1} E_1S \xrightarrow{K_2} E_2S
\]  

(4.12)
According to the following definitions:

\[
K_1 = \frac{[E_i][S]}{([E_i][S] + [E_2][S])}
\] (4.13)

and

\[
K_1 = \frac{[E_i][S]}{[E_i][S]} \quad K_2 = \frac{[E_2][S]}{[E_i][S]}
\] (4.14)

it can be shown that

\[
K_i = \frac{1}{(1 + K_2)K_1}
\] (4.15)

Depending on the kind of effect of viscosity on \(K_2\), \(K_1\) can either increase or decrease upon viscosity variation provided that \(K_2\) is large enough compared to unity.

Since \(K_i\) of H36Q is similar to that of WT, \(K_2\) of WT should not be very large. If it is assumed that \(K_1\) is insensitive to mutagenesis but \(K_2\) becomes zero in H36Q due to removal of the second step, then \(K_1 = 3400\) M\(^{-1}\) (MgATP) and 1280 M\(^{-1}\) (AMP) for both WT and His-36 mutants, and \(K_2 = 0.84\) (MgATP) and 1.1 (AMP) for WT and 0.13 (MgATP) and 0 (AMP) for H36N. When the relative viscosity is raised to 2.5 (30% sucrose), the \(K_i\) values of WT reduced to ca. 20% as shown in Figure 35,
which means that $K_2$ is raised to ca. 10 for WT.

A more general scheme is described by eq. 4.16, in which $E_2$ exists

![Diagram](image)

\begin{equation}
K_1 = \frac{1+K'_2}{(1+K_2)K_1}
\end{equation}

(4.17)

where $K'_2 = [E_2]/[E_1]$. Eq. 4.17 reduces to eq. 4.15 if $K'_2 << K_2$. If $K'_2 = K_2$, $K_1$ will not be affected very much by the viscosity-sensitive conformational changes even if the magnitude of $K'_2$ and $K_2$ is not small. Conformational changes may also take place after the formation of the ternary complex. However, they may not be detectable in our viscosity variation experiments if they are not sensitive to viscosity change.

It has been suggested that the equilibrium point of the conformational isomerization could be shifted by viscosity variation in favor of the more compact conformational state (Lee & Timasheff, 1981; Pocker & Janjic, 1987; Gates & Northrop, 1988). Since the conformational change of AK (and kinases in general) has been shown to involve closing of an open cleft
(Anderson et al., 1979), it is reasonable that increasing viscosity enhances the conformational change (i.e., favors the more compact form). The viscosity effect on conformational steps has been observed previously in other enzymes: aminoglycoside nucleotidyltransferase 2'-I (Gates and Northrop, 1988), adenosine deaminase (Kurz et al., 1987), and chicken liver NADP malic enzyme (Grissom and Cleland, 1988).

**Kinetic and Catalytic Properties of AK.** Since a conformational change can be clearly detected by proton NMR upon binding of the first substrate and the second substrate (Sanders et al., 1989), and since the random mechanism has been well established for AK (Rhoads and Lowenstein, 1968), a partial kinetic scheme for WT AK can be established as shown in the solid lines of Scheme III, where $E_1$, $E_2$ and $E_3$ represent different conformational states of the enzyme. Although AK-MgATP and AK-AMP ($E_2A$ and $E_2B$, respectively) have qualitatively similar, but quantitatively different conformations (Sanders et al.,

![Scheme III](image-url)
Figure 38. Qualitative free energy diagram of WT AK. The dotted lines indicate the effects of increasing viscosity detected in the results of this work. It should be noted that although the step $E_2AB \rightarrow E_3AB$ is not shown as a rate-limiting step, the possibility of its being a viscosity independent rate-limiting step cannot be ruled out.
1989), they are kinetically indistinguishable in a random mechanism and are designated by the same conformational state of the enzyme. The dashed lines in Scheme III represent the steps which may or may not exist. A particularly interesting question is the fate of $E_{1}AB$ for H36Q: conversion to $E_{2}AB$, to $E_{3}AB$, or to products directly.

Our results suggest that in the kinetic scheme shown in Scheme III, the diffusion steps (strictly speaking the step $EMgATP + AMP$ according to the way the experiments were performed, possibly including other diffusion steps), the conformational change induced by the first substrate, and the chemical step are all partially rate-limiting, as illustrated by the qualitative free energy diagram in Figure 38. The steps affected by increasing viscosity are indicated by dotted lines: the energy barriers of the diffusion step(s) and the first conformational step are increased, and the energy of $E_{2}A$ is stabilized relative to $E_{1}A$. It should be noted that the actual kinetic scheme could be more complicated than that shown in Scheme III, since ADP can exchange between the two sites (Vasavada et al., 1984; Nageswara Rao et al., 1978), and AMP can also bind to the ATP site with a low affinity (Rhoads & Lowenstein, 1968; Sanders et al., 1989).

Quantitative determination of the microscopic rate constants will require detailed investigation using the isotope partition method (Rose et al., 1974), stopped-flow kinetics, and other biophysical method such as line-shape analysis of $^{31}P$ NMR spectra (Vasavada et al., 1984). The results described in here have set a good stage for these analyses, particularly since some of these techniques cannot differentiate a conformational step from an overall binding step.

**Impact on Future Site-Specific Mutagenesis Studies on AK.**
Because of the complication by the conformational change in our viscosity variation studies, only has qualitative information about rate-limiting steps been obtained. However, identification of the three kinetically significant steps are important for proper interpretation of the kinetic data from site-specific mutagenesis studies (whether the product releasing is also rate-limiting or not is unknown and thus it is not explicitly considered here). If the $k_{cat}$ is not perturbed (e.g., within 2x), it can be concluded that neither the chemical step nor the first conformational step is perturbed, unless both steps are equally perturbed in opposite direction. To rule out the latter possibility, the substrate system dAMP + MgATP can be used, which is known to give rate-limiting chemical step for WT. If the $k_{cat}$ is somewhat perturbed (e.g., within 10x), either the chemical step and/or the conformational step can be involved and use of substrate analogues and viscosity-dependent kinetics should be conducted to determine which step is perturbed. If the $k_{cat}$ is greatly reduced (e.g., > 100x), it is unlikely to be mainly caused by perturbation in the conformational step. The main problem in this case will be whether the global conformation of the enzyme has been perturbed. These analysis and cautions have been employed in the interpretation of the data in Chapter III.
CHAPTER V

EPIMERIZATION OF SCREW SENSE ISOMERS OF CrATP

A. INTRODUCTION

Stereochemical analysis has traditionally provided fundamental information about transition states and intermediates in enzymatic and nonenzymatic reactions. In enzymatic reactions, stereochemical analysis generates information about both the chemical reaction mechanisms and specific binding interactions between substrates or inhibitors and enzymic binding sites. In this chapter we will focus on the stereochemical specificity of adenylate kinase (AK) towards its substrate MgATP.

Nucleotides such as ATP exist in cells predominantly as complexes with Mg(II), or to certain extent with other divalent metal ions, and for AK the Mg(II) complex reacts fastest (Noda, 1973). When Mg(II) forms a complex with ATP, a number of regional isomers such as bidentate and tridentate complexes can be formed. Each of bidentate and tridentate isomers can in turn form different stereo isomers (Cleland, 1986). For example, two screw sense stereo isomers, A and A isomers, are predicted for the β,γ-bidentate complex (Cleland, 1986):
When the α-phosphate becomes coordinated in MgATP, endo and ex isomers result from each bidentate isomer (Cleland, 1986):

![Diagram of MgATP isomers](image)

It is designated as Δ Exo, Δ Endo, Λ Endo, and Λ Exo for the complex with AMP attached to the oxygen 1, 2, 3, and 4, respectively.

It is expected that only a single screw sense isomer of MgATP will be a substrate for a given enzyme, since the signature of an enzyme-catalyzed reaction is its absolute stereospecificity. However, because of the rapid equilibrium between the isomers, one can not tell which one is the substrate. Three approaches have been used to answer this question. One method is to prepare substitution-inert complexes with metal ions such as Cr(III) or Co(III) (Cleland & Mildvan, 1979; Cleland, 1982; Dunaway-Mariano and Cleland, 1980a). Unlike Mg(II), these metal ions form coordination isomers with half-lives of days or weeks rather than fractions of second. The pure isomers thus can be isolated by use of chromatographic methods (Cleland, 1982; Gruys & Schuster, 1982; Gruys
et al., 1986) and applied for stereospecificity studies (Dunaway-Mariano and Cleland, 1980b; Cleland, 1986; Dunaway-Mariano, 1986).

The second way to identify the active isomer of MgATP complex is to use chiral thionucleotides such as ATPβS (Eckstein, 1983; 1985). Since Mg(II) prefers to bind to oxygen rather than sulfur by a factor of at least 31,000, while Cd(II) shows a ~ 60-fold preference for sulfur over oxygen (Pecoraro et al., 1984), the active isomer can be deduced from kinetic studies of the chiral thionucleotides with both Mg(II) and Cd(II) (Cohn, 1982; Eckstein, 1983; 1985).

Thirdly, the stereospecificity can also be elucidated by ESR studies of Mn(II) complex of oxygen-17 labeled ATP (Reed and Leyh, 1980; Leyh et al., 1982; Reed et al., 1986). By use of this method, inhomogeneous broadening due to unresolved superhyperfine coupling between the unpaired electron spin of Mn(II) and the nuclear spin of oxygen-17 (I=5/2) can be directly observed in EPR spectra in favorable cases (Reed and Leyh, 1980). This method however differs from the other two methods described above in that only ground state of the enzyme-substrate complex can be studied unless suitable transition state analogues are available.

The stereospecificity of muscle adenylate kinase (AK) has been studied by all of these three techniques. It was shown by Dunaway-Mariano and Cleland (1982b) that rabbit muscle AK preferentially turns over the Δ isomer of β,γ-bidentate Cr(III)(H2O)4ATP (abbreviated as CrATP). Kalbitzer et al. (1983) showed that the specificity of porcine muscle AK for the diastereomers of ATPβS is reversed from Sp to Rp on replacing Mg(II) by Cd(II), and that (Rp)- but not (Sp)-[β-17O]ATP caused a line broadening in the Mn(II) EPR spectrum of the AK-MnATP complex. The
results from all three approaches were used by the authors to conclude that the pro-R oxygen of the β-phosphate group of ATP is coordinated to the metal ion at the active site of AK.

Two problems remain unresolved. Since the condition used in the EPR study was ground state instead of transition state, the stereospecific coordination observed would suggest that the triphosphate binding region is held tightly in the correct mode by the chiral environment of active site before the catalytic reaction takes place. This result of AK is different from that obtained on creatine kinase, which showed that the stereochemical requirement is expressed only in the transition state analogue complex E-MnADPγS-nitrate-creatine, not in the ground state complex E-MnADPαS (Reed et al.; 1986). The stereochemical requirement at ground state as suggested by Kalbitzer et al. (1983) would predict that AK should not bind the A isomer of β,γ-bidentate CrATP preferentially. To test this prediction, we carried out the inhibition studies on porcine muscle AK by use of pure screw sense isomers of β,γ-bidentate CrATP (the K_i has been reported only for the mixture of stereoisomers of CrATP (Dunaway-Mariano and Cleland, 1982)), and we found that porcine muscle AK has similar affinities for both the "right" and "wrong" isomers.

For those kinases with no stereochemical requirement at the ground state, the "wrong" isomers will bind as nonproductive complexes. In the case of AK, not only wrong stereoisomers, but also wrong geometric isomers, may bind to the enzyme, since the K_i of bidentate and tridentate CrATP are the same (11 μM, as mixtures of stereoisomers in both cases) (Dunaway-Mariano and Cleland, 1980b). This suggests that in the case of MgATP, which exists as an equilibrium mixture of predominantly bidentate
and tridentate isomers (Huang and Tsai, 1982; Pecoraro et al., 1984), only ca. 20% of the mixture may exist in the correct mode (Δ, β, γ) and bind to AK productively. If the nonproductive AK-MgATP complexes can only dissociate, at any time, 80% of the active sites of AK are occupied by these competitive inhibitors. Thus, it is important to ask whether a nonproductive complex at the ground state can be converted to a productive complex without going through the process of dissociation and reassociation. In an effort to understand this problem, we investigated the effect of porcine muscle AK on the rate of epimerization between the Δ and the Λ isomers of β,γ-bidentate CrATP. The question to be answered is whether AK allows, enhances, or inhibits the rate of epimerization.

B. RESULTS AND DISCUSSION

Separation and Stability of Pure Isomers of CrATP. We used the HPLC procedure (Gruys & Schuster, 1982; Gruys et al., 1986) to separate the isomers 1-4 of CrATP (Figure 39). As established previously (Dunaway-Mariano, 1986), 1 and 4 are, respectively, pseudoaxial and pseudoequatorial conformers of the Δ isomer whereas 2 and 3 are, respectively, pseudoaxial and pseudoequatorial conformers of the Λ isomer. Since these isomers may undergo conformational isomerization (1 → 4 or 2 → 3), epimerization (1,4 → 2,3), and hydrolysis (or dissociation), and these processes are very sensitive to pH, temperature, ionic strength, etc. (Gruys et al., 1986), it is important to address these properties under our experimental conditions.
Figure 39. HPLC profile and structures for isomers 1-4 of Cr(III)(H2O)4ATP. The drawing of isomer structures and the assignment of the peaks of the HPLC profile were based on the work by Dunaway-Mariano and co-workers (Dunaway-Mariano, 1986).
Gruys et al. (1986) studied the equilibrium and rates of interconversion between conformational isomers in the pH range 5.3-6.2. According to their report, the epimerization is negligible relative to conformational isomerization in this range, but above pH 6.2 epimerization starts to occur at a significant rate. Under our conditions, epimerization and conformational isomerization occurred at comparable rates in the pH range 5.8-6.1, as shown in Figure 40. Although the resolution in Figure 40 was not optimal, it clearly shows that isomer 1 was converted to isomer 4 (right-hand side peak) and isomers 2 and 3 (central peaks). The first order rate constants of the disintegration of CrATP isomers, based on the decrease of the peak intensity (Figure 41), at pH 6.1 (70 mM MES buffer, 0.1 mM CrATP; see Chapter II for details), 25 °C, are summarized in Table 13. (0.017, 0.013, 0.008, and 0.010 min⁻¹) for isomers 1, 2, 3, and 4, respectively. These values were in the range suitable for steady state kinetics and for monitoring the epimerization by HPLC. Thus we carried out all experiments in the pH range 5.8-6.1. Under such conditions the activity of AK was still at more than 50% of the optimal activity (k_cat = 420 s⁻¹ compared with 670 s⁻¹ at optimal pH).

In addition, the rates for the disintegration of the four CrATP isomers suggest that the pseudoaxial isomers are less stable than the pseudoequatorial isomers (based on the order of the stability isomer 1 < isomer 4 and isomer 2 < than isomer 3), and the Δ isomer is also slightly less stable than the Λ isomer (based on the order of stability isomer 1 < isomer 2 and isomer 4 < isomer 3).

K_i of Δ and Λ Isomers of CrATP. First, the inhibition pattern by CrATP before separation of isomers was shown to be competitive by
Figure 40. HPLC profiles showing the time courses of conformational isomerization and epimerization of the isomer 1 of CrATP. The reaction condition was 0.1 mM CrATP in 70 mM MES buffer, pH 6.1, 25 °C. The mobile phase was 0.01 M methanesulfonic acid. The flow rate was 1 ml/min and chart speed 1 mm/min.
Figure 40
Figure 41. Plots of log(peak height %) vs. time for isomers 1-4 of Cr(III)(H$_2$O)$_4$ATP in conformational isomerization and epimerization reactions. The reaction condition was 0.1 mM CrATP in 70 mM MES buffer, pH 6.1, 25 °C. The mobile phase was 0.01 M methanesulfonic acid. The flow rate was 1 ml/min and chart speed 1 mm/min.
Table 13. Summary of data on the disintegration an inhibition properties of isomers 1-4 of CrATP. $k_{(1)}$, $k_{(2)}$, $k_{(3)}$, and $k_{(4)}$ represent the first order rate constants for the disintegration of isomer 1, 2, 3, and 4, respectively.

<table>
<thead>
<tr>
<th>Disintegration</th>
<th>Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>First order constant (min$^{-1}$)</td>
<td>(μM)</td>
</tr>
<tr>
<td>$k_{(1)}$</td>
<td>17</td>
</tr>
<tr>
<td>$k_{(2)}$</td>
<td>13</td>
</tr>
<tr>
<td>$k_{(3)}$</td>
<td>8</td>
</tr>
<tr>
<td>$k_{(4)}$</td>
<td>10</td>
</tr>
</tbody>
</table>
varying both the concentrations of substrate (MgATP) and inhibitor, as
shown in Figure 42A. The competitive inhibition constant $K_i$ for the
mixture of the four isomers was determined by the slope replot (the inlet of
Figure 42A). Since the inhibition is competitive, the $K_i$ can also be
determined according to the following equation (Todhunter, 1979):

$$K_i = \frac{K_m}{V_m[S] \text{slope}}$$

(5.1)

in which, $[S]$ is a constant substrate concentration (0.250 mM in this case)
and the slope is obtained from the plot of $1/v$ vs. $[I]$ at the constant
substrate concentration. Since this method is time-efficient and thus it
reduces the chance of epimerization of the purified screw sense isomers,
the $K_i$'s for each of the $\Delta$ and $\Lambda$ isomers were determined by this method.

For determination of the $K_i$ of each of the screw sense isomers,
equal amounts of the conformers (isomers 1 and 4, or isomers 2 and 3)
were mixed before kinetic studies. Figure 42B shows the plots of $1/v$ vs.
$[I]$ ($\Delta$ isomer (1+4) and $\Lambda$ isomer (2+3)) at fixed concentration of MgATP.
The results of the inhibition studies are listed in Table 13. The $K_i$ values
thus obtained indicate that there is no significant discrimination in
stereospecificity toward the screw sense isomers in the ground state.
Therefore, the stereospecificity that AK preferentially turns over the $\Delta$
isomer in the presence of AMP is resulted from that AK expresses its
stereochemical requirement only at the transition state.
Figure 42. Plots of the inhibition of AK by CrATP. A: mixture of all isomers at varying concentration of [MgATP]; the inset shows the slope replot. B: Δ isomer (1 + 4) and Λ isomer (2 + 3) at 0.250 mM MgATP.
The result is at odd with the observation of stereospecific interaction between the metal ion and the pro-R oxygen of the β-phosphate of ATP in the AK-MnATP complex (Kalbitzer et al., 1983). It should be noted that the same paper also reported lack of coordination between Mn(II) and the γ-phosphate of ATP, which also contradicts the results of recent NMR studies (Ray et al., 1988).

Epimerization Between Δ and Λ Isomers. The above result suggests that the AK-Δ and AK-Λ complexes have similar energies with the energy for the AK-Λ complex slight higher than that for the AK-Δ complex. To determine whether AK affects the energy barrier between the Δ and the Λ isomers of CrATP, we monitored the epimerization of each isomer, 1-4, by HPLC in the absence and presence of AK. Figure 43 shows the result of isomer 4. In Figure 43A (in the absence of AK) isomer 4 was converted to isomer 1 (conformational isomerization) and isomer 2 (epimerization). There was also a trace of isomer 3 formed but the rate of its formation is slower than that for isomer 2. This is reasonable considering that the formation of isomer 2 from isomer 4 requires a simple rotation of the bond between the γ-P and the bridge oxygen connecting γ-P and β-P while the formation of isomer 3 from isomer 4 requires a similar rotation as well as a conformational isomerization (see Figure 39). In the presence of AK (Figure 43B), similar processes occurred, except that epimerization was now slightly faster than conformational isomerization, and that small amounts of unidentified byproducts were formed. Figure 44 shows the plots of the time course of epimerization for all isomers. Since we are only interested in the epimerization, and there are insufficient data to determine quantitative rate constants for all processes (epimerization, conformational
Figure 43. HPLC profiles showing the effect of AK on the epimerization and conformational isomerization of the isomer 4 of CrATP. The reaction condition was 0.1 mM CrATP 4 alone (A) and in the presence of AK (B) in 70 mM MES buffer, pH 5.9, 25 °C. The mobile phase was 0.01 M methanesulfonic acid. The flow rate was 1 ml/min and chart speed 1 mm/min.
isomerization, and potential side reactions in the presence of AK), we plot the intensity ratios $[1+4]/[2+3]$ as a function of time for isomers 2 and 3, and $[2+3]/[1+4]$ for isomers 1 and 4.

The results in Figure 44 demonstrate that all isomers of CrATP can undergo epimerization at the active site of AK. The epimerization seems faster in the presence of AK, and the enhancement was retarded by addition of ATP as shown in Figure 45. Since in solution, the energy of the $\Delta$ isomer is slightly higher than that of the $\Lambda$ isomer while in the active site of AK the energy of the $\Lambda$ isomer is slightly higher than that of the $\Delta$ isomer, some changes of the rate of epimerization is expected. However, we do not consider the enhancement by AK significant. It is no more than a factor of two and could be at least partially contributed by unidentified side reactions of the starting isomer as shown in Figure 43. Thus, we conclude that AK allows, but does not enhance or inhibit epimerization of CrATP. The same conclusion also applies to the process of conformational isomerization.

**Effect of AMP on Epimerization.** Since the $\Delta$ isomer is readily turned over by AK in the presence of AMP (Dunaway-Mariano and Cleland, 1980b), isomer 2 ($\Lambda$ isomer) was used to examine the effect of AMP on the rate of epimerization. Figure 46 shows the time course for the epimerization of isomer 2 alone (Figure 46A), in the presence of AK (Figure 46B), and in the presence of AK and saturating concentration of AMP (Figure 46C). Although the resolution of this set of data was not optimal and the AMP peak obscured the peak of isomer 4, plots of the decay of the peak height of isomer 2 as shown in Figure 47 suggests that AMP does not affect the rate of epimerization. Again the small enhancement in the presence of AMP is insignificant, and could be contributed by AK-
Figure 44. Plots of the time course of epimerization for CrATP isomer 1 (A), isomer 2 (B), isomer 3 (C), and isomer 4 (D), in the absence (open circles) and presence (closed circles) of AK. The ratios were calculated from the peak areas measured by enlarging, cutting, and weighing the paper. Possible errors in the measurement of peak areas are ±7%.
Figure 45. Plots of the time course of the disintegration of the isomer 4 of CrATP, showing the retardation of the AK-enhanced effect by ATP. (a) CrATP alone; (b) CrATP + AK; (c-e) same as B, with 0.1, 0.2, and 0.4 mM ATP present. A control experiment showed no effect of ATP in the absence of AK.
Figure 46. HPLC profiles of the time course of the reactions of the isomer 2 of CrATP, showing the effect of saturation of the AMP site of AK. (A) CrATP (2) alone; (B) CrATP (2) + AK; (C) CrATP (2) + AK + AMP. The reaction condition was 0.1 mM CrATP (2) and 2 mM AMP in 70 mM MES buffer, pH 6.0, 25 °C.
Figure 47. Plots of peak height as a function of time from Figure 46. Curves A-C represent the isomer 2 of CrATP in A-C, respectively, of Figure 46. Curves D and E represent the appearance of isomer 4 in A and B, respectively, of Figure 46.
catalyzed reaction between AMP and the Δ isomer of CrATP (formed from epimerization of the Δ isomer). The result is consistent with the observation in the deuterium NMR studies that addition of AMP does not increase the rigidity of bound AMPPCP or MgAMPPCP (Sanders and Tsai, 1989b). Even though an analogue was used in both systems, the results suggest that binding of both substrates (AMP and MgATP) is still not sufficient to induce the enzyme to express its stereochemical requirement.

Significance on the Mechanism of AK. The above results suggest that the triphosphate site of AK is not stringent in the ground state and allows epimerization and conformational isomerization between isomers of CrATP. In the case of MgATP the wrong screw sense isomer, or even the wrong geometric isomers, may be able to "equilibrate" with the correct isomer while bound to the active site, without going through the process of dissociation and reassociation. This process allows the enzyme to "capture" different isomers of MgATP in solution and utilize them with optimal catalytic efficiency.

It is also reasonable that AK does not catalyze the process of epimerization, since there should be no evolutionary pressure to improve this process. Under standard assay conditions the $k_{cat}$ of AK is 670 s$^{-1}$. The rate of exchange between MgATP and Mg(II) + ATP has been found to be $1.5 \times 10^3$ s$^{-1}$ at 30 °C (Vasavada & Nageswara Rao, 1984), $2 \times 10^4$ s$^{-1}$ (Bryant, 1972) or $1.2 \times 10^3$ s$^{-1}$ (Diebler et al., 1960). Since the epimerization requires breakage of a single bond (the bond between Mg(II) and an oxygen of β-phosphate of ATP) whereas the exchange process requires breakage of an extra bond (the bond between Mg(II) and an oxygen of β-phosphate of ATP), the rate of epimerization should be faster
than that for the exchange process, and thus the epimerization in solution between the Δ and Λ isomers of MgATP should be faster than the turnover rate of AK (since even the exchange rate is faster than the turnover rate).
LIST OF REFERENCES


APPENDIX. MEDIA, BUFFERS, AND REAGENTS

A. FOR MOLECULAR BIOLOGY

Media

1. Glucose/minimal medium plates
   Autoclave the following reagents separately, and cool before mixing aseptically:

   M9 salts with 15g agar 1 liter
   1 M MgSO4 1 ml
   0.1 M CaCl2 1 ml
   20% glucose 10 ml

   M9 salts
   Na2HPO4 6g/liter
   KH2PO4 3g
   NH4Cl 1g
   NaCl 0.5g

   Store at 4 °C.

2. 2XTY medium

   Bacto tryptone 16g/liter
   Bacto yeast extract 10g
   NaCl 5g

   Sterilize by autoclaving.

3. L-broth

   Bacto tryptone 10g/liter
   Bacto yeast extract 5g
   NaCl 5g

   Sterilize by autoclaving.

4. H plates

   Bacto tryptone 10g/liter
   NaCl 8g
   Agar 15g

   Sterilize by autoclaving.

5. H top agar

   Bacto tryptone 10g/liter
   NaCl 8g
Agar
8g
Sterilize by autoclaving.

6. SOB medium
- Bacto tryptone 5g/200ml
- Yeast extract 1.00g
- NaCl 0.12g
- KCl 0.10g

Adjust to pH 6.9 and autoclave.
- MgCl\(_2\) 0.41g/10ml
- MgSO\(_4\) 0.24g

Filtrate into the above medium.

Buffers and solutions

1. TE buffer
- 10 mM Tris-HCl
- 1 mM EDTA

Adjust to pH 8.0 with HCl and sterilize by autoclave.

2. TFBI
- KOAc 0.59g/200ml
- KCl 1.49g
- CaCl\(_2\)\(2\)H\(_2\)O 0.29g
- MnCl\(_2\)\(2\)H\(_2\)O 1.98g
- Glycerol 30g (24ml)
- H\(_2\)O 176ml

Adjusted to pH 5.8 with acetic acid, filtrate, and store at 4°C.

3. TFBII
- MOPS 0.42g/200ml
- KCl 0.15g
- CaCl\(_2\)\(2\)H\(_2\)O 2.21g
- Glycerol 30g (24ml)
- H\(_2\)O 176ml

Adjusted to pH 5.8 with HCl, filtrate, and store at 4°C.

4. TEAB buffer
- 0.01 M triethylamine, adjust to pH 7.0 to 7.5 with dry ice.

5. PEG/NaCl
- Polyethylene glycol 6000 20.0g/100ml
- NaCl 14.6g

Sterilize by autoclaving.

6. TE-saturated phenol solution
Add solid phenol reagent to the TE buffer and dissolve the phenol in boiling water bath. Store at 4°C in dark.

7. LSI
- 50 mM glucose
25 mM Tris-HCl (pH 8.0)
10 mM EDTA
Sterilize by autoclaving.

8. LSII
0.2 M NaOH
1% SDS

B. For Enzyme Purification, Kinetics, and Protein Chemistry

1. DB (dialyzing buffer)
   NH$_4$Ac
   KCl
   EDTA
   0.7g/4 liters
   3.0g
   0.2g
No need to adjust pH.

2. CBA (column buffer A)
   30 mM Tris-HCl, 50 mM NaCl, 1 mM DTT, 1 mM EDTA, pH 7.5

3. CBB (column buffer B)
Same as CBA, except 0.5 M NaCl.

4. CBC (column buffer C)
   5 mM imidazol-HCl, 1 mM EDTA, 1 mM DTT, pH 6.9.

5. HGM
   Hexokinase
   Glucose 6-phosphate dehydrogenase
   500 units/0.5ml
   250 units
Dissolved in RB (reaction buffer) with 1 mM DTT. Keep on ice.

6. MOPS buffer
   0.075 M MOPS and 0.002 M DTT (or DTE), pH 7.0.

7. PLM
   Pyruvate kinase
   Lactic dehydrogenase
   2.5 mg/0.5ml
   2.5 mg
Dissolve in RB with 1 mM DTT. Keep on ice.

8. RB
   0.075 M Tris-HCl and 0.065 KCl, pH 8.0.

9. RS1
   ATP
   AMP
   NADH
   PEP
   DTT
   119 mg/10ml
   73 mg
   16 mg
   27 mg
   16 mg
Store at -65 °C.

10. RS2
    MgCl$_2$
    102 mg/10ml
Store at -65 °C.
11. RS3

- NADH: 16 mg/10ml
- PEP: 27 mg
- DTT: 16 mg

Store at -65 °C.

12. RS4

- NADP: 52 mg/10ml
- Glucose: 117 mg
- DTT: 16 mg

Store at -65 °C.

13. SB

20 mM Tris-HCl, 0.14 M NaCl, pH 7.5.

14. SNB

30 mM Tris-HCl, 1 mM DTT, 1 mM EDTA, pH 7.5.