BIOCHEMICAL INVESTIGATIONS ON ADENOSINE KINASE AND ITS MUTANTS

By

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ABSTRACT

In the present study, adenosine kinase (AK) was purified from a number of different sources (e.g. bovine liver, Syrian hamster liver, kidney and heart; Chinese hamster ovary cells and human placenta). The enzyme from bovine liver and Syrian hamster liver was purified to apparent homogeneity by a combination of ion-exchange chromatography, affinity chromatography and gel filtration chromatography. The purified enzyme showed a single band of 40 kDa in SDS-polyacrylamide gel electrophoresis, which was similar to that reported from other sources in the literature. A number of biochemical and enzymatic characteristics of AK were investigated.

The reliability and reproducibility of the AK assay as established in previous studies was determined. The apparent Km of partially purified AK from Syrian hamster liver for adenosine was determined to be 0.16 uM, which is consistent with earlier reports. A novel result obtained in these studies is that AK activity was found to be completely dependent upon the presence of phosphate or other pentavalent ions. AK from different sources did not show any activity in the Tris-HCl buffer, pH 7.0, but the activity increased

dramatically upon the addition of phosphate and it reached a maximum at 2 mM phosphate. There was no inhibition of AK activity when the phosphate concentration was increased up to 100 mM. AK could also be activated by substituting phosphate with either arsenate or vanadate, which have similar chemical structures to phosphate.

The temperature inactivation kinetics of AK showed that AK from human fibroblast cells had higher thermal resistance than AK from Chinese hamster ovary cells at 50°C. The presence of phosphate had no effect on the thermal stability of AK.

Antibodies to purified AK from Syrian hamster liver were raised in both rabbits and guinea pigs. Antiserum from the rabbit which gave the strongest response was used for further studies. AK was recognized by and reacted specifically with the antiserum at a dilution of up to 1:16,000. The AK antibody which was covalently bound to Protein A Sepharose beads immunoprecipitated a 40 kDa protein from radio-labelled Chinese hamster ovary or baby hamster kidney cell extracts. In addition, this antibody preparation immunoprecipitated AK activity from Syrian hamster liver extracts. However, immunoblotting showed that all the antisera could not react with AK from Chinese hamster ovary cells and human cells, suggesting a strong species specificity.

A partial protein sequence of AK was obtained by microsequencing of a cyanogen bromide fragment of purified AK

from Syrian hamster liver. The sequence was Tyr-Val-Asp-Ser-Leu-Phe-Gly-Ala-Glu-Thr-Glu-Ala-Ala-Leu. Degenerate DNA probes for this sequence can now be made and used for either screening of cDNA libraries or carrying out PCR experiments to facilitate the cloning of the AK gene(s).

Finally, conditions for selection of revertants from AK^- mutants of Chinese hamster ovary cells have been developed. The AK^+ revertants from AK^- mutants can be obtained using adenosine, alanosine, uridine and erythro-9-(2-hydroxyl-3-nonyl) adenine in the growth medium. Three revertants have been isolated. These revertants regained their AK activities and lost their drug-resistance at same time. This method can also be used in the future to clone the AK gene by transfecting AK^- mutants with foreign DNA.

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ABBREVIATIONS

AK:	Adenosine kinase
AMP:	Adenosine 5'-monophosphate
ATP:	Adenosine 5'-triphosphate
BSA:	Bovine serum albumin
CHO:	Chinese hamster ovary
CM Sepharose:	Carboxymethyl Sepharose
CNBr:	Cyanogen bromide
CPM:	Counts per minute
DEAE Sephacel:	Diethylaminoethyl Sephacel
DNA:	Deoxyribonucleic acid
EHNA:	Erythro-9-(2-hydroxyl-3-nonyl) adenine
EMS:	Ethyl methanesulfonate
GTP:	Guanosine 5'-triphosphate
HAT:	Hypoxanthine-aminopterin-thymidine
HF:	Human fibroblast
hr:	Hour(s)
IgG:	Immunoglobulin G
IMP:	Inosine 5'-monophosphate
kDa:	Kilodalton
MEM:	Minimal essential medium
min:	Minute(s)

6-MeMPR:	6-Methylmercaptopurine ribonucleoside
mRNA:	Messenger ribonucleic acid
PBS:	Phosphate buffered saline
PRPP:	Phosphoribosylpyrophosphate
rRNA:	Ribosomal ribonucleic acid
SDS:	Sodium dodecyl sulphate
SDS-PAGE:	SDS polyacrylamide gel electrophoresis
TBS:	Tris buffered saline
TEMED:	N,N,N',N'-tetramethylethylenediamine
Tris:	Tris(hydroxymethyl)aminomethane
tRNA:	Transfer ribonucleic acid
u:	Micro (10^{-6})
V:	Volt(s)
V_o:	Initial velocity
WT:	Wild type

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INTRODUCTION

During the past two decades, much work has been done on somatic cell genetics by means of studies with mutants resistant to different drugs. Such studies have played an important role in the research and development of different areas, such as somatic cell genetics, cell biology, mutagenesis, cancer research and pharmacology [Jackson *et al.*, 1989; Pillwein, *et al.*, 1990]. Analysis of the drug-resistant mutants can lead to a detailed understanding of the cellular metabolism of the drugs and corresponding analogs in cells. These studies can also lead to a better understanding of the molecular mechanism of action of different drugs. In addition, some of the drug-resistant mutants have proven useful as *in vitro* models for studying human genetic diseases and investigating resistance to chemotherapeutic agents [Ullman *et al.*, 1976]. It is anticipated that knowledge gained from such studies may lead to the rational design of useful drugs in addition to providing important basic information about the eukaryotic cells [for review, see Gupta, 1989].

There are several advantages of using established cell lines for genetic studies [Siminovitch, 1976; Puck and Kao, 1982]. These include: (i) because of their clonal origin, the

established cell lines provide a source of genetically uniform cells; (ii) in comparison with the generation time of a whole organism, the doubling time of somatic cells (generally between 12 to 24 hr) is much shorter and hence genetic and biochemical investigations involving mutant isolation and characterization can be conveniently carried out; (iii) the composition of the medium can be controlled so that different cells can be investigated under defined environmental conditions.

During the past three decades, a large number of cell lines from many different sources and from many different tissues have been established. Among them, Chinese hamster ovary (CHO) cells have been broadly exploited for genetic studies in different areas [Siminovitch, 1976; Puck and Kao, 1982; Gupta, 1989]. CHO cells show many favourable characteristics for genetic studies. First, CHO cells can be grown under a variety of culture conditions, such as in monolayer, in suspension culture and in soft agar. Secondly, CHO cells have a very high plating efficiency (near 100%) and a short generation time (about 12 hr) so that a large cell population can be obtained in a short time. Thirdly, many recessive mutants have been isolated from CHO cell lines, suggesting that they are functionally hemizygous at many genetic loci and that such mutants can be easily studied in CHO cell lines.

1.1 Selection of Drug Resistant Mutants and Mechanisms of Drug Resistance in Mammalian Cells.

Drug resistant mutants can be isolated mainly by two types of procedures, a single-step protocol or a multi-step protocol. In the single-step protocols drug resistant mutants can be acquired following a single exposure of cells to a certain concentration of the drug. The Luria-Delbruck fluctuation test indicated that mutants selected in the single-step protocol are already present in the cell population and drugs cannot induce these mutations *in vivo* [Luria and Delbruck, 1943]. In the multi-step protocol, the somatic cells are exposed to gradually increasing concentrations of the drugs and different levels of resistant mutants can be isolated by increasing the drug concentrations [Gupta, 1981]. In some cases, these kinds of mutants can revert back to non-resistant cells in the absence of selection pressure, suggesting that gene amplification is involved in developing the resistance, because gene amplification could lead to drug resistance by increasing the level of the target protein that is inhibited by the drugs [Schimke, 1988].

Different kinds of mechanisms can be involved in producing drug resistance in somatic cells during the selection of drug resistant mutants. The following mechanisms are the most common ones:

(a) A mutated plasma membrane protein which leads to the decreased intracellular level of the drug by either increasing the efflux of the drug or decreasing the uptake of the drug.

(b) Mutation of the target enzyme for the drug so that the enzyme is not affected or inhibited by the drug.

(c) Mutation or inactivation of the certain enzyme(s) active on the drug so that the drug cannot be converted into its cytotoxic form.

(d) An increased level of the cellular target protein for the drug due to gene amplification.

(e) Increased conversion of the drug into an inactive form.

(f) Creation of an alternate metabolic pathway to bypass the metabolic step inhibited by the drug.

1.2 Physiological Importance of Adenosine.

Adenosine has a multitude of biological and biochemical effects [Fox and Kelley, 1978]. It causes cellular toxicity and immunosuppression, alters cell morphology, acts as a vasodilator, stimulates hormone secretion, serves as a neurotransmitter and has an assortment of other physiological effects, including activation of adenylate cyclase, blocking pyrimidine biosynthesis and inhibition of phosphoribosyl-pyrophosphate (PRPP) synthesis [Fox and Kelley, 1978]. Adenosine analogs such as 5'-N-ethylcarboxamidoadenosine, 5-methylthioadenosine and 2-chloroadenosine have been widely used to study the role of adenosine receptors in mammalian cells [Olsson and Pearson, 1990; Stiles, 1992]. Other adenosine analogs have cytotoxic, anticancer, and antiviral effects. Adenosine can cross the cell membrane by either

simple diffusion or transportation into cells through specific nucleoside channels in the plasma membrane [Kwong *et. al.*, 1988; Gu *et al.*, 1993]. This nucleoside is also known to exert its actions through activation of specific receptors which have been characterized with the use of adenosine agonists and antagonists [Bruns *et al.*, 1983; Stiles, 1992]. Once inside the cells, adenosine can be deaminated by adenosine deaminase or phosphorylated by adenosine kinase (AK). Adenosine in higher concentrations is toxic to mammalian cells in culture. The most likely mechanism for the cellular toxicity of adenosine is a depletion of cellular pyrimidine nucleotides, UTP and CTP, due to the inhibition of *de novo* pyrimidine biosynthesis. The addition of uridine in the culture medium reverses the toxicity of adenosine in most cell lines [Ullman *et al.*, 1976]. On the other hand, adenosine has been recently approved as a therapeutic agent for use in treatment of paroxysmal supraventricular tachycardia because of its effective termination of tachycardia in which the atrioventricular node forms part of reentrant circuit [Mosqueda-Garcia, 1992].

1.3 Metabolism of Purines in Mammalian Cells.

Mammalian cells have two routes for the synthesis of purines *in vivo*: the *de novo* biosynthetic pathway and the salvage pathway [Fig. 1]. In the *de novo* biosynthetic pathway, inosine 5'-monophosphate (IMP) is synthesized from ribose-5-phosphate in a process that involves 11 enzymes. The

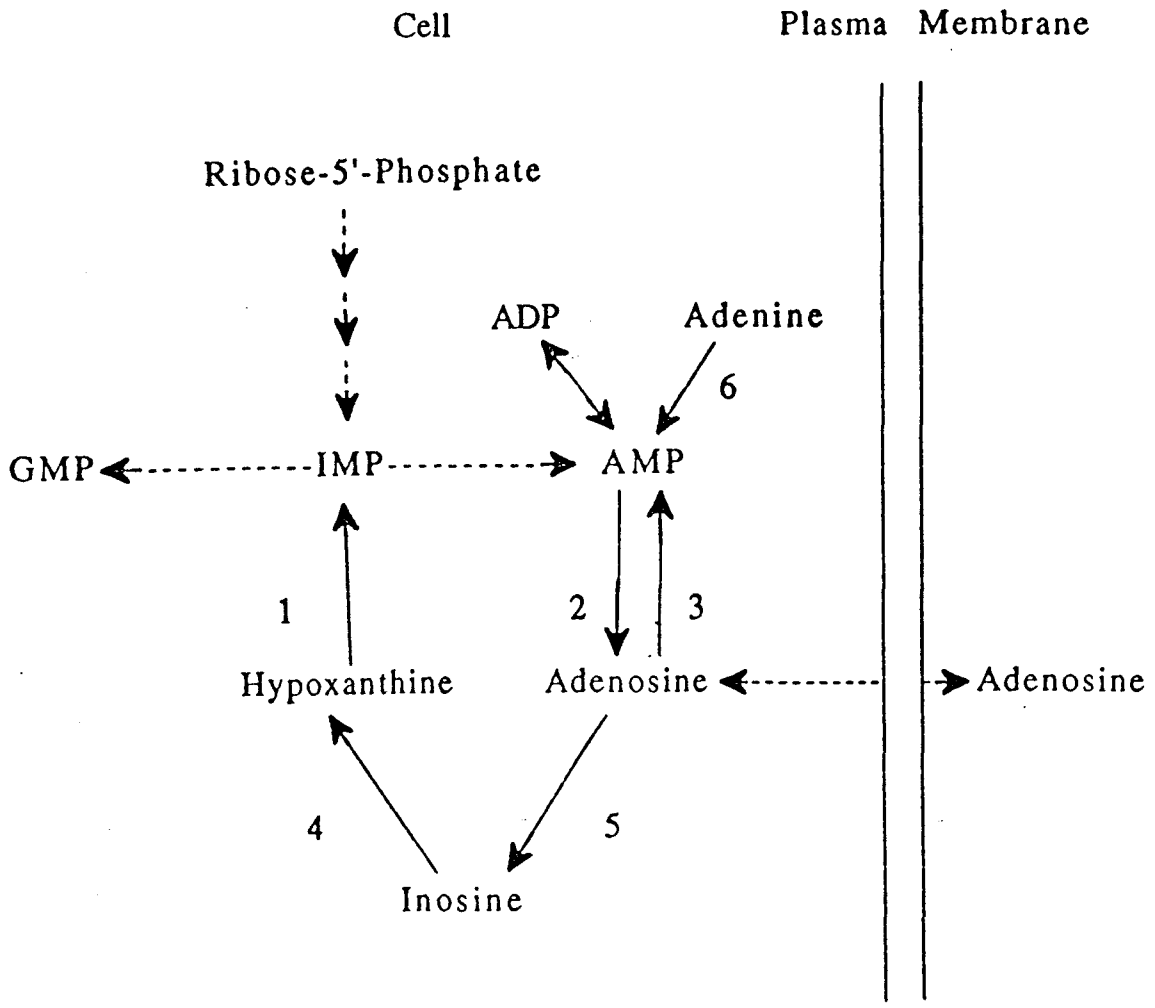


Fig. 1 The purine metablolism pathways in mammalian cells. ----: the *de novo* purine biosynethetic pathway; ____: the salvage purine biosynthetic pathway. 1, hypoxanthine phophoribosyltransferase (HPRT). 2, 5′-nucleotidase and non-specific phosphatase. 3, adenosine kinase. 4, purine nucleoside phosphorylase. 5, adenosine deaminase. 6, adenine phosphoribosyltransferase.

de novo pathway is highly regulated because it is an energy expensive process, entailing the hydrolysis of six pyrophosphate bonds (standard free-energy change equals -7.3 kcal/mol). The first two enzymes of purine biosynthesis, PRPP synthetase and PRPP amidotransferase, are regulated by feedback inhibition by various nucleotide effectors. The two branchpoint enzymes, which lead to the synthesis of AMP and GMP from IMP, are also highly regulated by AMP and GMP, respectively. The fact that GTP is required for AMP synthesis and ATP is obligatory for the conversion of IMP to GMP ensures a balanced supply of purine nucleotides for the cell.

The second pathway by which purine nucleotides are generated is the salvage pathway in which nucleotides are synthesized from pre-formed purine bases or nucleosides which may come from the breakdown of nucleotides and nucleic acids from cell lysis, as well as from the diet. The salvage process is much more energy efficient in contrast to the *de novo* biosynthetic pathway [Deussen *et al.*, 1993]. Both AK (ATP:adenosine 5'-phosphotransferase, EC 2.7.1.20) and adenosine deaminase play important roles in the salvage pathway of purine biosynthesis [Pawelczyk *et al.*, 1992].

In addition, enzymes of the purine synthesis pathway are very important in pathological studies and the rational design of drugs [Ghose *et al.*, 1989; Renouf *et al.*, 1989]. Deficiency of one of two enzymes in purine metabolism, adenosine deaminase or purine nucleoside phosphorylase, causes an ultimately fatal immune dysfunction (severe combined

immunodeficiency disease, SCID) because of the cellular toxicities of adenosine, deoxyadenosine and deoxyguanosine [Agarwal *et al.*, 1976; Coleman *et al.*, 1978].

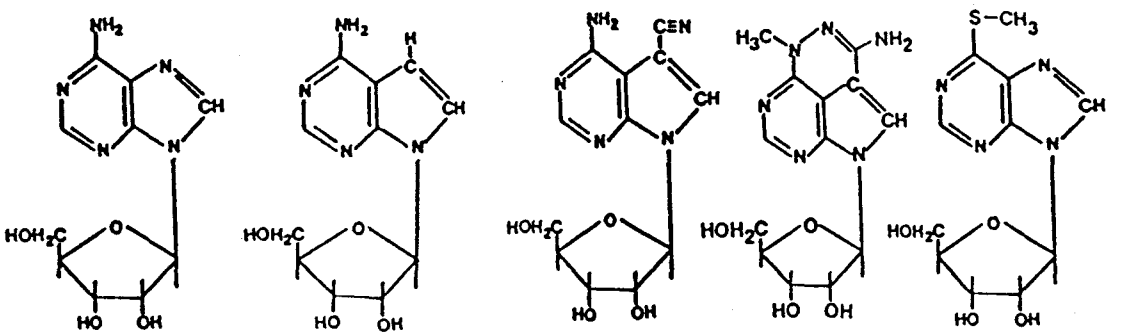
By virtue of the fact that mammalian cells possess two routes for producing purine nucleotides, the *de novo* pathway can be blocked with purine synthesis inhibitors, such as methotrexate and amethopterin, and cells can still survive and grow with proper supplies of purines in the culture medium. This is the basis for the application of hypoxanthine-amethopterin-thymidine (HAT) medium, which is widely exploited in pharmacologic and genetic manipulation [Littlefield, 1964]. Furthermore, under normal conditions, cells can meet their purine and pyrimidine requirement by the *de novo* biosynthetic pathway. Therefore, mutants of the different enzymes in the salvage pathways can be selected easily by using specific analogs. If the endogenous biosynthetic pathway is blocked by specific nucleoside analogs, revertants regaining these enzymes could be readily selected from the mutants lacking these enzymes. The convenient selection for mutants either in the forward or reverse direction is unique to purine/pyrimidine salvage pathway enzymes. This unique property has made these enzymes the targets for numerous genetic, biochemical and molecular biological investigations in somatic cells, such as first reports on mutant selection [Szybalski *et al.*, 1962] and gene transfer [McBride and Ozer, 1973], the development of hybridoma technology [Kohler and Milstein, 1975], as well as the development of carcinogen

screening systems in somatic cells [Li *et al.*, 1988].

1.4 Physiological Characteristics of Adenosine Kinase and Its Role in the Mutants Resistant to Purine Analogs.

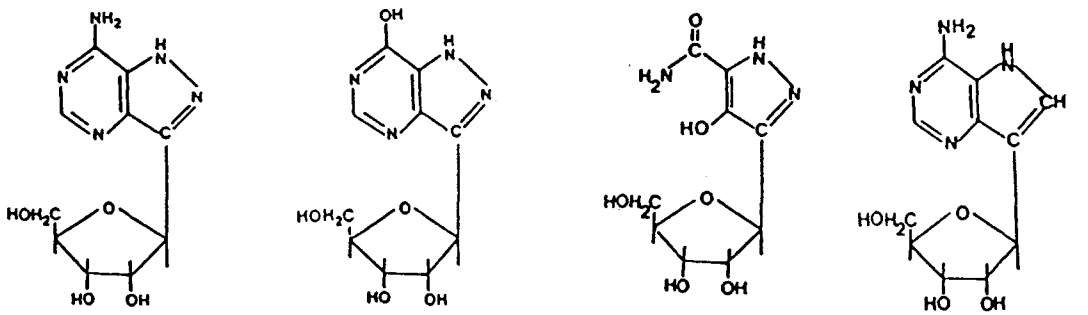
AK is one of the key enzymes in the salvage pathway of purine biosynthesis in the mammalian cell. It catalyzes the phosphorylation of adenosine to AMP. Previous biochemical studies indicated that this enzyme exhibits a very broad substrate specificity and is involved in the conversion of an important group of nucleoside analogs to their phosphorylated derivatives [Miller *et al.*, 1979b; Lin, *et al.*, 1988] [Fig. 2]. This group of compounds includes the pyrrolopyrimidine nucleosides toyocamycin, tubercidine and sangivamycin; the pyrazolo-pyrimidine nucleosides formycin A and formycin B, and a large number of other nucleoside derivatives, such as 6-methyl-mercaptopurine riboside (MeMPR), pyrazofurin and 8-aza-adenosine. These substrates can also be divided into two categories, N-nucleosides and C-nucleosides. N-nucleosides, which include adenosine, inosine and most other nucleosides, possess a N-C glycosyl bond [Fig. 2]. In contrast, C-nucleoside analogs, such as formycin A and formycin B, contain a C-C glycosyl bond [Fig. 2]. Bennett and Hill proposed that substrates for AK must have a 2'-hydroxyl group which is trans to the β-glycosyl linkage [Bennett and Hill, 1975]. This hypothesis was supported by later research done by Miller *et al.* [1979b], who examined 119 nucleosides and nucleoside analogs. They found that adenosine,

Chemical Structures of Adenosine and N-Nucleoside Analogs



Adenosine Tubercidin Toyocamycin TCN 6-MeMPR

Chemical Structures of C-Nucleoside Analogs



Formycin A Formycin B Pyrazofurin 9-DEAZA Adenosine

Fig. 2 Chemical structures of adenosine, N-nucleoside analogs and C-nucleoside analogs.

8-azaadenosine, toyocamycin, and sangivamycin were among the best substrates for AK. In addition, it was found that with regard to the substrate efficiency (Vmax/Km), the substrate efficiency of adenosine was more than 10^4 times that of 2'-deoxyadenosine and more than 10^6 times of that of adenine arabinoside [Miller *et al.*, 1979b].

Phosphorylation of all of the above analogs via AK is an essential prerequisite for their biological and cellular activities [Gupta, 1989]. After phosphorylation, the phosphorylated derivatives can substitute for AMP, ADP and other nucleotides in a wide variety of cellular reactions. Studies have shown that phosphorylated toyocamycin can induce selective inhibition of mature rRNA formation via rapid incorporation into RNA [Cohen and Glazer, 1985]. The incorporation of phosphorylated toyocamycin significantly inhibits the formation of mature 28S and 18S rRNA, probably by interfering with methylation of adenosine residues during RNA processing, while the processing of 45S RNA to the 38S RNA is not affected by the incorporation of toyocamycin [Suhadolnik, 1979].

The pyrazolo-pyrimidine nucleoside formycin A and formycin B are structural analogs of adenosine and inosine, respectively. Because of its close structural similarity to adenosine, phosphorylated formycin A is incorporated into DNA, various kinds of RNA, cAMP and NAD^+ [Suhadolnik, 1979]. A number of experiments indicate that at least partial cellular phosphorylation of the inosine analog formycin B is carried

out via AK before its incorporation into RNA [Mehta and Gupta, 1985], although earlier studies showed that formycin B was not phosphorylated in mammalian cells [Umezawa *et al.*, 1967]. Since formycin B shows a potent growth inhibitory effect against *Leishmania donovani*, a protozoan that causes visceral disease in humans, there is much interest in its metabolism in *Leishmania donovani* and mammalian cell lines. However, the exact cytotoxic mechanisms of both formycin A and formycin B remain to be elucidated. In addition, AK is also one of the main enzymes involved in controlling the intracellular adenosine concentration because of the cellular toxicity of adenosine above a certain level [Newby, 1985; Sciotti and Van Wylen, 1993].

AK was first identified in yeast and mammalian tissues by Capputo [1951]. This enzyme is one of the most abundant nucleoside kinases present in mammalian tissues [Miller *et al.*, 1979a]. Since the 1970s, AK has been partially purified or purified into homogeneous form from many different sources (see Table 1 for detail). The molecular mass of AK varies from 34 to 56 kDa, depending upon the different sources [Table 1]. Even from one source, the molecular mass of AK varies from SDS-PAGE to column chromatography, suggesting that AK is not a globular protein or certain cofactors bind to AK (molecular mass of AK from rabbit liver is 34 kDa by Sephadex G-100 column but 51 kDa by SDS-PAGE) [Miller *et al.*, 1979a] [Table 1]. Previous research also suggested that AK is a single polypeptide. ATP is the most efficient phosphate donor

Table 1. Size variation of adenosine kinase purified from different sources.

Source	Molecular mass (kDa)	Methods	Reference
Human placenta	40 38	SDS-PAGE sucrose-gradient	Andres and Fox, 1979 Yamada *et al.*, 1982
Human liver	40	SDS-PAGE	Yamada *et al.*, 1982
Rabbit liver	40 51 34	SDS-PAGE SDS-PAGE Sephadex G-100	Miller *et al.*, 1979 Yamada *et al.*, 1982
Leishmania donovan	38	SDS-PAGE	Datta *et al.*, 1987
Mongolian Gerbil liver	40	SDS-PAGE	Yamada *et al.* , 1982
Syrian hamster liver	43 40	SDS-PAGE	Juranka *et al.*, 1985
Bovine liver	43	SDS-PAGE	Noda *et al.*, 1989
Murine leukemia L1210 cells	56	Sephadex G-100	Chang *et al.*, 1980
Mouse liver	40	SDS-PAGE	Yamada *et al.*, 1982

for the enzyme, and GTP can substitute weakly in this regard. The evidence that both dATP and araATP exhibited decreased activity relative to ATP indicates the importance of the 2'-hydroxy group on the nucleoside triphosphate [Miller *et al.*, 1979b]. In addition, the purine nucleoside triphosphates are more efficient than the pyrimidine ones. Magnesium ion is essential for AK activity, but the enzyme is inhibited by higher concentration of magnesium ion [Miller *et al.*, 1979; Chang *et al.*, 1980].

Based on kinetic studies on the interactions between AK and different adenosine analogs, it has been suggested that AK has two adenosine binding sites: the catalytic site and the regulatory site [Hawkins and Bagnara, 1987; Lin *et al.*, 1988]. The catalytic site has high affinity for adenosine, while the regulatory site has a lower affinity for adenosine, which inhibits the rate of adenosine phosphorylation *in vivo*. At a lower concentration, adenosine binds only to the catalytic site, following Michaelis-Menten kinetics. At higher concentrations, adenosine also binds to the regulatory site and inhibits AK activity, referred to as substrate inhibition. The regulatory site is not the ATP binding site, since neither ATP nor GTP could provide protection afforded by adenosine against 5,5'-dithiobis(2-nitrobenzoic acid)-mediated oxidation of thiol groups in AK [Hawkins and Bagnara, 1987]. Previous studies have shown that at adenosine concentrations above 1 mM, the increase of AK activity by adenosine analogs is due to the competition of adenosine analogs with adenosine for

binding at the adenosine regulatory site. This competition results in a decrease in the adenosine-mediated substrate inhibition, and a net increase in AK activity [Lin *et al.*, 1988]. Substrate inhibition by adenosine is affected by pH, AMP concentration and free magnesium ion concentration [Hawkins and Bagnara, 1987]. Adenosine shows strong inhibition of AK activity at pH 7.4, moderate inhibition at pH 8.0 and no inhibition at pH 6.0. AMP, depending upon the concentrations of both adenosine and free magnesium ion, could decrease the substrate inhibition. In contrast, free magnesium ion enhances the substrate inhibition by adenosine [Hawkins and Bagnara, 1987]. Earlier studies also indicate that thiol groups of cysteine residues are involved in the catalytic and regulatory process [Bhaumik and Datta, 1992]. Studies on AK from human erythrocytes demonstrate that the regulatory site, which contains a functional sulfhydryl group, can be protected from -SH inactivating agents by adenosine. Thiol group titration using cysteine-directed reagents has shown that the AK from *Leishmania donovani*, contains cysteine residues which are essential for the functional activity of the enzyme [Hawkins and Bagnara, 1987; Bhaumik and Datta, 1992].

The mechanism of substrate recognition and phosphorylation of various nucleoside or analogs by AK is far from clear. By studying the kinetic properties of AK from L1210 cells, it was shown that phosphorylated AK was an obligatory intermediate in the reaction catalyzed by AK (Chang

et al., 1983). AK was phosphorylated when it was incubated with ATP. In addition, phosphorylated AK transferred phosphate to adenosine in the absence of ATP and $MgCl_2$. This study suggested a random-bi ordered-bi route model as the AK reaction mechanism. Two substrates, adenosine and MgATP, bind in a random order to AK, followed by the sequential dissociation of MgADP and AMP. In contrast, other studies supported a reaction sequence in which adenosine is the first substrate to bind and AMP the last product to be released [Hawkins and Bagnara, 1987; Bhaumik and Datta, 1988; Rotllan and Miras Portugal, 1985]. In addition, by using bi-substrate analogs of AK, Bone *et al.* [1986], showed that two bi-substrate analogs, Ap_4A and Ap_5A, were the strongest inhibitors of AK activity, suggesting that the enzyme carries out phosphoryl transfer directly from ATP to adenosine. Bi-substrates were chemicals in which two adenosine residues were joined by a polyphosphate bridge with various length. A more recent study indicates that the enzyme in anoxic hepatocytes catalyzes an adenosine-AMP exchange reaction in the absence of ATP, suggesting the existence of a phosphorylated enzyme intermediate but through a mechanism different from that of previous studies [Bontemps *et al.*, 1993a; 1993b]. While it is possible that AK from different sources may catalyze the phosphorylation reaction via different reaction mechanisms, it is more likely that these differences result from difficulties in the interpretation of the kinetic data. These difficulties are largely due to the properties of the enzyme, such as the

inhibition caused by high concentration of adenosine and excess free magnesium ion, pH of the assay and its stability in diluted solution, as well as ratios among ATP, magnesium, and adenosine.

1.5 Previous Work on Mutants Affecting AK in Mammalian Cells.

Much work has been done on the isolation and characterization of mutants which are affected in AK. Many different types of mutants in various cell lines, especially in CHO cell lines, have been isolated. Based on their genetic and biochemical characteristics, three different kinds of mutants affected in AK have been described [Gupta and Mehta, 1986a]. A brief summary of the work on these mutants is given below [for review, see Gupta, 1989].

Class A mutants which have completely lost AK activity are the most common type of AK mutants. These mutants have been selected in many different cell types by using a variety of purine nucleoside analogs. They exhibit high degree of cross resistance to both N- and C-nucleoside derivatives which are phosphorylated via AK (viz. toyocamycin, tubercidin, 6-MeMPR, pyrazofurin, formycin A, etc.). The drug resistant property of these mutants behaves recessively in cell hybrids formed with the parental sensitive cells. One interesting phenotype of this group of mutants is their unusually high spontaneous mutant frequency (10^{-3} to 10^{-4}) in CHO cells, suggesting the presence of a mutational hot spot within the AK gene or within the regulatory element of the AK gene.

Class B mutants affected in AK have been obtained using formycin A and formycin B as selective drugs [Mehta and Gupta, 1983]. These mutants show increased resistance to various C-nucleoside analogs (viz. pyrazofurin, 9-deazaadenosine, etc.) but no significant cross resistance to various N-nucleoside derivatives (e.g. tubercidin, toyocamycin, TCN, 6-MeMPR). An interesting property of these mutants is that the phosphorylation of N-nucleoside derivatives *in vivo* was not reduced, even though no AK activity could be detected in cell extracts of Class B mutants under the different experimental conditions tested so far. Drug resistant phenotype of these mutants is exhibited codominantly with AK activity in cell hybrids formed with the parental sensitive cells. The mutation frequency of this group of mutants ranges from 10^{-5} to 10^{-6} which is normal in mammalian cell lines.

Class C mutants have been selected using the inosine analog formycin B [Mehta and Gupta, 1986]. Similar to the class A mutants, these mutants exhibit a recessive phenotype in cell hybrids and show increased resistance to both N- and C-purine nucleoside analogs. However, the degree of resistance to various C- and N-nucleoside analogs in class C mutants is much lower in comparison to the class A mutants. The main distinguishing characteristic of the class C mutants is that they contain a substantial amount of AK activity. But the enzymes from the mutant cells, which exhibit increased resistance, show lower affinity for the adenosine analogs, suggesting an alteration of the substrate binding site in AK.

The mutant frequency of class C mutants is similar to that of class B mutants.

A novel class of arabinofuranosyladenine-resistant ($araA^r$) mutants of baby hamster kidney (BHK) cells was reported by Juranka *et al.* [1984]. These mutant cells were extremely sensitive to adenosine in culture and AK derived from these cells showed altered kinetic properties and an altered pH optimum, suggesting that a mutation within the AK structural gene was likely responsible for this kind of mutant [Juranka and Chan, 1985; Juranka *et al.*, 1984].

In addition to the AK mutants discussed above, the pyrrolopyrimidine nucleoside toyocamycin exhibits some unique characteristics in the selection of AK mutants. It was found in our laboratory that the minimum growth inhibitory concentration of toyocamycin varied in a linear manner with the cell number over a wide range, suggesting that the cytotoxicity of toyocamycin results from either stoichiometric binding or incorporation into a cellular component. Furthermore, second-step mutants selected by an exposure of single-step selected mutants to higher concentrations of toyocamycin showed no increased cross resistance to other adenosine analogs, such as tubercidin. This result suggested that the second-step mutation affected a specific metabolic step or the gene responsible for the cellular toxicity of toyocamycin. Further studies with these second-step mutants could be very useful in understanding the mechanism of cellular action of toyocamycin.

Immunological characteristics of AK from the class A, B, and C mutants have been investigated using an antibody raised against AK from CHO cell lines [Gupta and Mehta, 1986b]. This antibody appeared to pull out AK activity from cell extracts and cross reacted with a protein that has a similar molecular mass (about 38 kDa) to AK. Using immunoblotting techniques, studies on different mutants showed that a similar amount of AK antibody cross-reacting protein with identical electrophoretic mobility was present in class A, B and C mutants, suggesting that missense types of mutations within the AK structural gene may occur in all three classes of AK mutants [Gupta and Mehta, 1986b]. In contrast, immunoblotting studies on AK^- arabinofuranosyl-adenine-resistant ($araA^r$) mutants of BHK cells indicated that there was no detectable AK protein in the mutant cell lysates [Juranka and Chan, 1985].

1.6 The Purpose of the Project.

Based on the information available in this area and the previous work in our laboratory as stated above, a number of questions can be asked. First, what is the genetic and molecular basis for the unusually high frequency of class A AK^- mutants in CHO cells? Second, what is the genetic and biochemical basis of the codominant drug resistant phenotype of class B mutants and the phosphorylation of N-nucleoside analogs in such cells? Third, what is the mechanism of the cellular toxicity of toyocamycin? Fourth, what is the genetic basis of the unusual characteristic of AK in class C mutants?

The key point to answer the above questions is to clone the AK gene(s) from wild type CHO cells as well as mutants. Once the DNA sequence of AK gene becomes known, many of these questions can be answered by determining the specific changes in AK that have occurred in the different classes of mutants. The AK gene has not been cloned from any species so far, even though this protein was partially or completely purified from many sources several years ago (for details, see previous discussion) [Table 1]. The AK locus has been assigned to chromosome 10 in human cells [Chan *et al.*, 1978; Klobutcher *et al.*, 1976], chromosome 14 in mouse cells [Leinwand *et al.*, 1978; Samuelson and Farber, 1985] and it is not X-linked in CHO cells [Gupta and Siminovitch, 1978].

There are at least three ways to clone the AK gene. The classic way is to purify AK protein and get a partial protein sequence by microsequencing. From this sequence, degenerate oligonucleotide probes could be designed which can be used to screen cDNA libraries to obtain a AK-specific clone. The second way is to raise antibodies against AK. A cDNA library of CHO cells can be made in an expression vector. AK antibodies can be used as a probe to screen the cDNA library. A positive colony indicates that the fusion protein translated includes at least partial AK protein and the vector contains at least a partial AK gene. The third way is to transfect AK^- mutant cells with expression vectors containing cDNA from normal human cells. By growing the transfected cells in selective medium where only AK^+ cells can survive, AK^+

transfectants that have acquired AK from the vectors could be obtained. Spontaneous revertants should arise in this experiment at much lower frequency and should be relatively easy to distinguish based on the absence of vector sequence and human DNA. In an effort to clone the AK gene in the long run, I have pursued all three approaches. Although substantial progress has been made, the main focus of my work has been on purification and characterization of AK from a number of sources. AK has been purified and partially purified from a number of sources. By using purified AK from Syrian hamster liver, a partial protein sequence of AK was obtained and antibodies were raised in both rabbits and guinea pigs. Furthermore, enzymatic properties of AK was investigated by using partially purified AK. Finally, conditions to selected AK^+ revertants from AK^- mutants in CHO cells were developed.

MATERIALS AND METHODS

2.1. Materials.

Adenosine 5'-triphosphate (ATP), lanthanum chloride, adenosine 5'-monophosphate (AMP) Sepharose 4B, 6-methyl-mercaptopurine ribonucleoside (6-MeMPR), DEAE-Sephacel, CM Sepharose CL-6B were purchased from Sigma Chemical Co., St. Louis, MO. Toyocamycin was obtained from the Drug Synthesis and Chemistry Branch of the National Cancer Institute, National Institute of Health, Silver Spring, MD. Tris base and bovine serum albumin (BSA) were the products of Boehringer Mannheim Corporation, Indianapolis, IN. Erythro-9-2-hydroxy-3-nonyl) adenine (EHNA) was a product of Burroughs Wellcome Co. [2,8,-^{3}H]Adenosine (30.0 Ci/mmol), was purchased from Du Pont, Boston, MA. Protein A Sepharose CL-4B was the product of Sigma Chemical Co., St. Louis, MO. Goat anti-rabbit (or anti-guinea pig) horseradish peroxidase was purchased from Bio-Rad Laboratories, Richmond, CA. Probe-Design protein digestion kit was the product of Promega Corporation, Madison, WI. Potassium chloride, sodium chloride, and other common chemicals were purchased from BDH Inc., Toronto.

The protein standards used for protein molecular mass determination in SDS-polyacrylamide gels (phosphorylase b, 94 kDa; bovine serum albumin, 67 kDa; ovalbumin, 43 kDa; carbonic

anhydrase, 30 kDa; soybean trypsin inhibitor, 20.1 kDa; and alpha-lactalbumin, 14.4 kDa) were purchased as a kit from Pharmacia LKB Biotechnology Inc., Piscataway, NJ.

2.2. Methods.

2.2.1. Adenosine Kinase Activity Assay.

The activity of AK was determined using the method described previously by Gupta and Siminovitch [1978]. The reaction mixture in a total volume of 250 ul contained (final concentration) 50 mM potassium phosphate, pH 7.0; 0.25 mM $MgCl_2$; 2.5 mM ATP; 43 uM adenosine (2.5 uCi [^{3}H]adenosine with unlabelled adenosine). It was incubated in a 33°C water bath. The reaction was initiated by adding the protein sample (maximum 50 ul). An aliquot of 50 ul was taken every five minutes and delivered immediately into 1.0 ml of ice cold 0.1 M lanthanum chloride. The precipitated [^{3}H]AMP was collected on GF/C glass microfibre filters and washed with 30 ml of distilled water. The glass microfibre filters were dried and counted in aqueous counting scintillant. The background radioactivity was obtained by using a reaction mixture without the protein sample and this radioactivity data was subtracted from all other experimental data. The AK activity was linear with time and protein concentration under the experimental conditions. To study the Km of AK for adenosine, AK was assayed at varied concentrations of labelled adenosine from 16 to 96 uM. Each study was performed in triplicate. Double-reciprocal plots of initial velocity versus substrate

concentration were linear under experimental conditions. Kinetic data were fitted to the simple Michaelis-Menten equation [Rudolph and Fromm, 1979; Cleland, 1979].

2.2.2. Phosphate-Dependent Adenosine Kinase Activity.

The same procedures were used as described previously for every step of the assay, except for the buffer. In contrast to the normal AK assay, the phosphate buffer was replaced with 20 mM Tris-HCl, pH 7.0, with the addition of phosphate to reach different final concentrations of phosphate ranging from 0.1 mM to 100 mM. The same amounts of AK and other reagents were added to the different Tris-phosphate solutions in the assay. AK assay with 20 mM Tris-HCl, pH 7.0, was used as a control. Each study was carried out in triplicate.

2.2.3. Effect of Different Ions on Enzymatic Activity of Adenosine Kinase.

The same procedures were used as described previously for each step of the assay, except for the buffer. The buffer used in the assay was 20 mM Tris-HCl, pH 7.0 instead of phosphate buffer in the normal AK assay. Different amounts of various chemicals were added to the reaction solution in a final concentration varied from 0.1 to 100 mM. The AK assay with 20 mM Tris-HCl, pH 7.0, was used as a negative control and the assay with 20 mM Tris-HCl, 50 mM phosphate, pH 7.0 was used as a positive control. Each assay was carried out in triplicate (or duplicate in some cases).

2.2.4. Protein Determination.

Protein concentration was determined by the Bradford assay [Bradford, 1976]. A standard curve was plotted using bovine serum albumin as the standard protein. The sample protein concentration was determined by the linear part of the standard curve.

2.2.5. Experimental Approach for AK Purification.

Purification of AK was performed by a method similar to that used by Andres with some modifications [Andres and Fox, 1979]. All the procedures for AK purification were carried out at 4°C or on ice [Fig. 3] unless otherwise stated. All the solutions were autoclaved. The material (liver in most cases) was homogenized in the presence of isolation buffer (0.25 M sucrose, 1 mM EDTA, 10 mM Tris-HCl, pH 7.2, 0.1 mM phenylmethylsulfonyl fluoride), and the homogenate was centrifuged at 30,000 x g for 1 hr. The precipitate was discarded and the pH of supernatant was adjusted to 6.0 with 1.0 M HCl. CM-Sepharose and DEAE-Sephacel were previously treated with 0.5 M sodium acetate, pH 6.0, and then equilibrated in 10 mM sodium acetate, pH 6.0. After rocking with an equal volume of CM-Sepharose for 90 min, the sample was filtered using a Buchner funnel and then mixed with the same amount DEAE-Sephacel. After rocking for 2.5 hr, the sample was filtered again and applied to a 20 ml 5'-AMP Sepharose affinity column which was previously equilibrated with 10 mM sodium acetate, pH 6.0. The column was washed with

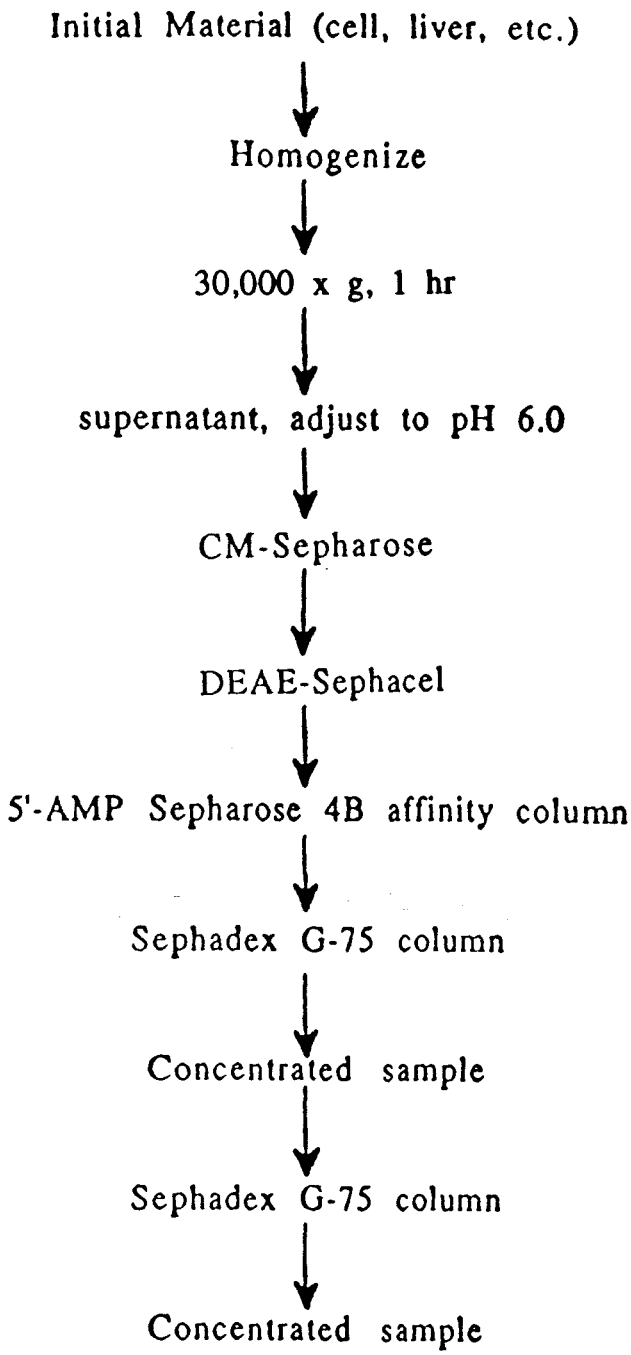


Fig. 3 Schematic flow-chart of adenosine kinase purification procedure. The detail procedures are described in "Materials and Methods".

three different kinds of buffers: 140 ml of 10 mM sodium acetate, pH 7.0; 40 ml of 10 mM Tris-HCl, pH 7.4, 1 M KCl; 20 ml of 0.1 M Tris-HCl, pH 7.4, 5 mM ATP. AK was eluted with the elution buffer containing 0.1 M Tris-HCl, pH 7.4, 5 mM ATP, 5 mM adenosine, 5 mM $MgCl_2$. Approximately 20 fractions (1.5 ml per fraction) were collected and characterized by enzymatic assay and SDS-polyacrylamide gel electrophoresis. Fractions containing AK activity were pooled and concentrated in an Amicon concentration cell equipped with a PM-10 membrane. The pooled partially purified AK sample was frozen at -70°C.

The concentrated elution sample containing AK activity from the 5'-AMP Sepharose affinity column was applied to one Sephadex G-75 column (0.75 x 100 cm). Elution rate was controlled by a Gilson Minipuls II pump. Fractions were collected on a Bio-Rad Model 2110 Fraction Collector. After about 4 hr running at a flow rate of 16.5 ml per hr with a buffer of 0.1 M Tris-HCl, pH 7.4, 0.25 M NaCl, 1 mM $MgCl_2$, AK was successfully separated from the high molecular mass protein but only partially separated from the medium molecular mass protein. In order to get better separation, two Sephadex G-75 columns connected in series (0.75 x 100 cm each) were used to separate the proteins. After more than 9 hr running with the same buffer at a flow rate of 10.5 ml per hr, three different proteins were separated. AK assay was carried out with every third fraction. Fractions containing AK activity were pooled and concentrated in an Amicon concentrator

equipped with a PM-30 membrane. The concentrated AK was stored at -70°C.

2.2.6. Protein Microsequencing.

In order to get pure protein for CNBr digestion, partially purified AK was resolved in a SDS-polyacrylamide gel and transferred to PVDF membrane, according to the standard procedures described later. The membrane was stained in freshly prepared 0.025% Coomassie Blue in 50% methanol for 10 min and destained in 50% methanol for 30 min. The band corresponding to AK was cut out and placed in a 1.5 ml Eppendorf tube. 200 ul of CNBr solution (10 mg/ml in 70% formic acid) was added to each tube and these tubes were incubated overnight at room temperature. The CNBr solutions were transferred to new Eppendorf tubes and dried with a nitrogen stream in a fume hood. At the same time, peptide elution from the PVDF membrane was performed by adding 200 ul of peptide elution solvent to each tube containing the PVDF membrane segment (1 ml elution solvent contained 700 ul isopropanol, 2 ul trifluoroacetic acid, 1 ul lysine, 1 ul thioglycolic acid and water). These tubes were occasionally vortexed. After incubating at room temperature for 2 hr, the peptide elution solvent was transferred to the tubes containing dried CNBr solution. The membrane segments were extracted another time by adding 200 ul of peptide elution solvent to each tube and incubating for another 2 hr. The elution solvent was evaporated using a nitrogen stream in a

fume hood and 25 ul of 2 x loading buffer was added to the dried sample before the samples were boiled for 2 min. The eluted sample was resolved on a specific SDS-polyacrylamide gel (top: 2.0 cm of 4% acrylamide stacking gel; middle: 1.0 cm of 10% acrylamide spacing gel; bottom: 4.2 cm of 16.5% acrylamide separating gel). The SDS-polyacrylamide gel was prepared 3 hr before use and was pre-run for 30 min at 40 V with the addition of thioglycolic acid to the cathode buffer (200 ul of the 100 mM stock thioglycolic acid solution per 100 ml buffer). The cathode buffer contained 0.1 M Tris base, 0.1 M Tricine and 1% SDS, while the anode buffer consisted of 0.2 M Tris-HCl, pH 8.9. The electrophoresis was carried out under a constant voltage of 80 V, then 200 V for the separating gel until the dye front reached the bottom of the separating gel. Transfer of the peptides from the gel to the PVDF membrane was performed under a constant current of 150 mA for 20 min, following a constant current of 200 mA for 20 min. Transfer buffer consisted of 25 mM Tris-HCl, pH 8.0, 195 mM glycine and 20% methanol. After the transfer, the PVDF membrane was stained in freshly prepared 0.1% Coomassie Blue in 50% methanol for 10 min and destained with 50% methanol for 30 min. Four major bands were seen on the PVDF membrane and these peptide bands were cut out for protein microsequencing.

2.2.7. Raising Adenosine Kinase Antibody.

Purified homogenous AK was used to raise antibody in rabbits and guinea pigs. A solution of 0.6 ml containing

about 0.5 mg of protein emulsified with the same volume of either complete (first injection) or incomplete (following three injections) Freund's Adjuvant was injected intradermally at multiple sites on the back of one male rabbit or two female guinea pigs. Three booster injections of the same amount of antigen were given with an interval of four weeks. A total of three rabbits and four guinea pigs were used to raise antibodies against AK. Before each injection, 1 ml of blood was taken out from the marginal ear vein for the antibody assay. About 0.5 ml of serum from 1 ml blood was obtained each time after incubation of the blood at 37°C for 40 min and centrifugation for 30 min at 4°C. Prior to the first antigen injection, the rabbits and guinea pigs were bled for normal control serum. After four injections, the two rabbits were bled and 60 ml of antiserum was obtained per rabbit. The antiserum was aliquoted and kept at -70°C. About 15 ml of antiserum was obtained per guinea pig.

2.2.8. Coupling of AK antibodies with Protein A Sepharose Beads.

Approximately 2 ml of AK antiserum from one rabbit and 1 ml of Protein A Sepharose beads were mixed and incubated at room temperature for 1 hr with gentle rocking. After washing twice with 10 volumes of 0.2 M sodium borate (pH 9.0) by centrifugation at 3,000 x g for 5 min, the beads were resuspended in 10 volumes of 0.2 M sodium borate (pH 9.0). The equivalent of 10 ul of beads was removed. Solid dimethyl

pimelinidate was added to the resuspended beads to reach the final concentration of 20 mM. After gently rocking for 30 min at room temperature, the equivalent of 10 ul of the coupled beads was removed. The coupling reaction was stopped by washing the beads once with 0.2 M ethanolamine (pH 8.0). The beads were gently rocked with 0.2 M ethanolamine (pH 8.0) for 2 hr at room temperature. After the final wash with PBS, the beads were resuspended in PBS and kept at 4°C. The Protein A-AK antibodies beads were used to carry out the immunoprecipitation test described later. The efficiency of coupling can be determined by running the two equivalents of 10 ul beads taken previously on a SDS-polyacrylamide gel (10% acrylamide).

2.2.9. SDS-Polyacrylamide Gel Electrophoresis.

SDS-PAGE was carried out as described by Laemmli [Laemmli, 1970]. Electrophoresis was performed in a Bio-Rad Mini-protean II apparatus. Separating gels contained 10% (w/v) acrylamide, o.27% (w/v) N,N'-methylene bisacrylamide, 0.375 M Tris-HCl, pH 8.8, 0.1% (w/v) glycerol and 0.025% TEMED, 0.075% (w/v) ammonium persulfate and 0.10% (w/v) SDS. The solution was degassed before the addition of TEMED and SDS, as well as freshly prepared ammonium persulphate. The stacking gel contained 5% (w/v) acrylamide, 0.13% (w/v) N,N'-methylene bisacrylamide, 0.125 M Tris-HCl, pH 6.8, 0.1% (w/v) SDS, 0.05% (w/v) glycerol, 0.075% (w/v) TEMED and 0.064% (w/v) ammonium persulphate.

Samples for electrophoresis were mixed with an equal volume of 2 x electrophoretic sample buffer, which contained 0.125 M Tris-HCl, pH 6.8, 2% (w/v) SDS, 20% (w/v) glycerol, 2.5% (v/v) 2-mercaptoethanol, 0.002% (w/v) bromophenol blue. The samples were boiled for 3 min in order to denature the proteins in the samples. Electrophoresis running buffer consisted of 0.3% (w/v) Tris base, 1.44% (w/v) glycine and 0.1% (w/v) SDS. After applying the samples, a constant voltage of 80 V was used initially until the dye front reached the boundary between stacking and separating gels. Then, a constant voltage of 200 V was applied until the dye front reached the bottom of the gel.

After running the gel for approximate one hr, the gel was taken out and placed in the staining solution containing 0.25% (w/v) Coomassie Blue, 50% (v/v) methanol, 10% (v/v) glacial acetic acid. After staining for 1 hr with shaking, the gel was destained overnight in the destaining solution, which consisted of 50% (v/v) methanol and 10% (v/v) glacial acetic acid. Gels were dried on Whatman #3 chromatography paper using a Bio-Rad gel dryer.

2.2.10. Immunoblotting.

Electrophoretic transfer of protein from SDS-polyacrylamide gels to nitrocellulose sheets was carried out by the method of Towbin *et al.* [Towbin *et al.*, 1979]. Protein samples (cell lysates, liver extract, purified protein) were resolved on 10% SDS-polyacrylamide gels, using

the method described previously. When the electrophoretic run was finished, the gel was taken out and placed onto the sheet of nitrocellulose membrane. The complex of gel-nitrocellulose membrane was sandwiched between two sheets of Whatman #3 chromatography paper and then put in plastic grids with the polyacrylamide gel facing the negative electrode. The whole complex was then locked and transferred into a Bio-Rad transblot chamber which was filled with the transfer buffer [25 mM Tris base, 195 mM glycine, 20% (v/v) methanol]. The proteins in the gel were transferred to nitrocellulose membrane under a constant current of 200 mA for 2 hr. The nitrocellulose membrane was treated with 3% BSA in Tris-buffered saline (TBS) for 30 min and then washed three times with TBS. TBS contains 0.8% (w/v) NaCl, 0.02% (w/v) KCl and 25 mM Tris-HCl, pH 7.4. The nitrocellulose membrane was then rocked for 1 hr with properly diluted rabbit (or guinea pig) antiserum in 3% BSA/TBS, before washing three times with TBS. After it was incubated for 1 hr with properly diluted horseradish peroxidase-conjugated goat anti-rabbit (or guinea pig) IgG in 3% BSA/TBS, the membrane was rinsed in TBS with three changes. The final colour reaction was carried out with a mixture of 6 ml of TBS, 3 ul of hydrogen peroxide (30%) and 1 ml of HRP stocking buffer (6 mg of HRP per ml of methanol). The preimmune serum was served as a control in the above experiments.

2.2.11. Electroelution of AK from SDS-Polyacrylamide Gels.

The protein samples (partially purified AK samples in most cases) were resolved in 12% SDS-polyacrylamide gels using the same method described previously. Once the proteins had been separated by gel electrophoresis, the protein bands were visualized by soaking the gel in 0.25 M KCl overnight at 4°C. The protein band of interest were cut off, collected and frozen at -20°C.

Protein elution from bands of SDS-polyacrylamide gels was performed using a Bio-Rad Model 422 Electro-Eluter assembly. Protein elution buffer consisted of 25 mM Tris base, 192 mM glycine and 0.1% (w/v) SDS. The membrane caps were soaked at 60°C in the protein elution buffer for 1 hr prior to use. Pre-wetted membrane caps were placed in the bottom of silicone adaptors. After filling them with the elution buffer, the adaptors with membrane caps were slid into the bottom of the glass tubes with frits. The adaptors were partially pulled up and down several times in order to expel all the air bubbles inside the silicone adaptors. The SDS-polyacrylamide gel slides corresponding to the protein of interest were cut into small pieces. These pieces were loaded into glass tubes of the protein elution assembly. The entire assembly was placed into the Bio-Rad buffer chamber filled with the elution buffer. A stirring bar was put in the bottom of the buffer tank and kept stirring during the run. Protein elution was carried out at the constant current of 10 mA per glass tube for 4 hr. After the elution was completed, the buffer left in

the glass tubes to the level of the frits was removed and discarded. The adaptors, together with the membrane caps, were dismantled from the bottom of the glass tubes. The solution left in the adaptors with the membrane caps contained the eluted protein. The caps were rinsed twice with the elution buffer and combined with the eluted protein solutions. The eluted protein was stored at -70°C.

2.2.12. Cell Culture and Cell Lines.

CHO wild type cells, CHO DR31 cells, Hela cells, BHK cells, and human fibroblast cells were used in this study. The cells were routinely grown in monolayer culture in alpha minimal essential medium supplemented with 5% fetal calf serum and maintained at 37°C in a 5% CO_2 environment. The doubling time of CHO cells is about 14 hr under these conditions. Sterile phosphate-buffered saline (PBS), which consisted of 0.02% (w/v) KCl, 0.8% (w/v) NaCl, 0.144% (w/v) disodium hydrogen orthophosphate and 0.024% (w/v) potassium dihydrogen phosphate, pH 7.2, was used to rinse cells. Cells were harvested by trypsinization with 1 ml of 0.125% trypsin in PBS per dish for 3 min and washed with PBS before they were transferred to new culture dishes. The cell number was counted using a Coulter Electronic Counter.

2.2.13. Selection of AK^+ Revertants from AK^- Mutants.

2.2.13.1. Plating Efficiencies.

Plating efficiencies were determined by plating 250 cells

at different concentrations of the desired drug in 60-mm tissue culture dishes containing 7 ml of the growth medium (including control dishes which did not contain any drug). After two weeks of incubation at 37°C, the dishes were stained with 0.5% methylene blue in 50% methanol. The colonies were counted. The relative plating efficiencies were determined as the ratios of the number of colonies at a given drug concentration to that obtained in the absence of the drug.

2.2.13.2. Selection of AK^+ Revertants from AK^- Mutants.

AK^- mutants from CHO DR31 cell lines were used in revertant selection. After treatment overnight with 200 ug/ml ethyl methanesulfonate, cells (about 10^6 cells per 100-mm dish) were seeded in minimal alpha-medium with 5% fetal calf serum, containing 2 x 10^{-5} M adenosine, 0.7 mg/ml alanosine, 1 x 10^{-3} M uridine and 20 mg/ml EHNA. The dishes were incubated at 37°C in a CO_2 incubator for about 15 days and surviving colonies were transferred separately to new tissue culture dishes. Each colony was grown in normal minimal alpha-medium with 5% fetal calf serum for freezing, AK enzymatic activity assays and drug resistant tests.

2.2.13.3. Determination of the Degree of Drug Resistance (or Minimum Inhibitory Concentration).

The degree of resistance of mutant cell lines towards different drugs was measured by mixing 250 cells in 0.5 ml of growth medium with 0.5 ml of various concentrations of the

drugs in the growth medium in the well of 24-well tissue culture dishes. The final drug concentrations were half of those before adding the cells. The dishes were incubated at 37°C for one week. After they were stained for about 30 min with 0.5% methylene blue in 50% methanol and washed with deionized water, the number of colonies per well was counted. The degree of drug resistance was determined by the relative plating efficiencies which was the ratio between the number of colonies at a given drug concentration to that obtained in control cells plated in the absence of drug.

2.2.14. Preparation of Cell Extracts.

Two 100-mm dishes of culture cells (about 5 X 10^6 cells per dish) were harvested by adding 1 ml of 0.125% trysin in PBS to each dish for 3 min, centrifuged and suspended in 1 ml of PBS. After rinsing twice with PBS, the suspension was sonicated four times at medium output for 30 sec on a Sonic Dismembrator (Fisher model 300) with 2 min intervals between each sonication. The extract was centrifuged at 30,000 x g for 30 min. The supernatant was transferred to another Eppendorf tube and frozen at -70°C. This supernatant (crude cell extract) could be used for AK assay and immunoblotting.

2.2.15 Labelling of Tissue Culture Cells with [^{35}S]Methionine.

For metabolic labelling of tissue culture cells in monolayer cultures, logarithmically growing cultures (nearly

75% confluent dishes) were used for labelling. After the normal medium was removed, the monolayer was washed once with PBS. Approximately 3.0 ml of methionine-free medium was added per 100-mm dish. After incubation at 37°C in a CO_2 incubator for 1 hr, 10 uCi of [^{35}S]methionine was added per 100-mm dish. The cells were incubated for another 4 hr before removing the medium. The monolayer was rinsed twice with PBS and incubated on ice for 15 min after adding 1 ml of RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% DOC, 0.1% SDS, 50 mM Tris-HCl, pH 7.5). The cells were scraped from the plate with a rubber policeman and transferred to an Eppendorf tube. After spinning for 15 min at 10,000 x g at 4°C, the lysate (supernatant) was transferred to a new microtube and used for immunoprecipitation. Alternatively, cells were lysed by sonication in the presence of 0.1 M potassium phosphate, pH 7.0

2.2.16. Immunoprecipitation.

2.2.16.1. Immunoprecipitation of Labelled Cell Extracts.

AK-polyclonal antibodies covalently bound to Protein A beads, were used for immunoprecipitation. One 100-mm dish of cells was labelled with [^{35}S]methionine as described previously. The cells were lysed in 1 ml of RIPA buffer (150 mM NaCl, 1.0% NP-40, 0.5% DOC, 0.1% SDS, 50 mM Tris-HCl, pH 8.0) or sonicated in 1 ml of 0.1 M potassium phosphate buffer, pH 7.0 and the supernatant was transferred to another tube after centrifugation for 15 min at 4°C. An aliquot of 250 ul

was taken out and mixed with 50 ul of Protein A-antibody beads. The mixture was gently rocked at 4°C for 4 hr or overnight before centrifugation for 1 min. The supernatant was discarded by aspiration and the beads were washed three times with 1 ml of RIPA buffer (or 1 ml of 0.1 M potassium phosphate buffer, pH 7.0 if the cell extracts were sonicated in the phosphate buffer, pH 7.0). The last wash was removed as completely as possible. Then, 30 ul of 2 x loading buffer was added to the beads and the sample was boiled for 5 min. The sample was then spun at 10,000 x g for 3 min and the supernatant was loaded onto the SDS-polyacrylamide gel. After electrophoresis, the gel was immobilized on a sheet of Whatman paper using a Bio-Rad gel dryer and exposed overnight to an X-ray film.

2.2.16.2. Immunoprecipitation with Syrian Hamster Liver Extract.

Seventy microliters of Protein A-AK antibodies beads were incubated with 250 ul of Syrian hamster liver extract. As a control, Protein A Sepharose beads were incubated with preserum of the rabbit for 2 hr and the beads were then washed three times with 0.1 M potassium phosphate, pH 7.0. Then these Protein A Sepharose beads were mixed with 250 ul of Syrian hamster liver extract. After gently rocking at 4°C for 24 hr, both kinds of the beads were washed three time with 0.1 M potassium phosphate, pH 7.0. The beads (including the beads incubated with preserum) were then used for AK assay.

3. RESULTS AND DISCUSSION

3.1. Isolation and Purification of Adenosine Kinase from Different Sources.

3.1.1 Results.

3.1.1.1 General Description of the Purification of AK.

The general method of AK purification was derived from the work of Andres and Fox with some modifications [Andres and Fox, 1979] [Fig. 3]. AK was purified from different sources, including bovine liver, CHO cells, human placenta, Syrian hamster liver, Syrian hamster heart, and Syrian hamster kidney. Chinese hamster would have been the best source for AK purification because of the origin of CHO cell lines. However, due to the unavailability of Chinese hamsters, Syrian hamster, which is a closely related species and is readily available, became the main source for AK purification. Bovine liver, due to its easy availability, was used to find the optimum conditions for the purification of AK. Because mutants affected in AK have also been isolated in our laboratory from human cell lines, human placenta was also used as a source to purify AK.

Because Syrian hamster liver was used as the main source for AK purification, the following discussion applies mainly

to the results of AK purification from Syrian hamster liver. The first three steps in the purification procedure involving high speed centrifugation, CM-Sepharose chromatography and DEAE-Sephacel chromatography, did not produce an appreciable purification (only 4-fold purification as shown in Table 2). However, these steps were retained since they removed bulk of the other proteins. The high speed centrifugation after homogenization of the initial material resulted in the separation of the extract (supernatant) from all the membraneous and undissolved materials. Since the isoelectric pH of AK was approximately 6.0, the pH of the extract was adjusted to pH 6.0 so that AK bound to neither of the ion exchange resins in the ion exchange chromatographic steps. Those proteins carrying either positive or negative charges bound to the CM-Sepharose or DEAE-Sephacel, respectively and were discarded after centrifugation. The affinity chromatography was the key step to the purification of AK [Table 2 and Fig. 4]. The last wash with the Tris buffer containing ATP was critical to the elution of majority of the 55-kDa contaminating protein, which seemed to bind specifically to the 5'-AMP Sepharose column. As indicated in Fig. 4, the third wash, which corresponded to the fractions numbered from 1 to 10, washed out the majority of the 55 kDa protein (especially shown in fraction 2, 3 and 4). As shown in fraction 1' and 2' in Fig. 4, AK was eluted immediately from the 5'-AMP Sepharose column after the addition of elution buffer containing adenosine, the substrate for AK. A mixture

Table 2. Purification of adenosine kinase from Syrian hamster liver. The detail procedures are described in the section of "Material and Methods".

Purification steps	Total volume (ml)	Protein concentration (mg/ml)	AK specific activity (I.U./mg)	Purification (fold)	Yield (%)
Supernatant after 30,000xg	10	180	0.0362	1.0	100%
Extracts after CM-Sepharose	11	87.5	0.043	1.2	63.5%
Extracts after DEAE-Sepharcel	11	18.9	0.143	4.0	45.7%
Sample after the 5'-AMP affinity column	0.5	2.45	3.06	84.0	5.8%

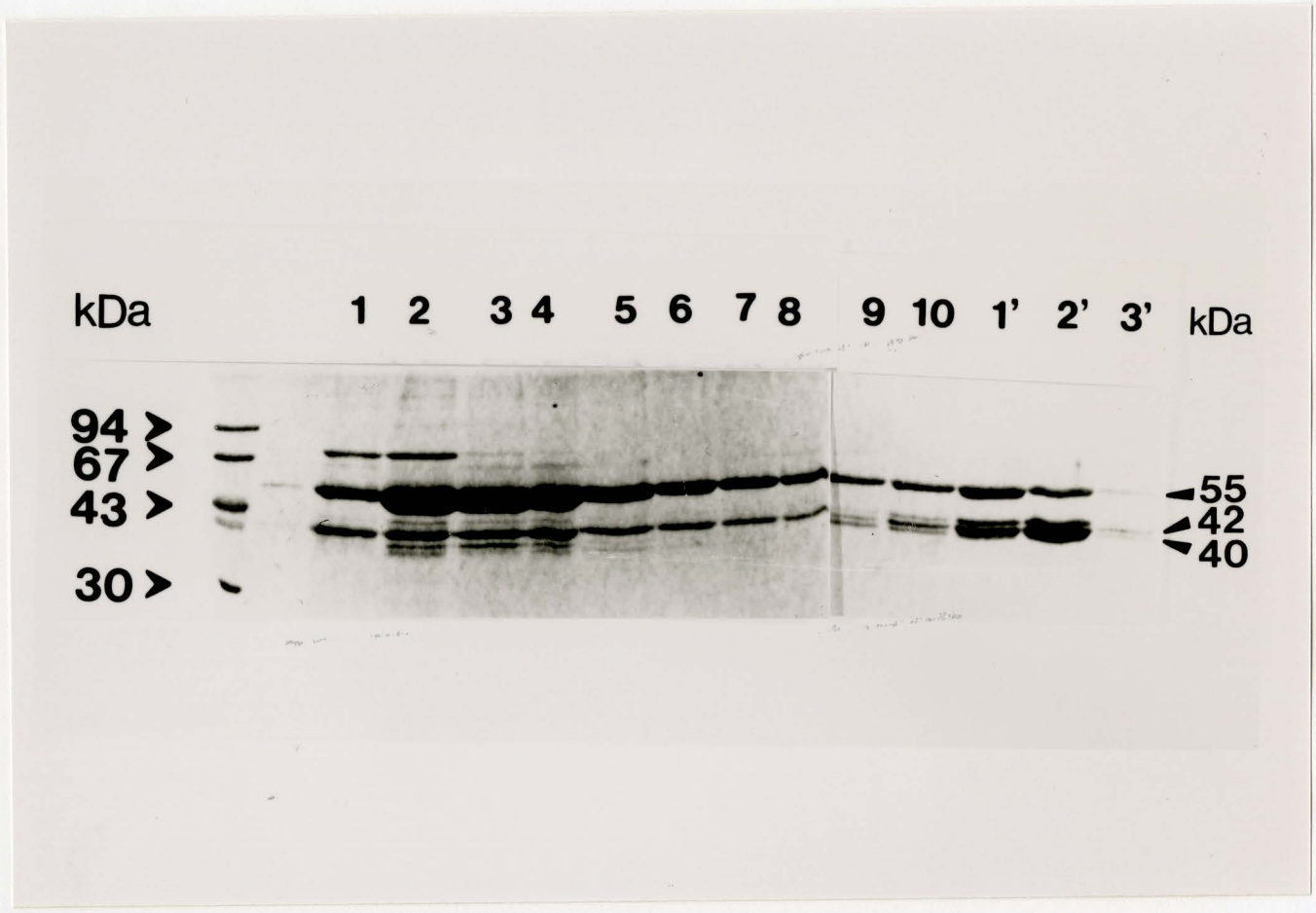
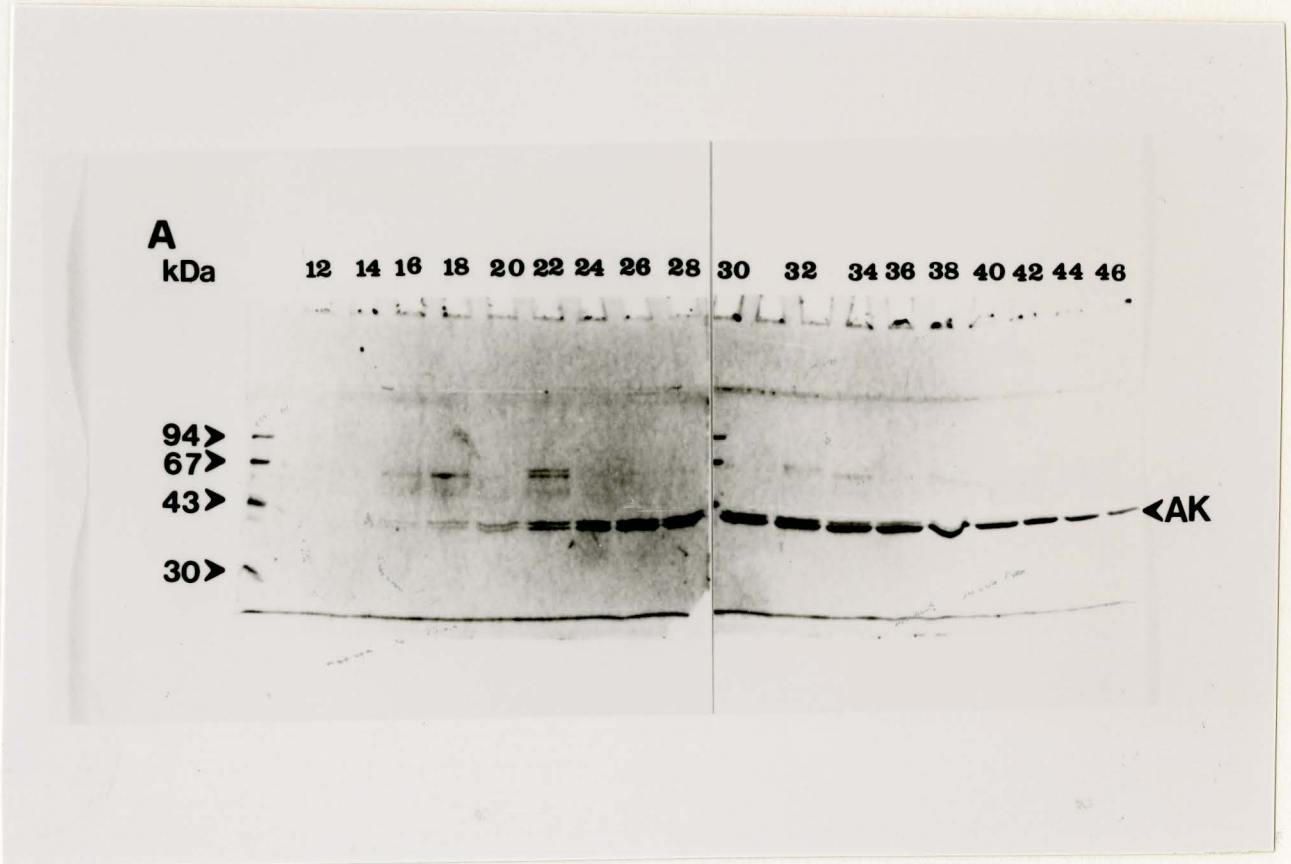
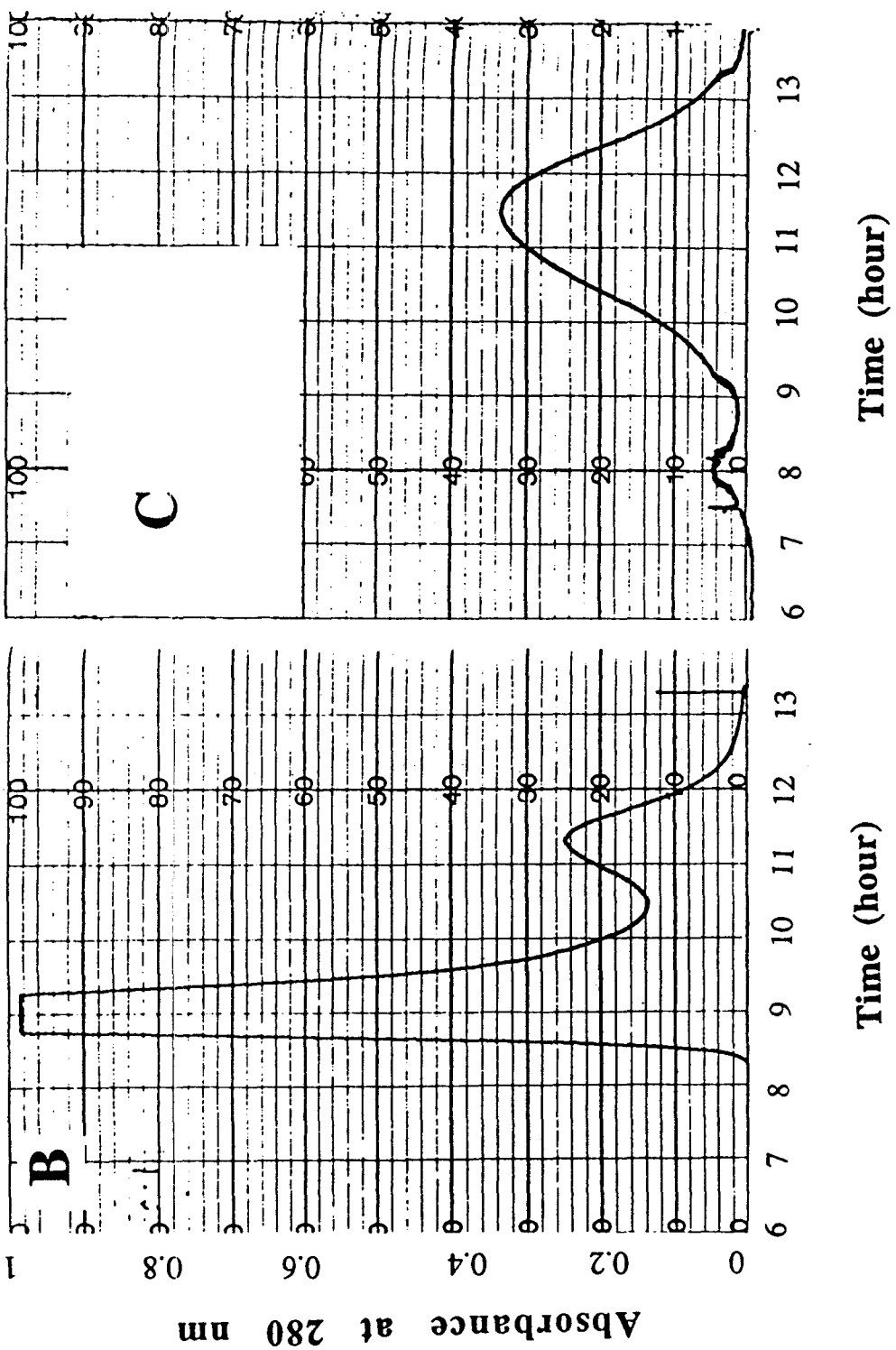


Fig. 4 SDS-PAGE analysis of protein fractions obtained after the 5'-AMP Sepharose column. Proteins were visualized by Coomassie Blue staining. The numbers shown above the lanes indicate the sequence of the fractions. Fractions numbered from 1 to 10 corresponded to the third washing. Fractions numbered from 1' to 3' corresponded to the elution of AK from the column.

of three different proteins, corresponding to 55, 42, and 40 kDa, were obtained in the elution of Syrian hamster liver sample from the 5'-AMP Sepharose column. In general, a mixture of two to five proteins, depending upon the sources, were obtained and further purification was required to obtain pure enzyme. Several different methods, such as ion-exchange chromatography, preparative gel electrophoresis and molecular sieve chromatography, were tested to further purify the AK sample obtained from the 5'-AMP Sepharose affinity column. Of these, gel filtration chromatography was found to be the best method to purify AK. Fractions, such as fraction 1' and 2' in Fig. 4, were pooled and concentrated for further AK purification. Two rounds of Sephadex G-75 chromatography were required for the purification. The first round of Sephadex G-75 chromatography separated AK activity from the 55 kDa protein. As shown in Fig. 5B, two peaks were obtained and the first peak corresponds to the 55 kDa protein (SDS-PAGE analysis, results not shown) which did not show any AK activity. AK activity was retained in the second peak. Fractions with AK activity were pooled and concentrated before being applied to the same column again. Fig. 5C shows that in the second round of Sephadex G-75 chromatography, the 55 kDa protein (the first small peak) was completely separated from the 42 kDa and 40 kDa protein which corresponded to the second peak as shown by SDS-PAGE analysis in Fig. 5A. As seen, the 40 kDa protein eluted somewhat later than the 42 kDa protein and an apparently homogeneous 40 kDa protein was obtained in

Fig. 5 Sephadex G-75 chromatography on purification of adenosine kinase. A, SDS-polyacrylamide gel electrophoretic analysis of the fractions obtained from second round of Sephadex G-75 column chromatography. The numbers indicated the sequence of the fractions. B, First round of Sephadex G-75 chromatography for AK purification. C, Second round of Sephadex G-75 chromatography for AK purification using the concentrated sample from fractions exhibiting AK activity from the first round of Sephadex G-75 column chromatography. The protein concentration was determined by the absorption at 280 nm (in arbitrary units).


A
kDa
12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44 46
94>
67>
43>
30>
<AK


B
C
Absorbance at 280 nm
Time (hour)
Time (hour)

fraction 40 to 46. It could be however that the 42 kDa band was not apparent because of the low protein concentration. Enzymatic activity assays of different fractions indicated that only fractions containing the 40 kDa protein exhibited AK activity. In addition, by comparing the AK activity and protein ratio between two proteins (40 and 42 kDa) among different column fractions, it was inferred that the 40 kDa protein was AK, because the increase of AK activity was related to the increasing concentration of the 40 kDa protein. Fractions containing apparently homogenous 40 kDa protein confirmed above result, demonstrating that the 40 kDa protein was AK. The final enzyme preparation obtained from two rounds of running two-serially connected Sephadex G-75 columns exhibited a single protein band in SDS-gel electrophoresis (fraction 40 to 46 in Fig. 5A). However, this purification step led to a large loss in the enzyme yield, indicating that it could only be used when a large amount of partially purified material was available.

3.1.1.2 Purification of Adenosine Kinase from Different Sources.

The results of the AK purification from Syrian hamster liver are summarized in Table 2 with an 84-fold purification. Typical fractions from different purification steps are shown in Fig. 6. Large amount of other proteins were removed after the sample was treated with CM-Sepharose (lane e in Fig. 6) and DEAE-Sephacel (lane f in Fig. 6). After seven rounds of

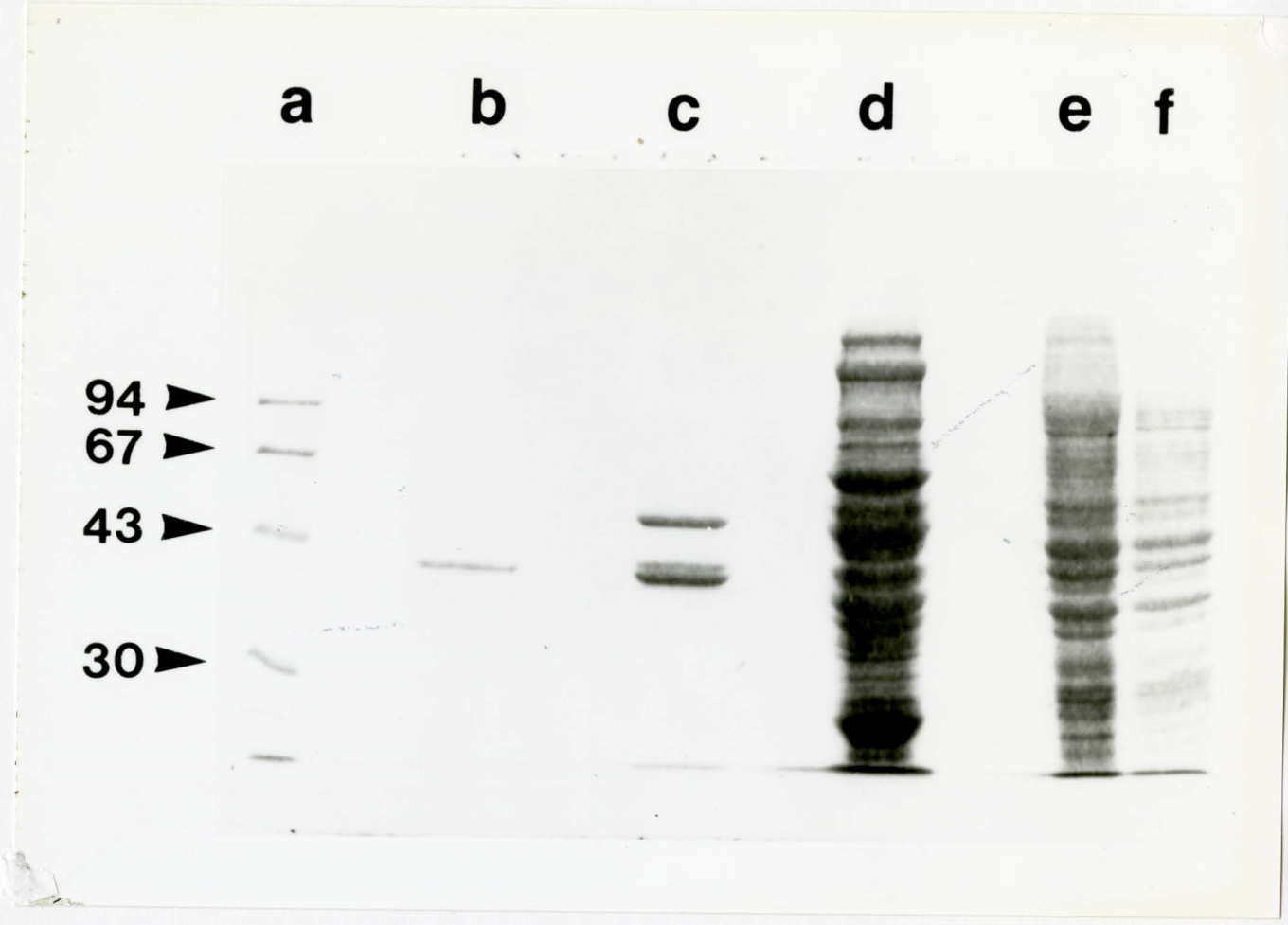


Fig. 6 SDS-PAGE analysis of adenosine kinase purification from Syrian hamster liver. a. low molecular mass markers; b, purified AK from Syrian hamster liver; c, partially purified AK eluted from the 5'-AMP Sepharose affinity column; d, total liver extracts after 30,000 x g centrifugation; e, liver extracts after treatment with CM-Sepharose; f, liver extracts after treatment with DEAE-Sephacel.

purification using the same procedure, 8 ml of elution sample (samples like fraction 1' and 2' in Fig. 4) was pooled from fractions after the 5'-AMP Sepharose column with a protein concentration of 100 mg/ml. The pooled sample contained three proteins, corresponding to 55, 42 and 40 kDa, as shown in lane c in Fig. 6. This sample was concentrated before being applied to the Sephadex G-75 column. As shown in lane b in Fig. 6, apparently homogenous AK from Syrian hamster liver was obtained after two rounds of Sephadex G-75 column chromatography with a final 254-fold purification. Silver nitrate and Coomassie Blue staining procedures produced similar results. The resulting enzyme preparation was estimated at least 95% pure, and had a specific activity of 9.3 I.U./mg (1 I.U. means that 1 umol product, such as AMP, is formed per minute) and a molecular mass of 40 kDa, which was consistent with that of AK purified from other sources [Fig. 7] [Yamada *et al.*, 1982].

Starting with 380 g of bovine liver, similar result was obtained as that from Syrian hamster liver, except that elution fractions from the 5'-AMP Sepharose affinity column were found to contain two proteins, corresponding to 55 kDa and 40 kDa, as shown in lane b in Fig. 7. A 0.6 ml sample with the protein concentration of 600 mg/ml was obtained after pooling and concentrating the column fractions from the 5'-AMP Sepharose affinity column. The sample was further purified by two rounds of Sephadex G-75 column chromatography and only the 40 kDa protein exhibited AK activity, which was in agreement

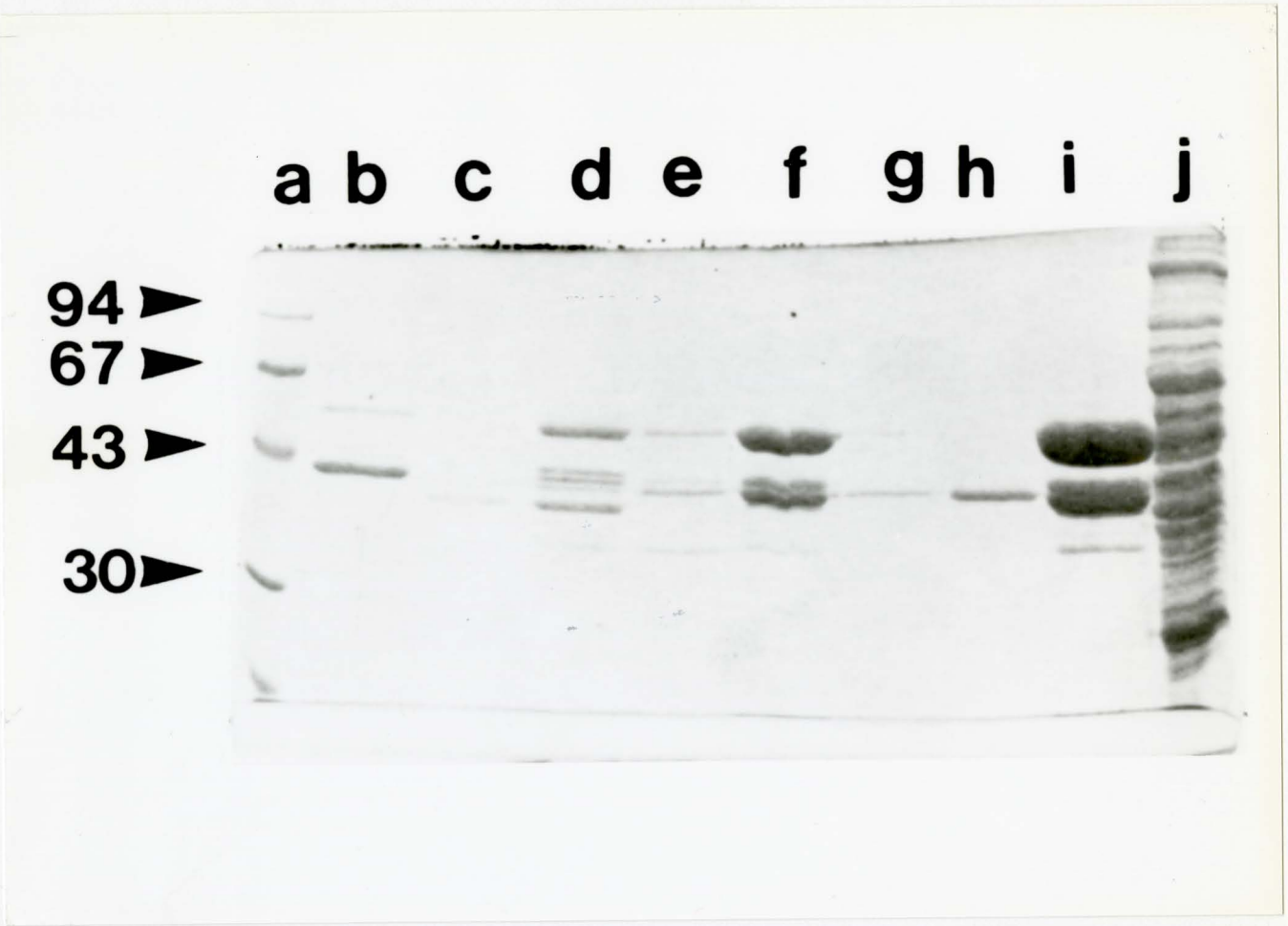


Fig. 7 SDS-PAGE analysis of adenosine kinase from different sources. a, low molecular mass markers; b, bovine liver; c, CHO WT cells; d, human placenta; e, Syrian hamster heart; f, Syrian hamster kidney; g, Syrian hamster liver; h, Syrian hamster liver; i, Syrian hamster liver sample after the elution from the 5'-AMP affinity column; j, Syrian hamster liver after treatment with DEAE Sephacel.

with previous results [Noda *et al.*, 1989].

Because of the limitation of obtaining a large amount of cells, AK purification from CHO cells was not very successful. The eluted fractions from the 5'-AMP Sepharose column had very low protein concentration and exhibited little AK activity (lane c in Fig. 7). This sample contained five different protein bands, corresponding to 55, 42, 41, 40 and 38 kDa (all five could be seen on the SDS-polyacryamide gel, but only the 38 kDa band is seen in the picture). To obtain AK from CHO cells for microsequencing, samples obtained after DEAE-Sephacel treatment were run on the preparative SDS-polyacrylamide gel, and the protein band corresponding to AK was excised, and proteins were electroeluted from the gels. However, results from protein microsequencing indicated that the eluted sample consisted of a mixture of several proteins, because several amino acids were found to be released during each cycle of protein microsequencing.

Human placenta had less AK in comparison with other sources with regard to the specific activity of AK in the extracts. Using similar amount of initial material as bovine liver (about 400 g), eluted fractions from the 5'-AMP Sepharose column contained very low AK activity. The pattern of these elution fractions, as shown in lane d in Fig. 7, were similar to those of CHO cells, which contained five different protein bands, corresponding to 55, 42, 41, 40 and 38 kDa. The molecular mass of adenosine kinase from human placenta, which was around 40 kDa, was consistent with

previous result [Table 1].

Fig. 7 illustrates a comparison of either partially or totally purified AK from different sources, such as bovine liver, CHO cells, human placenta, and Syrian hamster heart, kidney, and liver. The molecular mass of AK from different sources was around 40 kDa, consistent with the results from most of the other papers, except the result based on the data from gel filtration chromatography [Table 1 and Yamada *et al.*, 1982]. The molecular mass of AK from bovine liver was slight higher than that from the other four sources, while AK from CHO cells might have the lowest molecular mass among the five sources [Fig. 7].

3.1.2 Discussion.

The purification of AK constituted the main work for the first part of this project. The purified enzyme, a prerequisite for both protein microsequencing and raising AK antibody, provided two means for cloning the AK gene. Therefore, much effort had been made to purify AK from different sources in the past two years. The experimental approaches to purify AK appeared to be varied from paper to paper in previous studies [for details, see references listed in Table 1]. But there were some common features among these procedures used by previous studies to purify AK. First, DEAE-Sephacel and CM-Sepharose, or other similar ion exchange resins, were employed to eliminate the bulk of other proteins from the extracts. Secondly, a 5'-AMP Sepharose (or Agarose)

affinity column, which was included in every approach, was the critical step to purify this enzyme [Spychala and Fox, 1989]. Finally, further purification was necessary for the sample eluted from the 5'-AMP Sepharose affinity column, because in general, the elution was a mixture of several different proteins, depending upon the sources. It was shown in this study that the elution from the affinity column contained a mixture of two different proteins (55 and 40 kDa respectively) if the initial material was bovine liver, three different proteins (55, 42 and 40 kDa respectively) if the initial material was Syrian hamster kidney, heart or liver, five different proteins (55, 42, 41, 40 and 38 kDa respectively) if the initial material was CHO cells or human placenta [Fig. 4 and Fig. 7]. These proteins appeared to contain AMP-binding sites because they could only be eluted from the 5'-AMP Sepharose column by the addition of ATP or adenosine in the elution buffer [Spychala and Fox, 1989]. This may also be due to the similarity in chemical structures of adenosine, ATP and AMP. Results from additional purification steps showed that only the 40 kDa protein exhibited AK activity. It should be mentioned that using bovine liver extracts as the initial material, the 42 kDa protein did not appear in the elution from the affinity column, suggesting a species difference between bovine and other sources. On the other hand, since this 42 kDa protein was recognized by AK antibodies against AK from Syrian hamster liver (discussed in Section 3.3), it is suspected that this protein, though lacking AK activity, may

be related to AK.

The addition of 5 mM adenosine in the elution buffer could efficiently elute AK from the 5'-AMP Sepharose column, suggesting that AMP competed with adenosine for the same binding site, whereas ATP could not efficiently elute AK from the 5'-AMP Sepharose column. It should be mentioned that in the literature for the purification of AK, the purificatons ranged from several hundred to ten thousand folds (see references listed in Table 1). But this difference is believed to result from the difference in specific activity of the enzyme in the initial materials. The purified enzyme preparation from different sources has similar specific activity ranging from 2.2 to 9.3 I.U./mg [Yamada *et al.*, 1982].

The values of molecular mass of AK from bovine, Chinese hamster ovary cells, Syrian hamster, and human were similar, although AK from bovine liver was slightly higher than that obtained from the other sources. The value was close to 40 kDa, which was in good agreement with the results from most other papers [Table 1]. The value of molecular mass of AK was higher if gel filtration chromatography was used as a criterion, suggesting that AK molecule might not be a globular molecule. The molecular mass of AK based on gel filtration column was less precise than those based on SDS-PAGE, because the previous method depends upon the shape of the molecule, and the amino acid composition of AK shows that AK contains the normal amount of basic and acidic amino acids, suggesting

that it should migrate properly in SDS-polyacrylacrimde gel [Yamada *et al.* 1982]. Furthermore, some differences in the molecular masses of AK from different sources could also result from the posttranslational modification of this enzyme [Juranka and Chan, 1985].

3.2 Biochemical Studies on Adenosine Kinase.

3.2.1 Result.

3.2.1.1 Adenosine Kinase Assay and its Km.

The average data of five independent AK enzymatic assays is shown in Fig. 8, using same amount of partially purified AK solution from Syrian hamster liver. The standard deviations of these five repeat assays were below 8%, indicating the reproducibility of the AK assay. Under the experimental condition used, the phosphorylation of [^{3}H]adenosine into [^{3}H]AMP was linear for at least 20 min. In addition, results of the AK assays using different amounts of AK indicated that the initial velocity of the reaction was proportional to the enzyme concentration, confirming the reliability of the assay [Fig. 9].

Double-reciprocal plots of initial velocity versus substrate concentration were linear under the experimental condition, as indicated in Fig. 10. Kinetic data were fitted to the simple Michaelis-Menten equation at low adenosine concentration (20 mM). The apparent Km of partially purified AK from Syrian hamster liver for adenosine was 0.16 uM, which

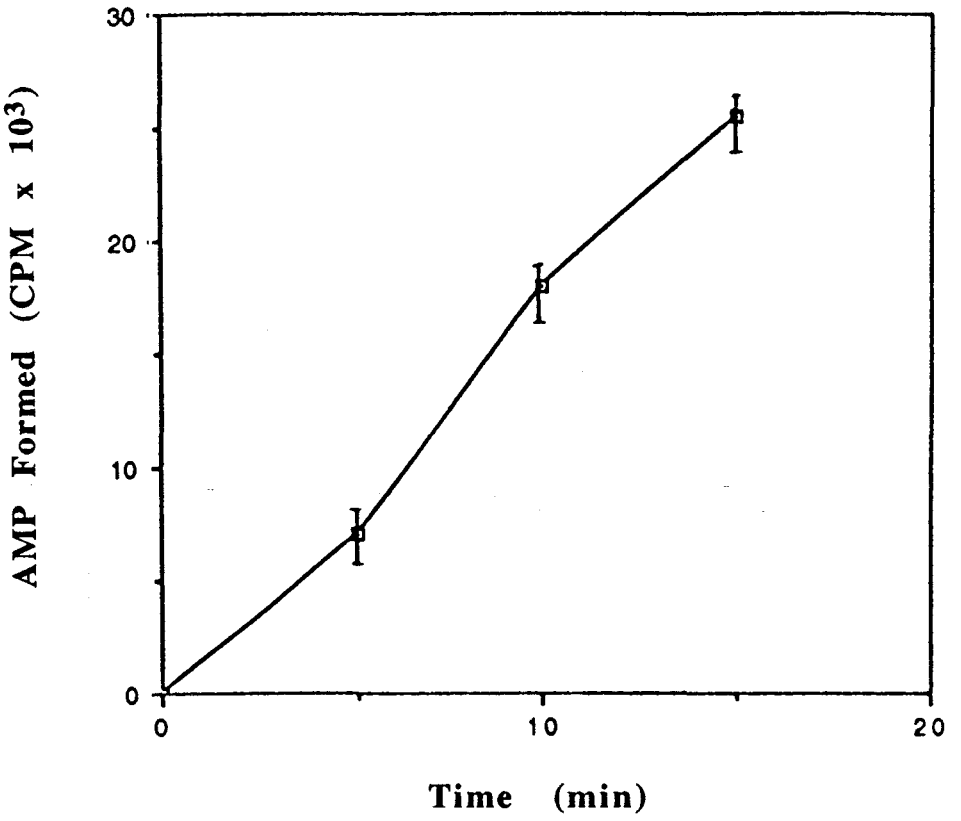


Fig. 8 Time course of adenosine kinase reaction. Five independent AK assays were carried out under identical conditions. The averages of the five assays and their standard deviations are shown in this figure.

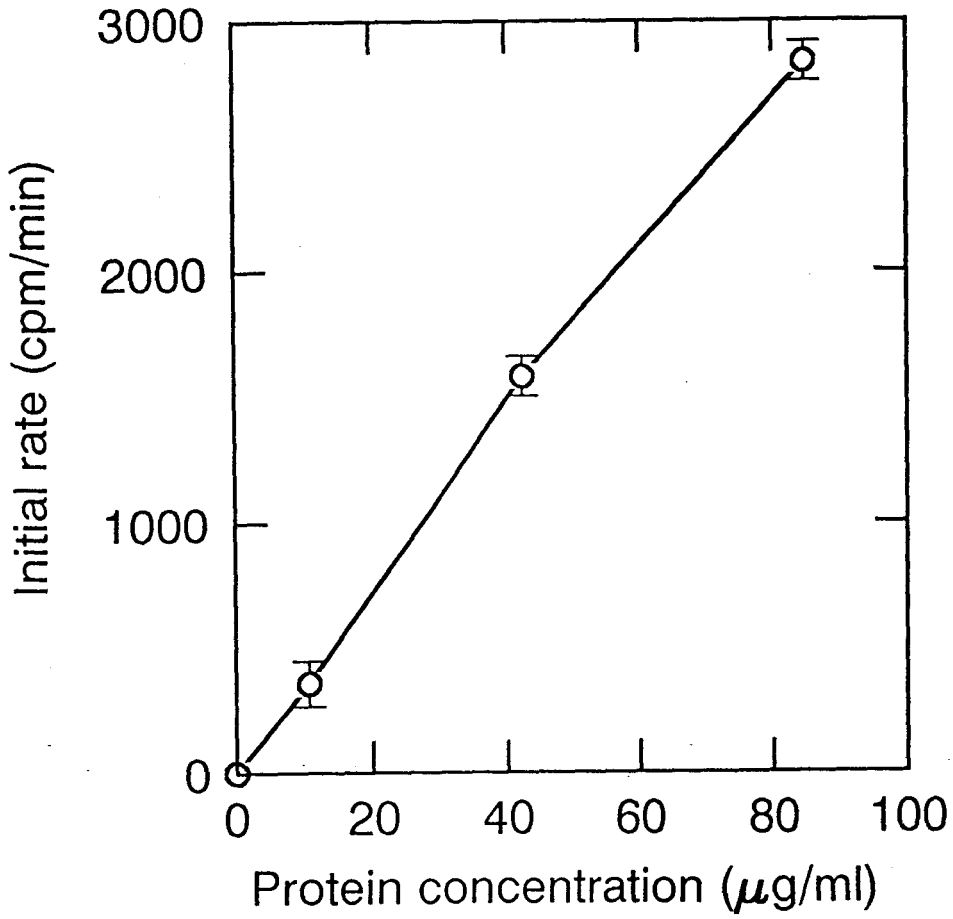


Fig. 9 The enzyme concentration-dependence of adenosine kinase initial velocity. The assays were carried out with three different concentrations (10.6, 42.4 and 84.8 ug/ml) of the partially purified enzyme from the elution of the 5'-AMP Sepharose column. Buffer and concentrations of other factors were kept constant in all three sets of experiments. The averages of three identical experiments and their standard deviations are shown in the figure.

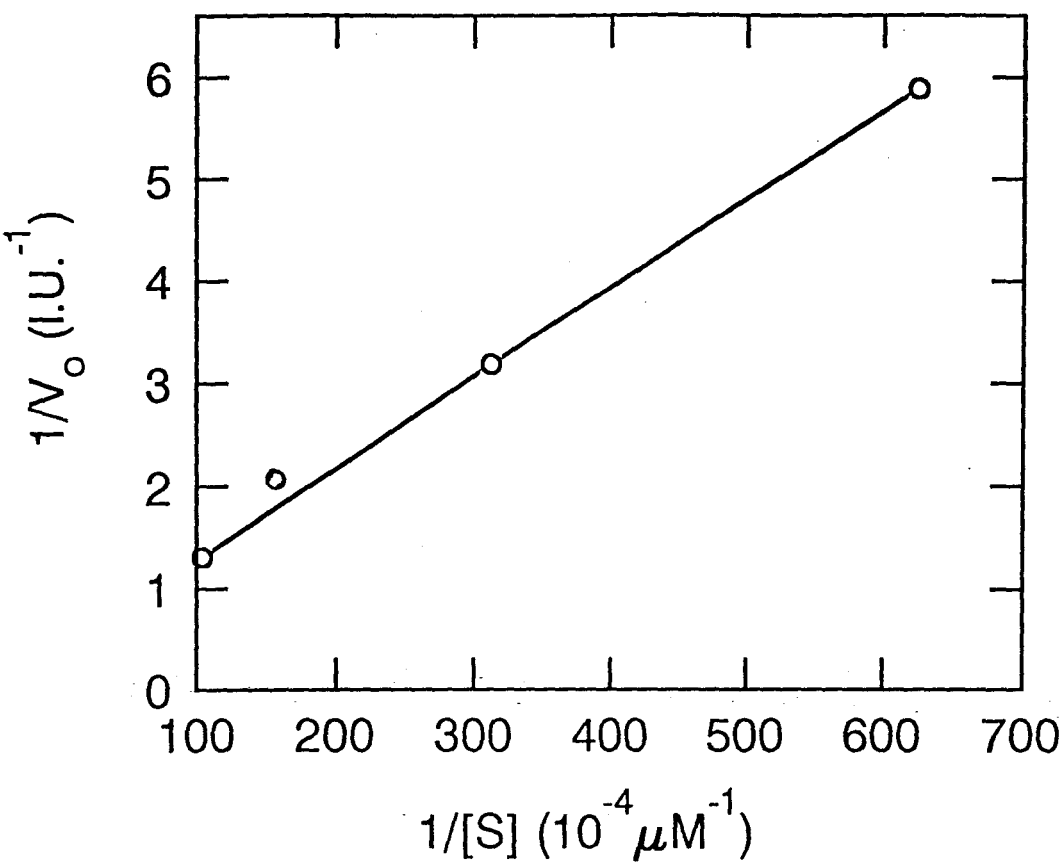


Fig. 10 Determination of apparent Km of adenosine kinase for adenosine. Double-reciprocal plot of initial velocity versus adenosine concentrations ranging from 16 to 96 uM is shown in th figure. The apparent Km for adenosine, derived from the plot, was 0.16 uM. The experiments were performed at the standard conditions as describe under "Material and Methods". One I. U. means the formation of 1 umol AMP per min.

was consistent with previous reports [Yamada, *et al.*, 1982; Rotllan and Miras Portugal, 1985; Fisher and Newsholme, 1984]. At higher concentration, an inhibition of AK activity by adenosine was observed. With 86 mM adenosine in the solution, the initial velocity of AK activity was only 60% of that under 43 mM adenosine.

3.2.1.2 Effect of pH on Adenosine Kinase Activity.

The effect of pH on the AK activity was also investigated in this project. The AK assay was carried out at standard conditions except that the pH of the potassium phosphate buffers ranged from pH 5.0 to pH 8.5 (this pH range was chosen because of the limitation of the buffer capacity of phosphate). As seen in Fig. 11, the AK activity gradually increased from pH 5.0 to pH 7.5 and reached a maximum at pH 8.0, followed by a slight decrease of the activity at pH 8.5. This result was in agreement with some previous reports. AK from *Leishmania. donovani* showed pH optima from 7.5 to 8.5, depending upon the ratio of ATP to Mg^{2+} [Datta *et al.*, 1987].

3.2.1.3 Phosphate-Stimulated Adenosine Kinase Activity.

The effect of phosphate on the enzyme activity is shown in Fig. 12. By using different buffer system (Tris and phosphate), it was found that in the Tris buffer, the enzyme activity averaged only 2% of the control, which is hardly distinguishable from the background with no enzyme [Fig. 12]. The addition of phosphate to the assay solution increased the

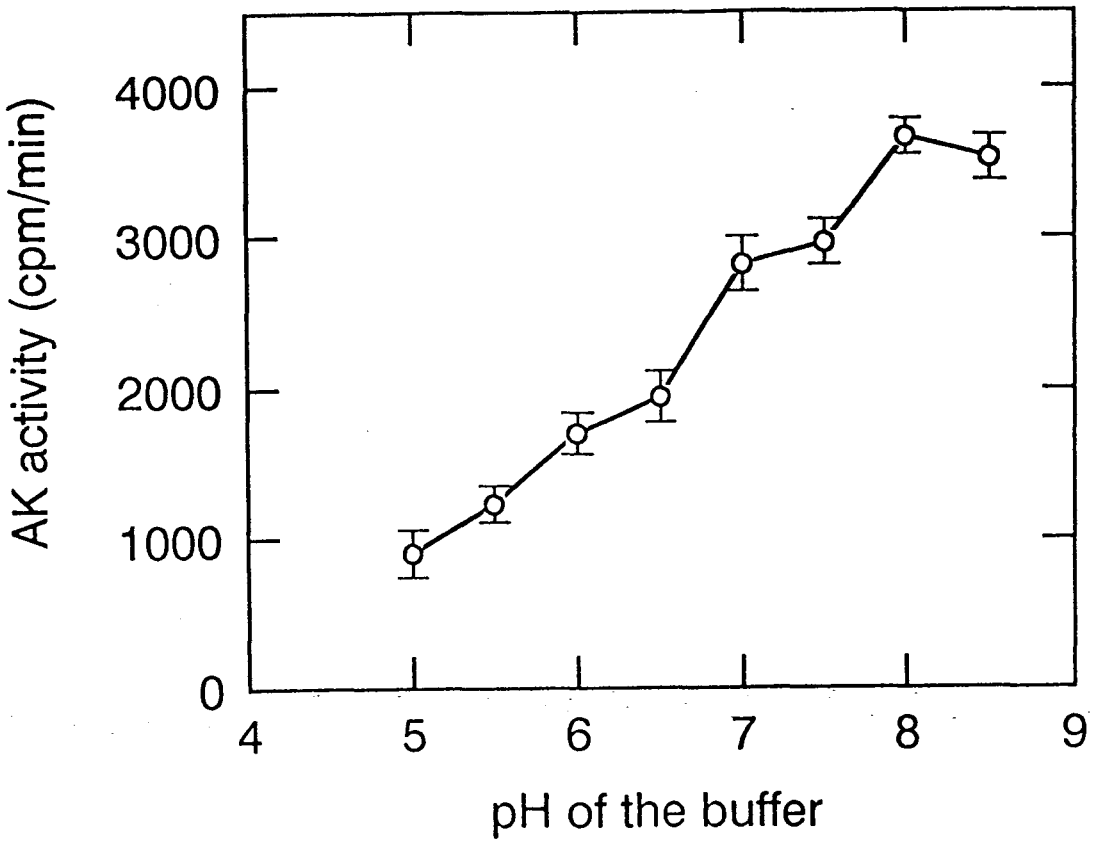


Fig. 11 Effect of pH on the adenosine kinase activity. The pH of the buffers varied between 5.0 to 8.5 by changing the ratios between diphosphate and monophosphate in the buffer and the AK activity was measured under standard conditions (except for the pH of the buffer) using same amount of enzyme. The averages of three identical experiments and their standard deviations are shown in the figure.

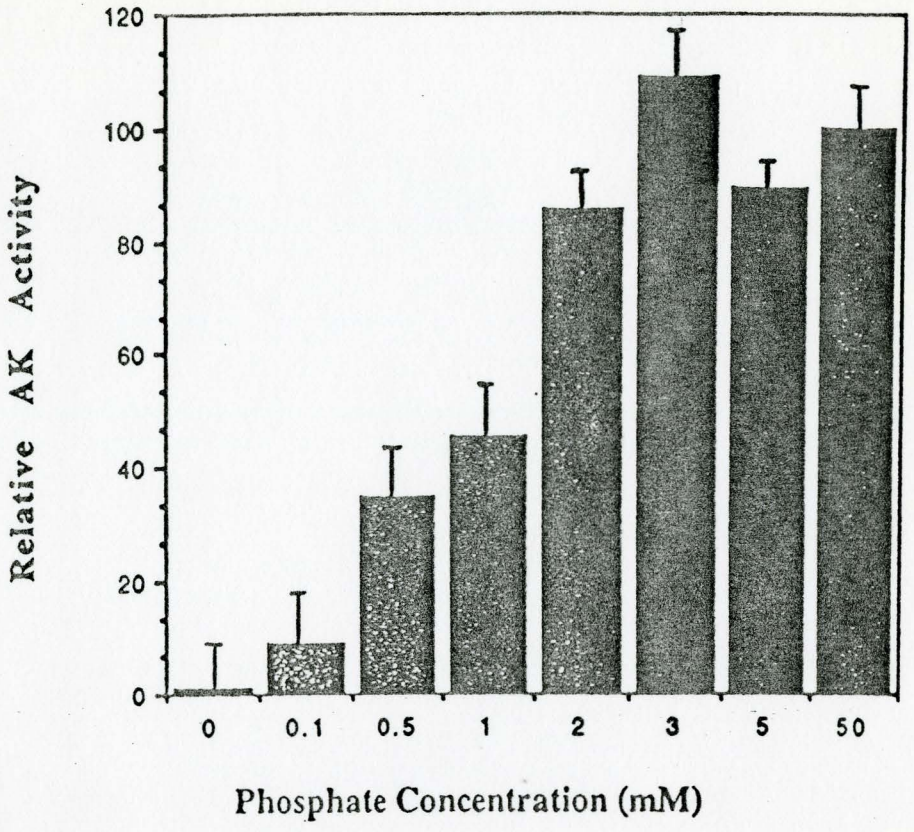


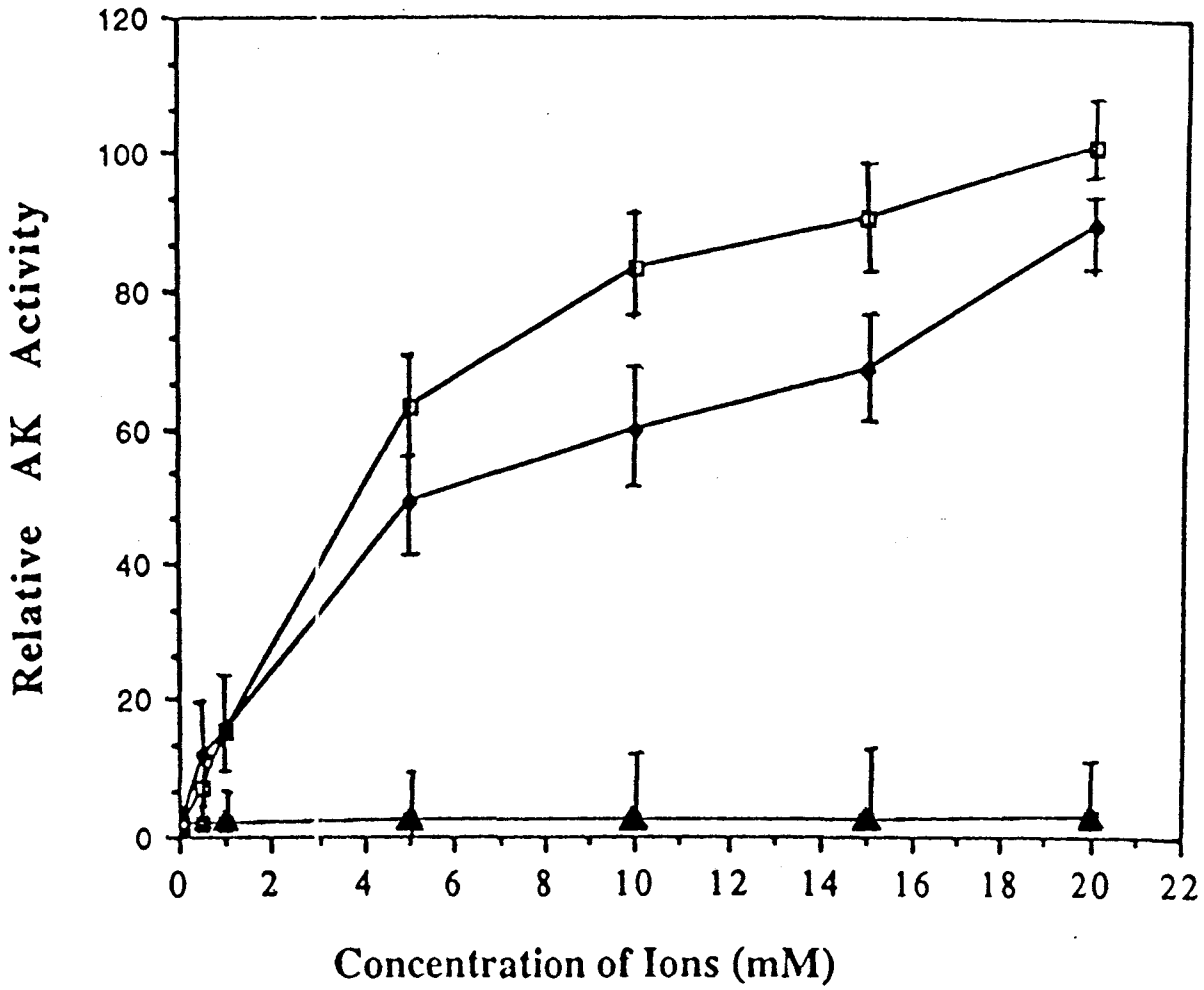
Fig. 12 Effect of phosphate on the adenosine kinase activity. AK assays were performed in 20 mM Tris-HCl, pH 7.0 with the addition of different amounts of phosphate ranging from 0 to 5.0 mM. Same amount of enzyme was used in all the experiments. AK activity was measured as percentage of activity observed in a buffer containing 50 mM phosphate (i.e. similar to that in the standard assay condition).

enzyme activity dramatically. A maximum stimulation was obtained with the addition of 3 mM phosphate. There was no obvious inhibition to AK activity when phosphate concentration reached up to 100 mM. The phosphate requirement was an unexpected result and the exact reason of this dependence is not clear. AK can also be activated by the addition of vanadate or arsenate in the Tris buffer.

3.2.1.4 Effect of Different Ions on the Enzyme Activity.

Since the dependence of AK activity upon phosphate has not been reported previously, it is of interest to find out whether phosphate is the sole ion required for AK activity. Several kinds of ions were tested to try to restore AK activity in the Tris buffer. These ions includes K^+, Na^+, Fe^{2+}, Fe^{3+}, Cu^{2+}, Ca^{2+}, Zn^{2+}, Mn^{2+}, Cl^-, acetate, sulphate, sulphite, nitrate, nitrite, carbonate. The observed re-stimulation of AK activity depended markedly on the chemical structures of ions added. There was no cation, which could activate AK in the Tris buffer, as indicated in Fig. 13. It was found that only vanadate and arsenate could activate AK as shown in Fig. 13. These two chemicals have similar chemical structures as phosphate. But, the concentrations of ions needed for the maximum activation of AK was different among phosphate, vanadate, and arsenate. It required 2 mM sodium phosphate or 20 mM sodium arsenate to restore 100% of AK activity, while 20 mM sodium vanadate could only restore

Fig. 13 Effect of different ions on the adenosine kinase activity. AK assays were carried out in 20 mM Tris-HCl, pH 7.0 with the addition of different concentrations of various ions. The control reaction was carried out with the addition of 50 mM phosphate. AK activity for different ions was indicated as the percentage of the control AK activity. AK assays were carried out in triplicate with the addition of sodium arsenate and sodium vanadate, while AK assay was carried out in duplicate with the addition of other ions. The error bars show the standard deviations. □ , sodium arsenate; ♦ , sodium vanadate; ▲ , other ions, which include K^+, Na^+, Fe^{2+}, Fe^{3+}, Cu^{2+}, Ca^{2+}, Zn^{2+}, Mn^{2+}, Cl^-, acetate, sulphate, sulphite, nitrate, nitrite, carbonate, as well as sodium antimonate.


Relative AK Activity
120
100
80
60
40
20
0
0
2
4
6
8
10
12
14
16
18
20
22
Concentration of Ions (mM)

87% of AK activity [Fig 12 and Fig. 13]. Sodium antimonate, which also has a chemical structure similar to phosphate, could not be tested due to its very low solubility in the aqueous solution.

3.2.1.5 Temperature Inactivation Kinetics of Adenosine Kinase.

The purpose of comparing the thermal stability of AK between CHO cells and human fibroblast (HF) cells is that this difference could be used to distinguish AK from CHO cells and AK from human cells. The human AK gene could be cloned by transfecting AK^- CHO cells with human DNA, followed by the selection of AK^+ cells under the conditions described in Section 3.5. Only those cells, which regained their AK activities either by means of spontaneous mutation or by capture of the AK gene from the transfected human DNA, could survive under the selective medium. Different thermal stabilities of AK could be used to distinguish colonies which regained CHO AK by spontaneous mutation, and colonies which regained AK activities from the human AK gene(s). The later ones could then be used to clone the human AK gene. With regard to this purpose, CHO cell extracts and human fibroblast cell extracts were used to investigate the thermal properties of AK. Cell extracts were incubated at 45°C or 50°C for a certain time period before measuring the remaining AK activity. AK from CHO cells and HF cells showed differences in the thermal inactivation kinetics at 50°C, as indicated in

Fig. 14. After incubating at 50°C for 4 or 8 minutes, in comparison with controls, 53% or 23% of the AK activity remained in the HF cell extracts respectively, while only 27% or 7% of the AK activity remained in the CHO cell extracts respectively, indicating that AK from HF cells was more thermostable in comparison with AK from CHO cells at 50°C [Fig. 14]. However, there was no significant difference of thermal stability when AK from CHO cells and human cells was tested at 45°C [Fig. 14].

Since AK showed phosphate dependent activity, experiments were done to determine whether phosphate affects the conformational stability, or thermal sensitivity, of AK at higher temperature. Experimental results in Fig. 15 shows that there was no significant difference in terms of thermal sensitivity of AK in either Tris buffer or phosphate buffer at 50°C, even though AK activity is dependent upon phosphate, suggesting that phosphate did not affect the conformational stability of AK.

3.2.2 Discussion

Earlier studies have shown that there is a very complex relationship betweem AK activity and the concentrations of various reactants, viz. free magnesium ions, free ATP, MgATP complex, adenosine and AMP [Andres and Fox, 1979; Miller *et al.*, 1979c]. High concentration of either free magnesium ions, free ATP, adenosine or AMP could inhibit AK activity [Miller *et al.*, 1979b; 1979c]. But combination of certain

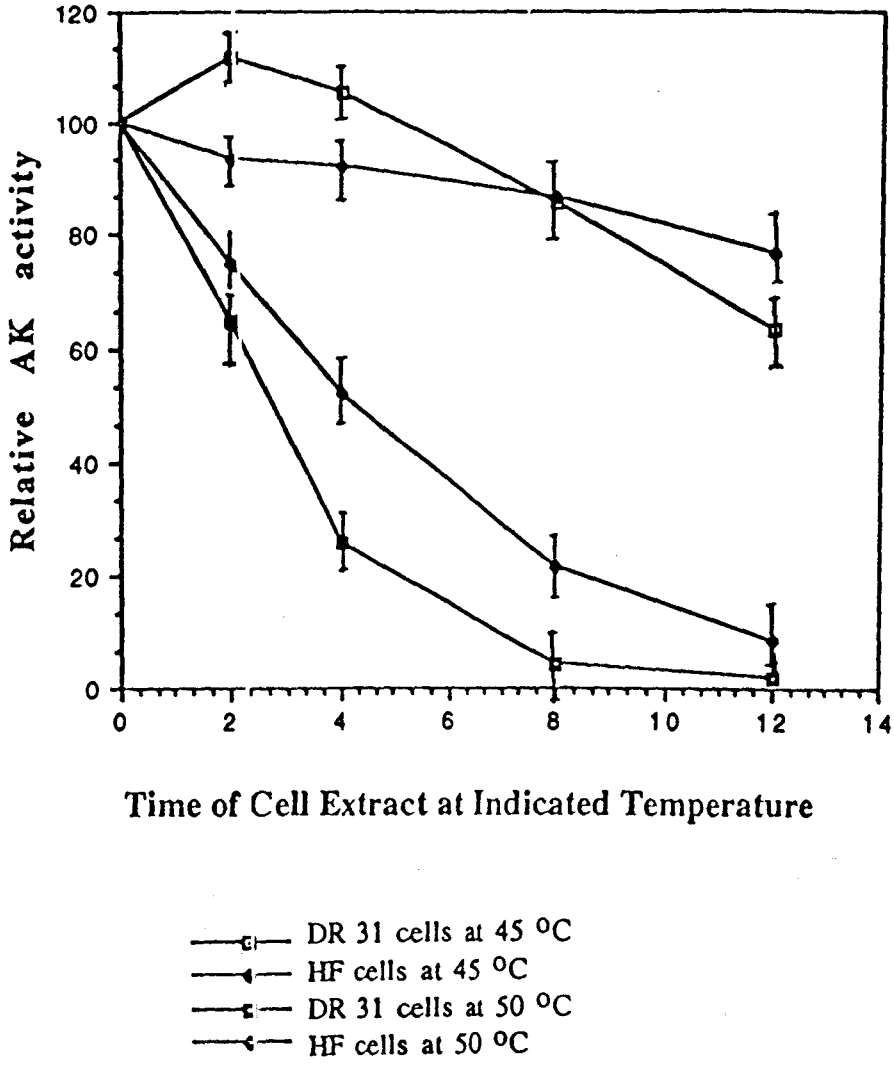


Fig. 14 Thermal inactivation kinetics of adenosine kinase from CHO cells and HF cells. The cell extracts from CHO and HF cells were incubated at 45 and 50°C without substrates for the indicated period and then assayed for their remaining activity under the standard conditions described under "Material and Methods". The AK activity is shown as the percentage of the control assay, in which the extracts were not exposed in high temperature.

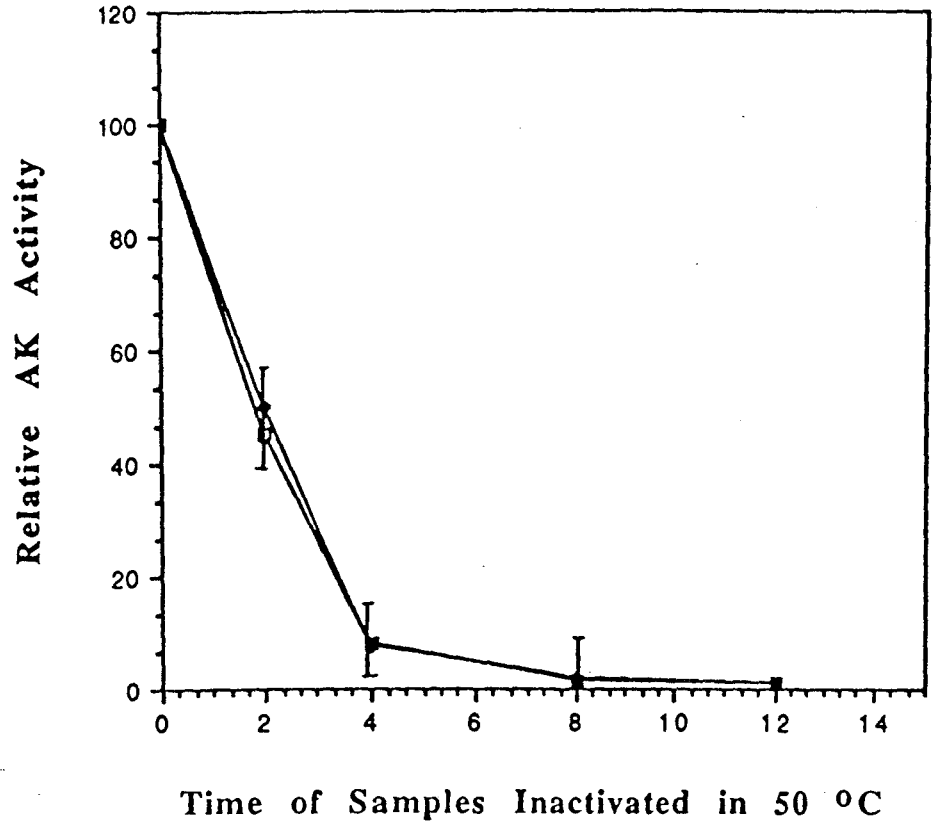


Fig. 15 Thermal inactivation kinetics of partially purified adenosine kinase from Syrian hamster liver in different buffers. The samples were incubated at 50°C with either phosphate or Tris buffer for the indicated period and then assayed for their remaining AK activity under the standard conditions described under "Material and Methods". The AK activity is shown as the percentage of the control AK assay with non-heated enzyme. ◘ , sample in 10 mM potassium phosphate buffer, pH 7.0; ♦ , sample in 20 mM Tris-HCl, pH 7.0.

factors could stimulate AK activity. For example, higher concentration of AMP could release the inhibition to AK activity by the high concentration of adenosine [Hawkins and Bagnara, 1987].

The observation that the enzyme from Syrian hamster was inhibited by the higher concentration of adenosine is similar to the findings with the enzyme from human placenta [Andres and Fox, 1979], human erythrocyte [Hawkins and Bagnara, 1987], and rabbit liver [Miller *et al.*, 1979b], but it differs from the enzyme from *Leishmania donovani* , which is not inhibited by adenosine [Datta *et al*, 1987].

Magnesium ions are essential for AK activity, because its removal after dialysis overnight, leads to loss of AK activity. By addition of Mg^{2+} to the dialyzed preparation, the activity could be restored to 80% of the original activity. The magnesium dependence observed in this project is in good agreement with the earlier results on the divalent cation dependence of AK activity [Miller *et al.*, 1979c; Hawkins and Bagnara, 1987].

It was intriguing to find that AK activity was absolutely dependent upon phosphate. Furthermore, the lack of activity of AK in the Tris buffer could be reversed by the addition of vanadate and arsenate, which have similar chemical structures to phosphate. This novel property of AK has not been reported previously. It is possible that low concentrations of phosphate which lead to partial AK activity were present in most earlier enzyme preparations when Tris buffers were used.

On the basis of the observation that AK requires phosphate or phosphate-like anions for its activity, it is reasonable to suggest that these anions contain a certain chemical structure or configuration which plays an important role in the enzyme catalytic process because other ions which have different chemical structures could not substitute phosphate. Alternatively, there is a regulatory site to which these compounds bind to activate the enzyme. However, such anions do not have a general stabilizing effect on the enzyme since the thermal stability of AK was not affected by their presence as shown in Fig. 15.

The purpose for comparison of the thermal stability of AK between CHO cells and human fibroblast cells was that this difference could be used to distinguish AK from CHO cells with that from human cells. Human DNA can be transfected into AK^- CHO cells and AK^+ cells can be selected from the population of cells, as described in the later section. The difference in the thermal stability of AK from CHO cells and human cells can then be used to distinguish colonies, which regain their AK activities from human gene, as opposed to those which regain AK activities by spontaneous reversion. The experiment on AK stability showed that human AK was slightly more thermal stable than AK from CHO cells [Fig. 14]. Combined with other markers (such as the presence of human Alu sequence), this property can be used to distinguish transfectants containing the human AK gene from spontaneous revertants in order to clone the AK gene.

3.3 Immunological Studies on Adenosine Kinase.

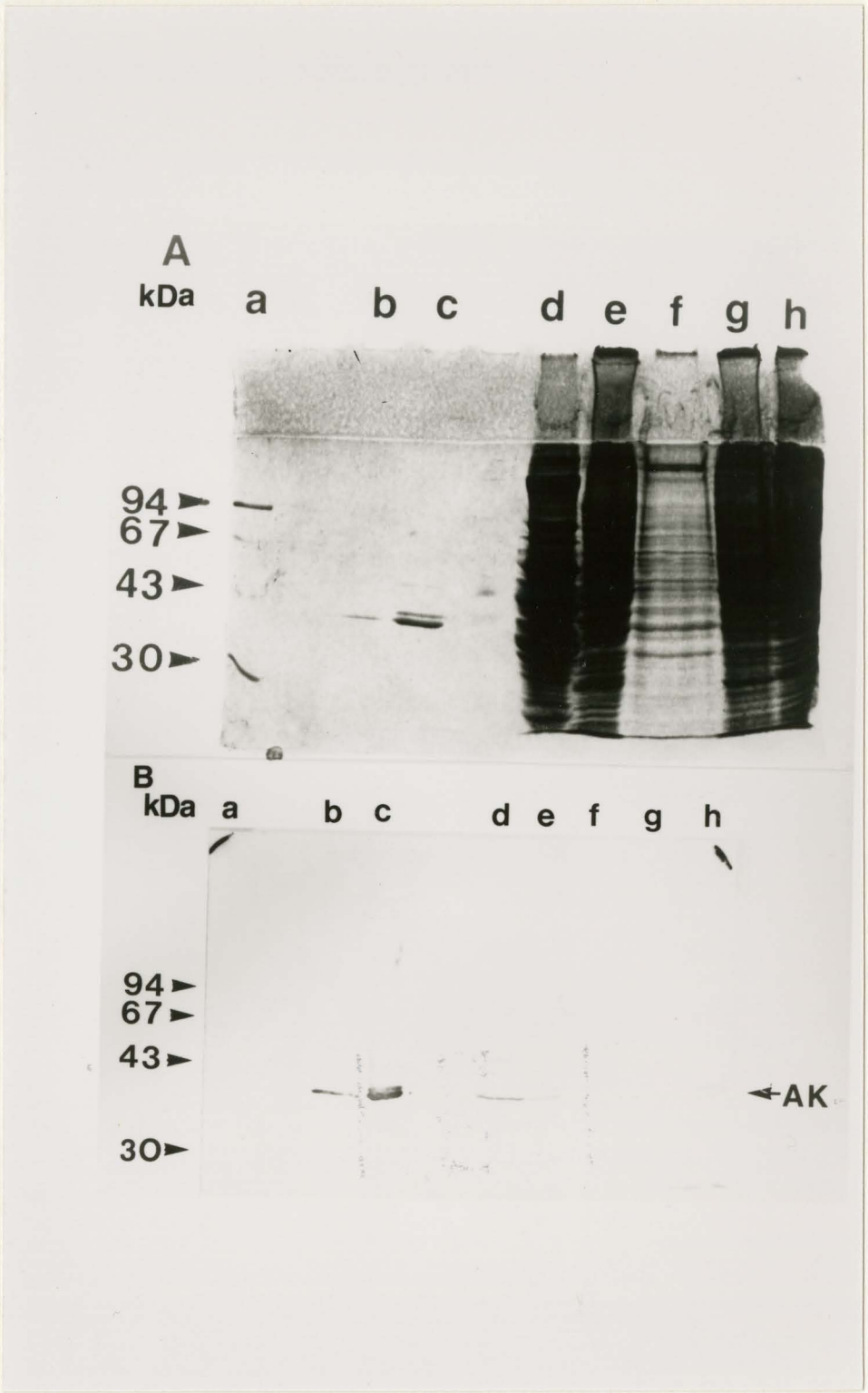
3.3.1 Result

A total of three rabbits and four guinea pigs were used to raise antibodies against AK purified from Syrian hamster liver. Except for one rabbit, all of the other animals showed good immunoresponses to the injected protein. In immunoblots of total cellular proteins from Syrian hamster liver extract, diluted rabbit antiserum recognized a protein with the right molecular mass after the second injection. This band did not show up using the original preserum. This band did show up by using up to 1:16,000 diluted antiserum. The third and forth injections showed slightly higher titers of this antibody in the serum. The animals were bled two weeks after the last booster shot. The sera were aliquoted and stored at -70°C. These sera from different animals were used for the following work.

3.3.1.1 Immunological Reactivity of Antisera Towards Adenosine Kinase From Different Sources.

In immunoblots containing purified AK, partially purified AK, and the total extracts from different species, the AK antibodies reacted specifically to Syrian hamster AK, indicating that right antibodies were obtained from the K2 rabbit [Fig. 16]. The antibodies also showed weak reactivity towards AK from BHK cell extracts. However, the antibodies did not cross-react with AK from CHO cells and human cells.

Fig.16 Immunoblot analysis of different adenosine kinase samples using K2 antiserum. Appropriate amount of different samples were applied to different lanes. SDS-PAGE and immunoblot analysis were carried out as under the standard conditions described under "Materials and Methods". Panel A shows Coosmassie blue staining of the gel while in panel B, immunoblot of the samples is shown. a, low molecular mass markers; b, purified AK from Syrian Hamster liver; c, partially purified AK from Syrian hamster liver (sample eluted from the 5'-AMP Sepharose 4B column); d, Syrian hamster liver extracts; e, BHK (baby hamster kidney) cell extracts; f, Hela cell extracts; g, CHO (Chinese hamster ovary) cell extracts; h, BHK cell extracts.


A
kDa
a
b
c
d
e
f
g
h
94
67
43
30
B
kDa
a
b
c
d
e
f
g
h
94
67
43
30
AK

To check the antibody titer and specificity, the following experiments were carried out. In immunoblots using a series of different concentrations of antiserum from the K2 rabbit, antiserum with dilutions of up to a 1 to 16,000 still specifically reacted with the AK band from Syrian hamster as shown in Fig. 17. In addition, the background reactivity of the antisera decreased quickly, as the dilution factor increased, except for the stable AK band [Fig. 17]. The immunoblot experiments with a 1 to 5,000 dilution of antisera from another rabbit and four guinea pigs also gave similar results [Fig. 18]. Higher dilution of these antisera was not tested. It was noticeable that all these antisera could also bind to a 42 kDa protein, which was eluted together with AK from the 5'-AMP affinity column [Fig. 16, Fig, 17, and Fig. 18]. At higher dilutions of AK antibody, somewhat decreased immunological reactivity to this protein was observed.

3.3.1.2 Immunoprecipitation of Native and Denatured AK.

The experiments described above indicated that the antibody obtained from the rabbit did not recognize AK from other species, but did recognize AK from Syrian hamster. This was an unexpected result. The following experiments were carried out to further examine the specificity of the antiserum. Antiserum from the K2 rabbit was used in all of the following experiments, since it gave the strongest response among all the animals. Total proteins from the K2 rabbit antiserum was covalently bound to Protein A Sepharose

Fig. 17 Immunoblot analysis of K2 rabbit antiserum with different dilution. Total cellular proteins from Syrian hamster liver extracts were transferred to nitrocellulose membrane under the standard conditions described under "Material and Methods". Strips derived from same nitrocellulose membrane were reacted with different dilutions of the antiserum. a, control preimmune serum with a 1: 2,000 dilution; b, antiserum with a 1: 1,000 dilution; c, antiserum with a 1:2,000 dilution; d, antiserum with a 1:4,000 dilution; e, antiserum with a 1:8,000 dilution; f, antiserum with a 1:16,000 dilution.

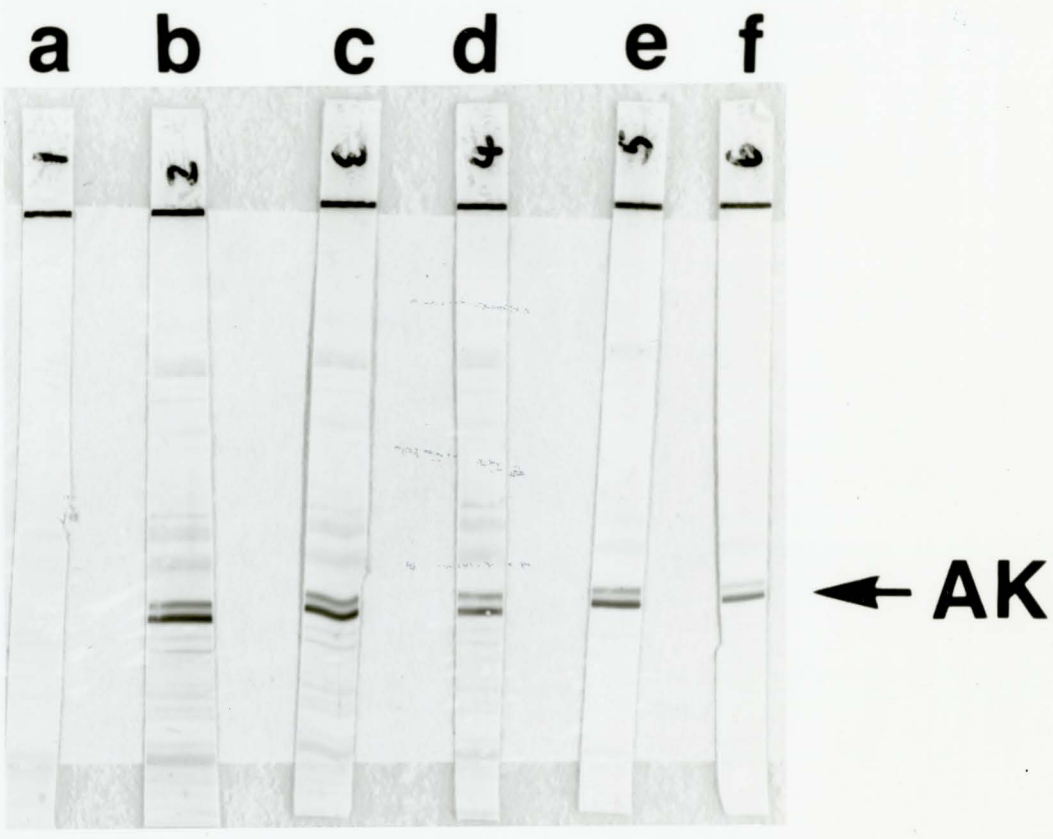

a
b
c
d
e
f
1
2
3
4
5
6
AK

Fig. 18 Immunoblot analysis of antiserum from other animals. Immuoblot analysis was performed under the standard conditions described in "Materials and Methods". Strips derived from same nitrocellulose membrane were used in this experiment. -, control preimmune serum with a 1:2,000 dilution; +, antiserum with a 1: 5,000 dilution. a, serum from K1 rabbit; b, serum from H1 guinea pig; c, serum from H2 guinea pig; d, serum from H3 guinea pig; e, serum from H4 guinea pig.

beads, as shown in Fig. 19. In immunoprecipitation experiments, these beads precipitated a 40 kDa protein from both [^{35}S]labelled CHO and BHK cell extracts, which had similar molecular mass as AK [Fig. 20]. The K2 antiserum was also tested for its ability to immunoprecipitate AK from Syrian hamster liver extracts. As illustrated in Fig. 21, the antibody bound to Protein A beads was able to precipitate native AK from Syrian hamster liver extracts. The bead pellet showed strong AK activity in contrast to the control bead pellet bound with preimmune serum, confirming that the 40 kDa protein immunoprecipitated was AK.

3.3.2 Discussion.

One of the main purposes for raising antibody has been to employ it in future studies to screen cDNA express libraries to isolate clone(s) for the AK gene(s). In addition, antibody has many other useful applications, such as immunoreactivity to identify the protein in blots, or to make affinity columns for protein purification, as well as localization of the subcellular site of the protein. The AK antibody which was obtained previously in the laboratory by using AK from CHO cells [Gupta and Mehta, 1986b] did not bind to purified AK from Syrian hamster liver in immunoblots, suggesting a species specificity in immunune recognition. Further, the protein recognized by the antibody raised previously is a 38 kDa protein, whose molecular mass is lower than that observed in the present work. In the view of these observations, it is

Fig. 19 SDS-PAGE analysis of covalent binding reaction between Protein A Sepharose beads and adenosine kinase antiserum. 10 ul of each sample was applied to different wells. a, low molecular mass markers; b, sample of beads mixed with the antiserum before the covalent reaction; c and d, sample of the beads mixed with the antiserum after the covalent reaction; e, supernatant of the reaction mixture after the covalent reaction; f, supernatant of the mixture before the reaction.

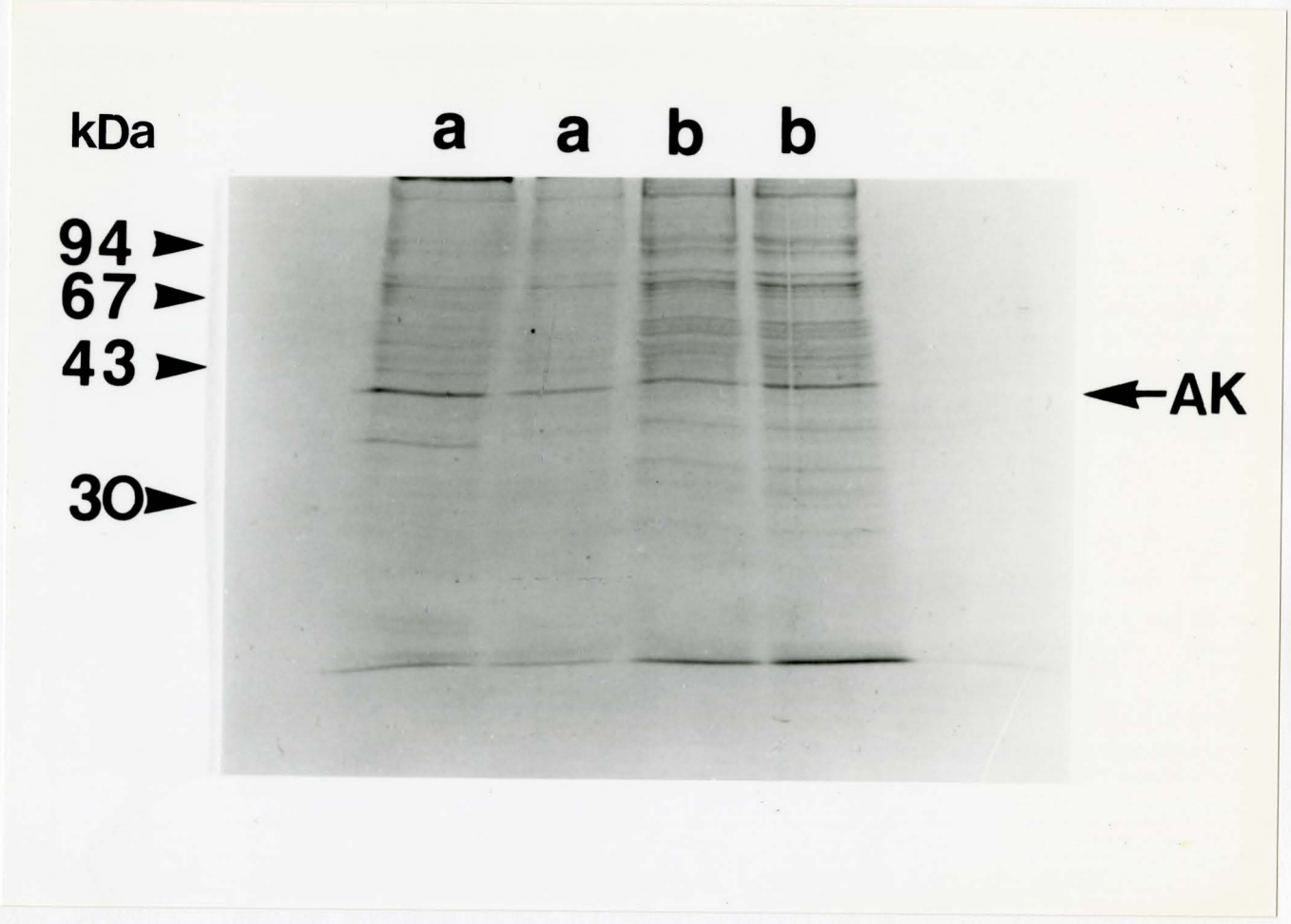


Fig. 20 Immunoprecipitation of a protein of 40 kDa from labeled cell extracts. The experiment was carried out as described in "Material and Methods". The immunoprecipitated proteins were separated on SDS-PAGE gels and autoradiographed. a, immunoprecipitation from [^{35}S] methionine-labeled CHO cell lysates; b, immunoprecipitation from [^{35}S] methionine-labeled BHK cell extracts.

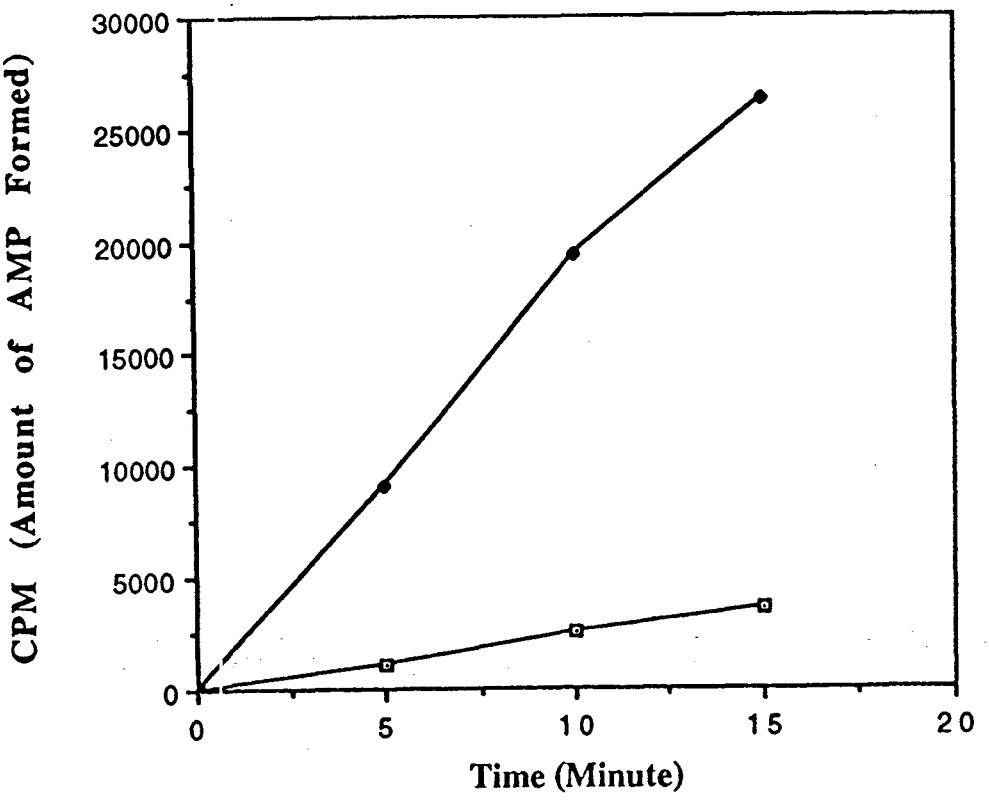


Fig. 21 Immunoprecipitation of adenosine kinase activity from Syrian hamster liver extracts. Cell lysates of Syrian hamster liver were immunoprecipitated with K2 rabbit antiserum or control serum as described in "Materials and Methods". The immunoprecipitates were analyzed for AK activity as described in "Materials and Methods". —•— , antiserum; —□— , control serum.

not entirely clear whether the antibody raised earlier recognized AK or another protein. Therefore, much effort has been spent on successful preparation and characterization of new polyclonal antibodies in this project.

Immunoblot reactivity indicated that all of the antisera raised could recognize and bind to purified AK from Syrian hamster. Of these, the K2 rabbit antiserum showed the strongest reponse and it could react with AK at a dilution of up to 1:16,000. The sera from the other rabbit and four guinea pigs also showed good reactivities towards AK. However, all of these antibodies failed to recognize AK from CHO cells or human cells in immunoblots. The absence of any background activity in immunoblots indicated that the antibody did not show cross-reactivity with some other proteins in these species. In contrast to the results of immunoblots, results of immunoprecipitation experiments indicated that the K2 antibody specifically pulled down a protein of 40 kDa from CHO and BHK cells and pulled out AK activity from Syrian hamster liver extracts, suggesting that the antibody was able to recognize the native AK (as opposed to denatured AK in immunoblots) from these species.

The high degree of antigenic specificity displayed by these antibodies suggested that these antibodies were directed toward a structural domain that differed in different species. The species specificity of AK antibody has also been reported previously. Studies done by Juranka and Chan [1985] showed that the antibodies against AK from CHO cells could not

recognize AK from BHK cells. In another study, an antibody raised against *Leishmania donovani* AK was absolutely specific for this parasite and did not cross-react with AK from other species [Bhaumik and Datta, 1989]. The present findings regarding the immunospecificity of AK antibodies are in accordance with these reports and indicate that the primary structure may show large differences between closely related species.

The AK antibodies raised here in addition to reacting with AK also cross-reacted with a protein of 42 kDa. Similar cross-reactivity was also noted in an earlier study [Juranka and Chan, 1985]. One possibility was that this cross-reactivity was due to the contamination of antigen with the 42 kDa protein. Even though only apparently homogenous AK protein of 40 kDa was used to immunize the animals, the contamination of a trace amount of the 42 kDa protein was still possible. Another possibility was that this protein contains an antigenic domain which could be related to AMP binding domain. This AMP binding domain might be recognized by the AK antibodies. This view was supported by the observation that the 42 kDa protein was co-eluted with AK from the 5'-AMP Sepharose column [Fig. 4]. The exact relationship between AK and the 42 kDa protein should be of interest to investigate in future studies.

3.4 Partial Amino Acid Sequence of Adenosine Kinase by Microsequencing.

3.4.1 Result

One of the approaches to clone the AK gene involves obtaining a partial sequence of AK protein, followed by screening cDNA libraries with degenerate oligonucleotide probes which are based on this protein sequence. Purified AK in either native form or denatured form is a prerequisite for protein microsequencing. As described in the previous section (Section 3.1), native AK can be purified by an approach combining ion exchange chromatography, affinity chromatography and gel filtration chromatography. Denatured AK can also be purified readily using preparative SDS-PAGE. In the later case, partially purified AK from the 5'-AMP Sepharose column (a mixture of three different proteins for Syrian hamster liver sample) was separated in SDS-PAGE and the band corresponding to AK was excised. Denatured AK was obtained by electroeluting AK from the gel strips. Both purified native AK and denatured AK from Syrian hamster liver were sent for protein microsequencing. However, the results of the protein microsequencing indicated that the amino-terminal of AK was blocked.

Because of above reason, several ways were examined to digest AK into specific fragments suitable for protein microsequencing. One of the preferred methods for obtaining such fragments involves the digestion with cyanogen bromide

(CNBr) which cleaves a polypeptide chain on the N-terminal side of methionine (Met) residues [Matsudaira, 1990]. Both purified native AK and denatured AK obtained by electroelution were used for CNBr digestion. As shown in Fig. 22, four different large fragments, termed fragment A, B, C and D, were obtained after the CNBr cleavage of denatured AK from Syrian hamster liver. Similar results were obtained when native AK from Syrian hamster liver was used. The 17 kDa protein observed in Fig. 22 was believed to be a partially digested fragment because this fragment was not present in the other similar experiments. The CNBr fragments had molecular masses between 5 to 10 kDa. The fragments immobilized on the PVDF membrane were excised and sent for protein microsequencing. A partial protein sequence of fragment A, which had the highest molecular mass among the four fragments, was obtained. The sequence of 14 amino acids from the N-terminal end of fragment A thus obtained was as follows: Tyr-Val-Asp-Ser-Leu-Phe-Gly-Ala-Glu-Thr-Glu-Ala-Ala-Leu. Results of protein sequence from other fragments were not clear and several amino acids were released from each cycle of protein microsequencing. This might be due to the poor separation of these fragments on the SDS-polyacrylamide gel, which could lead to a mixture of different fragments when the bands were excised for protein microsequencing.

3.4.2 Discussion

The main purpose of protein microsequencing of AK is to

Fig. 22 SDS-PAGE analysis of adenosine kinase fragments digested by CNBr. The protein fragments were transferred to PVDF membrane, which is shown in the figure. The protein digestion with CNBr, SDS-PAGE separation, and transfer were carried out as described in "Materials and Methods". The three lanes contained similar samples. The four CNBr fragments were termed fragment A, B, C, and D.

obtain a partial protein sequence which can be used to clone the AK gene in the future. The amino-terminal of AK from Syrian hamster liver was found to be blocked in the present study. Earlier studies have shown that the amino-terminal modification of proteins occurs in a majority of proteins in cells and the modification normally leads to a blockage of the N-terminal end [Tsunasawa and Sakiyama, 1984; Brown and Roberts, 1976]. The blockage of the amino-terminal end of AK has not been reported previously, even though much work has been done on the amino acid composition of AK from different sources [Table 1; Yamada *et al.*, 1982]. CNBr cleavage of AK gave rise to four fragments ranging in molecular masses from 5 to 10 kDa. An amino acid sequence of 14 amino acids from fragment A was obtained. This sequence did not match any protein sequences which have been published. The CNBr digestion pattern of AK protein suggested that there were at least three methionine residues in the AK protein. The partial sequence of AK protein has not been reported previously. An attempt has been made to achieve better separation of the CNBr fragments for protein microsequencing.

Based on the partial protein sequence of AK, degenerate oligonucleotide probes with both direction could be deduced. These probes could be used for screening cDNA libraries or carrying out PCR experiments to clone the AK gene. The DNA sequence from the positive colonies or PCR products should match the sequence which is used as probes. The full length of the AK gene should be obtained once partial DNA sequence of

the AK gene is known.

3.5 Isolation of AK^+ Revertants from AK^- CHO Mutants.

3.5.1 Result.

It is important to find out the conditions for the selection of revertants from AK^- mutants, since this method could be used to clone the AK gene, as discussed in the "Introduction" section. In the present study, the selection of AK^+ revertants from AK^- CHO mutants was achieved by using alpha-medium with the addition of four chemicals. This selective medium consists of alanosine to block *de novo* AMP biosynthesis, adenosine to provide a salvage route for AMP biosynthesis via the adenosine kinase reaction, and uridine to alleviate the cellular toxicity caused by adenosine at the concentration employed, as well as EHNA, erythro-9-(2-hydroxy-3-nonyl)adenine, to inhibit adenosine deaminase which converts adenosine to inosine. Therefore, AK become an essential enzyme for cells to generate AMP when other pathways to produce AMP are blocked by the additions of above chemicals and in this selective medium only AK^+ cells should survive. The AK^+ revertants, which have acquired adenosine kinasse, are expected to lose their resistance to adenosine analogs such as toyocamycin, since AK can convert these analogs into cytotoxic forms by phosphorylation, and this property can be used as a marker to test for the reversion. Before the selection of revertants, minimum

inhibitory concentrations of the four chemicals were determined individually by testing the plating efficiency of DR31 CHO cells at different concentrations of chemicals in 24-well dishes in order to find out the optimum concentrations for the selection of revertants. The final concentrations of each component used for selection in the alpha-minimal medium supplemented with 5% FCS were: 20 mM adenosine, 0.7 mg/ml alanosine, 1 mM uridine and 20 mg/ml EHNA. A total of 23 colonies were picked at a mutation frequency of 1.1 X 10^{-6}, after culturing AK^- DR31 mutants in this selective medium for two to three weeks (10^6 cells/60-mm dish). Each colony was grown and expanded in normal non-selective medium for AK activity assay and drug resistance test. Both WT cells (AK^+) and DR31 AK^- mutants were used as controls in all of the experiments. Out of 23 colonies, three colonies showed both AK activities in enzyme assays and decreased resistance to adenosine analogs in the resistance test. As shown in Fig. 23, colony No. 16, 18 and 22 exhibited drug resistance up to 1 ng/ml of toyocamycin, which was similar to that of WT cells. In contrast, colony No. 10 showed resistance up to 200 ng/ml of toyocamycin, which was similar to that of AK^- mutants. In addition, results of AK assays using revertant cell extracts showed that cells from colony No. 16, 18, 22, 10 contained 104%, 30%, 40% and 9% of AK activities respectively, in comparison to WT AK^+ cells, indicating that the previous three colonies (No. 16, 18 and 22) were AK^+ revertants from AK^- mutants [Fig. 24]. The above experimental results indicated

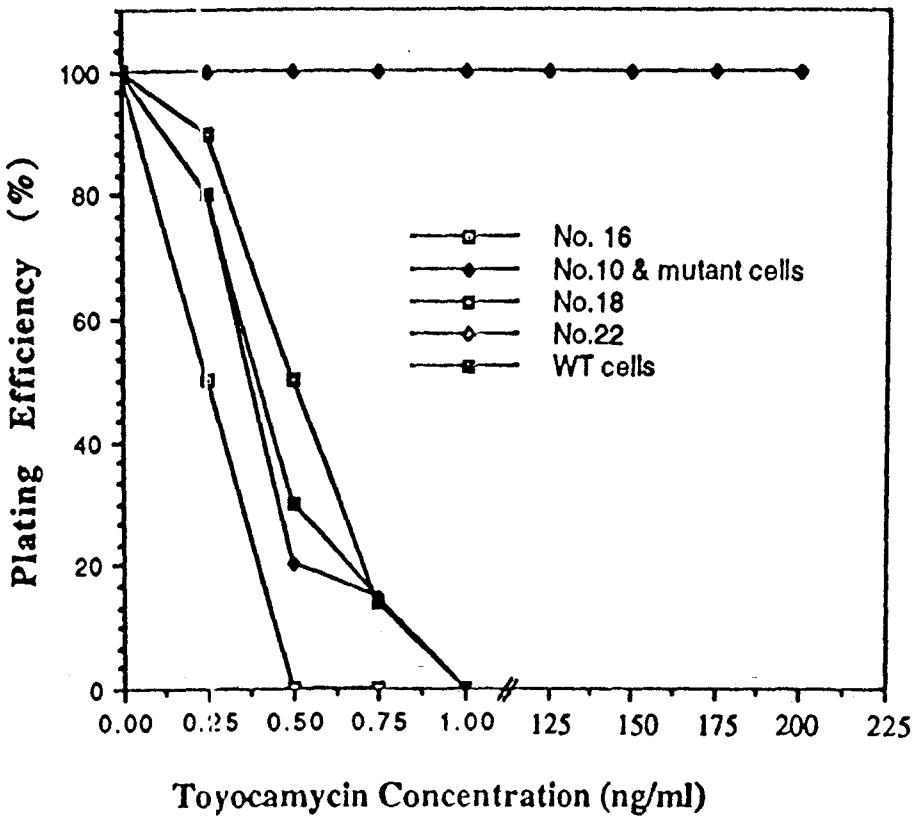


Fig. 23 Dose-response curves of revertants, WT cells and AK⁻ mutants in presence of different concentrations of toyocamycin.

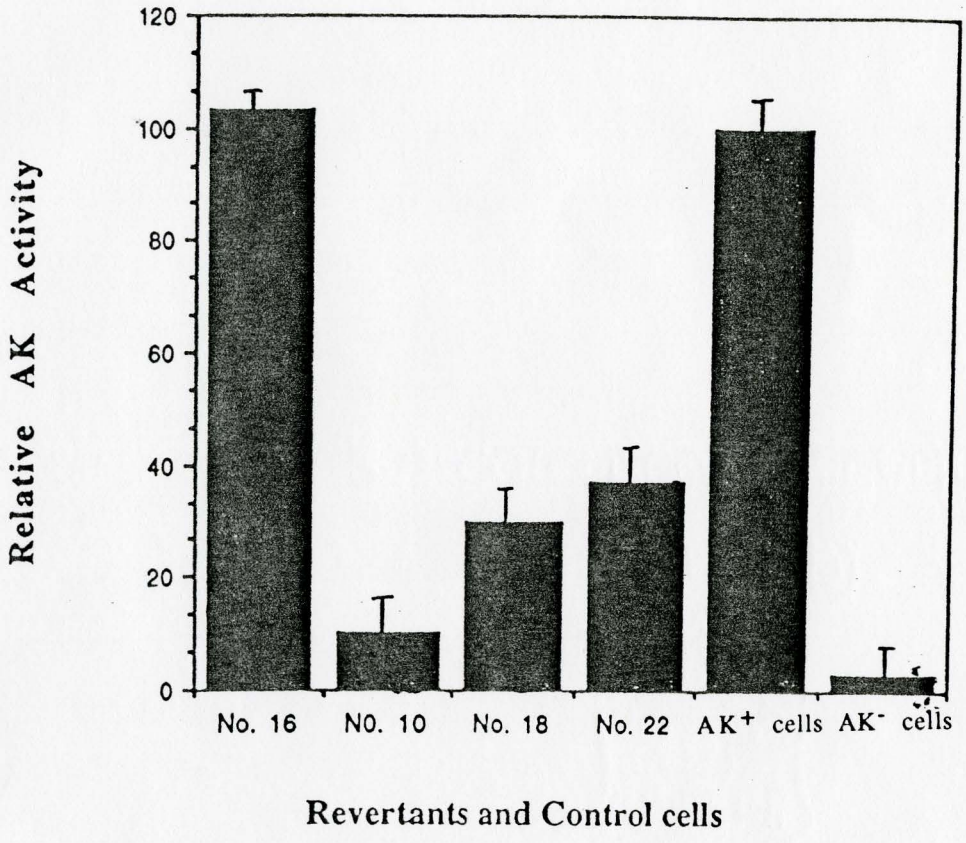


Fig. 24 Adenosine kinase activity analysis in revertants. The revertants were assayed as described in "Materials and Methods". The AK activity is indicated as the relative activity in comparison to that presented in the same number of WT cells.

that under the selective condition developed in this study, revertants, which have regained various levels of AK activities and at the same time lose their drug resistant properties, can be selected from AK^- mutants [Fig. 23 and Fig. 24].

3.5.2 Discussion

Selection of AK^+ revertants from AK^- mutants with transfection of human DNA is another possible way for cloning the AK gene from the human genome, as mentioned in the introduction part. In the present study, as the first step in this direction, conditions to isolate AK^+ revertants from AK^- CHO mutants were developed. Previous work suggested that it was difficult to select revertants from AK^- mutants because many other purine salvage enzymes might be involved in the reversion [Pang *et al.*, 1989; Chan *et al.*, 1978]. It was found that adenylosuccinate synthetase activity was elevated in revertants and none of them exhibited AK activity, when only alanosine, adenosine and uridine were used in the medium to select revertants from AK^- BHK cells [Pang *et al.*, 1989]. However, Progress has been made in the selection of revertants from AK^- mutants in our laboratory. This was accomplished by developing a selective medium, in which alanosine is used to block *de novo* biosynthetic pathway to generate AMP, while adenosine is used to provide the extracellular source for cell to produce AMP via AK [Chan *et al.*, 1978; Pang *et al.*, 1989]. In addition, EHNA is employed to prevent the conversion of

adenosine to inosine, and uridine is added to alleviate the cellular toxicity caused by high concentration of adenosine in the medium [Ullman *et al.*, 1976]. By using this selective medium, AK^+ revertants were successfully isolated from AK^- CHO mutants. Revertants regained various levels of AK activities ranging from 30% to 104% in comparison with AK activity of WT cells. At same time, these revertants lost their drug resistant properties, as expected. The exact nature of the AK^+ revertants remaines to be elucidated.

This selective medium could be used to clone the AK gene by isolating AK^+ transformants in this medium from AK^- CHO mutants, which are transfected with human DNA. AK activity and the resistance to toyocamycin can be used as criteria to distinguish AK^+ colonies from AK^- colonies. The identification of true transformants from possible spontaneous revertants could be achieved by the analysis of the thermal kinetic difference between human AK in transformants and Chinese hamster AK in spontaneous revertants, as described in Section 3.2. Further, human DNA could be separated from hamster DNA by the unique human markers, such as Alu sequence. Therefore the human AK gene could be identified from the human DNA fragments, which are transformed into AK^- CHO mutants.

4. CONCLUSION AND FUTURE PROSPECTS

4.1 Conclusion.

Several purposes of this project have been achieved in the work described in this thesis. AK has been purified to an apparently homogenous form from bovine liver and Syrian hamster liver, partially purified from CHO cells, human placenta, and Syrian hamster kidney and heart. Purified AK from Syrian hamster liver showed a molecular mass of 40 kDa, and a specific activity of 9.3 I.U./mg, which is in agreement with previous studies [Yamada *et al.*, 1982]. Adenosine kinases from different sources, such as bovine liver, human placenta, CHO cells and Syrian hamster kidney and heart, were also around 40 kDa, which is in accordance with earlier reports [Table 1 and Yamada *et al.*, 1982]. AK from Syrian hamster liver, CHO cells and human fibroblasts has been used to carry out further studies on the biochemical and immunological characteristics of this enzyme.

An interesting finding in this work is that AK activity is completely dependent upon the presence of phosphate. This novel property has not been reported previously. Ion-replacement studies indicated that phosphate can be substituted only by structurally similar anions, such as vanadate and arsenate. However, thermal kinetics studies

showed that phosphate does not affect the thermal stability of AK. The exact function of phosphate in the AK catalyzed reaction is still unknown.

AK antibodies raised in both rabbits and guinea pigs showed strong specificity to AK from Syrian hamster. Immunoblot analysis shows that antiserum from the K2 rabbit recognized specifically, and reacted strongly with AK, at a dilution of up to 1:16,000. Antisera from other animals also showed similar results. In addition, AK antibodies bound covalently to Protein A beads could pull down AK activity from Syrian hamster liver extract and a 40 kDa protein from CHO and BHK cell extracts. These results strongly indicated that these antibodies are directed specifically against AK. However, results of immunoblots also showed that these antibodies cannot recognize AK from human or CHO cells, even though Syrian hamster and Chinese hamster are closely related species. Furthermore, AK antisera also cross-reacted with a 42 kDa protein, which possesses an AMP binding site similar to AK. Further studies are required to elucidate the mechanism of the antibody specificity of AK.

A partial protein sequence of Syrian hamster AK has been obtained from one of the CNBr fragments of AK. The sequence is Tyr-Val-Asp-Ser-Leu-Phe-Gly-Ala-Glu-Thr-Glu-Ala-Ala-Leu. This is the first report of protein sequence of AK from any sources, even though this enzyme has been purified from many sources. This sequence can be used for the synthesis of degenerate oligonucleotide probes, which can be used to screen

cDNA libraries to clone the AK gene(s).

Conditions for selection of AK^+ revertants from AK^- CHO mutants have been developed. Four chemicals, which play different functions in the selective medium, were used to select revertants. They are adenosine, alanosine, uridine and EHNA. The revertant selection approach can also be used to clone the AK gene by means of transfection. Three revertants, which regain AK activities and lose their drug-resistant properties, have been isolated from AK^- mutants.

In general, several novel properties of AK were characterized in this project and progress toward cloning the AK gene has been made in three areas, include raising AK antibodies, obtaining the partial protein sequence of AK and developing conditions for selection of AK^+ revertants from AK^- mutants.

4.2. Future Prospects.

Previous studies on AK mainly focused on two major areas. One is genetic and kinetic properties of AK from different sources [Gupta, 1989]. The other is the clinical importance of AK, mainly focusing on human AK [Pillwein *et al.*, 1990; Sciotti and Van Wylen, 1993; Deussen *et al.*, 1993]. Little work has been done on the research of AK at the molecular level in different kinds of cells, such as the molecular mechanisms of different AK mutants and the regulation of AK at the transcriptional or translational level. Efforts should be made in the studies of AK using molecular biological tools.

The crucial study on the research of AK in the future is to clone the wild type AK gene, since the AK gene has not been cloned from any sources. As mentioned earlier, partial protein sequence of AK from Syrian hamster liver was obtained. Based on the fourteen amino acid sequence obtained, degenerate oligonucleotide primers can be synthesized to screen cDNA libraries and carry out PCR experiments. The other progress made in this study, obtaining of AK antibodies and development of conditions for selection AK^+ revertants from AK^- mutants, could also be used to clone the AK gene, as described previously.

Once the DNA sequence for a wild type AK gene in CHO is known, further work will focus on identifying genetic alterations in different kinds of AK mutants isolated previously. DNA sequences of a large number of class A, B and C mutants of AK as mentioned in the "Introduction", as well as other novel AK mutants, would be identified by PCR or screening cDNA libraries. Large amounts of interesting and important information could be obtained by comparing the DNA sequences among different mutant AK genes and the wild type AK gene. The unusual characteristics of these three different kinds of AK mutants could be explained at the genetic level, such as the unusual high mutation frequency of class A mutants, the codominant behaviour of class B and unique properties of AK in class C mutants. The difference of DNA sequences among the WT gene(s) and mutant genes should be of much importance for understanding AK function.

Finally, the AK gene can be also studied at the genomic level. Since AK plays an important role in purine metabolism [Gupta, 1989], it is obvious that AK level should be highly regulated. Identification of the AK gene at the genomic level will open a new area to understand the regulation of AK at the molecular level.

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